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RENATA DO SOCORRO BARBOSA CHAVES

AVALIAÇÃO DA ATIVIDADE LARVICIDA DO EXTRATO BRUTO ETANÓLICO E ÓLEOS ESSENCIAS DAS FOLHAS DE *Origanum majorana* L. E *Origanum vulgare* L. FRENTE AO *Aedes* a*egypti* (Linnaeus, 1762)

Macapá
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Orientadora: Sheylla Susan Moreira da Silva de Almeida.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

µg	Micrograma
µL	Microlitro
% AA	Porcentagem de atividade antioxidante
ATCC	American Type Culture Collection
BHI	Brain Heart infusion
CBM	Concentração Bactericida Mínima
CIM	Concentração Inibitória Mínima
CG-EM	Cromatografia Gasosa acoplada ao Espectrômetro de Massa
CI	Concentração Inibitória
$CL_{50\%}$	Concentração Letal média
CLSI	Manual Clinical and Laboratory Standards Institute
Cm	Centímetro
CHIKV	Vírus Chikungunya
DENV	Vírus da Dengue
DMSO	Dimetilsufóxido
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EBE	Extrato Bruto Etanólico
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuaria
g	Grama
IEPA	Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá
IBGE	Instituto Brasileiro de Geografia e Estatística
IK	Índices de Kolvats
KPa	Quilopascal
mg	Miligrama
MHA	Ágar Mueller-Hinton
mL	Militro
Nm	Nanometro
OEs	Óleos essenciais
OMS	Organização Mundial da Saúde
ºC	Grau Celsius
PECD	Programa Estadual de Controle da Dengue (PECD)
ppm	Parte por milhão
SPSS	Statical Package for Social Science

SPJ	Science Partner Journal
UFC/mL	Unidade Formadora de Colónia por mililitro
WHO	World Health Organization
λ	Comprimento de onda
ZIKV	Vírus Zika

Avaliação da Atividade Larvicida do Extrato Bruto Etanólico e Óleos Essenciais das Folhas de *Origanum majorana* L. e *Origanum vulgare* L. Frente ao *Aedes aegypti* (Linnaeus, 1762)

RESUMO

Introdução: *Origanum majorana* L. e *Origanum vulgare* L. pertencentem à família Lamiaceae e são popularmente conhecidas como manjerona e orégano, respectivamente. **Objetivo:** Avaliar a atividade larvicida de *O. majorana* L. e *O. vulgare* L. frente ao *Aedes aegypti*. **Metodologia:** o bioensaio larvicida foi realizado com larvas no terceiro estádio de *A. aegypti*; as identificações dos componentes dos óleos por Cromatografia Gasosa Acoplada à Espectrofotômetro de Massas; o extrato bruto etanólico por avaliação fitoquímica; a atividade microbiológica pelo método de microdiluição com bactérias *Pseudomona aeruginosa, Escherichia coli* e *Staphylococcus aureus*; o antioxidante foi avaliado pelo método de sequestro do radical 2,2-difenil-1-picril-hidrazila e a atividade citotoxica foi realizada utilizando *Artemia salina*. **Resultados e discussões:** Os óleos de manjerona e orégano apresentaram elevada atividade larvicida com CL_{50} de 74,63 e 56,00 $\mu g.mL^{-1}$ após 24h, CL_{50} de 20,27 e 15,69 $\mu g.mL^{-1}$ respectivamente, após 48h. O extrato de manjerona apresentou boa atividade com LC_{50} de 219,14 e 79,40 $\mu g.mL^{-1}$ após 24h e 48h respectivamente. Os constituintes majoritários encontrados nos óleos de manjerona e orégano foram pulegona (57,05%), γ-terpinene (27,18%), respectivamente. No extrato foram encontrados açúcares redutores, taninos, fenóis, depsideos e depsedonas, esteroides e triterpenóides. A *P. aeruginosa e E. coli* foram mais suscetíveis nos óleos e no extrato com CIM de 31,25 $\mu g.mL^{-1}$. Apenas o óleo de orégano frente *S. aureus* com CIM de 500 $\mu g.mL^{-1}$. Na CBM do óleo de orégano, as concentrações de 250, 500 e 1000 $\mu g.mL^{-1}$ inibiram o crescimento de *P. aeruginosa*, *S. aureus* e *E. coli*, respectivamente. No óleo de manjerona as concentrações de 1000 e 500 $\mu g.mL^{-1}$ inibiram o crescimento de *P. aeruginosa* e *E. coli*. Não houve atividade antioxidante das plantas, porém houve atividade citotóxica dos óleos orégano e manjerona frete a *A. salina* com elevado LC_{50} de 38,11 e 172,6 $\mu g.mL^{-1}$ e baixo LC_{50} de 946,9 $\mu g.mL^{-1}$ do extrato. **Conclusões:** Desse modo, os óleos e o extrato obtidos das folhas de *O. vulgare* L. e *O. majorana* L. apresentam potenciais para o desenvolvimento de larvicidas naturais.

Palavras-Chave: *Manjerona; orégano; febre da dengue; larvas; metabólitos secundários.*

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Evaluation of the Larvicidal Activity of Ethanolic Crude Extract and Essential Oils of *Origanum majorana* L. and *Origanum vulgare* L. Leaves Against *Aedes aegypti* (Linnaeus, 1762)

ABSTRACT

Introduction: *Origanum majorana* L. and *Origanum vulgare* L. belong to the family Lamiaceae and are popularly known as manjorana and oregano, respectively. **Objective**: To evaluate the larvicidal activity of *O. majorana* L. and *O. vulgare* L. against *Aedes aegypti*. **Methodology**: the larvicidal bioassay was performed with larvae in the third stage of *A. aegypti*; the identifications of the components of the oils by Gas Chromatography Coupled to the Mass Spectrophotometer; the crude ethanolic extract by phytochemical evaluation; the microbiological activity by the microdilution method with bacteria *Pseudomona aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*; the antioxidant was evaluated by the sequestration method of the 2.2-diphenyl-1-picryl-hydrazyl radical and the cytotoxic activity was performed using *Artemia saline*. **Results and discussion**: Manjerona and oregano oils presented high larvicidal activity with LC_{50} of 74.63 and 56.00 µg.mL^{-1} after 24h, LC_{50} of 20.27 and 15.69 µg.mL^{-1} respectively, after 48h. Manjerona extract showed good activity with LC_{50} of 219.14 and 79.40 µg.mL^{-1} after 24h and 48h, respectively. The major constituents found in the manjerona and oregano oils were pulegone (57.05%), γ-terpinene (27.18%), respectively. In the extract were found reducing sugars, tannins, phenols, depsides and depsedones, steroids and triterpenoids. *P. aeruginosa* and *E. coli* were more susceptible in oils and extract with MIC of 31.25 µg.mL^{-1}. Only the oil of oregano opposite *S. aureus* with MIC of 500 µg.mL^{-1}. In the MBM of oregano oil, concentrations of 250, 500 and 1000 µg.mL^{-1} inhibited the growth of *P. aeruginosa*, *S. aureus* and *E. coli*, respectively. In manjerona oil the concentrations of 1000 and 500 µg.mL^{-1} inhibited the growth of *P. aeruginosa* and *E. coli*. There was no antioxidant activity of the plants, but there was cytotoxic activity of oregano and manjerona oils at *A. salina* with high LC_{50} of 38.11 and 172.6 µg.mL^{-1} and low LC_{50} of 946.9 µg.mL^{-1} extract. **Conclusions**: In this way, the oils and extract obtained from the leaves of *O. vulgare* L. and *O. majorana* L. present potential for the development of natural larvicides.

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1 INTRODUÇÃO

A dengue continua se apresentando como um agravo que provoca relevantes efeitos negativos sobre a situação epidemiológica, social e econômica, ocorrendo em ampla distribuição geográfica e causando formas graves e letais (BARRETO; TEXEIRA, 2008; WHO, 2019a; GÓMEZ-DANTÉS et al., 2011; WHO, 2019b; ORRICO, 2015).

O *Aedes aegypti* (Linnaeus, 1762) é um importante vetor relacionado à saúde pública, transmissor da dengue, febre amarela, chikungunya e zika, possui ampla distribuição geográfica, nas áreas tropicais e subtropicais situadas entre os paralelos de latitudes 45° Norte e 40° Sul e nas zonas isotermais intermediadas a 20ºC. O mosquito possui hábito antropofílico, apresenta grande capacidade de adaptação a criadouros artificiais o que possibilita o aumento de sua população, o que leva ao aparecimento de epidemias, como as da dengue (BESERRA et al., 2006; ZARA et al., 2016).

O mosquito para se desenvolver depende fortemente de alguns fatores climáticos, como altas temperaturas e umidade. Tais fatores associados ao desenvolvimento urbano descontrolado, à resistência aos inseticidas e à baixa eficácia de controle, contribuíram para o aumento do *A. aegypti* (MANEERAT; DAUDÉ, 2016; GRECHA et al., 2015).

O ciclo de vida do *A. aegypti* é constituído por quatro fases: ovo, larva, pupa e adultos. O desenvolvimento ocorre por meio de metamorfose completa (Holometabolia). As fases de ovo, larva e pupa ocorrem na água, enquanto a fase adulta é terrestre (NUNES, 2015).

O ovo do *A. aegypti* possui contorno alongado e fusiforme, com comprimento médio de 1 a 0,44 mm são depositados pela fêmea sobre superfície da água, são depositados individualmente, são brancos e se tornam de coloração preta e brilhante. O embrião se desenvolve em 48 horas, na ausência de água (secos) ficam ávidos num período de até 450 dias (NUNES, 2015; GUIMARÃES et al., 2015).

Na fase larval (4 estádios – 5 à 10 dias) ocorre a alimentação e o crescimento. Para a alimentação a larva usa o material orgânico presente no criadouro, fatores como temperatura, quantidade de alimento e de larvas determinam o período até a próxima fase. A larva é dividida em cabeça, tórax e abdômen (8 segmentos). No abdômen há brânquias que são responsáveis pela regulação osmótica e o sifão usado para respiração larvária, essa respiração que ocorre nas superfícies da água, as larvas se deslocam forma de S e possuem fotofobia (CASTRO-JUNIOR et al., 2013; NUNES, 2015).

A fase de pupa (2 a 3 dias) sendo ela transitória, não há necessidade de alimentação nesse período, o corpo se divide em cefalotórax e abdômen, fazendo um formato de vírgula,

sendo que a respiração é mantida através de trombetas, como o ovo, a pupa de cor branca se torna enegrecida. O mosquito adulto emerge aos poucos até atingir a superfície da água (NUNES, 2015).

Após 24 horas da mudança para mosquito os órgãos reprodutivos tornam-se aptos, tanto do macho quanto da fêmea já podem cruzar. A primeira da inseminação da fêmea é a última também (CASTRO-JUNIOR et al., 2013; GUIMARÂES et al., 2015).

O tamanho do *A. aegypti* adulto mede em torno de 5 mm e possui o corpo segmentado. No exoesqueleto recoberto de quitina predomina a coloração negra, mas nas pernas apresenta manchas brancas e os segmentos tarsais são listrados de cor branco/prateado. Outra característica marcante é o desenho em forma de lira no tórax (CANTIONÍLIO; SILVA, 2013; CASTRO-JUNIOR et al., 2013).

Com o passar dos anos, o mosquito *A. aegypti*, transmissor da doença sofreu diferentes mutações, o que fortaleceu o vírus já existente, ocasionando o surgimento de variações do vírus, além de trazer como consequência o aparecimento de outras doenças como a febre amarela e, atualmente, o zika vírus (ZIKV) e o vírus *chikungunya* (CHIKV). As primeiras infestações do vírus da dengue possuem registros que datam do final do século XIII, na ilha de Java (Sudoeste da Ásia) e na Filadélfia (EUA). Entretanto, foi apenas a partir do século XX que a dengue passou a ser reconhecida como doença pela Organização Mundial de Saúde (OMS) (NOWAK; RAGONHA, 2018).

O vírus da dengue (DENV) foi isolado pela primeira vez em 1943 (DENV 1) no Japão e em 1945 (DENV 1) no Havaí. O DENV-2 foi isolado em Nova Guiné em 1944. Por fim o DENV-3 e DENV-4 foram isolados nas Filipinas em 1956 (SILVA, 2013) e desde então, espalha-se infectando as populações no mundo todo. Até o ano de 2006, quatro sorotipos de dengue tinham sido isolados: DENV 1, 2, 3 e 4. Porém, em 2007, pesquisadores que examinaram amostras virais de dengue encontraram um vírus coletado durante um surto no estado de Sarawak, na Malásia, os mesmos sequenciaram o vírus e descobriram que este é distinto dos outros quatro tipos, denominando-o de DENV-5 (SPJ, 2013).

A incidência de dengue no mundo aumentou em mais de 30 vezes nos últimos 50 anos. A Organização Mundial de Saúde (OMS) estima que anualmente entre 50 a 100 milhões de infecções ocorram em mais de 100 países (OMS, 2018). A manifestação severa da doença, antes denominada Febre Hemorrágica da Dengue (FHD), foi identificada pela primeira vez nos anos 50 durante uma epidemia nas Filipinas e Tailândia. Atualmente a dengue grave tem se tornado uma das principais causas de hospitalizações e morte de crianças na maioria dos países da Ásia e América Latina (BRASIL, 2017).

Desde 2015, o vírus DENV, ZIKV e Chikungunya (CHIKV) circulam no Brasil e co-infecção pelo *A. aegypti* com esses arbovírus poderiam favorecer a transmissão de um vírus específico, refletindo a mudança no padrão epidemiológico. Contudo, quando o vetor foi co-infectado com dois ou três arbovírus (DENV-CHIKV e DENV - ZIKV) não foram observadas vantagens para nenhum destes vírus (LE-COUPANEC et al., 2017; GÖERTZ et al., 2017).

O Brasil é um país endêmico de dengue e ao longo dos anos tem enfrentado vários surtos causados por diferentes sorotipos do vírus. Mais recentemente, entre 2015 e 2016, o país registrou uma média anual de 1.586.155 casos prováveis (BRASIL, 2016a; BRASIL, 2017). Nas três primeiras semanas de 2016, o número de notificações de casos de dengue no Brasil cresceram 48% quando comparadas ao mesmo período de 2015. Nos primeiros 20 dias do ano, 15 Estados apresentaram aumento nos casos de dengue (CANCIAN, 2016).

No entanto, em 2017, dois ou quatro anos após a provável introdução do vírus Zika (ZIKV) (ZANLUCA et al., 2015; FARIA et al., 2016), o Brasil experimentou uma diminuição na dengue para 252.054 casos, além de redução do número de dengue grave e óbitos (BRASIL, 2018).

Contudo, em relação à dengue, as causas desse declínio ainda não são totalmente compreendidas. De fato, os dados atuais sobre imunidade de rebanho, reações cruzadas entre Vírus da dengue (DENV) e ZIKV, ecologia de mosquitos, medidas de controle de vetores e fatores ambientais não são suficientes para explicar o cenário da dengue em 2017 (LOPES et al., 2018).

O Estado do Amapá está localizado na região Norte do país e tem como capital a Cidade de Macapá, atualmente o Estado possui 16 municípios. De acordo com dados do IBGE (2015) sua população estimada está em torno de 766.679 habitantes para o ano de 2015. Possui uma área total de 142.828,520 km², sendo uma média de 4,69 km² por habitante. O rendimento médio per capita de seus habitantes no ano de 2014 era de 753 reais por domicílio (IBGE, 2015).

No Amapá, de acordo com a coordenação do Programa Estadual de Controle da Dengue (PECD), durante a década de 80, por diversas vezes foi detectada a presença do *A. aegypti* no Estado pelo serviço de vigilância de portos, aeroportos e fronteiras. (MONTEIRO, 2014). A proliferação da doença no município de Macapá causa preocupação para a sociedade, e os métodos de controle do vetor não são eficientes, devido ao crescimento desordenado da cidade (TOSTES et al., 2016).

Apesar do alto índice de ocorrências de dengue no município e por ser considerado um dos grandes problemas de saúde pública no estado do Amapá, as discussões em torno do uso de tecnologias avançadas para o monitoramento da dengue ainda são insipientes.

As atividades humanas prevalecem na proliferação do mosquito que transmite o vírus, e isso é visível quando se observa o descaso da população ao deixar objetos que podem servir de criadouros para mosquito em seus quintais ou até mesmo na sua própria rua (DUARTE et al., 2016).

As epidemias de dengue refletem na economia dos países em virtude da mortalidade e morbidade, que provoca absenteísmo no trabalho e nas escolas, além de repercutir negativamente no setor turístico e provocar colapso dos serviços de saúde, em decorrência da alta demanda por atendimento de paciente (MARTELLI et al., 2015; BRASIL, 2009; PESSOA et al., 2016).

A infecção pelo vírus dengue pode ser assintomática ou sintomática. A primeira manifestação é a febre que tem duração de dois a sete dias, geralmente alta (39ºC a 40ºC), de início abrupto, associada à cefaleia, à adinamia, às mialgias, às artralgias e a dor retroorbitária (BRASIL, 2016b).

Considerando que o mosquito possui hábitos oportunistas, a infestação é mais intensa em ambientes urbanos com alta densidade populacional e ocupação desordenada, onde as fêmeas possuem mais oportunidades de alimentação e dispõem de mais criadouros para ovoposição. Acrescenta-se ainda que, é crucial a eliminação de recipientes, reservatórios ou quaisquer lugares, tanto favoráveis, quanto os não convencionais para a ovoposição e desenvolvimento das fases aquáticas do vetor, visto que, o vetor é de fácil adaptação e tem escolhido locais menos prováveis para se desenvolver. A utilização de inseticidas, quando permitida, contribui para eliminação do vetor (WHO, 2019b).

