
Article

Essential oil of *Lippia origanoides* H.B.K.: a promising compound acetylcholinesterase inhibitors for the treatment of Alzheimer's disease

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Abstract: Alzheimer's disease is characterized by a progressive decline of cognitive functions. The class of drugs used for the treatment are acetylcholinesterase inhibitors. Essential oils have contributed to folk medicine and discovery of new drugs for a long time. The purpose of the study was to investigate the in vitro and in silico the anti-acetylcholinesterase activity, as well as acute toxicity of the essential oil of *Lippia origanoides*. EOLO was obtained by hydrodistillation and analyzed by gas chromatography-mass spectrometry. The inhibition assay of acetylcholinesterase enzyme activity was evaluated in vitro, as well as in silico by docking. The effects of EOLO on hematological, biochemical and behavioral parameters were analyzed in mice. We expose that EOLO shows good anti-acetylcholinesterase activity and low toxicity, possibly resulting from the action of the majority compounds thymol, carvacrol and p-cymene. The anti-acetylcholinesterase potential in vitro demonstrating a 70% inhibition. The docking results elucidated the participation of the major phenolics in AChE inhibition by interacting with the catalytic cavity of AChE. The acute oral toxicity test classified as low toxicity. These results contribute to expand the knowledge about essential oil of *Lippia origanoides*. Therefore, appears to be promising for herbal medicine production with anti-acetylcholinesterase and antioxidant activity.

Keywords: Acetylcholinesterase; Dementia; Essencial oil

1. Introduction

It has been estimated that more than 50 million people will have developed dementia in 2020, with more than 60% of all cases being associated with Alzheimer's disease (AD) [1,2]. AD is a neurodegenerative disease characterized by a progressive decline of cognitive functions, especially memory impairment [2].

The complete mechanism of the pathogenesis of the multifactorial disease is unsolved [3]. The "cholinergic hypothesis," suggests that the progressive degeneration of cholinergic neurons is the main factor contributing to AD, remains one of the major theories to explain the origin of this disease [4].

The decline of the acetylcholine concentration in the brain of AD patients is further amplified by the activity of neuronal acetylcholinesterase (AChE), which regulates the termination of the synaptic signal by hydrolyzing the neurotransmitter acetylcholine secreted in the inter-synaptic cleft.

As a result, the inhibition of AChE has become a promising therapeutic strategy for treating the symptoms of AD. The use of cholinesterase inhibitors reduces symptoms by increasing the concentration of acetylcholine in the brain, which, in turn, improves patient memory and cognitive function.

Donepezil, galantamine, and rivastigmine are currently the most used commercial inhibitors for the treatment of AD [2]. However, the use of these drugs is hampered by severe dose-dependent side effects. In addition to these respective side-effects, the short half-life of some inhibitors such as rivastigmine and physostigmine also jeopardizes their long-term therapeutic use.

The use of medicinal plants by man in the treatment of diseases is as old as his existence [5]. However, due to lack of correct orientation, these practices can become quite dangerous, since many plants have difficult identification, chemical composition variable, or still relative toxicity [6,7,8]. With the advent of modernity, medicine has been making great strides, but medicinal plants still play an important role in world health, since most drugs evaluated as therapeutic agents are derived from natural products [9]. Among the vastness of vegetable products, essential oils deserve special attention. These are complex mixtures of hydrocarbons and oxygenated hydrocarbons from the isoprenoid routes, consisting mainly of monoterpenes and sesquiterpenes [10].

Reports in the literature have evidenced the importance of the essential oil of the species *Lippia origanoides* Humboldt, Bonpland, and Kunth (HBK), belonging to the family *Verbenaceae*, for traditional medicine, where the leaves or aerial parts of the species are used for the treatment of respiratory and gastrointestinal tract infections and is also used to relieve uterine cramps, vaginal diseases, menstrual disorders, fever, and as a general antiseptic for mouth, throat and vaginal infections and wound healing [11]. The pharmacological studies have shown the importance of this species as antibacterial, antifungal, antiparasitic, antihypertensive and spasmolytic activity [12,13,14]. However, there is a gap in studies on pharmacological potentials and possible toxicological effects.

This work aims to evaluate the acetylcholinesterase inhibitor activity in vitro and in silico of essential oil of *Lippia origanoides* (HBK), as well as acute toxicity by biochemical, hematological, physiological, and behavioral parameters in female Swiss rats, to estimate the 50% median lethal dose (LD50) based on the Organization for Economic Cooperation and Development (OECD) Guide 423.

The main highlights are that the essential oil of *Lippia origanoides* HBK (EOLO) exerted an inhibitory effect on acetylcholinesterase as well as its major compounds (carvacrol, thymol, and p-cymene). The acute oral toxicity test classified the oil as low toxicity and no changes were observed.

2. Materials and Methods

Collection of plant material

Collection of the leaves of *L. origanoides* H.B.K. was carried out in the month of May 2013, in the municipality of José de Freitas (latitude 04°45'23 "south and longitude 42°34'32" west), Piauí, Brazil. The determination of the species was carried out by the botanist Fátima Salmena Pires and an exsiccata is deposited in the Herbarium "Graziela Barroso" of the biology department of the Federal University of Piauí under the number TEPB 09205.

Essential oil extraction

Biomass of the aerial parts (leaves and fine branches) of the plant was used. The biomass was dried at room temperature. The sample was chopped in small pieces and subjected to four hours of Clevenger type hydrodistillation. The oil was stored in an amber glass bottle and kept under refrigeration at about 4 ° C.

Characterization of essential oil

The volatile constituents were analyzed on a GC-17A Shimadzu gas chromatograph coupled to a GCMS-QP5050A mass spectrometer equipped with J & W Scientific DB-5 HT capillary column (95% methylpolysiloxane and 5% phenyl, 30 m long, 0.25 mm internal diameter and 0.1 µm film thickness of the fixed phase). Most volatile constituents were identified by comparing the mass spectra obtained with the Wiley229® computer library records, as well as by comparing the respective retention indices (RI), compared to the available literature spectra [15].

Anticholinesterase potential

Qualitative evaluation of the acetylcholinesterase inhibitory activity of the essential oil of *Lippia organoides* H.B.K.

