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Chemical composition and larvicidal activity of *Citrus limonia Osbeck* bark essential oil

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Aedes aegypti mosquito arouses the interest of public authorities, as it is a vector for four diseases (dengue, zika, chikungunya, and yellow fever) and one of the ways to combat it is through insecticides. In this study, the main constituent, the predominant class of essential oil extracted from the husks of *Citrus limonia* Osbeck was identified and evaluated to know if it has biological activity against larvae in the third stage of *Ae.aegypti*. gas chromatography coupled with mass spectrometry (CG-MS); larvicidal activity as described by the World Health Organization (WHO) was evaluated and the lethal concentration (LC_{50}) from the Probit model was calculated. The results show that the oil consists mainly of limonene, beta-Pinene, meta-Cymene, beta.-Phellandrene and alpha-Pinene, in which the predominant class was monoterpenes and the lethal concentration, CL_{50}, was 67.18 µg.mL^{-1}. Therefore, the oil has potential larvicidal activity.

Key words: Volatile compounds, monoterpenes, *aedes aegypti*, limonene, natural insecticide.

INTRODUCTION

Aedes aegypti draws the public attention authorities. The interest in that mosquito is based on the principle that it is necessary to combat it, due to the diseases it transmits (dengue, zika, chicungunya and yellow fever) and the outbreaks and deaths that those diseases have caused in recent years (Bhatt et al., 2013). In this manner, to

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understand better the dimension of the problem, between 2013 and 2015 in Brazil, two million people were diagnosed with dengue and one million were infected with the zika virus, thus resulting in the largest outbreak reported in that country (Brasil, 2017; Cardoso et al., 2015). In conseqeunce to the fast spread of these diseases, especially the Zika virus, the World Health Organization (WHO) decreed in February 2016 emergency of Global Public Health when the relationship of that disease with the cases of microcephaly registered in Brazil was verified (Brasil, 2017). So, faced with this scenery, the authorities sought to minimize the number of cases through public awareness policies, showing the risks, symptoms and measures that prevent the mosquito's proliferation, and in the development of vaccines; until now there is only for yellow fever (Brasil, 2020; Rothman, 2004). While the development of vaccines for other diseases, such as dengue, zika and chicungunya, are still in the testing phase (Pang and Loh, 2017; Tripp and Ross, 2016), combating the vector mosquito remains the most effective control, be it in the larval or adult phase.

In the literature, there are reports of combating the *Ae.aegypti* mosquito using two methods: Predation and chemical insecticides. In the predation method, fish species (*Trichogaster trichopteros* and *Astyanax fasciatus*) were used to prey on larvae in a certain period of time (Cavalcanti et al., 2007). Chemical insecticides, on the other hand, generally use compounds based on carbamates, pteroids and organophosphates to kill larvae or adult insects at low concentrations, which is recommended by WHO (Govindarajan and Benelli, 2016). It is a fact that between the two methods the most effective are the insecticides, as these act on both larvae and adults. However, studies show that the intensive use of these compounds provoked attacks on non-target organisms and increased the resistance of the mosquito population (da Rocha Voris et al., 2018), which limits their use and encourages the search for other insecticides preferably, of plant origin that are effective and have less impact on the environment.

Among the larvicides of plant origin, the most studied are those based on essential oils. As these compounds are formed by complex structures, it is believed that one of its components, be it the majority or the minority, is responsible for biological activity. Among the compounds that have proven larvicidal activity, either alone or as the main constituent of an essential oil, limonene stands out (Cavalcanti et al., 2004; Estevam et al., 2016; Giatropoulos et al., 2012; Gomes et al., 2019; Millezi, 2014; Santos et al., 2011). In citrus species, this component is present around 80% (Ladaniya, 2008) so that a large part of the plants have larvicidal activity.

Among the various species of citrus plants, whose limonene is the main component, there is *Citrus limonia* Osbeck, known as pink lemon or china lemon; this species of plant has been widely cultivated in orchards and nurseries, due to the early maturation of its fruits and the economic values at the beginning of the harvest (Reda et al., 2005). Studies of the chemical composition revealed that the leaves and peels of the *C. limonia* Osbeck fruit consist, respectively, of 40 and 82% of limonene (Cavalcanti et al., 2004; Estevam et al., 2016; Millezi, 2014). In addition, this species of plant has some known biological activities which are: Trypanocidal, antibacterial, leishmanicidal (Estevam et al., 2016; Millezi, 2014) and larvicidal (Cavalcanti et al., 2004). It was emphasize that the larvicidal activity of the essential oil was demonstrated in the aerial parts (leaves and branches) of this plant, in which the low lethality against *Ae.aegypti* larvae in the 3rd stage was proven (Cavalcanti et al., 2004). Although the essential oil extracted from the leaves has a low larvicidal activity, until now there are no studies of this activity for the fruit peels. Thus, according to the view talked here before, in this study the main constituent was identified, the predominant class of essential oil was extracted from the husks of *Citrus limonia* Osbeck and assessed if it has biological activity against larvae in the third stage of *Ae.aegypti.*

MATERIALS AND METHODS

Obtaining and extracting essential oil

Fruit collection was carried out in a rural area of São Brás dos Macacos, municipality of São José de Ribamar-MA, Brazil, in January 2017, geographic coordinates latitude 02° 35'51, 8''S and Longitude 44° 09 '33, 3''W, and certified by the Laboratory of Botanical Studies with number 11.170. After collection, the shells were removed with a stylus.

