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CICERA JANAINE CAMILO

INVESTIGAÇÃO DA INFLUÊNCIA DE ESTUDOS ETNOBIOLÓGICOS NO DESENVOLVIMENTO DE BIOINSETICIDAS E PERFIL TOXICOLÓGICO DO EXTRATO AQUOSO DE *Lippia sidoides* CHAM.

CRATO – CE
- 2023 –

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Tese apresentada ao Programa de Pós-graduação em Etnobiologia e Conservação da Natureza (UFRPE, UEPB, URCA e UFPE) como parte dos requisitos para obtenção do título de doutora.

Orientador:

Prof. Dr. José Galberto Martins da Costa

Universidade:

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Dedico a minha família.

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SUMÁRIO

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ANEXOS

Camilo, Cicera Janaine; Dra.; Universidade Federal Rural de Pernambuco; fevereiro, 2023; Investigação da influência de estudos etnobiológicos no desenvolvimento de bioinseticidas e perfil toxicológico do extrato aquoso de *Lippia sidoides* Cham. José Galberto Martins da Costa

RESUMO

O uso de pesticidas sintéticos é comum para manutenção da produção agrícola mundial. No entanto, esses insumos geram efeitos negativos ao meio ambiente e ao homem, dentre eles podem ser citados a contaminação de ambientes aquáticos e terrestres, toxicidade em animais dispersores, como abelhas, e acúmulo no sangue do homem e animais, podendo gerar problemas no sistema nervoso central (SNC), entre outros. Esse trabalho teve como objetivo realizar um levantamento sobre as principais indicações etnobotânicas de espécies do gênero *Lippia* sp., com destaque para o uso como inseticida e praguicida, e relacionar a influência dessas indicações com os avanços em estudos com atividade pesticida do gênero. Além disso, um perfil toxicológico da espécie *Lippia sidoides* Cham foi investigado com diferentes metodologias, com o intuito de assegurar o uso dessa espécie na medicina tradicional e em estudos futuros. O levantamento bibliográfico demonstrou que espécies de *Lippia* são comumente utilizadas pelas populações para tratamento de diversas enfermidades, sendo a preparação de chás através das folhas a principal forma de uso. *L. alba* e *L. janvica* são as espécies mais citadas para uso medicinal, assim como, se destacam para uso como repelente e inseticida. Os estudos inseticidas com o gênero *Lippia* sp indicam as espécies *L. sidoides*, *L. alba* e *L. janvica* como as mais investigadas. No estudo toxicológico foi possível verificar que o extrato aquoso de *L. sidoides* não apresenta perfil toxicológico pelos diferentes métodos utilizados, demonstrando seguridade para uso entre as comunidades tradicionais e promissor para o desenvolvimento de estudos científicos na área inseticida.

Palavras-chave: Fitoinseticidas; *Lippia sidoides;* Toxicidade; Etnobiologia.

Camilo, Cicera Janaine; Dra.; Universidade Federal Rural de Pernambuco; fevereiro, 2023; Investigation of the influence of ethnobiological studies on the development of bioinsecticides and toxicological profile of the aqueous extract of *Lippia sidoides* cham. José Galberto Martins da Costa

ABSTRACT

The use of synthetic pesticides is common for maintaining world agricultural production. However, these inputs generate negative effects on the environment and on humans, among which can be mentioned the contamination of aquatic and terrestrial environments, toxicity in dispersing animals, such as bees, and accumulation in the blood of humans and animals, which can cause problems in the System Central Nervous system (CNS), among others. This work aimed to carry out a survey on the main ethnobotanical indications of species of the genus *Lippia* sp., with emphasis on their use as an insecticide and pesticide, and to relate the influence of these indications with the advances in studies with the pesticidal activity of the genus. In addition, a toxicological profile of the *Lippia sidoides* Cham species was investigated using different methodologies, with the aim of ensuring the use of this species in traditional medicine and in future studies. The bibliographic survey showed that *Lippia* species are commonly used by populations to treat various diseases, with the preparation of teas through the leaves being the main form of use *L. alba* and *L. janvica* are the most cited species for medicinal use, as well as stand out for use as a repellent and insecticide. Insecticide studies with the genus *Lippia* sp indicate the species *L. sidoides*, *L. alba* and *L. janvica* as the most investigated. In the toxicological study it was possible to verify that the aqueous extract of *L.sidoides* does not present a toxicological profile by the different methods used, demonstrating safety for use among traditional communities and promising for the development of scientific studies in the insecticide area.

Keywords: Phytoinsecticides; *Lippia sidoid*es; toxicity; ethnobiology**.**

1. INTRODUÇÃO GERAL

1.1. Objetivos e questionamentos

Os produtos naturais têm contribuído significativamente na indústria farmacêutica para descoberta de novas substâncias ativas provenientes do seu metabolismo secundário, de forma que esses constituintes químicos apresentam propriedades biológicas que atuam como protetores contra vários patógenos e no tratamento de doenças (AZAM *et al*., 2016). Assim, uma das ferramentas para o desenvolvimento de estudos em bioprospecção com novas substâncias oriundas de vegetais, tem sido os trabalhos etnofarmacológicos que fornecem sobre o uso, partes vegetais utilizadas, formas de preparo e indicações de suas propriedades medicinais. (CUNHA; BORTOLOTTO, 2011).

A etnofarmacologia pode ser considerada um método eficaz no direcionamento de seleção de plantas para o desenvolvimento de estudos biológicos mais aprofundados (TAN *et al*., 2015). Dentre as utilizações bioativas das plantas a atividade repelente e/ou inseticida é bastante citada em comunidades locais que as utilizam para o controle de diferentes pragas como alternativa aos produtos sintéticos (ALMEIDA NETO *et al*., 2017).

O advento da tecnologia inseriu no mercado produtos sintéticos que atuam como inseticidas. Os pesticidas ainda são considerados necessários para o aumento e a manutenção da produção agrícola mundial, contudo, o seu uso em larga escala e de forma indiscriminada tem promovido inúmeros impactos negativos ao meio ambiente e ao homem (BARBOZA *et al*., 2018). No Brasil, o consumo desse insumo equivale a 20% de todos os agrotóxicos produzidos no mundo, provocando diversos problemas ambientais e a contaminação dos alimentos. Em resposta a essa problemática o desenvolvimento e o uso de defensivos agrícolas naturais ganham destaque, principalmente pela necessidade de se consumir alimentos cada vez mais saudáveis (VIEIRA *et al*., 2016).

Diante das inúmeras desvantagens observadas pelo uso de pesticidas sintéticos a formulação de bioinseticidas é considerado alternativa viável à sua substituição. Isso por que, a variabilidade de substâncias químicas encontradas nos vegetais permite que estes possam, a partir de preparações adequadas, apresentar características como baixa toxidade, possibilidade de agirem por diferentes mecanismos de ação, serem considerados ecologicamente corretos, econômicos e biodegradáveis (SOSA *et al*., 2018; MIRESMAILLI; ISMAN, 2014).

Apesar das vantagens relacionadas a produção de bioinseticidas ainda existem muitas dificuldades na sua produção. Segundo Isman, (2017) a produção de inseticidas botânicos está relacionada muitas vezes a condução das pesquisas, uma vez que, somente a investigação da atividade inseticida não é suficiente para elaboração de bioproduto, existindo diversos estudos com extratos vegetais pra essa finalidade, mas nenhuma investigação sobre métodos de extração, formulação de extratos estáveis físico-quimicamente, rendimentos, composição química e perfil toxicológico, sendo estes os principais impasses para produção.

Dentre a variedade de espécies vegetais que são utilizadas como inseticidas, as do gênero *Lippia* sp. tem demonstrado potencial para o desenvolvimento de bioinseticidas de baixo custo e biodegradáveis. Diversos estudos etnobotânicos indicam que espécies deste gênero são utilizadas na repelência de mosquitos, baratas e carrapatos, sendo as folhas a parte da planta mais utilizadas (ALMEIDA NETO *et al*., 2017; FARIAS *et al*., 2016). Lima *et al*., (2011) mostraram que o óleo essencial da espécie *Lippia sidoides,* conhecida popularmente como "alecrim-pimenta", tem efeito repelente contra *Tenebrio molitor*, uma espécie de larva que ataca a farinha de mandioca.

Nesta perspectiva, este trabalho teve como objetivos: (1) realizar um levantamento de trabalhos publicados acerca de indicações etnobotânicas sobre espécies do gênero *Lippia*, com foco em seu uso como inseticida/praguicida, relacionando-os com os avanços nas pesquisas com bioinseticidas e (2) determinar o perfil toxicológico para o extrato aquoso de *L. sidoides* a fim de assegurar seu uso na medicina tradicional e no desenvolvimento de novos estudos.

1.2.Estratégias de pesquisa

Para o desenvolvimento desta pesquisa foi realizado um estudo de revisão acerca das principais indicações etnobotânicas e do desenvolvemento de estudos pesticidas com gênero *Lippia* sp. pelo mundo. O objetivo desse levantamento bibliográfico foi traçar uma relação entre as indicações populares e o desenvolvimento de estudos inseticidas com o gênero. Para isso, foram utilizadas as bases de dados internacionais Science Direct, PubMed, Web of Science and Scopus. A partir da determinação de critérios, os artigos referentes as indicações etnobotânicas e estudos inseticidas foram selecionadas e os dados foram extraídos e distribuídos em tabelas. A análise dos dados permitiu traçar uma relação direta entre as linhas de pesquisas.

O segundo artigo está baseado na produção de extratos a partir da técnica de atomização em *spray dryer*. O método de secagem escolhido é uma das técnicas mais desenvolvidas na industria de fitoterapicos, devido a sua capacidade de proporcionar ao produto maior estabilidade microbiológica e controle dos suas caracteristicas finais, como higroscopicidade e densidade (OLIVEIRA; PETROVICK, 2010). A adição de adjuvantes de secagem aos extratos permite a eliminação de caracteristicas indejaveis no produtos final, como baixa compressibilidade e compactabilidade. Assim, o emprego de adjuvantes de secagem como o dioxido de silico coloidal e celulose microcristalina interferem na biodispolinilidade do produto final. Esses adjuvantes, tem sido amplarmente utilizados na industria por apresentar bons resultados, especialmente na redução da agregação do produto nas paredes do equipamento (JONAT et al., 2006; VASCONCELOS *et al*., 2005).

Para determinar perfil de segurança do extrato da espécie *Lippia sidoides* foram desenvolvidos diferentes métodos que contemplassem as diversas possibilidades de toxicidade que o extrato pode ter. Assim, foram realizados ensaios com zebrafish, que é considerado um modelo para ensaios em laboratórios de pesquisas, por ter reprodução rápida, serem pequenos e econômicos (Horzmann; Freeman, 2018).

Drosophila melanogaster (Meig.) é muito utilizada como modelo alternativo ao uso de mamíferos, isso porque, 70% dos genes envolvidos em alguma patogenia humana apresentam homólogos funcionais em *D. melanogaster* (UGUR *et al*., 2016). Além disso, apresenta alta taxa reprodutiva, similaridade fisiológicas ao corpo humano e pequeno porte (UGUR et al, 2016). A simplicidade estrutural observada em eritrócitos torna essas células bons modelos para investigação de extratos oxidativos e que podem causar danos celulares irreversíveis (GARCIA, 2014). O microcrustáceo *Artemia salina* Leach é utilizado em laboratórios como um bioindicador, por apresentar respostar rápidas e nítidas a pequenas variações no ambiente em que está inserido. Sua utilização em ensaios de toxicidade tem como objetivo identificar uma resposta biológica a partir da análise dos fatores morte ou vida da espécie em curto período (MEYER, 1982).

A cromatografia líquida acoplada à espectrometria de massas (LC/MS) é, atualmente, a técnica que possibilita a análises de diversas substâncias com ampla caracterização de polaridade e massa molecular. Essa técnica tem permitido a identificação e separação de moléculas em curto prazo. Dessa forma, a análise química de extratos vegetais a partir dessa técnica é importante para identificar e relacionar a influência dos compostos na atividade biológica exercida pela planta (Coskun, 2016).

A partir da identificação dos compostos da classe dos flavonoides por LC/MS, fez se necessário a quantificação dessa classe química através do teste de *Folin-Ciocalteu*. Esse método baseia-se na reação de oxidação dos compostos fenólicos pelo reagente *Folin-Ciocalteu*, que possui uma coloração amarelada. Pelo processo de oxidação o reagente adquire coloração azulada, a quantificação é dada pelo método de espectrofotometria que vai medir a intensidade do produto de coloração azul através da sua absorbância.

Todas as análises foram realizadas em triplicata e os dados expressos como média (n=3) ± Desvio Padrão (SD) usando Análise de Variância (ANOVA) unidirecional e bidirecional sucedida pelo teste de Tukey para comparação múltipla para dados com distribuição normal e significativamente desvios padrão semelhantes com valores de $P < 0,05$; $P < 0,01$ e $P < 0,001$.

As análises estatísticas e a apresentação gráfica dos resultados foram realizadas no software GraphPad Prisma (versão 6.1).

1.3.Estrutura da tese

A partir da visão geral da pesquisa, o texto principal é dividido em três capítulos. O primeiro capítulo é uma fundamentação teórica que tem como perspectiva o embasamento da pesquisa sobre os eixos teórico em que a tese está fundamentada. Dessa forma, este capítulo aborda as problemáticas associadas ao uso de inseticidas sintéticos e a importância do uso de produtos naturais como bioinseticidas.

O capítulo traz a importância dos estudos etnobiológicos para seleção de espécies vegetais para o desenvolvimento de pesquisas no campo da bioprospecção. Além disso, o capítulo traz a importância de identificar espécies vegetais que apresentam perfil toxicológico, tanto para uso em comunidades locais, como para o desenvolvimentos estudos científicos que buscam a elaboração de produtos. Ainda no capítulo I é mostrado a relevância de se utilizar a técnica de atomização para secagem de extratos.

O segundo capítulo trata-se de um artigo de revisão intitulado "Traditional use of the genus *Lippia* sp. and pesticidal potential: a review". Esse artigo está baseado no preceito de que nos últimos anos vários trabalhos em etnobiologia foram publicados indicando o uso de espécies de *Lippia* sp. para diversos fins terapêuticos, e a partir destes, muitas pesquisas têm buscado a confirmação de tais usos por meio de estudos biológicos. Neste contexto, este capítulo tem o objetivo de relacionar estudos etonobiológicos que indicam os principais usos medicinais deste gênero no mundo, bem como, mostrar o avanço nas pesquisas especificamente em trabalhos inseticidas, dando ênfase a discussão de métodos utilizados e resultados obtidos. Desse modo, é possível verificar o avanço das investigações do potencial pesticida, pela influência direta de estudos etnobiológicos, além de avaliar os principais impasses que dificultam a elaboração de produtos inseticidas provenientes de espécies desse gênero.

O terceiro capítulo é um manuscrito original sobre a toxicidade da espécie alvo do projeto, intitulado "Analysis toxicity by different methods and anxiolytic effect of the aqueous extract *Lippia sidoides* Cham. O artigo tem como base o reconhecimento de que uso popular das plantas tem guiado a pesquisa farmacêutica voltada para combate de diferentes enfermidades. E, sabendo que *Lippia sidoides* Cham. apresenta grande versatilidade nos estudos etnobotânicos e tem sido muito utilizada no combate a insetos e como agente terapêutico, pesquisas biológicas são realizadas para avaliar seu potencial medicinal, no entanto, pouco se sabe sobre a segurança no uso de seus extratos, já que esta é a principal forma de utilização em comunidades locais. Como forma de contribuir para valorização e seguridade do uso tradicional desta espécie, esse capítulo tem como objetivo traçar um perfil de segurança no uso do extrato aquoso de *L. sidoides*, através de ensaios toxicológicos por diferentes métodos.

2. OBJETIVOS

➢ Geral

Avaliar os avanços nas pesquisas com bioinseticidas a partir de estudos etnobotânicos com o gênero *Lippia* sp., e determinar o perfil toxicológico do extrato aquoso de *Lippia sidoides.*

➢ Objetivos específicos

- Desenvolver um artigo de revisão sobre as indicações etnobotânicas do gênero *Lippia* e o desenvolvimento de estudos inseticidas com o gênero;
- Obter extratos secos por atomização em *spray dryer*;
- Caracterizar o extrato por Cromatografia Liquida de Alta eficiência acoplada a Espectrometria de Massas- CL/EM;
- Verificar a toxicidade do extrato aquoso por métodos *in vivo* (zebrafish, *Drosophila melanogaster* e *Artemia salina*);
- Verificar a toxicidade do extrato pelo método *in vitro* em células sanguíneas;
- Verificar o potencial ansiolítico via receptores GABA em zebrafish.

3. CAPÍTULO I

3.1. REFERÊNCIAL TEÓRICO

3.1.1. Prejuízos causados pelo uso de inseticidas sintéticos

A produção de alimentos teve aumento considerável com a implementação dos agrotóxicos. Com advento da chamada "revolução verde", ocorreram avanços tecnológicos agronômicos que aumentaram significativamente a produção agrícola em todo o mundo (LLEWELLYN, 2018). Desde então, o uso de inseticidas sintéticos tem sido a principal estratégia para o controle de pragas que causam prejuízos em lavouras.

Nesse contexto o agronegócio é uma das principais fontes de desenvolvimento do Brasil, devido ao clima tropical e ao solo fértil em boa parte do país. Um exemplo, é a soja que já chegou a 71% da produção, ocupando área de 1.62 milhões hectares, levando o Brasil, em tempos remotos, a se tornar o maior consumidor de agrotóxicos do mundo (WEI *et al*., 2011). Essa crescente produção em todo mundo levou ao aparecimento de diferentes pragas que causam, até hoje, perdas inestimáveis na agricultura, comprometendo a qualidade dos alimentos e devido ao uso de agrotóxicos, o surgimento de problemas à saúde dos consumidores.

A utilização dos inseticidas organofosforados e carbamatos foram por longo período a principal estratégia para o controle de pragas, atuando na inibição competitiva da acetilcolinesterase causando a morte do inseto (WISMER; MEANS, 2018). Esses produtos contêm substâncias altamente tóxicas como chumbo, mercúrio e arsênio que são absorvidas através da cadeia alimentar e podem causar diversos problemas à saúde (ZHANG et al., 2011).

Os trabalhadores que atuam na aplicação e fabricação são os principais atingidos pelo uso excessivo desses produtos tóxicos. Segundo Muñoz-Quezada *et al.*, (2016) essas pessoas tendem a ter menor desempenho neuropsicológico e motor, além de acumular na circulação sanguínea produtos tóxicos que pode provocar riscos à saúde irrecuperáveis (HAYAT *et al*., 2019). Recentemente, foi demonstrado que diferentes tipos de inseticidas em baixas concentrações podem causar danos no desenvolvimento embrionário de peixes-zebra, indicando o seu potencial tóxico (LIU *et al*., 2018).

A contaminação por inseticidas pode ocorrer através do contato direto do produto pelo indivíduo que atua na sua fabricação ou na aplicação, como também através do consumo por produtos contaminados. No trabalho de Lozowicka *et al*., (2016) foi verificado que frutas, como maçãs, peras e cerejas possuem níveis altos de contaminantes, especialmente os organofosforados e carbamatos, apresentando risco de intoxicação pela população consumidora. Não só as frutas e legumes entram na lista de alimentos contaminados, Dallegrave *et al*., (2018) mostraram que em carnes bovinas, leite, frango e peixes são encontrados resíduos de inseticidas, como também produtos da sua metabolização e que, apesar de não estarem em níveis excedidos do que é permitido, é uma problemática que deve ser considerada para que se possa promover a segurança alimentar.

A utilização de forma indiscriminada também pode promover a intoxicação em organismos não-alvos, de forma a ocasionar quadro de neurotoxicidade no sistema nervoso central-SNC de animais (RODRÍGUEZ *et al*., 2018). O trabalho de Christen e Fent, (2017) relata que populações de abelhas expostas a pesticidas sofrem alterações em genes reguladores do sistema imunológico, causando perdas populacionais e desequilíbrio na comunidade. Em outro estudo, foi demostrado que inseticidas sintéticos causam danos ao corpo, olhos e cabeça do peixe-zebra, sendo indicativo da sua toxicidade para ambientes aquáticos (LIU *et al*., 2018). Em ambientes aquáticos a presença de inseticidas promove a perda da biodiversidade pela contaminação do efluente, sendo demostrado que 52,4% dos níveis de inseticidas utilizados, excedem os limites específicos (STEHLE e SCHULZ, 2015).