The assay for inhibition of acetylcholinesterase (AChE) enzyme was performed according to Ellman and colleagues [16] with adaptations by Rhee and colleagues [17]. In this study, sample was applied to a thin-layer chromatography (TLC) plate, followed by sputtering with Ellman's reagent (5,5'-ditiobis-[2-nitrobenzoic acid, DTNB) and acetylthiocholine iodide in buffer and drying. After this procedure, the plate was sprayed with AChE enzyme (5 units/mL). After approximately 10 minutes, the enzymatic inhibition could be verified by the absence of the yellow color and the concomitant appearance of a white halo. Caffeine was used as a positive standard control.

Quantitative evaluation of the acetylcholinesterase inhibiting activity of the essential oil of *Lippia organoides* H.B.K.

The quantitative investigation was evaluated by means of the spectrophotometric assay. Initially, 1 mg of the sample (standard and EOLO) was weighed on an analytical balance, added to 1 mL of 50 mM Tris-HCl buffer solution, pH 8, 10% methanol, 500 μ L of sample was removed, and it was completed again with 500 μ L of 50 mM Tris-HCl buffer solution, pH 8, 10% methanol, and so on successively to obtain the concentrations of 62.5; 125; 250 and 500 μ g/mL.

From the first concentration 100 μ L was taken and placed in a test tube, adding 100 μ L of the enzyme 10U with buffer and albumin and 200 μ L of 50 mM Tris-HCl buffer, pH 8, 0.1% BS. This process was repeated for the remaining concentrations. All tests were performed in triplicate. As a blank, 100 μ L of 50 mM Tris-HCl, pH 8, 10 % methanol added to 300 μ L of 50 mM Tris-HCl, pH 8, 0.1% BSA was used. All samples were taken to the water bath for 5 minutes, with subsequent addition of 500 μ L of Buffer Solution with DTNB+ NaCl + MgCl₂.

The samples were read at 412 nm after the addition of 100 μ L of buffer solution with acetylcholine iodide and the initial absorbance was measured in the spectrophotometer, and after 5 minutes the final absorbance was measured again. The calculation of enzyme inhibition was obtained by the expression [18]:

$$\% \text{ inhibition} = 100 - (\text{sample reaction variation} / \text{control reaction variation} \times 100) \quad (1)$$

Simulation of the acetylcholinesterase mechanisms of essential oil *Lippia organoides* H.B.K.

The possible anti-acetylcholinesterase mechanism was elucidated by molecular docking through the evaluation of molecular interactions between their major components of EOLO and acetylcholinesterase. In view of this, the three-dimensional conformation of the molecules of arvacrol, thymol and p-cymene were retrieved from the ZINC database, <https://zinc.docking.org/>, under the respective record number 967563, 967597 and 968246. The crystal structure of recombinant human acetylcholinesterase in complex with Donepezyl was retrieved from the Protein Data Bank, <https://www.rcsb.org/>, under PDB registration 4EY7 and was prepared using UCSF Chimera.

Docking was performed using the SwissDock program, <http://www.swissdock.ch/docking>, taking into consideration the precise fit, flexible ligand, rigid protein and grid at coordinates X= 11.1529, Y= -55.8821, Z= - 23.9165; 15x15x15 for the E20 active site, as well as coordinates X= 23.1917, Y= -51.5844, Z=-10.2897; 15x15x15 for the NAG active site. The results were visualized using UCSF Chimera and Studio Discovery software.

Acute toxicity test of the essential oil of *Lippia organoides* H.B.K. - Class Test (OECD 423)

Animals

Swiss albino female mice weighing between 25 and 30 g, approximately 2 months old, were collected from the Central Biotherm of the Agricultural Sciences Center (CCA) of the Federal University of Piauí (UFPI). During the acclimation period, the animals were kept under monitored temperature conditions equivalent to 25 ± 1 °C, kept in a light / dark cycle of 12 hours. The animals had free access to water and food. All behavioral tests were carried out in silent rooms under the same conditions mentioned above and isolated from noise. All animals were treated according to the principles defined by the Brazilian College of Animal Experimentation (COBEA) and by the Brazilian legislation. All the experiments proposed were approved by the Animal Experimentation Ethics Committee of the Federal University of Piauí (protocol 064/14).

Drug administration

The animals were divided into 5 groups of 3 animals. The EOLO 300 and 2000 mg/kg doses, based on the Organization for Economic Cooperation and Development (OECD) guidelines 423 [19], were used orally and in a single dose for the evaluation of pharmacological screening behavior, occurrence of deaths, water and food intake. The control negative received 0.05% Tween 80 dissolved in 0.9% saline solution and Flumazenil (FLU) 2.5 mg/kg (Sigma Chem. Co., St. Louis, MO, USA) dissolution in distilled water and used as standards (open field test), of the through the same route of administration of the treated groups.

Analysis of physiological habits, weight and organ macromorphological analysis

Changes in the normal activities of the mice and their weights (g) were monitored, in addition to the weight of feed (g) and volume of water (mL) consumed and the weight of excreta (feces and urine) produced (g) daily. After 14 days of observation of the EOLO single dose toxicity assay at doses of 300 and 2000 mg/kg, the animals received ketamine (0.1ml/10g; i.p.) and were euthanized. For this, a macroscopic morphological analysis of the organs was made with the aid of a magnifying glass, in addition to weighing the brain, liver, lung, heart, kidneys, and spleen to determine the weights and verify whether or not there was a macroscopic morphological alteration in the organs evaluated.

Hippocratic Screening

Mice were observed in the first 24 hours and every two days for 14 days to evaluate the "Hippocratic screening", which provided a general estimate of the toxicity of the substance on the conscious state and general mood, activity and coordination of the motor system, reflexes and activities on the central nervous system and on the autonomic nervous system. The parameters analyzed were general appearance, vocal tremor, irritability, touch response, tail tightening response, abdominal writhing, gait, straightening reflex, body tonus, grasping strength, ataxia, tremors, convulsions, stimulation, "Straub" phenomena, sedation, hypnosis and anesthesia.

Acute toxicity study biochemical and hematological parameters

After 14 days of observation, the animals were anesthetized with ketamine (0.1mL/10g, i.p.), followed by the determinations of Resolution 1000/2012 of the Federal Council of Veterinary Medicine and blood collection was performed by puncture of the heart. The blood was conditioned in two types of tube: one with anticoagulant for the determination of hematological parameters, and the other, without anticoagulant, to obtain serum for the evaluation of biochemical parameters.

