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RICARDO DOS SANTOS ESTEVES

Bioformulação inseticida a partir de óleo essencial de espécie vegetal do Parque Nacional da Restinga de Jurubatiba.

Rio de Janeiro
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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia Vegetal e Bioprocessos da Universidade Federal do Rio de Janeiro – UFRJ, como parte dos requisitos necessários para a obtenção do título de grau Doutor em Biotecnologia Vegetal e Bioprocessos.

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RESUMO

Esteves, Ricardo dos Santos. Bioformulação inseticida a partir de óleo essencial de espécie vegetal do Parque Nacional da Restinga de Jurubatiba. Rio de Janeiro, 2023. Tese de doutorado do Programa de Pós-Graduação em Biotecnologia Vegetal e Bioprocessos, Decania do Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro.

O algodão (*Gossypium* spp.) é uma das culturas de fibra mais importantes na economia mundial, sendo o Brasil um dos cinco maiores do mundo em produção e exportação. O *Dysdercus peruvianus*, também conhecido como percevejo-manchador-do-algodão, é um dos insetos praga que atacam a plantação de algodão. Seu controle é feito através de inseticidas sintéticos que apresentam diversos danos ao meio ambiente, danos aos trabalhadores e a insetos não-alvo. Uma das alternativas seguras é o uso de óleos essenciais como forma de combate às pragas agrícolas. Porém, os óleos essenciais precisam ser viabilizados para aplicação em campo, já que suas características lipofílicas dificultam a aplicação. Nesse ponto a nanotecnologia é um caminho para atingir esse objetivo através da produção de uma nanoemulsão. Esse trabalho busca avaliar a atividade inseticida do óleo essencial e da nanoemulsão da *Persea venosa* frente ao *D. peruvianus*. As folhas frescas da espécie foram extraídas por hidrodestilação, e o óleo essencial obtido teve rendimento de 0,17%. A caracterização química foi realizada por CG-EM e CG-DIC apresentando as substâncias majoritárias β-cariofileno (43,78%), o óxido de cariofileno (8, 92%) e o α-humuleno (8,27%). Foi possível a produção de uma nanoemulsão contendo o óleo essencial da *P. venosa* através do método de baixo aporte energético por inversão de fases, apresentando um tamanho de partícula de 68,41 nm e índice de polidispersão de 0,266, se mantendo estável por um período de 120 dias. Foi realizado um ensaio para avaliar a atividade inseticida por contato utilizando tanto o óleo essencial quanto da nanoemulsão. Foram obtidos os resultados de DL_{50} do óleo essencial de 24,45 μg/μL e a DL_{50} da nanoemulsão foi de 28,73 μg/μL, podendo ainda ser observados os efeitos sub letais. Foi realizado um ensaio para avaliar a seletividade do óleo essencial frente *Apis mellifera* e *Partamona helleri*. Não foi observada mortalidade significativa nas abelhas, mas foram observadas diferenças significativas no consumo da dieta entre elas. Esse estudo mostra que tanto o óleo essencial da *P. venosa* e sua nanoemulsão tem atividade inseticida frente ao *D. peruvianus* e apresentam seletividade para o mesmo, não causando danos significativos nos insetos não-alvo, se mostrando um produto de fácil obtenção, eficaz e ecologicamente viável.

Palavras-chave: bioproduto; nanoemulsão; Lauraceae; atividade inseticida; seletividade.

ABSTRACT

Esteves, Ricardo dos Santos. Insecticide bioformulation from essential oils of plant species from Restinga de Jurubatiba. Rio de Janeiro, 2023. Ph.D. thesis of the Graduate Program in Plant Biotechnology and Bioprocesses, Dean of the Health Sciences Center, Federal University of Rio de Janeiro.

Cotton (*Gossypium* spp.) is one of the most important fiber crops in the world economy, with Brazil placed among the five largest in the world in terms of production and exports. The *Dysdercus peruvianus*, also known as the cotton-stainer-bug, is one of the insect pests that attack the cotton plantation. Its control is done through synthetic insecticides that present several damages to the environment, to workers and to non-target insects. One of the reliable alternatives is the use of essential oils as a way to control agricultural pests. However, essential oils need to be made viable for field application, since their lipophilic characteristics make this process unfeasible. At this point, nanotechnology is a way to achieve this goal with the production of a nanoemulsion. This work aims to evaluate the insecticidal activity of the essential oil and nanoemulsion of *Persea venosa* against *D. peruvianus*. The fresh leaves of the species were extracted by hydrodistillation, and the essential oil obtained had a yield of 0.17%. The chemical characterization was performed by GC-MS and GC-FID, showing the major substances β-caryophyllene (43.78%), caryophyllene oxide (8.92%) and α-humulene (8.27%). It was possible to produce a nanoemulsion containing the essential oil of *P. venosa* through the low energy input method by phase inversion, with a particle size of 68.41 nm and polydispersion index of 0.266, remaining stable for a period of 120 days. An assay was carried out to evaluate the insecticidal activity by contact using both the essential oil and the nanoemulsion. Results were obtained for the LD_{50} of the essential oil of 24.45 μg/μL, and the LD_{50} of the nanoemulsion was 28.73 μg/μL, and sub-lethal effects could also be observed. An assay was carried out to evaluate the selectivity of the essential oil against *Apis mellifera* and *Partamona helleri*. Significant mortality was not observed in bees, but differences in diet consumption were observed between them. This study shows that both the essential oil of *P. venosa* and its nanoemulsion has insecticidal activity against *D. peruvianus* and show selectivity for this insect by not causing damage to non-target insects, proving to be a product that is easy to obtain, effective and ecologically viable.

Keywords: bioproduct; nanoemulsion; Lauraceae; insecticidal activity; selectivity assay.

LISTA DE ILUSTRAÇÕES

Figura 1	Ciclo de vida de insetos do gênero *Dysdercus* spp.	p. 15
Figura 2	Estrutura química do DDT e do HCH.	p. 19
Figura 3	Estruturas químicas do Malation e Paration.	p. 20
Figura 4	Estruturas químicas do Carbaril e do Aldicarb.	p. 20
Figura 5	Estruturas químicas da Cipermetrina e da Deltametrina.	p. 21
Figura 6	Estruturas químicas do Imidacloprid e Triflumuron.	p. 22
Figura 7	Lagoa das Garças, PNRJ.	p. 25
Figura 8	*Persea venosa*.	p. 27

LISTA DE TABELAS

Tabela 1 Classificação dos pesticidas baseados na sua natureza química. p. 16
Tabela 2 Composição das formulações p. 31

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

a.C. Antes de Cristo

ha Hectare

DDT Diclorodifenil tricloroetano

DDD Diclorodifenil dicloroetano

HCH Hexacloro hexano

RCI Regulador de crescimento de insetos

PNRJ Parque Nacional da Restinga de Jurubatiba

UFF Universidade Federal Fluminense

UFRJ Universidade Federal do Rio de Janeiro

UFV Universidade Federal de Viçosa

UERJ Universidade Estadual do Rio de Janeiro

LTPN Laboratório de Tecnologia de Produtos Naturais

LABI – Laboratório de Biologia de Insetos

BraIn & Phy Lab Laboratório de Neurobiologia e Fisiologia de Invertebrados Brasileiros

EHL Equilíbrio Hidrófilo-Lipófilo

EDL Espalhamento dinâmico da luz

IP Índice de polidispersão

SUMÁRIO

1. INTRODUÇÃO ... 13
   1.1 Algodão ... 13
   1.2 *Dysdercus peruvianus* ... 14
   1.3 Pesticidas ... 15
      1.3.1 Inseticidas ... 18
         1.3.1.1 Organoclorados ... 18
         1.3.1.2 Organofosforados ... 19
         1.3.1.3 Carbamatos ... 20
         1.3.1.4 Piretroides ... 21
   1.4 Óleo essencial ... 22
   1.5 Nanotecnologia ... 23
      1.5.1 Nanoemulsões ... 23
   1.6 Parque Nacional da Restinga de Jurubatiba (PNRJ) ... 24
   1.7 Lauraceae ... 25
   1.8 Gênero *Persea* ... 26
   1.9 *Persea venosa* ... 26
2. JUSTIFICATIVA ... 28
3. OBJETIVOS ... 29
   3.1 Objetivo geral ... 29
   3.2 Objetivos específicos ... 29
4. MATERIAIS E MÉTODOS ... 30
   4.1 Coleta do material vegetal ... 30
   4.2 Extração dos óleos essenciais ... 30
   4.3 Caracterização dos óleos essenciais ... 30
   4.4 Preparo e caracterização das nanoemulsões ... 31
   4.5 Determinação do Equilíbrio Hidrófilo-Lipófilo (EHL) requerido ... 32
   4.6 Estabilidade das nanoemulsões ... 32
   4.7 Ensaio inseticida contra *Dysdercus peruvianus* ... 32
   4.8 Ensaio de seletividade com *Apis mellifera* e *Partamona helleri* ... 33
   4.9 Análise dos dados estatísticos ... 34
5. ARTIGO ... 35
   5.1 Artigo 1 ... 35
6. CONSIDERAÇÕES FINAIS ... 60
7. Referências ... 62
   Produtividade acadêmica 2019 - 2023 ... 71
8. ANEXOS ... 72
   8.1 Artigo 1 ... 72
   8.2 Artigo 2 ... 91
   8.2 Artigo 3 ... 92
   8.3 Artigo 4 ... 104
   8.4 Artigo 5 ... 115
   8.5 Artigo 6 ... 124
   8.6 Artigo 7 ... 132
   8.7 Artigo 8 ... 147

1. INTRODUÇÃO

1.1 Algodão

Segundo registros históricos, o algodão (*Gossypium* spp.) é usado por humanos como mortalhas de tecido de algodão em múmias Egípcias a mais de 5 mil anos a.C. e seu cultivo é datado a pelo menos 3 mil anos (LEE; FANG, 2015; RAJENDRAN; BIRAH; BURANGE, 2018). O algodão figura entre uma das mais importantes culturas de fibra mundial, tendo um papel muito importante na economia mundial. Além disso, a semente de algodão é usada para extração de óleo essencial e a torta da semente de algodão, subproduto da extração de óleo essencial, é usada para alimentação animal. Pertencente à família Malvaceae, o gênero *Gossypium* compreende mais de 50 espécies, tendo quatro delas altamente cultivadas para fins comerciais. A espécie *G. hirsutum* apresenta mais de 90% da área de algodão cultivada, seguida pela espécie *G. barbadens* (algodão egípcio), ocupando uma área de 8%. O restante é ocupado pelas espécies *G. arborium* e *G. herbaceum* (JUTURU; MEKALA; KIRTI, 2015).

O Brasil se encontra entre os 5 maiores produtores e exportadores de algodão do mundo. Desde a década de 50 a demanda mundial tem aumentado com um crescimento médio de 2% ao ano. Estima-se que o comércio mundial de algodão movimente cerca de US$ 12 bilhões, envolvendo cerca de 350 milhões de pessoas na sua produção (ABRAPA, 2023a, 2023b). Os custos de produção por hectare do algodão no Brasil no mês de dezembro de 2022 foram de mais de R$ 19 mil/ha e o valor de exportação na safra de 2021/2022 chegou a pouco mais de US$ 3 bilhões (ABRAPA, 2023c; IMEA, 2023).

O algodão é conhecido por hospedar mais de 1300 espécies de insetos. Dessas espécies, a grande maioria são de visitantes casuais e não se alimentam dos tecidos do algodão. Porém, o algodoeiro é atacado por diversos tipos de insetos praga diferentes, que podem trazer danos econômicos significativos às plantações. O controle dos insetos é feito em sua maioria com a aplicação de inseticidas sintéticos, mas também pode haver a utilização de inimigos naturais (RAJENDRAN; BIRAH; BURANGE, 2018).

No algodoeiro, as pragas são umas das principais ameaças ao cultivo, principalmente pelo alto poder de multiplicação e de disseminação dos insetos, reduzindo os ganhos e afetando a produção. Os principais insetos-praga são: a broca-da-raiz (*Eutinobothrus brasiliensis*), a lagarta-rosca (*Agrotis ipsilon*), os tripes (*Thrips* spp.), os pulgões (*Aphis gossypii* e *Myzus persicae*), a cochonilha (*Phenacoccus solenopsis*), o curuquerê-do-algodoeiro (*Alabama*

argillacea), a mosca-branca (*Bemisia argentifolii* e *Bemisia tabaci*), o bicudo-do-algodoeiro (*Anthonomus grandis*), a lagarta-das-maçãs (*Chloridea virescens*), a *Helicoverpa armigera*, a lagarta-militar (*Spodoptera frugiperda*), a lagarta rosada (*Pectinophora gossypiella*), os ácaros rajado, vermelho e branco (*Tetranychus urticae*, *Tetranychus ludeni* e *Polyphagotarsonemus latus* respectivamente), o percevejo-rajado (*Horcia nobilellus*), o percevejo-manchador-do-algodão (*Dysdercus* spp.) e os percevejos-da-soja (*Nezara viridula* e *Euschistus heros*) (SORIA et al., 2016; ALMEIDA; SOARES; ALBUQUERQUE, 2019; EMBRAPA, 2020).

1.2 *Dysdercus peruvianus*

Dentre todas as espécies de insetos citadas no tópico anterior, que podem atuar como praga do algodão, as espécies do gênero *Dysdercus* estão entre as mais importantes (SILVA et al., 2021). O gênero *Dysdercus*, que apresenta mais de 300 espécies, pertence à ordem Hemiptera, subordem Heteroptera e são caracterizados como percevejos manchadores. O ataque desses insetos pode causar perdas na produtividade que podem estar associadas a fatores como redução do teor de óleo essencial, podridão e redução de peso das sementes causada pela deposição de dejetos e da fitofagia, além da introdução de vírus e bactérias (MILANO et al., 1999; GALLO et al., 2002; ROSADO et al., 2019).

O gênero *Dysdercus* é encontrado nos trópicos de cada continente (RAJENDRAN; BIRAH; BURANGE, 2018). Já a espécie *Dysdercus peruvianus* tem distribuição nos países da América do Sul (CABI, 2019). Os ovos do inseto apresentam 1,5 x 0,9 mm de tamanho e coloração branca a laranja de acordo com a idade dos ovos. Existem cinco instares das ninfas até atingirem a fase adulta, apresentando coloração vermelha e preta gradualmente escurecida (Figura 1). Os insetos adultos apresentam 15 mm de comprimento e 4,5 mm de largura. As fêmeas do gênero podem fazer a postura de 300 a 450 ovos. Após a eclosão dos ovos, cerca de 5 dias após a postura, os insetos levam em torno de 20 dias para atingirem o estágio de adultos. Quando adultos, são capazes de voar por até 15 km de distância (RAJENDRAN; BIRAH; BURANGE, 2018).

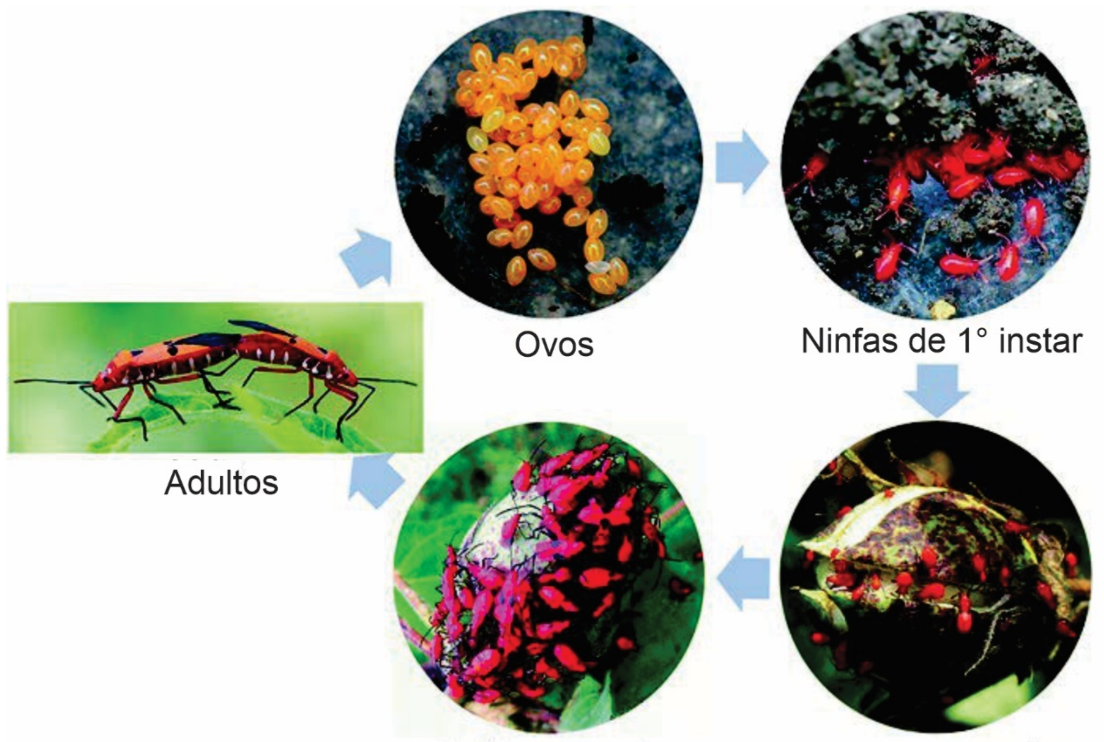

Figura 1: Ciclo de vida de insetos do gênero *Dysdercus* spp. Fonte: adaptado de RAJENDRAN; BIRAH; BURANGE, 2018.

Tendo em vista a capacidade de reprodução e desenvolvimento do inseto, seu poder de locomoção, habilidade para se alimentar de outras espécies de plantas e de sobreviver apenas de água na idade adulta após a temporada do algodão (RAJENDRAN; BIRAH; BURANGE, 2018; CABI, 2019), se torna mais urgente o controle do *D. peruvianus* de forma a reduzir os impactos causados na plantação.

1.3 Pesticidas

Pesticidas são produtos utilizados para repelir, destruir ou controlar as pragas. Eles são compostos de diversas subclasses que são divididas baseadas em seus alvos, como por exemplo os insetos (inseticidas), os fungos (fungicidas) e as ervas-daninha (herbicidas). Na tabela 1, são mostrados os pesticidas classificados quanto ao seu grupo químico. Cada uma dessas classes pode ser subdividida conforme a sua origem (biopesticidas) ou método de aplicação (fumigante) e são utilizados tanto para a agricultura quanto para o uso doméstico (RICHARDSON et al., 2019; BOLZONELLA et al., 2019). Insetos praga podem causar uma grande destruição em lavouras, podendo levar a perdas irreparáveis em sua produção e a forma mais comum de se controlar os insetos é a aplicação de inseticidas sintéticos (MA et al., 2020). Para garantir que o controle desses insetos seja adequado dentro de um período conveniente, os produtores utilizam grandes quantidades de inseticidas, excedendo as concentrações recomendadas para o

controle das pragas (BOEHM et al., 2003). Foi estimada uma média de 2 milhões de toneladas de pesticidas utilizados por ano no mundo todo para o controle de diversos tipos de pragas agrícolas, tendo o Brasil como um dos principais consumidores de pesticidas desde 2008 (SYAFRUDIN et al., 2021; INCA, 2022).

Tabela 1. Classificação dos pesticidas baseados na sua natureza química.

N°	Grupo químico	Nomes dos produtos químicos
1	Organoclorados	DDT, DDD, Dicofol, Eldrin, Dieldrin, Clorobenzoato, HCH, Metoxicloro Aldrin, Clordano, Heptacloro, Endosulfan, Isodrin, Isobenzan, Toxafeno, Cloropropilato.
2	Organofosforados	Dimefox, Mipafox, Metil Paration, Ronel, Fenitrotiona, Dicrotofós, Forato, Fenthion, Coumafós, Temefós, Diclorvós, Dipterex, Fosfomidon, Demeton, Metil-oxidemeton, Malation, Dimetoato, Metrifonato.
3	Carbamatos	**Metil** Carbaril, Carbanolato, Propoxur, Dimetan, Dimetilan, Isolan, Carbofurano, Pirolan, Aminocarb, Aldicarb. **Tio** Vernolato, Pebulato, Diallate, Molinato, Butilato, Cicloato, Trialato, Tioureia. **Ditio** Thiram, Ferbam, Amobam, Nabam, Zineb, Maneb, Ziram Polyram, Dithane M- 45.
4	Piretroides	Aletrina, Bifentrina, Dimetrina, Tetrametrina, Piretrina, Cicletrina Furetrina, Fenvalerato, Alfametrina, Decametrina, Cipermetrina.
5	Fenilamidas	**Carbanilatos** Barban, Carbetamida, Cloroprofam, Profam, Fenil Ureia, Lufenuron, Monuron, Diuron, Fluometuron, Cloroxuron, Neburon, Bromuron. **Acilanalida** Propanil, Solan, Dicril, Karsil, Propacloro, Alacloro, Butacloro. **Toluidinas** Trifluralina, Dipropanil, Benefin, Orizalina, Isopropanil, Nitralin. **Acetamida** Difenamida.
6	Cloro fenoxi	2,4-D (2,4-ácido acético dicloro fenoxi). 2,4 5 T(2,4 5-ácido acético tricloro fenoxi). Diclorprop, Mecoprop, Erbin, Sesone.
7	Triazinas	Atrazina, Simazina, Ametrina, Atraton, Clorazina, Cinazina, Ciprazina, Metribuzin, Propazina, Terbutina, Simetrina.
8	Ácidos benzóicos	Dicamba, Diclorobenzil, Cloroambina, Tricamba, Neptalan, Bromoxinil.

N°	Grupo químico	Nomes dos produtos químicos
9	Ftalimidas	Captan, Diflotan, Folpet.
10	Bipiridílios	Paraquate, Diaquate.
11	Outros	Pentaclorofenol, Fluoroacetato, Acetato de fenilmercúrio, Fosfato de etilmercúrio, Cloreto de metilmercúrio, Arsenato de sódio, Arsenato de cálcio, Arsenato de chumbo, Ácido cacodílico, Fosfeto de alumínio, Fosfeto de zinco.

Fonte: adaptado de JAYARAJ; MEGHA; SREEDEV, 2016.

Existe uma preocupação global quanto à exposição de trabalhadores das lavouras aos pesticidas, que pode ser classificado como um problema de saúde pública (MSIBI et al., 2020). Segundo registros da Organização Internacional do Trabalho, são registrados em torno de 70 mil casos de intoxicação por pesticidas, que podem evoluir para óbito, além de mais de 7 milhões de casos de doenças agudas e crônicas não fatais. A OMS registrou 20 mil mortes por ano decorrentes do uso de pesticidas (INCA, 2022). A exposição a esses pesticidas ocorre na manipulação das substâncias durante o preparo e aplicação sem o uso de equipamentos de proteção individual adequado, na limpeza do equipamento utilizado para aplicar os pesticidas, no hábito dos trabalhadores de reentrar no local contaminado e na lavagem das roupas contaminadas. Também são fontes de contaminação a reutilização das embalagens e seu armazenamento e descarte inadequados, além de possível contaminação de lençol freático e posterior consumo da água contaminada (INCA, 2021).

O registro mais antigo que se tem notícia de substâncias químicas sendo utilizadas para o controle de pragas é de 2500 a.C. e foi atribuído aos Sumérios, que fizeram a utilização de enxofre para combater insetos (CARNEIRO, 2006). Somente na época da II Guerra Mundial que os estudos para combater insetos praga realmente despontou com a descoberta do diclorodifenil tricloroetano, mais conhecido como DDT, inseticida sintético da classe dos organoclorados. Seu uso aumentou de forma expressiva devido a sua eficácia contra vários tipos de praga, utilizando uma baixa dosagem do produto. Após o grande sucesso, vieram também os impactos causados no meio ambiente e nos humanos pelo uso excessivo do DDT, causando seu banimento do uso na agricultura e doméstico. Durante esse tempo foram descobertos outros tipos de inseticidas, como os organofosforados, os piretroides e carbamatos, que também são conhecidos por causarem danos ao meio ambiente e aos seres humanos (SYAFRUDIN et al., 2021).

Mesmo sendo desenvolvidos para afetar as pragas agrícolas, os pesticidas também acabam afetando organismos não-alvo, e assim, comprometendo toda a sustentabilidade do ecossistema (SERRÃO et al., 2022). Não bastasse toda contaminação anteriormente descrita, os insetos, um dos principais alvos dos pesticidas, têm desenvolvido resistência aos mesmos, gerando um aumento ainda maior na concentração usada *in loco* e em número de aplicações. Esse uso indevido gera apenas uma maior quantidade de insetos resistentes e uma maior contaminação do meio ambiente, além de afetar também os seres humanos (RICHARDSON et al., 2019; SERRÃO et al., 2022).

1.3.1 Inseticidas

Inseticidas são substâncias químicas ou biológicas utilizadas para controlar insetos, resultando na morte ou prevenindo a ação destrutiva dos insetos (WARE; WHITACRE, 2004). Os primeiros inseticidas utilizados datam da China antiga e depois da Pérsia durante a Idade Média. Eles utilizavam as flores do piretro-da-Dalmácia que continha 1,5% da substância ativa piretrina (OBEREMOK et al., 2015). Como já foi mencionado antes, somente após a II Guerra Mundial é que possibilitou toda a revolução química do controle de insetos, sendo o DDT o primeiro inseticida (WARE; WHITACRE, 2004). Para exemplificar, serão descritas algumas das classes de inseticidas mais conhecidas.

1.3.1.1 Organoclorados

Organoclorados são substâncias compostas majoritariamente de átomos de carbono, hidrogênio e cloro, podendo apresentar outro heteroátomo como oxigênio (WARE; WHITACRE, 2004; SINGH et al., 2016). São substâncias que podem ser absorvidas pela pele, pulmões e pelo estômago, tendo seu poder de absorção aumentado por lipídios ou solventes apolares (SINGH et al., 2016). Os inseticidas dessa classe apresentam alta persistência no meio ambiente. Existem diversos produtos dessa classe, porém os mais conhecidos são o DDT e o hexaclorociclohexano (HCH) (Figura 2) (JAYARAJ; MEGHA; SREEDEV, 2016).

Quando foi inventado, o DDT foi muito utilizado para o tratamento de malária, até ser proibido na maioria dos países desenvolvidos (JAYARAJ; MEGHA; SREEDEV, 2016). No Brasil, a proibição se deu em 1985 após a proibição do seu uso nas pastagens em 1971 (LIGNANI; BRANDÃO, 2022). Os organoclorados já foram banidos em vários países por apresentarem muitos efeitos tóxicos para o meio ambiente e para a população e por serem substâncias extremamente persistentes no meio ambiente (WARE; WHITACRE, 2004;

JAYARAJ; MEGHA; SREEDEV, 2016; SINGH et al., 2016). O DDT e o HCH possuem mecanismos de ação bastante similares. Essas substâncias agem na abertura dos canais de sódio, causando uma alteração no equilíbrio dos íons sódio e potássio nos axônios de forma a não deixar que ocorra normalmente a transmissão do impulso nervoso. A diferença entre as duas substâncias é que o mecanismo de ação do HCH ocorre mais rápido do que o do DDT. Esse efeito ocorre tanto em insetos quanto em mamíferos (WARE; WHITACRE, 2004; REZENDE-TEIXEIRA et al. 2022).

Cl Cl Cl Cl Cl DDT

Cl Cl Cl Cl Cl Cl HCH

Figura 2: Estruturas químicas do DDT e do HCH. Fonte: Autor.

1.3.1.2 Organofosforados

Os organofosforados são ésteres dos ácidos fosfóricos, fosfínicos, fosfônicos e fosforosos, podendo conter cadeias adicionais de grupos fenoxi, cianeto e tiocianato (SIDHU et al., 2019). Podem ser classificados como os inseticidas mais tóxicos da classe para os vertebrados (WARE; WHITACRE, 2004). Além de serem os componentes majoritários em herbicidas e inseticidas, os organofosforados também estão presentes nos chamados “gases asfixiantes” Sarin, Soman e Tabun (WARE; WHITACRE, 2004; SIDHU et al., 2019).

As substâncias pertencentes a essa classe são quimicamente instáveis e apresentarem pouca persistência no meio ambiente. Elas se degradam por hidrólise quando expostos à luz, ao ar e ao solo (JAYARAJ; MEGHA; SREEDEV, 2016). Essas características fizeram com que os organofosforados substituíssem os organoclorados na agricultura (WARE; WHITACRE, 2004). Os organofosforados agem inibindo irreversivelmente a enzima acetilcolinesterase, o que leva a um aumento significativo da acetilcolina nas fendas sinápticas, gerando uma alta estimulação dos receptores colinérgicos que pode levar à morte (WARE; WHITACRE, 2004; REZENDE-TEIXEIRA et al. 2022). Os representantes mais conhecidos dessa classe são o Malation e o Paration (Figura 3).

Malation

Paration

Figura 3: Estruturas químicas do Malation e do Paration. Fonte: Autor.

1.3.1.3 Carbamatos

Assim como os organofosforados, os carbamatos também tem como mecanismo de ação a inibição da acetilcolinesterase, porém de forma reversível, pela carbamilação de um grupo hidroxil presente no resíduo de serina do sítio ativo da enzima. Apesar dessa diferença, os sintomas observados pela intoxicação com carbamato são iguais aos observados pela intoxicação com organofosforados (REZENDE-TEIXEIRA et al. 2022). Os carbamatos começaram a ser produzidos em meados dos anos 1950 e atualmente tem apresentam um volume de uso na agricultura e doméstico maior do que os organofosforados e organoclorados pelo fato de apresentarem baixa toxidez oral e dérmica em mamíferos e amplo espectro para o controle de insetos (WARE; WHITACRE, 2004; OLIVEIRA et al., 2020).

Apesar de terem sido inicialmente usados como inseticida, os carbamatos, quando em doses terapêuticas, são aplicados como medicamentos para tratamento quimioterápico (etil carbamato ou uretano), doenças de Alzheimer e Parkinson (rivastigmina), ansiedade e tensão muscular (meprobamato e seus derivados), dentre outros. Mesmo apresentando propriedades medicinais, seu uso majoritário é como inseticida e rodenticida. O carbaril e o aldicarb são alguns dos representantes dessa classe (Figura 4) (OLIVEIRA et al., 2020).

Carbaril

Aldicarb

Figura 4: Estruturas químicas do Carbaril e do Aldicarb. Fonte: Autor.

1.3.1.4 Piretroides

Os piretroides são substâncias sintéticas derivadas das piretrinas, encontradas nas flores do *Chrisanthemum cinerariaefolium*. As substâncias encontradas nessa espécie são muito fotossensíveis e foram substituídas pelos derivados sintéticos, quando ainda achavam que os piretroides eram seguros para os humanos e animais. Desde então os piretroides vem sendo utilizados no mundo inteiro como inseticidas devido à sua alta eficácia e baixa toxidez quando comparados com os organofosforados e os carbamatos. Além do uso na agricultura, os piretroides também têm sido aplicados tanto no uso doméstico quanto em formulações repelentes e em xampus (SINGH et al., 2022).

O mecanismo de ação dos piretroides se faz via abertura dos canais de sódio e cloreto, interferindo na transmissão do sinal. Apesar de se parecer com o mecanismo de ação do DDT, seus poucos efeitos tóxicos em seres humanos e baixa persistência no meio ambiente, fizeram dos piretroides, um dos inseticidas mais usados nos últimos anos. Em torno de 25% do mercado mundial de inseticidas é dominado pelos piretroides. Na figura 5 são mostrados dois dos piretroides mais conhecidos (WARE; WHITACRE, 2004; RICHARDSON et al., 2019; SINGH et al., 2022).

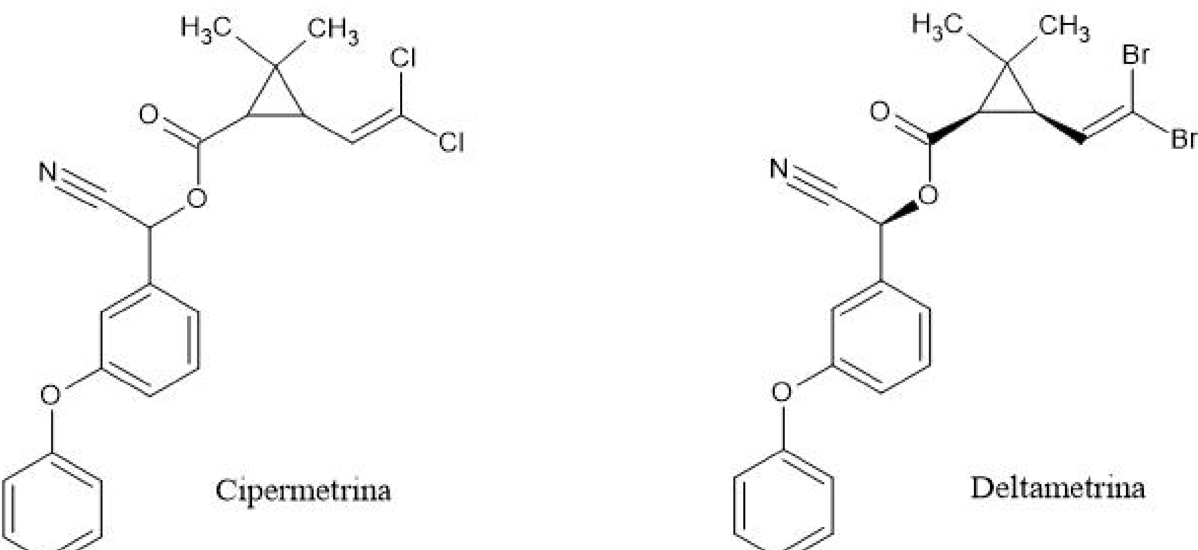

Figura 5: Estruturas químicas da Cipermetrina e da Deltametrina. Fonte: Autor.

Dentre as várias outras classes de inseticidas existentes, também são encontrados os neonicotinoides e os reguladores de crescimento dos insetos (RCI) como o imidacloprid e o

triflumuron, respectivamente (Figura 6). Os neonicotinoides agem como agonistas dos receptores nicotínicos da acetilcolina e apesar de apresentarem alta seletividade para insetos e versatilidade no modo de aplicação, o imidacloprid foi um dos responsáveis pela mortandade de abelhas em diversos países. Os RCIs podem atuar como os análogos ou agonistas hormonais, a exemplos da ecdisona e hormônio juvenil, ou como, no caso do triflumuron, substâncias que inibem a produção da quitina, que leva o inseto a apresentar problemas durante o período da muda, podendo causar sua morte (BATRA et al., 2005; HAN; TIAN; SHEN, 2018).

Imidacloprid

Triflumuron

Figura 6: Estruturas químicas do Imidacloprid e Triflumuron. Fonte: Autor.

Visando buscar novas alternativas que sejam menos tóxicas para o meio ambiente e para os mamíferos, que apresentem seletividade para os insetos-alvo e que não causem dano aos insetos não alvo, diversos autores vêm buscando alternativas ao uso de pesticidas sintéticos. Essas alternativas estão, cada vez mais, girando em torno das substâncias derivadas de plantas, como os óleos essenciais (PAVELA, BENELLI, et al., 2020; MA, JIA, et al., 2020; MACHADO, FOLLY, et al. 2023; VIANA, MACHADO, et al., 2023).

1.4 Óleo essencial

Os óleos essenciais são líquidos aromáticos lipofílicos extraídos de partes de plantas aromáticas (ASBAHANI et al., 2015). São caracterizados pela sua volatilidade, odor e são compostos por metabólitos secundários como mono e sesquiterpenos, fenilpropanoides e outros. São geralmente obtidos por hidrodestilação ou arraste a vapor e podem ser encontrados nos mais diversos produtos farmacêuticos e cosméticos, além de flavorizantes em alimentos (BAKKALI et al., 2008; ADORJAN; BUCHBAUER, 2010).

Óleos essenciais são biosintetizados em partes diferentes da planta como flores, folhas, frutos, sementes e outros. Por serem substâncias lipofílicas, são solúveis em solventes orgânicos e insolúveis em água. Fatores extrínsecos como época do ano, clima, temperatura e tipo de solo,

e intrínsecos como os diferentes órgãos produtores de óleo essencial, podem interferir tanto na composição química quanto no rendimento do óleo essencial (ASBAHANI et al., 2015; LI et al., 2020).

Apesar de apresentarem diferentes tipos de atividade biológica, dentre elas a atividade inseticida, a viabilidade do óleo essencial para que seja aplicado *in loco* fica prejudicada pelo fato dele não ser solúvel em água. Nesse ponto, a nanotecnologia entra como uma forma de viabilizar a aplicação de óleos essenciais em diversas áreas (PAVONI et al., 2019; FOLLY et al., 2021; MACHADO et al., 2023).

1.5 Nanotecnologia

Por definição, a nanotecnologia significa a capacidade de controlar ou reestruturar a matéria nos níveis atômicos e moleculares na faixa de tamanho de 1 a 100 nm (HULLA; SAHU; HAYES, 2015; BHUSHAN, 2017). Apesar da grande explosão de aplicações envolvendo nanotecnologia nos últimos anos, o conceito de nanotecnologia é bem mais antigo. O ganhador do Prêmio Nobel de 1925, Richard Zsigmondy, foi o primeiro a propor o conceito de nanômetro, ao medir partículas coloidais de ouro usando um microscópio (HULLA; SAHU; HAYES, 2015). Ao final do século XX, ocorreu a descoberta de novos materiais, processos e fenômenos que aconteciam em escala nanométrica e o desenvolvimento de novas técnicas teóricas e experimentais para pesquisa. Tudo isso tornou possível o desenvolvimento de sistemas e materiais nanoestruturados inovadores. Além disso, toda a característica físico-química desses sistemas e materiais puderam e estão sendo explorados na aplicação comercial e como uma forma inovadora de performance, com o intuito de trazer benefícios para a sociedade (BHUSHAN, 2017).

Problemas relacionados ao meio ambiente, medicina e indústria foram solucionados pelo avanço da integração da nanotecnologia em sistemas maiores, que incluem eletrônica, sistemas de carreamento de drogas, biotecnologia, tecnologia da informação, dentre outros (BHUSHAN, 2017). Em relação ao meio ambiente, a nanotecnologia tem sido bastante aplicada para o manejo de pragas na agricultura. As nanoemulsões, nanopartículas e nanocápsulas tem sido ferramentas inovadoras como forma de aplicação de substâncias com atividade pesticida (PAVONI et al., 2019).

1.5.1 Nanoemulsões

Nanoemulsões são sistemas coloidais termodinamicamente instáveis, porém isotrópica e cineticamente estáveis, onde uma fase aquosa e a outra oleosa se encontram em dispersão e são estabilizados por surfactantes. As nanoemulsões podem ser do tipo óleo em água (O/A), se o óleo estiver disperso na água, ou água em óleo (A/O), se a água estiver dispersa no óleo. Elas são sistemas que apresentam tamanho de partícula geralmente variando entre a faixa de 20 a 200 nm e que apresentam um grande potencial de aplicação na indústria farmacêutica, de alimentos, agroquímica e outros (McCLEMENTS, 2012; BARRADAS; DE HOLANDA E SILVA, 2020).

As nanoemulsões podem ser preparadas por dois métodos: alto ou baixo aporte energético. Para o alto aporte energético, são usados geradores de ultrassom e homogeneizadores de alta pressão, causando o rompimento das gotículas e formando a nanoemulsão. O método de baixo aporte energético utiliza as propriedades físico-químico do sistema para determinar o tamanho de partícula, com a vantagem de utilizar equipamentos simples para obter a nanoemulsão. (BARRADAS; DE HOLANDA E SILVA, 2020; FENG et al., 2020). Uma das formas de utilizar o método de baixo aporte energético é a obtenção da nanoemulsão por inversão de fases. Esse é o método de escolha para ser aplicado a materiais termolábeis como os óleos essenciais. Ele consiste na adição de um dos componentes, com temperatura constante, a uma mistura do resto dos outros componentes, como por exemplo, a adição de água à mistura de óleo essencial e surfactantes (FENG et al., 2020).

Nosso grupo de pesquisa vem demonstrando o uso de óleos essenciais nanoemulsionados aplicados a diferentes alvos (PASSOS et al., 2020; FOLLY et al., 2021; MACHADO et al., 2023; VIANA et al., 2023; ESTEVES et al., 2023). Esses óleos essenciais são extraídos de espécies vegetais obtidas em uma região específica no Estado do Rio de Janeiro chamado Parque Nacional da Restinga de Jurubatiba.

1.6 Parque Nacional da Restinga de Jurubatiba (PNRJ)

O PNRJ foi criado em 1998 e está situado na região Norte do Rio de Janeiro abrangendo os municípios de Carapebus, Quissamã e Macaé. A Restinga de Jurubatiba possui quase 15 mil ha, 44 km de extensão costeira e 18 lagoas em seu território (Figura 7) (SANTOS et al., 2009; LUZ et al., 2022). Foi realizado um estudo na região que mostrou as espécies mais utilizadas pela população local. Foram registrados o uso de 119 espécies pertencentes a 49 famílias e 100 gêneros vegetais com as finalidades alimentares, medicinais, ornamentais,

tecnológicas, higiênicas, de construção e de combustível (SANTOS, FEVEREIRO, et al., 2009). Um outro estudo informa que existem atualmente no PNRJ, mais de 600 espécies vegetais (CASTILHORI, CALLADO, LIMA, 2021).

Figura 7: Lagoa das Garças, PNRJ. Fonte: http://www.icmbio.gov.br/parnajurubatiba/guia-do-visitante.html

A vegetação de restingas apresenta grande variedade de formações vegetais, apresentando herbáceas, arbustos e florestas com dossel de até 20 metros (GOMES et al., 2007). Esse tipo de formação de vegetais se dá devido a uma combinação de fatores físico-químicos como temperatura elevada, alta exposição a luminosidade, alta salinidade, terreno arenoso e alta deposição de salsugem (COGLIATTI-CARVALHO et al., 2001).

Dentre as várias famílias que já foram encontradas e descritas no PNRJ, foi escolhida uma espécie da Família Lauraceae, exemplificando, nesse trabalho, o potencial das espécies que lá habitam.

1.7 Lauraceae

A família Lauraceae apresenta distribuição pantropical, ocorrendo principalmente na América, Ásia tropical, Austrália e Madagascar. A família conta com quase 3 mil espécies em 68 gêneros (KROPF; QUINET; ANDREATA, 2015; THE PLANT LIST, 2023). No Brasil são registrados 27 gêneros e 466 espécies (FLORA E FUNGA DO BRASIL, 2023).

