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COMPOSIÇÃO QUÍMICA E EFEITOS NEUROFARMACOLÓGICOS DE ÓLEOS ESSENCIAIS DA AMAZÔNIA BRASILEIRA

ANTÔNIO QUARESMA DA SILVA JÚNIOR

SANTARÉM – PA
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Tese de doutorado apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia da Rede BIONORTE, na Universidade Federal do Oeste do Pará (UFOPA) como requisito para a obtenção do Título de Doutor em Biodiversidade e Biotecnologia.

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RESUMO

O objetivo deste trabalho foi determinar os constituintes voláteis e o perfil neurofarmacológico de óleos essenciais (OE) de plantas aromáticas na região do Baixo Amazonas, bem como realizar estudos de docagem molecular com os compostos majoritários dos OE, como forma de elucidar a interação ligante-receptor com a enzima acetilcolinesterase (AChE). Neste estudo, foi realizada uma triagem de OE a partir do Banco do Laboratório de Bioprospecção e Biologia Experimental (LabBBEx) da Universidade Federal do Oeste do Pará (UFOPA), onde um total de doze óleos foram testados em relação à sua capacidade de inibição da AChE. Destas amostras, 5 foram selecionadas para continuidade dos estudos, devido apresentarem efeito inibitório frente a enzima, sendo estas: *Lippia origanoides*; *Curcuma longa*, *Zingiber officinale*, *Myrcia sylvatica* e *Lippia alba*, esta última sendo três quimiotipos (citral, carvona e linalol). Os testes anticolinesterásicos foram realizados *in vitro*, através de bioautografia e teste quantitativo de Ellman. A composição química dos OE foi determinada por cromatografia gasosa acoplada à espectrometria de massas (CG-EM), e a docagem molecular foi realizada com os componentes majoritários dos OE, como forma de avaliar a interação destes com o sítio ativo da AChE. Este trabalho está dividido em quatro capítulos. No Capítulo 1, foi avaliada a atividade anticolinesterásica do OE de três quimiotipos de *Lippia alba*: citral, carvona e linalol, bem como associação entre estes. Todos os quimiotipos apresentaram atividade significativa, com destaque para o quimiotipo carvona (IC_{50} = 0,1 μg/mL). O estudo de docagem demonstrou interações entre os constituintes principais e os resíduos da AChE, indicando o potencial biotecnológico para a espécie. No Capítulo 2 foi abordada a atividade anticolinesterásica dos OE de *Curcuma longa* e *Zingiber officinale*, testados isoladamente e em associação. Os óleos individuais demonstraram inibição frente a AChE com IC_{50} de 0,33 e 0,38 μg/mL, respectivamente, enquanto a associação reduziu o efeito inibitório (IC_{50} = 0,64 μg/mL). Os constituintes majoritários apresentaram interações no sítio ativo da AChE. No Capítulo 3 foi investigado se o óleo essencial de *Myrcia sylvatica* apresenta além da atividade anticolinesterásica, efeito ansiolítico e antidepressivo por inalação do óleo em modelo animal, por se tratar de uma espécie que vem sendo usada pela população para fins de promoção de bem-estar. O óleo reduziu significativamente os comportamentos ansiosos e depressivos, sem alterar a memória,

aprendizado ou coordenação motora. Além disso, apresentou atividade anticolinesterásica *in vitro* (IC_{50} = 0,47 μg/mL), indicando potencial terapêutico para condições cognitivas relacionadas à AChE. No Capítulo 4, foi explorado o efeito inibitório sobre a AChE do OE de *Lippia origanoides,* do constituinte majoritário isolado (carvacrol) e de sua nanoformulação. A análise de docagem molecular indicou interações dos compostos principais com a AChE. Testes *in silico* mostraram perfis farmacocinéticos favoráveis, incluindo boa penetração na barreira hematoencefálica. A nanoformulação produzida com o OE de *Lippia origanoides* apresentou estabilidade e maior bioatividade frente a AChE em relação ao óleo individual e ao constituinte majoritário carvacrol, destacando a viabilidade do OE como candidato promissor no desenvolvimento de terapias naturais para doenças neurodegenerativas. Os resultados obtidos evidenciam o potencial neurofarmacológico de óleos essenciais de plantas aromáticas da Amazônia, principalmente no desenvolvimento de terapias alternativas para a Doença de Alzheimer e outros distúrbios neurológicos como ansiedade e depressão.

Palavras-chave: Colinesterase; Alzheimer; Ellman; Demência; Óleo essencial.

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ABSTRACT

The aim of this study was to determine the volatile constituents and neuropharmacological profile of essential oils (EO) from aromatic plants in the Lower Amazon region, as well as to conduct molecular docking studies with the major EO compounds to elucidate ligand-receptor interactions with the enzyme acetylcholinesterase (AChE). A screening of essential oils was carried out using the Bioprospecting and Experimental Biology Laboratory (LabBBEx) collection at the Federal University of Western Pará (UFOPA), in which a total of twelve oils were tested for their AChE inhibitory capacity. Of these, five samples were selected for further study due to their inhibitory effect on the enzyme: *Lippia origanoides*, *Curcuma longa*, *Zingiber officinale*, *Myrcia sylvatica*, and *Lippia alba* (with three chemotypes: citral, carvone, and linalool). Anticholinesterase tests were performed *in vitro* using bioautography and Ellman's quantitative assay. The chemical composition of the EOs was determined by gas chromatography-mass spectrometry (GC-MS), and molecular docking was carried out with the major components to assess their interactions with the active site of AChE. This work is divided into four chapters. Chapter 1 evaluates the anticholinesterase activity of *Lippia alba* essential oil from three chemotypes: citral, carvone, and linalool, as well as their combinations. All chemotypes showed significant activity, with carvone being the most potent (IC_{50} = 0.1 μg/mL). Docking studies revealed interactions between the main constituents and AChE residues, indicating the species' biotechnological potential. Chapter 2 addresses the anticholinesterase activity of *Curcuma longa* and *Zingiber officinale* essential oils, tested both individually and in combination. The oils showed AChE inhibition with IC_{50} values of 0.33 and 0.38 μg/mL, respectively, while the combination resulted in reduced inhibitory effect (IC_{50} = 0.64 μg/mL). The major constituents interacted with the AChE active site. In Chapter 3, the essential oil of *Myrcia sylvatica* was investigated not only for anticholinesterase activity but also for anxiolytic and antidepressant effects through inhalation in an animal model, since this species is traditionally used by the local population for well-being. The oil significantly reduced anxiety- and depression-like behaviors without impairing memory, learning, or motor coordination. Additionally, it demonstrated *in vitro* anticholinesterase activity (IC_{50} = 0.47 μg/mL), suggesting therapeutic potential for cognitive conditions related to AChE. Chapter 4 explores the AChE inhibitory effect of *Lippia origanoides* essential oil, its isolated major compound (carvacrol), and its nanoformulation. Molecular docking analysis indicated

interactions between the main compounds and AChE. *In silico* tests showed favorable pharmacokinetic profiles, including good blood-brain barrier penetration. The nanoformulation of *Lippia origanoides* EO showed enhanced stability and higher bioactivity against AChE compared to the individual oil and carvacrol, highlighting its potential as a promising candidate for the development of natural therapies for neurodegenerative diseases. The results demonstrate the neuropharmacological potential of essential oils from Amazonian aromatic plants, particularly in the development of alternative therapies for Alzheimer's disease and other neurological disorders such as anxiety and depression.

Keywords: Cholinesterase; Alzheimer's; Ellman; Insanity; Essential oil.

LISTA DE FIGURAS

Capítulo 1

Figura 1 – Chemical structure of the majority components of the main chemotypes of *L. alba*. (A) = citral – neral; (B) citral – geranial; (C) = carvone and (D) = linalool. Source: NIST ... **33**

Figura 2 – Direct bioautography on the silica gel chromatoplate (TLC) of the anticholinesterase activity of *Lippia alba* essential oil (100 Ml/Ml) and physostigmine samples. LA1 = *Lippia alba* citral; LA2 = *Lippia alba* carvone; LA3 = *Lippia alba* linalool; Controls: (B) blank, (NC) methanol negative control, and (PC) physostigmine positive control (100 μg/Ml). Inhibition halos were measured in cm. ……..… **42**

Figura 3 – Anticholinesterase activity (IC_{50}) of *Lippia alba* essential oil samples and their respective mixtures (pools). Control = physostigmine; LA1 = *Lippia alba* citral; LA2 = *Lippia alba* carvone; LA3 = *Lippia alba* linalool; Pool 1 = LA1 + LA2 + LA3; Pool 2 = LA1 + LA3; Pool 3 = LA1 + LA2; Pool 4 = LA2 + LA3. ……... **43**

Figura 4 – Re-docking of donepezil and *h*AChE (4EY7 and donepezil). In green, experimental pose and, in red, predicted *in silico* pose. **RMSD:** 0.334**;** ΔG= -11.237 kcal/mol. **43**

Figura 5 – Donepezil and AchE. ……..... **44**

Figura 6 – Statistical analysis of the binding energy values from donepezil compared to those of the phytochemicals.

Figura 7 – Donepezil and AchE.

Figura 8 – Physostigmine and AchE.

Figura 9 – 2D Diagram of the interactions between carvone (A) and elemol (B) and amino acid residues from *h*AChE (4EY7).

Figura 10 – 2D diagram of the interactions between limonene (A) and γ-muurolene (B) and amino acid residues from *h*AChE (4EY7).

Figure 11 – 2D diagram of the interactions between neral (A) geranial (B) and amino acid residues from *h*AChE (4EY7).

Figure 12 – 2D diagram of the interactions between 1,8-cineole (A) linalool (B) and amino acid residues from *h*AChE (4EY7).

Capítulo 2

Figure 1. Direct bioautography on silica gel chromatoplates (TLC) of the anticholinesterase activity of essential oil samples from *C. longa* and *Z. officinale* and the physostigmine standard. CL = *Curcuma longa*; ZO = *Zingiber officinale*; Controls: (B) blank, (CN) negative control: methanol, and (CP) positive control: physostigmine (100 μg/mL).

Figura 2 – Anticholinesterase Activity (IC_{50}) of Essential Oil Samples from *C. longa*, *Z. officinale*, and the Respective Mixture (Pool). CL = *Curcuma longa*; ZO = *Zingiber officinale*; Pool = Mixture of the two oil samples*: C. longa* + *Z. officinale*.

Figure 3: 2D diagram illustrating the interactions between donepezil and the acetylcholinesterase (AChE) enzyme (4EY7).

Figure 4: 2D diagram of the interactions between α-Phellandrene (A) and α-Zingiberene (B) with amino acid residues of hAChE (4EY7).

Figure 5: 2D diagram illustrating the interactions between ar-Turmerone (A) and β-Sesquiphellandrene (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

Figure 6: 2D diagram illustrating the interactions between Camphene (A) and 1,8-Cineole (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

Figure 7: 2D diagram illustrating the interactions between the compounds (*E,E*)-α-Farnesene (A) and (*E*)-γ-Atlantone (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

Figure 8: 2D diagram illustrating the interactions between Neral (A) and Geranial (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

Capítulo 3

Figure 1. Time (in seconds) spent in the open arms of the elevated plus maze by animals exposed for 5 min to inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (2 s) (n = 8). Values represent the mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, compared to the negative control group using ANOVA and Tukey's test. NC = negative control, PC = positive control. EMS: *Myrcia sylvatica* essential oil.

Figure 2. Number of entries into the open arms of the elevated plus maze by animals exposed for 5 min to inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (n = 8). Values represent the mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, compared to the negative control group using ANOVA and Tukey's test. NC = negative control, PC = positive control. EMS: *Myrcia sylvatica* essential oil.

Figure 3. Time spent (seconds) in the light compartment of the light–dark box test by animals exposed to 5 min of inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (n = 6). Values represent the mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, compared to the negative control group using ANOVA and Tukey's Test. NC = negative control, PC = positive control. EMS: *Myrcia sylvatica* essential oil.

Figure 4. Immobility time (seconds) in the tail suspension test of animals exposed to 5 min of inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (n = 5). Values represent the mean ± standard deviation. * $p < 0.05$ ** $p < 0.01$, compared to the negative control group using ANOVA and Tukey's Test. NC = negative control, PC = positive control. EMS: *Myrcia sylvatica* essential oil.

Figure 5. Latency time to immobility during forced swimming of animals exposed to inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (n = 6) for 5 min. The values represent the mean ± standard deviation. *$p < 0.05$, **$p < 0.01$, compared to the negative control group using ANOVA and Tukey's test. NC = negative control, PC = positive control. EMS: *Myrcia sylvatica* essential oil.

Figure 6. Percentage of alternation in the arms of the Y-maze in animals exposed to 5 min of inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (n = 8). Values represent the mean ± standard deviation. $p < 0.05$; $p < 0.01$, compared to the negative control group using ANOVA and Tukey's Test. NC = negative control. EMS: *Myrcia sylvatica* essential oil.

Figure 7. Average time in seconds spent to locate the platform from the four reference points in the Morris water maze of animals exposed to 5 min of inhalation of the essential oil of

Myrcia sylvatica at doses of 0.1%, 1%, and 2% (n = 8). Values represent the mean ± standard deviation. $p < 0.05$, $p < 0.01$, compared to the negative control group using ANOVA and Tukey's Test**.** NC = negative control. EMS: *Myrcia sylvatica* essential oil.

Figure 8. Mean time in seconds spent on the rotarod by animals exposed to 5 min of inhalation of *Myrcia sylvatica* essential oil at doses of 0.1, 1, and 2% *(n* = 8). Values represent the mean ± standard deviation. $p < 0.05$, $p < 0.01$, compared to the negative control group using ANOVA and Tukey's test. NC = negative control. EMS: *Myrcia sylvatica* essential oil.

Figure 9. Direct bioautography on silica gel chromatoplates (CCD) of the anticholinesterase activity of the essential oil of *Myrcia sylvatica* and the standard physostigmine. (B) blank, (CN) negative control: methanol, (CP) positive control: physostigmine (100 μg/mL). MS = EO of *M. sylvatica*.

Capítulo 4

Figure 1: Macroscopic characteristics (translucency) of the *Lippia origanoides* formulation with 1% essential oil and 9% polysorbate 20.

Figure 2: Temperature influence (25-75 ºC) in the droplet size distribution by the intensity of the *Lippia origanoides* formulation.

Figure 3: Zeta potential distribution of the *Lippia origanoides* formulation before (A), and after (B) thermal stress.

Figura 4 – Anticholinesterase Activity (IC_{50}) of Samples: LOEO = oil essential of *L. origanoides*; CVC = carvacrol and NF = nanoformulation. Control = = physostigmine. The data are presented as mean ± standard deviation. Statistical analysis was performed using ANOVA followed by a Tukey post-hoc test, with significance defined at $p < 0.05$.

Figure 5: Statistical analyses of the binding energy values of donepezil compared to the major compounds of *Lippia origanoides* essential oil. Statistical analysis was performed using ANOVA followed by a Tukey post-hoc test, with significance defined at $p < 0.05$.

Figure 6: 2D diagram illustrating the interactions between donepezil and the acetylcholinesterase (AChE) enzyme (4EY7).

Figure 7: 2D diagram of the interactions between carvacrol (A), *p*-cymene (B), thymol (C), γ-terpinene (D), Linalool (E) and γ-gurjunene (F), with the acetylcholinesterase (AChE) enzyme (4EY7).

LISTA DE TABELAS

Revisão Bibliográfica

Tabela 1 – Estudos sobre atividade anticolinesterásica de óleos essenciais, publicados nos últimos anos.

Capítulo 1

Tabela 1- Main studies that evaluated the biological activities of the essential oil from different chemotypes of *L. alba*, published in the period 2020-2024.

Tabela 2 - Mixtures obtained using essential oil of the three chemotypes of *Lippia alba.*

Tabela 3 – Volatile constituents and essential oil yield of three samples *Lippia alba* and their mixtures (pools).

Tabela 4 – Screening of the most abundant constituents of *Lippia alba*.

Tabela 5 – *In silico* prediction of pharmacokinetic properties of the natural compounds from *Lippia alba*.

Capítulo 2

Tabela 1 - Volatile Constituents and Yield of the Essential Oils from *C. longa* and *Z. officinale* and the Mixture of Both Samples (Pool).

Tabela 2 - Binding Energy Values of the Major Constituents of *Zingiber officinale*, *Curcuma longa*, and Pool.

Tabela 3 - *In silico* pharmacokinetic prediction of the major constituents of *Zingiber officinale* and *Curcuma longa*.

Capítulo 3

Tabela 1 - Volatile constituents of *M. sylvatica* essential oil.

Capítulo 4

Tablela 1 - Volatile constituents of *Lippia origanoides* essential oil.

Tabela 2 - Average droplet size and polydispersity index of the *Lippia origanoides* formulation at rising temperatures (25-75 ºC).

Tabela 3 - Macroscopic characteristics, average size, polydispersity index, zeta potential, and conductivity before and after thermal stress (25 ºC).

Tabela 4 - In silico pharmacokinetic prediction of the major constituents of *Lippia origanoides* essential oil.

SUMÁRI

O

1. INTRODUÇÃO.....17
1.2. OBJETIVO GERAL.....19
1.3. OBJETIVOS ESPECÍFICOS.....19
2. REVISÃO BIBLIOGRÁFICA.....20
2.1. Óleos essenciais – características gerais (adicionar tópico sobre biossíntese).....20
2.2. Biossíntese de óleos essenciais.....21
2.3. Doença de Alzheimer.....22
2.4. Tratamento farmacológico da Doença de Alzheimer.....23
2.5. Enzima Acetilcolinesterase.....26
2.6. Óleos essenciais como potenciais inibidores colinesterásicos.....27
2.7. Docagem molecular.....33
3. CAPÍTULOS.....35
3.1. CAPÍTULO 1 - Molecular modelling and anticholinesterase activity of the essential oil from three chemotypes of *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson (Verbenaceae).....35
3.2. CAPÍTULO 2 - Essential oils of *Curcuma longa* L. and *Zingiber officinale* R. (Zingiberaceae): anticholinesterase activity and molecular modeling – Artigo submetido na revista *Ouricuri*, em Novembro de 2024 – Qualis B1);.....62
3.3. CAPÍTULO 3 - Anxiolytic, Antidepressant, and Anticholinesterase Effects of Essential Oil from *Myrcia sylvatica* (G.Mey.) DC.....85
3.4. CAPÍTULO 4: Essential Oil of *Lippia origanoides* Kunth: Nanofomulation, Anticholinesterase Activity, and Molecular Docking.....115
4. DISCUSSÃO INTEGRADORA.....144
5. CONCLUSÃO.....146
5. REFERÊNCIAS (citadas na introdução e na revisão bibliográfica).....147
6. ANEXOS 156

Esta Tese foi elaborada de acordo com as nomas da Instrução Normativa nº 04 do PPG-BIONORTE que estabelece critérios para defesa de tese no âmbito do programa. A IN pode ser consultada no link: IN nº 04 - PPG-BIONORTE

1. INTRODUÇÃO

Dentre os produtos naturais conhecidos por fornecerem grande interesse para as indústrias alimentícias, farmacêuticas e cosméticas, os óleos essenciais, são descritos como produtos com grande potencial. Estes óleos são constituídos por uma diversidade de constituintes químicos tais como hidrocarbonetos terpênicos, álcoois simples e terpênicos, aldeídos, ésteres, cetonas, fenóis, éteres, óxidos, peróxidos, furanos, ácidos orgânicos, lactonas, cumarinas, até compostos com enxofre (BORUGĂ et al., 2024). A distinta composição química de cada óleo essencial caracteriza sua atividade biológica (HARO-GONZÁLEZ et al., 2021), a qual poderá resultar da ação sinérgica de todos seus compostos ou apenas dos seus constituintes majoritários (ZHU et al., 2022). Sendo assim, o conhecimento da composição química e avaliação das atividades biológicas de óleos essenciais são importantes para o desenvolvimento de novos produtos.

Considerando a extensão e a riqueza da biodiversidade amazônica, assim como, a necessidade de promover o desenvolvimento sustentável com valorização da diversidade química e biológica, o Laboratório de Bioprospecção e Biologia Experimental da Universidade Federal do Oeste do Pará (LabBBEx/UFOPA) vem desenvolvendo um banco de óleos essenciais com plantas nativas da Amazônia ou cultivadas em sistemas agroflorestal. Este banco possui aproximadamente cem amostras de óleos essenciais pertencentes a diferentes famílias botânicas. Sendo, portanto, uma fonte para muitos estudos e valorização da floresta em pé.

Neste trabalho, foi avaliado o potencial neurofarmacológico de amostras de óleos essenciais contidas no banco do LabBBEx/UFOPA. Inicialmente foi realizada uma pré-seleção das amostras de óleo baseado na composição química, rendimento e disponibilidade da planta, bem como, levantamento na literatura referente a estudos anteriores de óleos essenciais como inibidor da enzima acetilcolinesterase (AChE). Foi selecionado um total de doze amostras de óleos essenciais: *Lippia origanoides* (LO); *Piper marginatum* (PM); *Myrcia sylvatica* (MS); *Myrcia multiflora* (MM); *Conobea scoparioides* (CS); *Pectis elongata* (PE); *Protium heptaphyllum* (PH); *Copaifera sp* (CS); *Aniba rosaeodora* (AR); *Curcuma longa* (CL), *Zingiber officinale* (ZO) e *Lippia alba* (LA) esta última sendo utilizados três diferentes quimiotipos: Carvona, Linalol e Citral.

Das amostras selecionadas cinco apresentaram forte atividade inibitória sobre a enzima AChE sendo estas: LO, MS, CL, ZO, e os três quimiotipos de LA. Seguindo assim,

para os estudos da quantificação da atividade anticolinesterasica e modelagem molecular dos constituintes majoritários sob a enzima AChE.

A presente tese foi dividida em IV capítulos. No **capitulo I** foi avaliada a atividade anticolinesterásica do óleo essencial dos três quimiotipos de *Lippia alba* individualmente e em combinação, bem como, foi determinado o padrão de interação entre o receptor da AChE e os principais constituintes presentes nas amostras de óleo através de Docagem molecular. O **capítulo II** foi avaliado se os óleos essenciais e misturas destes obtidos de rizomas de *Curcuma longa* e *Zingiber officinale*, apresentam atividade inibitória frente a AChE e foi determinado o padrão de interação entre o receptor da AChE e os principais constituintes presentes nas amostras de óleo através de estudo de docagem molecular. No **capítulo III** foi investigado se o óleo essencial de *Myrcia sylvatica* apresenta além da atividade anticolinesterásica o potencial ansiolítico e antidepressivo por inalação do óleo em modelo animal, por se tratar de uma espécie que vem sendo usada pela população para fins de promoção de bem-estar. Sabe-se que a aromaterapia no Brasil é considerada uma prática integrativa e complementar (PIC), utilizada para promover ou melhorar a saúde, o bem-estar e a higiene (NASSIF et al., 2022). E sendo esta, uma espécie facilmente encontrada em savanas da Amazônia e sem estudos quanto ao uso do óleo essencial no Sistema Nervoso Central, os dados podem ser importantes inclusive para preservação da espécie que vem constantemente sofrendo com ação de queimadas na Amazônia.

No **Capítulo IV**, foi avaliada a atividade anticolinesterásica do óleo essencial de *Lippia origanoides*, seu principal constituinte isolado (carvacrol) e a nanoemulsão do óleo essencial. A nanoemulsão apresentou maior bioatividade e estabilidade, além de aumentar a biodisponibilidade do óleo essencial. O estudo de docagem molecular indicou fortes afinidades de ligação entre os compostos do óleo e a AChE, corroborando seu potencial terapêutico para doenças neurodegenerativas, como a Doença de Alzheimer. Os resultados também indicaram que os principais constituintes do óleo apresentam boa absorção e penetração na barreira hematoencefálica, favorecendo seu uso em tratamentos de doenças neurológicas.

Dessa forma, a busca por novas substâncias com ação anticolinesterásica é justificada, sendo os óleos essenciais uma promissora fonte de novas moléculas bioativas, uma vez que, devido ao seu baixo peso molecular e alta lipofilicidade, os constituintes voláteis dos óleos essenciais tendem a atravessar facilmente a barreira hematoencefálica (LUKAS, 2021), possibilitando a descoberta de novas substâncias para utilização ou complementação por exemplo no tratamento da Doença de Alzheimer.

1.2. OBJETIVO GERAL

Determinar os constituintes voláteis e o perfil neurofarmacológico de óleos essenciais (OE) de plantas aromáticas na região do Baixo Amazonas, bem como realizar estudos de docagem molecular com os compostos majoritários dos OE, como forma de elucidar a interação ligante-receptor com a enzima acetilcolinesterase (AChE).

1.3. OBJETIVOS ESPECÍFICOS

- Selecionar amostras de óleos essenciais no banco do LabBBEx/UFOPA;
- Determinar os constituintes voláteis, bem como o teor dos óleos essenciais selecionados;
- Determinar a atividade anticolinesterásica dos óleos;
- Realizar estudos de docagem molecular dos componentes majoritários das amostras de óleos que apresentaram inibição frente a enzima AChE;
- Investigar o potencial ansiolítico e antidepressivo e influência sobre cognição e coordenação motora do óleo essencial de *Myrcia sylvatica;*
- Desenvolver e avaliar uma nanoemulsão contendo o óleo essencial de *Lippia origanoides*.

2. REVISÃO BIBLIOGRÁFICA

2.1. Óleos essenciais – características gerais (adicionar tópico sobre biossíntese).

Os óleos essenciais (OE), também chamados de óleos voláteis ou essências, são definidos como uma complexa mistura de substâncias voláteis, lipofílicas, geralmente odoríferas e líquidas, oriundas do metabolismo secundário de plantas (THAKUR et al., 2021). São solúveis em solventes orgânicos como éter, álcool, clorofórmio, benzeno e apresentam solubilidade limitada em água. Podem ser produzidos em todos os órgãos da planta, porém é mais comum serem encontrados nas partes aéreas, composta de flores e folhas (SIMÕES & SPITZER, 2004; RÍOS, 2016). São armazenados no interior de estruturas especializadas como células secretoras, cavidades, canais, células da epiderme ou tricomas glandulares (MANSOURI et al., 2023, TEIXEIRA et al., 2013). Naturalmente, os OE desempenham um papel vital na proteção e propagação de plantas, atuando como antimicrobianos, repelentes para herbívoros e agentes atrativos para insetos que auxiliam na polinização (AYAZ, et al., 2017).

Os OE podem ser extraídos de qualquer parte vegetal, entretanto quando obtido de diferentes órgãos de uma mesma planta podem apresentar características químicas e organolépticas distintas (SIMÕES; SPITZER, 2004). Os óleos essenciais são compostos por misturas extremamente complexas de compostos e contêm vários componentes em concentrações variáveis. Os componentes fundamentais dos OE são compostos aromáticos, terpenos/terpenoides e moléculas alifáticas, principalmente de baixo peso molecular (BAKKALI, et al. 2008).

Alguns óleos essenciais são reconhecidos pela sua fragrância, no entanto muitos apresentam propriedades medicinais (SIMÕES; SPITZER, 2004) como potencial ansiolítico (CHIOCA et al., 2019; DINIZ et al., 2020), analgésico (NASIRI; MAHMODI; NOBAKHT, 2016), calmante, sedativo (PERRY; PERRY, 2016), antimicrobiano (SILVA JÚNIOR et al., 2019), antisséptico (ALMEIDA et al., 2023), antidepressivo (PERAZZO et al., 2018), anti-inflamatório (ALI et al., 2022), anticolinesterásico (ROMERO et al., 2023) entre outros.

Nesse sentido, os óleos essenciais devido às suas propriedades naturais, têm sido amplamente utilizados devido a gama de aplicações e atividades biológicas apresentadas por seus componentes químicos, que devido ao seu baixo peso molecular e capacidade de penetração via membranas biológicas os levaram a ser importantes ferramentas na busca de novas moléculas bioativas, incluindo doenças neurodegenerativas como a Doença de Alzheimer (PICHERSK; NOEL; DUDAREVA, 2020; AYAZ, et al., 2017).

2.2. Biossíntese de óleos essenciais

Quimicamente, os óleos essenciais (OE) são misturas complexas de substâncias voláteis que variam desde hidrocarbonetos terpênicos, álcoois simples e terpênicos, aldeídos, cetonas, fenóis, ésteres, éteres, óxidos, peróxidos, furanos, ácidos orgânicos, lactonas, cumarinas, até compostos contendo nitrogênio e enxofre, metabólitos que conferem suas características organolépticas. Os principais esqueletos carbônicos que sustentam essa diversidade de substâncias pertencem, majoritariamente, a dois grupos: os fenilpropanóides e, principalmente, os terpenoides (SIMÕES & SPITZER, 2004; BAKKALI et al., 2008).

Os terpenoides, uma das maiores classes de metabólitos em produtos naturais, são compostos formados por unidades isoprênicas (com cinco carbonos) e classificados conforme o número dessas unidades: hemiterpenos (C5), monoterpenos (C10), sesquiterpenos (C15), diterpenos (C20), sesterpenos (C25), triterpenos (C30) e tetraterpenos (C40) (BASER & BUCHBAUER, 2010; PAVARINI et al., 2012). A biossíntese dos esqueletos carbonados dos terpenoides ocorre a partir da condensação das unidades isoprênicas 3,3'-dimetilalil pirofosfato (DMAP) e 3-isopentenil pirofosfato (IPP). Essas unidades são formadas por duas rotas biossintéticas: a via do mevalonato (MVA), que utiliza o ácido (3R)-mevalônico como intermediário, e a via do metileritritol fosfato (MEP), descoberta mais recentemente (ABREU & HEGGIE, 2007; DEWICK, 2009).

A condensação cabeça-cauda entre DMAP e IPP forma o geranil pirofosfato (GPP), precursor dos monoterpenos. A adição sequencial de unidades IPP a essa cadeia gera o farnesil pirofosfato (FPP), o geranilgeranil pirofosfato (GGPP) e o geranilfarnesil pirofosfato (GFPP), precursores dos sesquiterpenos, diterpenos e sesterpenos, respectivamente. Já os triterpenos e tetraterpenos são formados por condensações cauda-cauda de duas unidades FPP ou GGPP, respectivamente. Nos OE, predominam os terpenos formados por condensações cabeça-cauda, em especial os monoterpenos e sesquiterpenos, que, por possuírem baixo peso molecular, são facilmente extraídos por destilação a vapor. Os diterpenos, por sua vez, geralmente requerem extração com solventes orgânicos (SIMÕES & SPITZER, 2004).

Os monoterpenos são os compostos mais representativos nos OE, podendo representar até 90% de sua composição. São altamente voláteis e os principais responsáveis pelas fragrâncias características das plantas, justificando seu uso na perfumaria e como especiarias (BAKKALI et al., 2008; GUNATILAKA, 2008).

Além da diversidade estrutural, a biossíntese dos OE é regulada por fatores ambientais, desenvolvimento da planta e estímulos externos, o que resulta em diferentes perfis químicos entre espécies e entre órgãos de uma mesma planta. A atividade das enzimas terpene

sintases (TPS), por exemplo, desempenha papel crucial na formação dos diferentes compostos voláteis (DUDAREVA et al., 2013; GERSHENZON; DUDAREVA, 2007).

Recentemente, avanços em engenharia genética têm possibilitado a produção heteróloga de OE e seus precursores em microrganismos, com o objetivo de aumentar a disponibilidade e a padronização de compostos bioativos, ampliando seu uso na indústria farmacêutica e alimentícia (GUPTA et al., 2020).

A compreensão dos processos biossintéticos é essencial para a bioprospecção racional de OE com potencial terapêutico. Entre suas muitas atividades farmacológicas, destaca-se o uso de monoterpenos e sesquiterpenos em doenças neurodegenerativas, como a Doença de Alzheimer. Estudos demonstram que essas moléculas atuam como inibidores da acetilcolinesterase, agentes antioxidantes e neuroprotetores, mostrando-se promissores na modulação de processos fisiopatológicos do sistema nervoso central (AYAZ et al., 2017; ROMERO et al., 2023).

2.3. Doença de Alzheimer

A doença de Alzheimer (DA) é uma das principais causas de demência no mundo, é uma síndrome de desordem progressiva crônica, que tem como característica a destruição de neurônios colinérgicos, causando perda de memória, déficits cognitivos e alterações comportamentais (HOLTZMAN, et al. 2011; NEBEL, et al. 2018). Como sintomas, em um nível moderado, há problemas de linguagem, alterações de humor, perda de motivação, desorientação, efeitos cognitivos, incapacidade de manter o autocuidado, perda de iniciativa, comprometimento da memória, pensamento abstrato e problemas comportamentais (BURNS; ILIFFE, 2019; CHOPRA et al., 2018).

A maior parte dos casos da doença surge esporadicamente, sendo sua causa atribuída a uma combinação de fatores genéticos, fatores endógenos, ambiente exógeno e muitos outros fatores de risco, apenas cerca de 1% dos casos está associada à DA autossômica dominante de início precoce causada por mutações nos genes PSEN1, PSEN2 ou APP (WATTMO; WALLIN, 2017; TWOHIG; NIELSEN, 2019; VRABEC; BLUNDEN; CAHLÍKOVÁ, 2023). Uma das principais percepções que está mudando em relação as abordagens diagnósticas e terapêuticas da doença é o fato que as alterações patológicas subjacentes à degeneração cerebral e à perda cognitiva começam a ser são observadas em pacientes com pelo menos 10 a 20 anos antes do início da demência (HOLTZMAN, *et al.* 2011).

Assim, a idade é o maior fator de risco para o desenvolvimento da doença, ocorrendo um aumento exponencial conforme a idade, e aproximadamente dobrando o risco a cada 5

anos após os 65 anos (ALEWIJN et al., 2015; QUERFURTH, LAFERLA, 2018). A doença afeta 10% das pessoas com mais de 65 anos e cerca de 40% com mais de 80 anos (SERENIK; VITAL, 2008). Sendo está a causa mais comum de demência, atualmente estima-se que 50 milhões de pessoas em todo mundo sofra dessa patologia, e a estimativa é que esse valor alcance 150 milhões até 2050 (VAZ; SILVESTRE, 2020; ALZHEIMER'S DISEASE INTERNATIONAL., 2023).

Além do fator idade, estima-se que dos 5,3 milhões de pessoas com idade igual ou superior a 65 anos com a doença de Alzheimer nos Estados Unidos, 3,3 milhões são mulheres e 2,0 milhões são homens (ALZHEIMER'S ASSOCIATION, 2017). Dessa forma, observa-se que a DA, afeta consideravelmente mais as mulheres, além disso, elas apresentam um risco maior de desenvolver a doença ao longo da vida (ALZHEIMER'S ASSOCIATION, 2023).

Apesar de alguns mecanismos de patogênese e progressão da doença serem ainda desconhecidos, tem-se conhecimento que as características neuropatológicas das DA, acontece pela deposição extracelular de amiloide β (Aβ), na forma de placas, além da associação a uma variedade de diferentes tipos de placas senis, acúmulo de filamentos anormais da proteína tau, formando consequentemente um emaranhados neurofibrilares intracelulares (NFT), ocasionado assim, perda neuronal e sináptica, ativação da glia e inflamação (SELKOE, 2015; DETURE; DICKSON, 2019). Além disso, em nível celular, a DA está correlacionada à diminuição das taxas de acetilcolina (ACh) no processo sináptico, reduzindo a neurotransmissão colinérgica cortical, além de outros neurotransmissores como noradrenalina, dopamina, serotonina, glutamato e substância P em menor extensão. Alguns estudos mostram a redução do número de vários receptores nicotínicos e muscarínicos (M2) de ACh que estão localizados nas terminações colinérgicas pré-sinápticas, correndo preservação dos receptores muscarínicos (M1 e M2) pós-sinápticos (JUNIOR et al. 2016).

O diagnóstico da doença de Alzheimer é clínico-biológico, sendo necessário a presença de um fenótipo clínico específico da doença (fenótipo positivo) e evidência de biomarcador da patologia (amilóide positivo e tau positivo) (DUBOIS et al., 2021). Para isso é necessário a realização de um exame microscópico de múltiplas regiões do cérebro empregando métodos de coloração que podem detectar alterações neuropatológicas do tipo Alzheimer (MONTINE, et al. 2012).

2.4. Tratamento farmacológico da Doença de Alzheimer

Apenas quatro fármacos são aprovados para o tratamento da doença de Alzheimer, pela Food and Drug Administration (FDA) segundo Vaz e Silvestre (2020). Destes, três atuam como inibidores da enzima acetilcolinesterase (AChE) (donepezil, galantamina e

rivastigmina) e um antagonista do receptor N-metil-D-aspartato (NMDA) (memantina) que utilizando isolado e em terapia combinada com donepezil (aprovado em 2014) (HUISA, et al., 2019). Contudo, esses medicamentos trazem contribuições e benefícios moderados no controle dos sintomas. Além disso, eles não atuam prevenindo a perda neural, atrofia cerebral, e por consequência, a deterioração progressiva da cognição dos indivíduos (Alzheimer's Association, 2020).

A tacrina (etrahidroaminoacridina) foi a primeira droga aprovada para o tratamento da DA, em 1993 pela FDA, sendo um composto sintético com estrutura simples e com forte atividade inibitória da AChE, contudo em função de sua hepatotoxicidade a mesma foi removida do mercado (CHENG et al., 2019, TAQUI et al. 2022). Apesar de não está sendo utilizada atualmente, existem alguns estudos sobre o uso de derivados da substância, que buscam utilizar a síntese de ligantes direcionados a múltiplos alvos (MTDL) com base na estrutura da tacrina para obter maior eficácia inibitória (TAQUI et al. 2022).