Predictive general assessment of neurotoxic activity of essential oil *Lippia origanoides* H.B.K. in the Central Nervous System (CNS) in mice

The open field test

The animals' motor activity was verified by means of an open field made of acrylic (transparent walls and black floor, 30x30x15 cm) and divided into 9 equal quadrants, based on the model described by [20]. After 30 minutes of treatments, the animals, one at a time, were placed in the center of the open field where the number of crosses with four legs (spontaneous locomotor activity), number of self-cleaning behavior (grooming) and number of surveys (rearing), without leaning against the wall, were observed during the time of 5 minutes.

Statistical Analysis

The data were obtained as mean \pm standard error of the mean (E.P.M.), and were evaluated by Analysis of Variance (ANOVA) followed by the t-Student-Newman-Keuls test as post hoc. The statistic was performed using the *GraphPad Prism* version 5.01 software.

3. Results

3.1. Characterization of essential oil

The results of chromatographic analyzes of the essential oil of *L. origanoides* H.B.K. carried out in the sample are shown in Table 1 and Figure 1. A variety of constituents were observed, totaling 34 substances representing 99.23% of the total oil. Carvacrol 20.28%, thymol 30.55%, and p-cymene 11.68% corresponded to more than 60% of the total composition, which thus corresponded to the three major compounds.

Table 1. Constituents of the essential oil of *L. origanoides* H. B. K.

Retention Time	Compound	IK (lit.)	IK (calc.)	Area (%)
5,660	α -pinene	939	931	0,70
6,065	camphene	953	946	0,11
7.207	myrene	991	1991	1,61
7.682	α - felandreno	1005	1004	0,16
7,882	Careno	1011	1009	0,35
8,084	α -terpinene	1018	1015	0,21
8,378	p-cymene	1026	1023	11,68
8,555	N.I			
8,607	1,8 cineol	1033	1029	1,49
9,580	γ -terpinene	1054	1056	0,15
10,755	benzene-1-isopropenyl-2-naphthyl	--	1087	0,27
11,121	Linalol	1098	1098	0,76
14,432	4-terpineol	1177	1176	2,76
14,990	α -terpineol	1189	1189	0,33
16,934	Timyl-methyl-ether	1232	1233	5,34
17,328	Carvacrol-methyl-ether	1244	1242	0,35
19,250	Phenol-5-methyl-2-(1-methylether) - thymol	--	1286	0,63
19,628	Thymol	1290	1294	30,55
19,806	N.I			0,40
20, 065	Carvacrol	1298	1304	20,28
22,220	Timila acetate	1355	1356	4,53
22,804	Cycloisotirane	--	1367	0,10
23,007	Carvacrol Acetate	1371	1372	0,70
23,170	α -copaene	1376	1375	1,40
25,078	trans-caryophyllene	1418	1419	6,28
25,176	α -bergamotene	1436	1435	0,46
26,006	2-methoxy-4-ethyl-6-methylphenol	--	1442	0,37
26,510	α -humulene	1454	1454	2,31
27,471	Nafitalene	1441	1476	0,16
27,817	3-tert-butyl-4-methoxyphenol	--	1484	1,29
28,472	α - muurulene	1499	1500	0,25
28,744	β -bisabolene	1509	1508	0,44

29,418	δ -cadinene	1524	1524	0,96
30,780	N.I			
31,859	Caryophyllene oxide	1581	1584	1,49
32,899	Humulene Oxide	1606	1610	0,41
35,429	1,6-dimethylnaphthalene	--	1676	0,19
43,621	N.I			
Total				99,23%

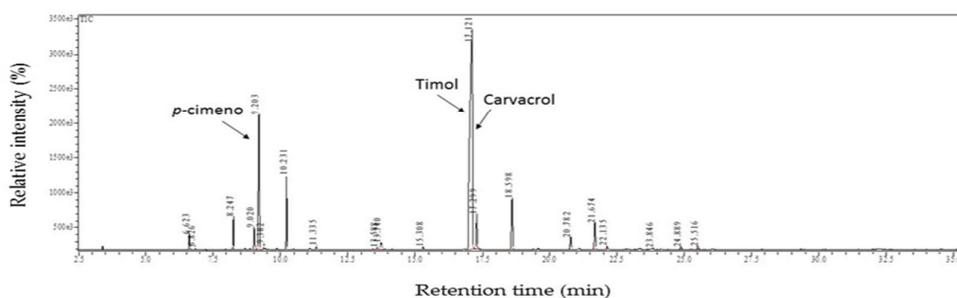


Figure 1. Total ion chromatogram (TIC) of volatile constituents, with majorities identified.

3.2. Anticholinesterase potential of *Lippia organoides* H.B.K. essential oil

3.2.1. Qualitative evaluation of the acetylcholinesterase inhibiting activity

The qualitative analysis revealed that the essential oil of *L. organoides* H.B.K exhibit its positive result for acetylcholinesterase inhibition, as shown in Figure 2.

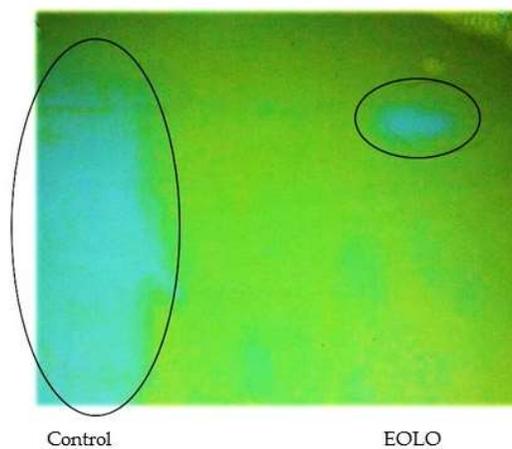


Figure 2. Qualitative result on the acetylcholinesterase inhibitory activity of EOLO.

