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LIZANDRA LIMA SANTOS

Estudo fitoquímico e biológico do óleo essencial e extrato bruto etanólico das folhas de *Pogostemon cablin* (Blanco) Benth

Macapá
2019

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Amapá para obtenção do Título de Mestre em Ciências Farmacêuticas.

Orientadora: Sheylla Susan Moreira da Silva de Almeida

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BANCA EXAMINADORA

Aluna: Lizandra Lima Santos

Orientadora: Sheylla Susan Moreira da Silva de Almeida

Profa. Dra. Sheylla Susan Moreira da Silva de Almeida / Presidente
Professor Titular do Curso de Farmácia da Universidade Federal do Amapá, UNIFAP.

Profa. Dra. Elizabeth Viana Moraes da Costa / Membro Titular
Professor Titular do Curso de Farmácia da Universidade Federal do Amapá, UNIFAP.

Prof. Dr. Raimundo Nonato Picanço Souto / Membro Titular
Professor Titular do Curso de Farmácia da Universidade Federal do Amapá, UNIFAP.

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SÍMBOLOS, SIGLAS E ABREVEATURAS

µL – Microlitro

% AA – Porcentagem de atividade antioxidante

ATCC – American Type Culture Collection

CBM – Concentração bactericida mínima

CIM – Concentração inibitória mínima

GC-MS – Gas Chromatography coupled to the Mass Spectrometer

CI_{50} – Concentração Inibitória 50%

CL_{50} – Concentração Letal que causa mortalidade de 50% dos indivíduos

CLSI – Manual Clinical and Laboratory Standards Institute

cm – Centímetro

CO_2 – Dióxido de carbono

DMSO – Dimetilsufóxido

DPPH – 2,2-difenil-1-picrilhidrazil

FIOCRUZ – Fundação Oswaldo Cruz

HAMAP – Herbário Amapaense

IEPA – Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá

RI – Índices de Retenção

mg – Miligrama

CMH – Caldo Müeller-Hinton

mL – Mililitro

mm – Milímetro

OE – Óleos Essenciais

OMS – Organização Mundial da Saúde

ppm – partes por milhão

SPSS – Statical Package for the Social Science tR – Tempo de retenção

WHO – World Health Organization

RESUMO

AVALIAÇÃO DO POTENCIAL LARVICIDA DO ÓLEO ESSENCIAL E EXTRATO BRUTO ETANÓLICO DAS FOLHAS DE *Pogostemon cablin* (Blanco) Benth NO CONTROLE DO *Aedes aegypti*.

RESUMO

Introdução: O *Pogostemon cablin* (Blanco) Benth, popularmente conhecida como Oriza e Patchouli é uma planta da espécie da família Lamiaceae, que possui diversas atividades biológicas incluindo antimicrobiana, antioxidante, analgésica, anti-inflamatória, antitrombótica, antidepressiva, citotóxica e potencial entomotóxico reconhecido que servem como estratégia alternativa para o controle químico do *Aedes aegypti*. **Objetivo:** Realizar o estudo químico e avaliação da atividade antioxidante, citotóxica, antimicrobiana e larvicida do óleo essencial e extrato bruto etanólico de *P. cablin*. **Metodologia:** A identificação dos metabólitos foi realizada por testes fitoquímicos, quantificação de fenólicos totais para os extratos, e GC-MS para o óleo essencial. A atividade larvicida foi realizada frente as larvas do *A. aegypti*. A atividade antioxidante foi avaliada por meio da capacidade sequestrante do 2,2-difenil-1-picril-hidrazila (DPPH). Quanto a avaliação microbiológica, utilizou-se a técnica de diluição em microplacas contra três bactérias. A atividade citotóxica foi avaliada frente às larvas de *Artemia salina.* **Resultados e discussões:** A espécie *P. cablin* apresentou os seguintes compostos majoritários no óleo essencial: álcool de patchouli (33,25%), Seyshellene (6,12%), α-bulnesene (4,11%), Pogostol (6,33%) e Norpatchouleno (5,72%), no extrato as classes de compostos: esteroides e triterpenóides, depsídeos e depsidonas, que em sinergia com as demais substâncias potencializaram a significativa ação larvicida da espécie com CL_{50} de 28,43 $\mu g.mL^{-1}$ para óleo, e CL_{50} de 63,91 $\mu g.mL^{-1}$ para o extrato, em 24h. Não houve atividade antioxidante, todavia o oléo essencial apresentou atividade antimicrobiana frente a todas bactérias testadas com CIM e CBM de 62,5 $\mu g.mL^{-1}$ e o extrato apresentou inibição do crescimento bacteriano frente a *E. coli* com CIM de 31.25 $\mu g.mL^{-1}$. O óleo essencial demonstrou expressiva ação toxica com CL_{50} de 24,25 $\mu g.mL^{-1}$ e o extrato moderada ação toxica com CL_{50} de 257.93 $\mu g.mL^{-1}$. **Conclusões:** A espécie *P. cablin* demonstrou significativo potencial larvicida, com ação antimicrobiana, ausência de ação antioxidante e elevada toxicidade.

Palavras-Chave: Biocida, Patchouli, Oriza, Plantas medicinais, Lamiaceae, Controle vetorial

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ABSTRACT

EVALUATION OF THE LARVICIDAL POTENTIAL OF THE ESSENTIAL OIL AND CRUDE ETHANOLIC EXTRACT OF THE LEAVES OF *Pogostemon cablin* (Blanco) Benth IN THE CONTROL OF *Aedes aegypti*.

Introduction: *Pogostemon cablin* (Blanco) Benth, popularly known as Oriza and Patchouli is a plant of the family Lamiaceae, which has several biological activities including antimicrobial, antioxidant, analgesic, anti-inflammatory, antithrombotic, antidepressant, cytotoxic and recognized entomotoxic potential that serve as alternative strategy for the chemical control of *A. aegypti*. **Objective:** To carry out the chemical study and evaluation of the antioxidant, cytotoxic, antimicrobial and larvicidal activity of the essential oil and crude ethanolic extract of *P. cablin*. **Methodology:** The identification of metabolites was performed by phytochemical tests, quantification of total phenolics for extracts, and GC-MS for essential oil. The larvicidal activity was performed against the larvae of *A. aegypti*. The antioxidant activity was evaluated by the sequestering ability of 2,2-diphenyl-1-picryl-hydrazyl (DPPH). As for the microbiological evaluation, the technique of dilution in microplates against three bacteria was used. The cytotoxic activity was evaluated against larvae of Artemia salina. **Results and discussion:** The species P. cablin presented the following major compounds in the essential oil: patchouli alcohol (33.25%), Seyshellene (6.12%), α-bulnesene (4.11%), Pogostol 33%) and Norpatchouleno (5.72%), in the extract the classes of compounds: steroids and triterpenoids, depsides and depsidones, which in synergy with the other substances potentiated the significant larvicidal action of the species with LC_{50} of 28.43 $\mu g.mL^{-1}$ for oil, and LC_{50} of 63.91 $\mu g.mL^{-1}$ for the extract, in 24 h. There was no antioxidant activity, however, the essential oil presented antimicrobial activity against all bacteria tested with MIC and CBM of 62.5 $\mu g.mL^{-1}$ and the extract showed inhibition of bacterial growth against E. coli with MIC of 31.25 µg.mL -1 . The essential oil showed a significant LC_{50} toxicity of 24.25 $\mu g.mL^{-1}$ and the moderate extract LC_{50} toxicity of 257.93 µg.mL-1. **Conclusions:** The *P. cablin* species showed a significant larvicidal potential with antimicrobial action, absence of antioxidant action and high toxicity.

Keywords: Biocide, Patchouli, Oriza, Medicinal plants, Lamiaceae, Vector control.

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1 INTRODUÇÃO

1.1 INSETICIDAS DE ORIGEM VEGETAL

Os mosquitos possuem destaque no cenário das doenças infectoparasitárias em virtude do seu desempenho como vetores em uma variabilidade de organimos patogênicos. O mosquito *A. aegypti* é um destes, visto que é responsável pela transmissão da Dengue, Zika e Chikungunya, doenças estas relacionadas com casos epidêmicos em diversos países, principalmente os situados nas zonas tropical e sobtropical do planeta (MARCOMBE et al., 2018).

Inseticidas sintéticos são comummente usados no controle vetorial do *A. aegypti* e geralmente são aceitos como eficazes, mas cancerígenos e perigosos para o meio ambiente e organismos não-alvo. O composto *N,N*-dietil-3-metilbenzamida, também conhecido como DEET, é um produto com expressiva eficiência para repelência de insetos (DENNIS; VOSSHALL, 2018) . No entanto, devido a neurotoxicidade aliado as reivindicações ambientais, a população passou a se preocupar com seu uso generalizado (LEGEAY et al., 2018).

Todavia, existe uma crescente resistência dos mosquitos aos inseticidas sintéticos, além dos impactos negativos ao meio ambiente. Assim, é importante realizar a busca por métodos alternativos para serem usados no controle do *A. aegypti,* que sejam eficientes, de baixo custo, biodegradáveis e mais seletivos (BRITO, 2015).

As plantas possuem mecanismos contra ação de insetos sendo capazes de sintetizar, a partir de diferentes vias metabólicas, compostos de defesa como metabólitos secundários e proteínas que agem como toxinas inseticidas. São essas substâncias de origem vegetal que possuem potencial entomotóxico reconhecido e despertam interesse de vários pesquisadores na busca por alternativas para o controle químico do *A. aegypti* (RAMOS, 2016).

Geralmente os compostos químicos são extraídos dos óleos essências e extratos de plantas com potencial repelente e inseticida, e estudados para formulação de produtos que sejam eficazes no controle do *Aedes*. Os produtos naturais de origem vegetal basicamente não apresentam toxicidade para os seres vivos, e pelo fato deles serem biodegradáveis, evitam a contaminação do meio ambiente. Diferente dos

produtos sintéticos, aos quais os insetos se tornam cada vez mais resistentes, são tóxicos e poluentes (BUSATO et al., 2015).

Pesquisas para o controle de vetor identificaram a presença de compostos químicos em geral monoterpenos, bem como sesquiterpenos, os quais possuem significante toxicidade contra insetos, mas negligenciável toxicidade para animais. (SANTOS; ALMEIDA, 2015; SANTANA, et al., 2015; MAKETE, 2016). A mistura desses compostos fornece as plantas seu odor característico, proteção contra herbívoros e patógenos, competição entre plantas, atrativo para polinizadores e dispersores de sementes e microorganismo simbiontes (NEVES et al., 2014). Tais compostos químicos são de interesse da comunidade científica para formulação de biocidas ativos e não poluentes.

Assim, com as dificuldades operacionais e econômicas geradas pela crescente resistência dos mosquitos aos inseticidas sintéticos, os métodos alternativos ganham novo destaque e passam a ter maior atenção por se mostrarem mais eficientes e baratos, visto que são obtidos de recursos renováveis, rapidamente degradáveis e apresentam várias substâncias que atuam simultaneamente, fazendo com que o desenvolvimento da resistência dos insetos a essas substâncias ocorra de forma muito lenta (MAKETE, 2016).

1.2 METABÓLITOS SECUNDÁRIOS

Entende-se como metabolismo o conjunto de reações químicas (anabólicas, catabólicas ou de biotransformação) que ocorrem continuamente no interior das células. Nas células vegetais, o metabolismo normalmente é dividido em primário e secundário. O metabolismo primário possui função essencial no vegetal, tais como a fotossíntese, a respiração e o transporte de solutos; os compostos deste metabolismo distribuem-se de maneira universal nas plantas. Em contraponto, o metabolismo secundário ocorre de maneira diferente em cada planta, não se distribuindo universalmente; possui importante função ecológica nas plantas, tais como proteção contra herbívoros e patógenos, competição entre plantas, atrativo para polinizadores e dispersores de sementes e microorganismo simbiontes (REZENDE et al., 2016).

As plantas possuem uma variedade de metabólitos secundários com ação antioxidante, como por exemplo, os terpenoides e aos fenilpropanoides presentes nos óleos essenciais, que agem inibindo a formação de radicais livres (TOHIDI; RAHIMMALEK, 2017).

Existem três grandes grupos de metabólitos secundários: terpenos, compostos fenólicos e alcaloides. Os terpernoides possuem estruturas mais variadas de produtos vegetais naturais, seu nome se deve ao fato de que os primeiros membros da classe foram isolados a partir da terebintina. São formados através da justaposição sucessiva de isopentenilpirofosfato (IPP-C_5) do qual se originam os demais terpenos (monoterpenos (C_{10}), sequiterpenos (C_{15}), diterpenos (C_{20}), triterpenos (C_{30}) e tetreterpenos (C_{40}) (SOUSA, 2015).

