UNIVERSIDADE FEDERAL DO AMAPÁ
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LETHICIA BARRETO BRANDÃO

AVALIAÇÃO DO POTENCIAL LARVICIDA NO CONTROLE DE *Aedes aegypti* DO ÓLEO ESSENCIAL E EXTRATOS BRUTOS ETANÓLICO E AQUOSO DAS FOLHAS DE *Tridax procumbens* L.

Macapá
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Orientador: Sheylla Susan M. S. de Almeida

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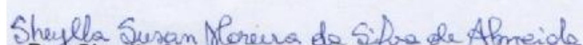

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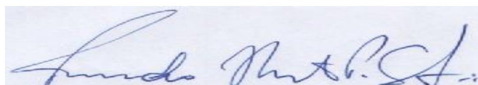

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Dedico este trabalho a comunidade cientifica.

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SÍMBOLOS, SIGLAS E ABREVIATURAS

μL – Microlitro

% AA – Porcentagem de atividade antioxidante

ATCC – American Type Culture Collection

BM – Biomassa do vegetal

BU – Base úmida

CBM – Concentração bactericida mínima

CIM – Concentração inibitória mínima

GC-MS – Gas Chromatography coupled to the Mass Spectrometer

CI_{50} – Concentração Inibitória 50%

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

CLSI – Manual Clinical and Laboratory Standards Institute

cm – Centímetro

CO_2 – Dióxido de carbono

DMSO – Dimetilsufóxido

DPPH – 2,2-difenil-1-picrilhidrazil

FIOCRUZ – Fundação Oswaldo Cruz

HAMAP – Herbário Amapaense

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

IK – Índices de Kolvats

ISO – International Standart Organization

KPa – Quilopascal

mg – Miligrama

CMH – Caldo Müeller-Hinton

mL – Mililitro

mm – Milímetro

OE – Óleos Essenciais

OMS – Organização Mundial da Saúde

ppm – partes por milhão

SPSS – Statical Package for the Social

Science tR – Tempo de retenção

WHO – World Health Organization

AVALIAÇÃO DO POTENCIAL LARVICIDA NO CONTROLE DE *Aedes aegypti* DO ÓLEO ESSENCIAL E EXTRATOS BRUTOS ETANÓLICO E AQUOSO DAS FOLHAS DE *Tridax procumbens* L.

RESUMO

Introdução: A *Tridax procumbens* L., pertence à Asteraceae e é vulgarmente conhecida por erva-de-touro. Partes da planta são utilizadas na medicina popular para tratamento de diferentes patologias. **Objetivo:** Avaliar o potencial antioxidante, citotóxico e larvicida do óleo essencial (OE) e extratos bruto etanólico (EBE) extrato bruto aquoso (EBA) das folhas de *Tridax procumbens*, assim como, identificar as principais classes de metabólitos secundários. **Metodologia:** A atividade antioxidante foi avaliada pelo método de sequestro do radical 2,2-difenil-1-picrilhidrazil; A atividade citotóxica foi avaliada utilizando *A. salina;* A atividade microbiológica dos extratos foram realizadas pelo método de microdiluição com bactérias *Escherichia coli*, *Pseudomonas aeruginosa* e *Staphylococcus aureus*. O bioensaio larvicida foi realizado com larvas do mosquito *A. aegypti* e a identificação dos metabólitos foi realizada por testes fitoquímicos, quantificação de fenólicos totais para os extratos, e GC-MS para o óleo. **Resultados e discussão:** A análise fitoquímica dos extratos mostrou a presença de depsídeos e depsidonas, flavanoides, fenóis e taninos e alcaloides, proteínas eaminoácidos, saponinas. A análise fitoquímica do OE mostrou a presença de 20 compostos, sendo os principais: o timol e γ-Terpinene. Os resultados microbiológicos para o EBA foram positivos, sendo possível identificar a CIM e CBM, nas concentrações testadas, para as três bactérias utilizadas. Para a atividade citotóxica frente à *A. salina* apresentou baixa atividade. O EBA apresentou boa atividade larvicida quando comparado a literatura e ao EBE. O OE apresentou atividade antioxidante com IC_{50} de 194,51 μg.mL^{-1}, demonstrando atividade nas maiores concentrações testadas. Apresentou baixa atividade citotóxica frente à *A. salina*, com LC_{50} de 1238,67 μg.mL^{-1}, demonstrando atoxicidade do óleo testado. O OE apresentou boa atividade larvicida quando comparado a literatura, com LC_{50}= 79,0 μg.mL^{-1} em 24 horas e CL_{50}= 69,15 μg.mL^{-1} em 48 horas. **Conclusão:** Foi possível identificar que tanto o EBA e OE das folhas de *Tridax procumbens* apresentam potencial para o desenvolvimento de inseticidas naturais.

Palavras-Chave: Erva de Touro; Asteraceae; Ensaio biológico.

Agradecimentos: CAPES, CNPq e FAPEAP.

ABSTRACT

EVALUATION OF LARVICIDE POTENTIAL IN THE CONTROL OF Aedes aegypti ESSENTIAL OIL AND GROSS ETHANOLIC AND AQUEOUS EXTRACTS OF THE LEAVES OF *Tridax procumbens* L.

SUMMARY

Introduction: *Tridax procumbens* L., belongs to the Asteraceae and is commonly known as bull weed. Parts of the plant are used in folk medicine. **Objective:** To evaluate the antioxidant, cytotoxic and larvicidal potential of essential oil (EO) and crude ethanol extracts (EBE) from the leaves of *T. procumbens*, as well as to identify the main classes of secondary metabolites. **Methodology:** The antioxidant activity was evaluated by the sequestration method of the 2,2-diphenyl-1-picrylhydrazyl radical; Cytotoxic activity was assessed using *A. salina*; The microbiological activity of the extracts were performed by the microdilution method with bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The larvicidal bioassay was performed with larvae of the *A. aegypti* mosquito and the identification of the metabolites was performed by phytochemical tests, quantification of total phenolics for the extracts, and GC-MS for the oil. **Results and discussion:** Phytochemical analysis of the extracts showed the presence of depsidones and depsidones, flavanoids, phenols and tannins and alkaloids, proteins and amino acids, saponins. The phytochemical analysis of OE showed the presence of 20 compounds, being the main ones: thymol and γ-Terpinene. The microbiological results for the EBA were positive, and it was possible to identify the MIC and CBM, at the concentrations tested, for the three bacteria used. For the cytotoxic activity against *A. salina* presented low activity. EBA showed good larvicidal activity when compared to literature and EBE. OE presented antioxidant activity with IC_{50} of 194.51 $\mu g.mL^{-1}$, demonstrating activity in the highest concentrations tested. It presented low cytotoxic activity against *A. salina*, with LC_{50} of 1238.67 $\mu g.mL^{-1}$, demonstrating the toxicity of the oil tested. OE presented good larvicidal activity when compared to the literature, with LC_{50} = 79.0 $\mu g.mL^{-1}$ in 24 hours and LC_{50} = 69.15 $\mu g.mL^{-1}$ in 48 hours. **Conclusion:** It was possible to identify that both the EBA and EO of the leaves of *T. procumbens* present potential for the development of natural insecticides.

Keywords: Erva de touro; Asteraceae; Biological assay.

Acknowledgments: CAPES, CNPq and FAPEAP.

1 INTRODUÇÃO

1.1 PLANTAS MEDICINAIS NO BRASIL

O Brasil é o país com a maior biodiversidade do planeta, com base nisto, em junho de 2006, o governo federal através do Decreto nº 5.813 aprovou a Política Nacional de Plantas Medicinais e Fitoterápicos onde seu objetivo visa garantir à população brasileira o acesso seguro e o uso racional de plantas medicinais e fitoterápicos, promovendo o uso sustentável da biodiversidade, o desenvolvimento da cadeia produtiva e da indústria nacional (BRASIL, 2009).

Neste sentido, o Brasil possui uma rica história de uso das plantas medicinais no tratamento dos problemas de saúde da população, uso este construído com base na experiência popular, sendo transmitido através de gerações (NUNES; BERNARDINO; MARTINS, 2015).

O conjunto de plantas medicinais que perfazem o arsenal médico de uma dada cultura é o resultado de um longo e dinâmico processo de validação (ARAÚJO et al., 2014). As populações humanas que ocupam florestas tropicais convivem com a grande diversidade destes ambientes e desenvolvem, cada qual à sua maneira, formas de explorá-los para sua sobrevivência. De seu repertório cultural, destaca-se o conhecimento sobre o uso de plantas para fins medicinais (PINTO, 2006).

Através de recomendações da Organização Mundial da Saúde (OMS) e das Conferências de Saúde (BRASIL, 2006) muitas plantas medicinais e seus fitoterápicos foram inseridos na saúde pública brasileira a partir da década de 1980, por iniciativa de alguns gestores e/ou profissionais de saúde. Este uso de práticas alternativas em saúde tem persistido, entre outros motivos, pela dificuldade no acesso à assistência de saúde por parte da população, que não tem suas demandas e necessidades atendidas, as quais são parcialmente supridas pelo uso das terapias (REZENDE; COCCO, 2002).

A partir dessa constatação, visualiza-se que esses agentes e serviços possam ser organizados em arranjos produtivos, para a produção de bens e serviços, aproveitando todo o potencial da biodiversidade, com a implantação de políticas públicas em plantas medicinais e fitoterápicos que conduzam ao desenvolvimento econômico, tecnológico e principalmente social do país (TORRES, 2015).

Dentre as espécies de plantas medicinais, as pertencentes a família Asteraceae vem sendo cada vez mais estudadas devido suas propriedades e benefícios a saúde humana.

1.2 FAMÍLIA ASTERACEAE

A família Asteraceae é a maior família das Eucotiledôneas (ARAÚJO, 2014). Apresenta cerca de 1.600 gêneros e cerca de 23.000 espécies conhecidas, agrupadas em três subfamílias e 17 tribos, segundo Andenberg (2007). Espécies da família Asteraceae são cultivadas como ornamentais, medicinais, apícolas, oleaginosas, aromáticas, inseticidas e comestíveis. Além disso, muitas espécies são invasoras de lavouras e tóxicas ou potencialmente tóxicas para animais e para o homem (BERETTA; KOPP; MELZIG, 2008).

Várias características morfológicas em Asteraceae suportam seu monofiletismo, entre elas a presença de um capitulo altamente modificado, ovário bicarpelar ínfero que se desenvolve em uma cipsela com ovulo basal e reto e antera sinânteras. Asteraceae é formada por plantas herbáceas a arbustivas, principalmente, que se caracterizam por apresentar caule e ramos tetrangulares, quando jovens, flores fortemente zigomorfas, bilabiadas, e ovário estilete ginobásico (JANSEN e PALMER, 1988). Segundo a classificação taxonômica proposta por Dahlgren, a família Asteraceae pertence à ordem Asterales.