Apesar dos inseticidas botânicos terem sido as primeiras formas de combate a pragas, devido ao seu baixo potencial de ação, foi necessário utilizar altas concentrações, fazendo com que houvesse a substituição por produtos sintéticos. Nos últimos 30 anos houve uma retomada na pesquisa com inseticidas botânicos, desde então, várias espécies vegetais têm sido avaliadas quanto ao seu potencial inseticida, entre elas destacam-se *Azadirachta indica* A. Juss. (nim), *Symphytum officinale* L (confrei), *Vernonia condensata* Baker (boldo-baiano), *Stryphnodendron adstringens* (barbatimão), *Lippia* spp. (SANTOS *et al*., 2017; ROCHA; SUJII, 2019; OLIVEIRA *et al*., 2018).

Os produtos naturais são considerados alternativas adequadas por possuírem baixa toxidade, possibilidade de agirem por diferentes mecanismos de ação, serem considerados ecologicamente corretos, econômicos e biodegradáveis (SOSA *et al*., 2018). Essas características estão relacionadas a sua variabilidade química, em contraste aos inseticidas sintéticos que são geralmente baseados em único componente ativo, o que limita sua ação e facilita o desenvolvimento de resistência pelos insetos (MIRESMAILLI; ISMAN, 2014).

A resistência observada para boa parte dos inseticidas sintéticos, está associada a capacidade que um grupo de organismos tem de tolerar altas doses de um produto tóxico para maioria dos outros organismos da população. Geralmente, associa-se a resistência dos insetos aos inseticidas a alta frequência de utilização dos produtos, o que proporciona a seleção de uma parcela da população que é resistente. Os mecanismos envolvidos para diminuição da eficiência dos inseticidas são: (i) modificações comportamentais, que ajudam a reconhecer a presença do produto tóxico; (ii) modificações nos sítios alvos dos inseticidas; (iii) resistência metabólica, pelo aumento da capacidade de desintoxicação (MOREIRA et al, 2012).

Dessa forma, torna-se importante o aprofundamento de estudos com espécies vegetais que possuem potencial para o desenvolvimento de bioinseticidas.

3.1.2. Toxicidade de espécies vegetais

O reino vegetal tem contribuído de forma significativa para o fornecimento de substâncias úteis ao tratamento de doenças que acometem o homem a muitos anos. A utilização de plantas para fins medicinais pela população é registrada desde antigas civilizações na Índia, Egito e Grécia (ALVES, 2013). A ação medicinal dos vegetais está relacionada aos compostos produzidos pelo seu metabolismo secundário, substâncias que são reconhecidas por serem seletivas e desempenharem papel importante na evolução e adaptação dos vegetais ao ambiente (BORGES; AMORIM, 2020).

O uso medicinal dos vegetais, muitas vezes é seguido sem nenhuma preocupação com efeitos tóxicos, isso porque, existe uma percepção por parte da população de que produtos de origem natural não apresentam riscos à saúde (BEDNARCZUK et al, 2010). Dessa forma, a utilização das plantas na terapêutica deve ser conduzida a partir de um conhecimento prévio das características do vegetal, modo de uso e preparo, sendo a ingestão, inalação e contato as principais formas de desencadear uma reação indesejável (JESUS; SUCHARA 2013).

Diversas espécies utilizadas na medicina tradicional têm algum tipo de toxicidade registrada, entre elas podem ser citadas: *Anarcadium occidentale* L (caju) suas diversas partes podem provocar queimaduras na pele e mucosa; *Allamanda catartica* L (dedal de dama) todas as partes podem provocar problemas gastrointestinais; *Artemisia absinthium* L(sintro, absinto) tem efeito convulsivante, alucinógeno e abortivo; *Solanum cnnitum* Lam (Jurubeba) seus frutos causam náuseas, dores abdominais e são abortivos (CAMPOS et al, 2016).

Neste contexto, é importante o desenvolvimento de estudos toxicológicos com espécies vegetais para assegurar seu uso de forma adequada, assim como, buscar nestas espécies a comprovação de sua utilização, através da investigação de substâncias ativas para o desenvolvimento de fármacos.

As principais formas de determinar a toxicidade de um produto é através da realização de ensaios *in vivo* e *in vitro*, esses testes são uma base para o desenvolvimento de fármacos, visto que, é a partir deles que é possível determinar de forma antecipada os riscos que as substâncias podem provocar ao homem (SILVA et al, 2021). Entre os ensaios preliminares mais executados está o teste de letalidade em *Artemia salina* que visa ter um reconhecimento prévio da capacidade da amostra ser tóxica em concentrações elevadas ou não (FERREIRA et al, 2017).

Modelos que utilizam células de mamíferos avaliam a viabilidade celular a partir do contato com os extratos. Esse modelo é bastante difundido por ter uma variedade de células que podem ser investigadas, além de ser reprodutível, rápido e sensível (BEDNARCZUK et al, 2010).

Podem ser citadas como modelos, ensaios com células sanguíneas, células de defesa e células cancerígenas. Os ensaios com células são fáceis de serem manipuladas e dão suporte para estudos *in vivo*, auxiliando na redução do número de animais para o desenvolvimento da pesquisa (MORALES, 2008)

Os ensaios *in vivo* dividem-se em modelos que abordam diferentes formas de toxicidade, sendo os principais: toxicidade aguda, subcrônica e crônica. A realização desses ensaios é feita em animais e deve ser antecipada por um delineamento experimental e aprovação em comitê de ética em experimentação animal (LICIO, 2013). No Brasil, os experimentos com animais são guiados pelo CONCEA (Conselho Nacional de Experimentação Animal) que estabelece os critérios para criação e desenvolvimento de estudos com animais em laboratórios. Outras instituições estão envolvidas na regulamentação do uso de animais, tais como: *Food and Drug Administration* (FDA); Agência Nacional de Vigilância Sanitária (ANVISA); *European Medicines Agency* (EMA); e *Organisation for Economic Co-operation and Development* (OECD).

De maneira geral, espécies do gênero *Lippia* sp. têm sido investigadas quanto ao seu potencial tóxico, sendo os ensaios com camundongos os mais desenvolvidos. Nas espécies *L. gacilis* e *L. citiodora* a administração oral dos seus óleos essenciais não causam alterações comportamentais e fisiológicas nos animais, não apresentado toxicidade aguda (SPYRIDOPOULOU et al, 2021; GUILHON et al, 2011). Em outro estudo verificou-se que o extrato aquoso de *L. javanica* pode causar hemorragias e danos ao órgão de camundongos após 48 de exposição do produto (MADZIMURE et al, 2011). Os resultados obtidos em ensaios toxicológicos variam de acordo com o método utilizado, via de administração, dose, frequência de administração e tempo de exposição do animal ao produto (ALMEIDA et al, 2010). Dessa forma, é comum a realização de mais de um ensaio para mesma espécie com a finalidade de ter maior conhecimento sobre a capacidade toxicológica dos diferentes produtos que podem ser extraídos de uma mesma espécie.

3.1.3. Vantagens na utilização de *spray drying*

A principal técnica utilizada atualmente para produção de extratos em forma de pó é a atomização. Esse processo consiste em três etapas principais, que envolvem o aumento da superfície de contato, transferência de calor e evaporação do solvente (OLIVEIRA; PETROVICK, 2010).

Os benefícios desta técnica estão relacionados ao controle do tamanho, forma e morfologia da partícula, tais características estão diretamente relacionadas ao aumento da estabilidade de produtos naturais, especialmente extratos vegetais, sendo esta uma das principais vantagens desta técnica (ARPAGAUS *et al*., 2018). A obtenção de extratos em forma de pó ocorre através do aparelho *spray dryer* (Figura 1), que além de permitir maior estabilidade da amostra, facilita a padronização dos princípios ativos do extrato, o que aumenta o valor agregado do produto (SOUZA, 2009). Quando comparado a outros métodos de secagem, esta técnica se destaca por ser capaz de transformar qualquer tipo de líquido, seja soluções, emulsões, dispersões, lamas, pastas etc., em partículas sólidas ajustáveis (ARPAGAUS *et al*., 2018).

Figura 1: Mini Spray Dryer ADL 311S Labmaq do Brasil
Fonte: Camilo, CJ. 2020.

Diversos tipos de extratos e substâncias já foram secas e encapsuladas por atomização, em estudo recente Santos *et al*., (2019) mostram o aumento da estabilidade de extratos da amora-preta, assim como a preservação da sua composição química a partir da utilização desta técnica. Óleos ricos em carotenoides também foram transformados em pó permanecendo com alta estabilidade química em um período de seis meses (CHUYEN *et al*., 2019).

A atomização é uma das técnicas de secagem que se destaca na indústria, por ser um processo rápido, contínuo, econômico e por não expor o produto a elevadas temperaturas, diminuindo desta forma a sua degradação. Além disso, a obtenção em forma de pó permite que os extratos possam ser transformados em diferentes formulações. É uma técnica bastante utilizada, devido à facilidade nas configurações, a capacidade de produzir pós de um tamanho específico de partículas e controle da umidade (ENGEL *et al*., 2017). Dessa forma, a produção de extratos em forma de pó é uma alternativa para superar problemas de estabilidade, assim como pode ser usada como técnica para aumento da produção.

3.1.4. A etnobiologia na seleção de plantas inseticidas com potencial para bioprospecção

A biodiversidade é fonte inestimável de informação e matéria-prima que suporta vários sistemas médico-tradicionais ao redor do mundo. Nesse sentido o conhecimento tradicional vem sendo há muito tempo disseminado de maneira informal através da medicina popular, que por muitas vezes é a única prática disponível para manutenção da saúde de certos grupos étnicos (LINHARES *et al*., 2014). Apesar da influência industrial, boa parte da população ainda faz uso de plantas medicinais para cura e tratamento de diferentes enfermidades (BADKE *et al*., 2016). Assim, o resgate do conhecimento popular sobre o uso de vegetais, que ocorre através da etnobiologia, tem proporcionado a descoberta de novos compostos ativos. Sendo a etnobotânica uma estratégia de grande valor para seleção de plantas em estudos mais aprofundados.

Dentro da etnobiologia, o domínio cultural é caracterizado como itens organizados de acordo com regras ou critérios culturalmente determinados pelos povos, neste caso, o domínio de “plantas repelentes ou inseticidas” é categorizado pelo critério de uso (ALMEIDA NETO *et al*., 2017). Esses vegetais apresentam grande importância para diversos grupos culturais, uma vez que estão acessíveis e podem ser utilizadas contra diversos tipos de pragas e vetores de doenças (FARIAS et al., 2016). As diversas comunidades que utilizam as plantas como inseticida, possuem algumas vantagens, dentre elas está o baixo ou nenhum custo para o seu desenvolvimento, uma vez que estes inseticidas são produzidos através de recursos renováveis que estão disponíveis na natureza. Assim, o uso de plantas como inseticidas por pequenos produtores tem sido alvo de pesquisas que acreditam que esses vegetais possam ser futuramente uma alternativa ao uso de pesticidas sintéticos. Contudo, ainda existe restrições de uso, limitando-se apenas há pequenos produtores, isso se dá por falta de estudos que viabilizem a produção de inseticidas naturais em grande escala (MORAIS, 2011).

As pesquisas com plantas para o controle de pragas se baseiam na produção e caracterização química de extratos e frações, que são posteriormente avaliados quanto à sua atividade biocida (JIANG *et al*., 2018; PAZ *et al*., 2018). Uma das vantagens em utilizar produtos de origem natural para o controle de pragas é a seletividade do produto e o seu potencial em ser biodegradável, diminuído os danos causados ao meio ambiente e animais.

Diversos trabalhos mostram o potencial de espécies vegetais para esse fim, dentre elas destacam-se o gênero *Lippia* sp. As pesquisas com *L. sidoides* estão direcionadas ao uso do óleo essencial, puro ou associado a nanoformulações, e seus componentes majoritários. Em estudo recente foi verificado que a nanoformulação a base do óleo é ativa contra *Sitophilus zeamais*, importante praga na cultura do milho (OLIVEIRA *et al*., 2018). Outras atividades, como antifúngica e anti-inflamatória já foram registradas para essa espécie, mostrando seu potencial terapêutico (MONTEIRO *et al*., 2007; FONTENELLE *et al*., 2007).

A ação inseticida verificada em óleos essenciais de espécies de *Lippia* sp. e outras espécies está relacionada a composição química, que apresenta como principais constituintes o eugenol, 1,8-cineol e o timol. Foi verificado que a ação larvicida ocorre em menos tempo, quando comparada a outros óleos essenciais extraídos das espécies de *S. aromaticum* e *H. martiusi* que possuem constituição química diferente das espécies de *Lippia* sp. (COSTA *et al*., 2005). Em relação aos compostos fixos presentes em extratos da *L. sidoides* foi relatado sua atuação sobre diferentes funções em diversos organismos, assim o uso de extratos pode ser considerado como estratégia de manejo a insetos-pragas, atuando na repelência, inibição da oviposição, diminuição na alimentação, além de alterações no sistema hormonal (SANTOS, 2017). Santiago *et al*., (2008) mostraram que o extrato aquoso das folhas de *L. sidoides* afeta a postura e a viabilidade de *Spodoptera frugiperda*, confirmando o potencial desta espécie para o desenvolvimento de bioinsseticidas.

Considerando o potencial inseticida de espécies vegetais como a *Lippia sidoides*, estudos que visam a elaboração de produtos que apresentem características, como baixa toxidade e estabilidade, que permitam ser considerados bons inseticidas é de suma importância para manutenção do desenvolvimento agrícola, sem gerar problemas ao homem e ao meio ambiente.

3.1.5. Gênero *Lippia* sp.

O gênero *Lippia* sp. (Verbenaceae) compreende aproximadamente 200 espécies distribuídas pelos continentes Americano e África. Representada por pequenas árvores, ervas e arbustos, as espécies de *Lippia* são aromáticas e apresentam diversos usos medicinais (STASHENKO et al, 2014; GOMES et al, 2011). Por ter muitas espécies utilizadas na medicina popular e pela presença de diversos quimiotipos no gênero, muitas pesquisas têm sido desenvolvidas para avaliar diferentes atividade biológicas dessas espécies (GOMES et al, 2011).

Na medicina popular *Lippia* spp. são usadas para tratar doenças do trato gastrointestinal, dermatites, doenças respiratórias e dores de cabeça. A principal parte utilizada são as folhas através de chás e maceração mecânica (CAMILO et al, 2022). Em relação as atividades biológicas investigadas, a antimicrobiana é a mais registrada em artigos acadêmicos. Trabalhos com *L. alba* mostram potencial do seu óleo essencial frente a bactérias Gram-positivas e Gram-negativas, a ação do óleo inibi o crescimento de bactérias que estão relacionadas com a deterioração de alimentos, formação de biofilmes e doenças infecciosas, sendo os compostos neral, citral e geraniale majoritários (MOTA et al. 2018; TOFIÑO-RIVERA et al. 2016; MACHADO et al. 2014).

Dentre outras atividades, a antioxidante, citotóxica e inseticida são as mais citadas para o gênero. Alguns trabalhos avaliaram a capacidade do óleo essencial de *L. alba* em inibir o crescimento de células tumorais, no qual foi possível observar concentrações inibitórias de 45 a 64 µg/mL (SANTOS et al. 2016). O químiotipo citral da mesma espécie demostra eficiência na inibição de células de carcinoma epitelioide do colo do útero humano, esse resultado foi relacionado ao composto citral presente em seu óleo essencial (MESA-ARANGO et al. 2009).

O óleo essencial de *L. origanoides* rico em carvacrol (48,31%) e timol (8.78%) apresenta alta atividade de sequestro do radical livre DPPH, com IC_{50} de 8,4 µg/mL (MAR et al. 2018). Diferentes químiotipos dessa mesma espécie foram avaliados quanto a sua capacidade antioxidante, sendo verificado o quimiotipo timol com maior capacidade de sequestro do radical DPPH, com inibição de 77.5% (SILVA et al. 2017).

Lippia sidoides conhecida popularmente como alecrim-pimenta, tem grande representatividade para região Nordeste brasileira por ser uma das plantas inseridas no RENISUS (Relação Nacional de Plantas de Interesse ao SUS). Ela é representada por arbustos ramificados e aromáticos, sendo suas folhas usadas em forma de chá para tratar rinites e alergias (MATOS, 2007). Devido a sua diversidade de aplicações, especialmente a antisséptica, essa espécie tem importância em áreas como a farmácia, medicina e odontologia (GUIMARÃES et al, 2015).

A espécie (figura 2) apresenta grande quantidade de estudos sobre seus óleos essenciais, demonstrando ter atividade antioxidante, antimicrobiana (COSTA et al, 2018) e inseticida (CARVALHO et al, 2003). Seu óleo essencial apresenta alto rendimento em plantas jovens, e tem como compostos majoritários o timol, carvacrol e 1,8-cineol (FIGUEIREDO et al, 2009; GUIMARÃES et al, 2014). O extrato etanólicos das suas folhas apresenta compostos da classe dos flavonoides, quinonas e naftoquinonas, compostos estes, responsáveis por diferentes atividades biológicas (COSTA et al, 2001). Embora sejam pouco os estudos com extratos fixos de *L. sidoides*, a presença desses compostos sugerem que esta planta apresenta potencial farmacológico, especialmente nas atividades antimicrobiana e antioxidante.

Figura 2: Espécie cultivada de *L. sidoides*

Fonte: Camilo, C.J. 2020.

Estudos com os extratos aquoso e etanólico das folhas de *L. sidoides* demonstraram ter atividade antibacteriana contra *Aeromonas hydrophila* em concentração de 520 µg mL^{-1} (MAJOLO et al, 2020). Em outro estudo, também com extratos aquoso e etanólico, foi observada capacidade de impedir a peroxidação lipídica e potencial antioxidante total das amostras. Os resultados estão associados a presença de flavonoides nas amostras (Lima et al, 2015). O óleo essencial e o extrato etanólico das folhas de *L. sidoides* apresenta atividade considerada contra espécies de *Candida*, com valores superiores ao observado para o fármaco de referência fluconazol (FARIAS et al, 2012).

Em relação a estudos inseticidas, grande parte das pesquisas são realizadas com o óleo essencial da espécie que apresenta ação em diferentes espécies de pragas. Podem ser citadas atividade frente a larvas e ovos de *Aedes aegypti* (LIMA et al, 2013); fumigante frente *Cryptotermes brevis* e *Sitophilus zeamais* (SANTOS et al, 2017; OLIVEIRA et al, 2018); e toxicidade de contato em *Microcerotermes indistinctus* (BACCI et al, 2015).

Diante dos estudos que demonstram potencial inseticida do óleo essencial da espécie *L. sidoides* e dos pouco trabalhos nessa área com extratos aquosos e etanólicos torna-se importante estudos de investigações aprofundados sobre o potencial inseticida dos seus extratos fixos, assim como, a sua caracterização química. Assim como, demostrar que esta espécie pode ser segura para uso pelas populações locais.