O estudo da família Lauraceae é importante devido a dois aspectos: primeiro pela sua representatividade em número de indivíduos e riqueza de espécies realizados em áreas florestais nas regiões Sudeste e Sul. Segundo por sua importância econômica, uma vez que algumas espécies têm sido utilizadas para fabricação de produtos culinários, marcenaria, construção civil, além de outras espécies serem usadas de acordo com o conhecimento tradicional (KROPF; QUINET; ANDREATA, 2015).

A respeito da produção de metabólitos secundários, a família apresenta as neolignanas como um potencial quimio-taxonômico significante. Apresenta também alcaloides isoquinolínicos e os óleos essenciais como representantes da família. A composição do óleo essencial apresentou o β-cariofileno e o 1,8-cineol como as substâncias predominantes nas espécies de Lauraceae (DAMASCENO et al., 2019).

1.8 Gênero *Persea*

O gênero *Persea* teve sua primeira descrição em 1754 publicada por Miller, o qual, posteriormente em 1768 descreveu a *Persea americana* como única espécie representante desse gênero. Anterior a Miller, Linnaeus publicou em 1753 sobre uma espécie chamada *Laurus indica* que, posteriormente, em 1825, essa espécie foi validada como *Persea indica* por Sprengel. *Persea americana* hoje é popularmente conhecida como abacateiro, é cultivada mundialmente e tem o México como seu maior produtor (GALINDO-TOVAR; OGATA-AGUILAR; ARZATE-FERNANDEZ, 2008).

Em 1836, Nees publicou um manuscrito sobre as Lauraceae, contendo o gênero *Persea* dividido em dois subgêneros: o *Gnesiopersea* e o *Eriodaphne*. Posteriormente, vários autores sugeriram outras subdivisões, porém Kopp, em 1966, publicou um manuscrito com os membros Americanos do gênero *Persea*. Nele, os dois subgêneros *Persea* e *Eriodaphne* podem ser reconhecidos morfologicamente se o perianto é patente ou refletido na ântese, pelo número de sacos de pólen por antera e pelo crescimento de pelos do gineceu e do perianto. Nesse trabalho também foram sugeridas 4 seções dentro do gênero *Persea* subgênero *Eriodaphne* que foram as seções *Aurateae* Kopp, *Eriodaphne*, *Hexanthera* Mez, *Mutisaea* Kopp (DUDAS; ROHWER; RUDOLPH, 2016).

1.9 *Persea venosa*

A espécie é nativa e endêmica do Brasil, sendo conhecida como pau-de-andrade ou canela-sebo na região Sul do país e como canela-seda-branca (Figura 8) no Rio de Janeiro e é nativa do Rio Grande do Sul. Tem ocorrência nos estados de Minas Gerais, Rio de Janeiro, Espírito Santo, Paraná e Santa Catarina (MENDES et al. 2013). Segundo Rodrigues e colaboradores (1998), a *P. venosa* é uma espécie que ocorre em terrenos de brejo, apresentando ramos angulosos no ápice, retos, grossos, glabros e com cicatrizes foliares junto à sua base. A florada da espécie acontece entre os meses de setembro a dezembro e a frutificação ocorre de novembro a fevereiro no Rio Grande do Sul (RODRIGUES et al., 1998).

Figura 8: *Persea venosa*. Fonte: Próprio autor

Apesar da pouca quantidade de estudo sobre os usos dessa espécie, podemos encontrar que a casca da *Persea venosa* é usada pela medicina tradicional como tratamento para úlceras, feridas e como cicatrizante. Porém, até o momento, não há estudos que assegurem seu uso na medicina tradicional (MENDES et al. 2013). O perfil químico do óleo essencial foi descrito por Silva e colaboradores (2019) da espécie encontrada em região de Cerrado. Os autores encontraram o espatulenol, epóxido de humuleno e óxido de cariofileno como substâncias majoritárias. (SILVA et al., 2019).

Nesse trabalho, foram demonstradas a caracterização química do óleo essencial das folhas da espécie *Persea venosa*, coletada no Parque Nacional da Restinga de Jurubatiba. Também foram mostrados o processo de obtenção e caracterização físico-químico da nanoemulsão obtida com o óleo essencial, a atividade inseticida tanto do óleo essencial quanto

da nanoemulsão frente a uma das pragas do algodão e a seletividade do óleo essencial frente a duas espécies de insetos não-alvo.

2. JUSTIFICATIVA

A importância do algodão para a economia mundial e do controle de pragas que atacam as plantações a cada safra justificam esse trabalho. O controle dos insetos é feito à base de inseticidas e inimigos naturais, incluindo o tratamento das sementes com inseticidas (RAJENDRAN; BIRAH; BURANGE, 2018). O uso de inseticidas sintéticos teve a sua explosão após a descoberta do DDT na época da II Guerra Mundial. Porém, os danos causados ao meio ambiente logo começaram a aparecer, como também danos causados aos trabalhadores rurais. As doenças causadas por contaminação por inseticidas podem ser consideradas como problemas de saúde pública (MSIBI et al., 2020; SYAFRUDIN et al., 2021). Ainda assim, seu uso é amplamente difundido entre os agricultores e, no Brasil, nos últimos anos ocorreu um grande aumento de registros de inseticidas sintéticos. Dos 499 novos registros, 422 (84,5%) foram de inseticidas sintéticos (REZENDE-TEIXEIRA et al. 2022).

Portanto, novas formas de controle para insetos devem ser discutidas. Os óleos essenciais vêm ganhando cada vez mais espaço como formas alternativas e mais seguras para o controle de insetos. Com isso, o estudo de novas espécies vegetais pode gerar a descoberta de novas moléculas bioativas, e com isso, um novo leque de estudos de formulações para gerar novos bioprodutos. O Parque Nacional da Restinga de Jurubatiba é uma fonte abundante de espécies a serem estudadas e o Laboratório de Tecnologia de Produtos Naturais vem fazendo isso durante os últimos anos. Espécies com atividade inseticida já foram descritas antes pelo nosso grupo, tanto frente ao *Dysdercus peruvianus* quanto a outros tipos de pragas e vetores. Para exemplificar temos as espécies *Myrciaria floribunda* e *Manilkara subsericea* apresentando atividade inseticida contra o *D. peruvianus*, a espécie *Ocotea pulchella* apresentando atividade moluscicida contra *Biomphalaria glabrata* e as espécies *Ocotea indecora*, *Annona acutiflora* e *Xylopia ochrantha* apresentando atividade larvicida contra *Aedes aegypti* (FERNANDES et al., 2014; TIETBOHL et al., 2014; PASSOS et al., 2020; FOLLY et al., 2021; MACHADO et al., 2023; VIANA et al., 2023).

3. OBJETIVOS

3.1 Objetivo geral

Esse trabalho tem como objetivo geral avaliar a atividade inseticida do óleo essencial das folhas e da nanoemulsão da espécie *Persea venosa*.

3.2 Objetivos específicos

- Extrair e caracterizar quimicamente o óleo essencial da espécie estudada;
- Preparar e caracterizar uma emulsão com o óleo essencial extraído;
- Avaliar a atividade inseticida do óleo essencial e da nanoemulsão;
- Avaliar os efeitos subletais no ensaio com o inseto-praga *Dysdercus peruvianus*;
- Avaliar a toxidez do óleo essencial frente a *Apis mellifera* e *Partamona helleri*.

4. MATERIAIS E MÉTODOS

4.1 Coleta do material vegetal

A folha da espécie *Persea* venosa foi coletada no Parque Nacional da Restinga de Jurubatiba (PNRJ) no Rio de Janeiro. A coleta e a pesquisa foram autorizadas pelo SISBIO/ICMBio (13659-14) e SisGen (A0D648D). A identificação da espécie foi realizada pelo botânico Professor Dr. Marcelo Guerra Santos da Universidade Estadual do Rio de Janeiro (UERJ). A herborização e a exsicata foram depositadas no Herbário da Faculdade de Formação de Professores (FFP) da UERJ sob o número de registro RFFP: M.G.Santos 2336.

4.2 Extração dos óleos essenciais

Após a coleta, as folhas frescas de *Persea venosa* (2.971,0 g) foram trituradas com água destilada em um liquidificador e transferidas para um balão de fundo redondo de 5,0 L, que foi colocado sobre uma manta de aquecimento. A esse balão foi acoplado um aparato do tipo Clevenger e sobre ele foi acoplado um condensador de bolas. O óleo essencial foi extraído por hidrodestilação durante 4 horas. Ao final da extração, o óleo essencial foi seco com sulfato de sódio anidro e acondicionado em frasco de vidro âmbar e armazenados sob refrigeração (-20 °C) para futuras análises.

4.3 Caracterização dos óleos essenciais

O óleo essencial foi analisado por CG-EM-QP500 (SHIMADZU), um cromatógrafo a gás equipado com um detector de massas por ionização por elétrons. As condições da cromatografia gasosa (CG) foram: temperatura de injeção: 260 °C; gás de arraste: Hélio; taxa de fluxo: 1 mL/min e injeção *spliless*. A temperatura do forno, inicialmente, foi de 60 °C e depois aumentou até 260 °C a uma taxa de 3 °C/min. Cada amostra foi diluída em CH_2Cl_2 (1:100 mg/mL) e foi injetada em uma coluna DB-5 (id = 0,25 mm, comprimento = 30 m, espessura = 0,25 mm). As condições da espectrometria de massas (EM) foram: voltagem: 70 eV e taxa de varredura: 1 scan/s. A composição percentual dos óleos essenciais foi calculada pelo método de normalização das áreas de pico obtido por cromatografia gasosa. A identificação das substâncias foi 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). O padrão de fragmentação do espectro de massas foi comparado com bibliotecas de espectro de massa do *National Institute*

of Standards and Technology (NIST). A abundância relativa das substâncias químicas foi obtida usando a cromatografia gasosa equipada com um detector de ionização de chamas (CG-DIC), sob as mesmas condições utilizadas anteriormente e com a temperatura do detector de ionização de chamas em 290 °C. As porcentagens dessas substâncias foram obtidas usando o método de normalização da área do pico do DIC.

4.4 Preparo e caracterização das nanoemulsões

A nanoemulsão da espécie foi preparada pelo método do baixo aporte energético com inversão de fases, com algumas modificações (OSTERTAG; WEISS; MCCLEMENTS, 2012). Onze formulações foram preparadas para conter 5% (p/p) de óleo essencial, 5% (p/p) de uma mistura de surfactantes (polissorbato 20 e monooleato de sorbitano 80) como fase oleosa, e 90% (p/p) de água destilada como fase aquosa. Inicialmente a fase oleosa foi homogeneizada por 30 minutos com um agitador magnético (600 rpm) à temperatura ambiente. Em seguida, a água destilada foi adicionada gota a gota sob a fase oleosa, em agitação constante, por mais 60 minutos. A composição das formulações está descrita na tabela 2.

Para a análise das formulações, o tamanho da gotícula (nm) e o índice de polidispersão (IP) foram determinados pela técnica de Espalhamento Dinâmico da Luz (EDL) em um Nanosizer (Malvern, UK). As formulações preparadas foram diluídas em água destilada (1:50) e após a análise dos parâmetros uma formulação foi selecionada com base nos critérios de tamanho de partícula < 200 nm e índice de polidispersão < 0,300. Todas as análises foram realizadas em triplicata e os valores foram representados em forma de média e desvio-padrão.

Tabela 2: Composição das formulações

Formulações	FA (%) (p/p)	OE (%) (p/p)	Polissorbato 20 (%) (p/p)	Monooleato de sorbitano 80 (%) (p/p)
F1	90,0	5,0	5,0	0,0
F2	90,0	5,0	4,5	0,5
F3	90,0	5,0	4,0	1,0
F4	90,0	5,0	3,5	1,5
F5	90,0	5,0	3,0	2,0
F6	90,0	5,0	2,5	2,5
F7	90,0	5,0	2,0	3,0
F8	90,0	5,0	1,5	3,5
F9	90,0	5,0	1,0	4,0
F10	90,0	5,0	0,5	4,5
F11	90,0	5,0	0,0	5,0

FA: fase aquosa; OE: óleo essencial.

4.5 Determinação do Equilíbrio Hidrófilo-Lipófilo (EHL) requerido

As formulações foram preparadas tendo uma faixa de EHL entre 4,3 (monooleato de sorbitano 80) e 16,7 (polissorbato 20). O valor EHLr referente a mistura dos surfactantes de cada formulação foi calculada pela equação (a)

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

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

Foram preparadas, a partir da formulação selecionada previamente, três formulações do óleo essencial e acondicionadas em frasco de vidro âmbar (5 mL) com batoque e tampa e armazenadas em locais com temperatura ambiente de 25 °C, em geladeira (4 °C) e em estufa (42 °C) por um período de 120 dias. Os aspectos macroscópicos das nanoemulsões avaliados foram: cor, separação de fase, cremagem, floculação e sedimentação. As análises de tamanho de partícula e índice de polidispersão foram realizadas conforme descrito no item 4.5.

4.7 Ensaio inseticida contra *Dysdercus peruvianus*

Esta etapa do trabalho foi desenvolvida em colaboração com o grupo de pesquisa liderado pela Dra. Denise Feder, do Laboratório de Biologia de Insetos (LABI), o qual vem colaborando conosco nessa linha de pesquisa. Os ensaios biológicos com os insetos foram feitos em conjunto com o doutorando Raul Vitor da Cruz Apolinário (LABI), do Departamento de Biologia Geral da Universidade Federal Fluminense (GBG/UFF).

As colônias de *D. peruvianus* foram mantidas a 26 ± 1 °C e umidade relativa do ar de 60% pelo Laboratório de Biologia de Insetos (LABI) da Universidade Federal Fluminense (UFF) como descrito por Fernandes e colaboradores (2013) e Gonzalez e colaboradores (2014). O uso de patrimônio de biodiversidade genética foi autorizado sob o número (A0E95C4) Sistema de Gerenciamento do Patrimônio Genético Brasileiro e de Conhecimento Tradicional Associado (SISGEN) do Ministério do Meio Ambiente Brasileiro.

Para avaliar a DL_{50} e os efeitos sub-letais, amostras do óleo essencial da *P. venosa* e de sua nanoemulsão (contendo 5% do óleo essencial) e do β-cariofileno e de sua nanoemulsão (contendo 5% do β-cariofileno) foram aplicados topicamente a ninfas de 4° instar de *D. peruvianus* escolhidos aleatoriamente. Inicialmente, 1 mL do óleo essencial foi pesado, correspondendo a 0,90 g. Desse valor, foi realizada uma diluição seriada em acetona pura (Merck, Darmstadt, FRG) do óleo essencial, resultando nas concentrações de 450; 220; 112,5; 56,25; 28,12 e 14,6 µg/mL. A nanoemulsão da *P. venosa* foi diluída em água destilada para as concentrações finais de 50; 25; 12,5; 6,25; 3,12 e 1,56 µg/mL, expressos em massa de óleo essencial. Feito isso, 1 µL de cada diluição foi aplicada topicamente na cutícula dorsal dos insetos do grupo testado (FERNANDES et al., 2013, TIETBOHL et al., 2014). O grupo de controle do solvente recebeu a aplicação tópica de 1 µL de acetona. O grupo de controle positivo recebeu a aplicação tópica de 1 µL de uma solução inseticida de Triflumuron (Hades, Dominus) diluída em 1 mL de água. O grupo controle da nanoemulsão recebeu a aplicação tópica de 1 µL da nanoemulsão branco, contendo apenas água e os surfactantes e produzida seguindo a mesma metodologia previamente descrita. O grupo de controle negativo não recebeu nenhum tratamento. A avaliação biológica dos tratamentos foi realizada durante todo o tempo necessário para o desenvolvimento das ninfas do 4° instar até chegarem ao estágio adulto (20 dias). Os parâmetros avaliados foram a letalidade (mortalidade dos insetos após 20 dias de tratamento) e efeitos subletais. Todos os experimentos foram realizados em triplicata com grupo de 30 insetos cada (n = 90). Os dados de mortalidade foram corrigidos com o uso da Fórmula de Abbott (Abbott, 1925).

4.8 Ensaio de seletividade com *Apis mellifera* e *Partamona helleri*

Esta etapa do trabalho foi desenvolvida em colaboração com o grupo de pesquisa liderado pela Dr. Eugênio Oliveira, do Laboratório de Fisiologia e Neurobiologia de Invertebrados (*BraIn & Phy Lab*), o qual vem colaborando conosco nessa linha de pesquisa. Os ensaios biológicos com os insetos foram feitos em conjunto com o Dr. Luis Viteri e o aluno de

iniciação científica Thiago Svacina, do Departamento de Entomologia da Universidade Federal Viçosa (DDE/UFV).

O ensaio seguiu a metodologia conforme descrito previamente por Britto e colaboradores (2021). As abelhas forrageiras *A. mellifera* e *P. helleri* foram expostas oralmente a quatro diferentes concentrações de óleo essencial de *P. venosa* (1,0 e 0,1 mg/mL) e de β-Cariofileno (0,5 e 0,05 mg/mL). Tanto o óleo essencial quando o β-cariofileno foram diluídos em solução de sacarose (50% [p/v]), Tween 20 (1,25% [v/v]) e DMSO (1,5% [v/v]) e ofertado às abelhas em micro-tubos Eppendorf de 2 mL, inseridos no fundo de recipientes de plástico de baixa densidade (capacidade de 250 mL). O grupo de controle branco continha somente a solução de sacarose, Tween e DMSO nas mesmas concentrações citadas acima. Ao grupo de controle negativo foi ofertada apenas a solução de sacarose. Cada recipiente de plástico foi como uma unidade experimental contendo 10 abelhas em 5 replicatas (n = 50). Foram utilizadas 5 colônias de abelhas diferentes para cobrir os efeitos de variação entre as colônias.

As abelhas tiveram jejum de 1 hora antes de terem acesso ao alimento, visando equalizar o consumo durante o experimento. Após 5 horas do início da exposição, foram oferecidas às abelhas a dieta descontaminada (solução de sacarose) e a mortalidade e o consumo da dieta foram analisados após 1, 3, 5 e 24 horas de exposição desde o início do experimento. As abelhas foram mantidas em incubadoras de demanda bioquímica de oxigênio (DBO) (32 °C para *A. mellifera* e 28 °C para *P. helleri*). As abelhas foram consideradas mortas se elas não conseguissem se mexer após serem gentilmente encostadas com um pincel de cerdas finas.

4.9 Análise dos dados estatísticos

Os resultados do ensaio inseticida e dos efeitos subletais foram analisados usando ANOVA e o teste de Tukey. As diferenças entre os grupos controle e tratado foram consideradas significantes quando $P < 0,05$ (ARMITAGE et al., 2002). O *software SAS system* 9.0 foi usado para estimar as curvas dose-resposta do ensaio inseticida utilizando a análise de Probit. Foi realizado uma ANOVA de medidas repetidas para analisar as diferenças significativas entre o consumo de alimentos dos insetos não-alvo.

5. ARTIGO

O artigo "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" foi publicado no periódico Industrial Crops and Products (Fator de impacto = 6,449 ; DOI: https://doi.org/10.1016/j.indcrop.2023.116348) em Janeiro de 2023.

Outros artigos publicados com dados obtidos através de projetos paralelos a este trabalho estão apresentados como anexos.

5.1 Artigo 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

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Abstract

Cotton is one of the most significant commodities in the whole world, so the protection of this kind of crop against pest insect infestations is essential. This work aims to evaluate the insecticidal potential of the essential oil of the leaves of *Persea venosa* Nees & Mart. and its nanoemulsion against the cotton stainer bug. Fresh leaves of *P. venosa* were submitted to hydrodistillation and analyzed by gas chromatography (GC) coupled to a mass spectrometer (MS). The plant species presented the substance β-caryophyllene, Caryophyllene oxide, α-humulene and α-copaene as the major compounds, comprising 43.78, 8.92, 8.27 and 5.90% of the essential oil, respectively. The nanoemulsion was obtained by low energy input, using the phase inversion method, generating a nanoemulsion with a particle size of 68.41 nm. The insecticidal assays were conducted to evaluate the lethality and sub-lethal damages at the cotton-stainer-bug. The essential oil and nanoemulsion presented LD_{50} of 24.45 μg/μL and 28.73 μg/μL respectively. Pure oil and the dilution at a concentration of 450 μg/μL caused 100% mortality of the insects on the first day. An assay was performed with β-caryophyllene, which also showed 100% mortality on the first day of treatment, indicating that the major substance could be responsible for insect mortality. The nanoemulsion showed 55% of mortality after 20 days. Delays in insect development were observed in all samples tested. The essential oil showed selectivity to *D. peruvianus* by not causing significant harm to *Apis mellifera* and *Partamona helleri.* This research shows promising feasibility results for the production of a nanoemulsified essential oil with selective insecticidal activity to control a pest of agricultural importance.

Keywords: Brazilian flora, crop protection, Lauraceae, botanical pesticide, nanotechnology.

1 Introduction

Dysdercus peruvianus is a phytophagous insect known as the cotton-stained bug (Rosado et al., 2019). The *Dysdercus* genera are constantly associated with the economic loss of cotton crops (*Gossypium* sp.) since they feed on flowers, seeds, and cotton bolls in development, leading to immature lint and secondary fungal infections (Rajendran et al., 2018; Rosado et al., 2019). The capacity of the adult insects to fly freely, and lay 300 to 450 eggs at once within five days of hatch interval, makes it imperative to seek new control strategies (Rajendran et al., 2018).

Conventional pesticides (insecticides, fungicides, and herbicides) are commonly used to manage crop pests. However, they can be toxic to humans and non-target organisms, such as beneficial pollinators. Usually, they contain high environmental persistence and induce the emergence of resistant insects (Tomé et al., 2017). Plant-based products are currently in the spotlight for the development of new eco-friendly alternatives to reduce, assist or mitigate agricultural necessities associated with nanostructured systems. They enable the plant-derived metabolites to become novel, effective solutions (Mustafa and Hussein, 2020; Pavoni et al., 2019). Bioactive oils, such as essential oils (EO), are a good source of sustainable insecticidal phytochemicals (Pavoni et al., 2019; Tortorici et al., 2022). However, essential oils are composed of low-weight lipophilic terpenoids (i.e., monoterpenes and sesquiterpenes) and phenylpropanoids. That physicochemical characteristic makes the use and exploration of the biorational potential of these compounds unfeasible (Barradas and de Holanda e Silva, 2021).

Nanoemulsion (NE) is a promising way to enable the use of essential oils as insecticides (Barradas and de Holanda e Silva, 2021; Sharma et al., 2020). The NEs are composed of two immiscible phases. The oil phase is dispersed in the aqueous phase in reduced size and stabilized by surfactants (McClements, 2012). In general, the criteria to classify a dispersion as a nanoemulsion are the characteristic blue color, droplet sizes between 20 and 200 nm, despite other authors suggesting a larger droplet size of 500 nm, and polydispersity index with values lower than 0.300 (McClements, 2012; Mustafa and Hussein, 2020; Sharma et al., 2020).

Persea venosa Nees & Mart. (Lauraceae) is a native and endemic plant species from Brazil, with southeastern and southern geographical occurrence, and can be found in the Rio de Janeiro State's Atlantic Forest in coastal areas (Mendes et al., 2013; Quinet A et al., 2015). It is popularly known in the southern region as "Pau-de-Andrade" or "Canela-sebo" and "Canela-

seda-branca" in the southeastern sandbanks (Mendes et al., 2013). The decoct of the barks is traditionally used in folk medicine as cicatrizing in wounds and ulcers (Mazza et al., 2000).

The insecticidal effects of essential oils and their nanoemulsion have already been described in literature (Pavoni et al., 2019). To ensure the safety of the insecticidal activity evaluations, assays on non-target organisms must be realized in order to avoid any potential undesired effect mediated by these biorational pest control tools (Haddi et al. 2020). Machado et al. (2023) showed the safety of *Ocotea indecora* nanoemulsified essential oil. Duarte et al. (2022) demonstrated the safety of *Annona acutiflora* and *Syderoxylum obtusifolium* essential oils. Therefore, this work contributes to the literature on the biological and biotechnological potential of the essential oil of *Persea venosa* and the latent need to manage the cotton crop pest *D. peruvianus*. The ecotoxicological assay of the essential oil were performed against two pollinator bees, *Apis mellifera* and *Partamona helleri*.

2 Materials and Methods

2.1 Plant material

Persea venosa aerial parts were collected at the Restinga de Jurubatiba National Park, RJ, Brazil, under SISBIO/ICMBio authorization number 13659-12 and SISGEN authorization number A0D648D for scientific activities. Botanist Marcelo Guerra Santos, a Professor at Universidade do Estado do Rio de Janeiro (UERJ), identified the species. A voucher specimen was deposited in the herbarium at UERJ's Faculdade de Formação de Professores (Teacher's Training College) under registration number M.G.Santos 2336.

2.2 Essential oil extraction and chemical characterization

The essential oil was obtained from fresh *P. venosa* leaves (2.971 kg) by the hydrodistillation method in a Clevenger-type apparatus for four hours. The leaves were crushed in a blender with distilled water and then transferred to a 5.00 L round bottom flask placed over a heating mantle. The Clevenger was placed over the flask, and a bubble condenser was placed over the Clevenger to start the hydrodistillation. The oil was collected after 4 hours, dried over anhydrous sodium sulfate, and stored at 4 ºC until further analysis. The essential oil's chemical characterization was performed by a SHIMADZU GCMS-QP500 (Shimadzu Corporation, Kyoto, Japan) gas chromatograph (GC) equipped with a mass spectrometer (MS) detector by electron impact ionization. The essential oil was also submitted to a GC-2014 (SHIMADZU)

gas chromatograph equipped with a flame ionization detector (GC-FID) for quantification analysis. Helium was used as the carrier gas, and the injection temperature was 260 ºC. The flow rate was 1 mL/min with splitless injection. The oven temperature was started at 60 ºC and was increased at a rate of 3 ºC/min to 290 ºC. One microliter of the sample was dissolved in CH_2Cl_2 (1:100 mg/mL) and injected into an RTX-5 column for MS (id = 0.25 mm, length = 30 m, thickness = 0.25 mm). The mass spectrometry was performed at 70 eV and a scan rate of 1 scan/s. The arithmetic index (AI) was calculated by interpolating the retention times of a standard mixture of aliphatic hydrocarbons (C7-C40) and analyzed under the same conditions. The chemical identification was performed by comparing the mass spectrum's obtained AI and fragmentation patterns with literature data (Adams, 2017). The fragmentation profiles of the substances were also compared with the mass spectrum database of the National Institute of Standards and Technology, Gaithersburg, MD, US (NIST). The relative abundance of the chemical constituents was obtained using flame ionization gas chromatography (GC-FID) under the same conditions as the GC-MS and a flame ionization detector (FID) temperature of 290 ºC. The percentages of these substances were obtained using the FID peak area normalization method.

2.3 Nanoemulsion preparation and characterization

The nanoemulsion was obtained by low energy input by the phase inversion method as described by Ostertag et al. (2012) with few modifications. Eleven formulations were prepared to contain 5% (w/w) of essential oil, 5% (w/w) of a surfactant's mixture (polysorbate 20 and sorbitan monooleate 80) as oil phase, and 90% (w/w) of distilled water as the aqueous phase. The surfactant mixture's relative hydrophilic-lipophilic balance (HLB) was calculated with a range between 4.3 to 16.7. Same methodology was used to prepare a β-caryophyllene nanoemulsion.

Initially, the oily phase (essential oil and the surfactants) was homogenized on a magnetic stirrer for 30 minutes at 600 rpm (rotations per minute) at room temperature (25 ºC). After that, the distilled water was slowly added, and the magnetic agitation continued for 60 min. The formulations were diluted with distilled water (1:50) and analyzed by dynamic light scattering (DLS) using a Nanosizer (Malvern, UK). The parameters evaluated were droplet size (nm) and polydispersity index (PdI).

2.3.1 Nanoemulsion stability

Three nanoemulsions were prepared and stored at three different locations with controlled temperatures: room temperature (25 ºC), refrigerator (4 ºC), and lab oven (42 °C). The parameters evaluated were particle size (nm), PdI, and visual parameters such as the Tyndall effect, turbidity, clarity, or creaming effect.

2.4 Insecticidal activity against *Dysdercus peruvianus*

The *D. peruvianus* colony was reared at 26 ± 1°C and 60% relative humidity at the Laboratory of Insect Biology (UFF) as described by Fernandes et al. (2013) and Gonzalez et al. (2014). Genetic biodiversity property was authorized under number (A0E95C4) at the Brazilian Genetic Heritage and Associated Traditional Knowledge Management System (SISGEN) of the Brazilian Ministry of Environment.

To evaluate the LD_{50}, and the sub-lethal effects, samples of *P. venosa* essential oil and its nanoemulsion (containing 5% of essential oil), standard grade β-Caryophyllene (Sigma-Aldrich) and its nanoemulsion (containing 5% of β-Caryophyllene) were topically applied on randomly chosen fourth instar *D peruvianus* nymphs.

The essential oil was previously weighed, and 1 mL corresponded to 0.90 g. From 0.90 g of the pure essential oil, a serial dilution in pure acetone (Merck, Darmstadt, FRG) produced final concentrations of 450, 225, 112.5, 56.25, 28.12, and 14.06 µg/µL. The *P. venosa* nanoemulsion was diluted with distilled water, and the concentrations tested were 50, 25, 12.5, 6.25, 3.12, and 1.56 µg/µL, expressed in essential oil weight. Subsequently, 1µL of each dilution was applied topically to the dorsal cuticle of the insects in the experimental groups (Fernandes et al., 2013, Tietbohl et al., 2014). The solvent control group received a topical application (1 µL) of acetone. The positive control group received a topical application of 1 µL of a solution of the insecticide triflumuron in 1 mL of water (Hades, Dominus). The nanoemulsion control group only received a topical application (1µL) of the blank nanoemulsion. The negative control group did not receive any treatment at all. The biological evaluation of the different treatments was performed during the entire time (20 days) required for development from the fourth instar nymphs to the adult stage. The recorded parameters were lethality (mortality during 20 days of treatment), and sub-lethal effects. All experiments were performed with groups of thirty insects, each in triplicate (n = 90). The mortality data was corrected using Abbott's formula (Abbott, 1925).

2.5 *Apis mellifera* and *Partamona helleri* selectivity assay

This bioassay followed the methodology previously described by Britto et al. (2021). Foragers of *A. mellifera* and *P. helleri* were orally exposed to four different concentrations 1.0; 0.1 mg/mL of the *P. venosa* essential oil and 0.5; 0.05 mg/mL of β-Caryophyllene. Both the essential oil and β-Caryophyllene were diluted in sucrose solution (50% [m/v]), Tween 20 (1.25% [v/v]), and DMSO (1.50% [v/v]) and offered to bees in 2 mL Eppendorf microtubes inserted into low-density plastic containers (250 mL capacity). Blank control had a sucrose solution containing 1.50% (v/v) DMSO and 1.25% (v/v) Tween 20 (negative control). The negative control had only the sucrose solution. Each plastic container was used as an experimental unit containing ten bees in five replicates (n=50), and five colonies were used to cover the effects of intercolonial variation.

The bees fasted for one hour before accessing the food, aiming to equalize consumption during the experiment. Five hours after the beginning of exposure, the bees were provided with an uncontaminated diet (sucrose solution), and mortality and diet consumption were recorded after one, three, five, and 24 hrs of exposure from the beginning of the bioassay. The bees were maintained in a biochemical oxygen demand (BOD) incubator (32 ºC for *A. mellifera* and 28 ºC for *P. helleri*). The bees were considered dead if they could not move after being gently prodded with a fine-hair brush.

2.6 Statistical data analysis

Insecticidal assay and sub-lethal effects results were analyzed using ANOVA and Tukey’s test. Differences between control and treated groups were considered significant when $P < 0.05$ (Armitage et al., 2002). SAS System 9.0 software was used to estimate the dose-response curves of the insecticidal assay by Probit analysis. A repeated measures ANOVA was realized to evaluate significant differences between non-target insects' food consumption.

3 Results

3.1 Essential oil extraction and characterization

The essential oil presented a 0.17% (w/w) of yield and bright yellow color. A total of 94.68% were identified, as shown in Table 1. The major compounds were β-Caryophyllene (43.78%), Caryophyllene oxide (8.92%), α-humulene (8.27%), and α-copaene (5.90%). Sesquiterpenes hydrocarbons were a major class identified, comprising 85.63% of the essential oil.

Table 1: Chemical characterization of the *Persea venosa*'s essential oil from leaves by GC-MS and GC-FID.

	RT (min)	AI EXP	AI LIT	SUBSTANCE	%
1	22.037	1362	1373	α-Ylangene	0.48
2	22.299	1368	1374	α-Copaene	5.90
3	22.915	1383	1389	β-Elemene	0.90
4	24.070	1411	1417	β-Caryophyllene	43.78
5	25.524	1447	1452	α-Humulene	8.27
6	25.701	1451	1458	allo-Aromadendrene	3.31
7	26.354	1467	1478	γ-Muurolene	4.17
8	26.577	1473	1481	Germacrene D	3.18
9	26.897	1481	1489	β-Selinene	1.31
10	27.004	1484.	1496	Viridiflorene	1.57
11	27.191	1488	1498	α-Selinene	1.70
12	27.333	1492	1500	α-Muurolene	1.86
13	27.886	1505	1513	γ-Cadinene	2.17
14	28.096	1511	1522	δ-Cadinene	2.50
15	28.240	1515	1528	cis-Calamene	1.67
16	28.824	1530	1537	α-Cadinene	1.01
17	28.987	1534	1545	Selina-3,7(11)-diene	0.73
18	29.603	1550	1559	Germacrene B	1.12
19	30.495	1573	1582	Caryophyllene oxide	8.92
20	32.858	1636	1638	epi-α-Cadinol	0.13
Total identified					94.68
Sesquiterpenes hydrocarbons					85.63
Oxygenated sesquiterpenoids					9.05

*RT: Retention time. AI exp: Experimental arithmetic index; AI lit: Literature arithmetic index.

3.2 Nanoemulsion characterization

Only five (F1-F5) of the eleven formulations prepared for the DLS analysis were kept stable. The other ones (F6-F11) showed phase separation and were not analyzed. All stable formulations presented reduced size particle and polydispersity index within the range that classify a nanoemulsion. Formulation 3 (F3) showed the smaller particle size among all other and, for that, this formulation was chosen to carry out the subsequent assays. The chosen nanoemulsion presented a particle size of 68.41 nm and a PdI of 0.266 (figure 1). It also presented a yellowish color due to the essential oil color, but when diluted, it showed a translucent bluish color, characterized by the Tyndall effect, and showed no creaming effect. Size distribution by intensity is observed in figure 2. It shows that size distribution (PdI) is homogenous at the chosen formulation (F3).

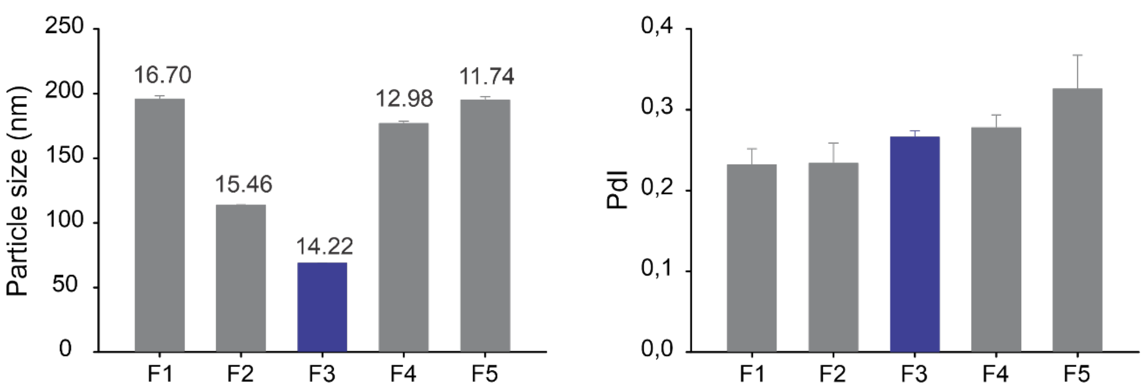


Figure 1: Particle size (nm) and PdI of the *Persea venosa* formulations. Above each particle size, the graph bar is the respective formulation's relative HLB value.

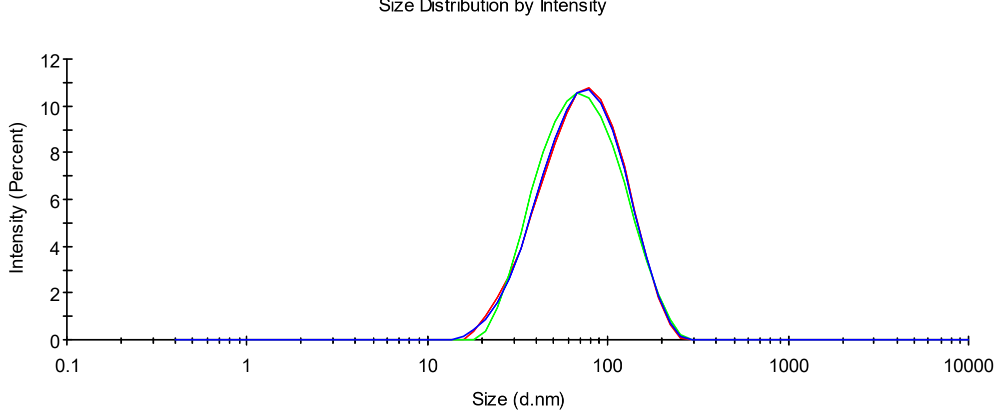


Figure 2: Size distribution by the intensity of the *Persea venosa* leaves essential oil F3 formulation.

3.2.1 Nanoemulsion stability assay

The stability assay was carried out using the chosen formulation (F3). This assay had 120 days, and the results are shown in figure 3. According to the data, the room temperature and refrigerator samples maintained their particle size and PdI within the established range of nanoemulsion until the end of the assay. The sample from the lab oven showed, at the end of the assay, an increase in the particle size and a decrease in PdI, indicating a destabilization in the nanoemulsion. In addition, samples from room temperature and refrigerator maintained their physical appearances, with a yellowish color and no phase separation or creaming effect. On the other hand, the lab oven sample did not maintain its initial physical characteristic at the

end of the essay. It presented a slightly darker yellow color and showed signs of the creaming effect.

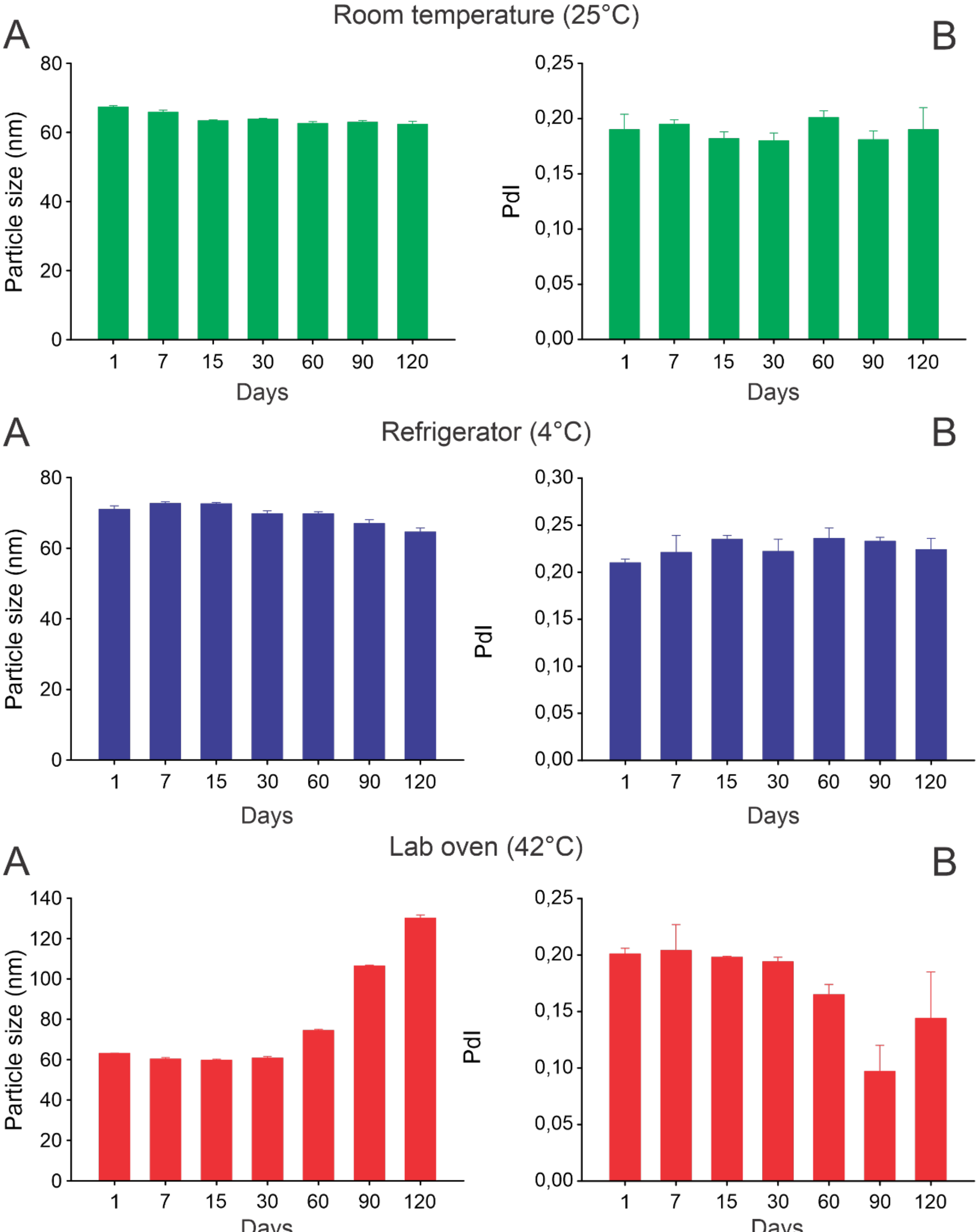


Figure 3: Particle sizes (A) and PdIs (B) of the nanoemulsion stability assay.

3.3 Insecticidal activity

The toxicity of the *P. venosa*'s essential oil and nanoemulsion are shown in figure 4. The essential oil presented an LD_{50} of 24.45 (18.87 – 30.13) µg/µL, and the nanoemulsion presented an LD_{50} of 28.73 (20.05 – 49.83) µg/µL. After the first day of treatment, the essential oil and pure β-Caryophyllene showed a 100% mortality rate. On the second day of treatment, the 450 µg/µL concentration also showed a 100% mortality rate. Positive control (triflumuron) only showed 100% mortality at the treatment's end.

