

Larvicidal effect of dried leaf extracts from *Pinus caribaea* against *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae)

Efeito larvicida dos extratos de folhas secas de *Pinus caribaea* contra *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae)

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ABSTRACT

In this study, the larvicidal activity of dried leaf extracts from *Pinus caribaea* Morelet against *Aedes aegypti* was evaluated for the first time. *Pinus caribaea* extracts were obtained by macerating dried leaves in alkaline hydroethanol, ethanol and acetone solutions followed by evaporation under reduced pressure. The lignin content was quantified using the thioglycolic acid complexation method. Lethality bioassays (LC₅₀ and LC₉₀) were carried out in accordance with the recommendations of the World Health Organization. The results showed that the acetone extract from *Pinus caribaea* was more active, and that larvicidal activity was associated with lignin concentration.

Key-words: *Aedes aegypti*. *Pinus caribaea*. Larvicide. Extract.

RESUMO

Neste trabalho foi avaliada pela primeira vez a atividade larvicida contra *Aedes aegypti* de extratos obtido a partir de folhas secas de *Pinus caribaea*. Os extratos de *Pinus caribaea* foram obtidos a partir da maceração de folhas secas em solução hidroetanólica alcalina, etanol e acetona, seguido de evaporação sob pressão reduzida. O teor de lignina foi quantificado usando o método de complexação com ácido tioglicólico. Os ensaios de letalidade foram conduzidos de acordo com a recomendação da Organização Mundial da Saúde. Os resultados demonstraram que o extrato obtido com acetona foi mais ativo, e a atividade larvicida esta associada com a concentração de ligninas.

Palavras-chaves: *Aedes aegypti*. *Pinus caribaea*. Larvicida. Extratos.

Aedes aegypti is the primary vector of the dengue viruses, a group of four serotypes of single-stranded RNA virus³. The spread of the dengue virus via its *Aedes* mosquito vector throughout most of the tropics has led to worldwide resurgence of the epidemic dengue, including dengue hemorrhagic fever⁵.

Insecticides are one of the major tools for controlling vector populations and for reducing the transmission of human pathogens¹⁸. However, continuous use of one insecticide may cause changes to susceptibility status for many mosquito species. Over recent years, resistance has been reported worldwide in several *Aedes aegypti* populations^{8 11 14 16 24}.

One alternative for avoiding resistance among mosquitoes could be the use of products involving insecticidal substances from plants. Such substances have several advantages compared with the use of synthetic insecticides. Natural insecticides are obtained from renewable resources and are quickly degradable in the environment²². Thus, several studies worldwide have

reported on the use of plant extracts as potential larvicides for many mosquito species^{1 20 25}. One of the most promising groups of plants that have been studied is the Meliaceae family, for example the species *Azadirachta indica*^{17 19 26}. These plants are known to possess substances such as limonoids that are effective against many mosquito species.

Lignins are another important type of plant substance. These are polymers that accumulate in the walls of plant cells, thereby giving such plants great rigidity. They are important for water and nutrient transportation^{13 27}. Furthermore, it has already been proven that lignin accumulations in wood protect it against fungus and bacteria, and also against attacks by herbivores¹⁰.

The chemical structure of these macromolecules includes phenylpropanoid units called C₆C₃ or simply C₉ that occur irregularly in the chain. They originate from condensation of coumaryl, coniferyl and sinapyl alcohols²³.

Studies have shown that lignin from some plant species induces toxic effects on *Aedes aegypti* larvae²⁸ and antifungal activity on *Microsporium canis*, and *Thrichophyton rubrum*¹⁵.

Pinus species (Pinaceae) are known to contain a variety of important chemical compounds such as lignin. *Pinus caribaea* Morelet, known as Brazilian pine, has worldwide economic importance since it is extensively used in the wood industry and for obtaining cellulose. Its resin has also been used to manufacture gums, cleaning materials, etc²¹.

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Financial support: UNISUL/FAPESC

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Received in 06/04/2009

Accepted in 20/07/2009

In plantation areas in Brazil, *Pinus caribaea* leaves are discarded in the environment. Although the possibilities for their use in mosquito control are still unknown, they could form an abundant source material for such applications.

For this reason, the aim of this study was to evaluate the larvicidal effect of *Pinus caribaea* leaf extracts, based on their lignin content, against *Aedes aegypti*.