3.2.2. Quantitative evaluation of the acetylcholinesterase inhibitory activity

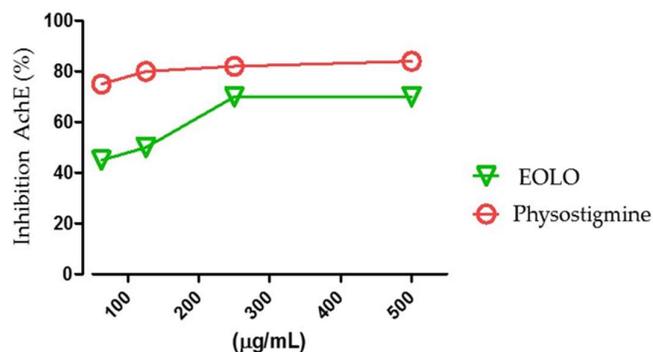


Figure 3. Quantitative evaluation of the anticholinesterase activity of EOLO compared to the standard physostigmine.

The EOLO showed favorable inhibitory potential in the quantitative evaluation when compared to the effects of the standard physostigmine, as per Figure 3. It was testified that the oil at concentrations of 62.5; 125; 250 and 500 µg/mL inhibited AchE activity by 45%, 50%, 70% and 70%, respectively, demonstrating in quantitative terms a potential range of action for this pharmacological activity. EOLO proved active in the quantitative assay by showing an IC₅₀ value of 387.5 µg/mL. However, this result was lower when compared to physostigmine, the drug used as a positive control, which showed an IC₅₀ value of 105.6 µg/mL.

3.2.3. Simulation of the mechanisms of essential oil *Lippia origanoides* H.B.K. on acetylcholinesterase

The docking analysis evidenced the various conformations exhibited by carvacrol, thymol and p-cymene when interacting with AChE. Table 2 presents the values of Gibbs free energy (ΔG) and FullFitness in Kcal/mol observed during the study of the most stable interactions with the E20 and NAG active site regions of AChE. It is possible to note ΔG and FullFitness values equivalent to -6.64 and -1809.77 Kcal/mol for carvacrol, -6.54 and -1807.8 Kcal/mol for thymol; -6.15 and -1797.26 Kcal/mol for p-cymene with the E20 site of the protein structure, while at the NAG site the respective molecules manifested values of -5.70 and -1801.69 Kcal/mol; -5.71 and -1801.54 Kcal/mol; -5.68 and -1794.36 Kcal/mol.

Table 2. Estimated values of binding energy ΔG and FullFitness in Kcal/mol obtained from the interaction between the molecules and the E20 and NAG active sites of AChE.

Molecule	E20		NAG	
	ΔG	FullFitness	ΔG	FullFitness
Carvacrol	-6.64	-1809.77	-5.70	-1801.69
Thymol	-6.54	-1807.83	-5.71	-1801.54
P-cimene	-6.15	-1797.26	-5.68	-1794.36

The Figure 4 announces the pharmacophore map of interact with the E20 functional site by, elucidating the interactions with amino acid residues. Carvacrol and thymol were observed to perform hydrogen bonds with this region of the enzyme. Carvacrol can interact with AChE through hydrogen bridges at Ser203, Pi-akil at Phe338, Trp86, Hsd447, hydrogen-carbon bridge at Gly121, Pi-sigma at Hsd447 and Pi-Pi docking at Trp86 And Tyr337. Similarly, thymol interacts by hydrogen bridges in Ser203 and

Gly122, Pi-akil in Phe338, Hsd447. In turn, p-cymene showed Pi-akil bonds in Phe338 and Hsd447, Pi-Pi docking in Trp86, Tyr337.

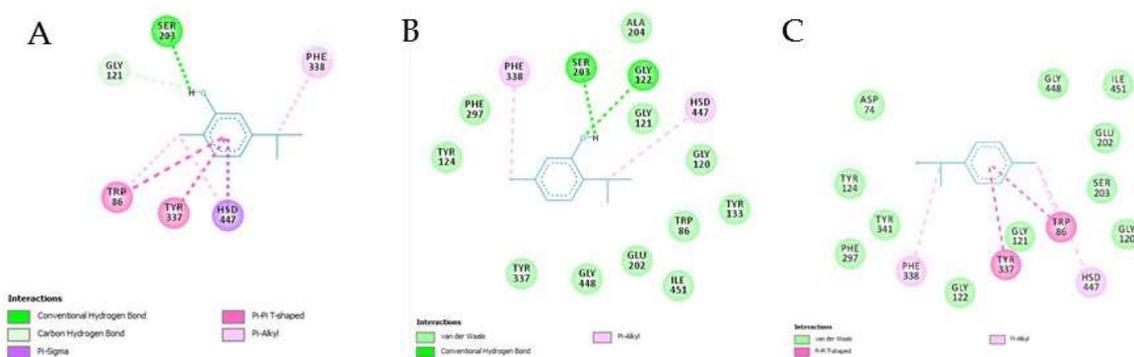


Figure 4. Schematic representations of the molecular interactions of carvacrol (A), thymol (B) and p-cymene (C) with amino acid residues of the E20 site of AchE.

In the NAG binding pocket, the carvacrol molecule demonstrated hydrogen bridge binding in Glu351, Akil and Pi- hydrogen donor in Leu353; thymol showed hydrogen bonds in Phe346, Pi-sigma and Akil in Pro344, and p-cymene interactions Akil in Leu289, Pi-sigma in Glu292 and Pi- hydrogen donor in Gly445. The 2D interaction diagrams and positioning of phytoconstituents in NAG are illustrated in Figure 5.

In pocket E20, carvacrol interacted via hydrogen and Pi-akil bridging with the catalytic triad region (Ser203 and Hsd447), the anion cavity (Gly121) of CAS, the choline binding site (Trp86), and the peripheral region via its binding to Trp86. The thymol molecule demonstrated hydrogen bridges with PAS amino acid residues in the triad and oxyanion cavity (Ser203, Gly122), as well as p-cymene showed interactions with the catalytic cavity and peripheral anion. At the NAG site the molecules did not show binding to key amino acid residues for protein function, but demonstrated effective binding to amino acid residues proximal to the catalytic triad region, acyl binding cavity and PAS. The molecules coupled to Donepezil target amino acid residues (Trp86, Phe338, Tyr337).