A galantamina é um alcaloide da família Amaryllidaceae, sendo um IAChE seletivo, reversível e competitivo. Apesar de sua capacidade inibitória comparativamente limitada, serve muito bem como um modulador alostérico do receptor nicotínico neuronal de ACh. Assim como a tacrina, e o constante desenvolvimento de medicamentos está ocorrendo uma grande mudança no uso da galantamina. Uma série de compostos sintetizados pela combinação de galantamina com memantina mostrou um notável perfil neuroprotetor tanto pela inibição da AChE quanto pela atenuação da neurotoxicidade induzida por N-metil -d -aspartato (SIMONI et al., 2012; TAQUI et al., 2022).

No caso da rivastigmina ela é um derivado fenilcarbamato, sendo denominado como um inibidor pseudo-irreversível da AChE em razão de seus efeitos prolongados no plasma além do intervalo previsto (MEHTA et al. 2012). Ela apresenta propriedades inibidoras da BuChE e da AChE. Entre os três AChEi de segunda geração, este é o único que está disponível tanto em formulação oral quanto transdérmica. A eficácia de um fármaco administrado depende em grande parte da disponibilidade de uma proteína carreadora no sangue (TAQUI et al. 2022).

Já o donepezil é um inibidor potente, seletivo e não competitivo da AChE sendo a base de piperidina (TAQUI et al. 2022). Acredita-se que o donepezil não atue apenas no nível do neurotransmissor, mas também no nível molecular e celular em quase todos os estágios envolvidos na patogênese da DA. Estes incluem, mas não estão limitados a induzir uma isoforma neuroprotetora da AChE, bloqueando vários aspectos da cascata excitotóxica

induzida pelo glutamato, mitigando os efeitos do estresse oxidativo e reduzindo a expressão de citocinas inflamatória.

No caso da memantina ela é uma droga antagonista, não-competitiva, voltagem-dependente do receptor N-metil-D-aspartato (NMDA), que bloqueia os efeitos patológicos dos níveis elevados de glutamato (DANYSZ et al., 2000). A mesma pode ser utilizada como monoterapia ou em combinação com inibidores da colinesterase (HUISA, et al. 2019). Como citado por Vaz e Silvestre (2020) desde 2020 nenhum medicamento havia sido provado para o tratamento da DA, apesar dos inúmeros estudos e o desenvolvimento de terapias modificadoras para retardar o avanço da doença. Contudo, em 2021 e 2023, por meio do processo de aprovação acelerada o FDA realizou a aprovação para o uso de dois novos medicamentos para o tratamento da doença.

Em junho de 2021, foi realizada a aprovação da utilização do Aduhelm, uma imunoterapia da Biogen com um anticorpo monoclonal IgG1 humano que induz a depuração dos depósitos β-amilóides associados à doença de Alzheimer no cérebro. Esse medicamento, não atua nos sintomas cognitivos da doença, mas sim por meio, da remoção do β-amilóide como um biomarcador substituto para a taxa de progressão da DA (TANZI, 2021). A decisão de aprovar este medicamento foi altamente controversa e levou à renúncia de três conselheiros da FDA em função da ausência de evidências de que o medicamento seja eficaz, uma vez que os ensaios clínicos deram resultados conflitantes sobre sua eficácia (MAHASE, 2021).

Já o medicamento Leqembi (lecanemab-irmb), foi aprovado em 6 de janeiro de 2023, após resultados satisfatórios alcançados após a fase III de estudo do medicamento, em que os pesquisadores avaliaram a eficácia do Leqembi em um estudo duplo-cego, utilizando placebo com controle, de grupos paralelos, para encontrar a dose de 856 pacientes com doença de Alzheimer. O tratamento foi iniciado em pacientes com comprometimento cognitivo leve ou estágio de demência leve da doença e presença confirmada de patologia beta-amilóide, os quais o medicamento aprovado, possui indicação. Os pacientes a dose aprovada de lecanemab-irmb - 10 miligramas/quilograma a cada duas semanas obteve uma redução estatisticamente significativa na placa amilóide cerebral em comparação com o placebo, que não apresentou redução da placa beta-amilóide, justificando assim a aprovação do medicamento (CANADY, 2023).

Como o medicamento Aduhelm, o Leqembi, esse é capaz de eliminar as placas Aβ e retardar o declínio da cognição em pacientes com DA em estágio inicial. No entanto, o tratamento está acompanhado de efeitos adversos, e mais estudos sobre sua segurança e eficácia ainda são precisos para que seu uso seja reforçado (REARDON, 2023).

Apesar do elevado número de casos e da natureza multifatorial da DA, atualmente só existe uma medida de tratamento adotada pelos órgãos de saúde, baseada na chamada hipótese colinérgica de disfunção cognitiva. Essa hipótese indica que boa parte dos declínios cognitivos da DA é ocasionada pela diminuição dos níveis do neurotransmissor Acetilcolina (ACh) no cérebro, levando ao desenvolvimento dos vários sintomas da doença, como a perda de memória e de cognição. Muitos estudos comprovam essa hipótese, onde os níveis de ACh podem diminuir até 85% no cérebro de pessoas acometidas com a DA.

De forma geral, como a DA não tem cura, o tratamento da mesma consiste em aumentar as taxas de ACh, inibindo a ação da enzima acetilcolinesterase (AChE). Os medicamentos citados anteriormente, como rivastigmina, donepezila, galantamina e tacrina (este último usado com restrições) – tem mecanismos de ação baseados na inibição da enzima AChE, onde ajudam a diminuir a progressão neurodegenerativa da Doença de Alzheimer, o que torna a enzima AChE como um dos principais alvos terapêuticos para tratamento da DA.

2.5. Enzima Acetilcolinesterase

A acetilcolina é biossintetizada por meio da acetil-coenzima A (acetil-CoA) e colina por ação da enzima colina-acetiltransferase (JUNIOR et al. 2020). A enzima acetilcolinesterase faz parte da superfamília de proteínas dobradas a/b hidrolase, um grupo definido por homologia estrutural comum e inclui as colinesterases, carboxilesterases e lipases, sendo está alvo de estudos para o tratamento da DA (SINGH, et al. 2013). Essa enzima tem como principal função o término da transmissão do impulso nervoso nas sinapses colinérgicas pela rápida hidrólise da acetilcolina (ACh) (MUKHERJEE, et al. 2017).

Conforme Junior e colaboradores (2004) esse neurotransmissor está presente no cérebro e nas junções neuromusculares, formando parte do sistema nervoso parassimpático. Entre seus efeitos existe a contração dos músculos lisos, dilatação dos vasos sanguíneos e regulação da taxa de batimentos cardíacos; no cérebro engloba as sinapses associadas ao controle motor, memória e cognição. Fisiologicamente sua principal função é a rápida hidrólise da acetilcolina na sinapse e junção neuromuscular, resultando na terminação do impulso nervoso (JOHNSON; MOORE, 2006).

A enzima alvo AChE consiste em um desfiladeiro estreito com dois sítios de ligação de ligantes separados. Na medicina, os inibidores da AChE são usados principalmente no tratamento da doença de Alzheimer (DA), glaucoma, bloqueio neuromuscular em anestesia cirúrgica e miastenia gravis. Em vertebrados, dois tipos de enzimas colinesterase estão presentes, acetilcolinesterase (AChE) e butirilcolinesterase (BuChE), ambas catalisam eficientemente a hidrólise da acetilcolina. A AChE está intimamente relacionada com a

butirilcolinesterase. As duas enzimas são distinguidas com base nas especificidades do substrato, distribuição nos tecidos e sensibilidade aos inibidores (LANE; POTKIN; ENZ, 2015; ZIMMERMAN; SOREQ, 2016; BLICHFELDT-LAURIDSEN; HANSEN 2021)

Segundo Singh e colaboradores (2013) a AChE é composta basicamente por dois sítios, sendo estes: tríade catalítica e sítio aniônico periférico. Dessa maneira, o sítio ativo da AChE é uma tríade catalítica constituída de resíduos de aminoácidos serina (Ser-200), histidina (His-440) e glutamato (Glu-327). O mecanismo de hidrólise de AChE engloba o ataque nucleofílico da serina ao carbono carbonílico da ACh, ocasionando um intermediário tetraédrico estabilizado por ligações de hidrogênio, gerando uma colina livre e serina acetilada. Ao finalizar o processo, a hidrólise do grupo acetila da serina pela água realiza a recuperação do sítio catalítico da enzima (JUNIOR et al. 2004).

Nos tratamentos de Alzheimer a atividade acetilcolinesterase aumentada ocasiona a quebra do neurotransmissor acetilcolina e causa o declínio no nível de acetilcolina no cérebro. Outra relação entre a enzima e a DA é o envolvimento parcial da enzima na formação de placas amiloides e emaranhados neurofibrilares. Foi constatado que a AChE promove a agregação de fragmentos de peptídeos β-amilóides constituindo um complexo com as fibrilas em crescimento (SINGH et al. 2013). Devido à sua função no desenvolvimento da proteína amiloide e na hidrólise do neurotransmissor ACh, a inibição da enzima tida como uma estratégia promissora no tratamento da DA (BALLARD, 2022).

Os inibidores da colinesterase se ligam reversivelmente aos sítios ativos das enzimas AChE/BChE e, com isso, inibem a degradação hidrolítica de um importante neurotransmissor ACh, implicado na neurotransmissão. Por consequência, a concentração sináptica de ACh aumenta, resultando no alívio dos sintomas da DA (AYAZ et al., 2017).

2.6. Óleos essenciais como potenciais inibidores colinesterásicos

Nos últimos anos, muitos estudos tem relatado a eficácia óleos essenciais como inibidores da enzima acetilcolinesterase. Na tabela 1, estão descritos alguns trabalhos que já avaliaram a atividade anticolinesterásica de óleos essenciais provenientes de variadas espécies vegetais. Estes estudos foram encontrados nas plataformas de busca PubMed, Science Direct, e Scielo, com os descritores “essential oil anticholinesterase activity”, de 2015 a 2025.

Tabela 1 – Estudos sobre atividade anticolinesterásica de óleos essenciais, publicados nos últimos anos.

Nº	**Título**	**Autor(es) e ano**	**Tipo de estudo**	**Composto majoritário**	**Parte da planta usada**	**Valores de IC_{50}**
1	Chemical profile and therapeutic potential of	FERREIRA et al.	*In vitro* (método	*Limoneno*	*Casca*	*50-150 mg/L*

	the essential oil and nanoemulsions of Citrus x sp (Tanja Lemon).	(2024)	de Ellman)			
2	Extratos florais do domínio Cerrado e inibição da acetilcolinesterase	MENEZES FILHO (2023)	*In vitro* (método de Ellman)	*Limoneno*	*Flores*	*10-50 µg/mL*
3	O Potencial Nutracêutico de *Pereskia grandifolia* Haw.(Cactaceae).	LAVERDE JÚNIOR (2022)	*In vitro* (método de Ellman)	*β-cariofileno*	*Folhas*	*90,4 µg/mL*
4	Plantas brasileiras com ação anticolinesterásica	LOPES et al. (2022)	*In vitro (método de Ellman)*	*δ-cadineno*	*Folhas, casca, sementes ou vagens*	*25 µg/mL,*
5	Chemical composition and anticholinesterase activity of *Lepisanthes rubiginosa* (Roxb.) Leenh.	SALLEH et al., (2022)	*In vitro (método de Ellman)*	*Chavibetol*	*Folhas*	*77,2-89,2 µg/mL*
6	Chemical constituents and anticholinesterase activity of the essential oil of Algerian *Elaeoselinum thapsioides* (Desf.) maire	SMAILI et al., (2022)	*In vitro* (método de Ellman)	*Mirceno*	*Partes aéreas*	*100 µg/mL*
7	Chemical composition, antioxidant and anticholinesterase activity of the essential oil of *algerian cachrys* sicula L.	TAHAR et al., (2022)	*In vitro* (método de Ellman)	*Mirceno*	*Partes aéreas*	*100 µg/mL*
8	O uso do óleo essencial de *Rosmarinus officinalis* L. no paciente com Alzheimer	DIAS; MENEZES FILHO (2021)	*In vitro* (método de Ellman)	*Cineol, Cânfora, Borneol, Verbenona, α-Pineno, β-Pineno*	*Flores, folhas e caule*	*36,2%-45,9%*
9	Leaf morphology of *Raulinoreitzia crenulata* and antioxidant activity of its essential oils	MENEZES-FILHO et l. (2021)	*In vitro* (método de Ellman)	*Limoneno*	*Folhas e galhos*	*10-50 mg/mL*

10	Chemical Composition and Anticholinesterase Activity of the Essential Oil of Leaves and Flowers from the Ecuadorian Plant *Lepechinia paniculata* (Kunth) Epling.	PANAMITO et al., (2021)	*In vitro* (método de Ellman)	*Cineole e β-Pinene.*	*Folhas e flores*	*38-47 µg/mL*
11	Enantiomeric Composition, Antioxidant Capacity and Anticholinesterase Activity of Essential Oil from Leaves of Chirimoya (*Annona cherimola* Mill.)	VALAREZO et al. (2022)	*In vitro* (método de Ellman)	*Germacrene D*	*Folhas*	*41,51 µg/mL*
12	Essential oil of *Cymbopogon citratus* cultivated in Egypt: seasonal variation in chemical composition and anticholinesterase activity.	MADI et al., (2021)	*In vitro* (método de Ellman)	*Citral*	*Folhas*	*2,86 mg/mL*
13	Identificação dos constituintes químicos e ensaio biológico do óleo essencial *de Pectis elongate* kunth (Asteraceae)	NASCIMENTO et al. (2021)	*Geranial (50,3%) e neral (35,2%),*	*Geranial e neral*	*Folhas, flores e galhos*	*250 µg/mL*
14	Chemical composition and anticholinesterase inhibitory activity of *Pavetta graciliflora* Wall. ex Ridl. essential oil. Zeitschrift fur Naturforschung.	SALLEH & KHAMIS, (2020)	*In vitro* (método de Ellman)	*β-cariofileno*	*Folhas*	*10 mg/mL*
15	Chemical Composition, in vitro Trypanocidal and Antibacterial Activities of the Essential Oil from the Dried Leaves of *Eugenia dysenterica* DC from Brazil	SANTOS et al. (2020)	*In vitro* (método de Ellman)	*Limoneno, citral, caryophyllene oxide, e trans-caryophyllene*	*Folhas*	*Acima de 100 µg/mL*
17	Emulsion incorporating *Eugenia dysenterica* aqueous extract entrapped in chitosan microparticles as a novel topical treatment	SILVA et al. (2020)	*Estudo in vitro* (método de	*Limoneno*	*Folhas*	*Acima de 100 µg/mL*

	of cutaneous infections.		Ellman)			
17	Chemical composition, enantiomeric analysis and anticholinesterase activity of *Lepechinia betonicifolia* essential oil from Ecuador	CALVA et al., (2022)	*In vitro* (método de Ellman)	*β-pinene*	*Folhas e flores*	*74,97 µg/mL*
18	Chemical Composition, Enantiomeric Distribution and Anticholinesterase and Antioxidant Activity of the Essential Oil of *Diplosthephium juniperinum*	SALINAS et al., (2022)	*In vitro* (método de Ellman)	*α-pinene*	*Folhas*	*67,20-89,00 µg/mL*
19	Chemical Composition and Biological Activity of Essential Oil from Leaves and Fruits of Limoncillo (*Siparuna muricata* (Ruiz & Pav.) A. DC.)	MOROCHO et al., (2023)	*In vitro* (método de Ellman)	*α-pinene e limonene*	*Folhas e frutos*	*52,98-98,84 µg/mL*
20	Chemical Characterization and Biological Activity of the Essential Oil from *Araucaria brasiliensis* Collected in Ecuador	JARAMILLO et al., (2022)	*In vitro* (método de Ellman)	*Beyerene e kaurene*	*Folhas*	*95,7 µg/mL*
21	Chemical composition, in vitro antioxidant, anticholinesterase, and antidiabetic potential of essential oil of *Elaeagnus umbellata* Thunb	NAZIR et al., (2021)	*In vitro* (método de Ellman)	*Humulene epoxid*	*Fruto*	*48-90 µg/mL*
22	Study of Essential Oil Isolated from Achiote (*Bixa orellana*) Leaves: Chemical Composition, Enantiomeric Distribution and Antimicrobial, Antioxidant and Anticholinesterase Activities	VALAREZO et al., (2023)	*In vitro* (método de Ellman)	*Germacrene D*	*Folhas*	*39,45 µg/mL*
23	Chemical Composition, Enantiomeric Distribution and Biological Activity of	VALAREZO et al., (2023)	*In vitro* (método de	*Caryophyllene*	*Folhas*	*133,5 µg/mL*

	Essential Oil from *Morella pubescens* (Humb. & Bonpl. ex Willd.) Wilbur.		Ellman)			
24	Assessment of anti-cholinesterase activity and cytotoxicity of cagaita (*Eugenia dysenterica*) leaves	GASCA et al. (2024)	*In vitro* (método de Ellman)	*Volatizes do óleo das folhas de Eugenia dysenterica*	*Folhas*	*10-50 µg/mL*
25	Atividade antimicrobiana de extratos etanólicos de *Banisteriopsis anisandra* (Malpighiaceae) e interações medicamentosas	FERREIRA; MENDES-COSTA (2019)	*In vitro* (método de Ellman)	*alfa-pineno*	*Folhas*	*50 µg/mL*
26	Antimicrobial activity and phytochemical prospection of vegetal extracts of *Ocotea silvestris* Vattimo-Gil and *Ocotea diospyrifolia* (Meisn.) against serotypes of *Salmonella* of poultry origin	WEBER et al. (2018)	*In vitro* (método de Ellman)	*Folhas*	*α-pineno, β-pineno, e linalol*	*50 e 100 µg/mL*
27	Activity against *Aedes aegypti* mosquito, larvicidal. estudos etnofarmacológicos de óleos essenciais com atividade larvicida contra o mosquito aedes aegypti	VORIS et al. (2017)		*Methyl-eugenol e Anethol*	*Folhas*	*25 mg/L*
28	*Rhanterium epapposum* Oliv. essential oil: Chemical composition and antimicrobial, insect-repellent and anticholinesterase activities.	DEMIRCI et al., (2016)	*In vitro* (método de Ellman)	*Camphene*	*Partes aéreas*	*10-50 µg/mL*

Fonte: o próprio autor.

Esses estudos fornecem um bom indicativo de que os óleos essenciais têm sido bastante investigados quanto à sua capacidade em inibir a enzima acetilcolinesterase.

Os inibidores da acetilcolinesterase (IAChEs) são a principal classe de fármacos atualmente utilizadas para o tratamento da fase demencial da DA, sendo a galantamina a única substância de ocorrência natural. No entanto, várias espécies de plantas produtoras de diversas

classes de alcaloides, cumarinas, terpenos e polifenóis têm sido avaliados quanto à sua atividade anti-AChE, tornando-se potenciais candidatas a novos medicamentos anti-DA (SANTOS et al. 2018).

A diversidade da estrutura dos IAChEs conhecidos e as perspectivas de explorar modos de ação distintos têm incentivado o estudo fitoquímico de várias espécies vegetais e de microorganismos, que possam subsidiar novos modelos de substâncias anticolinesterásicas. Neste sentido, várias exemplares da biodiversidade têm sido analisadas em função do seu uso popular ou de dados etnobotânicos (JUNIOR, et al. 2020).

A história da descoberta de fármacos demonstra que as plantas são altamente ricas sendo fontes na busca de novos compostos ativos e se com isso, tornaram um desafio para a indústria farmacêutica moderna. Muitas drogas sintéticas devem sua origem à medicina complementar baseada em plantas (BARBOSA FILHO et al., 2021).

Segundo Kim e colaboradores (2012) os compostos químicos derivados de plantas que possuem propriedades potencialmente promotoras da saúde, são denominados de fitoquímicos. Entre os fitoquímicos de interesse emergente estão a curcumina, o resveratrol e as catequinas do chá verde, com base em estudos epidemiológicos que sugeriram uma relação positiva entre o consumo desses compostos e a prevenção da DA (SINGH et al. 2018).

Um estudo realizado por Trevisan e Macedo (2013) com plantas brasileiras mostrou excelentes resultados para as espécies *Amburana cearensis*, *Lippia sidoides*, *Paullinia cupana*, *Plathymiscium oribundum* e *Solanum asperum*.

Enquanto outras estudos trazem o levantamento de diversas plantas que podem ser utilizadas, Filho e colaboradores (2022) realizou a pesquisa de 309 plantas pertencentes a 92 famílias botânicas testadas contra a inibição da acetilcolinesterase e constatou que maioria dos extratos vegetais testados apresentaram atividade inibitória contra a acetilcolinesterase e podem ser considerados para mais estudos no tratamento da DA. Em particular, as espécies pertencentes a Amaryllidaceae, Apiaceae, Asteraceae, Fabaceae e Fumariaceae, que até então, foram as mais estudadas (BARBOSA FILHO et al. 2022).

No caso do uso de óleos essenciais, se verifica que estes podem ter a capacidade de atravessar facilmente a barreira hematoencefálica em alto nível de translocação para o cérebro, apresentando uma potencial estratégia de tratamento para a DA (MIYAZAWA, et al. 2016). Vários estudos demonstram a eficácia dos óleos essenciais derivados de plantas de *Marlierea racemose*, *Eucalyptus globulus*, *Salvia leriifolia* e *Citrus libanotis* na melhora da disfunção cognitiva e déficit de memória em diferentes modelos de demência (AYAZ et al., 2017).

Talic e colaboradores (2023) ao estudar a atividade antioxidante e a inibição da acetilcolinesterase do óleo essencial de *Lavandula angustifolia* Mill. constatou que os extratos polares possuem bom poder antioxidante, enquanto o óleo essencial pode ser considerado um bom inibidor da AChE. Martins e colaboradores (2021) também obteve resultados positivos ao estudar o óleo essencial de *Siparuna guianensis* Aubl. constantando que a mesmo pode ser uma nova fonte de timidina e derivados capazes de inibir reversivelmente a AChE apresentando potenciais propriedades neuroprotetoras para serem aplicadas no tratamento da doença de Alzheimer.

Karakaya e colaboradores (2019b) relatam que as famílias Apiaceae e Lamiaceae têm se destacado pelo alto teor de compostos fenólicos e óleos essenciais, pois as mesmas possuem eficácia significativo no sistema nervoso central. Como exemplo, tem-se os estudos realizados com os óleos essenciais de *Zosima absinthfolia* (Apiaceae) e *Rosmarinus officinalis* (Lamiaceae) que evidenciaram atividade anticolinesterásica significativa (LOŽIENĖ et al., 2018, KARAKAYA et al., 2019a, PULOK et al., 2017).

Embora muitos estudos já tenham comprovado o potencial de óleos essenciais em inibiram a AChE, é importante também que seja esclarecido de que forma os componentes químicos dos OE podem ser ligar ao sítio ativo da enzima e inativá-la. Uma da maneiras mais eficazes de se determinar os mecanismos de interação de substâncias químicas aos seus alvos moleculares é a docagem molecular.

2.7. Docagem molecular

O design computacional de medicamentos é um campo em rápido crescimento que leva à redução de custos e tempo de desenvolvimento de medicamentos. Hoje, um grande número de estruturas 3D de proteínas é determinado e depositado no Protein Data Bank (PDB). Assim, o número de moléculas bioativas com base na análise estrutural da droga, auxiliada por computador, está em constante crescimento. Dentre os métodos mais eficazes no planejamento teórico de fármacos destaca-se o docking molecular, procedimento que busca prever o modo de ligação de uma pequena molécula (ligante) no sítio ativo de uma macromolécula alvo (receptor) e que foi aplicado e vem sendo aplicado em diversos estudos (SILVA et al., 2014; KARAKAYA, et al., 2019a; SILVA et al., 2019).

O processo de docking molecular é útil para detectar a interação de ligação de um ligante com um receptor de proteína no nível molecular e atômico. É comumente usado na descoberta de drogas modernas para entender a eficiência da ligação de um composto químico nas interações droga-receptor, para ajudar a determinar a seletividade de uma droga para diferentes alvos e a potência de diferentes drogas em um alvo. As ferramentas de ancoragem

podem gerar uma série de conformações de ligação para posicionar adequadamente o ligante dentro do local de ligação definido ou previsto da proteína, e as funções de pontuação traduzem adequadamente as geometrias em valores de energia que quantificam as interações. Por esse motivo, um pré-requisito fundamental para a realização do docking molecular é o conhecimento da estrutura tridimensional da proteína-alvo (SALMASO; MORO, 2018; SIVAKUMAR et al. 2020).

O algoritmo de docking molecular consiste principalmente em duas etapas: um mecanismo de busca para encontrar conformações, orientações e posições otimizadas para energia do ligante em solução induzida por ligação de proteína e uma função de pontuação para dar um valor a cada pose com base nas energias previstas contatos atômicos (ABAGYAN; TOTROV, 2023). O processo envolve a amostragem do espaço conformacional e a identificação das soluções de pontuação corretas definidas pela paisagem de energia livre e a análise e avaliação do conjunto completo de soluções (SIVAKUMAR et al., 2023).

Usando a docking molecular Fernández-Galleguillos e colaboradores (2023) analisaram o perfil fenólico e a composição das partes aéreas de três espécies nativas utilizadas na medicina tradicional no Altiplano Andino do norte do Chile, *Clinopodium gilliesii* (Benth.), *Mutisia acuminata* Ruiz & Pav. var. hirsuta e a *Tagetes multiflora* (Kunth), constatando que o extrato de *T. multiflora* tem a maior atividade anti-AChE e BChE em comparação com *C. gilliesii* e *M. acuminata*, contudo, em comparação com o controle de galantamina essa atividade foi considerada fraca. Karakaya e colaboradores (2019a) também utilizou essa técnica para análise de partes da planta de *Zosima absinthifolia*, constatando no estudo que a fração diclorometano da fruta apresentou a melhor inibição contra a enzima butirilcolinesterase em relação a acetilcolinesterase.

De maneira geral, estudos de docagem molecular são ferramentas muito importantes, não somente para reforçar atividades biológicas de compostos bioativos, mas também para entendermos melhor como as moléculas interagem com um determinado sítio de ligação. Uma vez que a atividade farmacológica de uma determinada substância está intimamente relacionada à ligação desta ao receptor celular, os estudos de docking molecular se tornam essenciais em estudos de bioprospecção de novas moléculas bioativas.

3. CAPÍTULOS

3.1. CAPÍTULO 1 - Molecular modelling and anticholinesterase activity of the essential oil from three chemotypes of *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson (Verbenaceae)

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Molecular modelling and anticholinesterase activity of the essential oil from three chemotypes of *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson (Verbenaceae)

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ABSTRACT

Lippia alba (Mill.) N.E. Brown (Verbenaceae), popularly known as "erva cidreira", is one of the most used plants in Brazilian folk medicine. The species has several chemotypes and its volatile constituents have already been characterized, and present different chemical markers with known pharmacological properties, such as analgesic, sedative and antifungal properties. The objective of this study was to evaluate the anticholinesterase activity (AChE) of the essential oil of three chemotypes of *Lippia alba* and, by using molecular anchoring, determine the best receptor-ligand interaction energies of the main constituents present in the samples of oil. The essential oils were obtained via hydrodistillation (LA1 and LA2) and steam drag (LA3), and their volatile constituents determined using GC-MS. For the determination of anticholinesterase activity, direct bioautography and colorimetry assays based on Ellman's method were used. Molecular docking was performed using a multiple solution genetic algorithm and Merck molecular force field 94 (MMFF94) as the scoring function. In the main constituents of the oil samples, three chemotypes were identified for *L. alba*: LA1 is rich in citral, LA2 is rich in carvone and LA3 is rich in linalool. All L. *alba* chemotypes showed AChE enzyme inhibition with an IC_{50} of 3.57 μg/mL (LA1), 0.1 μg/mL (LA2) and 4.34 μg/mL (LA3). The molecular docking study complemented the results of the experiment and demonstrated significant interactions between the main constituents of the oils and the amino acid residues of the AChE enzyme. Irrespective of the chemotype, *Lippia alba* presents biotechnological potential for the discovery of anticholinesterase substances, with the chemotype LA2 (rich in carvone) being the most active.

1. Introduction

Lippia alba (Mill.) N.E.Br. ex Britton & P. Wilson, popularly known as lemon balm and/or false melissa, is an aromatic plant that belongs to the Verbenaceae family and is widely distributed in several tropical and subtropical regions of the Americas. Since ancient

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Molecular modelling and anticholinesterase activity of the essential oil from three chemotypes of *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson (Verbenaceae)

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Abstract

Lippia alba (Mill.) N.E. Brown (Verbenaceae), popularly known as "erva cidreira", is one of the most used plants in Brazilian folk medicine. The species has several chemotypes and its volatile constituents have already been characterized, and present different chemical markers with known pharmacological properties, such as analgesic, sedative and antifungal properties. The objective of this study was to evaluate the anticholinesterase activity (AChE) of the essential oil of three chemotypes of *Lippia alba* and, by using molecular anchoring, determine the best receptor-ligand interaction energies of the main constituents present in the samples of oil. The essential oils were obtained via hydrodistillation (LA1 and LA2) and steam drag (LA3), and their volatile constituents determined using GC-MS. For the determination of anticholinesterase activity, direct bioautography and colorimetry assays based on Ellman's method were used. Molecular docking was performed using a multiple solution genetic algorithm and Merck molecular force field 94 (MMFF94) as the scoring function. In the main constituents of the oil samples, three chemotypes were identified for *L. alba*: LA1 is rich in citral, LA2 is rich in carvone and LA3 is rich in linalool. All *L. alba* chemotypes showed AChE enzyme inhibition with an IC_{50} of 3.57 µg/mL (LA1), 0.1 µg/mL (LA2) and 4.34 µg/mL (LA3). The molecular docking study complemented the results of the experiment and demonstrated significant interactions between the main constituents of the oils and the amino acid residues of the AChE enzyme. Irrespective of the chemotype, *Lippia alba* presents biotechnological potential for the discovery of anticholinesterase substances, with the chemotype LA2 (rich in carvone) being the most active.

Keywords: erva cidreira; aromatic plant; acetylcholinesterase inhibitors.

1. Introduction

Lippia alba (Mill.) N.E.Br. ex Britton & P. Wilson, popularly known as lemon balm and/or false melissa, is an aromatic plant that belongs to the Verbenaceae family and is widely distributed in several tropical and subtropical regions of the Americas. Since ancient times, traditional communities have used this plant species in their daily lives, giving it a significant relevance in historical, cultural and medicinal contexts [1]. Generally, *L. alba* is cultivated in domestic environments and is traditionally prepared as an infusion, maceration, decoction, and used in compresses, baths or extracts to relieve stress, insomnia and the symptoms of flu and colds [2]. Other uses also include cases of diarrhea, cramps, bronchitis, hypertension, headache and liver disorders [3,4].

Many studies have attributed pharmacological activities to the essential oil of this plant such as antibacterial [5, 6], anesthetic [7] antiparasitic [8], antiviral [9], antioxidant, sedative/relaxing [10, 11], antispasmodic [12], and anxiolytic [13] activities, in addition to efficacy in aromatherapy to reduce psychological stress [14]. The essential oil of *L. alba* is composed of a variety of substances, being citral (mixture of geranial and neral isomers) one of the main components responsible for its characteristic aroma; however, other compounds may be present in the composition of the essential oil, since the same species can present different chemical types (chemotype).

These quantitative and qualitative variations in the chemical composition of *L. alba* essential oil have been widely proven, and have led to the classification of the species into different chemotypes according to its main constituents [15], the most described in the literature being citral, carvone and linalool (Figure 1). However, 1,8-cineole, myrcene and β-elemene also occur [16, 17, 18]. In the Amazon, there is a report of the chemotypes citral [6], carvone-limonene [19, 20], myrcene-citral [21], citral-carvone-limonene [22], limonene-1,8-cineole, carvone-limonene-germacrene D, and citral-germacrene D [16].

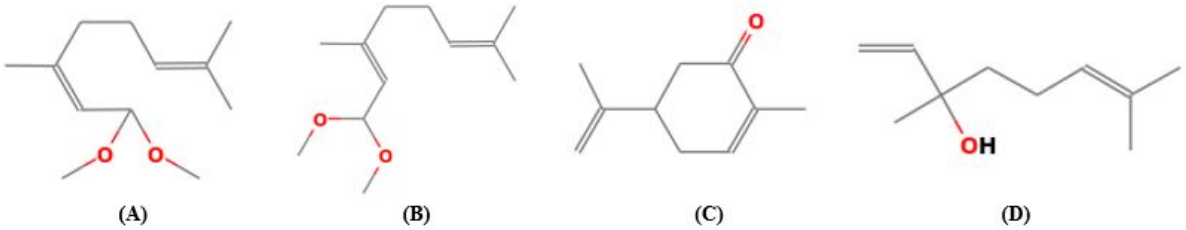


Figure 1 - Chemical structure of the majority components of the main chemotypes of *L. alba*. (A) = citral - neral; (B) citral - geranial; (C) = carvone and (D) = linalool. Source: NIST.

The occurrence of chemotypes in this species have aroused the interest of the academic community since, depending on the chemical composition, the biological activity of a

plant species can undergo considerable changes. Therefore, it is important to evaluate the effect of different chemotypes of *L. alba* in different biological activities. As the species has been used by communities without regards to its chemical composition, this has led to inappropriate use of the species. In Table 1, it is possible to observe the studies that have been carried out with the different chemotypes of *L. alba.*

In this brief review (Table 1) of studies involving *L. alba* essential oil that have been carried out in the last five years (2020-2024) and are available on the PubMed search platform, it can be noted that there is a lack of studies that prove the action of this plant on the central nervous system (CNS). Many diseases that affect the CNS have no cure and, among the pathologies that affect the CNS, Alzheimer's disease (AD) stands out. Its treatment is based on the use of drugs that inhibit the enzyme acetylcholinesterase (AChE), which is an important natural organic substance responsible for hydrolyzing the neurotransmitter acetylcholine, and is often used in prospective studies of plant species with therapeutic action for neurodegenerative pathologies such as AD [23].

Thus, since comparative studies between the different chemotypes of *L. alba* are still incipient, the objective of this study was to evaluate whether the essential oil of three chemotypes of *Lippia alba* have an inhibitory effect on the enzyme acetylcholinesterase. Currently, a gap remains in the literature regarding the use of the different chemotypes of *L. alba* as acetylcholinesterase inhibitors or for other pharmacological activities. As the species is already used by the population, mainly as an alternative treatment for various types of diseases and without chemotype discrimination, this study shows that the inhibitory effect of *L. alba* essential oil on the enzyme acetylcholinesterase is dependent on the chemical constitution of the chemotype used.

Since it is an aromatic plant with a high essential oil content, *L. alba* can be used in aromatherapy or for the development of more sustainable chemical products, thus reducing environmental impacts and saving materials. Therefore, the use of essential oils for the development of phytoproducts favors green chemistry, a segment of chemistry that seeks alternative processes that generate less pollution and less waste, and presents high energy efficiency, in addition to valuing the use of renewable raw materials and generating biodegradable and safe products for society and the environment.

In addition to knowledge about the activity of the chemical constituents of *L. alba* in the inhibition of acetylcholinesterase, it is important to understand its mechanism of action and its pharmacokinetic properties. Thus, in the present work, we also conducted a study on the interaction between the main compounds of each oil and the human acetylcholinesterase

enzyme, as well as a detailed *in silico* pharmacokinetic study in order to evaluate the probability of these compounds being absorbed orally and reaching the central nervous system to finally interact with the molecular target and present an effective pharmacological response.

Table 1: Main studies that evaluated the biological activities of the essential oil from different chemotypes of *L. alba*, published in the period 2020-2024.

Number	Author(s)	Title	Chemotype(s)	Biological activity evaluated	Year	Reference
1	Barbosa et al.	*In vitro* anthelmintic activity of *Lippia alba* essential oil chemotypes against *Haemonchus contortus*.	Citral and carvone	Anthelminth	2023	[24]
2	Bonilla-Carvajal et al.	Essential Oil of Carvone Chemotype *Lippia alba* (Verbenaceae) Regulates Lipid Mobilization and Adipogenesis in Adipocytes.	Carvone	Lipid mobilization and adipogenesis	2022	[25]
3	Quintero et al.	Immunomodulatory, trypanocide, and antioxidant properties of essential oil fractions of *Lippia alba* (Verbenaceae).	Citral and carvone	Trypanocide and immunomodulator	2021	[26]
4	Lima et al.	Insecticidal activity of a chemotype VI essential oil from *Lippia alba* leaves collected at Caatinga and the major compound (1,8-cineole) against *Nasutitermes corniger* and *Sitophilus zeamais.*	1,8-cineol	Insecticide	2021	[27]
5	Borges et al.	Effect of *Lippia alba* (Mill.) N.E. Brown Essential Oil on the Human Umbilical Artery.	Citral	Vasorelaxant in human umbilical arteries (HUA)	2022	[28]

Table 1: (Continued)

Number	Author(s)	Title	Chemotype(s)	Biological activity evaluated	Year	Reference
6		Ovicidal effect of essential oils of *Lippia alba*, *Lippia sidoides* and *Lippia gracilis* on the acanthocephalan *Neoechinorhynchus buttnerae* (Eoacanthocephala: Neoechinorhynchidae).		Ovicide (*Neoechinornchus buttnerae*)	2022	[29]
7	Postay et al.	The effectiveness of surfactants applied with essential oil of *Lippia alba* in the anesthesia of Nile tilapia (*Oreochromis niloticus*) and their toxicity assessment for fish and mammals.	Linalool	Anesthetic/Toxicity	2021	[30]
8	Gomes et al.	*Lippia alba* and *Lippia gracilis* essential oils affect the viability and oviposition of *Schistosoma mansoni.*	Citral	Anthelminth	2022	[31]
9	Filho et al.	Chemical composition and biological activities of the essential oils from *Lippia alba* and *Lippia origanoides.*	Citral/Limonene	Antioxidant; antimicrobial and acute toxicity	2023	[32]
10	de Lima et al.	Eugenol and *Lippia alba* essential oils as effective anesthetics for the Amazonian freshwater stingray *Potamotrygon wallacei* (Chondrichthyes, Potamotrygonidae).	Eugenol	Anesthetic	2021	[33]

Table 1: (continued)

Number	Author(s)	Title	Chemotype(s)	Biological activity evaluated	Year	Reference
11	Pagotti et al.	Trypanocidal Activity of *Dysphania ambrosioides*, *Lippia alba*, and *Tetradenia riparia* Essential Oils against *Trypanosoma cruzi.*	Linalool	Trypanocide	2021	[34]
12	Nonato et al.	Comparative analysis of chemical profiles and antioxidant activities of essential oils obtained from species of *Lippia* L. by chemometrics.	Citral	Antioxidant	2022	[35]
13	de Brito et al.	Identification of Bioactive Compounds against *Aedes aegypti* (Diptera: Culicidae) by Bioassays and in Silico Assays.	Citral	Repellent	2021	[36]
14	Tabari et al.	Acaricidal activity, mode of action, and persistent efficacy of selected essential oils on the poultry red mite (*Dermanyssus gallinae*).	Carvone-Limonene	Acaricide	2020	[37]

Source: Authors (2024).

