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Chemical Study, Antioxidant Analysis and Evaluation of the Larvicidal Potential against *Aedes aegypti* Larvae of Essential Oil of *Ocimum basilicum* Linn

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RDSR and SSMDSDA planned all experiments. Authors RDSR and ABLR supported the chemical study, antioxidant activity and larvicidal potential. Author RNPS supported the study of the larvicidal potential. Authors RDSR and SSMDSDA wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The purpose of this research was to accomplish chemical study, antioxidant analysis and evaluation of the larvicidal potential against *Aedes aegypti* larvae of essential oil from the leaves of *O. basilicum* Linn.

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Location of the Study: Pharmacognosy and Phytochemistry Laboratory, Department of Biological and Health Sciences, Federal University of Amapá (UNIFAP), between July 2013 and March 2014. Arthropoda Laboratory, Department of Biological and Health Sciences, Federal University of Amapá (UNIFAP) between September 2013 and March 2014.
Methodology: The essential oil was obtained by hydrodistillation; the identification and quantification of components was achieved with the use of GC-MS analysis. The antioxidant activity was evaluated by the method of sequestration of DPPH. The essential oil was tested in the third larval state of the development of the mosquito *Aedes aegypti*. The third larval instar were exposed to different concentrations of the oil (500, 400, 300, 200 and 130 ppm) in triplicates.
Results: Chromatographic analysis identified that the major constituents found in essential oil of *O. basilicum* were limonene (13%), 1,8-cineole (15%), linalool (20%) and methyl chavicol (45%). In trials of free radicals sequestration, the essential oil showed (AA%) 67.35±1.11 in the highest concentration and inhibitory concentration, IC_{50} value of 61.517 mg/mL. The essential oil of *O. basilicum* showed larvicidal potential with CL_{50} of 67.22 ppm.
Conclusion: A more detailed study should be done to verify the larvicidal potential and biological mechanism of action, as several authors claimed that the constituent of essential oils affect the nervous system of the mosquito *Aedes aegypti* and the action mechanism is not yet fully elucidated. New studies demand the development of tests using samples of lower concentrations to verify the degree of toxicity in other animal species, including man, and preparation of formulations that may function as a natural alternative to combat mosquito larvae.

Keywords: Essential oil; aromatic plant; Lamiaceae. secondary metabolites.

1. INTRODUCTION

The use of medicinal plants and their derivatives for the treatment of diseases is an ancient practice, and is currently in expansion all over the world [1]. It is estimated that over the 2000 the products made from medicinal plants, about 30 billion dollars are circulated. In this context, the World Health Organization (WHO) estimates that 80% of the population depends on traditional medicine, especially in emerging countries. Herbal medicine has emerged as a drug option well accepted and accessible to people of the world, and in the case of Brazil it meets the local needs of hundreds of municipalities in primary health care [2].

It is estimated that there are 350 000 plant species in the world, whereas only 10% of these have been studied and evaluated for their pharmacological properties. Brazil has the highest plant diversity on the planet and a rich source of new drugs. The introduction of exotic species in the country was a result of the cultural miscegenation involving Africans and Europeans, and on the other hand, there is the legacy of Amerindian culture in the use of medicinal plants that are few studied [3].

The International Standard Organization (ISO) defines Essential Oils (EO) as volatile products obtained from plant parts that are extracted through steam distillation technique, in most cases, and also by pressing the pericarp of citrus fruits. Essential oils are usually complex mixtures of volatile substances, lipophilic, odoriferous and liquid in nature. They are associated with various activities necessary to the survival of the plant in its ecosystem functions, playing a key role in the defense against microorganisms and predators, and also in attracting insects and other fecundation agents. In traditional medicine, essential oils have been used for a long time [4,5].

The volatile oils are rarely found in gymnosperms (except conifers). Among angiosperm monocots, the occurrence is relatively rare, except Poaleae (especially, species of *Cymbopogon* and *Vitiveria*) and Zingiberaceae (species of Curcuma and Alpinia, among others). However, plants rich in volatile oils are abundant among the dicotyledonous angiosperms such as the families Asteraceae, Apiaceae, Lamiaceae, Lauraceae, Myrtaceae, Myristicaceae, Piperaceae and Rutaceae [6].

Studies have shown that the toxicity of some compounds of volatile oils constitute a protection against pests and weeds. For example, menthol and menthone are inhibitors of the growth of several types of larvae. There is also evidence that some insects use parts of these oils to exert a protective action against predators. Thus, the vapors of certain substances such as α-pinene and citronella can cause some irritation to a predator to make him give up his prey [6].