Os compostos fenólicos formam um grupo bastante presente no cotidiano, mesmo não sendo percebido. Dessa forma, muito do sabor, odor e coloração de diversos vegetais evidencia as características deste grupo. São importantes para a proteção das plantas contra fatores ambientais e bióticos adversos, bem como a conquista do ambiente terrestre pelas plantas. Quimicamente, são formados por pelo menos um anel aromático no qual ao menos um hidrogênio é substituído por um grupamento hidroxila. Existem duas rotas principais que estes são sintetizados: a via do ácido chiquímico e a via do ácido mevalônico, a qual é menos significativa (VEGGI, 2013).

Outra classe de metabolismo secundário importante são os alcaloides, que dispõe da presença de substâncias que atuam no sistema nervoso, sendo muitas delas largamente utilizadas como venenos ou alucinógenos. Quimicamente, são formados por compostos orgânicos cíclicos com pelo menos um átomo de nitrogênio no seu anel, onde na maioria possuem caráter alcalino devido a presença do átomo N representar um par de elétrons não compartilhados (VIZZOTTO et al., 2010).

Nesse contexto, análise química dos metabólitos secundários e do material botânico quer seja folhas, frutos, galhos e flores, fornecem informações imprescindíveis para formulação de produtos naturais com alta qualidade farmacognóstica, por exemplo.

1.3 FAMÍLIA LAMIACEAE

As plantas da família Lamiaceae (Labiatae) apresentam importância agrícola e são bastante utilizadas na culinária, medicina tradicional, indústria farmacêutica e cosmética. A Lamiaceae é uma das maiores famílias Angiosperma, apresenta distribuição comospolita incluindo aproximadamente 300 gêneros e 7.500 espécies em todo o mundo. No Brasil há ocorrência de cerca 350 espécies distribuídas em 26 gêneros (FERREIRA, 2016).

Estas espécies são herbáceas ou arbustivas com folhas simples, sem estípulas, com limbo inteiro, denteado, serreado, lobado ou partido, com filotaxia oposta cruzada, sendo menos frequentemente verticiladas ou alternas e raramente compostas. Apresentam caule e ramos tetrangulares, quando jovens, flores fortemente zigomorfas, bilabiadas, e ovário estilete ginobásico (SOARES, 2017).

No âmbito químico e econômico a família Lamiaceae possui potencial para extração de óleos essenciais, que são produzidos a partir de pêlos e tricomas glandulares, e seus estudos são direcionados para avaliação dos constituintes presentes nos óleos. Com a caracterização e qualificação dos OE têm-se a expectativa de estabelecer relação entre a possível aplicação de um produto de origem natural a problemas que acompanham a humanidade desde tempos antigos, por exemplo, a infestação de lavouras, controle bacteriano, fungicida, repelente e inseticida, desde que sejam testados através de ensaios *in vivo* e *in vitro*, e que tenham comprovação científica (ARAÚJO et al., 2018).

As substâncias encontradas nas plantas da família Lamiaceae possuem diversas atividades biológicas incluindo antimicrobiana, antioxidante, analgésica, anti-inflamatória, antiplaquetária, antitrombótica, afrodisíaca, antidepressiva, antimutagênica, antiemética, fibrinolítica e citotóxica (CHAKRAPANI et al., 2013; DONGARE et al., 2014; BEEK; JOULAIN, 2018). Além de propriedade inseticidas, antibacterianas, antifúngicas (KUMARA; ANURADHA, 2011; WAN et al., 2016) e potencial entomotóxico reconhecido que servem como estratégia alternativa para o controle químico do *A. aegypti* (MARCOMBE et al., 2018).

1.3.1 *Pogostemon cablin* (Blanco) Benth

P. cablin é uma planta da espécie da família Lamiaceae, tropical perene que se originou na Índia e é cultivada na Indonésia e Malásia, de forma intensiva, já na América do Sul a planta é cultivada no Paraguai e no Brasil, chamando a atenção por possuir um óleo essencial com odor característico, persistente e canforáceo (MOURA et al., 2016).

Figura 1 – *Pogostemon cablin* (Blanco) Benth

Fonte: Autor (2018)

Popularmente conhecida como Oriza e Patchouli, é uma planta arbustiva com altura até aproximadamente 1,0 m, cultivada preferencialmente em clima quente e úmido com solos ricos em matéria orgânica. O caule é ereto, grosso, quadrangular e ramificado na parte superior. As folhas apresentam textura aveludada, lisa, cobertas com glândulas de óleo em ambas as faces, fortemente aromáticas, medindo de 5 a 10 cm de comprimento por 3 a 7 cm de largura (MOURA et al., 2016). Tradicionalmente usada para fins medicinais, principalmente para o tratamento de enjoo de criança, dor de cabeça e problemas cardíacos (BEEK; JOULAIN, 2018).

A importância das espécies do gênero *Pogostemon* está relacionada à alelopatia, sendo encontrado por Kusuma e Mahfud (2017) 19 compostos do óleo essencial da espécie *P. cablin*, tendo como compostos majoritários o patchoulol (26,2%), δ-guaiene (14,69%), α-guaiene (12,18%), α-gurjunene (11,13%), seychellene (8,42%), viridiflorol (5,93%), β-caryophyllene (4,63%) e β-patchoulene (2,87%).

A composição do óleo de patchouli é complexa como muitos óleos essenciais, mas distinta porque se constitui em grande parte de sesquiterpenos. O patchoulol, um sesquiterpeno oxigenado, é o seu maior constituinte e é o principal responsável pela típica nota do patchouli. O óleo contém um grande número de outros hidrocarbonetos sesquiterpenos tais como, α, β, σ – patchoulenos, α-bulneseno, α- guaieno e

seicheleno, com estruturas claramente relacionadas ao patchoulol e outros sesquiterpenos. O acúmulo e a biosíntese do patchoulol e sesquiterpenos relacionados nas folhas de patchouli foi estudada por meio de sua morfologia (CHAKRAPANI et al., 2013).

1.4 ATIVIDADES BIOLÓGICAS

A potencialidade biológica dos óleos essenciais e extratos vegetais está relacionada aos constituintes químicos (SANTOS; ALMEIDA, 2015). Como os vegetais possuem em sua estrutura química uma mistura complexa de diversos compostos, a definição exata daqueles que atuam como mecanismos de ação antioxidante, citotóxico, amtimicrobiana e larvicida é uma tarefa complexa, pois os efeitos biológicos podem ser resultados do componente majoritário ou da ação sinérgicas destes constituintes (NASIR et al., 2015).

Os Antioxidantes são substâncias capazes de desacelerar ou retardar a velocidade de oxidação de um material oxidável. Através de um ou mais mecanismos, os antioxidantes permitem que ocorra a redução da oxidação, como por exemplo, por meio da inibição de radicais livres e complexação de metais (FREITAS et al., 2014).

Naturalmente, os radicais livres são produzidos endogenamente ou adquiridos de forma exógena, e seu excesso gera o estresse oxidativo. Assim, os antioxidantes naturais provenientes de plantas medicinais têm despertado interesse farmacêutico por se tratar de uma alternativa de substituição dos antioxidantes sintéticos que são suspeitos de induzir o câncer (CHEN et al., 2013).

Dentre os diferentes métodos existentes para avaliar a capacidade antioxidante de um material vegetal destacam-se o ensaio do DPPH (1,1-difenil-2-picrilhidrazil), no qual o antioxidante reage com o radical DPPH, convertendo-o em sua forma reduzida (1,1-difenil-2-picrilhidrazina). Nesta reação, a solução metanólica de DPPH, inicialmente de coloração violeta, torna-se amarelada e o grau deste descoramento indica a habilidade do antioxidante em sequestrar o radical livre (HUSSAIN et al., 2011).

Outra atividade biológica importante utilizada para verificar a toxicidade de óleos e extratos vegetais é o bioensaio com *Artemia salina,* que é uma análise preliminar que visa verificar a taxa de letalidade em face aos microcrustáceos, afim de determinar a dose efetiva para matar 50% das larvas de *A. salina* (RAMOS; RODRIGUES; ALMEIDA, 2014).

A importância deste ensaio de toxicidade deve-se ao fato de que vários autores buscam correlacionar a toxicidade sobre *A. salina* com atividades anticancerígena, antifúngica, inseticida e tripanossomicida (VIEIRA et al., 2015; JEONG et al., 2013). Além de ser um econômico método aplicado a verificação da toxicidade de óleos bioativos de plantas medicinais.

O bioensaio toxicológico com *A. salina* é pertinente, pois os efeitos que um composto produz nestes microcrustáceos são aplicáveis ao ser humano, sendo necessário apenas a correção matemática para determinar a dose por unidade de superfície corporal adequada, ou seja, os efeitos tóxicos no homem são aproximadamente semelhantes aos observados nos animais utilizados em laboratório (SIMÕES; ALMEIDA, 2015).

O emprego de novos produtos naturais com atividade antimicrobiana tem despertado interesse dos pesquisadores em virtude de muitas bactérias possuirem resistência aos antibióticos utilizados para o tratamento de diversas infecções (RASHED et al., 2013). Sendo assim, medicamentos à base de compostos naturais com potencial antimicrobiano surgem como alternativa fitoterápica para auxiliar nos cuidados as doenças infecciosas.

Os óleos essenciais possuem compostos que atuam como agentes antimicrobianos, que agem afetando tanto o invólucro externo quanto o citoplasma das células bacterianas, sendo a membrana celular o primeiro alvo. Isto ocorre devido à hidrofobicidade destes e de seus componentes, que ocasiona a difusão através da bicamada fosfolipídica (NAZZARO et al., 2013).

Os constituintes químicos dos óleos essenciais atuam com mecanismo de ação antimicrobiana sobre as bactérias interligado à perturbação da membrana citoplasmática, danos nas proteínas da membrana, coagulação do citoplasma, alteração no fluxo de elétrons, interrupção da força próton motriz, alteração do transporte ativo e redução do pool de ATP intracelular (BURT, 2004; NAZZARO et al., 2013).

Em virtude da ação biocida que os vegetais apresentam, o estudo para o desenvolvimento fitoterápicos com ação ovicida, larvicida, inseticida e repelente contra *A. aegypti* têm dispertado interesse da comunidade científica. A maioria dos estudos é realizada a partir de extratos brutos e óleos essenciais, sendo que, na maioria destes casos, não se conhece o composto responsável pela atividade apresentada. Muitos produtos à base de plantas apresentam compostos ativos, que

agem sinergicamente ou de forma isolada, possuindo características que podem ser eficientes para o controle e monitoramento das populações de mosquitos (NEVES et al., 2014).

Muitos estudos comprovam a atividade de óleos essenciais e extratos vegetais no controle de diferentes espécies de mosquito (COSTA et al., 2016; LIU et al.,2015; MISSAH, 2014) incluindo o *A. aegypti* (NASIR et al., 2015; MARCOMBE et al., 2018). O estudo das atividades larvicida, inseticida e repelente dos compostos extraídos dos vegetais tem sido uma alternativa aos produtos sintéticos para o controle de vetor, visto que não existe vacina contra a dengue, sendo esta tratada apenas com medicamentos que amenizem os sintomas.

1.5 AEDES (STEGOMYIA) AEGYPTI

A ocorrência do *A. aegypti* foi primeiramente descrita no Egito por Linnaeus, em 1762, o que lhe conferiu seu nome específico. Foi reconhecido como transmissor da febre amarela em 1881, por Carlos J. Finlay. Em 1906, Brancroft publicou as primeiras evidências de que o mosquito também era o vetor de dengue, fato posteriormente confirmado por Agramonte, em 1906, e por Simmons, em 1931 (BRAGA; VALLE, 2007). No Brasil, recentemente ocorreu a entrada do vírus chikungunya e Zika, em setembro de 2014 e maio de 2015, respectivamente; ambos transmitidos também pelo *A. aegypti* (VALLE et al., 2016).

O mosquito *A. aegypti* mede menos de 1 centímetro e possui uma aparência inofensiva, é de cor preta com listras brancas no corpo e nas pernas. Sua picada não dói e nem coça. O ciclo de vida do *A. aegypti* compreende quatro fases: ovo, larva, pupa e adulto. Os ovos do mosquito transmissor são depositados em condições adequadas, ou seja, em lugares quentes e úmidos como aqueles próximos a linha d'água. (SANTANA et al., 2015).

A etologia do *A. aegypti* influencia na sua ampla dispersão, favorecida nos ambientes urbanos, preferencialmente nas condições domiciliares e peridomiciliares ofertado pelo modo de viver do homem. Seus criadouros são preferencialmente recipientes artificiais, como aqueles abandonados a céu aberto, reservatório de água de chuva ou para armazenar água para uso doméstico. Este vetor prefere reproduzir em reservatórios de águas limpas, embora possa se adaptar às novas situações impostas pelo homem, adaptando-se a outros tipos de criadouros, como por exemplo, bromélias e esgotos a céu aberto encontrados em vários centros urbanos. A presença

dos criadouros em ambiente de convívio com o homem favorece a rápida proliferação da espécie, por dois aspectos: condições ideais para reprodução e fontes de alimentação (ZARA et al., 2016).