As plantas da família Asteraceae são conhecidas pelas propriedades terapêuticas, cosméticas e aromáticas. Já é relatado na literatura o uso medicinal dessa família como antihelmíntico, antiinflamatório, adstringente, colestérico, antihemorrágico, antimicrobiano, diurético, analgésico e antiespasmódico (PORTILLO et al., 2001; ISCAN et al., 2006; ABAD; BERMEJO, 2007; BENEDEK et al., 2007; JEON et al., 2008).

Dentre as espécies dessa família, destaca-se a *Tridax procumbens* que veem sendo estudada devido suas características e propriedades terapêuticas benéficas ao homem, sendo encontrada nas florestas brasileiras.

1.2.1 *Tridax procumbens* Linn

Tridax procumbens L., pertence à Asteraceae e é popularmente conhecida por erva-de-touro, destaca-se por ser uma planta amplamente invasora e por sua capacidade em eliminar as espécies adjacentes, sobressaindo em seu habitat. Essa espécie é originária da América Central (KISSMANN e GROTH, 1999). Trata-se de uma espécie herbácea, anual

ou bianual, nativa e de distribuição desconhecida nas regiões brasileiras, possui a característica de se instalar em áreas com lavouras anuais ou perenes, áreas destinadas à fruticultura, entre outras. Partes da planta são utilizadas na medicina popular, além do uso como inseticida. Fornece recursos alimentares para abelhas-europa.

Quanto as suas características morfológicas, é uma planta que apresenta caule verde ou com pigmentação avermelhada, ramos prostrados ascendentes capazes de originar raízes e recobertos por pilosidade branca. Folhas opostas cruzadas, curto-pecioladas e com o limbo ovalado ou em forma de losango, com as margens irregularmente serreadas e as faces também revestidas por pilosidade. Inflorescência terminal do tipo capítulo isolado (MOREIRA e BRAGANÇA, 2011).

Capítulos oblongos assentados sobre um longo eixo piloso e margeados por brácteas verdes que protegem numerosas flores de sexo diferenciado. As flores masculinas são periféricas e possuem lígula tridentada, podendo ocorrer também lígulas bidentadas ou inteiras, coloração amarela ou branca. No centro do capítulo inserem-se flores hermafroditas com corola amarela. Na maturação rompem-se as brácteas que margeiam o capítulo para dar lugar à disseminação dos frutos do tipo aquênio, coroados por um tufo de pelos sedosos. Pode ser identificada em campo por meio do conjunto formado pelos capítulos abertos com coloração amarelada e branca, associados a capítulos globosos frutificados, exibindo numerosos pelos sedosos (JACHAK et al., 2011).

A *T. procumbens* é encontrado em todo o mundo e tem sido usado para tratar anemia, resfriados, inflamações e hepatopatias na América Central (Taddei e Rosas-Romero, 2000). Na Guatemala, o *T. procumbens* é usado como tratamento antibacteriano, antifúngico e antiviral (CACERES et al., 1998), bem como para vaginite, dor de estômago, diarréia, inflamações da mucosa e infecções de pele (TADDEI e ROSAS-ROMERO, 2000). O suco da folha é usado para tratar feridas e parar o sangramento (CACERES et al., 1998). Um estudo feito em Chiquimula, Guatemala, mostrou que mulheres grávidas que amamentam que sofrem de anemia podem reduzir seus sintomas usando *Tridax* (Calderón, resultados não publicados). Esta espécie também é usada no tratamento de infecções gastrointestinais e respiratórias, hipertensão arterial e diabetes (PÖLL, 2005, GIOVANNINI et al., 2016. PARDESHI e BHIUNGADE, 2016). Na Guatemala, toda a planta é usada para o tratamento de infecções por protozoários (CACERES et al., 1998; BERGER et al., 1998, MARTÍN-QUINTAL et al., 2009, GAMBOA-LEON et al., 2014, EBILOMA et al., 2017), incluindo malária, leishmaniose e disenteria. Extratos aquosos de *T. procumbens* têm forte atividade anti-plasmodial contra parasitas *P. falciparum* resistentes à cloroquina (APPIAH-

OPONG et al., 2011); tem atividade contra o *Trypanosoma brucei*, propriedades antibacterianas e cicatrizantes de feridas (KORAM et al., 2014, AGYARE et al., 2016).

1.3 COMPOSTOS QUÍMICOS DA *Tridax procumbens*

Segundo Santos et al., (2013) a natureza várias espécies de plantas que apresentam diferentes compostos químicos que se subdividem em dois grupos, os metabólitos primários e secundários.

O metabolismo primário compreende várias reações químicas envolvidas na transformação de moléculas em unidades constitutivas essenciais das células. Já o metabolismo secundário não se relaciona diretamente à manutenção de vida do organismo produtor. Vegetais e alguns micro-organismos apresentam esse metabolismo diferenciado, em que os produtos formados, apesar de não ser essencial ao organismo produtor, garante vantagens para perpetuação e sobrevivência da espécie – são os metabolitos secundários (ARRAIAS, 2012).

A composição de metabólitos secundários nos tecidos da planta pode influenciar diretamente na qualidade e toxicidade dos alimentos produzidos para humanos e animais (GOMES; LIMA, 2014). A grande utilidade e diversidade de metabolitos secundários despertam o interesse de pesquisadores da atualidade que visam os vegetais como fonte promissora de novas moléculas uteis ao homem. A indústria agronômica, alimentícia e farmacêutica tem grande interesse na descoberta dessas moléculas, pois estas, devido a suas propriedades, possuem uma elevada importância comercial (ARRAIAS, 2012).

A origem dos metabólitos secundários pode ser resumida a partir do metabolismo da glicose via dois intermediários principais: o ácido chiquímico e o acetato. Apesar do precursor em comum, alguns desses metabolitos não derivam apenas de um desses intermediários, sendo estes resultantes da combinação de unidades de ácido chiquímico com unidades do acetato. Logo, os metabolitos se diferenciam seguindo a via metabólica em: derivados do acetato via ciclo do ácido cítrico; derivados do acetato via mevalonato e produtos da condensação do acetato (ARRAIAS, 2012).

Diante disso, para se avaliar os metabolitos presentes nas plantas são necessários processos de extração e fracionamento. Os extratos são preparações concentradas, que possuem consistências diversas, que conforme Santos et al., (2013) é possível extrair tanto de folhas secas, ou quando suas amostras vegetais são conduzidas através de maceração para obter extratos com princípios ativos. O produto é obtido pela passagem de um solvente, como por exemplo, a água ou o álcool etílico, através de partes de planta moída ou não, de modo a se retirar os princípios ativos contidos no vegetal (GOMES; LIMA, 2014).

Dentre os metabolitos encontrados na *T. procumbens* destaca-se os hidrocarbonetos e ácidos graxos de cadeia longa. Neofitadieno e hexadecanóico ácido foram encontrados como componentes principais no estudo realizado por Rajasekaran e Duraikannan (2012). O extrato de acetato de etila das partes aéreas continham uma mistura de ácidos graxos, compostos aromáticos, poliaromáticos ácidos carboxílicos e fenóis. O mesmo extrato das flores continha uma mistura complexa de hidrocarbonetos. Ambos os extratos, revelaram uma mistura complexa de açúcares. Entre eles, d-sacarose, galactose, frutose, galacturônico ácido, glicose, d-manopiranosídeo, d-alose, xilitol e arabinopiranose foram identificados. Outros derivados de pirimidina, carbazol, indol e curto ácidos carboxílicos de cadeia também foram identificados (ADJAGBA et al., 2014).

1.4 CICLO DE VIDA DO *Aedes (Stegomyia) aegypti* (LINAEUS, 1972), (DIPTERA: CULICIDAE)

Aedes aegypti (Linnaeus, 1762) (Diptera: Culicidae) é um mosquito de origem africana, provavelmente da região da Etiópia, considerado um importante vetor do vírus que causa a dengue nas Américas e da febre amarela na América Central e no Sul e no Oeste da África. Pertence ao Reino Animalia, Filo Arthropoda, Classe Insecta, Ordem Diptera, Família Culicidae, Gênero *Aedes*, Subgênero *Stegomyia* (MONTEIRO, 2014).

O ciclo de vida desse inseto compreende quatro fases de desenvolvimento: ovo, larva (com quatro estádios), pupa e adulto (LOZOVEI, 2001). As formas imaturas se desenvolvem em água doce, parada e com pouca matéria orgânica, embora tenha sido verificado também em ambientes poluídos (CLEMENTS, 1999). As fêmeas adultas possuem hábitos diurnos com pico das atividades hematofágicas e postura durante os períodos matutino e vespertino (FORATTINI, 2002).

O período larvário do *A. aegypti* é compreendido em quatro estádios (L1, L2, L3 e L4), ou seja, ocorrem três mudas, até que a larva de 4º estádio, o mais longo, que originará a pupa. Em média, a fase larvária ocorre em cinco dias, sendo influenciada pela temperatura, luz, densidade larvária do criadouro e da disponibilidade de alimentos. Após a fase larval vem o estádio da pupa, que dura de dois a três dias. É nesta fase que ocorre a metamorfose no mosquito (MONTEIRO, 2014). Os adultos vivem por cerca de 35-40 dias e se alimentam de substâncias açucaradas, como néctar e seiva. (BARBOSA, 2014).

O *A. aegypti* é considerado atualmente um dos principais problemas em saúde pública, devido ao seu papel como transmissor da dengue e febre amarela urbana. Ao longo de sua evolução, desenvolveu um comportamento estritamente sinantrópico e

antropogênico, sendo considerada a espécie de mosquito mais dependente do ambiente urbano. Seu habitat está intimamente ligado às condições domiciliares ou peridomiciliares ofertadas pelas populações humana (MONTEIRO, 2014).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o potencial larvicida no controle de *Aedes aegypti* do óleo e extratos brutos etanólico e aquoso das folhas de *Tridax procumbens* L.

