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Essential Oil of *Ocimum basilicum* against *Aedes aegypti* and *Culex quinquefasciatus*: Larvicidal Activity of a Nanoemulsion and In Silico Study

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Abstract: Diseases transmitted by vectors such as *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae) have been responsible for endemics and epidemics in several countries, causing irreparable damage to human health. For this reason, vector control is one of the main strategies to prevent the contamination and dissemination of these etiological agents. In this study, the essential oil (EO) of *Ocimum basilicum* was obtained by hydrodistillation, and the compounds were identified by GC/MS. A nanoemulsion was obtained through a low-energy input method and evaluated by photon correlation spectroscopy. Bioassays were performed against 4th instar larvae of *A. aegypti* and *C. quinquefasciatus*. Furthermore, additional in silico studies of biological activity prediction and molecular docking for the acetylcholinesterase enzyme and juvenile hormone protein targets were performed with the 53 identified compounds of the EO. The major compounds identified in greater quantity were linalool (32.66%) and anethole (32.48%). The obtained nanoemulsion had an average size diameter between 244.6 and 280.4 nm and a polydispersity index below 0.250 during the entire storage period. The nanoemulsion was tested at concentrations of 10, 20, 30, 40, and 50 mg/L and the following activity values were observed: LC_{50} = 42.15 mg/L and 40.94 mg/L and LC_{90} = 50.35 mg/L and 48.87 mg/L for *A. aegypti* (24 h and 48 h); LC_{50} = 39.64 mg/L and 38.08 mg/L and LC_{90} = 52.58 mg/L and 54.26 mg/L for *C. quinquefasciatus* (24 h and 48 h). The in silico results showed better activity values for linalool, anethole, carvone, α-selinene, eugenol, and limonene. The α-selinene compound showed the best binding affinity with the insect acetylcholinesterase enzyme (−9.1 Kcal) in molecular docking, showing the importance of antagonist compounds in elucidating the mechanism of action for the investigated targets. Thus, the studied nanoemulsion was considered active against the tested species, becoming a potential alternative as an ecological bioinsecticide due to bioactivity and simplicity of formulation.

Keywords: natural compounds; volatiles; nanoemulsion; phenylpropanoid; terpene

1. Introduction

Aedes aegypti serves as the primary transmitter of chikungunya, zika, and dengue; *Culex quinquefasciatus* acts as the vector for lymphatic filariasis, encephalitis, and Nile fever, outlining a challenging scenario for public health [1]. Both mosquitoes are widely found in tropical countries since favorable environmental and social conditions (e.g., temperature, poor population education) contribute to their development and proliferation. The lack of vaccines for many of the tropical diseases associated with these mosquitoes makes it necessary to focus on prophylactic strategies to reduce the vector population [2,3].

The predominant strategy for managing *A. aegypti* and *C. quinquefasciatus* mosquitoes entails employing chemical insecticides to curb mosquito proliferation [4]. However, the continued use of synthetic chemicals has become a significant challenge after the emergence of resistant insect populations. For this reason, a great increase in cases of the pathologies transmitted by this vector has been observed, mainly in Brazil and Latin American countries [5,6].

Alternative approaches to vector control have been proposed with different actions and mechanisms. Chemical control of the vectors through utilizing bioactive compounds from plants and tools of medicinal chemistry and molecular modeling leads to promising potential molecules that target insect receptors [7]. This approach is innovative since it allows for the achievement of good alternatives to overcome resistance and reduce damage caused to people and the environment. For example, an extract of the species *Ocimum basilicum* is widely cited in the literature, with promising results [8–10].

The species *O. basilicum* is traditionally used as an analgesic, anti-inflammatory, antimicrobial, antioxidant, anti-ulcerogenic, cardiac stimulant, ad chemo-modulator of ills of the central nervous system (CNS), such as depression, hepatoprotective, hypoglycemic, hypolipidemic, and immunomodulatory properties [9], also as a repellent due to its characteristic odor, and mainly as an insecticide, as shown by studies against several species [11]. It is an herb shrub ranging from 50 to 130 cm in height, with an annual cycle. It can be found in several countries, and the most studied constituent is its essential oil, extracted mainly from leaves and flowers. This species belongs to the Lamiaceae family and is popularly known as "basil" and "alfavaca", widely used in cooking due to its aromatic characteristics and flavor [12].

The essential oil (EO) extracted from the leaves has been analyzed for its larvicidal activity against *A. aegypti*, showing the potential of this EO in blocking the development of vectors [13]. However, it is worth mentioning that the low polarity of EO components makes them unfeasible in an aqueous environment, the commonplace of larvae development, making the technological development of aqueous formulations necessary [14] (Table S1).

In this context, alternatives such as the development of nanoemulsions have been considered to be very promising; since many EO pesticides of natural origin are poorly soluble in water, the development of aqueous nanoemulsions could be a viable alternative, including for the application of EO in aquatic environments [15]. Nanoemulsions represent emerging delivery systems for bioactive compounds, composed of immiscible liquids and often stabilized by one or more surfactants [16].

In this case, they are called oil-in-water nanoemulsions or aqueous nanoemulsions. In addition to the correct choice of surfactants and/or other components, the preparation method is another essential factor for forming nanoemulsions [17]. Taking this into account, nanoemulsions can be generated through either high- or low-energy input methods [18]. The latter entails spontaneous formation or phase inversion to achieve stable systems, yielding outstanding results at a low cost and in an eco-friendly manner. Given that biodegradable ingredients can be utilized to formulate nanoemulsions with natural components, this method plays a significant role in the green concept. Hence, this study aims to assess the larvicidal activity of the aqueous nanoemulsion of *O. basilicum* EO using the low-energy method against *A. aegypti* and *C. quinquefasciatus*.

2. Materials and Methods

2.1. Botanical Material

The *O. basilicum* leaves used in the extraction processes were collected in Macapá (Amapá, Brazil). The collection was carried out during lower rainfall in a family farming area. This specimen was identified by comparison with an authentic sample, incorporating an exsicata in the Amapaense Herbarium of the Institute of Scientific and Technological Research of the State of Amapá (HAMAB/IEPA), under registration number 68922.

2.2. Preparation and Characterization of Botanical Material

The leaf samples of *O. basilicum* were dried in an air-circulation oven for five days at 35 °C and then crushed in a knife mill (Tecnal, model TE-631/3, Piracicaba/SP, Brazil) at a speed of 2251 rpm for 10 min. The moisture content was analyzed by using a moisture analyzer (model IV2500, GEHAKA, Duquesa de Goiás, Real Parque, São Paulo, Brazil).

2.3. Extraction of the EO

The samples were subjected to hydrodistillation in modified Clevenger-type glass systems for 3 h, as proposed by Silva Junir et al., 2021 [19].

2.4. Chemical Composition Analysis

The chemical compositions of the EOs of *O. basilicum* were analyzed using a Shimadzu GCMS-QP2010 gas chromatography system equipped with an Rtx-5MS capillary column (30 m × 0.25 mm; 0.25 µm film thickness) (Restek Corporation, Bellefonte, PA, USA) coupled to a mass spectrometer (GC/MS) (Shimadzu, Kyoto, Japan), as proposed by Silva Junir et al., 2021 [19]. The components were identified by comparing (i) the experimental mass spectra with those compiled in libraries (reference) and (ii) their retention indices to those found in the literature [20,21].

2.5. Preparation of Nanoemulsions

The nanoemulsion was crafted through a low-energy method. The oil phase comprised EO and the surfactant, and it was formulated by combining these components and subjecting them to intense stirring for 2 min using a vortex stirrer (model AP59-Phoenix). The total mass reached 4 g, consisting of 5% (*w*/*w*) *O. basilicum* EO, 5% (*w*/*w*) polysorbate 20, and 90% (*w*/*w*) deionized water.

The deionized water was added dropwise while continuously stirring the system, and after its complete addition, the nanoemulsion was stirred for 2 min. The nanoemulsion was stored in a screw-top glass vial at room temperature (20 ± 2 °C) for further characterization.

2.6. Physicochemical Characterization of Nanoemulsion

The droplet size distribution (droplet size and polydispersity index) and zeta potential of the nanoemulsion were determined using a Zetasizer ZS (Malvern, UK). Prior to each analysis, it was diluted with deionized water (1:20) and evaluated after 0, 1, and 14 d of preparation. The measurements were performed in triplicate, and the results were expressed as the mean ± standard deviation.