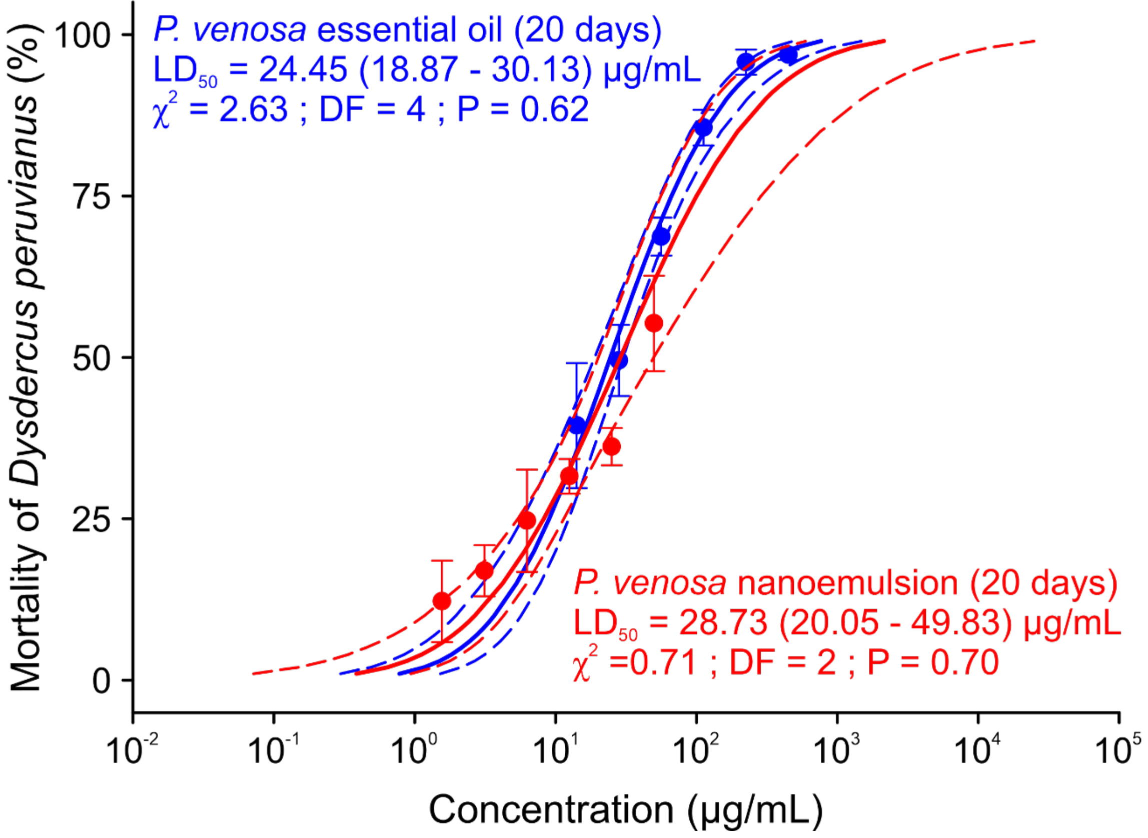


Figure 4: Toxicity of the *Persea venosa*'s essential oil (blue) and nanoemulsion (red) against *Dysdercus peruvianus*, based in dose-response curve. Solid lines denote the lethal dose (LD) values based in dose-mortality bioassays using probit analysis. The inferior and superior limit (dotted lines) represent 95% confidence intervals for the LD estimations. Symbols (± standard error) represent the average mortality recorded for each insecticide concentration testes.

As seen in figure 5, *P. venosa* nanoemulsion was more effective than β-caryophyllene nanoemulsion. On the last day of treatment, *P. venosa* and β-caryophyllene nanoemulsion presented 55.28 ± 7.39% and 16.18 ± 2.22% of mortality, respectively. Only *P. venosa* nanoemulsion presented a significant difference compared to all samples (df = 3; $F = 27.2$; $P < 0.001$), as shown in Table 2.

Table 2: One-way ANOVA between nanoemulsion samples.

Comparison	Diff of Means	p	q	P
P. venosa nano vs. Negative control	14.585	4	10.959	**<0.001***
P. venosa nano vs. Nanoemulsion control	14.551	4	10.993	**<0.001***
P. venosa nano vs. Beta-caryophyllene nano	11.730	4	8.814	**0.001***
Beta-caryophyllene nano vs. Negative control	2.855	4	2.145	0.472
Beta-caryophyllene nano vs. Nanoemulsion control	2.821	4	2.120	0.481
Nanoemulsion control vs. Negative control	0.0339	4	0.0255	1.000

*Significant at P < 0.05.

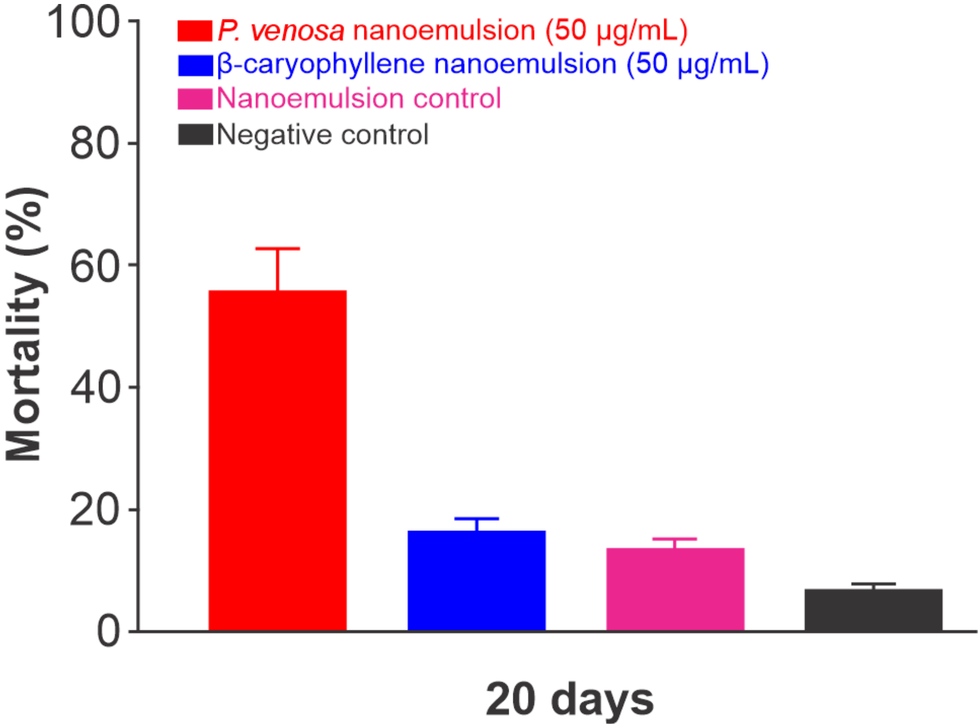
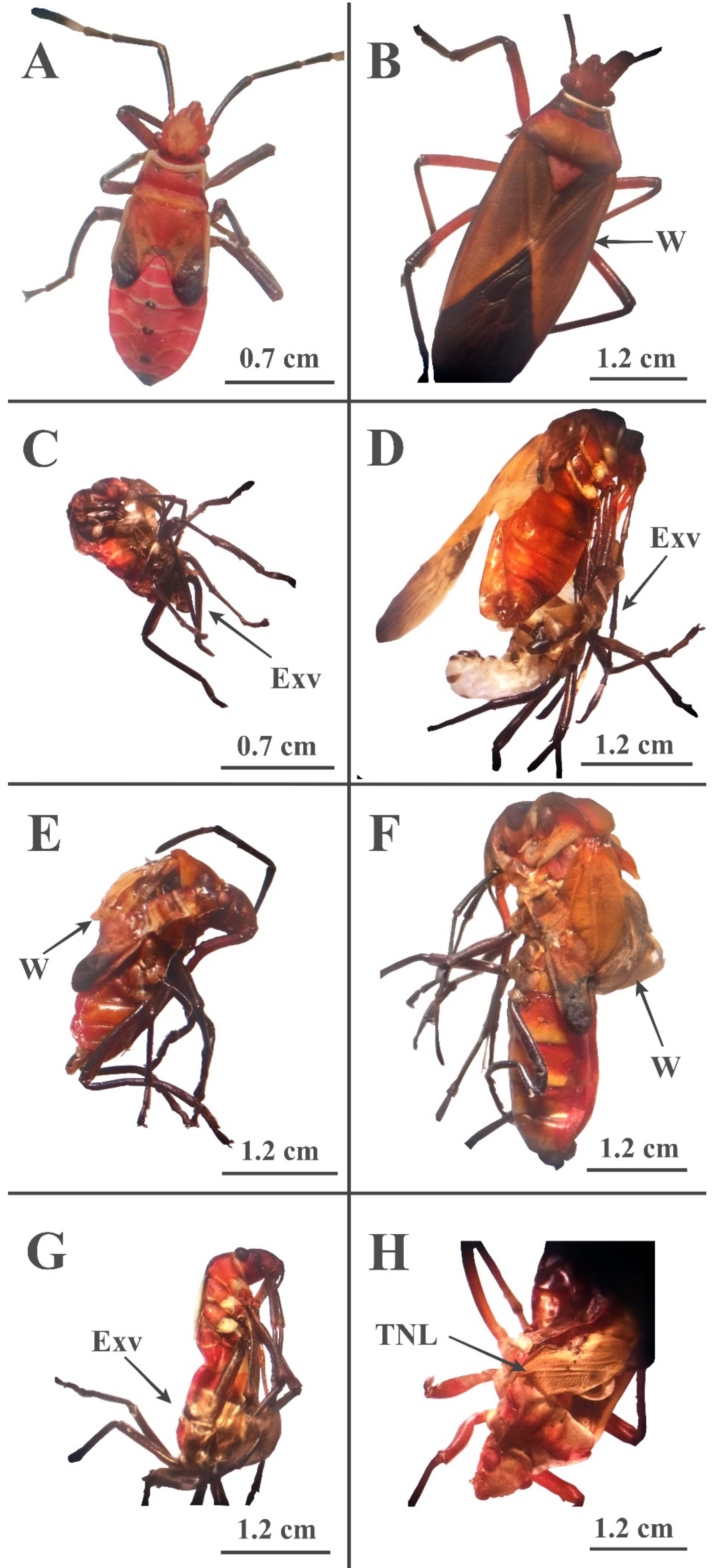


Figure 5: Mortality efficiency of *Persea venosa* and β-caryophyllene nanoemulsion against *Dysdercus peruvianus*.

Despite the high mortality rate, sub-lethal effects could be noticed in the surviving insects (Figure 6). Insects were identified with tanning problems (darkening of the new cuticle), some of them were trapped in the exuviae (Fig 6 C), and some remained at the 5th stage. At the highest concentrations, there were no surviving insects. Significant differences were observed at 56.25 and 28.12 µg/µL concentrations (df = 9, F = 12.748, P < 0.001). At the 225.0 and 112.5 µg/µL concentrations, there were 2.22% (P =0.972) of the 5th instar nymph trapped in the exuviae (Fig 6C). Both these concentrations showed a high percentage of mortality rate (98% and 89%, respectively). There was no interference at the oviposition of the remaining adults at the lower concentrations tested and nanoemulsions.


A
0.7 cm
B
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1.2 cm
C
Exv
0.7 cm
D
Exv
1.2 cm
E
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1.2 cm
F
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1.2 cm
G
Exv
1.2 cm
H
TNL
1.2 cm

Figure 6: Stereoscopic micrography (x15) of *Dydercus peruvianus* insects submitted to treatments with the *Persea venosa*'s essential oil and nanoemulsion. A – fifth-instar nymph from the negative control group; B – adult from the negative control group; C - fifth-instar nymph trapped in exuviae, from the treatment with 225 µg/µL of essential oil; D – adult trapped in exuviae, from the treatment with 112.5 µg/µL of essential oil; E – adult containing deformed wings, from the treatment with 56.25 µg/µL of essential oil; F – adult with deformed wings, from the treatment with 28.12 µg/µL of essential oil; G – adult trapped in exuviae, from the treatment with 50 µg/µL of nanoemulsion containing essential oil; H - adult presenting problems in the tanning process, from the treatment with 50 µg/µL of nanoemulsion containing essential oil. W (wings), Exv (exuviae) and TNL (tanning lacking).

3.4 *Apis mellifera* and *Partamona helleri* selectivity assay

For *A. mellifera*, at first, the bees fasted more than the control group, but at the third hour of the assay, the food consumption of both the treated and the control groups decreased at the same levels, and when the food changed to a clean diet, levels of consumption increased to the same levels of the beginning of the assay (figure 7).

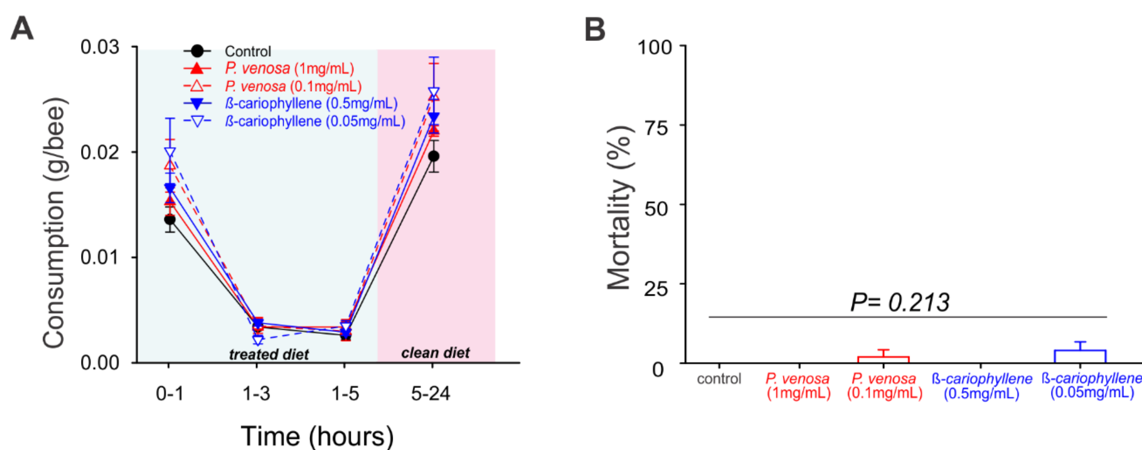

Figure 7: *Apis mellifera* consumption (A) of *Persea venosa* essential oil and β-caryophyllene, and mortality rate (B).

Differently for *P. helleri*, at first, consumption of the food impregnated with the *P. venosa*'s essential oil and β-Caryophyllene was lower than the blank control group. Later, the food changed to the uncontaminated diet, and the bees of the treated group fed more than the control group and even more than at the beginning of the assay (figure 8).

Partamona helleri

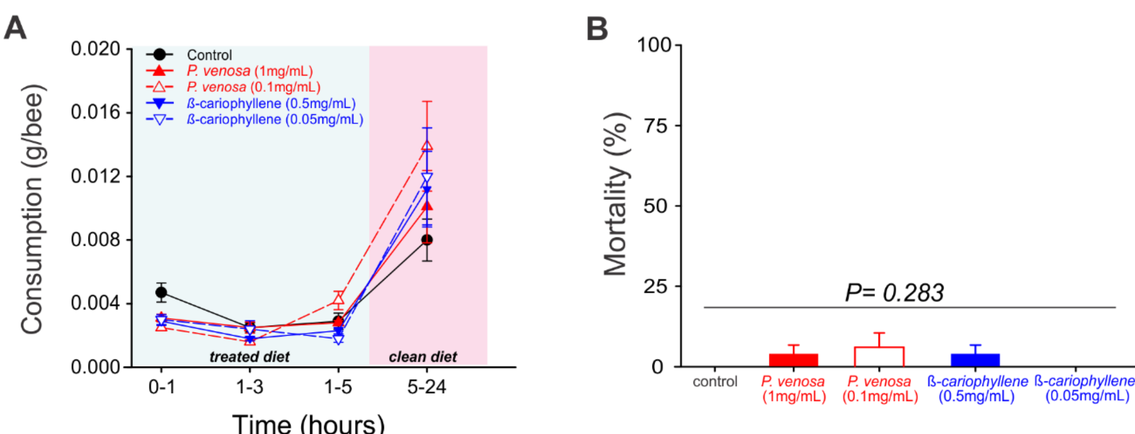

Figure 8: *Partamona helleri* consumption (A) of the *Persea venosa*'s essential oil and β-caryophyllene, and mortality rate (B).

There was no significant difference in the mortality rate between the treated and the control groups, showing that the tested samples were selective to both *A. mellifera* and *P. helleri* bees. There were differences in the amount of food consumed, the contaminated food consumed, and the concentration of the contaminated food consumed between the species in all evaluated hours of exposure, as shown in Table 3. Also, *A. mellifera* had a higher food consumption than *P. helleri*.

Table 3: Repeated measures analysis of variance (ANOVA) between subjects for the food consumption of *Apis mellifera* and *Partamona helleri*.

Variation source	df	F	*P*
between subjects			
Sample (Sa)	2	1.58	0.2161
Concentration (C)	1	0.67	0.4183
Specie (S)	1	183.44	**<0.0001***
Sa vs C	2	0.50	0.6069
Sa vs S	2	0.77	0.4702
C vs S	1	0.03	0.8553
Sa vs C vs S	2	0.01	0.9915
Error	48	-	-

Within-subject effects	df_{den}	df_{num}	Wilks' lambda	F	*P*
Time (T)	46	3	0.079	178.55	**<0.0001***
T vs Sa	92	6	0.844	1.35	0.2417
T vs C	46	3	0.928	1.18	0.3263
T vs S	46	3	0.176	71.66	**<0.0001***
T vs Sa vs C	92	6	0.937	0.51	0.7993
T vs Sa vs S	92	6	0.675	3.33	**0.0052***
T vs C vs S	46	3	0.912	1.47	0.2345
T vs Sa vs C vs S	92	6	0.756	2.30	**0.0413***

*Significant at P < 0.05

4 Discussion

Although few authors have published papers containing information about *Persea venosa* (Linsingen et al., 2006, Santos et al., 2012), recently, Silva et al. (2019) published the composition of the *P. venosa* essential oil from the leaves. The individuals were found in a mountain region in Minas Gerais state in Brazil. So, to our knowledge, this is the first study of the insecticidal activity evaluation of the *P. venosa*'s essential oil and its nanoemulsion.

The yield of the *P. venosa*'s essential oil from leaves shown in this work was 0.17% (w/w) of raw weight. Silva et al. (2019) obtained a 0.28% of yield on a dry-weight basis. Both studies are from plants from Brazil but in different regions. Despite a small similarity in minor compounds, major substances were different, showing Spathulenol as a major component in the previous study. They also showed that the major chemical class was the oxygenated sesquiterpenes, while in this work, the major chemical class was the sesquiterpenes hydrocarbons. This suggests that extrinsic factors, such as climate, in the different regions can influence the biosynthesis pathways of the secondary metabolite (Morais, 2009, Li et al., 2020). The major substances found in this research were β-Caryophyllene, Caryophyllene oxide, α-Humulene, and α-Copaene. Scora and Scora (2001) found β-caryophyllene as a major compound in three of the eight *Persea* species studied. Nasri et al. (2022) showed that, of seven varieties of *Persea americana*, two contained β-Caryophyllene as the major substance. Also, Padalia et al. (2009) presented a chemical characterization of the essential oil of *Persea duthiei* leaves, fruit, and flower, showing the presence of β-Caryophyllene in all of them. These authors also showed the presence of Caryophyllene oxide, α-Humulene, and α-Copaene in several analyzed species.

The nanoemulsions were prepared to enhance the bioavailability of the essential oil, since oils are not soluble in water. The low energy input by the phase inversion method was selected because it requires simple equipment and does not use organic solvents or heat treatment that could affect the amount of essential oil inside the obtained nanoemulsion, which maintains the physicochemical properties of the product, and still is considered eco-friendly (Ostertag et al. 2012, Folly, et al. 2021). The choice to use two surfactants was to ensure the formation and stabilization of the nanoemulsion (McClements, 2012). Therefore, the stability assay helped to observe this effect, showing that two of the treatment proved to be more stable over the months than when heat was applied. When the nanoemulsion is submitted to a heat treatment, the effect of Ostwald ripening is favored, so the smaller particles are dissolved and

deposited on the larger particles (Tadros et al., 2004, McClements, 2012, Pavoni et al., 2019). All formulations prepared using the same conditions as F3 resulted in light yellow nanoemulsions with a translucent bluish appearance due to the Tyndall effect, similar characteristics as described by Fernandes et al. (2014).

Insecticidal activity assays of the essential oil and its nanoemulsion were performed and presented an LD_{50} of 24.45 µg/µL and 28.73 µg/µL, respectively. This is the first report of the insecticidal activity of the *P. venosa*'s essential oil and its nanoemulsion. Nascimento et al. (2020) tested the essential oil of *Ocotea elegans* against *D. peruvianus*. This is a plant species from the same family as *P. venosa*. They showed an LD_{50} of 94.91 µg/µL on the last day of treatment. Apolinário et al. (2020) showed the activity of *Pilocarpus spicatus* against the same insect tested in this study. That plant species presented an LD_{50} of 90 µg/µL. Both *O. elegans* and *P. spicatus* were collected from the same region of *P. venosa* but are from different genera and show different chemical compositions. *P. venosa* was the most active when comparing its insecticidal activity against *D. peruvianus*. Fernandes et al. (2014) found that the nanoemulsion of the *Manilkara subsericea* extract presented a lethality of 66% in 30 days of treatment against *D. peruvianus*. The *P. venosa*'s nanoemulsion presented a mortality rate of 55.28% in 20 days of the assay against the same insect. This study also shows the insecticidal activity of β-caryophyllene isolated, which corroborates with Ma et al. (2020), that showed the insecticidal activity of the *Cephalotoxus sinensis*'s essential oil major substances, β-Caryophyllene and Caryophyllene oxide being amongst them. Kim and Lee (2014) showed that α-humulene had relevant insecticidal activity against *Sitophilus zeamais* and *Tribolium castaneum*. Both nanoemulsions presented activity against the insect, but the nanoemulsion of the β-caryophyllene showed lower insecticidal activity when compared to the *P. venosa*'s essential oil nanoemulsion and did not present significant difference when compared to the samples of the control groups. This effect can be explained by the synergistic effect that a variety of essential oil substances present (Lu et al. 2019). These results showed the potential of using the nanoemulsified essential oil over the essential oil itself as an insecticidal agent, since they presented similar LD_{50}. The nanoemulsified essential oil is a bioproduct ready to be applied in the field without using organic solvents.

One of the advantages of using plant-based insecticides is that not only the lethality has an interesting outcome, but also sub-lethal effects can be important ways to stop a crop pest from acting on a plantation (Abdelgaleil et al., 2022). Apolinário et al. (2020) demonstrated, using electron micrography, the sub-lethal damages that *P. spicatus* caused in *D. peruvianus* at

low concentrations of the essential oil. Fernandes et al. (2014) observed changes in molt and metamorphosis processes in insects treated with the nanoemulsion. This study also showed delays in the development of the insects with all the samples tested. Lower concentrations of the essential oil and nanoemulsion were capable of cause enough harm to *D. peruvianus* after the end of the assay. Sub-lethal effects can be caused by the action of the mono and sesquiterpenes in the essential oil. They can interfere with the hormone regulation of insects during its development or disrupt the synthesis of chitin (Apolinário et al., 2020, Abdelgaleil et al., 2022).

The data present in this work corroborates with Pereira et al. (2020), which showed that β-caryophyllene was more selective to *A. mellifera* and *P. helleri* bees than other extracts and the Imidacloprid insecticide. This study showed that both the essential oil and β-caryophyllene were selective to the bees, with a high survival rate, compared to the insecticidal activity against *D. peruvianus*. The essential oil dose applied at the assay was approximately ten times higher than the value of LD_{85} (113.5 μg/μL), and the β-Caryophyllene dose applied was half of the essential oil dose, to simulate the amount of β-Caryophyllene at the essential oil. Pereira also showed that Imidacloprid, a neonicotinoid insecticide, had 100% lethality, and so did other researchers (Goulson, 2013, Laycock et al., 2014, Tomé et al., 2015). This work also corroborates with Britto et al. (2021), showing that the diet consumption of *A. mellifera* was higher than *P. helleri*, despite the feeding reduction observed in both bees. All these results corroborate the data present in the literature and assure the need of novel plant-based insecticides to protect crops and non-target animals. In addition, our group published a recent study (Machado, 2023) showing that a nanoemulsion, prepared with the same methodology as this work did not cause mortality in *A. mellifera*.

5 Conclusion

This study presented the essential oil's chemical composition, the bioproduct preparation as a nanoemulsion, and the stability of the nanoemulsion. Then, this work also showed the lethal and sublethal effects of the essential oil and nanoemulsion on the cotton pest *D. peruvianus*. It could be demonstrated that the nanoemulsion of *P. venosa* had similar effectivity as the essential oil but used a considerably lower amount of essential oil. Furthermore, it presented an assay on non-target insects, stating that the essential oil and the nanoemulsion are lethal to the crop pest and caused no significant harm to two pollinator bees, *Apis mellifera* and *Partamona helleri*. These results confirm the safety of the insecticidal

potential of a *P. venosa*'s bioproduct against *D. peruvianus*. Since this is a Lauraceae species with little data available in the literature, these findings could help bring more investment in new studies on the protection of the flora. Furthermore, it shows that new plant-based products can be used to control agricultural pests.

Authors' contribution

Ricardo dos Santos Esteves: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Raul Apolinário:** Methodology, Investigation, Formal analysis. **Francisco Paiva Machado:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - review. **Diogo Folly:** Conceptualization, Methodology, Investigation, Formal analysis. **Valéria Costa Rocha Viana:** Conceptualization, Methodology, Investigation, Formal analysis **Ana Paula Soares:** Methodology, Investigation. **Luis Oswaldo Viteri Jumbo:** Methodology, Investigation, Formal analysis. **Thiago Svacina:** Methodology, Investigation, Formal analysis. **Marcelo Guerra Santos:** Methodology, Botanic investigation. **Eduardo Ricci-Junior:** Methodology, Investigation, Resources. **Eugênio E. Oliveira:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review. **Denise Feder:** Conceptualization, Formal analysis, Resources. **Leandro Rocha:** Conceptualization, Methodology, Investigation, Formal analysis, Resources, Writing – review, and editing.

Conflict of interest

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

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6. CONSIDERAÇÕES FINAIS

Nesse trabalho, o óleo essencial de *Persea venosa* teve um rendimento de 0,17% e apresentou as substâncias majoritárias β-cariofileno (43,78%), o óxido de cariofileno (8,92%) e o α-humuleno (8,27%). Silva e colaboradores (2019) avaliaram o óleo essencial dessa mesma espécie, porém coletada em região de Cerrado e obtiveram 0,28% de rendimento e as substâncias majoritárias foram o espatulenol, o epóxido de humuleno e o óxido de cariofileno. Isso mostra diferença na produção de metabólitos que a mesma espécie pode ter quando habita duas regiões diferentes, sugerindo que o clima nessas regiões pode influenciar a rota biosintética dos metabólitos secundários.

Foi possível produzir uma nanoemulsão do óleo essencial da *Persea venosa* com 68,41 nm de diâmetro e índice de polidispersão de 0,266 através do método de baixo aporte energético por inversão de fases. É um método que usa equipamentos simples e não utiliza solventes orgânicos ou calor, que poderia afetar a quantidade de óleo essencial dentro da nano emulsão obtida e o método mantém as características físico-químicas do produto e ainda é considerado biologicamente amigável. Foi possível também a avaliação da estabilidade da nanoemulsão e ela se mostrou estável por 120 dias, tanto a 4 °C quanto a 25 °C.

O óleo essencial e a nanoemulsão apresentaram atividade inseticida contra *Dysdercus peruvianus*. Os valores de DL_{50} foram de 24,45 e 28,73 µg/µL para óleo essencial e nano emulsão, respectivamente. São valores muito próximos que mostram a viabilidade do óleo essencial na forma de nano emulsão. Foi possível demonstrar também que a nanoemulsão do óleo essencial teve maior atividade inseticida do que a nanoemulsão da substância majoritária β-cariofileno. Uma realidade é o efeito sinérgico que alguns óleos essenciais apresentam e isso pôde ser evidenciado quando a atividade do óleo essencial nanoemulsionado é 3x maior do que a substância majoritária nanoemulsionada. Além disso foi possível observar diversos danos sub letais nos insetos sobreviventes, que impedem que os insetos se alimentem do algodão e causem mais danos na plantação. Foi feito também um estudo de seletividade com as abelhas *Apis mellifera* e *Partamona helleri* para avaliar se o óleo essencial tem o poder de causar mortalidade em insetos não-alvo. O óleo essencial não causou mortalidade significativa em ambas as abelhas a ponto de classificar o óleo essencial como tóxico. Houve uma diferença no consumo da dieta entre as abelhas, que corrobora com o que foi encontrado em estudos prévios.

Esse trabalho mostrou o potencial biotecnológico encontrado no Parque Nacional da Restinga de Jurubatiba, através da viabilização da produção de um óleo essencial nanoemulsionado com atividade inseticida e sem causar maiores danos em insetos não alvo de uma espécie vegetal nativa e endêmica do Brasil, mostrando o potencial encontrado no Parque Nacional da Restinga de Jurubatiba. Os resultados aqui encontrados mostram que há a possibilidade de produzir uma alternativa aos inseticidas sintéticos, que vem aumentando em números de produção, registros e uso nas plantações. Apesar disso, ainda há necessidade de serem realizados estudos sobre toxidez do óleo essencial em linhagens celulares humanas e em modelos de animais para atestar a viabilidade desse óleo essencial e de outros estudados para que esses produtos possam substituir os inseticidas sintéticos.

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8. ANEXOS

8.1 Artigo 1

Characterization and inhibitory effects of essential oil and nanoemulsion from *Ocotea indecora* (Shott) Mez in *Aspergillus* species

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Article

Characterization and Inhibitory Effects of Essential Oil and Nanoemulsion from *Ocotea indecora* (Shott) Mez in *Aspergillus* Species

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Abstract: The *Aspergillus* genus, the etiological agent of aspergillosis, is an important food contaminant and mycotoxin producer. Plant extracts and essential oils are a source of bioactive substances with antimicrobial potential that can be used instead of synthetic food preservatives. Species from the Lauraceae family and the *Ocotea* genus have been used as traditional medicinal herbs. Their essential oils can be nanoemulsified to enhance their stability and bioavailability and increase their use. Therefore, this study sought to prepare and characterize both nanoemulsion and essential oil from the *Ocotea indecora*'s leaves, a native and endemic species from the Mata Atlântica forest in Brazil, and evaluate the activity against *Aspergillus flavus* RC 2054, *Aspergillus parasiticus* NRRL 2999, and *Aspergillus westerdjikiae* NRRL 3174. The products were added to Sabouraud Dextrose Agar at concentrations of 256, 512, 1024, 2048, and 4096 µg/mL. The strains were inoculated and incubated for up to 96 h with two daily measurements. The results did not show fungicidal activity under these conditions. A fungistatic effect, however, was observed. The nanoemulsion decreased the fungistatic concentration of the essential oil more than ten times, mainly in *A. westerdjikiae*. There were no significant changes in aflatoxin production.

Keywords: Flavi section; fungistatic; aflatoxin; natural products; sesquirosefuran; sesquiterpenes

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1. Introduction

One of the most critical problems reported in human history is hunger. Even with all the breakthroughs and innovations since the implementation of agriculture, it was not enough to free us from this malady. In 2022, almost 830 million people, about 10% of the global population, suffered from hunger [1].

The Food and Agriculture Organization (FAO) estimates that up to 25% of the world's cereal grains are contaminated by fungi and/or mycotoxins in the field or during storage [2,3], leading to a mass wastage of food.

Members of the genus *Aspergillus* spp. have the strongest ecological link to the human food supply [4]. The genus presents a worldwide distribution, with 339 species [5], divided

into sections and clades. The clade *A. flavus*, in section Flavi, is important for containing the most common agents of superficial and invasive aspergillosis [6].

The species of the *A. flavus* clade are still linked to the production of mycotoxins, secondary metabolites with harmful toxic effects on animals and humans [7]. The main mycotoxins produced by fungi of the genus *Aspergillus* spp. are aflatoxins (AF) and difuranocoumarins, with rigid and flat structures that form the four major substances: B1, B2, G1, and G2 [8]. This chemical stability gives them high resistance to heat treatments, extreme pH values, high pressures, and food-grade chemical treatments [9], making them also detectable at various levels of the production chain [7].

The ingestion of AFB1 can lead to acute conditions associated with cellular damage and metabolism disruption. Chronic conditions can cause damage to the metabolization organs, especially those related to nephrotoxicity and hepatotoxicity, including oncogenesis [10,11].

Such hazardous substance, as expected, has a strict limit for concentrations in each medium, particularly for food supplies. The limits are determined by legislation worldwide and vary for each country. The Brazilian legislation, aligned with the MERCOSUL designations, established similar limits to the most accepted international recommendations, the ones in the *Codex Alimentarius* [12]. The limits range from 5 to 20 μg/kg, except for those outlined for children, with a limit of 1 μg/kg [13].

Inhibiting the growth and mycotoxin production of the food-related strains, therefore, shows itself as an essential strategy to combat worldwide hunger and is a matter of public health. These issues interconnect with the United Nations 17 Sustainable Development Goals for 2030, principally goals 2 and 3, respectively, "Zero Hunger" and "Good Health and Well-Being" [14].

Fungal and aflatoxin contamination can occur before, during, or after harvest, especially during storage and processing. Methods for preventing contamination can be divided into pre-harvest, harvest, and post-harvest strategies [15].

Pre-harvest factors include seed and cultivation conditions and the prevention of fungal infestations, considering environmental factors that influence infection [16]. Most of the threats, however, are post-harvest factors. These range from harvesting patterns to transport to the consumer, with a particular focus on storage [15]. About 25% of harvested fruits and vegetables are lost due to diseases, mainly caused by fungi. Although more severe in developing countries, it is not insignificant in developed countries, as the annual economic loss of the United States is around US$ 1 billion [16].

The usual method to prevent fungal contaminations in pre- and post-harvest is using chemical additives. The environmental and human health impact and the possible development of new resistant strains have forced the industry to seek new strategies as "clean label" alternatives [17].

Products extracted from plants and their formulations are classified mainly by the Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS) [18]. These products can be used in Europe and the USA as antimicrobial agents in pre- and post-harvest and food and feed additives, with a wide acceptance by consumers [19].

Among the plant-based products that can be obtained, essential oils (EO) are an option with proven methodologies and recognized potential, aiming to isolate the volatile chemicals found in low concentrations in plants. EOs stand out among plant derivatives because they often present biological activity [20]. In addition, they can be used in formulations such as nanoemulsions (NE), that may enhance their properties.

Nanoemulsions, like traditional emulsions, are dispersions of one immiscible liquid in another. Both are kinetically stable, but thermodynamically unstable systems. To reduce the natural tendency of phase separation by creaming, flocculation, coalescence, or Ostwald ripening, surfactants are ordinarily used to decrease the interfacial tension and improve the long-term stability of the nanodispersions [21].

The principal difference between nanoemulsion and traditional emulsion is that nanoemulsion droplets are on a nanometer scale, between 20 and 200 nm. Another difference

is the translucid monophasic appearance, often with the bluish color of the Tyndall optical effect of light dispersion [22].

The conventional applicability of nanoemulsions is to enable oily phases, such as essential oils, in aqueous matrices. Recently, nanoemulsified essential oils have been spotlighted for developing new antimicrobial products [23]. They are a promising tool for antimicrobial delivery, and among the advantages are bioavailability enhancement, increased stability, a larger surface area, and improved bioactivity, allowing more effective interactions with microorganisms [24,25].

Ocotea indecora (Lauraceae) is a native endemic plant from Brazil. Species from this family and genus have been used as traditional medicinal herbs. Regarding *Ocotea* genus, they present several groups of secondary metabolites recognized in the literature, especially steroids, terpenoids, and alkaloids [26]. This plant species is found in the remaining Mata Atlântica forest in Brazil's south and southeast regions, especially in the sandbank areas of Rio de Janeiro state [27]. However, the coastal ecosystem where most of the current population resides is the highest deforested biome in the country, having less than 7% of its original area [28]. The devastation associated with the endemic nature of the species highlights the urgency and importance of its study.

The main metabolite related to the *Ocotea indecora*'s essential oil from leaves is the furanosesquiterpene sesquirosefuran. Even though most of the reports are about insecticidal effects [29–31], furanosesquiterpenes have several activities recognized in the literature as antioxidant, herbicidal, antibacterial, chemo-preventive agents, and principally for this study, antifungal [32,33]. Thus, essential oils and their possible effects are important research proposals for the biotechnological development of sustainable products.

The objectives of this work were to evaluate the effectiveness of the *O. indecora* leaves' essential oil and nanoemulsion on the growth of *A. flavus* (RC 2054), *A. parasiticus* (NRRL 2999), and *A. westerdjikiae* (NRRL 3174), and the inhibition of aflatoxins.

2. Results

2.1. Essential Oil Extraction and Chemical Characterization

The EO yielded 0.4% and allowed the identification of three substances, totaling 91.93% of the oil (Table 1). The sesquirosefuran (86.13%) was widely predominant, followed by the sesquiterpenes hydrocarbons: (Z)-β-farnesene (3.33%) and allo-aromadendrene (1.55%).

Table 1. Chemical characterization of the EO by GC-MS and GC-FID.

	RT	AI_{exp}	AI	Substances	%
1	26.970	1448	1440	(Z)-β-Farnesene [a]	3.33965
2	27.323	1457	1458	allo-Aromadendrene [a]	1.55898
3	30.802	1545	1549	Sesquirosefuran [b]	86.13331
		Total identified			91.03194
		Sesquiterpene hydrocarbons			4.89863
		Sesquiterpene oxygenated			86.13331

RT: retention index; AI: arithmetic index; AI_{exp} arithmetic index calculated. [a] Identified from Adams [34]. [b] Identified from Pherobase (El- Sayed) [35].

2.2. Nanoemulsion Preparation, Characterization, and Thermal Stress Stability

The NE presented a white-bluish coloration (Figure 1), and the dynamic light scattering (DLS) analysis showed a mean droplet size (nm) of 103.4 ± 0.9. The polydispersity index (PdI) was 0.268 ± 0.010, and the zeta potential was −32.83 ± 0.8208 after preparation at room temperature (25 °C). The thermal stress stability of the nanoemulsion was realized initially at room temperature (25 °C) and increased to 65 °C at 10 °C/analysis. The size distribution by intensity represented in Figure 2 shows no relevant alterations in the nanoemulsion's stability, while the droplet size and PdI values at each temperature can be seen in Table 2, showing statistical differences above 35 °C. After the thermal stress, no

macroscopic changes were observed and the droplet size was 89.38 ± 2.153, the PdI was 0.246 ± 0.003, and the zeta potential was −34.85 ± 0.5284.

Figure 1. Nanoemulsion: bluish-white color characteristic of the Tyndall effect with 2% of NE.

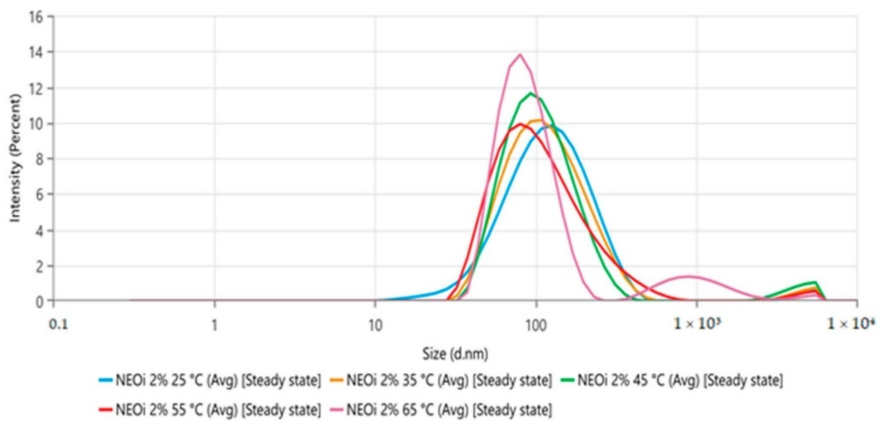

Figure 2. NE thermal stress (25–65 °C) size distribution by intensity.

Table 2. NE mean size and PdI values from the thermal stress stability study (25–65 °C).

Temperature (°C)	Average Size (nm)	Polydispersity Index
25	103.40 ± 0.90	0.239 ± 0.010
35	102.90 ± 0.519	0.247 ± 0.015
45	98.97 ± 0.312 *	0.241 ± 0.018
55	91.58 ± 0.515 *	0.252 ± 0.009
65	89.38 ± 2.15 *	0.246 ± 0.003

* The values were recognized as statistically significant at a confidence interval of 95%.

2.3. *Inhibitory Effects on Aspergillus Strains*

2.3.1. *Aspergillus flavus*

The effects of the EO at 4096 µg/mL and the NE at 256, 512, 1024, and 2048 µg/mL on the growth of the *A. flavus* strain are presented in Table 3.

Table 3. Diameter (mm) of *A. flavus* colonies in the presence of EO and NE at 48, 72, and 96 h.

Bioproduct	48 h	72 h	96 h
Control	20.90 ± 1.838	32.75 ± 3.942	43.35 ± 3.470
EO 4096 μg/mL	17.20 ± 3.960	32.90 ± 0.990	42.30 ± 2.121
NE 256 μg/mL	20.00 ± 1.212	31.90 ± 2.402	43.30 ± 1.900
NE 512 μg/mL	18.50 ± 1.769	30.60 ± 1.200	42.30 ± 1.513
NE 1024 μg/mL	16.50 ± 0.854	28.50 ± 1.114	40.00 ± 1.682
NE 2048 μg/mL	15.00 ± 2.287 *	28.50 ± 2.921	37.80 ± 3.651

* The values were recognized as statistically significant at a confidence interval of 95%.

The colonies developed in the presence of the EO and the NE, demonstrating no fungicidal activity at the tested concentrations. The EO also showed no significant reduction ($p > 0.05$) compared to the control groups, evidencing no fungistatic activity.

As seen in Table 3, there was only a significant reduction up to 48 h (df = 5; F = 4.11; $p = 0.021$), but only for the NE in the concentration of 2048 μg/mL ($p = 0.020$). The statistical analysis for the 48 h incubation is shown in Table 4. No statistical significance was observed for the 72 h ($p = 0.210$) and 96 h ($p = 0.135$) incubations.

Table 4. One-way ANOVA between *A. flavus* colonies with or without the EO and the NE.