2.2 OBJETIVOS ESPECÍFICOS

Identificar as principais classes de metabólitos secundários do óleo essencial e extratos brutos etanólico e aquoso da espécie *Tridax procumbens* L.;

Avaliar a atividade antioxidante do óleo essencial e extratos brutos etanólico e aquoso da espécie vegetal estudada;

Avaliar a toxicidade do óleo essencial e extratos brutos etanólico e aquoso da espécie em analise contra larvas de *Artemia salina*;

Analisar a atividade larvicida do óleo essencial e extratos brutos etanólico e aquoso da espécie em estudo contra larvas de *A. aegypti*;

Larvicidal evaluation against *Aedes aegypti* and antioxidant, cytotoxic and microbiological potential of the aqueous and ethanolic extracts of leaves of the *Tridax procumbens* L. plant species

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Larvicidal evaluation against *Aedes aegypti* and antioxidant, cytotoxic, and microbiological potential of the aqueous and ethanolic extracts of leaves of the *Tridax procumbens* (L.) L. plant species

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ABSTRACT: The present study compared the antioxidant, cytotoxic, microbiological and larvicidal potential of crude ethanolic extracts and aqueous of the leaves of *Tridax procumbens*, as well as, identified the main classes of secondary metabolites. The antioxidant activity was evaluated by the sequestration method of the 2,2-diphenyl-1-picrylhydrazyl radical. Cytotoxic activity was assessed using *Artemia salina*. The microbiological activity was performed by the microdilution method with bacteria *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The larvicidal bioassay was performed with larvae of the *Aedes aegypti* and the identification of the metabolites was performed by phytochemical tests. The phytochemical analysis of the extracts showed the presence of depsides, flavonoids, phenols, tannins, alkaloids, proteins, amino acids, and saponins. The crude aqueous extract presented good larvicidal activity when compared to the ethanol that had LC_{50}=86.53 μg.mL^{-1}. The microbiological results for the former were positive. For the three bacteria used, where the smallest MIC is 31.25 μL.mL^{-1} and CBM= 1.25 μL.mL^{-1}, it was present for the *E. coli* bacteria in the aqueous and ethanolic bacteria. For cytotoxic activity, it was non-toxic (LC_{50}=1323 μL.mL^{-1}). Thus, it was possible to identify better larvicidal, microbiological and antioxidant potential for crude aqueous extract when compared to ethanol.

Keywords: Erva de Touro; Asteraceae; Biological Assay.

1. INTRODUCTION

Many plant species have been used, from antiquity, for medicinal purposes in the search for treatment and cure of diseases, a factor that has spread throughout the world in an empirical way, passing from generation to generation. In this perspective, man has shown an interest in his well-being and quality of life, motivating the population and the scientific community to seek and identify new substances from the plant species that are beneficial to the human organism.

Among the species, there are those belonging to the family Asteraceae that have been used as raw material by the pharmaceutical industry for the creation and development of natural products due to its beneficial properties to humans. The Asteraceae family emerges as the origin of the center of the most primitive genera in Brazil [2]. However, the phylogenetic, taxonomic and chemosystematic information are incipient, reflecting the presence of little-known genera [3]. Ethnobotanical and ethnopharmacological studies indicate the therapeutic interest of species of this family through the medicinal use that traditional communities have in their social practices [4].

Across the world, the genus *Tridax* has been used to treat different diseases, there are several popular reports that point to the utility of this plant to circumvent innumerable diseases. It has components that have hypotensive effects [5], hepatoprotective action [6], immunomodulatory activity, antibiotic, antioxidant, anti-inflammatory and antitumor [7, 8, 9, 10].

Dengue, Chikungunya fever and Zika can be considered infectious diseases, transmitted by the same vector, the mosquito *Aedes aegypti*. These diseases are circulating at the same time in Brazil, putting public health on alert [11]. The control of *A. aegypti* can be accomplished through the elimination of larvae reproduction sites, as well as biological and chemical control by the use of insecticides [12].

This study aimed to evaluate the larvicidal potential against *A. aegypti* and the antioxidant, cytotoxic, and microbiological activity of the ethanolic crude and aqueous extracts of *Tridax procumbens*, as well as to identify the main classes of secondary metabolites present. Considering the use of this species by the population, the potential of the Asteraceae family and the need for studies, it aimed at identifying the advantages through biological activities.

2. MATERIAL AND METHODS

2.1. Plant material

Specimens of *T. procumbens* were collected in Fazendinha district (00°02'23 "S and 51°06'29" O) in Macapá-Amapá Municipality. Two samples of the species were deposited in the Amapaense Herbarium (HAMAB) of the Institute of Scientific and Technological Research of the State of Amapá (IEPA) under registration number 019178.

2.2. Vegetable extract

The ethanolic crude extract (EBE) was prepared by maceration, an exhaustive method in which 100 g of powdered leaves were placed in 1000 mL of ethanol. The macerate was stored at room temperature for 7 days, with occasional stirring. The extract was filtered and placed in a vacuum rotary evaporator at a temperature of 50 °C, under a pressure of 500 to 750 mmHg. The extraction process was repeated with the residue three times.

The aqueous crude extract (EBA) was prepared by maceration, an exhaustive method in which 100 g of powdered leaves were placed in 1000 mL of distilled water. The aqueous extract was then boiled for 30 minutes, cooled to 30 °C, and thereafter sieved and filtered on filter paper to a volume of 500 mL of aqueous extract.

2.3. Qualitative phytochemical analysis

The qualitative phytochemical analyzes of EBE and EBA were carried out using methods for detection of cardiac glycosides, catechins, flavonoids, sesquiterpene lactones and other lactones, purines, anthraquinones, steroids and triterpenoids, depsides and depsidones, polysaccharides, phenols and tannins, proteins and amino acids, organic acids, saponins and alkaloids [13].

2.4. Quantitative phytochemical analysis

2.4.1. Total phenolic content

The quantification of total phenolics was determined by the Folin-Ciocateu method, with some modifications [14]. Initially, an aqueous solution of gallic acid (5000 μg.mL^{-1}) was prepared for successive dilutions. Then, the calibration curve was performed at concentrations ranging from 10 to 500 μg.mL^{-1}. At these concentrations, aliquots of 400 μL of Folin-Ciocateu (10%) were added, followed by the addition of 1600 μL of Na_2CO_3 (75 g.L^{-1}). The mixture was incubated at 25 °C for 2 hours and the absorbance was measured in a spectrophotometer with a wavelength of 760 nm. After reading the calibration curve, an

aqueous solution of EBE (1 $mg.mL^{-1}$) was prepared and from this, 200 µL was added in a 10 mL flask followed by the addition of 400 µL of Folin-Ciocateu (10%), and 1600 µL Na_2CO_3 (75 $g.L^{-1}$) for quantification of the total phenolic content in the sample. The results were expressed as mg equivalent of gallic acid per gram of extract (mg EAG/g). The analysis was performed in triplicate and the value presented as the mean (± standard deviation).

2.5. Determination of antioxidant activity

2.5.1. Sequestration of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH)

The quantification of the antioxidant activity performed by the DPPH assay was based on the methodology proposed by Chen, Berlin e Froldi [15], Lopes-Lutz et al. [16], Hajlaoui et al. [17] with some adaptations relative to laboratory conditions. Initially, a methanolic solution of DPPH (stock solution) was prepared at a concentration of 40 $µg.mL^{-1}$, which was kept under the light. Then, six concentrations (7.81, 15.62, 31.25, 62.5, 125 and 250 $µg.mL^{-1}$) of EBE and EBA and the positive control (ascorbic acid and gallic acid) were prepared. For the evaluation, 2.7 mL of the DPPH stock solution was added to a test tube, followed by the addition of 0.3 mL of the EBE solution in different concentrations. The white solution was prepared, it being a mixture of 2.7 mL of methanol and 0.3 mL of a methanolic solution of each concentration of EBE evaluated. After 30 minutes, spectrophotometer readings (Biospectro SP-22) were performed at 517 nm wavelength [18]. The assay was performed in triplicate and the calculation of the percentage of antioxidant activity (%AA) was calculated with the following equation [15]:

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

%AA = percentage of antioxidant activity

Abs_{sample} = Sample Absorbance

Abs_{white} = white Absorbance

$Abs_{control}$ = Absorbance Control

The IC_{50} value was also calculated, denoting as the concentration of a sample required to decrease the absorbance at 517 nm by 50%. The IC_{50} was expressed in $µg.mL^{-1}$.

2.6. Cytotoxic activity

The cytotoxicity of the extracts was evaluated against larvae of *Artemia saline* Leach based on the study of Araújo et al. [19]. A solution of 250 mL of synthetic sea salt at 35 $g.L^{-1}$ was prepared, in which 25 mg of *A. salina* eggs were exposed to artificial lighting within 24 hours for hatching of the larvae (nauplii). The nauplii were then separated and placed in a dark environment at room temperature for another 24 hours, to reach methanuplion stages.

The stock solution was prepared to contain 0.06 g of the essential oil, 28.5 mL of the solution of synthetic sea salt and 1.5 mL of Tween 80, added to facilitate solubilization. Later, at the end of the dark period, they were selected and divided into 7 groups with 10 methanuplia in each test tube. In each group, aliquots of the stock solution (100, 75, 50, 25 and 2.5 μL) were added and the volume was made up to 5 mL with synthetic sea salt solution. Solutions with final concentrations of 40, 30, 20, 10 and 1 $\mu g.mL^{-1}$ were obtained in triplicates. In the end, the number of non-survivors for LC_{50} determination was counted using the SPSS® software PROBIT analysis.

2.7. Antimicrobial activity

2.7.1. Bacterial strains and culture conditions

The evaluation of the antimicrobial activity of EBA and EBE obtained from *T. procumbens* leaves were tested against two gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 25922, *Escherichia coli* ATCC 8789) and a gram-positive bacterium (*Staphylococcus 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.

2.7.2. 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 of the Clinical and Laboratory Standards Institute [20], with adaptations.

The bacteria were initially reactivated from the stock cultures and kept in BHI broth for 18 h at 37 °C. After bacterial growth, an inoculum was prepared in 0.9% saline solution for each microorganism. It was adjusted to the McFarland 0.5 scale, subsequently diluted in BHI and tested at 2×10^6 $UFC.mL^{-1}$ concentration.

For the determination of MIC, the extracts were diluted in 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 extracts at the concentration of 2000 $\mu g.mL^{-1}$. 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 $CFU.mL^{-1}$) adjusted according to the previous item was added to each well, resulting in a final volume of 0.2 mL. Control of the culture environment, control of the extracts, the negative control (DMSO 2%)

and the positive control were done using amoxicillin (50 µL.mL^{-1}). The experiments were carried out in triplicates. After incubation of the microplates in an incubator at 37 °C for 24 hours, the plates were read in ELISA reader (DO630nm).

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. The MBC was established as the lowest concentration of each extract capable of completely inhibiting microbial growth in Petri dishes after 24-48 hours of growth.

2.7.3. Statistical analysis of microbiological assays

All experiments were performed in triplicate, with the respective results categorized in Microsoft Excel (Version 2010 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 $p < 0.001$.

2.8. Larvicidal Activity

The larvae of *A. aegypti* used in the bioassays came from the colony maintained in the insectary of the Medical Entomology Laboratory of the Institute of Scientific and Technological Research of Amapá (IEPA), in the 3rd young stage. The biological tests were conducted in a room (3m x 4m) with controlled climatic conditions: temperature of 25±2 °C, relative humidity of 75± 5% and a photoperiod of 12 hours.

The methodology used followed the standard protocol of the World Health Organization (WHO) [21, 22] with modification in the test vessel. After preliminary tests, the aqueous solutions were selected at the concentrations: 20, 40, 60, 80, 100 µg.mL^{-1} pre-solubilized in 5% Tween 80.