For the identification of essential oils and extracts, various techniques have been used, as well as to separate and purify them, such as high-performance liquid chromatography, gas chromatography, high-resolution gas chromatography coupled to mass spectrometry and nuclear magnetic resonance (NMR). NMR has been used with advantage over other spectroscopic techniques in the sense of requiring no pretreatment of the sample, and also because a detailed spectral analysis of the hydrogen spectrum can provide information about the structure and the chemical composition of the major chemical constituents present [7].

The plants of the family Lamiaceae (Labiatae) have agricultural importance and are widely used in cooking, traditional medicine and pharmaceutical and cosmetics industries. The family is distributed worldwide with approximately 300 genera and 7.500 species. However, in Brazil there is an occurrence of about 350 species in 26 genera [8]. Lamiaceae family has chemical and economical potential for extraction of essential oils (produced by hairs and glandular trichomes) and studies are aimed at evaluating the constituents present in the oils.

The species *O. basilicum* Linn. is popularly known as “alfavaca, alfavaca-cheirosa, alfavaca da América, alfavaca de vaqueiro, alfavaca do mato, basílico-grande, basilicão, basílicum-grande, erva-real, folhas largas de cozinheiros, manjericão, manjericão da folha branca, manjericão da folha larga, manjericão de molho, quioiô”. It is largely grown in Brazil in home gardens for medicinal use and flavor, being marketed in fresh form at open markets and grocery stores [9].

Considered an aromatic, annual, erect, branched shrub, measuring about 30-50 cm tall, native from tropical Asia and introduced in Brazil by the Italian colony, it presents simple leaves, membranous, with wavy margins and prominent veins, 4-7 cm long. In traditional medicine is used as restorative, which relieves spasms, low fever and improves digestion, in addition to effectively combat bacterial infections and intestinal parasites [10,11].

The biosynthesis of antioxidant secondary metabolites in plants is significantly increased when they absorb at wavelengths 300-400 nm, thus providing a level of protection against harmful oxidants (free radicals). Medicinal plants are used as antioxidants in traditional medicine, where the therapeutic properties would be sustained in part by their ability to sweep free radicals that may be associated with many diseases. The antioxidants work to fight diseases linked to premature aging of cells relative to chemical processes, such as emphysema, cirrhosis and arteriosclerosis. These diseases are due to inefficiency of antioxidant protection [12,13].

The resistance development of the dengue vector to chemical insecticides and their toxicity motivates the search for new natural insecticides. The use of natural products in the formulation of new insecticides as an alternative for control of mosquito and disease vectors is crucial, with the advantage that the natural agents are biodegradable [14].

Dengue is a viral infection transmitted by the mosquito *Aedes aegypti* (Diptera: Culicidae) and it is caused by a flavivirus with four different antigen and distinct serotypes (DENV 1-4). Due to the negligence, it's estimated that 2.5 billion people worldwide are under infection risk of dengue infection, in which approximately one billion live in urban areas in tropical and subtropical countries of South and Southeast of Asia and Western Pacific regions of America [15-17]. This study aimed to accomplish chemical study, antioxidant analysis and evaluation of the *Aedes aegypti* larvicidal potential of essential oil from the leaves of *O. basilicum* Linn.

2. MATERIALS AND METHODS

2.1 Collection of Species

Species of *O. basilicum* Linn were collected in the city of Macapá-AP and later sent to the Herbarium of the Institute of Scientific and Technological Research of the Amapá State (IEPA) for procedures of taxonomic identification and preparation of voucher specimen.

2.2 Obtaining the Essential Oil

Leaves were treated (washed and dried) for extracting essential oils. The essential oil was obtained by hydrodistillation (under temperature of 100℃) in Clevenger type apparatus for 3h. [18].

The characterization of the profile of the samples was determined by gas chromatography (GC), according methodology described by Barbosa et

al. (2006). The oil analysis was performed by gas chromatography coupled to mass spectrometry (GC-MS), using equipment Shimadzu model GCMS-QP 5050A. It was employed a DB-5HT column (J & W Scientific), 30 m long, 0.32 mm in diameter, film thickness of 0.10 microns and nitrogen as carrier gas. The operating conditions of the gas chromatograph were: Column internal pressure of 56.7 kPa, split ratio 1:20, gas flow in the column of 1.0 mL/min. (210℃), injector temperature 220℃, detector's interface temperature of 240℃ (GC-MS). The initial column temperature was 60℃ followed by an increase of 3℃/min up to 240℃ and kept constant for 30 min. The mass spectrometer was programmed to perform readings in a range 29-400 Da in 0.5 s, with ionization energy of 70 eV. It was injected 1 µL of each sample at a concentration of 10,000 ppm dissolved in hexane. The identification of the components was based on comparison of the retention indices (RI) and mass spectra of each substance with the literature data.