Diante dos desafios de controle do vetor e de um quadro grave e preocupante em relação às arboviroses delineado pela expansão destes vírus em todo o mundo, torna-se imprescindível a adoção de estratégias específicas, com maiores investimentos em métodos adequados. Assim, em face do atual cenário de surtos e epidemias de Zika, chikungunya e dengue, este estudo torna-se relevante, pois propicia uma estratégia de redução do contato do mosquito com o homem.

1.6 POTENCIALIDADE BIOCIDA DA FAMÍLIA LAMIACEAE PARA O CONTROLE DO *Aedes aegypti*

O controle do *Aedes* constitui um grande desafio, principalmente nos países em desenvolvimento. Existem recursos financeiros destinados ao controle do vetor por meio de implementação de programas, porém, muitas vezes não se tem alcançado sucesso. Aspectos relacionados a falta de coleta de lixo e intermitência no abastecimento de água, são fatores que influenciam diretamente nos métodos de controle tradicionais do *Aedes* (ZARA et al., 2016).

Como alternativa de controle químico de origem natural, surgem os fitoterápicos que atuam como mecanismos de ação inseticida, larvicida e repelente. O uso de inseticidas para controle de populações de mosquitos adultos (adulticidas) e na sua forma larvária (larvicidas) pode ser feito por meio do tratamento focal e perifocal e da aspersão aeroespacial de inseticidas em ultra baixo volume (UBV). Os repelentes podem ser aplicados na pele do indivíduo afim de repelir os mosquitos e evitar picadas (MAKETE, 2016).

Segundo Cheng et al. (2003) substâncias com valores de CL_{50} (concentração letal da morte de 50%) menores que 100ppm são considerados bons agentes larvicidas. Ramos (2014) em seus estudos constatou que as plantas *Ocimum gratissimum, Ocimum basilicum, Pogostemon heyneanus* e *Hyptis crenata* apresentaram significativo potencial larvicida com valores de $CL_{50\ (ppm)}$ de 76.6, 67.2, 69.9 e 89,4 respectivamente. Nasir et al. (2015) comprovaram que a espécie *Mentha piperita* é altamente tóxica para larvas do mosquito *A. aegypti* atingindo a aproximadamente 90% da mortalidade larval.

Silva et al. (2014) buscaram avaliar o potencial larvicida em larvas de libélulas, no qual os resultados foram considerados altamente potentes dos óleos das espécies *Hesperozygis ringens* (Bentham) Epling e *Ocimum gratissimum* com CL_{50} de 62,92; 75,05 µL respectivamente. Veloso et al. (2015) constataram em seu estudo eficiente ação larvicida das espécies *Ocimum basilicum* L. (manjerição) e *Cymbopogon nardus* L. (capim citronela) frente as larvas do *A. aegypti*, ressaltando que as alíquotas de 5,0, 7,5 e 10,0 µL do óleo essencial do capim citronela foram mais eficientes apresentando a partir da segunda época de avaliação 100% de larvas mortas. Alguns extratos e nanopartículas de prata sintetizada (AgNP) das folhas de Leucas aspera foram estudadas quanto ao seu potencial larvicida contras as larvas do *A. aegypti,* na qual os extratos das folhas de AgNP sintetizado apresentaram maior mortalidade larval (CL_{50}= 8,5632 ppm; CL_{90}= 21, 5686 ppm), os demais extratos de Leucas áspera também apresentaram alta mortalidade larval (SUGANYA et al., 2013) Resultados estes que corroboram com o uso potencial da espécie para atividade larvicida e repelente em trabalhos futuros para elucidação de mecanismo de ação biológica.

A toxicidade de uma substância química não está necessariamente associada à morte dos insetos, pois outros aspectos podem estar vinculados a esta ação, como a repelência, deterrência e antibiose (efeito adverso na sua biologia). Para ser considerado um bom inseticida ou 'inseticida ideal', vários fatores devem ser levados em consideração, como a eficácia em baixas concentrações, ausência de toxicidade frente a mamíferos e animais superiores, ausência de fitotoxicidade, fácil obtenção, manipulação e aplicação, viabilidade econômica e não possuir efeito cumulativo no homem e animais (NEVES et al., 2014).

Diversos estudos comprovam a atividade inseticida de óleos essenciais e extratos vegetais no controle de diferentes espécies de hospedeiros (SANTANA et al., 2015; RAFSHANJANI et al., 2014; LIU, et al., 2014). Savaris et al., (2012) em seu estudo que avaliou atividade inseticida de *Cunila angustifólia* constataram que todas as doses de óleo essencial da espécie apresentaram eficiência de 100% na mortalidade de adultos de *A. obtectus*, 24 horas após a exposição aos insetos. A espécie *Foeniculum vulgare,* também da família Lamiaceae, apresentou 93% de mortalidade frente ao *Tribolium castaneum* H., resultado constatado por Brito (2015).

Segundo o levantamento bibliográfico realizado por Swamy e Sinniah (2015) várias plantas da família Lamiaceae produzem óleo essencial com atividade inseticida, como o de hortelã, orégano, tomilho, sálvia. Dentre os compostos com atividade

inseticida pode-se citar o terpenóide mentol, encontrado nas plantas do gênero mentha, que se mostram como potente inseticida com inibidor do crescimento de larvas. Além dos monoterpenóides fenólicos, timol e carvacrol que possuem atividade antioxidante e inseticida.

Os óleos essenciais extraídos de plantas diferentes têm sido relatados por ter propriedades larvicida e repelente contra *A. aegypti* (KUMAR et at., 2011; HANAN, 2013). Porém, há escassez de estudos que comprovem a atividade repelente da Família Lamiaceae frente ao *A. aegypti,* na qual abre a possibilidade de novas investigações para complementar as pesquisas relacionadas as atividades biológicas desta família.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Realizar o estudo químico e avaliação da atividade antioxidante, citotóxica, amtimicrobiana e larvicida do óleo essencial e extrato bruto etanólico de *Pogostemon cablin* (Blanco) Benth.

2.2 OBJETIVOS ESPECÍFICOS

- ✓ Obter o óleo essencial e extrato bruto etanólico de *P. cablin;*
- ✓ Analisar a composição química do óleo essencial e extrato bruto etanólicos da espécie;
- ✓ Avaliar a atividade antioxidante dos óleo essencial e extrato bruto etanolico da espécie;
- ✓ Avaliar a atividade citotóxica do óleo essencial e extrato bruto etanólico frente a larvas de *Artemia salina* Lench;
- ✓ Avaliar a atividade antimicrobiana do óleo essencial e extrato bruto etanólico frente as bactérias gram-negativas *Pseudomonas aeruginosa* ATCC 25922, *Escherichia coli* ATCC 8789 e gram-positiva *Staphylococcus aureus* ATCC 25922;
- ✓ Avaliar a atividade larvicida do óleo essencial e extrato bruto etanólico frente as larvas de *A. aegypti;*

3 CAPÍTULO 1

Evaluation of the larvicidal potential of the essential oil *Pogostemon cablin* (Blanco) Benth in the control of *Aedes aegypti*

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Article

Evaluation of the larvicidal potential of the essential oil *Pogostemon cablin* (Blanco) Benth in the control of *Aedes aegypti*

Lizandra Lima Santos [1]*, Lethicia Barreto Brandão [1], Rosany Lopes Martins [1], Erica de Menezes Rabelo [1], Alex Bruno Lobato Rodrigues[1], Camila Mendes da Conceição Vieira Araújo[2], Talita Sobral[2], Allan Kardec Ribeiro Galardo[2], Sheylla Susan Moreira da Silva de Ameida[1]

[1] Laboratory of Pharmacognosy and Phytochemistry, Federal University of Amapa, Highway Jucelino Kubistichek, Km 02, 68.9022-80, Macapa, Brazil; lizandralsantos@hotmail.com; lethiciabrandão12@gmail.com; rosyufpa@gmail.com; ericamrabelo@gmail.com; alex.rodrigues@unifap.br; sheyllasusan@yahoo.com.br

[2] Laboratory of Medical Entomology of the Institute of Scientific and Technological Research of the State of Amapa, Highway Jucelino Kubistichek, Km 10, 68.9034-19, Macapa, Brazil; camilamendes.cva@gmail.com, talitaferz@gmail.com; allangalardo@gmail.com

* Correspondence: lizandralsantos@hotmail.com;

Abstract: The objective of this work was to collect information on the chemical constituents that demonstrate the larvicidal activity against *Aedes aegypti*, as well as the antioxidant, microbiological, and cytotoxicity potential of the essential oil of *Pogostemon cablin* leaves. The chemical characterization was performed by gas chromatography coupled to mass spectrometer (GC-MS). The larvicidal activity was performed according to the protocol of the World Health Organization. The antioxidant activity was evaluated through the sequestering capacity of 2,2-diphenyl-1-picryl-hydrazine (DPPH). As for the microbiological evaluation, the microdilution technique was used, according to the protocol of the Clinical and Laboratory Standards Institute. The cytotoxic activity was evaluated against the larvae of *Artemia salina*. The species *P. cablin* presented the following compounds: Patchouli alcohol (33.25%), Seyshellene (6.12%), α-bulnesene (4.11%), Pogostol (6.33%), and Norpatchoulenol (5.72%), which was in synergy with the other substances may significantly potentiate the larvicidal action of the species with the LC_{50} of 28.43 $\mu g \cdot mL^{-1}$. There was no antioxidant activity, however, it presented antimicrobial activity against all bacteria tested with Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of 62.5 $\mu g \cdot mL^{-1}$. The species demonstrated significant toxic action with LC_{50} of 24.25 $\mu g \cdot mL^{-1}$. Therefore, the *P. cablin* species showed significant larvicidal potential, antimicrobial activity, the absence of antioxidant action, and high toxicity.

Keywords: Biocide; patchouli; Oriza; vector control; Lamiaceae.

1. Introduction

The use of medicinal plants is an ancient practice used by populations to cure various diseases. This practice is expanding all over the world. Medicinal plants constitute an important readily available resource found in popular markets, backyards and areas of native vegetation. Brazil is a living pharmacopeia, due to its size and variety of ecosystems, which offers rich possibilities that can meet the needs of diverse communities, especially those in need [1].

Among these medicinal plants, the study of plant extracts, as well as their essential oils (EO), appears as an expectation to find substances with biocidal activities that can be selected for use in future formulations of a commercial product.

In this context, the species of the Lamiaceae family present huge potential for the obtention of essential oils, which have several biological functions in folk medicine to treatment of several conditions, such as burns, headache, colic, fever, as well as reports of antiviral activities against influenza, insecticide, insect repellent, antibacterial and anti-parasitical [2].

The *P. cablin*, popularly known as Oriza and Patchouli is an evergreen tropical species of plant, originated in Southeast Asia that belongs to the family Lamiaceae, which is currently being extensively cultivated in Malaysia, Indonesia, the Philippines, China, India, Seychelles, and Brazil [3]. The importance of the species of the genus *Pogostemon* is related to allelopathy. Kusuma and Mahfud [4] found 19 compounds of the EO of *P. cablin*. The major compounds were patchoulol (26.2%), δ-guaiene (14.69%), α-guaiene (12.18%), α-gurjunene (11.13%), seychellene (8.42%), viridiflorol (5.93%), β-caryophyllene (4.63%) and β-patchoulene (2.87%).

The composition of the *P. cablin* oil is complex, like those of many other essential oils, but distinct because it is constituted largely by sesquiterpenes. Patchoulol, an oxygenated sesquiterpene, is the major constituent and is primarily responsible for its characteristic aroma.

The oil contains a large number of other hydrocarbons sesquiterpenes such as, α, β, σ - patchoulenos, α-bulneseno, α-guaieno, and seicheleno, with structures related to patchoulol and other sesquiterpenes. The accumulation and biosynthesis of patchoulol and related sesquiterpenes in *P. cablin* leaves were studied by their morphology [5].

The substances found in this species have several biological activities described in the literature, such as antioxidant, analgesic, anti-inflammatory, antiplatelet, antithrombotic, aphrodisiac, antidepressant, antimutagenic, antiemetic, fibrinolytic and cytotoxic [6-9]. In addition to the insecticide, antibacterial, and antifungal proprieties [6,10,11]. Their recognized entomotoxicity suggest possible applicability as an alternative strategy for the chemical control of *A. aegypti* [12].

A. aegypti control has been a major challenge, especially in developing countries, where usually there is the presence of financial resources for the vector control through the implementation of programs that often fail due to aspects related to the lack of garbage collection and intermittent water supply, that are factors that directly influence the traditional *A. aegypti* control methods [13].

In view of the operational and economic difficulties generated by the increasing resistance of mosquitoes to the synthetic insecticides, alternative methods are becoming more prominent, efficient, and cheaper since they are obtained from renewable resources that are rapidly degradable and have several substances which act simultaneously, causing insect resistance to these substances to occur slowly [14].