For each repetition of treatment, 25 larvae were used, pipetted to a 100 mL beaker containing distilled water. Then, the larvae were removed from the beaker into the test vessel, thus minimizing the time between the preparation of the first and last samples. The safety of the solvent in the employed concentration was verified, being also present in the replicates of the control. During the experiment, the average water temperature was 25 °C. After 24 and 48 hours the dead larvae were counted, being considered as such all those unable to reach the surface.

2.9. Statistical analysis

The analysis of the data was performed through analysis of variance (ANOVA) and the Tukey test to identify significant differences between the averages, using the BioEstat program. Differences that presented probability levels less than and equal to 5% ($p \leq 0.05$) were considered statistically significant. The results were expressed as mean ± standard deviation (SD).

3. RESULTS

3.1. Qualitative phytochemical analysis

The phytochemical analysis of EBE and EBA of *T. procumbens* leaves showed the presence of depsides, depsidones, flavonoids, phenols, tannins, proteins, amino acids, saponins, and alkaloids, among 14 constituents that were tested. As can be identified in Table 1 below.

Table 1. Comparison between the chemical constituents of EBE and EBA of leaves of *T. procumbens*.

TEST	Results	
	EBA	EBE
Cardiac glycosides	-	-
Catechins	-	-
Flavonoids	+	+
Sesquiterpenolactones and other lactones	-	-
Purines	-	-
Anthraquinones	-	-
Steroids and triterpenoids	-	-
Depsides and depsidones	+	+
Polysaccharides	-	-
Phenols and catheter tannins	+	+
Proteins and amino acids	-	+
Organic acids	-	-
Saponins	-	+
Alkaloids	+	+

Parameters: present (+); absent (-).

The EBE and EBA showed similarity in the compounds identified in the phytochemical tests. As observed in them, only two compounds, saponins and proteins, and amino acids were identified, which were identified only in EBA.

3.2. Quantitative phytochemical analysis

Table 2 shows the total phenolic content found in the leaves of *T. procumbens*.

Table 2. Total phenolics of EBE and EBA from leaves of *T. procumbens.*

Extracts	Phenolic compounds (mg GAE/g)	Phenolic compounds (%)
EBE	37.5 µg	3,75
EBA	10.36 µg	1,036

It can be observed that, in Table 2, EBE presented a higher percentage of phenolic constituents in relation to EBA, when compared the percentage of phenolic compounds in the extracts. The evidence that the extracts present % of phenolic compounds, EBE equal to 3.75% and EBA equal to 1.036%, it suggests positive antioxidant activity.

3.3. Larvicidal activity against Aedes aegypti

The larvicidal activity of leaf extracts of *T. procumbens* was tested at five different concentrations: 20, 40, 60, 80, 100 µg.mL^{-1} at 24 and 48 hours, as can be seen in the table.

Table 3. Percentage of mortality (%) of *A. aegypti* larvae in different concentrations of EBE and EBA of the leaves of *T. procumbens* in 24 and 48 hours.

	Larvicidal activity (%) in EBE		Larvicidal activity (%) in EBA	
Concentrations (µg.mL^{-1})	**24 h**	**48 h**	**24h**	**48h**
Control (-)	0	0	0	0
Control (+)	100	100	100	100
20	12	12	25,32	32
40	13,2	16	29,32	33,32
60	14,64	18,64	40	44
80	18,4	18,64	49,2	50,64
100	22,4	22,6	52	52
CL_{50}	247,23	261,70	89,28	86,52
CL_{90}	486,98	536,58	220,55	259,53

Source: Own author. * p <0.001 statistically different in relation to extracts and their concentrations.

According to the table, the concentrations of 80 and 100 μg.mL^{-1} of the EBA showed larvicidal activity after 24 h, with mortality higher than 50%. The bioassay determined the LC_{50} of the EBE 247.23 μg.mL^{-1}, with R^2 = 0.956 and the EBA the LC_{50} was 89.28 μg.mL^{-1} with the Regression Coefficient (R^2) = 0.904.

Mortality at 48 hours against *A. aegypti* from EBA and EBE can be seen in table 3. According to the graph, concentrations of 60, 80 and 100 μg.mL^{-1} of the EBA showed better larvicidal activity after 48h, they being higher than 50%. The bioassay determined the LC_{50} of EBE 261.70 μg.mL^{-1}, with R^2 = 0.950 and EBA 86.52 μg.mL^{-1} with R^2 = 0.880.

3.4. Cytotoxic activity with Artemia salina

Another analysis was the toxicity of EBA and EBA against *A. salina*, where it is possible to evaluate, in a preliminary way, the toxicity of the substances against marine organisms [23]. The data in Table 4 demonstrate a 3.3% mortality at the concentration of 1000 μg.mL^{-1} in both extracts, as it can be seen below.

Table 4 – Percentage of mortality of *A. salina* larvae of *T. procumbens* extracts at different concentrations.

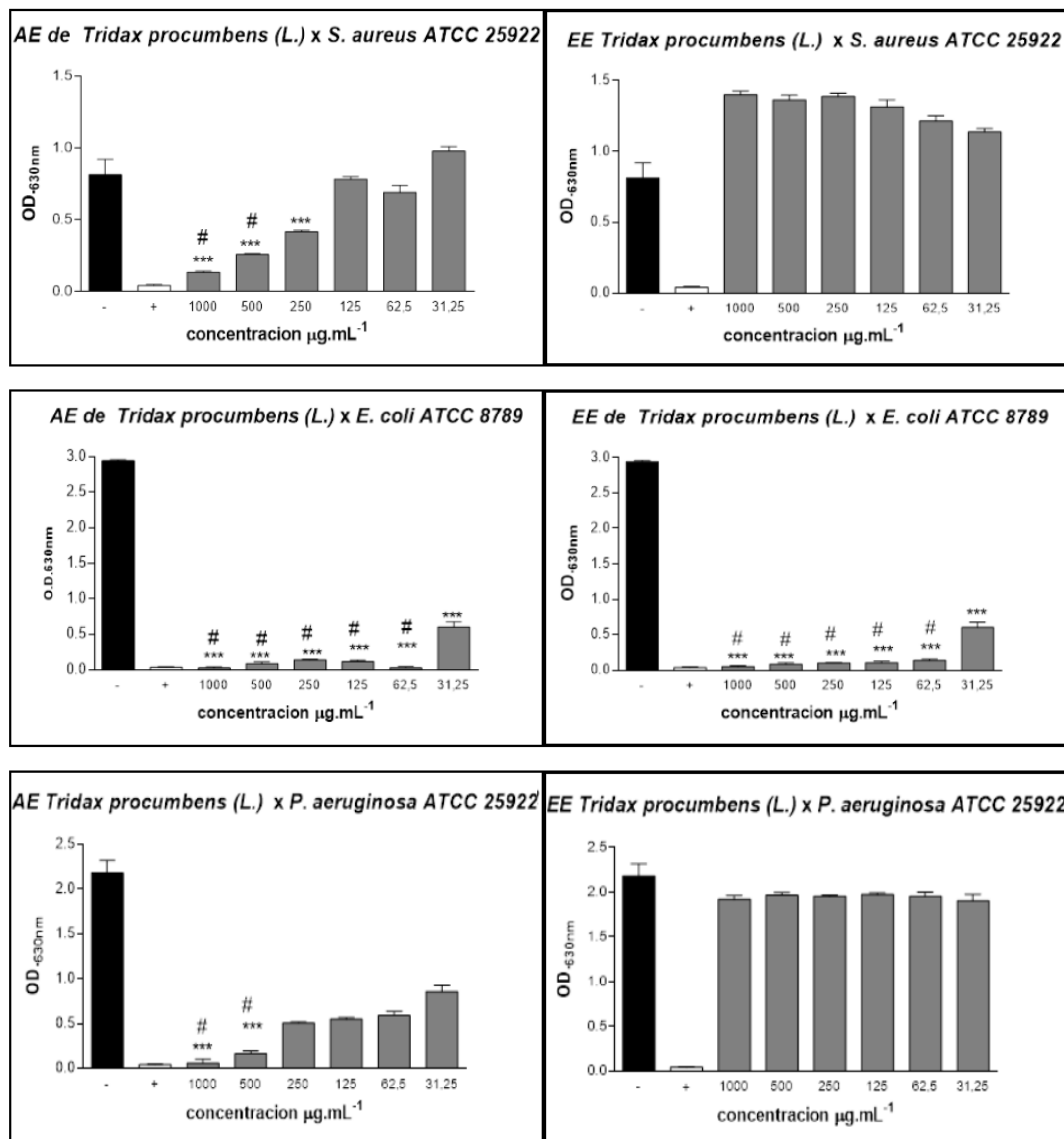
Concentrations of extracts (μg.mL-^{1})	**Mortality (%)**	
	EBE	**EBA**
50	0	0
100	0	0
250	0	0
500	0	0
750	0	0
1000	3.3	3.3
LC_{50}	1.323 μg.mL^{-1}	1.323 μg.mL-^{1}
LC_{50} ($K_2Cr_2O_7$)	12.60 μg.mL^{-1}	12.60 μg.mL^{-1}

3.5. Microbiological activity

Image 1 refers to the microbiological activity of EBA and EBE of *T. procumbens*. The results showed that Gram-negative bacteria *E. coli* and *P. aeruginosa* and Gram-positive bacteria *S. aureus* showed susceptibility to *T. procumbens* extracts. EBA presented the best activity with minimum inhibitory concentration (MIC) of 250 μg.mL^{-1} and in 500 μg.mL^{-1} presented the best Minimum Bactericidal Concentration (MBC) when compared to EBE for *S. aureus* bacteria. A similar result was found in the *E. coli* bacteria, where both extracts had a MIC of 31.25 μg.mL^{-1} and MBC of 62.5 μg.mL^{-1}, demonstrating susceptibility to *T. procumbens* extracts. As for *P. aeruginosa* bacteria, only EBA presented bacteriostatic and

bactericidal activity with MIC and MBC of 500 µg.mL^{-1}, as it can be seen in the following image.

Image 1. Sensitivity Test (MIC) and (MBC) the EBA and EBE of *T. procumbens* against the *E. coli, S. aureus* and *P. aeruginosa.*



Source: Own author. Substance test (■), BHI with 2% DMSO (□) and Amoxiline (■). * P <0.001 statistically significance in relation to the negative control, # *p* <0.001 statistically significance in relation to the positive control.

3.6. Antioxidant activity by the DPPH method

The antioxidant capacity of natural products may be related to the composition of phenolic compounds, which causes the interruption of the free radical chains in the initiation stage of the oxidative process, leading to degradation [24]. Among the several ways to evaluate the antioxidant potential of natural products, it is the method of sequestration of the

DPPH radical. This antioxidant activity is measured by the consumption of DPPH, the higher the intake of DPPH in a sample, the lower the IC_{50} and the greater its activity.