2.3 Analysis of Antioxidant Activity

Evaluation of antioxidant activity was based on the methodology proposed by Sousa et al. [19], Lopes-Lutz et al. [20] and Andrade et al. [21] on the consumption of DPPH with some modifications.

A methanol solution of DPPH at the concentration of 40 µg/mL was prepared. The essential oils were diluted in methanol concentrations 100, 50, 25, 10 and 1 µg/mL. For the evaluation, 2.7 mL of the stock solution of DPPH were added to a test tube, followed by addition of 0.3 mL of the essential oil. In parallel, a blank test was prepared, using a mixture of 2.7 mL of methanol and the methanolic solution of compounds evaluated. After 30 minutes, readings on a spectrophotometer (Biospectro SP-22) at a wavelength of 517 nm were performed [22]. The antioxidant activity was calculated according to the equation proposed by Sousa et al. (2007)[19]:

$$(\mathrm{AA\%}) = 100 - \left\{\left[\left(\mathrm{Abs_{sample}} - \mathrm{Abs_{blank}}\right)100\right] / \mathrm{Abs_{control}}\right\}$$

2.4 Larvicidal Activity of the Essential Oil

2.4.1 Larvae

The larvae of *Aedes aegypti* Linneu used in bioassays came from the insectary of the Laboratory of Arthropods, Federal University of Amapá, all the F_6 generation, the young third stage.

2.4.2 Bioassays

The biological tests were conducted at the Arthropoda Laboratory of the Federal University of Amapá, Macapá in a room (3 m x 4 m) with controlled climatic conditions: Temperature 25±2℃, relative humidity of 75 ±5%, 12 hours photoperiod.

The methodology followed the standard protocol WHO [23-25] with modifications in container testing. Concentrations of 500, 400, 300, 200 and 130 ppm of essential oil from the leaves of the species *O. basilicum* were selected, to test the toxicity to mosquito species larvae used in this study.

A stock solution was prepared with 465 mg of essential oil, pre-solubilized in Tween 80 and dissolved in 93 mL of water to obtain a concentration of 5000 ppm. From this solution, a dilution series were prepared in order to obtain concentrations of 500, 400, 300, 200 and 130 ppm. For each treatment replicate, 10 larvae were used, pipetted into a beaker containing 100 mL of distilled water. Then, the larvae were removed from the beaker to the test container, thereby minimizing the time between the preparation of the first and the last sample. A control solution was used to verify if the solvent was affecting the larvae mortality. During the experiment, the average water temperature was 25°C. The dead larvae were counted after 24 and 48 hours considering all those unable to reach the surface. The data of mortality (%) versus concentration (ppm) were analyzed using the SPSS program for Probit graph to determine the lethal concentration that causes 50% mortality of the population (LC50).

2.5 Statistical Analysis

Statistical analysis was performed by analysis of variance (ANOVA). Significant differences between means were determined by Tukey test.

3. RESULTS AND DISCUSSION

In the GC-MS analysis of the essential oil of *O. basilicum*, four compounds were found and identified, representing 93% of the oil characterized as monoterpenes. The chemical components were: limonene (1), eucalyptol (2), linalool (3), and methyl chavicol (4). The peaks of

the compounds in the chromatogram and their structural formulas may be observed in the Fig. 1.

The obtained mass spectra were compared with the mass spectra of monoterpenes found in Wily/MBP GC-MS system equipment library. This comparison and identification of constituents present in essential oil of *O. basilicum* are shown in Fig. 2, and their retention times and respective percentages are shown in Table 1.

The profile of fragmentation observed in Peak (1) shows the peak at *m/z* 121 which is related to the loss of the methyl group and the peak at *m/z* 93 can be associated to the structure $C_7H_9^+$, which is formed by isomerization followed by cleavage allylic. It is also observed that the fragment mass *m/z* 136 refers to the molecular ion observed commonly in olefins. The base ion peak with the mass *m/z* 68 is a fragment of the *retro*-Diels-Alder reaction, whose products fragments are shown in Fig. 3.

Table 1. Compounds identified as monoterpenes in essential oil (OE) of *O. basilicum*. The retention time - t_R (min.) were obtained by GC-MS

Compound	t_R peaks OE *O. basilicum*	Relative percentage (%)
Limonene	8.48	13
Eucalyptol	8.60	15
Linalool	11.06	20
Metylchavicol	15.15	45
Total		93