Thus, in view of the mosquito control challenges and a critical scenario regarding the arboviruses, outlined by the spread of *A. aegypti*-transmitted diseases worldwide, it is essential to adopt specific strategies with greater investments in new methods. Due to the current outbreaks and epidemics of yellow fever, Zika, chikungunya, and dengue, which has victimized thousands of people.

In this context, the present study becomes relevant, since the species *P. cablin* possesses biocidal potential. However, there are still few studies regarding the insecticidal and larvicidal activity of the plant over this vector; as well as sources of preliminary information regarding the search of strategies to reduce the contact of the mosquito with humans, such as the insertion of herbal medicines into the public health system, which would assist in the quality of the health primary care as vector control.

It is also worth noting that for the formulation of a commercial product, it is important to carry out a study of biological activities such as antioxidant, microbiological and cytotoxic as a complement to prove the phytotherapeutic potential of the species.

Therefore, the present study aimed to collect information on chemical constituents that demonstrate the larvicidal, antioxidant, antimicrobial and cytotoxicity activities of the EO of the leaves of *P. cablin*.

2. Material and Methods

2.1. Plant material

The species *P. cablin* was collected in the district of Fazendinha (00°02'23" S and 51°06'29" O) in the Macapa Municipality, in the Amapa State, Brazil. For the taxonomic identification, samples of the species were deposited in the Amapaense Herbarium (HAMAB) of the Institute of Scientific and Technological Research of the State of Amapa (IEPA) under the registration number 019183.

2.2. Essential oils

The leaves of *P. cablin* were dehydrated in an incubator with air circulation at 36 °C, after dried they were crushed in an electric mill. The EO was extracted by hydrodistillation at 100 °C in Clevenger-type apparatus for three hours [15].

2.3. Chemical analysis

The chemical composition of the EO was determined by gas chromatography coupled to mass spectrometer (GC-MS), using a model GCMS-QP 5050A, manufactured by Shimadzu company (Kyoto, Japan), under the following conditions: DB-5HT column of the brand J and W Scientific, with length of 30 m, diameter of 0.32 mm, film thickness 0.10 µm, and nitrogen as carrier gas, according to Martins et al. [16].

The apparatus operated under internal column pressure of 56.7 kPa, split ratio 1:20, the gas flow in the column was of 1.0 $mL.min^{-1}$ (210 °C), injector temperature of 220 °C, and in the spectrum of the mass of 240 °C. The initial temperature of the column was 60 °C with an increase of 3 $°C.min^{-1}$, until reaching 240 °C, kept constant for 30 minutes.

The mass spectrometer was programmed to perform readings in a range of 29 to 400 Da, at intervals of 0.5 seconds, with ionization energy of 70 eV. 1 µL of each sample with a concentration of 10.000 ppm dissolved in hexane was injected.

The identification of individual components was based on the comparison of their retention index (RI) and mass spectra with the literature [17]. The RI was calculated relative to a number of n-alkanes (C8-C40, Sigma-Aldrich, St. Louis, MO, USA) using the Van Den Dool and Kratz equation [18].

2.4. Larvicidal activity

The larvae of *A. aegypti* employed in the larvicidal test were from a colony kept in the insectary of the Medical Entomology Laboratory of the Institute of Scientific and Technological Research of the State of Amapa (IEPA). The biological assays were conducted under controlled climatic conditions with a temperature of 25±2 °C, relative humidity of 75±5% and a photoperiod of 12h. The methodology adopted followed the World Health Organization standard protocol [19] with a slight modification regarding the test vessel.

The EO of *P. cablin* (0.09 g) was dissolved in 85.5 mL of distilled water and 4.5 mL of Tween 80; and for the negative control, it was used respectively Tween 80 with distilled water (1%), and the larvicidal esbiothrin as the positive control.

After the preliminary tests, the aqueous solution was diluted in the following concentrations: 100, 60, 40, 20, 10, 1 $\mu g.mL^{-1}$. Each concentration was tested in triplicate, and 25 larvae of the *A. aegypti* mosquito in the 3rd young stage (L3) were used. They were pipetted into a 100 mL beaker containing distilled water, then they were transferred into the test vessels, minimizing the time between the preparation of the first and last samples. During the experiment, the average water temperature was 25 °C. After 24 and 48 hours, the dead larvae were counted, being considered as such all those unable to reach the surface.

2.5. Determination of antioxidant activity

The evaluation of the antioxidant activity was based on the sequestering ability of 2,2-diphenyl-1-picrylhydrazyl (DPPH), as proposed by Chen, Berlin and Froldi [20] and Lopez-Lutz et al. [21] with modifications. The antioxidant activity was calculated [22] as follows:

$$(\%AA) = 100 - \{[(Abs_{sample} - Abs_{white})100]/Abs_{control}\}$$

%AA – percentage of antioxidant activity

Abs_{sample} – Sample absorbance

Abs_{white} – White absorbance

$Abs_{control}$ – Control absorbance

A methanolic solution of DPPH at the concentration of 40 $\mu g.mL^{-1}$ was prepared. The EO was diluted in methanol at the following concentrations: 7.81; 15.62; 31.25; 62.5; 125 and 250 $\mu g.mL^{-1}$. The antioxidant activity evaluation was made in triplicate

with a volume of 0.3 mL of EO solution per tube, added to 2.7 mL of the DPPH solution. In parallel, the negative control of each concentration was prepared. For positive control, ascorbic acid was used under the same conditions of EO preparation. After 30 min of incubation at room temperature and protected from light, the spectrophotometer, of the manufacturer Biospectro, model SP-22 (Curitiba, BRA) was measured at wavelength 517 nm in a quartz cuvette.

2.6. Antimicrobial activity

2.6.1. Bacterial strains and culture conditions

Two gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 25922, *Escherichia coli* ATCC 8789) and a gram-positive bacterium (*Staphylococcus aureus* ATCC 25922) were used to test the antimicrobial activity of the EO of *P. cablin* leaves.

From a stock culture in BHI (Brain Heart Infusion) with 20% glycerol stored at -80 °C the activation of each microorganism as performed by transferring 50 µL of this culture into 5 mL of sterile BHI broth followed by incubation for 24 hours at 37 °C.

2.6.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The determination of the MIC and the MCB was performed using the microplate dilution technique (96 wells) according to the protocol established by the Clinical and Laboratory Standards Institute [23], with adaptations. Initially, the bacteria were reactivated in BHI broth, for 18 h at 37 °C. After bacterial growth, a 0.9% saline an inoculum adjusted to the McFarland 0.5 scale was prepared for each microorganism, and subsequently diluted in BHI and tested at 2 x 106 CFU.mL^{-1}.

For the determination of MIC, the EO was diluted in Dimethyl sulfoxide (2% DMSO). The first well column of the plate was filled with 0.2 mL of the EO at the concentration of 2000 µg.mL^{-1}, the other wells were filled with 0.1 mL of 0.9% NaCl. Subsequently, base two serial dilutions were performed in the ratio of 1: 2 to 1: 128 dilution in a final volume of 0.1 mL. Cells (2 x 106 CFU.mL^{-1}) with 0.1 mL adjusted according to the previous item were added to each well, resulting in a final volume of 0.2 mL. Control of the culture environment, EO control, and negative control (DMSO 2%) were performed. For positive control, amoxicillin (0.5 µg.mL^{-1}) was used. The experiments were carried out in triplicates. The microplates were incubated at 37 °C for 24 hours, after this time, the plates were read in an ELISA reader (OD 630 nm).

The MIC was considered to be the lowest concentration of the test substance in which there was no significant bacterial growth compared to the negative control (comparison between the values of D.O.630 by the Bonferroni test with 99% confidence interval).

The MCB was determined based on the results obtained in the MIC test. Microplate wells were spread in Müller-Hinton agar and incubated at 37 °C for 24 h. The MBC was established as the lowest concentration of EO capable of completely inhibiting microbial growth in Petri dishes after 24-48 hours of growth.

2.7. Cytotoxic activity

The cytotoxicity of the EO was evaluated against larvae of *Artemia saline* [24, 25] with adaptations. A solution of 250 mL of synthetic sea salt at 35 $g.L^{-1}$ was prepared, in which 25 mg of *A. salina* Leach eggs were exposed to artificial lighting within 24 hours for hatching of the larvae (nauplii). The nauplii were then separated and placed in a dark environment at room temperature for a further 24 h to reach the methanauplii stage.

A stock solution was prepared to contain 0.06 g of the EO, 28.5 mL of the solution of synthetic sea salt and 1.5 mL of Tween 80 added to facilitate solubilization. For the negative control, it was used respectively Tween 80 with solution saline (5%), and the ($K_2Cr_2O_7$) Potassium dichromate (1%) as the positive control. Later, at the end of the dark period, they were selected and divided into 7 groups with 10 methanauplii in each test tube. In each group it was added aliquots of the stock solution of 100, 75, 50, 25 and 2.5 µL and completed the volume to 5 mL with a solution of synthetic sea salt, obtaining solutions with final concentrations of 40, 30, 20, 10 and 1 $\mu g.mL^{-1}$ in triplicates.

2.8. Statistical analysis

The results obtained from the bioassays were expressed through Averages and Standard Deviation, categorized in Microsoft Excel (Version 2010 for Windows, Redmond, WA, USA). The graphs were built on GraphPad Prism software (Version 6.0 for Windows, San Diego, CA, USA). Significant differences between treatments were assessed using the ANOVA test One criterion and the Tukey test using the BioEstat program (Version 5.0 for Windows, Belem, BRA). The LC_{50} values were determined in the PROBIT regression, through the SPSS statistical program (Version

21 for Windows, Chicago, USA). Differences that presented probability levels less than or equal to 5% ($p \leq 0.05$) were considered statistically significant.

3 Results

3.1. Chemical analysis

For GC-MS analysis of EO of *P. cablin*, it was possible to identify 29 compounds divided between sesquiterpenes and oxygenated terpenes, according to table 1.

Table 1. Substances identified by GC-MS analysis of *P. cablin* essential oil.

Peak	RT (min)	RI	Compounds	Relative percentage
1	23.942	1382	β-patchoulene	0.46%
2	24.183	1387	β-elemene	0.88%
3	25.275	1412	Cycloseychellene	0.65%
4	25.467	1417	(*E*)-caryophyllene	0.65%
5	26.217	1434	α-guaiene	2.99%
6	26.817	1448	Seychellene	6.12%
7	27.042	1453	α-humulene	0.42%
8	27.292	1459	α- patchoulene	3.59%
9	27.558	1465	9-epi-(*E*)-caryophyllene	1.24%
10	27.875	1473	β-chamigrene	0.14%
11	28.450	1486	β-selinene	0.19%
12	28.750	1493	Aciphyllene	0.54%
13	28.875	1496	Viridiflorene	0.57%
14	29.042	1500	α-bulnesene	4.11%
15	29.708	1516	7-epi-α-selinene	0.15%
16	31.042	1549	Elemol	0.16%
17	31.942	1571	Norpatchoulenol	5.72%
18	32.342	1580	Caryophyllene oxide	3.86%
19	32.458	1583	Globulol	1.79%
20	32.950	1595	Fokienol	0.67%
21	33.442	1607	Humulene epoxide II	0.72%
22	34.267	1629	Junenol	1.87%
23	34.717	1640	*allo*-aromandendrene epoxide	1.82%
24	35.742	1666	Pogostol	6.33%
25	36.308	1681	Patchouli alcohol	33.25%
26	37.083	1701	Thujopsenal	2.06%
27	37.758	1719	*Z*- α-atlantone	1.44%
28	38.225	1732	Isobicyclogermacrenal	0.77%
29	39.925	1777	Squamulosone	0.97%
Compounds identified				84.13%
Unidentified compounds				15.87%

RI = Retention Index of Van den Dool and Kratz (1963); RT = Retention Time

Among the compounds identified, the following constituents were found: Seyshellene (6.12%), α-bulnesene (4.11%), Norpatchoulenol (5.72%), Pogostol (6.33%) and Patchouli alcohol (33.25%), shown in figure 1.