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5. **CAPÍTULO II: Traditional use of the genus *Lippia* sp. and pesticidal potential: a review**

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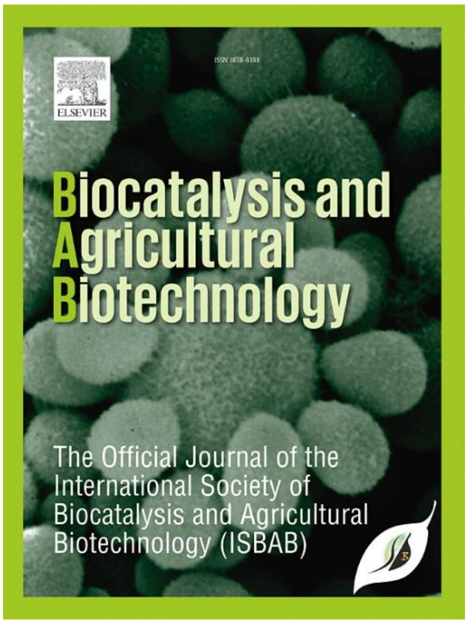

Traditional use of the genus *Lippia* sp. and pesticidal potential: a review

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Abstract

The understanding of popular knowledge has guided the studies involving promising natural products for the development of botanical insecticides, through ethnobotanical research. The frequent records of scientific research with the genus *Lippia* demonstrate a high insecticidal potential for the species, with prospects of new products for the market. Therefore, it is important to recognize the contribution of traditional knowledge and use it in plant selection for in-depth research that can lead to the elaboration of a final product. Thus, the aim of this research was to analyze existing relationships between ethnobotanical studies and research related to the control of insects, mites and ticks with the genus *Lippia* sp., as well as to verify the difficulties and perspectives for the development of new products derived from species of this genus for the fight pests. The main indications in the ethnobiological survey were medicinal for diseases of the gastrointestinal tract (34.2%), respiratory system (27.1%) and nervous system (22.18%), in addition to these, six species had indications for use as repellent. For the pesticide survey, the essential oils of the species *L. alba* and *L. sidoides* were the most investigated, being thymol and carvacrol, the most frequently identified compounds. The species *L. alba* was the most cited in both surveys, demonstrating an influence between the indications of traditional uses and biological investigations for the genus.

Keywords: Ethnobotany; botanical insecticides; industry; perspectives.

1. Introduction

Insects, mites, and ticks belonging to various species are associated with diseases such as allergies, dengue, malaria, zica, chikungunya, and yellow fever, as well as causing potential losses in agriculture and livestock (Moreau et al. 2020; Amoabeng et al. 2020; Chagas et al. 2016). The current research challenge in controlling these pests is to understand their diversity and adaptability which results in frequent increases in resistance and common non-target organism toxicity. Therefore, the control through synthetic or semi-synthetic products has become difficult, allowing practices associated with integrated pest management, which reduces the use of insecticides, pesticides, and decreases environmental impacts (Lefebvre et al. 2015).

Among the main causes that promote the use of synthetic insecticides limitation are the resistance acquired by pests to these products, with some chemical classes such as pyrethroids and organophosphates exhibiting greater development of resistance profile (Gandhi et al. 2016). According to Sparks et al. (2020), there has been an increase in resistance in recent years, with some pests being found to be resistant to 40 or more different insecticides; this probably occurs from the inappropriate use of agronomic practices and such as the overuse of synthetic products.

Considering this perspective, the investigation of plant species use as insecticide agents has become a viable alternative. Ethnobiological research brings, through traditional knowledge, important contributions in the selection of plants with the most varied therapeutic purposes; thus, guiding more specific chemical and biological studies through indications for use (Araújo et al. 2008). As for the insecticide potential, ethnobotanical studies have been contributing with indications of species for this purpose, although still very discreetly. Some plant species have been mentioned and investigated, both regarding their preventive use for some diseases and their insecticidal potential, among which we can highlight *Azadirachta indica* (neem), *Symphytum officinale* L. (confrei), *Vernonia condensata* Baker (boldo-baiano), *Stryphnodendron astringens* Mart. (barbatimão), and *Lippia* sp. (Santos et al, 2017a; Rocha and Sujii, 2019; Oliveira et al. 2018).

Despite the diversity of published studies on the insecticidal potential of plant species, there are still few botanical insecticides in the market. According to Isman (2017), the difficulty in their production is related to the research conduct, since only the investigation of insecticidal activity is not enough for product development. According to the author, there are several studies with plant extracts for this purpose; however, there are no detailed investigations on extraction methods, physicochemical stability, yields, and chemical composition of extracts, considering that these are the main impasses for the production of botanical insecticides.

The genus *Lippia* sp., belonging to the family Verbenaceae J. St. -Hil, and whose life form ranges from shrubby, herbaceous and subarbaceous, includes approximately 200 species distributed across regions of South America, Central America and Tropical Africa, with Brazil being considered the main center of richness in rupestrian fields and savannas (Terblanché and Kornelius, 1996). Ethnobotanical studies indicate that species of this genus are used in repelling mosquitoes, cockroaches and ticks, with leaves being the most used part of these plants (Almeida Neto et al. 2017; Farias et al. 2016), which justifies the interest of scientifically investigating its applicability as an insecticide.

This research aims to analyze the relationship between ethnobotanical indications for insecticide use with the genus *Lippia* and the development of research with biological activity related to the control of insects, mites and ticks.

2. Methodology

This search was conducted using the Science Direct, PubMed, Web of Science and Scopus databases; in addition, articles in English, Spanish, and Portuguese were also considered. Search were conducted on ethnobiological and medicinal data of the genus by using the following terms: "ethnobiology", "ethnobotany", "traditional medicine", "traditional use", whether or not associated with the word *Lippia*. A second search was conducted regarding biological assays by using the terms "insecticide", "acaricidal", "pesticide", whether or not associated with the word *Lippia*. Review papers, monographs, dissertations, theses, papers published in event annals, and books were excluded. Studies that did not include the scientific name of the insect, mite, or tick were also excluded.

The inclusion criteria were time span of 10 years for both ethnopharmacology data and biological test research. The ethnobiological data which contained the following information were considered relevant: scientific name of the plant, place where the study was conducted, therapeutic indication, part of the plant used, and preparation form. The data were tabulated in the order mentioned above. Some studies did not present the part used, or the form of preparation. As for the biological test data, the following information was considered relevant: insecticidal test, efficient concentration, chemical composition, and scientific name of the insect/mite/tick under study. After analysis of the papers, the data were tabulated with the scientific name of the plant, part used for extraction, bioactivity, main components, and their respective citations. The scientific names of the plant species were confirmed by the sites "The plant list" and "Tropicos".

3. Result and discussion

3.1. Ethnobotany of the genus *Lippia*

The search resulted in 92 articles out of which 70 contained the criteria for this work. Due to its wide distribution, the genus *Lippia* has medicinal importance that is recognized and spread throughout different regions of the world. The therapeutic potential of *Lippia spp*. is supported by several scientific publications that report both the ethnobiological use, indicating a high number of citations, and the investigations related to the proof of medicinal effects in general for species of this genus (Pascual et al. 2001; Ribeiro et al. 2014). Due to the diversity of ethnobiological properties reported and the indications for use against insects, mites and ticks, there is a growing interest and need for more specific studies in favor of this use.

Most of the works have as main uses the medicinal category for *Lippia* species. The most common form of preparation was the tea obtained from the decoction and infusion of the leaves. Seventeen species were recorded in this analysis, among which *L. alba* (26 articles) and *L. janvica* (16 articles) had the highest number of medicinal indications. *L. alba* presented indications for the treatment of gastrointestinal problems, such as diarrhea, colic, pain and indigestion. In addition to these, it can be indicated in the treatment of fever, pneumonia and as tranquilizers. The species *L. janvica* had the highest number of indications for diseases related to the respiratory system, such as bronchitis, flu and colds, nasal congestion and rhinitis, being the leaves the most used part.

Based on the data collected, it was found that the species are mainly used in American and African countries, regions with limited medical resources. Of the wide variety of therapeutic uses, diseases related to the gastrointestinal tract (34.2%), respiratory system (27.1%) and nervous system (22.18%) stand out.

Among the analyzed species, as can be seen in Table 1, six of them had indications as repellents against lice and diseases related to flies, with L. janvica having the highest number of indications for this purpose (Mhlongo and Wyk. 2019; Mavundza et. al. 2011; Corrigan et al. 2011; Magwede et al. 2019; Nyahangare et al. 2015). For repellent use, the fresh leaves of *L. janvica* were the parts of the plant most used from its burning to generate smoke or in the form of teas and mixtures. The other species, *L. alba*; *L. rehmannii*, *L. nodiflora*, *L. gracillis* and *L. microphyla* also present the leaves as the most used plant part in the form of maceration. These species are used as natural insecticides and for the control of mites and against diseases caused by flies. The African continent has the highest number of indications for these uses

Table 1. Traditional use of the genus *Lippia*. *continued*

Species	State / country	medicinal indications	used part	Method of preparation and use	References
***Lippia alba* (Mill.) N.E. Br. ex Britton & P. Wilson**	Minas Gerais/Brasil	Soothing	-	-	Conde et al. 2014
	Crato, Juazeiro, Barbalha/ Brasil	Abdominal spasms, vomiting, diarrhea, labyrinthitis, nervousness	Leaves	Infusion	Bitu et al. 2015
	Colares, Pará/Brasil	Anthelmintic, diarrhea, colic	Root, leaves	Tea	Ritter, 2012
	Paraná/ Brasil	Diseases of the respiratory system, diseases of the gastrointestinal system, diseases of the cardiovascular system	leaves, floral parts	Infusion	Bolson et al, 2015
	Santa Catarina/ Brasil	Cough	Leaves	Infusion	Tribess et al, 2015
	Antioquia/ Colombia	Snake bite	Whole plant	oral decoction	Vásquez et al, 2015
	Juruena, Mato Grosso, Brasil	Soothing, depression, stress, fatigue, hypertension, insomnia, malaise, local pain, fever, diarrhea, vomiting, stomach, intestine, intoxication, by digestion, colic, flu, cough	-	-	Bieski et al. 2015
	Santa Isabel, Amazonas, Brasil	Fever	Leaves	oral infusion	Frausin et al. 2015
	Maragogipe, Bahia, Brasil	Relief from stress, cough, colic, indigestion, gas, high blood pressure; high cholesterol; fever	-	-	Santana et al. 2016
	Rio Jauaperi, Amazonas, Brasil	soothing, insomnia, pain, fever, flu	Leaves	Oral	Pedrollo et al. 2016

a and b correspond to works that have the same author name and year of publication

Table 1. Traditional use of the genus *Lippia*. *continued*

Species	State / country	medicinal indications	used part	Method of preparation and use	References
	Caiena and Saint-Laurent du Maroni, Guiana Francesa	Flu	Leaves	Decoction, oral infusion	Tareau et al. 2017
	Araguaia, Mato Grosso, Brasil	High blood pressure, tranquilizer, insomnia, fever	Aerial parts	infusion	Ribeiro et al. 2017
	Ubatuba, São Paulo, Brasil	Digestive, high blood pressure, soothing	Leaves	infusion, oral	Yazbek et a. 2019
	Mon Sate, Ásia	eye disease	-	-	Kyaw et al. 2021
	Sangay, Morona Santiago, Equador	Stomach problems, diarrhea, body pain	Leaves	Decoction	Caballero-Serrano et al. 2019
	Maranhão, Brasil	diarrhea and dysentery	Leaves	-	Neiva et al. 2014
	Demerval Lobão, Piauí, Brazil	Soothing	Leaves	Tea	Aguiar et al. 2012
	Bananal, Mato Grosso, Brasil	Soothing, flu, cough, headache, pneumonia	-	-	Migueís et al. 2019
	Manacapuru, Amazonas Brasil	Stomach pain, soothing, to give, sleep, fever	Leaves	Tea	Vásquez et al. 2014
	Rangamati, Bangladesh	insect repellent, lice	Leaves	-	Rudra et al. 2020
	Manejo, Lima Duarte, Brasil	digestive, soothing	Leaves	Tea, infusion	Oliveira et al. 2012

a and b correspond to works that have the same author name and year of publication

Table 1. Traditional use of the genus *Lippia*. *continued*

Species	State / country	medicinal indications	used part	Method of preparation and use	References
	Abreu e Lima, Pernambuco, Brasil	Disorder of the nervous system; complications of pre and postpartum; indigestion; delayed menstruation; menstrual cramps; anemia; hypertension; migraine; intoxication.	Leaves	Infusion, decoction	Rodrigues et al. 2014
	Imperatriz, Maranhão, Brasil	Antispasmodic, sedative, Inflammation	Leaves	Infusion	Penido et al. 2016
	Barbalha, Ceará, Brasil	Flu, Fever, Cough with discharge	Leaves	Tea, infusion	Lemos et al, 2016
	Caaguazú, Paraguai	stomach antispasmodic	Leaves	Tea, infusion	Soria et al. 2020
	Salobrinho, Bahia	Abdominal pain, tranquilizer, fever, stomach pain, poor digestion, dizziness, high blood pressure, gas, scarring, diarrhea, abdominal pain, headache, appetite increase, flu, bloated stomach, vermifuge, Inflammation, high blood pressure, pain belly, gas, calming, poor digestion	Leaves, stalk, Stalks	Tea, juice	Feijó et al. 2013
***Lippia turbinata* Griseb.**	Bahía Blanca Argentina	Digestive	Leaves e floral tops	Tea	Michetti et al. 2019
	Gran Chaco, Argentina	Rheumatism	Leaves	Infusion, ingestion	Suárez. 2019
***Lippia integrifólia* (Griseb.) Hieron**.	Bahía Blanca Argentina	Digestive	Leaves e floral tops	Tea	Michetti et al. 2019
***Lippia javanica* (Burm.f.) Gaertn.**	Nkonkobe África do Sul	Coughs, colds, bronchitis, fever, ulcer	Leaves	Decoction	Asowata-Ayodele et al. 2016
	Amandawe África do Sul	Heartburn, bone repair, fever, trauma, poison, headache, wounds, colds and flu, musculoskeletal,	-	-	Mhlongo and Wyk. 2019

a and b correspond to works that have the same author name and year of publication

Table 1. Traditional use of the genus *Lippia*. *continued*

Species	State / country	medicinal indications	used part	Method of preparation and use	References
		inflammation, hysteria and shock, eye problems, congested nasal passages, lice			
	KwaZulu-Natal, África do Sul	Repellent	Fresh leaves	Smoke	Mavundza et al. 2011
	Maputaland África do Sul	Respiratory infections	Leaves e roots	Steam inhalation	York et al. 2011
	Mpoza, África do Sul	Coughs, colds, bronchial problems	Leaves (50g)	Tea infusion	Gail et al. 2015
	Santa Lúcia, África do Sul	Fever, Repellent	Leaves	Bath with tea, Burning leaves	Corrigan et al. 2011
	Vhembe, África do Sul	Repellant, medicine	Leaves	Tea	Magwede et al. 2019
	Sekhukhune e Waterberg, África do Sul	Fever	Fresh leaves	Inhalation	Semenya and Maroyi 2019
		Tuberculosis	Fresh leaves	Tea	
	Manica, República de Moçambique	venereal diseases	Root	oral decoction	Bruschi et al. 2011
	Butambala, Uganda	Tuberculosis	Leaves	-	Bunalema et al. 2014
	Amatole, Cape Province, South Africa	Boils, scabies, sores	Leaves	Infusion	Afolayan et al. 2014
	Mpigi e Butambala, Uganda	HIV treatment	Leaves, root bark	-	Nyamukuru et a. 2017
	província de Limpopo, África do Sul	Tuberculosis, Asthma, fatigue, fever, labored breathing, nasal congestion, pneumonia, rhinitis, sinusitis, wheezing	Leaves	Decoction	Semenya and Maroyi. 2020

a and b correspond to works that have the same author name and year of publication

Table 1. Traditional use of the genus *Lippia*. *continued*

Species	State / country	medicinal indications	used part	Method of preparation and use	References
	província de Limpopo, África do Sul	Rhinitis	Root	Crushed mixture, ingested nasally	Semenya and Maroyi. 2018[a]
	Província de Limpopo, África do Sul	Asthma	Leaves	Mixed and crushed dry	Semenya and Maroyi. 2018[b]
	Matobo, Kadoma, Chiredzi, Muzarabani, Zimbábue	Against ticks, fleas, lice	Leaves, branches	Crushed and mix with water	Nyahangare et al. 2015
***Lippia gracillis* Schauer**	Crato, Juazeiro, Barbalha, Brasil	Weakness, fever, wound healing, pain, poor appetite, flu, cough, sore throat, gastritis, rheumatism	Leaves, seeds	infusion	Bitu et al. 2015
	Sitio Carão, Sitio Letreiro	Myiasis	Leaves	Maceration	Silva et al. 2014[b]
***Lippia nodiflora* (L.) Michx**	Kodagu, Karnataka, Índia	diabetes, antiseptic	Whole plant	Decoction, vegetable paste	Lingaraju et al. 2013
	Thanjavur, Índia	memory loss cold	Stem bark (30g) Leaves (15g)	Maceration, boiling	Rajalakshmi et al. 2019
	Nelliyampathy colinas Kerala, Índia	Gonorrhea, Asthma, Mucous diarrhea, Skin diseases, Dandruff	Leaves	paste	Vijayakumar et al. 2015
	Pachamalai de Tamil Nadu, Índia	Dandruff, lice, rheumatism	Leaves	paste	Prabhu et al. 2021
***Lippia rehmannii* H.Pearson**	Sekhukhuneland, África do Sul	Food, neutralize smell, repellent	Leaves	Tea, food mixes, dry plant	Mogale et al. 2019
***Lippia multiflora* Moldenke**	Togo, África Ocidental	Malaria	Leaves	oral decoction, oral infusion	Koudouvo et al. 2011

a and b correspond to works that have the same author name and year of publication

Table 1. Traditional use of the genus *Lippia*. *continued*

Species	State / country	medicinal indications	used part	Method of preparation and use	References
	Bacia do Congo	hypertension	Leaves	Decoction	Tchicaillat-Landou et al. 2018
	Região Marítima, Togo	liver diseases	Leaves	Decoction	Kpodar et al. 2016
	Benin, África Ocidental	Candidiasis	Leaves	Oral decoction, topical use	Fanou et al. 2020
	Lomé, Assahoun, Tsevié, Atakpamé, Sokodé, Togo	Central nervous system disorder	Stem bark	Decoction, oral maceration	Kantati et al. 2016
***Lippia kituiensis* Vatke**	Loitoktok, Quênia	induce vomiting	Leaves	-	Muthee et al. 2011
	Machakos Condados de Makueni, Quênia	chronic joint pain	Roots	oral infusion	Wambugu et al. 2011
	Condado de Kajiado, Quênia	Respiratory problems, measles, protects cattle from ectoparasites	Leaves	-	Kimondo et al. 2015
***Lippia berlandieri* Schauer**	Xalpatlahuac, Guerrero, México	menstrual cramps, Stomachache	Whole plant	oral infusion	Juárez-Vázquez et al. 2013
***Lippia grandifolia* Hochst. ex A.Rich.**	Butambala, Uganda	Tuberculosis	Leaves	-	Bunalema et al. 2014
***Lippia chevalieri* Moldenke**	Guiné-Bissau, África Ocidental	High fever associated with chills	Roots	-	Catarino et al. 2016
	Senegal, África Ocidental	amoeba infections, diarrhea, common cold, cough, gastrointestinal disorder	Leaves, flowers	Decoction	Diop et al. 2018

a and b correspond to works that have the same author name and year of publication

Table 1. Traditional use of the genus *Lippia*. *continued*

Species	State / country	medicinal indications	used part	Method of preparation and use	References
	Daoudabougou, Bamako, sul de Bamako, Dioila, Mali	wellness, nutrition, diet, supplement	Leaves	Decoction	Nergard et al. 2015
***Lippia micromera* Schauer**	Caiena and Saint-Laurent du Maroni, Guiana Francesa	The flu	Leaves	Oral decoction	Tareau et al. 2017
***Lippia plicata* Baker**	Bié, Angola	Sore throat, muscle relaxant	Leaves, root	Decoction, mouthwash, massage	Novotna et al. 2021
***Lippia microphylla* Cham**	Sitio Carão, Sitio Letreiro	Myiasis	Leaves	Maceration	Silva et al. 2014[b]
***Lippia origanoides* Kunth**	Manacapuru, Amazonas Brasil	Stomach pain, gastritis, malaria	Leaves	Tea	Vásquez et al. 2014
***Lippia brasiliensis* (Link.) T. Silva**	Caaguazú, Paraguai	stomach antispasmodic	Leaves	Tea, decoction, maceration in cold water	Soria et al. 2020

a and b correspond to works that have the same author name and year of publication

3.2. Insecticide studies evaluation with the genus *Lippia*

The search resulted in 110 original articles, among which 71 satisfied the criteria favorable for this study. Despite the large number of species present in the genus, only 19 of them were investigated for some insecticidal/acaricidal activity. Table 2 lists the species of the genus *Lippia*, activity and chemical composition, and methods used in their respective studies, including different species of mites, insects, and ticks. Figures 1 and 2 show the structures of the chemical compounds identified from this survey.

