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Melaleuca quinquenervia (Cav.) S.T. Blake (Myrtales: Myrtaceae): Natural alternative for mosquito control

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ABSTRACT

Objective: To evaluate an essential oil with larvicide, adulticide and growth inhibitory activity against *Aedes aegypti, Aedes albopictus* and *Culex quinquefasciatus* mosquitoes, of medical importance.

Methods: Standardized methodology by WHO was used to determine the levels of susceptibility of mosquito larvae exposed to the essential oil. To evaluate the adulticide activity with the essential oil at different doses, bottles were impregnated according to the methodology CDC. To determine the development inhibitory activity of *Melaleuca quinquenervia* (*M. quinquenervia)* oil in three mosquito species, third instar larvae were exposed to the LC_{50} and LC_{90} dose (calculated for each population) of *M. quinquenervia* oil in glass containers with a capacity of 500 mL. After 24 h exposure, the dead larvae were discarded. The mortality of larvae and pupae were recorded on a daily basis.

Results: The calculated LC_{50} indicates an order of effectiveness of preferential oil for *Culex quinquefasciatus* (LC_{50} = 0.002 1%), *Aedes aegypti* (LC_{50} = 0.004 7%) and *Aedes albopictus* (LC_{50} = 0.004 9%).

Conclusions: The adulticide activity was achieved with impregnated bottles at 40 and 50 mg/mL with the three mosquitoes species. In larvae, a growth inhibition was detected when exposed to sublethal doses. The results indicate that *M. quinquenervia* is a plant with promising environmentally sustainable source for vector control.

1. Introduction

Culex quinquefasciatus (*Cx. quinquefasciatus*), *Aedes albopictus* (*Ae. albopictus*) and *Aedes aegypti* (*Ae. aegypti*) are within the entomological fauna of mosquitoes, vectors responsible for the maintenance and transmission of viruses such as West Nile [1], Dengue [2], Chikungunya [3] and Zika [4] in America region. Increasing population densities, high levels of unemployment, poverty, and lack of political will, among others, are factors that favor the circulation and maintenance of these endemic diseases in communities of developing countries [5].

Unfortunately for many of vector-borne diseases, vaccine candidates are not available, being the chemical control the basic measure to reduce mosquito populations and thus the incidence of disease. This reduction is usually transient without a thorough understanding of ecological aspects of the species responsible for transmission: behavior, habitat preferences and susceptibility to insecticides applied, among others [6–8].

The increased resistance to synthetic insecticides in these vectors of medical importance in Cuba was detected at laboratory level since the late 1990s [7,9]. While it is true that in periods of high infestation, insecticide application is the measure that reduces the incidence of diseases, it is also necessary to study alternative control, with a comprehensive approach to delay or

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reduce the resistance to synthetic insecticides in field mosquito's population.

Melaleuca quinquenervia (*M. quinquenervia)* (Cav.) S.T. Blake (Myrtales: Myrtaceae) is a plant considered for vector control, because of its proven insecticidal activity, being widely distributed, having complementary utilities such as medicinal or food and be environmentally sustainable. This plant is a tree widely distributed in Asian countries and parts of America [10–12].

After its introduction in Cuba, this plant has become an invasive specie in the wetlands of the Ciénaga of Zapata, and it has caused losses to the botanical biodiversity as a result of its high reproductive potential and its ability to withstand long dry periods [13].

Despite its adverse effects on the ecosystem, its essential oil and various extracts show a potential as antiprotozoal [14], antimalarial [15], bactericide, fungicide [16] and insect repellent [17].