Comparison	Diff of Means	*p*	q	*p*
Control 48 h vs. EO	3.700	6	3.055	0.322
Control 48 h vs. 2048 ppm	5.900	6	5.524	0.020 *
Control 48 h vs. 1024 ppm	4.400	6	4.119	0.104
Control 48 h vs. 512 ppm	2.400	6	2.247	0.620
Control 48 h vs. 256 ppm	0.900	6	0.843	0.989

* The values were recognized as statistically significant at a confidence interval of 95%.

The percentages of reductions are shown in Figure 3, demonstrating the intensity and duration of the effects correlated to the tested concentrations. The highlight is the only significant inhibition that reached a 28.2% reduction in the colony diameter.

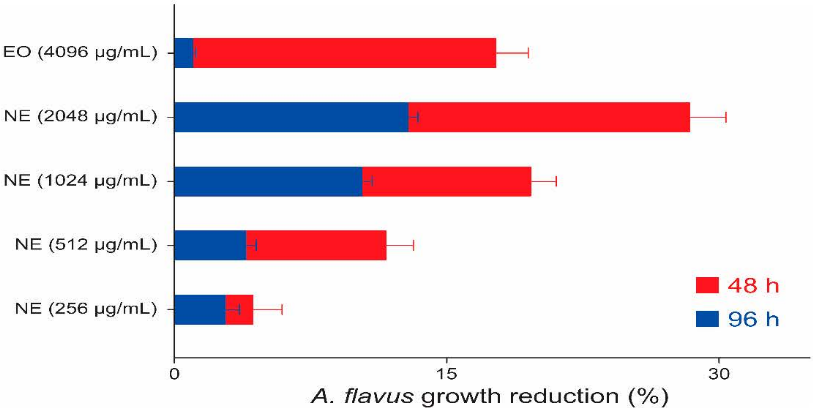

Figure 3. Inhibition of the *A. flavus* colonies by the concentrations of EO and NE at 48 and 96 h.

Figure 4 compares the control colony and the colony with NE at 2048 μg/mL, showing a significant result. The photos were taken with a 48 h growth time and no difference was observed in either the macroscopic or microscopic morphology.

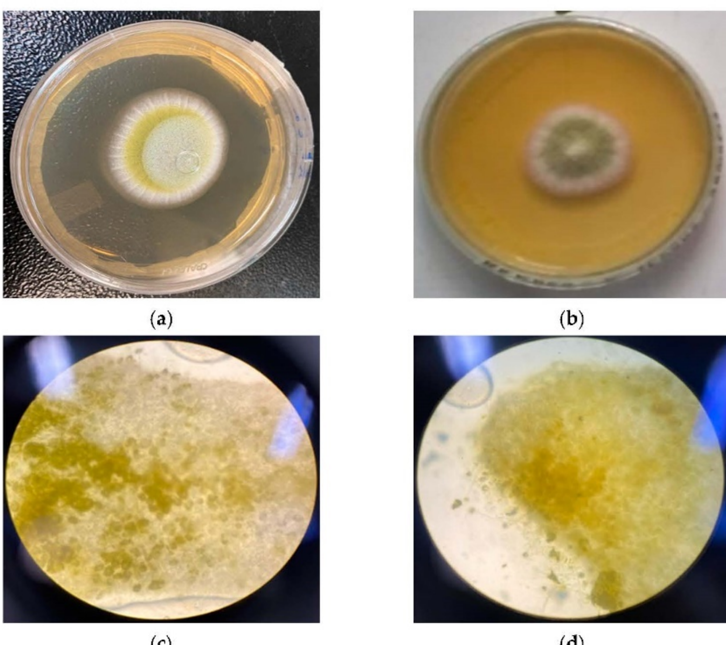

Figure 4. Macroscopic and microscopic comparison between the control colony and the colony with the NE at 2048 µg/mL at 48 h: (**a**) *A. flavus* colony, (**b**) *A. flavus* colony in the presence of the NE at 2048 µg/mL, (**c**) microscopy of *A. flavus*, and (**d**) microscopy of *A. flavus* in the presence of the NE at 2048 µg/mL.

The mycotoxicological analysis, on average, showed that the production in all concentrations of the bioproduct did not show a difference with the control groups. The results are shown in Table 5.

Table 5. AFB1 production by *A. flavus* colonies in the presence of EO and NE at 48, 72, and 96 h.

Bioproduct	Concentration *
Control	9.06 ± 1.50 µg/kg
EO 4096 µg/mL	9.81 ± 1.09 µg/kg
NE 256 µg/mL	9.79 ± 1.07 µg/kg
NE 512 µg/mL	9.55 ± 1.61 µg/kg
NE 1024 µg/mL	9.48 ± 1.72 µg/kg
NE 2048 µg/mL	9.38 ± 1.52 µg/kg

* No significant difference observed in the Tukey's test ($p > 0.05$) for the different concentrations.

2.3.2. *Aspergillus parasiticus*

The trials' results are shown in Table 6, demonstrating the effects of the EO at 4096 µg/mL and the NE at 256, 512, 1024, and 2048 µg/mL on the *A. parasiticus*' growth.

Table 6. Diameter (mm) of *A. parasiticus* colonies in the presence of the EO and the NE at 48, 72, and 96 h.

Bioproduct	48 h	72 h	96 h
Control	21.75 ± 2.453	35.60 ± 4.510	45.30 ± 6.056
EO 4096 μg/mL	18.20 ± 0.141	31.40 ± 2.828	41.30 ± 3.110
NE 256 μg/mL	19.20 ± 1.852	32.30 ± 3.676	43.10 ± 3.105
NE 512 μg/mL	17.10 ± 3.804	28.20 ± 4.518	42.40 ± 1.952
NE 1024 μg/mL	16.20 ± 1.735 *	28.10 ± 2.797	38.50 ± 2.835
NE 2048 μg/mL	13.30 ± 1.873 *	27.90 ± 3.579	37.90 ± 1.411

* The values were recognized as statistically significant at a confidence interval of 95%.

The colonies showed growth in the presence of the EO at 4096 μg/mL, and compared to the control groups, showed no significant difference ($p > 0.05$). This demonstrates the essential oil concentration's lack of fungicidal and fungistatic activity.

The growth of colonies in the presence of the NE showed no fungicidal activity and no significant effects were observed at both 72 h ($p = 0.093$) and 96 h ($p = 0.164$) of incubation. However, inhibition was observed in the first 48 h (df = 5; F = 9.96; $p < 0.001$).

While the NE concentrations of 256 and 512 μg/mL showed no effects even at 48 h, in the 1024 and 2048 μg/mL concentrations, a significant reduction in colonies' growth was observed in the timeframe ($p = 0.003$ and $p < 0.001$, respectively), as shown in Table 7.

Table 7. One-way ANOVA between *A. parasiticus* colonies after 48 h of incubation with the EO and the NE.

Comparison	Diff of Means	*p*	q	*p*
Control 48 h vs. EOOi	0.392	6	2.932	0.361
Control 48 h vs. 2048 ppm	1.017	6	8.627	<0.001 *
Control 48 h vs. 1024 ppm	0.844	6	7.159	0.003 *
Control 48 h vs. 512 ppm	0.333	6	2.827	0.396
Control 48 h vs. 256 ppm	0.280	6	2.373	0.569

* The values were recognized as statistically significant at a confidence interval of 95%. The duration and intensity of the effects are shown in Figure 5, especially the NE at 1024 μg/mL and 2048 μg/mL, which reached, respectively, 25.5% and 38.9% of inhibition.

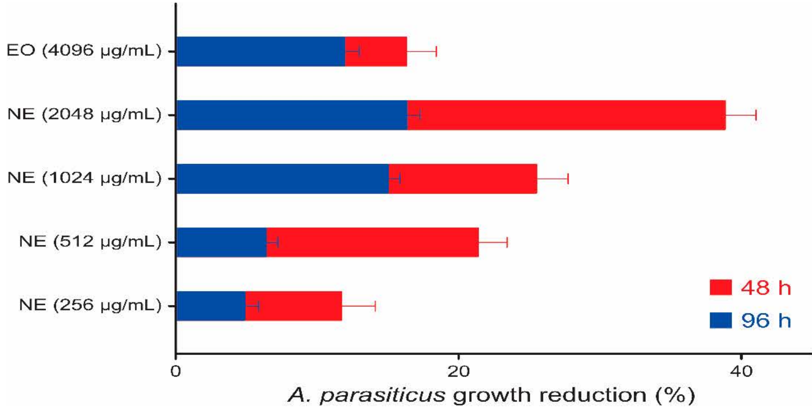

Figure 5. Inhibition of the *A. parasiticus* colonies by a concentration of the EO and the NE at 48 and 96 h.

The macroscopic and microscopic comparisons of the control colonies with the colonies in the presence of the NE at 2048 µg/mL at 48 h are shown in Figure 6, but no relevant morphological differences could be seen.

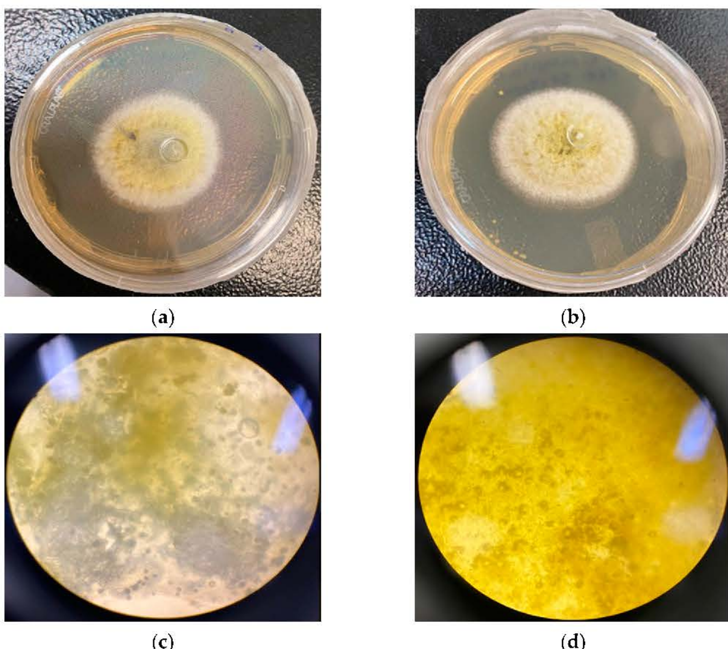

Figure 6. Macroscopic and microscopic comparison between the control colony and the colony with the NE at 2048 µg/mL at 48 h: (**a**) *A. parasiticus* colony, (**b**) *A. parasiticus* colony in the presence of the NE at 2048 µg/mL, (**c**) microscopy of *A. parasiticus*, and (**d**) microscopy of *A. parasiticus* in the presence of the NE at 2048 µg/mL.

The AFB1 detection for all concentrations of the bioproduct again showed no activity compared to the controls. The quantification is shown in Table 8.

Table 8. AFB1 production by *A. parasiticus* colonies in the presence of EO and NE at 48, 72, and 96 h.

Bioproduct	Concentration *
Control	31.71 ± 4.67 µg/kg
EO 4096 µg/mL	36.24 ± 5.35 µg/kg
NE 256 µg/mL	33.81 ± 4.28 µg/kg
NE 512 µg/mL	33.67 ± 3.64 µg/kg
NE 1024 µg/mL	31.88 ± 2.36 µg/kg
NE 2048 µg/mL	30.14 ± 3.64 µg/kg

* No significant difference observed in the Tukey's test ($p > 0.05$) for the different concentrations.

2.3.3. *Aspergillus westerdjikiae*

Table 9 shows the effects on the growth of the *A. westerdjikiae* strain caused by the EO at 4096 µg/mL and the NE at 256, 512, 1024, and 2048 µg/mL.

Table 9. Diameter (mm) of *A. westerdjikiae* colonies in the presence of the EO and the NE at 48, 72, and 96 h.

Bioproduct	48 h	72 h	96 h
Control	11.90 ± 0.779	20.20 ± 1.922	31.00 ± 1.858
EO 4096 µg/mL	9.80 ± 0.990	20.20 ± 1.556	29.20 ± 1.273
NE 256 µg/mL	6.60 ± 1.735 *	16.50 ± 2.227	25.10 ± 3.509
NE 512 µg/mL	6.40 ± 1.682 *	16.30 ± 1.744	24.20 ± 3.064
NE 1024 µg/mL	6.30 ± 1.539 *	15.60 ± 1.800 *	21.80 ± 2.987 *
NE 2048 µg/mL	3.70 ± 2.894 *	11.20 ± 3.764 *	18.70 ± 4.414 *

* The values were recognized as statistically significant at a confidence interval of 95%.

The growth of the control groups and the EO at 4096 µg/mL showed no significant difference ($p > 0.05$) in the tested strain, indicating no fungicidal or fungistatic effect caused by the concentration of the tested EO. The NE in all concentrations also demonstrated a lack of fungicidal activity, but it did show fungistatic activity, as seen in Table 10.

Table 10. One-way ANOVA between *A. westerdjikiae* colonies with the EO and the NE in all incubation periods.

Comparison	48 h				72 h				96 h			
	Diff of Means	*p*	q	*p*	Diff of Means	*p*	q	*p*	Diff of Means	*p*	q	*p*
Control vs. EOOi	2.100	6	1.989	0.723	3.553×10^{-15}	6	2.506×10^{-15}	1.000	1.775	6	0.947	0.982
Control vs. 2048 ppm	8.200	6	8.805	<0.001 *	9.000	6	7.198	0.003 *	12.275	6	7.422	0.002 *
Control vs. 1024 ppm	5.600	6	6.013	0.011 *	4.600	6	3.679	0.035 *	9.175	6	5.548	0.019 *
Control vs. 512 ppm	5.500	6	5.906	0.013 *	3.900	6	3.119	0.303	6.775	6	4.097	0.107
Control vs. 256 ppm	5.300	6	5.691	0.016 *	3.700	6	2.959	0.352	5.875	6	3.552	0.195

* The values were recognized as statistically significant at a confidence interval of 95%.

In the first 48 h, all concentrations led to a significant reduction (df = 5; F = 9.53; $p < 0.001$), approximately 44% to 48%, except the concentration of 2048 µg/mL that reached 69% inhibition. The reduction by NE at 512 and 256 µg/mL decreased to nearly 20% after 48 h, while the 1024 µg/mL and 2048 µg/mL concentrations remained significant at 72 h (df = 5; F = 6.28; $p = 0.004$) and 96 h (df = 5; F = 7.03; $p = 0.003$), when they decreased to, respectively, 29.6% and 39.7% inhibition. All the reductions are graphically demonstrated in Figure 7.

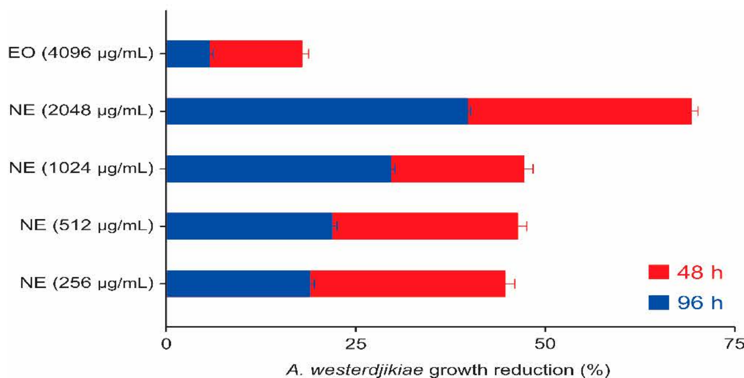

Figure 7. Inhibition of the *A. westerdijikiae* colonies by a concentration of EO and NE at 48 and 96 h.

The photos in Figure 8 compare the colonies after 48 h of incubation in the presence of the NE at 2048 µg/mL with the control colonies. The macroscopic and microscopic morphological comparison showed no observable difference.

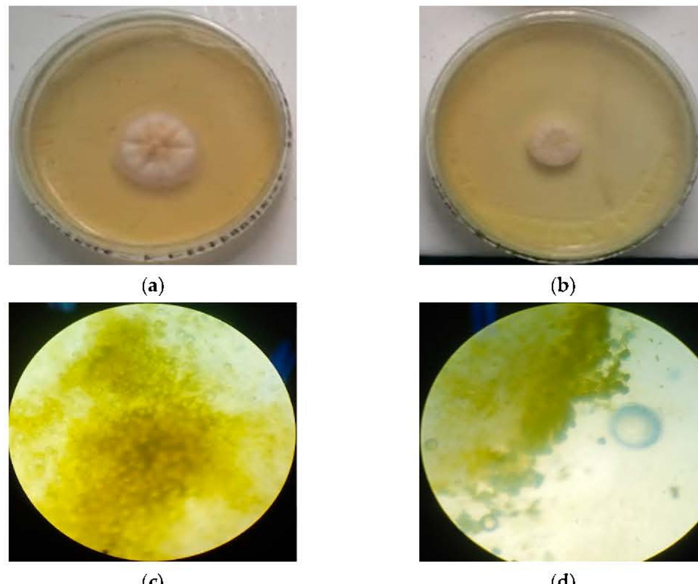

Figure 8. Macroscopic and microscopic comparison between the colonies with or without the NE at 2048 µg/mL at 48 h: (**a**) *A. westerdjikiae* colony, (**b**) *A. westerdjikiae* colony in the presence of the NE at 2048 µg/mL, (**c**) microscopy of *A. westerdjikiae*, and (**d**) microscopy of *A. westerdjikiae* in the presence of the NE at 2048 µg/mL.

As for the production of mycotoxins, the average of each strain in the presence of the products had no observable effects compared to the control production. The analysis outcome is demonstrated in Table 11.

Table 11. AFB1 production by *A. westerdjikiae* colonies in the presence of EO and NE at 48, 72, and 96 h.

Bioproduct	Concentration *
Control	15.79 ± 3.18 µg/kg
EO 4096 µg/mL	16.09 ± 2.86 µg/kg
NE 256 µg/mL	15.59 ± 2.54 µg/kg
NE 512 µg/mL	14.83 ± 2.73 µg/kg
NE 1024 µg/mL	13.92 ± 2.54 µg/kg
NE 2048 µg/mL	13.52 ± 2.48 µg/kg

* No significant difference observed in the Tukey's test ($p > 0.05$) for the different concentrations.

3. Discussion

The essential oil from the leaves of *O. indecora* showed approximately 86% of sesquirosefuran, corroborating with the *O. indecora* chemical profile and sesquirosefuran amount (88–92%) described previously by other authors [29,31].

The sesquirosefuran is a furanosesquiterpene registered under CAS number 39007-93-7, with the molecular formula $C_{15}H_{22}O$ and a molecular weight of around 218 u [36]. It has a log *p* of 5.847 and a TPSA equal to 13.140; then, according to the Pfizer Rule, it is

likely to be toxic, especially as an inhibitor of some CYP family enzymes and a probable human hepatotoxic substance. The sesquirosefuran, however, is unlikely to cause severe skin, respiratory, and eye damage [37].

In this way, it is possible that the bioproduct can be used as an external preservative, as long as it does not involve direct consumption, and reinforcing that it is important to choose carefully where to use it in order not to cause unwanted damage to fauna, flora, and workers.

The sesquirosefuran has few reports about the molecule bioactivity, mainly insecticide activity [29,31], and none related to its antifungal potential. Sesquiterpenes and furanosesquiterpenes, in turn, have several recognized activities, and among them, Marongiu et al. [33] demonstrated activity against several *Aspergillus* sp. by essential oils rich in furanosesquiterpenes.

The furansesquiterpenes action mechanisms are not yet fully elucidated, but several studies describe antifungal effects related to the furan group in natural molecules [38,39]. Loi et al. [40], however, describe sesquiterpenes isolated from different plants capable of altering the mitochondrial function of mammals. The mitochondrial membrane potential is maintained in healthy individuals by an electrochemical gradient. The mechanism of action is not clearly understood, but it is theorized that a disturbance in the protons of the osmotic balance affects the electrochemical potential. As ATP levels decrease, metabolism and functions reduce until cell death, thus explaining the observed fungistatic effect.

The effect observed in mammal mitochondria is possibly similar in fungal mitochondria, as Campbell et al. [41] related similarities between both. This would explain the fungistatic effect and the absence of morphological alteration, as the inhibition could be a decrease in the metabolism caused only by the reduction in the ATP concentration.

Despite this report, *O. indecora*'s EO did not demonstrate a fungicidal or fungistatic effect at 4096 μg/mL and could not inhibit the aflatoxin production in the three *Aspergillus* strains tested. However, when the EO is nanoemulsified, the results are promising. The NE at 2048 μg/mL showed a fungistatic effect in the three strains, at least at 48 h, while the EO at a concentration two times higher did not show the same capacity.

The production of the EO nanoemulsion is an exciting tool to enable these lipophilic matrices into viable products and has been widely used in pharmaceutical and food industries [23]. In this study, the *O. indecora*'s EO was nanoemlusified by a low-energy approach to maintain the chemical profile since it does not use heat treatment in the nanoemulsification process, which avoids thermal degradation and volatilization of the terpenoids present in the EO [42].

There are two prevailing forces crucial to the stability of nanoemulsified systems: the gravitational (droplet size and weight) and the electrostatic repulsion (zeta potential). Together, they influence the physicochemical maintenance of a nanodispersion's collective parameters [21,43]. The prepared nanoemulsion showed a bluish-white appearance consistent with the Tyndall effect, a reduced droplet diameter of 103.4 ± 0.9, and a 0.268 ± 0.010 PdI. As for the surface charge, one of the parameters related to the nanodroplets' stability, the NE zeta potential, was -32.83 ± 0.8208, indicating good short-term stability, with coulombic repulsion between the negatively charged nanodroplets, and in that way, favoring the droplets' Brownian motion [43]. Due to all these parameters, the product was characterized as a conventional monodispersed nanoemulsion [22].

The different nanoemulsion physicochemical properties, such as the reduced droplet diameter, may justify the higher effect of the NE in comparison to the EO. The increased fungistatic effect may be associated with higher bioavailability of the substances present in the EO. Furthermore, the increase of hydrophilicity and the nanoscale of the particles can lead to higher dispersion and stability in the medium [24] and may facilitate the absorption and metabolization of the substances present in the bioproduct [25]. The activity of the EO can be optimized in this way, by increasing the contact between the metabolites in the essential oil and the fungal cells.

Comparing the reduction pattern, a dose-dependent response of the NE against the strains was observed (Figures 3, 5 and 7). The fungistatic effect for the *A. flavus* strain was not significant below 2048 µg/mL but became significant upon reaching this concentration. The turning point for the *A. parasiticus* strain was even lower, as at 1024 µg/mL it had already shown a significant difference.

The comparison was even wider for the *A. westerdjikiae* strain, as all concentrations showed significant activity in the first 48 h, and the higher ones up to 96 h. The greater susceptibility of *A. westerdjikiae* to bioproducts was already reported in other studies, such as those of Rodrigues et al. [44] and Schlösser and Prange [45].

Even though all concentrations of the NE were active against the *A. westerdjikiae* strain, the dose-dependent effect was still visible. The NE showed activity that the OE in a concentration 16 times higher did not, therefore endorsing the potential caused by the nanoemulsification process.

The observed correlation between dosage and effect, and the NE showing a better result than the EO, even though it was in lower concentrations, indicate how the formulation can trigger and potentiate effects, whether expected or not, as observed in the study by Do Carmo Silva [46].

However, despite the potential, no concentration of the NE showed fungicidal activity in any of the strains. A concentration higher than 2048 µg/mL may be necessary to increase the bioavailability of the molecule even more to cause a proper fungicidal effect.

Another limitation observed was the drastic decrease in activity after 48 h of incubation as, after this point, only a fungistatic effect was observed in the colonies of the *A. westerdjikiae* strain and only with the NE at 1024 and 2048 µg/mL. This behavior may be due to the consumption of all the present NE by the fungal colonies, and only the residual effect was observed afterward. Another possibility is degradation over time in the incubation temperature and/or medium.

However, in the thermal stress stability study of the *O. indecora* nanoemulsion, only temperatures from 35 °C slightly reduced the droplet size (nm). As the incubation temperature was 25 to 30 °C, it is unlikely that a significant degradation occurred. The droplet reduction is probably associated with the increased solubility of essential oil terpenoids due to the higher temperature in the aqueous phase [47].

Regarding the size homogeneity of the nanodroplets, there was no statistical significance ($p < 0.05$) in the polydispersity index between all temperatures analyzed (25 to 65 °C). The zeta potential after the thermal stress was -34.85 ± 0.5284, suggesting no relevant alteration in the nanodroplets' surface charge [43]. Both parameters reassured the improbability of degradation.

While the bioproduct showed a short period of a fungistatic effect and no fungicidal effect, it was still significant. The activity was seen in the exponential phase of growth for the three strains and, even though it did not establish a stationary range, the reduction in the log phase points to a potential for concomitant use with other antifungal products. The promising potential in associated substances of plant origin and commercial products was also described by Chagas [48].

Another point worth noting is that high-complexity food matrices can lead to reduced effectiveness of antimicrobials. Therefore, a larger amount of preservative than the one used in vitro tends to be necessary to achieve the same results. Higher concentrations, however, can impair the organoleptic properties of foods. To avoid this problem, lower concentrations of bioproducts with fungistatic effects can be used [39]. This highlights the use of nanoemulsions as food preservatives, as long as they are obtained from safe plant derivatives, since the present work demonstrated that they have exactly this desired capacity.

As for the quantification of AF, the variations were considered insignificant, according to statistical results, regardless of the concentration. However, there are reports of the chemical properties of the AFs being modified in different ways after incubation with plant

extracts, including removal of the furan double bond in AFB1 and modification of the lactone ring, resulting in a significant decrease in cytotoxicity and carcinogenicity [49].

About the variations, considering the high sensitivity of the AF production [50], the oscillation may be due to the alteration in the fungal metabolism and its metabolites [51].

When comparing the results obtained with other descriptions in the literature, it is observed that this is the first study to report the antifungal properties of *Ocotea indecora* and its major substance, sesquirosefuran. Other studies on the genus have been carried out, however, few species have a chemical composition similar to the *O. indecora*. Furthermore, most trials are on the clinically important leviduriform fungal species.

Focusing only on species with a high content of sesquiterpenes in their EO and their activities on filamentous fungi, the studies by Prieto et al. [52] stand out, where *Ocotea macrophylla* is observed with 70% of the constituents being sesquiterpenes, and this oil demonstrates antifungal activity against species of the genus *Fusarium*. Another significant trial is that carried out by Mezzomo et al. [53], who observed *Ocotea puberula*'s EO with 17% of sesquiterpenes and reported weak antifungal activity in two *Aspergillus* species, *alternate* and *flavus*.

The low antifungal effect was, therefore, concordant with the literature. However, the potentiation demonstrated by the nanoemulsification of the *O. indecora* essential oil qualifies the nanoemulsion as a fungistatic agent for the tested strains. Combined with their biodegradation and metabolization properties, the nanoemulsion was demonstrated as an appropriate option for pre-harvest treatment that, if well-used, will not compromise the environment or the workers' and consumers' health.

4. Materials and Methods

4.1. Plant Material

The fresh leaves of *O. indecora* were collected in the Restinga of Jurubatiba National Park, Carapebus, RJ, Brazil ("22°12.683′ S", "41°35.283′ O", "22°12.703′ S", and "41°35.336′ O"). The obtention and research of the plant material were authorized by SisBio/ICMBio (13659-14) and SisGen (A0D648D). The species was identified, and a voucher specimen was deposited at Universidade Estadual do Rio de Janeiro—Faculdade de Formação de Professores (UERJ—FFP) herbarium, under registration number RFFP: 16.873.

4.2. Essential Oil Extraction and Chemical Characterization

Fresh leaves of *O. indecora* (250 g) were crushed into a blender with distilled water, transferred to a 5.0 L round-bottom flask, and subjected to hydrodistillation in a Clevenger-type apparatus for 4 h. After that, the essential oil was dried over anhydrous sodium sulfate and stored in an amber glass vial at 4 °C.

The essential oil was analyzed in a GC-MS QP2010 (Shimadzu) gas chromatograph equipment coupled with a mass spectrometer and a GC-2014 (Shimadzu) gas chromatograph equipped with a flame ionization detector (FID). The chromatographic conditions were: a 260 °C injector temperature, with the carrier gas helium, the flow rate was 1 mL/min, and the split ratio was 1:40. Initially, the oven temperature began at 60 °C and then increased to 290 °C (3 °C/min rate). The essential oil (1 µL) was dissolved in dichloromethane (1:100 µL) and injected into a DB-5 column for MS (0.25 mm ID, 30 m in length, 0.25 µm film thickness). The mass spectrometry conditions were 70 eV electron ionization and a 1 scan/s scan rate. The GC-FID analysis was similar to the MS, except for the injection in a DB-5 column (0.25 mm ID, 30 m in length, 0.25 µm film thickness) and the FID temperature at 290 °C. The arithmetic index (AI) was calculated by interpolating the retention times of a mixture of aliphatic hydrocarbons (C7–C40) and analyzed under the same chromatographic methods. Substances were identified by comparing their retention indices and mass spectra with those reported in the literature [34,35]. Compounds' mass spectra fragmentation pattern was also compared with NIST mass spectrum libraries. GC-FID performed the relative abundance of the chemical constituents under the same

conditions as GC-MS. The FID peak area normalization method obtained the percentages of these compounds.

4.3. Nanoemulsion Preparation, Characterization, and Thermal Stress Stability

The formulation of the nanoemulsion of *O. indecora* was previously described by Machado et al. [29]. The nanoemulsion was prepared by the low-energy method. The NE aqueous phase was 96% (*w/w*) of distilled water, and the oil phase was made up of 2% (*w/w*) of EO, and 2% (*w/w*) of the surfactants polysorbate 20 and sorbitan monooleate 80 in a 4:1 proportion, respectively. The oil phase was homogenized in a vortex for 1 min, and then the aqueous phase was slowly dripped into the oil phase in continuous agitation.

The droplet size (nm), zeta potential (ZP), and polydispersity index (PdI) from the NE diluted with distilled water (1:40) were characterized by dynamic light scattering (DLS) in a Zetasizer Advance Lab Blue (Malvern Instruments®, Worcestershire, UK).

The *O. indecora* nanoemulsion was submitted to thermal stress after preparation to assess the trend of the nanoemulsion droplet size, zeta potential, and polydispersity index with a temperature increment from 25 to 65 °C, with an increase of 10 °C between analyses. The characterization was realized under the same conditions described above.

4.4. Fungal Strains

The strains used were *A. flavus* RC 2054, *A. parasiticus* NRRL 2999, and *A. westerdjikiae* NRRL 3174, all reference strains known to produce aflatoxin B1 (AFB1).

4.5. Inoculation and Incubation Conditions

The methodology was adapted from Rodrigues et al. [54], with the direct addition of the bioproduct in the medium and then needle-inoculating the strains.

The EO samples were diluted with 10% dimethylsulfoxide (DMSO) and tested at a 4096 μg/mL concentration in the culture medium. As for the NE, the concentrations of 256, 512, 1024, and 2048 μg/mL were tested in the medium.

The medium used was Kasvi's Sabouraud Dextrose Agar (SDA), with a standardized volume of 25 mL being used for each culture, thus allowing control of the study concentrations [55]. The EO and the NE samples were added to the SDA in sufficient volume to reach the determined concentrations.

The Petri plates with the bioproducts were needle-inoculated centrally with 10 μL of the spore suspension. The plates were incubated at 25–30 °C for 96 h, with daily observation and diameter measurement.

Plates containing SDA medium without essential oil were used as a control group (Control 1), and the diluents, DMSO, and a nanoemulsion without the EO were used as a second control group (Control 2).

4.6. Growth Assessment

The diameter of the growing colonies was measured daily for four days. The percentages of inhibition of diameter growth (PIDG) values were determined according to the equation below:

$$\mathrm{PIDG}(\%) = 100 \times \frac{(\text{Diameter of control} - \text{Diameter of sample})}{\text{Diameter of control}} \quad (1)$$

4.7. Microscopic Evaluation

A 1 mm piece of the colonies was collected for each plate of the three strains for the observed points. The pieces were then placed in proper slides for the optical microscope with one drop of distilled water and then set with the coverslip. The slides were observed in a Nikon ALPHAPHOT-2 YS2-H at 1000× magnification.

4.8. Aflatoxin Analyzes

The detection and quantification of aflatoxins was performed based on Geissen [56], taking a three-point sample for each plate with fungal growth and placing it in microtubes in duplicates. Later, 0.5 mL of chloroform was added to each microtube and then centrifuged at 4000 rpm for 10 min. After precipitation, the extract was removed and transferred to another microtube to dry and the contents were resuspended with 70% methanol. The quantification was realized by HPLC.

4.9. Statistical Analyses

Data evaluations were performed by analysis of variance (ANOVA). Data were transformed using the logarithmic function, log10 (x + 1), before ANOVA, and when necessary, the data were transformed with a root square to obtain a homogeneous data distribution. Tukey's test was used to compare the enumeration data of the different concentrations of the evaluated products, the different presentations of the tested products, and the variations in incubation and growth times. All analyses were based on evaluating these substances' fungicidal or fungistatic potential. Analyses were conducted using the PROC GLM computer program in SAS (SAS Institute, Cary, NC, USA).

5. Conclusions

The *O. indecora* essential oil did not show fungicidal potential at the concentrations tested against *A. flavus* RC 2054, *A. parasiticus* NRRL 2999, and *A. westerdjikiae* NRRL 3174. However, the *O. indecora* nanoemulsion showed fungistatic potential in *Aspergillus* strains. At 256 and 512 µg/mL, the EO affected the growth of the *A. westerdjikiae* strain for 48 h. At 1024 µg/mL, the EO inhibited the growth in both *A. parasiticus* and *A. westerdjikiae* for 48 and 96 h. The higher concentration, 2048 µg/mL, showed activity in all three strains up to 48 h, and up to 96 h for the *A. westerdjikiae*.

The nanoemulsion of the *Ocotea indecora* can be considered a fungistatic agent for the tested strains, and the formulation of the essential oil in the nanoemulsion triggered and potentiated effects that otherwise would not be significant.

The aflatoxin evaluation indicated that the products in the tested concentration had no significant effect on the production of secondary metabolites by the studied strains.

The study indicated an exciting line of research not only in the prospection of natural products but also for the application of nanoemulsions as a bioactive carrier, especially those that have, by nature, hydrophobic properties, and the results should be applied to other promising plant derivatives.

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8.2 Artigo 2

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

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Article 1

Molluscicidal and cercaricidal effects of *Myrciaria floribunda* essential oil nanoemulsion 2 3

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8.2 Artigo 3

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

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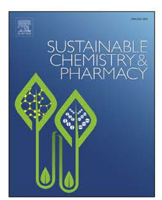

Green Nanobioinsecticide of a Brazilian endemic plant for the *Aedes aegypti* control

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ABSTRACT

The search for sustainable alternatives for larval control of the *Aedes aegypti* (Diptera: Culicidae) mosquito, the vector that transmits Dengue Fever, is urgently needed in tropical and subtropical regions. This work aims to realize the chemical characterization of the essential oil from *Xylopia ochrantha* Mart. Leaves, prepare a stable nanoemulsion, and determine its hydrophilic-lipophilic balance (HLB) and larvicidal activity against the 3rd instar larvae of *Aedes aegypti*. Aerial dried parts of *Xylopia ochrantha* were collected at Restinga de Jurubatiba National Park (Brazil), and the essential oil was obtained by hydrodistillation using a Clevenger-type apparatus. The chemical characterization was done by gas chromatography, revealing germacrene D (17.8%), bicyclogermacrene (17.4%), and δ-elemene (13.9%) as the major compounds. The nanoemulsion prepared by the low-energy method presented a droplet size of 75.56 nm, a polydispersion index of 0.271, and a relative hydrophilic-lipophilic balance (HLB) value of 14.22. The LC_{50} was 192.5 μg/mL within 48 h against the 3rd instar *Ae. Aegypti* larvae. This study concluded that the nanoemulsion obtained from the essential oil of *X. ochrantha* leaves proved helpful for controlling this vector, which is responsible for causing diseases with a great impact on public health. Moreover, it gives visibility to the *restinga,* an important ecosystem. It points to the possibility of developing environmentally friendly products to help solve significant public health problems.

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1. Introduction

At present, neglected tropical diseases (NTDs), a group of 20 diseases, are responsible for aggravating the health of more than 1 billion people worldwide, particularly those living in poor sanitation and inadequate housing (Reed and McKerrow, 2018; WHO, 2020). In particular, Dengue Fever is one of the NTDs with an increasing prevalence and geographic range, being ranked as one of the diseases with the highest public health burden in subtropical and tropical regions (Vuitika et al., 2022; Wong et al., 2022). This disease can be caused by one of the 4 known serotypes of Dengue viruses (DENV-1-4) which is transmitted mainly by the *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) mosquito (Wong et al., 2022). It is estimated that more than 150 countries are at risk of infection by this arboviral disease (Aungtikun and Soonwera, 2021). In 2019, there were 3,190,851 cases of dengue fever, the highest number of cases reported in the Americas in the last four decades. Currently, this disease has reached 2,548,167 cases (Pan American Health Pan American Health Organization/World Health Organization, 2022). The prevention, control, and eradication of NTDs is the new pathway outlined by the World Health Organization to achieve the Sustainable Development Goals (SDGs) by 2030 (WHO, 2020). However, difficult mosquito vector control, increasing uncontrolled urbanization, poverty, human migration, and climate change exacerbate the epidemiological scenario of this disease in affected regions (Wong et al., 2022).

In the absence of basic sanitation conditions in many endemic regions and of an effective vaccine for the four circulating serotypes of the Dengue virus (Vuitika et al., 2022), mosquito vector control is currently the main preventive strategy to control this human health issue (Aungtikun and Soonwera, 2021). There are various options for vector control, but chemical control using products with larvicidal, adulticidal, and repellent action is the most widely used and disseminated. However, the widespread resistance of vectors to chemical compounds and the environmental and toxicological effects associated with these products urgently require the search for effective and eco-friendly alternatives (Aungtikun and Soonwera, 2021; Folly et al., 2021).

In this way, the bioactivity present in aromatic plants is shown to be an attractive avenue in the search for sustainable solutions to combat the mosquito vector. This subject has mobilized many researchers, and several studies show the action of secondary plant metabolites with activity against *Ae. Aegypti* larvae (Martins et al., 2021; Oliveira et al., 2017; Folly et al., 2021; Aungtikun and Soonwera, 2021), which can open up a whole new panorama in the fight against the mosquito vector study. In addition, it is known that plants' bioactive substances are often biodegradable with low or no toxicity to mammals and have activity against insects due to their different chemical structures (Aungtikun and Soonwera, 2021; Folly et al., 2021). Thus, compounds of plant material appear to be an environmentally safe and promising alternative. Alcala-Orozco et al. (2019) highlight the importance of research for developing products from plants, especially those derived from essential oils (EO) that have become a field of study, given the low predisposition of EOs to generate phenomena of resistance in organisms in general. EOs have potential applications in agriculture and the pharmaceutical industry due to their antibacterial, antifungal, anti-inflammatory, or antioxidant properties (Plata-Rueda et al., 2020; Alcala-Orozco et al., 2019). However, the volatile and hydrophobic nature of EOs precludes their application in aqueous systems, which is where the early stages of *Ae. Aegypti* mosquitoes develop (Folly et al., 2021). Therefore, the nanoformulation of EOs emerges as a strategy to address this identified problem and that can increase their stability and effectiveness if they are nanoencapsulated. The advantage of this type of preparation lies in its droplet size on a nanometric scale, which provides better physicochemical properties and can improve biological activity (Gonçalves-Esteves et al., 2001; Pires and Moura, 2017).

Particles with a nanometric or submicrometric size that have a high surface-to-volume ratio stand out and have key differences when compared to materials of another type and confer changes in magnetic, electronic, biochemical, and optical properties. These factors determine optimal performance and release mechanisms (Bobo et al., 2016). Additionally, nano products or nanomaterials confer favorable organoleptic characteristics, greater absorption capacity, greater bioavailability, and controlled release of actives. Another advantage refers to improving the solubility of hydrophobic substances in water. Not to mention the kinetic stability acquired by these products (Porto et al., 2020, Vuitika et al., 2022). All these advantages characterize them as innovative and versatile products. Nanotechnology has put different products on the market in different areas. Different types of nanosystems are described in the literature for the controlled release of drugs, such as polymeric, lipidic and magnetic nanoparticles, cyclodextrins, nanocrystals, and liposomes, among others (Siqueira et al., 2019; Singh et al., 2017; Rai et al., 2018). In this context, nanoemulsions appear. They are biphasic or triphasic dispersions of two immiscible liquids, water, and oil, stabilized by one or more surfactants, of amphiphilic character, on a nanometric scale. The production of nano pharmaceuticals is based on techniques for manufacturing and applying nanostructures with controlled shapes and sizes. In the case of nanoemulsions, they are widely used as vehicles in the pharmaceutical, cosmetic, chemical and food industries (Porto, Almeida e Vuitika et al., 2022; Singh et al., 2017). These attributes make nanotechnology multifunctional and novel.

Nevertheless, no nanoemulsions are available on the market to be applied in the field for vector control (Pavela, 2015). This is a space that must be filled. Thus, trials that propose the development of these formulations are both appropriate and welcome.

Bioactive products prepared with EOs are considered an option for controlling or eliminating different target organisms (Bobo et al., 2016; Duarte et al., 2015; Botas et al., 2017) that can be vectors or transmitters of diseases or even agricultural pests. This reinforces the conduction of research that eliminates or even controls these agents from natural products elaborated with nanotechnology. Data show that there has been a global interest in integrative practices in controlling disease vectors, and many of them involve natural products as bioactive agents (Folly et al., 2021; Oliveira et al., 2017; Bobo et al., 2016; Duarte et al., 2015). Access to plant material requires a broad, holistic view. There must be an interdisciplinary contribution for scientists to work with nature's resources to preserve its diversity with wisdom (Sachs, 2002). This demonstrates the importance of developing and executing a project that brings sustainable solutions in both the human and environmental spheres.