2. Materials and Methods

2.1 Plant Material

The aerial parts (leaves, flowers and thin branches) of two specimens of *L. alba* (Mill.) N.E.Br. ex Britton & P. Wilson (named LA1 and LA2) were collected in the village of Alter do Chão (2º30'31.0" S and 54º57'00.0" W), Santarém, Pará, Brazil, in the months of May and July 2021. Of these, exsiccates were prepared and deposited in the herbarium of the University of Juiz de Fora, Minas Gerais, under the number CESJ 65276, and the taxonomic confirmation was carried out by the specialist in Verbenaceae, Dr. Fátima Salimena. This research was registered in SisGen (Sistema Nacional de Gestão do. Patrimônio Genético e do Conhecimento Tradicional Associado) under the number A965D42.

The third sample of *L. alba* essential oil (LA3) was obtained from a commercial crop in Serra Negra, São Paulo (22°31'33.4" S 46°42'10.8" W) that was produced by DJUH Indústria e Comércio de Cosméticos Ltda. (batch: OMLA-0001/18), and was provided by Dr. Pedro Melillo Magalhães.

2.2 Acquisition of the essential oils

L. alba leaves (LA1 and LA2) were previously dehydrated in a forced air circulation oven at a temperature of 37 ± 2 °C and then subsequently subjected to the hydrodistillation process using a Clevenger-type apparatus for 120 min. The LA3 oil sample was obtained from dehydrated aerial parts via steam distillation for 3 hours.

All the oil samples were subjected to centrifugation with anhydrous sodium sulfate to remove water. After centrifugation, the oil was removed with a pipette and stored in an amber bottle, hermetically closed, and stored in a refrigerator at 5 ºC until analysis. The yield was calculated based on the dry weight of the plant [38].

2.3 Analysis of the volatile constituents of the samples

The samples of the essential oil were analyzed in a gas chromatography system coupled to a mass spectrometer (GCMS-QP2010 Ultra, Shimadzu Corporation, Tokyo, Japan), equipped with an auto injector (AOC-20i) and CGMSsolution software, which contains databases of libraries [39] including FFNSC 2 [28] and a fused silica capillary column (Rxi-5ms, Restek Corporation, Bellefonte, PA, USA) of 30 m x 0.25 mm (diameter) x 0.25 μm (film thickness), coated with 5% diphenyl dimethylpolysiloxane. The analysis conditions were as follows: helium drag gas (99.995%); split ratio mode in the ratio of 1:20; injection of 1 μL of the sample (3 μL of the essential oil in 500 μL of hexane); ionization energy by electronic im-

pact (EI) 70 eV; injector temperature: 250 ºC; oven temperature program: 60-240 ºC; ion source temperature: 200 ºC; transfer line temperature: 250 ºC.

Quantitative data on the volatile constituents were obtained via peak area normalization using a gas chromatograph (GC 6890 Plus series, Agilent) coupled to a flame ionization detector (FID), which was operated under similar conditions to the GC-MS system. The mass spectra were obtained by automatic scanning at 0.3 scans/second, with mass fragments of 35-400 *m/z*. The compounds found in the ion chromatograms were identified by comparing the mass spectra (molecular mass and fragmentation pattern) with those found in the system's CGMSsolution library and by comparison with the retention indexes. The linear equation of Van den Dool and Kratz (1963) [40] was used to calculate the volatile components, with the use of a standard homologous series of C8-C20 n-alkanes (Sigma-Aldrich).

2.4 Determination of the cholinesterase inhibition

For the *in vitro* anticholinesterase assays, the enzyme acetylcholinesterase type VI-S, obtained from *Electrophorus electricus* (lyophilized powder, C3389-2Ku, Sigma-Aldrich, batch: SLBZ8573) was used. The standard used in the assays was the anticholinesterase inhibitor eserine (physostigmine) (Sigma-Aldrich, batch: BCBC4171V) diluted in methanol. A standard curve was used to define the concentration used in the tests.

2.4.1 Qualitative test on a thin layer chromatography (TLC) chromatoplate

For the qualitative testing of the samples of *L. alba* essential oil and the standard drug, an aluminum chromatoplate for TLC (ALUGRAM® Xtra SIL G, silica gel 60, 0.20 mm, Macherey-Nagel) was used based on the direct bioautography method of Marston et al. (2002) [41]. The enzyme was diluted in tris-HCl buffer, 50 mM, pH 7.8 in ultra-pure water to obtain a concentration of 4 U/mL, with the addition of bovine serum albumin (Sigma-Aldrich) at a ratio of 1:1. Samples of *L. alba* essential oil were diluted in methanol at a concentration of 100 µL/mL. Physostigmine at a concentration of 100 µg/mL was used as the standard. For the negative control, methanol was used. The colorimetric reagents of the test were naphthyl acetate (2.5 mg in methanol) and Fast Blue B salt (2.5 mg in ultrapure water). Both reagents were prepared and mixed immediately before use to prevent their decomposition.

To perform the test, the aliquots of 10 µL of the oil samples and controls were applied to the chromatoplates in duplicate, and allowed to stand for a period of 24 h for evaporation of the solvent. Subsequently, the plate was sprayed with the acetylcholinesterase enzyme solution (4 U/mL) and incubated in a humidity test chamber, without direct contact with moisture, at 37 °C for 20 minutes. Then, the plate was sprayed with the mixture of naphthyl acetate (2.5

mg) and Fast Blue B salt (2.5 mg) solutions to obtain the final results. The formation of a purple coloration occurred gradually, after 1 to 3 min.

2.4.2 Quantitative assay

The assay for the quantification of acetylcholinesterase inhibition of the samples was adapted from Ellman's method [42], with modifications, as described by [43]. In summary, three buffers were produced for the quantitative test, which were denominated A, B and C. These being: buffer A = 50 mM Tris/HCl, pH 8, dissolved in ultrapure water; buffer B = 0.1% bovine serum albumin in buffer A; and buffer C = 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$ dissolved in buffer A.

In a total volume of 1 mL, 415 µL of buffer A, 10 µL of the essential oil solution (diluted in methanol, buffer and Tween 80) at different concentrations (100, 50, 25, 12.5, 6.25 and 3.12 ug/mL), and 75 µL of acetylcholinesterase enzyme, containing 0.2 U/mL, were added. The samples were then incubated for 15 min at 25 °C. After incubation, 75 µL of a solution of 1.83 mM AChI (acetylthiocholine iodide) (Sigma-Aldrich, Steinheim, Germany) and 425 µL of 3 mM DTNB (5,5'-dithiobis[2-nitrobenzoic acid]) (Sigma-Aldrich, Steinheim, Germany) were added and the mixture was incubated for 30 min at 25 °C under a light source. The absorbance of the mixture was measured at 412 nm in a UV spectrophotometer (NOVA, 3300). Physostigmine was used as the standard drug and a dilution solution was used as negative control (buffer A, methanol and Tween 80 at a ratio of 2:2:1). The percentage of inhibition of enzyme activity was calculated according to the equation % = [(A0 – A1) / A0] * 100, where A0 was the absorbance of the control without the essential oil and A1 was the absorbance of the essential oil sample at different concentrations. All tests were performed in triplicate. The concentration of the sample that provided 50% inhibition (IC_{50}) was obtained by constructing graphs of the percentages of inhibition versus the concentration of the inhibitor. The non-linear regression parameters for the curve were plotted and the IC_{50} values were obtained using the Microsoft Excel 2019 software.

2.4.3. Evaluation of the possible synergistic effect of the essential oils

To evaluate the possible synergistic effect of the different essential oil samples of the chemotypes of *L. alba* on the acetylcholinesterase enzyme, mixtures of the three oil samples in different proportions were made, according to Table 2. The IC_{50} was determined using the same conditions mentioned above. The chemical composition of the mixtures was also evaluated using GC-MS in order to confirm the presence of the main constituents after mixing.

Table 2. Mixtures obtained using essential oil of the three chemotypes of *Lippia alba.*

Oil samples			
LA1	**LA2**	**LA3**	**Pool**
Proportion of essential oil in the mixture (µL)			
100	100	100	Pool 1
100	0	100	Pool 2
100	100	0	Pool 3
0	100	100	Pool 4

Source: Authors (2024).

LA1 = *Lippia alba* citral chemotype; LA2 = *Lippia alba* carvona chemotype; LA3 = *Lippia alba* linalool chemotype.

2.5 Molecular modelling and ADMET properties

2.5.1 Preparation of the ligands

The chemical structures of the main compounds of *Lippia alba* were obtained from the National Institute of Standards and Technology (NIST, available at https://webbook.nist.gov/cgi/cbook.cgi?ID=R185885). Subsequently, the compounds underwent optimization of their three-dimensional structure in the software ChemSketch (available at www.acdlabs.com) using the molecular mechanics method. Subsequently, the 2D structures were optimized using ChemSketch (available at www.acdlabs.com), that contains a 3D optimization algorithm modified from a molecular mechanics package (CHARMM) that consider angle bending, bond stretching, internal rotation, and van der Waals non bonded interactions [44]. During the 3D optimization, the stereo bonds of compounds with well-defined stereochemistry were maintained and were not replaced by single bonds.

2.5.2 Preparation of the AChE crystal structure

The crystallographic structure of human acetylcholinesterase (*h*AChE) complexed with the inhibitor donepezil was obtained from the Protein Data Bank (PDB, available at https://www.rcsb.org/), under the PDB code 4EY7 [45]. Then, the crystallographic complex was treated with the software BIOVIA Discovery Studio® v. 20.1.0, whereby the water molecules [46], the enzymatic co-factors and the co-crystallized ligand were removed, leaving only the chain of interest containing the binding site with donepezil.

2.5.3 Re-docking

In order to validate the molecular docking of the main compounds of the studied plant species, donepezil was anchored to the 4EY7 binding site through the DockThor web server (https://www.dockthor.lncc.br/v2/).

The molecular docking was performed using a multiple solution genetic algorithm and Merck molecular force field 94 (MMFF94) as the scoring function. The in-house program, PdbThorBox, is applied to set the protein atoms, the partial charges and complete missing side chains of the protein file [47], according to MMFF94 force field.

At this stage, the modules "Rotatable Bonds Enable all" and "Add Hydrogen Disabled" were used to prepare the binder on the server. Regarding anchoring, the grid box dimension was 20x20x20 Å and the pre-selected algorithm precision settings were conserved. After completing the molecular docking, the results were filtered by setting the modules "RMSD to cluster conformers" equal to 2 and "Number of binding modes" equal to 10.

The RMSD (root-mean-square deviation) value was calculated via the Discovery Studio Software. To analyze the docked pose, donepezil was used as a reference molecule. Via the "Structure" > "RMSD" > "Heavy Atoms" module, it was possible to obtain the RMSD value report, which was obtained from the comparison between the pose with the best score of re-docking and donepezil. Molecular docking parameterization was established based on an RMSD value of below 1.5 Å, which indicates that the molecular docking protocol can be used for docking of other ligands [48].

2.5.4 Molecular docking

The molecular docking of the main compounds of *Lippia alba* with a crystal structure (PDB: 4EY7) followed the same method of the re-docking step with the co-crystallized ligand, with exception of the RMSD calculation. At this step, physostigmine, a well-known cholinesterase inhibitor, was used as control of molecular docking study. The DockThor program generated ten energetically favorable conformations of the ligands in the active site of the enzyme. Via DockTScore, which is a linear empirical function coupled to the DockThor portal, binding affinities were predicted, with lower values indicating high binding affinity for *h*AChE. The interpretation and visualization of the molecular interactions were performed using the Discovery Studio software.

2.5.5 Pharmacokinetic prediction

The screened phytochemicals were subjected to pharmacokinetic analysis using the online prediction tool Swiss ADME (available at http://www.swissadme.ch/). This program

provides the physicochemical properties and ADME (absorption, distribution, metabolism, and excretion) parameters associated with the pharmacokinetics of each compound. Data calculated included the number of rotatable bonds (NRB), hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), $LogP_{o/w}$ values as a measure of lipophilicity, gastrointestinal (GI) absorption, and blood brain barrier (BBB) permeation. SwissADME predicts human gastrointestinal absorption (HIA) and BBB through the BOILED-Egg model, a classification chart, which defines favorable and unfavorable zones in the physicochemical space of lipophilicity versus polar surface area, for passive diffusion through of the two physiological barriers [48].

2.6 Statistical analysis

Data from the quantitative anticholinesterase assay were analyzed using the Prism 5 software with one-way ANOVA, followed by the Tukey test with multiple comparisons, at a significance level of $p<0.05$. The GraphPad Prism 8.0.1 software was used for the statistical evaluation of binding affinity values through a one-Way ANOVA, which was followed by Sidak's multiple comparison test in order to evaluate and infer differences to the chosen pivot molecule.

3. Results

3.1 Volatile constituents of the essential oil from the three chemotypes of *Lippia alba*

The volatile constituents of the essential oils of *L. alba* samples and their quantities are presented Table 3. According to their main constituents, *L. alba* was classified into three distinct chemotypes: citral chemotype – LA1; carvone chemotype – LA2 and linalool chemotype – LA3. As its main constituents, LA1 presented neral (23.84%), geranial (32.31%), γ-muurolene (7.67%) and limonene (7.02%); LA2 presented carvone (30.72%), 1,8-cineole (14.37%) and limonene (10.3%), and LA3 presented as its main constituent linalool (68.31%). The essential oil content measured in g/g dry weight, which ranged from 1.1 to 3.7% according to the chemotype. Table 3 also shows the volatile constituents of the different mixtures of the *L. alba* essential oil.

Table 3 – Volatile constituents and essential oil yield of three samples *Lippia alba* and their mixtures (pools).

Essential oils			LA1	LA2	LA3	Pool 1	Pool 2	Pool 3	Pool 4
Yield			3.7%	1.1%	1.3%*	-	-	-	-
Constituents	RI_{calc}	RI_{lit}	(%)						
α-Pinene (HM)	932	932	-	0.56	-	0.19	-	-	-
Sabinene (HM)	972	969	-	3.74	-	1.48	0.86	3.02	3.31

6-methyl-5-Hepten-2-one (HM)	983	983	1.9	-	-	-	-	-	-
Mircene (HM)	989	988	0.9	3.89	-	1.73	-	1.79	1.9
δ-3-Carene (HM)	1012	1008	-	-	-	-	-	-	-
p-Cymene (HM)	1023	1020	0.77	-	-	-	-	0.94	-
Limonene (HM)	1027	1024	**7.02**	**10.3**	-	**6.11**	**0.94**	**7.43**	**5.88**
1,8-Cineole (OM)	1030	1026	-	**14.37**	-	**5.99**	**-**	**11.72**	**12.86**
trans-β-Ocimene (HM)	1046	1044	0.53	0.96	1.98	0.61	-	-	-
γ-Terpinene (HM)	1057	1054	2.78	-	-	0.77	-	-	-
cis-Sabinene hydrate (OM)	1065	1065	-	0.52	-	-	-	-	-
cis-Linalool oxide (OM)	1071	1067	-	-	1.81	-	0.62	-	0.62
trans-Linalool oxide (OM)	1088	1084	-	-	1.58	-	0.56	-	0.59
Linalool (OM)	1104	1095	0.77	0.75	**68.31**	**28.11**	**47.53**	**0.89**	**42.7**
endo-Fenchol (OM)	1106	1114	-	-	2	-	-	-	-
trans-Verbenol (OM)	1143	1140	-	0.73	-	-	-	-	-
δ-Terpineol (OM)	1165	1162	-	0.51	-	-	-	-	-
E-Isocitral (OM)	1181	1177	0.63	-	-	-	-	-	-
α-Terpineol (OM)	1189	1186	-	2.14	-	0.76		0.57	0.58
Myrtenol (OM)	1195	1194	-	0.69	0.92	-	-	-	-
β-Cyclocitral (OM)	1208	1217	-	-	2.29	-	-	-	-
Citronelol (OM)	1227	1223	1.17	-	-	-	-	0.52	-
Neral (OM**)**	1241	1235	**23.84**	-	-	**8.06**	**14.36**	**16.94**	**-**
Carvone (OM)	1243	1239	0.8	**30.72**	-	**11.53**	**-**	**16.62**	**18.47**
Geraniol (OM)	1253	1249	1.14	-	-	-	-	-	-
Geranial (OM)	1272	1264	**32.31**	-	-	**11.23**	**22.1**	**21.25**	
Piperitenone (OM)	1339	1340	-	1.34	-	-	-	-	-
β-Cubebene (HS)	1390	1387	-	0.58	-	-	-	-	-
β-Elemene (HS)	1391	1389	0.64	0.79	2.78	0.9	-	-	-
***E*-Caryophyllene** (HS)	1419	1417	0.58	-	**4.14**	0.92	0.99	-	1.02
γ-Muurolene (HS)	1481	1478	**7.67**	**6.45**	**4.13**	**4.82**	**1.98**	**2.48**	**2.16**
α-Zingiberene (HS)	1494	1493	1.15	-	-	-	-	-	-
Cubebol (OS)	1514	1514	0.58	1.14	-	0.57		0.53	-
Elemol (OS)	1549	1548	**5.39**	**5.21**	-	3.29	1.62	2.54	0.65
Guaiol (OS)	1597	1600	-	0.53	-	-	-	0.55	-
Cedr-8(15)-en-9-α-ol (OS)	1650	1650	-	1.3	-	-	-	0.61	-
8-Cedren-13-ol (OS)	1696	1688	-	1.61	-	-	-	0.51	-
Curcumenol (OS)	1736	1733	-	0.6	-	-	-	-	-
Hydrocarbon monoterpenes (HM)			**19.02**	**18.95**	**9.18**	**30.73**	**18.31**	**20.92**	**27.92**
Oxygenated monoterpenes (OM)			**45.65**	**34.12**	**55.08**	**30.73**	**45.75**	**36.61**	**41.88**
Hydrocarbon sesquiterpenes (HS)			**15.21**	**15.16**	**27.54**	**13.36**	**18.31**	**5.23**	**13.96**

Oxygenated sesquiterpenes (OS)	**3.8**	**22.75**		**10.24**	**9.15**	**26.15**	**6.04**
Esters	**19.02**	**18.95**	**9.18**	**30.73**	**18.31**	**20.92**	**27.92**
Others							
Total (%)	**91.3**	**91.0**	**91.8**	**87.07**	**91.56**	**88.91**	**90.74**

Source: Authors (2024).

RI_{calc} = Calculated retention time; RI_{lit} = Retention time claimed in the literature.

*Yield reported by the producer. LA1 = *Lippia alba* citral; LA2 = *Lippia alba* carvone; LA3 = *Lippia alba* linalool; Pool 1 = LA1 + LA2 + LA3; Pool 2 = LA1 + LA3; Pool 3 = LA1 + LA2; Pool 4 = LA2 + LA3.

3.2. Qualitative assay of AChE inhibitory activity

Figure 2 shows the halos of inhibition of *L. alba* essential oil samples using the enzyme acetylcholinesterase by direct bioautography assay. All essential oil samples of the different chemotypes (LA1, LA2 and LA3), in addition to the physostigmine standard, showed inhibition halo formation in the chromatoplate.

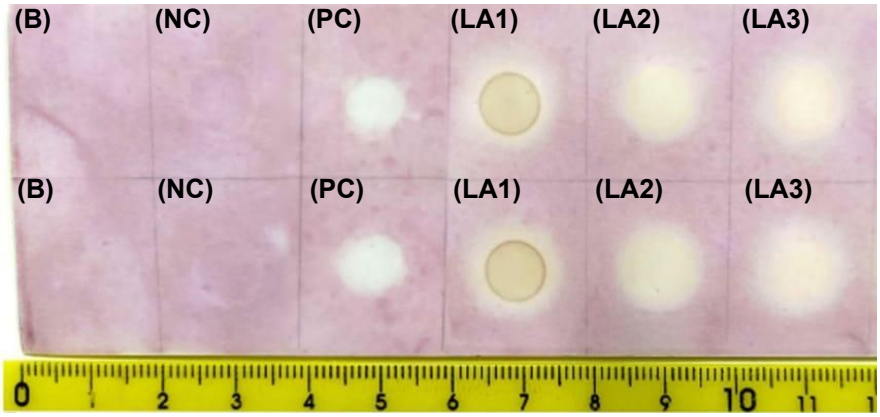


Figure 2 – Direct bioautography on the silica gel chromatoplate (TLC) of the anticholinesterase activity of *Lippia alba* essential oil (100 µL/mL) and physostigmine samples. LA1 = *Lippia alba* citral; LA2 = *Lippia alba* carvone; LA3 = *Lippia alba* linalool; Controls: (B) blank, (NC) methanol negative control, and (PC) physostigmine positive control (100 µg/mL). Inhibition halos were measured in cm.

3.3. Quantitative assay of AChE inhibitory activity

According to the results of Figure 3, it is possible to observe that all samples of *L. alba* essential oil and the different mixtures (pool) showed inhibitory activity against acetylcholinesterase, with an IC_{50} that ranged from 0.1 to 4.3 µg/mL.

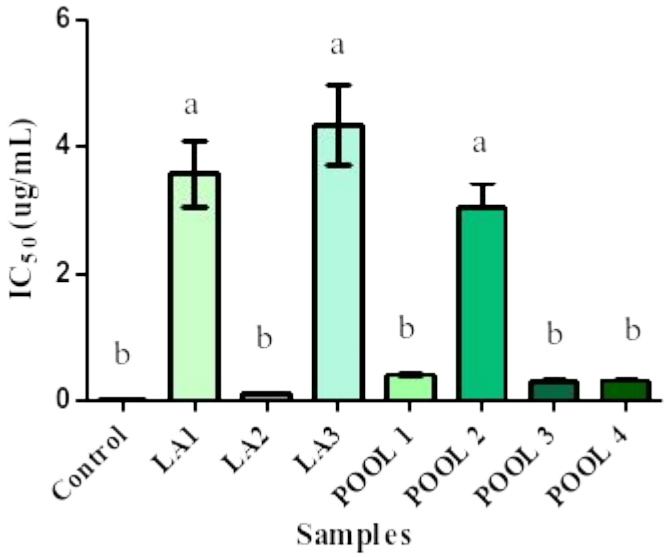


Figure 3 – Anticholinesterase activity (IC_{50}) of *Lippia alba* essential oil samples and their respective mixtures (pools). Control = physostigmine; LA1 = *Lippia alba* citral; LA2 = *Lippia alba* carvone; LA3 = *Lippia alba* linalool; Pool 1 = LA1 + LA2 + LA3; Pool 2 = LA1 + LA3; Pool 3 = LA1 + LA2; Pool 4 = LA2 + LA3.

3.4. Redocking of donepezil into the active site of AChE (PDB ID: 4EY7)

The human AChE enzyme co-crystallized with donepezil (PDB ID: 4EY7) was used as a virtual target to classify the main constituents of the essential oil samples according to their binding affinities. First, the anchoring procedures were validated by precisely refitting the co-crystallized donepezil into the *h*AChE model to better compare our anchoring results. Donepezil was anchored against AChE with the same parameters and was observed to have -11.237 kcal/mol as the binding affinity (Figure 4 and Table 4). The energy calculation for all anchor complexes was evaluated using the MMFF94 force field as the scoring function.

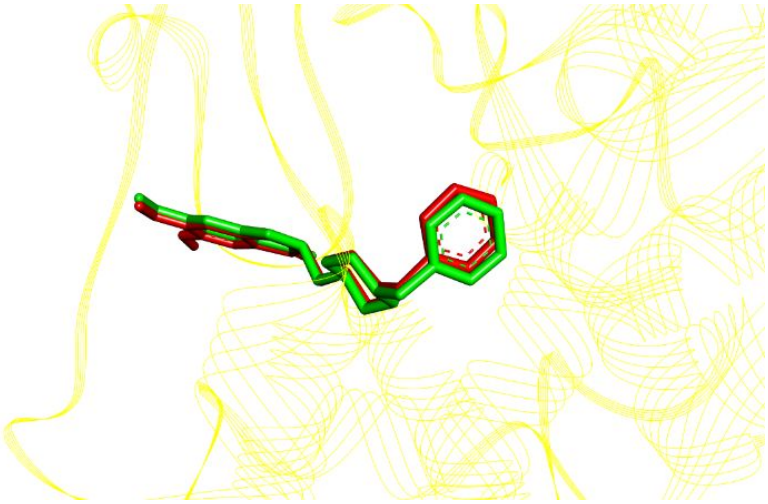


Figure 4 – Re-docking of donepezil and *h*AChE (4EY7 and donepezil). In green, experimental pose and, in red, predicted *in silico* pose. **RMSD:** 0.334**;** ΔG= -11.237 kcal/mol.

Figure 5 shows the binding pocket and target residues involved in the binding interaction of donepezil.

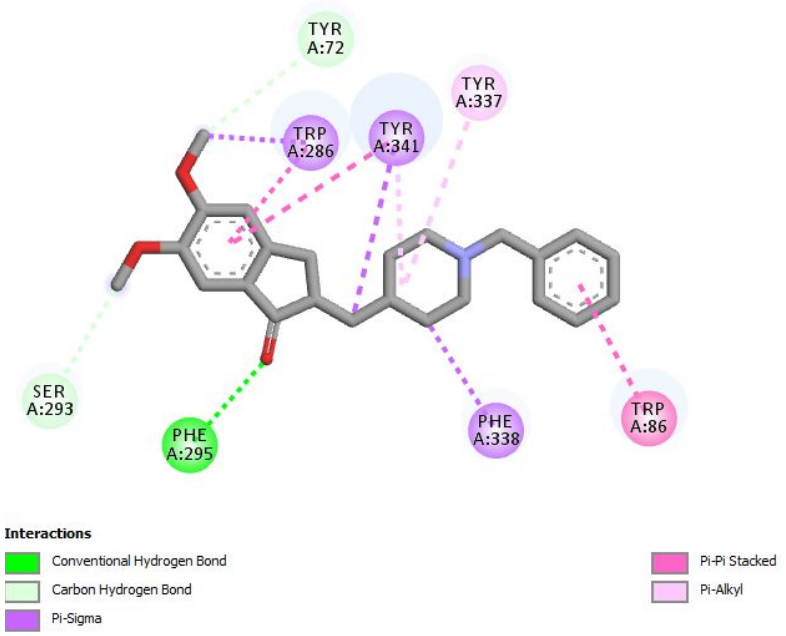


Figure 5 – Donepezil and AChE.

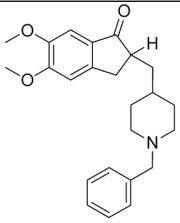
3.5 Molecular docking validation

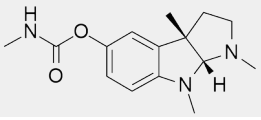
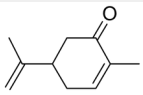
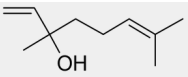
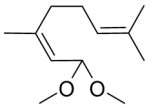
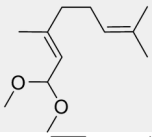
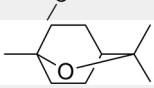
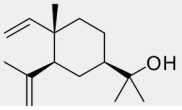
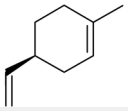
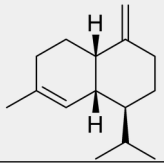
In addition to re-docking of donepezil to *h*AChe, we also used physostigmine as a positive control for molecular docking studies. We used the same donepezil coordinates, as there is no co-crystal with human acetylcholinesterase deposited in the database. The best pose showed binding affinity of -9,146 Kcal/mol as can be seen in Table 4.

3.6. Binding affinity and binding pocket analysis of the phytochemicals

The energy calculation for all anchor complexes was evaluated using the MMFF94 force field as the scoring function. This study evaluated the results of ten poses obtained through molecular docking on the DockThor Portal, and we considered the ranked pose with the highest binding affinity value. The results can be viewed in Table 4.

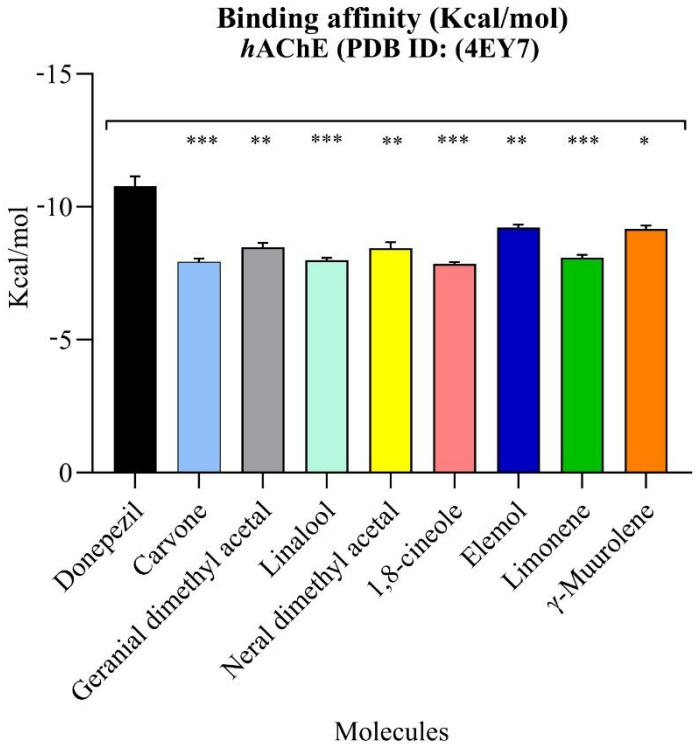
Table 4 – Screening of the most abundant constituents of *Lippia alba*.

Ligand	Chemical structure	AChE binding affinity (kcal/mol)
Donepezil (Pivot)		-11.237

Physostigmine		-9,146
Carvone		-7.951
Linalool		-8,114
Neral		-8.218
Geranial		-8.476
1,8-Cineole		-7.960
Elemol		-9.252
Limonene		-8.194
γ-Muurolene		-9.134

In relation to the chemotypes present in *L. alba*, the binding affinity values ranged from -8,095 to -9,217 Kcal/mol exhibited by the coupling of limonene and elemol, respectively. Figure 6 correlates the average binding affinity values of the phytochemicals with the reference ligand donepezil. It is noted that there was a statistically significant difference between all molecules and donepezil. However, as can be seen in the 2D diagrams (Figures 7-12), the compounds established important interactions with some of the amino acid residues present in the active site of AChE enzyme and which participate in the complexation with donepezil.

Figure 6 – Statistical analysis of the binding energy values from donepezil compared to those of the phytochemicals.



3.7. Binding pocket analysis of the phytochemicals

The docking complexes of the main constituents were examined in order to interpret the binding conformation pattern within the active site of AChE (4EY7) compared to donepezil. Figure 7 shows the binding pocket and target residues involved in the binding interaction of donepezil.

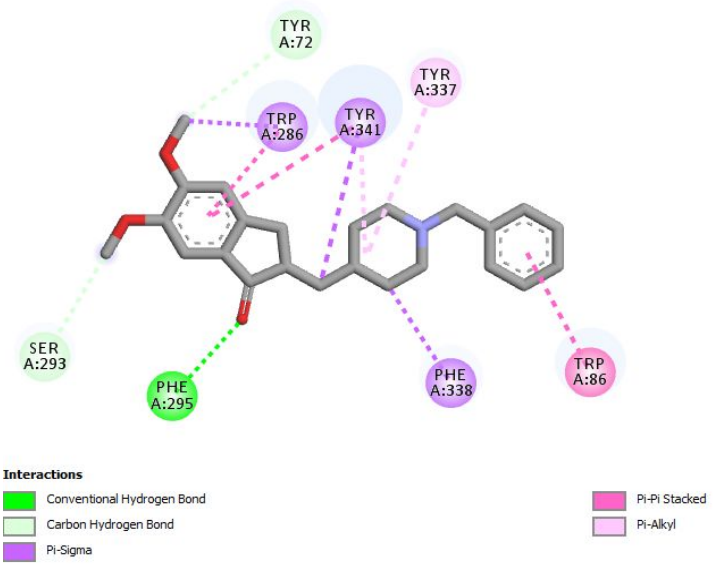


Figure 7 – Donepezil and AChE.

Figure 8 presents the involved amino acids residues of *h*Ache with physostigmine. Previous studies showed that its accommodation to the active site is mediated especially by hydrophobic interactions [49].

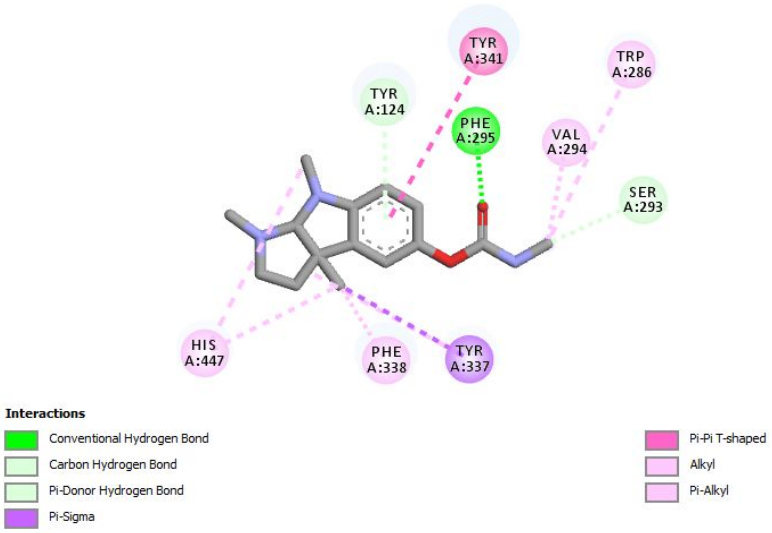


Figure 8 – Physostigmine and AChE.

Figure 9 shows the interaction diagram with amino acid residues of the monoterpenes carvone (A) and elemol (B).

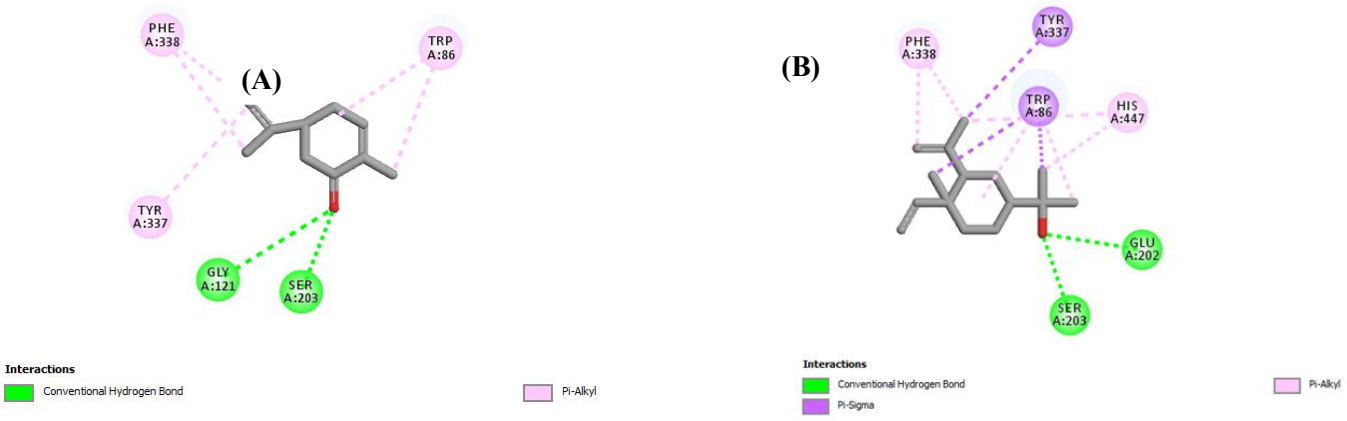


Figure 9 – 2D Diagram of the interactions between carvone (A) and elemol (B) and amino acid residues from *h*AChE (4EY7).

Figure 10 shows the bonds made with the amino acid residues of AChE by the terpene hydrocarbon limonene (A), and sesquiterpene γ-muurolene (B).

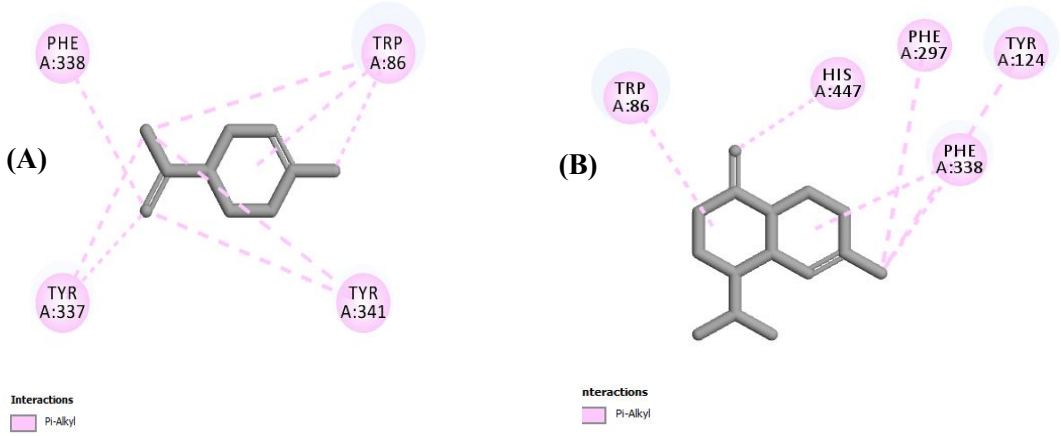


Figure 10 – 2D diagram of the interactions between limonene (A) and γ-muurolene (B) and amino acid residues from *h*AChE (4EY7).

