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Larvicidal activity, molluscicide and toxicity of the essential oil of *Citrus limon* peels against, respectively, *Aedes aegypti*, *Biomphalaria glabrata* and *Artemia salina*

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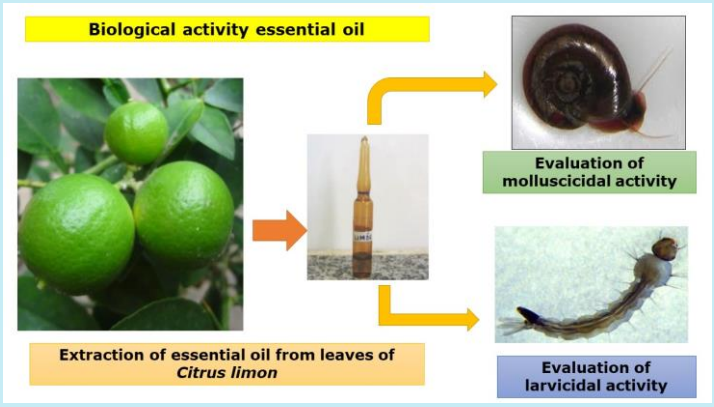
ABSTRACT: In this present work, we tested the larvicidal activity, molluscicide and toxicity of the oil extracted from *Citrus limon* peels, respectively against third stage larvae of *Aedes aegypti*, snail *Biomphalaria glabrata*, and *Artemia salina*. For this, we extract the essential oil by hydrodistillation. Then, we identified and quantified the components by gas chromatography coupled to mass spectrometry (GC-MS). We tested the larvicidal and molluscicidal activity, respectively, using the method adopted by the Brazilian Ministry of Health and the World Health Organization. We calculated the lethal concentration (LC_{50}) from the Probit method for the three biological activities with 95 %. The results of the chromatographic analysis showed that the oil has 58.81% of Limonene (major constituent) and 0.11% α-Mulene (minority component). The essential oil presented lethal concentration (LC_{50}) for larvicidal activity, molluscicide and toxicity, respectively at 15.48, 13.05 and 743.35 mg·L^{-1}. Therefore, the essential oil is active against larvae of *A. aegypti* and snail *B. glabrata* and non-toxic against larvae of *A. salina*.



1. Introduction

The essential oils extracted from citrus plants arouse in man the interest in identifying his constituents and the possible applications to society. Among the identified compounds, the main ones are limonene, β-myrene, α-pinene, p-cymene, β-pinene, terpinolene[1-3], and its applications are in medicine, food, cosmetics, detergents, aromatherapy, inhibition of pathogens and control of insects.

Among the major chemical constituents found in citrus, we highlight limonene. In the nomenclature adopted by IUPAC (International Union of Pure and Applied Chemistry), it has the name of 4-isoprenyl-1-methyl-cyclohexene and constitutes more than 300 plant species. In addition, it has two enantiomers which are S - (-) - limonene R - (+) - limonene, in which R - (+) -

limonene is the major component of oils from lemon peel (*Citrus limon*) and orange peel and of the essential oil of *Carum carvi*, being responsible for the prevention of dehydration and inhibition of microbial growth[4].

In the literature, it was observed that the limonene was found to have larvicidal and molluscicidal activity against *Dysmicoccus brevipes*[5] and *Lymnaea acuminata*[6]. Based on this information, we asked: did the essential oil extracted from the bark of *C. limon* have larvicidal activity against the third stage larvae of *Aedes aegypti* and molluscicide against the snail *Biomphalaria glabrata*, both disease vectors, respectively, dengue and schistosomiasis? Therefore, the fight against the vectors of dengue and schistosomiasis occur through, respectively, larvicides and synthetic molluscicides. Among the larvicides and molluscicides recommended by the World Health Organization (WHO), are, respectively, the temephos and niclosamide[7]. However, the use of these larvicides and molluscicides provokes resistance of larvae and snails, low selectivity, environmental contamination and high cost[8-10]. Thus, it is recommended studies of extracts of plants[7] and larvicides.

