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Cumulative mortality of *Aedes aegypti* larvae treated with compounds

Mortalidade acumulativa de larvas de Aedes aegypti tratadas com compostos

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ABSTRACT

OBJECTIVE: To evaluate the larvicidal activity of *Azadirachta indica*, *Melaleuca alternifolia*, *carapa guianensis* essential oils and fermented extract of *Carica papaya* against *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae).

METHODS: The larvicide test was performed in triplicate with 300 larvae for each experimental group using the third larval stage, which were exposed for 24h. The groups were: positive control with industrial larvicide (BTI) in concentrations of 0.37 ppm (PC1) and 0.06 ppm (PC2); treated with compounds of essential oils and fermented extract, 50.0% concentration (G1); treated with compounds of essential oils and fermented extract, 25.0% concentration (G2); treated with compounds of essential oils and fermented extract, 12.5% concentration (G3); and negative control group using water (NC1) and using dimethyl (NC2). The larvae were monitored every 60 min using direct visualization.

RESULTS: No mortality occurred in experimental groups NC1 and NC2 in the 24h exposure period, whereas there was 100% mortality in the PC1 and PC2 groups compared to NC1 and NC2. Mortality rates of 65.0%, 50.0% and 78.0% were observed in the groups G1, G2 and G3 respectively, compared with NC1 and NC2.

CONCLUSIONS: The association between three essential oils from *Azadirachta indica, Melaleuca alternifolia, Carapa guianensis* and fermented extract of *Carica papaya* was efficient at all concentrations. Therefore, it can be used in *Aedes aegypti* Liverpool third larvae stage control programs.

DESCRIPTORS: ***Aedes*, growth & development. Plant Oils, toxicity. Insect Control. Disease Vectors.**

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RESUMO

OBJETIVO: Avaliar a eficácia de composto de óleos essenciais de *Azadirachta indica*, *Melaleuca alternifolia, Carapa guianensis* e extrato fermentado de *Carica papaya* sobre larvas de *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae).

MÉTODOS: O ensaio larvicida foi realizado em triplicata com 300 larvas para cada grupo experimental utilizando larvas de terceiro estádio, as quais foram expostas por 24h, em 2013. Os grupos foram: controles positivos com larvicida industrial (BTI) nas concentrações de 0,37 ppm (CP1) e 0,06 ppm (CP2); tratado com composto de óleos essenciais e extrato fermentado na concentração de 50,0% (G1); tratado composto e óleos essenciais e extrato fermentado na concentração de 25,0% (G2); tratado com composto de óleos essenciais e um extrato fermentado na concentração de 12,5% (G3); controle negativo com água (CN1) e controle dimetil sulfóxido (CN2). As larvas foram monitoradas a cada 60 min através de visualização direta.

RESULTADO: Larvas dos grupos CN1 e CN2 não tiveram mortalidade durante o período de 24h de exposição, mas os grupos CP1 e CP2 apresentaram taxa de mortalidade de 100% em relação a CN1 e CN2. Os tratamentos G1, G2 e G3 exerceram atividade larvicida de 65,0%, 50,0% e 78,0%, respectivamente, quando comparados a CN1 e CN2.

CONCLUSÕES: A associação entre os três óleos essenciais de *Azadirachta indica, Melaleuca alternifolia, Carapa guianensis* e extrato fermentado de *Carica papaya* foi eficiente em todas as concentrações testadas, podendo ser utilizado no controle de larvas de terceiro estádio de *A. aegypti* linhagem Liverpool.

DESCRITORES: *Aedes*, crescimento & desenvolvimento. Óleos Vegetais, toxicidade. Controle de Insetos. Vetores de Doenças.