Figure 11 shows the bonds made with the amino acid residues of AChE by the oxygenated monoterpenes neral (A) and geranial (B).

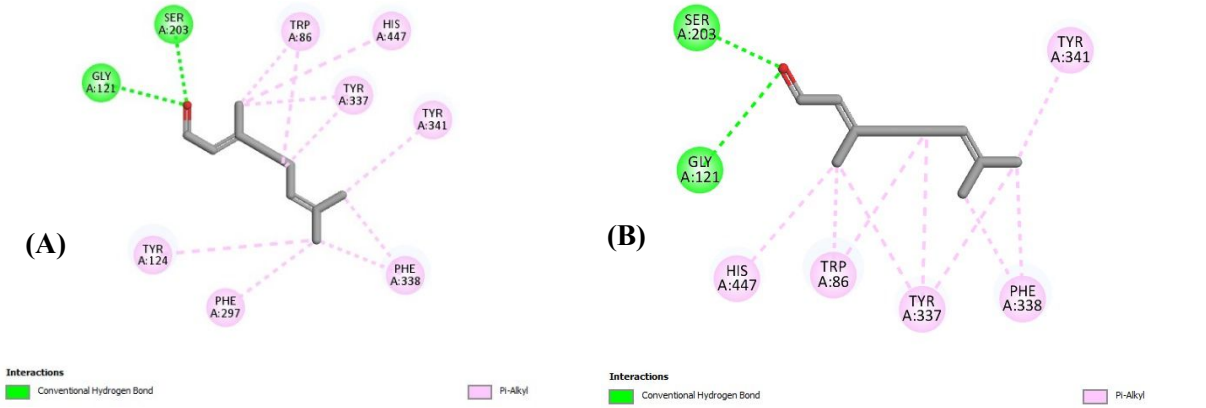


Figure 11 – 2D diagram of the interactions between neral (A) geranial (B) and amino acid residues from *h*AChE (4EY7).

Figure 12 shows the bonds made with the amino acid residues of AChE by the cyclic compound 1,8-cineole (A) and by the monoterpene linalool (B).

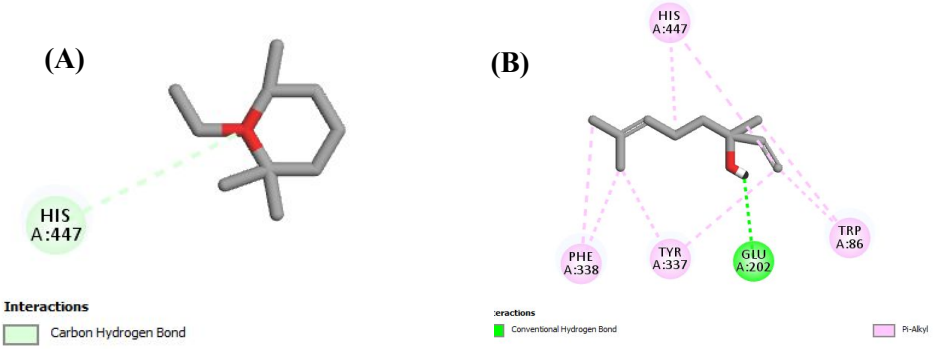


Figure 12 – 2D diagram of the interactions between 1,8-cineole (A) linalool (B) and amino acid residues from *h*AChE (4EY7).

3.6. Pharmacokinetic prediction

The screened phytochemicals were subjected to pharmacokinetic analysis using the online prediction tool Swiss ADME. The results are presented in Table 5.

Table 5 – *In silico* prediction of pharmacokinetic properties of the natural compounds from *Lippia alba*.

Ligand	MW (g/mol)	NRB	HBA	HBD	Log $P_{o/w}$	GI absorption	BBB permeation
Donepezil	379.49	6	4	0	4.00	High	Yes
Carvone	150.22	1	1	0	2.44	High	Yes
Linalool	154.25	4	1	1	2.66	High	Yes
Neral	198.30	6	2	0	3.05	High	Yes
Geranial	198.30	6	2	0	3.07	High	Yes
1,8-Cineole	154.25	0	1	0	2.67	High	Yes
Elemol	222.37	3	1	1	3.77	High	Yes
Limonene	136.23	1	0	0	3.35	Low	Yes
γ-Muurolene	204.35	1	0	0	4.18	Low	No

MW = molecular weight; NRB = number of rotatable bonds; HBA = number of hydrogen bond acceptors; HBD = hydrogen bond donors; Log $P_{o/w}$ = octanol/water partition coefficient; GI = relative gastrointestinal absorption and BBB = blood brain barrier.

4. Discussion

The objective of this study was to evaluate whether the essential oil from the three chemotypes of *Lippia alba* and mixtures of these has an inhibitory effect on the enzyme acetylcholinesterase (AchE), as well as to determine via the molecular anchoring technique the best energies of receptor-ligand interaction of the main constituents present in the oil samples with AChE. In this regard, it has been shown that, according to the main constituents (citral chemotype - LA1; chemotype carvone - LA2 and chemotype linalool - LA3), the chemotypes significantly ($p \leq 0.05$) inhibited the action of AChE, with the chemotype rich in carvone (LA2) with an IC_{50} of 0.10 µg/mL ± 0.006 being the most active in relation to the others (LA1 IC_{50} 3.57 µg/mL ± 0.51 and LA3 IC_{50} 4.34 µg/mL ± 0.63). These results of anticholinesterase action can be seen in figures 2 and 3. In addition, it was observed that the mixtures containing LA2, these being pool 1 (IC_{50} 0.41 µg/mL ± 0.008); pool 3 (IC_{50} 0.31 µg/mL ± 0.023) and pool 4 (IC_{50} 0.31 µg/mL ± 0.035) stood out in relation to the mixture containing only the

chemotypes LA1 and LA3 (Figure 3). Although all chemotypes showed significant inhibitory action ($p \leq 0.05$) of AChE, the presence of carvone seems to potentiate this action. The molecular docking study complemented the results of the experiment and demonstrated that there are significant interactions between the main constituents of the oils and the amino acid residues of the AChE enzyme.

The molecular anchoring experiment is a technique that consists of predicting the orientation and binding conformation of ligands in the active region of target proteins. In this study, the human AChE enzyme, co-crystallized with donepezil (PDB ID: 4EY7), was used as a virtual target to classify the main constituents of the essential oil samples according to their binding affinities. This crystal was chosen since donepezil is commonly known as a cholinesterase inhibitor and is used in the treatment of Alzheimer's disease as a standard drug. First, the anchoring procedures were validated by precisely refitting the co-crystallized donepezil into the *h*AChE model to better compare our anchoring results. Donepezil was anchored against AChE with the same parameters and was observed to have -11.237 kcal/mol as the binding affinity (Figure 4). The energy calculation for all anchoring complexes was evaluated using the MMFF94 force field as the scoring function. Based on the results of the energy values, eight main constituents of the oil samples (carvone, linalool, neral dimethyl acetal, geranial dimethyl acetal, 1,8-cineole, elemol, limonene, γ-murolene) showed energy values that were close to that of donepezil, though they were significantly different ($p \leq 0.05$) (Table 4 and Figure 6).

Physostigmine is a drug widely used in the treatment of Alzheimer's disease. In this study, it was found that the interactions with the catalytic site are mostly hydrophobic, with only one hydrogen bond between the carbamate group and Phe295. π-alkyl and alkyl interactions are observed with residues Trp286, Val294, Phe338 and His447. Other interactions, such as π-π-shaped and π-sigma, are shown with residues Tyr341 and Tyr337, respectively. The compounds γ-muurolene and elemol presented binding affinity values very close to those calculated for physostigmine.

The docking complexes of the main constituents were examined in order to interpret the binding conformation pattern within the active site of AChE (4EY7) when compared to donepezil. Figure 7 shows the binding pocket and target residues involved in the binding interaction of donepezil. As noted, ten amino acid residues are the counterpart of the binding site: Trp286, Tyr72, Tyr124, Tyr341, Phe338, Tyr337, Glu202, Ser203, Trp86 and His447. Natural compounds that have the potential to bind to these residues can be considered AChE inhibitors.

As seen in Figure 9 (A), carvone, a main constituent of LA2, interacts with Gly121 through hydrogen bonding, unlike donepezil. This result also differs from that established by Wojtunik-Kulesza et al. (2017) [50], who via molecular docking evaluated the interactions of different monoterpenes, such as carvone, that are present in vegetable oils and showed that the carbonyl of hydrogen-bonded carvone interacts with Tyr337 [51]. Various reasons can be given to explain these divergences, which include the choice of crystallographic structure and the molecular docking protocol. On the other hand, in our study, the π-alkyl and π-sigma interactions of carvone with trp86, Tyr337, His447 and Phe338 residues are the same as those of donepezil (Figures 9 (A) and 8).

The hydrocarbons terpenes, limonene (figure 10 [A]), present in LA1 and LA2, and γ-muurolene (figure 10 [B]), present in LA1, LA2 and LA3, are apolar compounds, so they bind to AChE only through π-alkyl hydrophobic interactions. Monoterpene limonene interacts with Phe338, Trp86, Tyr341 and Tyr37 while the sesquiterpene γ-muurolene interacts with five residues: Phe338, Trp86, His447, Phe297, Tyr124 (Figure 10 [B]). As seen in Figure 11, the oxygenated monoterpenes, neral (figure 11 [A]) and geranial (figure 11 [B], which are main constituents of LA1, interact by bonding of hydrogen, π-alkyl and hydrogen-carbon bonding. The monoterpene neral interacts through hydrogen bonding with Ser203 and Gly121, while geranial interacts with the residue of Ser125. The π-alkyl interactions for neral occur via the residues Phe297, Phe338 and Tyr124, while for the compound geranial, the interactions occur via the residues Tyr341, Phe338, His447 and Tyr337.

Cyclic constituents, such as 1,8-cineole (figure 12 [A]) found in essential oils, have been reported to be AChE inhibitors. The synergistic associations of these sesquiterpenes may be responsible for their inhibitory action [52]. Linalool (figure 12 [B]) interacts with a hydrogen bond of the AChE in Glu202 and via π-alkyl hydrophobic interactions with the residues Trp86, Hit447, Tyr337, Phe338, similarly to donepezil (Figure 12). These interactions are in agreement with the results of the biological evaluations and confirm the significant activity of the essential oil of the different *L. alba* chemotypes when tested against AChE.

Previous studies have reported that the inhibitory activity against the enzyme acetylcholinesterase is related to the high content of monoterpenes in the chemical composition, such as 1,8-cineole, α-pinene, linalool and caryophyllene oxide [53,54]. In our study, 1,8-cineole and α-pinene are present in the chemotype LA2 and linalool and caryophyllene oxide are present in chemotype LA3. Although α-pinene and caryophyllene oxide do not exceed 2% of the constituents found, 1,8-cineole is one of the main constituents of chemotype LA2 and the mixtures (pools 1, 3 and 4). Linalool is present as a main component in the LA3 chemo-

type. One study that evaluated the anticholinesterase activity of the essential oil of a plant native to Malaysia (*Pseuduvaria macrophylla*), which does not have monoterpenes in its composition, showed a weak inhibition against acetylcholinesterase and butyrylcholinesterase [55]. In other words, the volatile constituents of essential oils can have different biological properties and the synergism between the constituents is important for potentiating the anticholinesterase effect.

In addition, other studies have reported that the essential oil of citrus species that present limonene, citronellol, o-cymene and 1,8-cineole in their chemical composition tend to exhibit strong inhibition of acetylcholinesterase [56,57], which corroborates the findings of our research. One study that evaluated the anticholinesterase action of monoterpenes highlighted S-carvone and linalool among the main inhibitors found, as well as fenchone, γ-terpinene, geraniol, estragol and camphor [58].

Studies evaluating the anticholinesterase activity of *Lippia alba* essential oil [23,24] and its extracts [25] have shown the species to have a potential cholinesterase inhibitory effect. The variety of effects attributed to *Lippia alba* is a result that is related in part to its volatile constituents. Morais et al. (2022) [59], who evaluated the anticholinesterase activity of *L. Alba* chemotypes, presented different results in relation to the present study, since the chemotype with the lowest IC_{50} was citral, unlike in our research, in which the carvone chemotype was the most active. However, it is important to note that the other constituents present in the essential oil differ from that presented in this study. Since essential oils are mixtures of several compounds that can act synergistically and antagonistically with each other, the efficacy of the samples as acetylcholinesterase inhibitors may also vary depending on the percentage of constituents that are in smaller quantities, which could explain the difference between these two studies. In addition, it is important to highlight that many factors can influence the variation of the chemical constitution and, consequently, the biological activity of a plant species. Thus, it is almost impossible to obtain the same results, or even similar ones, despite using the same chemotype of a plant.

Pharmacokinetic analyses of natural compounds are important steps in the classification of molecules based on absorption, distribution, metabolism, and excretion (ADME). The main constituents of the chemotypes LA1, LA2 and LA3 showed properties similar to oral drugs and follow Lipinski's rule of five. The logP value of all the constituents is less than five, the hydrogen bond donor and acceptor atoms are in the optimal range, and the molecular weights are less than 500 Da. These results corroborate those found by Awasthi et al. (2017) [53], who predicted the pharmacokinetic characteristics of 25 promising terpenoids, including

carvone, limonene, linalool and geraniol, against Alzheimer's disease. In addition, drugs that act on the central nervous system have significantly reduced molecular weights compared to other therapies. Previous work has reported a cutoff for blood-brain barrier (BBB) penetration of up to 400 Da. Penetration into the CNS requires a sum of heteroatoms of five or less and has significantly fewer rotatable bonds than other classes of drugs, usually the number of rotatable bonds is five or less. These drugs have less than three H-binding donors and the number of H-binding acceptors is less than seven. All of the compounds mentioned above fit these criteria, so they are able to permeate the BBB and reach the target. However, limonene and γ-muurolene do not meet the water solubility criteria, so these compounds probably have low oral bioavailability and are not suitable as potential oral drug candidates. Thus, six of the main compounds of *Lippia alba* have great physicochemical properties that can be developed as a potential oral drug for Alzheimer's disease.

5. Conclusion

The essential oil of *Lippia alba*, which has chemotypes that are rich in citral, carvone, and linalool, has inhibitory action against the enzyme acetylcholinesterase, with the chemotype that is rich in carvone being the most active. The molecular docking study complemented the experimental results and demonstrated significant interactions between the main constituents of essential oils and the amino acid residues of the AChE enzyme. The pharmacokinetic studies revealed that six of the main constituents from *L. alba* have properties that are similar to oral drugs and follow Lipinski's rule of five. In addition, their reduced molecular weights allow these compounds to achieve the central nervous system. Thus, six of the main compounds of *Lippia alba* have great physicochemical properties that can be developed as a potential oral drug for Alzheimer's disease.

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Author contributions

RHVM was responsible for the development of the concept and hypotheses of this research, writing of the manuscript, raw material used for the samples and data analysis. AQSJ conducted the analyses of the volatile constituents of the essential oil samples and conducted the experiments of the anticholinesterase tests, data analysis and drafted the manuscript. KAS conducted the biological experiments and contributed to the writing of the manuscript. LEMB was responsible for the development of the concept and hypotheses for this research, performed the data analysis and edited the manuscript. ASB was responsible for determining the chemical composition of the oil samples. GBS was responsible for the molecular modeling and contributed to the writing of the manuscript. GSR was responsible for molecular modeling and determination of the pharmacokinetic parameters of chemical compounds. All authors read and approved the final version of the manuscript.

Declaration of interests

The authors declare that there are no conflicts of interest.

Data availability statement

The data associated with this study has not been deposited in a publicly available repository; however, it can be provided upon request.

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3.2. CAPÍTULO 2 - Essential oils of *Curcuma longa* L. and *Zingiber officinale* R. (Zingiberaceae): anticholinesterase activity and molecular modeling – Artigo submetido na revista *Ouricuri*, em Novembro de 2024 – Qualis B1);

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Manuscrito elaborado de acordo com as normas da revista *Ouricuri*, Qualis B1 (Classificações de periódicos quadriênio 2017 – 2020). O manuscrito encontra-se atualmente em revisão por pares, e está disposto na íntegra, conforme foi submetido.

Abstract

This study aims to evaluate the anticholinesterase activity of *Curcuma longa* and *Zingiber officinale* rhizomes essential oils obtained from both individually and in combination, as well as to evaluate the interaction pattern between acetylcholinesterase (AChE) and the main constituents present in the oil's samples. Anticholinesterase activity was assessed using bioautography and Ellman methods (IC_{50}). Molecular docking studies were performed by simulating oils' major constituents docking against the AChE enzyme, and pharmacokinetics were evaluated using SwissADME and PreADMET programs. The essential oils of *C. longa* and *Z. officinale* showed strong inhibition of the AChE enzyme, with IC_{50} values of 0.33 and 0.38 μg/mL, respectively. The combination of the oils in a 1:1 ratio maintained the major components of both oil samples; however, the anticholinesterase activity was reduced (IC_{50} 0.64 μg/mL). The molecular docking assay complemented the experimental studies, demonstrating a strong interaction between the essential oil's major constituents and the amino acid residues of AChE responsible for interaction with donepezil (standard drug). So this study showed that the major compounds of *C. longa* and *Z. officinale* have potential use in future clinical studies as active principles against Alzheimer's disease.

Palavras-chave: Acetylcholine; Alzheimer; Acetylcholinesterase; Molecular Docking; Rhizomes.

Introduction

Acetylcholinesterase (AChE) is a crucial enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh) at cholinergic synapses. These synapses, widely distributed in the central nervous system (CNS) and peripheral nervous system (PNS), play a key role in transmitting messages from one neuron to another. This function is critical for maintaining numerous human physiological processes, such as memory formation and learning (Azizi et al., 2022).

Several drugs target cholinergic synapses, acting on the AChE enzyme either by inhibiting or reactivating it or interacting with ACh receptors as agonists or antagonists. Donepezil, galantamine, and rivastigmine are noteworthy among these drugs. They are used as AChE inhibitors for the treatment of Alzheimer's disease (AD), a neurodegenerative

disorder characterized by the progressive loss of neurons in the central nervous system structures. AD leads to gradual functional impairments that can result in dementia, with memory loss and cognitive decline (Graff-Radford et al., 2021).

The treatment of this disease consists of alleviating the symptoms, primarily through increasing ACh levels in the brain by using acetylcholinesterase enzyme inhibitors (Briggs et al., 2016). Alzheimer's disease treatment is mainly pharmacological, and the currently used medications are still limited and cause numerous undesirable effects in patients. Therefore, research for new treatment alternatives and discovering new molecules that act as AChE inhibitors are crucial. Essential oils produced by the secondary metabolism of aromatic plants are gaining interest as a source of active molecules, either as functional dietary supplements due to their flavoring, antioxidant, and antimicrobial activities or as AChE inhibitors (Donato et al., 2020 ; Gao et al., 2020; Zhang e Kitts, 2021).

These biological properties may be associated with their chemical profiles, which typically vary between species in the number of components and their quantities, as well as their stereochemical structures or chemical variability. In the research for new bioactive substances, including AChE inhibitors, the species *Curcuma longa* L. and *Zingiber officinale* R., which belong to the Zingiberaceae family, stand out. They are widely used for their medicinal and culinary properties in various regions worldwide, including Brazil, where the population uses their rhizomes to treat gastrointestinal disorders and their anti-inflammatory potential (Dosoky e Setzer, 2018).

Curcuma longa, commonly known as turmeric or curcuma, is widely distributed in Southeast and East Asia and is rich in many trace elements beneficial to the human body, with curcumin being one of the most significant substances. This species exhibits various biological activities, as demonstrated by experimental studies, including anti-inflammatory and antioxidant effects, making it a potential therapeutic agent. Curcumin, one of its main compounds, is currently undergoing clinical trials in humans for various conditions, including Alzheimer (Vafaeipour et al., 2022). Another species with numerous applications is *Zingiber officinale*, known as ginger, which is used worldwide as an ingredient in Ayurvedic medicine and a well-known traditional phytotherapeutic remedy. It has anti-inflammatory, anti-ulcerogenic, anti-Alzheimer's, antioxidant, and neuroprotective activities in the central nervous system (CNS) (Choi et al., 2018).

Several studies have demonstrated the effectiveness of essential oils from these species in various biological activities, such as anti-rheumatic (Barão Paixão e Freire de Carvalho, 2021) anti-neoplastic (Lima et al., 2020), fungicidal, and antioxidant (Sharma et al., 2022), as

well as applications in aromatherapy (Toniolo et al., 2021). However, there are still few reports on the action of these oils in the central nervous system as inhibitors of cholinesterases. Studies indicate that essential oils can protect neurons from oxidative stress and reduce lipid peroxidation by neutralizing free radicals, and they also increase the activity of key antioxidant enzymes. Data also suggest that essential oils may have anticholinesterase properties, inhibiting the activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes, which have been pharmacological targets in the symptomatic treatment of Alzheimer's disease (Qneibi et al., 2023).

Due to the importance of AChE as a target for bioactive molecules, the present study aimed to evaluate whether the essential oils and their mixtures obtained from the rhizomes of *Curcuma longa* L. and *Zingiber officinale* R. exhibit inhibitory activity against AChE, assess the interaction pattern between the AChE receptor and the primary constituents present in the oil samples through molecular docking using a generic multiple-solution algorithm and the Merck molecular force field 94 (MMFF94) as the scoring function.

Methodology

Plant material and essential oil extraction

The rhizomes of *Curcuma longa* and *Zingiber officinale* were acquired from family farming in Belterra, Pará, Brazil (02°38'11" S and 54°56'14" W GPS), in May and June 2022, and were cleaned for subsequent essential oil extraction. This research was registered in the SisGen under number A965D42.

The essential oils from both samples were obtained by hydrodistillation using a Clevenger-type apparatus for 120 minutes. The rhizomes were first cut and ground in a domestic blender. The obtained essential oils were subjected to centrifugation with anhydrous sodium sulfate to remove water, and the yield was calculated based on the weight of the material used (Santos et al., 2004). The oil samples were placed in amber glass bottles, hermetically sealed, and stored at 5 ºC for subsequent analysis of volatile constituents and biological assays.

Essential oils Analysis

The essential oils were analyzed by gas chromatography system coupled to a mass spectrometer (GCMS-QP2010 Ultra, Shimadzu Corporation, Tokyo, Japan), equipped with an auto injector (AOC-20i) and CGMSsolution software, which contains spectra libraries (Adams, 2007), including FFNSC 2 (Mondello, 2011). A fused silica capillary column (Rxi-

5ms, Restek Corporation, Bellefonte, PA, USA) of 30 m x 0.25 mm (diameter) x 0.25 µm (film thickness), coated with 5% diphenyl dimethylpolysiloxane was used as stationary phase. The analysis conditions were as follows: helium drag gas (99.995%); split ratio mode at 1:20; injection of 1 µL of the sample (3 µL of the essential oil in 500 µL of hexane); ionization energy by electronic impact (EI) 70 eV; injector temperature: 250 ºC; oven temperature program: 60-240 ºC; ion source temperature: 200 ºC; transfer line temperature: 250 ºC.

Quantitative data on the volatile constituents were obtained via peak area normalization using a gas chromatograph (GC 6890 Plus series, Agilent) coupled to a flame ionization detector (FID), which was operated under similar conditions to the GC-MS system. The mass spectra were obtained by automatic scanning at 0.3 scans/second, with mass fragments of 35-400 *m/z*. The compounds found in the ion chromatograms were identified by comparing the mass spectra (molecular mass and fragmentation pattern) with those found in the system's CGMSsolution library and by comparison with the retention indexes. The linear equation of Van den Dool and Kratz (1963), was used to calculate the volatile components, with the use of a standard homologous series of C8-C20 n-alkanes (Sigma-Aldrich).

Determination of the cholinesterase inhibition

For the *in vitro* anticholinesterase assays, the enzyme acetylcholinesterase type VI-S, obtained from *Electrophorus electricus* (lyophilized powder, C3389-2Ku, Sigma-Aldrich, batch: SLBZ8573) was used. The eserine (physostigmine) diluted in methanol was used as standard anticholinesterase inhibitor (Sigma-Aldrich, batch: BCBC4171V). A standard curve was used to define the concentration used in the tests.

Qualitative test on a thin layer chromatography (TLC)

For the qualitative testing of *C. Longa* and *Z. officinale* essential oils and the standard drug, an aluminum chromatoplate for TLC (ALUGRAM® Xtra SIL G, silica gel 60, 0.20 mm, Macherey-Nagel) was used based on the direct bioautography method of Marston et al. (2002). The enzyme was diluted in tris-HCl buffer, 50 mM, pH 7.8 in ultra-pure water to obtain a concentration of 4 U/mL, with the addition of bovine serum albumin (Sigma-Aldrich) at a ratio of 1:1. Essential oils samples were diluted in methanol at a concentration of 100 µL/mL. Physostigmine at a concentration of 100 µg/mL was used as the positive control. For the negative control, methanol was used. The colorimetric reagents of the test were naphthyl acetate (2.5 mg in methanol) and Fast Blue B salt (2.5 mg in ultrapure water). Both reagents were prepared and mixed immediately before use.

To perform the test, the aliquots of 10 µL of the oil samples and controls were applied on chromatoplates in duplicate and allowed to stand for a period of 24 h for evaporation of the solvent. Subsequently, the plate was sprayed with the acetylcholinesterase enzyme solution (4 U/mL) and incubated in a humidity test chamber, without direct contact with moisture, at 37 °C for 20 minutes. Then, the plate was sprayed with the mixture of naphthyl acetate (2.5 mg) and Fast Blue B salt (2.5 mg) solutions to obtain the results. The formation of a purple coloration occurred gradually, after 1 to 3 min.

Quantitative assay

The assay for the quantification of acetylcholinesterase inhibition of the samples was adapted from Ellman's method, with modifications, as described by Albano and colleagues, 2012. In summary, three buffers were produced for the quantitative test, which were denominated A, B and C. These being: buffer A = 50 mM Tris/HCl, pH 8, dissolved in ultrapure water; buffer B = 0.1% bovine serum albumin in buffer A; and buffer C = 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$ dissolved in buffer A.

In a total volume of 1 mL, 415 µL of buffer A, 10 µL of the essential oil solution (diluted in methanol, buffer and Tween 80) at different concentrations (100, 50, 25, 12.5, 6.25 and 3.12 ug/mL), and 75 µL of acetylcholinesterase enzyme, containing 0.2 U/mL, were added. The samples were then incubated for 15 min at 25 °C. After incubation, 75 µL of a solution of 1.83 mM AChI (acetylthiocholine iodide) (Sigma-Aldrich, Steinheim, Germany) and 425 µL of 3 mM DTNB (5,5'-dithiobis[2-nitrobenzoic acid]) (Sigma-Aldrich, Steinheim, Germany) were added and the mixture was incubated for 30 min at 25 °C under a light source. The absorbance of the mixture was measured at 412 nm in a UV spectrophotometer (NOVA, 3300). Physostigmine was used as the standard drug and a dilution solution was used as negative control (buffer A, methanol and Tween 80 at a ratio of 2:2:1). The percentage of inhibition of enzyme activity was calculated according to the equation % = [(A0 – A1) / A0] * 100, where A0 was the absorbance of the control without the essential oil and A1 was the absorbance of the essential oil sample at different concentrations. All tests were performed in triplicate. The concentration of the sample that provided 50% inhibition (IC_{50}) was obtained by constructing graphs of the percentages of inhibition versus the concentration of the inhibitor. The non-linear regression parameters for the curve were plotted and the IC_{50} values were obtained using the Microsoft Excel 2019 software.

Evaluation of the possible synergistic effect of the essential oil mixture

To assess the possible synergistic effect between the chemical constituents of the essential oil samples on the enzyme acetylcholinesterase, a mixture was prepared in a 1:1 ratio (100 µL of *C. longa* oil + 100 µL of *Z. officinale* oil). The IC_{50} was then determined under the same conditions mentioned earlier. The chemical composition of the mixture was also evaluated to confirm the presence of the major constituents after mixing.

Molecular modelling and ADME properties

Preparation of the ligands

The chemical structures of the major compounds in the essential oil mixture of *Z. officinale* and *C. longa* were obtained in ".mol" format from the National Institute of Standards and Technology (NIST) database (available at https://www.nist.gov/). Subsequently, using ChemSketch software, the molecules had their 3D structures optimized by molecular mechanics methods.

Preparation of the crystallographic structure of hAChE (PDB: 4EY7)

To perform the molecular docking simulation, the crystallographic structure of human acetylcholinesterase (hAChE) was obtained from the Protein Data Bank (PDB) (//www.rcsb.org/) under ID code 4EY7. Subsequently, BIOVIA Discovery Studio® v. 20.1.0 was used to process the crystallographic complex. Thus, the co-crystallized ligand, enzymatic cofactors, and water molecules were removed, and the xyz coordinates of the donepezil binding site were determined.

Re-docking

The approved drug, donepezil, used here as a positive control, was docked to the protein binding site (PDB: 4EY7) via the DockThor web server (https://www.dockthor.lncc.br/v2/). Molecular docking was performed using a genetic algorithm with multiple solutions and the Merck Molecular Force Field 94 (MMFF94) as the scoring function. All of donepezil's rotatable bonds were considered at this stage, and the pre-selected precision settings of the algorithm, as well as the grid box size (20x20x20), were maintained.

The RMSD (Root Mean Square Deviation) value was calculated using Discovery Studio software. Thus, the molecular docking parameterization was established based on an RMSD value below 2.0 Å, indicating that the docking protocol can be used for docking other ligands.

ADME properties

The selected compounds were subjected to pharmacokinetic analysis using the online prediction tool Swiss ADME (available at //www.swissadme.ch/). This program provides physicochemical properties and ADME (absorption, distribution, metabolism, and excretion) parameters associated with the pharmacokinetics of each compound. Calculated data included lipophilicity values (LogPo/w) and blood-brain barrier (BBB) permeability. Additional properties such as plasma protein binding (PPB), permeability in MDCK and CACO-2 cells, and human gastrointestinal absorption (HIA) were assessed using the PreADMET server (https://preadmet.webservice.bmdrc.org/adme/).

Statistical analysis

Data from the quantitative anticholinesterase assay were analyzed using the Prism 5 software with one-way ANOVA, followed by the Tukey test with multiple comparisons, at a significance level of $p<0.05$. The GraphPad Prism 8.0.1 software was used for the statistical evaluation of binding affinity values through a one-Way ANOVA, which was followed by Sidak's multiple comparison test to evaluate and infer differences to the chosen pivot molecule.

Results and Discussion

Volatile Constituents of Essential Oils

The volatile constituents *C. longa* and *Z. officinale* rhizomes essential oils are presented in Table 1. The major constituents of *C. longa* oil were the sesquiterpenes ar-Turmerone (50.40%) and (*E*)-γ-Atlantone (17.00%). Additionally, the presence of the monoterpene α-Phellandrene (6.60%).

For *Z. officinale* oil, there is a greater predominance of monoterpenes, notably Camphene (10.10%) and Citral (isomers Neral and Geranial at 6.20% and 11.30%, respectively), which together account for 27.60%, although its major component is the sesquiterpene α-Zingiberene (20.00%). When essential oil samples of *C. longa* and *Z. officinale* were mixed in a 1:1 ratio (pool), the major components of both oils were retained (see Table 1).

The essential oils consist of lipophilic and low molecular weight compounds that can easily cross the blood-brain barrier, making them good candidates for neuroscience research. Historically, their value as sources of bioactive aromatic molecules and mixtures has been proven to be explored for industrial use as bioproducts in the pharmaceutical or food

industries. Despite the proven history of essential oils, chemical variability is the major obstacle to their use.

Table 1. Volatile Constituents and Yield of the Essential Oils from *C. longa* and *Z. officinale* and the Mixture of Both Samples (Pool).

Oil Samples			*Zingiber officinale*	*Curcuma longa*	Pool
Yield (%)			0,5	0,8	-
Constituents	**IR_{calc}**	**IR_{lit}**		**%**	
α-Pinene	932	932	2.70	-	1.50
Camphene	947	946	10.10	-	5.42
Myrcene	989	988	1.40	-	0.82
α-Phellandrene	1005	1002	-	6.60	1.87
p-Cymene	1023	1020	-	0.90	
β-Phellandrene	1029	1025	4.40	-	2.55
1,8-Cineole	1029	1026	8.80	2.40	5.75
Terpinolene	1087	1086	-	1.80	0.76
Linalool	1099	1095	0.90	-	0.45
Borneol	1165	1165	2.80	-	1.56
α-Terpineol	1190	1186	1.40	-	0.79
Neral	1242	1235	6.20	-	3.16
Geranial	1273	1264	11.30	-	5.81
γ-Muurolene	1476	1478	1.10		
ar-Curcumene	1483	1479	1.60	1.90	2.18
α-Zingiberene	1498	1496	20.00	2.20	10.06
(*E,E*)-α-Farnesene	1510	1505	7.30	-	3.74
β-Sesquiphellandrene	1526	1521	7.10	2.40	4.31
ar-Tumerol	1581	1582	-	1.27	0.63
ar-Turmerone	1675	1668	-	50.40	26.47
(*E*)-γ-Atlantone	1706	1706	-	17.00	9.43
Monoterpene hydrocarbons			27.50	9.30	12.92
Oxygenated monoterpenes			23.40	2.40	8.55
Sesquiterpene hydrocarbons			37.10	7.50	20.89
Oxygenated sesquiterpenes			-	68.70	44.84
Total (%)			**88.00**	**88.50**	**87.20**

RI_{calc} = Calculated retention time; RI_{lit} = Retention time claimed in the literature.
Pool = Mixture of the two oil samples: *C. longa* + *Z. officinale*.

As observed (Table 1), the major constituents of the essential oil obtained from *Curcuma longa* rhizomes are predominantly sesquiterpenes ar-Turmerone (27%) and (*E*)-γ-Atlantone (17%), corroborating other studies that describe this same characteristic (Zhang e Kitts, 2021; Lima et al., 2020; Hong et al., 2014). In contrast, the oil of *Z. officinale* shows a higher concentration of monoterpenes. Previous studies on these species reinforce this trend, with *Z. officinale* having a higher amount of monoterpenes, and *C. longa* higher

concentrations of sesquiterpenes (Akinyemi et al., 2018; Akinyemi e Adeniyi, 2018). However, when comparing the percentage of the major constituents, there is variability dependent on different factors, including cultivation type, plant age, and methods of extraction and characterization of the essential oil.

In the results obtained by Gerrini et al. (2023) with essential oil of *C. longa* collected in Macas, Morona-Santiago Province, Ecuador, sesquiterpenes were also the most abundant compounds, characterized mainly by oxygenated sesquiterpenes (66.29%), with ar-turmerone (23.35%), α- and β-turmerone (22.81% and 15.27%, respectively) being the most representative.

For the *Z. officinale* oil in this study, a higher predominance of monoterpenes is noted, with Camphene (10.1%) and Citral (isomers Neral and Geranial 6.2% and 11.3%, respectively) being prominent, totaling 27.6%. However, its major component is the sesquiterpene α-Zingiberene (20%). A study conducted by Wira Septama, (2023), with *Z. officinale* essential oil from Indonesia also identified α-Zingiberene as a major constituent, along with monoterpenes Citral and β-Sesquiphellandrene. When the essential oil samples of *C. longa* and *Z. officinale* were mixed in a 1:1 ratio (pool), the major components of both oils were maintained (Table 1).

Qualitative Assay of Essential Oil Samples Against AChE Enzyme

Figure 1 shows the inhibition halos of the essential oil samples of *C. longa* and *Z. officinale* against the acetylcholinesterase enzyme using the direct bioautography assay. All samples and standard physostigmine, exhibited inhibition halos on the chromatoplates, indicating the presence of chemical compounds with anticholinesterase activity in the essential oils of both species.

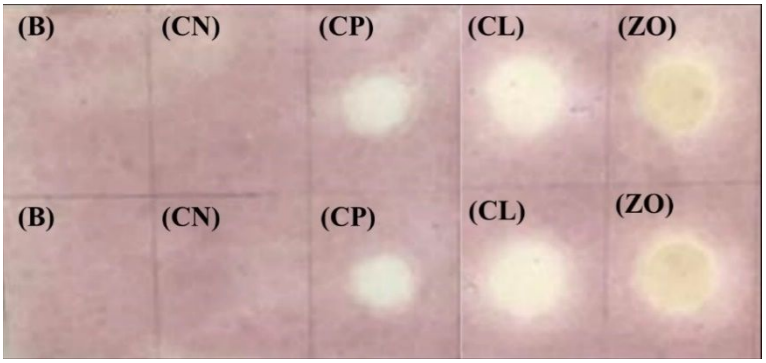


Figure 1. Direct bioautography on silica gel chromatoplates (TLC) of the anticholinesterase activity of essential oil samples from *C. longa* and *Z. officinale* and the physostigmine standard. CL = *Curcuma longa*; ZO = *Zingiber officinale*; Controls: (B) blank, (CN) negative control: methanol, and (CP) positive control: physostigmine (100 µg/mL).