MATERIAL AND METHODS

Pine leaves were collected in Rio do Pouso, a locality near Tubarão (28.28.00 S, 049.00.25 W), Santa Catarina, Brazil, in August 2006. Botanical identification was conducted by a specialist and an exsiccate was kept in the *Laelia purpurata* Herbarium of the University of Southern Santa Catarina. The leaves were kept for seven days at 35°C for dehydration and were then ground up. The resultant material was kept in covered plastic vials.

Leaf extraction. The extracts were obtained using three kinds of solvent: ethanol, acetone and alkaline hydroethanol solution (20/80; pH raised to 12 using NaOH). The ground leaves (300g) were mixed in 1.5 liter of these solvents and kept under constant agitation over a five-day period. The extracts obtained were filtered and the solvent was evaporated at 40°C under vacuum. The raw extract was stored in a refrigerator until analysis.

Lignin assay. To investigate the quantities of lignin in the extracts, assays were performed in accordance with the method described in Geiger et al⁹. An amount of 500mg of extract was incubated with 0.4ml of thioglycolic acid and 5ml of 2N HCl for five hours at 90°C. The suspension was filtered and the residue was filled with water until pH 5.0 was reached. The resultant material was suspended in 5ml of 2% NaOH, for 24 hours, at room temperature. The suspension was then filtered again and washed many times with 5ml aliquots of water. The thioglycolic-lignin complex was obtained by adding 5N HCl until pH 1.5 was reached. The insoluble complex was centrifuged at 4,500g for 20 minutes, washed with water and dried at 80°C for 48h. The material obtained was described in terms of percentage of lignin-thioglycolic complex.

Mosquito strains. The Rockefeller strain of *Aedes aegypti* was originally obtained from the Brazilian National Health Foundation (Fundação Nacional de Saúde), and was continuously maintained in our laboratory. The insects were reared under a photoperiod regime of 14 hours of light to 10 hours of darkness. The larvae were reared using powdered pet food (Purina® Cat Chow®) 0.2g/100ml, three times a week. Adult males and females were continuously provided with a 5% honey solution, while females were blood-fed on BALB/c mice twice a week in order to obtain eggs for colony development.

Larva susceptibility bioassay. The bioassays were performed in accordance with the World Health Organization protocols²⁹. The susceptibility of the larvae was assessed by exposing the population to several concentrations of *Pinus caribaea*. Late third or early fourth-instar larvae were collected using a Pasteur pipette, selected and transferred using a tiny

brush to plastic jars containing extracts of *Pinus caribaea* at concentrations ranging from 31.2 to 700ppm. The tests were conducted in quadruplicate: for each test, 25 larvae were kept in 100 ml of boiled water with the different extract concentrations. Control samples were incubated with the same concentration of ethanol and alkaline hydroethanol solution that was used to obtain the *Pinus caribaea* extracts. The percentage larva mortality was determined 24 hours after incubation, at a temperature of 25°C and relative humidity of 80 ± 10%, in a climate-controlled chamber (Eletrolab®, model 132FC). Larvae were considered dead if they demonstrated total absence of movement when lightly prodded with a teasing needle. The mortality rate was calculated in relation to the total number of *Aedes aegypti* larvae killed in each bioassay. The lethal concentrations (LC₅₀ and LC₉₀) were interpolated by Probit analysis, as described by Finney⁷.

RESULTS AND DISCUSSION

Crude extracts obtained from *Pinus caribaea* leaves showed resin characteristics with pH around 3.0. According to Saliba²³, the acid pH of such extracts is attributable to phenolic acid.

Table 1 shows the concentrations required for killing the *Aedes aegypti* larvae after 24 hours of exposure (LC₅₀ and LC₉₀, ppm), the correlation with lignin content (determined by means of thioglycolic complexation methods) and the total amount of extract material obtained from *Pinus caribaea* leaves. The low water solubility of ethanol and alkaline hydroethanol extracts limited the ability to obtain solutions with higher extract concentrations. It was therefore impossible to determine the LC₉₀ (Table 1).

TABLE 1

Larvicidal activity of *Pinus caribaea* extracts against the late third or early fourth instar larvae of *Aedes aegypti* after 24 hours of exposure, and correlation with lignin content and total extractive material.