Because of the importance that requires the search for natural alternatives for vector control, the objective was to determine the insecticidal activity of essential oil of (*M. quinquenervia)* on the vector species *Ae. albopictus, Cx. quinquefasciatus* and *Ae. aegypti.*

2. Materials and methods

2.1. Mosquito populations in the study

- Population Fraga 2012: *Ae. albopictus* specie collected at larval stage in Reparto Juan de Dios Fraga in the municipality of La Lisa, Havana Cuba in 2012.
- Population Regla 2013: *Cx. quinquefasciatus* specie collected in larval and pupal stage in the municipality Regla, Havana, Cuba, in 2013.
- Population Rockefeller: *Ae. aegypti*, laboratory reference strain susceptible to insecticides, supplied by the Center for Disease Control and Prevention (CDC), San Juan, Puerto Rico, 1996.
- Population Marianao 2013: *Ae. aegypti* specie strain collected in larval and pupal stages in 2013, during an intensive phase of vector control in the municipality of Marianao, Havana, Cuba.

The mosquito colonies were stabilized in the department insectarium Vector Control Institute of Tropical Medicine 'Pedro Kouri' Cuba, following the methodology of the Manual Technical Indications Insectarium [18] available on http://blue/bvs1/monografias/manual.pdf.

2.2. Bioassays to determine larvicidal activity of essential oils

Standardized methodology by WHO was used to determine the levels of susceptibility of mosquito larvae exposed to the essential oil [19].

The stock solutions were prepared in absolute ethanol. One ml of each concentration was added in a volume of 99 mL of water. A total of 125 larvae instar third or early fourth instar of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*, for each concentration were added. Each concentration had a control. Four replicates were done. Mortality was determined after 24 h and lethal concentrations (LC_{50} and LC_{90}) were calculated using the Probit test implemented in SPSS (version 11 for Windows).

2.3. Bioassays to determine the development inhibitory activities

Third instar larvae of three mosquito species were exposed to the LC_{50} and LC_{90} dose (calculated for each population) of *M. quinquenervia* oil in glass containers with a capacity of 500 mL. For each species, 150 larvae in 500 mL of water were used as control. After 24 h of exposure, the dead larvae were discarded and the survivors were added fishmeal as food. They remained in the water exposure until they reached the pupa state.

The mortality of larvae and pupae were recorded on a daily basis. The surviving pupae were separated by sex in separate vials until adult emergence. For the analysis of data normality the Kolmogorov–Smirnov tests and Shapiro–Wilk were used. Multifactor ANOVA was applied to the analysis of daily mortality of each state for all species (Statistica 7). Tukey post hoc test was used to identify differences between dose and immature stages.

2.4. Bioassays to determine adulticidal activity by impregnating bottles at different concentrations

To evaluate the adulticide activity with the essential oil at different doses, bottles were impregnated according to the methodology proposed [20]. Glass bottles of 250 mL capacity with frosted glass cover were used. The bottles were impregnated with 1 mL of each concentration of the oil, rotating them in every way until the acetone used as a solvent was evaporated. The bottles were covered with aluminum foil and kept uncovered overnight. Subsequently, they were capped until used. For each evaluated concentration, one control and four replicates were used. Fifteen females aged three days without blood feeding of each species were exposed. Every 5 min for 1 h mosquitoes knocked down were recorded.

Data of the doses that produced mosquito knockdown were analyzed with Probit test implemented in SPSS (version 11 for Windows).

3. Results

The *M. quinquenervia* essential oil showed larvicidal activity at the concentrations evaluated in three mosquito species (Table 1). The oil was more effective in *Cx. quinquefascitus* followed by *Ae. aegypti* and *Ae. albopictus* according to the LC_{50} values calculated.

In assessing the adulticide activity, in the Rockefeller population a 100% knockdown after 30 min was obtained when using the dose of 40 mg/mL. In populations of Marianao 2013, Regla 2013 and Fraga 2012, an increase in dose to 50 mg/mL was required to achieve the knockdown of 100% of the population in 30 min. The response of the three field populations was homogeneous in front of this oil despite the slight increase in the dose to achieve its toxic effect (Figure 1).

