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Chemical composition and larvicidal activity of essential oil of the bark of *Citrus sinensis* (L.) Osbeck

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Summary

Aim: We determined the chemical composition and larvicidal activity of the essential oil distilled from the *Citrus sinensis* (L.) Osbeck husks and the pattern of the major constituent. **Materials and methods:** For this, we distill the oil by hydrodistillation, identify the components by gas chromatography coupled with mass spectrometry (GC-MS), test the larvicidal activity against *Aedes aegypti* and calculate the lethal concentration at 50% (LC_{50}) by the Reed-Muench method and the confidence interval by the Pizzi method for both oil and standard. **Results:** it showed that the oil consists mostly of limonene and showed larvicidal activity (LC_{50} of 199.01 (± 2.10) μg·mL^{-1}) greater than the lemonade standard (126.03 (± 2.09) μg·mL^{-1}). **Conclusion:** Therefore, we conclude that distilled oil has the potential to replace chemical larvicides.

Keywords: Aedes aegypti, larvae, volatile compounds, *Citrus sinensis* (L.) Osbeck, distilled oil.

Resumo

Composição Química e atividade larvicida do óleo essencial das cascas do *Citrus sinensis* (L.) Osbeck

Objetivo: determinamos a composição química e a atividade larvicida do óleo essencial destilado das cascas do *Citrus sinensis* (L.) Osbeck e do padrão do constituinte majoritário. **Materiais e métodos:** para isso, destilamos o óleo por hidrodestilação, identificamos os componentes por cromatografia gasosa acoplada à espectrometria de massas (CG-EM), testamos a atividade larvicida contra o *Aedes aegypti* e calculamos a concentração letal a 50% (CL_{50}) pelo método Reed-Muench e o intervalo de confiança pelo método de Pizzi tanto para o óleo quanto para o padrão. **Resultados:** o óleo é constituído em sua maior parte por limoneno e apresentou atividade larvicida (CL_{50} de 199.01 (± 2,10) $\mu g \cdot mL^{-1}$) maior que o padrão limonento (126,03 (± 2,09) $\mu g \cdot mL^{-1}$). **Conclusões:** portanto, concluímos que o óleo destilado tem potencial para substituir os larvicida químicos.

Palavras-chave: Aedes aegypti, larvas, compostos voláteis, *Citrus sinensis* (L.) Osbeck, óleo destilado.

Resumen

Composición química y actividad larvicida del aceite esencial de corteza de *Citrus sinensis* (L.) Osbeck

Objetivo: determinamos la composición química y actividad larvicida del aceite esencial destilado de las cáscaras de *Citrus sinensis* (L.) Osbeck y el patrón del constituyente mayoritario. **Materiales y métodos:** para ello destilamos el aceite por hidrodestilación, identificamos los componentes mediante cromatografía de gases acoplada a espectrometría de masas (CG-EM), testeamos la actividad larvicida frente a *Aedes aegypti* y calculamos la concentración letal al 50% (CL_{50}) por el método Reed- Muench y el intervalo de confianza por el método de Pizzi tanto para aceite como para la muestra estándar. **Resultados:** el aceite consiste principalmente en limoneno y mostró actividad larvicida (CL_{50} de 199,01 (± 2,10) $\mu g \cdot mL^{-1}$) mayor que el estándar de limonada (126,03 (± 2,09) $\mu g \cdot mL^{-1}$). **Conclusiones:** por lo tanto, concluimos que el aceite destilado tiene el potencial de reemplazar los larvicidas químicos.

Palabras clave: Aedes aegypti, larvas, compuestos volátiles, *Citrus sinensis* (L.) Osbeck, aceite destilado.