O controle da incidência e de surtos de dengue está centrado na redução de infestações dos vetores, por meio de intervenções preventivas voltadas para os possíveis focos de transmissão da doença (TANNOUS, 2018).

A utilização de uma vasta gama de inseticidas sintéticos tem sido restrito recentemente, devido ao alto custo, ambiental de prejudicial efeitos, a sua natureza não biodegradável, e aumento a resistência à inseticida. Inseticidas como organoclorados e organofosforados são prejudiciais à saúde por contaminar o ar, à agua e os alimentos, enquanto que produtos derivados de plantas medicinais não promovem tamanha contaminação e tornam-se importantes alternativas para o controle de insetos resistentes (ZARA et al.; NIROUMAND et al., 2016).

No Brasil, o temefós foi o larvicida utilizado ao longo de 30 anos, mas teve seu uso restringido desde 2010, devidos os registros de populações de *A. aegypti* resistentes a esse composto (BRASIL, 2010). E em 2014 foi introduzido nos programas de controle o larvicida piriproxifen (BRASIL, 2014).

Os carbamatos são praguicidas derivados do ácido carbâmico, são compostos instáveis, com degradabilidade dependente de umidade, temperatura, luz e volatilidade. Estes agem inibindo a colisnesterase, cuja inibição pode ser reversível (CASIDA; QUISTAD, 1998; WARE, 2000; HEMINGWAY; RANSON, 2005).

O guia da OMS afirma que “poucos ingredientes ativos dos inseticidas estão disponíveis para o uso na saúde pública e, para minimizar o impacto da resistência aos inseticidas em um programa de controle, é necessário adotar decisões adequadas. Geralmente, a primeira opção a ser selecionada por um programa de controle é o inseticida mais econômico e mais eficaz contra a população de vetores, que possui também um baixo risco para aplicadores e transeuntes (BRASIL, 2016c).

Entre os produtos ecologicamente viáveis e eficazes para controle de pragas estão os óleos essenciais extraídos de plantas medicinais e/ou aromáticas, os quais podem ser usados para combate a diversos culicídeos (PAVELA, 2015b)

Os constituintes dos óleos essenciais são principalmente compostos lipofílicos que atuam como toxinas, impedimentos de alimentação e oviposição para uma ampla variedade de insetos e pragas (KOUL et al., 2008). Esses óleos são constituídos por misturas complexas (hidrocarbonetos oxigenados ou mono e sesquiterpenos alifáticos, aromáticos, etc.) de compostos majoritários como 1,8-cineol (alecrim, eucalipto), mentol (menta), carvacrol (orégano), timol (tomilho), eugenol (cravo e canela), etc. (PAVELA, 2007a). Geralmente, seus componentes majoritários atuam em sinergismo com outras substâncias em menores quantidades e são determinantes para se definir as propriedades biológicas dos óleos essenciais (BAKKALI et al., 2008).

Tais compostos têm sido investigados principalmente quanto à atividade larvicida sobre *A. aegypti*, uma vez que extratos orgânicos, óleos essenciais e derivados semissintéticos apresentam toxicidade contra larvas desta espécie (GOVINDARAJAN, 2011; WARIKOO et al.; FENECH et al., 2011; DOMINGOS et al., 2014; MEIRELES et al., 2016), pois provocam inibição da oviposição, alimentação, causam repelência, alteram o desenvolvimento, causam deformações, infertilidade, mortalidade, dentre outras (ROEL, 2001; AMER; MEHLHORN, 2006; COSTA et al., 2012; DELETRE et al., 2013; KAMIABI et al., 2013; PONTUAL et al., 2014; GUERRERO, 2016).

Além disso, apesar de inseticidas convencionais, serem baseados em um único composto ativo, inseticidas botânicos contendo misturas de compostos químicos podem afetar mais de um processo comportamental e fisiológico. Assim, a possibilidade de desenvolvimento de resistência por meio do mosquito para tais substâncias é muito baixa (NIROUMAND et al., 2016).

O emprego dessas substâncias para controle de pragas ou vetores tem diversas vantagens, já que eles são obtidos de fontes naturais renováveis, são biodegradáveis, não contaminam a água, solo e ar (PERON; FERREIRA, 2012).

A família Lamiaceae (MARTINOV,1820) compreende 240 gêneros e, aproximadamente, 7.200 espécies de distribuição praticamente cosmopolita (MARTIN et al., 2013; ADGABA et al., 2016). No Brasil, ocorrem 46 gêneros nativos e cerca de 524 espécies, com distribuição em regiões tropicais e subtropicais (HARLEY; PASTORE, 2012; PARTIDA et al., 2015).

As plantas da família Lamiaceae possuem grande diversidade morfológica, com hábito herbáceo, arbustivo ou arbóreo, são frequentemente odoríficas devido à presença de óleos voláteis e apresentam caule geralmente quadrangular em corte transversal. As folhas são opostas e geralmente decussadas. Flores bissexuadas, zigomorfas, cálice gamossépalo, geralmente pentâmero e mais ou menos tubuloso ou campanulado, persistente e ocasionalmente acrescente no fruto; corola gamopétala, pentâmera, zigomorfa ou actinomorfa, geralmente bilabiada, lobos imbricados. Fruto drupáceo ou esquizocarpo que se separa em quatro núculas unisseminadas; endosperma é escasso ou ausente (SILVA-LUZ, 2012; FERRAZ, 2016).

Na medicina popular, a família Lamiaceae ocupa o terceiro lugar em ordem de importância, com muitas espécies apresentando substâncias biologicamente ativas (HARLEY et al., 2004). Algumas espécies desta família são comumente usadas na medicina popular como antimicrobiano, anti-séptico, no tratamento de infecções do trato respiratório, dermatoses e feridas (SANTIN, 2013; SHENOY et al., 2009; AZAD et al., 2014).

A literatura relata que o gênero *Origanum* Linnaeus, 1753 (Lamiaceae) é caracterizado por um grande número de atividades biológicas, incluindo os efeitos antioxidantes, antiinflamatórios e anticolinesterásicos, assim como as atividades contra o envelhecimento e a doença neurodegenerativa (LOIZZO et al., 2009).

Origanum majorana Linnaeus, 1753 pertence à família Lamiaceae, considerada uma das mais importantes ervas termoculinárias com grande importância econômica e industrial (SELLAMI et al., 2009). É conhecida como manjerona, uma erva perene, cespitosa, com caules grisáceo-tomentosos. Possui folhas simples, opostas, ovaladas ou arredondado-elípticas, verde-acinzentadas e pilosas, tendo aroma forte e agradável (USP, 2004). Flores esbranquiçadas, róseas ou violáceas, dispostas em glomérulos e reunidas em inflorescências paniculadas teminais (EMBRAPA, 2007). No Brasil é cultivada principalmente em hortas e jardins (BALDONI, 2017).

Contém uma série de compostos orgânicos, alguns dos quais foram encontrados para ter efeitos biológicos, incluindo carvacrol (UPADHYAY et al., 2017), flavonóides (HOSSAIN et al., 2014), taninos (KHAN et al., 2018), sitosterol (AFIFI et al., 2017), glicosídeos fenólicos e terpenoides fenólicos (MEDDAH et al., 2013).

Exibem uma série de atividades biológicas, incluindo antioxidante, antimicrobiano (IMTARA et al., 2018), antifúngico, espessamento da camada de muco intestinal (BASKARAN et al., 2016; WLODARSKA et al., 2015), podem prevenir doenças causadas pelo estresse oxidativo, como por exemplo, o câncer (ELANSARY; MAHMOUD, 2014).

O orégano, *Origanum vulgare* Linnaeus, 1753 (Lamiaceae), é uma planta herbácea, rasteira, de folhas verdes, pequenas e aromáticas, sendo considerado um dos condimentos mais tradicionais da culinária brasileira. Embora sua utilização ocorra em maior quantidade nas indústrias de alimentos, o orégano também é utilizado em indústrias farmacêuticas em razão de suas propriedades terapêuticas (SOUZA; STANDFORD, 2005).

Os metabólitos secundários como ácidos orgânicos, alcaloides, flavonoides, compostos fenólicos, terpenos, taninos condensados, antraquinonas, esteroides e triterpenoides, são conhecidos por apresentar efeitos antimicrobianos (TINTINO et al., 2015).

O uso das plantas medicinais é largamente atribuído ao tratamento de infecções que, por consequência chamou a atenção em pesquisas que visam determinar a atividade antimicrobiana dessas plantas para futuro isolamento dos princípios antimicrobianos ou na otimização do uso da planta (ALENCAR, 2014; BATINGAL et al., 2018)

Os óleos essenciais podem afetar 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 permitem que eles se difundam através da bicamada fosfolipídica (NAZZARO et al., 2013). Nem todos os mecanismos de ação agem em alvos específicos, extratos vegetais tendem a ter mais efeito sobre mais de um sítio de ação, em consequência outros mecanismos envolvidos (SILVA, 2010).

O uso indiscriminado de drogas antimicrobianas de natureza sintética é responsável pelo aparecimento de microrganismos resistente, tornando relevante como um problema mundial levando em considerações o risco para a saúde no desenvolvimento de doenças infecciosas de difícil tratamento e cura. Sendo assim, torna-se empírico a busca de fontes alternativas de compostos antimicrobianos (AMANCIO et al., 2015).

Pseudomonas aeruginosa é um microorganismo patogênico nosocomical, pertencente à família das gram-negativas, causadora de infecções que podem se desenvolver em todo o corpo humano, sendo mais patogênica em pacientes que

apresentam um quadro imunológico comprometido (GALES; REIS; JONES, 2001; FIGUEREDO et al., 2018).

O microrganismo *Echerichia coli* é um bacilo gram-negativo não esporulado, que habita normalmente o intestino da maioria dos animais, incluindo seres humano e pode indicar contaminação fecal devido às falhas higiênicas na manipulação de alimentos e ao uso de água contaminada (LIMA et al., 2017).

Staphylococcus aureus são cocos gram-positivos, que produzem enterotoxinas e tem sido frequentemente envolvido em surtos de toxinfecções alimentar, estando muito associado à manipulação inadequada dos alimentos, uma vez que, são comumente encontrados na pele, mucosas do trato respiratório superior e intestino de humanos (OLIVEIRA et al., 2010).

Sem antimicrobianos eficazes para a prevenção e tratamento de infecções, procedimentos médicos, como transplantes de órgãos, quimioterapia do cancro, diabetes gestão e cirurgia de grande porte (por exemplo, cesarianas ou substituições de quadril) se tornam de alto risco. A resistência antimicrobiana aumenta o custo dos cuidados de saúde com estadias mais longas em hospitais e cuidados mais intensivos necessários (WHO, 2019c).

A determinação da atividade antioxidante é largamente empregada devido sua simplicidade, rapidez e alta reprodutibilidade. O radical DPPH (violeta) é reduzido a hidrazina (amarelo) pelo composto antioxidante que age como doador de hidrogênios e a sua atividade é quantificada por espectrofotometria (MOKRANI; MADANI, 2016) com o uso de curva padrão de Trolox, um composto análogo a vitamina E, solúvel em fases polares e apolares (PIETTA, 2000).

Os diferentes tipos de radicais livres atuam nos organismos de maneiras distintas, exigindo que os antioxidantes sejam capazes de reduzi-los e dessa maneira neutralizar sua ação. Devido aos diferentes mecanismos de ação antioxidante, a atividade antioxidante de um composto pode ser determinada por inúmeros métodos in vitro, dentre os quais estão o sequestro dos radicais DPPH e ABTS, co-oxidação de β-caroteno e ácido linoleico, sequestro do peróxido de hidrogênio, sequestro do radical peroxil, entre outros (OLIVEIRA et al., 2009).

As plantas medicinais podem apresentar efeitos tóxicos decorrente da presença dos seus metabólitos secundários, esses vegetais utilizados inadequadamente ou utilizados sendo ignorado quanto esse efeito, mesmo apresentando eficácia terapêutica, torna-se um risco a saúde. Portanto, a investigação do potencial tóxico de plantas medicinais pode

esclarecer importantes aspectos farmacológicos de seus constituintes, propiciando uma utilização segura respeitando os riscos toxicológicos (SILVA et al., 2016).

Artemia salina é um organismo regulador hipo/ hiper-osmótico capaz de manter as concentrações de íons de hemolinfa, dentro de limites estreitos sobre uma gama de salinidade externa de NaCl a 0,26% em ambientes supersaturados (ATES et al., 2013; SANTOS, 2018).

Este ensaio se caracteriza como um teste preliminar, de baixo custo, rápido e que não exigi técnicas assépticas. Vários estudos tentam correlacionar a toxicidade sobre *A. salina* com atividades antifúngica, antiviral, antimicrobiana, antiparasitária, tripanossomicida, inseticida e antitumoral (AMARANTE et al., 2011; POMPILHO et al., 2014; MEYER et al., 1982).

Identificar novas estratégias que reduzam a possibilidade de disseminação e evolução de quadros de arboviroses na população mundial é essencial. E para tanto, ratifica-se a carência de ampliação de atividades interdisciplinares que sejam efetivas contra o mosquito transmissor da dengue (MARSHALL, 2010).

Mesmo com os avanços recentes na procura por vacinas para essas doenças, somente para a febre amarela existe disponibilidade de uma vacina de duração prolongada, cerca de 10 anos (ROTHMAN, 2004). Dessa forma, o combate ao vetor urbano continua sendo a medida de controle utilizada para se evitar essas arboviroses. Como os mosquitos adultos habitam em sítios de difícil acesso, o método de controle ideal é a eliminação de suas larvas. (COLLER, 2011). Dentre as técnicas utilizadas para o controle das larvas estão o emprego de peixes larvófagos das espécies *Gambusia afinis* e *Poecilia spp,* além do bioinseticida *Bacillus thuringiensis* H-14 e os inseticidas químicos das classes dos piretróides, carbamatos e organofosforados (LEFEVRE, 2003).

Todavia, com o uso continuado e prolongado de inseticidas sintéticos, tem motivado a seleção de indivíduos resistentes e a perda da eficiência, pois ao se reproduzirem, eles propagam os genes responsáveis pela resistência aos seus descendentes, e pouco a pouco, a população se torna mais tolerante e menos sensível ao produto. Além disso, outros efeitos indesejados, como a toxicidade aos seres humanos e a outros organismos não-alvo e também poluição ambiental (NICOLAU, 2013).

No combate dessa população resistente, há a necessidade da busca de novos compostos para a realização do manejo de inseticidas. Com isso, as plantas podem ser uma possibilidade, pois além de serem organismos que coevoluem com outros microrganismos e insetos, elas também são fontes naturais de substâncias inseticidas e antimicrobianas, visto que precisam se defender de um ataque patogênico (bactéria, fungos

e vírus) e de um ataque herbívoro. Diversos compostos voláteis (ácidos, aldeídos e terpenos) possuem grande importância por serem considerados compostos altamente bioativos, também denominados fitoalexinos (GRAYER, 2001).

A toxicidade de uma substância química para insetos não a qualifica necessariamente como praguicida. Para tal, devem estar associadas várias propriedades a esta atividade, como: eficácia em pequenas concentrações, baixa toxicidade para mamíferos e animais superiores, ausência de fitotoxicidade e biodegradabilidade. Estas propriedades são consideradas as ideais para inseticidas, porém dificilmente será encontrado um produto que agregue todas estas propriedades (NATAH; KUMAR, 1999; GARCEZ et al., 2013).

Nessa perspectiva, esse estudo possui como objetivo avaliar a atividade química e biológica frente as larvas de *A. aegypti* dos óleos essenciais das espécies *O. majorana* L. e *O. vulgare* L., buscando produzir informações essenciais para contribuir com o aumento da efetividade de ações de controle químico e prevenção da dengue. Aliado a essa aplicação, foi obtido tanto o perfil cromatográfico dos óleos essenciais de ambas as espécies, quanto o perfil fitoquímico do extrato de *O. majorana* L. Além disso, foi analisada a atividade antibacteriana, de citotóxicidade e potencial antioxidante tanto dos óleos quanto do extrato, a fim de avaliar o mecanismo de ação dos componentes orgânicos. Observa-se uma escassez de informações químicas e biológicas associadas à *O. majorana* L. e *O. vulgare* L. Estudos podem incentivar a utilização da espécie e de outras plantas medicinais da biodiversidade vegetal da floresta amazônica, tornando-se uma alternativa para países em desenvolvimento, como o Brasil (FONSECA; CUNHA, 2013).

2 OBJETIVOS

2.1 OBJETIVO GERAL

- Avaliar a atividade larvicida do extrato bruto etanólico e dos óleos essenciais obtidos das folhas de *Origanum vulgare* L. e *Origanum majorana* L. frente as larvas do terceiro estádio (L3) de *Aedes aegypti*.

2.2 OBJETIVOS ESPECÍFICOS

- Identificar a composição química dos óleos essenciais (OEs) das espécies vegetais em estudo por Cromatografia Gasosa Acoplada ao Espectrômetro de Massa (CG-EM);
- Identificar os grupos orgânicos presentes no Extrato Bruto Etanólico (EBE) obtido das folhas de *O. majorana* L. através de prospecção fitoquímica;
- Avaliar a atividade larvicida do EBE e dos OEs frente as larvas (L3) de *A. aegypti*;
- Analisar a atividade antimicrobiana do EBE e dos OEs contra as bactérias *Pseudomonas aeruginosa* (ATCC 25922), *Escherichia Coli* (8789) e *Staphylococcus aureus* (ATCC 25922) determinando Concentração Inibitória Mínima e Concentração Bactericida Mínima;
- Analisar o potencial antioxidante do EBE e OEs pelo método de sequestro do radical DPPH;
- Analisar a toxicidade do EBE e dos OEs em meio salino frente *Artemia salina* Leach.