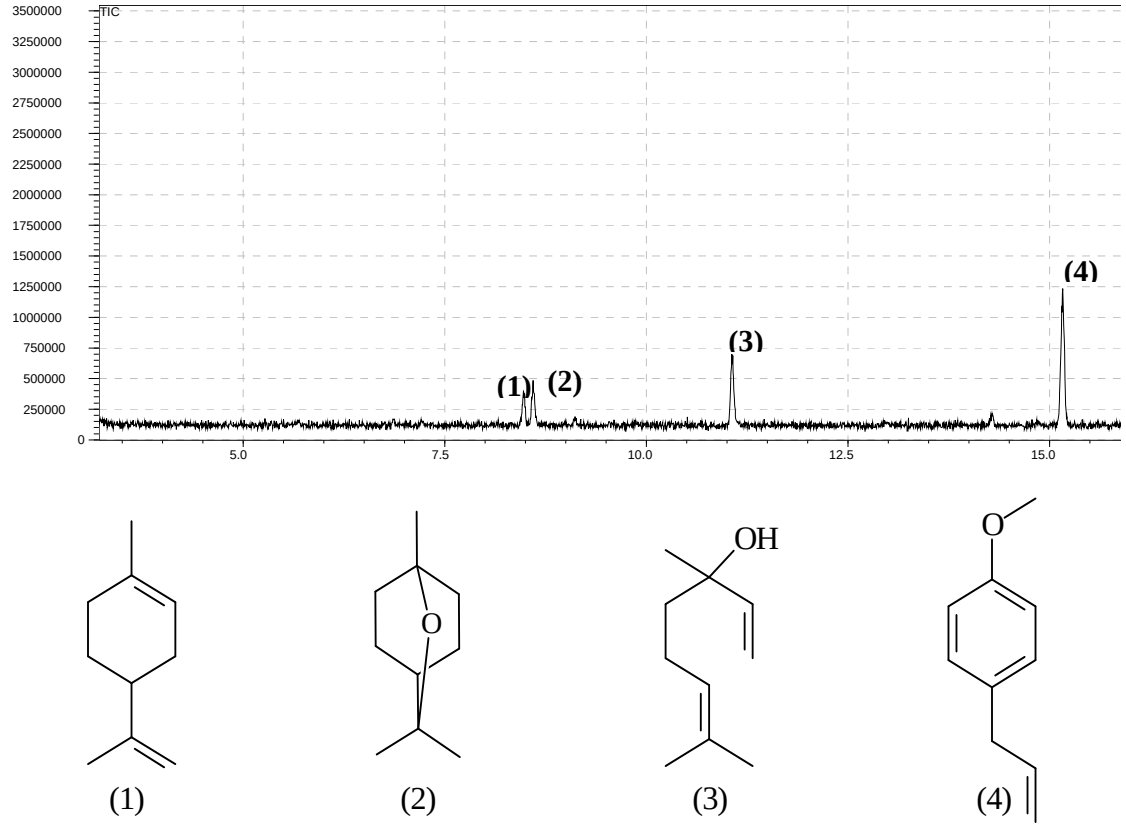
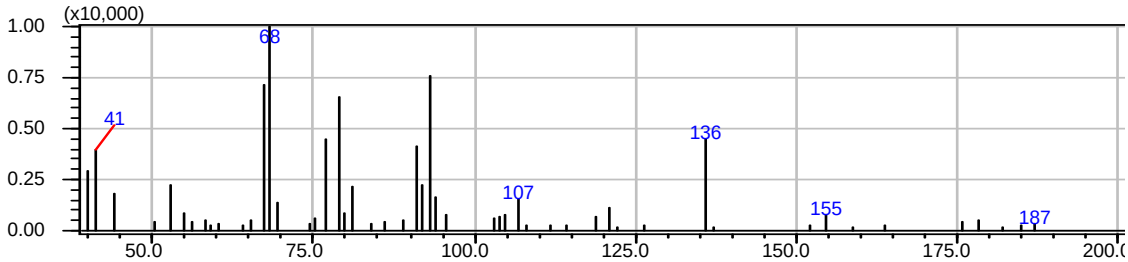
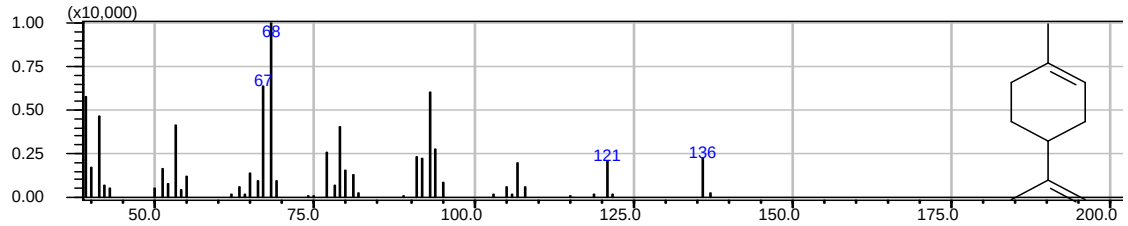


Fig. 1. Chromatogram obtained by GC of the essential oil of *O. basilicum.* Conditions: carrier gas: helium (He); initial temperature: 60℃; initia l time: 1.0 min.; column temperature rate increase: 3℃/min; final temperature: 240℃ final t ime at 240℃: 30.0 min.