Introduction

The methods used to control the mosquito or larvae of *Aedes aegypti* have advantages and disadvantages. Previous studies have shown that the method of predation by fish *Trichogaster trichopteros* and *Astyanax fasciatus* was effective only for larvae, but not for adults, while chemical insecticides based on carbamates, pteroids and organophosphates are effective for both at low con-centration, but its use is disadvantageous, as it causes resistance from mosquitoes, attacks on non-target organisms and contamination of the water flow [1-3]. Therefore, it is necessary to search for other larvicides that are effective and less dangerous to man and the environment.

Within this perspective, we highlight larvicides based on extracts or oils distilled from plants. Previous studies have shown the larvicidal activity of some plants [4-9], due to high biodegradability, low toxicity to mammals [10, 11], impediment of ovoposition, inhibition, growth and reproduction against mosquito species [12, 13] and in some cases with greater durability than synthetic products [14, 15]. Generally, the biological activity of a plant is attributed to the component in greater quantity, in which case limonene is highlighted.

Citric compounds, for the most part, consist of limonene [16]. Previous studies show that this compound alone or as a major constituent in plants has larvicidal activity against *Aedes aegypti* [6, 7, 17]. Among citrus species with high limonene content, we highlight *Citrus sinensis* (L.) Osbeck. In the literature, there are reports that the oil distilled from this plant has molluscicidal [18], antifungal [19], antimicrobial, antiaflatoxigenic and antioxidant activity [20].

Therefore, in view of the above, in this study we will determine the chemical composition and larvicidal activity of the oil distilled from the husks of *Citrus sinensis* (L.) Osbeck and the pattern of the major constituent against *Aedes aegypti*.

Material and methods

Obtaining and distilling essential oil

Sample collection took place similarly to the study by Gomes *et al.* [18], however with some modifications. We collected the samples (fruits), branches and leaves of the plant species *Citrus sinensis* (L.) Osbeck on the campus of the Federal Institute of Education, Science and Technology of Maranhão (IFMA) - Maracanã, located at Avenida dos Curiós, S / Nº, Vila Esperança, whose exsiccates presented identification number 01484 at "Herbário Ático Seabra" of the Universidade Federal do Maranhão.

To distill the essential oil, we used a Clevenger glass extractor that was attached to the 6000 mL round bottom flask connected to the electric blanket as a heat source. In each extraction routine, weigh and crush in an electric mill 300 g of the sample. After this step, we mix with 1:10 distilled water and place it in a round bottom flask connected to the extractor system. Then we turn on the electric blanket at a temperature of 100 °C. After 5 h, the distillation was extinguished by collecting the essential oil. This was dried by percolating over anhydrous sodium sulfate. These operations were performed in triplicates and the samples stored in amber glass ampoules under refrigeration to avoid possible losses of volatile constituents. For density determination, we used a pycnometer, while the yield was calculated from the volume of oil obtained and the base of the moisture-free material (B.L.U).

Chromatographic analysis GC/MS

We identified the constituents of the essential oil by gas chromatography coupled with mass spectrometry (GC-MS) equipped with the same configurations as the study by Gomes *et al.* [18]. The volatile constituents were observed in a Shimadzu gas chromatograph, coupled to a GC-MS mass spectrometer QP5050A, equipped with a BPX 5% phenylpolysphenylene-siloxane capillary column, (30 m length x 0.25 mm thick and 0. 25 μm of film thickness), using helium as carrier gas. The temperature of the injector and GC interface with selective detector was maintained at 280 °C in flow in the 2.7 $mL \cdot min^{-1}$ column, programmed to operate at 50 °C. For analysis, we inject aliquots with a volume of 1 mL in ethyl acetate. The components of the oil were identified by comparing these with data obtained from authentic substances existing in reference libraries.

Obtaining and cultivating *Aedes aegypti* larvae

We carried out the collection and cultivation of *Aedes aegypti* larvae according to the methodology proposed by Gomes *et al.* (2019) [6]. We collected the eggs at the Universidade Federal do Maranhão, Campus do Bacanga in São Luís Maranhão, through three black polyethylene buckets, with a capacity of 500 mL each, where we put water and insert two eucatex straws for the mosquito oviposition. We inspect the traps weekly to replace the reeds and collect the eggs. After this stage, we put the *Aedes aegypti* eggs to hatch at a temperature of 31 °C in a 200 mL polyethylene container with mineral water. We fed the larvae with cat food until they reached the third stage, when the experiments were carried out.