CAPÍTULO 1

Análise da Composição Química por CG-EM, Atividade Larvicida, Antimicrobiana, Citotóxica e Potencial Antioxidante dos Óleos Essenciais Obtidos das Folhas de *Origanum Vulgare* L. e Origanum majorana L.

4. 1 ARTIGO 1

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Evaluation of the Larvicidal Activity of the essential oil obtained from *Origanum vulgare* L. Leaves in the Control of *Aedes aegypti* Larvae

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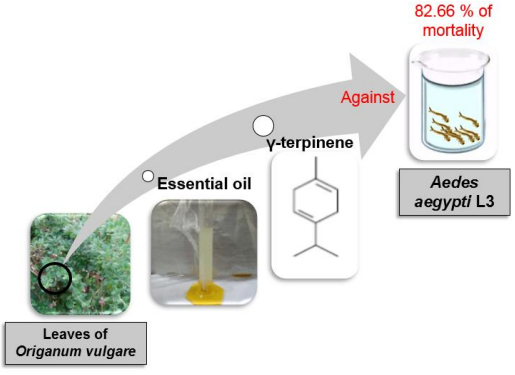
Abstract: This study evaluated the chemical composition of *Origanum vulgare* L. essential oil, its larvicidal, antimicrobial, cytotoxic activity and its potential antioxidant. The larvicidal activity was evaluated against *Aedes aegypti* larvae. Antioxidant the potential was evaluated from DPPH, cytotoxic activity was evaluated against Artemia salina and the antimicrobial test was against bacteria *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*. The main chemical components were γ-terpinene (27.18%), carvacrol (24.26%), thymol (6.67%), o-cymene (5.80%), p-cymene (5.53%), thymol, methyl ether, myrcene (3.94%) and δ-2-carene (3.49%). The results showed the larvicide activity presented a mean lethal concentration 74.63 μg.mL^{-1} and 20.27 μg.mL^{-1} at 24 and 48 hours, respectively. In the antimicrobial activity, the bacteria *P. aeruginosa* and *E. coli* presented minimal inhibitory concentration 31.25 μg.mL^{-1} when compared to the *S. aureus* bacterium with 500 μg.mL^{-1}. The minimum bactericidal concentration presented 250 μg.mL^{-1}, 500 μg.mL^{-1} for *P. auruginosa* and *S. aureus*, respectively. For *E. coli* presented 1000 μg.mL^{-1}. There was no significant antioxidant activity, but it had high cytotoxic activity against *A. salina* with mean lethal concentration 38.11 μg.mL^{-1}. Thus, it is observed that EO of *O. vulgare* L. caused toxic activity against larvae of *A. aegypti*, the bacterial gram- negatives and *A. salina.*

Keywords: dengue; lamiaceae; oregano; γ-terpinene; *artemia saline*

Graphical abstract



1. Introduction

Recently published data show an average 78% reduction of insects during the last 24 years [1]. This decline is mainly attributed to the extensive use of pesticides, applied both for agricultural and public health purposes [1]. The impacts of insect decline can be improved by climate change [2] and globalization that facilitates the migration of species [3].

Chemical insecticides are still widely used, but because of the resistance of *A. aegypti* populations to the use of these insecticides, government agencies have been trying to promote their substitution over the years. The vector control programs have used different strategies that are able to reduce the density of vector populations and, consequently, reduce the proliferation of diseases with the least possible impact on the environment [4].

A. aegypti is the vector responsible for the transmission of dengue fever, yellow fever, zika virus and chikungunya fever, Japanese encephalitis, and filariasis [5], leading to hundreds of deaths per year worldwide. It is estimated that it causes around 50 million symptomatic infections per year, thus being a worldwide public health problem [5].

It is a mosquito of domestic and diurnal habits, that proliferates in areas of greater population density, its development passes through four phases: egg, larva, pupa, and adult mosquito. Only the female bites the humans, because it needs blood to mature the eggs, and it is precisely through the bite of the female that the transmission of related diseases occurs [7].

There are several larvicides being studied for the control of the *A. aegypti* mosquito. Among them, the bacterial origin Garcez et al. [8], fungi Gomes et al. [9], EO Ferreira et al. [10], plant extracts Porto et al. [11] and seaweed Salvador et al. [12].

In addition to a growing concern about the use of synthetic chemicals used to prevent the growth of pathogenic microorganisms, especially in food products, there is a growing demand for products that use natural antioxidants, mainly bioactive substances extracted from plants [13-14-15].

Essential oils extracted from plants have antimicrobial activity against different microorganisms, most of them considered as an alternative to food preservation. [16].

The Botanical Family Lamiaceae, is composed of several species of plants with economic and medicinal interest. This Family, currently contains around 258 genera and 7,193 species. In Brazil, the presence of 23 genera and 232 native species occurs on average. They are cosmopolitan, originating in the Mediterranean regions, the Middle East and the subtropical mountains [37].

The *O. vulgare* L. is a plant that belongs to the family Lamiaceae Oliveira et al. [18], popularly known as "oregano", it is an aromatic herb native to Europe and regions of the Mediterranean, used as condiments [19]. The major chemical components of oregano essential oil are phenolic compounds, with carvacrol being the main compound, which is related to the antimicrobial activity attributed to oil [20].

In popular medicine Mitropoulou et al. [21] *O. vulgare* L. is considered an excellent substitute for cooking salt and can be used as a homemade seasoning, thus preventing chronic and cardiovascular diseases [22].

Much of the antioxidant activity associated with Lamiaceae family plants is due to the presence of phenolic compounds [23]. Aromatic herbs are also known as scented herbs, which have aspects associated with the constituents, since they have different flavors and aromas [24].

In the literature, there are few reports on the larvicidal activity against *A. aegypti* and on *A. salina* cytotoxicity, while some studies have been reported on the antioxidant and antimicrobial activity of the essential oils of this species.

Thus, the objective of this study was to evaluate the larvicidal activity against *A. aegypti* larvae, to identify the chemical composition, to evaluate the antimicrobial, antioxidant and cytotoxicity activity of the essential oil obtained from *O. vulgare* L. leaves.

2. Results

2.1. Identification of Chemical Compounds by GC-MS of O. vulgare L. Essential Oil

The chemical composition was determined by GC-MS, where the chromatogram can be observed in Figure 1. The chemical constituents identified 51.61% are hydrothermal monoterpenes, 36.22% are oxygenated monoterpenes and 2.99% are sesquiterpenes (Table 1). The major compounds of the oil are γ-terpinene

(27.18%) and carvacrol (24.26%), followed by the other main components thymol (6.67%), *o*-cymene (5.80%), *p*-cymene (5.53), thymol, methyl ether (4.06%), myrcen (3.94%), δ-2-carene (3.49%).

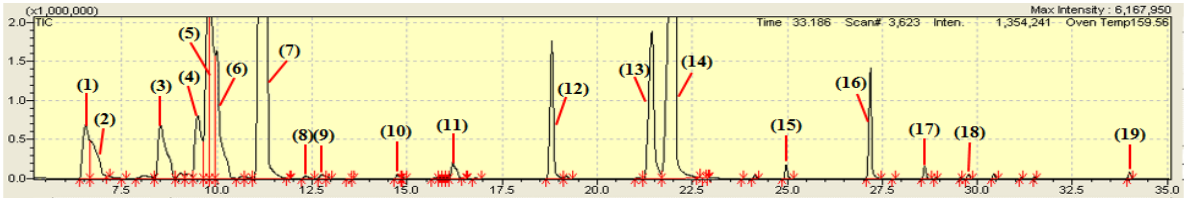


Figure 1. GC-MS chromatogram of *O. vulgare* L. essential oil. Notes: Gas drag: Helium (He); initial temperature 60 °C; initial time 1.0 min; the column temperature increased 3 °C/min. at 240 °C, maintained at this temperature for 30 min.

Table 1. Chemical composition of *O. vulgare* L. essential oil.

Nº	RI	KI	Compounds	Relative Percentage (%)	Identification*
1	6.658	930	α-thujene	2.74	MS, KI
2	8.091	939	α-pinene	2.82	MS KI
3	8.515	990	myrcene	3.94	MS, KI
4	9.500	1002	δ-2-carene	3.49	MS, KI
5	9.768	1026	*o*-cymene	5.80	MS, KI
6	9.991	1024	*p*-cymene	5.53	MS, KI
7	**11.195**	**1059**	**γ-terpinene**	**27.18**	**MS, KI**
8	12.339	1017	α-terpinene	0.11	MS, KI
9	12.766	1257	linalool acetate	0.16	MS, KI
10	14.732	1083	artemisia alcohol	0.11	MS, KI
11	16.202	1177	terpinen-4-ol	0.69	MS, KI
12	18.806	123 5	thymol, methyl ether	4.06	MS, KI
13	21.439	1290	thymol	6.67	MS, KI
14	**22.068**	**1298**	**carvacrol**	**24.26**	**MS, KI**
15	24.973	1372	carvacrol acetate	0.27	MS, KI
16	27.187	1419	Z-caryophyllene	2.50	MS, KI
17	28.615	1454	α-humulene	0.27	MS, KI
18	29.767	1481	germacrene D	0.09	MS, KI
19	34.001	1583	caryophyllene oxide	0.13	MS, KI
			monoterpenes hydrocarbon	51.61	
			monoterpenes oxide	36.22	
			sesquiterpenes	2.99	
			Total	90.82	

RI: retention indice. *Identification pathway of compounds, #to identification of compounds was performed by the mass spectrum (MS) of the Labsolutions GC-MS solution software version 2.50 SU1 (NIST05 and WILEY'S libraries of the 9th edition mass spectrum) and Kovats Indice (KI) [26].

2.2. Larvicidal activity

Table 2 shows the percentage mortality of *A. aegypti* larvae at different concentrations of *O. vulgare* L. EO in the 24 and 48 h exposure period. There was no mortality in the positive control groups.

The Probit test showed an LC_{50} of 74.63 μg.mL^{-1}, determination coefficient (R^2) of 0.974 and a *chi-square* quantitative evaluation of 0.125 in 24 h. After 48 h, LC_{50} of 20.27 μg.mL^{-1}, R^2 of 0.937 and *chi-square* of 0.170 in 48 h.

Table 2. Percentage mortality (%) of *A. aegypti* larvae produced by different concentrations of *O. vulgare* L. essential oil at different periods (24 and 48 h).

Concentrations (µg.mL^{-1})	24 h	48 h
Positive control	0	0
20	18.66*	52.66*
40	34.66*	60*
60	40*	69.33*
80	50.66*	76*
100	61.33*	82.66*

Source: study data; *deferential letters indicate a significant difference p <0.001; *chi-square* of 0.125 in 24 h*; chi-square* of 0.170 in 48 h.

2.3. Antimicrobial activity

Figure 2 shows the Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) found for *O. vulgare* L. EO. The results show that the gram-negative bacteria showed MIC = 31.25 µg.mL^{-1} compared to the gram-positive bacterium that presented MIC = 500 µg.mL^{-1}, being the most resistant to the negative control. In relation to CBM, the concentrations of the test solution for *P. aeruginosa* and *S. aureus* were equal to 250 µg.mL^{-1} and 500 µg.mL^{-1} respectively. While for *E. coli* the concentration was equal to 1000 µg.mL^{-1}, being the most resistant in relation to the positive control.

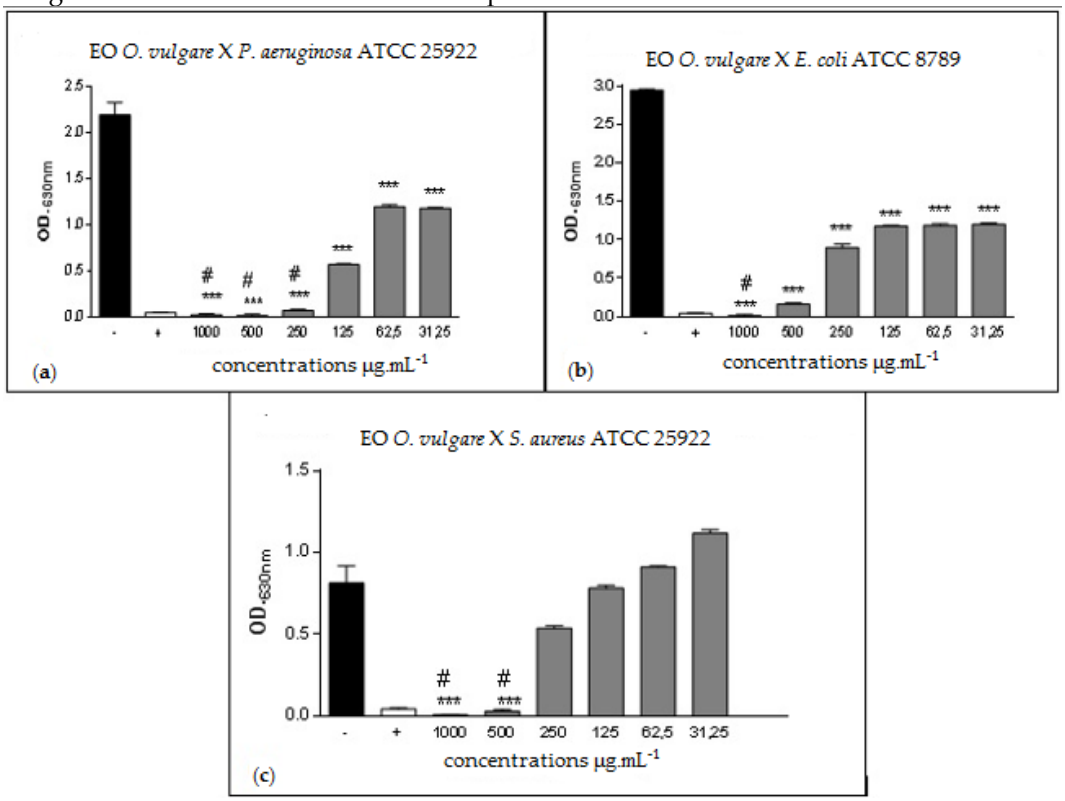


Figure 2. This is figure of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *O. vulgare* L.essential oil; (**a**) EO *Origanum vulgare* L. x *P. aeruginosa*; (**b**) EO *Origanum vulgare* L. x *E. coli*; (**c**) EO *Origanum vulgare* L. x *S. aureus*. Test substance of EO *O. vulgare* L. ( ); BHI with 2% DMSO ( ); Amoxilin ( ); *P <0.001 statistically significant in relation to the negative control; #p <0.001 statistically significant in relation to the positive control.

2.4. Antioxidant activity

The percentage antioxidant activity (%) is expressed in Table 3 and shows that *O. vulgare* L. EO presented a high IC_{50} = 541.16 µg.mL^{-1}. The percentage of antioxidant activity of ascorbic acid showed concentrations with IC_{50} = 16.71 µg.mL^{-1} (Table 4). The coefficient (R^2) = 0.9273 was determined.

Table 3. Mean and standard deviation of the percentage of antioxidant activity of *O. vulgare* L. essential oil in different concentrations.

Concentrations (µg.mL^{-1})	% AA
7.81	13.77± 0.68^{a}

15.62	16.58± 0.57[b]
31.25	17.34±0.99[c]
62.5	17.95±0.14[c]
125	19.95±0.28[d]
250	30.79±0.42[e]

Source: study data; different letters indicate that there was significant difference by the Tukey test (p≤0.05). Equation of the antioxidant activity Y = 0.0661x + 14.2292.

Table 4. Mean and standard deviation of the percentage of antioxidant activity of ascorbic acid at different concentrations.

Concentrations (μg.mL^{-1})	% AA
7.81	18.57± 0.52[a]
15.62	30± 0.10
31.25	99.93±0.02[c]
62.5	99.99±0.0[c]
125	99.99±0.0[d]
250	99.99±0.0[e]

Source: study data; different letters indicate that there was significant difference by the Tukey test (p≤0.05).

2.5. Cytotoxic activity

The percent cytotoxic activity of *O. vulgare* L. EO is shown in Table 5 and is expressed as the mean 24 h mortality readings. The concentrations of the oil presented LC_{50} = 38.11 μg.mL^{-1}, coefficient of determination = 0.883 and *chi-square* = 0.139.

Table 5. Mean and standard deviation of percentage of cytotoxic activity of *O. vulgare* L. essential oil at different concentrations again *A. salina*.

Concentrations (μg.mL^{-1})	% Mortality
Negative control	0±0.00a
50	50±0>00b
100	66.66±0.00c
250	75±0.00d
500	80±0.00e
750	83.33±0.00f
1000	85.71±0.00g

Source: study data; Values correspond to the mean and standard duration of triplicates; deferential letters indicate a significant difference (p <0.05).

3. Discussion

The chemical composition of EO obtained from leaves of *O. vulgare* L. corroborates the results obtained by Vieira [34] who reported that carvacrol (67.67%), γ-terpinene (7.45%), *o*-cymene (11.60%), linalool (2.42%), β-myrene (1.45%), trans-caryophyllene (1.30%), α-pinene (1.52%) are the main constituents of *O. vulgare* L. EO.