2.7. Larvicidal Bioassay

The *A. aegypti* (Rockefeller strain) and *C. quinquefasciatus* (Macapá strain) larvae were obtained from the Arthropod Laboratory of the Federal University of Amapá. The biological assay was performed under controlled conditions, with a temperature of 25 ± 2 °C, relative humidity of 75 ± 5%, and a photoperiod of 12 h. The experimental protocol was performed according to WHO (2005) with modifications. All experiments were performed in quintuplicate with 10 larvae in the fourth-young stage for each replicate, using the nanoemulsion of *O. basilicum* diluted at 10, 20, 30, 40, and 50 mg/L (expressed as essential oil content), for both *A. aegypti* and *C. quinquefasciatus*. The negative control was performed with distilled

water and 50 mg/L of polysorbate 20. The mortality was recorded after 24 h and 48 h of treatment. The positive controls were Pyriproxyfen (0.01 mg/L) and Temephos (3 µg/mL).

2.8. Statistical Analysis

The statistical analysis was conducted using GraphPad Prism 6.0 (San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests was used to compare the mortality among control groups and treated groups. A paired *t*-test was used to compare the mortality recorded between different exposure periods. Differences were considered significant when $p < 0.05$. Probit analysis was performed with a 95% confidence interval to determine the Chi-squared test and estimate the LC_{50} and LC_{90} using the software Statgraphics Centurion XV version 15.2.11 (Statpoint Technologies, Inc., Warrenton, VA, USA).

2.9. In Silico Analysis

2.9.1. Prediction of Biological Activity

When identifying the molecules of the constituents of the essential oil *O. basilicum*, the spectra of biological activities were predicted through the program PASS (Prediction of Activity Spectra for Substances), which has in its database about 250,000 compounds from the NCI Open Database, making predictions through complex surveys that combine probability ranges of active or inactive compounds, calculated to be revealed (Pa). The probability of not being revealed (Pi) ranges from 0 to 1, which is researched through parameters of ranges of the physical–chemical value, presence or absence of specific substructural fragment, and other research criteria [22].

2.9.2. Selection of the Proteins and Ligand Structures

The molecules with the best PASS results (activity spectrum prediction for substance) were selected, and the docking study with AutoDock 4.2/Vina 1.1.2 through the Pyrx graphical interface (version 0.8.30) was prepared using the Discovery Studio software 5.0. To validate the molecular docking method, compounds with crystallographic information were submitted to docking development until the spatial conformation was found using the AutoDock 4.2/Vina 1.1.2 software [23].

Macromolecular crystallographic structures were used with resolutions of 2.7 Å, 2.4 Å, and 1.87 Å, respectively, with two acetylcholinesterase enzymes (PDB 1QON) from *Drosophila melanogaster* in complex with tacrine [(9-3-iodobenzylamino)-1,2,3,4-tetrahydroacridine] (I40), and one of the recombinant humans (PDB 4EY6), complexed with (-)-galantamine (GNT), and also of the juvenile hormone protein (PDB ID 5V13) from *A. aegypti*, in complex with (2E, 6E)-9-[(2R)-3,3-dimethyloxyran-2-yl]-3,7-dimethylnone-2,6-methylene also using dienoate (JHIII), and ligands were used as positive controls of references of macromolecules I40, GNT and JHIII, and the commercial larvicide temephos as a positive control in the molecular docking study based on the standard protocol established by our group [24,25].

The validation of the ligand's molecular fit was performed by comparing the position of the crystallographic inhibitor and the position obtained for the same inhibitors (PDB ID structures: 1QON, 4EY6, and 5V13), based on the RMSD value (Table 1).

Table 1. Data from protocols used in the molecular docking validation.

Receptor	Ligand	Ligand Coordinates of the Grid Center	Grid Size (Points)
AChE (PDB ID 1QON)	9-(3-Iodobenzylamino)-1,2,3,4-tetrahydroacridine	X = 33.0302 Y = 67.5629 Z = 09.9828	25 x 23 y 12 z
AChE (PDB ID 4EY6)	(–)-Galantamine	X = 09.0708 Y = 60.8558 Z = 23.9190	29 x 18 y 12 z
Juvenile hormone (PDB ID 5V13)	Methyl(2E,6E)-9-[(2R)-3,3-dimethyloxiran-2-yl]-3,7-dimethylnona-2,6-dienoate	X = 251.9827 Y = 09.5063 Z = 353.313	34 x 25 y 19 z

2.9.3. Molecular Binding Affinity Statistics

In order to evaluate the affinity of the compounds, the data at each position calculated by the Pyrex software were typed in an Excel spreadsheet and analyzed by the one-way ANOVA followed by the Turkey test (5%); multiple comparisons test was performed using GraphPad Prism version 7.00 (GraphPad Software, La Jolla, CA, USA).

3. Results and Discussion

3.1. Chemical Composition

The yield obtained by the hydrodistillation technique was 0.6% (*w*/*w*) of *O. basilicum* EO. A similar result was found for the species in Oman, in the Middle East. According to Al Abbasy et al. (2015), it was 0.6% [26], while Sharopov et al. (2016) [27] obtained a yield of 0.5%, 0.6%, and 0.7% in different regions of Nepal, Tajikistan, and Yemen. Sajjadi [28] (2006) reported an essential oil yield of 0.5% in Iran. The yield *O. basilicum* depends on the genotype, and the result obtained in the present study is, therefore, as in the literature above, while others can reach 1.92% [29].

In addition to the characteristics of the subtypes, other factors influence the determination of the yield, such as seasonal variations, in which the distilled essential oil and its oil content varied from 0.5% to 0.8%. The maximum amounts were observed in winter and the minimum in summer. Another influencing factor is the time of harvest, where the highest yields of essential oil were obtained between 8 am and 12 pm. The severity of water stress and drying temperature (40 °C), as well as the plant part (aerial part, e.g., leaves, flowers, or stems), also influences phytochemicals [30,31].

In the phytochemical analysis of the species *O. basilicum* (Table 2), it presented 53 compounds, for which two compounds were identified as major, the monoterpene linalool (32.66%) and the aromatic compound anethole (32.48%); we believe that the results are correlated to the period of collection during a period of lower rainfall (Figure 1).

Table 2. Chemical composition of *Ocimum basilicum* essential oil.

RI_a	Constituents	Concentration (%)
831	3-hexanone	0.09
833	2-hexanone	0.12
835	3-hexanol	0.05
837	2-hexanol	0.07
867	Trans-2-hexenal	0.21
868	*Cis*-4-hexenol	0.21
925	α-thujene	0.19
932	α-pinene	0.61
947	Camphene	0.04
972	Sabinene	0.26
976	β-pinene	0.77
988	Myrcene	0.54
1004	4-carene	0.05
1015	α-terpinene	0.20
1023	p-cymene	0.24
1027	Limonene	6.65
1030	Eucalyptol	8.64
1034	*Trans*-β-ocimene	0.10
1044	β-ocimene	0.66

Table 2. *Cont*.

RI_a	Constituents	Concentration (%)
1056	γ-terpinene	0.48
1065	Sabinene hydrate	0.07
1087	Terpinolene	0.20
1099	Linalool	32.66
1144	Camphor	0.61
1166	δ-terpinolene	0.20
1177	L-4-terpineol	3.38
1190	α-terpineol	1.02
1199	Anethole	32.48
1210	Acetic acid	0.06
1219	*Trans*-carveol	0.05
1230	*Cis*-carveol	0.05
1243	Carvotanacetone	3.41
1286	Bornyl acetate	0.03
1337	Carveol acetate	0.04
1358	Eugenol	0.13
1385	β-bourbonene	0.05
1391	β-elemene	0.40
1420	*Trans*-caryophyllene	0.64
1435	*Cis*-α-bergamotene	0.06
1439	α-guaiene	0.08
1455	*Trans*-β-bergamotene	0.89
1482	β-cubebene	0.23
1487	β-selinene	0.78
1496	α-selinene	0.82
1506	δ-guaiene	0.28
1515	γ-cadinene	0.16
1562	*Trans*-nerolidol	0.07
1615	Epicubeol	0.10
1641	δ-cadinol	0.73
1651	β-eudesmol	0.10
Monoterpene hydrocarbons		11.26
Oxygenated monoterpenes		49.89
Sesquiterpene hydrocarbons		4.39
Oxygenated sesquiterpenes		1.00
Phenylpropanoids		32.61
Others		0.81
Total		99.96

RI_a: The Kovats Retention Indices were calculated from our analysis concerning a series of n-alkenes.