The results obtained after the determination of the antioxidant activity of the EBA and EBE in different concentrations are shown in Table 5. The EBA reached its maximum antioxidant activity (62.41%) in a concentration of 250 $\mu g.mL^{-1}$ and the EBE reached its maximum activity antioxidant (35.13%) in a concentration of 250 $\mu g.mL^{-1}$.

Table 5. - Mean and standard deviation of the percentage of antioxidant activity of extracts of *T. procumbens* and Ascorbic Acid at different concentrations.V.

Concentrations extracts	**% of antioxidant activity**		
	EBE	**EBA**	**Ascórbic A.**
7.81	12.13± 0.85	11.5±0.6	18.57±0.52
15.62	16.14± 0.8	17.14 ±0.85	30±0,10
31.25	16.24±0,0	20.31±0.35	99.93±0.02
62.5	17.55 ±0,0	29.23 ± 2.62	99.99±0.0
125	27,0 ± 0.85	56.36 ± 0.3	99.99±0.0
250	35.13 ± 1.24	62.41 ± 0.30	99.99±0.0
IC_{50}	403.54 $\mu g.mL^{-1}$	160.06 $\mu g.mL^{-1}$	16.71 $\mu g.mL^{-1}$

Equation of antioxidant activity of EBE Y= 0.0912x + 13.1969 and EBA Y= 0.2186x+15.0414.

The correlation between the antioxidant activity (%) and the extracts concentration showed a high IC_{50}, 403.54 $\mu g.mL^{-1}$ for EBE and 160.06 $\mu g.mL^{-1}$ for EBA, when compared with the ascorbic acid, with IC_{50} of 16.71 $\mu g.mL^{-1}$.

4. DISCUSSION

4.1. Quantitative and qualitative phytochemical analysis

Both extracts presented similarity in the compounds identified in the phytochemical tests. It was observed variation only in saponins, proteins, and amino acids, which were only identified in EBA. It was carried out to the phytochemical characterization of this species, where it was demonstrated the presence of alkaloids, carotenoids, flavonoids (catechins and flavones), saponins and tannins in aqueous extract [25].

The presence of saponins in the aqueous extract may be related to its lipophilic structure, which may justify its presence in EBA and absence in EBE. This can be affirmed in a study where it was possible to relate the presence of saponin in the aqueous extract with the factor of its structure presenting a lipophilic part called aglycone or sapogenin and a hydrophilic part composed of one or more sugars, making its interaction easier [26].

The positive result for saponins may represent a better performance of EBA in biological activities. The saponins may represent amphiphilic behavior, presenting the ability to form complexes with steroids, proteins and membrane phospholipids, it enabling diverse biological actions [27].

In addition to the presence of saponins, EBA showed positivity for proteins and amino acids. This positivity in the phytochemical test can be explained by the denaturation due to the temperature used in the extraction of EBA, denaturing the most sensitive amino acids. Amino acids are organic molecules attached to the same carbon atom (called carbon α) a hydrogen atom, an amine group, a carboxylic group, and an "R" side chain unique for each amino acid [28]. This side chain is what differs amino acids in their structure, size, electrical charges, and water solubility. This makes it possible for EBA to show more efficient biological results because its structure presents significant compounds.

Both EBE and EBA presented alkaloids in their composition. This compound represents a heterogeneous group of organic substances, in particular of vegetal origin, that is characterized by the presence of nitrogen [29]. It can be classified according to Its precursor molecules [30], such as derivatives of amino acids, peptides, and acetate. It has some biological activities found in the literature, such as the acetylcholinesterase inhibitory activity, antioxidant activity, and antineoplastic activity [31].

In addition to the alkaloids, the phytochemical screen identified the presence of depsides in its composition. depsides result from the esterification of two or more units derived from orselinic acid or beta-methyl-orselinic acid. Depsidones represent a group of compounds structurally related to the depsides, which are considered its precursors. These groups have been recognized for their antioxidant, antiviral, antitumoral, analgesic and antipyretic properties [32, 33, 34, 35].

The EBE presented a higher percentage of phenolic constituents in relation to the EBA. This may be related to the affinity of these substances whit polar solvents. Thus, it can improve their extraction, since most of the phenolic compounds are not found in the free state in nature, but in the form of esters or heterosides. However, they are soluble in water and in polar organic solvents [36].

The evidence that the extracts present % of phenolic compounds, EBE equal to 3.75% and EBA equal to 1.036%, suggest positive antioxidant activity. This may be related to these compounds, which are capable of inhibiting lipid peroxidation and lipoxygenase in vitro [37].

4.2. Larvicidal activity against Aedes aegypti

The bioassay determined the LC_{50} and R^2 of the EBA and EBE, determining the concentrations capable of causing mortality in 50% of the larvae and the regression coefficient, respectively. The LC_{50} of EBA = 86.52 $\mu g.mL^{-1}$ and R^2 = 0.880 was obtained evidencing the potential larvicidal effectiveness of this product. Substances with LC_{50} values below 100 $\mu g.mL^{-1}$ are regarded as a good larvicidal agent. While the EBE did not present LC_{50} 261.70 $\mu g.mL^{-1}$ satisfaction at the tested concentrations [38].

In the study conducted by Rajasekaran and Duraikannan [39], it was not possible to identify the effectiveness of *T. procumbens* EBA against *A. aegypti* larvae, whereas EBE showed high larval mortality at the highest concentrations, with a percentage equal to 84% of death [40]. These data corroborate the idea that the extract of this species presents a potential for larvicidal activity.

The best performance of EBA in relation to EBE may be related to saponiferous components, which are compounds that provide greater consistency in oxidation and are composed of sugars bound to triterpenes or steroids, such as aglycones. A mixture of saponins obtained from *Quillaja saponaria* showed important larvicidal activity [41], as well as, in the study by Moreira et al. [42] where it was possible to isolate three compounds of this class, isolated from *Ramalina usnea*, which had moderate larvicidal activity against *A. aegypti* and related this activity to the compound found in the plant.

However, it is difficult to associate only a single substance with a particular biological activity, because it is known that due to the diversity of compounds present in the extracts, often the biological action occurs due to a synergistic action of the constituents present. Therefore, to affirm that the action can come from only one substance, studies that evaluate action of the substance alone are needed [43, 44].

4.3. Cytotoxic activity on Artemia salina

It is possible to identify that both EBE and EBA did not present toxicity as the test performed on *A. salina* larvae. This can be reaffirmed by the study that classifies the plant extracts in degrees of toxicity according to interval. Extracts that are considered of high toxicity, exhibit CL_{50} below 100 $\mu g.mL^{-1}$, extracts of moderate activity exhibit LC_{50} between 500 and 1000 $\mu g.mL^{-1}$ and extracts with low toxicity present LC_{50} above 1000 $\mu g.mL^{-1}$ and are considered to be non-toxic [16].

In this sense, there are studies in the literature that show a good correlation between the toxicity tests on this species and their applicability in different biological activities, such as, antifungal, antimicrobial, parasiticidal, antitumor, among others, favoring the interest for

this species in future studies. And this value is below the toxicity standard of potassium dichromate with LC_{50} of 12.60 $\mu g.mL^{-1}$. This thought resembles studies that conclude that species of low lethality in A. salina are shown to have great potential for various biological activities [45].

4.4. Microbiological activity

The microbiological activity aims to evaluate the effect of the chemical constituents of the extracts and oils on the bacteria, in order to identify the way the bacterium is damaged. This may be interconnected to cytoplasmic membrane disturbance, membrane protein damage, cytoplasmic coagulation, electron flow alteration, disruption of proton power, impaired active transport, and reduction of intracellular ATP pool [46, 47]. The bactericidal and fungicidal activities occur by three general characteristics common to the two groups of tannins: complexation with metallic ions; antioxidant activity and free radical scavenger; ability to complex with other molecules, mainly proteins and polysaccharides [48].

The significant antimicrobial potential found in EBE against *E. coli* and *P. aeruginosa* resembles the study, where activity against *S. hemolytic*, *B. cereus*, *P. aeruginosa*, *E. coli*, and *C. albicans* was observed and with the study that also showed that ABA and EBE of the aerial parts presented antimicrobial activity on the same bacterium (*E. coli*) [49, 50].

EBA presented significant antimicrobial potential against the bacteria tested in this study. This result may be related to the secondary compounds found in the extract, such as saponins. Saponins are substances derived from the secondary metabolism of plants, mainly related to the defense system [51]. They are found in tissues that are more vulnerable to fungal, bacterial or predatory attacks of insects, being considered part of the plant defense system and indicated as "plant protection" [52]. This activity may be related, among other mechanisms, to interaction with membrane sterols [53].

4.5. Antioxidant activity by the DPPH method

Both extracts presented low antioxidant activity when compared to ascorbic acid. Although both extracts present compounds as alkaloids, which has in the literature identified antioxidant activity. One study showed in their experiment that variations in the content of the indole alkaloids, coronaridine, and voacangin in extracts of *T. catharinensis* did not alter the antioxidant potential in vitro [31].

However, it is possible to identify a significant difference when comparing the antioxidant activity of the two extracts, and it is possible to identify a better activity in the EBA.

In the EBA, the presence of saponins, proteins, and amino acids were detected. These compounds are supposedly related to the highest percentage of antioxidant activity when

compared to EBE [03]. At the concentration equivalent to 200 μg.mL^{-1}, the percentage of antioxidant activity was 62.41 ± 0.30, the estimated IC_{50} was 160.06 μg.mL^{-1} with a high statistical significance of the data (p-value = 0.0001).

In vitro evidence demonstrates the ability to capture hydroxyl radicals from the aqueous extract of *T. procumbens* [54]. It is attempted to explain this process through the use of a deoxyribose oxidation system by the free radical generated by reduced iron in the presence of hydrogen peroxide. Polyphenolic constituents, particularly flavonoids, represent an important form of antioxidant defense due to their chelating effects on metals. It is possible that plant extracts have a chelating effect on metal ions, especially Fe^{2+} ions, which are capable of generating ROS, peroxides which are implicated in many diseases including inflammatory diseases, from the Fenton reaction. By decreasing the bioavailable concentrations of Fe^{2+}, these substances can protect cells against oxidative damage, which would explain their positive performance in an antioxidant activity.

The extract of the leaves/flowers of *T. procumbens* presented better antioxidant activity (AA) compared to root and stem extracts, and the highest AA was obtained in the dynamic maceration extraction with IC_{50} of 17.80 μg.mL^{-1} [55], in contrast to the data found in the EBA and EBE of this study. This factor may be associated with the analytical conditions in which the species was submitted. The climatic conditions, soil, the period of collection, among other factors influence the presence or absence of the secondary compounds produced by the species.

Although the EBE presents better results in terms of total phenolic content in its composition, EBA may have presented a better antioxidant activity due to its solubilization occurring in the aqueous environment, contributing to the fact that some compound, in particular, is responsible for this activity more effectively. The literature suggests that there is some constituent that contributes particularly and more effectively to the free radical sequestering action when the result of % total phenols does not correspond proportionally to the antioxidant activity [56].