INTRODUCTION

The World Health Organization (WHO) has established various strategies for controlling the *Aedes aegypti,* population, especially in the use of chemical and biological products integrated with environmental management programs capable of eliminating the larval forms and adult insects.[31]

Conventional chemical insecticides used to control *Aedes aegypti* have encouraged the selection of resistant populations. Increasingly strong doses are needed, leading to toxic effects when accumulated in human and animal tissue, and to environmental contamination.[4,30] Ongoing use of biological control using the *Bacillus thuringiensis, israelenses* (BTI) variety also encourages the selection of resistant *A. aegypti* populations.[22]

Plant-based compounds are the main source of new molecules with the potential to be inserted into biological systems.[13] Natural insecticides meet the needs for alternatives to controlling resistant populations of *Aedes aegypti,* a vector for a variety of viruses. They can affect different stages of development through a variety of mechanisms.[18]

Azadirachta indica and the *Carapa guianensis* are from the Meliaceae family. There are various compounds that have a larvicidal action on *Aedes aegypti, A. albopictus* and *Culex*,[25,27] as well as acting as an insecticide, repellent, antifungal, antimicrobial, acaricide, antifeedant and growth regulator. They are effective at low concentrations and are, for mammals, of low toxicity.[17,18]

Melaleuca alternifolia belongs to the Myrtaceae family and is used for its antimicrobial, antiviral, antifungal, antiseptic, anti-inflammatory and healing actions.[10,15] The oxygenated monoterpenes present in *M. alternifolia* essential oil are toxic to *Aedes albopictus* larvae and lethal concentration (LC_{50}) of 267.13 ppm.[6]

Carica papaya is from the Caricaceae and has bactericidal and bacteriostatic properties, and is used as a dewormer, facilitating digestion, reducing lipid peroxidation and an antioxidant.[14] Fermented extract of *C. Papaya* leaf has larvicial, ovicidal and repellent actions against *Aedes aegypti*.[9]

In isolation, all of the components of the compound possess larvicidal properties, although there have been no studies on their efficacy when blended to form one single product.

The aim of this study was to evaluate the efficacy of the compound of *Azadirachta indica*, *Melaleuca alternifolia, Carapa guianensis* essential oils and fermented extract of *Carica papaya* on *Aedes aegypti* larvae (Linnaeus, 1762) (Diptera: Culicidae).

METHODS

The essential oils and fermented extract compound is a commercial product obtained from Gued's Biotecnologia®. Its formulation is as follows: essential oil from *Azadirachta indica* seeds 10.0%, essential oil from *Melaleuca alternifolia* fruit 0.3%, essential oil from *Carapa guianensis* 1.0%, bacterial fermented extract of *Carica papaya* fruit 5.0%.

The essential oil and fermented extract compound is immiscible in water and forms a film on the surface of the container, causing the larvae to die of asphyxiation. It needed to be dissolved in an organic solvent, dimethyl sulfoxide, to enable it to be mixed with water. This was tested separately to analyze its toxicity for *Aedes aegypti* larvae.

Aedes aegypti Liverpool colonies were established from strains from the *Universidade Federal Rural de Pernambuco*, Laboratory of Domestic Animal Parasitic Diseases insectarium, Recife, PE, Northeastern Brazil, 2013. They were kept in a room with controlled temperature of 28°C (SD = 1), 80.0% (SD = 5.0) relative air humidity and a natural 12/12h photoperiod cycle.

Plastic containers holding two liters of de-chlorinated water were used to hatch the larvae. They were fed industrialized powdered cat food.

The toxicological trials followed the methodology recommended by the WHO.[11,31] Three hundred larvae were collected and transferred to a disposable container holding 50 mL of de-chlorinated water (26°C to 28ºC) when they reached the third larval stage. Each test was conducted in triplicate, making 900 larvae in each experimental group, giving a total of 7,200 specimens. The larvae were exposed to the solutions for a 24h period and were monitored every 60 min. Larvae which survived the larvicide trial remained under observation until pupae and adult emerged. The behavioral parameters of the larvae were observed during the period of the experiment to verify alterations such as: stereotyped movement, forming clusters, agitation, lethargy, change of color, shedding exuviae and death.