Although the number of articles with pesticidal activity is higher than the ethnobotanical indications for this purpose, the species most cited in the survey are also the most investigated regarding their biological activity. In this way, the number of indications for a species in ethnobotanical surveys becomes a criterion that can lead researchers to select plants for further scientific investigations. This relationship becomes evident when we verify that the species *L. alba* is the most indicated for traditional use and the most studied in terms of its pesticidal activity.

As shown in table 2 *L. alba* is among the most investigated species in terms of pesticide activity, being also indicated in the ethnobotanical survey as a repellent. In general, the biological tests were carried out with the essential oil extracted from the leaves of this species, which demonstrates a good relationship with the ethnobotanical survey, which shows the leaves as the plant part most used by populations for mosquito repellency.

For the species *L. janvica*, the most indicated in the ethnobotanical survey for the use of insecticide and repellent, there was a positive relationship with scientific studies and, as in the ethnobotanical indications, biological tests were carried out with its leaves for the elaboration of aqueous extracts and powder mixtures.

The presence of essential oils in most studies for carrying out biological tests is related to the chemical composition of these products, which have proven to have pesticide activity. In addition, the repellent activity of volatile compounds can be observed through the form of use in ethnobotanical indications, which occurs by burning the leaves.

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
				Insects			
***Lippia javanica* (Burm.f.) Spreng.**	Leaves	Essential oil	*Sitophilus zeamais* Motsch	10 mg/mL	Adulticide	Perillaldehyde (44%); Limonene (24.1%)	Kamanula et al, (2017)
	Leaves	Aqueous extract	*Spodoptera frugiperda* J. E. Smith	10%	Larvicide	-	Phambala et al, (2020)
	Leaves	Dried leaf powder	*Callosobruchus maculatus* Fabr.	3g/10 g cowpea seeds	Adulticide Oviposition	* Camphor *α- Pineno	Mkenda et al, (2015a)
***Lippia origanoides* Kunth**	Leaves	Essential oil	*Cerataphis lataniae*	6.6 µg/mL	Adulticide	Carvacrol (48.31%);	Mar et al, (2018)
			Aedes aegypti L.	187.3 µg/mL	Larvicide	Cymene (9.11%)	
	Leaves	Essential oil	*Aedes aegypti* L.	53.79 mg/mL	Larvicide	Thymol (66.1%); *p*-Cymene (7.2%)	Ríos et al, (2017)
	Aerial parts	Essential oil	*Tribolium castaneum*	0.2 µL/cm^2	Repellent	-	Caballero-Gallardo et al, (2012)
	Whole plant	Essential oil	*Aedes aegypti* L.	390 ppm 1.000 ppm 1.000 ppm	Pupicide Adulticide Repellent	*p*-Cymene (8.7%); *y*-terpinene (5.1%)	Castillo et al, (2017)
	Leaves	Essential oil	*Aedes aegypti* L.	53.3 ppm	Larvicide	Carvacrol (32.3%); *p*-Cymene (12.0%)	Vera et al, (2014)

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
	Leaves	Essential oil	*Tribolium castaneum*	1.6 %	Repellent	Carvacrol (32.3 %);	Alcala-Orozco et al, (2019)
				20 µl/mL	Fumigant	Thymol (14.1 %)	
			Ulomoides dermestoides	1.6 %	Repellent		
				20 µl/mL	Fumigant		
	Leaves	Essential oil	*Myzus persicae* Sulzer	0.1%; 0.5%	Oviposition	Carvacrol (41,51%); *p*-Cymene (18,36%)	Teixeira et al, (2014)
	Leaves	Ethanol extract	*Rhyzopertha dominica* (F.)	95%	Adulticide	Alkaloids (0.0975 µl/mL) Phenols (0.0899 µl/mL Flavonoids (0.1055 µl/mL)	Flores et al, (2017)
	Stem and leaves	Essential oil	*Stegomyia aegypti*	0.50 mg/cm^2	Repellent	Thymol (50.1%); Carvacrol (14%)	Ramirez et al, (2012)
	Stem and leaves	Essential oil	*Stegomyia aegypti*	0.61 mg/cm^2	Repellent	Thymol (71%); Carvacrol (0.31%)	Ramirez et al, (2012)
***Lippia gracilis* Schauer**	Leaves	Essential oil	*Diaphania hyalinata*	20 µg/mg	Larvicide	Carvacrol (50.7%); Thymol (43.8%)	Melo et al, (2018)
	Leaves	Essential oil	*Nasutitermes corniger* Motschulsky	1,57 µg/mg	Mortality	Thymol (57.24%); Methyl-thymol (10.58%)	Lima et al, (2013a)
	Leaves	Essential oil	*Aedes aegypti* L.	0.039 mg/mL	Larvicide	γ -Terpineno (13.85%);	Galvão et al, (2019)

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
						p - Cymeny (10.70)	
	Leaves	Essential oil	*Liriomyza sativae* (Blanchard)	1.000 ppm 1.000 ppm	Larvicide Pupicide	-	Oliveira et al, (2020)
	Leaves	Essential oil	*Aedes aegypti* L.	0.292 mg/mL	Larvicide	1,8-Cineole (56.16%); α-Terpineol (12.09%)	Dias et al, (2015)
Lippia sidoides **Cham.**	Leaves	Essential oil	*Sitophilus zeamais* Motsch	3548 µL/ L	Fumigant	Thymol (68.45%); ρ-Cymene (10.66%)	Oliveira et al, (2018)
	Industrially obtained	Essential oil	*Cryptotermes brevis*	9.10 µg/mg	Fumigant	Thymol (76.3%); ρ-Cymene (10.0%)	Santos et al, (2017b)
	Leaves	Essential oil	*Sitophilus zeamais* Motsch	7.1 µg/ mg	Adulticide	Thymol (68.45%); ρ-Cymene (10.66%)	Oliveira et al, (2017)
	Leaves	Essential oil	*Nasutitermes corniger*	0.27 µg/mg	Mortality	Thymol (44.55%); ρ-cimene (25.25%)	Lima et al, (2013a)
	Leaves	Essential oil	*Rhodnius prolixus*	54.48 mg/cm^2 50 mg/cm^2	Nymphicidal Ovicidal	Thymol (69.91%); *o* –Cymene (14.84%)	Figueiredo et al, (2017)
	Industrially obtained	Essential oil	*Aedes aegypti* L.	25.5 µg/mL 276.8 µg/mL 66.4 µg/mL	Larvicide Pupicide Ovicidal	Thymol (83.24%); *trans*-Caryophyllene (5.77%)	Lima et al, (2013b)

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
				35.3 µg/mL	Oviposition		
	Leaves	Essential oil	*Tenebrio molitor*	8,04 µL/ L	Fumigation	Carvacrol (31.68%);	Lima et al, (2011a)
						ρ-Cymene (19.58%)	
	Leaves	Essential oil	*Amitermes cf. amifer*	2.43 µg/mg	Toxicity	Thymol (44.5%);	Bacci et al, (2015)
			Microcerotermes indistinctus	1.49 µg/mg	Toxicity	*p*-Cymene (25.2%)	
	Aerial parts	Essential oil	Coenagrionidae	51.65 µL/ L	Larvicide	Carvacrol (67.8%);	Silva et al, (2014a)
						p-Cymene (21.7%)	
	Leaves	Aqueous extract	*Trigona spinipes*	10%	Mortality	-	Correia-Oliveira et al, (2012)
	Industrially obtained	Essential oil	*Spodoptera frugiperda* J. E. Smith	3,21 mg/g	Nymphicidal	-	Lima et al, (2020)
			Podisus nigrispinus	28,43 mg/g	Nymphicidal		
	Industrially obtained	Essential oil	*Aedes aegypti* L.	36 ppm	Larvicide	-	Paula et al, (2011)
	Industrially obtained	Essential oil	*Nannotrigona* aff . *testaceicornis*	33.7 µg	Lethal toxicity	-	Matos et al, (2021)
***Lippia adoensis* Hochst.**	Leaves	Essential oil	*Callosobruchus maculatus* Fabr.	107 µL/ L	Fumigant	Eucalyptol (28.36%);	Adelani et al, (2016)
				30 µL /30 cm^{-2}	Repellent	*α*- Terpineol (25.99%)	

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
	Leaves	Essential oil	*Callosobruchus maculatus* Fabr.	10 µL/ g	Decrease resistance profile	-	Akami et al, (2019)
	Leaves	Essential oil	*Callosobruchus maculatus* Fabr.	10 g μL^{-1}	Mortality	-	Akami et al, (2017)
Lippia alba **(Mill.) N.E.Br. ex Britton & P.Wilson**	Leaves	Essential oil	*Sitophilus zeamais* Motsch	15.2 µL/mL	Toxicity	Carvona (63.47%);	Peixoto et al, (2015b)
			Tribolium castaneum	19.5 µL/mL	Toxicity	Geraniale (46.25%)	
	Leaves	Essential oil	*Aedes aegypti* L.	72.34 mg/mL	Larvicide	Carvona (35.3%); Limonene (35.0%)	Ríos et al, (2017)
	Industrially obtained	Essential oil	*Culex quinquefasciatus*	59.6 µl/L	Larvicide	Carvona (35.2%);	Benelli et al, (2018)
			Musca domestica	115 µl/L	Toxicity	Limonene (32.0%)	
	Leaves	Essential oil	*Nasutitermes corniger* (Isoptera: Termitidae)	2.15 µg/mg	Mortality	Carvona (62.86%); Limonene (26.51%)	Lima et al, (2013a)
	Leaves	Essential oil (Nanoemulsion)	*Aedes aegypti* L.	30.02 mg/L	Larvicide	Geranial (30.02%);	Ferreira et al, (2019)
			Culex quinquefasciatus	38.21 mg/L	Larvicide	Neral (25.26%)	
	Leaves	Essential oil	*Aedes aegypti* L.	0.0422 mg/mL	Larvicide	Carvone (38.3%); Limonene (31.8)	Vera et al, (2014)
	Leaves	Essential oil	*Callosobruchus chinensis* L	11049.2 µL kg^{-1}	Fumigant Repellent	-	Shukla et al, (2011)

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
				150 µL			
	Aerial parts	Essential oil	*Tribolium castaneum* Herbst	0.2 µL/cm^2	Repellent	Carvone (35.3%); Limonene (35.0%)	Caballero Gallardo et al, (2011)
	Leaves	Essential oil	*Spodoptera frugiperda* (J. E. Smith)	1.20 µg/ mg	Acute toxicity	Geraniol (47.0%); Neral (32.9%)	Niculau et al, (2013)
	Leaves	Aqueous extract	*Spodoptera frugiperda* J. E. Smith	2 mg/mL	Larvicide	-	Gualteros et al, (2019)
	Whole plant	Essential oil	*Aedes aegypti* L.	390ppm	Pupicide	Limonene (31.8%);	Castillo et al, (2017)
				1.000 ppm	Adulticide	carvone (38.3%),	
				1.000 ppm	Repellent		
	Leaves	Essential oil	*Bemisia tabaci*	1%	Repellent	-	Baldin et al, (2013)
	Stem and Leaves	Essential oil	*Stegomyia aegypti* L.	0.58 mg/cm^2	Repellent	-	Ramirez et al, (2012)
				0.62 mg/cm^2	Repellent		
	Leaves	Essential oil	*Sitophilus zeamais* M.	78 µL/L	Ingestion toxicity	1,8-Cineole (70.01%); γ-muurolene (9.24%)	Lima et al, (2021)
	Leaves	Essential oil	*Aedes aegypti*	113.99 µg/mL	Larvicide	β-Caryophyllene (26.08%); β-Elemene (10.96%)	Sobrinho et al. (2021)
		Essential oil	*Aedes albopictus*	275.1 µg/mL	Larvicide	β-Caryophyllene (26.08%)	Sobrinho et al. (2021)

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
						β-Elemene (10.96%)	
***Lippia multiflora* Moldenke**	Leaves	Essential oil	*Callosobruchus maculatus* Fabr.	20 µL	Mortality	-	Ilboudo et al, (2015)
	Leaves	Essential oil	*Bemisia tabaci*	0.4 µL/L	Fumigant	Linalool (46,6%); (*E*) -nerolidol (16,5%)	Tia et al, (2011)
***Lippia pedunculosa* Hayek**	Leaves	Essential oil	*Aedes aegypti* L.	58 ppm 0.49 ppm	Larvicide Repellent	Piperitenone oxide (70.35%); Limonene (25.10%)	Nascimento et al, (2016)
***Lippia turbinata* Griseb.**	Leaves	Ethanol extract	*Acromyrmex lundi* Guérin	5 mg/mL	Anti-foraging	-	Napal et al, (2015)
	Aerial parts	Essential oil	*Plodia interpunctella*	432.97 mg/L	Larvicide	-	Corzo et al, (2020)
***Lippia junelliana* (Moldenke) Tronc.**	Leaves	Essential oil	*Aedes aegypti* L.	0.005 $\mu L/cm^2$	Repellent	Camphor (20.7%); Limonene (19.4%)	Gleiser et al, (2011)
***Lippia integrifolia* (Griseb.) Hieron.**	Leaves	Essential oil	*Aedes aegypti* L.	0.11 $\mu L/cm^2$	Repellent	Camphor (26.5%); Methylheptenone (24.9%)	Gleiser et al, (2011)
	Aerial parts	Essential oil	*Triatoma infestans*	0.5%	Repellent	Borneol (8.72%); Lippifoli-1(6)-en-5-one (8.71%)	Lima et al, (2011b)
***Lippia graveolens* Kunth**	Aerial parts	Essential oil	*Sitophilus zeamais* Motsch	600 µL/L	Fumigation	Thymol (22.8%); Carvacrol (22.7%)	Peschiutta et al, (2016)

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
	Leaves	Essential oil	*Ulomoides dermestoides*	0.002 µL/cm^2	Repellent	*trans-β*-caryophyllene (11.3%); *p*-cymene (11.2%)	Cervantes-Ceballos et al, (2015)
***Lippia berlandieri* Schauer**	Leaves	Essential oil	*Culex quinquefasciatus*	6.5 µg/mL 181 µg/mL	Larvicide Pupicide	Carvacrol (57.5%); Thymol (32.8%)	Andrade-Ochoa et al, (2018)
***Lippia Schaueriana* Mart. ex Schauer**	Leaves	Essential oil	*Liriomyza sativae*	1000 ppm	Larvicide	-	Oliveira et al, (2020)
***Lippia palmeri* S. Watson**	Leaves	Essential oil	*Prostephanus truncatus* *Sitophilus zeamais* Motsch	320.52µL/L 441.45 µL/L	Mortality Mortality	Thymol (58.9%); *ρ*- Cymene (21.8%)	Martínez-Evaristo et al, (2015)
	Leaves	Essential oil	*Zabrotes subfasciatus*	1.35 µL/g	Mortality	*p*- Cymene (33.7%); Carvacrol (18.3%)	Ortega-Nieblas et al, (2014)
***Lippia grata* Schauer**	Leaves	Essential oil	*Aedes aegypti*	36.28µg/mL	Larvicide	Thymol (73.49%); 1,8-Cineole (13.58%)	Felix et al, (2021)
			Mites				
***Lippia origanoides* Kunth**	Leaves	Essential oil	*Tetranychus urticae* Koch	25.1 µg/mL	Adulticide	Carvacrol (48.31%); Cimeno (9.11%)	Mar et al, (2018)
	Leaves	Ethanol extract	*Tetranychus cinnabarinus* (Boisduval)	20%	Mortalidade	Alkaloids; tannins; flavonoids; saponins	Sivira et al, (2011)
***Lippia gracilis* Schauer**	Leaves	Essential oil	*Raoiella indica*	499 mg / mL	Toxicity	Thymol (52.41%);	Santos et al, (2019)

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
			Aceria guerreronis	4.28 mg / mL	Toxicity	Thymol de metyl (10.81%)	
	Leaves	Essential oil	*Tetranychus urticae*	0.06 µL L^{-1}	Fumigant	Carvacrol (61%);	Born et al, (2018)
			Neoseiulus californicus	1.2 µL L^{-1}	Fumigant	*p-* cymene (11%)	
***Lippia alba* (Mill.) N.E.Br. ex Britton & P.Wilson**	Industrially obtained	Essential oil	*Dermanyssus gallinae*	77.7 µg/ mL	Toxicity	Carvona (35,2%); Limonene (32,0%)	Tabari et al, (2020)
***Lippia graveolens* Kunth**	Leaves	Essential oil	*Varroa destructor*	1.16mL	Mortality	Carvacrol (59.29%); *p-* Cymeno (28.65%)	Romo-Chacón et al, (2016)
				Ticks			
***Lippia javanica* (Burm.f.) Spreng.**	Leaves	Extrato aquoso	*Boophilus*	20%	Mortality	*Verbascosídeo, apigenin, luteolin, diosmetina, Chrysoeriol	Madzimure et al, (2011)
			Rhipicephalus evertsi evertsi		Mortality		
			Rhipicephalus appendiculatus		Mortality		
			Hyalomma		Mortality		
			Amblyomma		Mortality		
***Lippia origanoides* Kunth**	Leaves/ Inflorescence	Essential oil	*Rhipicephalus (Boophilus) microplus*	3.10 mg/mL	Mortality	Carvacrol (49.7%); *p-* cymene (13.3%)	Chagas et al, (2016)
***Lippia gracilis* Schauer**	Leaves/ Inflorescence	Essential oil	*Rhipicephalus (Boophilus) microplus*	3.21 mg/mL	Mortality	Carvacrol (40.4%); *p-* cymene (11.4%)	Chagas et al, (2016)
	Leaves	Essential oil	*Rhipicephalus (Boophilus) microplus*	1.31 mg/mL	Larvicide	Thymol (59.26%); Metil-thymol (8.32%)	Cruz et al, (2013)

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
	Leaves	Essential oil	*Rhipicephalus (Boophilus) microplus*	1.02 mg/mL	Larvicide	Thymol (59.26%); *β*- caryophyllene (8.57%)	Costa-Júnior et al, (2016)
	Leaves	Essential oil	*Rhipicephalus microplus*	4,66 mg mL^{-1}	Change in ovaries	Thymol (59.26%); *β*-Caryophyllene (8.57%)	Penha et al, (2021)
	Leaves	Essential oil	*Rhipicephalus microplus*	6,65 mg/mL	Oocyte morphology	Carvacrol (35.28%); γ-Terpinene (21.11%)	Penha et al, (2021)
Lippia sidoides **Cham.**	Leaves	Essential oil	*Rhipicephalus sanguineus* *Amblyomma cajennense*	11.56 mg/mL 15.70 mg/mL	Larvicide Larvicide	Thymol (69.9%) *o*- Cymene (14.84%)	Gomes et al, (2014)
	Leaves/ Inflorescence	Essential oil	*Rhipicephalus (Boophilus) microplus*	27.67 mg/mL	Mortality	Thymol (64.5%); *p*- cymene (11.7%)	Chagas et al, (2016)
	Leaves	Essential oil	*Rhipicephalus (Boophilus) microplus*	0.93 mg/mL	Larvicide	Thymol (54.40%); *p*-Cymene (19.18%)	Soares et al, (2016)
	Leaves	Essential oil	*Rhipicephalus (Boophilus) microplus*	3.36 mg/mL	Larvicide	Thymol (38.68%); *p*-Cymene (34.11)	Soares et al, (2016)
	Leaves	Essential oil	*Rhipicephalus (Boophilus) microplus*	3.90 mg/mL	Larvicide	Thymol (64.82%); *p*-Cimene (13.89%)	Soares et al, (2016)
	Leaves	Essential oil	*Rhipicephalus (Boophilus) microplus*	2.99 mg/mL	Larvicide	Carvacrol (43.69%); *p*-Cymene (17.83%)	Soares et al, (2016)