In the study by Vazirian et al. [35] *O. vulgare* L. EO presented 0.5% yield, a complex mixture of oxygenated monoterpenes (59.25%), monoterpene hydrocarbons (18.71%), with a small amount of oxygenated sesquiterpenes (12.86%) and sesquiterpene hydrocarbons (5.02%). In the identification of 37 components, thymol (37.13%), γ-terpinene (9.67%), carvacrol (9.57%), carvacrol methyl ether (6.88%), *cis*-bisabolene (6.80%), eucalyptol, *p*-cymene (3.58%), and elemol (2.04%) as predominant components.

The major compounds do not differ significantly between cultivation systems and types of fertilization, and crops in both the field and greenhouse favor the production of the major constituents of essential oil, however the presence of solar radiation in the field provides a greater difference of the major compounds in relation to the greenhouse [36].

The larvicidal activity observed in *O. vulgare* L. EO can be explained by the chemical composition of this oil and the action or effect of most of the compounds [37-38] since there was no mortality in the positive control.

According to Cheng et al. [39] and Pavela [40], essential oils with LC_{50} <100 ppm have received much attention as bioactive compounds potentially useful against insects showing a broad spectrum of activity against insect pests, low toxicity in mammals and rapid degradation in the environment.

In the study by Leite et al. [41] *O. vulgare* L. essential oil was evaluated against the A. aegypti mosquito and exhibited 100% inhibition with LC_{50} of 14.91 μg.mL $^{-1}$, whereas the isolated α-pinene compound had LC_{50} of 9.59 μg.mL^{-1}.

The toxic potential of essential oils against *A. aegypti* may change according to intrinsic and extrinsic factors in different plant species, plant parts, geographical conditions, season of occurrence, precipitation, percentage of humidity, temperature, sunlight, larvae, and the tests used, in general, with the purpose of inducing various larval responses [42].

Kiran et al. [43] showed that germacrene D isolated from *Chloroxylon swietenia* DC showed an LC_{50} value of 63.6 mg/mL when tested against larvae of the fourth instar of *A. aegypti*. LC_{50} value of 59.5 mg/mL when tested against larvae of the fourth instar of *A. Stephensi* in 24 h. Tiwary et al. [44] (LC_{50} = 49 ppm), *A. aegypti* (LC_{50} = 54 ppm), and *A. stephensi* (LC_{50} = 58 ppm) were observed in the essential oil of *Zanthoxylum armatum* against different mosquito species, including *C. quinquefasciatus* ppm).

Recently Govindarajan et al. [45] identified by GC-MS the essential oil of *O. vulgare* L. exhibiting larvicidal activity against the mosquitoes *Anopheles stepheensi*, *Anopheles subpictus*, *Culex quinquefasciatu*, and *Culex tritaeniorhynchus* with LC_{50} values of 67.00, 74.14, 80.35 and 84.93 μg.mL^{-1}, respectively.

According to Giatropoulos et al. [46], the high toxicity of its main components thymol, carvacrol, *p*-cymene, and γ-terpinene provided promising results on repellent activity against this mosquito, exhibiting high repellency due to the presence of carvacrol and thymol components.

In addition, Govidarajan et al. [45] extracted the major components of carvacrol (38.30%) and terpinen-4-ol (28.70%) from the essential oil of *O. vulgare* L., both exhibiting high toxicity against the larvae of four mosquitoes. The LC_{50} being 21.15 and 43.27 μg.mL^{-1} for *A. stephensi* CL_{50} 24.06 and 47.73 μg.mL^{-1} for *A. subpictus*, LC_{50} 26.08 and 52.19 μg.mL^{-1} for *C. quinquefasciatus* and LC_{50} 27.95 and 54.87 μg.mL^{-1} for *C. tritaeniorhynchus* , respectively.

The presence of 4-terpineol may have an important influence on the activity observed since it is terpenic alcohol. It presents polar hydroxyls capable of making hydrogen bonds and can act to induce deformations in cell membranes, thus altering its permeability. In the case of the larvae, it was observed that the larval activity of the larvae was higher than that of the larvae [47-48-49].

Although to date, major substances have been found to be responsible, in addition to the synergism of multiple substances that may actually be very important in terms of biological activity, including substances in small quantities [50].

Regarding antimicrobial activity, *O. vulgare* L. EO exhibited a strong bacteriostatic effect (MIC) in all bacteria. On the bactericidal effect (MBC), the bacterium *P. aeruginosa* and *S. aureus* had a higher toxic effect. Whereas *E. coli* showed to be more resistant to the oil, which, according to Aust [51] classifies for essential oils the determination values of MIC up to 500 μg.mL^{-1} as strong, between 600 and 1500 μg.mL^{-1} moderately and above 1500 μg.mL^{-1} is weak.

In addition, Londeiro and Carrion [52] classifies as bactericidal a product when it causes the death of bacteria through mechanisms with irreversible inhibition of DNA replication, whereas bacteriostats are those that inhibit the growth of the bacteria in the medium.

Studies suggest that gram-positive bacteria are more susceptible to essential oils than gram-negative bacteria [53-54]. In the result reported by Guterres [55] the concentrations of *O. vulgare* L. essential oil varying between 0.4 and 250 μg.mL^{-1} showed on the bacterium *P. aeruginosa* inhibitory effect with MIC and CBM equivalent of 250 μg.mL^{-1}, *E. coli* had MIC of 0.9 μg.mL^{-1} and CBM 1.9 μg.mL^{-1}, whereas *S. aureus* showed susceptibility with MIC and CBM of 3.9 μg.mL^{-1}.

Essential oils and their constituents, such as terpenoids, carvacrol, and thymol, do not have a mechanism of action against the bacteria well understood by Simões et al. [56] and Marques et al. [57]. However, this may occur because the cell wall of the gram-positive bacterium, that consists of peptidoglycan (90-95%) along with teicoic acid and proteins bound to it and as the major components of the EOs are hydrophobic, they interact with the cell membrane and thus pass easily through the cytoplasm [58].

Recently, Lima et al. [59] evaluated the antibacterial activity of phytochemicals carvacrol and thymol against *E. coli* bacteria with concentrations ranging from 1 to 2,048 μg.mL^{-1}. It showed as results for MIC of carvacrol concentrations of 64 μg.mL^{-1} for the strain 65 and 128 μg.mL^{-1} for the C-18, C-20, C-21, C-24, C-25.

And C-24 strains, and for thymol, MIC was at concentrations of 1.024 μg.mL^{-1} for strain C-25, 512 μg.mL^{-1} for strains C-18, C-21, 24 and C-65; 256 μg.mL^{-1} for the C-24 strain and 128 μg.mL^{-1} for the C-20 strain.

The presence of terpenes has been shown to have antimicrobial activity [60-61]. According to Badia [58] although the cell wall of the gram-negative bacteria has a more complex structure, formed by proteins and lipopolysaccharides and hydrophilic. Lima et al. [59] explain that the biological action of both phytochemicals can be attributed to the induction in the cell membrane deformations that both cause, altering its permeability.

The synergism, which is the increase in the activity of compounds when applied together, is shown as an alternative to potentiate the antimicrobial action of these substances. The essential oils, when used in association with this, have a greater effect against the microorganism, thus achieving results that are more effective and more promising than when tested in isolation [62-63-64].

This difference in antimicrobial activity between the different studies may be related to the bacterial strains used, since strains of the same species may present different resistance [65-66].

O. vulgare L. EO was subjected to antioxidant action screening by the elimination of the DPPH radical and total phenolic content (lower than ascorbic acid) (Table 3 and 4). The results shown in Table 3 show that the oil concentrations did not provide a 50% reduction because it did not exhibit significant radical scavenging activity, presenting high LC_{50} = 541.16 μg.mL^{-1}. According to Nascimento et al. [67] the higher the DPPH consumption in a sample, the lower the LC_{50} and the higher the antioxidant activity.

The DPPH consists of the reduction of the 2.2-diphenyl-1-picrylhydrazyl radical that absorbs at 515 nm and when receiving an electron or a hydrogen radical is stable and with the disappearance of the absorption that can be evaluated by the decrease of the absorbance [68-69].

The results of this study corroborate with the results presented by Souza [70] because *O. vulgare* L. oil also presented low antioxidant activity with IC_{50} of 106.10 μg.mL^{-1}. The results found to differ from previous studies reporting high antioxidant activity for EO of oregano, which may have occurred due to the alteration in the chemical composition, resulting from cultivation, processing or storage [70].

However, Vazirian et al. [35] observed IC_{50} = 2.5 μg mL^{-1} for EO of oregano, the antioxidant activity presented by EO was higher than that of the standard antioxidants used (Vitamin E and butylhydroian anolol). Souza [69] reports that high antioxidant activity may be associated with the concentration of phenolic compounds in the oil. Henn et al. [71] observed an IC_{50} of 174.17 μg.mL^{-1}, classifying EO of oregano as a potent antioxidant against the inhibition of fatty acid oxidation.

Recently Hajlaoui et al. [72] attributed the low antioxidant activity of essential oils of the *Origanum* species to its result due to the weak presence of the main component terpinen-4-ol. Given that it corroborates with the chemical composition of the species of this study, with terpinen-4-ol representing only 0.64% of the essential oil.

In the present study, it is possible to evaluate the effect of anaerobic bacteria on *A. salina* L. (Brine shrimp), a microcrack of the order Anostraca used as a bioindicator of toxicity and has the advantage of simplicity of handling, rapidity of tests and low cost [73-74]. This assay allows the evaluation of toxic effects, is considered essential as a bioassay in the study of compounds with potential biological activity [75-76].

Interpreting the results of Table 5, all concentrations of *O. vulgare* L. essential oil against *A. salina* presented a high toxic effect, due to the LC_{50} for each one of the concentrations were> 50 μg.mL^{-1}. The event that Silva [77] classifies that cytotoxicity is elevated with LC_{50} between 0 and 100 μg.mL^{-1}, moderate with LC_{50} between 100 and 500 μg.mL^{-1}, is considered weak if the LC_{50} is between 500 and 1000 μg.mL^{-1}, and has no toxicity when the LC_{50} is greater than 1000 μg.mL^{-1}.

Few preliminary studies of *A. salina* were found for *O. vulgare* L.essential oil. In a study carried out by Leite et al. [41], the essential oil of *O. vulgare* L. and the isolated α-pinene presented bioactivity against *A. salina* with values of LC_{50} between 9.59 and 253.43 μg.mL^{-1} respectively, since this result can be explained by the richness of this oil in phenolic compounds such as carvacrol and thymol [72].

In contrast to the Begnini et al. [78] the high cytotoxicity effect of *O. vulgare* L. essential oil at 50 μg.mL^{-1} concentration in the HT-29 cell line was reported due to the presence of the 4-terpineol component, inducing the inhibition of the growth of cancer cells.

Aydin, Seker [79] and Arunasree [80] observed that the cytotoxicity effect of *O. vulgare* L. essential oil is due to the action of its main phenolic components, such as carvacrol and thymol, as they exhibit anticancer activity and significant antimutagenicity when assayed.

4. Materials and methods

4.1. Plant material

The leaves of O. vulgare L. were collected in Fazendinha district (00 "36'955" S and 51 "11'03'77" W) in the Municipality of Macapá, Amapá. Five samples of the plant species were deposited in the Amapá Herbarium (HAMAB) of the Institute of Scientific Research and Technology of the State of Amapá (IEPA).

4.2. Obtaining the essential oil

O. vulgare L. essential oil (EO) was obtained by hydrodistillation using the Clevenger type apparatus for a period of 2 h. [25]. EO was kept under refrigeration (4 °C).

4.3. Identification of the chemical composition by gas chromatography coupled to mass spectrometer (GC-MS)

The EO analysis was performed by Gas Chromatography coupled to the Mass Spectrometer (GC-MS) of the Museu Paraense Emílio Goeldi. The Shimadzu equipment, GCMS-SHIMADZU QP 5000, was used. A molten silica capillary column (OPTIMA®-5-0.25 μm), 30 m long and 0.25 mm in internal diameter and nitrogen as carrier gas, was used. The operating conditions of the gas chromatograph were: internal column pressure 67.5 kPa, division ratio 1:20, gas flow at column 1.2 mL/min (210 °C), injector temperature 260 ° C, temperature detector or interface (CG-MS) of 280 °C. The initial column temperature was 50 ºC, followed by an increase from 6 ºC/min to 260 ºC kept constant for 30 min. The mass spectrometer was programmed to perform readings at intervals of 29-400 Da, 0.5 s with ionization energy of 70 eV.

The identification of the chemical compounds present in EO was made from the comparisons of the Retention (RI) and Kovats (KI) indices of the homologous series of n-alkanes (C_8-C_{26}) and the literature [26]. In addition to the identification made by combining the spectra obtained by the analysis performed in the Labsolutions GC-MS version 2.50 SU1e software equipment of the mass spectra of the NIST05 and WILEY'S libraries.

4.4. Larvicidal activity against A. aegypti

The larvae of *A. aegypti* (Rockefeller strain) used in the bioassay came from the colony maintained at the Institute of Scientific and Technological Research (IEPA). The methodology used followed [27] with adaptations.

The procedure started with the separation of 18 beakers of 50 mL and in each becker were added 25 larvae of the third instar of *A. aegypti*. Then they were reserved in a room with conditions of ambient temperature between 25 to 30ºC and photoperiod of 12 h.

Preparation of the samples started after 24 h. The stock solution was prepared with 4.5 mL of 5% Tween 80, 85.5 mL of distilled water and 0.09 g of the *O. vulgare* L. EO sample. The positive control was prepared with 17.5 mL of Tween 80 dissolved in 350 mL of distilled water.

In the bioassay, 18 beakers of 100 mL of glass were organized into six groups. The mother solution was distributed as follows: in group 1 it was added 10 mL, in group II it was added 8 mL, in the beakers of group III it was added 6 mL, in those of group IV it was added 4 mL and in the group There was added 2 mL. In group VI it was added 80 mL of positive control solution.

Then, 80% distilled water from the total volume plus 25 larvae of *A. aegypti* were added to each beaker. Group, I had a total of 100 $\mu g.mL^{-1}$, the II group with 80 $\mu g.mL^{-1}$, the III group with 60 $\mu g.mL^{-1}$, the IV group with 40 $\mu g.mL^{-1}$ and the V group with a total of 20 $\mu g.mL^{-1}$ of test solution. After 24 and 48 h, the number of larvae mortality was counted, being considered as dead all those unable to reach the surface.

4.4.1. Larvicidal Statistical Analysis

The experiment was carried out in triplicate. The larval mortality efficiency data were calculated in percentages using the Abbott formula and later tabulated in Microsoft Excel (Version 2013 for Windows). LC_{50} (lethal concentration causing 50% mortality in the population) was analyzed by the IBM SPSS® program [version 21.0; SPSS Inc., Chicago, IL, USA]. The results were shown in the table 2 and the differences that presented probability levels $p \leq 0.001$ were considered statistically significant.

4.5. Antimicrobial Activity

4.5.1. Microorganisms

The antimicrobial EO test obtained from *O. vulgare* L. leaves was tested in vitro against two gram-negative bacteria (*P. aeruginosa* ATCC 25922 and *E. coli* ATCC 8789) and a gram-positive bacterium (*S. aureus* ATCC 25922).

For each microorganism, a stock culture was set in BHI medium (Brain Heart infusion) with 20% glycerol and stored at -80 °C. An aliquot of 50 μL of this culture was inoculated into 5 mL of sterile BHI broth medium and incubated for 24 h at 37 °C.

4.5.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC were determined using the microplate dilution technique (96 wells) according to the protocol established by CLSI [28], with adaptations.

Bacteria were initially reactivated from the stock cultures, kept in BHI broth, for 18 h at 37 °C. Subsequently, bacterial growth was prepared in 0.9% saline inoculum for each microorganism, adjusted to the McFarland 0.5 scale, then diluted in BHI and tested at 2 x 10^6 UFC.mL^{-1} concentration.

In determining MIC, EO was diluted with Dimethyl sulfoxide (2% DMSO). Each well of the plate was initially filled with 0.1 mL of 0.9% NaCl, except for the first column, which was filled with 0.2 mL of EO at the concentration of 2000 μg.mL^{-1}. 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. After this process, 0.1 mL of cells (2 x 106 CFU mL^{-1}) added in each well related to the second preceding item, resulting in a final volume of 0.2 mL. Control of the culture medium, EO control, negative control (DMSO 2%) and amoxicillin (50 μg.mL^{-1}) was used for positive control. After incubation of the microplates in an incubator at 37ºC for 24 hours, the plates were read in ELISA reader (DO 630nm).

The determination of MBC was performed 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. CBM was established as the lowest concentration of EO capable of completely inhibiting microbial growth in Petri dishes after 24-48 h of growth.

4.5.3. Statistical analysis of microbiological assays

All experiments were performed in triplicate with the respective results categorized in Microsoft Excel (Version 2013 for Windows) and later 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. The data were considered statistically significant when $p < 0.001$.

4.6. Antioxidant activity

The antioxidant quantitative test was based on the methodology recommended by Andrade et al. [29], Lopes et al. [30] and Sousa et al. [31] by the use of 2.2-diphenyl-1-picryl- hydrazine (DPPH) with adaptations.