Other phytochemical components found in the EO of the species *O. basilicum* in moderate to minimum amounts, such as eugenol (0.13%), limonene (6.65%), alpha-selinene

(0.82%), and beta-selinene (0.78), are associated with several biological activities, including insecticides, larvicides, herbicides, and others, such as insulation of cDNA isolation, antioxidants, and resistance to fungal pathogens [32,33].

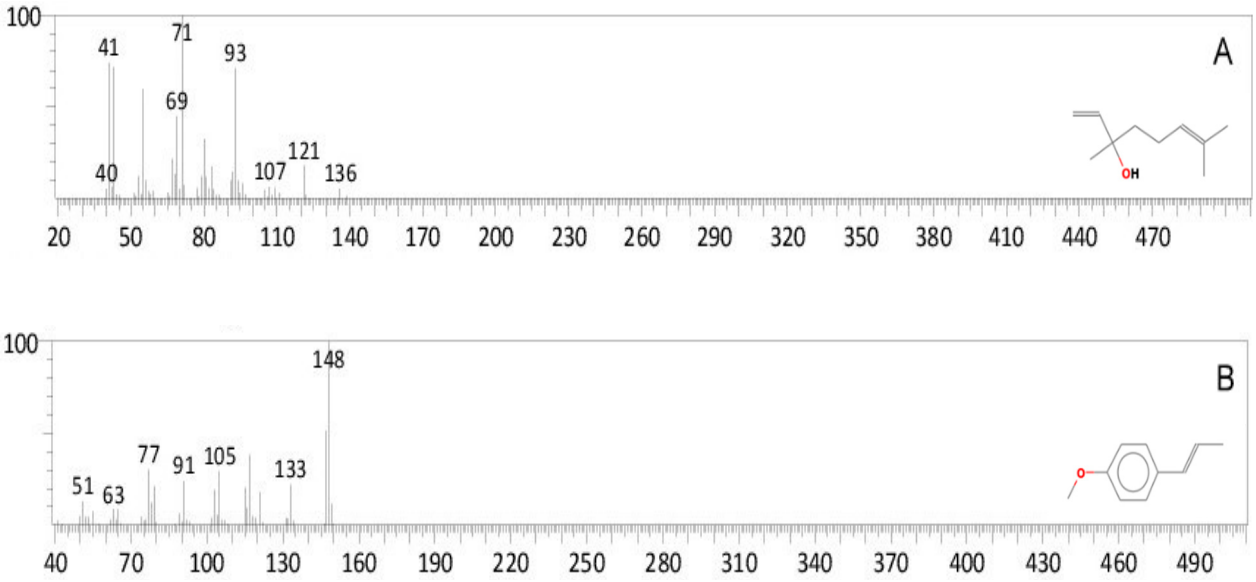


Figure 1. Mass spectrum of the two major compounds of *O. basilicum*: (**A**) linalool and (**B**) anethole.

When the phytochemical components were analyzed, the composition showed major components being constituted by the compounds of linalool and anethole, which has been observed in studies with biological functionality and activities. Linalool is a component found in most *O. basilicum* [29] subtypes and a highly desired component in the food industry as medicinal ingredients, flavors, fragrances for perfumes, household products, such as soaps, detergents, shampoos, and lotions, and serves as a valuable reagent in the synthesis of vitamin E [34].

It is reported in the literature that linalool showed activity against *A. aegypti* at different stages of development. In contrast, anethole showed action against *Ceratitis capitata, Sitophilus oryzae*, *Callosobruchus chinensis*, *Lasioderma serricorne*, *Bactrocera dorsalis*, *Bactrocera cucurbitae*, and *Aedes caspius* [35,36]. Segundo Hashem (2018) [37], who studied nanoemulsions of the EO of *Pimpinella anisum* against *Tribolium castaneum*, observed anethole impregnation with nanoemulsion particles through the cuticle of *T. castaneum* adults and was able to exert profound effects on insects.

3.2. Preparation and Physical Characterization of Nanoemulsion

The nanoemulsion prepared with EO of *O. basilicum* with a value of 16.5 of hydrophilic-lipophilic balance (HLB) presented a milky bluish and fluid aspect without sedimentation or phase separation. It was observed that nanoemulsions showed physico-chemical characterization when prepared with polysorbate 20 as a surfactant, generating more excellent stability after the first to the fourteenth days of preparation; the potential zeta showed that its surface load was negative, ranging from −15.7 to −18.6. However, the droplets had average sizes below 300 nm, and the polydispersity index was found below, as shown in Table 3 and Figure 2.

Table 3. Physicochemical characterization of nanoemulsions containing *O. basilicum* EO.

Days	Size (nm)	Polydispersity Index	Zeta Potential (mV)
D0	278.3 ± 1.102	0.231 ± 0.021	−18.6 ± 0.265
D1	244.6 ± 3.102	0.152 ± 0.022	−17.1 ± 0.0577
D14	280.4 ± 2.957	0.209 ± 0.015	−15.7 ± 0.289

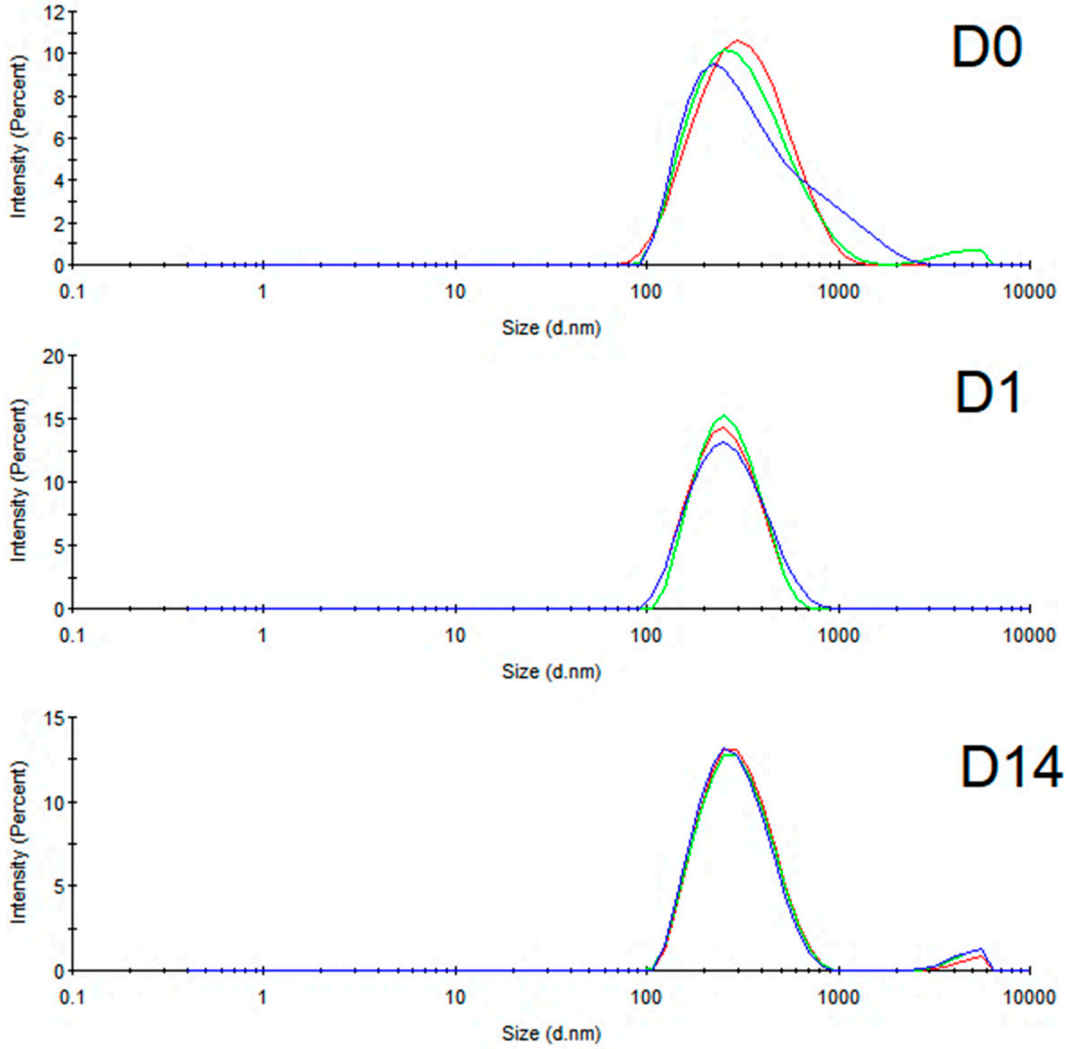


Figure 2. Droplet size distribution of the nanoemulsion prepared with *O. basilicum* and polysorbate 20. Mean droplet size: Day D0: 278.3 ± 1.102 nm; Day D1: 244.6 ± 3.102 nm; Day D14: 280.4 ± 2.957 nm. Polydispersity index: Day D0: 0.231 ± 0.021; Day D1: 0.152 ± 0.022; Day D7: 0.209 ± 0.015.