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
	Leaves	Essential oil	*Rhipicephalus microplus* *Dermacentor nitens*	11.13 µL/mL 5.59 µL/mL	Larvicide Larvicide	Thymol (67.70%); Carvacrol (6.30%)	Gomes et al, (2012)
	Industrially obtained	Essential oil	*Rhipicephalus microplus*	40.0 µL / mL	Larvicide	Thymol (69.91%); *o*-Cymene (14.84)	Monteiro et al, (2014)
	Leaves	Essential oil	*Rhipicephalus microplus*	2,80 mg mL^{-1}	Change in ovaries	Thymol (38.68%); *p*-Cymene (34.11%)	Penha et al, (2021)
	Leaves	Essential oil	*Rhipicephalus microplus*	4,31 mg mL^{-1}	Change in ovaries	Thymol (64.82%); *p*-Cymene (13.89%)	Penha et al, (2021)
***Lippia alba* (Mill.) N.E.Br. ex Britton & P.Wilson**	Leaves	Essential oil	*Rhipicephalus (Boophilus) microplus*	8,8 mg/mL	Larvicide	Carvone (63.47%); Geranial (46.25%)	Peixoto et al, (2015a)
	Leaves/inflorescence	Essential oil	*Rhipicephalus (Boophilus) microplus*	10,78 mg/mL	Mortality	Carvone (61.7%); Limonene (17.5%)	Chagas et al, (2016)
	Leaves	Essential oil	*Rhipicephalus microplus*	0.47 mg/cm^2	Repellent	Carvone (62.8%); Limonene (26.5%)	Lima et al, (2016)
	Leaves	Essential oil	*Rhipicephalus microplus*	0.20 mg/cm^2	Repellent	Carvone (52.6%); Limonene (26.6%)	Lima et al, (2016)
***Lippia graveolens* Kunth**	Leaves	Essential oil	*Rhipicephalus (Boophilus) microplus*	2,5%	Larvicide	Thymol (24.59%); Carvacrol (24.54%)	Martinez-Velazquez et al, (2011)

Table 2. Pesticide activity and chemical composition of extracts from species of the genus *Lippia* sp. *continued*

Species	Used part	Type of extract	Organisms	Concentration effective	Effect	Identified/isolated compound	References
	Leaves	Essential oil	*Rhipicephalus microplus*	10%	Adulticide	-	Flores-Fernández et al, (2016)
***Lippia triplinervis* Sin. *Lippia microcephala* Cham**	Aerial parts	Essential oil	*Rhipicephalus microplus*	2.5 mg / mL	Larvicide	Carvacrol (31.9%); Thymol (30.6%)	Lage et al, (2013)
	Aerial parts	Essential oil	*Rhipicephalus microplus*	71.9 mg / mL	Adulticide	Carvacrol (31.9%); Thymol (30.6%)	Monteiro et al, (2021)

*Authors do not report percentage of compounds

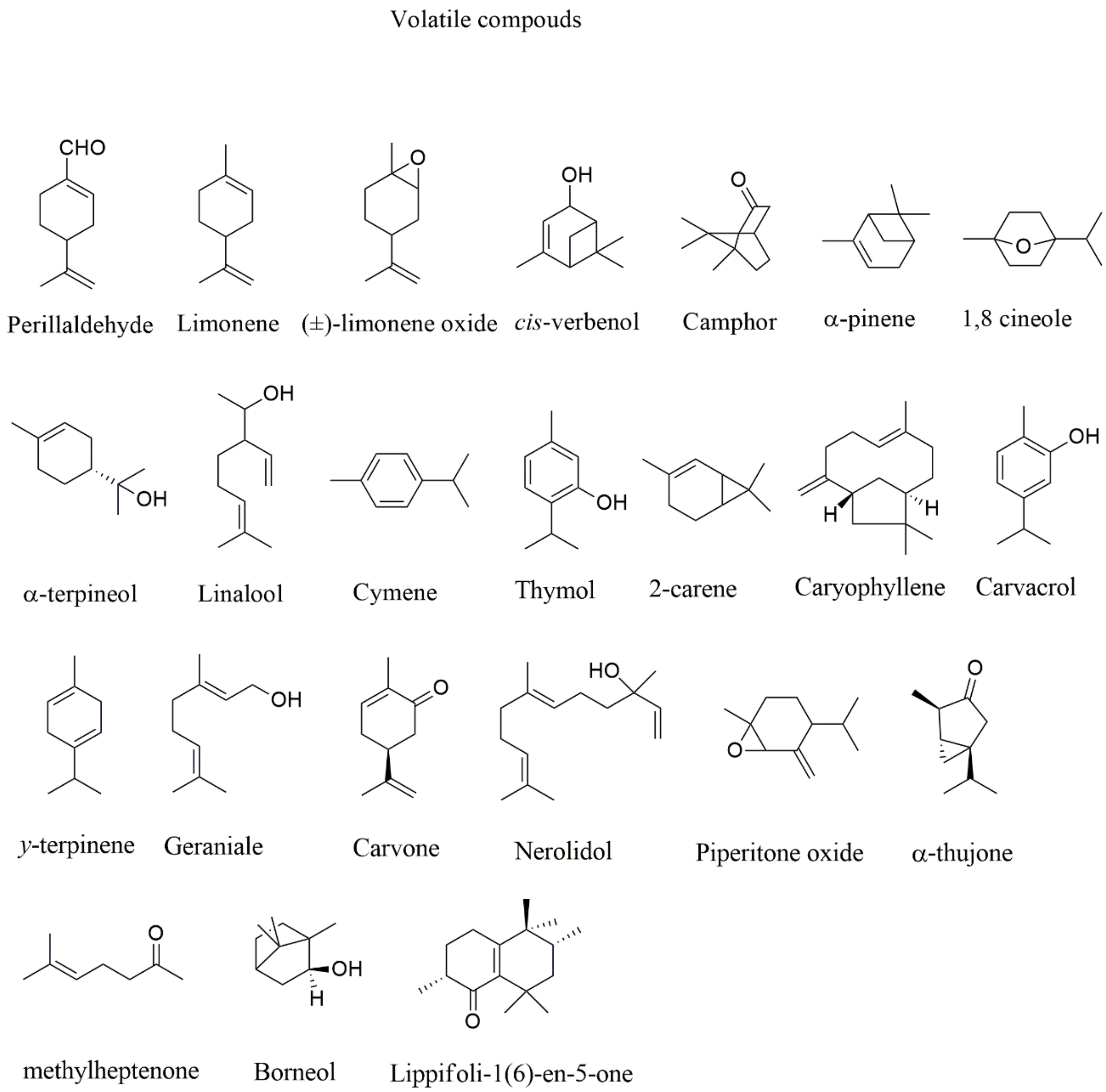

Figure 1. Chemical structure of volatile compounds.

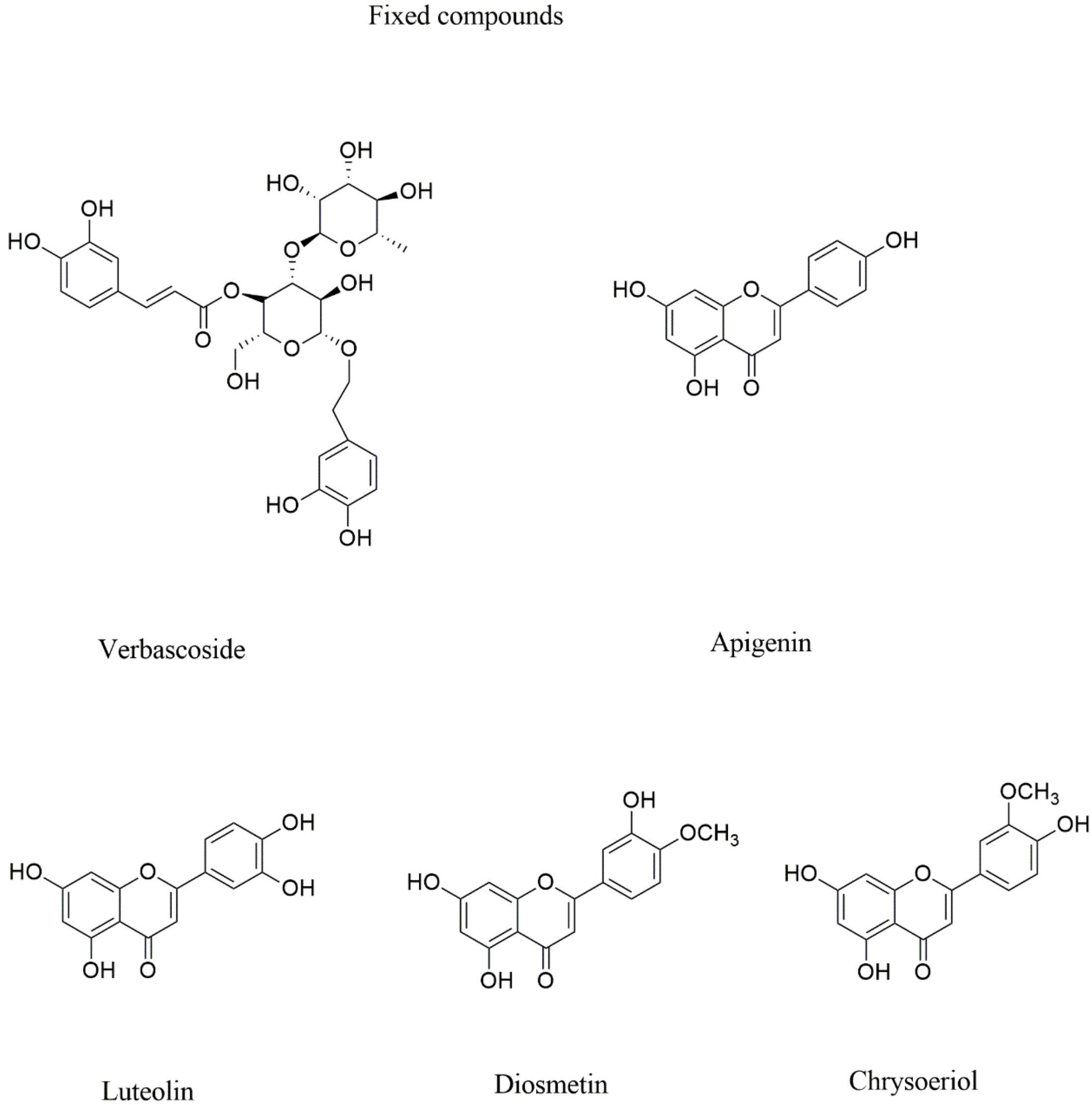

Figure 2. Chemical structures of the compounds identified in this study.

From the data obtained in this survey, the potential that *Lippia* species have to act as good biopesticides is evident. The species *L. alba* obtained the largest number of works carried out, being carvone, the main compound identified in its essential oil. Peixoto et al, (2015)b state that the essential oils of this species, rich in monoterpernes, have rapid insecticidal activity and can act in less than 20 hours after exposure. The effect of *L. alba* essential oil can occur through different techniques, such as repellency, fumigation, toxicity and mortality (Table 2).

The toxic activity of *L. alba* essential oil is more efficient when compared to Croton rudolphianus essential oil against *S. zeamais* with LC_{50} results of 15.2 µL/mL and 70.64 µL/mL, respectively (Ribeiro et al. 2020). The high repellent potential of *L. alba* can be confirmed in the study by Caballero-Gallardo et al. (2011), where the essential oil showed greater repellency than the positive control of IR3535 [ethyl 3-(N-acetyl-N-butylamino).

The essential oil of *L. origanoides* exhibits greater potential for repellency and mortality against *T. Castaneum*, when compared to other essential oils of species such as *Elettaria cardamomum* (L.) Maton and *Salvia officinalis* (L.) (Alcala-Orozco et al. 2019). The authors suggest that this species is promising for use in techniques such as integrated pest management. In another study, the essential oil of *L. berlandieri* was more efficient against larvae of *C. quinquefasciatus* when compared to the essential oils of *Citrus aurantifolia*, *Cuminum cyminum*, *Syzygium aromaticum*, *Laurus nobilis*, proving to be a promising species against the pest. (Andrade-Ochoa et al. 2018).

Among the compounds that have pesticidal activity, carvone has shown efficiency for the control of different pests. When evaluated against *R. microplus* this compound has an LC_{50} of 9.9 mg/mL (Peixoto et al. 2015)a. The repellent action of carvone was confirmed against *T. castaneum* at a concentration of 0.2 µL/cm^2, maintaining its action at 95% after 4 hours of exposure. (Caballero-Gallardo et al. 2011). Vera et al. (2014) state that the presence of these compounds and other moterpenes are responsible for the insecticidal action of *Lippia* species.

3.2.1. Relationship between activity and chemical composition

As seen in table 2, the constituents identified in the most frequent essential oils were thymol and carvacrol, with ocerrences in 8 and 6 species respectively, recognized as chemical markers of the genus *Lippia*, and with a wide biological action on different pests. The toxic potential of thymol against the mites *Aceria guerreronis* and *R. indica* was investigated by Santos et al. (2019), who obtained LC_{50} of 5.34 mg/mL and 9.03 mg/mL, respectively. However, in another study against *C. brevis* species, thymol showed a lethal dose of 8.20 µg/mL in a direct contact assay (Santos et al. 2017). In the same study, the essential oil from *L. sidoides* presented a lethal dose close to thymol, with a value of 9.33 µg mg^{-1}; such results are considered significant in combating these and other pests.

The compound thymol can act in inhibiting the acetylcholinesterase enzyme in mite species, exhibiting a positive relationship between acaricidal activity and enzyme inhibition. Structurally, inhibitory effect may be related to oxygenate functions of this compound (Cardoso et al. 2020).

L. gracilis essential oil (carvacrol chemotype) exhibited 90% mortality in *D. hyalinata* larvae at a dose of 16.72 μg mg^{-1} (Melo et al, 2018). In another study, carvacrol caused 100% mortality of *R. microplus* larvae at a concentration of 2.5 mg / mL (Novato et al, 2018), demonstrating that this compound may have a direct connection with larvicidal activity. Regarding the action mechanism, carvacrol can act in modifying the morphology of tick ovaries, preventing their reproduction (Souza et al, 2019); therefore, it could be used as a control for this pest.

The action mechanisms related to essential oils and their components can occur through genetic modifications, as demonstrated by Akami et al, (2019), in which essential oil of *L. adoensis* acts in inhibiting the P450 and GST genes (encode the synthesis of phase I and II detoxification enzymes, respectively) in the insect *C. maculatus*, (figure 3). These genes are responsible for the development of resistance mechanism which occurs in most beetles treated with DDVP (2,2 dichlorovinyl dimethylphosphate), which is used for its elimination from the cell.

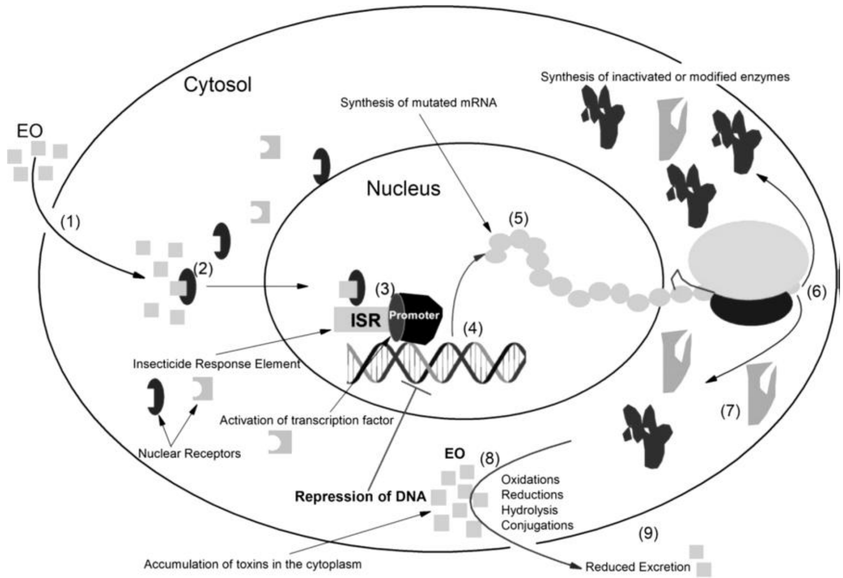

Figure 3. Mechanism of action of *L. adoenses* essential oil on *C. maculatus* (Akami et al. 2019).

Volatile compounds can also cause neurotoxic action on insects and mites through enzyme inhibition. Direct relation between the activity of *L. origanoides* essential oil against the pests *T. urticae* and *C. lataniae*, and the inhibition of the acetylcholinesterase enzyme, was observed by Mar et al (2018), suggesting that the insecticidal and acaricidal actions may have occurred through this pathway, since the results were similar for both pests.

A suggestion was made by Corzo et al. (2020) that *L. turbinata* essential oil may prevent or cause delay in ecdysis in *P. interpunctella* larvae through inhibition of neuropeptide precursors that regulate post embryonic development, most notably PloinOK, PloinETH and PloinCZ. This study found that the coefficient of variability of the groups not treated with the essential oil was 1.07 ± 0.08, while the treated group had a coefficient of 0.63 ± 0.16, showing a greater potential for inhibition.

Binary combination between isolated components of essential oils, as in the case of carvacrol:thymol (1:1) and 1,8-cineol:thymol (1:1), proves to be more efficient presenting higher toxicity against *T. molitor* larvae, when compared to essential oil and isolated components (Lima et al. 2011a).

The essential oils of *L. gracilis*, chemotypes thymol and carvacrol, exhibited strong activity against *D. hyalinata* larvae, showing lethal doses of 5.90 μg/mg and 4.56 μg/mg, respectively, revealing variation in the results based on the evaluated chemotype. When these compounds were tested in isolation, they showed significant difference in the results, with 0.94 μg/mg for carvacrol and 2.99 μg/mg for thymol, which are higher potentials than those obtained from essential oils (Melo et al, 2018). These results demonstrate the participation of the chemotype in the biological activity of the species.

The fixed chemical composition extracts were also investigated for their insecticidal/acaricidal potential, and their composition associated with activity; among the classes of compounds that have been identified in different *Lippia* species are alkaloids, flavonoids, and saponins (Flores et al, 2017). The ethanolic extract of L. origanoides acts on oviposition and causes mortality of *T. cinnabarinus* at concentrations of 15 and 20%; the same study reports the presence of the classes of compounds mentioned above, which are probably responsible for the activity of this species extract (Sivira et al. 2011).

3.2.2. Analysis of the results from the methods used

Several methods are used to evaluate the ability of a plant species to inhibit the development of pests. In this survey, larvicidal testing is the most widely used, since it is one of the most efficient ways to reduce the incidence of adult mosquitoes, and, consequently, disease transmission (Pavela, 2015). In addition to this, tests involving toxicity by contact or ingestion feature as the main ways of eliminating pests.

A way to verify the insecticidal ability of the extracts is through the use of different methodologies which can target different life stages of the insect. One example is the distinct studies conducted with *A. aegypti*, which was the most studied species for the genus *Lippia*. Several studies presented different methodologies which evaluated the mortality of the insect at different life stages; therefore, the low lethal concentration values observed, regardless of the method used, indicate the variability of the extracts in acting as good insecticides against this species.

The larvicidal capacity against *A. aegypti* observed by most of the species studied is directly related to their chemical composition. The activity of the compounds carvacrol and thymol (Table 2), which are presented mostly as majorities, has already been proven in the work of Silva et al. (2017), with lethal concentrations of 51 and 58 ppm respectively, justifying the action of the essential oils.

Another factor to be considered among the methodological analyses are the studies dealing with different exposure periods of the natural product to the pest. This method will be able to determine how long the natural product acts efficiently. For example, this is the case of the study with the essential oil of *Lippia alba* against *T. castaneum*, which demonstrated that even after 4 h, the essential oil was able to maintain 96% repellency activity at a concentration of 0.2 μL cm2 (Caballero-Gallardo et al. 2011). This result was related to the compound Carvone (35.3%) which showed itself as the majority, obtaining 100% repellency at the highest concentration tested.

4. Toxicity in the genus *Lippia*

Considering the pesticidal activity spectrum observed for the genus *Lippia*, there is a need to understand the capacity of cytotoxic activity in the different models which these species can develop; therefore, we discuss some results that deal with the cytotoxic activity of *Lippia* species in models commonly used in current research.