Extract	Lignin (%)	Extractive material (%)	LC ₅₀ ppm	LC ₉₀ ppm
Acetone	45.0 ± 2.6	9.5	92 (73-112)	760 (834-2207)
Ethanol	21.7 ± 1.2	10.7	713 (622-826)	**
Alkaline hydroethanolic	14.8 ± 0.3	16.8	2051* (1615-2875)	**

Level of confidence 95%, LC₅₀: lethal concentration that causes 50% of mortality, LC₉₀: lethal concentration that causes 90% of mortality.

*extrapolated from regression linear obtained from Probit, **not determined.

The results showed that the alkaline hydroethanol solvent had the greatest ability to extract total substances from *Pinus caribaea* leaves (16.8%). This result can probably be attributed to the intermediate polarity and elevated pH of the solvent, which can increase the solubility of phenolic acid components. However, the lignin content obtained was very low (14.8%), regardless of the low lignin solubility in water. Ethanol and acetone demonstrated lower ability to extract the total substances, but good ability to extract lignin. Better results were obtained with acetone, since 45% of the lignin content was obtained from the total substances extracted. According to Saliba²³, this is attributable to the higher lignin solubility in acetone.

Alkaline hydroethanol, ethanol and acetone extracts gave LC_{50} of 2051 (1615-2875) ppm, 713 (622-826) ppm and 92 (73-112) ppm respectively. The acetone extract was 22 times more potent than the alkaline hydroethanol extract and eight times more potent than the ethanol extract. The LC_{50} from acetone extracts (92ppm) was similar to that described by Ansari et al, who tested a pine oil from *Pinus longifolia* against larvae of urban mosquitoes in India such as *Anopheles culicifacies*, *Culex quinquefasciatus* and *Aedes aegypti*². The data on the larvicidal activity showed LC_{50} of 82.1ppm for *Aedes aegypti*, 85.7ppm for *Culex quinquefasciatus* and 112.6ppm for *Anopheles stephensi*.

In this study, the quantity of lignin was assayed because of its importance as a larvicide. These chemical compounds were also tested against other mosquito species. David et al⁴ and Tilquin et al²⁸ reported that lignin extracted from leaves demonstrated a toxic effect on *Aedes aegypti* larvae. The correlation between lignin content and larvicidal activity can be seen better in **Figure 1**.

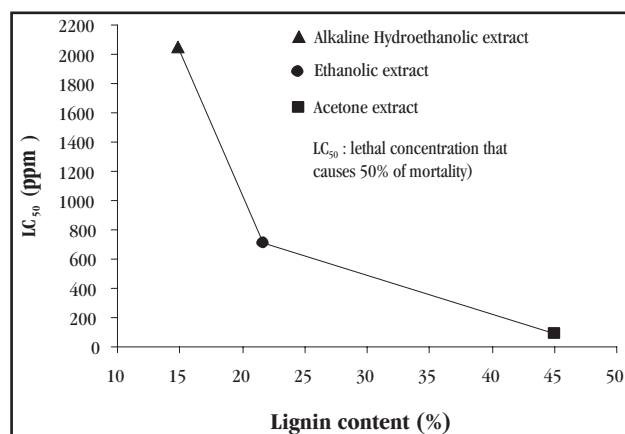


FIGURE 1

Lignin contents correlation with larvicidal activity of *Pinus caribaea* extracts.

These results showed that greater lignin content in the extract promoted increased larvicidal activity. However, the decrease in LC_{50} was not linear with the increase in lignin content, and this result suggests that the activity was dependent not only on lignins, but also perhaps on other chemical constituents of *Pinus*, such as phenolic acid derivatives.

Komalamisra¹² classified the larvicidal activity of plants as effective when LC_{50} was lower than 750ppm, moderate when it was 50-100ppm and high when it was lower than 50ppm. Thus, the activity of the acetone extracts from *Pinus caribaea* was considered moderate, while the ethanol extract was effective and alkaline hydroethanol extracts showed low activity. Comparing the activity of *Pinus caribaea* acetone extracts to that of other plants, like Meliaceae, this could be a good source for larvicidal development. Moreover, the Environmental Protection Agency of the United States has classified pine lignins in toxicity category IV, with very low acute toxicity: LC_{50} of 2-5g/kg for rats and 1-3g/l for fish⁶.

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