There are two essential points in the manual of the World Health Organization (WHO)[11] on the efficacy of a plant's molluscicidal activity, although the same does not exist for larvicidal activity. One is about the activity of the extracts. These are considered to be active when the 24 h shellfish mortality is equal to or greater than 90% at the concentration of 20 ppm for extracts and 100 ppm for the raw vegetable. Another is on toxicity and field studies. Even if the natural molluscicides are biodegradable, within the values required by the WHO, they may present risks[7].

In this context, in our toxicity study, we chose to carry it out with *Artemia salina* for two reasons. One, low cost, easy manipulation and a good indication of non-target organisms[12,13]. Another study, due to the good results of plants with the molluscicidal activity that used *A. salina* in the toxicities test[14,15]. Hence, in view of the above, we chemically characterized the essential oil extracted from the bark of *C. limon* and tested the larvicidal activity against larvae in the third stage of *A. aegypti*, molluscicidal activity against the snail *B. glabrata* and toxic activity against larvae *A. salina*.

2. Materials and methods

2.1 Obtaining essential oil

We collected the fruits, branches, and leaves of *C. limon* in the district of Sá Viana (January and June 2010), in the peripheral region of São Luís/MA, directly from the lemon tree, which is free of agricultural pesticides. In the Seabra Attic Herbarium (SLS) of the Federal University of Maranhão (UFMA), we identified this species from the observation and comparison with the part already identified in the herbarium under registration number 100379 (family *Rutaceae*, genus *Citrus*). After this step, we removed the fruit peels with a stylet.

To extract the essential oil, we used a glass Clevenger extractor coupled to a 1000 mL round bottom flask and to an electric blanket as a heat source. To each essential oil extraction routine, we weigh and grind in an electric sample mill 30 g. After this step, we mixed the sample with distilled water in the proportion 1:10 and placed in a round bottom flask, coupled to the extractor system. Then we switched on the electric blanket and set the temperature to 100 °C. After 5 h the distillation was stopped, and the essential oil was collected. The oil is dried by means of percolation in anhydrous sodium sulfate. We performed these operations in triplicates and stored the samples in ampoules of amber glass under refrigeration (temperature of 15 °C) to avoid possible losses of volatile constituents. So, we determined the density of the essential oil extracted from the use of a 1.0 mL pycnometer, previously dried, tared and calibrated.

2.2 Chemical analysis

For the chemical analysis, we used the gas chromatographic technique coupled to the electron impact mass spectrometer and ion trap analyzer (GC/MS). The equipment used was of the Varian 2100 brand, using helium as drag gas with flow in the column of 1mL min^{-1}; injector temperature 270 °C, split 1:50; (15 m × 0,25 mm) with stationary phase VF-1ms (100% methylsiloxane 0.25 µm) and oven temperature programming of 60 to 200 °C with a heating rate of 8 °C min^{-1} and 200-290 °C with heating rate of 15 °C min^{-1}. In the mass spectrometer the manifold, ion trap, and transfer line temperatures were 50, 190 and 200 °C, respectively. 1.0 µL (automatic injector CP-8410) aliquots of the samples diluted in the proportion of

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20 µL in 1.5 mL of hexane were injected. We have identified the components of the oil from the comparison of these with the data obtained from authentic substances in reference libraries[16].

2.3 Collection and cultivation of Aedes aegypti *larvae*

In this way, we collected eggs at the Federal University of Maranhão, Bacanga Campus in São Luís Maranhão, through traps called ovitraps. These consist of black polyethylene pails with a capacity of 500 mL each, where we put water and insert two eucatex vanes into the mosquito. We inspect the traps weekly for replacement of the reeds and egg collection. After this step, we placed *A. aegypti* eggs to hatch at a temperature of 31 °C in a 200 mL polyethylene vessel with mineral water. We fed the larvae with cat food until they reached the third stage, when the experiments were done.