When examining the droplet size of the *O. basilicum* essential oil nanoemulsions, according to the graphs in Figure 2, after one day of storage (day 1), a narrower base of the curve was observed when compared to the day of preparation (day 0). Also, the small, abundant peak related to larger droplets, observed on day 0, disappeared after one day of storage.

This behavior explains the reduction in the polydispersity index and the parameter related to homogeneity in the droplet size distribution, and the low values are associated with monodispersed systems. Then, after 14 days of storage (day 14), even the most abundant droplet population remained narrow, and larger particles were generated, responsible for the slight increase in the polydispersity index.

The surfactant polysorbate 20 was used at a concentration of 5%; in this concentration, it can favor an increase in the size of droplet nanoemulsions in the range of 120 to 505 nm when there is an increase in the concentration of the surfactant from 0.75 to 5% (w/w). The excess of surfactant can cause flocculation in oil droplets generated by the depletion effect; the presence of surfactants in the micelles of the aqueous phase can cause the exclusion of surfactant molecules between the oil droplets when they are at a distance less than the diameter of the micelles of the surfactant due to osmotic pressure and eventually cause the flow of particles [38,39].

The dynamic light scattering analysis showed that the droplets had an average size of about 268 nm during the 14 days, in which they were divided into times, D0, D1, and D14, while the polydispersity index was around 0.221. After one day of storage, a slight size reduction was observed, accompanied by a decrease in the polydispersity index. After 14 days of storage, the size and polydispersity index returned to values close to those observed on the day of preparation. The importance of the zeta potential did not change significantly.

Concerning the physical–chemical characterization, when observed, the nanoemulsion containing EO *O. basilicum* showed a bluish reflection, which is characteristic of this type of colloidal system consisting of the Tyndall effect, making it a valuable macroscopic indicator of the generation of droplets to be analyzed. The average droplet in this study was 268.0 ± 2.387, so the variation in the average size of an adequate nanoemulsion must have a size between 20 and 500 nm and also contain kinetic stability and transparency for the criteria to be obeyed [40].

Although the size of the nanoemulsions is still in agreement with studies on the characterization of a nanoemulsion, despite this, still above expectations, it would be below 100 nm. Possibly, the increase in the size of the droplets depends directly on some physical–chemical variables (nature of the components, composition, temperature, and pressure), as well as related to the preparation: concentration of the surfactant, the addition of the reagents, interfacial tension oil/water. However, the droplets remained stable during the analyzed period and in determining the various properties [40,41].

The values of the polydispersity index in this work showed that an average of 0.177 ± 0.058 indicates kinetic stability, with an almost monomodal distribution. However, a change was observed during the analysis of the days since the border between the two regions is quite sharp, mainly caused by a "transient polydispersity", which, possibly due to the osmotic swelling, is still incomplete due to its high viscosity, and the micelles remain fixed as they swell with the aqueous phase [40,41], considering the amplitude of the repulsions due to the high concentration of counter ions.

Corroborating these data, the zeta potential showed an opposing average of −17.1 ± 0.204. However, the surfactant used was the non-ionic polysorbate 20, demonstrating adequate stability even during prolonged storage, which, according to the literature, is −30 mV to 30 mV, and the negative zeta potential is due to the adsorption of anionic species derived from materials used to produce nanoemulsions, such as free fatty acids (COO-) in oils or hydroxyl ions (OH-). However, the size of the droplets showed little variation, and the drastic behavior of a decrease and initial increase influenced this variation at higher temperatures, which may be due to the better solubilization of the compounds in the external phase and/or to the volatilization.

We can analyze the influence of temperature on the size of the nanoemulsion droplets, as shown in Figure 3. There was a slight decrease in the size of the nanoemulsion in the increase in temperatures in a range of 25–50 °C; from this temperature to 60 °C, the size was maintained. After this temperature, it tended to decrease, with a notable reduction in the average size after 70 °C, reaching a minimum value at the end of the analysis, confirming the correlation between the temperature and the size of the nanoemulsions [42,43].

According to the literature, the heat treatment to evaluate the alteration in the system depends on the concentration of the surfactant, which shows that the heated systems remain optically opaque and with high turbidity at a concentration of 0.5 to 10% w/w of surfactant; however, when the system has 1 to 4% w/w of surfactant, a layer of cream on top of a milky white part is characteristic, generating large droplets that, in diffused light, migrate to cream quickly [42,43].

The surfactant used in this study was polysorbate 20, a non-ionic small-molecule surfactant, which contains a hydrocarbon tail composed of 12 carbon atoms, performing symmetrical correspondence when combined to generate a monophasic region [44]; in this case, it is the monoterpene structures that have ten carbons in their isoprenoid chain, found as major compounds in *O. basilicum* oil, generating nanoemulsions with larger sizes.

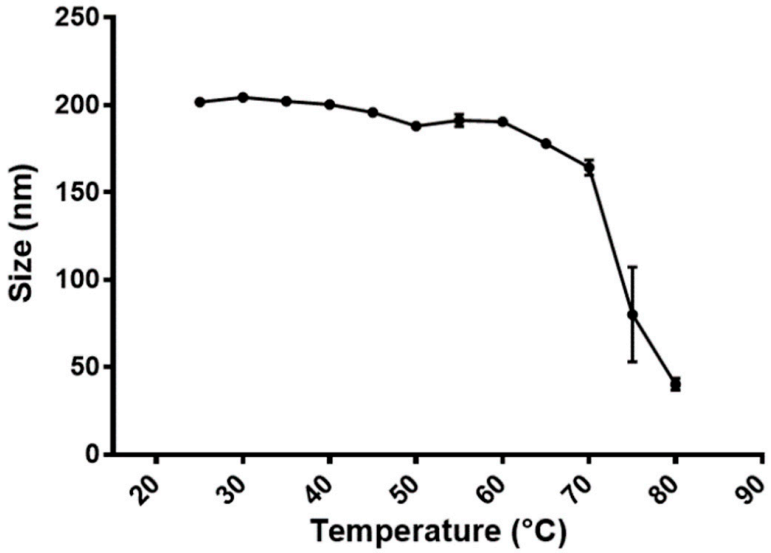


Figure 3. Influence of temperature on droplet size of the nanoemulsion prepared with EO of *O. basilicum*. Each measurement represented mean ± standard deviation.

According to Ostertag (2012) [45], the size of the droplets depends on some factors. First, for the type of oil, in this case, the essential oil *O. basilicum* has a majority of phytochemical composition monoterpene components, which have an isoprene chain of 10 carbons, according to a type of surfactant. Polysorbate 80 < polysorbate 20 < polysorbate 85, polysorbate 20 was used in this study, and the third was the concentration of surfactant, which was 5% and the fourth location of the surfactant: surfactant initially in oil < surfactant initially in water. All of these contributed to the formation of the size of the nanoemulsions on the studied days.

Therefore, the growth in the size of the droplets is directly related to an increase in temperature, causing the bioactivity to be impaired; maintaining the droplet size up to 60 °C can be an advantage for applications in the field of larvicidal nanoemulsions; potentially, they would remain in the environment properly. Therefore, in general, considering that in this study, most droplets remained the same size (268.0 ± 2.387 nm) and had droplet distribution profiles and a polydispersity index similar to the limits observed in the literature for stable systems, they are suitable for in vivo use and in vitro studies and as for potential larvicidal activity.