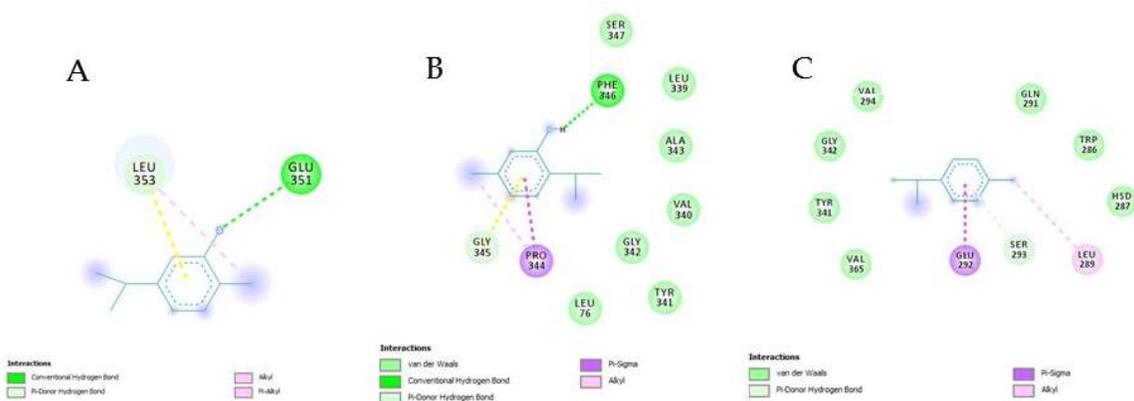


Figure 5. Schematic representations of the molecular interactions of the carvacrol (A), thymol (B) and p-cymene (C) molecules with amino acid residues of the NAG site of AchE.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity test of the essential oil of *Lippia origanoides* H.B.K. - Classroom Test (OECD 423)

The results of the acute toxicity, performed at the intermediate and maximum doses, 300 and 2000 mg/kg, respectively, no deaths of the animals exposed to EOLO were observed in the tested concentrations. In general, no significant changes were observed in the quantitative parameters of water consumption (mL), feed intake (g) and excreta production (g) during the 14-day observation period, as can be observed in Table 3.

Table 3. Effect of oral administration of EOLO in a single dose on the daily physiological habits of the animals.

Groups n = 3	Water consumption (mL)	Feed intake (g)	Excreta production (g)
Control	23,54 ± 0,22	9,39 ± 0,32	3,22 ± 0,22
EOLO 300	18,89 ± 2,22	7,10 ± 1,05	2,61 ± 0,38
EOLO 2000	21,67 ± 0,1	9,54 ± 0,62	2,72 ± 0,49

The results of the weighing showed that there were no statistically significant changes ($p < 0.005$) in this parameter after the acute treatment period or with the increase in the concentration of the administered doses (300 and 2000 mg/kg). The total weight results of the animals are shown in Figure 6.

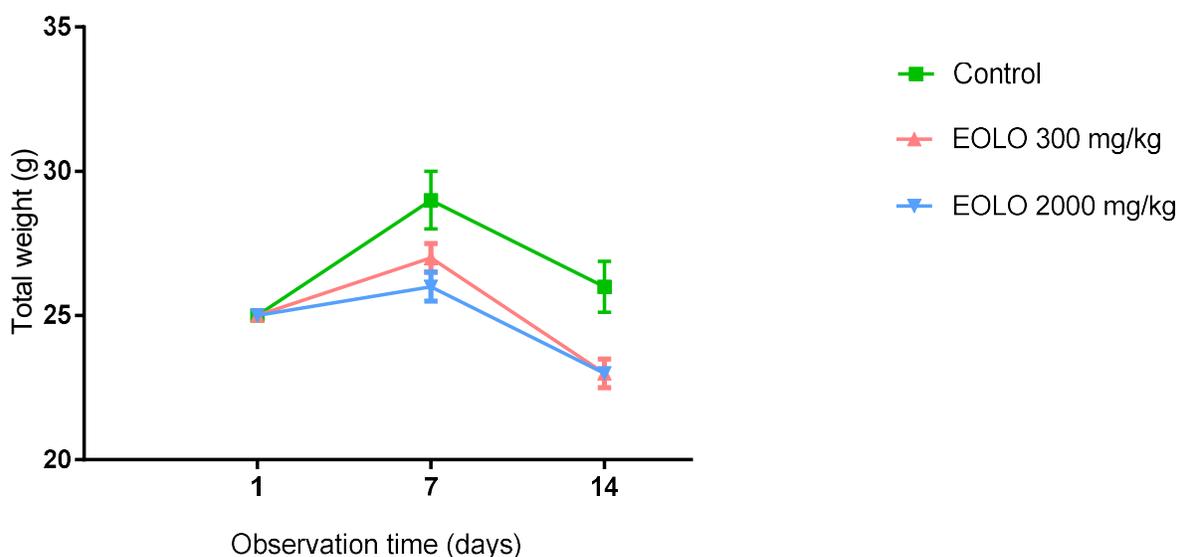


Figure 6. Effect of single dose administration of EOLO on animal body weight. Averages of body weight of mice treated with EOLO observed for 14 days.

There was no statistically significant variation in the organ weights of animals treated with different doses of EOLO compared to the control group after single dose (acute) treatment observed for 14 days (Table 4). In the macroscopic evaluation of the removed and studied organs (brain, spleen, heart, liver, lung, and kidneys) of the animals submitted to a single dose (acute) treatment with EOLO at doses of 300 and 2000 mg/kg, no significant visual changes were observed when compared to the control group, which reinforces the findings in the other parameters regarding the low toxicity of this oil.

Table 4. Weighing of organs treated with *Lippia origanoides* essential oil (EOLO) in acute toxicity.

	Heart	Lung	Kidney	Liver	Brain	Spleen
Control	0,175 ± 0,003	0,422 ± 0,102	0,486 ± 0,117	1,568 ± 0,437	0,478 ± 0,081	0,153 ± 0,028
300 mg/kg	0,140 ± 0,006	0,340 ± 0,089	0,407 ± 0,045	1,332 ± 0,128	0,464 ± 0,031	0,140 ± 0,050
2000 mg/kg	0,166 ± 0,034	0,530 ± 0,293	0,478 ± 0,091	1,615 ± 0,493	0,458 ± 0,038	0,231 ± 0,044

3.3.2. Effects of EOLO on hematological and biochemical parameters of treated mice

The evaluation of the hematological parameters can be observed in Table 5, did not show significant differences when compared with the control, about the parameters: red cell values, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), platelets, leukocytes, neutrophils, eosinophils and lymphocytes. However, animals treated with 300 mg/kg of EOLO had lower levels of neutrophil and platelet concentration than the control ($p < 0.05$).