Restinga de Jurubatiba National Park is a sandbank ecosystem inserted in the Atlantic Forest domain, considered an integral protection unit in Brazil. The *restingas* are vast regions of coastal plains with sandy soil (Costa and Dias, 2001). The climate is humid trop-

ical, but with a relatively low rate of free water in the soil, as much of the rainfall that falls is percolated into the water table. These regions present complex environmental mosaics with a rich diversity of fauna and flora. These different vegetal formations have potential use for several purposes due to their economic potential, tourism due to their natural beauty, the fishing productivity of their lagoons, and also due to the most varied metabolites produced by the species present in this environment (Cogliatti-Carvalho et al., 2010; Santos et al., 2009; Gonçalves-Esteves et al., 2001). This ecosystem has an extensive knowledge gap, home to many plant species that arouse interest in scientific research on their potential biological use (Costa and Dias, 2001). In this environment, some native species had identified essential oils' chemical constituents that showed proven bioactivity (Folly et al., 2021; Araújo et al., 2019). Among the many families that populate this coastal ecosystem in Rio de Janeiro, Brazil, the Annonaceae family is represented by six genera and nine species: *Anaxagorea dolichocarpa*, *Annona acutiflora*, *A. glabra*, *A. montana*, *Duguetia sessilis*, *Guatteria nigrescens*, *Oxandra nitida*, *Xylopia sericea* and *X. ochrantha* (Lobão et al., 2005). This family has a distribution subtropical and tropical throughout the world (JolyAnnonaceae, 2002) and comprises about 135 genera and over 2500 species (Shakri et al., 2020).

The genus *Xylopia* includes about 160 species distributed throughout the Americas, Africa, and Asia. Brazil has about 25 of these species. This genus can produce metabolites such as alkaloids, amides, lignoids, acetogenins, and terpenoids (Silva et al., 2015). Several studies cited in a review showed the EO's biological activities of different *Xylopia* species, such as antioxidant, antimicrobial and antitumor properties. In traditional medicine, especially in Africa, different plant parts such as leaves, fruits, roots or seeds, among others, have been used to treat fever, cough, and skin infections (Shakri et al., 2020). The specie *Xylopia ochrantha* Mart. Is an endemic specie in Brazil, found in the states of Rio de Janeiro, Espírito Santo, and Pará, located mainly in sandbanks (Costa and Dias, 2001). In Rio de Janeiro, it is known by the popular name of Imbiu-prego. A NE made with the essential oil from the leaves of *X. ochrantha* caused mortality in different snail species of the genus *Biomphalaria* (Araújo et al., 2019). As long as we know, never before was a nanoemulsion made with the essential oil from the leaves of *X. ochrantha* tested against 3rd instar *Ae. Aegypti* larvae. This study aimed to realize the chemical characterization of the essential oil from *X. ochrantha* leaves, prepare a stable nanoemulsion, and determine its hydrophilic-lipophilic balance (HLB) and larvicidal activity against 3rd instar *Ae. Aegypti* larvae.

2. Material and methods

2.1. Plant material

Aerial parts of *Xylopia ochrantha* Mart., Annonaceae, were collected in Brazil, at the municipality of Carapebus (RJ) at Restinga de Jurubatiba National Park in clusia scrub vegetation (22°12.696′S – 41°35.322′O and 22°12.673′S – 41°35,249′O), during the day in June 2019. The botanist, Dr. Marcelo Guerra Santos, identified the plant material and deposited a voucher specimen (register number RFFP 15.326) at Faculdade de Formação de Professores (UERJ, Brazil). The studies were carried out according to the authorization from SISBIO/ICMBio under register number 13.659–12, and SisGen number A0D648D.

2.2. Essential oil extraction

The extraction of EO from *X. ochrantha* (1.621 g) was made with dried leaves, which were kept in an oven at 40 °C for 24 h. Then they were turbocharged (Skymsen® turbocharger) with distilled water. The material was divided into equal portions, placed in 5 L distillation flasks with enough water to cover the plant material, and subjected to extraction by hydrodistillation using a Clevenger-type apparatus for 4 h. At the end of extraction, the essential oil was collected and stored under refrigeration (4 °C) in an amber flask for further identification of chemical constituents, preparation of nanoemulsions, and biological tests.

2.3. Chemical analysis

The *X. ochrantha* essential oil was characterized by gas chromatography (GC) coupled with mass spectrometer (MS) model GC-MS QP 2010 (Shimadzu) and in gas chromatograph equipped with a flame ionization detector (FID) model GC-2014 (Shimadzu). The GC conditions were: Helium as carrier gas with a flow rate of 1 mL/min with split injection with a ratio 1:40. The injector temperature was 260 °C. The initial oven temperature was 60 °C, then increased to 290 °C at a 3 °C/min rate. Then 1 μL of the essential oil, dissolved in dichloromethane (1:100 mg/μL) was injected in RTX-5 column for MS (0.25 mm ID, 30 m in length, 0.25 μm film thickness). The electron ionization from the mass spectrometry was 70 eV, with a 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 the aliphatic hydrocarbons mixture (C9–C30) analyzed under the same condition previously described. The substances were identified by comparing their retention indices and mass spectra with those reported in the literature (Adams, 2017). MS fragmentation pattern of compounds was also compared with NIST mass spectrum libraries. The FID peak area normalization method obtained the analysis and percentages of these compounds.

2.4. Nanoemulsification method and relative hydrophilic-lipophilic balance (HLB)

The NEs were prepared with some modifications by the low-energy input method described by Ostertag et al. (2012). The formulations oil phase consisted of 5% (w/w) of EO, 5% (w/w) of surfactants (sorbitan monooleate 80 and polysorbate 20), and the aqueous phase was 90% (w/w) of distilled water. The surfactants and essential oil were weighed on an analytical balance (Shimadzu®, AY220 and homogenized simultaneously in a 15-place magnetic stirrer (Mag-multi®, Marte) for 30 min. After this time, 4.5 mL of distilled water was added dropwise in each flask that remained under stirring for 1 h (Fernandes et al., 2013). The final weight of the nanoemulsion was 5 mL.

The *X. ochranta* essential oil formulation study is represented in Table 1. Eleven formulations with different ratios of surfactants (polysorbate 20 and sorbitan monooleate 80) to achieve a hydrophilic-lipophilic balance (HLB) range between 4.3 and 16.7 were prepared.

Droplet size and PDI of the nanoemulsions were determined by dynamic light scattering (DLS) in a Zetasizer Advance Lab Blue (ZetaPlus® (BrookhavenInst. Corp., USA), characterize and select the suitable final formulation composition. Each emulsion was diluted in distilled water (1:25). The analyses were performed in triplicate. The results were expressed as a function of mean size ± mean standard deviation (Fernandes et al., 2013). In addition, the macroscopic characteristics of formulations, such as homogeneity, phase separation, cream forming, sedimentation, translucency, and bluish reflection, were verified due to the Tyndall effect (Ferreira et al., 2010).

2.5. Nanoemulsion stability

Another batch of NEXO was prepared and characterized under the same conditions described in item 2.4. After nanoemulsion preparation, the short-term thermal stress study was conducted to evaluate the influence on macroscopic characteristics, droplet diameter, polydispersity index, and zeta potential. The nanoemulsions were diluted in distilled water (1:20) and submitted to a temperature range of 25 °C–75 °C. The temperature was increased 10 °C at each analysis interval. All analyses were performed in triplicate. After that, a newly prepared nanoemulsion was compared to six months NEXO stored in an amber glass flask at room temperature (25 °C ± 2), to evaluate its particle growth, PDI, and zeta potential (ZP). Several parameters, such as appearance (bluish reflex, homogeneity), phase separation, presence of cremation, and sedimentation, were evaluated macroscopically.

2.6. Aedes aegypti larvae strain

The eggs of *Ae. Aegypti* were supplied by the Laboratory of Physiology and Control of Vector Arthropods (LAFICAVE-IOC, Rio de Janeiro, Brazil). Rockefeller strains (temephos/deltamethrin susceptible) were used for the assays. A filter paper containing around one thousand adherent eggs was used for egg hatching. It was immersed in a beaker of dechlorinated water at 37 °C and then placed in an incubator at 26 °C for 50 min.

The hatched larvae were transferred to a tray containing 300 mL of water with 300 mg of shredded fish feed (Poytara Tropical Flakes®, Rio de Janeiro, Brazil). The larvae were maintained at 25 ± 2 °C, under 75 ± 5% relative humidity, 12 h light-dark cycle and larval feeding. The experimental protocol was performed according to WHO (WHO, 2005). Larvae at the third instar (L3) were used in all bioassays.

2.7. Larvicidal bioassay

The experiments were carried out in disposable 50-mL polypropylene plastic containers, each containing 10 mL of the solutions test with ten 3rd instars *Ae. Aegypti* larvae. The plastic containers' trays were maintained at 26 ± 1 °C (Prado et al., 2019). Larvicide assay protocol was carried out following WHO (2005). All experiments were performed in triplicate with ten larvae in each replicate (n = 30). The selected nanoemulsion of *X. ochrantha* was diluted in distilled water at 400, 300, 200, and 100 μg/mL (expressed in essential oil). The negative control group was treated with distilled water, and the positive control was imidacloprid at 0.9 μg/mL. The blank nanoemulsion (without essential oil) at the same concentration as the nanoemulsion was also evaluated. The levels of mortality were recorded after 24, 48, and 144 h of exposure.

2.8. Acute oral toxicity single dose in Danio rerio

The experiment followed the animal welfare regulations, The ARRIVE guidelines (Kilkenny et al., 2010), and approved by the Ethics Committee of Instituto Vital Brazil protocol number 003/2019. 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 °C, ammonia = 0 μg/mL, photoperiod (light/dark) = 12 h/12 h. Ten animals were randomly distributed in equal numbers in five experimental tanks (15 × 8 × 12 cm) containing 1 L of dechlorinated water. In the negative control, no fish received the treatment. The tests were performed in triplicate. The fish were fed with tropical fish feed (Tetra-

Table 1
Nanoemulsion formulations of *X. ochranta* essential oil.

Formulations	O.P. (%, p/p)	A.P. (%, p/p)	Essential oil (%, p/p)	Polysorbate 20 (%, p/p)	Sorbitan monooleate 80 (%, p/p)
1	10.0	90.0	5.0	5.0	0.0
2	10.0	90.0	5.0	4.5	0.5
3	10.0	90.0	5.0	4.0	1.0
4	10.0	90.0	5.0	3.5	1.5
5	10.0	90.0	5.0	3.0	2.0
6	10.0	90.0	5.0	2.5	2.5
7	10.0	90.0	5.0	2.0	3.0
8	10.0	90.0	5.0	1.5	3.5
9	10.0	90.0	5.0	1.0	4.0
10	10.0	90.0	5.0	0.5	4.5
11	10.0	90.0	5.0	0.0	5.0

*O.P., oil phase; A.P., aqueous phase.

Color Flakes, Tetra) previously triturated two times a day. They received no food 12 h before starting and during the first 12 h of the tests.

Animals were weighed before and after the experiment. The dose corresponding to 200 μg/mL of *X. ochantra* EO contained in NE/animal was administered orally (gavage), with a micropipette containing 4 μL of NEXO/animal. The clinical signs related to balance, swimming behavior, ventilatory function, skin pigmentation, and visible abnormality were observed at the intervals 0, 3, 24, 30, and 48 h after oral administration (OECD, 2019). To verify mortality, the fish were considered dead when there was no response to mechanical stimuli and the operculum movements were not detected. The animals were euthanized at the end of the experiment with a eugenol solution.

2.9. Statistical analysis

Estimating test Chi-squared, LC50 estimations were performed by Probit analysis using the SAS® software (SAS Institute Inc., 2018). ANOVA (two-way) followed by Tukey's test was performed using Graphpad Prism 8®. Differences when $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Essential oil extraction and chemical profile

The chemical characterization allowed the identification of 24 compounds (Table 2), comprising 90.3% of the composition (Fig. 1). The essential oil showed the presence of sesquiterpenes as the main chemical class, representing 69.8% of the components. Among them, 66.7% were shown to be sesquiterpene hydrocarbons, and 3.1% were oxygenated sesquiterpenes. There were also 20.5% of monoterpenes, of which 19.7% were monoterpenes hydrocarbons, and 0,8% were oxygenated monoterpenes.

The major compounds were Germacrene D (17.8%), Bicyclogermacrene (17.4%), and δ-elemene (13.9%). Together, they represented almost 50% of the essential oil chemical composition. β-Caryophyllene (6.9%), β-phellandrene (5.3%), Sabinene (4.6%), and β-pinene (4.5%) were also significant in this sample. *X. ochrantha* is a plant rich in hydrocarbon terpenoids. They are mostly sesquiterpenes, although monoterpenes are a relevant part of this EO's chemical profile. Sesquiterpenes are the most varied terpenoids. Their different chemical structures arouse great interest among researchers. As well as their structures, their properties are also quite varied and even involve repellent activity and natural pesticides. This circumstance points to a potential for biological activity in this species. These metabolites are lipophilic and volatile. Because of this, they can penetrate the insect's body and cause metabolic changes (Senthil-Nathan, 2020; Vera et al., 2019).

Table 2
Chemical profile of essential oil of *Xylopia ochrantha* (leaves).

RI EXP	RI LIT	Substances	%
935	932	α-Pinene	2.7
975	969	Sabinene	4.6
979	974	β-Pinene	4.5
1007	1002	α-Phellandrene	1.7
1030	1025	β-Phellandrene	5.3
1048	1044	(E)-β-Ocimene	0.9
1179	1174	Terpinen-4-ol	0.8
1335	1340	δ-Elemene	13.9
1377	1374	α-Copaene	1.2
1394	1389	β-Elemene	1.7
1421	1417	β-Caryophyllene	6.9
1435	1434	γ-Elemene	1.2
1448	1448	*cis*-Muurola-3,5-diene	0.7
1453	1452	α-Humulene	1.1
1461	1458	allo-Aromadendrene	0.6
1478	1478	γ-Muurolene	1.3
1483	1480	Germacrene D	17.8
1492	1493	*trans*-Muurola-4 (14),5-diene	0.6
1499	1500	Bicyclogermacrene	17.4
1525	1522	δ-Cadinene	1.4
1544	1542	*cis*-Sesquisabinene hydrate	0.9
1578	1577	Spathulenol	1.5
1585	1586	Gleenol	0.9
1592	1590	Globulol	0.7
		Total Identified	90.3
		Monoterpenes hydrocarbons	19.7
		Oxygenated Monoterpenes	0.8
		Sesquiterpenes hydrocarbons	66.7
		Oxygenated Sesquiterpenes	3.1

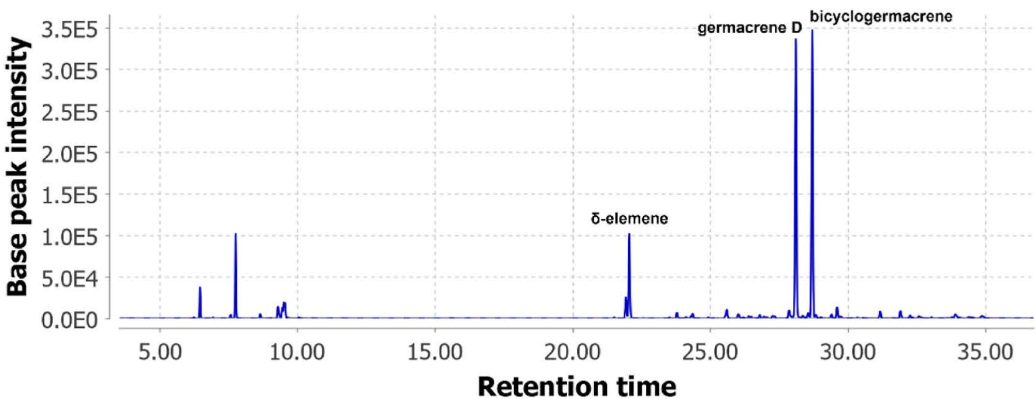

Fig. 1. *Xylopia ochrantha* leaves essential oil chemical profile by GC-MS.

Hydrodistillation of *X. ochrantha* dried leaves yielded 0.82% (w/w) of transparent essential oil. This process is widely used to extract EOs and has been the first choice by many researchers (Machado et al., 2023; Anjos et al., 2022; Folly et al., 2021; Oliveira et al., 2017). In this way, volatile compounds can be preserved. One of the advantages of this methodology is to avoid overheating the plant material and to use low-cost equipment (Kant and Kumar, 2022). Araújo and coworkers (2019) obtained a yield of 0.2% (w/w) from fresh leaves of *X. ochrantha*. The differences between the results are due to the state of the material used at the extraction time. Hydrodistillation made from dried leaves had a higher yield than fresh leaves. Santos et al. (2004) pointed out that the yield of essential oil extracted from plant biomass can be calculated based on dry material or moisture-free basis (MFB) and moist material or moist basis (MB). One point that stands out is that the method using the MFB is standardized and can be repeated at any time without significant deviations. In contrast, the MB method is imprecise, has no reproducibility, and induces significant variations due to not considering the true amount of dry biomass used. That led us to believe we made the right choice to dry the plant material.

3.2. Nanoemulsification method and relative hydrophilic-lipophilic balance (HLB)

NEs' preparation method involved low energy, organic solvent-free, and no use of heat. The surfactants used are biodegradable. According to Folly et al. (2021), this method has more advantages than the high-energy input because it minimizes the chances of degrading or volatilizing natural substances from the EO. The simplicity of preparation, low cost, and the ability of the method to produce smaller droplets are advantages of the low-energy input method (Pavoni et al., 2019). Small details such as low electricity consumption and biodegradable components make the process more sustainable and attractive. Because of that, it is considered an eco-friendly method. This approach has recently aroused great interest in research due to its easy reproducibility on an industrial scale (Solans and Solé, 2012).

All formulations were prepared using different blends of surfactants to establish the best combination between them. For an adequate formulation, the right selection of surfactants is a critical step (Rai et al., 2018; Singh et al., 2017). Another criterion chosen by us in the experimental design was the proportion of surfactants used. The amount of surfactants added to the formulation corresponded to the amount of EO used. Thus, the ratio of surfactant to oil (SOR) was 1:1, i.e., (SOR) = 1, an important parameter for obtaining nanoemulsions (Ostertag et al., 2012). The main collective parameters to characterize a formulation as nanoemulsion are droplet size, and polydispersity index (PDI). The best combination of these characteristics indicates kinetic stability (Oliveira et al., 2017). Several authors have described that a nanoemulsion must have nanodroplets in the range of 20–200 nm and PDI <0.3 to be considered a monodisperse system (Matosdos et al., 2020). These were this study's criteria for considering a formulation as a nanoemulsion.

In this study, 11 formulations were prepared, the formulations 1–4 showed a bluish reflection characteristic of Tyndall's effect in nanodispersed systems. The quantification of formulation sizes resulted in average droplet sizes between 75 and 303 nm (Table 3). Formulations 2 and 3 were considered nanoemulsions for fitting the criteria established above. The most suitable composition for the *X. ochrantha* essential oil was formulation 3 with the ratio of polysorbate 20 and sorbitan monooleate 80 of 4:1. This proportion indicates a better capacity to reduce the interfacial tension of the droplets from the oil and water (Fig. 2). It presented the smallest droplet size (74.56 ± 1.93 nm), and 0.271 polydispersity index. The EO HLB was 14.22, suggesting the oil's hydrophilic nature (Marhamati et al., 2021). The influence of the surfactant type and the oil's hydrophilic-lipophilic balance interferes with nanoemulsion formation

Table 3
Droplet size, polydispersity index (PDI), and hydrophilic-lipophilic balance (HLB) values of NE made with *X. ochrantha* E.O.

Formulation	Droplet size (nm)	PDI	HLB
1	303.0 ± 6.809	0.241 ± 0.042	16.70
2	147.7 ± 2.301	0.292 ± 0.007	15.46
3	74.5 ± 1.939	0.271 ± 0.007	14.22
4	244.4 ± 3.585	0.373 ± 0.027	12.98

Fig. 2. *Xylopia ochrantha* nanoemulsion (formulation 3).

due to the surfactants' amphiphilic character that decreases the surface tension between the two immiscible phases. Consequently, nanoscaled droplets, spread in a monomodal pattern along the surface through Brownian motion, can overcome the force of gravity, leading to kinetic stability that prevents phenomena such as sedimentation, creaming, or coalescence, from occurring (Pavoni et al., 2019; Rai et al., 2018).

The other formulations (5–11) were discarded from de DLS analysis due to their macroscopic characteristics, indicating a system containing larger droplets, milky and turbid appearance, absence of blue reflection, and/or showing phase separation after preparation.

Colloidal dispersions are considered the most versatile tool in modified-release system research (Pavoni et al., 2019). The surface properties and nano-scale of droplets are of great significance in amplifying these products' physical and biological behavior (Sharma et al., 2020). According to that, NEs prepared with EOs are considered a novel sustainable approach to control the mosquito *Ae. Aegypti* due to enabling lipophilic matrices in aqueous media.

3.3. Nanoemulsion stability

Fig. 3 shows the thermic stress test in NEXO as a function of temperature and its influence on droplet size and PDI. The NEXO showed no droplet size or PDI alterations up to 45 °C (84 ± 2 nm, and 0.26 ± 0.01 PDI). After that, a slightly decreased tendency in the nanodroplet size of 76.73 ± 1.36 nm at 55 °C, and 72.14 ± 1.60 nm at 65 °C was observed. The PDI values were stable at 0.24 for both temperatures. When the temperature was raised to 75 °C the mean size again decreased to 67.48 ± 0.65 nm with PDI value increasing to 0.25. This behavior may be associated with the Ostwald ripening (OR) effect, the main phenomenon involved in the essential oil NEs destabilization (Pavoni et al., 2019).

At the first moment, the slight decrease in droplet size observed in the thermal stress study could be explained by the functionalized oxygenated terpenoid fraction (4%) of the essential oil becoming more soluble in the aqueous phase due to the continuous in-

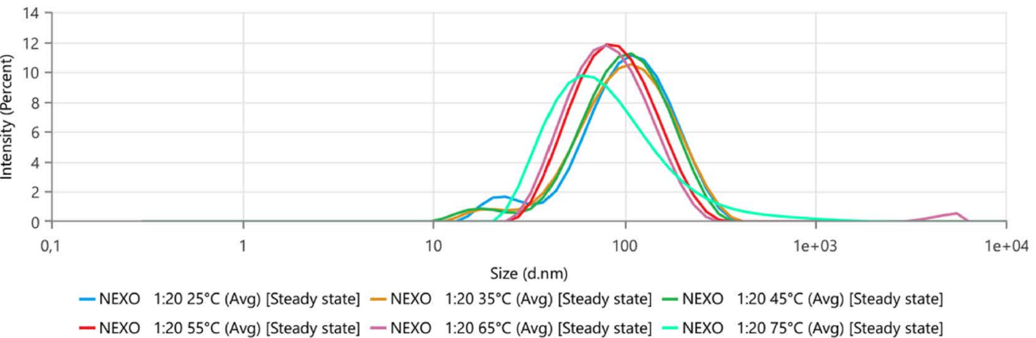

Fig. 3. *Xylopia ochrantha* nanoemulsion (NEXO) size distribution by intensity thermal stress stability.

crease in system temperature, as the HLB 14.2 suggests a relatively hydrophilic characteristic to the *X. ochrantha* essential oil (Marhamati et al., 2021). Then as the dispersed system cools down to room temperature (25 °C), the formerly aqueous phase soluble fraction will tend to mass transfer into more lipophilic droplets of a larger radius. That resulted in decreased droplet size homogeneity, becoming a more polydispersed system, as observed in the post-heated NEXO droplet size of 72.31 ± 1.50 nm, and 0.295 ± 0.009 PDI after returning to 25 °C (Table 4) (Marhamati et al., 2021; Koroleva et al., 2021).

Despite the tendency for the nanoemulsion to destabilize at higher temperatures (>65 °C), NEXO can be considered a stable dispersed system when occasionally exposed to temperatures above room temperature (25 °C). Thus, it is worth mentioning that the shelf life of the *X. ochrantha* nanoemulsion may decrease when stored at higher temperatures due to effects such as the OR collision of the nanodroplets by the accelerated Brownian motion (Cossetin et al., 2021). Additionally, there was no difference in the macroscopic characteristics of the NE (bluish reflection, transparency) after heating.

Regarding the NEXO stability through time, Table 5 compares the mean size (nm), PDI, and ZP of a nanoemulsion after preparation and other stored at room temperature (25 °C) for 180 days. Despite, the size, PDI and ZP of the nanoemulsion freshly prepared showed a statistical difference concerning the NE stored at room temperature (25 °C) after 180 days ($p < 0.05$). The NEXO may be considered a stable kinetic stable colloid system once, after 180 days, it did not alter the macroscopic characteristics and maintained the nanoemulsion's collective main parameters in an acceptable range of values (size < 200 nm, < 0.3 PDI, and ZP ± 5 mV).

The surface charge is an important parameter in the evaluation of nanoemulsion stability. Zeta potential (ZP) values of ±30 mV indicate good stability (Rai et al., 2018). In this sense, after preparation, the X. ochrantha NE showed mean ZPs −25.15 ± 0.65 mV. In addition, when compared to the nanoemulsion stored at room temperature (25 °C) after 180 days showed a ZP of −19.38 ± 0.46 mV. Also, the NE ZP before thermal stress (ZP: −26.71 ± 1.09) showed no variation compared to after stress (ZP: −24.56 ± 1.51). When the ZP values vary ±5 mV indicates rapid droplet aggregation. In general, NEXO provided satisfactory ZP values suggesting efficient coulombic repulsion between the nanodroplets contributing partially to the stability over the time observed (Rai et al., 2018).

3.4. Larvicidal assay

NEXO was assayed against 3rd instar *Ae. Aegypti* larvae to evaluate its potential lethality. This strategy ensures that their reproductive cycle is not reached. The negative control showed 0% mortality, and the white control showed low rates from the second day onwards. The positive control (imidacloprid) at 0.9 μg/mL presented 100% mortality in 24 h. After 48 h of exposition, the 400 μg/mL dilution had a mortality rate of 83.3%, the 300 μg/mL dilution was 63.3%, the 200 μg/mL dilution was 53.3%, and the 100 μg/mL dilution was 23.3%. It is possible to observe how continued exposure to the product increased the toxic effects on larvae. The NEs at all concentrations were statistically significant compared to all control groups, except for the nanoemulsion at 100 μg/mL ($p > 0.05$). The LC_{50} was 192.5 (146.6–238.5) μg/mL within 48 h (Fig. 4).

Among the many studies published to verify the bioactivity of EOs, few use nanostructured systems, such as NEs. Generally, the authors use DMSO, solvents, and surfactants to enable the diffusion of the EOs in water or in a water-soluble medium (Nascimento et al., 2017). Our work was not limited to dispersing EOs in water using these technical adjuvants. NEXO was developed for this purpose. The NE circumvents the volatility characteristic of EOs. This technology can gradually release its actives, to promote a sustained effect. Within the concept of integrated practices for vector control, NEXO may be a tool to be used in this type of management (Sharma et al., 2020; Bobo et al., 2016). The development of NEs promotes modifications in surface properties such as charge, permeability, thickness, rheology, and, most importantly, environmental response capacity (Rai et al., 2018).

In a total of 90.3% of components identified in this essential oil, the three majority components were the Sesquiterpenes hydrocarbons D-germacrene (17.8%), Bicyclegermacrene (17.4%) and δ-Elemene (13.9%). These 3 components, added together, accounted for 49.1% of all the essential oil components. For this reason, we believe these substances are responsible, at least partly, for the larvici-

Table 4
Xylopia ochrantha nanoemulsion heating (25–75 °C) influence on mean size (nm), polydispersity index (PDI), and zeta potential (ZP).

Thermal stress stability			
Thermal stress	Average size (nm)	PDI	ZP
Before	84.41 ± 1.25	0.264 ± 0.017	−26.71 ± 1.09
After	72.31 ± 1.50	0.295 ± 0.009	−24.56 ± 1.51

*PDI: polydispersity index; ZP: zeta potential.

Table 5
Xylopia ochrantha nanoemulsion (formulation 3) stability after 6 months of preparation.

Long term stability			
Days	Size (nm)	PDI	ZP
0	86.62 ± 0.37	0.266 ± 0.017	−25.15 ± 0.65
180	69.77 ± 0.72	0.235 ± 0.018	−19.38 ± 0.46

*PDI: polydispersity index; ZP: zeta potential.

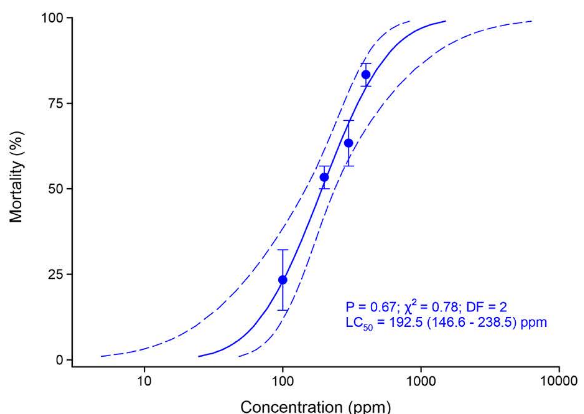

Fig. 4. Dose-response curve of *X. ochrantha* against *Ae. Aegypti* in 48 h.

dal activity found. That hypothesis is suggested by published data (Santana et al., 2015; Bortolucci et al., 2019). In addition, Albuquerque et al. (2022) highlight that β-germacrene-D-4-ol, showed larvicidal activity and oviposition deterrence.

EOs can be used as environmentally sustainable alternatives to synthetic insecticides. The new trend in the search for insecticides that fit into the eco-friendly concept lies in nano products (Senthil-Nathan, 2020). Specially NE prepared with biodegradable surfactants appear as a promising alternative. These products have a high potential to be used in many applications, easiness of formulation, and industrial scale-up (Pavoni et al., 2019). Another advantage is the reduced costs and biodegradable raw materials added to the preparation with low-energy methods. Moreover, sustainable biodiversity management can obtain a nanoemulsion made with leaves of X. ochrantha. When proposing applied research with vegetal species, as is the case of the *restingas*, it is important to carry out management that preserves the concept of standing trees (Oliveira et al., 2017) to obtain the plant material. This is what happens when leaves are used to extract EOs from this environment. Such sustainable management does not require the felling of trees to obtain plant material. Another point is that *restingas* are often the target of real estate development, and valuing the biodiversity of this ecosystem through promising products is a beautiful contribution of science to the preservation of the environment.

Although there is not much data described on larvicidal activity of the genus *Xylopia,* some studies can be found. One of them shows larvicidal action of *X. frutecens* and *X. laevigata* against *Ae. Aegypti* (L3). The authors concluded that the genus did not show larvicidal activity at concentrations below 1000 μg/mL (Nascimento et al., 2017). In our work, nanoemulsion of *Xylopia ochrantha* leaves essential oil showed lethality at 192.5 μg/mL concentrations against the same target. It suggests that it is a species of this genus with promising potential against mosquitoes.

3.5. Acute oral toxicity in Danio rerio

D. rerio, indicated as an animal model for toxicological (OECD, 2019) and ecotoxicological (ABNT, 2016) studies, was used as a non-target aquatic organism. The applied dose (200 μg/mL) corresponds close to NEXO LC_{50} after 48 h (192.5 μg/mL) facing the third instar *Ae. Aegypti* larvae. This way, it was possible to correlate the quantity needed to obtain a larvicidal effect and its toxic effect on non-target organisms.

As a result, no deaths occurred. No clinical signs indicating changes in parameters related to balance, swimming behavior, ventilatory function, skin pigmentation, visible abnormality (OECD, 2019), and weight were observed at the end of 48 h of observation. It was found that under the study conditions, NEXO is not toxic to *Danio rerio*, at the same concentration that causes 50% of mortality in 3rd instar *Ae. Aegypti* larvae. This makes it possible to prospect for other organisms besides aquatic ones. This study indicates no toxicity in mammals, with a concrete result. Still, other studies should be conducted on the same or other models to confirm the safety observed in this experiment.

4. Conclusions

As long as we know, a nanoemulsion made with the essential oil from the leaves of *X. ochrantha* was tested for the first time against 3rd instar *Ae. Aegypti* larvae. The present research also showed that essential oils and nanotechnology could be used in the service of human being to face many adversities that devastate the world and harm people's quality of life.

Towards sustainability NE has been tested against the zebrafish model and is safe for use in this non-target aquatic organism. In addition, this type of research enables the development of innovative products that are maybe safer for human use and less harmful to the environment. New nano products have been developed to adapt to a new world order, which considers sustainability in its different spheres. Besides, this way points to a future of sustainability, as it proposes sustainable management of this ecosystem that preserves the environment.

The NE obtained showed desirable characteristics, nanodroplets below 100 nm, monodisperse and stable long-term. Our results indicate the possibility of developing eco-friendly products to assist in controlling the vector-borne *Ae. Aegypti* and supporting the public health against several arboviruses.

Author statement

Valéria Viana: Investigation, Writing – Original Draft. **Francisco Machado:** Writing Review & Editing, Visualization. **Ricardo Esteves:** Formal analysis, Writing Review & Editing. **Andres Duarte:** Visualization. **Jairo Enríquez:** Investigation, Methodology. **Milton Campaz:** Investigation, Formal analysis. **Eugenio E. Oliveira:** Investigation, Formal analysis. **Marcelo Santos:** Investigation, Data curation. **Eduardo Ricci:** Conceptualization, Methodology. **Bettina Ruppelt:** Investigation, Funding acquisition. **Leandro Rocha:** Project Administration, Funding acquisition, Investigation, Writing Review & Editing.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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8.3 Artigo 4

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

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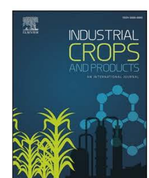

Nanoemulsion of *Ocotea indecora* (Shott) Mez essential oil: Larvicidal effects against *Aedes aegypti*

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A R T I C L E I N F O

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A B S T R A C T

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.

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

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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 10–15 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). They are kinetically stable and thermodynamically unstable, with droplet size between 20 and 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 ("22°12.683′S", "41°35.283′O", "22°12.703′S" and "41°35.336′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 h. The essential oil obtained was stored at 4 °C 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 min. 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.

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 1 h, 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 sirup (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 h, bees that were fed the nanoemulsion-contaminated diet received an uncontaminated diet (sugary solution only), and mortality was recorded 24 h 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

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

design and the responses in the dependent variable's droplet size and PdI. Fig. 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, Fig. 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 (Fig. 2A and D). The RPM did not influence the diameter of the droplets or PdI but indicated better values with lower RPM (Fig. 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. Fig. 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.

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.

3.5. *Transmission electron microscopy (TEM)*

Fig. 3 B 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.

3.6. *Larvicidal activity against Aedes aegypti*

Fig. 4 A 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 μg/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 μg/mL (LT_{50} = 96 [92 –100] h) and 200 μg/mL (LT_{50} < 24 h). As demonstrated in (Fig. 4B), further estimations of NEOI-OPT toxicity were achieved when the exposure periods were 48 h and 144 h, resulting in median lethal-concentration (LC_{50}) of 26.8 (21.5–32.6) μg/mL for the exposure period of 144 h and of 61.4 (52.4–72.0) μg/mL when the larvae were exposed to 48 h (Table 4).

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 Fig. 5D, E, and F. Control *Ae. aegypti* larvae showed no alteration in morphology after the incubation period (Fig. 5A, B, and C).

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

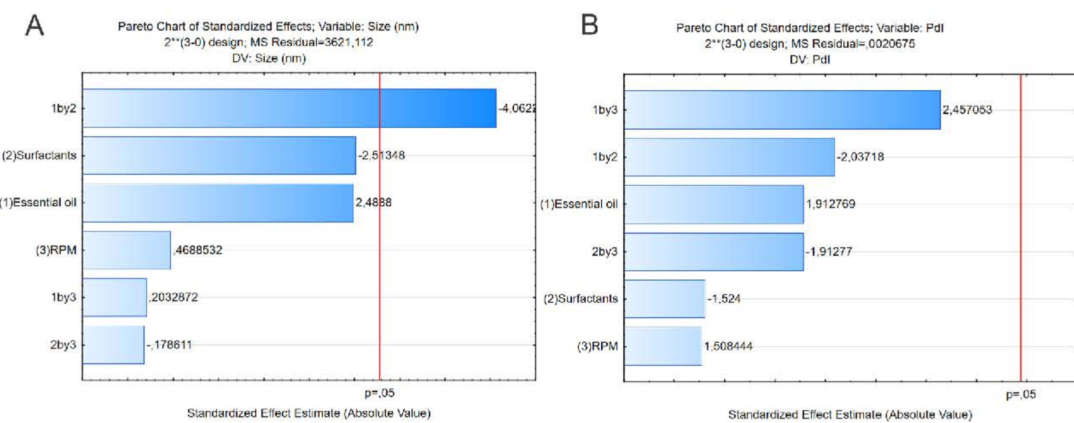


Fig. 1. Pareto charts for the variable (A) droplet size and (B) polydispersity index.

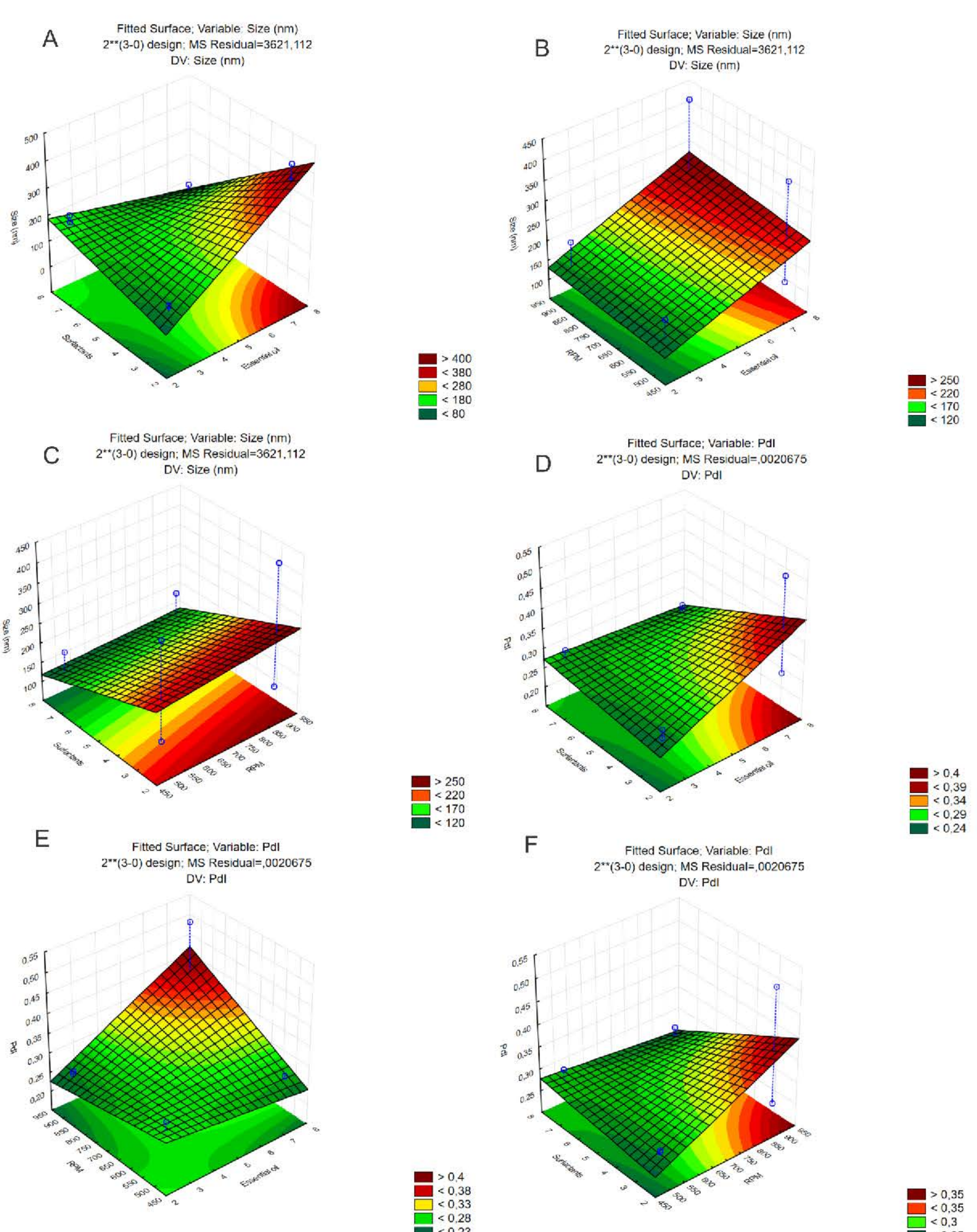
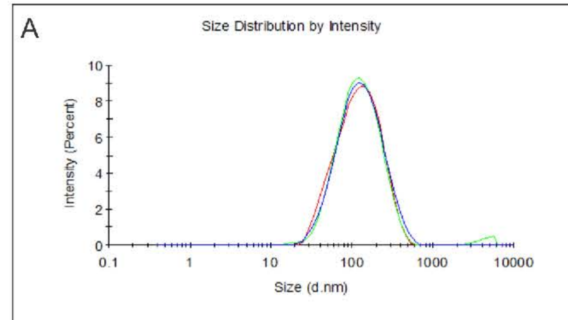


Fig. 2. Surface graph for (A, B, and C) mean size (nm) and (D, E, and F) polydispersity index from the 2^3 experimental designs.

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 (Fig. 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 (Fig. 6B).

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



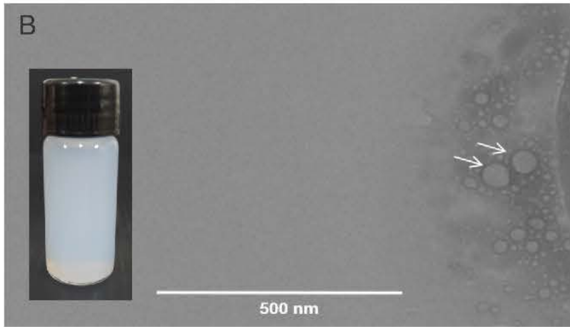


Fig. 3. (A) NEOI-OPT size distribution by intensity; (B) TEM image of the NEOI-OPT, arrows show spheric droplets with approximately 100 nm.

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).

based on approximately four times the LC_{50} after 24 h. There was 100 % of survival after 24 h in the groups treated with the NEOI-OPT, control, and nanoemulsion blank.