Ribeiro et al. [57] identified in his study that *T. procumbens* species may present better antioxidant results because in aqueous solution, O^{2-} can be converted to hydrogen peroxide (H_2O_2) from the partial reduction of molecular oxygen by two electrons. By making this conversion occur enzymatically, through the action of superoxide dismutase (SODs), xanthine oxidase, amino acid oxidase and NAD(P)H oxidase. H_2O_2 diffuses easily through cell membranes [58, 59] and may generate the hydroxyl radical (OH) in the presence of transition metals, such as Fe^{+2}, Fe^{+3} or Cu^{+2} [60].

5. CONCLUSION

The chemical composition of extracts of *T. procumbens* indicated the presence of 08 substances, in total. The EBA presented depsides and depsidones, flavonoids, phenols and tannins, proteins and amino acids, saponins, and alkaloids in its composition. While EBE demonstrated depsides, depsidones, phenols, cathemical tannins, and alkaloids in its extract.

In the study, the aqueous extract presented a better larvicidal potential, antioxidant by the DPPH radical capture method when compared to EBE and below adequate when compared to the ascorbic acid standard. Both extracts presented low toxicity to *A. salina*. The aqueous extract presented better microbiological potential compared to EBE. These results highlight the advantages of this species for the development of natural products, which can be developed for the benefit of society, without causing negative impacts on the environment.

It was possible to observe that EBA is a promising natural larvicidal agent for use in *A. aegypti* larval growth sites. New studies should be developed to combat this vector since it is related to diseases considered serious public health problems. Therefore, for the development of commercial production of natural origin it is important to deepen the studies on the toxicity towards the human being and to the environment.

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Larvicidal evaluation against *Aedes aegypti* and antioxidant and cytotoxic potential of the essential oil of *Tridax procumbens* L. leaves

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Larvicidal evaluation against *Aedes aegypti* and antioxidant and cytotoxic potential of the essential oil of *Tridax procumbens* L. leaves

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ABSTRACT: The present study evaluated the antioxidant, cytotoxic and larvicidal potential of the essential oil of *Tridax procumbens* leaves, as well as, it identified the compounds present in their essential oils. The antioxidant activity was evaluated by the sequestration method of 2,2-diphenyl-1-picrylhydrazyl radical, the cytotoxic activity was evaluated using *Artemia salina*, the larvicidal bioassay was performed with larvae in the third stage of development of the *Aedes aegypti* mosquito, and the identification of the metabolites was performed by gas chromatography coupled to the mass spectrometer (GC-MS). The phytochemical oils analysis showed the presence of 20 compounds, being the main ones: thymol and γ-Terpinene. It presented antioxidant activity with IC_{50} of 194.51 $\mu g.mL^{-1}$, demonstrating antioxidant activity in the highest concentrations tested. It presented low cytotoxic activity against *A. salina*, with LC_{50} of 1238.67 $\mu g.mL^{-1}$, demonstrating atoxicity in the concentrations tested. The essential oil presented good larvicidal activity when compared to the literature, with LC_{50} = 79.0 $\mu g.mL^{-1}$ in 24 hours and LC_{50} = 69.15 $\mu g.mL^{-1}$ in 48 hours. In this way, it was possible to identify that the essential oils of *T. procumbens* leaves presented potential for the development of natural insecticides, demonstrating a satisfactory antioxidant activity and low toxicity to *A. salina*.

Keywords: Erva de Touro, Asteraceae, Biological Test.

1. INTRODUCTION

Several plant species, including those belonging to the Asteraceae family, have been studied and used as raw material by the pharmaceutical industry for the creation and development of natural products, with natural biodegradable pesticides and anticancer activities.

The *Tridax* genus has been used to treat different diseases; there are several popular reports that point to the usefulness of this plant to circumvent innumerable diseases. In traditional medicine, it is used as antidiabetic [1], antifungal [2], repellent [3] and immune [4]. In addition, it has a healing activity and promotes hair growth [5].

In addition to pharmacological effects, some plants are being studied because of their insecticidal properties. Botanical insecticides are compounds that result from the secondary metabolism of plants [6], they compose their own chemical defense against herbivorous insects, possess compounds capable of interfering in basic biochemical processes with physiological and behavioral consequences on insects. This type of insecticide has several advantages such as rapid action and degradation, low to moderate toxicity to mammals, increased selectivity and low phytotoxicity [7].

Among the vectors of diseases in Brazil, the mosquito *Aedes aegypti* stands out, it is a transmitter of diseases such as Dengue, Chikungunya, and Zika. The control of this vector can be accomplished through the elimination of larvae reproduction sites, and its biological and chemical control can be performed through the use of insecticides [8]. In view of the operational and economic difficulties generated by the increasing resistance of mosquitoes to synthetic insecticides, alternative methods are becoming more prominent and are becoming more efficient and cheaper since they are obtained from renewable resources that are rapidly degradable and have several substances which act simultaneously, making the resistance of insects to these substances occur very slowly [4]. Many studies seek to evaluate the behavior of this vector against the formulation of products with plant origin, in order to identify species that effectively combat *A. aegypti.*

The Tridax procumbens species presents as a potential alternative to combat these vectors, through their studies with extracts. However, its yield for essential oil is low, in this study the yield of essential oil was 0.02%.

Considering the use of this species by the population, the potential of the Asteraceae family and the need for studies aimed at identifying the advantages through biological activities, the present study aimed to evaluate the larvicidal, antioxidant, and cytotoxic potential of the essential oil of *Tridax procumbens*, as well as to chemically identify the compounds present in this plant.

2. MATERIAL AND METHODS

2.1. Plant material

Specimens of *T. procumbens* were collected in Fazendinha district (00 ° 02'23 "S and 51 ° 06'29" O) in Macapá-Amapá Municipality. Two samples of the species were deposited in the Amapaense Herbarium (HAMAB) of the Institute of Scientific and Technological Research of the State of Amapá (IEPA) under registration number 019178.

2.2. The obtaining process of the essential oil

The process of obtaining the Essential Oil (EO) was carried out at the Laboratory of Pharmacognosy and Phytochemistry of the Federal University of Amapá (UNIFAP), where the leaves of the plant species were dehydrated in a greenhouse with air circulation at 36 ºC. The EO was extracted by hydrodistillation in a Clevenger-type apparatus at 100 ° C for two hours [9]; it was stored in an amber bottle and cooled to -20 ° C in the dark.

2.3. Qualitative phytochemical analysis

The phytochemical evaluation of the essential oil was determined by gas chromatography coupled to the mass spectrometer (GC-MS) in equipment of the Shimadzu brand, model GCMS-QP 5050A, in a DB-5HT column of the J & W Scientific brand, with a length of 30 m, a diameter of 0.32 mm, a film thickness of 0.10 μm and nitrogen as a carrier gas. Thus, the operating conditions of the gas chromatograph were: internal pressure of the column of 56.7 kPa, split ratio of 1:20, the gas flow in the column of 1.0 mL.min^{-1}. (210 ° C), the temperature in the injector of 220 ° C, temperature in the detector or in the interface (GC-MS) of 240 ° C. The initial column temperature was 60 ° C, followed by an increase of 3 ° C. min $^{-1}$. up to 240 ° C, it is held constant for 30 min. The mass spectrometer was programmed to perform readings in a range of 29 to 400 Da, at intervals of 0.5 s, with ionization energy of 70 eV.

1μL of each sample with a concentration of 10,000 ppm dissolved in hexane was injected. The identification of the constituents was based on the comparison of the Kolvats Index (IK) and mass spectra of each substance with the literature data.

2.4. Determination of the antioxidant activity

2.4.1. Sequestration of the 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The quantification of the antioxidant activity performed by the DPPH assay was based on the methodology proposed by Sousa et al. [11], Lopes-Lutz et al. [12] and Andrade et al. [13] with some adaptations to laboratory conditions. Initially, a methanolic solution of DPPH (stock solution) was prepared at a concentration of 40 μg.mL^{-1}, which was kept under the

light. After that, six concentrations (7.81, 15.62, 31.25, 62.5, 125 and 250 $\mu g.mL^{-1}$) of EO and of the positive control (ascorbic acid and gallic acid) were prepared. For the evaluation, 2.7 mL of DPPH stock solution was added to a test tube, followed by the addition of 0.3 mL of the EO solution in different concentrations. Then, the white solution was prepared, this being a mixture of 2.7 mL of methanol and 0.3 mL of a methanol solution of each EO concentration evaluated. After 30 minutes, spectrophotometer readings (Biospectro SP-22) were performed at 517 nm wavelength [14]. The assay was performed in triplicate and the calculation of the percentage of antioxidant activity (% AA) was calculated with the following equation [11]:

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

%AA = percentage of antioxidant activity

Abs_{sample} = Sample Absorbance

Abs_{white} = white Absorbance

$Abs_{control}$ = Absorbance Control

The IC_{50} value was also calculated, it denoting as the concentration of a sample required to decrease the absorbance at 517 nm by 50%. The IC_{50} was expressed in $\mu g.mL^{-1}$.

2.5. Cytotoxic activity

The cytotoxicity of the oil was evaluated against larvae of Artemia Saline Leach-based study, with adaptations [15]. A solution of 250 mL of synthetic sea salt at 35 $g.L^{-1}$ was prepared. In this, 25 mg of *A. salina* eggs were incubated and exposed in artificial lighting within 24 hours for the larvae hatching (nauplii stage). The nauplii were then separated and placed in a dark environment at room temperature for another 24 hours to reach methanuplion stages. The stock solution was prepared to contain 0.06 g of the essential oil; 28.5 mL of the solution of synthetic sea salt and 1.5 mL of Tween 80 were added to facilitate solubilization. Subsequently, at the end of the dark period, they were selected and divided into 7 groups with 10 methanuplia in each test tube. In each group were added aliquots of the stock solution (100, 75, 50, 25 and 2.5 µL) and the volume was supplemented to 5 mL with a solution of synthetic sea salt, obtaining solutions with final concentrations of 40, 30, 20, 10 and 1 $\mu g.mL^{-1}$, in triplicates. In the end, the number of non-survivors for LC_{50} determination was counted using the SPSS® software PROBIT analysis.

2.6. Larvicidal Activity

The larvae of *A. aegypti* used in the bioassays came from the colony maintained in the Medical Entomology Laboratory of the Institute of Scientific and Technological Research of Amapá (IEPA), in the 3rd young stage. The biological tests were conducted in a room (3m x 4m) with controlled climatic conditions: temperature of 25 ± 2 ° C, relative humidity of 75 ± 5% and a photoperiod of 12 hours.

The methodology used followed the standard protocol of the World Health Organization WHO [16, 17] with modification in the test vessel. After preliminary tests, the aqueous solutions were selected at the concentrations: 20, 40, 60, 80, and 100 $mg.mL^{-1}$, they were pre-solubilized in Tween 80 at 5%.