A methanolic solution of DPPH (stock solution) was prepared at the concentration of 40 μg.mL^{-1}, which was kept under the light. The EOs were diluted in methanol at concentrations 5, 2.5, 1.0, 0.75, 0.50 and 0.25 μg.mL^{-1}. For the evaluation of the test, 0.3 mL of the oil solution was added to a test tube, followed by the addition of 2.7 mL of the DPPH solution. The blank was prepared from a mixture with 2.7 mL of methanol and 0.3 mL of the methanol solution of each EO concentration evaluated. After 30 min. the readings were performed on a spectrophotometer (Biospectro SP-22) at a wavelength of 517 nm. The test was performed in triplicate and the calculation of the percentage of antioxidant activity (%AA) was calculated with the following equation 1:

$$(\%AA) = 100 - \left\{\frac{[(Abs_{sample} - Abs_{white}).100]}{Abs_{control}}\right\} \qquad (1)$$

AA% - Percentage of antioxidant activity

Abs_{sample} – Sample Absorbance

Abs_{white}- White Absorbance

$Abs_{control}$- Control Absorbance

4.7. Cytotoxic Activity Front Artemia salina Leach

The *A. salina* cytotoxicity assay was based on the technique of Araújo et al. [32] and Lôbo et al. [33] with adaptations. An aqueous solution of artificial sea salt was prepared (35 gL^{-1}) at pH 9.0 for incubation of 45 mg of *A. salina* eggs, which were placed in the dark for 24 h for the larvae to hatch (nauplii), then the nauplii were exposed to artificial light in 24 h, period to reach the stage methanuplii. The stock solution was prepared to contain 0.06 g of EO, 1.5 mL of Tween 80 and 28.5 mL of saline to facilitate solubilization of the same. The test tubes were marked up to 5 mL.

The methanauplia were selected and divided into 7 groups of 10 subjects in each test tube. Each group received aliquots of the stock solution (2500, 1875, 1250, 625, 250, and 125 μL), which were then filled to a volume of 5 mL with the sea salt solution to produce final solutions with the following concentrations: 1000, 750, 500, 250, 100 and 50 $\mu g.mL^{-1}$. The tests were performed in triplicates. For control of the test, saline solution was used. After 24 hours, the number of dead was counted. The lethal concentration causing 50% mortality in the population (LC_{50}) was determined by Probit analysis using SPSS Software (version 20.0; SPSS Inc., Chicago, LI, USA). The data were considered statistically significant when $p<0.05$.

5. Conclusion

The EO obtained from the leaves of *O. vulgare* L. was evaluated against *A. aegypti* larvae and showed good potential against *A. aegypti* larvae with mortality from the concentration of 20 $\mu g.mL^{-1}$. The main components identified by GC-MS were γ-terpinene (27.18%), carvacrol (24.26%), thymol (6.67%), *o*-cymene (5.80%), *p*-cymene (5.53), thymol, methyl ether 4.06%), myrcene (3.94%), δ-2-carene (3.49%). The antimicrobial activity revealed that *O. vulgare* L. EO exhibited higher antimicrobial efficiency for *P. aeruginosa* and *S. aureus* bacteria than for *E. coli* bacteria, is more resistant to oil. EO did not present antioxidant activity by DPPH radical capture method. However, it showed high cytotoxic activity against *A. salina*. Thus, these results point to the importance of preliminary tests such as selection of the biological potential of herbal products, in order to enhance the importance of the study of new natural products with larvicidal activity for *A. aegypti* as a method that causes less impact to the environment.

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4.2 ARTIGO 2

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PLOS ONE

Evaluation of larvicidal potential against larvae of *Aedes aegypti* (Linnaeus, 1762) and of the antimicrobial activity of essential oil obtained from the leaves of *Origanum majorana* L.

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Abstract

This study evaluated the larvicidal activity of *Origanum majorana* Linnaeus essential oil, identified the chemical composition, evaluated the antimicrobial, cytotoxic and antioxidant potential. The larvicidal activity was evaluated against larvae of the third stage of *Aedes aegypti* Linaeus, whereas the chemical composition was identified by gas chromatography coupled to mass spectrometer, the antimicrobial activity was carried out against the bacteria *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus auereus*, the antioxidant activity was evaluated from of 2.2-diphenyl-1-picryl-hydrazila sequestration and *Artemia salina* Leach artcytotoxicity. Regarding to the results, the larvicidal activity showed that *O. majorana* L. essential oil caused high mortality in *A. aegypti* L. larvae. In the chromatographic analysis, the main component found in *O. majorana* L. essential oil was pulegone (57.05%), followed by the other components verbenone (16.92%), trans-p-menthan-2-one (8.57%), iso-menthone (5.58%), piperitone (2.83%), 3-octanol (2.35%) and isopulegol (1.47%). The antimicrobial activity showed that *E. coli* and *P. aeruginosa* bacteria were more sensitive to oil than *S. aureus*, which was resistant at all concentrations. Essential oil did not present antioxidant activity, but it has high cytotoxic activity against *A. salina L.*

Introduction

Dengue remains an important public health problem in Brazil, even after the introduction and recent dissemination of the Zika and chikungunya [1,2] viruses. The disease presents a great epidemic potential, affecting all regions of Brazil [3,4].

The *A. aegypti* mosquito (Linnaeus, 1762) is a vector of viruses that cause diseases known as dengue, chikungunya, and zika [5]. It has holometabolic development, with egg, larva, pupa and adult phases. Because it is a mosquito highly adapted to the urban environment, its most common breeding sites are artificial containers that accumulate water, such as bottles, tires, cans, and pots [6].

Among the control policies adopted in Brazil, the mechanical control is carried out by ACE (Agents to Combat Endemics), with the participation of the population, aiming at the protection, destruction or adequate allocation of potent breeding sites. The intensive collaboration of the population is crucial to hinder the proliferation and installation of the mosquito. In addition, it reinforces the need for adequate sanitary conditions in the cities, eliminating stocks of water that allow eggs to hatch. An important strategy is the promotion of educational actions during home visits made regularly by the health agents [7].

The spread and flow of various serotypes of the dengue virus over the years also have a significant influence on epidemics, as well as an increase in cases diagnosed for the most severe form of the disease. These factors demonstrate the importance of introducing preventive measures in order to reduce dengue rates [8].

Origanum majorana L. belongs to the Lamiaceae family, and it contains several terpenoids, which are isolated from aerial parts of the *Origanum* plant and exhibit antimicrobial, antiviral and antioxidant properties, without toxic effects [9,10]. In folk medicine, *O. majorana* L. is used for cramp, depression, migraine, nerve headaches [9].

The antimicrobial and antioxidant properties of many spices and their essential oils have been known for a long time, but only in recent years have consumers given proper attention to the use of these substances [11]. Because many plants are toxic to mosquitoes, the mixture of essential oils may represent an efficient outlet for this problem, compared to the *A. aegypti* mosquito [12].

In the literature, there are no reports on larvicidal activity against *A. aegypti* and cytotoxicity against *A. salina*, and few studies have been reported on the antioxidant and antimicrobial effects of the essential oils of this species.

Therefore, the objective of this study was to evaluate the larvicidal activity against *A. aegypti*, to determine the chemical composition, to evaluate the antimicrobial activity against E. coli, P. aeruginosa and S. aureus bacteria, to determine the antioxidant potential through the sequestration of DPPH and cytotoxicity against *A. salina* of *O. majorana* L. essential oil.

Materials and methods

Plant material

The leaves of *O. majorana* L. were collected in the district of Fazendinha (00 "36'955" S and 51 "11'03'77" W) in the Municipality of Macapá, Amapá. Five samples of the plant species were deposited at the Amapaense Herbarium (HAMAB) of the Institute of Scientific Research and Technology of Amapá (IEPA).

Essential oil obtaining

The essential oil (EO) was obtained by the hydrodistillation process using the Clevenger type apparatus, 131 g of *O. majorana* L. dried leaves were dried at 45 °C for a period of 2 h [13]. The EO was kept under refrigeration (4ºC).

Identification of the chemical composition by gas chromatography coupled to mass spectrometer (GC-MS)

The EO analysis was performed by Gas Chromatography coupled to the Mass Spectrometer (GC-MS) of the Museu Paraense Emílio Goeldi. The Shimadzu equipment, model GCMS-QP 5000 A was used. A fused silica capillary column (OPTIMA®-5-0.25 μm) was used. It has 30 m of length and 0.25 mm of internal diameter and nitrogen as carrier gas. The operating conditions of the gas chromatograph were: internal column pressure 67.5 kPa, division ratio 1:20, gas flow at column 1.2 $mL.min^{-1}$ (210 °C), injector temperature 260 °C, temperature detector or interface of 280 °C. The initial column temperature was 50 °C, followed by an increase from 6 $°C.min^{-1}$ to 260 °C kept constant for 30 min. The mass spectrometer was programmed to perform readings at intervals of 29-400 Da, at intervals of 0.5 s 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 the chemical compounds present in the EO was made from the comparisons of the Indices of Retention (IR) and Kovats (IK) of the homologous series of n-alkanes (C8-C26) and the literature [17]. Identification was also made by combining the spectra obtained by the analysis performed on the Lab solutions GC-MS version 2.50 Sigma – Aldrich, St. Louis, MO, USA e software equipment of the mass spectra of the NIST05 and WILEY'S libraries.

Larvicidal activity against *A. aegypti* larvae

The *A. aegypti* L. larvae used in the bioassay came from the colony (strain Rockfeller) kept in the Medical Entomology Laboratory of the Institute of Scientific and Technological Research of the

State of Amapá (IEPA). The methodology used followed the World Health Organization standard protocol [18] with adaptations.

The procedure started with the separation of 18 beakers of 50 mL and in each Becker, there were added 25 larvae of the third instar of *A. aegypti* L.. Then they were reserved in a room with conditions of ambient temperature between 25 to 30 ºC and photoperiod of 12 h.

Preparation of the samples started after 24 h. The stock solution was prepared with 4.5 mL of Tween 80, 85.5 mL of distilled water and 0.09 g of the EO sample of *O. majorana* L. The positive control was prepared with 17.5 mL of Tween 80 dissolved in 350 mL of distilled water, and the larvicidal esbiothrin as the positive control.

After the preliminary tests, the aqueous solution was diluted in the following concentrations: 100, 80, 60, 40, 20, 10, and 1 $\mu g.mL^{-1}$. Each concentration was tested in triplicate, and 25 larvae of the *A. aegypti* L. 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 ºC. After 24 and 48 h, the dead larvae were counted, being considered as such, all those unable to reach the surface.

Statistical analysis

The experiment was carried out in triplicate. The larval mortality efficiency data were calculated in percentages using the Abbott formula and later tabulated in Microsoft Excel (Version 2013 for Windows). LC_{50} (lethal concentration causing 50% mortality in the population) was analyzed by the IBM SPSS® program [version 21.0; SPSS Inc., Chicago, IL, USA]. The results were shown in the table. Differences that presented probability levels $p \leq 0.001$ were considered statistically significant.

Antimicrobial activity

Microorganisms

The antimicrobial EO test obtained from *O. majorana* L. leaves was tested in vitro against two gram-negative bacteria (*P. aeruginosa* ATCC 25922 and *E. coli* ATCC 8789) and a gram-positive bacterium (*S. aureus* ATCC 25922).

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

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

The MIC and MBC were determined using the microplate dilution technique (96 wells) according to the protocol established by Clinical and Laboratory Standards Institute [19], with adaptations.

Bacteria were initially reactivated from the stock cultures, kept in BHI broth, for 18 h at 37 °C. Subsequently, bacterial growth was prepared in 0.9% saline inoculum for each microorganism, adjusted to the McFarland 0.5 scale, then diluted in BHI and tested at 2 x 106 UFC.mL^{-1} concentration.

In determining the MIC, the EO was diluted in Dimethylsulfoxide (2% DMSO). Each well of the plate was initially filled with 0.1 mL of 0.9% NaCl, except for the first column, which was filled with 0.2 mL of the EO at the concentration of 2000 μg.mL^{-1}. 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. After this process, 0.1 mL of cells (2 x 106 CFU mL $^{-1}$) added in each well related to the second preceding item, resulting in a final volume of 0.2 mL. Control of culture medium, control of EO, and negative control (DMSO 2%) were performed. And for the positive control, amoxicillin (0.5 μg.mL^{-1}) was used. After incubation of the microplates in an incubator at 37 ºC for 24 hours, the plates were read in ELISA reader (OD 630nm).

The determination of MBC was performed 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 concentration of EO capable of completely inhibiting microbial growth.

Statistical analysis

All experiments were performed in triplicate with the respective results categorized in Microsoft Excel (Version 2013 for Windows) and later 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. The data were considered statistically significant when $p < 0.001$.

Antioxidant activity

The antioxidant quantitative test was based on the methodology recommended by Sousa et al. [20], Lopes-Lutz et al. [21] and Andrade et al. [22] by the use of 2.2-diphenyl-1-picryl-hydrazila (DPPH) with adaptations.

A methanolic solution of DPPH (stock solution) was prepared at the concentration of 40 μg.mL^{-1}, which was kept under the light. The EOs were diluted in methanol at concentrations 7.81;

15.62; 31.25; 62.5; 125 and 250 μg.mL^{-1}. For the evaluation of the test, 0.3 mL of the oil solution was added to a test tube, followed by the addition of 2.7 mL of the DPPH solution. White was prepared from a mixture with 2.7 mL of methanol and 0.3 mL of the methanol solution of each EO concentration as measured. After 30 min the readings were performed on a spectrophotometer (Biospectro SP-22) at a wavelength of 517 nm. The test was performed in triplicate and the calculation of the percentage of antioxidant activity (% AA) was calculated with the following equation 1:

$$(\%AA) = 100 - \left\{\frac{[(Abs_{sample} - Abs_{white}) \cdot 100]}{Abs_{control}}\right\} \qquad (1)$$

AA% - Percentage of antioxidant activity
Abs_{sample} – Sample Absorbance

Abs_{white}- White Absorbance

$Abs_{control}$- Control Absorbance

Cytotoxic activity front *Artemia salina* Leach

The cytotoxicity assay against *A. salina* L. Leach was based on the technique of Araújo et al. [23] and Lôbo et al. [24] with adaptations. An aqueous solution of artificial sea salt was prepared (35 g.L^{-1}) at pH 9.0 for incubation of 45 mg of *A. salina* L. eggs, which were placed in the dark for 24 h for the larvae to hatch (nauplii), then the nauplii were exposed to artificial light in 24 h, period to reach the stage methanuplii. The stock solution was prepared to contain 0.06 g of EO, 28.5 mL of solution of synthetic and 1.5 mL of Tween 80 to facilitate solubilization of the same. The test tubes were marked up to 5 mL. 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.

The methanauplia were selected and divided into 7 groups of 10 subjects in each test tube. Each group received aliquots of the stock solution 100, 75, 50, 25, and 2.5 μL, which were then filled to a volume of 5 mL with the sea salt solution to produce final solutions with the following concentrations 40, 30, 20, 10, and 1 μg.mL^{-1}. The tests were performed in triplicates. For the test control, saline solution was used. After 24 h, the number of dead was counted.

Results and discussion

Identification of chemical compounds by GC-MS of the *Origanum majorana* L. essential oil

This result corroborates with other studies that have shown that environmental factors may affect certain chemical compounds, while in others they have no influence on their production [22,23].

The chemical composition was determined by GC-MS, where the chromatogram can be observed in Fig. 1.

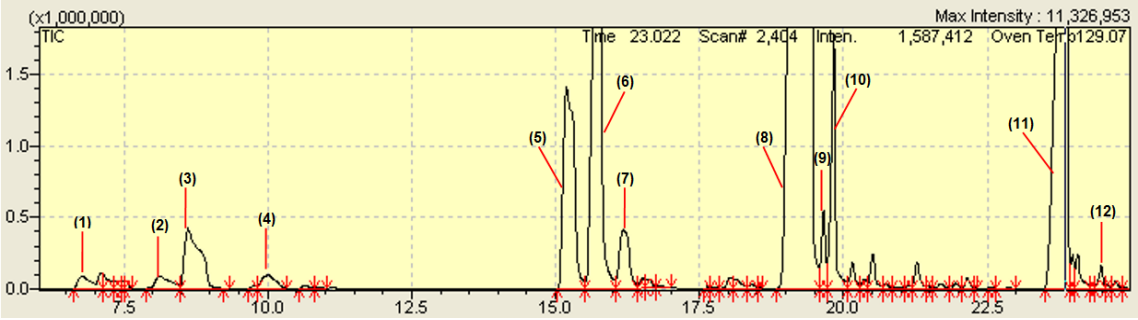

Fig. 1. Obtaining Gas Chromatography of *O. majorana* L. essential oil. Gas Trap: Helium (He); initial temperature 60 °C; initial time 1.0 min; the column temperature increased 3 °C / min. at 240 °C, maintained at this temperature for 30 min.

On the chemical composition of the EO of *O. majorana* L. (Table 1), 95.8% are oxygenated monoterpenes and only 1.38% are monoterpene hydrocarbons. The major component of EO is pulegone (57.05%), followed by other components verbenone (16.92%), trans-menthone (8.57%), cis-menthone (5.58%), piperitone (2.83%), 3-octanol %) and isopulegol (1.47%).

Tabela1. Chemical composition of *O. majorana* L. essential oil.

Nº	IR	IK	Compounds	Relative Percentage (%)	Identification *
1	6.776	939	α-pinene	0.39	MS, IK
2	8.127	979	β-pinene	0.50	MS IK
3	8.617	991	3-octanol	2.35	MS, IK
4	9.995	1029	limonene	0.49	MS, IK
5	15.185	1162	iso-menthone	5.58	MS, IK
6	15.688	1199	trans-*p*-menthan-2-one	8.57	MS, IK
7	16.174	1149	isopulegol	1.47	MS, KI
8	**19.448**	**1237**	**pulegone**	**57.05**	**MS, IK**
9	19.655	1165	lavandulol	0.77	MS, IK
10	19.883	1252	piperitone	2.83	MS, IK
11	23.873	1205	verbenone	16.92	MS, IK
12	24.482	1161	nonen-1-al-(2E)	0.26	MS, IK
			monoterpenes hydrocarbon	95.8	
			monoterpenes oxide	1.38	
			Total	97.18	

The identification path of the compounds. tR: retention time. The identification of the compounds was performed by the mass spectrum (GC-MS) of the Labsolutions CG-EM software version 2.50

SU1 (NIST05 and WILEY'S libraries of the 9th edition mass spectrum) and Kovats Index (KI) in literature [14].