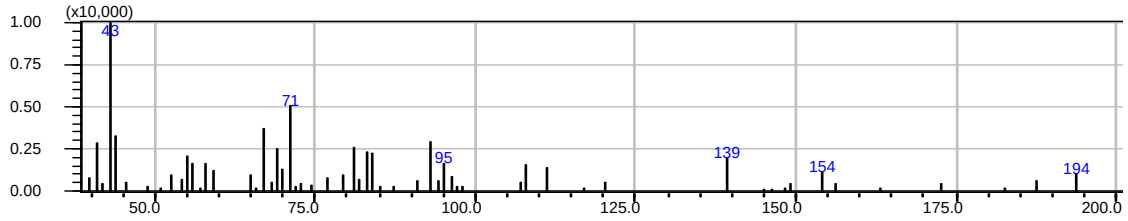
Peak (1). Limonene



Mass spectrum library



Peak (2). Eucalyptol



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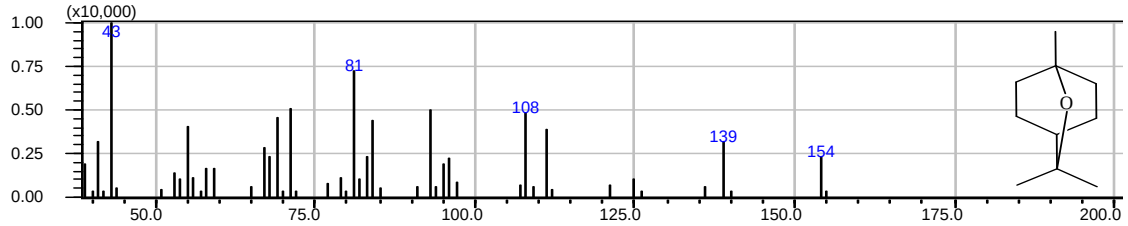
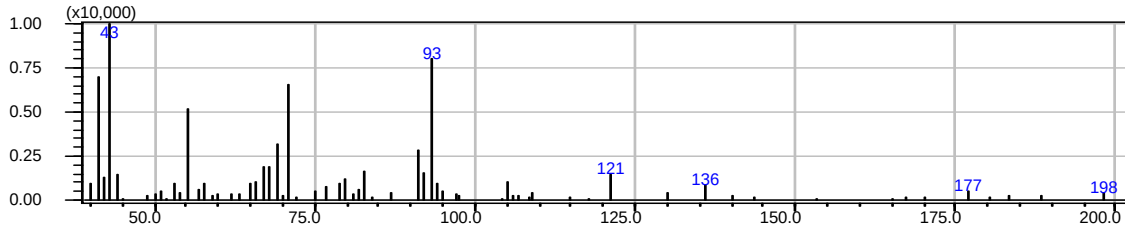
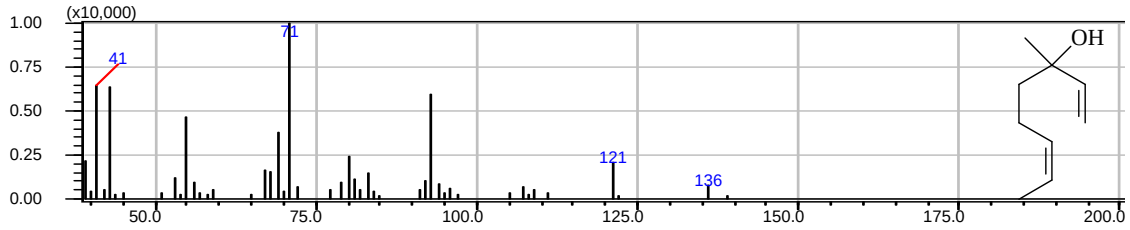