Table 5. Effects of oral EOLO after single dose administration on hematological parameters of female Swiss mice.

Parameters	Control	EOLO 300	EOLO 2000
Red blood cells (mm ³)	7,23 ± 0,21	6,16 ± 0,12	6,33 ± 0,46
Hemoglobin (g/dL)	9,66 ± 0,08	8,46 ± 0,08	8,7 ± 0,83
Hematocrit (%)	32,46 ± 0,80	27,33 ± 0,18	28,93 ± 1,93
MCV (fL)	45,00 ± 0,81	44,33 ± 0,88	45,66 ± 1,33
MCHC (%)	29,50 ± 0,60	30,83 ± 0,17	29,93 ± 0,78
Platelets (mm ³)	996,5 ± 0,50	913,00 ± 3,00 ^a	975,5 ± 0,50
Leukocytes (mm ³)	4,71 ± 0,07	2,57 ± 0,51	2,93 ± 0,71
Neutrophils (%)	22,33 ± 4,80	11,66 ± 3,66 ^a	21,00 ± 1,00
Eosinophils (%)	1,33 ± 0,33	0,33 ± 0,33	1,33 ± 0,88
Lymphocytes (%)	76,33 ± 5,04	88,00 ± 4,00	78,00 ± 0,57

^a $p < 0.05$ versus Control (ANOVA followed by the t-Student-Newman-Keuls test as *post hoc* test). MCV (Medium Corpuscular Volume); MCHC (Mean Corpuscular Hemoglobin Concentration); Control (0.05% Tween 80 dissolved in 0.9% saline); EOLO (essential oil of *Lippia origanoides* H.B.K. 300 and 2000 mg / kg).

The biochemical parameters presented in Table 6 show no statistically significant differences were observed between the EOLO and the control groups ($p < 0$) in the urea, creatinine, alanine aminotransaminase (ALT) and aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, albumin and globulin parameters ($p < 0.05$). However, lower values of creatinine and alkaline phosphatase were identified in treated animals at a dose of 300 mg/kg compared to the control ($p < 0.05$), as well as a decrease in the ALT value in treated animals at the dose of 2000 mg/kg, in relation to the control ($p < 0.05$). As for AST aspartate, the mice treated with the doses of 300 and 2000 mg/kg presented higher values in comparison to the control negative ($p < 0.05$).

Table 6. Effects of oral EOLO after single dose administration on the biochemical parameters of female Swiss mice.

Parameters	Control	EOLO 300	EOLO 2000
Urea (mg dL ⁻¹)	38,60 ± 4,74	39,30 ± 1,50	28,16 ± 0,67
Creatinine (mg dL ⁻¹)	0,195 ± 0,005	0,105 ± 0,005 ^a	0,25 ± 0,05 ^a
AST (U L ⁻¹)	133,35 ± 11,45	146,50 ± 18,80 ^a	145,05 ± 19,05 ^a
ALT (U L ⁻¹)	57,45 ± 10,65	55,05 ± 0,05	38,30 ± 2,40 ^a
Alkaline Phosphatase (U L ⁻¹)	198,50 ± 0,50	157,50 ± 0,07 ^a	218,7 ± 0,14 ^a
Total protein (g dL ⁻¹)	6,16 ± 0,17	5,90 ± 0,52	6,33 ± 0,67
Albumin (g dL ⁻¹)	2,60 ± 0,17	3,33 ± 0,28	2,76 ± 0,08
Globulin (g dL ⁻¹)	3,56 ± 0,31	2,56 ± 0,62	3,56 ± 0,76

^ap <0.05 versus Control (ANOVA followed by Student-Newman-Keuls t-test as post hoc test). AST (Aspartate aminotransferase); ALT (Alanine aminotransferase). Control (0.05% Tween 80 dissolved in 0.9% saline); EOLO (essential oil of *Lippia origanoides* H.B.K. 300 and 2000 mg / kg).

3.4. General predictive evaluation of essential oil neurotoxicity activity of *Lippia origanoides* H.B.K. (EOLO) in the Central Nervous System (CNS) in mice

3.4.1. Effect of EOLO on the spontaneous movement test (Open Field)

In the analysis of spontaneous locomotor activity (crossing) (Figure 7), the results showed that EOLO did not alter the locomotor activity in any of the doses tested at 300 mg/kg (45.33 ± 2.83) and 2000 mg/kg (52.33 ± 2.50) when compared with the group that only received the vehicle (43.67 ± 1.66). Significant effects were detected with the dose 2000 mg/kg, when compared with to positive control flumazenil (32.63 ± 3.21) [p<0.05], as expected.

The grooming reflex was also analyzed in this test (Figure 8). In this parameter there was no significant change in the groups treated with EOLO at the doses tested 300 mg/kg (6.66 ± 0.70) and 2000 mg/Kg (6.00 ± 0.67) when compared to the group that received only the vehicle (8.83 ± 0.67). Significant effects were detected with the positive control flumazenil (4.12 ± 0.81), when compared with the group that only received the vehicle [p<0.05], as expected.

The third parameter analyzed in this test was the number of rearing under the back legs (Figure 9). The results showed that EOLO in the two doses tested 300 mg / kg (3.66 ± 0.67) and 2000 mg / kg (4.33 ± 0.33) did not change this parameter when compared to the control group (4.0 ± 0.2). Significant effects were detected with the positive control flumazenil (4.12 ± 0.81), when compared with the group that only received the vehicle and the doses tested at 300 mg/kg and 2000 mg/kg [p<0.05], as expected.

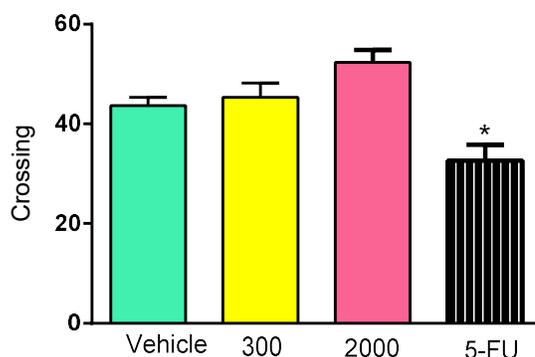


Figure 7. Effect of the essential oil of *Lippia origanoides* H.B.K on the spontaneous locomotor activity (crossing) of the animals in the open field test.