Lima et al. [24], reports that piperitone has three organic functions in its chemical structure and it can be used for the synthesis of other compounds. Piperitone is derived from the metabolic pathway for the formation of piperitenone oxide, in which cis-pulegone is also, derived [25]. Macêdo et al. [26] observe that the variations of the active components of the plant are important parameters to correlate the activities, such as antibacterial and insecticide.

In addition, a number of biotic factors such as plant/ microorganism Stoppacher et al. [27], plants/ insects Kessler and Baldwin [28] plant interactions, age and stage of development. As well as abiotic factors such as luminosity Takshak and Agrawal [29], temperature, precipitation, nutrition, time and harvest time Bitu et al. [30], they may present correlations with each other, acting together, and they may exert a joint influence on chemical variability and yield of essential oil [26].

Larvicidal activity of essential oil of the *Origanum majorana* L. against *Aedes aegypti* larvae

The results of the larvicidal activity of this study show that *O. majorana* L. EO is active against *A. aegypti* larvae.

A fact that Komalamisra et al. [31], Magalhães et al. [32] and Dias et al. [33], classified with the values of the minimum lethal concentration that eliminates 50% of the population (LC_{50}) as a criterion for the activity. Because if LC_{50} <50 μg.mL^{-1}, the product is considered very active, if 50 <LC_{50} <100 μg.mL^{-1} the product is considered active, and when LC_{50}> 750 μg.mL^{-1} the product is considered inactive.

The percentage of dead *A. aegypti* larvae is shown in Table 2, at different EO concentrations of *O. majorana* L. in the 24-48 h exposure period. There was no mortality in the control group. Through the Probit test, LC_{50} = 56.008 μg.mL^{-1}, determination coefficient (R^2) = 0.917 and quantitative evaluation (X^2) = 0.844 in 24 h. After 48 h at LC_{50} = 15.696 μg.mL^{-1}, X^2 = 0.572 and R^2 = 0.835.

Table 2. Percentage of dead larvae (%) of *A. aegypti* produced by different concentrations of *O. majorana* L. essential oil in 24-48 h.

Concentrations (μg.mL^{-1})	24 h	48 h
Control	0^{a}	0^{a}
20	16^{b}	57.33^{b}
40	40^{c}	76^{c}
60	40^{d}	77.33^{d}

80	65.32[e]	82.66[e]
100	78.62[f]	94.66[f]

The values correspond to the mean and standard deviation of triplicates. Different letters indicate a significant difference ($p < 0.001$).

There were no reports of studies on the larvicidal activity of *O. majorana* L. essential oil against *A. aegypti* larvae.

According to Cantrell et al. [34], larvicidal compounds act by absorption through the cuticle, via the respiratory tract, and/or enter by ingestion via the gastrointestinal tract. Once inside the larva, the substances may reach the site of action or may cause systemic effects by diffusion in different tissues [35].

Studies on the insecticidal effect of *Mentha* spp. reported that menthol, mentona, pulegone and carvone help to clarify the mechanisms of action on insects [36]. Previous studies indicate that limonene, camphene, and verbenone have insecticidal insect activity [37].

Some EOs are known to cause dissuasive or anti-eating behavior in insects suggesting a neurotoxic action Satyan et al. [38], while some act as growth-regulating insects through analogous effects or antagonistic endogenous hormones. In the present study, it was found that even short-term exposure of larvae to lethal doses can dramatically increase their mortality over time and thereby reduce the total number of viable adults, leading to a possible reduction in total populations [39].

Microbiological activity of *Origanum majorana* L. essential oil

In relation to the microbiological activity, it was possible to verify that gram-negative bacteria were more sensitive to *O. majorana* L. EO than gram-positive bacteria.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) that were identified for *O. majorana* L. EO can be verified in Fig. 2. The results show that gram-negative bacteria were more sensitive to EO presenting MIC = 31.25 $\mu g.mL^{-1}$ compared to the negative control. The MBC for *E. coli* was at the concentration of 500 $\mu g.mL^{-1}$ and for *P. aeruginona* was at the concentration of 1000 $\mu g.mL^{-1}$ in relation to the negative control (amoxicillin). While the *S. aureus* bacterium did not present MIC and neither MBC.

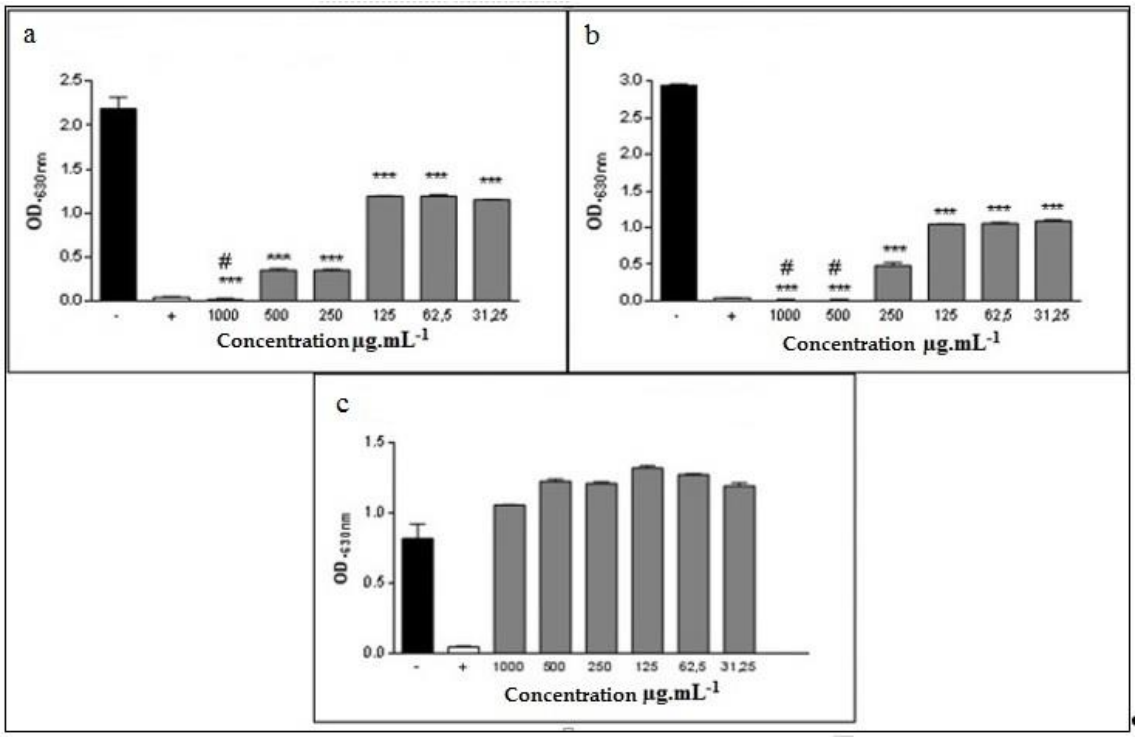

Fig 2. This is figure of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *O. majorana* L. essential oil. a) EO *Origanum majorana* L. against *P. aeruginosa* ATCC 25922. b) EO *Origanum majorana* L. against *E. coli* ATCC 8789. C) EO *Origanum majorana* L. against *S. aureus* ATCC 25922. Test substance of essential oil of the *O. majorana* L. (▢), BHI with 2% of 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.

According to Rosato et al. [40], the antibacterial activity in gram-negative bacteria occurs due to the high percentage of oxygenated monoterpenes present in the EO and consequently the synergism between these components. On the other hand, bacteria can also respond to adverse conditions in a transient way, through so-called stress tolerance responses. Bacterial stress tolerance responses include structural and physiological modifications in the cell, and complex genetic regulatory machines mediate them [41].

In the study by Duru et al. [42] pulegone showed high antimicrobial activity, particularly against Candida, albicans and Salmonella typhimurium. Pulegone is classified as a monoterpene, in the same way as carvone. It can be obtained from a variety of plants [43,44]. Menthone is a common volatile compound in Lamiaceae, which may also be active against a large number of bacteria, such as *E. coli* and *Enterococcus faecalis* [45,46]. Some studies have argued that monoterpenes can cross cell membranes and interact with intracellular sites critical for antibacterial activity [47].

However, reports of non-adaptation or cross-adaptation of bacteria to sublethal concentrations of major constituents of essential oils have also been reported [48]. Cross-resistance can occur when different antimicrobial agents attack the same target in the cell, reach common route of access to the respective targets or initiate a common pathway for cell death, ie, the resistance mechanism is the same for more than one antibacterial agent [49].

Essential antioxidant activity of essential oil of *Origanum majorana* L. by DPPH radical capture method

Many antioxidants derived from natural products demonstrate neuroprotective activity in vitro and/ or in vivo models such as flavonoid phenolic compounds [50].

Table 3 shows the percentage of antioxidant activity of *O. majorana* L. EO.

Table 3. Mean and standard deviation of the percentage of the antioxidant activity of *O. majorana* L. essential oil at different concentrations.

Concentrations (μg.mL^{-1})	% AA
7.81	16.95±0.20^{a}
15.62	17.15±0.17^{b}
31.25	17.48±0.37^{b}
62.5	18.98±0.56^{c}
125	19.28±0.75^{d}
250	21.85±2.55^{e}

The values correspond to the mean and standard deviation of triplicates. Different letters indicate that there was significant difference of Tunkey test ($p \leq 0.05$).

For the EO concentrations IC_{50} = 16.83 μg.mL^{-1} was compared with ascorbic acid (vitamin C) which showed IC_{50} = 16.71 μg.mL^{-1} as shown in Table 4. The absorbance of EO was Y = 0.0196x + 17.0078 and the coefficient (R^2) = 0.9600.

Table 4. Mean and standard deviation of the percentage of antioxidant activity of ascorbic acid (vitamin C) in different concentrations.

Concentrations (μg.mL^{-1})	% AA
7.81	18.57± 0.52^{a}
15.62	30± 0.10^{b}
31.25	99.93±0.02^{c}
62.5	99.99±0.0^{d}
125	99.99±0.0^{d}
250	99.99±0.0^{d}

The values correspond to the mean and standard duration of triplicates. Different letters indicate that there was a significant difference ($p \leq 0.05$).

The percentage of antioxidant activity of the essential oil showed a high IC_{50} = 1683 μg.mL^{-1}, whereas ascorbic acid presented 16.71 μg.mL^{-1} [51]. According to Rodrigues [52] the higher the consumption of DPPH for a smaller sample will be its IC_{50} and the greater its antioxidant capacity.

According to Beatović et al. [53], the antioxidant capacity of EO is related to its main compounds. However, this study did not present antioxidant activity. The importance concerning the

performance of antioxidants depends on the factors types of free radicals formed; where and how these radicals are generated; analysis and methods for identifying damage, and ideal doses for protection [54].

Toxicity to *Artemia saline* essential oil of *Origanum majorana* L.

A. salina is a microcrustacean used in fish feed, and it is widely used in toxicological studies because of the low cost and easy cultivation. Several studies have attempted to correlate toxicity on *A. salina* with antifungal, virucidal, antimicrobial, trypanosomicidal and parasiticidal activities. Lethality assays are performed in toxicological tests and the median lethal concentration (LC_{50}), which indicates death in half of a sample, can be obtained [55].

Table 5 shows the percentage of cytotoxic activity of *O. majorana* L. EO and the mean mortality readings after the 24 h period are expressed. The oil concentrations presented LC_{50} of 172.6 $\mu g.mL^{-1}$, the coefficient of determination (R^2) of 0.883 and X^2 of 1.915.

Table 5. Mean and standard deviation of percentage of cytotoxic activity of *O. majorana* L. essential oil at different concentrations.

Concentrations ($\mu g.mL^{-1}$)	% Mortality
Negative control	0±0,00a
50	9.37±0,00b
100	48.48±0,00c
250	67.39±0,00d
500	75.40±0,00e
750	80.26±0,00f
1000	83.51±0,00g

The values correspond to the mean and standard duration of triplicates. Different letters indicate a significant difference ($p < 0.05$).

According to Nguta et al. [56], both organic extracts and aqueous extracts with LC_{50} values of less than 100 μg/mL show high toxicity, LC_{50} between 100 and 500 μg/mL exhibited moderate toxicity, LC_{50} between 500 and 1000 μg/mL presented low toxicity and LC_{50} above 1000 μg/mL are considered to be non-toxic (non-toxic).

The lethal concentration of mortality against the *A. salina* larvae of this assay showed moderate cytotoxic activity. In order to evaluate the cytotoxicity of a given sample, it is possible to elucidate the cytotoxic effect of the cytotoxic mechanism and the mechanism of action of different compounds during their interaction with the tissues [57].

Conclusions

The results of the present study demonstrated that OE obtained from dry leaves of *O. majorana* L. showed good larvicidal activity against *A. aegypti* larvae with mortality from the concentration of 20 $\mu g.mL^{-1}$ in 48 h. In relation to the chemical analysis, it presented a mixture of monoterpenes, with the major constituent being pulegone (57.05%), followed by the other constituents verbenone (16.92%), trans-menthone (8.57%), cis-menthone), piperitone (2.83%), 3-octanol (2.35%) and isopulegol (1.47%). The oil showed satisfactory antimicrobial activity against *P. aeruginosa* and *E. coli* bacteria. In addition, despite the lack of antioxidant activity by the DPPH radical capture method, the oil showed moderate cytotoxic activity against *A. salina*. In summary, these results provide subsidies for future EO *O. majorana* L. studies in order to enhance the use of organic compounds with larvicidal activity against the *A. aegypti* mosquito, as well as the importance of the study of bioactive plant products that do not pollute the environment and that do not cause harm to human health.

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CAPÍTULO 2

Análise Fitoquímica, Atividade Larvicida, Antimicrobiana, Citotóxica e Potencial Antioxidante do Extrato Bruto Etanólico Obtido das Folhas de *Origanum majorana* L.

4.1 ARTIGO 3

Artigo em fase de submissão para o periódico Molecules (ISSN 1420-3049) qualis B1 em Farmácia, segundo a classificação do quadriênio 2013-2016 da plataforma Sucupira – CAPES.

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Article

Larvicidal evaluation of the ethanolic crude extract obtained from the leaves of *Origanum majorana* L. against larvae of *Aedes aegypti* (Linnaeus, 1762)

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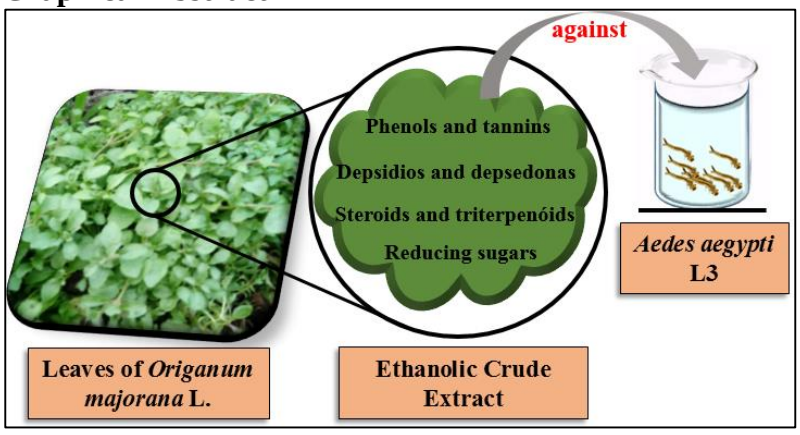
Abstract: This research evaluated the chemical composition of the crude ethanolic extract of *Origanum majorana* L. and its larvicidal, antimicrobial, cytotoxic and antioxidant potential. The larvicidal activity was evaluated against *Aedes aegypti*. The antioxidant activity was evaluated from 2.2-difenil-1-picril-hidrazila sequestration, the cytotoxic activity was evaluated against *Artemia salina* and the antimicrobial test was evaluated against bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*. The secondary metabolites identified were reducing sugars, tannins, phenols, depsides, depsidones, steroids and triterpenoids. The results showed that *A. aegypti* larvae were sensitive to letal concentration in 219.147 μg.mL^{-1} and letal concentration in 79.4 μg.mL^{-1} at 24 and 48 hours, respectively. In the antimicrobial activity the bacteria *P. aeruginosa* and *E. coli* are more sensitive with inhibitory concentration in 31.25 μg.mL^{-1}. *S. aureus* bacteria did not show inhibitory concentration at concentrations and there was no bactericide concentration at any of the ethanolic crude extract of *O. majorana* L. concentrations. In addition, there was no significant antioxidant activity and ethanolic crude extract had no cytotoxic activity against *A. salina* with concentrations equal to 946.9 μg.mL^{-1}. Thus, the crude ethanolic extract of O. majorana L. was observed to cause activity against larvae of A. aegypti and a relative antimicrobial activity.

Keywords: lamiaceae; manjerona; secondary metabolites; vector of dengue.

Graphical Abstract



1. Introduction

One of the major and challenging public health problems has been combating vector-borne diseases, including malaria, dengue, chikungunya, leishmaniasis, and yellow fever [1,2].

A. aegypti (Linnaeus, 1762) belongs to the Arthropoda phylum, to the Insecta class, to the Diptera order, to the Culicidae family and to the Culicinae subfamily [3]. Currently, *A. aegypti* is recognized as the main vector of Dengue, the most common arbovirus in the world, with about 3.9 million people exposed to infection risk and an estimated 390 million cases annually, mainly in the tropics and subtropical [4,5,6].