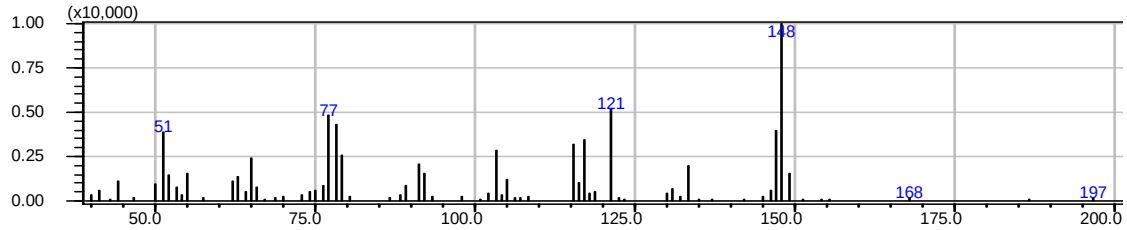
Peak (3). Linalool



Mass spectrum library



Peak (4). Metyl chavicol



Mass spectrum library

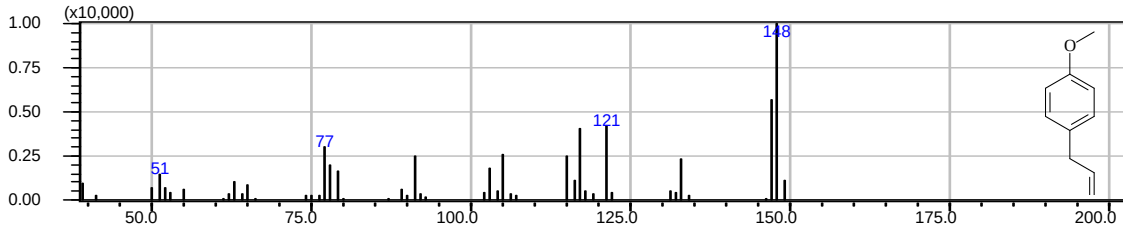


Fig. 2. Mass spectra of the essential oil obtained by GC-MS in comparison with spectra obtained from the library product

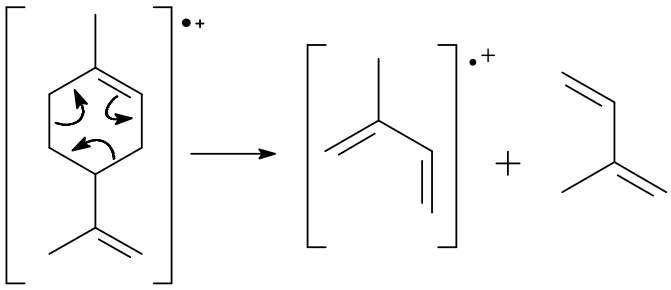


Fig. 3. Fragmentation of limonene by *retro*-Diels-Alder reaction

Cyclic alkenes usually show a distinct molecular ion peak. The retro Diels-Alder reaction of limonene undergoes rearrangement in which a fragment is a neutral molecule isoprene [26].

In the inspection of the mass of Peak (2), the identification of molecular ion peak in the spectrum can be stated with respect to the compound of molecular weight 154.25 g/mol, which corresponds to the compound 1,8-cineole. Analyzing the fragmentation profile of the compound and comparing them with the library's equipment, it equates to the 1,8-cineole represented by typical ionic fragments, as shown in Fig. 4.

The fragment of mass *m/z* 154[M^{+}] is the molecular ion, whereas the fragment *m/z* 139

[$M^{\cdot+}$-15], corresponding to loss of methyl (-CH_3). The fragment with *m/z* 43 is the base peak ion, which is a very stable fragment, characteristic of stability by resonance.

The peaks with the ratio *m/z* 136 and 121 were produced by fragmentation of the molecule with loss of water, followed by loss of methyl radical ($^{\cdot}CH_3$). The output of ethane from the fragment of *m/z* 121 generates the cation with *m/z* 93. It is also observed in the allylic breaking represented by the peak at *m/z* 69, forming a cation and another fragment as radical. The base ion peak appears at *m/z* 71 and is formed by mass loss in relation to the molecular ion, as shown in Fig. 5.

The delocalization of the positive charge between the unsaturated carbon and oxygen which supports the charge is the reason for the high stability of the fragment among the others produced.

The main peak is the one with mass-charge ratio of *m/z* 148 which represents the molecular ion, confirming the compound of formula $C_{10}H_{12}O$. The peak of mass *m/z* 133 corresponds to the loss of a methyl [$M^{\cdot+}$-15], wherein the peak of mass-charge ratio *m/z* 117 refers to $C_9H_9^+$ fragment generated from a rearrangement of the molecular ion with consequent loss of a neutral molecule H_2CO and a radical $^{\cdot}H$. The peak of