2.4 Test of larvicidal activity

We prepared a 1,000 $mg \cdot L^{-1}$ stock solution from the 50 mg weighing of the oil into a solution of 49.75 mL of distilled water and 0.25 mL of Tween-80. From this, we prepared five solutions at the concentrations 5, 10, 30, 50 and 70 $mg \cdot L^{-1}$. For each concentration, we used ten larvae and 30 mL of each solution in the cited concentrations. We performed all the tests in triplicate and as negative control we used a solution formed by 49.75 mL of water to 0.25 mL of Tween-80, and as a positive control, a solution of Temephos ([4-(4-dimethoxyphosphinothioyloxyphenyl) sulfanylphenoxy]-dimethoxy-sulfanylidene-λ^5-phosphane), which is equivalent to the concentration used by the National Health Foundation (Funasa) for the larvicidal control of the vector, in addition to Novaluron (N-[[3-chloro-4-[1,1,2-trifluoro-2-(trifluoromethoxy)ethoxy]phenyl]carbamoyl]-2,6-difluorobenzamide) at 0.02 $mg \cdot L^{-1}$, a dose adopted by the Brazilian Ministry of Health, which indicates by the World Health Organization in the range of 0.01 to 0.05 $mg \cdot L^{-1}$.

2.5 Malacological investigation

From this, we collected the samples of snails in the natural breeding sites of the neighborhood Sá Viana, the periphery of São Luís, Maranhão. The catch was carried out during rainy periods, with the use of PPEs (personal protective equipment), such as glove, boot seven leagues, and metal tongs. The collection technique consists of scraping the submerged areas with the shell and the collected snails were placed in a glass container with a lid, with water from the breeding site itself[17]. The search of the same ones was realized in several points of each breeding place, in order to obtain a good sampling. After collection, these were labeled by the breeder and taken to the laboratory for identification and analysis. From the technique of dissection of the genital apparatus[18], we identified the snails as belonging to the family *Planorbidae*, genus *Biomphalaria*, species *B. glabrata*.

2.6 Snails positivity test

For instance, we placed five snails in clear glass vials (30 mL capacity) with 25 mL of dechlorinated water, that is 5 mL per snails, brought to light exposure (100 W lamps), at a distance of 30 cm, during 1 h, to stimulate the release of cercariae[19]. After exposure, the glasses were taken for analysis by means of a stereoscopic magnifying glass 8x. Those that were parasitized (positive) were labeled and separated for future individual analysis in order to verify which was contaminated and those that did not show signs of infection by the trematode in the period of 30 days were selected for the molluscicidal activity test. The period of analysis of the snails was every 7 days, for one month (30 days) to confirm the absence of larval stages.

So, after the positivity test, we placed the snails in polystyrene containers with dechlorinated water and fed with hydroponic lettuce for future test of molluscicidal activity.

2.7 Molluscicidal activity test

The molluscicidal activity was performed according to a manual described by the World Health Organization. To do this, we placed 10 adult snails, negative for *Schistosoma mansoni* in each beaker containing 500 mL of a solution obtained from the dilution of each oil with distilled water and 0.15 mL of Tween 80 (surfactant) at the concentrations of 100, 75, 50, 25, and 10 mg L^{-1}, obtaining at the end a proportion of 50 mL of solution for each snail and feeding them with hydroponic lettuce *ad libittum*[20]. They were exposed to the solution for 24 h at room temperature. After this period, the snails were

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removed from the solution and the snails were washed twice with dechlorinated water, placed in each beaker containing 500 mL of dechlorinated water, fed with hydroponic lettuce and observed every 12 h (method recommended 24 h) for four days to assess mortality. To confirm the activity, we observe the mollusks. If the cephalopods mass is retracted into the shell, release the hemolymph, or swell and extend the cephalopod out of the shell, it is considered dead[21].

2.8 Toxicity test with Artemia salina

So that, the *Artemia salina* Leach cysts were transferred to an aquarium containing the synthetic saline solution (60 g of sea salt / liter of distilled water) and oxygen saturation, obtained with the aid of an air pump. The aquarium was divided into two interconnected compartments, the cysts remaining in one of the compartments, leaving the second compartment under artificial illumination of a 100 W lamp. After 24 h, the cysts hatched, the larvae migrated to the lighted compartment because they had phototropism positive. These were transferred to an aquarium containing synthetic saline and kept in incubation for another 24 h under the same lighting and oxygenation conditions. The methodology used was described by Meyer *at al*.[22] but with modifications.