3.3. Larvicidal Bioassay

According to the biological assay of larvicidal activity of the nanoemulsion prepared with *O. basilicum* essential oil against *A. aegypti*, there was no mortality in the control group and treated at 10 mg/L after 24 and 48 h of exposure. A percentage of mortality higher than 50% was observed only in the group treated with the nanoemulsion at 50 mg/L (98.0 ± 4.472% in 24 h and 100 ± 0% in 48 h) (Table 4).

Regarding the mortality recorded for every experiment (24 and 48 h) in *Ae. aegypti*, there was a significant difference between the control group and the groups treated for periods of 24 h (F = 228.1, df = 6 and $p < 0.0001$) and 48 h (F = 228.1, df = 6 and $p < 0.0001$), being that this difference is related to concentrations of 40 and 50 mg/L, see Table 3. The mortality in the treatments showed no significant difference (t = 1.238, df = 4, and $p = 0.2835$) between 24 and 48 h of exposure.

Table 3 shows the larvicidal activity of the nanoemulsion prepared with *O. basilicum* essential oil against *C. quinquefasciatus*. There was no mortality in the control group after 24 and 48 h of exposure. A percentage of mortality higher than 50% was observed only in the group treated with the nanoemulsion at 50 mg/L (86 ± 11.40% in 24 h and 90 ± 10% in 48 h).

Table 4. Mortality levels (±SD) after treatment of *A. aegypti* and *C. quinquefasciatus* four-instar young with nanoemulsion of the *O. basilicum* essential oil.

Concentration (mg/L)	*Aedes aegypti*		*Culex quinquefasciatus*	
	Mortality (%)			
	24 h	48 h	24 h	48 h
10	0 ± 0 a	0 ± 0 a	0 ± 0 a	4 ± 0.548 a
20	2 ± 0.447 a	2 ± 0.447 a	0 ± 0 a	4 ± 0.58 a
30	4 ± 0.548 a	4 ± 0.548 a	26 ± 0.548 b	30 ± 10.00 b
40	20 ± 10.00 b	30 ± 12.25 b	44 ± 15.17 c	44 ± 15.17 b
50	98 ± 4.47 c	100 ± 0 c	86 ± 11.40 d	90 ± 10.00 c
C1	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
C2	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
Positive Control *	100 ± 0.00 a	100 ± 0.00 a	100 ± 0.00 a	100 ± 0.00 a
Positive control **	100 ± 0.00 a	100 ± 0.00 a	100 ± 0.00 a	100 ± 0.00 a

C1: Negative control with distilled water. C2: Negative control with polysorbate 20 at 50 mg/L. Different letters at the same line indicate a statistically significant difference (Bonferroni's multiple comparison test, $p < 0.05$). Pyriproxyfen (0.01 mg/L) *; Temephos (3 µg/mL) **.

The mortality was recorded in every experiment (24 and 48 h) in *Cx. quinquefasciatus*, and there was a significant difference between the control group and the groups treated for periods of 24 h (F = 97.69, df = 6 and $p < 0.0001$) and 48 h (F = 80.26, df = 6 and $p < 0.0001$), being that this difference is related with concentrations of 30, 40, and 50 mg/L (Table 3). The mortality obtained in the treatments showed a significant difference (t = 4.0, df = 4, and $p = 0.0161$) between 24 and 48 h of exposure.

After 24 h of treatment of *A. aegypti* larvae with the nanoemulsion tested, the Probit analysis of the data indicated that the percentage of deviance explained by the model was 89.8832%, and the adjusted percentage was 79.5538%. The *p*-value for the model and *p*-value for the residuals were, respectively, <0.0001 and 0.2705. The Chi-squared test ($\chi2 = 34.8066$, df = 1, and $p < 0.0001$) indicated that mortality is dose-dependent with concentration. The estimated LC_{50} and LC_{90} values with the lower and upper confidence limits are, respectively, 42.1538 (37.795–46.8778) and 50.3545 (45.9357–62.8197) mg/L (Table 5). After 48 h of treatment of *A. aegypti* larvae with the nanoemulsion tested, the Probit analysis of the data indicated that the percentage of deviance explained by the model was 91.3904%, and the adjusted percentage was 81.6312%.

Table 5. Estimative of Probit analysis of LC_{50} and LC_{90} against *A. aegypti* and *C. quinquefasciatus* larvae in bioassays containing nanoemulsion of *O. basilicum*.

Species	Time (Hours)	LC_{50} (mg/L)	LC_{90} (mg/L)	$\chi2$	*p* Value
Aedes aegypti	24	42.15 (37.79–46.87) *	50.35 (45.93–62.81) *	34.80	<0.0001
	48	40.94 (36.64–45.42) *	48.87 (44.63–60.28) *	37.45	<0.0001
Culex quinquefasciatus	24	39.64 (34.52–46.00) *	52.58 (46.16–69.15) *	27.48	<0.0001
	48	38.08 (32.36–45.55) *	54.26 (46.47–74.23) *	23.54	<0.0001

$p > 0.0001$ = represents heterogeneity in the population of tested larvae. * Confidence Limits (95%). $\chi2$ = Chi square.

The *p*-value for the model and *p*-value for the residuals were, respectively, <0.0001 and 0.3170. The Chi-squared test ($\chi2 = 37.458$, df = 1, and $p < 0.0001$) indicated that mortality is dose-dependent with concentration. The estimated LC_{50} and LC_{90} values with the lower and upper confidence limits are 40.9486 (36.642–45.4246) and 48.8715 (44.6334–60.2878) mg/L.

After 24 h of treatment of *C. quinquefasciatus* larvae with the nanoemulsion tested, the Probit data analysis indicated that the percentage of deviance explained by the model was 95.4732%, and the adjusted percentage was 81.5795%. The *p*-value for the model and *p*-value for the residuals were, respectively, <0.0001 and 0.7284. The Chi-squared test ($\chi 2 = 27.4869$, df = 1, and $p < 0.0001$) indicated that mortality is dose-dependent with concentration. The estimated LC_{50} and LC_{90} values with the lower and upper confidence limits are 39.6465 (34.5242–46.0088) and 52.5835 (46.1684–69.1584) mg/L. After 48 h of treatment of *Cx. quinquefasciatus* larvae with the nanoemulsion tested, the Probit data analysis indicated that the percentage of deviance explained by the model was 93.3927%, and the adjusted percentage was 77.526%.

The *p*-value for the model and *p*-value for the residuals were, respectively, <0.0001 and 0.6446. The Chi-squared test ($\chi 2 = 23.5443$, df = 1, and $p < 0.0001$) indicated that mortality is dose-dependent with concentration. The estimated LC_{50} and LC_{90} values with the lower and upper confidence limits are 38.0837 (32.3653–45.5584) and 54.2618 (46.475–74.2333) mg/L.

In larvicidal activity, according to Cheng et al. (2003) [46], essential oils with $LC_{50} > 100$ mg/L are considered inefficient, those with $LC_{50} < 100$ mg/L are deemed efficient, and those with $LC_{50} < 50$ mg/L are considered highly efficient. Consequently, the nanoemulsion can be regarded as promising according to literature data.

When comparing the larvicidal activity obtained with the data in the literature, it is possible to observe that the results obtained in this study were significant. According to a study by Sundararajan et al. (2018) [41], where they prepared a larvicidal nanoemulsion with *O. basilicum* essential oil against *C. quinquefasciatus*, the following LC_{50} and LC_{90} values were reported according to different larval instars, as follows: second instar (LC_{50} = 36.53 mg/L and LC_{90} = 100.03 mg/L) and third instar (LC_{50} = 38.89 mg/L and LC_{90} = 105.94 mg/L). Although the LC_{50} values after 24 h of treatment were similar to those observed in the present study, the LC_{90} values were two-times higher for the same species.

The larvicidal activity of *Citrus sinensis* EO and R-limonene complexes at a concentration of 30 ppm induced 100% mortality of *A. aegypti* larvae after 24 h and their LC_{50} = 21.5 ppm) and R-limonene at 50 ppm (LC_{50} = 26.8 ppm). In a study by Silva (2008) [47], R-limonene reached an LC_{50} 37 $\pm$ 2.08 ppm. Ramos (2016) [48] demonstrated that the essential oil of *O. basilicum* against *A. aegypti* obtained LC_{50} = 75.58 mg/L after 24 h of treatment and 67.22 mg/L after 48 h.