For each repetition of treatment, 25 larvae were used, pipetted to a 100 mL beaker containing distilled water. Then, the larvae were removed from the beaker into the test vessel, thus minimizing the time between the preparation of the first and last samples. The safety of the solvent in the employed concentration was verified, being also present in the replicates of the control. During the experiment, the average water temperature was 25 ° C. After 24 and 48 hours, the dead larvae were counted, being considered as all those that can not reach the surface. 2.7 Statistical analysis

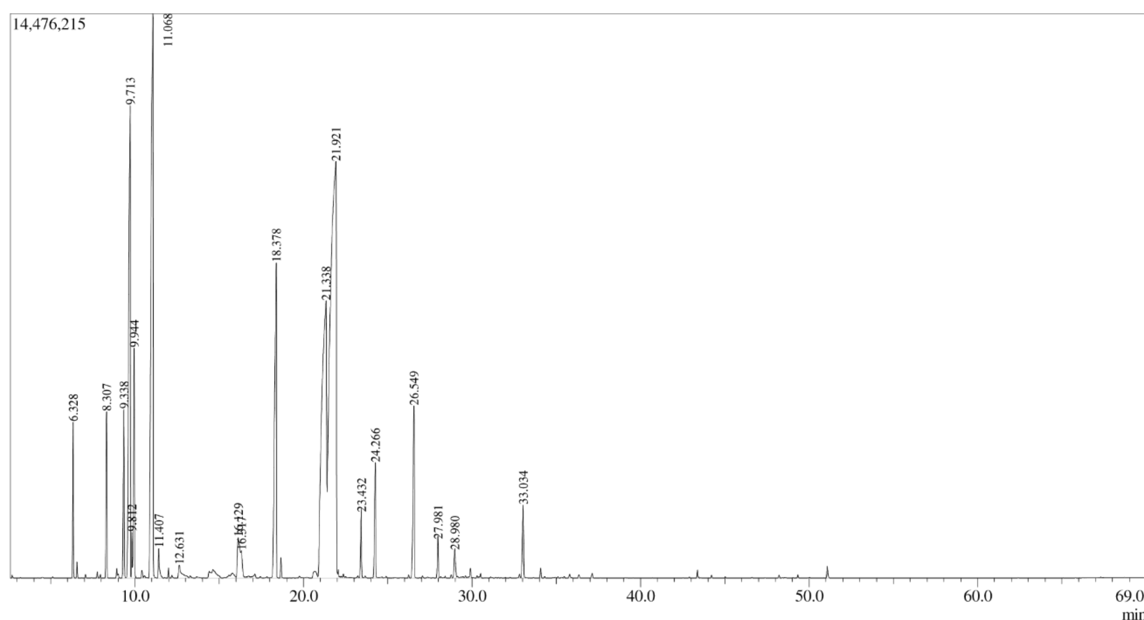
Data analysis was performed through analysis of variance (ANOVA) and the Tukey test, aiming to identify the significant differences among averages, using the BioEstat program. Differences that presented probability levels less than and equal to 5% ($p \leq 0.05$) were considered statistically significant. The results were expressed as mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1. Qualitative phytochemical analysis

The GC-MS chromatogram of the EO obtained is shown in Figure 1, where it is possible to identify the peaks and compounds identified in the sample.

Figure 1 – Chromatogram of compounds identified in GC-MS analysis of *T. procumbens* essential oil



In the GC-MS analysis of the essential oil of *T. procumbens*, it was possible to identify 20 compounds divided between sesquiterpenes and oxygenated terpenes, according to Table 1.

Table 1 – Substances identified in GC-MS analysis of *T. procumbens* essential oil.

Nº	t_R(min)	IK*	Compounds	Relative percentage
1	6.328	1002	α-Phellandrene	1.33
2	8.307	990	β–Myrcene	1.82
3	9.338	1001	(+)-2-Carene	2.19
4	9.713	1022	*o*-Cymene	10.27
5	9.812	1024	Limonene	0.38
6	9.944	1026	1,8-Cineole	2.37
7	11.068	1054	γ-Terpinene	15.93
8	11.407	1098	Sabinene hydrate	0.40
9	12.631	1098	Sabinene hydrate	0.22
10	16.129	1177	Terpinen-4-ol	1.22
11	16.317	1171	Umbellulone	0.53
12	18.378	1244	Carvacrol, methyl ether	7.70
13	21.338	1290	Thymol	16.52
14	21.921	1299	Thymol	31.70
15	23.432	1352	Thymol acetato	0.86
16	24.266	1352	Thymol acetato	1.69
17	26.549	1424	β–Caryophyllene	2.75
18	27.981	1454	α–Humulene	0.53
19	28.980	1489	Butylated hydroxy anisole	0.51
20	33.034	1583	Caryophyllene oxide	1.09

IR= Retention index of Van den Dool e Kratz (1963); t_R= Retention time. Identification of the compounds was performed by the mass spectrum (MS) OF THE LIBRARY SOFTWARE Lansolutions GC-MS solution version 2.50 SU1 (NIST05 and WILEY'S Library of Mass spectra 9th Edition).

Among the major compounds of *T. procumbens* oil, Thymol (48.22%), γ-Terpinene (15.93%) and *o*-Cymene (10.27%) are the most important.

There are few EO studies of the *T. procumbens* species. It is a plant native to the African and Asian continents, found easily in countries like India, Malaysia and China. In the study by Manjamalai, Valavil and Grace [18], it was possible to identify compounds such as Sabinene hydrate, α-Phellandrene, β-Caryophyllene, Limonene and *o*-Cymene in *T. procumbens* EO.

In the study, the *Eupatorium ballotifolium* Kunth, belonging to the same family as *T. procumbens*, the family Asteraceae, shows similarity in its majority compound [19]. In the two species, Thymol and *o*-Cymene, were identified among the major compounds of the EOs analyzed. These compounds are mentioned in the literature as promising biological agents in different activities, demonstrating the promising potential of the *T. procumbens* species, which presents about 47% Thymol in its composition.

3.2. Larvicidal activity against Aedes aegypti

The larvicidal activity of *T. procumbens* EO was tested in five different concentrations: 20, 40, 60, 80, 100 μg.mL^{-1} at 24 and 48 hours, as can be seen in table 2.

Table 2 - Percentage mortality (%) of *A. aegypti* larvae at different concentrations of essential oil of *T. procumbens* leaves at 24 and 48 hours.

	Larvicidal activity (%)	
Concentrations (μg.mL^{-1})	**24 h**	**48 h**
Control (-)	0	0
Control (+)	100	100
20	6,6	13,32
40	13,32	21,32
60	40	45,2
80	41,2	45,2
100	60	65,2

According to table 2, the concentrations of 80 and 100 μg.mL-1 presented larvicidal activity after 24 h, with a mortality greater than 50%, the bioassay determined the LC_{50} of 86.163 μg.mL^{-1}, with R^2 = 0.936. In 48 hours, EO presented activity at concentrations 60, 80 and 100 μg.mL^{-1}, the LC_{50} of 76.524 μg.mL^{-1} and R^2 = 0.923.

In the literature, there are not many studies on essential oil against *A. aegypti* of this plant species. However, its insecticidal effect is confirmed in the literature through the extracts of the species, where it is documented the potential larvicidal activity against *A. aegypti* and *Culex quinquefasciatus*. Sakthivadivel and Daniel [20] reported in their study that the crude petroleum extract of *T. procumbens* has larvicidal activity against *Culex*

quinquefasciatus with LC_{50}> 200 ppm and median mortality against *A. aegypti* and *Anopheles stephensi*.

However, in recent studies on the larvicidal activity of EO components against mosquito species, monoterpenes such as *o*-cymene, limonene, β-pinene, (-) - β-pinene, α-terpinene, β-terpinene and thymol, which are compounds found in the sample of *T. procumbens*, are considered compounds with great larvicidal potential, having action on one or more mosquito species [21].

3.3. Cytotoxic activity with Artemia salina

EO to *A. salina* toxicity was performed to preliminarily assess the toxicity of the substances. The data in Table 3 demonstrate the mortality of *A. salina* at different concentrations, as can be seen below.

Table 3 – Percent mortality of *A. salina* larvae of *T. procumbens* oil at different concentrations.

Oil Concentrations (μg.mL-1)	% mortality
50	0
100	0
250	0
500	0
750	0
1000	6.6
LC_{50}	1238.67 μg.mL^{-1}
LC($K_2Cr_2O_7$)	12.60 μg.mL^{-1}

The data in table 2 shows 6.6% mortality at the concentration of 1000 μg.mL^{-1} in EO. In addition, with LC_{50} equal to 1238.67 μg.mL^{-1}, it indicates low toxicity to *A. salina*. This value is above the toxicity standard of potassium dichromate with LC_{50} of 12.60 μg.mL^{-1}.

It is possible to identify that OE had no toxicity in the test performed with *A. salina* larvae. This can be reaffirmed by a study that classifies the essential oils in degrees of toxicity and according to the interval. In which the extracts with LC_{50} below 100 μg.mL^{-1} are considered to have high toxicity, for moderate activity, the extracts present LC_{50} between 500 and 1000 μg.mL^{-1} and for low toxicity, the extracts present LC_{50} above 1000 μg.mL^{-1} and are considered nontoxic [12]. Reaffirming the toxicity of the oil tested, which presented a value of LC_{50} = 1238.67 μg.mL^{-1}, above the standards considered non-toxic.

In this sense, there are papers in the literature that show a good correlation between the toxicity tests on this species and their applicability in different biological activities, such

as antifungal, antimicrobial, antitumor, among others, favoring interest in this species in future studies.

This thought resembles studies that conclude that the species present cytotoxicity, and it demonstrates great potential for diverse biological activities [22].

Because it is a preliminary analysis, the low toxicity to *A. salina* may represent that the species present low toxicity to the environment and to the human being, being able to become a promising larvicidal agent.

3.4. Antioxidant activity

Among the several ways to evaluate the antioxidant potential of natural products, it is the method of sequestration of the DPPH radical.

The method of sequestration of the DPPH radical is among the several ways to evaluate the antioxidant potential of natural products. This antioxidant activity is measured by the consumption of DPPH, the higher the intake of DPPH in a sample, the lower the IC_{50} and the greater its activity.

The results obtained after the determination of the antioxidant activity of EO at different concentrations are shown in Table 4.

Table 4 - Mean and standard deviation of the percentage of antioxidant activity of *T. procumbens* essential oil in different concentrations.