The experimental groups were organized as followed: treated with *A. indica, M. alternifolia* and *C. guianensis* essential oils and bacterial fermented extract of *C. papaya* in concentrations of 50.0% (G1), 25.0% (G2) and 12.5% (G3) a positive control with *Bacillus thuringiensis* serotype *israelensis* (BTI) industrial larvicide at concentrations of LC_{90} 0.37 ppm (PC1) and LC_{50} 0.06 ppm (PC2) and a negative control with de-chlorinated water (NC1) and negative control with dimethyl sulfoxide (NC2).

The data concerning the compound's efficacy were expressed using statistics describing centrality and dispersion trends (mean and standard deviation). The non-parametric Kruskal-Wallis and the Dunn post-hoc tests were used in order to analyze significance between the results and see which groups differed between themselves. The non-parametric Chi-square test (χ^2) was used in the analyses regarding the behavior of the larvae during the 24h period. The GraphPad Software, Inc., 2000 program was used for analyses with a significance level of 0.05.

RESULTS

During the larvicide test, the behavior of the larvae in groups G1, G2 and G3 altered, $p < 0.05$, compared to that of those in NC1 and NC2 groups, 60 min after exposure to the compound. Movement gradually decreased, the larvae formed clusters and were lethargic, remaining immobile even when touched after three hours. The larvae in the positive control groups, PC1 and PC2, became lethargic two hours after exposure, remaining inert to touch and with dark, rigid cephalic capsule (Table).

The larvae in the negative control group NC1 and NC2 were fed and developed into pupae and adults within 72h of the experiment. However, the surviving larvae in G1 and G2 did not shed their exuviae and did not develop into pupae and adults during the 21 days following exposure. Compounds in concentrations of 50.0% and 25.0% inhibited their development.

Larvae in groups treated with the compound (G1, G2 and G3) had mortality rates of 65.0%, 50.0% and 78.0%, respectively, in the first ten hours of exposure, whereas the mortality rate in the positive control groups (PC1 and PC2) was 100%, $p < 0.05$, compared with NC1 and NC2. Larvae died in all of the treated groups. However, after 24h, the group with the most efficacious treatment was G3, in which 100% of the larvae died, comparable to groups PC1 and PC2.

Larvae in the negative control groups using water (NC1) and dimethyl sulfoxide (NC2) did not die in the 24h following exposure. The dimethyl sulfoxide used

Table. Mortality rate of *Aedes aegypti* larvae evaluated over a 24h period, and treated with different concentrations of a compound of *Azadirachta indica, Melaleuca alternifolia, Carapa guianensis* essential oils and *Carica papaya* bacterial fermented extract. Recife, PE, 2013.

Time (h)	Experimental group							
	G1	G2	G3	PC1	PC2	NC1	NC2	p
2	0[a]	1[ab]	2[ab]	92[b]	90[ab]	0[b]	0[b]	0.0032
6	21[ab]	25[ab]	66[ab]	99[a]	99[a]	0[b]	0[b]	0.0028
10	65[ab]	50[ab]	78[ab]	100[a]	100[a]	0[b]	0[b]	0.0028
16	68[ab]	78[ab]	97[a]	100[b]	100[b]	0[b]	0[b]	0.0028
20	73[ab]	79[ab]	100[a]	100[b]	100[b]	0[b]	0[b]	0.0028
24	83[a]	81[a]	100[b]	100[b]	100[b]	0[b]	0[b]	0.0028

G1: Treated with 50.0% compound; G2: Treated with 25.0% compound; G3: Treated with 12.5% compound; PC1: BTI LC_{90} 0.37 ppm; PC2: BTI LC_{50} 0.06 ppm; NC1: Control, water; NC2: control, dimethyl sulfoxide; $p < 0.05$ according to the Kruskal-Wallis non parametric and Dunn post-hoc tests.
Different letters in the same row represent statistical significance.

in diluting the compound did not provoke mortality in the NC2 group, indicating that it had no effect on larvae development or death in groups G1, G2 and G3.

A. aegypti larvae were susceptible to the compound of *A. indica, M. alternifolia* and *C. guianensis* essential oils and *C. papaya* fermented extract, especially at concentrations of 12.5%.