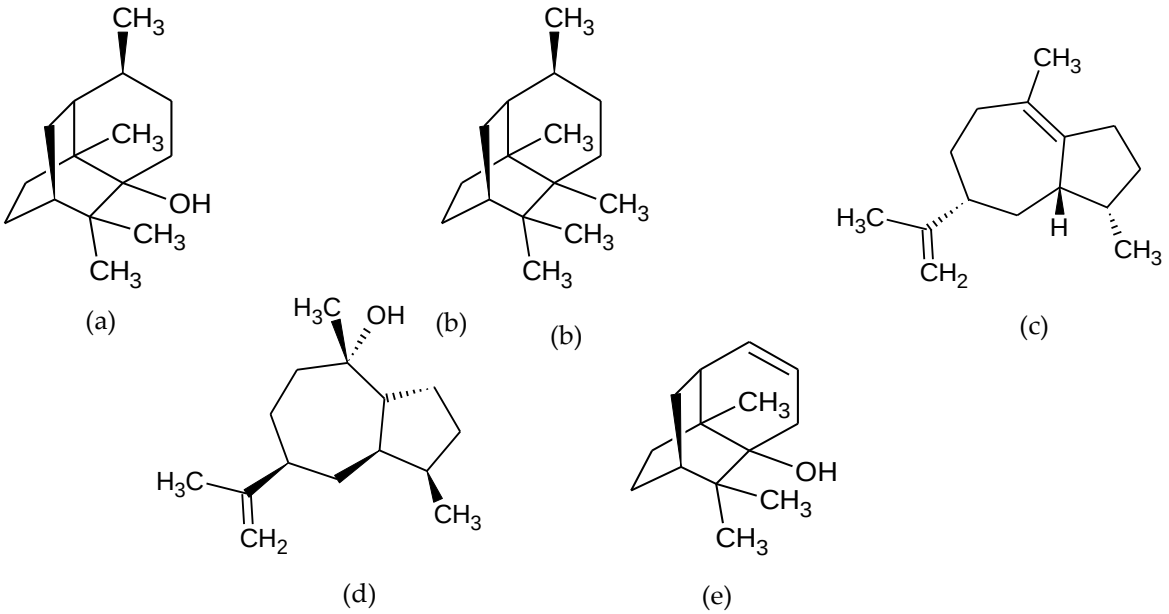

Figure 1. Molecular structure of the major compounds of *P. cablin* essential oil. (a) Patchouli alcohol (33.25%), (b) Seyshellene (6.12%), (c) α-bulnesene (4.11%), (d) Pogostol (6.33%) and (e) Norpatchoulene (5.72%).

3.2. Larvicidal activity

The *P. cablin* EO presented excellent larval mortality at low concentrations. And this is shown the table 2 regarding a period of 24 and 48 hours.

Table 2 - Percentage of mortality (%) of *A. aegypti* larvae in different concentrations of essential oil of *P. cablin* in two periods.

Concentrations	**Larvicidal activity (%)**	
(μg.mL^{-1})	**24 h**	**48 h**
Control (-)	0.0	0.0
20	38.0	70.66
40	52.0	89.33
60	92.0	97.33[a]
80	94.66[a]	98.66[a]
100	96.0[a]	98.66[a]
LC_{50} (EO)	28.43 μg.mL^{-1}	
LC_{50} (Control +)	0.0034 μg.mL^{-1}	

(a) statistically significant in relation to the positive control.

Essential oils with LC_{50} below 100 ppm are considered good agents with larvicidal potential [26]. According to the table 2, the results demonstrated that the EO of *P. cablin* presents a significant larvicidal effect with LC_{50} of 28.43 μg.mL^{-1}, p-value

<0.05 and coefficient of determination (R^2) of 0.0963 in 24 h, result with a high value when compared to the standard larvicidal Esbiothrin, with LC_{50} of 0.0034 μg.mL^{-1}.

3.3. Antioxidant activity

The correlation between the antioxidant activity (%) and the EO concentration presented a high IC_{50} value with 329.81 μg.mL^{-1} when compared to the standard Ascorbic acid (vitamin C) with IC_{50} of 16.71 μg.mL^{-1}. The results showed that the species under study did not present antioxidant activity, since the IC_{50} of the correlation between antioxidant activity (%) and the EO concentration was higher than that of the positive control, besides the DPPH consumption was smaller than 50% in all the concentrations tested.

3.4. Antimicrobial activity

In the evaluation of the antimicrobial activity, the microorganisms *P. aeruginosa* (ATCC 25922), *S. aureus* (ATCC 25922) and *E. coli* (ATCC 8789) were used for the experiments. The results were expressed as Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) according to figure 2.

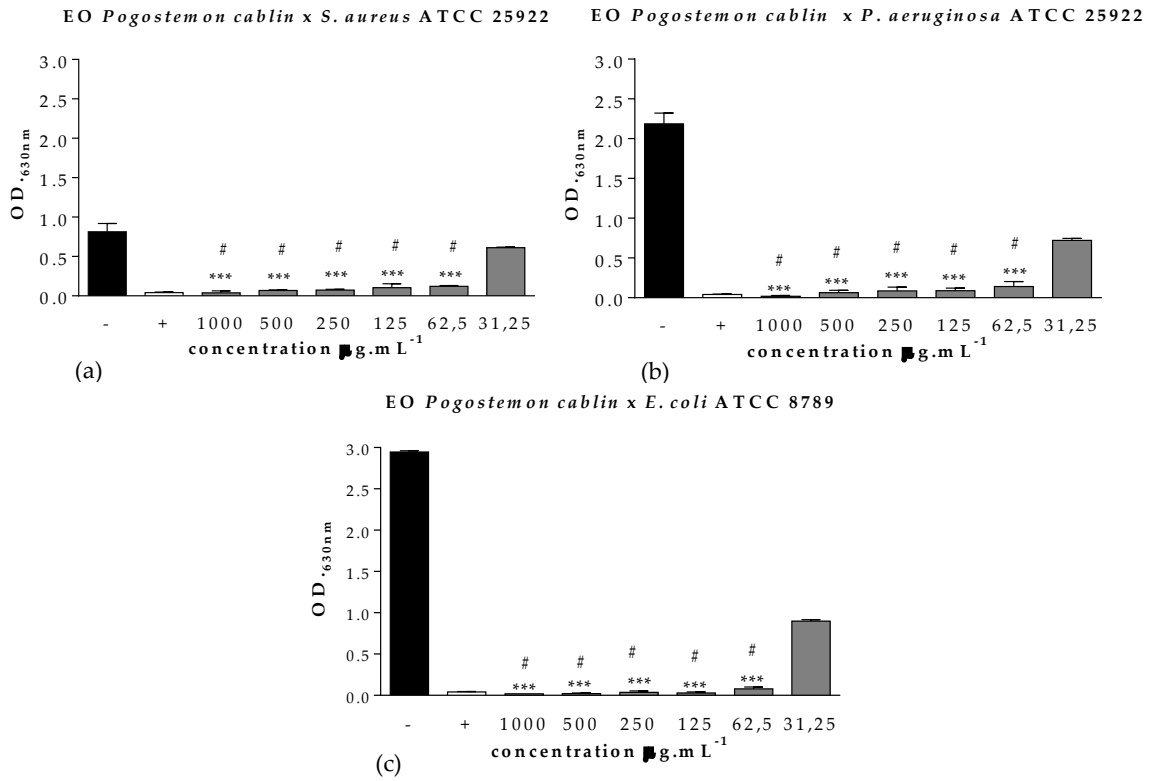

Figure 2. MIC and MBC of the EO of *P. cablin* against (a) *S.aureus,* (b) *P. aeruginosa* and (c) *E. coli.* Source: Own author. Substance test (□), BHI with 2% DMSO (■) and Amoxiline (□). ***P <0.001 statistically significant in relation to the negative control, # p <0.001 statistically significant in relation to the positive control.

The vitro antimicrobial activity, assays demonstrated that the bacteria *S. aureus, P. aeruginosa*, and *E. coli* bacteria were susceptible to the EO of *P. cablin* with a MIC and MBC the concentration of 62.5 $\mu g.mL^{-1}$, which corresponds to a high value when compared to Amoxiline that presented MIC and MBC at the concentration of 0.048 $\mu g.mL^{-1}$.

3.5. Cytotoxic activity

Table 4 shows the average mortality rates obtained after a 24 hour period of exposure to the EO of *P. cablin*. against *A. salina*.

Table 3. Mortality percentage of *A. salina* larvae due to exposure to the essential oil of *P. cablin*

Concentrations ($\mu g.mL^{-1}$)	**Mortality (%)**
Control negative	0.0%[a]
1	3.3%[a]
10	20.0%[a]
20	45.0%[b]
30	56.6%[c]
40	76.6%[d]
LC_{50}(EO)	24.25 $\mu g.mL^{-1}$
LC_{50} ($K_2Cr_2O_7$)	12.60 $\mu g.mL^{-1}$

Different letters indicate that there was a significant difference between the concentrations ($p < 0.05$)

The data in Table 4 show that the EO of *P. cablin* presents a LC_{50} 24.25 $\mu g.mL^{-1}$, p-value <0.05 and coefficient of determination R^2 of 0.902. This value is above the toxicity standard of potassium dichromate with LC_{50} of 12.60 $\mu g.mL^{-1}$, however, the EO of *P. cablin* has a high toxic action, because according to Martins et al.[16] pure substances extracted from plants are considered toxic when LC_{50} <100 $\mu g.mL^{-1}$, and nontoxic with LC_{50} >1000 $\mu g.mL^{-1}$.

4. Discussion

The study for the development of herbal remedies with larvicidal action against *A. aegypti* is recent, beginning in the 1980s, in order to isolate and characterize such bioactive substances. Most of the studies are carried out from raw extracts and essential oils. In most of these cases, the compound responsible for the activity is not known because its action occurs more effectively when grouped with other substances.

Many herbal products have active compounds, which act synergistically or in isolation, having characteristics that can be effective for the control and monitoring of mosquito populations [27].

Out of the compounds found in the chemical analysis of *P. cablin*, patchouli alcohol was the major constituent with a relative percentage of 33.25%, followed by the compounds Seyshellene (6.12%), α-bulnesene (4.11%), Norpatchoulene (5.72%) and Pogostol (6.33%). An approximate amount of the main subtance of the present study was found in study of Albuquerque et al. [28], which identified patchouli alcohol as the predominant compound in its oil, with a relative percentage of 36.60%.

The amount of patchouol found in this species cultivated in the state of Amapá-Brazil, differs from many studies in the literature that have also highlighted patchouli alcohol as the major compound [4-6, 8, 9, 28, 29], due to several factors, including the genetic factor, climate, soil conditions, cultural management and nutrition, where the last one is considered the most important, since deficiency or excess of nutrients may interfere with the production of the active substances in the species [30].

The insecticidal potential of essential oils is related to chemical constituents [31]. Since essential oils have a complex mixture of several compounds, the exact definition of those that act in the chemical control of immatures becomes a complex task because the biological effects may be a result of the major component or the synergistic action of these constituents [32].

However, there are reports in the literature indicating that sesquiterpenes have several bioativities [33, 34] , such as patchoulol present insecticidal activity against *A. aegypti* larvae, as evidenced by the study conducted by Autran et al. [35], which analyzed the essential oils of leaves, stems, and inflorescences of *Piper marginatum*, finding 40 chemical components, and among them the patchoulol was the major component that presented potent larvicidal activity with LC_{50} of 20 ppm, similar to the results of the present study, in which the EO of *P. cablin* had a significant larvicidal effect against larvae of *A. aegypti* with LC_{50} of 28.43 $\mu g.mL^{-1}$, and also occurs the production of patchoulol as the major constituent. Different from this study, Paulraj [36] found a high LC_{50}, with a value of 200 in the larvicidal analysis of the EO of *P.cablin* against *A. aegypti.*

As for the antioxidant analysis, Tohidi et al. [37] emphasize that the samples with high antioxidant potential have the capacity of sequestering free radicals and possess low IC_{50} values. Thus, a small amount of sample is capable of decreasing the

initial concentration of the DPPH radical by 50%, ie, inhibiting the radical oxidation by 50%. Thus, from the results observed in this study, there was no antioxidant activity, since the correlation of the IC_{50} and between the antioxidant activity (%) and EO concentration was 329.81 $\mu g.mL^{-1}$ was higher when compared to the standard ascorbic acid (vitamin C) with IC_{50} of 16.71 $\mu g.mL^{-1}$.

Studies related to the antioxidant activity performed with essential oils of the Lamiaceae family, including the *P. cabin* species, also showed a high IC_{50} value with 225.7 $\mu g.mL^{-1}$ and did not present a significant antioxidant capacity through analysis by the DPPH method, data similar to the results of this study [38].

For Beatović et al. [39], the antioxidant capacity of the EO is related to its major compounds. The content of these phytochemicals in the plants is largely influenced by genetic factors, environmental conditions, besides the degree of maturation and plant variety, among others. It is also emphasized that the antioxidant activity is influenced by the lipid substrate used in the test, the solvent and the extraction technique employed.

In relation to the antimicrobial activity, the EO of *P. cablin* presented antimicrobial action with MIC and MBC at the concentration of 62.5 $\mu g.mL^{-1}$. Studies conducted by Adhavan et al. [40] on the antimicrobial activity of nanoemulsions from three different genera of *Pogostemon*, showed that the essential oil of *P. cablin* presented antimicrobial activity at the concentration of 12.5 $mg.mL^{-1}$ for the *S. aureus* bacterium, a result above the concentration found in this one study.