4.1. Cell culture

Frequently, investigations into the cytotoxic profile of essential oils from various *Lippia* species have been conducted on cancer cell lines as well as on healthy cells. As an example, *L. gracilis* essential oil exhibits cytotoxicity in hepatocellular carcinoma and chronic myelocytic leukemia cell lines with IC50 of 4.93 to 22.92 g/mL, respectively; this effect occurs through alterations in cell cycle progression, caused by a halt in the G1 phase. Probably, this result is associated with the mixture of constituents found in the oil (Ferraz et al. 2013).

Another study showed that the essential oil (920 µg/mL) of *L. citriodora* showed genotoxic effect in Jurkat cells within only 20 minutes of incubation. Citral, the major component of the oil, was also evaluated demonstrating non-concentration-dependent genotoxic action (Fitsiou et al. 2017). This species is abundant in acteoside, a phenylethanoid glycoside that acts as an antioxidant by decreasing the activity of Reactive Oxygen Species (ROS) from tumor cells. The action of this compound was isolated and investigated against A5 skin carcinoma cells by Cheimonidi et al. (2018), and they could observe a toxic effect by increasing ROS.

L. alba essential oil (citral chemotype) causes significant antiproliferative effect in K562 cells, inducing 77% of cell death at the concentration of 45 µg/mL. The mechanism involved in this activity occurs by activation of p53, which causes apoptosis due to the release of reactive oxygen species (García et al. 2017). In human breast cancer and adenocarcinoma cells, essential oil from the leaves of this species exhibited cytotoxic activity with IC50 of 63.9 µg/mL and 100 µg/mL, respectively (Santos et al. 2016). Based on this result, the oil was considered promising for the treatment of cancers, since it exhibited no cytotoxic action on healthy cells.

In colon carcinoma cell lines, the essential oils of *L. sidoides* (19.05 µg /ml), L. salviifolia (30.20 µg /ml), and *L. rotundifolia* (36.30 µg /ml) reduced cell viability, while not affecting normal cells (Gomide et al. 2013). *L. multiflora* essential oil exhibited antiproliferative activity in prostate cancer cell lines and in bone metastasizing cells, as well as anti-inflammatory and antioxidant activity. These activities were associated with the monoterpene compounds which compose the essential oil of the species (Bayala et al. 2014).

L. nodiflora essential oil associated with silver nanoparticles has a dose-dependent cytotoxic effect towards MCF-7 cancer cell line, exhibiting an IC_{50} of 40 μg/ml after 24 hours of treatment (Sudha et al. 2017). The ethanolic extract of this same species promotes morphological distortions in lung cancer cells, probably triggering an apoptotic pathway (Vanajothi et al. 2012).

4.2. Conventional *in vivo* methods

To evaluate the toxic ability of *L. sidoides* essential oil in mammals, Lima et al. (2013)b conducted assays with rats, in which moderate toxicity was demonstrated for the species, with an LD_{50} value of 2,624 $mg.kg^{-1}$. Authors claim that this result may be associated with the type of methodology used, or even with the difference in the chemical composition of the species. Another study to determine the toxic capacity in rats was conducted with the aqueous extract of the species *L. javanica*, which showed mortality within 48 h after ingestion, from the observation of hemorrhages on the serosal surfaces of the organs and effusions in the pleural and abdominal cavities (Madzimure et al. 2011).

Oral administration of *L. citiodora* essential oil does not cause toxicity in mice, with no changes observed in body weight, liver, and spleen. Regarding the animal's health, no signs of discomfort, pain or behavioral deviation were observed (Spyridopoulou et al. 2021). The acute toxicity of L. *gracilis* oil in mice was evaluated by oral administration of different doses, and no toxic effect was observed regarding the following parameters: gastric lesions and bleeding, behavioral alteration, presence of convulsion, death or presence of gastric ulcer (Guilhon et al. 2011).

Different extracts of *L. origanoides* were evaluated for toxicity in mice. It was observed that the hexanic extract at a dose of 1000 mg/Kg caused the death of the animal within 30 mim after administration, while the aqueous, methanolic, and ethyl acetate extracts had a lethal dose >2000 mg/Kg, causing 33% death of the animals. The composition of the hexanic extract may be the justification for higher toxicity, with thymol (33.40%), m-cimen-8-ol (16.37%), methyl salicylate (10.48%), carvacrol (6.75%) and linalool (5.17%) being found as majority constituents (González-Trujano et al. 2017). On the other hand, the essential oil of *L. origanoides* does not cause any toxic effect in relation to these animals; in the study by Andrade et al. (2014) the animals treated with the oil demonstrated to have no behavioral changes, no weight changes, and no damage to the organs, heart, liver, and kidney.

4.3. Alternative methods

Among the alternative methods tested with the genus *Lippia*, the assays with the enzyme acetylcholinesterase can be highlighted. Mar et al. (2018) demonstrated that L. origanoides oil has inhibitory activity on the enzyme acetylcholinesterase; this activity was considered important to demonstrate a possible mechanism of action in insects. However, further studies are needed in order to demonstrate that the used dose of the oil will not affect non-target organisms. Also with the same species, inhibition of this enzyme was observed in vitro; however, when tested directly on *S. zeamais* adults no activity was detected, showing that there was no direct relationship between insecticidal activity and enzyme inhibition (Peschiutta et al. 2016). Other study tested the essential oil of *L. timoides* as an inhibitor of acetylcholisterase activity in vitro, and the results showed that the action of the oil may be associated with the thymol component (Silva et al. 2019).

Galvão et al. (2019) reported that *L. gracilis* essential oil showed LC_{50} of 30.31 ppm against *Artemia sp. nauplii*. According to the authors, this concentration is higher when compared to the concentration needed to kill *A. aegypti* larvae. Acute toxicity tests in juvenile tambaqui were performed with *L. alba hydrolate*, which showed increased mortality with increasing concentrations tested. The IC_{50} observed was 7.43% for the hydrolate (Maia et al. 2019). These results can show low toxicity of the investigated oils.

5. Perspectives

The biopesticide potential for the genus Lippia is demonstrated by the large number of works published in recent years, so it is necessary to develop more in-depth studies that can elucidate the mechanisms of action against these organisms, isolate the active substances and develop means that allow greater stability of essential oils.

Despite the physical-chemical stability of essential oils being an obstacle to the development of effective formulations, the use of nanomaterials has been shown to be efficient in increasing specificity, reducing impacts on non-target organisms and increasing the stability of these products, protecting their active ingredient for longer. This area of research has progressed in recent years and may be a viable alternative for the development of more effective products.

6. Conclusion

The gathering of ethnobotanical data on the genus Lippia is important for directing further research with these species. The main findings show that the main indications for use are in the gastrointestinal, respiratory and nervous systems. Regarding pesticide data, essential oils were the most investigated, with thymol and carvacrol being the main compounds identified. The information obtained in this work is useful for directing future research that seeks viable alternatives for pest control with natural products

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6. CAPÍTULO III- Analysis toxicity by different methods and anxiolytic effect of the aqueous extract *Lippia sidoides* Cham

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Analysis toxicity by different methods and anxiolytic effect of the aqueous extract *Lippia sidoides* Cham.

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Abstract

Lippia sidoides Cham. (Verbenaceae) is a species often mentioned in traditional medicine due to the medicinal properties attributed to its leaves, which include antibacterial, antifungal, acaricidal and antioxidant. Several of these actions have been scientifically proven, according to reports in the literature; however, little is known about toxicological aspects of this plant. This work included studies to determine the chemical composition and toxicity tests, using several methods aiming to evaluate the safety for use of the aqueous extract of *L. sidoides* leaves, in addition, the anxiolytic effect on adult zebrafish was investigated, thus contributing to the pharmacological knowledge and traditional medicine concerning the specie under study. The chemical profile was determined by liquid chromatography coupled to mass spectrometry-HPLC/MS with electrospray ionization. Toxicity was evaluated by zebrafish, *Drosophila melanogaster*, blood cells, and *Artemia salina* models. 12 compounds belonging to the flavonoid class were identified. In the toxicity assays, the observed results showed low toxicity of the aqueous extract in all tests performed. In the analysis with zebrafish, the highest doses of the extract were anxiolytic, neuromodulating the GABAa receptor. The obtained results support the safe use of the aqueous extract of *L. sidoides* leaves for the development of new drugs and for the use by populations in traditional medicine.

Keywords: Aqueous extract; *L. sidoides*; Flavonoids; Toxicological profile

Introduction

Natural products of vegetal origin are recognized for the variety of chemical substances present in their parts, and for their broad property of performing pharmacological activities[1]. Although the industrial influence, medical plants are still used to cure and treat different diseases by the majority of population[2]. The medical use can occur through different ways of preparations, whether in the form of teas, stews, baths, among others[3]. These plants represent considerable importance for many different cultural groups, since they are accessible and can be used against several types of pests and diseases[4].

In this regard, recognizing the potential of a particular plant species to present some type of toxic reaction among its users is important, whether for the development of new drugs or for home use based on preparations; consequently, an evaluation of the relationship between risk and benefit is necessary to improve medical plant use, which means that the development of complete toxicological studies to help ensure the use of these species is extremely important.

The search for anxiolytic drugs has become relevant in current research, given that the most used drug in the clinic is Diazepam[5], but clinical uses of benzodiazepines are limited by their side effects such as psychomotor impairment, sedation, myorelaxation, ataxia, amnesia, physical and psychological dependence[6]. Benzodiazepines act through benzodiazepine receptors present on the pentameric GABAa complex.

The genus *Lippia*, related to the family Verbenaceae, contains about 200 species distributed in various parts of the world. The species Lippia sidoides, popularly known as “Alecrim-pimenta” (rosemary pepper), is an aromatic plant which can be found in the Northeast region of Brazil[7]. In traditional medicine, this species is used to treat inflammation, wounds, mycoses, and acnes[8]; it is also part of the list of Brazilian medical plants, which are considered of interest by RENISUS (The National List of Medicinal Plants of Interest to the Brazilian Unified Health System (SUS)).

Many studies have demonstrated the pharmacological potential of products extracted from *L. sidoides*, such as antimicrobial activity[9,10], acaricidal[11], anti-inflammatory, antioxidant, and gastroprotective[12]; these activities are generally associated with the essential oil, with some chemical components highlighted: E-caryophyllene, thymol, 1,8-cineol, β-myrcene, and other important substances.

Lippia essential oils are associated with different biological activities, such as antimicrobial. In randomized studies have verified the effect of mouthwash based on *L. sidoides* essential oil to reduce dental plaque, gingival inflammation and gingival bleeding. The random choice of patients made it possible to verify the efficiency of the oil treatment over the course of seven days. In one of the studies, a decrease in the presence of Streptococcus mutans among treated patients is demonstrated[13,14]. This information refers to the fact that there is little research demonstrating the biological capacity of its fixed extracts, especially related to toxicity[15-16].

The aqueous extract of *L. sidoides* leaves has compounds such as quercetin, luteolin and taxifolin, which are responsible for important pharmacological activities. Among these activities, anxiolytic activity was recorded by quercetin and luteolin, which may have their effect due to interactions with GABAergic receptors[17,18].

Knowing the pharmacological potential that the species *L. sidoides* presents and its variability of chemical compounds considered active, it is important to develop new studies that can consider this species as a future phytotherapic.

Considering the importance of its extracts for the development of scientific studies, and the necessity to assure the use of this species by the population in traditional medicine, the present study approaches the toxicological profile of the aqueous extract of *Lippia sidoides* leaves by different evaluation methods and its anxiolytic effect on adult zebrafish via GABAa receptor.

Materials and Methods

Obtaining plant material and preparing the extract

Experimental research and field study, including collection of plant material, followed institutional, national and international guidelines and legislation. Permission to collect leaves of *Lippia sidoides* was obtained from the authority of the Regional University of Cariri and the registration is in the Genetic Heritage Management Council of Brazil (code A2B7A05) and in the Herbário Caririense Dárdano de Andrade Lima, from URCA, under number 3038.

Lippia sidoides leaves were collected from the medicinal plant garden of the Universidade Regional do Cariri-URCA; latitudinal coordinates 7º14'20.1" S, and longitudinal coordinates 39º24'53.1 W. The collecting was performed on November 2019 at 3 h in the afternoon.

Fresh leaves were macerated with hexane to remove lipidic components and then subjected to agitation with water in a refrigerated Shaker incubator (NT 715 Novatecnica) using the following parameters: Temperature: 50 ºC; Rotation: 180 rpm; Agitation time: 4 h/day for 7 d.

The yield corresponded to 4L of aqueous solution, which was dried by atomization until obtaining the powder form with the Mini-spray dryer MSDi 1. 0 Mini-spray dryer (Labmaq do Brasil), using a 1.2 mm nozzle, under the following operational conditions: a) flow control: 200 mL/h; b) inlet temperature: 120±2º C; c) outlet temperature: 88±2º C; d) atomization air flow: 45 L/min; e) blower flow: 1.80 m3/min.

HPLC-MS-ESI analysis

The extract was analyzed by HPLC system - Shimadzu, using analytical chromatographic column C18 (Kromasil - 250 mm x 4.6 mm x 5 µm), coupled to a mass spectrometer (Ion-Trap AmazonX, Bruker) with Electrospray Ionization (ESI). The sample was solubilized in methanol (1mg/mL), after which it was filtered on PVDF (Poly-vinylidene Fluoride) filters with a mesh size of 0.45 µm. The chromatographic method used methanol (solvent B) chromatographic grade and ultrapure water type I (Milli-Q) with formic acid (0.1% v/v) (solvent A), in concentration gradient (5 to 100% of B in 95 min). The injection volume was 10 µL and flow rate was 0.6 mL/min. In the mass spectrometer, the samples were subjected to sequential fragmentation in MS3. The parameters used were: capillary 4.5 kV, end plate offset 500 V, nebulizer gas 35 psi, dry gas (N2) with flow rate 8 mL/min and temperature 300 °C. The sample was analyzed in negative ionization mode and the identification of the compounds was based on the data (MS/MS) as reported in the literature.

Total flavonoid quantification

The quantification of flavonoids was performed according to the methodology described by Kosalec et al (2004)[19] with adaptations. The extract was prepared at an initial concentration of 20 µg / mL and diluted by 10; 5; 2 and 1 µg / mL in tubes with a final volume of 50 mL. Into these tubes, a total of 760 µL of methanol, 40 µL of 10% potassium acetate, 40 µL of 10% aluminum chloride, and 1,120 mL of water were added; the samples were incubated at ambient temperature, and readings taken in a UV-Visible spectrophotometer at 415 nm. The analysis was performed in triplicate, and the flavonoid content calculated from the calibration curve using quercetin (QE), with results expressed as µg eq.Q/g for extract.

Toxicity analysis in *Drosophila melanogaster*

Rearing and stocking of *Drosophila melanogaster*

Drosophila melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. Flies were reared in 340 mL glass containers with the medium containing: (83 % corn paste, 4 % sugar, 4 % freeze-dried milk, 4 % soybean meal, 4 % wheat bran, and 1 % salt). When cooking the mixture, 1 g Nipagin (Methylparaben) was added. After cooling in the growth containers, 1 mL of solution containing Saccharomyces cerevisiae was added. The flies were kept at 25 °C and 60 % relative humidity. All tests were performed with the same strain[20].

Mortality Test

Tests were performed according to the method proposed by Cunha et al, (2015)[20] with some modifications. Adult flies (males and females) were placed in 130 mL glass containers (6 cm high and 6.5 cm in diameter), containing filter paper at the bottom. For the control, 1 mL of 20 % sucrose in distilled water was added on this paper. For the other groups different concentrations of the extract ranging from 2000 to 4000 µg/mL were added. During the entire procedure, a 12-hour light/dark cycle was maintained with a controlled temperature of 25 °C, and 60% relative humidity. The experiment was performed in triplicate in which each "n" was composed of two containers, with 20 flies placed in each of them. Readings to verify mortality were taken every 3, 6, 12, 24 and 48 h.

Negative Geotaxis Test

Determination of the damage to locomotor ability was performed using the negative geotaxis assay. Each group of live flies exposed to the different concentrations of the extract at reading times of 3, 6, 12, 24 and 48 h were led to the bottom of the containers, and after 1 min the number of flies that reached 8 cm in height from the container were counted. The trials were repeated twice at 1 min intervals[21].

Cytotoxic activity on erythrocytes

Preparation of human erythrocytes and ethics statement

The erythrocyte samples were donated from the blood bank of the Laboratory Escola de Análises Clínicas de Biomedicina, with the consent and approval of the responsible researcher, José Walber Gonçalves Castro- CRBM 9815. The developed method was reviewed and approved by the biomedicine clinical analysis committee from the Doctor Leão Sampaio University Center. The procedures were performed according to the laboratory immunohematology manual-Ministry of Health, Brazil.

The blood used was type O^+, which was initially homogenized in sodium citrate before the procedure. In a test tube, 900 µL of saline solution was pipetted, followed by 100 µL of whole blood in sodium citrate. The red blood cell washing procedure was performed by centrifuging the tube at 3500 rpm for 15 s, discarding the supernatant at the end of each centrifugation. The washing process was repeated 6 times, removing as much of the supernatant as possible with absorbent paper on the last wash. At the end the RBCs were homogenized with 900µL of saline.

Cytotoxicity analysis

Blood samples were collected, prepared, and exposed to different concentrations of the extract (10, 25, 50, 100, 250, 500, 1000 µg/mL). The solutions remained in a 37 ºC water bath for 30 min, after which 2100µL of 0.9% saline solution was added to the blood. Following this, the samples were centrifuged, and the supernatant was read in a UV-visible spectrophotometer at a wavelength of 540nm. The negative control contained only the RBCs and 0.9% saline solution[22].

Morphological analysis

The samples treated with the aqueous extract of *L. sidoides* and the positive control were prepared and fixed on slides for microscopic analysis. The images were obtained by smears of erythrocytes stained by conventional staining and compared using specific software for erythrocyte morphological analysis based on morphological changes described in the literature.

Cytotoxic activity against *Artemia salina*

A. salina eggs were incubated in artificial seawater under light at 28°C. After 24 h of incubation, larvae were collected with a Pasteur pipette, and kept for another 24 h under the same conditions to reach the largest stage. The sample was dissolved in Tween 80 and serially diluted (1000, 250, 125, 100, 75 µg/ml) in seawater; Next, 10 larvae were added to each set of tubes containing the samples. A control was run with potassium dichromate. 24 h later, the number of survivors was counted[23].

Zebrafish Bioassays

Zebrafish

Adult zebrafish (ZFa) animals between 60 and 90 d (0.4 ± 0.1 g), from the wild, of both sexes, were obtained from a commercial supplier (Fortaleza, CE). The animals were kept in glass aquarium (n=5/L), at a temperature of 25 ± 2°C, in light-dark cycles for 24 h. Water was treated with antichlorine. The bioassays performed are in accordance with the Ethical Principles of Animal Experimentation, and were approved by the Ethics Committee for Animal Use (CEUA) of the Ceará State University (UECE) (04983945/2021). The procedures for performing this test are in accordance with the ARRIVE guide. After the experiments, the animals were sacrificed by freezing and immersed in ice water (2-4ºC) for 10 min until loss of opercular movements.

Locomotor activity (Open Field Test)

Animals were given the sample application, and subsequently submitted to the open field test[24] to assess whether there was a change in the motor system, either by sedation and/or muscle relaxation. Animals (n=6/group) were intraperitoneally treated with the extracts (40; 200 and 400 mg/kg; 20 µL; i.p), and group with vehicle (DMSO 3%). One group of animals (n=6/group) without treatments (Naive) was included. After 30 min of the treatments, the animals were added into Petri dishes (10 x 15 cm) containing the same aquarium water, marked with four quadrants, and analyzed locomotor activity by counting the number of line crossings (CL). Using the CL value of the Naive group as a baseline (100%), the percentage of line crossings (CL%) was calculated individually for 0-5 min.

Acute toxicity 96h

The acute toxicity study was conducted against adult zebrafish according to the Organization for Economic Cooperation and Development Standard Method[25] to determine the LD50-96h. Mortality was controlled every 12 h after the beginning of the tests. The animals (n=6/group) were treated intraperitoneally with 20 µL of the extracts (40; 200 and 400mg/kg; 20 µL; i.p), vehicle (DMSO 3%). After 96 h, number of fish deaths in each group were counted and the lethal dose capable of killing 50 % of the animals (LD_{50}) was determined using the Trimmed Spearman-Karber method with 95% confidence interval[26].