For the evaluation of the lethality of *A. salina* Leach, 20 mg of the oil was added to 0.02 mg of Tween 80, the volume was filled to 2 mL with artificial saline. This dilution was done to obtain a 10 mg mL^{-1} stock solution and a concentration of 0.1% Tween 80. Samples of 5, 50, 250 and 500 µL of this stock solution were transferred to vials with 5 mL of final solution, obtaining concentrations of 10, 100, 500 and 1000 $mg \cdot L^{-1}$, respectively. Ten larvae in the nauplii phase were transferred to each flask. White (saline) was made with 20 µL and the negative control (saline and 0.1% Tween 80) was made with 20 µL. After 24 h of incubation, the live larvae were counted, considering those microcrustaceans that did not move during observation and with slight agitation of the flask. Thus, we adopted the criterion established by Amarante *et al*. [23], which consider LC_{50} samples less than 100 mg L^{-1}, highly toxic; with LC_{50} between 100 and 500 $mg \cdot L^{-1}$, moderately toxic; and LC_{50} greater than 500 $mg \cdot L^{-1}$ nontoxic.

2.9 Statistical analysis

The statistical test used was Anova of single factor and Tukey's posterior test to identify significant differences. For the calculation of the lethal concentration (LC_{50}), we used the Probit method[24]. For mortality results, we expressed these results with mean ± standard deviation. For all statistical tests, we considered the significance of $p \leq 0.05$.

3. Results

3.1 Evaluation of the chemical characteristics of the essential oils obtained by gas chromatography coupled to mass spectroscopy (GC/MS)

Before identifying and quantifying the components present in the oil, we performed the kinetic test for extraction in the time interval of 0.5 to 5 h to verify the best performance. From this test, we verified that from 3 to 5 h, the oil yield remained constant, obtaining a volume of 0.35 mL. We calculated the extraction yield from the mass we used, which was 30 g of the material, volume obtained after extraction, of the density measurement, which was 0.823 $g \cdot mL^{-1}$ and the formula expressed by the Brazilian Pharmacopoeia[52]. From this, the result obtained was, respectively in the ratio mass / volume and mass / mass, of 1.17% and 0.96%.

From this study, we identify and quantify the components present in the oil. The result of the GC-MS analysis showed 23 peaks, which indicated the presence of 23 compounds (Fig. 1). In comparing the mass spectra of the constituents with the NIST 8 library, we identified the 15 compounds (Table 1). Based on the results, we observed that the major component is Limonene (58.81%) and the minority is the α-Mulene (0.11%) (Table 1).

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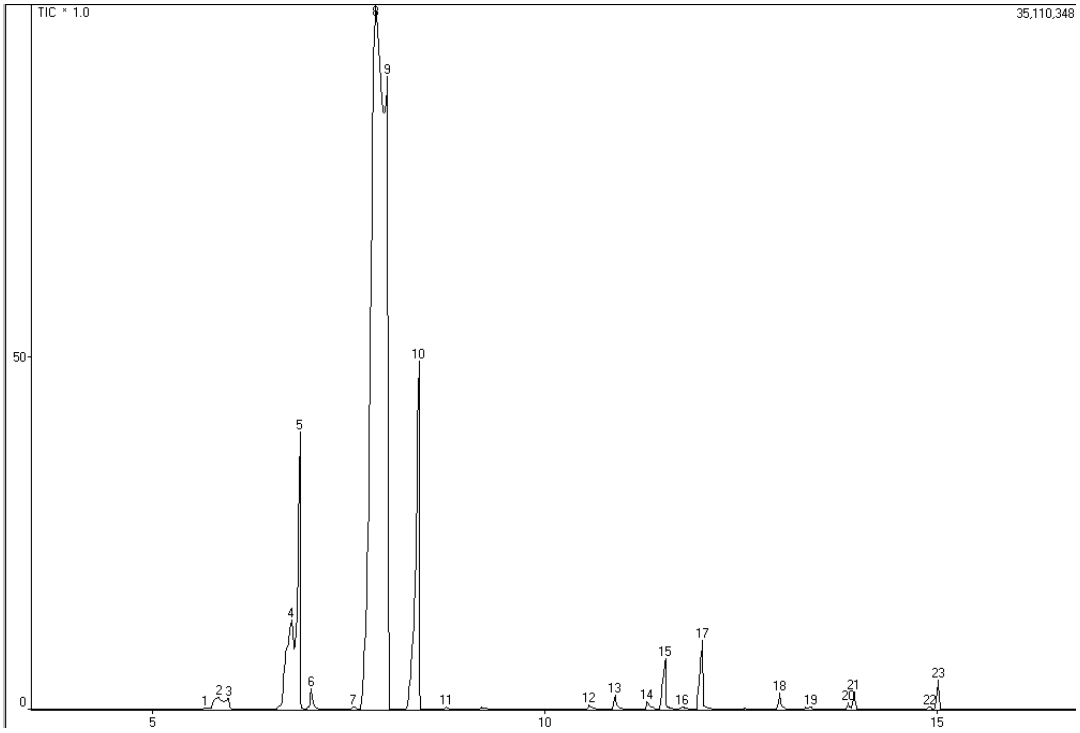