4. 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 (Gonçalves et al., 2018). However, Nascimento et al. (2020) and Figueiredo 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 Figueiredo 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 (Fig. 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

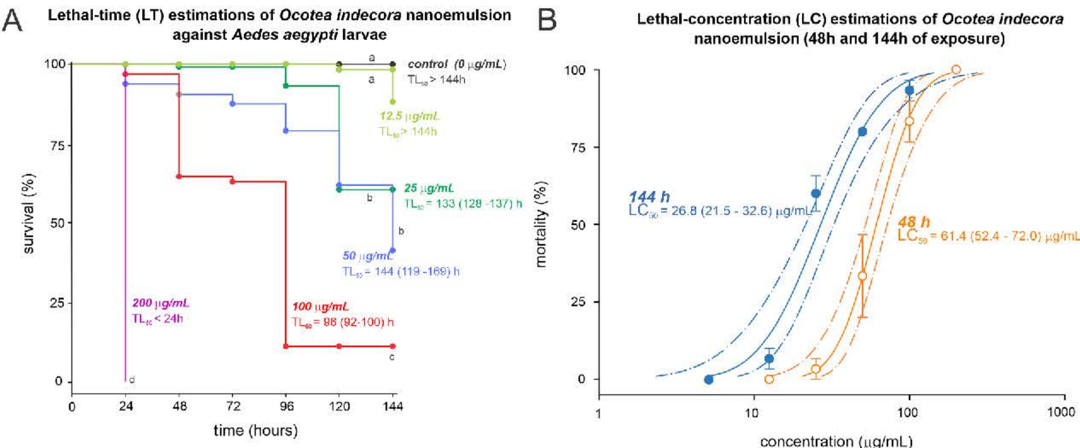


Fig. 4. Lethal-time (A) and lethal concentrations (expressed in essential oil) (B) of optimized *Ocotea indecora* nanoemulsion in *Aedes aegypti* larvae.

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

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 (Fig. 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

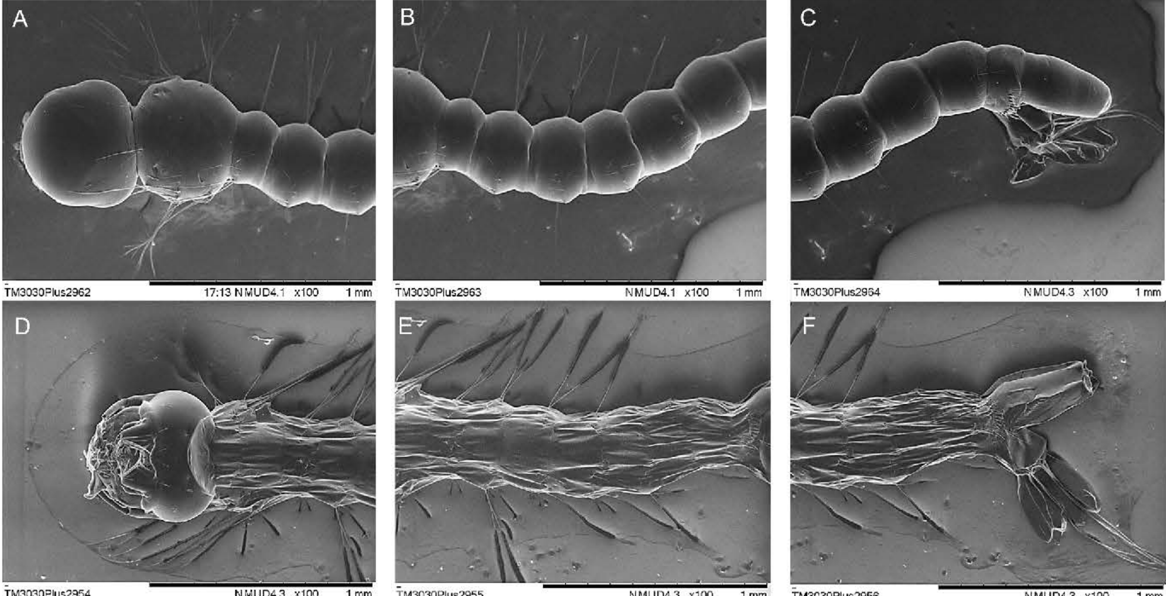


Fig. 5. Scanning electron micrograph of *Ae. aegypti* larvae (L3) without treatment showing no alteration on the head and thorax (A), abdomen segments (B), siphon, and anal papillae (C). Larvae exposed to the NEOI-OPT at 250 µg/mL showed cuticle alterations on the head and thorax (D), abdomen segments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

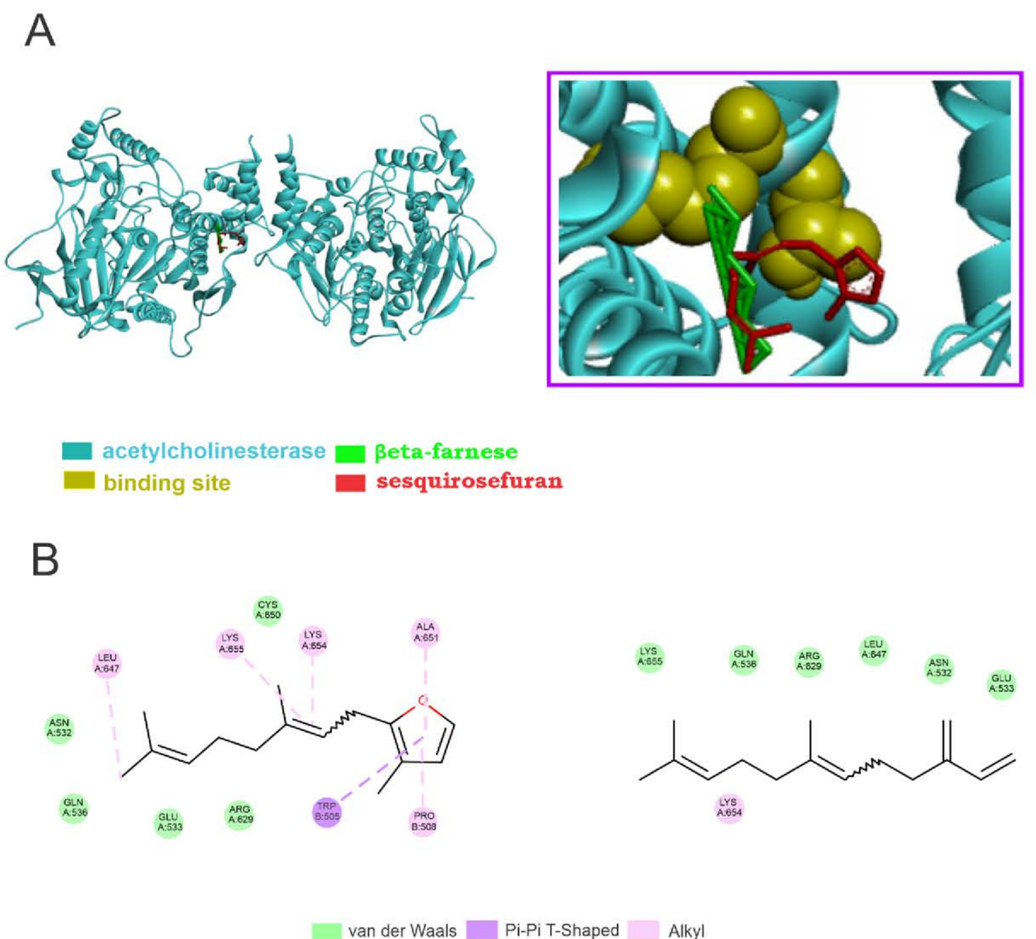


Fig. 6. *Ocotea indecora* major constituents bind to acetylcholinesterase enzyme related to *Aedes aegypti* (A) Docking views of the sesquirosefuran and β-farnesene with acetylcholinesterase binding sites. (B) 2D interaction maps of acetylcholinesterase enzyme interaction sites with sesquirosefuran,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

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 (Fig. 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 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 48 h to 26.8 μg/mL after 144 h). 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 h. 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 the *Aedes aegypti*.

CRediT authorship contribution statement

Francisco P. Machado: Conceptualization, Investigation, Methodology, Funding Acquisition, Supervision, Writing-Reviewing, and Editing; **Diogo Folly:** Investigation; **Jairo J. Salas Enriquez:** Investigation, formal analysis; **Cícero B. Mello:** Investigation, formal analysis, Funding Acquisition; **Ricardo Esteves:** Investigation; **Raquel S. Araújo:** Investigation; **Pedro F. S. Toledo:** Investigation, formal analysis; **Javier G. Mantilla-Afanador:** Investigation, formal analysis; **Marcelo G. Santos**: Investigation, formal analysis; **Eugenio E. Oliveira:** Investigation, formal analysis, Funding Acquisition, supervision; **Eduardo Ricci Junior:** Investigation, formal analysis, Funding Acquisition, supervision; **Leandro Rocha:** Conceptualization, Investigation, Funding Acquisition, Software, Supervision, Writing-Reviewing, and Editing.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2022.116031.

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8.4 Artigo 5

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

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

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ABSTRACT

This work describes the chemical composition of the essential oil from *Annona acutiflora* and its larvicidal nanoemulsion. The volatile oil was extracted by hydrodistillation. The chemical characterization/quantification was performed by gas chromatography, revealing α-santalene, Bicyclogermacrene, and α-zingiberene as the major compounds. The nanoemulsion was prepared by low energy method. It was assayed against *Aedes aegypti* larvae, and *in vitro* anticholinesterase assay was carried out. This nanoemulsion presented a droplet size around 170 nm and PdI value around 0.170. The assay against larvae showed LC_{50} and LC_{90} values after 48 h, respectively, to 21.3 and 37.1 ppm. The anticholinesterase assays showed an IC_{50} value around 295.6 ppm. These results demonstrate that the method satisfactorily generated fine liquid oily nanodroplets dispersed in aqueous media. Moreover, to our knowledge, this is the first report of the chemical composition-from this species, being of great interest for new further integrative practices of vector control.

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1. Introduction

The World Health Organization (WHO) classifies dengue, Zika, and Chikungunya as neglected tropical diseases. The difficulty of epidemiological data is a consequence of global underreporting. Among American countries, Brazil has the highest number of confirmed Zika, Chikungunya, and dengue fever. These diseases also can be correlated with other syndromes, as can be observed in the case of Zika and microcephaly in new-borns (1,2).

Synthetic insecticides have been used for the control of the mosquito *Aedes aegypti* (Linnaeus). However, the development of resistance and contamination of the environment by these insecticides makes it necessary to find other safer and more effective ways to restrain this insect. Bioinsecticides, such as those prepared with essential oils, are considered an option to control the mosquito *Ae. aegypti*. The advantages of essential oils include quick degradation in the environment, relatively low toxicity in mammals, and less mosquito resistance (3–5).

The life cycle of *Ae. aegypti* consists of four basic stages: egg, larva, pupa, and adult (6). Among these stages, three of them occur in the water. For this reason, the preparation of nanoemulsions is viable for the dispersion of essential oils in aqueous matrices. In this context, a nanoemulsion can improve essential oils' bioactivity and stability as a bioinsecticide product (5,6). This formulation design may also induce a controlled release and remain the physical characteristics of the botanical material used in its production (7).

The Annonaceae family is represented by 29 genera, with 392 species in Brazil being one of the most prominent families among the angiosperms (8). The *Annona* genus presents a considerable wealth of species principally in the Amazon region and the Atlantic Forest. It is known for its edible fruits, like custard apple (*A. squamosa*) and soursop (*A. muricata*). Furthermore, some species supply carpentry wood and usable roots as cork (*A. glabra, A. crassiflora*); others species are considered medicinal (*A. spinescens, A. foetida*), ornamentals (*A. cacans*), and the essential oils demonstrated different pharmacological activities like anticancer (*A. pickelii, A. salzmannii* and *A. muricata*), antifungal (*A. salzmannii*), anti-inflammatory (*A. sylvati ca*); other species were utilized for the dengue vector control activity (*A. crassifora, A. glabra*, and *A. muricata*) (8–10).

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Annona acutiflora Mart., an endemic species from Brazil, and commonly known as Araticum or Guiné (11), is distributed in the Brazilian states of Bahia, Espírito Santo, and Rio de Janeiro (12). Its leaves contain the essential oil located in the spongy parenchyma, while the palisade parenchyma stores more mucilage (13). Furthermore, it is used in quilombola communities as ritualistic and a potentially medicinal species to treat inflammation and stomach pains (14).

This work describes for the first time the chemical composition of the essential oil of *A. acutiflora* leaves and the obtainment of its green nanoemulsion with larvicidal activity.

2. Materials and methods

2.1. Plant material

Aerial parts of six different specimens of *Annona acutiflora* were collected in Brazil, at the municipality of Carapebus (Rj) at Restinga de Jurubatiba National Park, at the following coordinates: 22°12.593'S–41°35.373′W; 22°12.690'S-41°35.234′W; 22°12.698'S-41°35.289′W; 22° 12.687'S–41°35.289′W; 22°12.669'S–41°35.251′W; 22° 22.699'S–41°35.273′W. The botanist Dr. Marcelo Guerra Santos identified the plant material and deposited a voucher specimen (register number RFFP 13.789) at Faculdade de Formação de Professores (UERJ, Brazil). The studies were carried out according to the authorization from SISBIO/ICMBio under the register number 13,659–12 and SisGen number A0D648D.

2.2. Essential oil extraction

The fresh leaves of *Annona acutiflora* (2893 g) were fragmented with distilled water. Then, the plant material (413 g) was placed in a 5 L distillation flask with 2 L water. The material was subjected to extraction by hydrodistillation using a Clevenger type apparatus for 4 h. This method was repeated 7 times to extract all plant material. After the extractions, the essential oil was collected, filtered over anhydrous sodium sulphate, and stored in an amber flask at 4°C until use.

2.3. Gas-chromatograph analysis

The essential oil was analysed using a GC-MSQP2010 (Shimadzu) gas chromatograph, equipped with a mass spectrometer and a GC-2014 (Shimadzu) gas chromatograph equipped with a flame ionization detector (FID).

GC-MS conditions were as follows: injector temperature 260°C, carrier gas Helium; flow rate 1 ml/min and split injection with a split ratio of 1:40. The oven temperature was initially 60°C and then increased to 290°C at a rate of 3°C/min. One microliter of the essential oil dissolved in dichloromethane (1:100) was injected into an RTX-5 column (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.

GC-FID conditions included injector temperature 260°C, carrier gas Helium, flow rate 1 ml/min, and split injection with a split ratio of 1:40. The oven temperature was at first 60°C and then raised to 290°C at a rate of 3°C/min. One microliter of the essential oil dissolved in dichloromethane (1:100) was injected into an RTX-5 column (0.25 mm ID, 30 m in length, 0.25 µm and film thickness). The flame ionization detector (FID) temperature was 290°C.

The identification of compounds was performed by comparison of Arithmetic Index (AI), determined relative to the retention times of a mixture of a series of aliphatic hydrocarbons (C9 – C30) (15). Identification of substances was accomplished by comparing their retention indices and mass spectra with those reported in the literature (16). 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 (CG/FID) under the same conditions of GC-MS. Analysis and percentages of these compounds were obtained by the FID peak area normalization method.

2.4. Nanoemulsion preparation

The nanoemulsion was prepared by the following composition: 5% (w/w) of essential oil, 5% (w/w) of polysorbate 20, and 90% (w/w) of distilled water. The oily phase (essential oil and surfactants) was kept under constant stirring for 30 minutes. Then, the aqueous phase was added dropwise through a continuous flow rate. Finally, the system was maintained under moderate stirring for 60 minutes more. The final mass of the nanoemulsion was 10 g.

2.5. Characterization of nanoemulsion

Analysis of droplet size distribution of the nanoemulsion was carried out by dynamic light scattering (DLS) using a Zetasizer ZS (Malvern, UK). Samples were diluted in distilled water (1:25), and measurements were performed in triplicate.

2.6. Droplet growth (DG.)

Droplet growth was determined as follows:

DG = 100 x [mean droplet size (d_y) – mean droplet size (d_x)]/mean droplet size (d_x). where d_y is the mean particle size after Y days of storage, and d_x is the initial

mean droplet size on the range of X → Y days. The analysis was performed in triplicate, and the result was expressed as the mean ± standard deviation (17).

2.7. Larvicidal bioassay

Aedes aegypti larvae (Rockefeller strain) were obtained from the Arthropoda Laboratory (Universidade Federal do Amapá, Brazil). The biological assay was performed under controlled conditions, being larvae kept at 25 ± 2°C, the relative humidity of 75 ± 5%, and a 12 h light: dark cycle. Experimental evaluation was performed according to the World Health Organization protocol with some modifications (18). All experiments were performed in triplicate (n = 10) with late-3rd instar larvae in each sample. The nanoemulsion was diluted in distilled water at 12.5, 25.0, and 50.0 ppm (concentration expressed as *A. acutiflora* essential oil content in aqueous media). The control group was constituted by distilled water and polysorbate 20, in the same concentration used to prepare the nanoemulsion. Mortality levels were recorded after 24 and 48 h of exposure.

2.8. Acetylcholinesterase Inhibitory Assay

Anticholinesterase activity was measured using the method described by Ellman (19) with some modifications (20). The essential oil from leaves was dissolved in methanol to produce a sample solution (5 mg/mL). Methanolic dilutions were prepared to obtain different concentrations (10, 50, 100, 200, and 400 ppm) for the assay. The lyophilized enzyme was dissolved in sodium phosphate buffer (100 mM pH 7.5) to produce an 80 U/ml stock solution containing 0.1% of BSA. The enzyme stock solution was kept in the freezer. For the assay, the enzyme stock solution was dissolved in the phosphate buffer. DTNB was dissolved in the same buffer, and ATCI was dissolved in deionized water. The inhibitory assay was performed by adding 340 μL of sample solution to 1660 μL of phosphate buffer and 200 μL of AChE 0.51 U/mL. This solution was incubated for 15 min. Then, 1000 μL of 0.68 mM DTNB and 200 μL of 17 mM ATCI were added. The absorbance was measured at 412 nm for 1.5 min at 0.5 min intervals in Spectrophotometer (Shimadzu UV-1601). No spontaneous hydrolysis of the substrate was observed with the use of this phosphate buffer. The percentage inhibition of enzyme activity was calculated by comparison with the negative control (methanol). Sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentages against essential oil concentrations and calculated using Excel 2003 software (Microsoft Corp., Seattle, WA). Physostigmine, dissolved in methanol, was used as a positive control. Each measurement was made in triplicate.

2.9. Statistical analysis

Estimation of test Chi-squared, LC_{50}, and LC_{90} values were carried out by Probit analysis using the software Statgraphics Centurion XV version 15.2.11 (Statpoint Technologies, Inc., Warrenton, VA). ANOVA (two-way) followed by Tukey's HSD was performed using the software R (R Core Team, 2016), and differences were considered significant when $p < 0.05$.

3. Results and discussions

3.1. Essential oil analysis

The essential oil yield was 0.17% (w/w) and stayed in the genus range, from 0.10% to 0.15% (21). It presented a transparent, slightly yellowish aspect. The essential oil's chemical characterization allowed the identification of 30 compounds, comprising 89.2% of the composition (Figure 1). The essential oil showed the presence of sesquiterpenes as the main chemical class, representing 88.9% of the components. Of these, 83.2% were shown to be sesquiterpene hydrocarbons, and 5.7% were shown to be oxygenated sesquiterpenes. The presence of 0.3% monoterpene hydrocarbons was also detected. The major compounds were the following: α-santalene (15.5%), bicyclogermacrene (12.5%), α-zingiberene (8.7%), (E)-β-farnesene (8.2%), α-trans-bergamotene (6.0%) and germacrene D (5.7%) (Table 1).

Thang *et al.* (22) described the higher proportions of the components in the essential oil of *A. glabra* leaves as β-caryophyllene (21.5%), germacrene D (17.7%), α-cadinol (5.4%), β-elemene (5.2%), and α-phellandrene (4.3%). Besides, the essential oil of *A. muricata* leaves contained the terpenes α-pinene (9.4%), β-pinene (20.6%), ρ-mentha -2,4 (8)-diene (9.8%), β-elemene (9.1%), germacrene D (18.1%) and in *A. reticulata* were germacrene D (22.8%), β-elemene (16.6%) and β-caryophyllene (8.3%).

In another study realized by Garg and Gupta (23), the major terpenes of *A. squamosa* essential oil of leaves were β-caryophyllene (23.0%), germacrene D (21.3%), Bicyclogermacrene (8.5%), and β-elemene (7.8%).

Recently, in a review study of the *Annona* genus by Kusmardiyani (10), major compounds were found as follows: *Annona foetida* – (E)-caryophyllene, Bicyclogermac rene and α-copaene; *Annona pickelii* – Bicyclogermacrene,

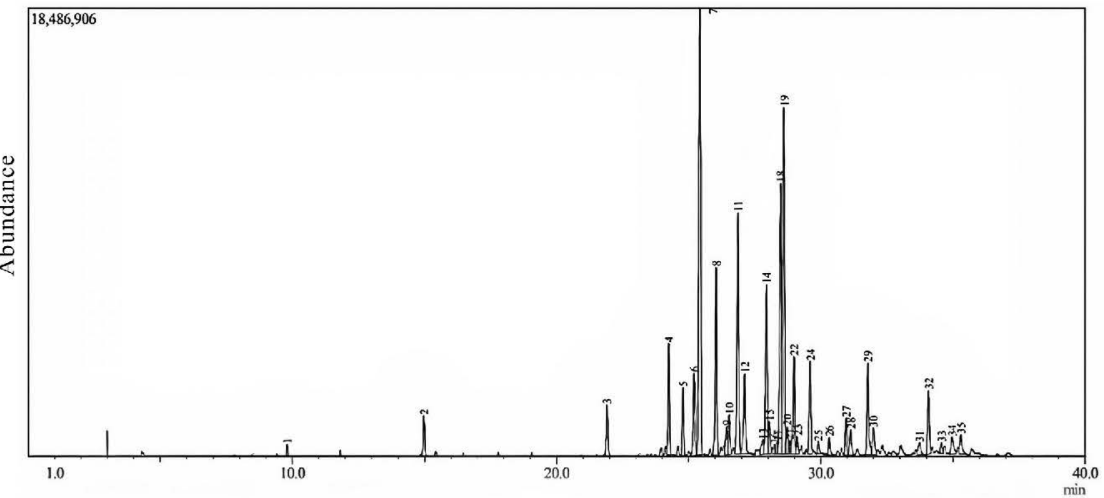

Figure 1. GC chromatogram of the essential oil from *Annona acutiflora* leaves.

Table 1. Chemical characterization of the essential oil from leaves of *A. acutiflora*.

Peak	RI_{lit} RI_{exp}	Compound	%
1	10441046	(E)- β-ocimene	0.3
3	13351338	σ-elemene	1.5
4	13891393	β-elemene	3.4
5	14051405	Sesquithujene	2.0
6	14111416	(Z)-α-bergamotene	2.7
7	14161421	α-santalene	15.5
8	14321436	(E)-α-bergamotene	6.0
10	14451448	β-santalene	1.4
11	14541456	(E)-β-farnesene	8.2
12	14581463	allo-Aromadedrene	3.8
14	14801483	Germacrene D	5.7
17	14921493	σ-selinene	0.5
18	14931496	α-zingiberene	8.7
19	15001499	Bicyclogermacrene	12.5
20	15021502	(E)-β-guaiene	1.0
21	15051505	(E,E)-α-farnesene	0.5
22	15051509	β-bisabolene	3.6
23	15051512	α-cuprenene	0.6
24	15211525	β-besquiphellandrene	3.0
25	15291532	(E)-γ-bisabolene	0.6
26	15321543	γ-cuprenene	0.7
27	15591559	Germacrene B	1.3
28	15611564	(E)-Nerolidol	1.0
29	15771580	Spathulenol	3.4
30	15821586	Caryophyllene oxide	1.3
	Monoterpenes hydrocarbons		0.3
	Oxygenated monoterpenes		-
	Total monoterpenes		0.3
	Sesquiterpenes hydrocarbons		83.2
	Oxygenated sesquiterpenes		05.7
	Total sesquiterpenes		88.9
	Total		89.2

(E)-caryophyllene, σ-cadinene, α-copaene, and allo-aromadendrene; *A. reticula* – (E, E)-farnesyl acetate, ar-turmerone, benzyl benzoate, and γ-terpinene.

The essential oil of *Annona acutiflora* presents Bicyclogermacrene, germacrene D as major compounds, while and β-elemene, Spathulenol, Caryophyllene oxide were found as minors' compounds, corroborating the previous studies with the essential oil of the genus *Annona* (22,23). However, there are qualitative and quantitative differences between these essential oils. Also, it showed (E)-β-farnesene; meanwhile, other species had (E.E)-farnesyl acetate, a derivative from farnesene (10).

In addition, it is the first time that was described α-santalene and α-zingiberene, as major compounds in essential oil from the leaves of the *Annona* genus.

3.2. Nanoemulsion characterization

In this report, the essential oil was nanoemulsified by low energy, organic solvent-free, and no-heating method. This method has some advantages against the high-energy input. Among them, it minimized chances to degrade or volatilize natural compounds while maintaining their physicochemical properties and being also considered ecofriendly. These characteristics are advantageous when working with essential oil (24,25).

Although some authors suggest that a nanoemulsion must have a droplet size with values less than 500 nm, a suitable criterion indicates an upper limit of 200 nm for size and polydispersity index below 0.3 (26,27). Therefore, the population of *A. acutiflora* essential oil nanodroplets generated in aqueous media can be properly considered a satisfactory nanoemulsion. It shows droplet size values of 171.1 ± 1.159, PdI of 0.171 ± 0.011, and zeta potential of −15.0 ± 0.529 after preparation. After 48 h of storage, the characteristics were also associated with the larvicidal assay's duration time (Table 2).

3.3. Larvicidal assay

The nanoemulsion was assayed against the 3rd instar of *Aedes aegypti* larvae to evaluate its potential lethality. The results are shown in Table 3. After 24 h, the LC_{50} and the LC_{90} were, respectively, 36.0 (23.2–64.0) ppm and 66.1 (48.3–179.8) ppm. After 48 h, the LC_{50} and the LC_{90} were, respectively, 21.2 (11.2–31.8) ppm and 37.0 (28.3–84.1) ppm (Table 4).

The potential of this essential oil after its nanoemulsification can be demonstrated by comparing it with literature data. According to Thang (22), the present study's major compounds were also found in other essential oils from Annona's genus. When the larvicidal assay was realized with the *Annona salzmannii* and *Annona pickelii*, it did not show a significant activity, despite having some of the major compounds of the *Annona acutiflora*, suggesting that the larvicidal activity may be related with minor terpenes in the essential oil (28). Other genus species present larvicidal activity, as shown in the literature (29,30).

Magalhães et al. (2010) described the *Guarea convergens* branches essential oil effects in third instar larvae of *Aedes aegypti* with LC_{50} of 141.1 μg/mL (21). Although the major compound in *G. convergens* (26.3%) essential oil corroborates with the α-santalene predominance in *A. acutiflora* (15.5%) does not ratify the larvicidal activity of the current study (LC_{50} = 36 ppm) after 24 h of exposure. This fact suggests the important role of other compounds in the larvicidal activity of *A. acutiflora* essential oil. Other reports realized by Pavela (2015) and Chung et al. (2020) described the presence of germacrene D in essential oils presenting LC_{50} with values ranging from 10 to 82 ppm in mosquitoes' larvae (31,32). Otherwise, Kiran et al. (2006) verified the LC_{50} of the germacrene D isolate showing 63.6 μg/mL in the third instar of *A. Aegypti* larvae after 24 h (33). The α-zingiberene present in *Zingiber officinale* essential oil showed LC_{50} of 46 ppm (31). The sesquiterpene bicyclogermacrene was described by Govindarajan and Benelli (2016) in *Anopheles subpictus*, *Aedes albopictus*, and *Culex tritaeniorhynchus* larvae with LC_{50} of 10.3, 11.1, and 12.5 μg/mL, respectively (34).

According to Montenegro (35), a larvicidal product is considered promising when the mortality range after 48 h reaches the following parameters: >75% (promising), >50% and <75% (partially promising), >25% and <50% (weakly promising) and <25% (inactive). In this context, this nanoemulsion can be classified as a promising product, presenting 100% of larvae mortality at 50 ppm concentration and LC_{90} of 37 ppm after 48 h of exposure.

Natural products such as extracts or essential oils are a promising alternative to synthetic insecticides (36). They are complex mixtures containing several active

Table 2. Droplet size (nm), polydispersity index (PdI) and Zeta potential of the bionanoemulsion of *A. acutiflora* essential oil after 48 h of preparation.

	0 h	24 h	48 h
Droplet size (nm)	171.1 ± 1.159	224.0 ± 10.460	250.1 ± 4.677
PdI	0.171 ± 0.011	0.235 ± 0.018	0.277 ± 0.011
Zeta potential	−15.0 ± 0.529	−17.8 ± 0.173	−9.41 ± 0.802

Table 3. Average mortality of *Aedes aegypti* larvae treated with the nanoemulsion of *Annona acutiflora* leaf oil.

	Average mortality (%)	
Concentration (ppm)	24 h	48 h
50.0	73.3 ± 0.58	100.0 ± 0.0
25.0	30.0 ± 0.0	56.7 ± 1.52
12.5	16.6 ± 0.58	26.7 ± 0.58
Control	0 ± 0.0	6.7 ± 0.58

Table 4. Lethal concentration (ppm) of the bionanoemulsion from *A. acutiflora* leaves in *Aedes aegypti* larvae.

	LC_{50}	LC_{90}	X^2	*p*
24 h	36.0 (23.2–64.0)	21.2 (11.2–31.8)	7.35114	0.0067
48 h	66.1 (48.3–179.8)	37.0 (28.3–84.1)	14.3505	0.0002

substances with different modes of action (37). That may have a synergistic effect, improving efficiency and decreasing resistance mechanisms insects (36).

3.4. Acetylcholinesterase inhibitory assay

Plants produce essential oils as a chemical defence against insects, usually inhibiting the acetylcholinesterase enzyme, as described by many authors (38). Monoterpenes like 1.8-cineole, camphor, α-pinene, β-pinene, borneol, linalool, menthone, carvone, and anethole are known for their anticholinesterase activity (39,40). To determine the mechanism of insecticidal action of this essential oil, an evaluation of this enzyme's inhibitory activity was carried out. As a result, we obtained an IC_{50} value of 295.6 ppm (278.0–315.8). The enzymatic inhibition assay showed that 10.0 ± 0.7% ($p < 0.05$) of decay in enzyme activity was observed after exposure to 200 ppm of the essential oil. In a previous paper, the *Annonaceae* species *Pseuduvaria macrophylla*'s essential oil also showed a weak anticholinesterase inhibition of 32.5% with a 1000.0 ppm concentration (39). The *A. acutiflora* essential oil demonstrated acetylcholinesterase inhibition in a concentration eight times greater than the larvicidal activity. This result suggests that the larvicidal mechanism of action of the nanoemulsion from *Annona acutiflora* may not be through the inhibition of the acetylcholinesterase enzyme. Another possible mechanism of action associated with terpenes' insecticidal activity that constitutes essential oils is related to GABA, octopaminergic systems (41).

4. Conclusion

This is the first report of the chemical composition from the essential oil of *Annona acutiflora* leaves, which was used to prepare a larvicidal potential nanoemulsion against *Aedes aegypti* larvae (LC_{50}48 h = 21.2 ppm). The utilization of low energy, non-heating, solvent-free method and the utilization of biodegradable ingredients is under eco-friendly concepts and goals for a sustainable world. Therefore, this study shows the potential of the *A. acutiflora* as a source for a biotechnological product with insecticide activity and opens the perspective for integrative practices of vector control with this kind of herbal colloid.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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8.5 Artigo 6

Phenolic substances and cyanogenesis in galled and non-galled tissue of the fern species *Microgramma vacciniifolia*

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Original Article

Phenolic substances and cyanogenesis in galled and non-galled tissue of the fern species *Microgramma vacciniifolia*

Substâncias fenólicas e cianogênese em tecidos galhados e não galhados da samambaia *Microgramma vacciniifolia*

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Abstract

Galls, neo-formed plant structures that can occur in different organs, are generated by species-specific interaction with an inducing organism. Inducers manipulate the metabolism of its host. *Microgramma vacciniifolia* (Langsd. & Fisch.) Copel. is a Neotropical epiphytic fern that hosted two stem galls, one induced by a midge species (Diptera) and other by a micromoth species (Lepidoptera). The aim of this study was to evaluate the impact of these two gall-inducing insects on the biochemistry of phenolic acids and the cyanogenesis in galls, stems and leaves of *M. vacciniifolia*. High performance liquid chromatography (HPLC) indicated a total of 14 phenol derivatives, including caffeic and coumaric acid. Principal Coordinates Analysis (PCoA) of the phenolic substances indicated three groups consisting (1) non-galled stems and micromoth-induced galls; (2) midge-induced galls; (3) midge-induced galls with parasitoids. Regarding the frequency of cyanogenesis assessed by the picrate paper test, the chi-squared test showed significant difference between fertile leaves (8.3%), sterile leaves (27.7%), non-galled stems (0%) and galls. Among galls, only the midge-induced galls analyzed were cyanogenic (15%). Our results indicated that the different gall-inducers (midge and micromoth) promote species-specific alterations to the phenolic substance composition of the host fern.

Keywords: Cecidomyiidae, chemical ecology, fern-insect interactions, micromoth, midge.

Resumo

Galhas são estruturas vegetais neo-formadas que ocorrem em diferentes órgãos. Elas são geradas por uma interação espécie-específica com um organismo indutor. Os indutores manipulam o metabolismo do hospedeiro. *Microgramma vacciniifolia* (Langsd. & Fisch.) Copel. é uma samambaia epífita neotropical que hospeda duas galhas caulinares, uma induzida por uma espécie de mosquito (Diptera) e outra por uma micromariposa (Lepidoptera). O objetivo deste estudo foi avaliar o impacto desses dois insetos indutores de galhas na bioquímica dos ácidos fenólicos e da cianogênese em galhas, caules e folhas de *M. vacciniifolia*. Em análise de cromatografia líquida de alta eficiência (CLAE) foi possível indicar a presença de um total de 14 derivados fenólicos, incluindo ácido cafeico e ácido cumárico. Análise das Coordenadas Principais (ACoP) indicou três grupos (1) caules não galhados e galhas induzidas pela micromariposa; (2) galhas induzidas pelo mosquito; (3) galhas induzidas pelo mosquito com parasitoides. Em relação a frequência da cianogênese analisada com o teste do papel picrato, o teste do qui-quadrado apresentou diferença significativa entre as folhas férteis (8,3%), folhas estéreis (27,7%), caules não galhados (0%) e galhas. Entre as galhas, somente aquelas induzidas pelo mosquito foram cianogênicas (15%). Os resultados encontrados indicam, ao menos para as substâncias fenólicas, que os insetos indutores de galha (mosquito e micromariposa) promovem alterações espécie-específica na composição química da samambaia hospedeira.

Palavras-chave: Cecidomyiidae, ecologia química, interações samambaias-insetos, micromariposa, mosquito.

1. Introduction

Galls, neo-formed plant structures that can occur in different organs, are generated by species-specific interaction with an inducing organism (Mani, 1964; Isaias et al., 2014). Gall morphology can be considered an extended phenotype of the galler (Nyman and Julkunen-Titto, 2000). Inducers establish a parasitic relationship with

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the host plant and manipulate its metabolism, quantitatively and qualitatively regulating the production of substances to create better food and shelter (Mani, 1964; Nyman and Julkunen-Titto, 2000; Raman, 2007; Guiguet et al., 2016). Phenolic substances, tannins, flavonoids and alkaloids accumulate mainly in the gall outer cortex, as a chemical defense or protection against UV radiation, while sugars and proteins are accumulated in gall tissues around the larval chamber (Isaias et al, 2014).

Santos et al. (2019) report galls on 93 host species, belonging to 41 genera of ferns and lycophytes. In Brazil, galls have been recorded on 21 fern species (Santos and Maia, 2018; Farias et al., 2020; Lehn et al., 2020). Galls have been registered in four *Microgramma* C. Presl (Polypodiaceae) species, *M. squamulosa* (Kaulf.) de la Sota, *M. vacciniifolia* (Langsd. & Fisch.) Copel., *M. percussa* (Cav.) de la Sota (Santos et al., 2019) and *M. mortoniana* de la Sota (Lehn et al., 2020)

Microgramma vacciniifolia is a Neotropical epiphytic fern. Two different galls, one ovoid and the other ellipsoid, have been recorded on the stems of this species. The ovoid gall is induced by a midge known as *Primadiplosis microgrammae* (Cecidomyiidae - Diptera) (Maia and Santos, 2011), which is attacked by two parasitoid wasps: Torymidae and Tetrastichinae (Eulophidae). The ellipsoid gall is induced by the micromoth species *Tortrimosaica polypodivora* (Lepidoptera: Tortricidae) (Brown et al., 2004), attacked by the parasitoid wasp Cheloninae (Braconidae) (Maia and Santos, 2015).

One form of direct or indirect defense or resistance against herbivores in plants is the production of secondary metabolites (Boege and Marquis, 2005). Among plant chemical defenses, cyanogenic glycosides (CNglcs) are recognized as a direct, constitutive plant defensive trait (Kautz et al., 2014). When plant tissues are injured by herbivores, CNglcs (stored in the vacuoles) come into contact with β-glucosidase and α-hydroxynitrile lyases which hydrolyze the CNglcs and release hydrogen cyanide (HCN) in a process called cyanogenesis (Vetter, 2000; Zagrobelny et al., 2004; Oliveros-Bastidas and Alonso-Amelot, 2010), which can be polymorphic in a same cyanogenic plant population (Hegnauer, 1977). HCN is extremely toxic to various organisms, inhibiting, for example, oxygen reduction in cytochromes present in the respiratory electron transport chain (Francisco and Pinotti 2000). However, Zagrobelny et al. (2004) reported that cyanogenic glycosides can act as both feeding deterrents and phagostimulants for specialist herbivores that eat plants containing CNglcs.

Similarly, phenolic substances also play a role in plant defense by repelling herbivores or affecting their digestive systems via toxic and ovicidal substances, as well as components that affect insect photosensitivity (Harborne et al., 1999; Mello and Silva-Filho, 2002; Zagrobelny et al., 2004). Phenolic acids such as *p*-coumaric acid and caffeic acid are frequently found in ferns and other plants (Bohm and Tryon, 1967; Summers and Felton, 1994). The toxicological effect of caffeic and chlorogenic acid against insect herbivores is due primarily to their ability to act as prooxidants, inducing oxidative stress in insect herbivores (Summers and Felton, 1994).

Some studies focus on the chemical strategies used by ferns against herbivores, such as the production of phenolic and cyanogenic substances. Balick et al. (1978) reported condensed tannins in all 26 fern species on which arthropod damage was recorded, and only 3% of 100 species tested positive for cyanogenesis. According to the authors, it is unlikely that they play a significant role as defensive substances in the ferns examined. However, Cooper-Driver and Swain (1976) suggest that cyanogenic polymorphism in bracken fern (*Pteridium aquilinum* (L.) Kuhn) has a positive role in determining the degree of herbivore predation. The cyanogenic leaves of this species suffered less damage from chewing herbivores than acyanogenic leaves (Cooper-Driver and Swain, 1976; Schreiner et al., 1984). The defense syndrome of some tropical ferns combines low nutritional quality, high phenol concentrations, and many trichomes (Farias et al., 2019).

Research on chemical alterations in galls on ferns and lycophytes remains scarce. Patra et al. (2009) evaluated the biochemical changes during gall development in *Selaginella* species. These authors found that mature galled tissue had a higher sugar and total phenol content than non-galled tissue at the same stage.

In angiosperm-insect systems authors have found a slight increase in some phenolic substances in galled tissue, while others absent in non-galled tissue were detect in galls and, that qualitative and quantitative changes in the gall chemical profile depend on the inducing insect (Nyman and Julkunen-Titto, 2000). These results suggest that galling insects control phenolic biosynthesis in their hosts (Hartley, 1998; Nyman and Julkunen-Titto, 2000). Research on cyanogenesis in galled and non-galled tissues is still scarce.

Santos et al. (2005) studied cyanogenesis in 19 species of ferns and lycophytes over a one-year period, in a rocky outcrop in southeastern Brazil. Among the species analyzed, *Microgramma vacciniifolia* was found to be cyanogenic throughout the study period, with the following cyanogenesis frequencies: stem (10%), fertile leaves (40%) and sterile leaves (47%). The effects of herbivorous stress were not evaluated. Peres et al. (2009) isolated and identified in *M. vacciniifolia* phenolic substances and ethyl esters of carboxylic acids. However, none of the studies analyzed variations in the chemical profile between the galled and non-galled tissue of *M. vacciniifolia*.

The hypotheses to be tested are (1) the gallers of *M. vacciniifolia* stem promote a qualitative alteration of phenolic substances in their galled tissues, and (2) The frequency of cyanogenesis in *M. vacciniifolia* stems and galls is lower than that of leaves.

Thus, the aim of this study was to evaluate the impact of two gall-inducing insects on the biochemistry of phenolic acids of the host fern, and the prospection of cyanogenesis in galled and non-galled tissue of *M. vacciniifolia*.

2. Materials and Methods

Microgramma vacciniifolia samples were collected in a Myrtaceae "thicket" community (22°57'39.6"S 42°51'43.0"W), a dense shrubby vegetation growing on

the beach ridges from the restinga (sandy coastal plains) vegetation of the Maricá Environmental Protection Area in Rio de Janeiro state, Brazil. The voucher specimens (M.G. Santos 2250) were deposited in the FFP-UERJ Herbarium (RFFP).

2.1. Profile of the phenolic substances

The samples were collected in one day of March 2015 (rainy season) from seven different individuals of *M. vacciniifolia* and mixed into one composite sample, placed in plastic bags and taken to the Laboratório de Tecnologia de Produtos Naturais of the Universidade Federal Fluminense. The extraction was carried in the same day and the profile of the substances was evaluated one week later. We analyzed galls induced by midge (MIG), galls induced by micromoth (MMIG), non-galled stems near of the midge induced galls (SNM) and non-galled stems near of the micromoth induced gall (SNMM).

Methanolic extracts were obtained at 60 °C under magnetic stirring, for five minutes (min.), using a 1:10 solution (1 g of fresh plant material in 10 mL of methanol). The chromatographic profile of the substances was evaluated by high performance liquid chromatography (HPLC) (Shimadzu LC-20A), using the following analytical conditions: a Phenomenex® Synergi Hydro RP18 (250 mm x 4.6 mm i.d. particle size 4 µm) column, Jasco® chromatograph, and flow rate of 1.0 mL/min. Mobile phase: MeOH + 0.1% TFA 0.1% (B) and H_2O + 0.1% TFA (A). Gradient elution was 0-20% B (20 min), 20% B (isocratic, 2 min), 20-50% B (38 min), and 50-100% B (5 min). The analysis lasted 65 min and the wavelength of the diode array detector (DAD) was fixed at 285nm. The retention times and peaks were used as indicative to differentiate between substances. Peak purity measurement was used, indicating that each peak corresponds to only one substance. The UV-spectra of the separated substances were analyzed in line with Campos and Markham (2007).