Quantitative Assay of Essential Oil Samples Against AChE Enzyme

According to Figure 2, it is evident that all essential oil samples tested individually and in combination (pool) exhibited inhibitory activity against acetylcholinesterase, with IC_{50} values ranging from 0.33 to 0.64 µg/mL. The *C. longa* essential oil showed an IC_{50} of 0.38 ± 0.002 µg/mL, indicating strong inhibition of the acetylcholinesterase enzyme. On the other hand, *Z. officinale* essential oil was more active, with an IC_{50} of 0.33 ± 0.018 µg/mL, showing a significant difference compared to *C. longa* ($p \leq 0.05$), suggesting that the chemical composition of both oils influences their inhibitory activity against the AChE enzyme. The IC_{50} for the mixture of essential oils was 0.64 µg/mL.

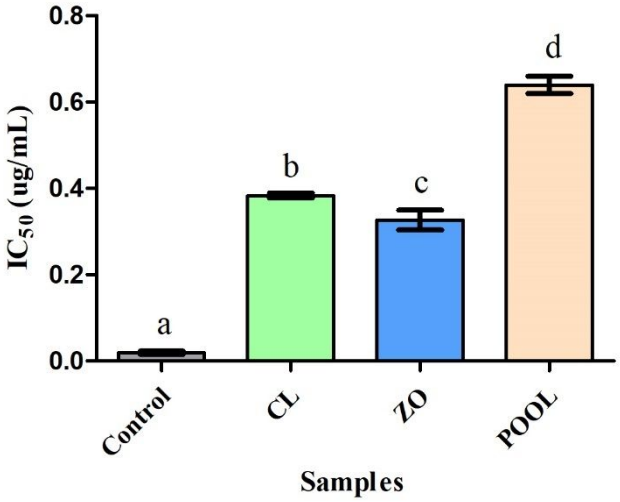


Figura 2 – Anticholinesterase Activity (IC_{50}) of Essential Oil Samples from *C. longa*, *Z. officinale*, and the Respective Mixture (Pool). CL = *Curcuma longa*; ZO = *Zingiber officinale*; Pool = Mixture of the two oil samples*: C. longa* + *Z. officinale*.

Both oils tested significantly inhibited ($p \leq 0.05$) AChE activity. *Z. officinale* (IC_{50} 0.33 ± 0.018 µg/mL) oil was more active than *C. longa* (IC_{50} 0.38 ± 0.002 µg/mL). Regarding the combination of essential oils in a 1:1 ratio (pool), a reduction in AChE inhibition was observed (0.64 ± 0.021 µg/mL). Essential oil combinations can synergistically or antagonistically affect a given biological activity (Karakaya et al., 2020). Therefore, it is essential to evaluate the oils separately and in combination.

Some studies indicate that the combined effect of essential oils plays a critical role in the effectiveness of their biological activities, sometimes enhancing or reducing the biological activity. For example, the binary combination of essential oils from *Lavandula dentata*, *Origanum majorana*, and *Thymus serpyllum* enhanced antibacterial activity (Ouedrhiri et al., 2016), and the combination of essential oils from *Thymus vulgaris* and *Lippia berlandieri* also enhanced antimicrobial activity (Silva et al., 2016). On the other hand, the combination of

essential oils from *Croton argyrophyllus* and *Croton tetradenius* reduced insecticidal activity (Cruz et al., 2020), and the combination of essential oils from *Callistemon lanceolatus*, *Ocimum gratissimum*, *Cymbopogon winterianus*, *Cymbopogon flexuosus*, *Mentha longifolia*, and *Vitex negundo* reduced antioxidant and antimicrobial activities (Sharma et al., 2020).

In this study, the essential oils were mixed in equal proportions, and a reduction in anticholinesterase activity was observed compared to the individual oils. However, in the 1:1:1 mixture of essential oils from three chemotypes of *Lippia alba*, the addition of the carvone chemotype enhanced AChE inhibition (Silva Junior et al., 2024). This demonstrates the need for caution regarding essential oil mixtures. Their effects can be influenced positively or negatively depending on their chemical variability, botanical species, and types of combinations.

When essential oils are mixed (pool) or combined, it is important to analyze the constituents individually and in combination. It is observed that, in most cases, the combination of essential oils reduces biological activity compared to the effect of the individual oil.

Molecular docking

The energy calculation for all docking complexes was evaluated using the MMFF94 force field as the scoring function. The binding energy values of the major constituents of the essential oils ranged from -9.448 kcal/mol to -7.960 kcal/mol. For α-Zingiberene (ZO) present in the *Z. officinale* oil, the energy value was closest to that of donepezil, corresponding to -11.237 kcal/mol (Table 2).

Table 2: Binding Energy Values of the Major Constituents of *Zingiber officinale*, *Curcuma longa*, and Pool.

Molecule	**Binding Affinity (kcal/mol)***
Donepezil	-11.237
α-Phellandrene (CL)	-8.214
α-Zingiberene (ZO)	-9.448
ar-Turmerone (CL)	-9.221
β-Sesquiphellandrene (ZO)	-9.293
Camphene (ZO)	-8.072
1,8-Cineole (CL E POOL)	-7.960
(*E,E*)-α-Farnesene (ZO)	-9.297
(*E*)-γ-Atlantone (CL)	-9.149
Geranial (ZO)	-8.363
Neral (ZO)	-8.218

*Mean (n=8) CL = *Curcuma longa*; ZO = *Zenziber officinales*; *Donepezil* = Standard drug.

It is noted that all compounds exhibited statistically different binding affinities compared to donepezil. However, as observed in the 2D interaction diagrams (Figure 4 to Figure 8), all show interactions with key amino acids that are also present in the interaction of donepezil with the enzyme's active site, indicating that they may share common binding sites with one of the principal and well-known acetylcholinesterase inhibitors (Peitzika e Pontiki, 2023). Molecular docking experiments are one approach to studying the binding conformation of ligands within the active site of target proteins, and they serve as a crucial tool in Computer-Aided Drug Design.

In this study, the human AChE enzyme co-crystallized with donepezil (PDB ID: 4EY7) was used as a virtual target to rank the major constituents of the essential oil samples from *C. longa* and *Z. officinale* according to their binding affinities. This crystal was chosen because donepezil is commonly known as an acetylcholinesterase inhibitor and is used as a standard medication in the treatment of Alzheimer's disease. According to the statistical analyses of binding affinity values obtained from donepezil, compared to the major compounds of the essential oil from the rhizomes of *C. longa* and *Z. officinale*, significant differences were observed in the molecular docking of donepezil (Figure 3). However, according to the interaction diagrams, the compounds displayed common interaction sites with donepezil.

Analysis of the binding sites of major compounds with the AChE enzyme

The initial procedure involved docking, which was validated through the accurate re-docking of the co-crystallized donepezil into the *h*AChE model to better compare the docking results. Donepezil was docked to AChE using the same parameters and was found to have a binding affinity of -11.237 kcal/mol and RMSD = 0.334 Å. Figure 3 shows the binding pocket and target residues involved in the binding interaction of donepezil.

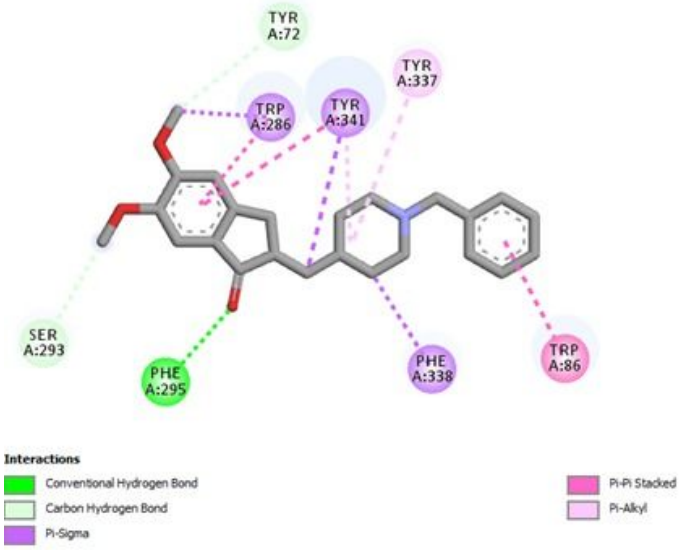


Figure 3: 2D diagram illustrating the interactions between donepezil and the acetylcholinesterase (AChE) enzyme (4EY7).

The major compounds (Table 1) from each oil, docked with AChE, including α-Phellandrene, α-Zingiberene, ar-Turmerone, β-Sesquiphellandrene, Camphene, 1,8-Cineole, (*E,E*)-α-Farnesene, (*E*)-γ-Atlantone, Geranial, and Neral, were examined to interpret the binding conformation patterns within the active site of AChE (4EY7), in comparison to the donepezil pattern.

In the binding pocket with the specific residues involved in donepezil interaction, eight residues were observed as counterparts in the binding site: Trp286, Tyr72, Tyr341, Phe338, Tyr337, Ser293, Trp86, and Phe295. It was noted that five different types of interactions exist among the various chemical bonds. A π-alkyl interaction is observed at Tyr337, a hydrogen bond at Phe295, a conjugated interaction between aromatic rings Trp86, three π-sigma bonds Trp286, Tyr341, Phe338, and two carbon-hydrogen bonds Ser293 and Tyr72 (Figure 3).

Thus, natural compounds that show potential interaction with these residues can be considered promising AChE inhibitors. Dai et al. (2022), highlight how traditional Chinese medicine through various natural compounds has contributed to AChE inhibition. A total of sixty-six compounds with BACE1 and AChE inhibitory activity were analyzed, identifying nine terpenoid compounds, three alkaloids, fourteen coumarin compounds, twenty-seven flavonoids, twelve polyphenolic compounds, and one peptide compound.

Figure 4 shows the interaction diagram with amino acid residues of the compounds α-Phellandrene (A) and α-Zingiberene (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

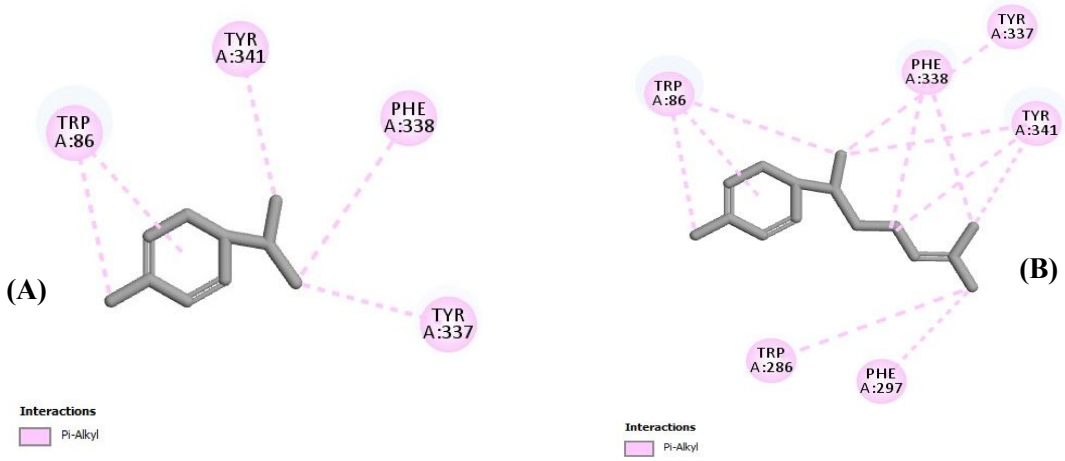


Figure 4: 2D diagram of the interactions between α-Phellandrene (A) and α-Zingiberene (B) with amino acid residues of hAChE (4EY7).

For the monoterpene α-Phellandrene, interaction with four amino acid residues was observed, which are also noted for donepezil: Tyr341, Phe338, Tyr337, and Trp86. In the case of the sesquiterpene α-Zingiberene, interactions occurred with Trp286, Tyr341, Phe338, Tyr337, Phe297, and Trp86 (Figure 4). Both molecules bind through π-alkyl interactions. It is

observed that the α-Phellandrene molecule interacts with amino acid residues Phe338, Tyr337, and Trp86 with the anionic subsite (Dvir et al., 2010; Johnson and Moore, 2006). In contrast, for α-Zingiberene, Trp286 is one of the residues in the peripheral anionic site (Wang et al., 2017; El-Sayed et al., 2019; Kuzu et al., 2019).

Dvir et al. (2010) and Johnson, Moore (2006), also mention Tyr86, Tyr337, and Phe338 as the anionic subsite, responsible for the catalytic machinery and binding to choline. Two phenylalanine amino acid residues (Phe295 and Phe297) create an additional obstacle in the AChE structure. They form a pocket responsible for substrate selectivity, preventing access to larger members of the choline ester series, with one of these amino acids being Phe297 in this molecule.

Figure 5 shows the interaction diagram with amino acid residues of the compounds ar-Turmerone (A) and β-Sesquiphellandrene (B) with the acetylcholinesterase (AChE) enzyme (4EY7). For the compound ar-Turmerone, π-alkyl interactions were observed binding to the sites Phe297, Tyr124, Trp286, Phe338, Tyr337, Trp86, and Tyr341, with a π-sigma bond at Trp86 remaining the same as in donepezil. This molecule shows the presence of the peripheral anionic site of AChE Trp286 (Wang et al., 2017; El-Sayed et al., 2019; Kuzu et al., 2019), the anionic subsites Tyr337, Phe338, and Trp86, and one of the amino acid residues Phe297 (Dvir et al., 2010; Johnson and Moore, 2006). β-Sesquiphellandrene binds to the sites Trp86, Tyr337, Tyr341, Phe338, and Trp286 through π-alkyl interactions (Figure 5).

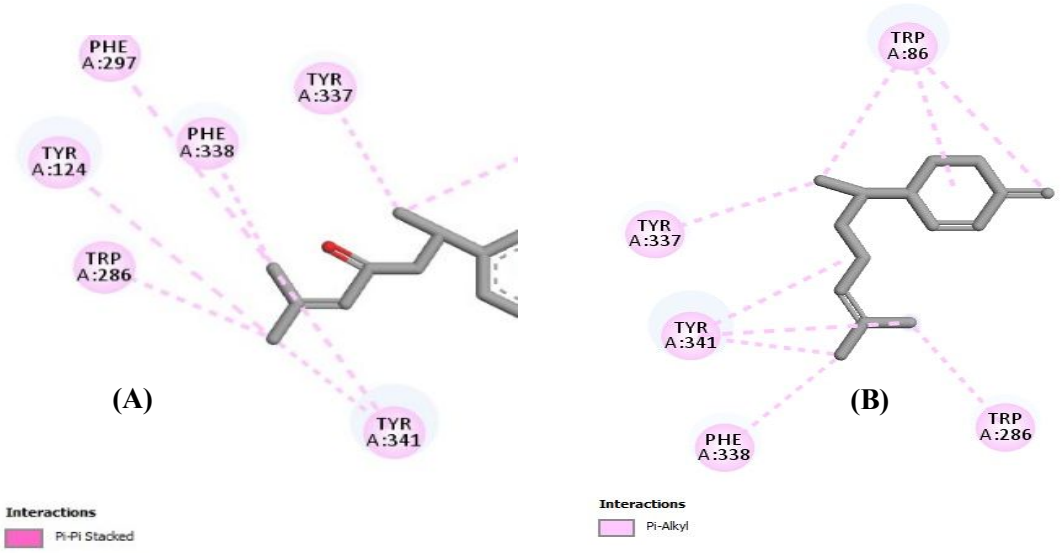


Figure 5: 2D diagram illustrating the interactions between ar-Turmerone (A) and β-Sesquiphellandrene (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

Figure 6 shows the interaction diagram with amino acid residues of the compounds Camphene (A) and 1,8-Cineole (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

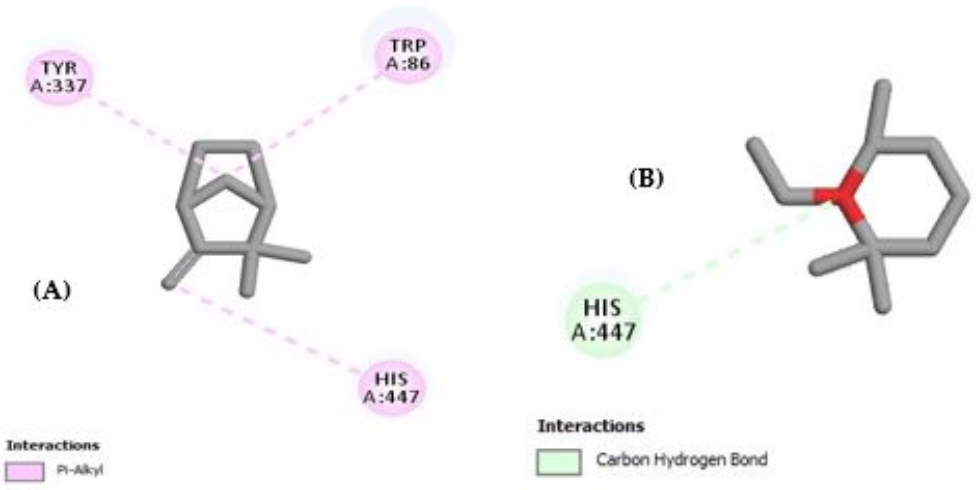


Figure 6: 2D diagram illustrating the interactions between Camphene (A) and 1,8-Cineole (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

For the monoterpene Camphene, a π-alkyl interaction is observed for the Tyr337, Trp86, and His447 sites. While for 1,8-Cineole, it binds to His447 through a carbon-hydrogen bond (Figure 6). The His447 site obtained in both molecules is one of those that form the catalytic triad of AChE (Riaz et al., 2020). In this compound, the presence of two more sites, Tyr337 and Trp86, which are anionic subsites of the enzyme, is also observed (Dvir et al., 2010; Johnson and Moore, 2006). Figure 7 shows the interaction diagram with amino acid residues of the compounds (*E,E*)-α-Farnesene (A) and (*E*)-γ-Atlantone (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

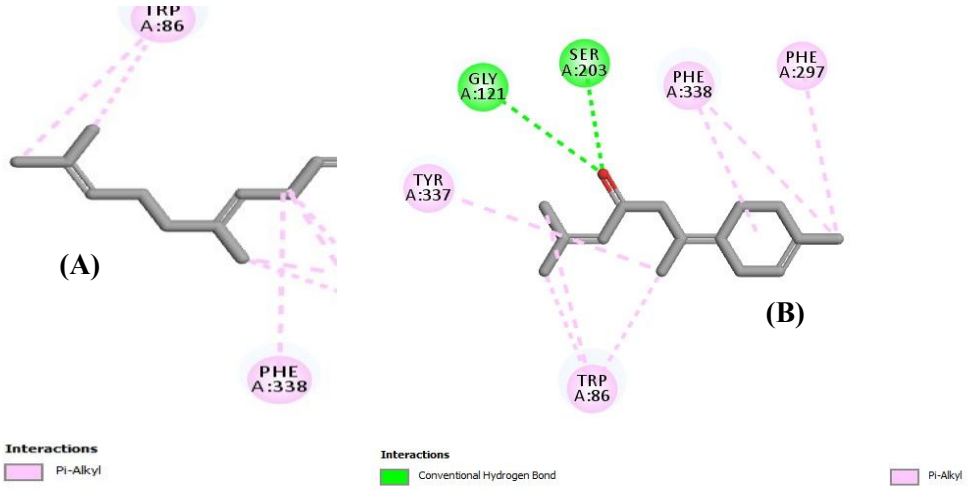


Figure 7: 2D diagram illustrating the interactions between the compounds (*E,E*)-α-Farnesene (A) and (*E*)-γ-Atlantone (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

The compound (*E,E*)-α-Farnesene interacts through π-alkyl bonding with Trp86, Trp286, Tyr337, Tyr341, Phe338, and Phe297. The bonds Trp86, Tyr337, and Phe338 are part of the anionic subsite of acetylcholinesterase. The compound (E)-γ-Atlantone interacts

with two hydrogen bonds of AChE at Gly121 and Ser203, while the other bonds are π-alkyl, involving Tyr337, Phe338, Trp86, and Phe297 (Figure 7). The presence of Ser203, a component of the catalytic triad, is observed, along with bonds at Trp86, Tyr337, and Phe338, which make up the anionic subsite.

It is also noted that Gly121 is one of the components of the oxyanion region, providing hydrogen bond donors that stabilize the tetrahedral transition state of the substrate. Therefore, this can be considered one of the molecules with the best potential for AChE inhibition. As Wojtunik-Kulesza et al. (2017), mention, one of the most important factors in molecular docking results is the interactions with amino acid residues forming anionic and esteratic subsites.

Figure 8 shows the interaction diagram with amino acid residues of the compounds Geranial (A) and Neral (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

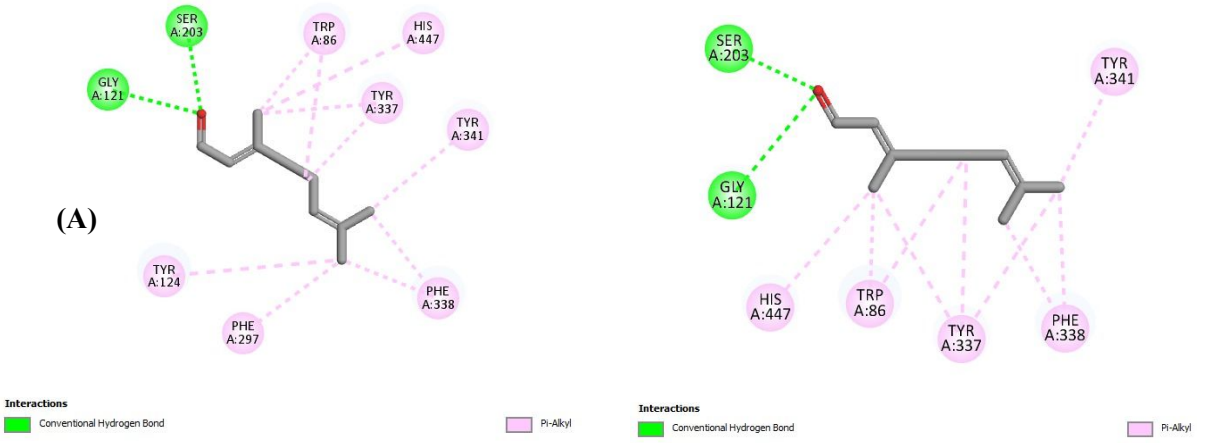


Figure 8: 2D diagram illustrating the interactions between Neral (A) and Geranial (B) with the acetylcholinesterase (AChE) enzyme (4EY7).

In Figure 8, the compound Geranial makes two carbon-hydrogen interactions (Trp86 and Gly121), two π-alkyl interactions (His447 and Phe338), two π-sigma interactions (Tyr337 and Tyr341), and one interaction with hydrogen (Ser125). It is observed that one of the bonds is part of the catalytic triad His447, and additional bonds are forming the anionic subsites Trp86, Phe338, and Tyr337, as well as the bonds Ser125 and Tyr341 which are part of the peripheral anionic site (Wang et al., 2017; El-Sayed et al., 2019; Kuzu et al., 2019).

For Neral, interactions are observed with hydrogen at Ser203 and Gly121, a carbon-hydrogen bond with His447, and π-alkyl bonds at Tyr124, Phe338, and Phe297 in AChE. The His447 bond observed in the molecule is part of the AChE catalytic triad. At the same time, Tyr124 is part of the peripheral anionic site, Phe338 is part of the anionic subsite, and Phe297 is one of the phenylalanine amino acid residues (Dvir et al., 2010; Johnson and Moore, 2006).

In this context, considering the bonds found between the sites in the above molecules, it is important to recognize that the active site of AChE is a deep cleft of 20 Å, with three amino acids (Ser203, Gly334, and His447) forming the catalytic triad. This creates a static site where acetylcholine is hydrolyzed into acetate and choline (Riaz et al., 2020).

The anionic subsites comprise bonds involving Trp86, Tyr133, Tyr337, and Phe338, responsible for the catalytic machinery and binding to choline. Additionally, an extra obstacle in the AChE structure is created by two phenylalanine residues (Phe295 and Phe297), forming an acyl pocket responsible for substrate selectivity, preventing access to larger members of the choline ester series. The enzyme also has an oxyanion hole created by Gly121, Gly122, and Ala204, which provides hydrogen bond donors that stabilize the tetrahedral transition state of the (Wang et al., 2017; El-Sayed et al., 2019; Kuzu et al., 2019).

It is important to highlight that although the major compounds used in the docking study did not show significant differences in binding with AChE, the quantitative test demonstrated that the essential oil of *Z. officinale* exhibited a better percentage of inhibition, with a significantly lower IC_{50} compared to *C. longa*. This difference can partly be explained by the fact that the oil from *Z. officinale* contains compounds with higher binding affinities to the AChE enzyme (α-Zingiberene, β-Sesquiphellandrene, and (E,E)-α-Farnesene).

The mixture of oils showed a significantly higher IC_{50} compared to the individual oils. This increased inhibition may be attributed to antagonistic effects or a reduction in the percentage of major chemical compounds in the mixture.

ADME Properties

The major constituents were subjected to pharmacokinetic analysis using the online prediction tools Swiss ADME and the PreADMET server. The results are presented in Table 3.

Table 3: *In silico* pharmacokinetic prediction of the major constituents of *Zingiber officinale* and *Curcuma longa*.

[a]

Molecule	Absorption				"Distribution"	
	LogP[a]	P_{Caco-2}[b]	HIA (%)[b]	P_{MDCK}[b]	LPP (%)[b]	BHE[a]
α-Zingiberene	4,47	23,40	100	61,24	100	15,19
Geranial	3,07	52,00	100	51,83	100	2,19
Neral	3,05	52,00	100	51,83	100	2,19
(*E,E*)-α-Farnesene	4,96	23,40	100	66,48	100	21,31
1,8-Cineole	2,67	21,89	100	233,71	100	1,46
β-Sesquiphellandrene	4,56	23,40	100	61,85	100	20,19
Camphene	3,43	23,49	100	300,51	100	5,75
ar-Turmerone	3,84	55,50	100	144,71	100	2,29
(*E*)-γ-Atlantone	4,04	55,65	100	135,92	100	5,25
α-Phellandrene	2,97	23,41	100	267,70	100	7,17

Analysis conducted on the SwissADME web server (http://www.swissadme.ch/index.php#); [b] Analysis conducted on the PreADMET web server (https://preadmet.webservice.bmdrc.org/adme/).

Pharmacokinetic prediction of natural compounds is crucial in classifying molecules considering absorption, distribution, metabolism, and excretion (ADME), based on the physicochemical and structural properties of the compounds under study. The lipophilicity value (LogP) is closely related to the absorption of small molecules. The values for the studied compounds are below five, which is the ideal range for hydrophilic-lipophilic balance for oral solubility and absorption (Lipinski, 2004). Consequently, the predicted human gastrointestinal absorption (HIA) for all molecules was 100%, meaning all evaluated compounds are highly likely to be absorbed by the gastrointestinal tract upon oral administration.

The Caco-2 and MDCK (Madin-Darby canine kidney) cell models have been recommended as reliable in vitro models for predicting oral drug absorption. Therefore, in silico predictions based on in vitro Caco-2 and MDCK cell assays are relevant for absorption predictions, through PCaco-2 and PMDCK parameters. Values considered as high permeability are above 70 nm/s, and considering this cutoff, the compounds with the highest probability of permeation are camphene, ar-turmerone, (E)-γ-Atlantone, and α-Phellandrene. The other compounds are considered to have moderate permeability, with values between 4 and 70 nm/s (Yamashita et al., 2004; Irvine et al., 1999; Yee et al., 1997).

Generally, compounds with more than 90% plasma protein binding (PPB) are classified as strongly bound, while less than 90% are weakly bound. The calculated PPB value for the major constituents studied was 100.00%, indicating that they are strongly bound compounds,

and therefore, free forms will be less available in the systemic circulation (Wojtunik-Kulesza et al,. 2017).

The penetration of the blood-brain barrier (BBB) is represented as BBB=[brain]/[blood], where [brain] and [blood] are the steady-state concentrations of radiolabeled compounds in the brain and peripheral blood. Predicting BBB penetration means assessing whether the compounds cross the blood-brain barrier. This is crucial in the pharmaceutical sphere as active compounds in the CNS must permeate through the barrier and reach the target enzyme. According to Xiao-lei et al. (2005), values above 2.0 are suitable for high CNS absorption, and therefore, all evaluated compounds showed adequate properties for BBB permeation. Thus, the main compounds from *Zingiber officinale* and *Curcuma longa* exhibit excellent physicochemical properties, potentially making them promising molecules for developing oral medications for Alzheimer's disease.

Conclusion

When tested individually, the essential oil obtained from the rhizomes of *C. longa* and *Z. officinale* exhibits inhibitory action against the enzyme acetylcholinesterase. However, when these oils were mixed, the inhibitory effect was reduced. Combining essential oils can be a great way to enhance their therapeutic benefits and create unique aromas. However, not all essential oils are compatible, and some combinations may lead to undesirable interactions or reduce the therapeutic effect, as observed with the mixture of essential oils from *C. longa* and *Z. officinale*.

The experimental results were further validated by molecular docking studies, which demonstrated significant interactions between the major constituents of the essential oils and the amino acid residues of the AChE enzyme. In addition, the in silico pharmacokinetic studies played a crucial role in our research, revealing that the major compounds of *C. longa* and *Z. officinale* possess suitable characteristics for oral absorption. This information is vital for professionals in the field, as it indicates the potential use of these essential oils in future clinical studies as active principles against Alzheimer's disease.

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3.3. CAPÍTULO 3 - Anxiolytic, Antidepressant, and Anticholinesterase Effects of Essential Oil from *Myrcia sylvatica* (G.Mey.) DC

SILVA JÚNIOR, A.Q.da; GARCIA, M.M.; FARIAS, W.S.; SOUSA, D. J.A.; BARROSO, A.S.; FIGUEIREDO, P.L.B.; BIANCHI, G.S.; OLIVEIRA, R.B.; MOURÃO, R.H.V.

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Anxiolytic, Antidepressant, and Anticholinesterase Effects of Essential Oil from *Myrcia sylvatica* (G.Mey.) DC

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Abstract: Aromatic plants are rich sources of essential oils (EOs), recognized for their therapeutic properties due to their diversity of phytochemicals. This study investigated the anxiolytic and antidepressant effects of *Myrcia sylvatica* essential oil (MsEO) through inhalation in an animal model and its in vitro anticholinesterase (AChE) activity. The EO was obtained by hydrodistillation, and its volatile constituents were analyzed by GC-MS. Swiss mice were exposed to doses of 0.1%, 1%, and 2% of the EO via an inhalation apparatus. The anxiolytic activity was assessed using the elevated plus maze and light–dark box tests, while antidepressant activity was evaluated using the tail suspension and forced swimming tests. To examine potential side effects, the animals were subjected to rotarod, Y-maze, and Morris water maze tests to assess motor coordination, memory, and learning. Anticholinesterase activity was determined by direct bioautography and colorimetry based on the Ellman method. The results demonstrated that inhalation of MsEO at doses of 0.1% and 1% significantly reduced anxiety and depressive-like behaviors without impairing memory, learning, or motor coordination in the animals. Moreover, MsEO inhibited acetylcholinesterase with an IC_{50} of 0.47 µg/mL. These findings suggest that MsEO has potential therapeutic applications for anxiety and depression disorders, with additional anticholinesterase activity warranting further investigation in cognitive-related conditions.

Keywords: central nervous system; aromatherapy; anxiety; depression; Alzheimer's disease

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1. Introduction

Abstract

Aromatic plants are rich sources of essential oils (EOs), recognized for their therapeutic properties due to their diversity of phytochemicals. This study investigated the anxiolytic and antidepressant effects of *Myrcia sylvatica* essential oil (MsEO) through inhalation in an animal model and its in vitro anticholinesterase (AChE) activity. The EO was obtained by hydrodistillation, and its volatile constituents were analyzed by GC-MS. Swiss mice were exposed to doses of 0.1%, 1%, and 2% of the EO via an inhalation apparatus. The anxiolytic activity was assessed using the elevated plus maze and light–dark box tests, while antidepressant activity was evaluated using the tail suspension and forced swimming tests. To examine potential side effects, the animals were subjected to rotarod, Y-maze, and Morris water maze tests to assess motor coordination, memory, and learning. Anticholinesterase activity was determined by direct bioautography and colorimetry based on the Ellman method. The results demonstrated that inhalation of MsEO at doses of 0.1% and 1% significantly reduced anxiety and depressive-like behaviors without impairing memory, learning, or motor coordination in the animals. Moreover, MsEO inhibited acetylcholinesterase with an IC_{50} of 0.47 μg/mL. These findings suggest that MsEO has potential therapeutic applications for anxiety and depression disorders, with additional anticholinesterase activity warranting further investigation in cognitive-related conditions.

Keywords: central nervous system; aromatherapy; anxiety; depression; Alzheimer's disease

1. Introduction

Anxiety and depression are major global public health concerns, affecting approximately 300 million people worldwide. These disorders impair individuals' quality of life and burden healthcare systems substantially [1]. Conventional treatments often involve

psychotropic drugs, such as anxiolytics and antidepressants, which, despite their effectiveness, are frequently associated with adverse side effects [2]. Consequently, there is a growing interest in identifying alternative therapeutic agents derived from natural products.

In addition to being prevalent mental health disorders, anxiety and depression often coexist with neurodegenerative conditions, such as Alzheimer's disease (AD). Research indicates that up to 80% of individuals with AD experience symptoms of anxiety and depression, which may exacerbate cognitive decline and significantly impact quality of life [3,4]. Furthermore, the interplay between emotional and cognitive disorders is complex and bidirectional: anxiety and depression can precede or accelerate neurodegeneration. At the same time, the progression of conditions like AD may heighten emotional disturbances [5,6]. Therefore, addressing cognitive and emotional symptoms is critical in developing comprehensive therapeutic approaches.

Essential oils (EOs) from aromatic plants have gained attention for their psychoactive properties and potential therapeutic benefits in treating mental health disorders [7]. Specific constituents in EOs, such as terpenes, may exhibit anxiolytic, antidepressant, and anticholinesterase effects, supporting emotional and cognitive well-being [8,9]. Among these the essential oil of *Myrcia sylvatica* (G.Mey.) DC. (Myrtaceae), a native Amazonian plant, has shown promising therapeutic properties, including antioxidant and anti-inflammatory activities [10,11].

Myrcia sylvatica is particularly noteworthy due to the unique chemical composition of its essential oil, which lacks a substantial major component but instead comprises a diverse array of volatile compounds, including monoterpenes and sesquiterpenes. Previous studies have highlighted the biological activities of these constituents, including antimicrobial, anti-inflammatory, and neuroprotective effects [12,13]. This chemical complexity makes the oil an intriguing research subject, as its pharmacological effects are likely due to synergistic interactions among its components rather than the dominance of a single compound.

This study aimed to investigate the potential anxiolytic and antidepressant effects of *Myrcia sylvatica* essential oil (MsEO) when administered via inhalation in an animal model. Neurobehavioral tests, such as the elevated plus maze and forced swimming test, were used to assess these effects. Additionally, the in vitro anticholinesterase activity of MsEO was evaluated to explore its potential application in cognitive disorders, particularly those with comorbid symptoms of anxiety and depression. By correlating these findings, we aim to provide a clearer understanding of how MsEO may contribute to addressing the multifaceted nature of neurodegenerative and emotional disorders.

2. Materials and Methods

2.1. Plant Material and Essential Oil Extraction

Leaves of *Myrcia sylvatica* were collected in the Santa Rosa community (coordinates S 02° 30.464′ W 054° 50.931′), Everaldo Martins Road, Km 21, which connects the city of Santarém to the village of Alter do Chão, Pará, Brazil. A voucher was deposited in the herbarium of EMBRAPA/Eastern Amazon, Belém, Pará, Brazil, under the registration number IAN184696. The leaves were dried at 25 °C, ground, and subsequently used for essential oil extraction.

The MsEO was obtained by hydrodistillation in a modified Clevenger apparatus (Maia and Andrade, 2009) for 120 min. The EO was dehydrated with anhydrous sodium sulfate (Na_2SO_4) and stored in amber glass containers under refrigeration until use. The yield of the EO was calculated according to Santos et al. (2004) [14], based on the moisture-free biomass, using the ratio between the volume of oil obtained and the plant biomass used in the extraction.

2.2. Essential Oil Analysis

The essential oil was analyzed by a gas chromatography system coupled to a mass spectrometer (GCMS-QP2010 Ultra, Shimadzu Corporation, Tokyo, Japan), equipped with an auto injector (AOC-20i) and CGMSsolution software (Version 4.20), which contains spectra libraries [15] including FFNSC 2 [16]. A fused silica capillary column (Rxi-5ms, Restek Corporation, Bellefonte, PA, USA) of 30 m × 0.25 mm (diameter) × 0.25 µm (film thickness), coated with 5% diphenyl dimethylpolysiloxane was used as stationary phase. The analysis conditions were as follows: helium drag gas (99.995%); split ratio mode at 1:20; injection of 1 µL of the sample (3 µL of the essential oil in 500 µL of hexane); ionization energy by electronic impact (EI) 70 eV; injector temperature: 250 °C; oven temperature program: 60–240 °C; ion source temperature: 200 °C; transfer line temperature: 250 °C.

Quantitative data on the volatile constituents were obtained via peak area normalization using a gas chromatograph (GC 6890 Plus series, Agilent, Santa Clara, CA, USA) coupled to a flame ionization detector (FID), which was operated under similar conditions to the GC-MS system. The mass spectra were obtained by automatic scanning at 0.3 scans/second, with mass fragments of 35–400 *m*/*z*. The compounds found in the ion chromatograms were identified by comparing the mass spectra (molecular mass and fragmentation pattern) with those found in the system's CGMSsolution library and by

comparison with the retention indexes. The linear equation of Van den Dool and Kratz (1963) was used to calculate the volatile components, with the use of a standard homologous series of C8-C20 n-alkanes (Sigma-Aldrich, St. Louis, MO, USA).