Figure 1. *Citrus limon* peel essential oil GC/MS Chromatogram.

Table 1. Compounds identified in the essential oil sample from *Citrus lemon* peel.

Peak[1]	R.T(min)[2]	Components	Percentage (%)	Quality[3]
1	5.858	α-Pinene	0.72	95
2	6.775	Sabinene	5.08	92
3	6.875	β-Pinene	4.91	93
4	7.033	β-Myrcene	0.45	91
5	7.845	Limonene	58.81	90
6	8.408	γ-Terpinene	9.01	93
7	10.575	Terpine-4-ol	0.13	89
8	10.892	α-Terpinenol	0.31	96
9	11.308	Trans-geraniol	0.25	92
10	11.525	β-Citral (geranial)	1.11	93
11	12.000	α-Citral (geranial)	1.61	92
12	13.000	Geranial acetate	0.32	93
13	13.400	α-Mulene	0.11	85
14	13.950	Bergamolene	0.29	92
15	15.017	β-Bisabolene (geranial)	0.50	92

Note: [1]Number of the peak in the column elution order; [2]RT: Retention time of the compounds. Quality[3]: search index in the database that reflects the similarity of the mass spectrum obtained with the records in the libraries used.

3.2 Larvicidal activity, molluscicide and toxicity

We tested the larvicidal activity for concentrations of 5, 10, 30, 50 and 70 mg L^{-1}, molluscicide for 10, 25, 50, 75 and 100 mg L^{-1} and toxicity for 10, 100, 500 and 1000 mg L^{-1}. In all tests, we observed an increase in the percentage of mortality with the increase in concentration and absence of interference of the biological activity in the control test. Thus, they were higher in

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concentrations of larvicidal activities, molluscicides and toxicity, respectively 70, 50 and 1000 mg L^{-1}. In addition, we observed that there were no significant differences between treatments (Table 2). The LC_{50} values for larvicidal activities, molluscicides and toxicity are, respectively, 15.48, 13.05 and 743. 35 mg L^{-1} (Table 3).

Table 2. Results of the larvicidal, molluscicidal and toxicity activities of the essential oil of *Citrus limon* peels.

Larvicidal activity	
Concentration (mg L^{-1})	Mortality of larvae (%)
70	100.0 ± 0.0[a]
50	86.7 ± 0.4[a]
30	66.7 ± 0.4[a]
10	36.7± 0.4[a]
5	13.3± 0.4[a]
Molluscicidal activity	
Concentration (mg L^{-1})	Mortality of snails (%)
100	100.0 ± 0.0[a]
75	100.0 ± 0.0[a]
50	100.0 ± 0.0[a]
25	86.7 ± 0.4[a]
10	26.7 ± 0.4[a]
Toxicity activity	
Concentration (mg L^{-1})	Mortality of larvae (%)
1000	100.0 ± 0.0[a]
500	46.7± 0.4[a]
100	13.3 ± 0.4[a]
10	0.0 ± 0.0[a]
The mean values and the standard deviation of the measurements in triplicate. Different letters indicate significant differences ($p < 0.05$).	