The antimicrobial evaluation of EO of *P. cablin* in the studies conducted by Yang et al. [41] also showed good activity, with MIC at concentrations of 4.0, 5.0 and 4.0 $mg.mL^{-1}$ and MBC at concentrations of 2.0,> 10.0 and 6.5 $mg.mL^{-1}$ against *E. coli*, *P. aeruginosa* and *S. aureus*, respectively.

Therefore, the *in vitro* antimicrobial susceptibility testing of *P. cablin* essential oil has demonstrated a significant antimicrobial potential, as highlighted by Pattnaik et al. [42] that observed the inhibition of 20 bacteria promoted by the patchouli oil. This biological activity is directly related to the qualitative and quantitative composition of EO of *P. cablin*, which consists of more than 24 sesquiterpenes, in addition to mixtures of different mono-, sesqui- and di-terpene compounds [9].

The preliminary toxicological bioassay with *A. salina* allows to verify if the effects that a compound produces in these microcrustaceans are applicable to the human [43]. In this study, the EO of *P. cablin* presented significant toxic action (LC_{50} of 24.25 $\mu g.mL^{-}$

[1]). Similar data were found by Powers et al. [44] who studied the toxicity the EO of *P. cablin* to human breast tumor cell lines, in which in one of tumors found high toxicity with LC_{50} of 25.0 μg.mL^{-1}.

The significant toxicity of the EO of *P. cablin* is attributed to the chemical compounds that constitute it [45], that is, its oxygenated compounds (monoterpenes and sesquiterpenes) such as patchoulol, the main chemical component of the species. However, the essential oil may be more toxic than its isolated compounds, because of the synergistic effect among its constituents, which increases its effectiveness [46].

5. Conclusions

The chemical composition of the essential oil of *P. cablin* indicated the presence of 29 substances. The main components identified were patchouli alcohol (33.25%), Seyshellene (6.12%), α-bulnesene (4.11%), Pogostol (6.33%) and Norpatchouleno (5.72%).

The EO of *P. cablin* showed significant larvicidal potential and could be used to control *A. aegypti* mosquito larvae, suggesting the study with other vectors.

As for the antioxidant evaluation, there was no evidence of antioxidant activity by the DPPH method when compared to the vitamin C standard.

In the preliminary assessment of toxicity with *A. salina*, the EO presented significant toxic action, however, more comprehensive studies should be carried out to prove the toxicity of this species to humans and the environment.

The antimicrobial activity showed that the essential oil of *P. cablin* presented antimicrobial action with a MIC and MBC at the concentration of 62.5 μg.mL^{-1} against all the bacteria tested.

Thus, according to the literature consulted, the results of the biological activities of this study may be associated with the major compound of the species: patchoulol. However, the diversity of the chemical composition of this species is the most relevant factor associated with the biological actions proven by research that directs and supports the action of the major compounds alone or in combination (synergy).

The data show the relevance of the bioassays as a trial tool for the potential of the biological of the *P. cablin* species, as well as the importance of these as a source of biocidal compounds.

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Larvicidal activity against *Aedes aegypti* and antioxidant, cytotoxic and antimicrobial evaluation of the crude ethanolic extract of *Pogostemon cablin* (Blanco) Benth

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Article

Larvicidal activity against *Aedes aegypti* and antioxidant, citototoxic and antimicrobial evaluation of the crude ethanolic extract of *Pogostemon cablin* (Blanco) Benth

Lizandra Lima Santos [1]*, Lethicia Barreto Brandão [1], Rosany Lopes Martins [1], Erica de Menezes Rabelo [1], Alex Bruno Lobato Rodrigues[1], Camila Mendes da Conceição Vieira Araújo[2], Talita Sobral[2], Allan Kardec Ribeiro Galardo[2], Sheylla Susan Moreira da Silva de Ameida[1]

[1] Laboratory of Pharmacognosy and Phytochemistry, Federal University of Amapa, Highway Jucelino Kubistichek, Km 02, 68.9022-80, Macapa, Brazil; lizandralsantos@hotmail.com; lethiciabrandão12@gmail.com; rosyufpa@gmail.com; ericamrabelo@gmail.com; alex.rodrigues@unifap.br; sheyllasusan@yahoo.com.br
[2] Laboratory of Medical Entomology of the Institute of Scientific and Technological Research of the State of Amapa, Highway Jucelino Kubistichek, Km 10, 68.9034-19, Macapa, Brazil; camilamendes.cva@gmail.com, talitaferz@gmail.com; allangalardo@gmail.com
* Correspondence: lizandralsantos@hotmail.com;

Abstract: The present work aimed to evaluate the larvicidal, antioxidant, microbiological and cytotoxicity activity of the crude ethanolic extract of *Pogostemon cablin* (Blanco) Benth leaves. The chemical characterization was performed through staining and staining reactions for the detection of secondary metabolite classes. The larvicidal activity against *Aedes aegypti* was carried out according to the protocol of the World Health Organization. The antioxidant activity was evaluated by the sequestering ability of 2,2-diphenyl-1-picrylhydrazyl (DPPH). As for the microbiological evaluation, the microplate dilution technique was used against three bacteria, according to the protocol of the Clinical and Laboratory Standards Institute. *P. cablin* presented as classes of secondary metabolites: steroids and triterpenoids, depsides and depsidones, which in synergy with the other substances potentiated the larvicidal action of the species with an LC_{50} of 63.91 μg.mL^{-1} in 24 h. There was no antioxidant activity at the tested concentrations, however, it showed inhibition of bacterial growth against *E. coli* with MIC of 31.25 μg.mL^{-1}. The extract showed moderate toxic action with LC_{50} of 257.93 μg.mL^{-1}. Therefore, the *P. cablin* species showed significant larvicidal potential, with bacteriostatic action, the absence of antioxidant action and moderate toxicity.

Keywords: Biocide; patchouli; Oriza; vector control; Lamiaceae.

1. Introduction

Liquid and solid synthetic insecticides are used in vector control of *A. aegypti.* They are generally accepted as effective, but they are carcinogenic, hazardous to the environment and non-target organisms. The compound N, N-diethyl-3-methylbenzamide, also known as DEET, is the product with significant insect repellency efficiency [1]. However, due to neurotoxicity allied to environmental claims, the population began to worry about its widespread use. [2, 3].

In the last years, the secondary metabolites found in plants have aroused researchers' interest to be used as alternatives to chemical insecticides [4-6]. In fact, insecticides of botanical origin have several advantages such as rapid action and degradation, low toxicity to mammals, greater selectivity and low phytotoxicity [5,7].

The mosquito *A. aegypti* L. (Diptera: Culicidae) is the transmitter of dengue, yellow fever, Chikungunya, and Zika, which cause severe morbidity and mortality in humans [8-10]. The etiology of *A. aegypti* influences its wide dispersion, favored in urban environments, preferably in the domiciliary conditions offered by human's way of living. The presence of the breeding grounds in an environment of human conviviality favors the rapid proliferation of the species, due to two aspects: ideal breeding conditions and feeding sources [1].

The use of insecticides to control adult (adulticidal) and larval (larvicidal) mosquito populations can be done through focal and non-focal treatment by the aerospace spraying of ultra-low volume insecticides (UVB). Repellents can be applied to the individual's skin to repel mosquitoes and avoid stings [11].

However, there is increasing the resistance of mosquitoes to synthetic insecticides, as well as negative impacts on the environment. Thus, it is important to search for alternative methods to be used in the control of *A. aegypti*, which are efficient, low cost, biodegradable and more selective [12].

In this context, species of the Lamiaceae family present potential for obtaining essential oils and plant extracts, they have several biological functions used in the treatment of diseases in folk medicine, as well as reports of anti-influenza, insecticide, repellent, antibacterial and anti-intestinal parasite activities [13].

P. cablin is a species of the Lamiaceae family, popularly known as Oriza or Patchouli, traditionally used for medicinal purposes, especially for the treatment of nausea, headache and heart problems, as well as proven biological activities such as

antioxidant, analgesic, anti-inflammatory, antiplatelet, antithrombotic, aphrodisiac, antidepressant, ant mutagenic, antiemetic, fibrinolytic and cytotoxic [14-17].

Considering the potentiality of species of the Lamiaceae family and the need for more studies aimed at solving public health problems, in particular, those caused by the *A. aegypti* vector, it is important to adopt alternative strategies with greater investments in appropriate methods. Thus, the present research had the objective of studying the larvicidal activity against *A. aegypti* of the crude ethanolic extract of *P. cablin* leaves. This also includes antioxidant, microbiological and cytotoxic activities, which will serve as a complementary study to evaluate the potentiality of the species for future formulation of a natural biocide.

2 MATERIAL AND METHODS

2.1 Plant material

The species *P. cablin* was collected in Fazendinha district (0 ° 01'08 "S and 51 ° 06'17" O) in Macapá-Amapá Municipality, Brazil. For botanical identification, the sample of the species was sent to the Herbarium of the Institute of Scientific and Technological Research of the State of Amapá (IEPA), and it was registered under number 019183.

2.2 Vegetable extract

The leaves of *P. cablin* were oven dried at 50 ° C for a period of 48 hours and manually ground (400g of the plant material). The plant material was placed in a suitable vessel and ethyl alcohol (96%) was added until complete submersion. Every 3 days, the ethanol extract was filtered and placed in a rotary vacuum evaporator (totaling three extractions), under the following conditions: temperature of 50ºC and pressure of 500 to 760mmHg [18].

2.3 Qualitative phytochemical analysis

The qualitative phytochemical analysis of crude ethanolic extract was performed according to Barbosa et al. [19], in which methods of precipitation and staining reactions were applied to for the detection of cardiac glycosides, catechins, flavonoids, purines, anthraquinones, steroids and triterpenoids, depsides and depsidones, polysaccharides, phenols and tannins, proteins and amino acids, alkaloids, reducing sugars, azulenes, organic acids, and saponins.

2.4 Quantitative phytochemical analysis

The quantification of total phenolics was determined by the Folin-Ciocateu method, according to Amorim et al. [20], with modifications. An aqueous solution of gallic acid (5000 $\mu g.mL^{-1}$) was prepared for successive dilutions. Subsequently, the calibration curve was carried out at concentrations of 10 to 500 $\mu g.mL^{-1}$ and 400 µL of Folin-Ciocateu (10%) aliquots were added to 1600 µL of Na2CO3 (75 $g.L^{-1}$). The mixture was incubated at 25 ° C for 2 hours and the absorbance was measured in a spectrophotometer with a wavelength of 760 nm. After reading the calibration curve from the samples, an aqueous solution of extract (1 $mg.mL^{-1}$) was prepared with 200 µL added in a 10 mL flask, 400 µL of Folin-Ciocateu (10%) and 1600 µL Na2CO3 (75 $g.L^{-1}$), in triplicate, to quantify the total phenolic content. The results were expressed as mg equivalent of gallic acid per gram of extract (mg EAG/g).

2.5 Larvicidal Activity

The larvae of *A. aegypti* used in the bioassay were from the colony kept in the laboratory of the Medical Entomology of the Institute of Scientific and Technological Research of the State of Amapá (IEPA), where the larvicidal test was carried out. Biological assays were conducted under controlled climatic conditions with a temperature of 25±2 °C, relative humidity of 75±5 %, and a photoperiod of 12 h.

The methodology used followed the standard protocol of the World Health Organization [21] with modifications. The extract of *P. cablin* (0.09 g) was dissolved in 85.5 ml of distilled water and 4.5 mL of Tween 80. For the negative control, 1% Tween 80 and distilled water were used. As for the positive control, esbiothrin larvicide was used. The extract solution was separated in triplicates at concentrations of 20 to 100 $\mu g.mL^{-1}$ in Becker of 100 mL, and 25 larvae of the *A. aegypti* mosquito in the 3rd young stage (L3) were added. After 24 and 48 hours, the dead larvae were counted, they being considered as such all those that were unable to reach the surface.

2.6 Determination of antioxidant activity

The antioxidant activity was evaluated according to the methodology of Chen et al. [22] and Lopez-Lutz et al. [23] by the sequestering ability of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The antioxidant activity was calculated [24] as follows:

$$(\%AA) = 100-\{[(Abs_{sample} – Abs_{white})100]/Abs_{control}\}$$

%AA – percentage of antioxidant activity

Abs_{sample} – Sample absorbance

Abs_{white} – White absorbance

$Abs_{control}$ – Control absorbance

A methanolic solution of DPPH at the concentration of 40 $\mu g.mL^{-1}$ was prepared. The extract was diluted in methanol at different concentrations of 7.81 to 250 $\mu g.mL^{-1}$. Triplicates with 0.3 mL volume of the extract solution per tube were performed with 2.7 mL of the DPPH solution. In parallel, the negative control was prepared with 2.7 mL of methanol and 0.3 mL of the methanolic solution. For the positive control, ascorbic acid was used in the same conditions of preparation of extract. After 30 minutes of incubation at room temperature and protected from light, the absorbance was measured in a spectrophotometer (Biospectro SP-22) at wavelength 517 nm, in a quartz cuvette.