2.3. Animals

For the animal tests, Swiss male *Mus musculus*, approximately 60 days old and weighing between 35 and 45 g (n = 160), were used. The animals were provided by the Laboratory of Bioprospecting and Experimental Biology at the Federal University of Western Pará (UFOPA). They were housed in polypropylene cages, maintained at room temperature (25 ± 2 °C), with a controlled light–dark cycle of 12 h, and had ad libitum access to water and food. The research project was approved by the Animal Ethics Committee (CEUA/UFOPA), under Protocol No. 10004-2017.

2.4. Drugs and Treatment

To evaluate the MsEO effects, three animal groups, referred to as EMS, were exposed to doses of 0.1%, 1%, and 2% via inhalation (i.h.) using an inhalation apparatus. To validate the employed pharmacological procedures, the positive control group (PC) was administered the conventional anxiolytic diazepam (Compaz, Brazil—5 mg/kg) or the antidepressant imipramine (Funed, Brazil—15 mg/kg), and the negative control group (NC) was administered mineral water orally (p.o.), 60 min prior to the behavioral tests. These groups were also exposed to the inhalation apparatus, where a hydroalcoholic solution (20%) was administered.

2.5. Inhalation Apparatus

The inhalation apparatus consists of an acrylic box (36 × 30 × 29 cm), with the anterior and posterior walls featuring four 2 cm diameter holes. Cotton swabs soaked with the solutions to be evaluated were placed at these locations. The removable lid of the box has a plate with 30 ventilation holes, each 0.4 cm in diameter, to allow airflow. Each cotton swab was loaded with 2 mL of the solution according to the treatment group, and the eight swabs containing the essential oil (OE) were replaced every 10 min to maintain a homogeneous vapor concentration. The animals were placed individually in the apparatus for 5 min [17]. After each exposure, the inhalation apparatus was cleaned with a 10% ethanol solution. Following the treatment, the animals were subjected to their respective behavioral tests.

2.6. Behavioral Tests

2.6.1. Evaluation of Anxiolytic Activity

Elevated Plus Maze

The apparatus consists of a platform with two open arms (30 × 5 cm) perpendicular to two closed arms (30 × 5 × 25 cm). The maze height from the floor is 45 cm, and the open arms have a border of 0.5 cm [18]. After the treatments, the animals were individually placed at the maze's center, facing one of the closed arms. They were then observed for 5 min, during which their behavior was recorded on video. The traditional parameters for this model were recorded: the number of entries and the time spent in the open arms. An entry into the arm was considered when the animal placed all four paws inside the arm [19,20]. The apparatus was cleaned with 10% ethanol solution after each exposure.

Light-Dark Box Test

The apparatus used was an EP 158 Insight® model, made of acrylic with dimensions of 46 × 27 × 30 cm, consisting of a larger light compartment and a smaller dark compartment, with a passageway between them. Mice were individually placed in the light chamber and allowed to explore the apparatus for 5 min, during which their behavior was filmed. The time spent in each compartment was recorded, with the time spent in the illuminated chamber used as a parameter for anxiety [21]. Between each session, the apparatus was cleaned with 10% ethanol solution.

2.6.2. Evaluation of Antidepressant Activity

Tail Suspension Test

The animals were suspended by the tail using adhesive tape (approximately 1 cm from the tail tip) at a height of 50 cm above the bench for 6 min, during which the session was recorded on video. The total immobility time for each animal was recorded in seconds. Mice were considered immobile when they hung passively and remained completely still [22].

Forced Swimming

A cylindrical container with a height of 21 cm, a diameter of 20 cm, and a water column of 13 cm at a temperature of 25 ± 2 °C was used. The session lasted 6 min, during which the animal's behavior was recorded on video. The behavior analyzed was the immobility time, which was assessed during the last 4 min of the recording, as the first 2 min showed little immobility and would have interfered with the evaluation of the actual effect of the treatments [23].

2.6.3. Evaluation of Memory and Learning

Y-Maze Test

The apparatus consists of a wooden Y-shaped device with arms measuring 10 cm in width, 34 cm in height, and 43 cm in length, with an angle of 120° between the arms. The animals were placed individually in the maze with an accessible exploration time of 7 min and recorded on video. The sequence of entries into the arms of the maze, labeled as A, B, and C, was recorded during the last 5 min. The data were analyzed to determine the number of entries into the 3 arms without repetition (ABC, CAB, BCA, CBA, or ACB). Each mouse's alternation percentage was defined as the ratio between the actual number of alternations and the possible number (total number of arm entries minus two) multiplied by 100 [24]. Between tests, the entire maze was cleaned with 10% ethanol.

Morris Water Maze Test

A blue polyethylene circular container with a diameter of 80 cm and a height of 30 cm was used, previously filled with water at room temperature (25 ± 2 °C) before the training sessions. The container was divided into four imaginary quadrants, marked by four points: North (N), South (S), East (L), and West (O), which served as starting points for the animals during the test. A 10 cm × 20 cm submerged platform was hidden at a fixed location, one centimeter below the water surface. Non-toxic white paint was added to the water to make the surface opaque and prevent the platform from being visible.

A training session was conducted with the animals to help them learn how to locate the submerged platform. Each animal was placed individually in the water tank, facing the edge of the tank, and allowed to swim freely while using the reference points to search for the platform for 2 min. Animals that did not find the platform within the predetermined time were manually placed under the platform for 10 s to visualize the environment. All animals underwent four training sessions, each corresponding to a different starting point (N, S, L, and O). In the test session, which was conducted 24 h after the training session, the time (in seconds) it took for each animal to find the platform from each starting point was recorded [25].

2.6.4. Assessment of Motor Coordination

Rotarod Test

The rotarod (EFF 411 Insight®) was used at a speed of 10 revolutions per minute. A training session was conducted to allow the animals to adapt to the apparatus, divided into

three series of two minutes each, during which the animals were placed back on the rod as many times as necessary within the stipulated time. After 24 h, the animals were subjected to the test, and each animal was placed again on the rotating rod for three series of two minutes. The time each animal remained on the rod was recorded in seconds, and the result was obtained by calculating the average time across the three series. No additional placement on the rod was made if the animal fell [26].

2.7. Determination of the Cholinesterase Inhibition

For the in vitro anticholinesterase assays, the enzyme acetylcholinesterase type VI-S, obtained from *Electrophorus electricus* (lyophilized powder, C3389-2Ku, Sigma-Aldrich, batch: SLBZ8573) was used. The eserine (physostigmine) diluted in methanol was used as a standard anticholinesterase inhibitor (Sigma-Aldrich, batch: BCBC4171V). A standard curve was used to define the concentration used in the tests.

2.7.1. Qualitative Test on Thin Layer Chromatography (TLC)

For the qualitative testing of MsEO and the standard drug, an aluminum chromatoplate for TLC (ALUGRAM® Xtra SIL G, silica gel 60, 0.20 mm, Macherey-Nagel) was used based on the direct bioautography method of Marston et al. (2002) [27]. The enzyme was diluted in tris-HCl buffer, 50 mM, pH 7.8 in ultra-pure water to obtain a concentration of 4 U/mL, with the addition of bovine serum albumin (Sigma-Aldrich) at a ratio of 1:1. Essential oil samples were diluted in methanol at a concentration of 100 µL/mL. Physostigmine was used as the positive control at a concentration of 100 µg/mL. For the negative control, methanol was used. The colorimetric reagents of the test were naphthyl acetate (2.5 mg in methanol) and Fast Blue B salt (2.5 mg in ultra-pure water). Both reagents were prepared and mixed immediately before use.

To perform the test, the aliquots of 10 µL of the oil samples and controls were applied on chromatoplates in duplicate and allowed to stand for 24 h for solvent evaporation. Subsequently, the plate was sprayed with the acetylcholinesterase enzyme solution (4 U/mL) and incubated in a humidity test chamber, without direct contact with moisture, at 37 °C for 20 min. Then, the plate was sprayed with a mixture of naphthyl acetate (2.5 mg) and Fast Blue B salt (2.5 mg) solutions to obtain the results. The formation of a purple coloration occurred gradually, after 1 to 3 min.

2.7.2. Quantitative Assay

The assay for quantifying acetylcholinesterase inhibition of the samples was adapted from Ellman's method [28], with modifications described by [29]. In summary, three buffers were produced for the quantitative test, which were denominated A, B, and C. These were buffer A = 50 mM Tris/HCl, pH 8, dissolved in ultra-pure water; buffer B = 0.1% bovine serum albumin in buffer A; and buffer C = 0.1 M NaCl and 0.02 M MgCl2.6H2O dissolved in buffer A.

In a total volume of 1 mL, 415 µL of buffer A, 10 µL of the essential oil solution (diluted in methanol, buffer, and Tween 80) at different concentrations (100, 50, 25, 12.5, 6.25 and 3.12 ug/mL), and 75 µL of acetylcholinesterase enzyme, containing 0.2 U/mL, were added. The samples were then incubated for 15 min at 25 °C. After incubation, 75 µL of a solution of 1.83 mM AChI (acetylthiocholine iodide) (Sigma-Aldrich, Steinheim, Germany) and 425 µL of 3 mM DTNB (5,5′-dithiobis[2-nitrobenzoic acid]) (Sigma-Aldrich, Steinheim, Germany) were added and the mixture was incubated for 30 min at 25 °C under a light source. The absorbance of the mixture was measured at 412 nm in a UV spectrophotometer (NOVA, 3300). Physostigmine was used as the standard drug, and a dilution solution was used as negative control (buffer A, methanol, and Tween 80 at a ratio of 2:2:1). The percentage of inhibition of enzyme activity was calculated according to the equation % = [(A0 − A1)/A0] * 100, where A0 was the absorbance of the control without the essential oil and A1 was the absorbance of the essential oil sample at different concentrations. All tests were performed in triplicate. The sample concentration that provided 50% inhibition (IC_{50}) was obtained by constructing graphs of the percentages of inhibition versus the concentration of the inhibitor. The non-linear regression parameters for the curve were plotted, and the IC_{50} values were obtained using the Microsoft Excel 2019 software.

2.8. Statistical Analysis

The results were expressed as the mean ± standard deviation of the results for the respective experiments, using Analysis of Variance (ANOVA) and Tukey's test, where $p < 0.05$ was considered statistically significant.

3. Results

3.1. Volatile Constituents of the Essential Oil of M. sylvatica

The *M. sylvatica* leaf essential oil showed an average yield of 1.12%; its constituents are presented in Table 1. A total of 89.04% of the constituents were identified, with emphasis on the major compounds: β-Selinene (10.06%), *E*-Calamenene (6.38%), *ar*-Curcumene

(6.25%), 1,10;7,10-Bisepoxy-1,10-seco-calamenene (4.92%), *ar*-Tumerol (4.54%), and Cadalene (4.24%).

Table 1. Volatile constituents of *M. sylvatica* essential oil.

Constituents	I.R.Calc	I.R.Lit	Conc. (%)
α-Pinene	934	932	0.12
β-Pinene	978	974	0.03
Limona ketone	1130	1131	0.09
Nopinone	1137	1135	0.12
(E)-Pinocarveol	1138	1135	0.88
Pinocarvone	1162	1160	0.16
p-Mentha-1,5-dien-8-ol	1166	1166	0.73
p-Methyl-acetophenone	1183	1179	0.3
α-Terpineol	1191	1186	0.15
Myrtenol	1196	1194	1.12
Cumenol	1203	1996	0.06
Verbenone	1209	1208	0.53
α-Copaene	1377	1374	0.2
(E)-α-Bergamotene	1437	1432	0.14
(Z)-Muurola-3,5-diene	1451	1448	0.12
β-Santalene	1461	1457	0.11
Dehydrosesquicineole	1471	1469	0.08
(E)-Cadina-1(6),4-diene	1475	1475	0.34
ar-Curcumene	1484	1479	6.25
β-Selinene	1488	1489	10.06
(E)-Muurola-4(14),5-diene	1493	1493	0.28
β-Bisabolene	1509	1505	0.59
(E)-Calamenene	1525	1521	6.38
Zonarene	1527	1528	0.42
(E)-iso-ɣ-Bisabolene	1529	1528	0.13
(E)-Cadina-1,4-diene	1534	1533	0.17
Occidentalol	1542	1543	4.98
α-Calacorene	1544	1544	3.35
β-Calacorene	1564	1564	0.93
ar-Tumerol	1588	1588	4.54
Caryophyllene oxide	1584	1582	3.20
1,10;7,10-Bisepoxy-1,10-seco-calamenene	1587	1587	4.92
Guaiol	1603	1600	2.64
Humulene epoxide	1611	1613	2.38

α-Colocalene	1623	1622	0.29
1-epi-Cubenol	1630	1627	4.02
Caryophylla-4(12),8(13)-dien-5-β-ol	1637	1639	2.62
Cubenol	1644	1645	1.49
epi-α-Muurolol	1647	1640	1.07
Agarospirol	1650	1646	1.44
Isobornyl 5-hydroxy-isobutanoate	1654	1658	1.70
α-Bisabolol oxide	1657	1656	3.04
α-Bisabolol oxide B	1660	1655	4.36
(Z)-Calamenen-10-ol	1664	1660	0.55
14-Hydroxy-9-epi-(Z)-caryophyllene	1673	1668	0.46
Cadalene	1676	1675	4.24
Mustakone	1679	1679	3.02
α-Bisabolol	1687	1685	3.40
10-nor-Calamenen-10-one	1703	1702	0.28
ar-Curcumen-15-al	1713	1712	0.33
14-Hydroxy-α-humulene	1719	1713	0.23
Total (%)			**89.04**

RI_{calc} = Calculated retention time; RI_{Lit} = Retention time from the literature. Conc.(%) = percentage concentration of the constituents.

3.2. Evaluation of the Anxiolytic Activity of M. sylvatica Essential Oil

3.2.1. Elevated Plus Maze

MsEO treatment significantly increased the time spent in the open arms (at doses of 0.1% [7 s] and 1% [9 s]) [ANOVA: $F(4) = 19.77$, $p < 0.05$] (Figure 1) and the number of entries into the open arms [ANOVA: $F(4) = 19.73$, $p < 0.05$] (Figure 2) at doses of 0.1%(3 entries) and 1% (3.5 entries) ($p < 0.05$ vs. negative control [1.8 entries]). However, the 2% dose (1 entry) did not show a significant effect ($p > 0.05$).

Comparing the essential oil-exposed groups to the diazepam-treated group, the standard drug (positive control, PC) in the tests, the 1% dose did not show a significant difference ($p > 0.05$) in both parameters analyzed (Figures 1 and 2).

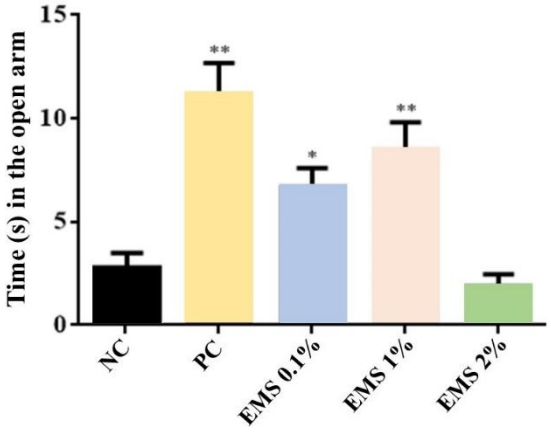


Figure 1. Time (in seconds) spent in the open arms of the elevated plus maze by animals exposed for 5 min to inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (2 s) (n = 8). Values represent the mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, compared to the negative control group using ANOVA and Tukey's test. NC = negative control, PC = positive control. EMS: *Myrcia sylvatica* essential oil.

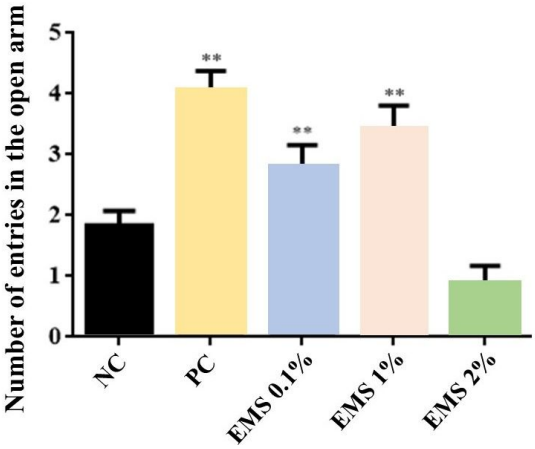


Figure 2. Number of entries into the open arms of the elevated plus maze by animals exposed for 5 min to inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (n = 8). Values represent the mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, compared to the negative control group using ANOVA and Tukey's test. NC = negative control, PC = positive control. EMS: *Myrcia sylvatica* essential oil.

3.2.2. Light–Dark Box

As shown in Figure 3, the time spent in the light compartment significantly increased [ANOVA: $F(4) = 6.75$, $p < 0.05$] when exposed to the MsEO at the 1% dose (76 s) ($p < 0.05$ vs. negative control). However, the 0.1% (49 s) and 2% (39 s) doses did not show a significant effect ($p > 0.05$ vs. negative control). When compared to the PC group (71 s), it was observed that the 1% dose did not show a significant difference ($p > 0.05$).

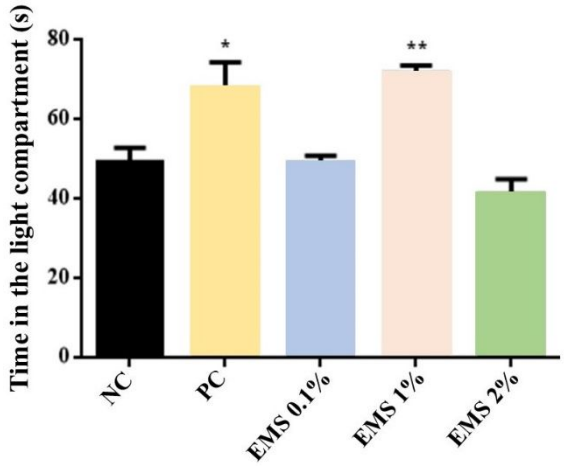


Figure 3. Time spent (seconds) in the light compartment of the light–dark box test by animals exposed to 5 min of inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (n = 6). Values represent the mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, compared to the negative control group using ANOVA and Tukey's Test. NC = negative control, PC = positive control. EMS: *Myrcia sylvatica* essential oil.

3.3. Evaluation of the Antidepressant Activity of Myrcia sylvatica Essential Oil

3.3.1. Tail Suspension Test

MsEO treatment significantly decreased [ANOVA: F(4) = 13.49, $p < 0.05$] the immobility time at the doses of 0.1% (75 s) and 1% (64 s) ($p < 0.05$ vs. negative control). However, the 2% (120 s) dose did not show a significant effect ($p > 0.05$) (Figure 4). When compared to the group treated with Imipramine (positive control), it was observed that the 0.1% and 1% doses did not present a significant difference ($p > 0.05$) in relation to the immobility time of the animals.

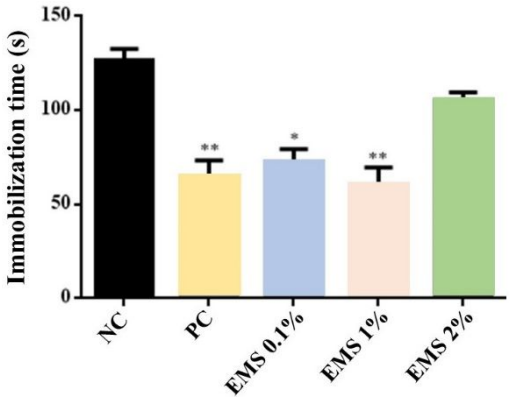


Figure 4. Immobility time (seconds) in the tail suspension test of animals exposed to 5 min of inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% ($n = 5$). Values represent the mean ± standard deviation. * $p < 0.05$ ** $p < 0.01$, compared to the negative control group using ANOVA and Tukey's Test. NC = negative control, PC = positive control. EMS: *Myrcia sylvatica* essential oil.

3.3.2. Forced Swimming Test

The treatment with essential oil significantly decreased [ANOVA: F(4) = 9.75, $p < 0.05$] the immobility time at concentrations of 0.1% (65 s) and 1% (53 s) ($p < 0.05$ vs. negative control), with no significant difference ($p > 0.05$) when compared to the CP group. However, the 2% (115 s) concentration showed no significant difference ($p > 0.05$) when compared to the negative control group (Figure 5).

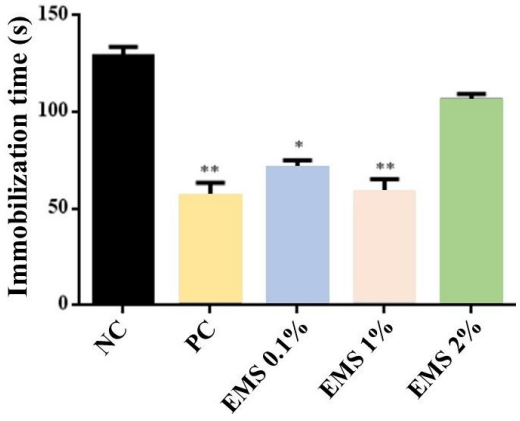


Figure 5. Latency time to immobility during forced swimming of animals exposed to inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (n = 6) for 5 min. The values represent the mean ± standard deviation. *p < 0.05, **p < 0.01, compared to the negative control group using ANOVA and Tukey's test. NC = negative control, PC = positive control. EMS: *Myrcia sylvatica* essential oil.

3.4. Evaluation of Memory and Learning

3.4.1. Y-Maze

MsEO treatment did not show a significant difference [ANOVA: F(3,28) = 0.689, p = 0.565] in the percentage of correct sequences when compared to the negative control group (Figure 6).

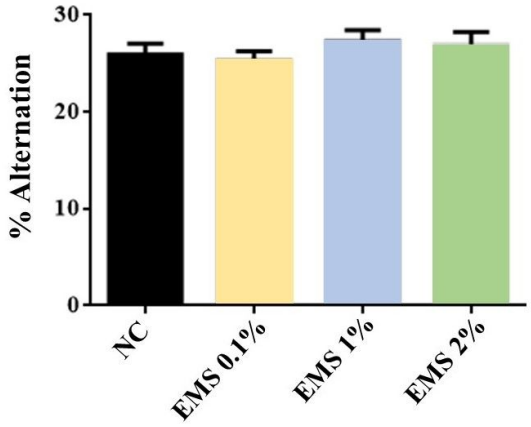


Figure 6. Percentage of alternation in the arms of the Y-maze in animals exposed to 5 min of inhalation of *Myrcia sylvatica* essential oil at doses of 0.1%, 1%, and 2% (n = 8). Values represent the mean ± standard deviation. p < 0.05 ; p < 0.01, compared to the negative control group using ANOVA and Tukey's Test. NC = negative control. EMS: *Myrcia sylvatica* essential oil.

3.4.2. Morris Water Maze

As shown in Figure 7, treatment with MsEO did not result in a significant difference [ANOVA: F (3,28) = 0.827, $p = 0.490$] in the average time to locate the platform across the four quadrants, when compared to the negative control group.

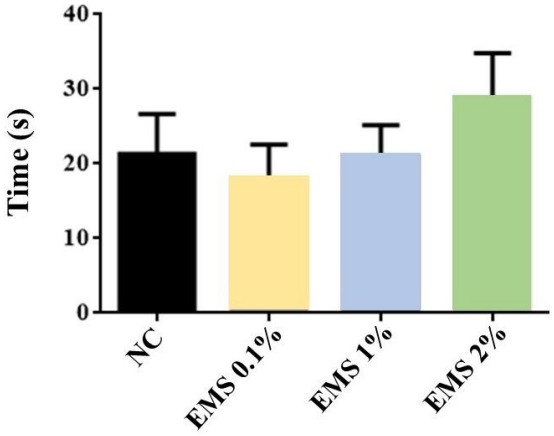


Figure 7. Average time in seconds spent to locate the platform from the four reference points in the Morris water maze of animals exposed to 5 min of inhalation of the essential oil of *Myrcia sylvatica* at doses of 0.1%, 1%, and 2% ($n = 8$). Values represent the mean ± standard deviation. $p < 0.05$, $p < 0.01$, compared to the negative control group using ANOVA and Tukey's Test**.** NC = negative control. EMS: *Myrcia sylvatica* essential oil.

3.5. Assessment of Motor Coordination

Rotarod Test

MsEO Inhalation did not alter the time spent on the rotarod [ANOVA: F (3,28) = 0.5490, $p = 0.6529$] when compared to the negative control group (Figure 8).

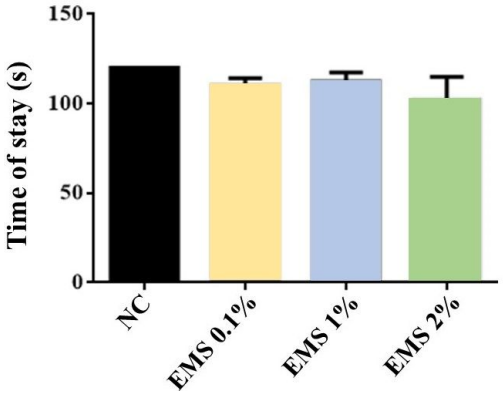


Figure 8. Mean time in seconds spent on the rotarod by animals exposed to 5 min of inhalation of *Myrcia sylvatica* essential oil at doses of 0.1, 1, and 2% *(n* = 8). Values represent the mean ± standard deviation. $p < 0.05$, $p < 0.01$, compared to the negative control group using ANOVA and Tukey's test. NC = negative control. EMS: *Myrcia sylvatica* essential oil.

3.6. Anticholinesterase Test—Qualitative and Quantitative

In Figure 9, the inhibition halos of the enzyme acetylcholinesterase are shown from the direct bioautography assay of MsEO, where we can observe the formation of an inhibition halo on the chromatographic plate. Regarding the quantitative assay, the IC_{50} found was 0.47 ± 0.015 μg/mL.

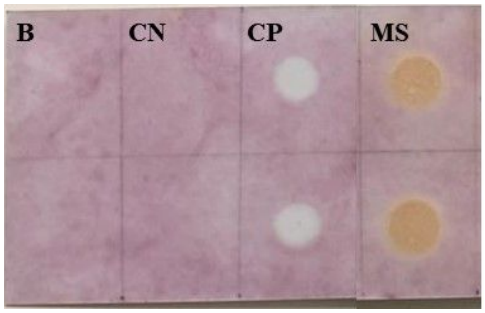


Figure 9. Direct bioautography on silica gel chromatoplates (CCD) of the anticholinesterase activity of the essential oil of *Myrcia sylvatica* and the standard physostigmine. (B) blank, (CN) negative control: methanol, (CP) positive control: physostigmine (100 μg/mL). MS = EO of *M. sylvatica*.

These results indicate that MsEO exhibits strong inhibition against the AChE enzyme. It is worth noting that the anticholinesterase potential of this species has not been described in the literature, making this the first report of the species being identified as an AChE inhibitor.

4. Discussion

Central nervous system (CNS) disorders affect millions worldwide, impairing various aspects of human behavior such as thought, emotions, memory, sensations, language, and movement [30]. Anxiety and depression disorders are the most common, affecting around 300 million people globally [31]. Moreover, there is a significant relationship between these disorders and Alzheimer's disease (AD). Studies show that patients with AD frequently experience symptoms of depression and anxiety, which may worsen disease progression and impact life quality [32,33]. Conventional drugs available for the treatment of these disorders have significant side effects, making adherence difficult for patients, and are considered inadequate [34].

In searching for new therapeutic tools, aromatic plants and their active compounds play an increasingly important role in medicine [35]. Essential oils (EOs) have been the subject of research in different behavioral models and administration methods, proving to be an alternative or complement to conventional anxiolytics and antidepressants [36,37]. These substances may offer a promising approach for managing depressive and anxious symptoms in AD patients, potentially improving their life quality and delaying the progression of

symptoms. In this context, the present study evaluated the anxiolytic, antidepressant, and anticholinesterase potential of MsEO through inhalation treatment, simulating aromatherapy, and using animal models in behavioral tests.

In the elevated plus maze test, the MsEO at doses of 0.1% and 1% increased the time spent and the number of entries into the open arms, indicating an anxiolytic effect. This model is widely used to investigate treatments with potential anxiolytic effects in rodents [38,39] and is based on the natural behavior of rodents' aversion to open and elevated spaces [40,41]. The anxiolytic effect of a drug is related to increased time spent (in seconds) and the number of entries into the open arms [20,41,42].

Complementing this finding, the light–dark box test was used based on the mice's aversion to brightly lit spaces versus their spontaneous exploratory behavior [43,44]. The anxiolytic effect is related to increased time spent in the light compartment [45,46]. The results showed that MsEO at a dose of 1% increased the time spent in the light compartment. Faturi et al. (2021) [47] found similar results regarding the dose response of the EO of *Citrus sinensis*, where inhalation of the intermediate dose increased the time spent in the light compartment. Thus, the results obtained in both methods indicate that MsEO exhibits an anxiolytic-type response, particularly at the 1% dose.

For pharmacological validation of the elevated plus maze and light–dark box tests, diazepam (5 mg/kg), a conventional anxiolytic, was administered orally, demonstrating its typical effects in both tests. This drug belongs to the class of benzodiazepines, which are CNS depressants. Its pharmacological action potentiates the effect of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), binding to the GABA-A receptor and increasing the frequency of opening the chloride channel, resulting in an increased influx of chloride through the receptor channel, facilitating rapid inhibitory transmission mediated by GABA and generating sedative and anxiolytic effects [41,48,49].

To evaluate antidepressant activity, the tail suspension and forced swimming tests were used. Although these tests do not fully reflect the symptoms of depression in humans, they are widely used to investigate compounds with antidepressant activity [50]. These tests are based on learned helplessness, where the animal, after an initial period of struggling, becomes immobile, reflecting a state of despair [51]. The administration of antidepressants increases the time the animal attempts to escape, reducing immobility [23,36]. The doses of 0.1% and 1% of MsEO reduced the immobility time, suggesting an antidepressant-like response to inescapable stress. These findings are particularly relevant in Alzheimer's disease,

where patients often exhibit depressive symptoms that can worsen the clinical condition and reduce quality of life [5].

Imipramine (15 mg/kg) was used for pharmacological validation of the tests, showing the expected effect of reducing immobility rates. As a tricyclic antidepressant, imipramine preferentially blocks serotonin and norepinephrine transporters, increasing the levels of these neurotransmitters in synapses [52].

Deficits in motor coordination and memory may accompany the anxiolytic and antidepressant effects of conventional medications [36,44]. Therefore, it was important to investigate whether the inhalation of MsEO affected motor coordination, memory, and learning, using the Y-maze, Morris water maze, and rotarod tests.

In the Y-maze test, inhalation of MsEO did not affect short-term memory based on exploring a new environment [53]. Similarly, in the Morris water maze, the EO did not interfere with spatial memory or learning, indicating that the rodents could learn how to find the platform to escape from the water [54]. These results are particularly relevant in the context of AD, which is characterized by cognitive and memory deficits, especially in the early stages. The preservation of short-term memory and spatial memory is crucial, as these cognitive domains are often affected in patients with Alzheimer's. The fact that MsEO did not show any negative interference in memory tests suggests a therapeutic potential that could be explored to develop interventions that improve cognitive function or delay the progression of symptoms in individuals with Alzheimer's. Therefore, using natural substances like EO could represent a promising alternative to managing cognitive deficits associated with the disease.

The rotarod test evaluates the integrity of motor function, assessing potential adverse effects on motor coordination [55]. In this test, no loss in motor performance was observed in the animals at any of the three doses of EO administered. Although the 2% dose did not show any anxiolytic or antidepressant effect, it did not interfere with motor coordination or the processes of memory and learning. These results are encouraging, as they suggest that MsEO could be a promising alternative with antidepressant effects without compromising essential cognitive functions, which is especially relevant in the context of neurodegenerative diseases such as Alzheimer's.

The MsEO administered via inhalation at 0.1% and 1% demonstrated anxiolytic and antidepressant potential without negatively affecting memory, learning, or motor coordination processes. In this administration method, the volatile substances reach the olfactory receptors, which are directly connected to the central nervous system, sending messages to the limbic system, where anxiety and emotions are often generated [56], leading to the release of

neurotransmitters such as serotonin, endorphins, and norepinephrine [57], which communicate with various systems in the body, affecting heart rate, blood pressure, and hormone release [58].

Volatile substances can also be absorbed by the nasal mucosa or through pulmonary blood vessels, spreading throughout the body and potentially crossing the blood–brain barrier, modulating signaling pathways involved in anxiety and depression [36,59]. This modulation is especially relevant in the context of Alzheimer's disease, where emotional dysfunction and anxiety are often observed in patients, exacerbating cognitive deficits.

The fact that MsEO does not affect memory suggests a therapeutic potential that could be explored to improve the quality of life of individuals with Alzheimer's, offering a natural alternative to conventional interventions. Several studies have demonstrated the anxiolytic and antidepressant potential of essential oils, such as Cananga odorata [60] and *Lavandula angustifolia* [61]. The effectiveness of essential oils is related to their distinct chemical composition [62], making chemical characterization crucial to ensure the standardization and reproducibility of their pharmacological effects [41].

The results of this study demonstrate the neuropharmacological potential of *Myrcia sylvatica* essential oil, though the absence of a clear dose–response relationship warrants further investigation. This characteristic may stem from the complex composition of the oil, where synergistic or antagonistic interactions between its constituents influence the observed effects. While statistically significant, the biological relevance of these findings must be interpreted cautiously, particularly given the multifactorial nature of the tested models. Future studies focusing on isolated components, their interactions, and validation in alternative models are necessary to understand better the mechanisms underlying these effects and confirm their clinical applicability.

MsEO demonstrated significant anticholinesterase activity, with an IC_{50} value of 0.47 µg/mL. This activity is particularly relevant in the context of neurodegenerative diseases such as Alzheimer's disease, where the inhibition of acetylcholinesterase (AChE) is a common therapeutic strategy. AChE is responsible for the degradation of the neurotransmitter acetylcholine, which is crucial for cognitive functions such as memory and learning [63]. The ability of MsEO to inhibit AChE suggests a promising potential for modulating acetylcholine levels, which could contribute to improving cognitive symptoms associated with Alzheimer's disease [64]. Furthermore, the effectiveness of essential oil, when compared to synthetic inhibitors, could offer a safer alternative with fewer side effects since many conventional drugs are associated with complications such as toxicity and drug interactions [65].

Investigating the chemical composition of the EO could shed light on the compounds responsible for this anticholinesterase activity, allowing for standardization and the use of the essential oil in therapeutic strategies for treating cognitive disorders.

The EO extracted from *M. sylvatica* leaves has a yield of approximately 1%, which is considered favorable for industrial-scale extraction [66]. It has a moss-green color, with spicy and woody fragrance notes [67], and a complex chemical composition, characterized by the presence of volatile compounds in low concentrations, with the major constituents being β-selinene (10.06%), (*E*)-calamene (6.38%), and *ar*-curcumene (6.25%). Although these compounds are present in low concentrations, it is possible that their synergistic effect contributes to the pharmacological activity of the essential oil. Future studies should investigate the role of individual components and their interactions in producing the observed effects.

The synergy between components of essential oils is a well-documented phenomenon in the literature, where the combination of bioactive substances can potentiate therapeutic effects compared to the effects of each isolated component [62,68]. Previous studies have indicated that the therapeutic action of essential oils, such as *Cananga odorata* and *Lavandula angustifolia*, can be significantly modulated by the interaction between their constituents, rather than depending on a single dominant compound [69,70]. This synergistic interaction may explain the efficacy of MsEO in anxiety, depression, and anticholinesterase models, despite the absence of a clear major component. Therefore, investigating the interaction between the components of MsEO and their impact on pharmacological effects is essential to understand better its therapeutic potential, especially in neurodegenerative diseases like Alzheimer's. Thus, no studies have been found in the literature on the anxiolytic and antidepressant effects of these isolated compounds or the anticholinesterase activity of this essential oil.

Several factors such as seasonality, soil and climate conditions, and plant phenology can affect the yield and chemical composition of essential oils, which in turn may influence their biological activity [71,72]. A study on the chemical stability of MsEO during seasonal and circadian periods reported a small quantitative variation between the rainy and dry seasons and at different collection periods. The chemical stability, highly pleasant aroma, significant pharmacological effects (anxiolytic and antidepressant response), and ability to inhibit acetylcholinesterase make this EO a promising therapeutic tool in aromatherapy, both as an adjunct in the treatment of anxiety and depression and in Alzheimer's disease [73].

Nevertheless, the results should be validated in other systems to confirm the therapeutic potential of MsEO. Limitations such as the small sample size and the absence of a clear dose–response relationship need to be addressed in future studies.

5. Conclusions

Our study demonstrated that *M. sylvatica* essential oil exhibits anxiolytic and antidepressant effects in an animal model without compromising the animals' motor coordination, memory, and learning abilities. This characteristic is particularly relevant, as many conventional drugs used to treat anxiety and depression disorders can cause unwanted side effects, such as cognitive and motor impairment.

The anticholinesterase activity of the oil further strengthens its potential in clinical contexts, especially in the management of neurodegenerative conditions such as Alzheimer's disease, where modulating acetylcholine levels is crucial for maintaining cognitive functions. Therefore, the inhalation of *M. sylvatica* EO may offer a helpful alternative or complementary option in the prevention or treatment of emotional and cognitive disorders.

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3.4. CAPÍTULO 4: Essential Oil of *Lippia origanoides* Kunth: Nanofomulation, Anticholinesterase Activity, and Molecular Docking

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Essential Oil of *Lippia origanoides* Kunth: Nanoformulation, Anticholinesterase Activity, and Molecular Docking

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Abstract: This study investigates the therapeutic potential of *Lippia origanoides* essential oil (LOEO) in neurological and pharmaceutical applications. The chemical composition of LOEO was analyzed using gas chromatography-mass spectrometry (GC-MS), revealing major constituents such as carvacrol, thymol, and γ-gurjunene, known for their antioxidant and antimicrobial properties. LOEO demonstrated significant acetylcholinesterase (AChE) inhibitory activity, particularly in a nanoformulation that enhances bioavailability and stability. Additionally, the major constituent carvacrol, when tested in isolation, also exhibited AChE inhibitory activity comparable to that of the nanoformulation. Molecular docking analysis indicated strong binding affinities between LOEO compounds and AChE, supporting its therapeutic potential for neurodegenerative diseases like Alzheimer's. Additionally, *in silico* pharmacokinetic predictions revealed favorable absorption and blood-brain barrier penetration profiles for key constituents. Despite promising results, the study acknowledges the need for *in vivo* validation and long-term stability assessments of the nanoformulation. Future research should focus on pharmacodynamic studies and evaluating the oil's effectiveness in animal models. These findings highlight LOEO as a valuable candidate for developing natural therapies for neurodegenerative diseases.