* $p < 0,05$, significantly different from control group and 2000 mg/kg (ANOVA followed by Student-Neuman-Keuls t-test as post hoc test). Vehicle (Tween 80 0.05% dissolved in 0.9% saline); EOLO 300 and 2000 mg/kg). Each column represents the mean \pm E.P.M. (n = 3).

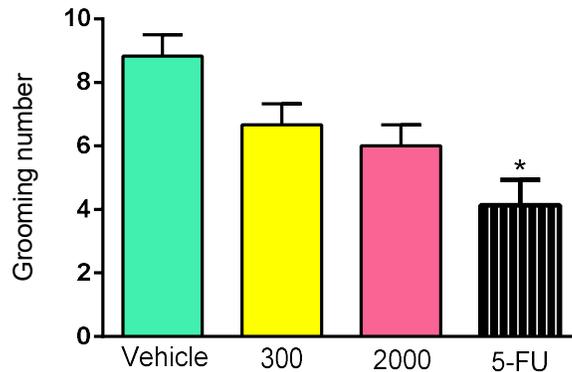


Figure 8. Effect of the essential oil of *Lippia origanoides* H.B.K on the grooming of the animals.

* $p < 0,05$, significantly different from control group (ANOVA followed by Student-Neuman-Keuls t-test as post hoc test). Vehicle (Tween 80 0.05% dissolved in 0.9% saline); EOLO 300 and 2000 mg/kg). Each column represents the mean \pm E.P.M. (n = 3).

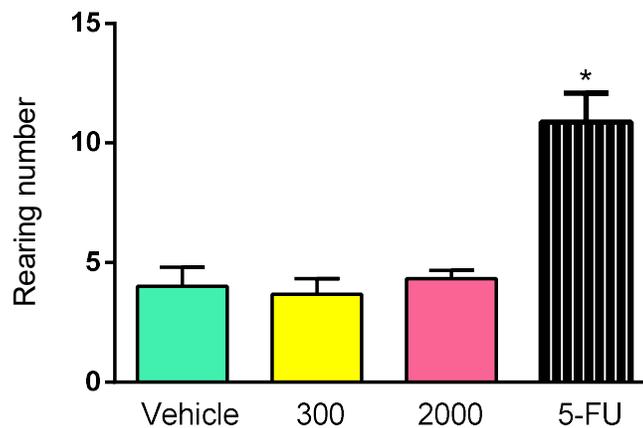


Figure 9. Effect of the essential oil of *Lippia origanoides* H.B.K. about the rearing of animals.

* $p < 0,05$, significantly different from control group, 300 and 2000 mg/kg (ANOVA followed by Student-Neuman-Keuls t-test as post hoc test). Vehicle (Tween 80 0.05% dissolved in 0.9% saline); EOLO 300 and 2000 mg/kg). Each column represents the mean \pm E.P.M. (n = 3).

4. Discussion

Thymol and carvacrol are known for their broad-spectrum antimicrobial activity which has been the subject of some investigations in vitro [21,22] and in vivo [23,24]. Some studies have reported that thymol and carvacrol effectively inhibit the growth of oral pathogens, presenting antibacterial, antifungal activity, potent activity against pro-

tozoa, anticarcinogenic effect, antiproliferative, anti-inflammatory, antiplatelet, antioxidant, larvicidal, and ovicidal activity, with the ability to inhibit the activity of acetylcholinesterase [25,26,27,28,29].

Other studies point out some of the main actions related to p-cymene, of which it is cited the use as a food product, herbicide, besides having antimicrobial activity, anti-inflammatory, antioxidant, antinociceptive, anticarcinogenic agent, gastroprotective among others [30,31,32,33,34,35].

The qualitative analysis revealed that the essential oil of *L. origanoides* H.B.K exhibits positive results for acetylcholinesterase inhibition). This statement is based on the visualization of the CCD plate that showed yellow coloration and white spots that indicate the inhibitory action on AChE, decreased hydrolysis of acetylcholine, and production of thiocolin that prevents the formation of a colored complex of thiocolin with Ellman's reagent [36,17]. Because of this, such a species becomes a candidate for a possible bio-guided study aiming at quantitative investigations as well as the isolation and structural elucidation of the active substances.

The search for AChE inhibitors is of great interest especially in the therapeutics of Alzheimer's disease (AD), the fourth significant cause of death in the world. Costimentally, acetylcholine levels in the synaptic process of AD patients are decreased, which causes reduced cortical cholinergic neurotransmission. AChE inhibition becomes a promising therapeutic approach by propitiating a decrease in the rate of acetylcholine hydrolysis, causing an increase in acetylcholine activity in the synaptic cleft and a cognitive improvement with an increase in central cholinergic function [37]. In light of this, products from natural sources have proven attractive for studies aimed at the treatment of AD because they provide milder side effects at therapeutic doses and thus may contribute to overcoming limitations, high hepatotoxicity, and low bioavailability of drugs that are already used in AD therapeutics [38,39].

Results of previous investigations conducted by Mar and colleagues [40] using the essential oil of *Lippia origanoides* Kunth converge with the observations obtained during the study, demonstrating the antiacetylcholinesterase potential of EOLO with $CI_{50} = 16.93 \mu\text{g} / \text{Ml}$. The significant difference between the reported CI_{50} value and that obtained during the present study ($387.5 \mu\text{g}/\text{Ml}$) may be associated with the distinct abiotic factors (growing conditions, altitude, climate, soil) indicated on the plants, or associated with the harvest time, part of the plant extract, and method of procurement. These factors exert influence on the production and proportion of secondary compounds, reflecting on the biological properties presented by EOLO [41].

The antiacetylcholinesterase potential reported for EOLO may be a consequence of the action of the majority phenolic monoterpenes carvacrol and thymol, which in investigations by Jukic and colleagues [28] performed inhibitory activity on AChE with IC_{50} of $63 \mu\text{g}/\text{L}$ and $740 \mu\text{g}/\text{L}$, respectively. These compounds can act synergistically among themselves or other fractions of oil constituents, producing desirable antiacetylcholinesterase effects [42].