2.7 Antimicrobial activity

2.7.1. Bacterial strains and culture conditions

Two gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 25922 and *Escherichia coli* ATCC 8789) and gram-positive bacteria (*Staphylococcus aureus* ATCC 25922) were used in this bioassay.

A stock culture in BHI (Brain Heart Infusion) environment, with 20% glycerol-preserved at - 80 ° C was prepared for each microorganism. An aliquot of 50 µL of this culture was inoculated into 5 mL of sterile BHI broth environment and incubated for 24 hours at 37 ° C.

2.7.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The microplate dilution technique (96 wells) was used for determination of MIC and MBC, according to the protocol established by the Clinical and Laboratory Standards Institute [25], with adaptations.

Initially, the bacteria were reactivated with stock cultures and kept in BHI broth for 18 h at 37 °C. The inoculum in 0.9% saline solution was prepared for each microorganism, adjusted for the McFarland 0.5 scale, followed by dilution in BHI and tested at 2 x 106 $CFU.mL^{-1}$.

For MIC determination, the extract was diluted in Dimethyl sulfoxide (2% DMSO). The first well column of the plate was filled with 0.2 mL of the extract solution at the concentration of 2000 µg.mL^{-1}, the other wells were filled with 0.1 mL of 0.9% NaCl. Subsequently, base two serial dilutions were performed in the ratio of 1: 2 to 1: 128 until the dilution in a final volume of 0.1 mL. The cells (2 x 106 CFU.mL-1) with 0.1 mL adjusted according to the previous item were added to each well, resulting in a final volume of 0.2 mL. There were performed the control of the culture environment, the control of extract, and the negative control (DMSO 2%). For positive control, amoxicillin (50 µL.mL^{-1}) was used. The experiments were carried out in triplicates. The microplates were incubated in an oven at 37 °C for 24 hours. After this time, the plates were read in ELISA reader (DO 630 nm).

The MBC was determined based on the results obtained in the MIC test. Microplate wells were replicated in Müller-Hinton agar and incubated at 37 ° C for 24 h. MBC was established as the lowest extract concentration capable of completely inhibiting microbial growth in Petri dishes after 24-48 hours of growth.

The results were categorized in Microsoft Excel (Version 2010 for Windows) and then, analyzed in GraphPad Prism software (Version 6.0 for Windows, San Diego California USA). Significant differences between the groups were verified using the One-way ANOVA test with Bonferroni post-test, considering $p < 0.001$.

2.8 Cytotoxic activity

The evaluation of the cytotoxic activity was performed against the larvae of *Artemia salina* Leach [26, 27] with adaptations. A solution of 250 mL of synthetic sea salt at 35 g.L^{-1} was prepared, 25 mg of exposed saline eggs were incubated in 24 h photoperiod to reach the methanuplion stage. The stock solution was prepared to contain 0.06 g of extract, 28.5 mL of the solution of synthetic sea salt and 1.5 mL of Tween 80. Seven groups of samples were divided in triplicate in the concentrations of 50 to 1000 µg.mL^{-1}, and 10 methanuplii were added in each test tube. For the negative control, it was used Tween 80 with solution saline (5%), and the ($K_2Cr_2O_7$) Potassium dichromate (1%) as the positive control. In the end, the number of non-survivors for LC_{50} determination was counted using the SPSS® software PROBIT analysis.

2.9. Statistical analysis

The data analysis was performed through analysis of variance (ANOVA) and the Tukey test, in the BioEstat program, in order to identify significant differences between the averages. The differences that presented probability levels less than and equal to 5% ($p \leq 0.05$) were considered statistically significant. The results were expressed as mean ± standard deviation (SD). The LC_{50} values were determined in the PROBIT regression, through the SPSS program (Statical Package for the Social Sciences).

3 Results

3.1 Qualitative phytochemical analysis

The phytochemical analysis of the extract of the leaves of *P. cablin* showed the presence of steroids and triterpenoids, depsides and depsidones, according to table 1.

Table 1 - Identification of the secondary metabolites of the extract of *P. cablin*

Tests	**Results**
Cardiac glycosides	-
Catechins	-
Flavonoids	-
Sesquiterpenolactones and other	-
lactones	-
Purines	-
Anthraquinones	-
Steroids and triterpenoids	+
Depsides e depsidones	+
Polysaccharides	-
Phenols and catheter tannins	-
Proteins and amino acids	-
Organic acids	-
Saponins	-
Azulenes	-
Alkaloids	-
Reducing sugars	-

Signal (+) indicates presence of secondary metabolite, while signal (-) indicates absence

The identification of steroids and triterpenoids is due to the result of the appearance of the staining that ranges from blue evanescence to persistent green, which occurs due to the loss of the hydroxyl that activates the conjugated system of the steroid nucleus [28]. While depsides are esters of two or more units of hydroxybenzoic acids, and depsidones are biogenetically derived from depsides through an intramolecular oxidative coupling [29], the positive result of this class is indicated by the appearance of green, blue or gray coloration.

3.2 Quantitative phytochemical analysis

Table 2 shows the total phenolic content found in *P. cablin* leaves. The content of phenolic compounds was 4.02%, a relatively low result in which may be related to the absence of antioxidant activity.

Table 2 - Total phenolics of extract from leaves of *P. cablin*

Ethanolic Extract	**Phenolic compounds (mg GAE/g)**	**Phenolic compounds (%)**
EE	40,27 µg/pipe	4,02

3.3 Larvicidal activity

The extract of *P. cablin* presented expressive larvicidal action, with LC_{50} of 63.91 $\mu g.mL^{-1}$ in 24 h, and LC_{50} of 64.58 $\mu g.mL^{-1}$ in 48 h, with R^2 of 0.914, result with a high value when compared to the standard larvicidal Esbiothrin, with LC_{50} of 0.0034 $\mu g.mL^{-1}$. These did not present statistical difference between the 24 h and 48 h periods with $p< 0.05$, as shown in table 3.

Table 3 - Percentage mortality (%) of *A. aegypti* larvae at different concentrations of *P. cablin* extract in two periods.

Concentrations ($\mu g.mL^{-1}$)	**Larvicidal activity (%)**	
	24 h	**48 h**
20	0.0	0.0
40	4.0	4.0
60	52.0	54.0
80	78.66	78.66
100	80.0	81.33
Control (-)	0.0	0.0
LC_{50} Control (+)	0.0034 $\mu g.mL^{-1}$	

Not present a statistically significant difference in relation to the positive control.

3.4 Antioxidant activity

In the evaluation of the antioxidant activity, the results showed the absence of this activity, since in the highest concentration (250 µg.mL^{-1}) the DPPH consumption was 24.52%, which was lower than the expected 50% of consumption, which caused a high IC_{50} of 900.98 µg.mL^{-1}, according to table 4.

Table 4 - Mean and standard deviation of the percentage of antioxidant activity of extracts of *P. cablin* in different concentrations.

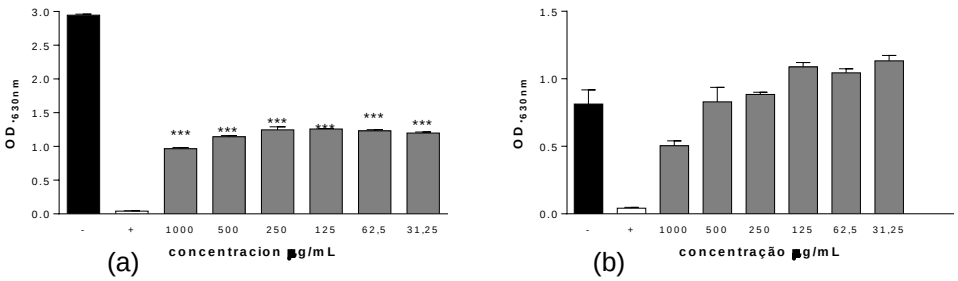
Concentrations (µg.mL^{-1})	% AA	% Ascorbic Acid
7,81	14.29± 0.25^{a}	18.57± 0.052
15,62	15.45± 0.73^{a}	29.95± 0.10
31,25	17.80±0.83^{b}	99.93±0.02
62,5	18.24 ±0.62^{b}	99.96 ±0
125	20.5 ± 0.29^{c}	99.99 ±0
250	24.52 ± 0.42^{d}	99.99 ± 0

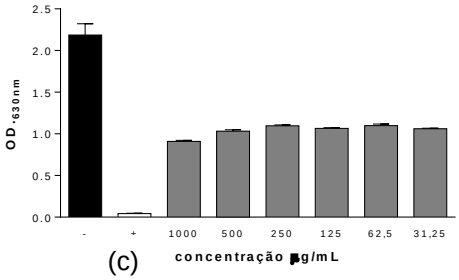
Different letters indicate that there was statistical difference between the concentrations p <0.05.

3.5 Antimicrobial activity

The antimicrobial activity showed that *P. cablin* extract had a better bacteriostatic potential against *E. coli* bacteria, with a MIC of 31.25 mg.mL^{-1} than for *S. aureus* and *P. aeruginosa* bacteria. Regarding MBC, extract did not demonstrate bactericidal activity against the bacteria under test, according to Figure 1.

Figure 1 - Sensitivity Test (MIC) and (MBC) OE of *P. cablin* against the *E. coli, S. aureus* and *P. aeruginosa.*





(c)

Figure 1. MIC and MBC of the EO of *P. cablin* against (a) *E. coli,* (b) *S.aureus* and (c) *P. aeruginosa*Source: Own author. Substance test (▩), BHI with 2% DMSO (■) and Amoxiline (☐). * P <0.001 statistically significant in relation to the negative control, # p <0.001 statistically significant in relation to the positive control.

3.6 Cytotoxic activity

In the evaluation of the cytotoxic activity, the extract of *P. cablin* presented moderate toxic action, with LC_{50} of 257.93 and R^2 0.981, p <0.05. Table 5 shows the mean mortality readings performed in the 24 hour extract exposure against *A. salina* larvae.

Table 5 - Percentage of mortality of *A. salina* larvae of *P. cablin* extract in different concentrations.

Concentrations (µg.mL^{-1})	Mortality (%)
50	0.0^a
100	15.0^a
250	56.6^b
500	63.3^c
750	100.0^d
1000	100.0^d
Control (-)	0.0^a
LC_{50} Control (+) **$K_2Cr_2O_7$**	12.60 µg.mL^{-1}

Different letters indicate that there was a significant difference between the concentrations (p <0.05)

4 Discussion

The study for the developament of biocidal herbs against *A. aegypti* is recent, beginning in the 1980s, in order to isolate and characterize such bioactive substances. Many plant-based products have active compounds that act synergistically or in isolation, and they have characteristics that can be effective in controlling and monitoring mosquito populations [30].

The plants have mechanisms against insect action and are able to synthesize, from different metabolic pathways, defense compounds as secondary metabolites and proteins that act as insecticidal toxins [18].

In this context, the phytochemical tests performed with *P. cablin's* extract, the classes of substances such as steroids and triterpenoids, depsids and depsidones showed positive results.

The identification of the steroids and triterpenoids is due to the result of the appearance of the coloration that goes from the blue evanescence to the persistent green, which occurs due to the loss of the hydroxyl that activates the conjugated system of the steroid nucleus in the reaction [31]. Steroids are derivatives of acetate, in which they act to reduce cholesterol absorption, reduce risks of cardiovascular diseases and inhibit the growth of malignant tumors [28]. The triterpenoids are a condensed compound derived from terpenoids and their biosynthetic source of isoprene. One of its main biological activities is the antispasmodic function, in which it has the function of relaxing the intestinal smooth muscle, reducing cramps [32].

In the staining reaction of depsides and depsidones, there was a positive result from the appearance of the greenish coloration. This class of metabolites consists of phenolic compounds of multiple properties such as antioxidant, antiviral, antibiotic, antitumor, allergen, inhibition of plant growth, anti-tuberculosis and enzyme inhibitory activity [33].

In the determination of the total phenolics, the extract of *P. cablin* presented 4.02%, a significantly low result, suggesting the absence of antioxidant activity, since the phenolic compounds present in the plants are related to the most abundant antioxidants. Therefore, the greater the number of phenolic compounds, the vegetable will have expressive antioxidant activity that contributes to the processes of inhibition of the risk of cardiovascular diseases and may act on oxidative stress, related to several chronic-degenerative pathologies, such as diabetes, cancer and inflammatory processes [34]. Currently, there is a shortage of studies indicating the content of phenolic compounds in the extract of *P. cablin.*

Studies have proven the activity of plant extracts in the control of different species of mosquito [35-37], including *A. aegypti* [1,4, 8-10, 12,13,30]. These plants synthesize several types of compounds that have recognized entomotoxic potential and arouse the interest of several researchers in the search for alternative strategies for the chemical control of *A. aegypti* [18].