Keywords: Carvacrol; acetylcholinesterase nanoemulsion.

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1. Introduction

Lippia origanoides Kunth, popularly known as wild oregano and Marajó sage, belongs to the Verbenaceae family and is widely distributed in tropical and subtropical regions of Latin America. This aromatic plant is valued for its medicinal properties, use as a condiment, and production of essential oils [1,2]. In traditional communities, *L. origanoides* has been used in infusions, decoctions, and compresses to treat various conditions, such

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1. Introduction

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The essential oil of *Lippia origanoides* (LOEO) typically presents carvacrol as its main component, which is responsible for many of the bioactive properties attributed to this plant [6,7]. Among these, antimicrobial, anti-inflammatory, and antioxidant activities stand out [8]. Specifically, the antioxidant activity of the LOEO has been associated with its ability to neutralize reactive oxygen species (ROS), reducing oxidative stress and protecting cells from damage. This antioxidant action may have direct implications for the prevention of neurodegenerative diseases [9], such as Alzheimer's disease (AD) [10].

The correlation between antioxidant and anticholinesterase activity has drawn increasing attention in scientific research. Studies indicate that compounds with antioxidant properties can inhibit the enzyme acetylcholinesterase (AChE) activity, which plays a critical role in the degradation of the neurotransmitter acetylcholine [11]. Reducing AChE activity results in higher acetylcholine levels in the central nervous system (CNS), which is essential for managing pathologies such as AD [12]. Therefore, exploring the anticholinesterase effects of essential oils and their bioactive constituents represents a promising approach to developing innovative therapies.

AD is one of the most prevalent neurodegenerative diseases and represents a significant public health challenge due to the lack of a cure and the limited treatment options available. Currently available drugs, such as AChE inhibitors, delay disease progression but show limited efficacy [13]. Among natural compounds with therapeutic potential, carvacrol has stood out for its ability to inhibit AChE activity and exert neuroprotective effects [14].

Although the LOEO and its main chemical constituents have demonstrated various biological activities, there are still gaps in the literature regarding its potential anticholinesterase activity and its application in nanostructured systems. Incorporating essential oils into nanoformulations, such as nanoemulsions, can enhance their stability, bioavailability, and efficacy while opening new perspectives for developing sustainable and effective pharmaceutical products. They are kinetic stable colloids often constituted by fine droplets of an oil dispersed in a external phase [15].

Despite the promising biological activities of LOEO, its practical application faces challenges related to volatility, low water solubility, and potential degradation under environmental conditions [15]. Nanoformulations, particularly nanoemulsions, offer an effective strategy to overcome these limitations by improving the stability, bioavailability, and controlled release of bioactive compounds. These systems enhance the solubility of hydrophobic molecules, protect them from degradation, and promote better absorption, which is especially relevant for neurological applications where effective blood-brain barrier penetration is required. Thus, incorporating LOEO into a nanoemulsion could optimize its therapeutic potential, making it a more viable candidate for neurodegenerative disease treatment.

In this context, the present study aimed to evaluate the anticholinesterase activity of the LOEO, the isolated constituent carvacrol, and a nanoformulation developed from this essential oil. Additionally, molecular modeling analyses were performed to investigate the interactions between the oil's main compounds and the AChE enzyme, and in silico pharmacokinetic studies were performed to assess the feasibility of oral absorption and potential CNS penetration.

2. Materials and Methods

2.1. Plant material and and obtaining the samples

The aerial parts (leaves and thin branches) of *L. origanoides* Kunth were collected in the village of Alter do Chão (2º30’31.0” S and 54º57’00.0” W), Santarém, Pará, Brazil, in May and July 2021. Vouchers were prepared and deposited in the herbarium of the University

of Juiz de Fora, Minas Gerais, under the number CESJ-64029. This research was registered in SisGen (Sistema Nacional de Gestão do. Patrimônio Genético e do Conhecimento Tradicional Associado) under the number A965D42.

The plant material (leaves and thin branches) was dehydrated at room temperature for 7 days, then the leaves were removed from the branches, manually crushed, and subjected to the essential oil extraction process. The essential oils from both samples were obtained by hydrodistillation using a Clevenger-type apparatus for 120 minutes. The obtained essential oils were subjected to centrifugation with anhydrous sodium sulfate to remove water, and the yield was calculated based on the weight of the material used [16]. The extraction resulted in an average yield of 3.3 mL of essential oil per 100 g of plant material. The oil samples were placed in amber glass bottles, hermetically sealed, and stored at 5 °C for subsequent volatile constituents and biological assay analysis. Additionally, the isolated compound carvacrol was obtained commercially from Sigma-Aldrich® with a purity of 99.99%.

2.2. Analysis of the volatile constituents of the samples

The essential oil was analyzed by a gas chromatography system coupled to a mass spectrometer (GCMS-QP2010 Ultra, Shimadzu Corporation, Tokyo, Japan), equipped with an auto injector (AOC-20i) and CGMSsolution software (Version 4.20), which contains spectra libraries [17] including FFNSC 2 [18], following the method described by us [19]. A fused silica capillary column (Rxi-5ms, Restek Corporation, Bellefonte, PA, USA) of 30 m × 0.25 mm (diameter) × 0.25 µm (film thickness), coated with 5% diphenyl dimethylpolysiloxane was used as stationary phase. The analysis conditions were as follows: helium drag gas (99.995%); split ratio mode at 1:20; injection of 1 µL of the sample (3 µL of the essential oil in 500 µL of hexane); ionization energy by electronic impact (EI) 70 eV; injector temperature: 250 °C; oven temperature was initially set at 60 °C, then increased to 240 °C at a rate of 3 °C/min, and held for 10 min; ion source temperature: 200 °C; transfer line temperature: 250 °C.

Quantitative data on the volatile constituents were obtained via peak area normalization using a gas chromatograph (GC 6890 Plus series, Agilent, Santa Clara, CA, USA) coupled to a flame ionization detector (FID), which was operated under similar conditions to the GC-MS system. The mass spectra were obtained by automatic scanning at 0.3 scans/second, with mass fragments of 35–400 *m*/*z*. The compounds found in the ion chromatograms were identified by comparing the mass spectra (molecular mass and fragmentation pattern) with those found in the system's CGMSsolution library and by

comparison with the retention indexes. The linear equation of Van den Dool and Kratz (1963) was used to calculate the retention index (RI) for each volatile components, with the use of a standard homologous series of C8-C40 n-alkanes (Sigma-Aldrich, St. Louis, MO, USA), following the equation displayed bellow.

$$RI = \left(\frac{100}{TR_{(HA)} - RT_{(HB)}} \right) x\, RT_{(X)} - TR_{(HB)} + IK_{(HB)}$$

Whereas $IK_{(HB)}$= Kóvalts index of hydrocarbon that elutes before the component; $TR_{(HA)}$ = retention time of hydrocarbon that elutes after the component, $TR_{(HB)}$= retention time of hydrocarbon that elutes before the component, $RT_{(X)}$ = retention time of the essential oil component.

2.3. Preparation, characterization, and thermal stress of the Lippia origanoides formulation

The nanoemulsification was realized by the low-energy phase inversion method [19]. The oil phase was composed of 1% (w/w) of LOEO and 9 % (w/w) of polysorbate 20 (Sigma-Aldrich, St. Louis, MO, USA), while 90% (w/w) of deionized water as the aqueous phase. The formulation was prepared by homogenizing the oil phase in a vortex homogenizer (AP56, Pheonix Luferco, Araraquara, SP, Brazil) for 2 min, then the aqueous phase was slowly dripped into the oil phase under constant homogenization. The resultant formulation was characterized in a Zetasizer Advance Lab Blue (Malvern Panalytical, UK) by its average droplet size (nm) and polydispersity index (PdI) by dynamic light scattering (DLS), and zeta potential (ZP) by electrophoretic light scattering (ELS). The refractive index of *L. origanoides* utilized was 1.52, glass cuvette (PCS8501) and DLS 90º detection angle of detection. The analysis was realized in triplicate without diluting the formulation. The colloidal behavior and nanometric parameters, including droplet size and polydispersity index (PdI), of the *Lippia origanoides* formulation were systematically evaluated across a temperature range of 25 to 75 °C, with analyses conducted at 10 °C intervals, and pos-heated formulation at 25 ºC to evaluate its thermal stability [20]. The zeta potential (ZP) and conductivity (mS/cm) were evaluated before and after the temperature increase.

2.4. Determination of the cholinesterase inhibition

For the *in vitro* anticholinesterase assays, the enzyme acetylcholinesterase type VI-S, obtained from *Electrophorus electricus* (lyophilized powder, C3389-2Ku, Sigma-Aldrich, batch: SLBZ8573) was used. The eserine (physostigmine) diluted in methanol was used as

standard anticholinesterase inhibitor (Sigma-Aldrich, batch: BCBC4171V). A standard curve was used to define the concentration used in the tests.

2.4.1. Quantitative assay

The assay for the quantification of acetylcholinesterase inhibition of the samples (Essential oil, nanoformulation, and isolated carvacrol) was adapted from Ellman's method [21], with modifications, as described by [22]. In summary, three buffers were produced for the quantitative test, which were denominated A, B and C. These being: buffer A = 50 mM Tris/HCl, pH 8, dissolved in ultrapure water; buffer B = 0.1% bovine serum albumin in buffer A; and buffer C = 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$ dissolved in buffer A. In a total volume of 1 mL, 415 µL of buffer A, 10 µL of the samples (diluted in methanol, buffer and Tween 80) at different concentrations (100, 50, 25, 12.5, 6.25 and 3.12 ug/mL), and 75 µL of acetylcholinesterase enzyme, containing 0.2 U/mL, were added. The samples were then incubated for 15 min at 25 °C. After incubation, 75 µL of a solution of 1.83 mM AChI (acetylthiocholine iodide) (Sigma-Aldrich, Steinheim, Germany) and 425 µL of 3 mM DTNB (5,5'-dithiobis[2-nitrobenzoic acid]) (Sigma-Aldrich, Steinheim, Germany) were added and the mixture was incubated for 30 min at 25 °C under a light source. The absorbance of the mixture was measured at 412 nm in a UV spectrophotometer (NOVA, 3300). Physostigmine was used as the standard drug and a dilution solution was used as negative control (buffer A, methanol and Tween 80 at a ratio of 2:2:1). The percentage of inhibition of enzyme activity was calculated according to the equation % = [(A0 – A1) / A0] * 100, where A0 was the absorbance of the control without the essential oil and A1 was the absorbance of the essential oil sample at different concentrations. All tests were performed in triplicate. The concentration of the sample that provided 50% inhibition (IC_{50}) was obtained by constructing graphs of the percentages of inhibition versus the concentration of the inhibitor. The non-linear regression parameters for the curve were plotted and the IC_{50} values were obtained using the Microsoft Excel 2019 software.

2.5. Molecular modelling and ADME properties

2.5.1. Preparation of the ligands

The chemical structures of the major compounds in the essential oil of *L. origanoides* were obtained in ".mol" format from the National Institute of Standards and Technology (NIST) database (available at https://www.nist.gov/). Subsequently, using ChemSketch software, the molecules had their 3D structures optimized by molecular mechanics methods.

2.5.2. Preparation of the crystallographic structure of hAChE (PDB: 4EY7)

To perform the molecular docking simulation, the crystallographic structure of human acetylcholinesterase (hAChE) was obtained from the Protein Data Bank (PDB) (//www.rcsb.org/) under ID code 4EY7. Subsequently, BIOVIA Discovery Studio® v. 20.1.0 was used to process the crystallographic complex. Thus, the co-crystallized ligand, enzymatic cofactors, and water molecules were removed, and the xyz coordinates of the donepezil binding site were determined.

2.5.3. Re-docking

The approved drug, donepezil, used here as a positive control, was docked to the protein binding site (PDB: 4EY7) via the DockThor web server (https://www.dockthor.lncc.br/v2/). Molecular docking was performed using a genetic algorithm with multiple solutions and the Merck Molecular Force Field 94 (MMFF94) as the scoring function. All of donepezil's rotatable bonds were considered at this stage, and the pre-selected precision settings of the algorithm, as well as the grid box size (20x20x20), were maintained.

The RMSD (Root Mean Square Deviation) value was calculated using Discovery Studio software. Thus, the molecular docking parameterization was established based on an RMSD value below 2.0 Å, indicating that the docking protocol can be used for docking other ligands.

2.5.4. ADME properties

The selected compounds were subjected to pharmacokinetic analysis using the online prediction tool Swiss ADME (available at //www.swissadme.ch/). This program provides physicochemical properties and ADME (absorption, distribution, metabolism, and excretion) parameters associated with the pharmacokinetics of each compound. Calculated data included lipophilicity values (LogPo/w) and blood-brain barrier (BBB) permeability. Additional properties such as plasma protein binding (PPB), permeability in MDCK and CACO-2 cells, and human gastrointestinal absorption (HIA) were assessed using the PreADMET server (https://preadmet.webservice.bmdrc.org/adme/).

2.5.5. Statistical analysis

Data from the quantitative anticholinesterase assay were analyzed using the Prism 5 software with one-way ANOVA, followed by the Tukey test with multiple comparisons, at a significance level of $p<0.05$. The GraphPad Prism 8.0.1 software was used for the statistical

evaluation of binding affinity values through a one-Way ANOVA, which was followed by Sidak's multiple comparison test to evaluate and infer differences to the chosen pivot molecule.

3. Results

3.1. Volatile Constituents of Essential Oils

The essential oil (EO) from *L. origanoides* leaves exhibited an average yield of 3.3%, and its constituents are presented in Table 1. A total of 95% of the constituents were identified, with emphasis on the major compounds: *p*-Cymene (11.1%), Thymol (10.2%), Carvacrol (41.2%), and γ-Gurjunene (11.8%). The chromatogram of the analysis is available as supplementary material, with the major compounds clearly indicated.

Table 1 - Volatile constituents of *Lippia origanoides* essential oil.

Constituents	**IR_{calc}**	**IR_{lit}**	**Conc. (%)**
α-Pineno	932	932	0,16
Mirceno	989	988	1,84
α-Terpineno	1016	1014	1,09
***p*-Cimeno**	1023	1020	**11,1**
1,8-Cineol	1029	1026	1,07
γ-Terpineno	1057	1054	3,49
Linalol	1099	1095	4,6
α-Terpineol	1190	1186	0,13
Éter metil Timol	1233	1232	1,56
Safrol	1287	1285	1,98
Timol	1292	1289	**10,2**
Carvacrol	1305	1298	**41,2**
(E)-Cariofleno	1419	1417	2,78
α-Humuleno	1453	1452	0,25
γ-Gurjunene	1494	1495	**11,8**
Óxido de Cariofileno	1582	1582	1,09
1-epi-Cubenol	1630	1627	0,28
Monoterpene hydrocarbons			18,75
Oxygenated monoterpenes			57,59
Sesquiterpene hydrocarbons			16,81
Oxygenated sesquiterpenes			1,37
Total (%)			95

RI_{calc} = Calculated retention time; **RI_{lit}** = Retention time claimed in the literature [17].
Conc.(%) = percentage concentration of the constituents.

3.2. Preparation, characterization, and thermal stress of the Lippia origanoides formulation

The macroscopic characteristics of the formulation were a translucent liquid with a bluish reflection when exposed to light (Figure 1). After the preparation, the average droplet size was 10.87±0.02 nm with PdI of 0.131±0.012.



Figure 1: Macroscopic characteristics (translucency) of the *Lippia origanoides* formulation with 1% essential oil and 9% polysorbate 20.

The formulation exhibited an increase in the mean size (nm) and polydispersity index (PdI) as the temperature increased (10 °C/intervals), demonstrating a monomodal behavior at all evaluated temperatures (Figure 2). However, there was a notable increase in the average size from 25 ºC (10.87±0.02 nm) to 75 ºC (66.09±0.373).

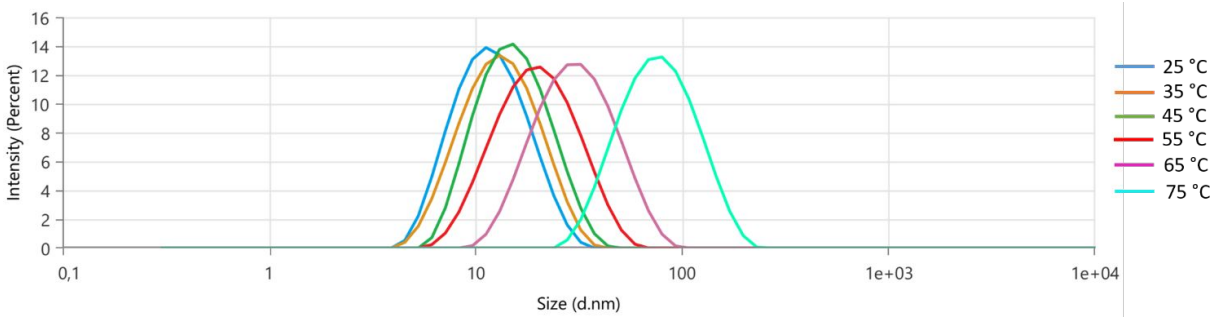


Figure 2: Temperature influence (25-75 ºC) in the droplet size distribution by the intensity of the *Lippia origanoides* formulation.

Table 2 shows each temperature's average droplet size (nm) and PdI values. After applying heat (25 to 75 ºC), the collective nanometric parameters were reassessed at room temperature (25°C). This resulted in no discernible macroscopic alteration and nanometric values similar to those observed before the heating process (Table 3). The increase in the mean size, PdI of the droplets and the colloidal behavior can be associated with the supersaturation of the external phase (aqueous phase) due to the increase in the solubility of the more electronegative substances (e.g. monoterpenoides and sesquiterpenoids), including the major compound (10,2 %) thymol (LogP 3.28). This response to the temperature increase is characteristic of the Ostwald ripening, a mass transference from smaller to higher mass droplets, a common instability mechanism of low-density colloidal systems (e.g. essential oils).

Table 2: Average droplet size and polydispersity index of the *Lippia origanoides* formulation at rising temperatures (25-75 ºC).

Temperature (°C)	Average droplet size (nm)	Polydispersity index
25	10.87±0.020	0.131±0.012
35	12.02±0.097	0.135±0.013
45	14.10±0.040	0.136±0.011
55	17.79±0.155	0.155±0.010
65	26.83±0.138	0.173±0.005
75	66.09±0.373	0.193±0.005

The data are presented as mean ± standard deviation (n=3).

The zeta potential (ZP) values (-1.634±0.464 and -1.866±0.482) indicate the nanodroplets are negatively charged (Table 2 and figure 3). The low magnitude of the ZP observed suggests a minor electrostatic repulsion, which can eventually lead to classical emulsion instability factors such as coalescence or aggregation. However, it is noteworthy that the occurrence of those specific colloidal destabilization events was not observed following the heating of the nanoformulation, indicating that it exhibits stability when briefly exposed to continuous temperatures.

The moderate increase in the conductivity after heating (0.280 to 0.370 mS/cm) suggests a higher free ion dissociation and presence in the external phase, possibly related to the phenol thymol (pKa 10.62) being ionized in the aqueous phase as the temperature increases.

Table 3: Macroscopic characteristics, average size, polydispersity index, zeta potential, and conductivity before and after thermal stress (25 ºC).

Thermal stress	Macroscopic characteristics	Average size (nm)	Polydispersity index	Zeta potential (mV)	Conductivity (mS/cm)
Before	Translucent	10.87±0.020	0.131±0.012	-1.634±0.464	0.2809
After	Translucent	10.16±0.050	0.069±0.011	-1.866±0.482	0.3709

The data are presented as mean ± standard deviation (n=3).

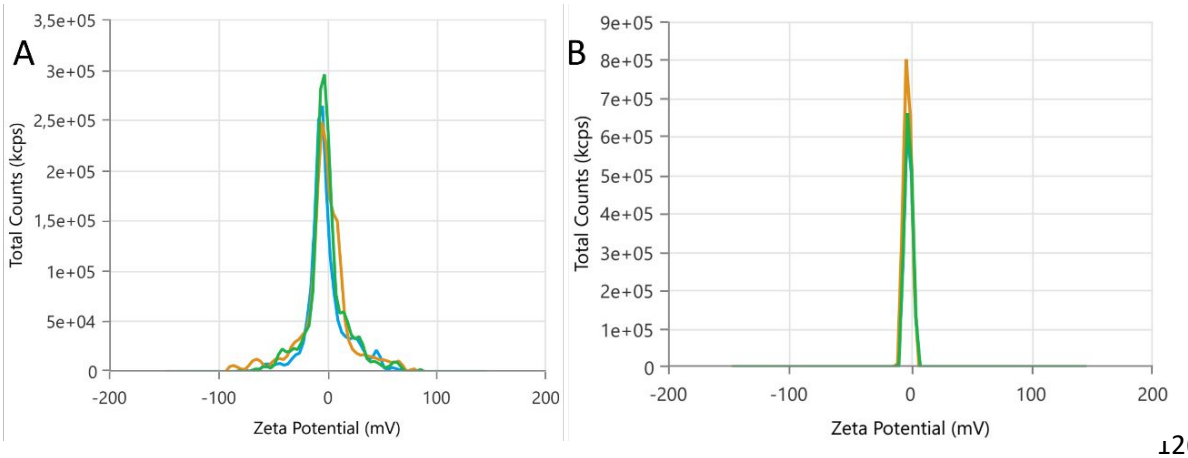


Figure 3: Zeta potential distribution of the *Lippia origanoides* formulation before (A), and after (B) thermal stress.

3.3. Determination of the acetylcholinesterase inhibition

The results demonstrate that all samples exhibited inhibitory activity, albeit with varying potencies compared to the control. Physostigmine, as expected, showed strong inhibitory activity, serving as a reference for the efficacy of the other samples. According to Figure 4, all samples *of Lippia origanoides* essential oil, carvacrol, and the nanoformulation exhibited strong inhibitory activity against acetylcholinesterase, with IC_{50} values ranging from 0.074 to 0.16 µg/mL. The Lippia origanoides essential oil showed an IC_{50} of 0.16 ± 0.036 µg/mL, while carvacrol showed an IC_{50} of 0.12 ± 0.006 µg/mL, and the nanoformulation had an IC_{50} of 0.074 ± 0.003 µg/mL. No significant difference was observed between the essential oil and carvacrol, nor between carvacrol and the nanoformulation ($p \leq 0.05$). However, a statistically significant difference was found between the essential oil and the nanoformulation, indicating that the nanoformulation exhibited greater inhibitory activity against the acetylcholinesterase enzyme.

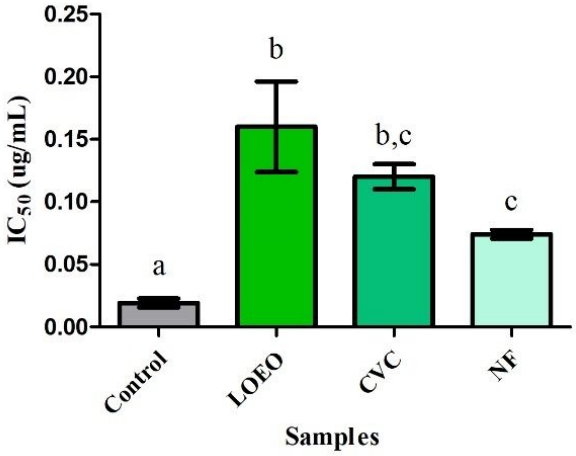


Figura 4 – Anticholinesterase Activity (IC_{50}) of Samples: LOEO = oil essential of *L. origanoides*; CVC = carvacrol and NF = nanoformulation. Control = = physostigmine. The data are presented as mean ± standard deviation. Statistical analysis was performed using ANOVA followed by a Tukey post-hoc test, with significance defined at $p < 0.05$.

3.4. Molecular docking

The energy calculation for all docking complexes was evaluated using the MMFF94 force field as the scoring function. The binding energy values of the major constituents of the essential oils ranged from -9.229 kcal/mol to -7.993 kcal/mol. All the major compounds of *Lippia origanoides* essential oil exhibited binding energy levels significantly lower and different ($p \leq 0.05$) compared to donepezil (-10.638 kcal/mol) (Figure 5). Among the compounds in the oil, γ-gurjunene demonstrated a statistically significant difference compared to the other constituents, with a binding energy of -9.229 kcal/mol.

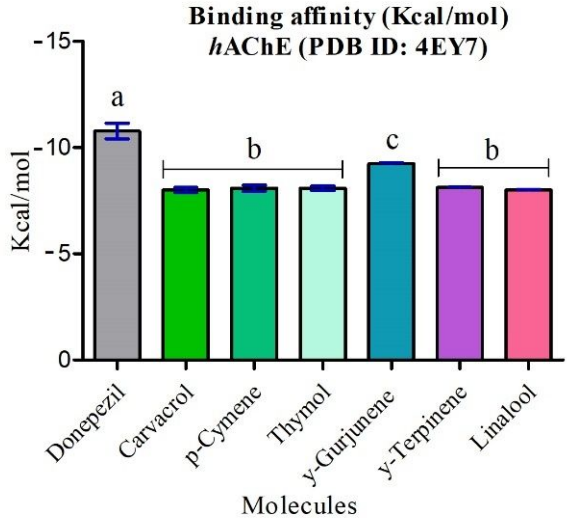


Figure 5: Statistical analyses of the binding energy values of donepezil compared to the major compounds of *Lippia origanoides* essential oil. Statistical analysis was performed using ANOVA followed by a Tukey post-hoc test, with significance defined at $p < 0.05$.

3.4.1. Analysis of the binding sites of major compounds with the AChE enzyme

The initial procedure involved docking, which was validated through the accurate re-docking of the co-crystallized donepezil into the *h*AChE model to better compare the docking results. Donepezil was docked to AChE using the same parameters and was found to have a binding affinity of -11.237 kcal/mol. Figure 6 shows the binding pocket and target residues involved in the binding interaction of donepezil.

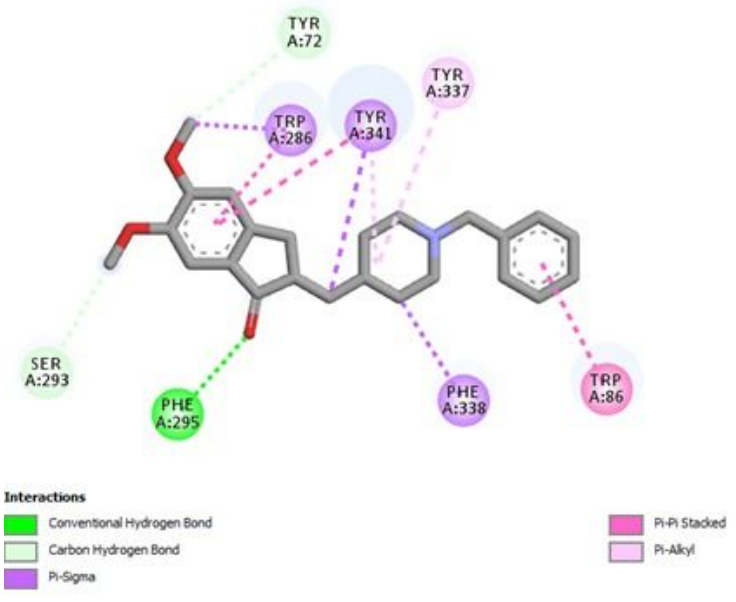
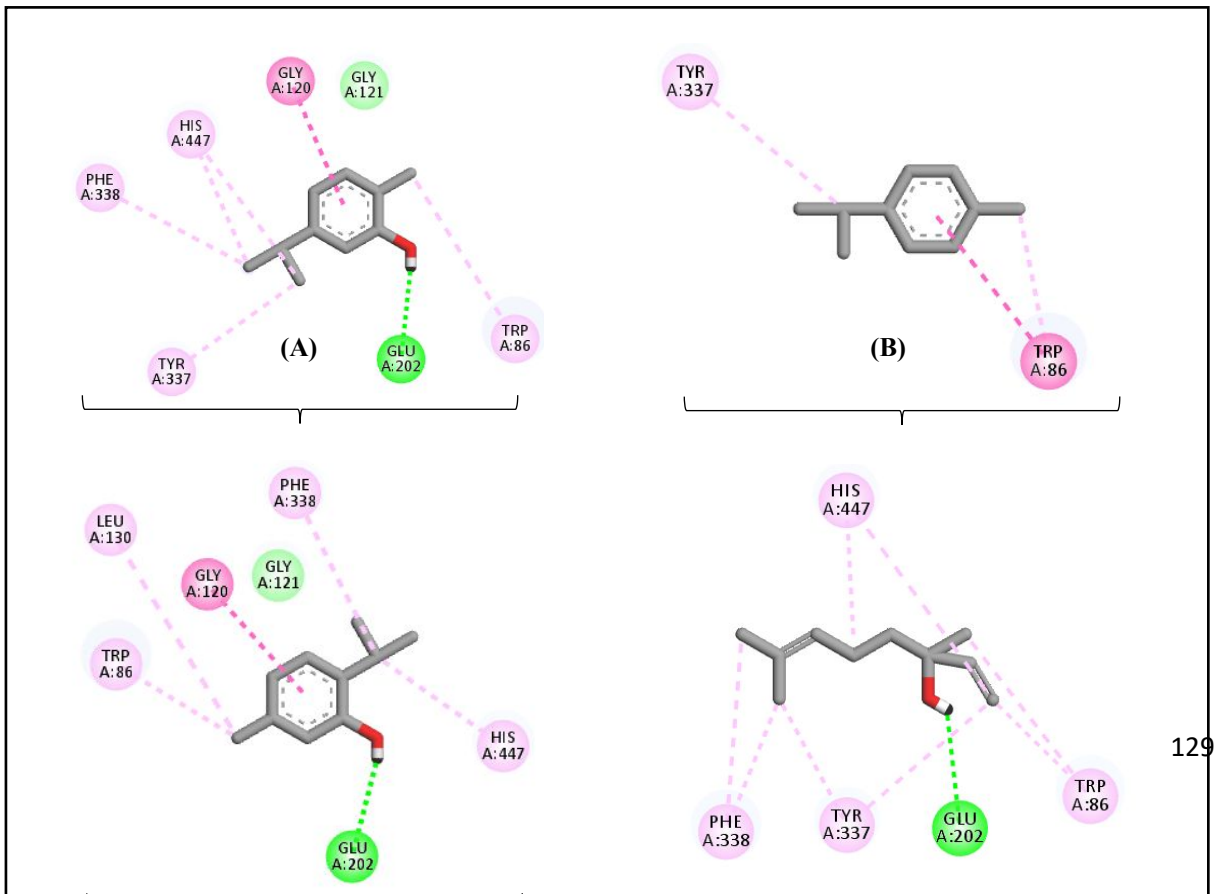


Figure 6: 2D diagram illustrating the interactions between donepezil and the acetylcholinesterase (AChE) enzyme (4EY7).

Figure 7 presents the interaction diagram between the acetylcholinesterase (AChE) enzyme (4EY7) and the amino acid residues of the compounds carvacrol (A), p-cymene (B), thymol (C), linalool (D), γ-terpinene (E), and γ-gurjunene (F).

The diagrams highlight specific bonds, such as hydrogen bonds, hydrophobic interactions, and other intermolecular forces, that contribute to the stabilization of the enzyme-compound complexes. Each panel (A-F) shows how the different compounds interact with the active residues of AChE, providing insights into the mechanism of enzyme inhibition. These interactions are crucial for understanding the potential efficacy of these compounds as AChE inhibitors, which may be relevant for the development of therapeutic agents in the treatment of cholinesterase-related diseases.



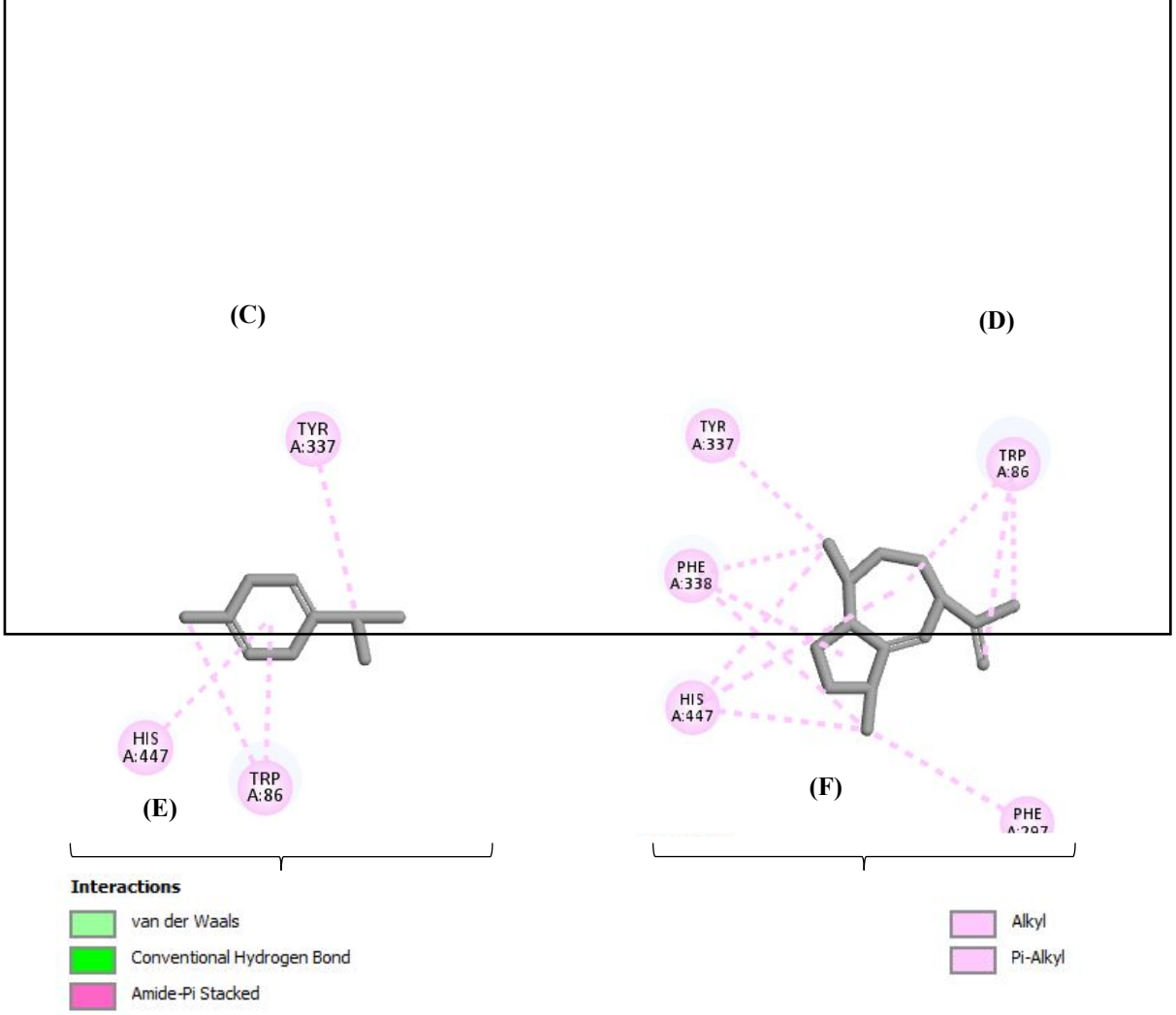


Figure 7: 2D diagram of the interactions between carvacrol (A), *p*-cymene (B), thymol (C), γ-terpinene (D), Linalool (E) and γ-gurjunene (F), with the acetylcholinesterase (AChE) enzyme (4EY7).

3.4.2. ADME Properties

Table 4 presents the in silico pharmacokinetic properties of the major constituents of LOEO, including carvacrol, p-cymene, thymol, γ-terpinene, linalool, and γ-gurjunene, predicted using the SwissADME and PxeADMET tools. The results indicate that all compounds exhibit high intestinal absorption (HIA = 100%) and permeability across the blood-brain barrier (BBB), suggesting good bioavailability and potential to reach the central nervous system. Additionally, the LogP values, ranging from 3.21 to 5.00, indicate that these compounds have suitable lipophilic characteristics for penetration into biological membranes. These favorable pharmacokinetic properties corroborate the results presented in Figure 4, where the compounds demonstrated anticholinesterase activity, suggesting that their inhibitory efficacy may be related to their absorption and distribution in the body. These data are essential for evaluating the therapeutic potential of these compounds in the development of pharmacological agents.

Table 4: In silico pharmacokinetic prediction of the major constituents of *Lippia origanoides* essential oil.

Molecule	Absorption				Distribution	
	LogP[a]	P_{Caco-2}[b]	HIA (%)[b]	P_{MDCK}[b]	LPP (%)[b]	BHE[a]
Carvacrol	3,81	38,0121	100	87,3307	100	6,38799
p-Cymene	3,9	23,4337	100	237,507	100	4,96983
Thymol	3,34	38,0122	100	87,3307	100	6,38802
γ-Terpinene	3,36	23,6401	100	244,909	100	8,03745
Linalool	3,21	29,355	100	115,421	100	6,12506
γ-Gurjunene	5	23,6411	100	57,0682	100	13,4717

[a] Analysis conducted on the SwissADME web server (http://www.swissadme.ch/index.php#); [b] Analysis conducted on the PreADMET web server (https://preadmet.webservice.bmdrc.org/adme/).