Another study aimed to generate a larvicidal nanoemulsion prepared with the EO of *O. basilicum* against *A. aegypti*. Although this study used polysorbate 20, the same non-ionic surfactant used to prepare the larvicidal nanoemulsion in the present study, the nano-emulsification method involves high-energy consumption and expensive equipment. However, no information on EO or surfactant concentration has been reported, and mortality is attributed to ready-made "double dilutions" [49,50]. In addition, it would be expected that a larvicidal bioassay using larvae before the third stage would lead to lower values of LC_{50} and/or LC_{90}, which was not observed when compared to this study. This more prominent larvicidal activity may result from a more monomodal droplet size distribution, as larger droplets can affect larvicidal activity.

Another advantage of the method used in the present study was the use of a superior hydrophilic surfactant that coincided with the HLB value needed for the EO of *O. basilicum*. In this context, alternatives such as the development of nanoemulsions have been considered very promising, including the use of *O. basilicum* EO as the best larvicidal agent [51]. This also indicates that oil nano-emulsification increased larvicidal activity, in keeping with the theoretical advantages of nanoemulsions.

The nanometric size of the droplets, often comprehended between 20 and 500 nm, may offer advantages, such as increased chemical and physical stability and even controlled release of the compounds [18]. The pesticide industry is increasingly interested in nano-emulsified systems containing natural substances with insecticidal activity. These products have several advantages compared to synthetic ones, such as not inducing resistance, possible biodegradability, and being less toxic to the environment. Thus, they can be

strategically used to control agricultural pests or vectors of diseases. In addition, a change in the profile of consumers has led to a search for "green products", indicating the potential of nanoemulsions containing natural products for the pesticide industry [52].

In addition to the biodegradable ingredients of herbal nanoemulsions, this method also plays a prominent role in the green concept. High energy can lead to the generation of fine droplets. In fact, it was successfully used for the preparation of a larvicidal nanoemulsion with *O. basilicum* EO [51]. However, methods that use high-energy inputs have disadvantages; therefore, it is worth mentioning that the use of low-energy methods is auspicious, as used in this study [53].

Another alternative with a good prospects is estimating the discovery of the possible targets of action of larvicidal activity in the insect through the design of in silico specification of the possible mechanisms of action and which of the components of the EO *O. basilicum* is inducing the insecticidal activity. Farag et al. (2016) [54] found that the essential oil of *O. basilicum* has an inhibitory activity of acetylcholinesterase (AChE), the leading indicator of the insecticidal activity of this species, so an in vitro test of some phytochemical components of the species was carried out.

3.4. Predictions In Silico of Biological Activity

The prediction of in silico biological activity estimates which possible mechanisms of action of certain molecules will be more promising to be specific for certain macromolecules in the insect, inducing its malfunction or inhibiting its action; that is, they carry out a preliminary in silico study to identify the enzymatic target of the most active compounds. They check if a given bioactive compound can inhibit natural biological activity, causing the insect's death [55].

The program used for this estimate was PASS online, which makes forecasts with values with Pa and Pi, indicative of Pa (activity) and Pi (inactivity). All the compounds identified in the sample that selected 53 molecules from the phytochemical components of the species under study were used, in comparison with the positive control, the market larvicide, the temephos, which has larvicidal action with the mechanism of action on the inhibition of the enzyme acetylcholinesterase (Table 6).

Table 6. Estimative prediction of in silico activity of phytochemical components of *O. basilicum*.

Compounds	Acetylcholine Neuromuscular Blocking Agent		Acetylesterase Inhibitor		Insecticide		Juvenile-Hormone Esterase Inhibitor	
	Pa [a]	**Pi** [b]	**Pa** [a]	**Pi** [b]	**Pa** [a]	**Pi** [b]	**Pa** [a]	**Pi** [b]
Temephos	0.260	0.255	0.509	0.031	0.780	0.002	0.038	0.005
Linalool	0.281	0.232	0.290	0.103	0.309	0.015	0.041	0.005
Anethole	0.594	0.025	0.446	0.041	0.416	0.005	0.043	0.005
Carvone	0.729	0.004	0.393	0.054	0.471	0.004	-	-
α-Selinene	0.741	0.004	0.274	0.117	0.475	0.004	-	-
Eugenol	0.630	0.014	0.229	0.167	0.444	0.005	-	-
Limonene	0.743	0.004	0.497	0.033	0.696	0.002	-	-

[a] Pa (probability to be active); [b] Pi (probability to be inactive).

The parameters evaluated in this methodological step were neuromuscular blockers of acetylcholine, acetyl-esterase inhibitors, insecticidal activity, and juvenile hormone esterase. When we checked the aspects of the neuromuscular blocker of acetylcholine, comparing it with the positive control, temephos, it is possible to verify that the anethole, carvone, α-Selinene, eugenol, and limonene presented values superior to the positive control, except the compound Linanool [27,33], one of the main compounds of the species.

Regarding the acetylesterase inhibitor, only anethole (0.446) and limonene (0.497) approached temephos, whose value was 0.509. As for insecticidal activity, only limonene (0.696) [56] showed proximity in the Pa value, close to the positive control (0.780); all other values presented Pa values above 0.400, which is considered good insecticidal activity.

3.5. Molecular Docking

The molecular docking uses a validation protocol established by the study group, in which the poses (conformation + orientation) of the ligands (I40, GNT, and JHIII) are analyzed within the active sites of the enzyme (PDB IDs 1QON, 4EY6, and 5V13, respectively, in the order mentioned). In this context, the parameter analyzed was the RMSD (mean deviation from the square root), less than or equal to 2.0 Å, considered more appropriate [57–60]. The values obtained between the reference ligands and the experimental ligands in this study were I40 (1.33 Å), GNT (1.21 Å), and JH31 (1.48 Å), as shown in Figure 4, which determine the best position of the initial structure at around the X-ray crystallographic complex, experimentally analyzing the most stable position so that there is no structural change between the proteins and the ligand reference when complexed with the macromolecule under study.

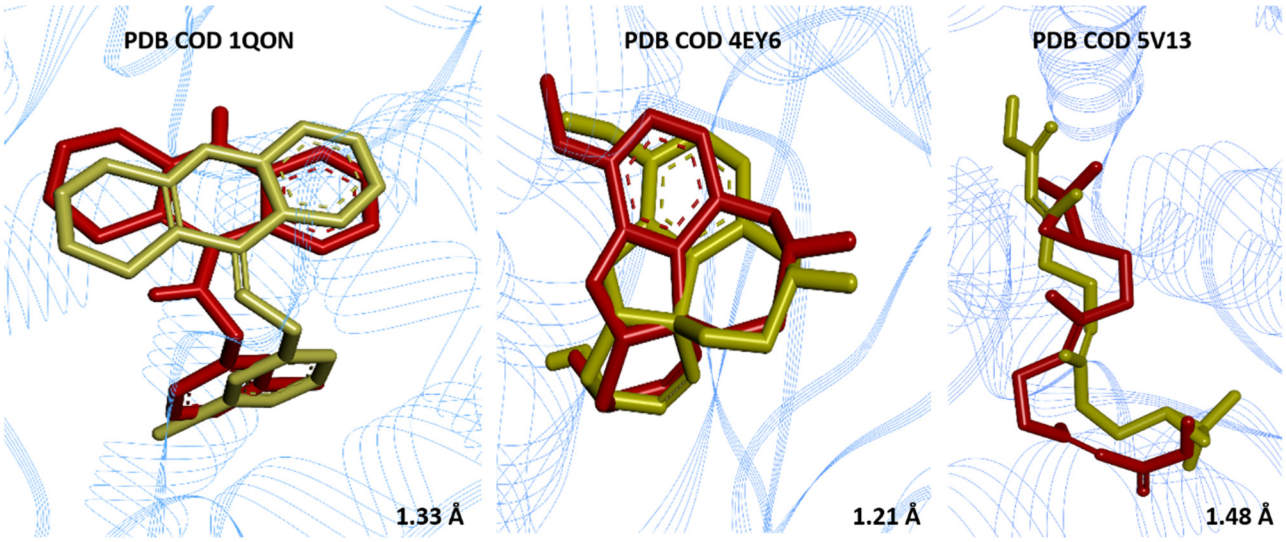


Figure 4. Overlays of crystallographic ligands poses: in (**A**) I40 (in red), with the calculated pose (in yellow), in (**B**) GNT (in red) with the calculated pose (in yellow), and in (**C**), JHIII (in red) with the calculated pose (in yellow).