Test of larvicidal activity

For the larvicidal test, we performed it according to the methodology proposed by Gomes and collaborators with some modifications [6]. Initially, we prepared

a 1.00 mg·L^{-1} stock solution by weighing 50 mg of the oil into a solution consisting of 49.75 mL of distilled water and 0.25 mL of Tween-80. From there, we prepare solutions in concentrations from 70 to 150 mg·L^{-1}. We used ten larvae for each concentration and 30 mL of each solution at the concentrations mentioned. We performed all tests in triplicate and as a 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- [(dimethoxyphosphorothioyl) oxy] phenyl} thio) phenyl dimethyl phosphate) to 100 mg·L^{-1}, equivalent to the concentration used by the Fundação Nacional de Saúde (FUNASA) for the larvicidal control of the vector. In an analogous way, we prepare the solutions for the standard of the major constituent, identified in the chromatographic analysis.

Statistical analysis

In this manner we performed the statistical analysis according to the methodology proposed by Gomes *et al.*[9, 21], using the Reed-Muench method. This method assumes that an animal may die or survive, respectively, a dose higher or lower than those stipulated. Basically, starting from a table with mortalities in the tested concentration, we constructed a graph of the percentage mortality according to the logarithm of the tested concentration, in which the intersection of the curves of live and dead accumulates shows the Lethal Concentration at 50% (LC_{50}) [22, 23].

To calculate the confidence interval, we use the Pizzi method [24], according to formula $2 \cdot 10^{SE}$, where the standard error (SE) is calculated from the formula ($SE^2 = 0.79 \cdot h \cdot R/20$), where R and h are determined, respectively, from the difference between the log of the dose that kills 75% and the log of the dose that kills 25% of the larvae and the average of the differences in the log values of the doses.

Performance and chromatographic analysis of essential oil

To determine the composition, distill the oil by hydrodistillation and identify the components by gas chromatography coupled to the mass spectrometer. The distillation result revealed an average yield and average density, respectively of 2.47% (m/m) and 0.850 g×mL^{-1}, while the chromatographic analysis showed us the presence of 15 compounds in which the largest were, respectively, limonene, linalool and β-Myrcene.

Larvicidal Activity

We investigated larvicidal activity by submitting ten larvae to concentrations of 70 to 150 μg·mL^{-1} for essential oil and 70 to 160 μg·mL^{-1} for the main component pattern identified in the GC / MS analysis over a 24 h period. Then, we performed the calculation for 50% lethal concentration (LC_{50}) from the intersection of the accumulated

dead and alive curves by the Reed-Muench method and the confidence interval by the Pizzi method. From this analysis, we observed that the essential oil distilled from the shells of *C. sinensis* (L) Osbeck (table 2 and figure 1) and the limonene pattern (table 3 and figure 2) showed larvicidal activity with 100% mortality, respectively, in the concentration 150 and 160 $\mu g \cdot mL^{-1}$, and LC_{50} and confidence interval, respectively, 99.01 (± 2.10) $\mu g \cdot mL^{-1}$ and 126.03 (± 2.09) $\mu g \cdot mL^{-1}$. As shown in the LC_{50} values, we observed that the oil has a higher lethality than the standard.

Table 1. Identification of compounds in the essential oil sample from *Citrus sinensis* (L) Osbeck.

Retention Time	Compounds	Percentage (%)
5155	α-Pinene	0.33
6350	β-Myrcene	1.90
6861	Octanal	0.93
7610	Limonene	89.55
8287	1-Octanol	0.46
8919	Linalool	5.36
8959	Nonanal	0.08
9866	Citronelal	0.06
10523	Terpineol	0.12
10873	α-Terpineol	0.39
10926	Decanal	0.25
11352	β-Citronelol	0.08
11643	Neral	0.13
12210	Citral	0.17
12496	Hexane cycle	0.20

Table 2. Mortality of larvicidal activity of the essential oil.