4. Discussion

This study aimed to explore the therapeutic potential of the essential oil of *Lippia origanoides* Kunth (LOEO), focusing on its acetylcholinesterase (AChE) inhibitory properties. The rationale for this study is based on the growing need to develop natural and effective treatments for neurodegenerative diseases, such as Alzheimer's, which are associated with neurotransmitter deficiency and oxidative stress. Additionally, the search for safe and sustainable therapeutic alternatives, especially those derived from plant sources, has gained prominence due to the side effects associated with synthetic drugs. LOEO, rich in phenolic compounds such as carvacrol and thymol, has shown significant potential for inhibiting AChE, a crucial enzyme in the pathogenesis of neurodegenerative diseases.

The research also investigated the feasibility of nanoformulations to improve the stability and bioavailability of these compounds, targeting pharmaceutical and clinical applications. Therefore, this study not only contributes to the understanding of the molecular mechanisms behind the biological activity of LOEO but also paves the way for the development of new therapies based on natural products, aligning with current demands for innovative and sustainable therapeutic solutions.

The analysis of the chemical composition reveals that the LOEO has a characteristic chemical profile, with carvacrol (41.2%), thymol (10.2%), p-cymene (11.1%), and γ-gurjunene (11.8%) as the main compounds, which is consistent with previous studies [8, 24]. These phenolic compounds are known for their potent antioxidant and antimicrobial activities, highlighting LOEO as a promising source of therapeutic agents for various clinical conditions. The results also indicate a good oil yield (3.3%), reinforcing its potential for commercial applications, especially in the pharmaceutical and food industries.

In terms of biological activity, LOEO demonstrated potent acetylcholinesterase (AChE) inhibitory activity, which is crucial in the context of treatments for neurodegenerative diseases such as Alzheimer's. Carvacrol, being the main component, plays a central role in this effect. AChE inhibition can be attributed to its ability to form π-π interactions with key residues of the enzyme, such as TRP86 and TYR337, as evidenced by molecular interactions.

However, the therapeutic contribution of LOEO is not solely due to carvacrol. γ-Gurjunene, although present in lower concentration, has a high binding affinity for AChE, indicating its significant contribution to the observed inhibitory activity. Thymol, in turn, complements the action of these compounds, enhancing the overall effect of LOEO. Thus, the therapeutic activity of the essential oil appears to result from a synergistic effect between these compounds, making its efficacy superior to that of any isolated compound.

Compared to other species of the *Lippia* genus, such as *Lippia alba* and *Lippia graveolens*, similar chemical profiles emerge, dominated by carvacrol and thymol. For instance, *Lippia graveolens* oil has been reported to contain carvacrol and thymol in concentrations ranging from 30% to 60%, depending on the plant's geographical origin and harvesting conditions [24, 25]. Similarly, *Lippia alba* often exhibits a chemotype rich in citral or carvone, but studies have also identified phenolic compounds like thymol in certain variants [26]. These comparisons highlight the intra-genus variability in essential oil composition, which is influenced by genetic, environmental, and extraction factors.

The therapeutic importance of phenolic compounds such as carvacrol and thymol lies in their ability to disrupt microbial membranes, neutralize free radicals, and inhibit enzymes like acetylcholinesterase (AChE). The prominence of carvacrol in LOEO, accounting for 41.2% of its composition, is particularly noteworthy as it exceeds concentrations typically observed in other *Lippia* species and other carvacrol-rich essential oils, such as those from *Origanum vulgare* [27, 28, 29]. Additionally, the presence of γ-Gurjunene, a sesquiterpene hydrocarbon, provides a unique chemical signature to LOEO, differentiating it from closely related species and potentially contributing to its bioactive profile. These findings reaffirm the value of *Lippia origanoides* as a promising source of bioactive compounds with diverse applications.

The analysis of the nanoformulation of LOEO reveals important aspects regarding its stability and physicochemical behavior under different thermal conditions, essential for its pharmaceutical and therapeutic applications. Initially, the formulation presented an average droplet size of 10.87±0.02 nm and a polydispersity index (PdI) of 0.131±0.012, characterizing a narrow distribution and a homogeneous formulation. PdI values below 0.2 are indicative of

good stability and uniformity of the particles, which is crucial for controlled release and efficient absorption of bioactive compounds, such as thymol, as one of the major components of the formulation [30, 31]. These parameters suggest that the formulation is suitable for therapeutic applications, where particle uniformity plays an important role in treatment efficacy.

The analysis of the temperature impact on droplet size revealed a notable increase in the average size, from 10.87±0.02 nm to 66.09±0.373 nm between 25 ºC and 75 ºC, along with a progressive increase in PdI. These results indicate that the formulation behaves in a monomodal way during thermal variation, with a tendency for droplet growth at higher temperatures. This phenomenon is a classic example of the Ostwald ripening process, where mass is transferred from smaller to larger droplets, resulting in increased instability of emulsions. This occurs due to the increased solubility of more electronegative substances, such as monoterpenes and sesquiterpenes present in *Lippia origanoides* essential oil [32, 33].

Moreover, the behavior of zeta potential (ZP) and conductivity of the formulation provides important insights into electrostatic stability and compression of the ionic layer around the nanoparticles. The negative zeta potential, -1.634±0.464 and -1.866±0.482, suggests that the nanoparticles are slightly negatively charged, which, under ideal conditions, would help prevent droplet coalescence. However, the low ZP magnitude indicates weak electrostatic repulsion between particles, which could favor aggregation or instability under some conditions. This phenomenon can be compensated by the presence of the surfactant (polysorbate 20), which contributes to the steric stabilization of the formulation, as recent studies on colloidal systems based on essential oils have suggested [34]. The moderate increase in conductivity from 0.280 mS/cm to 0.370 mS/cm after heating reinforces the idea that thymol, due to its high pKa, may ionize in the aqueous phase at higher temperatures, increasing ion dissociation and influencing the formulation's stability [35].

The thermal stability observed in the *Lippia origanoides* nanoformulation, which showed no significant changes in its macroscopic characteristics after exposure to varying temperatures, is an important advantage for its use in environmental conditions that may vary, especially in pharmaceutical formulations that require stability across different temperature ranges. The ability of the nanoparticles to maintain their characteristics even after thermal stress can be attributed to the synergistic interaction between the bioactive compounds of the essential oil and the surfactant used, which ensures the structural and functional stability of the formulation [36, 37, 38].

The combination of low polydispersity, thermal stability, and the presence of bioactive compounds such as thymol and carvacrol enhances the pharmacological potential of LOEO for therapeutic applications, such as neurological treatments or antimicrobial therapies. This research confirms the viability of the nanoformulation as a promising system for the efficient delivery of active ingredients, with a stability profile suitable for further investigation and clinical applications.

The results of the acetylcholinesterase (AChE) inhibition assay, presented in Figure 4, reveal a potent inhibitory activity of LOEO, carvacrol, and the nanoformulation against AChE. The IC_{50} values, ranging from 0.074 to 0.16 μg/mL, demonstrate that all tested formulations exhibit strong AChE inhibition. The nanoformulation, with the lowest IC_{50} value (0.074 ± 0.003 μg/mL), showed the highest activity, while carvacrol and L. origanoides essential oil exhibited similar values (0.12 ± 0.006 μg/mL and 0.16 ± 0.036 μg/mL, respectively). This finding suggests that the encapsulation LOEO in the nanoformulation enhances its bioactivity, possibly due to improved bioavailability and cellular uptake of the bioactive compounds, including carvacrol, which is known for its cholinesterase inhibitory properties [39, 40].

The observed increase in inhibitory activity of the nanoformulation compared to the free essential oil and carvacrol can be attributed to the enhanced solubility and stability provided by the nanoformulation. Nanoencapsulation is known to improve the dispersibility of hydrophobic compounds and facilitate their interaction with the enzyme, leading to more efficient inhibition [41, 42]. Several studies have highlighted that nanoparticles, particularly those made from surfactants like polysorbate 20, can significantly improve the pharmacokinetic properties of essential oils by protecting them from degradation and increasing their bioactivity against biological targets such as AChE [43, 44]. In this study, the nanoformulation's superior activity indicates its potential as a more effective therapeutic agent for conditions related to acetylcholinesterase dysfunction, such as Alzheimer's disease.

Additionally, the statistical significance found between the nanoformulation and the free LOEO points to the role of the nanoencapsulation in enhancing the therapeutic potential of the oil's bioactive compounds. While carvacrol, the major compound in the essential oil, already demonstrates promising AChE inhibitory activity, the nanoformulation likely provides a more sustained and controlled release of carvacrol and other constituents, enhancing their effects. This finding aligns with recent research that shows the ability of nanoformulations to significantly improve the biological activity of essential oils, including their antioxidant and anticholinesterase properties [45, 46].

In conclusion, the results underscore the importance of nanotechnology in enhancing the therapeutic efficacy of essential oils, particularly in neurological applications where acetylcholinesterase inhibition is a key therapeutic strategy. The improved AChE inhibition observed in the nanoformulation of LOEO provides compelling evidence for the development of more effective and targeted treatments for neurodegenerative diseases, offering a promising avenue for future research and pharmaceutical applications.

The analysis of the binding affinity levels of the main constituents of *Lippia origanoides* essential oil (LOEO) with the AChE enzyme revealed promising data regarding its inhibitory potential. Using the MMFF94 force field as the scoring function, the binding energy values of the compounds ranged from -9.229 kcal/mol to -7.993 kcal/mol, highlighting significant interactions with the enzyme's active site. Although all compounds showed lower binding energy values than the reference standard, donepezil (-10.638 kcal/mol), a statistically significant difference ($p \leq 0.05$) was observed among the LOEO constituents. Among them, γ-gurjunene stood out with the highest binding affinity (-9.229 kcal/mol), while linalool showed the lowest value (-7.993 kcal/mol). These results suggest that γ-gurjunene could play a relevant role as a natural inhibitor of AchE [47, 48].

The phenolic monoterpenes carvacrol (-8.003 kcal/mol) and thymol (-8.085 kcal/mol) also exhibited competitive binding affinity levels, attributed to their ability to form π-π interactions with key residues in the enzyme's active site, such as TRP86 and TYR337. These molecular interactions are essential for the stability of the enzyme-ligand complex, complementing evidence that phenolic compounds possess high anticholinesterase potential [48, 49]. Furthermore, molecular interactions revealed that LOEO compounds formed robust hydrophobic bonds and π-σ interactions, particularly with residues such as PHE295, TYR341, and TYR72. These interaction patterns stand out for their relevance in AChE inhibition, suggesting that the structural profile of the oil's constituents favors the formation of stable and effective complexes [49, 50].

The robust molecular interaction observed between γ-gurjunene and specific residues in the active site, such as PHE295, explains its higher binding affinity. In contrast, linalool, although forming bonds with catalytic residues, does not establish high-intensity π-π interactions, justifying its lower binding affinity. These results support the hypothesis that LOEO may exert synergistic effects due to the structural diversity of its constituents. For example, carvacrol and thymol, with their strong π-π and π-σ interactions, may enhance the overall inhibitory effect of the oil when combined with other constituents, such as γ-terpinene

and p-cymene, which exhibit moderate hydrophobic interactions and hydrogen bonding [50, 51].

The association between the binding affinity data and molecular interactions strengthens the understanding of LOEO's anticholinesterase potential. While γ-gurjunene stands out in terms of affinity, carvacrol and thymol provide additional advantages due to their diversified interactions, particularly with critical residues such as TYR341, TRP86, and PHE338. This synergistic profile suggests that LOEO, as a whole, could be a promising natural source of AChE inhibitors, combining significant affinity with effective molecular interactions. These factors are crucial for the development of natural therapies against neurodegenerative diseases, such as Alzheimer's disease [51, 52].

Additionally, the comparison with the standard inhibitor donepezil, which showed the highest binding affinity (-10.638 kcal/mol), reinforces the relative efficacy of LOEO compounds. Although none of the isolated constituents reached the same level of affinity, the combined interaction profile and binding affinity of LOEO constituents indicate a synergistic mechanism of enzymatic inhibition. This synergistic approach could be a significant advantage in terms of safety and therapeutic efficacy, especially when compared to synthetic drugs, which often have undesirable side effects[52, 53].

Besides acetylcholinesterase inhibition, carvacrol and thymol demonstrated additional neuroprotective properties that may contribute to the treatment of neurodegenerative diseases. Previous studies indicate that carvacrol has significant antioxidant activity, neutralizing free radicals and reducing oxidative stress, a key factor in the progression of diseases like Alzheimer's [8]. Additionally, carvacrol exhibits anti-inflammatory effects by inhibiting the release of pro-inflammatory cytokines and modulating signaling pathways such as NF-κB, which may help reduce neuroinflammation associated with neuronal damage [54]. Thymol, in turn, also has antioxidant and anti-inflammatory properties, protecting neuronal cells from oxidative damage and apoptosis. These combined effects suggest that LOEO, rich in carvacrol and thymol, may act in a multifaceted manner in protecting the central nervous system, not only by inhibiting AChE but also by mitigating cellular damage caused by oxidative stress and inflammation. These neuroprotective properties enhance the potential of LOEO as a promising therapeutic approach for neurodegenerative diseases.

The therapeutic activity of LOEO appears to result from a synergistic effect between its main compounds, such as carvacrol, thymol, and γ-gurjunene. Although carvacrol, as the major component, plays a central role in acetylcholinesterase (AChE) inhibitory activity, γ-gurjunene, with its high binding affinity to the enzyme, also makes a significant contribution.

Thymol, in turn, complements the action of these compounds, enhancing the overall effect of LOEO. Therefore, the therapeutic effect of the essential oil cannot be attributed to a single compound, but rather to the synergistic interaction between them, resulting in a more robust activity than that observed with any isolated compound.

These results, combined with the thermal and structural stability already discussed in other parts of this study, reinforce the potential of LOEO and its constituents as multifunctional agents for AChE inhibition. The ability to form stable, high-affinity complexes with the enzyme underlines its relevance as a natural alternative for the development of phytotherapeutic treatments against neurodegenerative diseases, such as Alzheimer's disease [54, 55].

The absorption parameters evaluated, such as LogP, PCaco-2, HIA (%), and PMDCK, indicate a favorable profile for the oral bioavailability of the major constituents of *Lippia origanoides* essential oil (LOEO). The LogP values, reflecting lipophilicity, ranged from 3.21 (linalool) to 5 (γ-gurjunene). LogP values close to 5 are generally considered the upper limit for good absorption, indicating that γ-gurjunene exhibits the highest lipophilicity among the compounds. This may facilitate its passage through lipid membranes but also suggests a higher risk of low aqueous solubility[56, 57]. On the other hand, compounds such as linalool (3.21) and carvacrol (3.81) demonstrate a more favorable balance between lipophilicity and aqueous solubility, aligning with desirable properties for effective drugs [58].

The PCaco-2 parameter, a predictor of intestinal permeability, indicated high values for carvacrol (38.0121), thymol (38.0122), and linalool (29.355), while γ-terpinene (23.6401), γ-gurjunene (23.6411), and p-cymene (23.4337) showed lower values. These results suggest that carvacrol and thymol have a greater ability to cross the intestinal barrier compared to the other constituents, which can directly influence the efficacy of the compounds when administered orally[59, 60]. All compounds exhibited 100% human intestinal absorption (HIA), corroborating their excellent oral bioavailability. Regarding the PMDCK parameter, which assesses permeability in renal cell cultures, γ-terpinene (244.909) and p-cymene (237.507) showed the highest values, indicating greater potential for absorption through alternative pathways[60].

For the distribution parameters, all compounds demonstrated high plasma protein binding (LPP), with values of 100%, indicating that most of these compounds would be bound to plasma proteins, reducing their free fraction for pharmacological activity. However, this can also act as a reservoir, prolonging the half-life of the compounds in the body [61, 62].

The blood-brain barrier penetration coefficient (BBB) revealed varying values among the compounds, with γ-gurjunene showing the highest value (13.4717), followed by γ-terpinene (8.03745). These results highlight γ-gurjunene's potential to efficiently cross the blood-brain barrier, which is highly relevant for neuroprotection and acetylcholinesterase (AChE) inhibition applications. On the other hand, p-cymene (4.96983) and linalool (6.12506) exhibited lower BBB penetration, which could be advantageous in applications where activity should be limited to the peripheral system[62, 63].

Overall, the in silico results suggest that the pharmacokinetic profile of LOEO constituents aligns with their previously observed bioactive properties, such as AChE inhibition. Compounds with higher absorption and distribution potential, such as γ-gurjunene, γ-terpinene, carvacrol, and thymol, may significantly contribute to the essential oil's efficacy in therapeutic contexts. Particularly, γ-gurjunene's ability to cross the BBB suggests a central role in LOEO's neuroprotective effect, while other compounds like carvacrol and thymol may act complementarily, providing structural stability and synergistic interactions with target receptors[63, 64].

These results emphasize the need for additional experimental studies to confirm the synergistic effects and bioavailability of the compounds in the body. Moreover, strategies such as nanoencapsulation can be employed to optimize the delivery of bioactive compounds, particularly those with high lipophilic properties, such as γ-gurjunene, ensuring their efficacy in therapeutic applications[64, 65].

The main strengths of this study include the comprehensive analysis of LOEO through a combination of chemical, biological, and pharmacokinetic techniques, which provide a detailed understanding of the therapeutic properties of LOEO and its nanoformulations. The use of nanoencapsulated formulations demonstrated not only the stability of the essential oil but also its increased efficiency in inhibiting AChE, highlighting its therapeutic potential in neurodegenerative diseases.

Additionally, the molecular modeling results provided precise insights into the interaction mechanisms of the key compounds with the enzymatic target. However, some limitations should be noted. Firstly, experimental data were primarily obtained in vitro, and clinical validation is essential to confirm the therapeutic efficacy of LOEO in humans. Furthermore, although the bioavailability results indicate that the compounds of LOEO have a good pharmacokinetic profile, absorption and distribution in the body need to be better understood through experimental studies. Future work should delve deeper into these issues and explore the applicability of nanoformulations in animal models.

5. Conclusions

This study confirms the potential of *Lippia origanoides* essential oil (LOEO) as a promising source of bioactive compounds with significant implications for pharmaceutical and neurological research. The chemical composition of LOEO, including carvacrol, thymol, and γ-gurjunene, along with its potent acetylcholinesterase (AChE) inhibitory activity, positions LOEO as a valuable candidate for therapies targeting neurodegenerative diseases, particularly Alzheimer's disease.

The results show that the LOEO nanoformulation offers additional benefits, such as enhanced bioactivity and stability of the bioactive compounds, highlighting the advantages of nanotechnology in improving bioavailability and treatment efficacy. Molecular docking analysis supports LOEO's AChE inhibitory potential, demonstrating strong binding affinities, while in silico pharmacokinetics indicate favorable absorption and good blood-brain barrier penetration.

Although the results are promising, the study acknowledges limitations, such as the lack of in vivo validation and the stability assessment of the nanoformulation. Future studies should prioritize in vivo pharmacodynamic investigations, evaluations in animal models, long-term stability testing, and the analysis of other pathways related to neurodegenerative diseases.

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4. DISCUSSÃO INTEGRADORA

Esta tese investigou sistematicamente o potencial neurofarmacológico de óleos essenciais (OEs) da Amazônia, com foco na inibição da acetilcolinesterase (AChE) e em efeitos psicoativos, integrando abordagens químicas, farmacológicas e computacionais. Os quatro capítulos revelaram descobertas complementares que não apenas validam o uso tradicional dessas plantas, mas também abrem novas perspectivas para aplicações terapêuticas inovadoras, especialmente em doenças neurodegenerativas e transtornos emocionais.

Sinergia entre Composição Química e Atividade Biológica

Os capítulos I e II destacaram a importância da variabilidade quimiotípica na atividade anticolinesterásica. Enquanto o óleo de *Lippia alba* rico em carvona (Capítulo I) mostrou maior inibição da AChE (IC_{50} 0,1 µg/mL) comparado aos quimiotipos de citral e linalol, os óleos de *Curcuma longa* e *Zingiber officinale* (Capítulo II) também exibiram potente atividade (IC_{50} 0,33 e 0,38 µg/mL, respectivamente). Curiosamente, a mistura desses óleos reduziu a eficácia, sugerindo interações antagônicas entre seus constituintes. Esses resultados reforçam que a atividade neurofarmacológica é fortemente influenciada pela composição química, com monoterpenos (como carvona e curcuminoides) desempenhando papéis-chave na ligação à AChE, conforme corroborado pelos estudos de docagem molecular.

Potencial Ansiolítico e Antidepressivo: Uma Abordagem Translacional

O Capítulo III explorou o óleo de *Myrcia sylvatica* (MsEO), que, além de inibir a AChE (IC_{50} 0,47 µg/mL), demonstrou efeitos ansiolíticos e antidepressivos em modelos animais *por inalação*, sem comprometer a cognição ou coordenação motora. Esse achado é relevante, dado o perfil de segurança do óleo e sua aplicabilidade em aromaterapia, alinhando-se às práticas integrativas do SUS. A ausência de um constituinte majoritário no MsEO sugere que seus efeitos resultam de sinergia entre componentes minoritários, um padrão distinto dos demais óleos estudados.

Inovações em Sistemas de Liberação: Nanoformulações

O Capítulo IV avançou na aplicação farmacêutica ao desenvolver uma nanoemulsão de *Lippia origanoides* (LOEO), que aumentou a estabilidade e biodisponibilidade do óleo, mantendo sua atividade anticolinesterásica. A docagem molecular identificou carvacrol como o principal composto ativo, com alta afinidade pela AChE e boa penetração na barreira hematoencefálica (*in silico*). Esse resultado destaca o potencial de nanoestruturação para superar limitações como volatilidade e baixa solubilidade, comum em OEs.

Lacunas e Direções Futuras

Apesar dos avanços, lacunas persistem:

1. **Validação *in vivo***: Os efeitos neuroprotetores dos OEs e da nanoemulsão de LOEO precisam ser testados em modelos de neurodegeneração (ex.: camundongos transgênicos para Alzheimer).
2. **Mecanismos de Sinergia**: Como a combinação de OEs pode modular a AChE e receptores GABAérgicos (ansiolíticos) ainda é pouco compreendido.

3. **Estudos de Toxicidade Crônica**: A segurança do uso prolongado, especialmente por inalação, requer investigação.
4. **Preservação da Biodiversidade**: A exploração sustentável de espécies como *Myrcia sylvatica* é urgente, dado seu risco por queimadas na Amazônia.

Esta tese demonstrou que os OEs amazônicos são fontes promissoras de moléculas multitarget, capazes de modular simultaneamente processos colinérgicos e emocionais. A nanoestruturação surge como estratégia viável para otimizar sua eficácia clínica. Futuros estudos devem focar em:

- **Tradução clínica**: Ensaios em humanos para ansiedade/AD.
- **Desenvolvimento de Fitofármacos**: Formulações combinatórias (ex.: carvona + carvacrol) ou nanoentregas.
- **Conservação Ecológica**: Parcerias com comunidades locais para cultivo sustentável.

Em síntese, os resultados aqui apresentados transcendem o âmbito acadêmico, oferecendo bases científicas para políticas públicas que integrem biodiversidade, saúde e inovação tecnológica.

5. CONCLUSÃO

Com base nos resultados obtidos nos estudos apresentados, destaca-se o potencial terapêutico de óleos essenciais de diversas espécies vegetais na inibição da acetilcolinesterase (AChE) e no tratamento de condições neurológicas e comportamentais. O óleo essencial de *Lippia alba*, com três diferentes quimiotipos, apresentou ação inibitória frente a enzima AChE com destaque para o quimiotipo rico em carvona (LA2), demonstrando potencial biotecnológico para a descoberta de novas substâncias anticolinesterásicas. Da mesma forma, os óleos essenciais de *Curcuma longa* e *Zingiber officinale* também demonstraram elevada atividade anticolinesterásica, com interação significativa de seus constituintes majoritários com o receptor da enzima AChE, reforçando que seus constituintes majoritários são responsáveis em parte, pela atividade biológica observada no óleo essencial e suas misturas.

O óleo essencial de *Myrcia sylvatica*, por sua vez, apresentou propriedades ansiolíticas e antidepressivas significativas quando administrado por inalação, além de atividade

anticolinesterásica relevante. Os testes comportamentais realizados em modelos animais demonstraram que o óleo foi capaz de reduzir comportamentos de ansiedade e depressão sem comprometer memória, aprendizado ou coordenação motora, destacando-se como uma promissora alternativa para o tratamento de transtornos emocionais.

Para o óleo essencial de *Lippia origanoides*, além do efeito inibitório sobre a AChE, foi usado para o desenvolvimento de uma nanoformulação, a qual apresentou estabilidade e potencializou a ação anticolinesterásica observada para o óleo essencial e seu constituintes majoritário carvacrol, o que destaca essa espécie como candidata ao desenvolvimento de terapias naturais voltadas para doenças neurodegenerativas, como o Alzheimer.

Portanto, os dados apresentados nesta tese reforçam a relevância das espécies aromáticas da Amazônia como fontes promissoras de compostos bioativos. O estudo da caracterização química dos óleos essenciais das espécies em estudo, aliados ao uso de tecnologias modernas, como a nanoformulação e os estudos de ancoragem molecular, abrem caminhos para o desenvolvimento de novos fitoterápicos para o tratamento de doenças neurodegenerativas como a Doença de Alzheimer e transtornos de humor como ansiedade e depressão. Esses resultados abrem perspectiva para a valoração das espécies aromáticas da Amazônia, podendo ser fontes importantes para a bioeconomia.

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6. ANEXOS

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

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

Comprovante de Cadastro de Acesso
Cadastro nº A965D42

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

Número do cadastro:	**A965D42**
Usuário:	**UFOPA**
CPF/CNPJ:	**11.118.393/0001-59**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie
Lippia alba
Lippia origanoidis
Myrcia sylvatica
Myrcia multiflora
Protium heptaphyllum
Curcuma longa
Copaifera multijuga
Pectis elongata
Aniba rosaeodora
Aeollanthus suaveolens

Título da Atividade:	**ÓLEOS ESSENCIAIS DE PLANTAS DA REGIÃO OESTE DO PARÁ: PROSPECÇÃO QUIMICA E ATIVIDADES BIOLÓGICAS**

Equipe	
Rosa Helena Veras Mourão	UFOPA
Antônio Quaresma da Silva Júnior	UFOPA
José Guilherme Maia	UFOPA
Adenilson Souza Barroso	UFOPA
Caio Pinto Fernandes	UNIFAP
Leonelde Erica Maduro Bouillet	UEPA
Maria José da Silva Fernandes	Universidade Federal de São Paulo
Contanza Rocha Jaules	Universidade Federal de São Paulo
Bernardo Baldiserotto	Universidade Federal de Santa Maria
Sandra Layse Ferreira Sarrazin	UFOPA
Ricardo Bezerra de Oliveira	UFOPA
Karina Aloântara de Souza	UFOPA
Lenice Vargas Flôres da Silva	UFOPA
Hugo Napoleão	Universidade Federal de Santa Maria
Mônica Camelo Pessôa de Azevedo Albuquer	UNIVERSIDADE FEDERAL DE PERNAMBUCO
Paulo Henrique Valença Nunes	UNIVERSIDADE FEDERAL DE PERNAMBUCO
Maria Beatriz Viana dos Santos	UFOPA
Walter Lucas Correa Santana	Universidade Federal do Oeste do Pará

Data do Cadastro: 02/06/2021 00:21:05
Situação do Cadastro: Concluído

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

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

Comprovante de Cadastro de Acesso
Cadastro nº AA0F83F

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

Número do cadastro:	AA0F83F
Usuário:	UFOPA
CPF/CNPJ:	11.118.393/0001-59
Objeto do Acesso:	Patrimônio Genético/CTA
Finalidade do Acesso:	Pesquisa

Espécie

Zingiber officinale

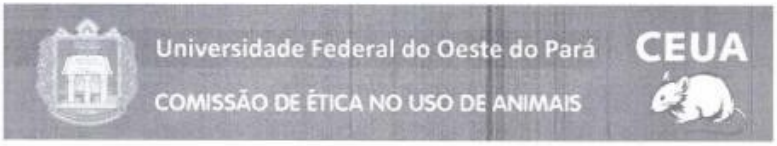
Fonte do CTA

CTA de origem não identificável

Título da Atividade: atividade anticolinesterasica de óleo essencial de Zingiber officinale

Equipe

Rosa Helena Veras Mourão	UFOPA
Antonio Quaresma da Silva Junior	Ufopa



CERTIFICADO

Certificamos que o Protocolo Nº 10004-2017, intitulado "Avaliação da atividade ansiolítica e antidepressiva do óleo essencial de *Myrcia sylvatica* (G. Mey.) D.C. Myrtaceae", sob a responsabilidade de Rosa Helena Veras Mourão, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal do Oeste do Pará-UFOPA.

CERTIFICATE

We certify that the protocol Nº 10004-2017, entitled "Avaliação da atividade ansiolítica e antidepressiva do óleo essencial de *Myrcia sylvatica* (G. Mey.) D.C. Myrtaceae", is in agreement with the Ethical Principles for Animal Research established by the National Council for Control of Animal Experimentation (CONCEA). This project was approved by the institutional Commission for Ethics



CERTIFICADO

XXVI SIMPÓSIO DE PLANTAS MEDICINAIS DO BRASIL

Certificamos que o trabalho intitulado **"Atividade anticolinesterásica de óleos essenciais dos rizomas de Curcuma longa L. E Zingiber officinale R. (Zingiberaceae)"** de autoria de **Antônio Quaresma da Silva Júnior, Rosa Mourão, Karina Alcântara, Leoneide Bouillet e Adenilson Barroso**, foi apresentado no dia 27 de outubro de 2022 na Modalidade Pôster, durante o **XXVI Simpósio de Plantas Medicinais do Brasil**, na Universidade Federal do Oeste do Pará.

Rosa Helena Veras Mourão
Presidente do XXVI Simpósio de
Plantas Medicinais do Brasil

SBPM
Sociedade Brasileira de Plantas Medicinais

Ministério da Saúde
FIOCRUZ
Fundação Oswaldo Cruz



ATIVIDADE ANTICOLINESTERÁSICA DE ÓLEOS ESSENCIAIS DOS RIZOMAS DE *Curcuma longa* L. E *Zingiber officinale* R. (ZINGIBERACEAE)

Antônio Quaresma da Silva Júnior[1,2]; Karina Alcântara de Sousa[1,3], Adenilson de Sousa Barroso[1]; Leoneide Erica Maduro Bouillet[1,3] e Rosa Helena Veras Mourão[1,2,3]

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A doença de Alzheimer (DA) é uma desordem neurodegenerativa caracterizada pela perda progressiva de neurônios nas estruturas do sistema nervoso central, resultando em alterações funcionais gradativas que podem levar à um quadro de demência, com perda de memória e cognição. O tratamento consiste em atenuar os sintomas, principalmente através do aumento das taxas do neurotransmissor Acetilcolina no cérebro, utilizando inibidores da enzima acetilcolinesterase (AChE). Porém, os fármacos utilizados no tratamento são onerosos e não manifestam nenhum efeito corretivo ou regressivo da doença, o que justifica a busca por novas substâncias com ação anticolinesterásica, sendo os óleos essenciais uma promissora fonte de novas moléculas bioativas por possuírem uma diversidade de substâncias em sua composição química. Dessa forma, o objetivo desse trabalho foi avaliar a atividade anticolinesterásica do óleo essencial dos rizomas de C*urcuma longa* L. e *Zingiber officinale* R., ambas muito utilizadas na forma de especiarias e por suas propriedades medicinais. Os óleos essenciais foram obtidos pelo método de hidrodestilação em aparelho do tipo Clevenger, a partir de rizomas frescos, adquiridos comercialmente em feiras livres de Santarém-PA. A composição química foi determinada por cromatografia gasosa acoplado a um espectrômetro de massas (GCMS-QP2010 Ultra - Shimadzu Corporation, Tokyo, Japan). A atividade anticolinesterásica foi avaliada

inicialmente de forma qualitativa, pelo método de bioautografia, conforme Marston et al. (2002). Em seguida foi utilizado o método quantitativo, de acordo com o método de Ellman et al. (1961) com modificações. Para ambos os testes, foi utilizada a enzima Acetilcolinesterase Tipo VI-S obtida de *Electrophorus electricus,* e como controle positivo, foi utilizado o fármaco Fisostigmina. No óleo essencial de *C. longa* foram identificados como componentes majoritários os sesquiterpenos ar-Turmerone (27%), (E)-γ-Atlantone (17%) e o monoterpeno α-Felandreno (6,6%). Enquanto que no óleo essencial de Z*. officinale* os compostos majoritários foram os sesquiterpenos: α-Zingibereno (20%), (E,E)-α-Farneseno (7,3%), β-Sesquifelandreno (7,3%), além dos monoterpenos Citral (17,5%) e Canfeno (10,1%). Ambos os óleos testados apresentaram atividade anticolinesterásica positiva no teste qualitativo, com a formação de halos de inibição na cromatoplaca. No teste quantitativo, o óleo essencial de *C. longa* e *Z. officinale* apresentaram IC_{50} de 0,5 e 0,4 mg/mL, respectivamente. De acordo com estes resultados, os óleos testados possuem compostos com ação anticolinesterásica, que possivelmente, devido ao seu baixo peso molecular e alta lipofilicidade, podem ter facilidade para atravessar a barreira hematoencefálica e inibir a ação da enzima acetilcolinesterase, o que estimula a continuidade dos estudos com as espécies supracitadas.

Palavras-chave: Acetilcolina; Alzheimer; Acetilcolinesterase; Rizomas.

ATIVIDADE ANTICOLINESTERÁSICA *IN VITRO* DOS ÓLEOS ESSENCIAIS DE DUAS PLANTAS COM ALTO TEOR DE CITRAL

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Órgão de fomento: FAPESPA

A doença de Alzheimer é uma patologia causada pelo déficit de atividade dos neurônios colinérgicos, levando à alteração de diversas funções cognitivas e desencadeando um quadro de demência e perda de memória. Atualmente é considerada um problema de saúde pública, com milhares de novos diagnósticos a cada ano. Embora não tenha cura, os sintomas da doença podem ser amenizados pela utilização de fármacos com ação inibitória sobre a enzima acetilcolinesterase, como forma de aumentar os níveis de acetilcolina no cérebro. No entanto, atualmente as opções de tratamento são limitadas e onerosas e não manifestam nenhum efeito corretivo ou regressivo da doença, o que justifica a busca por novas substâncias com ação anticolinesterásica. Dessa forma, o objetivo deste trabalho foi avaliar a atividade anticolinesterásica *in vitro* dos óleos essenciais de duas plantas bastante utilizadas

tradicionalmente como calmantes, e que possuem alto teor de citral: *Lippia alba* (Mill.) N. E. Brown e *Pectis elongata* Kunth. Os óleos essenciais foram obtidos pelo método de hidrodestilação, a partir de partes aéreas desidratadas, ambas oriundas de cultivos domiciliares de Santarém-PA. A composição química foi determinada por cromatografia gasosa acoplado a um espectrômetro de massas. A atividade anticolinesterásica foi avaliada inicialmente de forma qualitativa, pelo método de bioautografia, conforme Marston et al. (2002). Em seguida foi utilizado o método quantitativo, de acordo com o método de Ellman et al. (1961) com modificações. Para ambos os testes, foi utilizada a enzima Acetilcolinesterase Tipo VI-S obtida de *Electrophorus electricus*, e como controle positivo, foi utilizado o fármaco Fisostigmina. Além dos óleos, também foi avaliada a atividade do composto Citral isolado (95%, Sigma-Aldrich®). A composição química das espécies confirmou o alto teor de citral presente nos óleos essenciais, sendo 56,15% em *L. alba* e 93,6% em *P. elongata*. Além do citral, *L. alba* também apresentou como constituintes majoritários: γ-Muuroleno (7,67%); Limoneno (7,02%) e Elemol (5,39%). Todas as amostras testadas apresentaram atividade anticolinesterásica positiva no teste qualitativo, com a formação de halos de inibição na cromatoplaca. No teste quantitativo, os IC_{50} obtidos foram de 0,7 e 1,2 mg/mL, para *alba* e *P. elongata*, respectivamente, enquanto que para o composto citral isolado, o IC_{50} foi de 0,8 mg/mL. As diferenças entre as duas espécies podem indicar que no óleo da espécie *L. alba*, o citral pode agir em sinergismo com outros constituintes, potencializando sua ação anticolinesterásica. Os resultados indicam que o citral possui atividade anticolinesterásica significativa, o que estimula a continuidade dos estudos com os óleos e o composto isolado, uma vez que, devido à sua natureza lipídica e baixo peso molecular, os constituintes de óleos essenciais podem ter facilidade para atravessar a barreira hematoencefálica e agir como inibidores da enzima acetilcolinesterase.



Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação Tecnológica
IX Seminário de Pós-graduação
X Jornada Acadêmica da Ufopa

CERTIFICADO

Certificamos que **ANTÔNIO QUARESMA DA SILVA JÚNIOR**, CPF: 813.782.462-68, participou do evento IX Seminário de Pós-graduação, da Universidade Federal do Oeste do Pará, realizado durante o período de 09/11/2022 a 10/11/2022 apresentando trabalho: ATIVIDADE ANTICOLINESTERÁSICA IN VITRO DOS ÓLEOS ESSENCIAIS DE DUAS PLANTAS COM ALTO TEOR DE CITRAL, na modalidade de vídeo, com carga horária de 12 horas.

Santarém-Pará, 11 de novembro de 2022.

Lenise Vargas Flores da Silva
Pró-Reitora de Pesquisa, Pós-Graduação e Inovação Tecnológica
Portaria nº 520 de 30 de dezembro de 2019

Kelly Christina Ferreira Castro
Diretora de Pós-graduação
Portaria nº 233 de 29 de junho de 2022

IX SEMINÁRIO DE PÓS-GRADUAÇÃO

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