DISCUSSION

The compound of essential oils and bacterial fermented extract possessed hydro-soluble active substances with larvicidal properties on third stage *Aedes aegypti* Liverpool larvae. Such products, highly efficient, with low toxicity and little environmental contamination are preferred in studies on controlling culicidae larvae.[5,24]

The first sign of a product with larvicidal properties is decreased movement of the larvae.[26] Arruda et al[3] showed how the movement of *A. aegypti* larvae decreased when treated with *Magonia pubescens.* Such a decrease was also observed in *A.aegypti, Culex quinquefasciatus* and *Anopheles albimanus* larvae when exposed to BTI.[26]

The main active ingredient in *A. indica* essential oil is azadirachtin, which acts as a larvicide on *A. Aegypti* and is reported to cause irreversible physiological alterations.[7] Ndione et al[19] investigated the larvicidal action of *A. Indica* essential oil and found that 64.0% of fourth stage *A. Aegypti* larvae died at concentrations of 8 mg/L (1.0%), and 82.0% of larvae when the concentration was reduced to 3 mg/L (0.3%) in 24h exposure. This data showed the best performing larvicide in the G3, group treated with the lowest concentration of the compound.

The development of *A. aegypti* arvae exposed to *A. indica* was compromised. Azadirachtin blocks the synthesis and release of ecdysone,[13] impedes shedding the exuvia and causes the cuticle to deteriorate,[1] as well as blocking ecdysteroid protein receptors. This inhibits growth, and causes deformities, sterility and death in the larvae.[16,29]

Silva et al[28] studied the larvicide action of *C. guianensis* on all *A. Aegypti* Rockefeller larvae stages and reported that: LC_{90} and LC_{95} were 164 ppm and 182 ppm after 48h for first stage larvae; 212 ppm and 224 ppm for second stage; 210 ppm and 226 ppm for third stage; and 450 ppm and 490 ppm for fourth stage, respectively.[8] Third and fourth stage *Aedes albopictus, Culex* and *A. aegypti* larvae also died after using the oil from this plant at different dilutions.[25,27]

There are various species of *Melaleuca* spp with larvicide actions against *A. Aegypti*, inclduing *Melaleuca linariifolia, M. dissitiflora* and *M. quinquenervia,* the essential oils of which obtained mortality of more than 80.0% in concentrations of 0.1 mg/mL in 48h of exposure.[21] However, in a study of larvicides conducted by Amer & Mehlhorn,[2] *M. quinquenervia* oil in a 50 ppm solution caused mortality in 30.0% of third stage *A. Aegypti* larvae 24h after exposure.

Rawani et al[23] tested raw extract of *Carica papaya, Murraya paniculata* and *Cleistanhus collinus* on *Culex quinquefasciatus* larvae and observed the best larvicide activity in *Carica papaya*. This may be explained by the bioactive secondary metabolites in isolation or in combination. Kovendan[12] tested raw extract of *C. papaya* leaf in isolation and obtained 92.0% mortality in *A. aegypti* larvae at a concentration of 500 ppm.

Controlling *A. aegypti* larvae and adults and *Culex quinquefasciatus* larvae using extract of *C. papaya* seed is due to inhibition of amylase, which reduces life span and fecundity in adults, as well as provoking mortality in larvae.[20,23]

As in the above mentioned individual studies, in this article larvicidal activity remained even when associated with low concentrations of the essential oils and fermented extract, found in the commercial product; 1 mL contains 0.01 mg/L of *A. indica,* 0.003 mg/L of *M. alternifolia*, 0.01 mg/L of *C. guianensis* and 0.05 mg/L of *C. papaya.* This concentration is below those found used in isolation in the indexed journals, even when undiluted. Thus, the larvacidal efficacy remained.

To conclude, the mixture of *A. indica, M. alternifolia, C. guianensis* essential oils and *C. papaya* bacterial fermented extract act in synergy as a larvicide on *Aedes aegypti,* Liverpool at all concentrations in laboratory conditions. It is necessary to evaluate this compound against *A. aegypti* populations in the field and with larvae at other stages.

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