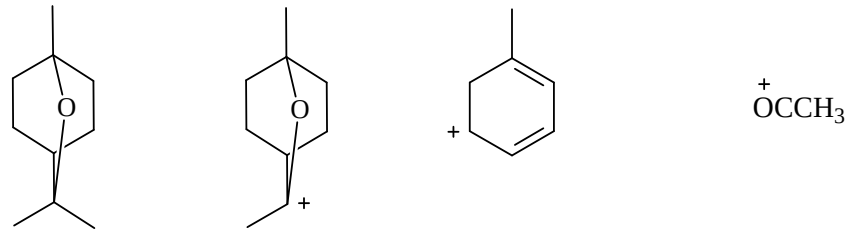


Fig. 4. Typical ionic fragments of 1,8-cineole

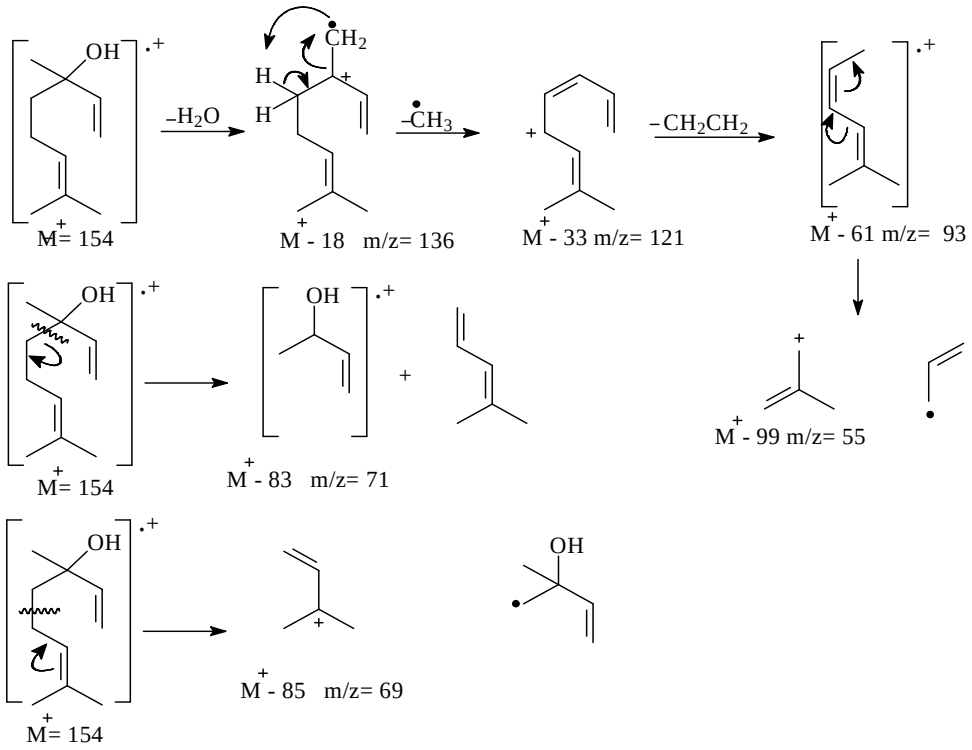


Fig. 5. Profile of fragmentation Linanol

mass *m/z* 105 indicates the $C_8H_9^+$ fragment by loss of CO, while the peak of *m/z* 91 correspondes to tropilic, classic ion of aromatic system. The fragment *m/z* associated with the aromatic cation $C_6H_5^+$ is generated by loss of a neutral molecule (H_2CO) and the side chain of the aromatic compound.

The mean values of the percentage of antioxidant activity of the essential oil of *O. basilicum* are shown in Table 2.

Table 2. Results of the antioxidant activity of the essential oil of *O. basilicum* species

Concentration (µg/mL)	(AA%)
100	67.35±1.109[a]
50	45.78±0.904[b]
25	35.77±2.609[c]
10	23.60±1.311[d]
1	17.98±2.695[e]

Different lowercase characters represent significant differences in antioxidant activity between concentrations of oil

The antioxidant activity (antioxidant capacity or potential) is a widely used parameter to characterize different biological materials. This activity relates to compounds capable of protecting a biological system against the harmful effects of processes or reactions that cause excessive oxidation involving oxygen reactive species [27]. According to Andrade et al. [21], the alcohols are the second most active class of oxygenated monoterpenes in antioxidant activity, following only the phenolic compounds.

The study of the chemical composition of the essential oil identified the presence of linalool (20%) which were responsible for significant antioxidant activity with *p* value <0.0001 in the concentration 100 µg/mL with (AA%) of 67,35±1,109. The linear regression calculation for the inhibition concentration of 50% (IC50) showed a value of 61.517 µg/mL, and high correlation coefficient (R^2) of 0.9919.

Graph 1 expresses the relation between the mortality of *Aedes aegypti* larvae and essential oil concentrations. Table 3 refers to the average mortality in the counts carried out in periods of 24 h and 48 h after the end of the experiment.

As the bioassay of essential oil from the leaves of the species *O. basilicum* with the *A. aegypti* larvae showed larvicidal effect with LC50 = 67.22 ppm, according to the literature [28], substances with values LC50 lower than 100 µg/mL are considered good larvicidal agents. The essential oil has great potential to be used as larvicidal agent against *A. aegypti* mosquito larvae, due to the presence of the major compounds of the class of monoterpenes reported in the literature for this activity.