Table 3. LC_{50} result of larvicidal activity, molluscicide and essential oil toxicity extracted from *Citrus limon* peel.

Biological Activity	**Lethal Concentration (LC_{50}) (mg L^{-1})**	**Lower Limit (mg L^{-1})**	**Upper Limit (mg L^{-1})**	**Standard deviation (SD)**	**R^2 (Correlation)**
Larvicidal	15.5	10.3	23.3	0.4	0.97
Molluscicidal	13.1	9.5	18.0	0.2	1
Toxicity	743.4	346.9	1593.0	0.7	1

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4. Discussion

From this study, we showed that the essential oil extracted from the bark of *C. limon* has larvicidal activity, molluscicide and toxicity. This may be a viable alternative to synthetic larvicides and molluscicides, since they would act against target organisms due to their toxicity against other organisms. We found that the percentage (m/v) of the oil yield was 1.17% and that the main components are limonene (major component) and α-Mulene (minor component).

The yield that we obtained in a time of extraction of 5 h was 1.17%, in which this value remained constant in the interval of 3 to 5 h. The study of the yield allows to evaluate the time necessary to conserve the best characteristics of the oil. According to Mouchrek Filho[25], quoted by Gomes *et al.*[26], a time of rapid extraction leads to a product with the predominance of more volatile constituents, but without the best characteristics. Otherwise, slow distillation overloads the product with undesirable flavors[54]. Generally, the best yields are obtained from the time of 3 h[26-30].

From the chromatographic method coupled to the mass spectrometer, we identified and quantified limonene as the major component of the essential oil extracted from *C. limon* peels. Thus, we confirm what has already been described in the literature regarding this component for citrus substances. However, we observe that the quantity of this differs from other works, where they are generally above[31-33] and others below 50%. The redirection of plant metabolic pathways to lead to the biosynthesis of different compounds[34-36] and abiotic factors are responsible for caused changes in the composition of *C. limon* essential oil.

In the study of larvicidal activity, the essential oil was active against the third stage larvae of *A. aegypti* in the concentration of 70 $mg{\cdot}L^{-1}$ with 100% mortality of the larvae tested. Although this result gives us a dimension of biological activity, a statistical calculation of the lethal concentration (LC_{50}) from the method of Finney[24] gave us an estimate of inferring this result for a population, considering a statistical distribution of the normal type. The result of LC_{50} was 15.48 $mg{\cdot}L^{-1}$. From this result, we compare the criteria adopted by Cheng *et al*. (2003), since up to the moment of this research there is no criterion established the World Health Organization[37] to consider active larvicidal activity or inactive. According to the Cheng *et al*.[38] the larvicidal activity of the essential oil is active when the LC_{50} <100 $mg{\cdot}L^{-1}$; inactive when LC_{50}> 100 $mg{\cdot}L^{-1}$ is highly active when LC_{50} <50 $mg{\cdot}L^{-1}$. Thus, from this classification the essential oil we extract from the bark of *C. limon* is considered highly active.

The larvicidal activity of the essential oils are influenced by several factors. A study by Fernandez *et al*. (2014)[39] showed that larvicidal activity is higher in spring, summer and autumn, and lower in winter, confirming the influence of seasonality; for Leyva *et al.*[40] synergistic action among metabolites (even in small proportions), the collection period and the extraction method are responsible for this; while other studies attribute this action to terpenes, alcohols, and aldehydes[41-43].