In Table 2 are shown knockdown time (TKN) calculated doses of 40 mg/mL for Rockefeller and 50 mg/mL in the rest of the evaluated populations. The times obtained (TKN_{50}) suggest

Table 1

Larvicidal activity of *M. quinquenervia* in populations of *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* used in the study.

Group	b[a] ± SE	LC_{50} (%)	LC_{90} (%)	χ^2 (*P*)
Rockefeller (*Ae. aegypti*)	12.85 ± 0.99	0.0047 (0.45%–0.51%)	0.0060 (0.58%–0.64%)	1.04 (0.400)
Marianao 2013 (*Ae. aegypti*)	2.63 ± 0.26	0.0047 (0.1 8%–1.20%)	0.0140 (0.75%–2.00%)	20.40 (0.000)
Regla 2013 (*Cx. quinquefasciatus*)	2.72 ± 0.21	0.0021 (0.11%–0.37%)	0.0064 (0.37%–3.20%)	15.98 (0.001)
Fraga 2012 (*Ae. albopictus*)	4.92 ± 0.73	0.0049 (0.45%–0.57%)	0.0089 (0.78%–1.10%)	4.11 (0.870)

[a] b refers to regression coefficient or slope.

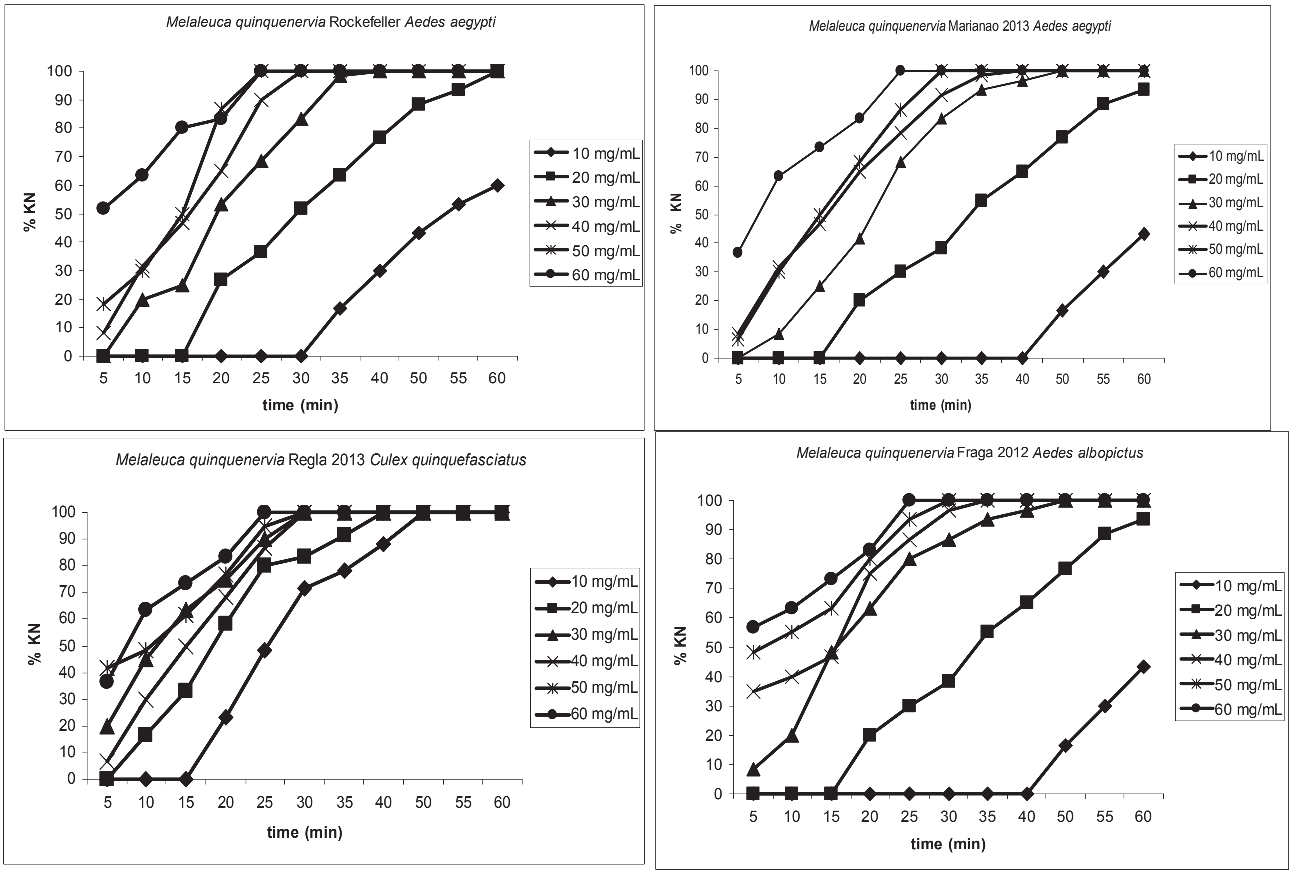

Figure 1. Knockdown percentage obtained during 1 h of exposure to different concentrations of *M. quinquenervia* oil in *Ae. aegypti, Cx. quinquefasciatus* and *Ae. albopictus*.

Table 2

Knockdown times obtained in populations of *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* used in the study, by impregnating bottles with *M. quinquenervia* oil.

Group	Doses (mg/mL)	b[a] ± SE	TKN_{50} (min)	TKN_{90} (min)	χ^2 (*P*)