The essential oil was extracted by hydrodistillation and the average yield was calculated from the density and weight measurements of the crude material. For extraction, 400 g of the samples was weighed and mix in 4000 mL of distilled water in a 1:10 ratio. Then, mixture was placed in a 1000 mL round-bottom flask and attached it to the Clevenger extractor under 100°C heating in an electric blanket for 4 h. After that time, the extracted oil was collected and dried by percolation in an anhydrous sodium sulfate solution. These operations were perform in triplicates and store the samples in amber glass ampoules under refrigeration to avoid possible losses of volatile constituents. A density pycnometer was used to measure density.

Chromatographic GC/MS analysis

In this manner, the components of essential oil by gas chromatography was identify coupled to mass spectrometry (GC/MS) in a gas chromatograph of the Shimadzu brand, coupled to a mass spectrometer of the model QP2020AS, using helium as carrier gas with flow in the 2.5 mL column .min^{-1}; injector temperature: 280°C, split 1:50; BPX 5% phenylpolysilphenylene-siloxane capillary column (30 m × 0.25 mm × 0.25 mm) and oven temperature programming from 60 to 280ºC. In the Mass Spectrometer, the temperature was 280°C and the recorded spectra were 35 to 550 m/z. We injected aliquots of 1 µL (automatic injector CP - 8410) of the samples diluted in the proportion of 20 µL

Table 1. Identification of the components present in the essential oil.

Compounds	Retention time (min)	Retention index	Standardized area (%)	Functional class
α-Pinene	3506	948	2.40	Monoterpene
β-Phellandrene	4024	964	3.01	Monoterpene
β -Pinene	4077	943	23.01	Monoterpene
β-Myrcene	4242	958	0.83	Monoterpene
m-Cymene	4725	1042	13.55	Monoterpene
Limonene	4789	1018	44.75	Monoterpene
Linalool	5834	1082	0.38	Monoterpene
1,2-Limonene oxide	6327	1031	0.98	Monoterpene
α-Pinocarvone	6771	1114	0.64	Monoterpene
Terpinen-4-ol	6988	1137	0.44	Monoterpene
α-Terpineol	7178	1143	0.77	Monoterpene
Myrtenol	7258	1191	1.20	Monoterpene
Cis-carveol	7583	1206	0.62	Monoterpene
(E)-Citral	7881	1174	0.75	Monoterpene
Carvone	7942	1190	0.64	Monoterpene
α-Bergamotene	10.496	1430	1.26	Sesquiterpene
β-Bisabolene	11.402	1500	1.66	Sesquiterpene
Caryophyllene oxide	12.375	1507	0.67	Sesquiterpene

in 1.5 mL of hexane. The oil components were identified by comparing their retention index with data obtained from authentic substances existing in NIST14 reference libraries.

Capture and creation of *Ae. aegypti*

The larvae were obtained through ovitraps in the period from January to February 2019. Ovitraps are prepared by the addition of water and two eucatex straws in polyethylene buckets with a capacity of 500 mL, where eggs are expected to be deposited by mosquito females. After hatching, the larvae in the 3rd stage were kept at room temperature 25 ± 2ºC and relative humidity of 70 to 80%, being fed with dog food.

Larvicidal bioassay

To carry out this experiment, the methodology described by the World Health Organization (World Health Organization, 2005) was followed. In the preliminary stage, ten larvae was transferred in the 3rd stage to disposable cups and expelled to three concentrations of oil (10, 50 and 100 μgmL^{-1}) in 24 h to check whether or not there was activity. After confirming this, five solutions of essential oil (50 to 130 μgmL^{-1}) dissolved in Dimethyl sulfoxide (DMSO) 0.1% was prepared. For each concentration tested, the negative control (DMSO 0.1%) was used with all tests performed in quintuplicates.

Statistical analysis

The average mortality data were submitted to Probit (Finney and Tattersfield, 1952) analysis for the calculation of LC_{50} and other statistics with 95% reliable limits, upper confidence limit, lower limit and chi-square calculated using the Medcalc 19.2 software with level of significance $p < 0.05$.

RESULTS

Oil extraction and chromatographic GC/MS analysis

The oil extracted by the hydrodistillation technique gave an average yield and density, respectively, of 2.54% (w/w) and 0.842 gmL^{-1}. In the chromatographic analysis by GC/MS, 18 compounds were identified (Table 1), where the five largest were, respectively, Limonene, β-pinene, m-Cymene, β-phellandrene and α-pinene and the class predominant was that of monoterpenes with 93.97%.

Larvicidal activity

In this study, it was observed that the essential oil extracted from the peels of *C. limonia* Osbeck has larvicidal activity (Table 2) CL_{50} 67.18 μg mL^{-1}, in an exposure time of 24 h, according to the criteria described by Cheng et al. (2003) that consider the activity when the lethal concentration (LC_{50}) is less than 100 μg mL^{-1}.