In this context, *P. cablin's* extarct presented a significant larvicidal potential with LC_{50} of 63.91 μg.mL^{-1} in 24h, since samples with LC_{50} below 100 μg.mL-1 are considered to be good larvicidal agents [38]. The significant larvicidal activity may be related to the class of terpenes identified in the preliminary chemical test of this species, considering that biocidal studies of the *P. cablin* [39-42] species considered the sesquiterpenes (among them patchouli alcohol) as main responsible for their larvicidal potentiality.

It is important to emphasize that to relate larvicidal activity to some chemical compound is still a complex task, since the biological effect may reflect the action of the major component or is the result of the synergistic action of the constituents.

As for the antioxidant analysis, Nascimento et al. [43] emphasize that the antioxidant test sample that has high potential in sequestering free radicals has a low IC 50 value. Thus, from a small amount of sample, there is a decrease in the initial concentration of the DPPH radical by 50%, to inhibit the radical oxidation by 50%. In conclusion, the results observed in this study did not demonstrate antioxidant activity, since the IC_{50} of the correlation between antioxidant activity (%) and the extract concentration was 900.98 μg.mL^{-1} when compared to the standard of ascorbic acid (vitamin C) with IC_{50} of 16.71 μg.mL^{-1}. These results are linked to the phytochemical profile of the species, in which Maqsood et al. [44] state that ketone or phenolic substances present in plants influence the antioxidant activity.

In this scenario of research related to the search of natural bioactive compounds, the use of new substances with antimicrobial activity has aroused the interest of the scientific community, because some bacteria have resistance to synthetic antibiotics [45]. Thus, drugs that are manufactured from natural compounds appear as a promising alternative for the effective treatment of infectious diseases.

The plant extracts have compounds with antimicrobial potential, they act with a mechanism of action on the bacteria interconnected to the disturbance of the cytoplasmic membrane, cytoplasmic coagulation, change in electron flow, disruption of proton power, alteration of active transport and reduction of intracellular ATP pool [46,47].

In this study, in the evaluation of the antimicrobial activity, the extract of *P. cablin* prevented the bacterial growth only against E. coli bacteria, with MIC 31.25 μg.mL^{-1}, and it showed no bactericidal activity. Liu et al. [48] found the anti-microbial activity of *P. cablin* extract against Rhizopus nigricans, demonstrating its efficacy against

infectious microorganisms. However, there are limited reports on the potentiality of the microbial activity of *P. cablin* extract, instigating further studies to clarify such biological activity.

The evaluation of the toxicity of a plant species is an important bioassay to verify if it can be used as herbal medicine. In this context, the preliminary toxicological bioassay with *A. salina* allows evaluating if the effects that a compound produces in these microcrustaceans are applicable to humans. It is necessary to make only mathematical corrections to verify the appropriate dose per unit of the body surface since the toxic effects caused in laboratory animals are approximately similar to those caused in humans [49].

The extract of *P. cablin* presented moderate toxicity, according to the classification of Lopez-Lutz et. al [23], in which high toxicity is considered LC_{50} values less than 100 $\mu g.mL^{-1}$, moderate toxicity between 100 and 500 $\mu g.mL^{-1}$, weak toxicity between 500 and 1000 $\mu g.mL^{-1}$, and LC_{50} above 1000 $\mu g.mL^{-1}$ are considered to be non-toxic.

The level of toxicity of a plant species depends on the chemical compounds that constitute it. In the species *P. cablin* it is estimated that the toxicity is related to the class of terpenes identified in its composition [50]. However, the plant extract of *P. cablin* may be more toxic than its isolated compounds, since the synergy between the substances potentiated the significant toxic action.

5 CONCLUSION

The preliminary chemical composition of *P. cablin* extract indicated the presence of the following classes of secondary metabolites: steroids and triterpenoids, depsides and depsidones.

P. cablin's extract demonstrated significant larvicidal potential and low toxicity in the LC_{50} found in this study, which can be used to control mosquito larvae without causing a cumulative effect in humans and the environment.

As for the antioxidant evaluation, there was no evidence of antioxidant activity by the DPPH radical capture method when compared to the vitamin C standard.

The antimicrobial activity showed that the extract of *P. cablin* presented a bacteriostatic action in the concentration of 31.25 $mg.mL^{-1}$ only against E. coli bacteria.

The data show the relevance of the bioassays as a screening of the biological potential of the *P. cablin* species, as well as the importance of these products as a

source of biocidal compounds. Also noteworthy is the lack of studies related to the biocidal activities of *P. cablin's* extract.

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5 CONSIDERAÇÕES FINAIS

A composição química do óleo essencial da *P. cablin* indicou a presença de 29 substâncias. Sendo que os principais compostos identificados foram o álcool de patchouli (33, 25%), Seyshellene (6,12%), α-bulnesene (4,11%), Pogostol (6,33%) e Norpatchouleno (5,72%). No extrato etanólico a composição química preliminar de *P.cablin* indicou a presença das seguintes classes de metabólitos secundários: esteróides e triterpenóides, depsídeos e depsidonas.

Quanto a avaliação antioxidante, o óleo essencial e o extrato etanólico de *P. cablin* não apresentou atividade antioxidante pelo método de captura do radical DPPH, quando comparado com o padrão vitamina C.

Na avaliação preliminar da toxicidade frente a *A. salina*, o óleo essencial apresentou expressiva ação tóxica e o extrato apresentou moderada ação tóxica, todavia estudos mais abrangentes devem ser realizados para comprovação da toxicidade desta espécie para o ser humano e meio ambiente.

A atividade antimicrobiana mostrou que o óleo essencial da *P. cablin* apresentou ação antimicrobiana na concentração de 62,5 $\mu g.mL^{-1}$ frente a todas as bactérias testadas. Já o extrato apresentou ação bacteriostática na concentração de 31,25 $\mu g.mL^{-1}$ somente frente a bactéria *E. coli*.

O extrato bruto etanólico e o óleo essencial de *P. cablin* demonstrou significativo potencial larvicida, podendo ser utilizado no controle das larvas do mosquito *A. aegypti*, sugerindo o estudo com outros vetores.

Os dados mostram a relevância dos bioensaios como uma triagem do potencial biológico da espécie *P. cablin*, bem como à importância destes produtos como fonte de compostos com ação biocida.

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ANEXOS

Anexo 1 – Normas de publicação da Revista Pharmaceuticals e Scientia Pharmaceutica

Submission Checklist

Please.

1. read the Aims & Scope to gain an overview and assess if your manuscript is suitable for this journal;
2. use the Microsoft Word template or LaTeX template to prepare your manuscript;
3. make sure that issues about publication ethics, research ethics, copyright, authorship, figure formats, dataand references format have been appropriately considered;
4. ensure that all authors have approved the content of the submitted manuscript.

Submission Process

Manuscripts for *Pharmaceuticals* should be submitted online at susy.mdpi.com The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the criteria to qualify for authorship) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the submission website. Once you have registered, click here to go to the submission form for *Pharmaceuticals*. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work and why it fits the scope of the journal. Confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
    - Front matter: Title, Author list, Affiliations, Abstract, Keywords
    - Research manuscript sections: Introduction, Results, Discussion, Materials and Methods, Conclusions (optional).

- Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest,References.

- **Graphical abstract:** Authors are encouraged to provide a graphical abstract as a self-explanatory image to appear alongside with the text abstract in the Table of Contents. Figures should be a high quality image in any common image format. Note that images displayed online will be up to 11 by 9 cm on screen and the figure should be clear at this size.
- **Abbreviations** should be defined in parentheses the first time they appear in the abstract, main text, and in figure or table captions and used consistently thereafter.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on Deposition of Sequences and of Expression Data.
- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
- **Research Data and supplementary materials:** Note that publication of your manuscript implies that you must make all materials, data, and protocols associated with the publication available to readers. Disclose at the submission stage any restrictions on the availability of materials or information. Read the information about Supplementary Materials and Data Deposit for additional guidelines.

Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.
- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, country, and all email addresses. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it

must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.

- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.
- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.
- **Conclusions:** This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.
- **Patents:** This section is not mandatory, but may be added if there are patents resulting from the work reported in this manuscript.

1.1.1. Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Acknowledgments:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published.

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  For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the CRediT taxonomy for the term explanation. For more background on CRediT, seehere. "**Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully**".
- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the choice of research project; design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. *Pharmaceuticals* does not publish studies funded by the tobacco industry. Any projects funded by pharmaceutical or food industries must pay special attention to the full declaration of funder involvement. If there is no role, please state "The sponsors had no role in the design, execution, interpretation, or writing of the study".
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- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files forEndnote and Zotero are available.

References should be described as follows, depending on the type of work:

 Journal Articles:
1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range. Available online: URL (accessed on Day Month Year).
 Books and Book Chapters:
2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.
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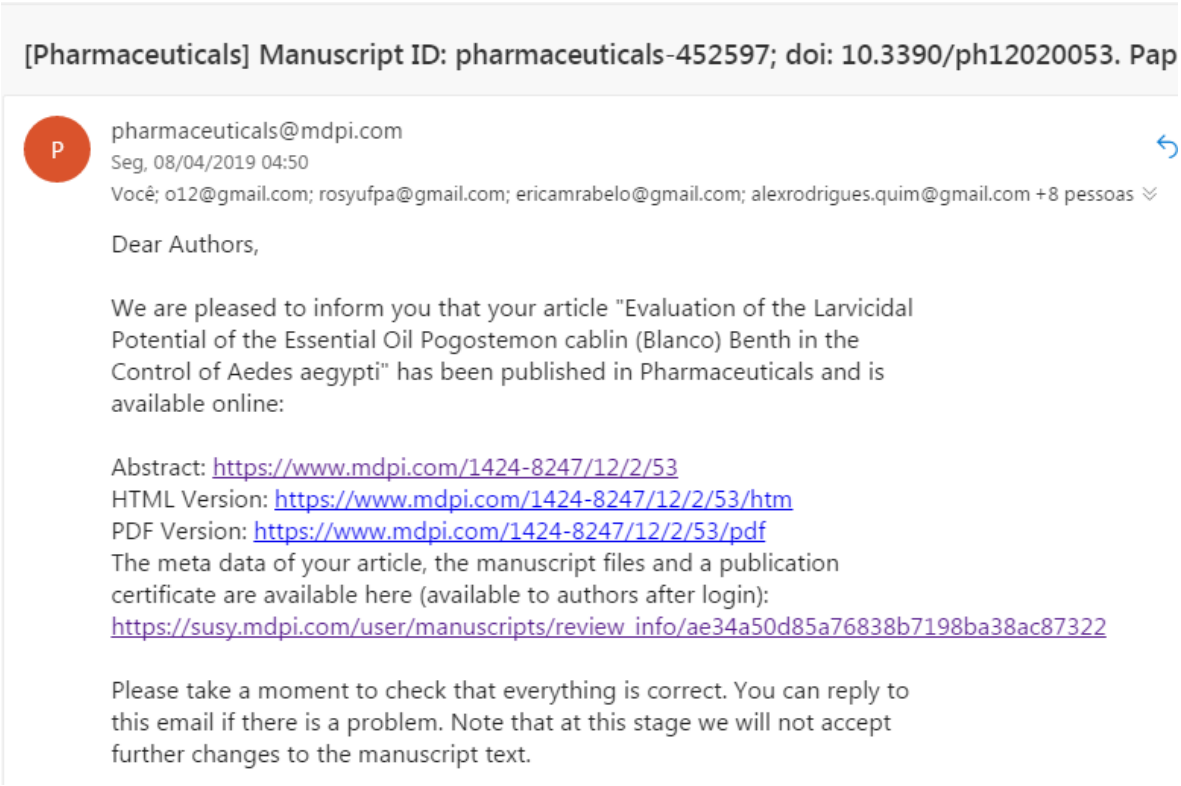
Preparing Figures, Schemes and Tables

- File for Figures and schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Pharmaceuticals* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.

- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

Anexo 2 – Confirmação de publicação do Artigo 1



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Dear Authors,

We are pleased to inform you that your article "Evaluation of the Larvicidal Potential of the Essential Oil Pogostemon cablin (Blanco) Benth in the Control of Aedes aegypti" has been published in Pharmaceuticals and is available online:

Abstract: https://www.mdpi.com/1424-8247/12/2/53
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Anexo 3 – Confirmação de submissão do Artigo 2

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Manuscript ID: scipharm-466990
Type of manuscript: Article
Title: LARVICIDAL ABCTIVITY AGAINST Aedes aegypti AND ANTIOXIDANT, CYTOTOXIC AND MICROBIOLOGICAL EVALUATION OF THE CRUDE ETHANOLIC EXTRACT OF Pogostemon
cablin Benth
Authors: Lizandra Lima Santos *, Lethicia Barreto Brandão, Rosany Lopes Martins, Erica de Menezes Rabelo, Alex Bruno Lobato Rodrigues, Camila Mendes da Conceição Vieira Araújo, Talita Fernandes Sobral, Allan Kardec Ribeiro