Rockefeller (*Ae. aegypti*)	40	3.86 ± 0.35	12.5 (9.0–16.8)	28.2 (20.4–59.4)	15.6 (0.004)
Marianao 2013 (*Ae. aegypti*)	50	3.32 ± 0.33	14.2 (12.8–15.8)	34.7 (29.5–43.4)	3.38 (0.490)
Regla 2013 (*Cx. quinquefasciatus*)	50	3.12 ± 0.31	9.6 (6.6–12.6)	25.8 (19.2–45.4)	8.89 (0.064)
Fraga 2012 (*Ae. albopictus*)	50	2.13 ± 0.28	6.6 (4.8–10.8)	26.4 (15.6–33.2)	18.4 (0.001)

[a] b refers to regression coefficient or slope.

that the oil acts relatively quickly after exposure in any of the three species tested.

With respect to the inhibitory activity of development, significant difference between individuals exposed to each lethal concentration and the control ($F = 4.8297$, $P = 0.00803$) was found, which makes evident the toxic effect of *M. quinquenervia* oil in mosquito larvae of the three species studied.

The analysis of mortality among immature stages showed significant difference between larvae and pupae, and between pupae and adults in all the mosquito species ($F = 6.8530$, $P = 0.00002$). The greatest lethal effect occurred in *A. albopictus*, followed by *Cx. quinquefasciatus* and *Ae. aegypti* (Figure 2). Only 4% of the surviving pupae of three mosquito species reached the adult stage. Male mosquitoes emerged exceeded 3 times the number of females. Total N of individuals