For these reasons, the results of our study confirm the larvicidal and insecticidal activity of limonene of *C. limon* essential oil against the genus *Aedes*. The study carried out by Campolo *et al*.[44] showed the activity of this oil against *Aedes albopictus* larvae with a lethal concentration (LC_{50}) of 145.27 mg L^{-1} after 24h exposure. However, the study of larvae, pulps and adults of *Aedes albopictus*[45] confirmed the activity of the oil with a lethal concentration (LC_{50}) of 35.99 and 34.89 $mg{\cdot}L^{-1}$ for the enantiometric forms of limonene, respectively, R - (+) - limonene and S - (-) – limonene. Finally, the study Amer and Mehlhorn[46] confirmed that activity against third-stage larvae of *A. aegypti* was higher at 24 h exposure time when compared to shorter times. Thus, the difference that these studies have with the results of our study is in the low value of the LC_{50} that we obtain. To explain this difference, we attribute seasonality, collection and extraction factors and synergism.

In relation to molluscicidal activity, the essential oil was active with LC_{50} 13.48 $mg{\cdot}L^{-1}$. In this case, to affirm this, we compare the result obtained from the LC_{50} with the criteria used by the WHO[11]. According to this criterion, the extract obtained from the plant is active when it causes mortality of 90% of the aquatic mollusks at the 24 h exposure time, under constant temperature and concentration up to 100 $mg{\cdot}L^{-1}$ [47]. Thus, we proved that the result we obtain from the LC_{50} of our study is within the limits established by the WHO.

Besides the molluscicidal activity which we show in this work, we observed in the literature that the limonene obtained from the *Carum cravi* seed powder showed activity against the snail *Lymnaea acuminata*[6]. Although this component caused mortality of *Lymnaea acuminata*, the inference of

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the lethal concentration, LC_{50}, for a population of these snails did not meet the criteria established by WHO[11], whose value was 130.61 mg·L^{-1} in the time of 24 h. In addition, this study left craving for the percentage absence of limonene contained in the extracts, in which it left doubts about its effectiveness.

In the literature there are other plants that have molluscicidal activity against *B. glabrata*, however, the effectiveness is in the lowest value of the lethal concentration (LC_{50}). The study of thirteen *Solanum* species revealed that the extracts of the species *S. asperum, S. diamantinese, S. paludosum, S. sisymbriifolium and S. stipulaceum* present activity with lethal concentration (LC_{50}) varying from 20 to 50 mg L^{-1} [48]; *Moringa oleifera* Lam seed extracts were active in the lethal concentration, LC_{50}, 419 mg L^{-1} [49]; extracts from the stems of *Melloa quadrivalvis* and *Tabebuia aurea* and whole plants of *Adenocalymma comosum, Arrabidaea parviflora, Cuspidaria argentea*, and *Clytostoma binatum* have activity with LC_{50} concentration varying from 5.2 to 37.5 mg L^{-1}, being the most active *Cuspidaria argentea*, LC_{50} 5.2 mg L^{-1} [50]; the essential oil of *Pimenta dioica* is active with LC_{50} 18.62 mg L^{-1}, however the toxicity study showed that it is highly toxic to other organisms[53]. Though, the *Ocotea bracteosa* essential oil has activity with concentration, LC_{50} 4.6 mg L^{-1} [51]. Although *O. bracteosa* essential oil has good results in lethal concentration values, the absence of a study of toxic activity in this study raises doubts about its effectiveness in a real system.

Hence, faced with this impasse, when performing the study of toxicity, we evaluated the possibility of the action of oil against non-target organisms. In our study, we used *A. salina* because of the following characteristics: formation of dormant cysts, low cost, easy manipulation in the laboratory and indication of interference with nontarget organisms[12,13]. Based on the toxicity parameters of Amarante *et al.*[23], the essential oil, with LC_{50} = 743.35 mg L^{-1}, is non-toxic (LC_{50}> 500 mg L^{-1}). In addition to our study, we found in the literature extracts from six plants of the family Bignoniaceae that have moderate to low toxicity with LC_{50} values varying from 485.5 to 815.4 mg L^{-1} [50].

5. Conclusions

In this manner, according to the results obtained in this study, we can conclude that the essential oil extracted from the shells of *C. limon* has larvicidal activity and molluscicide, respectively, third stage larvae of *A. aegypti* and snails *B. glabrata*, and non-toxic front *A. salina*. In addition, we identified and quantified the majority and minority components, which were, respectively, limonene and α-Mulene.

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