Dengue is a viral disease that can be found in every tropical and subtropical area of the globe, mostly in urban and peri-urban areas. The rapid increase in the global incidence of dengue in recent decades is related, among other factors, to rapid population growth and accelerated urbanization [7].

The application of chemical insecticides to combat vectors is the main type of control, but its use should be complementary to avoid selective pressure, in order to avoid increasing the population of resistant vectors [8,9].

In view of the challenges of vector control and a worrisome scenario in relation to the arboviruses outlined by the spread of these viruses worldwide, it is essential to adopt specific strategies, with greater investments inappropriate methods, that provide sustainability to the actions established by surveillance networks, and emphasize the analysis of their effectiveness [10].

Origanum majorana L. belongs to the Lamiaceae family of plants, considered one of the most important thermoculinary herbs of great economic and industrial importance [11]. Current data show that the essential oils of *Origanum* species are rich sources of compounds of particular biological importance, known for their antibacterial effect and antifungal activities, antispasmodic effects, inhibition of acetylcholinesterase, inhibition of lipid peroxidase, radical hijacking effect, and depressant activity [12].

In response to pathogenic attacks, plants produce secondary metabolites such as flavonoids, alkaloids, and terpenoids that coevolve with insects and microorganisms, becoming natural sources of insecticidal substances [13,14].

In this context, the objective of this study was to evaluate the larvicidal potential against larvae of the *A. aegypti* mosquito, to identify the chemical composition, to evaluate the antimicrobial activity against two gram-positive bacteria and one gram-negative, the cytotoxicity against *A. salina* Leach and the potential essential oil antioxidant obtained from *O. majorana* L. leaves.

2. Results

The secondary metabolites of *O. majorana* L. ECE were determined by a phytochemical screening and the main metabolites identified were reducing sugars, phenols, tannins, depsidases, steroids, and triterpenoids in Table 1.

Table 1. Classes of secondary metabolites of the ethanolic crude extract of *O. majorana* L.

Compound	Result + or –
Organic acids	-
proteins and amino acids	-
Polysaccharides	-
Reducing sugars	+
Phenols and tannins	+
Depsidios and depsedonas	+
Azulenes	-
Anthraquinones	-
Carotenoids	-
Derivatives of cumarin	-
Steroids and triterperoids	+
Purines	-
Alkaloids	-
Catechins	-
Cardiac glycosides	-

Sesquiterpenolactones	-
Flavonoids	-

Signal (+) response was positive/ signal (-) negative response.

2.1. Larvicidal Activity

The percentage of dead larvae of *A. aegypti* is shown in Table 2 at different ECE concentrations of *O. majorana* L. in the period of 24-48 h of exposure. There was no mortality in the control group. In the 24 h period the LC_{50} = 219.147 μg.mL^{-1}, determination coefficient (R^2) = 0.844 and X^2 = 0.487 was obtained. After 48 h there was LC_{50} = 79.4 μg.mL^{-1}, R^2 = 0.911 and X^2 = 0.472.

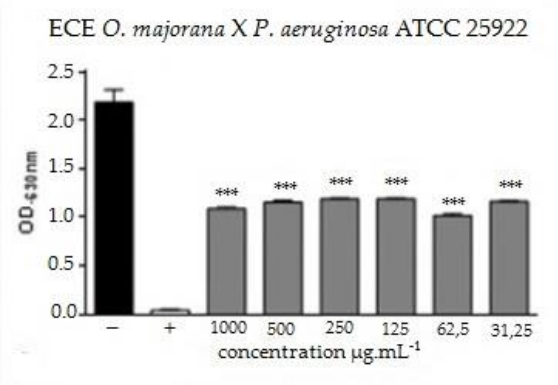
Table 2. Shows the percentage mortality (%) of *A. aegypti* larvae (L3) produced by different concentrations of *O. majorana* L. essential oil.

Larvicidal Activity (%)		
Concentrations (μg.mL^{-1})	**24 h**	**48 h**
Control	0	0
20	6.66	20
40	9.33	24
60	12	45.33
80	24[a]	53.33[a]
100	33.33[b]	62.66[b]
LC_{50} (Control +)	0.0034 μg·mL^{-1}	

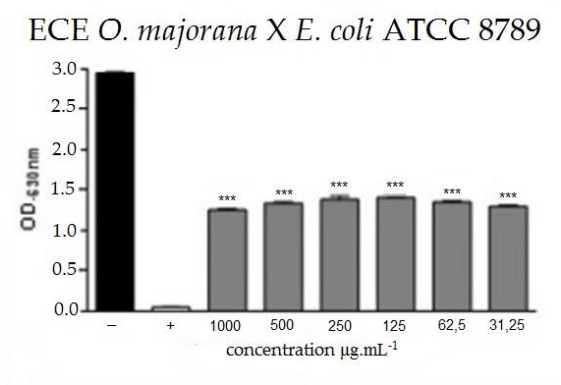
[a]Statistically significant in relation to the positive control..

2.2. Antimicrobial Activity

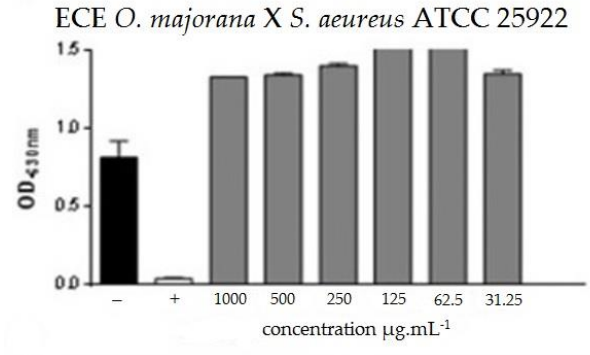
Figure 1 shows Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) identified for ECE *O. majorana* L. The results show that gram-negative bacteria were more sensitive in ECE *O. majorana* L. concentrations with MIC = 31.25 μg.mL^{-1}, whereas gram-positive bacteria had no MIC at any of the concentrations. In addition, there was no MBC at any of the ECE concentrations.



(a)



(b)



(c)

Figure 1. MIC and MBC of the ethanolic crude extract of *O. majorana* L. against (**a**) *P. aeruginosa*, (**b**) *E. coli*, (**c**) *S. aureus*. Source: own author. Test substance: of ECE ( ), BH with 2% DMSO ( ) and A oxiline ( ). ***P<0.001 statistically significant in relation to the negative control, #p<0.001 statistically significant in relation to the positive control.

2.3. Antioxidant Activity

The antioxidant activity of *O. majorana* L. ECE reached a maximum value of 38.77%. (Table 3). In relation to the percentage of antioxidant activity (%) and concentration, ECE presented a high IC_{50} = 399 μg.mL^{-1} compared to standard used in Table 3 which equation of the antioxidant activity Y = 0.0837x + 16.6115 and R^2 0.9665, ascorbic acid, which presented IC_{50} = 16.71 μg.mL^{-1} in Table 4.

Table 3. Shows of the percentage of antioxidant activity of *O. majorana* L. ECE at different concentrations.

Concentrations (μg.mL^{-1})	AA (%)
7.81	18.45%^{a}
15.62	18.61%^{a}
31.25	19.25%^{a}
62.5	20.69%^{a}
125	25.74%^{b}
250	38.77%^{c}

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

Table 4. Shows of the percentage of antioxidant activity of ascorbic acid at different concentrations.

Concentrations (μg.mL^{-1})	AA (%)
7.81	18.57%
15.62	30%
31.25	99.93%^{a}
62.5	99.99%^{a}
125	99.99%^{a}
250	99.99%^{a}

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

2.4. Cytotoxic Activity

Table 5 expresses the percentage of cytotoxic activity of *O. majorana* L. in the reading of the means of mortalities in the period of 24 h. The concentrations of the oil had LC_{50} = 946.9 μg.mL^{-1}, coefficient of determination (R^2) = 0.995 and X^2 = 0.042.

Table 5. Shows of the percentage of cytotoxic activity of *O. majorana* L. ECE at different concentrations.

Concentrations of ECE (μg.mL^{-1})	AA (%)
50	0.0%^{a}
100	0.0%^{a}
250	0.0%^{a}
5000	4.41%^{a}
750	29.62%^{b}
1000	54.34%^{c}
LC_{50} ($K_2Cr_2O_7$)	12.60 μg·mL^{-1}

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

3. Discussion

The results of the phytochemical study revealed the presence of phenols, tannins, steroids, triterpenoids, depsides, and reductive sugars in the ECE of *O. majorana* L. According to Carvalho, Gosmann, and Schenkel [15], phenols possess antioxidant activity, so that they may act to retard the development of pathologies caused by oxidase reactions. According to Santos et al. [16] and Oliveira, Ramos et al. [17], the tannins have potent antifungal activity due to the enzymatic inhibition process and complexation of the tannins to the fungal cell membrane.

Steroid metabolites and triterpenoids are biosynthetic products generated from isoprene. They have anti-inflammatory activity, but the mechanism is not well described [18]. Depsidones and depsidones are molecules of the phenolic nature that present antimicrobial, antitumor, antiviral, analgesic, antipyretic and antioxidant activity [19]. Reducing sugars are carbohydrates that have their free carbonyl group, capable of oxidizing in the presence of oxidizing agents in alkaline solution [20,21].

There are no reports of studies on the larvicidal activity of *O. majorana* L. ECE against *A. aegypti* larvae. According to Komalamisra et al. [22] classified the larvicidal activity of medicinal plant extracts as effective, moderate or high, depending on the value of LC_{50}% of the extract. The larvicidal activity is considered effective when the LC_{50}% of the extract is less than 750 ppm, moderate when between 50-100 ppm and high activity when less than 50 ppm. In this sense, the ECE of *O. majorana* L. was effective in periods of 24 and 48 h of exposure of *A. aegypti* larvae.

The insecticidal activity is the result of a series of actions and complex reactions between a toxic agent and the tissues of insects. This complex dynamic of toxicity can be simplified into three categories: penetration, activation (target site interaction) and detoxification [23]. To perform its insecticidal activity, the toxic substance has to penetrate the body of the insect, and this can occur through the alimentary canal, spiracles and/or integument [24].

The microbiological assays aim to investigate the antimicrobial potential of plant extracts since many compounds of secondary metabolism possess this activity [25].

The antimicrobial assay of *O. majorana* L. ECE inhibited the bacterial growth only of *P. aeruginosa* and *E. coli* at the concentration of 31.25 $\mu g.mL^{-1}$, which is the minimum concentration characterized by the extract as bacteriostatic. The main classes of compounds with antimicrobial effects can be divided into categories that include phenolic compounds, polyphenols, tannins and terpenoids [26,27,28].

These secondary metabolites act on the microorganisms affecting their structure and stability of the lipid bilayer, resulting in increased permeability of ions in the membrane, altering their electrochemical potential and their metabolic activities [29]. The use of synergism, as the combination of conventional antimicrobials with plant extracts, is also being investigated in order to obtain better results and potentiate its mode of action of antimicrobials [30,31].

However, ECE did not present MICs capable of lysing gram-positive *S. aureus* bacteria, this may be related to the morphological difference of the bacterial cell wall, altering its specificity to a binding site [32]. Bactericidal action can occur through different mechanisms of action such as inhibition of cell wall synthesis, inhibition of protein production, replication, inhibition of nucleic acids and transcription, plasma membrane damage and/or inhibition of the synthesis of essential metabolites [33]. In this sense, even if the organic groups are recognized by the antimicrobial action, there may be species-rich in phenols, tannins, polyphenols, and triterpenoids for this activity.

No antibacterial activity was observed at any concentration of *O. majorana* L. ECE against gram-positive and gram-negative. Antimicrobial resistance may be: 1) It may be acquired because of mutations that may occur during cell replication or are induced by mutagenic agents such as ionizing and non-ionizing radiation, alkylating agents or reactive oxygen species (ROS) [34]. 2) Acquired by the acquisition of exogenous genetic material previously present in other microorganisms that contain resistance genes that are propagated through horizontal gene transfer mechanisms [35] such as bacterial conjugation, transformation, and transduction [36].

In relation to the antioxidant activity of *O. majorana* L. ECE, the highest value presented was 38.77% of antioxidant capacity. It was observed that there was no required minimum reduction capacity of 50% (IC_{50}%) of the concentrations tested. According to Godoi et al. [37], the relationship between the percentage of the antioxidant activity and the EO concentration showed a high IC_{50} value of 399 $\mu g.mL^{-1}$ when compared with the standards, vitamin C with IC_{50} of 16.71 $\mu g.mL^{-1}$. This fact, Nascimento et al. [38] explains that the higher the consumption of DPPH for a sample, the lower its IC_{50} and consequently the greater its antioxidant activity.

An antioxidant substance can be defined as a chemical that inhibits the oxidation process, or any substance that, when present in low concentration, compared to that of the oxidizable substrate, significantly decreases or inhibits the oxidation of that substrate [39].

The most relevant classes of compounds are tannins, flavonoids, terpenoids and phenolic compounds [40]. Phenolic compounds have oxidative activity, being considered as potential agents for the prevention and treatment of diseases related to oxidative stress [41] and alternative sources for synthetic antioxidants [42].

It is common to use *A. salina* L. for the evaluation of cytotoxicity in studies of plant extracts indicated by popular medicine, either for the usual concentrations or for the monitoring of chromatographic fractionations in phytochemical studies [43].

However, there are no studies in the literature on the cytotoxic activity of ECE of *O. majorana* L. versus *A. salina*. Only at the concentration of 1000 $\mu g.mL^{-1}$ that the ECE presented mortality of 50% of the microcrustaceans. Amarante et al. [44] classified the degree of toxicity of organic extracts as non-toxic if the IC_{50} is equal to or above 1000 $\mu g.mL^{-1}$. Low toxicity if the IC_{50} exceeds 500 $\mu g.mL^{-1}$, moderate toxicity IC_{50} is between 500 $\mu g.mL^{-1}$ and 100 $\mu g.mL^{-1}$, and high toxicity are attributed if the values are below 100 $\mu g.mL^{-1}$.

4. Materials and Methods

4.1. Plant Material

The leaves of *O. majorana* L. were collected in the district of Fazendinha (00 "36'955" S and 51 "11'03'77" W) in the Municipality of Macapá, Amapá. Five samples of the plant species were deposited at the Amapaense Herbarium (HAMAB) of the Institute of Scientific Research and Technology of Amapá (IEPA).

4.2. Obtaining the Ethanol Crude Extract

The leaves were dried at 50 °C in a greenhouse to obtain the ethanolic crude extract (ECE) following the methodology proposed by [45]. The solvent used was ethanol at 96 °GL, the ratio was 1:3 relative to the feedstock. The vegetal material was filtered and evaporated in the rotating apparatus, model IRAHB05.06CN.

4.3. Phytochemical Screening

Phytochemical screening followed the methodology proposed by Matos [46], Simões [47] and Rosa [48] with adaptations, where it was submitted to phytochemical screening to detect the main classes of secondary metabolites.

4.4. Larvicidal Activity Against A. aegypti

The *A. aegypti* larvae used in the bioassay came from the colony kept in the Institute of Scientific and Technological Research Institute (IEPA). The methodology used followed [49] with adaptations.

The procedure started with the separation of 18 beakers of 50 mL and in each becker, 25 larvae of the third instar of *A. aegypti* were added. Afterward, they were reserved in a room with conditions of ambient temperature between 25 to 30 ºC and photoperiod of 12 h.

Preparation of the samples started after 24 h. The stock solution was prepared with 4.5. mL of 5% Tween 80, 85.5 mL of distilled water and 0.09 g of the ECE sample of *O. majorana* L. The negative control was prepared with 17.5 mL of Tween 80 dissolved in 350 mL of distilled water 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, and 1 $\mu g \cdot mL^{-1}$. 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 h, the dead larvae were counted, being considered as such, all those unable to reach the surface. 4.4.1. Larvicidal statistical analysis

4.4.1. Statistical analysis of larvicidal Activity

The experiment was carried out in triplicate. The larval mortality efficiency data were calculated in percentages using the Abbott formula, and later it was tabulated in Microsoft Excel (Version 2013 for Windows). The LC_{50} (lethal concentration causing 50% mortality in the population) was analyzed by the IBM

SPSS® program [version 21.0; SPSS Inc., Chicago, IL, USA]. The results were shown in the table. The differences that presented probability levels $p \leq 0.001$ were considered statistically significant.

4.5 Antimicrobial Activity

4.5.1. Microorganisms

The antimicrobial ECE test obtained from *O. majorana* L. leaves was tested in vitro against two gram-negative bacteria (*P. aeruginosa* ATCC 25922 and *E. coli* ATCC 8789) and a gram-positive bacterium (*S. aureus* ATCC 25922).

For each microorganism, a stock culture was set in BHI medium (Brain Heart infusion) with 20% glycerol and stored at - 80 °C. An aliquot of 50 µL of this culture was inoculated into 5 mL of sterile BHI broth medium and incubated for 24 h at 37 °C.

4.5.2. Determination of minimum Inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC were determined using the microplate dilution technique (96 wells) according to the protocol established by [50], with adaptations.

Bacteria were initially reactivated from the stock cultures, kept in BHI broth, for 18 h at 37 °C. Subsequently, bacterial growth was prepared in 0.9% saline inoculum for each microorganism, adjusted to the McFarland 0.5 scale, then diluted in BHI and tested at 2 x 106 UFC.mL^{-1} concentration.