Concentration ($\mu g \cdot mL^{-1}$)	Log of the concentration	Dead	Alive	Dead accumulated	Alive accumulated	Mortality (%)
150	2.1760	10	0	36	0	100
130	2.1139	8	2	26	2	80
120	2.0791	7	3	18	5	70
100	2.0000	5	5	11	10	50
90	1.9542	4	6	6	16	40
70	1.8450	2	8	2	24	20

Table 3. Mortality of the larvicidal activity of the limonene pattern.

Concentration ($\mu g \cdot mL^{-1}$)	Log of the concentration	Dead	Alive	Dead accumulated	Alive accumulated	Mortality (%)
160	2.2040	10	0	29	0	100
150	2.1760	7	3	19	3	70
130	2.1139	5	5	12	8	50
120	2.0791	3	7	7	15	30
100	2.0000	2	8	4	23	20
90	1.9542	1	9	2	32	10
70	1.8450	1	9	1	41	10

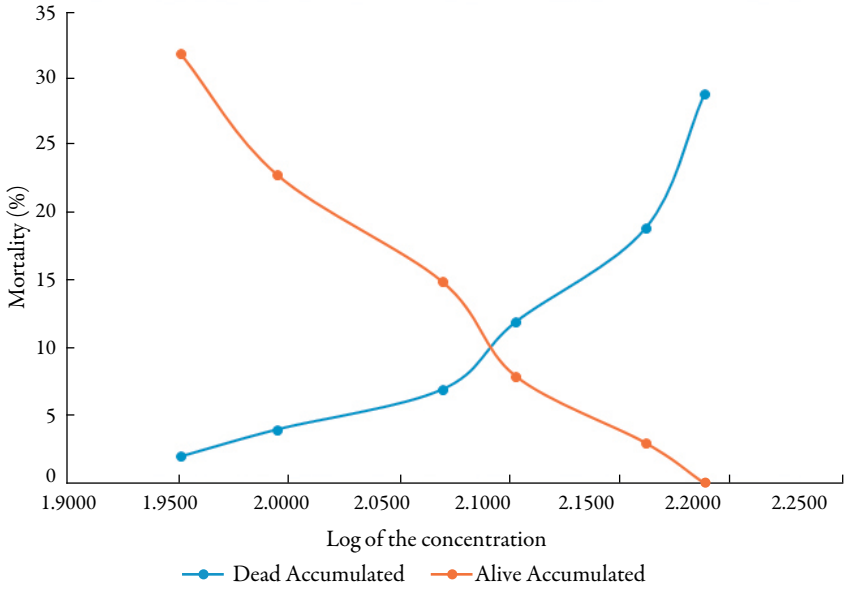

Figure 1. Estimate of the CL_{50} of the essential oil shells distillate of the *C. sinensis* (L) Osbeck through the method Reed-Muench.