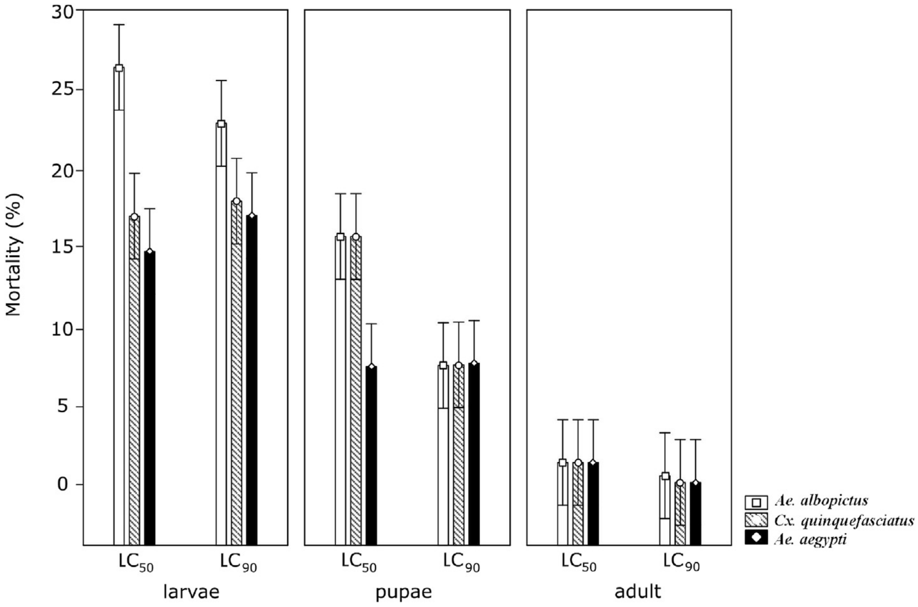

Figure 2. Mortality obtained by immature stages of mosquitoes *Ae. albopictus, Cx. quinquefasciatus* and *Ae. aegypti* species LC_{50} and LC_{90} exposed to doses *M. quinquenervia* oil. Graphic obtained by a Multifactor Anova (F = 2.0893, P = 0.0795 4). Error bars represent confidence intervals.

emerged was insufficient to study the effect of oil *M. quinquenervia* on fertility.

4. Discussion

It is understood as environmental sustainability: the exploitation of a biological system below its limit renewal without affecting adjacent diversity and ecosystem productivity [21]. A plant can be regarded as candidate for vector control, if in addition to its proven insecticidal activity, presents environmentally sustainable qualities.

M. quinquenervia stands out among the 100 most harmful to the ecosystem and of greatest concern to botanical species in Cuba. It invades about 40 000 ha in the swamps of Ciénaga of Zapata and Ciénaga of Majaguillar both in the province of Matanzas [22]. A form of exploitation of this renewable resource is to obtain its essential oil, which decrease the damage generated by their excessive growth [13].

The intensive search for alternative methods of vector controlling, and in this case, of plants with insecticidal activity have been focused mostly in plants where the essential oils and extracts have medicinal bioactivity or condiments utility [23–25]. The pesticide bioactivity depends largely on the botanical specie, extraction method, insects used and their susceptibility to synthetic insecticides [26,27].

WHO has not established diagnostic dose for the determination of the larvicidal activity of natural products. Authors like Komalamisra *et al.*, 2005 suggest that a natural product with $CL_{50} \leq 50$ mg/L is active and if the LC_{50} is between 50 mg/L and 100 mg/L is moderately active [28]. Moreover Ravi-Kiran *et al.*, 2006 [29] suggest that compounds with a $CL_{50} \leq 100$ mg/L present a significant larvicidal activity. In all our studies we have LC_{50} values below 50 mg/L, so the *M. quinquenervia* oil is active and has significant larvicidal activity for the species *Culex* and *Aedes* spp.

In numerous studies, insecticide action of plants is supported besides the bioassays, by enzymatic studies in the insects and chromatographic analysis that supporting the majority compound of the oils [30–33]. Authors recommended that because the mechanisms of action of secondary metabolites in many plants are different (inhibition of acetylcholinesterase, interrupting channels Na and K, blocking octopamine receptors) and similar to those used by synthetic insecticides in insects [34,35].

*M. quinquenervi*a produces different chemotypes, mainly based on the proportion of monoterpenes and sesquiterpenes 1.8 cineol and viridiflorol [10–12]. In the chemical characterization of the essential oil used in our studies, it was determined monoterpenes containing 1.8 cineol, α-pinene, β-pinene, α-terpineol, limonene and hydroxylated sesquiterpenoid viridiflorol, as majority compounds, all in a superior composition to 1% [36]. Several authors attribute this presence of metabolites in the essential oil of *M. quinquenervia*, to the insecticide action found in oils from other plants [23,37].