2.2. Cyanogenesis

The samples were collected on one day in June and one in August 2011 (dry season), and one day in March 2012 (rainy season), placed in plastic bags and taken to the Laboratório de Biodiversidade of the Faculdade de Formação de Professores (FFP), Universidade do Estado do Rio de Janeiro (UERJ). The analysis was conducted on the same day. Midge (N=20) and micromoth-induced galls (N=28) as well as non-galled stems near the former (N=20) and the latter (N=28) were used, in addition to sterile (N=47) and fertile (N=25) leaves. Samples were tested in isolation. Cyanogenesis was detected using the picrate paper test prepared by rectangular pieces of filter paper dripped with a saturated aqueous picric acid solution neutralized with $NaHCO_3$. The botanical sample was ground in a test tube and 3 drops of chloroform was added. Next, the picrate paper was putting inside of test tube, which was sealed with a stopper. A color change from yellow to reddish indicates a positive result for cyanogenesis (Harborne, 1984; Santos et al., 2005).

2.3. Statistical analyses

Principal Coordinates Analysis (PCoA) was performed based on presence and absence of the phenolic substances in galled and non-galled stems of *M. vacciniifolia*, using the Sørensen similarity index. The chi-squared test (X^2) was conducted to demonstrate the significant difference of cyanogenesis in different tissues of *M. vacciniifolia*. PAST (PAleontological STatistics) software version 3.10 was used for data analysis.

3. Results

Based on the retention times, a total of 14 phenolic substances were detected. Of these, 12 were found in non-galled stems (SNMM and SNM), 10 in midge-induced galls with parasitoids (MIGP), nine in micromoth-induced galls (MMIG), and six in midge-induced galls (MIG) (Table 1; Figures 1 and 2).

Wavelength analysis indicated that substances 1, 3 - 6, 8 and 9 are caffeic acid derivatives and substance 7 is more likely a coumaric acid derivative (Figures 1 and 2).

Figure 3 shows Principal Coordinates Analysis of the phenolic substances in galled and non-galled stems of *M. vacciniifolia* (Table 1). Axis 1 explains 55.8% and the axis 2 35.19% of the variance (total=90.99%). The axis 1 separates SNMM, SNM, MMIG and MIGP from MIG, the last forming an isolated group. Axis 2 separates SNMM, SNM and MMIG from MIGP (Figure 3). In MIGP two new substances (A and B) are present in the chemical profile, and substance 12 was only detected in non-galled stems (SNM and SNMM). Substances 4, 5, 7, 8 and 11 were detected in all the samples (Table 1).

With respect to cyanogenesis, the frequency differed significantly (X^2=27.02, DF=7, P=0.0003) between galls, non-galled stems, fertile and sterile leaves. None of the micromoth-induced galls or adjacent non-galled stems exhibited cyanogenesis. However, 15% of the midge-induced galls analyzed were cyanogenic. The sterile leaves were cyanogenic in 27.7%, and fertile leaves in 8.3% of the samples (Table 2).

4. Discussion

Analysis of the chemical profile of phenolic substances in galled and non-galled stems of the fern species *M. vacciniifolia* indicated changes in the relative amounts of these substances and in the induction of new substances in galled tissue. Nyman and Julkunen-Titto (2000) also observed a difference between the chemical profiles of galled and non-galled plant tissue in the sawfly-willow system. The authors found a slight increase in some phenolic substances in galls, while others absent in non-galled tissue were detected in galls. It has been suggested that plants raise the production of phenolic substances in tissue where galling insects have laid their eggs (Isaias et al., 2014) due a response to oxidative stress promoted by the insects (Soares et al., 2000).

In *Microgramma vacciniifolia*, the chemical composition of non-galled stems (SNM and SNMM) was similar to that

Table 1. Retention time (RT), signal area (SA) for compounds detected in the chromatograms of methanolic *Microgramma vacciniifolia* extracts. SNM (stem non-galled by midge), MIG (midge-induced galls), MIGP (midge-induced galls attacked by parasitoids), SNMM (stem non-galled by micromoth), MMIG (micromoth-induced galls), mAU (milli-Absorbance Units), min. (minutes), (-) not detected.

Compounds	SNM		MIG		MIGP		SNMM		MMIG	
	RT (min.)	SA (mAU)	RT (min.)	SA (mAU)	RT (min.)	SA (mAU)	RT (min.)	SA (mAU)	RT (min.)	SA (mAU)
A	-	-	-	-	9.560	211101	-	-	-	-
B	-	-	-	-	11.372	60028	-	-	-	-
1	25.519	70342	-	-	25.522	92303	25.542	41205	25.516	15704
2	29.058	43576	-	-	29.073	37250	29.053	37483	29.044	54020
3	29.947	29354	-	-	29.960	54360	29.950	25281	-	-
4	34.354	159806	34.345	215401	34.352	816033	34.345	91518	34.338	72466
5	35.763	75375	35.745	19447	35.751	116660	35.751	37081	35.754	20611
6	40.702	49040	-	-	-	-	40.702	49040	40.704	20637
7	41.176	144946	41.126	483588	41.140	633298	41.184	199151	41.169	118254
8	43.566	73625	43.534	574443	43.532	540211	43.537	241597	43.532	179910
9	47.196	91550	-	-	-	-	47.194	59084	47.180	37023
10	47.709	38641	47.196	21052	-	-	47.705	24772	-	-
11	50.635	21766	50.328	208457	50.319	68714	50.622	18536	50.323	62517
12	52.496	34240	-	-	-	-	52.490	50264	-	-

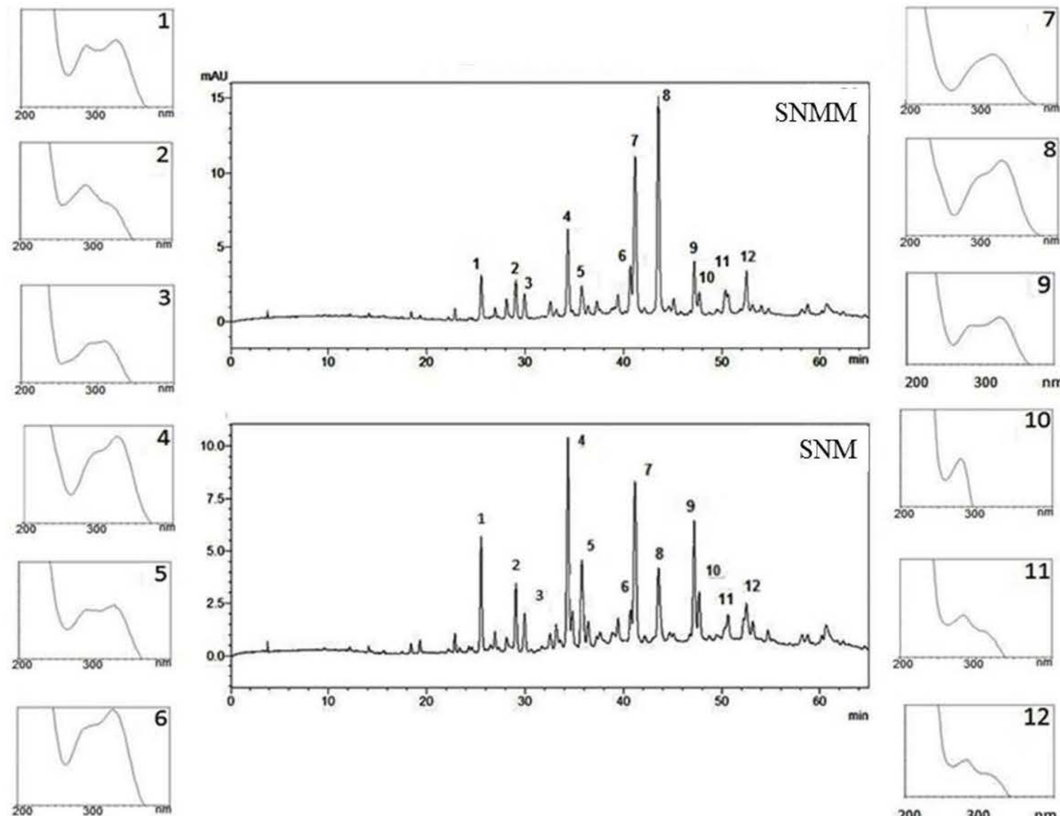

Figure 1. Chromatogram and ultraviolet (UV) spectra of methanolic *Microgramma vacciniifolia* extracts. SNMM (stem non-galled by micromoth), SNM (stem non-galled by midge).

of micromoth-induced galls (MMIG), but differed from MIG and MIGP. Galling insects control phenolic biosynthesis in their hosts (Nyman and Julkunen-Titto, 2000) and each inducer promotes species-specific alterations in the chemical composition of galled tissue (Hartley, 1998). Parasitoids (especially wasps) are the main natural enemies of gall-inducing insects, promoting a top-down effect on gall populations (Santos et al., 2012). The death of gall-inducing insects ends their manipulation of host plant metabolism, which may explains the different phenolic profiles between MIG and MIGP.

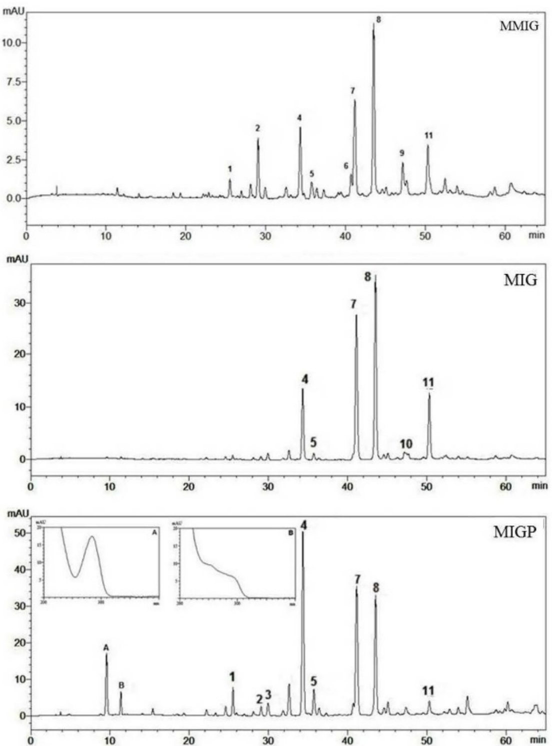

Figure 2. Chromatogram and ultraviolet (UV) spectra of methanolic *Microgramma vacciniifolia* extracts. MMIG (micromoth-induced galls), MIG (midge-induced galls), MIGP (midge-induced galls attacked by parasitoids).

Khattab and Khattab (2005) reported an accumulation of caffeic and coumaric acid in the galled leaves of *Eucalyptus obliqua* L'Hér. (Myrtaceae) when compared to non-galled leaves. Similary, Vereecke et al. (1997) found that caffeic acid was not detectable in non-galled tissues and appeared in significant concentrations in leafy galls of *Nicotiana tabacum* L. (Solanaceae). It is possible that caffeic and coumaric acid might accumulate as an expression of plant infection resistance (Matern and Kneusel 1988; Vereecke et al., 1997).

Cyanogenesis frequency differed in the non-galled tissue, galls and leaves of *M. vacciniifolia*. Santos et al. (2005) also reported the lower frequency of cyanogenesis in *M. vacciniifolia* stems than leaves, and its cyanogenic polymorphism, that is the variation of cyanogenesis profile between individuals and the organs of the same individual (Hegnauer, 1977; Oliveros-Bastidas and Alonso-Amelot, 2010).

All the stems near galls (SNM and SNMM) were non-cyanogenic. Research on plant species that exhibit cyanogenic polymorphism demonstrates that some herbivores prefer predating on non-cyanogenic individuals and avoid cyanogenic plants (Cooper-Driver and Swain, 1976; Bell, 2001). While all micromoth-induced galls were non-cyanogenic, some samples of midge-induce galls were cyanogenic. Cyanogenic potential depends on the concentration of cyanogenic precursors in each

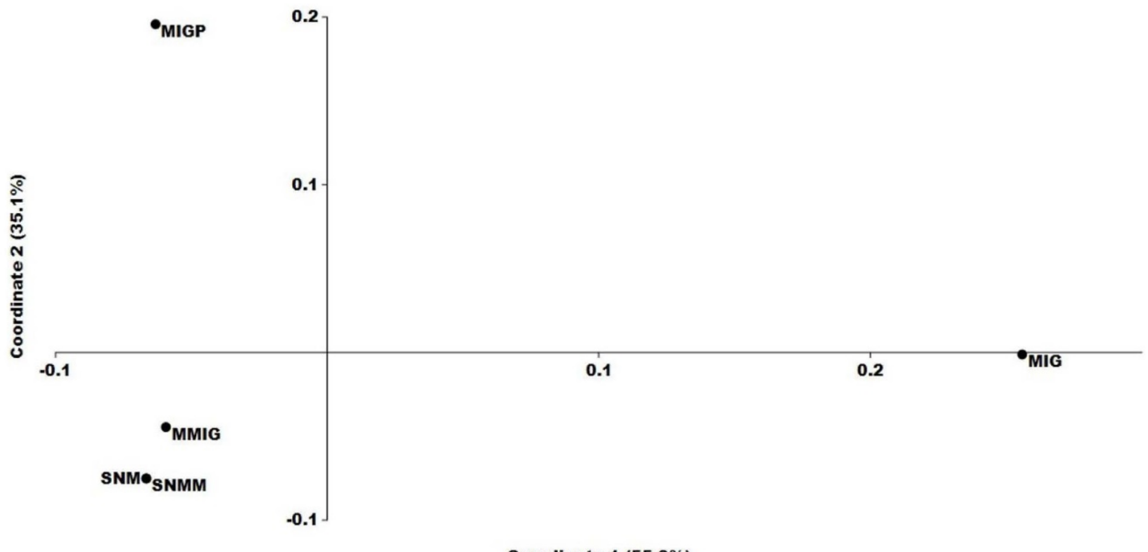

Figure 3. PCoA (Principal Coordinates Analysis) ordination diagram for the presence and absence of the phenolic substances in galled and non-galled tissue of the *Microgramma vacciniifolia*. SNM (stem non-galled by midge), MIG (midge-induced galls), MIGP (midge-induced galls attacked by parasitoids), SNMM (stem non-galled by micromoth), MMIG (micromoth-induced galls)

Table 2. Cyanogenesis in *Microgramma vacciniifolia*. N (Total number of cyanogenic samples/total number of samples analyzed). X^2=27.02 (DF=7 *P*=0.0003).

		Cyanogenic samples/total number of samples analyzed			N
		Jun 2011	Aug 2011	Mar 2012	
Individuals with midge-induced galls	Gall	0/6	3/9	0/5	3/20
	Non-galled stem	0/6	0/9	0/5	0/20
	Sterile leaf	0/6	5/9	1/4	6/19
	Fertile leaf	0/2	0/9	1/1	1/12
Individuals with micromoth-induced galls	Gall	0/10	0/11	0/7	0/28
	Non-galled stem	0/10	0/11	0/7	0/28
	Sterile leaf	2/10	3/11	2/7	7/28
	Fertile leaf	0/1	0/11	0/1	0/13

plant tissue as well as β-glucosidase and α-hydroxynitrile lyases activity (Zagrobelny et al., 2004; Kautz et al., 2014). Recent data show that plants express increased cyanogenic capacity (by induction β-glucosidases activity) in response to herbivore damage and jasmonic acid treatment (Kautz et al., 2014).

5. Conclusion

Our results indicate, at least for phenolic substances, that midge and micromoth gall-inducers promote species-specific alterations in the chemical composition of the *M. vacciniifolia*. In relation to cyanogenesis, stems non-galled and micromoth-induced galls were negative, whereas some midge-induced galls were positive, with greater frequency in leaves (fertile and sterile). The effects of this insect on cyanogenesis in the fern host remains to be tested.

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8.6 Artigo 7

Anti-*Leishmania amazonensis* activity of the terpenoid fraction from *Eugenia pruniformis* leaves

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Anti-*Leishmania amazonensis* activity of the terpenoid fraction from *Eugenia pruniformis* leaves

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Abstract: Leishmaniasis is caused by protozoan parasites belonging to the genus *Leishmania* and includes cutaneous, mucocutaneous and visceral clinical forms. Drugs currently available for leishmaniasis treatment present high toxicity, and development of parasite resistance. Plants constitute an important source of compounds with leishmanicidal potential. This study aimed to evaluate the anti-*Leishmania amazonensis* activity of the terpenoid fraction of *Eugenia pruniformis* leaves (TF-EpL). TF-EpL was active against the promastigote and intracellular amastigote forms of *L. amazonensis* with $IC_{50(24h)}$ value of 43.60µg/mL and 44.77µg/mL, respectively. TF-EpL altered the cell cycle of the parasite, increasing 2.32-fold the cells in the Sub-G0/G1 phase. TF-EpL also changed the ΔΨm and increased ROS and the number of annexin-V-PI positive promastigotes, which suggests incidental death. β-sitosterol, ursolic acid, corosolic acid and asiatic acid were isolated from TF-EpL. The results showed the antileishmanial activity of *E. pruniformis* terpenoids and its potential for further studies as a source of new drugs for leishmaniasis.

Key words: *Eugenia pruniformis*, *Leishmania amazonensis*, terpenoids, Leishmaniases.

INTRODUCTION

Leishmaniasis is a neglected tropical disease caused by unicellular protozoan parasites of the genus *Leishmania*, that affect 98 countries on 5 continents, with approximately 0.7 to 1 million cases of cutaneous leishmaniasis and 50.000 to 90.000 cases of visceral leishmaniasis occur every year. It causes several types of clinical manifestations and is classified as visceral, cutaneous and mucocutaneous forms. The pentavalent antimonials are the first-line drugs for leishmaniasis treatment, amphotericin B deoxycholate, liposomal amphotericin B and paromomycin, are secondary options for resistant cases (Burza et al. 2018). All these compounds have limitations in their use because of side effects, high cost, induction of parasite resistance and in-patient administration (Andrews et al. 2014). Miltefosine is the first oral treatment approved for leishmaniasis in India. However, it has a low efficacy against cutaneous leishmaniasis, besides being teratogenic (Kevric et al. 2015). Therefore, researches for new compounds are required.

Plants are an important source of biologically active compounds such as terpenoids that could be studied in the treatment of the neglected tropical diseases as leishmaniasis (Duarte et al. 2019, Ogungbe & Setzer 2013). Terpenoids

as β-sitosterol and ursolic acid have shown antileishmanial activity and were found in *Eugenia* genus (Myrtaceae) (Santos et al. 2008, Yamamoto et al. 2015, Frighetto et al. 2005), which in turn is represented by several species that have been used in folk medicine and are known for their antidiabetic, antirheumatic, antipyretic, antidiarrheal, anti-inflammatory, antifungal, antibacterial, antioxidant and cytotoxic activities (Reynertson et al. 2005, de Souza et al. 2018). Thus, species of this genus can be considered promising in order to obtain products to combat leishmaniasis and associate diseases.

Eugenia pruniformis, popularly known as "azeitoninha-da-praia", is distributed in sandbank vegetation along the Brazilian coast. The essential oil from the leaves of *E. pruniformis* has shown anticholinesterasic and antioxidant activities and β-caryophyllene as a major compound (Albuquerque et al. 2012). *E. pruniformis* has also shown wound healing activity in a skin rat model and the main flavonoid compounds present in the ethyl acetate extract are quercetin, kaempferol, and hyperoside (Albuquerque et al. 2016). Considering the anti-*Leishmania* potential of terpenes and the chemical diversity of the genus *Eugenia*, the present study aims to evaluate the anti-*Leishmania amazonensis* activity of the terpenoid fraction from *E. pruniformis* leaves (TF-EpL), as well as analyze its chemical composition.

MATERIALS AND METHODS

Plant material

Leaves and flowers of *Eugenia pruniformis* Cambess (Myrtaceae) were collected from four specimens in Restinga de Jurubatiba National Park, Rio de Janeiro State, Brazil, in open Clusia scrub vegetation (S22° 12'40.85"–W41° 35'14.61"; S22° 12'40.85"–W41° 35'14.61"; S22° 12'36.36"–W41° 35'20.18"; S22° 12'34.90"–W41° 35'21.04") (Authorization number for scientific activities: 13659-3 SISGEN). Plant material was identified by the botanist Dr. Marcelo Guerra and a voucher specimen (M.G. Santos 2206) was deposited at the herbarium of the Faculdade de Formação de Professores (Universidade Estadual do Rio de Janeiro, Brazil).

Extraction and isolation of terpenoids

The leaves were subjected to drying in a forced ventilation oven, with a temperature of approximately 35°C, for 48 hours. Then, dried and powdered leaves (1200 g) were exhaustively extracted by maceration in 96 % (v/v) ethanol and the filtrate was concentrated under reduced pressure (35°C). The resulting hydroethanolic extract (143.7 g) was resuspended in 2.0 L of ethanol 90 % (v/v) and then partitioned with n-hexane (2 x 1.0 L) to obtain 35.0 g of n-hexane extract, with the yield of 2.92 % w/w. For preparative isolation, the n-hexane extract (20.0 g) was purified with acetone to afford the terpenoid fraction (TF-EpL), which was chromatographed on a Silica Gel 60 column eluted with n-hexane, ethyl acetate and methanol, using an increasing polarity gradient to yield 81 fractions (1-89) that were combined with the aid of Thin Layer Chromatography (TLC) analysis. Further purification with n-hexane: ethyl acetate (9:1) solution of Fraction 14 gave compound 1 (20.0 mg). Fraction 40 was finally purified with ethyl acetate to yield compound 2 (43.0 mg). Fractions 54-59 were purified with ethyl acetate to afford compound 3 (25.0 mg). Final purification with ethyl acetate of Fractions 74-77 gave compound 4 (25.0 mg). Isolated compounds 1, 2, 3 and 4 were in the form of a white powder.

Structural elucidation of the terpenoids

All isolated compounds were identified by ^{1}H and ^{13}C NMR spectral data analysis, including 1D and 2D NMR experiments and mass spectrometry. The NMR spectra were recorded on a Varian VNMRS 500 MHz spectrometer operating at 500 (^{1}H) and 125 (^{13}C) MHz. Chemical shifts are reported as δ values (ppm) with the residual solvent signal as the internal reference, with *J* in Hz. Deuterated methanol (CD_3OD) was obtained from the Cambridge Isotope Laboratories (USA) and used for solubilization.

HPLC-Q-TOF/MS analyses were carried out by Infinit 1200 system hyphenated to a 6530 Accurate mass spectrometer (Agilent) equipped with a quadrupole-time-of-flight (Q-TOF). TF-EpL sample was dissolved in methanol at a concentration of 5 µg/mL. Separations were performed on a reverse-phase (C_{18}, 30 mm, 2.1 mm x 3.5 µm; Zorbax). Water (A) and acetonitrile (B) were used as mobile phases as follows: 0-30 min, 40 to 95 % B. The flow rate was 0.3 mL/min and the injection volume was 5 µL. Mass spectra were recorded in negative ion mode. The Q-TOF/MS data were acquired in negative mode over a *m/z* range of 100–600, at a rate of 2 spectra/sec. The MS profile was performed in full scan mode and displayed in TIC (Total Ion Current) chromatogram. The conditions were as follows: gas temperature 325 °C; gas flow: 8 L/min; nebulizer: 40 psig. The raw data were acquired and processed with software from Agilent Technologies.

Parasites

Leishmania amazonensis (WHOM/BR/75/Josefa) promastigotes were cultured at 26°C in Schneider insect medium (Sigma), 10 % fetal calf serum (Gibco, MD, US) and 20 µg/mL of gentamicin (Schering-Plough, RJ, Brazil).

Ethics Statement

All animal experiments were performed in strict accordance with the Brazilian animal protection law (Lei Arouca number 11.794/08) of the National Council for the Control of Animal Experimentation (CONCEA, Brazil). The protocol was approved by the Committee for Animal Use Ethics of the Universidade Federal do Rio de Janeiro (Permit Number: 128/15).

Antipromastigotes activity

Stationary-phase promastigotes were treated with 40, 20, 10 and 5 µg/mL of TF-EpL for 24 h at 26°C and then were added 20 µL/well of MTT solution (5 µg/mL) (Sigma) and incubated for 4 hours at 37°C with 5 % CO_2. The culture was centrifuged for 15 minutes at 4000 rpm 4°C. Then, the supernatant was removed, and 0.5 mL DMSO was added to each well to dissolve the resulting formazan crystals. The absorbance was measured at a wavelength of 595 nm. The results are expressed in the percentage of viable cells compared to untreated control, as reported by (Ceole et al. 2017).

Antiamastigotes activity

Infected mice peritoneal macrophage cultures were treated with different concentrations TF-EpL during 24 h at 35°C, 5 % CO_2. Then, cultures were washed and incubated with 0.01 % sodium dodecyl sulfate for 10 min followed by 1.0 mL of Schneider's medium supplemented with 10 % FCS, and maintained at 26°C for 2 days. The relative intracellular load of viable *L. amazonensis* amastigotes was measured, after promastigote transformation, using Alamar Blue (Invitrogen), as previously described (Alves Passos et al. 2017). Mean values were calculated in 3 independent experiments using 5 wells per condition.

Cytotoxicity for host macrophages

Mice peritoneal macrophages adhered to 96-well plates were treated with the TF-EpL for 24 h and cell viability was determined by XTT (Sigma), as previously described (Ferreira et al. 2017). The selectivity index (SI) was calculated through the division of the $CC_{50(24h)}$ by $IC_{50(24h)}$ values.

Cell Cycle Analysis

Promastigotes were treated or not with 43.60 µg/mL TF-EpL, 0.1 µg/mL amphotericin B and 1 % DMSO for 24 h. The cells were fixed in 70 % ice-cold methanol for 1 h at 4°C. Then, the cells were incubated in phosphate buffered saline (PBS) supplemented with 10 µg/mL propidium iodide (PI) and 20 µg/mL RNAse at 37°C, 45 min. Next, were measured using BD FACScalibur (Becton and Dickson) and analyzed using CellQuest software, as reported (Ferreira et al. 2017).

Measurement of mitochondrial membrane potential (ΔΨm)

Promastigotes were treated or not with 43.60 µg/mL TF-EpL for 24 h and then incubated with 5 µg/mL JC-1 staining solution (Sigma) for 10 min at 37°C. ΔΨm was measured on a BD FACScalibur (Becton and Dickson) and analyzed using CellQuest software, as described previously (Alves Passos et al. 2017).

Detection of Reactive Oxygen Species (ROS)

Promastigotes were treated or not with 43.60 µg/mL TF-EpL for 24 h at 26°C and then stained with 50 µM DCFDA (Sigma) for 30 min, and ROS was measured immediately using a BD FACScalibur (Becton and Dickson) and analyzed using CellQuest software, as reported (Eruslanov & Kusmartsev 2010).

Quantification of polar and neutral hydrophobic domains

Promastigotes (10^6 cells/mL) were treated with 43.60 µg/mL TF-EpL for 24 h at 26°C. Cells were centrifuged and resuspended in PBS. Nile Red (1 µg/mL, Sigma) was added and the cells were incubated at room temperature for 7 min. After three wash with PBS, the red and yellow fluorescence were measured immediately using BD FACScalibur (Becton and Dickson) and analyzed using CellQuest software, as reported (Greenspan et al. 1985).

Statistical analysis

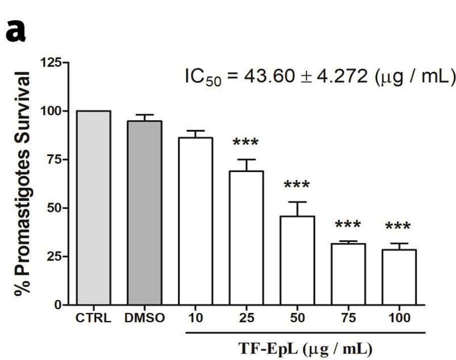
Data were analyzed using Student's t-test when comparing two groups or one-way ANOVA for more than two groups using the software GraphPad Prism. *P* values of less than 0.05 were considered significant.

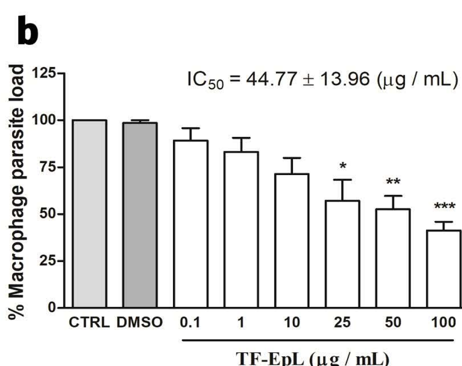
RESULTS AND DISCUSSION

Leishmaniasis is a neglected parasitic disease for which the current antileishmania therapies are complicated by drug toxicity, need for parenteral administration, high cost, increasing treatment failure rates, and emergence of drug resistance (Burza et al. 2018). Natural products extracted from a crude extract of plants or fractions are a good source of diverse chemical structures that show potent biological profile and pharmacological activities (Duarte et al. 2019, Ogungbe & Setzer 2013). In a search for new antileishmanial drugs and improve the chemical knowledge of *E. pruniformis*, the effect os TF-EpL on *L. amazonesis*, a Brazilian endemic species, was studied. In humans, this species may cause cutaneous leishmaniasis, a severe anergic diffuse cutaneous leishmaniasis and the visceral form of this disease (Burza et al. 2018).

The terpenoid fraction was obtained from the hydroethanolic extract of *E. pruniformis* leaves. In this study, the effect of this fraction TF-EpL on promastigotes and amastigotes forms of *L. amazonensis* was investigated, as well the cytotoxicity for murine macrophages. TF-EpL in the promastigote forms, which are present in the invertebrate host, presented an IC_{50} value of 43.60 ± 4.272 µg/mL (Fig. 1a), while in the intracellular amastigote forms, which belong to the vertebrate host, TF-EpL presented an IC_{50} of 44.77 ± 13.96 µg/mL (Fig. 1b).

The leishmanicidal effect of plant extracts is already described in the literature. Our group demonstrated that the crude extract of branches from *Guatteria latifolia* presents an IC_{50} value of 51.7 and 30.5 µg/mL against *L. amazonensis* promastigotas and amastigotes, respectively (Ferreira et al. 2017). Rodrigues et al. (2013) demonstrated that *E. uniflora L.* essential





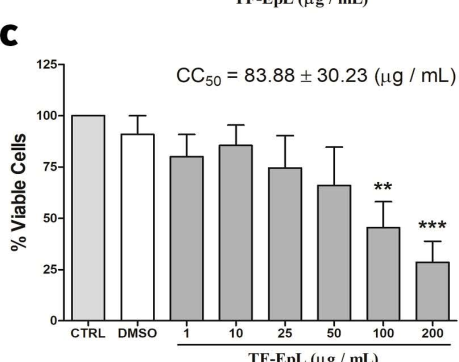


Figure 1. Inhibition of *L. amazonensis* proliferation by terpenoid fraction of *E. pruniformis* leaves (TF-EpL) and cytotoxicity against murine peritoneal macrophages *in vitro*. (a) Promastigotes (10^7 / mL) were grown in the presence or absence of TF-EpL, or diluent (DMSO), at the indicated concentrations, during 24 h. Parasite viability was checked by the dehydrogenases activity using the MTT assay. The results were expressed as the percentage of viable promastigotes in relation to the untreated control (100 %) and are shown as the mean ± SEM of 3 independent experiments. (b) Peritoneal macrophages (10^5) from BALB/c mice were infected with promastigotes at a ratio of 10 parasites to 1 macrophage for 24 h and were either left untreated or were treated with the indicated concentrations of TF-EpL for 24 h, washed, fed with Schneider's complete medium, and cultured at 26 °C. The macrophage parasite load was evaluated after 48 h using Alamar Blue. (c) Cells were cultured with 1, 10, 25, 50, 100 and 200 µg/mL TF-EpL for 24 h at 37 °C in 5 % CO_2. Viability was measured by the XTT method. The results represent the means ± SEM from 3 experiments performed in triplicate. $p < 0.05$ (*), $p < 0.001$ (**) and $p < 0.0001$ (***) compared to the control.

oil presented anti-*L. amazonensis* activity with IC_{50} of 1.75 μg/mL after 72h of treatment. However, the viability of the macrophages was altered, presenting significant toxicity at the concentration of 6.25 μg/mL, with an CC_{50} value of 45.3 μg/mL. TF-EpL also affects *L. amazonensis* strain MHOM/BR/77LTB0016, with an IC_{50} of 8.50 μg/mL after 24 hours of treatment (data not shown).

Analysis of results from the cytotoxicity assay, performed on mice peritoneal macrophages, revealed that TF-EpL was less toxic to the host cells than to parasite with the $CC_{50(24h)}$ value of 83.88 μg/mL (Fig. 1c). Thus, the TF-EpL showed SI values of 1.92 for promastigotes and 1.87 for intracellular amastigotes forms.

The effects of TF-EpL on the parasite and the cell cycle of *L. amazonensis* promastigotes were analyzed (Fig. 2). Untreated promastigotes (Fig. 2a), 1 % DMSO (Fig. 2b), 43.60 μg/mL TF-EpL (Fig. 2c) or 0.1 μg/mL amphotericin B (Fig. 2d), were labeled with propidium iodide in cell cycle solution and analyzed via flow cytometry. Our results showed that TF-EpL arrested *L. amazonensis* promastigote cycle, increasing 2.32-fold the number of cells in the sub-G0/G1 phase. Amphotericin B, used as a control, increased 2.18-fold the number of cells in the sub-G0/G1 phase and decreased 2.05-fold the number of cells in G0/G1 phase.

Leishmania presents a single mitochondria, an organelle that plays an important role in energy metabolism, and the dysfunction of this organelle can lead the parasite death. Thus, the parasite mitochondrial toxicity of TF-EpL was investigated by the reduction of the mitochondrial membrane potential (ΔΨm) using the JC-1 assay. Our data indicated that 43.60 μg/mL TF-EpL increased 1.04-fold the ΔΨm in comparison of untreated cells (Fig. 2e). Thus, TF-EpL affects parasite mitochondria, inducing its hyperpolarization, which is associated with apoptosis, as shown for *L. amazonensis* after treated with the hexane fraction of *Serjania lethalis* leaves (Alves Passos et al. 2017).

To elucidate the possible type of cell death was involved in the TF-EpL-treated promastigotes, the effects of treatment on ROS production and phosphatidylserine exposure were tested, by labeling with DCFDA probe and Annexin-V-PI, respectively (Fig. 2f and Fig. 3). Our results show that after 24 hours of treatment with 43.60 μg/mL TF-EpL, the ROS production increase 1.53-fold in a relation of control (Fig. 2f). TF-EpL treated promastigote increase 7.20-fold PI labeling (Fig. 3d), however, Annexin-V binding to TF of *E. pruniformis* TF-EpL treated promastigote was similar to the controls (Fig. 3e). The association between hyperpolarization and increased ROS generation may also result in the initiation and occurrence of necrotic cell death (Van Den Berghe et al. 2010). Moreover, TF-EpL increased 6.46-fold annexin-V-PI binding to the promastigotes (Fig. 3f).

In this paper, the changes in the parasite cell cycle were demonstrated, alterations in the ΔΨm and increased of ROS and percentage of annexin-V-PI labeling after TF-EpL treatment, indicating incidental death of *L. amazonensis* promastigotes (Proto et al. 2013). Corroborating our data, Ceole et al. (2017) demonstrated that nerolidol, the main constituent of *Piper aduncum* essential oil, has anti-*Leishmania braziliensis* activity, such as mitochondrial membrane depolarization, phosphatidylserine exposure, and cell size reduction, indicating incidental death process.

The effect of TF-EpL on the accumulation of lipid bodies with Nile Red was evaluated by flow cytometry for quantification of polar hydrophobic domains (red fluorescence) and neutral hydrophobic domains (yellow fluorescence). The treatment with 43.60 μg/mL of TF-EpL decreases 1.26-fold the accumulation

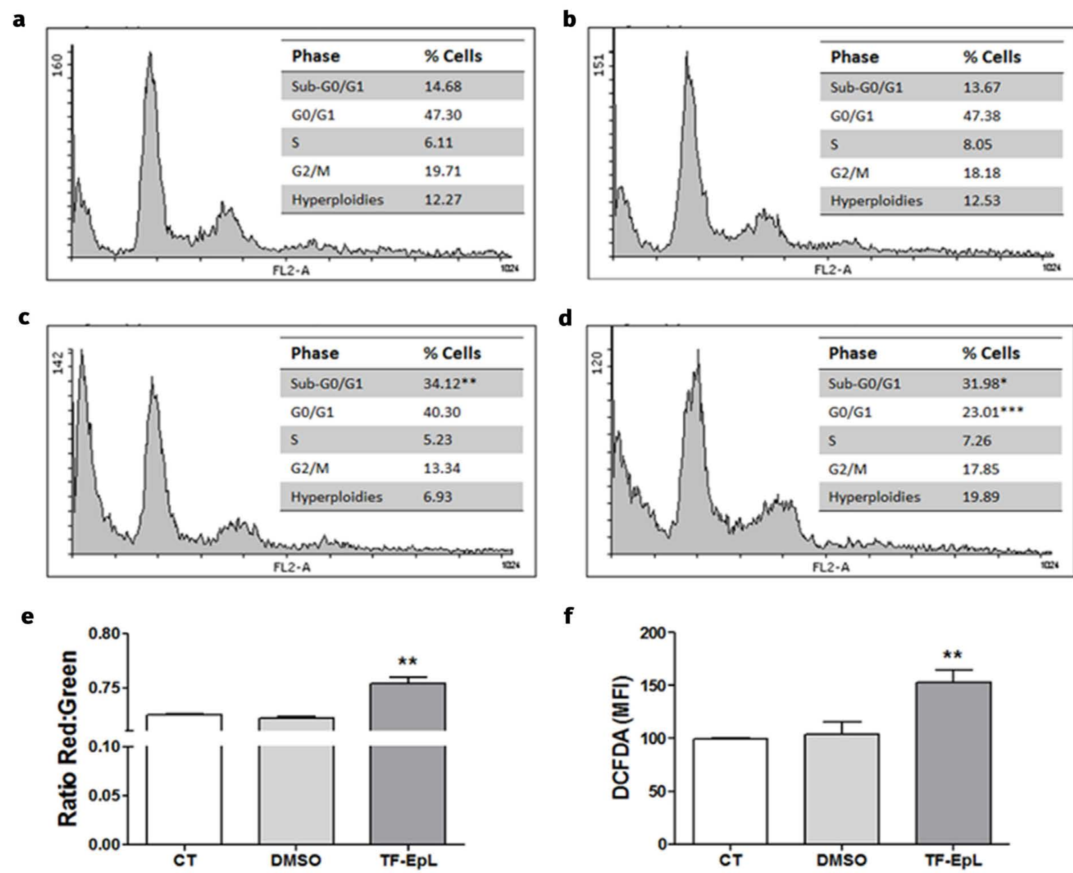


Figure 2. Evaluation of cell cycle, mitochondrial membrane potential (ΔΨm) and reactive oxygen species (ROS) production by *Leishmania amazonensis* promastigotes. Promastigotes were treated or not with TF-EpL and cell cycle was evaluated after 48h after PI staining by flow cytometry analysis. Untreated parasites control (a), 1 % DMSO (b), 43.60 µg / mL TF-EpL (c) and 0.1 µg/mL Amphotericin B (d). Representative plots of at least three independent experiments with similar results. (e) Promastigotes treated or not with 43.60 µg/mL TF-EpL was evaluated using a JC-1 assay. The results are expressed as red / green fluorescence ratios and represent the averages ± SEM from 3 independent experiments. (f) Promastigotes treated or not with 43.60 µg/mL TF-EpL or 1 % DMSO were then stained with 50 µM DCFDA and ROS was measured by flow cytometer. The results represent the means ± SEM from 3 experiments performed in triplicate. $p < 0.05$ (*), $p < 0.001$ (**), and $p < 0.0001$ (***) compared to the untreated control.

of neutral hydrophobic domains (Fig. 4a) but do not alter the polar hydrophobic domains or phospholipids (Fig. 4b). The reduction of neutral lipid in TF-EpL treated parasites suggested either inhibition or a deviation in the lipidic synthesis pathway (Ferreira et al. 2011).

The fractionation of TF-EpL led to the isolation of four compounds (1 – 4), identified by NMR (Table I) and HPLC-Q-TOF/MS. ^{1}H-NMR spectrum of 1 showed six methyl signals as two methyl singlets at δ_H 0.66 (s, H-16) and δ_H 0.99 (s, H-19), three methyl doublets at δ_H 0.79 (d, H-27), δ_H 0.81 (d, H-26) and δ_H 0.90 (d, H-21) and a methyl

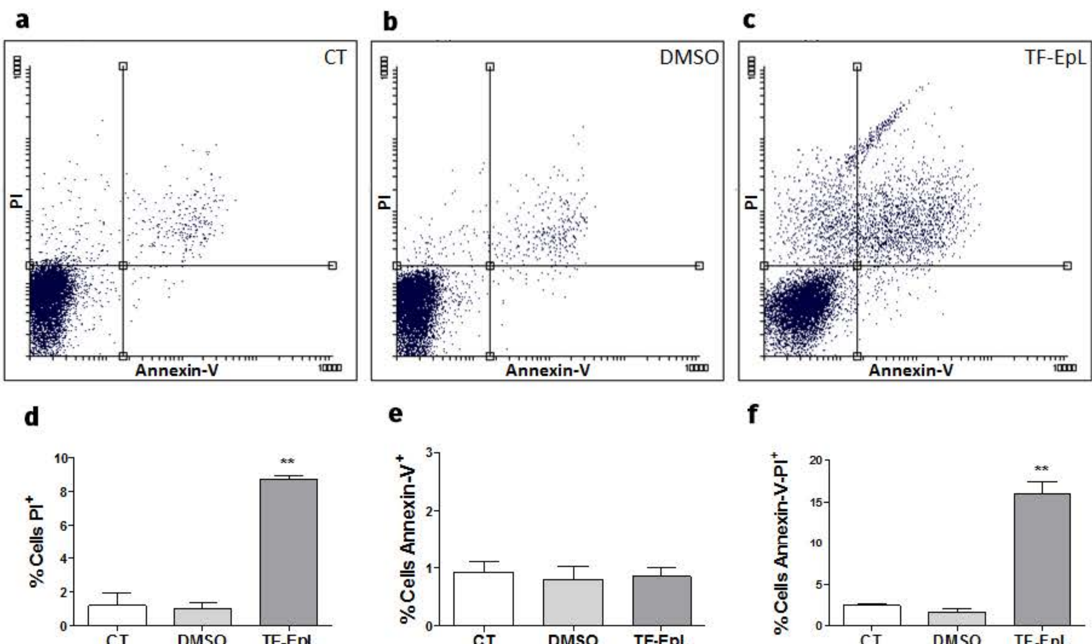


Figure 3. Assessment of Annexin-V expression on *Leishmania* induced by terpenoid fraction of *E. pruniformis* leaves (TF-EpL) treatment. Promastigotes were treated or not with TF-EpL and the expression of Annexin-V / Propidium Iodide (PI) measured. Untreated parasites control (a), parasites treated with 1 % DMSO (b) and 43.60 µg/mL TF-EpL (c) are shown as a representative result out of 3 independent experiments with similar results. The percentage of PI positive (d), Annexin-V positive (e) and Annexin-V-PI positive promastigotes (f), treated as above, shown as mean ± SEM of 3 independent experiments. $p < 0.001$ (**) compared to the control.

triplet at δ_H 0.84 (t, H-24). Also, a signal referent to the olefinic proton appeared at δ_H 5.33 (d, H-5) and a triplet of double doublets at δ_H 3.51 (tdd, H-3) of a proton connected to the C-3 hydroxy group. The ^{13}C-NMR spectrum of 1 exhibited typical twenty-nine signals of terpenes with a hydroxylated carbon at δ_C 71.83 (C-3), an olefinic carbons at δ_C 141.71 (C-5) and 121.71 (C-6), bound to the longer branch of the structure at δ_C 56.04 (C-17), a methyl carbons at δ_C 18.77 (C-28) and 11.85 (C-29) and terminal carbons of the main branch at δ_C 19.03 (C-19), 11.97 (C-24), 19.80 (C-26) and 19.38 (C-27). Afterward, the identification of compound 1 was followed using accurate mass measurements by HPLC-Q-TOF/MS analysis that presented deprotonated molecular ion peaks at *m/z*: 413.17091 $[M-H]^-$ ($C_{29}H_{50}O_1$). Analysis of data obtained associated with literature data (Aguirre et al. 2006) allowed the identification of 1 as β-sitosterol (Fig. 5).