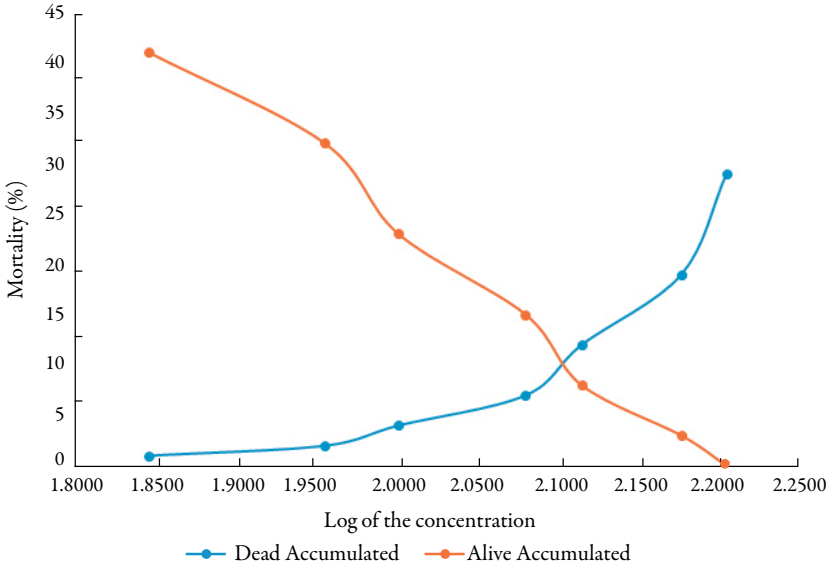

Figure 2. Estimate of the CL_{50} of the limonene pattern through the method Reed-Muench.

Discussion

Combating the larvae of the vector mosquito *Aedes aegypti* with chemical insecticides is the most effective way to prevent the spread of diseases. However, previous studies show that the use of these caused resistance in the mosquito population and reached non-target organisms, which, in some way, encouraged researchers to seek alternatives that impact less the environment and are more effective from plant-based insecticides. Here, we show that the essential oil distilled from the husks of *Citrus sinensis* (L.) Osbeck consists mostly of limonene and showed greater larvicidal activity against *Aedes aegypti* than the limonene standard. Therefore, distilled oil has the potential to replace the use of chemical larvicides.

In the first found, from the chromatographic analysis coupled to the mass spectrometer of the oil distilled from the shells *C. sinensis* (L.) Osbeck we identified 15 compounds in which limonene was present in greater quantity. The result of our study differs somewhat from previous studies, as in these the percentage of limonene ranged from 71.3 to 98% [18, 25-27]. Generally, we attribute this difference to the interference of some factors such as: seasonality, temperature, collection period, intensity of solar radiation, age, and development of plants, among others [28, 29].

In the second found, we confirmed the larvicidal activity of the oil by comparing the result of ours with a study with the criterion established by Cheng *et al.* (2003). According to these authors, a compound shows activity when the LC_{50} is less than 100 $\mu g \times mL^{-1}$ [30]. Taking this criterion into consideration, we observed that the standard was considered ineffective, as it exceeded the limit value. However, the results obtained in this study are in line with previous studies, in which they state that essential oils are more effective than the standard of the major component [31].

So, the evidence that the oil distilled from the shells of *C. sinensis* (L.) Osbeck showed larvicidal activity came from the observation of the main component identified in this study, limonene. Previous studies with other citrus species, whose limonene was the major constituent, showed larvicidal activity with LC_{50}, ranging from 15.48 to 37.03 $\mu g \cdot mL^{-1}$ [6.17]. However, the results obtained in this study differ from previous studies [17, 26, 27], in which the values obtained from the LC_{50} for distilled oil *C. sinensis* (L.) Osbeck ranged from 11.92 to 538 $\mu g \cdot mL^{-1}$. Although most of these studies do not present exactly the periods of the activity evaluation and due to the difference of seasons on the terrestrial globe in different hemispheres, we explain these differences from the seasonal variation, as previous studies have shown that larvicidal activity is greater in seasons spring and autumn when compared with summer and winter [32,33].

Despite the good results obtained in this study, we recommend for later studies the evaluation of the larvicidal activity of the other components of the oil in order to verify the influence of synergism.

However, a larvicidal activity described in our study just has shown the potential of the oil distilled from the shells of the *C. sinensis* (L.) Osbeck to replace chemical larvicides. However, further studies are needed to certify its safety for the environment and for man.

Conclusion

To sum up, the essential oil shells distillate of the *Citrus sinensis* (L.) Osbeck by hydrodistillation is formed in its most part by limonene. Furthermore, the essential oil represented a larvicidal activity moderated against the *Aedes aegypti* and greater than the limonene standard, which makes it a potential substitute for synthetic larvicides.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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