Anxiolytic activity (Light-Dark Test)

The animals' anxiety behavior was observed using a light-dark test. Similar to rodents, adult zebrafish naturally avoid illuminated areas[27]. The experiment was carried out in a glass aquarium (30 cm × 15 cm × 20 cm) divided into light and dark areas. The aquarium was filled with non chlorine tap water, which simulated a new shallow environment different from the conventional aquarium and capable of inducing anxiety behaviors. In animals (n = 6/group), 20 µL of the extract was administered intraperitoneally (i.p.) (40; 200 and 400 mg/kg; 20 µL). Negative and positive control groups consisted of 3% DMSO and 10 mg/kg DZP solution, respectively. After 1 h, the animals were placed individually in the clear zone, and the anxiolytic effect was measured based on the time spent in the clear zone of the aquarium within 5 min of observation[28].

Assessment of GABAergic neuromodulation

The anxiolytic action mechanisms of the extract were identified through pretreatment with flumazenil (a benzodiazepine channel antagonist)[29]. Zebrafish (n = 6/group) were pretreated with flumazenil (4 mg/kg; 20 µL; i.p.). After 15 min, the highest effective dose of the extract with an anxiolytic effect (400 mg/kg; 20 µL; i.p.) found in the pilot test was administered (see previous section). A group treated with 3% DMSO (vehicle; 20 µL; i.p.) was used as a negative control. DZP (10 mg/kg, 20 µL; i.p) was used as a positive control because it is an agonist of the Benzodiazepine binding channel in GABAa. After 30 min of treatment, the animals were submitted to the light/dark test as described in the previous section.

Statistical Analysis

All analyses were performed in triplicate and data expressed as mean (n=3) ± Standard Deviation (SD) using one-way and two-way Analysis of Variance (ANOVA) succeeded by Tukey's test for multiple comparison for data with normal distribution and significantly similar standard deviations with values of $P < 0.05$; $P < 0.01$ and $P < 0.001$. Statistical analyses and graphical presentation of the results were performed using GraphPad Prism software (version 6.1).

Results

Chemical profile by HPLC-MS-ESI and total flavonoid content

The chemical composition of *L. sidoides* extract was performed from negative mode HPLC-MS-ESI analysis from the data obtained in table 1. 12 compounds belonging to the class of flavonoids were identified through the molecular ion mass, error (ppm), and fragmentation profile analysis of the compounds, by comparing with literature data. The chemical structures of the identified compounds are shown in figure 1.

Table 1. Negative MS fragmentation and UV–vis absorption data of the compounds detected in *L. sidoides*.

Peak No.	t_R (min.)	*m/z* [M−H]⁻/	Molecular Formula	Error (ppm)	MS^2/MS^3	Tentative assignment	Ref.
1	43.2	303.0519	$C_{15}H_{12}O_7$	1.6	MS^2 [303.0]: 284.9; 176.9; 124.8	Taxifolin	61
2	43.4	449.1093	$C_{21}H_{22}O_1$	0.5	MS^2 [449.0]: 286.9 MS^3 [449.0→286.9]: 150.9 MS^4 [449.0→286.9→150.9]: 106.9	Eriodictyol-7-O-glicoside	62
3	47.2	451.1231	$C_{21}H_{24}O_1$	-2.6	MS^2 [451.0]: 288.9 MS^3 [451.0 →288.9]: 270.9; 166.8; 124.9	3-Hydroxyphlorizin	63; 64
4	48.5	447.0940	$C_{21}H_{20}O_1$	-1.3	MS^2 [447.0]: 284.9 MS^3 [447.0 →284.9]: 240.9; 198. 8; 174.9; 150.8; 132.9	Luteolin-6 -O-glicoside	62
5	51.9	435.1296	$C_{21}H_{24}O_1$	0.1	MS^2 [435.0]: 272.9 MS^3 [435.0 →272.9]: 166.8	Phloridzin	65; 63
6	52.6	431.0994	$C_{21}H_{20}O_1$	-2.4	MS^2 [431.0]: 268.9 MS^3 [431.0 →268.9]: 224.8	Emodin-8-O-glicoside	66
7	55.5	287.0596	$C_{15}H_{12}O_6$	-2.0	MS^2 [287.0]: 268.8; 150.8; 124.9; 106.9	Eriodictyol	67
8	60.6	271.0619	$C_{15}H_{12}O_5$	-2.2	MS^2 [270.9]: 176.8; 150.8; 118.9	Naringenin	67
9	62.3	285.0408	$C_{15}H_{10}O_6$	-1.3	MS^2 [284.9]: 266.9; 256.8; 242.9; 240.9; 216.9; 198.9; 174.9; 150.9; 132.9	Luteolin	63; 67
10	64.1	593.1489	$C_{27}H_{30}O_1$	-2.5	MS^2 [593.0]: 446.9; 284.9 MS^3 [593.0 →284.9]: 240.8; 198.7; 174.8; 150.9; 132.9	Luteolin-7-O-rutinoside	68; 69; 70
11	66.9	268.0459	$C_{15}H_{10}O_5$	-1.5	MS^2 [268.9]: 224.8; 226.9; 200.9; 150.9; 148.8	Apigenin	71; 72
12	70.0	313.0735	$C_{17}H_{14}O_6$	-1.3	MS^2 [313.0]: 297.9; 282.9; 268.9	Cirsimaritin	73

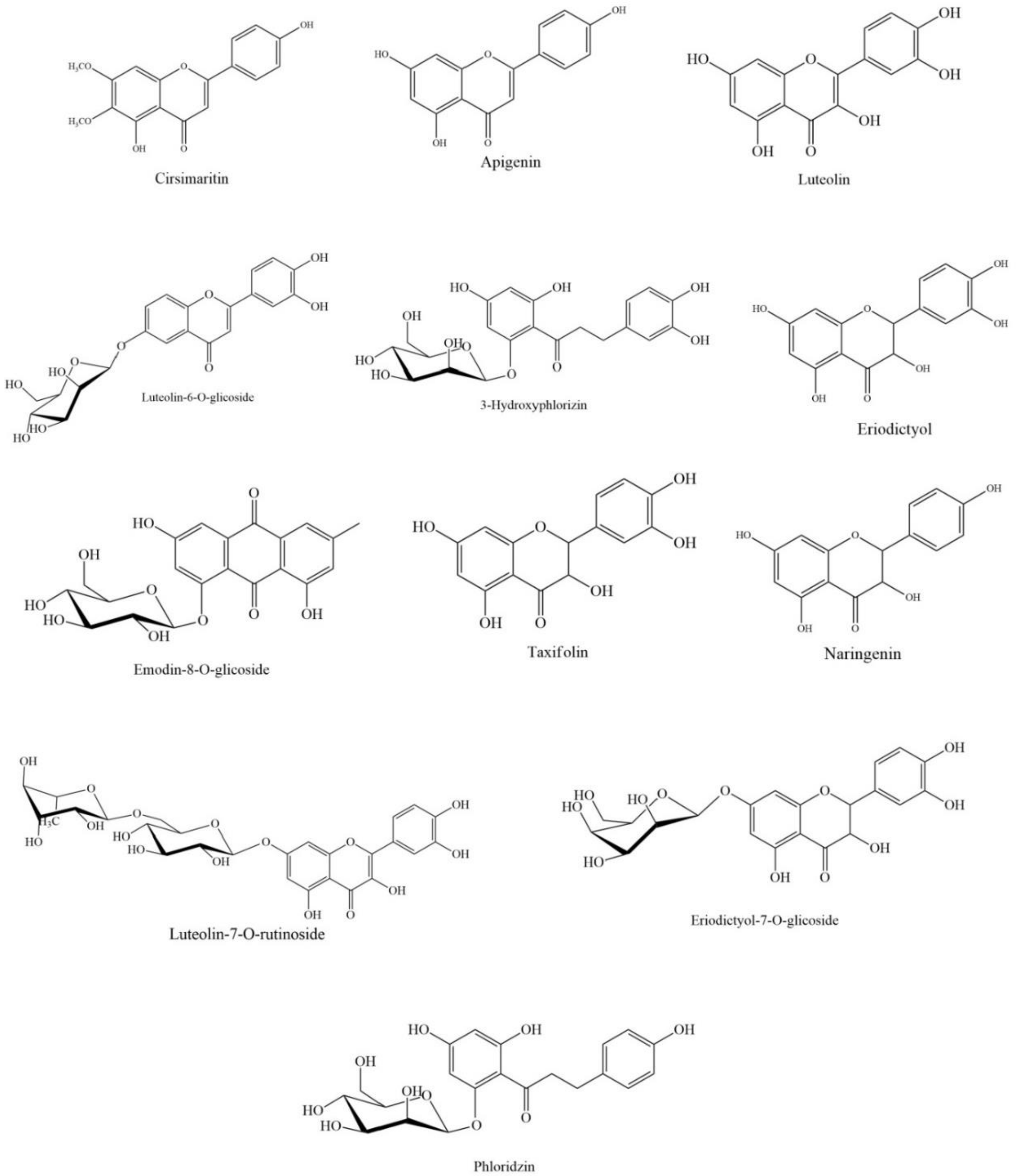

Figure 1. Identified compounds in the aqueous extract of *L sidoides*

The chromatographic profile demonstrated the presence of phenolic compounds in the extract. From this, a quantification of total flavonoids was performed in order to verify the estimated amount of this class in the extract. The value found was 6.3±0.8 µg eq.Q/g of the extract.

Toxicity in *Drosophila melanogaster*

Aqueous extract of *L. sidoides* exhibited the highest toxicity against *Drosophila melanogaster* at the concentration of 4000 µg/mL after 24 h of exposure. At concentrations of 2500 and 3000 µg/mL, an increase in the number of fly deaths could be observed after 48 h of exposure to the product. This result suggests that higher concentrations require less time of product exposure to cause mortality of *D. melanogaster* larvae. Figure 2 shows the number of deaths per concentration of the extract.

It was possible to observe that after the longest exposure time, even at the lowest concentrations, there was mortality. Thus, the results indicate a significant difference in the exposure time to the extract.

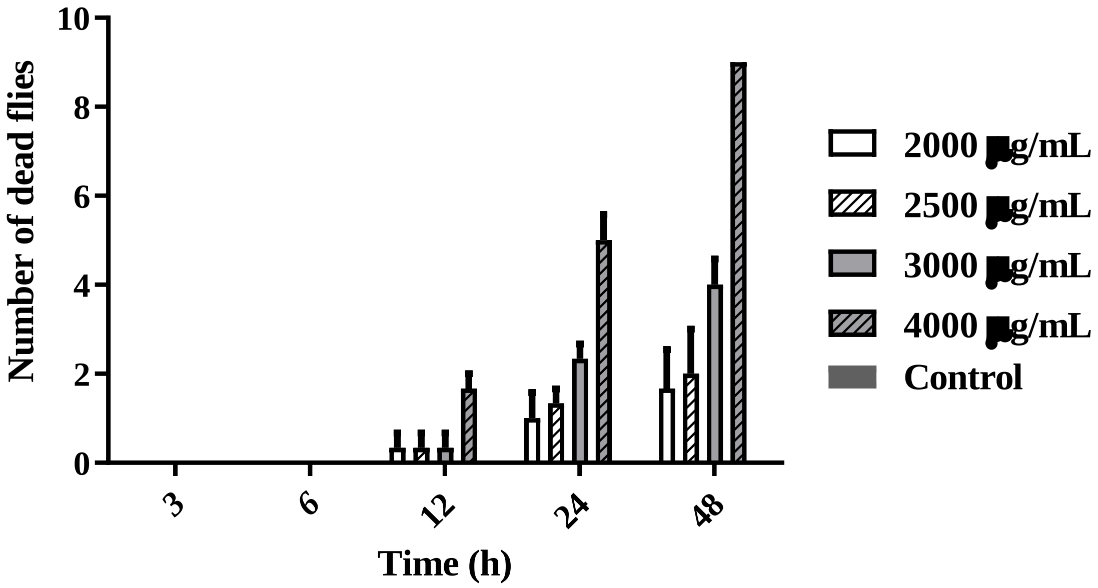

Figure 2. *L. sidoides* aqueous extract mortality tests against *Drosophila melanogaster*. The graph shows the mortality of *D. melanogaster* when exposed to concentrations of 2000, 2500, 3000, as well as 4000µL/mL of the extract at 48 h of exposure compared to the control group.

Negative geotaxis

Negative geotaxis results revealed that the extract showed damage to the locomotor system of the flies at the concentration of 4000 µg/mL after 12 h of exposure. The concentrations 2000, 2500, and 3000 µg/mL differed from the control group after 24 h of exposure. These results indicate a relationship between dose and damage to the locomotor system of the flies, as shown in Figure 3.

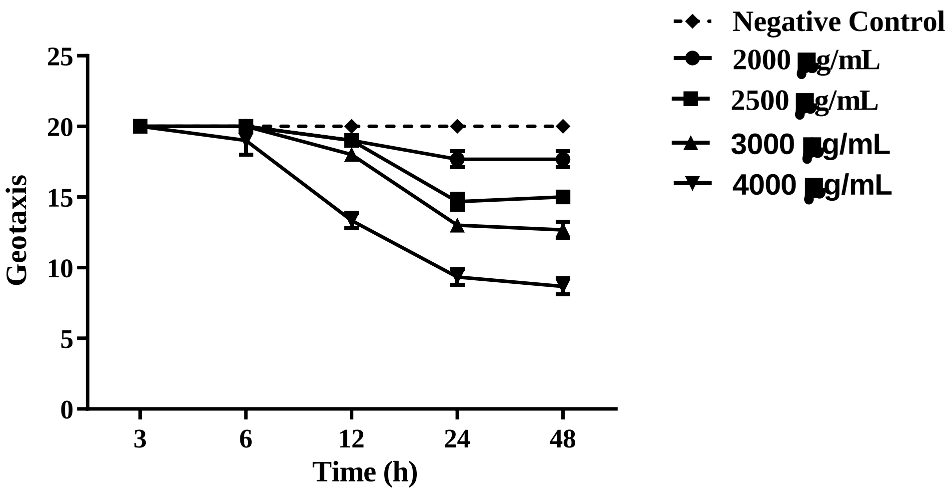

Figure 3. Negative geotaxis test in *Drosophila melanogaster* when exposed to different concentrations of *L. sidoides* aqueous extract in 48 h compared to the control group.

Cytotoxicity on erythrocytes

According to the results obtained in this study, the extract did not show cytotoxicity in blood cells, even at the highest doses (figure 4). When compared to the control with 0.9% saline, there was no significant difference in the different concentrations tested.

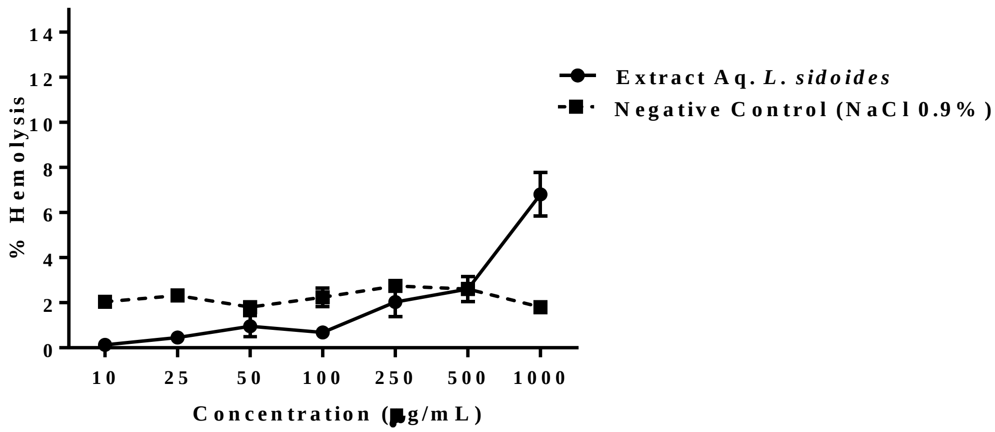

Figure 4. hemolytic effect on blood cells treated with aqueous extract of *L. sidoides*. The percentage of hemolysis was plotted for each concentration against the control with 100% hemolysis (NaCl 0.12%).

Morphological analysis

Comparing the morphological analysis of cells treated with *L. sidoides* extract, and the control group containing distilled water, no changes in cell structure were observed, demonstrating that different concentrations of *L. sidoides* aqueous extract do not morphologically affect blood cells (figure 5).

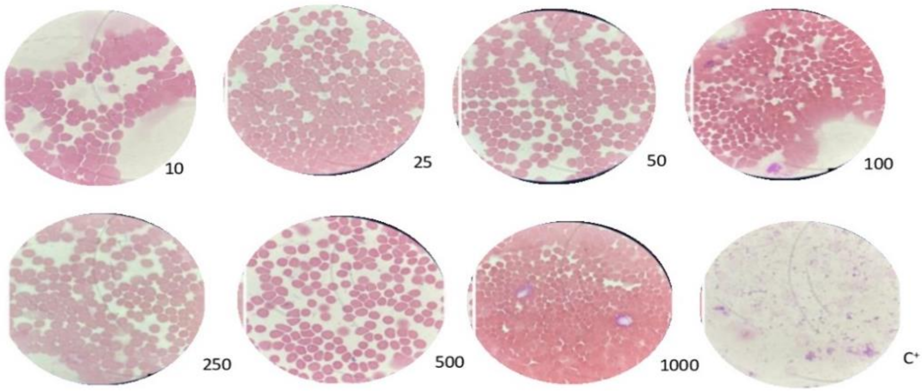

Figure 5. Comparison of erythrocyte morphology treated with different concentrations of *L. sidoides* aqueous extract and positive control (C^{+}) with distilled water.

Cytotoxic activity against *Artemia salina*

The assay against *Artemia salina* Leach revealed low toxicity at the concentrations tested, not being possible to determine the IC_{50} value.

Zebrafish Bioassays

Assessment of locomotor activity (Open Field Test)

According to the results of the open field test (Fig.6A), it was observed that the aqueous extract of *L. sidoides* did not cause a reduction in locomotion at the doses tested, significantly different from the effect of DZP ($^{\#\#\#\#}p<0,0001$ vs. DZP).

Anxiolytic activity (Light & Dark Test)

Higher doses of the extract increased the animals' permanence time in the clear area of the aquarium, similar to the group treated with DZP (*p<0.05 vs. Control) (Fig. 6B), indicating an anxiolytic effect of the aqueous extract of *L. sidoides* in adult zebrafish.

Assessment of GABAergic neuromodulation

The mechanism of anxiolytic action via GABA was determined by pretreatment with flumazenil. The highest dose of the extract with anxiolytic effect (400 mg/kg) and DZP (10 mg/kg) had the anxiolytic effect significantly blocked by flumazenil ($^{\#\#\#}p<0.001$; $^{\#\#\#\#}p<0.0001$ vs. *L. sidoides* and DZP), returning to present anxiety behavior when remaining most of the time in the dark area of the aquarium (Figure 6C).

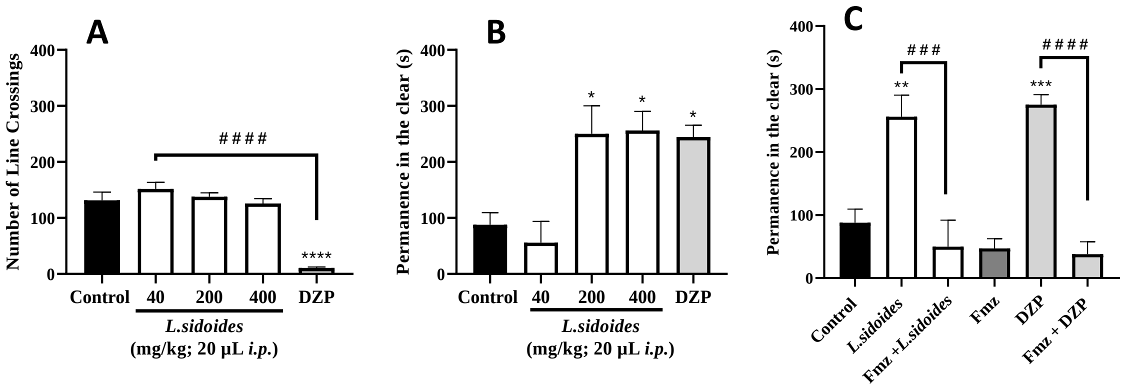

Figure 6. Effect of the aqueous extract of *L. sidoides* on the locomotor activity of adult zebrafish (*Danio rerio*) in the Open Field Test (0-5min) (A), in the light & dark test (B) and on the mechanism of action anisolytic (C). DZP - diazepam (10 mg/kg; 20 µL; i.p.). Control - 3% DMSO (20 µL; *i.p.*). Fmz - Flumazenil (4 mg/kg; 20 µL; *i.p.*). Values represent the mean ± standard error of the mean (S.E.P.M.) for 6 animals/group. ANOVA followed by Tukey (*p<0.05, **p<0.01, ****p<0.0001 vs. vehicle; ###p<0.001, ####p<0.0001 vs DZP or *L. sidoides*).