β-sitosterol was found in different species from *Eugenia* genus (Frighetto et al. 2005) and was active against *Leishmania infantum* in promastigotes and amastigotes forms (Santos et al. 2008) and against of *Leishmania tropica* promastigotes (Majid Shah et al. 2019). Besides, this phytosterol exhibited analgesic and anti-inflammatory activities that could be helpful for the treatment of secondary infections in leishmaniasis (Bin Sayeed et al. 2016).

The ^{1}H-NMR spectrum of 2 showed as main signals, the presence of five methyl as singlets at

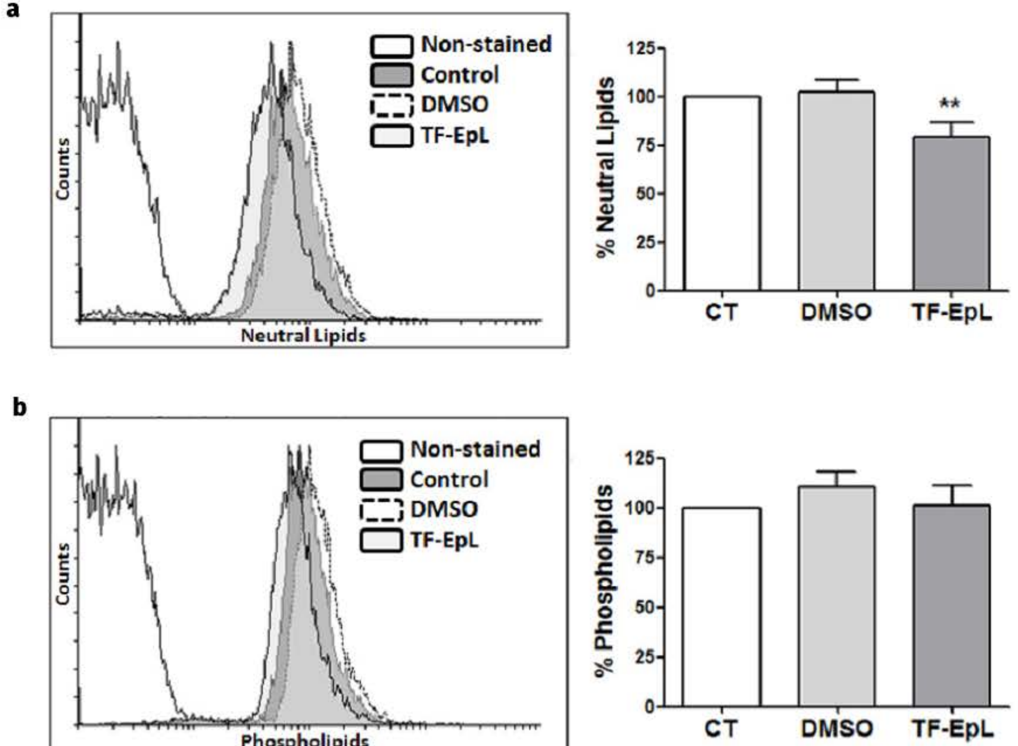


Figure 4. Effect of terpenoid fraction of *E. pruniformis* leaves (TF-EpL) on the synthesis of total lipids on *Leishmania*. Promastigotes were incubated in the presence or absence of 43.60 µg/mL TF-EpL for 24 h. Cells were labeled with Nile Red (1 µg/mL) and the percentage of neutral lipids (a) and phospholipids (b) were estimated in flow cytometry. Histograms are representative of 3 independent experiments with similar results. $p < 0.001$ (**), compared to control.

δ_H 1.26 (s, H-23), δ_H 1.25 (s, H-27), δ_H 1.08 (s, H-26), δ_H 1.04 (s, H-24) and δ_H 0.92 (s, H-25), a double doublet at δ_H 3.48 referent to H-3 of hydroxylated carbon and a triplet at δ_H 5.51 referent to H-12 bonded to sp^2 carbon. The combined two-dimensional ^{1}H-^{1}H data COSY showed important correlations as H-12 to H-11 (δ_H 5.51 and1.99), H-3 to H-2 (δ_H 3.48 and 1.84), H-18 to H-19 (δ_H 2.66 and 1.49), H-15β to H-27 (δ_H 2.35 to 1.25), H-20 to H-21α (δ_H 1.07 and 1.40). The ^{13}C-NMR spectrum of 2 showed thirty signals referents to a triterpenoid with the characteristic signals at δ_C 78.6 (C-3), 126.1 (C-12), 139.7 (C-13), 40.1 (C-19 and C-20) and 180.0 (C-28). Compound 2 was analyzed by HPLC-Q-TOF/MS and presented a deprotonated molecular ion at *m/z* 455.32534 $[M-H]^-$ ($C_{30}H_{48}O_3$). Comparing these spectral data to those reported in the literature (Gnoatto et al. 2008), compound 2 was identified as ursolic acid (Fig. 5).

Ursolic acid was identified in some *Eugenia* species as *E. gustavoides*, *E. florida* and *E. brasiliensis* and was reported to show anti-*Leishmania amazonensis* activity against promastigotes and amastigotes forms of the parasite ($IC_{50(24h)}$ = 6.4 µg/mL and $IC_{50(24h)}$ = 27.0 µg/mL, respectively) (Frighetto et al. 2005, Yamamoto

et al. 2015, Torres-Santos et al. 2004). Moreover, ursolic acid was also described to has activity against the visceral form of *Leishmania* (Jesus et al. 2017) and also anti-inflammatory property by suppression of NF-κB, AP-1, and NF-AT (Checker et al. 2012). Thus, β-sitosterol and ursolic acid may be responsible for the anti-parasitic activity of the TF-EpL which these compounds were isolated as main constituents. Furthermore, the anti-inflammatory activity is described in the literature for these isolated triterpenes, which are also known as antibacterial agents (Singh & Sharma 2015) and maybe useful to avoid secondary infections caused by leishmaniasis.

The ^{1}H-NMR spectrum of 3 showed as main signals, the presence of a triplet at δ_H 5.24 (t, H-12), a double double-doublet at δ_H 3.63 (ddd, H-2) referent to H-2 and H-3, bonded to hydroxylated carbons, a triplet at δ_H 2.91 (d, H-3) and singlet methyl of H-23, H-24, H-29 and H-30 at δ_H 1.02, 0.81, 0.97 and 0.87, respectively. The ^{13}C-NMR spectrum of 3 exhibited thirty signals characteristic of triterpenoids with typical signals at δ_C 68.1 (C-2), 86.1(C-3), 126.9 (C-12), 139.9 (C-13), 31.0 (C-23), 19.1 (C-24), 19.3 (C-29), 23.2 (C-30). Compound 3 was analyzed by HPLC-Q-TOF/MS and presented a deprotonated molecular ion peak at *m/z*: 471.14000 $[M-H]^-$ ($C_{30}H_{48}O_4$). Analysis of these spectral data compared to those reported in the literature (Aguirre et al. 2006) allowed the identification of compound 3 as corosolic acid (Fig. 5).

Corosolic acid was reported in *E. gustavoides* and asiatic acid was found in *E. gustavoides* and *E. crebinervis* (Frighetto et al. 2015). According to the literature, corosolic acid presented anti-inflammatory activity by different mechanisms such as attenuating of apoptotic and oxidative stress. Corosolic acid inhibits ethanol-induced apoptosis and increases the levels of tumor necrosis factor-α (TNF-α) and reactive oxygen species (ROS) *in vitro* (Guo et al. 2016). Furthermore, corosolic acid was effective as an anti-inflammatory agent through the arachidonic acid cascade inhibition, with similar potency to nimesulide (Aguirre et al. 2006).

Figure 5. Triterpenoids from *E. pruniformis* leaves. 1 – β-Sitosterol; 2 – Ursolic acid; 3 – Corosolic acid; 4 – Asiatic acid.

Table I. ^{1}H. and ^{13}C-NMR Data of Compounds 1, 2, 3 and 4, in ppm. Assignments of ^{13}C-NMR data were accomplished with the aid of DEPT, HSQC and HMBC experiments.

	β-sitosterol		Ursolic Acid		Corosolic Acid		Asiatic Acid	
Position	δ H	δ C	δ H	δ C	δ H	δ C	δ H	δ C
1		37.2		40.0		46.9		46.6
2		31.6	1.84	28.8	3.63 (ddd)	68.2	3.69 (ddd)	68.2
3	3.51 (m)	71.8	3.48 (dd)	78.8	2.91 (d)	83.1	3.36 (dd)	76.8
4		42.3		39.7		39.1		42.7
5	5.33 (d)	140.7		56.5		55.3		46.8
6		121.7	Hα 1.59 (t)	19.4		18.1		17.7
7		31.9	Hα 1.60 (t)	34.2		32.8		32.2
8		31.9		40.6		39.4		39.4
9		50.1	1.65 (t)	48.7		47.6		47.4
10		36.5		37.9		37.8		37.6
11		21.1	1.99 (m)	24.3		23.0		23.2
12		39.8	5.51 (t)	126.3	5.24 (t)	125.3	5.26 (t)	125.2
13		42.3		139.9		138.3		138.4
14		56.8		43.2		41.9		41.9
15		26.1	Hβ 2.35 (td)	29.3		27.8		27.7
16	0.66 (s)	28.2	2.15 (td)	25.6		23.9		23.9
17		56.0		48.7		47.6		48.4
18		36.1	2.66 (d)	54.2	2.21 (d)	53.0	2.21 (d)	52.8
19	0.99 (s)	19.0	1.49 (t)	40.1		39.0		39.0
20		34.0	1.07	40.1		39.0		39.0
21	0.90 (d)	26.1	Hα 1.40 (t)	31.7		30.4		30.3
22		45.8	1.96 (m)	37.9		36.7		36.7
23		23.1	1.26 (s)	29.5	1.02 (s)	27.9	3.50 (d)	64.9
24	0.84 (t)	12.0	1.04 (s)	17.2	0.81 (s)	16.1	0.70 (s)	12.5
25		29.2		16.3	1.01 (s)	15.8	1.03 (s)	16.2
26	0.81 (d)	19.8	1.08 (s)	18.1	0.85 (s)	16.4	0.85 (s)	16.4
27	0.79 (d)	19.4	1.25 (s)	24.6		22.7	1.14 (s)	22.7
28		18.8		180.2		180.2		180.2
29		11.9	1.03 (d)	18.2	0.89 (d)	16.2	0.89 (d)	16.2
30	-	-	0.98 (d)	22.1	0.97 (s)	20.1	0.97 (s)	20.1
U.S.	1.12-2.32 (m)							

U.S. = Undefined NMR Signals. Data were recorded at 500 (^{1}H) and 125 (^{13}C) MHz. *Some multiplicities are omitted due to overlapping events.

The ^{1}H-NMR spectrum of asiatic acid (4) exhibited also characteristic triterpenoids signals with the presence of H-12 as a triplet at δ_H 5.26, H-2 and H-3, bonded to hydroxylated carbons, as a double double-doublet at δ_H 3.69 and as a triplet at δ_H 3.36, respectively, and the singlet methyl of H-23, H-24, H-29 and H-30 at δ_H 3.27, δ_H 0.70, δ_H 0.89 and δ_H 0.97, respectively. The ^{13}C-NMR spectrum of 4 also showed a typical thirty signals of triterpenoids such as at δ_C 68.2 (C-2), 76.8 (C-3), 125.2 (C-12), 138.4 (C-13), 64.9 (C-23), at δ_C 12.5 (C-24), 16.2 (C-29), 20.1(C-30). These data, associated to that obtained from HPLC-Q-TOF with the quasi-molecular ion peak $[M-H]^-$ of 487.48385 ($C_{30}H_{48}O_5$) and those found in the literature (Aguirre et al. 2006), allowed the structural identification of asiatic acid (Fig. 5).

Asiatic acid possesses a wide spectrum of biological activities, notably anti-inflammatory (Hao et al. 2017) and antimicrobial effects (Harnvoravongchai et al. 2018). Hao and collaborators (Hao et al. 2017) show that asiatic acid significantly inhibited LPS-induced IL-6 and IL-8 expression levels in gingival tissues and significantly attenuated LPS-induced PGE2, NO, IL-6, and IL-8 production *in vivo*. Besides, asiatic acid displayed substantial inhibitory effects on *Clostridium difficile*, the causative agent of antibiotic-associated diarrhea, with the minimal inhibitory concentrations (MIC) value of 10.0 µg/mL, and also displayed an inhibitory effect on cell motility (Harnvoravongchai et al. 2018).

In conclusion, the results of our study show that TF-EpL have anti-*Leishmania amazonensis* activity of *in vitro*, which might be associated with promastigotes' incidental death, and its potential for further studies as a source of new drugs for leishmaniasis.

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RDDGA (PhD. student) contributed to collecting plant samples, running the laboratory work, analysis of the data and drafted the paper. MGS contributed to plant identification and herbarium confection. RSE contributed to the phytochemical analysis. VFA and GBB contributed to anti-promastigote assay in strain MHOM / BR / 77LTB0016. DCS contributed to anti-amastigote studies. CF, CLAP, and EF designed the leishmanicidal studies in strain WHOM / BR / 75 / Josefa analysis of the data and drafted the paper. ALMM contributed to the chromatographic analysis. APO and LMR designed the study, supervised the laboratory work and contributed to the critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

8.7 Artigo 8

Ocotea pulchella as an alternative against schitossomiasis: chemical analysis, development of nanoemulsion and biological control activity

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Ocotea pulchella as an alternative against schistosomiasis: chemical analysis, development of nanoemulsion and biological control activity

[*Ocotea pulchella* como una alternativa ante esquistosomiasis: análisis químico, desarrollo de una nanoemulsión y actividad en control biológico]

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Abstract: The aim of this work was to evaluate the potential of the essential oil (EO) from *Ocotea pulchella* leaves as an alternative in the control of schistosomiasis. It was tested *O. pulchella* EO nanoformulation to assess its activity against adult *Biomphalaria glabrata*, their spawning and *Schistossoma mansoni* cercariae. Additionally, the EO chemical composition was investigated by gas-chromatography. Nanoemulsion were elaborated by the low energy method. The adult mollusks, their spawning and cercariae were placed in contact with nanoemulsion to calculate lethal concentrations. Myristicin, bicyclogermacrene and α-Pinene were the main substances in the EO. Nanoemulsion caused mortality of adult *B. glabrata*, its egg embryos and *S. mansoni*. These results suggest the use of this nanoemulsion as an alternative in the control of the schistosomiasis cycle.

Keywords: Essential oil nanoemulsion; *Ocotea pulchella*; Mollusk control; Biomphalaria; *Schistosoma mansoni*; Schistosomiasis.

Resumen: El objetivo de este trabajo fue evaluar el potencial de los aceites esenciales (AE) de las hojas de *Ocotea pulchella* como una alternativa en el control de esquistosomiasis. Se probó una nanoformulación de AE de *O. pulchella* para evaluar su actividad ante adultos de *Biomphalaria glabrata*, sus huevos y cercarías de *Schistossoma mansoni*. La nanoemulsión fue elaborada por el método de baja energía. Los moluscos adultos, sus huevos y cercarías se colocaron en contacto con la nanoemulsión para calcular concentraciones letales. Los compuestos mayoritarios en el AE fueron miristicina, biciclogermacreno y α-pineno. La nanoemulsión causó mortalidad en adultos de *B. glabrata*, sus huevos y a *S. mansoni*. Los resultados sugieren el uso de esta nanoemulsión como una alternativa en el control del ciclo de esquistosomiasis.

Palabras clave: Nanoemulsión de aceites esenciales; *Ocotea pulchella*; Control de moluscos; Biomphalaria; *Schistosoma mansoni*; Esquistosomiasis.

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INTRODUCTION

Schistosomiasis is an acute and chronic parasitic disease caused by *Schistosoma trematodes* and transmitted by snails of various species. Nowadays, it is the second-largest infectious parasitic disease in the world after malaria. In total, 78 countries are affected, mainly in tropical and subtropical regions. In 2017, nearly 99 million people worldwide used treatment for schistosomiasis (WHO, 2018). The acute form of this disease causes symptoms such as fever, fatigue, malaise, myalgia, nonproductive cough. The later stages of this disease are characterized by abdominal pathologies such as diarrhea, hepatosplenomegaly, and diffuse abdominal pain. The chronic form of the disease occurs when *Schistosoma* lays its eggs, and host immune system reactions lead to hepatic, urinary, intestinal, and ectopic forms of the disease (Colley *et al.*, 2014).

Human schistosomiasis is caused by parasites of the genus *Schistosoma*, which use the mollusks of the genus *Biomphalaria* as intermediate hosts. In many cases, prevention methods such as eradicating these hosts using chemical pesticides are relevant to disease control. Niclosamide (Baylucide®, Bayer, Leverkusen, Germany) is the only commercially available molluscicide recommended by the World Health Organization (WHO) for large-scale use in schistosomiasis control programs (WHO, 1983). However, this substance is toxic to other organisms, and resistance to this agent makes it necessary to search for new drugs and elements to be used for intermediate host control (Inobaya *et al.*, 2014). Therefore, the WHO stimulates the search for alternative substances based on plant species (Ding-Feng, 2010). Because, plants are abundant in countries with endemic schistosomiasis and have different components in their extracts, which makes the appearance of resistance difficult (Tavares *et al.*, 2007).

Many plant extracts and oils exhibiting intrinsic molluscicidal activity may have problems with solubility in the aquatic environment due to the low polarity. In this context, nanotechnology has been used to circumvent this problem and promote the stability of active substances. There are currently several drug nanocarriers, including nanoparticles, nanoemulsions (NEs), and liposomes (Donsi & Ferrar, 2016).

Nanoemulsions have been widely used as nanocarriers of essential oils and hydrophobic substances. NEs are dispersions of nanometric oil droplets (20-200 nm) in water, stabilized by surfactants (Sagalowicz & Leser, 2010; Jaiswal *et al.*, 2015). Some key advantages of these nanocarriers are their easy preparation, simple composition, low production cost, industrial production possibility, and high thermodynamic stability (Tromer & Neubert, 2006; Campos *et al.*, 2012).

The species *Ocotea pulchella* is popularly known as "canelinha", "canela-preta", "canela-lageana" (Marques, 2001), and "inhumirin" (Quinet *et al.*, 2015). In Brazil, it is distributed in the North, Midwest, Southwest and South regions (Quinet *et al.*, 2015). Although botanical and agronomic studies are found for this species, little information is known about its chemical composition and biological importance. On the other hand, other species of the genus *Ocotea* have the chemical description of essential oils, which in turn have large compounds with molluscicidal activity (Rambo, 2014; Leite *et al.*, 2009). Thus, the aim of this work was to investigate the chemical composition of the essential oil from *O. pulchella* leaves and their activity in the control of schistosomiasis.

MATERIALS AND METHODS

Plant material

Fresh leaves from three specimens of *Ocotea pulchella* were collected from Restinga de Jurubatiba National Park, at the coordinates 22°12'697" S - 41°35'321 "W, 22 ° 12 688"S - 41 ° 35'324 "W, and 22 ° 12'692" S - 41 ° 35'331 "W. This work was realized under the authorization number 13659-12 (SISBIO) and A0D648D (SISGEN). The species was identified by the botanist Dr. Marcelo Guerra Santos, and a voucher sample was deposited in the herbarium of the Faculty of Teacher Education, under the number 16451 (RFFP) (Rio de Janeiro State University, Brazil).

Essential oil extraction

Leaves of *O. pulchella* (1810.9 g) were turbolized with distilled water. Then, the material was placed in three 5 L round bottom flask and hydrodistilled for 4 h in a Clevenger type apparatus. The essential oil obtained was stored at 4°C for further chemical analysis and development of NEs.

Gas chromatography analysis

Gas chromatography (GC) was performed under the following conditions: injector temperature, 260°C; GC/MS detector, 290°C; carrier gas, Helium; flow rate, 1 mL/min; Split, 1:40. The oven temperature was initially 60°C and then rose to 290°C at a rate of

3°C/ min. One microliter of each sample dissolved in dichloromethane (1:100 mg/ mL) was injected into a DB-5 column (0.25 µm x 30 m x 0.25 µm). Electron ionization by mass spectrometry (MS) was 70 eV, and the scanning speed was 1 Scan/ s. Retention rates (RI) were calculated by extrapolating the retention times of an aliphatic hydrocarbon mixture (C9-C30) analyzed under the same conditions (van der Dool & Kratz, 1963).

Substances were identified by comparing their arithmetic indices (AI) and mass spectra with those reported in the literature (Adams, 2007). The MS fragmentation pattern of the compounds was also compared to the NIST mass spectrum libraries (database program containing mass spectra of over 100,000 substances). Quantitative analysis of the chemical constituents was performed by flame ionisation gas chromatography (CG/FID), under the same conditions as GC/MS analysis. Percentages of these compounds were obtained by FID peak area normalisation method.

Nanoemulsification method and determination of hydrophilic-lipophilic balance (HLB)

Emulsification was produced by modifying the low energy method described by Ostertag *et al.* (2012).The emulsions were composed of 5% (w/w) *O. pulchella* oil, 5% (w/w) surfactants and 90% (w/w) water. Span 80 and Tween 20 were used as surfactants to prepare NEs. Several emulsions with HLB values ranging from 4.3 to 16.7 were prepared by mixing the surfactants in different proportions. For the preparation of the NEs, the oily phase consisting of *O. pulchella* oil and surfactants was homogenized by magnetic stirring (400 rpm) for 30 min at room temperature. After that, the aqueous phase (distilled water) was added to the magnetic stirring oil phase (400 rpm) for an additional 1 h. The formulations were analyzed for stability by droplet size analysis and polydispersion index (PDI) values. The formulation with the smallest droplet size and PDI values indicated the appropriate oil HLB.

The droplet size and polydispersity index (PDI) of the NEs were determined by photon correlation spectroscopy (Zetasizer ZS, Malvern, UK). Droplet measurements were performed in triplicate, and the mean droplet size was expressed as mean diameter ± standard deviation.

Molluscicidal Assays

The action of the selected NE was evaluated for biological control of *B. glabrata* using the methodology of Santos *et al.* (2017). Clams of size 10-12 mm were individually placed in 24-well plates and exposed to concentrations of 20, 40, 60, 80, 100 and 120 ppm in the final 2 mL NE volume. The mortality of the snail was compared with the negative controls: NE white (containing only distilled water and surfactants - 2 mL) and distilled water (2 mL), and with the positive control: niclosamide (2 mg/L, 2 mL). Mortality was assessed at 24 h and 48 h, and the absence of shell retraction and hemolymph release were the criteria to evaluate mortality.

Evaluation of ovicidal activity

On the initial day, the Styrofoam plates were deposited in the water of the *B. glabrata* rearing tanks for oviposition. After 48 h, the egg capsules were carefully removed from the Styrofoam and then accommodated in 24-well plates using the adapted methodology of Araújo *et al.* (2019). Then, the number of viable eggs at time zero was counted, and then 1000 µL of NE was added to the plate wells at concentrations of 20, 40, 60, 80, 100 and 120 ppm. After 24 h and 48 h of exposure, viable egg counts were repeated.

Evaluation of cercarial activity

In a 24-well plate, the amount of *S. mansoni* cercariae present in 1000 µL was initially estimated using 20 µL Lugol and counted in a stereomicroscope. Then, in another well of the plate, 1000 µL of *S. mansoni* cercaria suspension and 1000 µL of NE at 20, 40, 60, 80, 100, and 120 ppm were added. After that, 20 µL of 0.1% Trypan Blue dye was added. Dead blue cercariae were counted at 1 h, 2 h, 3 h, and 4 h.

Statistical analysis

Statistical analysis of the experiments was performed using the Prism 6 GraphPad program (GraphPad software), using one-way ANOVA, two-way ANOVA, followed by the Tukey test with a $p<0.001$. Linear regression with $p<0.0001$ and R2 = 0.9647 were used as significant statistical parameters.

RESULTS

Essential oil

The essential oil obtained from fresh leaves showed a translucent and transparent appearance with a volume of 19 mL and yielded 1.05%. In total, it was identified 20 substances (Table No. 1). There is a predominance of monoterpene hydrocarbons (41.87%), followed by 31.45% phenylpropanoid and 24.29% of hydrocarbon sesquiterepene. The main

substances were myristicin (26.34%), α-pinene (17.20%), and bicyclogermacrene (16.57%).

Table No. 1
Chemical composition of the essential oil of *Ocotea pulchella* (Mart.) leaves

AI LIT	AI EXP	SUBSTANCES	%
932	935	α-pinene	17.2
946	949	canphene	0.3
974	979	β-pinene	8.3
988	992	myrcene	1.2
1002	1008	δ-carene	9.5
1020	1026	cymene	2.3
1024	1030	limonene	0.9
1026	1032	cyneol	0.5
1086	1091	terpinolene	2.2
1335	1339	δ-elemene	0.6
1374	1378	α-copaene	0.3
1389	1395	β-elemene	0.6
1417	1423	β-caryophyllene	1.6
1492	1485	δ-selinene	2.0
1500	1503	bicyclogermacrene	16.6
1517	1508	myristicin	26.3
1522	1528	δ-cadinene	0.3
1568	1582	β-isoelemicine	5.1
1577	1586	spathulene	2.3
2042	2046	kaurene	1.9
		Total Identified	100.0
		Monoterpenes hydrocarbons	41.9
		Phenylpropanoid	31.4
		Sesquiterpenes hydrocarbons	24,3
		Diterpene	1.9
		Oxygenated Monoterpenes	0.5

Nanoemulsion
It was prepared 11 formulations with HLB values ranging from 16.7 to 4.3 (Table No. 2). Then the six formulations with the best HLB indices, which ranged from 10.5 to 13, were refined. The formulation selected presents an HLB value of 11.5, droplet size of 85.42 nm and PDI value of 0.284 (Table No. 3).

Molluscicidal assay
The evaluation of molluscicidal activity was performed to determine the lethality of the NE of the essential oil of *Ocotea pulchella* leaves in the schistosomiasis *B. glabrata* transmitting species. In the molluscicidal assay, the LC_{50} and LC_{90} values over the 24h period were LC_{50} = 45.78 ppm and LC_{90} = 64.14 ppm, while in the 48h period were LC_{50} = 38.58 ppm and LC_{90} = 42.97 ppm (Figure No. 1).

Ovicidal assay
It was evaluated the action of NE in the ovicidal assay, within 24h and 48h. It was observed LC_{50} =

33.53 ppm and LC_{90} = 39.25 ppm in 24h. At 48h, all tested concentrations of NE led the eggs to death (Figure No. 2).

Table No. 2
EHL values of different Nanoemulsion formulations from *Ocotea pulchella* and their respective percent composition

Formulation	EHL	TWEEN 20	Span 80	Water
1	16.70	0.250	0	4.5
2	15.46	0.225	0. 25	4.5
3	14.22	0.200	0. 50	4.5
4	12.98	0.175	0. 75	4.5
5	11.74	0.150	0.100	4.5
6	10.50	0.125	0.125	4.5
7	9.26	0.100	0.150	4.5
8	8. 2	0. 75	0.175	4.5
9	6.78	0. 50	0.200	4.5
10	5.54	0. 25	0.225	4.5
11	4.30	0. 0	0.250	4.5

Table No. 3
Droplet size, PDI and HLB values of the six *Ocotea pulchella* essential oil formulations

Formulation	Droplet size (nm)	PDI	EHL
F1	97.85	0.193	10,5
F2	99.85	0.207	11.0
F3	85.50	0.284	11.5
F4	87.09	0.282	12.0
F5	160.20	0.751	12.5
F6	93.43	0.295	13.0

Cercaricidal activity
The results of the cercaricidal activity of the NE during the 4h period were: LC_{50}/1h = 74.27 ppm, LC_{90}/1h = 121.79 ppm; LC_{50}/2h = 57.96 ppm, LC_{90}/2h = 98.12 ppm; LC_{50}/3h = 48.87 ppm, LC_{90}/3h = 89.01 ppm and LC_{50}/4h = 44.89 ppm, LC_{90}/4h = 86.01 ppm (Table No. 4 and Figure No. 3).

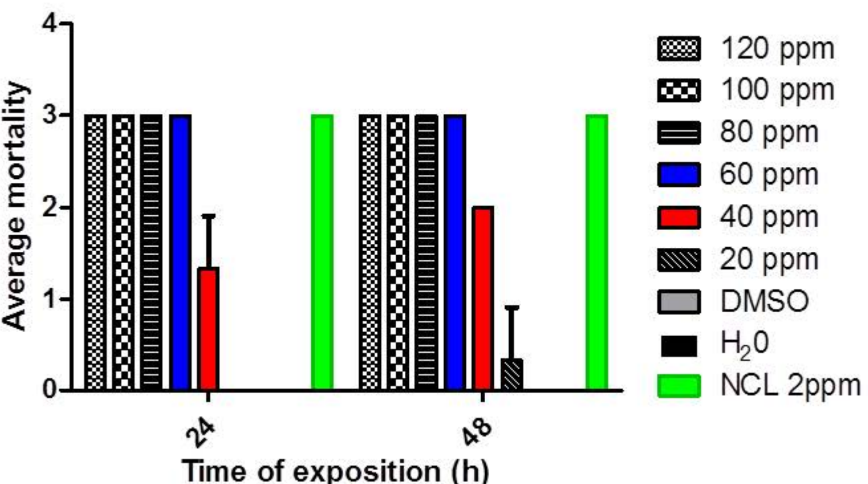

Figure No. 1
Dose-response relationship between mortality (percentage) of *Biomphalaria glabrata* (n = 3) and *Ocotea pulchela* nanoemulsion. This experiment was performed in triplicate on at least three separate days. Adult molluscicide test with *Biomphalaria glabrata* within 48 h of nanoformulation exposure. NCL - Niclosamide, DMSO-Dimethyl Sulfoxide

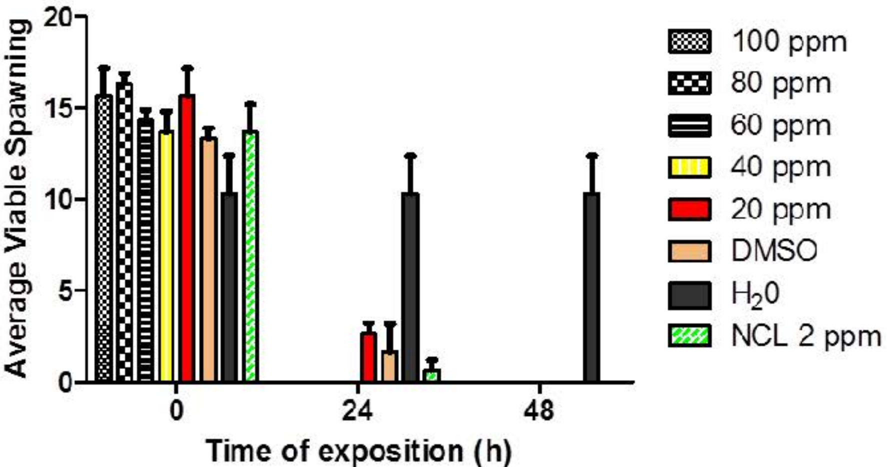

Figure No. 2
Concentration-response relationship between average viable spawning of *Biomphalaria glabrata* embryos and Ocotea pulchela nanoemulsion for 48 h of exposure. The results expressed in the graph represent the mean ± standard error. NCL - Niclosamide®, DMSO-Dimethyl Sulfoxide. $p<0.05$

Table No. 4
Lethal nanoemulsion concentrations (LC_{50} and LC_{90}) at 1 to 4 h exposure periods

Time of Exposition	LC_{50}	CL_{90}
1 h	74.27 ppm	121.79 ppm
2 h	57.96 ppm	98.12 ppm
3 h	48.87 ppm	89. 1 ppm
4 h	44.89 ppm	86. 1 ppm

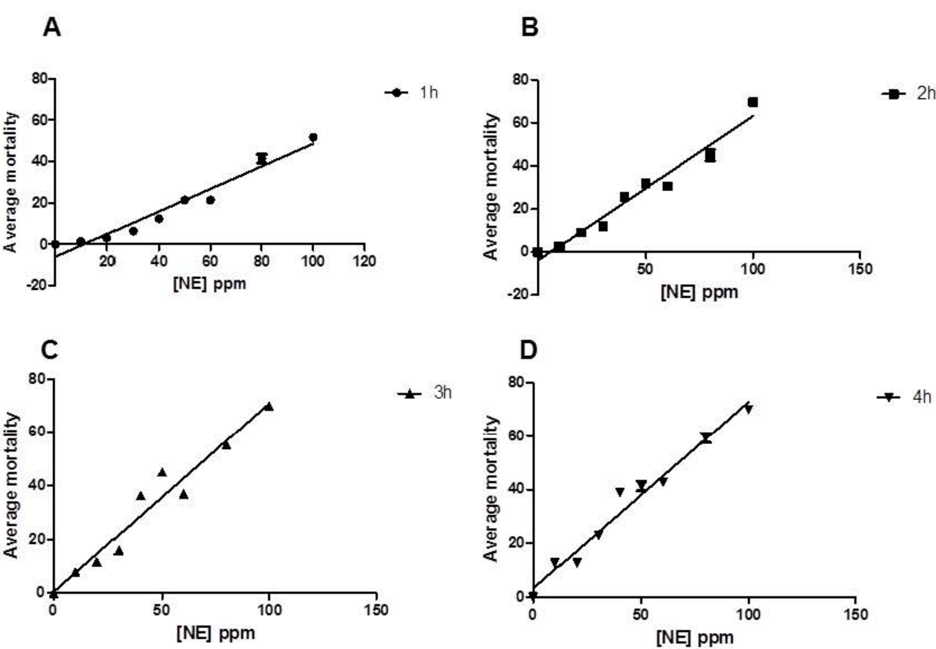

Figure No. 3
Concentration-response relationship between average mortality of *Schistossoma mansoni* cercariae and *Ocotea pulchela* nanoemulsion exposed for 1 h (A), 2 h (B), 3 h (C), and 4 h (D). The results expressed in the graph represent the mean ± standard error

DISCUSSION

Currently, the control of schistosomiasis includes several methods such as prevention, population control of vector mollusks, and the use of antiparasitic agents in the treatment of the disease (WHO, 2016). The control of intermediate hosts of parasites of the genus *Schistosoma*, such as *Biomphalaria glabrata*, is a widely used method, but it is dependent on the few commercially available molluscicidal agents, which also have restrictions, such as the occurrence of biological resistance to these agents, as well as their effects environmental impacts, negatively affecting the local ecosystem (Inobaya *et al*., 2014). Besides, the high risks of side effects and increased antiparasitic resistance also become sufficient reasons for the search for new agents that act on parasites in their various forms (Catanhede *et al*., 2010). Therefore, studies involving the search for new drugs with molluscicidal and/ or antiparasitic potential against *Schistosoma* are necessary. Preferentially, studies involving plant derivatives aiming at the following aspects: effectiveness, economic viability, low environmental impact, and generation of new technologies in the fight against schistosomiasis (Brasil, 2014; Nascimento *et al*., 2018).

The genus *Ocotea* is described in the literature about its broad pharmacological and biological activities, such as insecticide, larvicide, and anticholinesterase (Yamaguchi *et al*., 2012; Mossi *et al*., 2013; Scalvenzi *et al*., 2019). However, this study is a pioneer in the research of biological activity with *Ocotea pulchella* essential oil. The phytochemical analysis identified Myristicin, α-Pinene, and Bicyclogermacrene as the three main essential oil substances in *O. pulchella* leaves. Bicyclogermacrene and α-pinene were also found in other species of the genus between the two main essential oil substances, such as *Ocotea indecora*, *Ocotea caudata*, and *Ocotea morae*, while myristicin was first reported as the main substance in the essential oil of leaves of the genus *Ocotea* (Prieto *et al*., 2010; Chaverri *et al*., 2011; Rambo, 2014; Gil *et al*., 2016).

Myristicin (26.34%), α-pinene (17.20%), and bicyclogermacrene (16.57%) are the major components of the *O. pulchella* essential oil from leaves. These substances already have been described by having biological and pharmacological activities. The compound myristicin demonstrated insecticidal and acaricidal activity (Lichtenstein & Casida, 1963), monoaminoxidase inhibition (Truitt *et al*., 1963), and anticholinergic effect (McKenna *et al*., 2004). The monoterpene α-pinene showed nociceptive (Him *et al*., 2008), anti-inflammatory and chondroprotective activities (Rufino *et al*., 2014), as well as antifungal against *Candida albicans* and *Cryptococcus neoformans*, antibacterial against penicillin-resistant *Staphylococcus aureus* (Rivas da Silva *et al*., 2012) and molluscicide activity (Leite *et al*., 2009). This monoterpene also showed an anticholinesterase activity of IC_{50} = 0.96 mM (Zarrad *et al*., 2017). The substance bicyclogermacrene also demonstrated molluscicide activity against three different species of *Biomphalaria*, including *B. glabrata* (Araujo *et al*., 2019), and antifungal activity (Silva *et al*., 2007). These findings corroborate with the results found in this work and suggest a molluscicide activity against *B. glabrata*.

The NEs prepared in this study by modification of the low energy method of Ostertag *et al*. (2012), allowed better control of droplet size in the preparation of emulsions. The extremely low particle size provided greater resistance to the effects of cremation and sedimentation, presenting a lower interfacial tension. In addition, this system allows easier solubilization of the different substances (Salager *et al*., 2001; Solans *et al*., 2005).

The average particle size and polydispersion index values, besides the visual observations, were the parameters of physicochemical evaluation to choose the best proportion of surfactants in the NE. Results were obtained with the Nano SZS particle test. 90 (Malvern Instruments, Worcestershire, United Kingdom). The biodegradability of the formulation is another factor that minimizes the residual toxicity of molluscicidal agents to the environment and can be used to compare the mortality rate among *Biomphalaria* populations, optimizing the resistance to NE effect, which is not the case with Niclosamide (Mishra *et al*., 2018). Currently, the use of this product is effective, however, it is included in grade III chemical toxicity, killing both snails and eggs, as well as local faunas and flora. Also, *Biomphalaria* can shrink, bury itself in the substrate and still move away from the area of the highest concentration of dissolved products in water, even if it is in the soil (Oliveira-Filho, 2003).

NE is a product of operational simplicity, has been shown to be effective, has low reagent costs, has good dispersion in the aquatic environment, which makes this formulation have advantages over the use of Niclosamide. These objectives are guided by the schistosomiasis control program (PCE), 2008, in

which alternative research methods are suggested in plants with molluscicidal activities, confirming the need for efficient and ecologically acceptable molluscicides (Brasil, 2014).

NE molluscicidal activity showed LC_{50} = 45.78 ppm and LC_{90} = 64.14 ppm after 24h, which classifies a plant extract as active when it has an LC_{90}/24h value below 100 ppm (WHO, 1983).

In the cercariae activity assay, the NE presented LC_{90} = 86.69 ppm within the 4h period, proving to be effective when compared to the control group. The data obtained in this work demonstrated experimental similarity with the study by Albagouri *et al.* (2014). In their work, they presented results of different Sudanese plant species with cercaricidal activity showing LC_{50} and LC_{90} values below 100 ppm, corroborating with the results found in the present work.

Evaluations of the 24h period showed that 40 to 100 ppm concentrations led to 100% of the eggs to death, and the 20 ppm concentration obtained high but not total embryo mortality. In 48h, even the concentration of 20 ppm could kill 100% of the eggs. Watanabe (1997) reports the great importance of embryonic stage testing in *Biomphalaria* sp. serving as bioindicators in polluted waters and in tests performed as biomarkers in testing and mutagenicity.

The present study demonstrated the molluscicidal activity of the nanoemulsified essential oil of *O. pulchella* leaves in embryos and adult forms of schistosomiasis transmitters *B. glabrata*, as well as the cercaricidal activity. Thus, the biotechnological product developed from *O. pulchella* exhibited action at different stages of the schistosomiasis cycle, being a promising alternative in the control of this disease.

CONCLUSION

The chemical analysis of the essential oil from *Ocotea pulchella* leaves was performed for the first time, presenting α-pinene, bicyclogermacrene, and myristicin as main constituents. Therefore, it was described the effect of *O. pulchella* essential oil nanoemulsion on the control of neglected tropical and subtropical diseases, specifically against the species *Biomphalaria glabrata* and its embryos, both involved in *Schistosoma mansoni* schistosomiasis and cercariae transmission etiological agent of this disease. Thus, these results suggest the use of this product as a promising alternative for controlling the life cycle of *B. glabrata* species, the main vectors of schistosomiasis, and against its etiological agents, *S. mansoni.*

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