Molecular docking is a technique usually applied in the computational prediction of possible intermolecular interaction modes between a molecule, the ligand, and its receptor [37]. Through docking calculations, it was possible to detect considerable interactions between carvacrol, thymol, and p-cymene with AChE due to the ΔG and Full-Fitness energy values being on a negative scale, which denotes affinity that the molecules exhibit for the E20 and NAG protein regions, as well as the spontaneity of the interaction with these sites. In addition, the values can infer that during the intermolecular interaction process little or no energy expenditure can be demanded because there is a release of energy in the course of these interactions.

In the analysis, carvacrol had a higher docking score than the other structures used as ligands (Carvacrol > Thymol > p-cymene), showing ΔG of -6.64 with E20 and -5.70 with NAG, and the other molecules, values not too far apart. According to literature reports the binding energy of Donepezilla, an AD drug co-crystallized in the protein struc-

ture, is around -11.0 [43,44]. Although the scores of phytoconstituents did not exceed the values of Donepezil, they show interactive potential with AChE.

Based on crystallographic analyses there is a large cavity in the active site of AChE consisting of two distinct binding sites: the Ser-His-Glu catalytic site (CAS) present in the deeper region that is subdivided into catalytic triad (Glu334, Ser203, and His447), acyl binding cavity (Phe297 and Phe295), oxyanion cavity (Ala204, Gly120, and Gly121) and choline-binding site (Trp86), as well as is composed of the peripheral anion site (PAS), present at the entrance of the cavity, consisting of Tyr341, Trp286, Tyr124, Trp86, Tyr72 and Asp74 [45,46].

The presence of a hydroxyl group in the structure of carvacrol and thymol appears to favor interactions and possible inhibition of AChE, especially via hydrogen bridges [28], while the p-cymene benzene ring was responsible for most of the interactions visualized.

Given this, the major phenolics showed the ability to bind to AChE, and may be responsible for the antiacetylcholinesterase potential of the EOLO. Such a claim underscores the attractiveness that phytochemicals present on AD therapeutics since these inhibit AChE and compete with the enzyme substrate, causing stimulation of the cholinergic system, as well as hindering the formation of amyloid fibrils and stable AChE-A β -complexes [46]. In addition, phytochemicals may exhibit greater medicinal benefits as they have been shown to act on the two regions CAS and PAS essential for AChE catalytic activity.

The results of the acute oral toxicity of EOLO can be stated that is greater than 2000 mg/kg, and according to Guia 423 [19] the sample falls under category 5 (Globally Harmonized System standards), which chemicals with relatively low acute toxicity [19].

In the behavioral evaluation regarding the Hippocratic screening performed, the administration of EOLO at doses of 300 and 2000 mg/kg did not change the behavior of the animals in the treated groups when compared to the control group, and no clinical signs of toxicity. Thus, it is suggested that acutely administered EOLO did not present toxicity when administered in a single dose and was therefore orally tolerated in female mice. Although it is a promising result, it should be considered that the observed behavior is part of a preliminary assessment of the possible toxic properties of EOLO and that it provided information about the risks resulting from a single exposure, only in female animals.

Monitoring the animal's body mass is an important indicator for assessing the toxicity of an administered substance. As a general rule, most researchers consider weight gain or bodyweight loss of toxicological significance if the reduction is at least 10% less than the initial weight value of the animal [47,48]. Furthermore, there are no comparative studies on the effects of different dose levels of EOLO in mice in terms of body weight, which reinforces the need for the present study. The investigation of toxic effects, whether single or repeated dose, caused by possibly pharmacological substances, is of great relevance since they can interfere with various biological mechanisms, including the production of blood cells or the injury of noble organs, which reveal an important role in the vital functions of the body [49,50].

The biochemical parameters are essential as they provide information on the major toxic effects on tissues, specifically the effects on the kidney and liver. Some enzymes and proteins may be used to indicate hepatocellular effects (seen in ALT, AST, gamma-glutamyl transferase, and bilirubin). These results suggest a slight alteration of renal and hepatic function, so that the use of EOLO in an acute way may not be hepatotoxic, however, subchronic and chronic toxicity studies should be performed to better investigate its toxicity.

To identify possible changes resulting from the administration of the compound under study, it is necessary to perform behavioral tests. For this, the frequency of locomotion was recorded; which is the act of the animal moving with the trunk away from the ground using coordinated movements of the four legs, performing horizontal displacement on the base of the open field in which the animal has penetrated with all four

legs.

The EOLO at doses used did not interfere in the psychomotor activity, suggesting that it is not able to produce changes on the CNS in the animals, which reinforces the safety of this compound in non-clinical trials [51,52]. Throughout the treatment, and it is possible to suggest that EOLO does not interfere in emotional adaptation to a tense situation, however it should still be better investigated through other methodologies, such as the high cross maze or light / dark box. Suggesting that the oil does not affect motor coordination and maybe devoid of the side effects on the GABAergic system commonly observed in benzodiazepines [53,54].

With this, the search for therapeutic alternatives that are safer, more effective, and less expensive, as well as those that present low toxicity can combine in the search for the decrease in the use of associations, and the introduction of isolated and/or synthetic substances in therapeutics, being their application disseminated in the twentieth century. Thus, the constituents found in essential oils in small quantities are synthesized on a large scale, being used as raw material for research on their pharmacological activities on the central nervous system [55,56,57].

5. Conclusions

The essential oil of *Lippia organoides* HBK exerted an inhibitory effect on acetylcholinesterase by the methods used, this can serve as subsidies for the exploitation of pharmacological effects of the species, suggesting the application of Alzheimer's disease. The acute oral toxicity test classified EOLO as being of low toxicity and no changes in physiological, hematological, and biochemical parameters were observed in single-dose toxicity during the 14 days observation period. These results suggest feasibility for the pre-clinical future studies to complement the evaluation of toxicity in other species for a longer period and with repeated doses.

Supplementary Materials: Figure S1:: Mass spectra of the major compounds of the essential oil obtained by hydrodistillation of the aerial parts of *L. organoides*, Figure S2: Schematic representations of the molecular interactions of carvacrol (A), thymol (B) and p-cymene (C) with amino acid residues of the E20 site of AchE, Figure S3: Schematic representations of the molecular interactions of the carvacrol (A), thymol (B) and p-cymene (C) molecules with amino acid residues of the NAG site of AChE.

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Informed Consent Statement: Not applicable

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