DISCUSSION

Thus, the diseases transmitted by the *Ae. aegypti* mosquito arouse the interest of public authorities who are looking for ways to control them from combating the vector, whether in larvae or mosquitoes. However, one of the main means used in this control, larvicides or chemical insecticides, has the main disadvantage of

Table 2. Larvicidal activity of essential oil in a 24-h period.

Concentration (μg mL^{-1})	Mortality (%)	CL_{50} (μg mL^{-1}) confidence interval (IC 95%)	χ^2
50	28 ± 0.44		
70	48 ± 0.83		
90	68 ± 1.30	67.18(57.11 - 79.02)	0.92
110	94 ± 0.54		
130	100 ± 0.00		

Mortality was expressed as the mean ± standard deviation.

resistance to mosquitoes and damage to the environment. On the other hand, some essential oil-based larvicides have shown good results in controlling the vector mosquito. In this study, the main constituent, the predominant class of essential oil extracted from the husks of *C. limonia* Osbeck was identified and whether it has biological activity against larvae in the third stage of *Ae. aegypti* was evaluated. Here, it was shown that the essential oil is mostly made up of limonene, in which the monoterpernos class prevailed, and it was demonstrated that the oil has larvicidal activity against *Ae. aegypti*, and can be a potential substitute for chemical larvicides.

In the first finding, the oil was extracted by hydrodistillation and subjected to chemical analysis to identify the main component and the predominant class. From this analysis, it was found that the essential oil *C. limonia* Osbeck is made up mostly of limonene in which the monoterpernos class predominated. However, the amounts of limonene identified in this study differ from the amounts described in previous studies, where the values range from 33 to 82% (Cavalcanti et al., 2004; Estevam et al., 2016; Millezi, 2014). Although the result for the amount of limonene is within the expected value for citrus species (Ladaniya, 2008), the differences in the composition of the components identified in the oil are explained by some factors, such as collection period, extraction time, temperature, intensity of solar radiation (Gobbo-Neto and Lopes, 2007), seasonality (Silva et al., 2019), the age and development of plants (Gobbo-Neto and Lopes, 2007) among others.

In the second finding, it was demonstrated that the essential oil *C. limonia* Osbeck has larvicidal activity from the comparison of the result obtained in the LC_{50} with the criterion established by Cheng (2003), since there is no defined standard to evaluate larvicidal efficacy of an oil. Thus, according to these authors, an essential oil is active when the LC_{50} is less than or equal to 100 μg mL^{-1}.

The results obtained in this study also confirm the action of different types of monoterpenes with larvicidal activity. In previous studies, it is reported that isolated hydrocarbon monoterpenes (limonene and alpha-pinene) have greater larvicidal activity than oxygenated monoterpenes (carvone, terpinen-4-ol and α-terpineol) (Lucia et al., 2013; Santos et al., 2011), as well as it is also reported that these classes of compounds in essential oil have larvicidal activity (Dias and Moraes, 2014). In this study, these compounds were identified, but it turns out that the presence of the oxygenated monoterpenes in the essential oil reduced the potency of the hydrocarbon monoterpenes so that the essential oil had a low larvicidal activity, thus confirming the synergistic effect of other compounds. The evidence that the limonene present in greater quantity in the essential oil extracted from citrus peels has larvicidal activity is reported in studies with *Citrus limon* (CL_{50} 15.48 μg mL^{-1}) (Gomes et al., 2019), *C. sinensis* (CL_{50} 28.68 μg mL^{-1}) and *C. paradisi* (CL_{50} 37.03 μg mL^{-1}) (Giatropoulos et al., 2012) in which it has been shown that the most effective to date is *C. limon*.

These findings should be interpreted with caution, as the identification of the compounds by the GC/MS technique only shows that it is there, but does not indicate the exact quantity of each component. Regarding the larvicidal efficacy of essential oil, further studies are needed to qualify it as a substitute for the chemical larvicide. However compared to other citrus essential oils described in the literature, *C. limonia* Osbeck oil would not be indicated as the first option, due to the low larvicidal activity.

In summary, the demonstration of the chemical composition and larvicidal activity shows the potential of the essential oil *C. limonia* Osbeck, as well as opening the opportunity to investigate the mechanism of action and the development of products that help combat the transmission of diseases by the vector mosquito.

Conclusion

To sum up, the essential oil from *Citrus limonia* Osbeck peels was extracted by hydrodistillation, the chemical analysis and larvicidal activity was determined against *Ae. aegypti*. Chemical analysis by gas chromatography coupled to the mass spectrum showed the presence of eighteen compounds where the five largest were, respectively, limonene, beta-Pinene, meta-cymene, beta.-Phellandrene and alpha.-Pinene and the predominant class was of monoterpenes. The essential oil has low biological activity against larvae in the 3rd stage of *Ae. aegypti* when compared to other citruses described in the literature. Therefore, due to these characteristics, essential oil has potential biological activity and can replace synthetic larvicides.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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