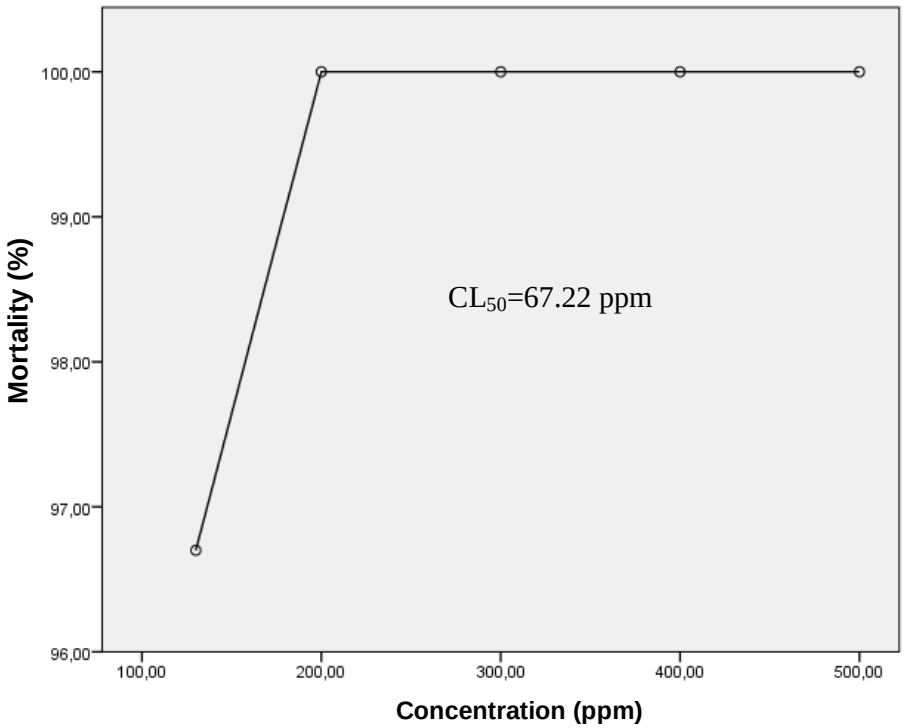
Studies by Furtado et al. [29] reported mean values of LC50 = 104 mg/mL, and Simas et al. [30] reported values below 100 ppm. The comparison to other studies showed higher activity than reported, thus indicating the possibility of other compounds to be responsible for the larvicidal activity of the essential oil or the existence of synergism between linalool and other compounds.

Several researches by various authors [31-34] have reported the larvicidal action of the species with average values of LC50 = 50 ppm, and assigning the presence of larvicidal potential of the major compound linalool. The results corroborate the research in that sense, as the values are in agreement with those reported in the literature. Considering seasonal factors, soil, radiation absorbed by the species in photosynthesis and environmental stress, these factors may influence the percent composition of chemical compounds during the process of production of secondary metabolites.

Table 3. Counting of average mortality of the mosquito *Aedes aegypti* larvae performed during periods from 24 and 48 h

Counting	Mortality (%)*					
	500 ppm	400 ppm	300 ppm	200 ppm	130 ppm	CL_{50} (ppm)
24 h	100[aA]	100[aA]	100[aA]	100[aA]	86[bB]	75.58
48 h	100[aA]	100[aA]	100[aA]	100[aA]	96.7[aB]	67.22

**Different lowercase characters represent significant differences in mortality (%) among the five concentrations of oil in the same counting. Different capital letters represent significant differences in mortality (%) between the two periods of counting*



Graph 1. Results of larvicidal activity of the essential oil of *O. basilicum* species as a function of mortality of the *Aedes aegypti* larvae

Monoterpenes, as well as some sesquiterpenes, in general, serve as repellents which have significant toxicity to insects, but negligible toxicity to mammals. Mixtures of these volatile compounds of low molecular weight, called essential oils, provide to plants such as peppermint (*Mentha piperita*), lemon (*Citrus limon*), basil (*Ocimum basilicum*) and sage (*Salvia officinalis*), their odoriferous characteristic and are commercially important for flavoring food and in the production of perfumes.

4. CONCLUSION

The great variability in the chemical composition of the essential oils is possibly due to their different origins, because the final content of secondary metabolites is influenced by several factors, including the location and the time of collection, stabilization processes and storage conditions, seasonality and among others.

A more detailed study should be performed to verify the larvicidal potential and biological mechanism of action, as several authors state that both plant extracts as the essential constituent act on the nervous system of the mosquito *Aedes aegypti* and the action mechanism is not yet fully understood. The development of new tests using samples of lower concentrations to verify the degree of toxicity in other animal species, including man, and preparation of new formulations that may function as a natural alternative to combat the mosquito larvae should be done in the future.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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