In relation to the acute toxicity test, the extract was not toxic against zebrafish up to 96h of analysis as shown in Table 2. This test allows establishing safe dose ranges and characterizing adverse effects for the use of extracts such as *L. sidoides*[30].

Table 2. Acute toxicity test results (96h).

Sample	Mortality				96h
	CN	D1	D2	D3	DL_{50} (mg/kg) / IV
Extract *L. sidoides*	0	0	0	0	> 400

CN- Negative control group: DMSO 3%. D1 - Dose 1 (40 mg/kg). D2 - Dose 2 (200 mg/kg). D3 - Dose 3 (400 mg/kg). LD50-Lethal dose to kill 50% of adult Zebrafish; IV-confidence interval.

Discussion

Several studies have demonstrated the chemical composition of *Lippia* species. Twelve compounds were isolated from the ethanolic extract of the *L. sidoides*, as follows: 3-O-acetyleolic acid, methyl 3,4-dihydroxybenzoate, lapachenol, tecomaquinone, tectoquinone, tectol, acetylated tectol, quercetin, luteolin, glucoluteolin, taxifolin, isolariciresinol, and lippsidoquinone, corroborating in part with the compounds identified in this study[31].

In phytochemical triage studies, *L. sidoides* extract exhibited secondary metabolite classes such as alkaloids, flavonoids, tannins, and terpenoids, which are associated with different biological activities[32]. Ethanolic extracts from six *Lippia* species were chemically investigated; among the compounds, naringenin, phloretin, (2S)- and (2R)-3′,4′,5,6-Tetrahydroxyflavanone-7-O-βglucopyranoside, (2S)- and (2R)-Eriodictyol 7-O-β-d-glucopyranoside, 6-Hydroxyluteolin-7-O-β-glucoside, aromadendrin, asebogenin, and sakuranetin were present in the *L. sidoides* extract[33]. This result may be associated with the type of extract, and that aqueous extracts of these species have a higher rate of quantification of flavonoids, which is associated with the polarity of the solvent and the quantification method used[34].

In the present study low toxicity of the aqueous extract of *L. sidoides* was demonstrated in different methods. The essential oil extracted from the leaves of this species has shown important pharmacological properties. In an analysis of the periodontal anti-inflammatory effect, it was verified that the nanostructured gel of the essential oil reduces tissue damage by decreasing the activity of the myeloperoxidase enzyme[35]. In another study, the essential oil of *L. siodides* showed anti-inflammatory activity by different models using mice, with thymol being the major representative in the chemical composition[36]. The toxicity results obtained in this study are essential to ensure the eventual use of this species in new works, especially in biological assays.

The presence of flavonoids in the chemical composition of the aqueous extract of *L. sidoides* can be associated with the low cytotoxic activity observed in this study. In concordance with this work, in Fernandes et al, (2017)[37] evaluated the ability of the flavonoid vitexin to cause genotoxicity in two *D. melanogaster* strains, and no significant changes were observed in the DNA of the species. Another study also investigated the flavonoids kaempferol, quercetin and quercetin 3β-d-glucoside for their genotoxic action; the results showed that the flavonoids do not induce somatic mutations in flies, with the exception of quercetin at the concentration of 50 μM[38].

In the study with *L. alba* essential oil is able to immobilize *D. melanogaster* flies after 150 min of exposure[39]. The oil effect was reversible, and no mortality of flies was observed, which is in agreement with our study. In another work it was shown that that the flavonoid hesperidin tends to decrease the deleterious effects caused to the locomotor system of flies by Fe action, as well as decreases the mortality of larvae exposed for long periods to this element[40].

From the results obtained for cytotoxicity on erythrocytes, no serious damage to the cell membrane was observed compared to the positive control of 0.12% saline. This result suggests that there is no cytotoxicity at the concentrations tested in human erythrocytes. In contrast, the essential oil of *L. microphylla* has moderate toxicity in erythrocytes of mice with 50% hemolysis at a concentration of 300 μg/mL[41]. In an osmotic stability study with the aqueous extract of *Lippia* sp., the ability of this extract to stabilize the erythrocyte cell membrane was observed, preventing the entry of NaCl and consequently its lysis[42]. These results indicate a possible relationship between the presence of flavonoids and the preservation of erythrocyte cell membranes against oxidative damage.

The presence of compounds such as flavonoids in extracts has been proven to be an important factor in stabilizing erythrocyte membranes exposed to factors that induce cytilysis; this response is associated with the antioxidative capacity that these substances play[43]. One of the factors that alter the erythrocyte membrane making it susceptible is the degradation of proteins. Flavonoids that present hydroxyl groups at C3 tend to have a higher protein anti-degrading activity[44], suggesting that the action of *L. sidoides* extract may be related to the presence of these compounds.

As observed in previous assays, the *L. sidoides* extract also showed low toxicity against A. salina larvae. Different results were observed in the study with the methanol/water extract of *L. multiflora* in which a high toxicity was observed, with an LC_{50} result of 1.1 μg/mL[45]. Analysis of methanolic extracts prepared from different parts (stem, leaf, and flowers) of *L. citriodora* showed that all extracts exhibited significant lethality against A. salina, with their composition being rich in tannins, polyphenols, triterpenes, catechins and alkaloids[46].

Essential oil of *L. alba* exhibited significant toxicity against *A. salina*, showing IC_{50} of 53.01 μg/mL; the result observed may be associated with terpenic compounds present in its essential oil[47]. Samples that present IC_{50} lower than 1000 μg/mL in cytotoxic analyses are considered significant according to the study by[21].

Locomotor activity is a behavioral analysis parameter used to evaluate chemicals acting on the central nervous system (CNS) in zebrafish[48]. From this perspective, regarding the biological effects reported for the genus *Lippia*, its properties on the central nervous system are highlighted by series of studies[49,50,51].

Differently from the results obtained, *Dianthus caryophyllus* essential oil was evaluated for its toxicity in juvenile zebrafish, showing LC_{50} value of 18.18 ± 5.52 $mg.L^{-1}$ after 96 h of exposure. The sensitivity caused by this oil can be associated to its major compound eugenol[52]. On the other hand, similar results were found with *Ocimum basilicum* essential oil showed no alteration in locomotor activity or death in adult zebrafish, even after 96 h of analysis, corroborating the results obtained in this study[53]. The zebrafish has been a wellaccepted model in vitro toxicological tests performed in laboratory[54]. According to the result, the aqueous extract did not cause motor impairment or any side effects in the adult zebrafish during the 96 h of analysis, unlike DZP which, in addition to causing changes in the animals' locomotion, is known in the clinic to cause side effects and dependence[55], for this reason the extract is promising for studies involving its effects on the CNS.

The anxiolytic effect of the extract was investigated through the light test on adult zebrafish. The treated animals showed an anxiolytic effect, as substances that fight anxiety increase the time spent by the animals in the clear region of the aquarium, while drugs that induce anxiety decrease it[28]. In the review study on the pharmacological effects of the *Lippia* genus on the central nervous system, several studies were identified demonstrating the sedative, anxiolytic, and anti-convulsant effects of plants of the genus, being responsible for these pharmacological actions nonvolatile substances such as phenylpropanoids, flavonoids and/or or inositols[56].

Several studies on the mechanism of action of anxiolytic compounds in adult zebrafish have used flumazenil to investigate its possible effects through modulation at the binding site of benzodiazepines on the GABAa receptor[55,57,58]. In this study, tests with flumazil were performed to verify its interference in the action of the extract. Pre-treatment with flumazenil reversed the activity of the extract, as the animals returned to show an anxious behavior similar to the control, thus, probably the anxiolytic effect of *L. sidoides* extracts may occur through interaction with the binding site of benzodiazepines[56].

The observed results show the potential of *L. sidoides* extract as an anxiolytic agent. However, there is still much to be explored about this activity, with regard to the prolongation of its effect. An alternative that can enhance the effect of this extract is its encapsulation in nanoparticles. Among the benefits of using nanoparticles is controlled delivery of the active ingrediente[59].

In a previous study, it was verified that the preparation of a nanogel containing thymol extracted from the essential oil of *L. sidoides* promotes the reduction of periodontitis in mice[60]. This result suggests that there may be a significant improvement in pharmacological activity when the substance is encapsulated in nanoparticles. Thus, this may be a new strategy for the continuation of this study. Aiming for significant improvements in results.

Conclusions

From the analysis of the results, it was possible to verify the presence of phenolic compounds of the flavonoid class present in the aqueous extract of *L. sidoides*; these compounds are involved in important biological activities as an antioxidant. In general, the extract did not show cytotoxic activity by the methods developed, as well as it was not toxic in adult zebrafish. The anxiolytic effect observed may be associated with GABAergic receptors. These findings suggest safety in the use of the species for the development of new studies for other biological activities, as well as for the development of specialized clinical trials, as well as for use by populations in traditional medicine.

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Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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7. CONSIDERAÇÕES FINAIS

Os dados oriundos da revisão mostraram que o gênero de *Lippia* apresenta ampla versatilidade, corroborada através da diversidade de indicações de uso e das partes vegetais utilizadas, demostrando que o conhecimento local/tradicional oferece um caminho positivo para a investigação cientifica das propriedades biológicas e que o conhecimento tradicional e a investigação científica caminham juntas. Os resultados das indicações etnobotânicas confirmam a importância do conhecimento popular para o direcionamento de novas pesquisas com o gênero *Lippia* e para maiores investigações do efeito inseticida..

No que diz respeito ao perfil toxicológico, o extrato de *Lippia sidoides* pode ser considerado seguro, tanto para o uso na medicina tradicional como para o desenvolvimentos de estudos futuros com a espécie. A composição química da espécie, rica em compostos fenólicos é uma justificativa coerente para o número de atividades biológicas observadas para a espécie e pela baixa toxicidade observada para os diferentes ensaios realizados.

8. PROPOSTAS DE INVESTIGAÇÕES FUTURA

Como perspectiva para continuidade dessa pesquisa está o desenvolvimento de ensaios par além da sua toxicidade, com intuito de investigar a ação inseticida em diferentes pragas que afetam a agricultura e são percursoras de doenças. Além disso, tem-se como proposta futura o isolamento de substâncias ativas, como os compostos fenólicos, e o desenvolvimento de nanoestruturas que possam melhorar as características de estabilidade e entrega dos extratos em organismos.

9. ORÇAMENTO DO PROJETO

Esse projeto foi financiado pela Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) por meio de bolsa para a aluna Cicera Janaine Camilo. As despesas para a coleta e produção dos extratos foram de 351,86 distribuídos em compras de solventes, reagentes e manutenção da plantação. Um total de 266,00 foram gastos para obtenção de material para realização dos ensaios de toxicidade, excluindo os ensaios com zebrafish que foi realizado por parcerias. Para as análises químicas foram gastos 330,00 com a compra de reagentes e solventes. Os equipamentos utilizados para as análises não foram contabilizados por ser disponibilizado pelo Laboratório de Pesquisa de produtos Naturais.

ANEXOS

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

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

Certidão

Cadastro nº A2B7A05

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**A2B7A05**
Usuário:	**Cicera Janaine Camilo**
CPF/CNPJ:	**049.866.693-03**
Objeto do Acesso:	**Patrimônio Genético/CTA**
Finalidade do Acesso:	**Pesquisa**

Espécie

Lippia sidoides

Fonte do CTA

CTA de origem não identificável

Título da Atividade:	**AVALIAÇÃO DO POTENCIAL INSETICIDA DO EXTRATO PADRONIZADO DE Lippia sidoides CHAM: UMA ALTERNATIVA AO USO DE INSETICIDAS SINTÉTICOS**

Equipe

Cicera Janaine Camilo	**INDEPENDENTE**

Data do Cadastro:	**13/08/2021 11:59:00**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **9:44** de **08/09/2022**.

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

Universidade Estadual do Ceará
Comissão de Ética para o Uso de Animais
Av. Dr. Silas Munguba, 1700 – Itaperi
CEP 60740-903 – fone 3101-9890
ceua.uece@uece.br – www.uece.br/ceua
Governo do Estado do Ceará
Secretaria da Ciência, Tecnologia e Educação Superior

CERTIFICADO

Certificamos que o Projeto intitulado **"Uso do Zebrafish (Danio rerio) como modelo alternativo para investigação do potencial farmacológico de produtos naturais e sintéticos"** registrado sob o número **04983945/2021**, tendo como pesquisador principal Jane Eire Silva Alencar de Menezes, está de acordo com os Princípios Éticos de Experimentação Animal adotados pela **Comissão de Ética para o Uso de Animais da Universidade Estadual do Ceará** (CEUA – UECE). Este certificado expira-se em 31 de Julho de 2023.

CERTIFICATE

We hereby certify that the Project entitled **"Use of Zebrafish (Danio rerio) as an alternative model for investigation of the pharmacological potential of natural and synthetic products"** registered with the protocol **04983945/2021**, under the supervision of Jane Eire Silva Alencar de Menezes, is in agreement with Ethical Principles in Animal Experimentation, adopted by the **Ethics Committee in Animal Experimentation of Ceará State University** (CEUA – UECE). This certificate will expire on July 31st, 2023.

RESUMO

Vigência do projeto	Início	Agosto/2021		Fim	31 de julho de 2023	
Espécie/Linhagem	*Danio rerio* Zebrafish (paulistinha)					
Número de animais	6000	Peso	0,5 g		Idade	90-120 dias
Sexo	3000	Feminino		3000	Masculino	
Origem	Aquário certificado					
Metodologia	X	Adequada			Não adequada	
Cronograma	X	Adequado			Ausente/ Não adequado	
Ofício de encaminhamento	X	Presente			Ausente/ Não adequado	
Orçamento	X	Adequado			Ausente/ Não adequado	
Financiamento	Órgão de fomento	Recursos de Pesquisa e Parcerias				
	Edital ou N. processo	-				

Fortaleza, 05 de agosto de 2021.

Vania Marilande Ceccatto
Presidente CEUA-UECE

UNIVERSIDADE REGIONAL DO CARIRI - URCA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE EXPERIMENTAÇÃO E USO DE ANIMAIS
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CEP 63105-000 – Crato - CE - Brasil
propg@urca.br - www.urca.br/ceua

Declaração

Declaro para devidos fins, que o projeto intitulado "Avaliação do potencial inseticida do extrato padronizado de *Lippia sidóides* Cham: Uma alternativa ao uso de inseticida sintético" - processo **00334/2019.2** foi APROVADO pela Comissão de Experimentação e Uso de animais-CEUA-URCA .

Roseli Barbosa

Coordenadora do CEUA-URCA

Crato, 22 de Junho 2021

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Traditional use of the genus *Lippia* sp. and pesticidal potential: A review

Cicera Janaine Camilo [a,*], Débora Odília Duarte Leite [b], Carla de Fatima Alves Nonato [c], Natália Kelly Gomes de Carvalho [d], Daiany Alves Ribeiro [a], José Galberto Martins da Costa [a,b,c,d,**]

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ABSTRACT

The understanding of popular knowledge has guided the studies involving promising natural products for the development of botanical insecticides, through ethnobotanical research. The frequent records of scientific research with the genus *Lippia* demonstrate a high insecticidal potential for the species, with prospects of new products for the market. Therefore, it is important to recognize the contribution of traditional knowledge and use it in plant selection for in-depth research that can lead to the elaboration of a final product. Thus, the aim of this research was to analyze existing relationships between ethnobotanical studies and research related to the control of insects, mites and ticks with the genus *Lippia* sp., as well as to verify the difficulties and perspectives for the development of new products derived from species of this genus for the fight pests. The main indications in the ethnobiological survey were medicinal for diseases of the gastrointestinal tract (34.2%), respiratory system (27.1%) and nervous system (22.18%), in addition to these, six species had indications for use as repellent. For the pesticide survey, the essential oils of the species *L. alba* and *L. sidoides* were the most investigated, being thymol and carvacrol, the most frequently identified compounds. The species L.*alba* was the most cited in both surveys, demonstrating an influence between the indications of traditional uses and biological investigations for the genus.

1. Introduction

Insects, mites, and ticks belonging to various species are associated with diseases such as allergies, dengue, malaria, zica, chikungunya, and yellow fever, as well as causing potential losses in agriculture and livestock (Moreau et al., 2020; Amoabeng et al.,

Abbreviations: UV- use value DDVP-2.2 dichlorovinyl dimethylphosphate, GST-glutathione S-transferase.

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Analysis toxicity by different methods and anxiolytic effect of the aqueous extract *Lippia sidoides* Cham.

Cicera J. Camilo[1], Débora O. D. Leite[2], Johnatan W. da S. Mendes[3], Alexandro R. Dantas[4], Natália K. G. de Carvalho[4], José W. G. Castro[5], Gerson J. T. Salazar[1], Maria Kueirislene Amâncio Ferreira[8], Jane Eire Alencar de Meneses[8], Antonio Wlisses da Silva[2], Helcio S. dos Santos[3], Josean F. Tavares[6], Joanda P. R. e Silva[6], Fabiola F. G. Rodrigues[5], Chunhoo Cheon[7], Bonglee Kim[7]✉ & José Galberto Martins da Costa[2,3,4]✉

Lippia sidoides **Cham. (Verbenaceae) is a species often mentioned in traditional medicine due to the medicinal properties attributed to its leaves, which include antibacterial, antifungal, acaricidal and antioxidant. Several of these actions have been scientifically proven, according to reports in the literature; however, little is known about toxicological aspects of this plant. This work included studies to determine the chemical composition and toxicity tests, using several methods aiming to evaluate the safety for use of the aqueous extract of *L. sidoides* leaves, in addition, the anxiolytic effect on adult zebrafish was investigated, thus contributing to the pharmacological knowledge and traditional medicine concerning the specie under study. The chemical profile was determined by liquid chromatography coupled to mass spectrometry-HPLC/MS with electrospray ionization. Toxicity was evaluated by zebrafish, *Drosophila melanogaster*, blood cells, and *Artemia salina* models. 12 compounds belonging to the flavonoid class were identified. In the toxicity assays, the observed results showed low toxicity of the aqueous extract in all tests performed. In the analysis with zebrafish, the highest doses of the extract were anxiolytic, neuromodulating the GABAa receptor. The obtained results support the safe use of the aqueous extract of *L. sidoides* leaves for the development of new drugs and for the use by populations in traditional medicine.**

Natural products of vegetal origin are recognized for the variety of chemical substances present in their parts, and for their broad property of performing pharmacological activities[1]. Although the industrial influence, medical plants are still used to cure and treat different diseases by the majority of population[2]. The medical use can occur through different ways of preparations, whether in the form of teas, stews, baths, among others[3]. These plants represent considerable importance for many different cultural groups, since they are accessible and can be used against several types of pests and diseases[4].

In this regard, recognizing the potential of a particular plant species to present some type of toxic reaction among its users is important, whether for the development of new drugs or for home use based on preparations; consequently, an evaluation of the relationship between risk and benefit is necessary to improve medical plant

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Declaration of consente

I declare that the study "Analysis toxicity by different methods and anxiolytic effect of the aqueous extract *Lippia sidoides* Cham" followed the guidelines proposed by the Declaration of Helsinki and is in accordance with the guidelines and regulations required by the Clinical Analysis Committee of Biomedicine of the Centro Universitário Doutor Leão Sampaio, for handling human samples.

José Walber Gonsalves Castro

Biomedical, hematologist in charge - CRBM 9815

March, 2021