After checking which possible phytochemical compounds isolated from *O. basilicum* oil with biological activity like the positive control, Temephos, molecular docking made it possible to compare the binding affinity with the reference ligands (I40, GNT, and JHIII), deposited in the macromolecules in the PDB (Protein Database), which carried out experimental studies on the inhibition of the enzyme acetylcholinesterase from the insect *Drosophila melanogaster*, fruit fly (PDB ID 1QON, I40), human acetylcholinesterase (PDB ID 4EY6, GNT), and juvenile hormone (PDB 5V13, JHIII). When analyzing the results, the ligand that showed the best binding affinity in the insect acetylcholinesterase enzyme was α-selinene (−9.1 Kcal/mol), and Temephos (−4.7 Kcal/mol) was superior to the positive control. However, it was closest to the I40 (−13.1 Kcal/mol) reference ligand of the macromolecule (Figure 5).

In the graph of the binding affinity with acetylcholinesterase in humans, it was possible to verify that only the α-selinene (−7.4 Kcal/mol) of the phytochemical compounds of the oil *O. basilicum* was superior to the reference ligand of the macromolecule (−6.9 Kcal/mol) and the positive control, Temephos (−3.2 Kcal/mol) (Figure 4). When analyzing that submitted to the fitting in *Homo sapiens* acetylcholinesterase and juvenile hormone of the insect *A. aegypti*, only the phytochemical compound α-selinene reached higher values in both of the reference ligands of the macromolecules (GNT) than observed for the temephos, positive control (Figures 6 and 7).

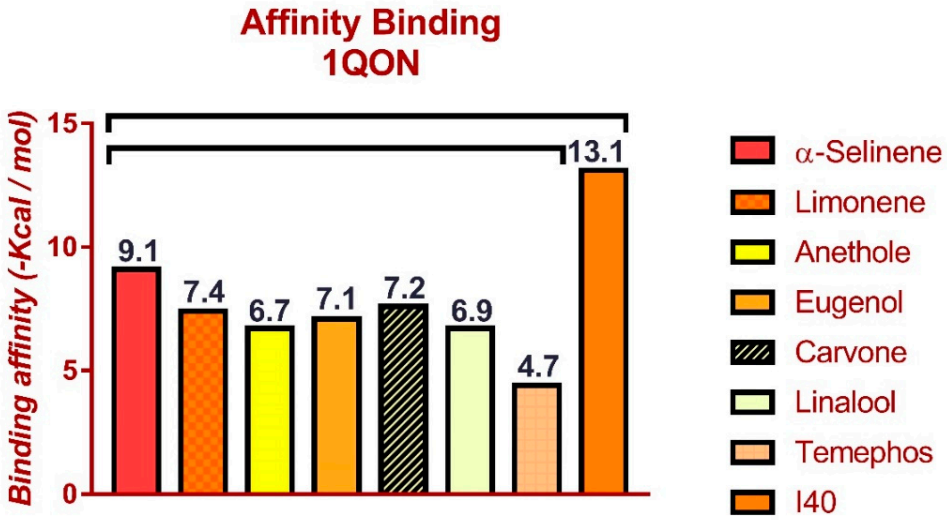


Figure 5. Results of binding affinity of the compounds with insect acetylcholinesterase receptor.

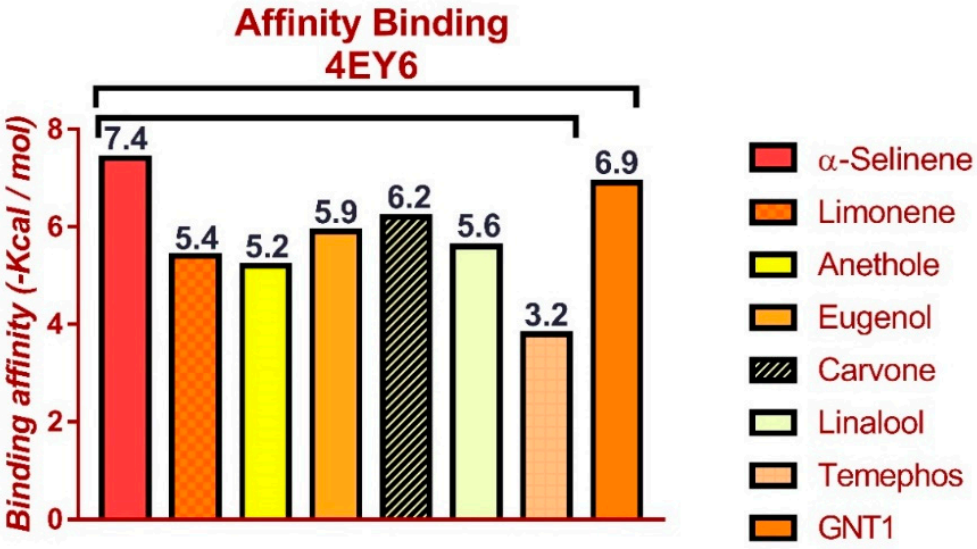


Figure 6. Results of binding affinity of the compounds with human acetylcholinesterase (PDB 4EY6).

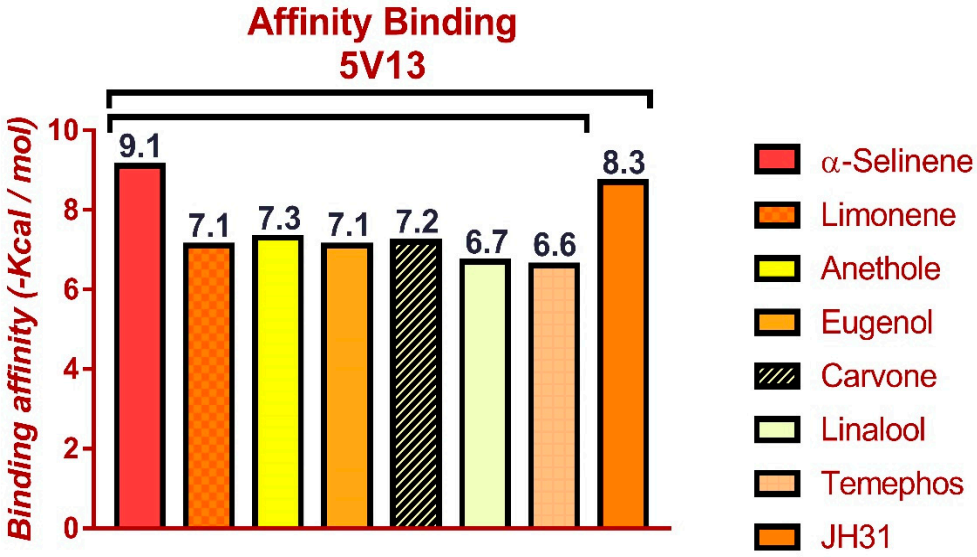


Figure 7. Results of binding affinity of the compounds with the juvenile hormone receptor (PDB 5V13).

According to Harel (2000) [61] and Costa (2019) [24], for the enzyme acetylcholinesterase of the fruit fly insect (*D. melanogaster*), inhibition was observed when binding with I40 (reference ligand). It showed interactions at the active site of the macromolecule mainly in amino acid residues Trp-83 and Trp-472, Phe-330, Tyr-71, Tyr-370 and Tyr-374, Tyr-324, and Phe-371. The interactions of the alpha-Selinene compound, which demonstrates the interactions of the best result of the binding affinity, confirms that this compound performed interactions with many amino acids of the macromolecule (PDB 1QON), which were Tyr-71, Tyr-370, Phe-374, Trp-83, and Trp-472, showing a possible inhibitor of the insect's acetylcholinesterase enzyme (Figure 8).