Oil Concentrations (µg.mL-1)	**% antioxidant activity**	
	Essential oil	A. Ascorbic
7.81	13.0± 1.63	18.57±0.52
15.62	13.9± 0.46	30±0.10
31.25	14.0±0.38	99.93±0.02
62.5	18.9 ±0.70	99.99±0.0
125	25.3 ± 0.24	99.99±0.0
250	67.1 ± 0.14	99.99±0.0
IC_{50}	194.51 µg.mL^{-1}	16.71 µg.mL^{-1}

Equation of the antioxidant activity of OE Y= 0.2190x + 7.4022

The EO achieved its maximum antioxidant activity (67.1%) at a concentration of 250 µg.mL^{-1} with the coefficient of determination (R^2) = 0.9347 and p-value equal to 0.0016. These data demonstrate that OE presented significant antioxidant activity at its highest concentration.

EO presented antioxidant activity with IC_{50} equal to 194.51 µg.mL^{-1}; however, this activity had below standard when compared to ascorbic acid. Although the EO presents

compounds like β-Caryophyllene and thymol that have in the literature identified antioxidant activity. The study by Pereira et al. [23] showed in its experiment that variations in the content of the indole alkaloids coronaridin and voacangin in the extracts of *T. catharinensis* did not alter the antioxidant potential in vitro. While Chizzola, Michitsch and Franz [24] verified high antioxidant properties in the extracts and EO of the leaves of *Thymus vulgaris*, which presented a greater amount of thymol in their constituents.

The isolated action of β-Caryophyllene or synergism among the major constituents of EO may also be related to the antioxidant activity identified in the study [25].

T. procumbens essential oils showed antioxidant activity, reducing levels of oxidative stress when using the DPPH assay. These essential oils appear to have higher antioxidant activity than ascorbic acid and in the study, it is pointed out that increasing the concentration of the essential oil consequently increases the antioxidant power. In the literature, *T. procumbens* is identified as a good candidate for the treatment of inflammation and cancer with fewer toxic effects [26], but these claims are not adequately researched and documented.

For example, *T. procumbens* has been shown to reduce inflammation when applied as a leaf plaster and has been shown to be effective in the treatment of neuropathic and inflammatory pain in rodent models [27]. However, much attention has been given to the antioxidant properties of the plant extracts and the identification of the compounds responsible for these activities. There are two studies that sought to evaluate the elimination of free radicals from *T. procumbens* extract of methanol and ethanol using DPPH and ABTS. In both assays, the extracts showed moderate free radical scavenging activity. The methanol extract showed higher antioxidant activity when compared to the 70% ethanol extract. The effects of the extracts were less potent compared to positive controls with ascorbic acid [28]. The isolated compounds exhibited a very mild effect on the free radicals in both assays.

4. CONCLUSION

The chemical composition of *T. procumbens* essential oil indicated the presence of 20 compounds in total. EO has as major compounds Thymol, γ-Terpinene, *o*-Cymene in its composition. The EO presented antioxidant activity by the DPPH radical capture method at the highest concentrations; however, it presents low activity when compared to ascorbic acid. It also presented low toxicity to *A. Salina*, serving as a preliminary analysis of the possible low toxicity of the species to the environment and to humans.

With these results, it is possible to observe that the EO presents as a great promising natural larvicidal agent for use in *A. aegypti* larvae growth sites, necessitating studies of technological bases for the development of finished products and evaluation of toxicity in humans.

The data show the importance of preliminary bioassays as a screening of the biological potential of plant products, as well as the importance of these products as a source of bioactive compounds and demonstrating the biological potential of this species.

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5 CONSIDERAÇÕES FINAIS

A composição química dos extratos de *T. procumbens* indicou a presença de 08 substâncias, ao total. O EBA apresentou depsídeos e depsidonas, flavanoides, fenóis e taninos, proteínas e aminoácidos, saponinas e alcaloides em sua composição. Enquanto que o EBE demonstrou Depsídeos e depsidonas, Fenóis e taninos catéquicos e alcaloides em seu extrato. Enquanto que o OE apresentou em sua composição química a presença de 20 compostos, ao total. O OE possui como compostos majoritários o Thymol, γ - Terpinene, o-Cymene em sua composição.

No estudo, o OE e o EBA apresentaram melhor potencial larvicida, antioxidante pelo método de captura do radical DPPH quando comparados ao EBE e abaixo do adequado quando comparado com o padrão de ácido ascórbico. Ambos extratos e óleo essencial apresentam baixa toxicidade frente à *A. salina*. O Extrato aquoso apresentou melhor potencial microbiológico comparado ao EBE. Esses resultados salientam as vantagens dessa espécie para o desenvolvimento de produtos naturais, que podem ser desenvolvidos em prol da sociedade, sem causar impactos negativos ao meio ambiente.

Foi possível observar que o EBA e o OE se apresentaram como promissores agentes larvicidas naturais para uso em locais de crescimento de larvas do *A. aegypti*. Novos estudos devem ser desenvolvidos no combate a esse vetor, haja vista, que ele estar relacionado a doenças consideradas graves problemas de saúde pública. Portanto para o desenvolvimento de produto comercial de origem natural é importante aprofundar os estudos acerca da toxicidade frente ao ser humano e ao meio ambiente.

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Anexo 1 – Normas de publicação da Revista Pharmaceuticals

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1. read the Aims & Scope to gain an overview and assess if your manuscript is suitable for this journal;
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1.1. Manuscript Submission Overview

1.1.1. Types of Publications

Pharmaceutics has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *Pharmaceutics* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on Supplementary Materials and references to unpublished data).

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- *Reviews:* These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA guidelines.

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1.1.3. Accepted File Formats

Authors must use the Microsoft Word template or LaTeX template 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 pharmaceutics@mdpi.com. Accepted file formats are:

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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/).

1.2. Manuscript Preparation

1.2.1. General Considerations

- **Research manuscripts** should comprise:
  - Front matter: Title, Author list, Affiliations, Abstract, Keywords
  - Research manuscript sections: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
  - Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.
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1.2.2. Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review,

meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.

- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, country, and all email addresses. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.
- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

1.2.3. 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.
- **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.
- **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.
- **Conclusions:** This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.
- **Patents:** This section is not mandatory, but may be added if there are patents resulting from the work reported in this manuscript.

1.2.4. Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Acknowledgments:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published.
- **Author Contributions:** Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the CRediT taxonomy for the term explanation. For more background on CRediT, see here. "**Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully**".
- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the choice of research project; design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. *Pharmaceutics* does not publish studies funded by the tobacco industry. Any projects funded by pharmaceutical or food industries must pay special attention to the full declaration of funder involvement. If there is no role, please state "The sponsors had no role in the design, execution, interpretation, or writing of the study".
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- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

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

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

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

Journal Articles:
1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range. Available online: URL (accessed on Day Month Year).
Books and Book Chapters:
2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.
Unpublished work, submitted work, personal communication:
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5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* stage of publication (under review; accepted; in press).
6. Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.
Conference Proceedings:
7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), 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:
8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.
Websites:
9. Title of Site. Available online: URL (accessed on Day Month Year).
Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as WebCite. Archived websites should be cited using the link provided as follows:
10. Title of Site. URL (archived on Day Month Year).
See the Reference List and Citations Guide for more detailed information.

1.3. Preparing Figures, Schemes and Tables

- File for Figures and schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Pharmaceutics* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).

- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

1.4. 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.

Unpublished Data

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).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Deposition of Sequences and of Expression Data

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.

1.5. Research and Publication Ethics

1.5.1. Research Ethics

1.5.2. 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/), 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.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

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 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.

You may refer to our sample form and provide an appropriate form after consulting with your affiliated institution. Alternatively, you may provide a detailed justification of why informed consent is not necessary. For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients' and any other individual's privacy, please do not send signed forms. The journal reserves the right to ask authors to provide signed forms if necessary.

1.5.3. Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must also have been conducted in accordance with relevant national legislation on the use of animals for research. For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [1].

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.

Pharmaceutics endorses the ARRIVE guidelines (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.

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.

1.5.4. 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.

1.5.5. Research Involving Plants

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 and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

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 be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from ‘Crown Mix’ (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

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

1.5.6. Publication Ethics Statement

Pharmaceutics is a member of the Committee on Publication Ethics (COPE). We fully adhere to its Code of Conduct and to its Best Practice Guidelines.

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 *Pharmaceutics* 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 *Pharmaceutics* 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.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- 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).
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our policy regarding publication of publishing addenda and corrections.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the Rights and Permissions page.
- Plagiarism, data fabrication and image manipulation are not tolerated.
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      Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

      Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

      If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, we may publish a correction or retract the paper.

- **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

  Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

  If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

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During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

1.7. English Corrections

To facilitate proper peer-reviewing of your manuscript, it is essential that it is submitted in grammatically correct English. Advice on some specific language points can be found here.

If you are not a native English speaker, we recommend that you have your manuscript professionally edited before submission or read by a native English-speaking colleague. This can be carried out by MDPI's English editing service. Professional editing will enable reviewers and future readers to more easily read and assess the content of submitted manuscripts. All accepted manuscripts undergo language editing, however **an additional fee will be charged** to authors if very extensive English corrections must be made by the Editorial Office: pricing is according to the service here.

1.8. Preprints and Conference Papers

Pharmaceutics accepts articles that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates *Preprints*, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* instructions for authors for further information.

Expanded and high quality conference papers can be considered as articles if they fulfil the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Pharmaceutics* does not publish pilot studies or studies with inadequate statistical power.

1.9. Qualification for Authorship

Each author is expected to have made substantial contributions to the conception or design of the work; acquisition, analysis, or interpretation of data; the creation of new software used in the work; and/or writing or substantively revising the manuscript. In addition, all authors must have approved the submitted version (and any substantially modified version that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even those in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. Note that acquisition of funding, collection of data, or general supervision of the research group do not, by themselves, justify authorship.Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgements.

More detailed guidance on authorship is given by the International Council of Medical Journal Editors (ICMJE).The journal also adheres to the standards of the Committee on Publication Ethics (COPE) that "all authors should agree to be listed and should approve the submitted and accepted versions of the publication. Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication (e.g. answering reviewers' comments)." [1]. We reserve the right to request confirmation that all authors meet the authorship conditions.

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.

1.10. Editorial Procedures and Peer-Review

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether they are properly prepared and whether they follow the ethical policies of the journal, including those for human and animal

experimentation. Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. After these checks, the *Managing Editor* will consult the journals' *Editor-in-Chief* or *Associate Editors* to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the potential impact of the work will be made at this stage. Reject decisions at this stage will be verified by the *Editor-in-Chief*.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past five years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

Optional Open Peer-Review

The journal operates optional open peer-review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open review at any time before publication, however once the paper has been published changes will only be made at the discretion of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee an impartial refereeing the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions*: The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.
- *Reconsider after Major Revisions*: The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. Usually, only one round of major revisions is allowed. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments.
- *Reject and Encourage Resubmission*: If additional experiments are needed to support the conclusions, the manuscript will

be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.

- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the Editor-in-Chief, Associate Editor, or Editorial Board member. The academic Editor being consulted will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage is final and cannot be reversed.

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Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.