In determining MIC, ECE was diluted with Dimethyl sulfoxide (2% DMSO). Each well of the plate was initially filled with 0.1mL NaCl 0.9%, except for the first column, which was filled with 0.2 mL of ECE at the concentration of 2000 µg.mL^{-1}. 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. After this process, 0.1 mL of cells (2x106 CFU.mL^{-1}) added in each well related to the second preceding item, resulting in a final volume of 0.2 mL. Control of the culture medium, ECE control, negative control (DMSO 2%) and amoxicillin (50 µg.mL^{-1}) was used for positive control. After incubation of the microplates in an incubator at 37 ºC for 24 h, the plates were read in ELISA reader (OD 630 nm).

The determination of MBC was performed 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 concentration of ECE capable of completely inhibiting microbial growth in Petri dishes after 24-48 h of growth.

4.5.3. Statistical analysis of microbiological assays

All experiments were performed in triplicate with the respective results categorized in Microsoft Excel (Version 2013 for Windows) and later 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. The data were considered statistically significant when $p < 0.001$.

4.6. Antioxidant activity

The antioxidant quantitative test was based on the methodology recommended by Sousa et al. [51], Lopes-Lutz et al. [52] and Andrade et al. [53] by the use of 2.2-diphenyl-1-picryl- hydrazine (DPPH) with adaptations.

A methanolic solution of DPPH (stock solution) was prepared at the concentration of 40 µg.mL^{-1}, which was kept under the light. The ECE were diluted in methanol at concentrations 7.81; 15.62; 31.25; 62.5; 125 and 250 µg·mL^{-1}. For the evaluation of the test, 0.3 mL of the oil solution was added to a test tube, followed by the addition of 2.7 mL of the DPPH solution. The blank was prepared from a mixture with 2.7 mL of methanol and 0.3 mL of the methanol solution of each ECE concentration evaluated. After 30 min, the readings were performed on a spectrophotometer (Biospectro SP-22) at a wavelength of 517 nm. The test was performed in triplicate and the calculation of the percentage of antioxidant activity (% AA) was calculated with the following equation 1:

$$(\%AA) = 100 - \left\{\frac{[(Abs_{sample} - Abs_{white}) . 100]}{Abs_{control}}\right\} \qquad (1)$$

AA% – Percentage of antioxidant activity
Abs_{sample} – Sample Absorbance
Abs_{white} – White Absorbance
$Abs_{control}$ – Control Absorbance

4.7. Cytotoxic Activity front Artemia salina Leach

The *A. salina* cytotoxicity assay was based on the technique of Araújo et al. [54] and Lôbo et al. [51] with adaptations. An aqueous solution of artificial sea salt was prepared (35 $g.L^{-1}$) at pH 9.0 for incubation of 45 mg of *A. salina* eggs, which were placed in the dark for 24 h for the larvae to hatch (nauplii), then the nauplii were exposed to artificial light in 24 h, period to reach the stage methanuplii. The stock solution was prepared to contain 0.06 g of ECE, 1.5 mL of Tween 80 and 28.5 mL of saline to facilitate solubilization of the same. The test tubes were marked up to 5 mL.

The methanauplia were selected and divided into 7 groups of 10 subjects in each test tube. Each group received aliquots of the stock solution (100, 75, 50, 25, and 2.5 µL), which were then filled to a volume of 5 mL with the sea salt solution to produce final solutions with the following concentrations: 40, 30, 20, 10, and 1 $\mu g \cdot mL^{-1}$ in triplicates. 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. After 24 h, the number of dead was counted.

4.8. 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 LC50 values were determined in the PROBIT regression, through the SPSS statistical program (Version 21 for Windows, Chicago, IL, USA). Differences that p

5. Conclusions

The ECE of *O. majorana* L. exhibited good activity against *A. aegypti* larvae exhibiting susceptibility from the concentration of 80 $\mu g.mL^{-1}$. Regarding the phytochemical analysis, it is identified secondary metabolites were reducing sugars, tannins, phenols, depsides, depsidones, steroids and triterpenoids. The antimicrobial activity revealed that the ECE of *O. majorana* L. exhibited higher bacteriostatic efficiency for bacteria *P. aeruginosa* and *E. coli* than for the *S. aureus* bacterium, which was resistant to oil. The concentrations of ECE did not present bactericidal activity. The ECE did not present antioxidant activity by the DPPH radical capture method nor any cytotoxic activity against *A. salina*. Therefore, the importance of this study extends the selection of herbal products with biological activity for insecticidal activity against *A. aegypti* larvae, in order to become a sustainable measure without damaging the environment and not causing harm to the health of the society.

Author Contributions: conceptualization, R.S.B.C.; methodology, R.S.B.C., S.S.M.S.A., P.C.C and A.K.R.G.; statistical analysis, R.L.M., A.B.L.R., A.F.F. and É.M.R., larvicidal testing, R.S.B.C.; supervision, S.S.M.S.A.

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6 CONSIDERAÇÕES FINAIS E PERSPECTIVAS.

Esse trabalho abriu perspectivas quanto ao uso de *Origanum majorana* e *Origanum vulgare* L. para avaliação de diferentes atividades biológicas, as quais se diferenciam do conhecimento empírico. Em relação a atividade larvicida tanto os óleos essenciais de *O. majorana* e *O. vulgare* L. quanto o extrato bruto etanólico de *O. majorana* demostraram resultados significativos, resultado associado a presença de terpenos. A dengue é um crescente problema de saúde pública no mundo devido o seu potencial de dispersão, pela rápida adaptação ao ambiente e hospedeiros, sejam eles vertebrados ou invertebrados, ou seja, essa rápida disseminação exige que novas politicas e intervenções alternativas sejam inseridas no contexto de combate e controle do vetor *A. aegypti.*

Apesar de ambos os óleos e o extrato etanólico não demostrarem potencial antioxidante, os óleos essencias demostraram forte citotoxicidade, evidenciando que nas folhas das espécies há grupos orgânicos com potenciais diferentes. O baixo potencial antioxidante no extrato bruto etanólico ocorreu devido a pouca presença de ácido fenólico e ausência de flavonóides. Essa variação da composição se deve a interferênça de fatores bióticos e abioticos na produção dos componentes orgânicos presentes nas folhas das plantas.

Os oleos essenciais de *O. vulgare* L. e *O. majorana* demostraram maior potencial como antimicrobianos contra *P. aeruginosa* e *E. coli* seguido do extrato bruto etanólico de *O. majorana*. Esse resultado se deve principalmente ao sinergismo dos principais componetes orgânicos com os que estão em menor concentração presentes tanto nos óleos quanto no extrato sendo responsáveis por sensibilizar e romper as camadas das mebranas das cepas.

S. aureus apresentou capacide adaptativa quando expostas às doses subletais dos óleos e do extrato. Esse resultado possivelmente se deve a adaptação cruzada entre os componentes dos óleos essenciais e pela ação conjunta dos principais componentes na membrana microbiana provocando alto estresse na cepa e como consequência sua resistência.

Futuramente outras atividades biológicas devem ser testadas utilizando os óleos essenciais e o extrato bruto das espécies em estudo. Existe a necessidade de caracterização fitoquímica e o estudo dos principais compostos orgânicos de forma isolada, com a finalidade de definir de fato quais são os componentes responsáveis por determinado mecanismo de ação fitoquímica e farmacológica.

Nos proximos estudos devem-se ser realizados outros testes toxocológicos que visem avaliar melhor a toxicidade dos óleos e dos extrato das espécies. Além disso, testar outras cepas bacterianas para determinar melhor a ação dos compostos dos óleos e do extrato são essencias, pois além desses resultados serem promissores se diferenciaram de alguns resultados relatados literatura e consequentemente essa singularidade pode definir novas características referente às plantas da família lamiaceae.

As espécies *O. majorana* e *O. vulgare* L. são nativas da região mediterrânea, são plantas perenes, aromáticas e cultivadas em várias partes do mundo para uso culinário e fitoterápico, inclusive no Brasil, dessa forma, a importância do estudo se baseia sobre esse aspecto para qualificação dos compostos orgânicos presentes na composição química das plantas para uso inseticida e em benefício geral. A validação dos óleos e do extrato como inseticida botânico são fundamentais para subtituição de inseticidas químicos em que atualmente as larvas do *A. aegypti* se encontram resistentes. Ações conjuntas de pesquisas e a padronização dos testes larvcidas que utilizam plantas tanto para controle do vetor quanto para prevenção da dengue devem ser levadas em consideração por serem alternativas promissoras e devem ser tomadas igualmente com responsábilidade para que sejam biodegradáveis sem impactos ambientais e que não causem danos a saúde humana.

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ANEXOS

ANEXO A – Comprovante de Cadastro ao Conselho de Gestão do Patrimônio Genético (SISGEN)

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº A5917DD

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

Número do cadastro:	A5917DD
Usuário:	Renata do Socorro Barbosa Chaves
CPF/CNPJ:	008.816.282-38
Objeto do Acesso:	Patrimônio Genético
Finalidade do Acesso:	Pesquisa

Espécie

Origanum majorana

Origanum vulgare

Título da Atividade:	AVALIAÇÃO QUÍMICA, ANTIOXIDANTE, CITOTÓXICA, ANTIMICROBIANA, LARVICIDA E REPELENTE DE ÓLEOS ESSENCIAIS E EXTRATOS BRUTOS ETANÓLICOS DAS ESPÉCIES Origanum majorana Linn E Origanum vulgare

Equipe

Renata do Socorro Barbosa Chaves	Unifap
Sheylla Susan Moreira da Silva de Almeida	Unifap

Data do Cadastro:	21/03/2018 08:45:22
Situação do Cadastro:	Concluído

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em 8:45 de 21/03/2018.

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

ANEXO B - Confirmação da submissão do Artigo 1 Qualis B1

[Pharmaceuticals] Manuscript ID: pharmaceuticals-477672 - Submission Received

Caixa de entrada ×

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Journal name: Pharmaceuticals
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Type of manuscript: Article
Title: Larvicidal evaluation of Origanum vulgare essential oils against of Aedes aegypti
Authors: Renata do Socorro Barbosa Chaves, Rosany Lopes Martins, Alex Bruno Lobato Rodrigues, Érica de Menezes Rabelo, Ana Luzia Ferreira Farias, Camila Mendes da Conceição Vieira Araújo, Talita Fernandes Sobral, Allan Kardec Ribeiro Galardo, Sheylla Susan Moreira da Silva de Almeida *
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Kind regards,

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ANEXO C - Confirmação da submissão do Artigo 2 Qualis A2

PLOS ONE: Notification of co-authorship on manuscript - [EMID:1b9bbb4d87d32001]

Caixa de entrada ×

PLOS ONE <em@editorialmanager.com> 14:21 (há 23 minutos)

para eu

inglês › português Traduzir mensagem Desativar para: inglês ×

PONE-D-19-08538
Larvicidal evaluation of the Origanum majorana L. Essential Oil against the larvae of the Aedes aegypti mosquito
Dr Sheylla Susan Moreira da Silva de Almeida

Dear Sra Renata Chaves,

You are receiving this email because you have been listed as an author on a manuscript recently submitted to PLOS ONE, which is entitled "Larvicidal evaluation of the Origanum majorana L. Essential Oil against the larvae of the Aedes aegypti mosquito".

The corresponding author for the submission process is: Dr Sheylla Susan Moreira da Silva de Almeida
The full author list for the submission is: Renata do Socorro Barbosa Chaves; Rosany Lopes Martins; Alex Bruno Lobato Rodrigues; Érica de Menezes Rabelo; Ana Luzia Ferreira Farias; Camila Mendes da Conceição Vieira Araújo; Talita Fernandes Sobral; Allan Kardec Ribeiro Galardo; Sheylla Susan Moreira da Silva de Almeida, Ph.D.

You are not required to confirm your co-authorship of this submission, but if you would like to add an ORCID iD please click the link below to confirm co-authorship and link your ORCID iD.
Yes, I am affiliated.

Please note that if you would like to link your ORCID iD to the submission, you will need to log in to your Editorial Manager account to do so. If you do not have an Editorial Manager account, you can register here: http://www.editorialmanager.com/pone/Default.aspx.

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ANEXO D – Norma da Revista Pharmaceulticals

ISSN: 1424-8247 – https://www.mdpi.com/journal/pharmaceuticals/instructions

pharmaceuticals

MDPI

Manuscript Submission Overview

Submission Process

Manuscripts for *Pharmaceuticals* should be submitted online at susy.mdpi.com (https://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 (https://susy.mdpi.com). Once you have registered, click here to go to the submission form for *Pharmaceuticals* (https://www.mdpi.com/user/manuscripts/upload/?journal=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.

Accepted File Formats

Authors must use the Microsoft Word template (../../../files/word-templates/pharmaceuticals-template.dot) or LaTeX template (https://www.mdpi.com/authors/latex) to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the editorial office pharmaceuticals@mdpi.com. Accepted file formats are:
Microsoft Word: Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, the *Pharmaceuticals* Microsoft Word template file (https://www.mdpi.com/files/word-templates/pharmaceuticals-template.dot) must be used. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.

LaTeX: Manuscripts prepared in LaTeX must be collated into one ZIP folder (include all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, please use the *Pharmaceuticals* LaTeX template files (https://www.mdpi.com/authors/latex). You can now also use the online application writeLaTeX (https://www.writelatex.com) to submit articles directly to *Pharmaceuticals*. The MDPI LaTeX template file should be selected from the writeLaTeX template gallery (https://www.writelatex.com/templates/mdpi-articletemplate/fvjngfxymnbr).

Supplementary files: May be any format, but it is recommended that you use common, non-proprietary formats where possible (see below for further details).

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.

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This journal automatically deposits papers to PubMed Central after publication of an issue. Authors do not need to separately submit their papers through the NIH Manuscript Submission System (NIHMS, http://nihms.nih.gov/ (https://www.nihms.nih.gov/)).

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.

Review manuscripts should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA (https://www.mdpi.com/editorial_process) guidelines.

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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.

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Front Matter

These sections should appear in all manuscript types
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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.

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 (https://www.crossref.org/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 –

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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).
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2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
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Conference Proceedings:

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Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional). Thesis:

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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.

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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.

Supplementary Materials, Data Deposit and Software Source Code

Data Availability

In order to maintain the integrity, transparency and reproducibility of research records, authors must make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal.

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository or uploading as supplementary information to the publication. The name and version of all software used should be clearly indicated.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable, however we recommend that common, non-proprietary formats are used where possible.

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Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

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New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.
New nucleic acid sequences must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.

New high throughput sequencing (HTS) datasets (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.

New microarray data must be deposited either in the GEO or the ArrayExpress databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.

New protein sequences obtained by protein sequencing must be submitted to UniProt (submission tool SPIN).
All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

Research and Publication Ethics

Research Ethics

Research Involving Human Subjects
When reporting on research that involves human subjects, human material, human tissues, or human data, authors must

declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975

(https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/ (https://www.wma.net/what-we-do/medicalethics/declaration-of-helsinki//)), revised in 2013. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. At a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Editors reserve the right to reject any submission that does not meet these requirements.
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A written informed consent for publication must be obtained from participating patients who can be identified (including by the patients themselves). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed informed consent from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. A template permission form (https://res.mdpi.com/data/mdpi-patient-consent-form.docx) is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission.
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Reduction in number of animals used, and

Refinement of experimental conditions and procedures to minimize the harm to animals.
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Manuscripts containing original descriptions of research conducted in experimental animals must contain details of approval by a properly constituted research ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.
Pharmaceuticals endorses the ARRIVE guidelines (www.nc3rs.org.uk/ARRIVE (http://www.nc3rs.org.uk/ARRIVE)) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at www.nc3rs.org.uk/ARRIVEchecklist (http://www.nc3rs.org.uk/ARRIVEchecklist).
1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf (http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf).

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.
An example of Ethical Statements:
The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

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Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity (http://www.cbd.int/convention/) and the Convention on the Trade in Endangered Species of Wild Fauna and Flora (http://www.cites.org/).

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oriza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may

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Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX , institute, city, country).

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The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Pharmaceuticals* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Pharmaceuticals* must abide to the following:

Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.

Authors should accurately present their research findings and include an objective discussion of the significance of their findings.

Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.

Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.

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Republishing content that is not novel is not tolerated (for example, an English translation of a paper that is already published in another language will not be accepted).

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1. Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

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John Doe[1¶], Antonie Data[1¶], Johannes van Stats[1,4¤a], Marie Testperson[2*], David Ribosome Jr.[3,5], Gregory H.T. McBio[4,4¤b], Angela Reviewerson[1,2&], Marina Measure[1&], on behalf of The Bunny Genome Sequencing Consortium[^]

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Abstract

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Materials and methods

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Whole genome RFLP analysis

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Table 1. This is the Table 1 Title.

	Chemical W	Chemical X	Chemical Y	Chemical Z
Chemical 1	Reaction 1W	Reaction 1X	Reaction 1Y	Reaction 1Z
Chemical 2	Reaction 2W	Reaction 2X	Reaction 2Y	Reaction 2Z
Chemical 3	Reaction 3W[a]	Reaction 3X	Reaction 3Y[b]	Reaction 3Z
Chemical 4	Reaction 4W	Reaction 4X	Reaction 4Y	Reaction 4Z
Chemical 5	Reaction 5W	Reaction 5X	Reaction 5Y	Reaction 5Z

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References

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92 1. Doe J, Data A, van Stats J, Testperson M, Ribosome D Jr,
93 McBio GHT, et al. This is the article title. PLoS ONE.
94 2017;12(12):e00000. doi: 10.1371/journal.pone.0000000
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Research Ethics

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1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf (http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf).

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1. Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

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