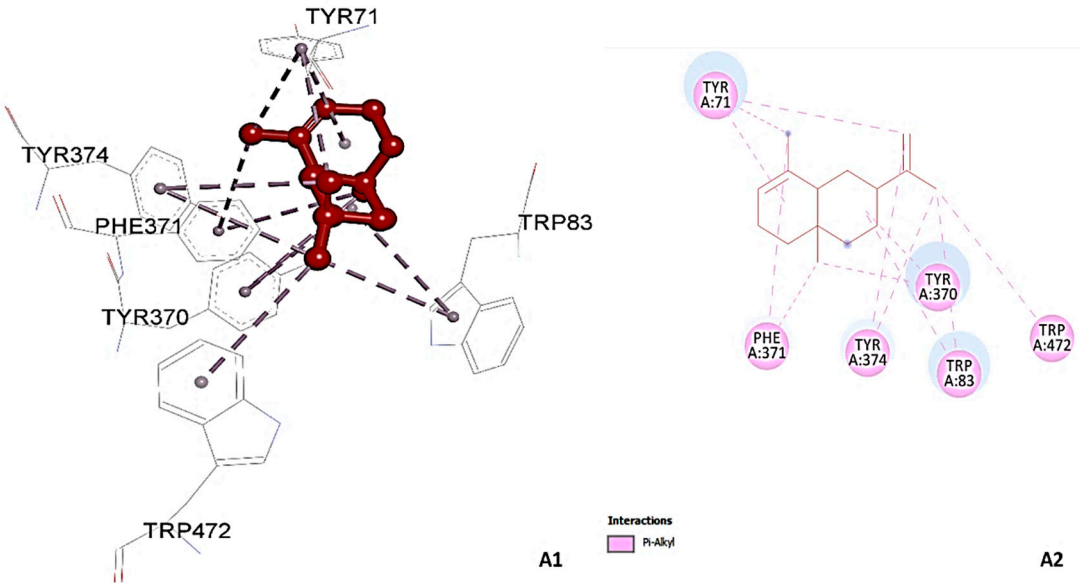


Figure 8. Interactions of the active site of the insect acetylcholinesterase (*D. melanogaster*) with the compounds α-Selinene 2D (**A1**) and 3D (**A2**).

The active site of human AChE contains a catalytic triad of amino acid residues: Ser-203, His-447, and Glu-334; other critical regions for inhibiting the enzyme are Gly-121, Gly-122, Ala-204, Trp-86, Trp-9, Trp-10, and Glu-202 [24,62–64]. When we analyzed the interactions in the enzyme acetylcholinesterase in humans, only α-selinene interacted with two amino acid residues (Tpr-86 and His-447) that are in distant positions in the amino acid chain, making it a compound with little activity on the enzyme in humans (Figure 9).

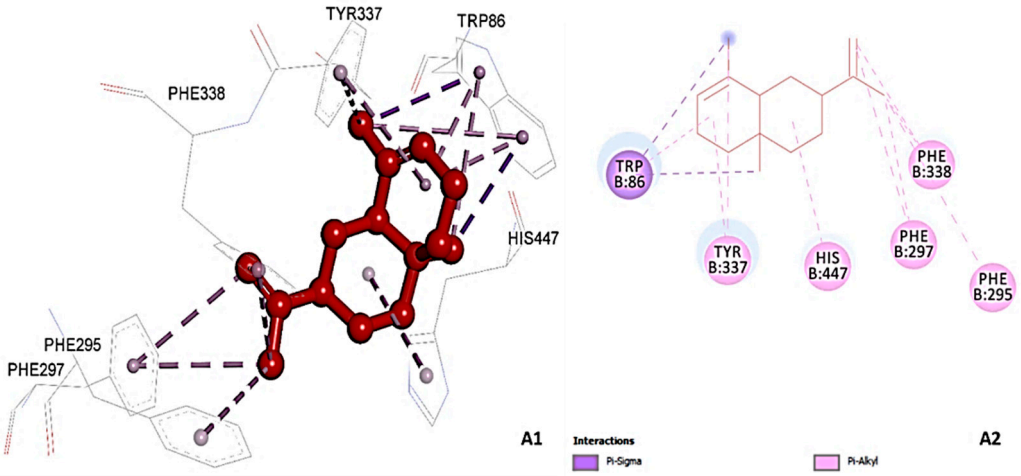


Figure 9. Interactions of the active site of the human acetylcholinesterase (*Homo sapiens*) with the compounds α-Selinene 2D (**A1**) and 3D (**A2**).

A study by Kim (2017), deposited in the PDB (Protein Data Base) using x-ray crystallography, demonstrated that in the complex with JHIII in the active site of the juvenile hormone of the species *A. aegypti*, being one of the main interactions with the epoxy group, it forms a hydrogen bond with the phenolic hydroxyl, with amino acid residue Try-129, and according to Ramos (2019) [25], amino acid residues in the structure of the active site are common, being Ser30-Ala38, Arg45-Glu51, Val60-Gln71, Phe123-Leu130, Val132-Arg136, Leu138-Arg143, and Val280-Trp286 for the β sheet between the Pro52-Pro55, Tyr72-Val73, Thr144-Val145, and Arg276-Gln279 amino acid residues. When analyzing the interactions with α-selinene, with the active site of the juvenile hormone of *A. aegypti*, the residues Tyr-33, -64, Leu-72, Trp-53, Val-65, -68, and Phe-144 were found to be similar to results from Costa (2019) [24], when he demonstrated the interactions with an active site complexed with JHIII, with possible inhibition of the active site of the juvenile hormone (Figure 10).

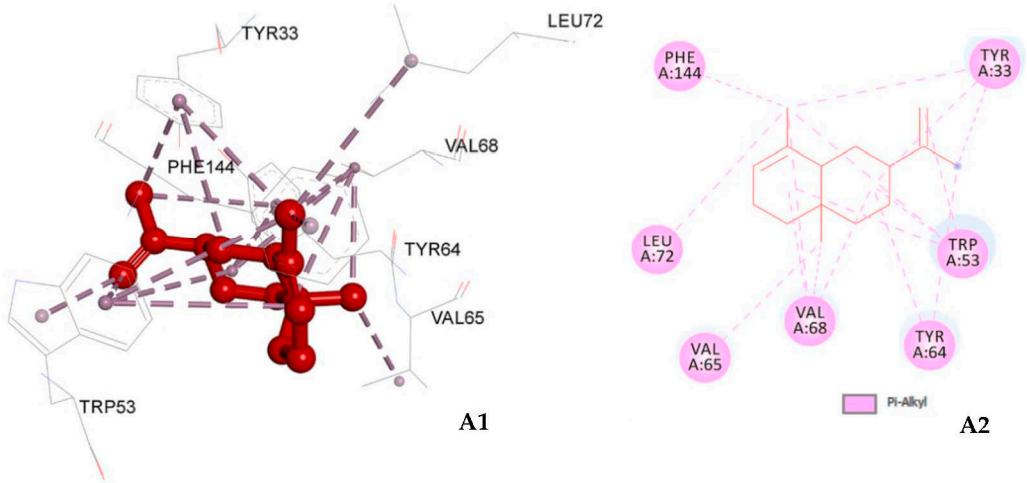


Figure 10. Interactions of the active site of the insect juvenile hormone receptor (PDB 5V13) with the compounds α-Selinene 2D (**A1**) and 3D (**A2**).

4. Conclusions

The nanoemulsion formulated in this study showed results in larvicidal bioassays with higher activity than *A. aegypti* and *C. quinquefasciatus* compared with data from the literature from independent tests performed against these larvae. In this study on the biological activity predictions of the compounds, the ones with the best values for probable activity (Pa) were linalool, anethole, carvone, α-selinene, eugenol, and limonene. In the molecular docking tests, the α-Selinene compound presented the best binding affinity with the insect's acetylcholinesterase enzyme, indicating its importance in the antagonistic action of the compounds present in the essential oil. In this sense, this study enabled the formulation of a low-energy, instrumental, and cost-effective nanoemulsion, which contains an ecological bias and could become a safe and efficient alternative for the control of disease vectors that affect thousands of people/animals around the world every year.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations11040097/s1; Table S1: Evidence of scientific studies on the insecticidal and repellent action of *O. basilicum* and other species of the same genus [65–72].

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writing—review and editing, E.L.V.d.S.d.S., J.N.C. and C.B.R.d.S. All authors have read and agreed to the published version of the manuscript.

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