There is no consensus on whether to attribute the insecticidal activity to the major components of oil, or one in particular. Certain metabolites isolated, produce an agonist effect when evaluated on their own, while others show a synergistic effect when combined with other components of oil [37,38].

In studies by Giatropoulus *et al.* 2012 [39] with a strain of *Ae. albopictus*, the α and β-pinene were higher when they were CL_{50} calculated for isolates and compared with CL_{50} three citrus oils which were isolated. This result demonstrated the synergistic role of components within an essence. Kim *et al.*, 2008 [40] found significant larvicide and adulticide activity of 1.8 cineol, compared with *Culex pipiens*, and Zahran *et al.*, 2011 at doses of 500 mg/mL detected larvicidal activity against this species and had not yet elapsed effective adulticide activity after 48 h of exposure [41]. Noleto-Diaz *et al.*(2015) although it doesn't evaluate isolated metabolites, of the five plants used in their study, *Eugenia piauhiensis* presents the lower CL_{50} value and the monoterpenes 1.8 cineol, α-pinene, β-pinene, α-terpineol and viridiflorol were the majority compounds in its essence [42].

In any case, due to the criteria variability of specialists in the field, complementary studies with isolated metabolites should be made. However, the results obtained show the insecticidal activity of metabolites present in the oil.

Bio-responses to phytochemicals may differ between larvae and adults because the adult insect is physiologically stronger, what could justify the increase in adults CL_{50} evaluated. There are papers in which the method of the impregnated bottles (CDC methodology) is used to evaluate the adulticide activity of plant oils. Articles that evaluate this type of activity are made by impregnating papers with solutions of essential oils or isolated metabolites but most without a standardized methodology [43,44]. The methodology of the bottles is a cheap, simple and easily applicable method under laboratory conditions and terrain.

In terms of adulticide activity of essential oils against mosquitoes, there are very few articles that allow comparison of results. In the Rockefeller population, with a dose of 40 mg/mL, the 100% knockdown of exposed females was obtained. The dose used in our work for the rest of the population (50 mg/mL = 5%) is in the range of those used in other studies, *e.g.* experiments conducted with aereosoles of *Melaleuca cajeputi* [45]. The slight dose increase may be related to the fact that three of the populations studied were collected in a period of high pesticide application and were resistant to some groups of insecticides [46]. Therefore, they are likely to have increased levels of detoxifying enzymes and antioxidant mechanisms, which could influence the increase in dose. This phenomenon of crossed response has been already described in many papers [47]. The possible implication of the mechanisms of metabolic action on those made up with the essential oil of *M. quinquenervia* should be studied with more detail, given the possibility of using this promising candidate for vector control. A variety of formulations with this oil could be used for controlling of field populations who do not show any specific type of enzyme activity, as other authors suggest [47].

With respect to the inhibitory activity of development, oil *M. quinquenervia* has a toxic effect on larvae exposed to cumulative sublethal doses, as reflected in the high mortality found in this immature stage, in dead or deformed pupae observed and inhibition of emergence of male were adhered to exuvias.

These results may be due to the disruption of the hormonal balance caused by some secondary metabolites in insects exposed to sublethal doses [48,49].

Molecular studies should be performed on possible sites of action and target organs. Most of the plants, which are inferred to have insecticidal activity against mosquitoes, have at least larvicidal activity, but few studies cover a wide bioactivity (larvicide, adulticide, inhibiting development and repellent) on the same plant. Our results allow recommending the use of *M. quinquenervia* oil for vector mosquito control. In this way it manages to give utility to an invasive plant of wetlands in the western part of our country and propose an alternative to control mosquito populations, contributing to an environmentally sustainable pest management.

Conflict of interest statement

The authors declare that they have no conflict interest.

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