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DITERPENOIDS FROM *Copaifera reticulata* DUCKE WITH LARVICIDAL ACTIVITY AGAINST *Aedes aegypti* (L.) (DIPTERA, CULICIDAE)

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SUMMARY

The objective of this study was to evaluate the larvicidal activity of diterpenoids obtained from the oil-resin of *Copaifera reticulata* against *Aedes aegypti* larvae, the principal vector of dengue and urban yellow fever. Four diterpenes were obtained from oil-resin extraction with organic solvents and subsequent chromatographic and spectroscopic procedures allowed to isolation and identification of these compounds as 3-β-acetoxylabdan-8(17)-13-dien-15-oic acid (**1**), alepterolic acid (**2**), 3-β-hidroxylabdan-8(17)-en-15-oic acid (**3**), and *ent*-agatic acid (**4**). Each compound was previously dissolved in dimethylsulphoxide, and distilled water was added to obtain the desired concentrations. Twenty larvae of third instars were placed into plastic beckers, containing the solution test (25 mL), in a five repetitions scheme, and their mortality, indicated by torpor and darkening of the cephalic capsule, was recorded after 48h. Probit analyses were used to determine lethal concentrations (LC_{50} and LC_{90}) and their respective 95% confidence intervals. This study showed that only diterpenoids **1** and **2** exhibited larvicidal properties with LC_{50} of 0.8 ppm and 87.3 ppm, respectively, revealing the former as the most toxic compound against third instars of *Ae. aegypti*. Therefore, this compound seems to be an interesting source for new metabolite to be exploited.

KEYWORDS*: Copaifera reticulata*; *ent*-Labdane Diterpenoids; Larvicidal activity; *Aedes aegypti*.

INTRODUCTION

Dengue is a viral disease that has major consequence in public health. This problem has grown dramatically in recent decades[30], and it is estimated that about two fifths of the world's population are at risk to be infected by dengue virus. According to the World Health Organization, the principal vector *Aedes aegypti* (Linnaeus, 1762), a highly anthropophilic species, has adapted to the urban environment by using artificial containers that collect rainwater or those for domestic water storage as its larval habitat[30]. The high population density of *Ae. aegypti* in the cosmotropical area has triggered several interventions by the public health authorities through synthetic insecticide application as the main means of control of the vector[30]. However, the mosquito resistance to organophosphates and carbamate insecticides[3,6,12,14], along with the need of looking for methods of control that are less or non-toxic to man and to environment, has stimulated the search for new means of *Ae. aegypti* control. From this point of view, plants may be an alternative as control agents because they constitute a rich source of bioactive chemicals[7,9,13,21,24].

During our screening procedures of plants for larvicidal activity we found that *Magonia pubescens* St. Hil (Sapindaceae) and *Copaifera reticulata* Ducke (Leguminosae) were the most active extracts exhibiting a great effect against *Ae. aegypti*[21,22] and *Culex quinquefasciatus*[23]. The larvicidal activity of *M. pubescens* extracts could be attributed to tannins, the majority compounds detected in these materials[21]. Moreover, the MeOH extract of oil-resin of *C. reticulata* showed an expressive activity against this larvae[22].

C. reticulata is a medium tree, known as "copaibeira" and "pau d'óleo", that is native to tropical regions of South America and grows abundantly in several States of Brazil, such as Pará, Amazonas and Ceará[10]. The oil-resin of this plant has been used mainly as healing, anti-inflammatory and antiseptic agent[1,10,18,27]. The main chemical constituents of the oil-resin are mono-, sesqui- and diterpenes, and many of biological activities can be attributed to these compounds[2,8,11,16,19,26].

The focus of the present study was the evaluation of larvicidal activity of *ent*-labdanes diterpenoids (**1**-**4**) obtained from the oil-resin of *C. reticulata* against *Ae. aegypti* larvae.

MATERIAL AND METHODS

Plant material: The oil-resin sample was collected at Jacundá, Pará, Brazil. The voucher specimen is deposited at the Herbarium of Departamento de Botânica, Universidade Federal de Goiás, Brazil.

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Isolation of compounds: The oil *in natura* (186.5g) was subjected to liquid-liquid partition with *n*-hexane and methanol resulting in hexane (162.3g) and methanol (22.8g) extracts. The latter was submitted to low-pressure silica gel column chromatography (CC) eluted with *n*-hexane to methanol gradient. The medium polarity fraction eluted with ethyl acetate was reiteratively chromatographed in silica gel column, using *n*-hexane, ethyl acetate and methanol gradient as mobile phase obtaining 18 fractions, being fractions 6 and 14 the diterpenoids **1** [(-)-3β-acetoxylabdan-8(17)-13-dien-15-oic acid] and **2** {alepterolic acid [(-)-3β-hydroxilabdan-8(17)-13-dien-15-oic acid]}, respectively. Subsequent CC procedure upon fraction 13 using *n*-hexane, ethyl acetate and methanol gradient as mobile phase allowed to get diterpenoid **3** [(-)-3β-hydroxilabdan-8(17)-en-15-oic acid] as a white amorphous powder. Finally, diterpenoid **4** (*ent*-agatic acid) was purified by preparative thin layer chromatography of fraction 9 with *n*-hexane: ethyl acetate (70:30) as mobile phase. Chemical structures of compounds are showed in Fig. 1.

Bioassays: Larvae of *A. aegypti* were obtained from a permanent colony, maintained since more than 10 years, at 28 ± 1 °C, 80 ± 5% and 12:12 (L:D)[20]. Each compound was dissolved in dimethylsulphoxide (DMSO), and distilled water was added to obtain a stock solution of 100 ppm; from this a series of dilutions was prepared. All experiments were carried out in an environmental chamber, kept under the same conditions as the colony. Replicates (n = 5), of 20 larvae, were used for each concentration of compounds **1** to **4**. Each group of larvae was placed into a test solution and their mortality, indicated by torpor and darkening of the cephalic capsule, was recorded after 48h. Control groups were exposed to a DMSO/distilled water solution.

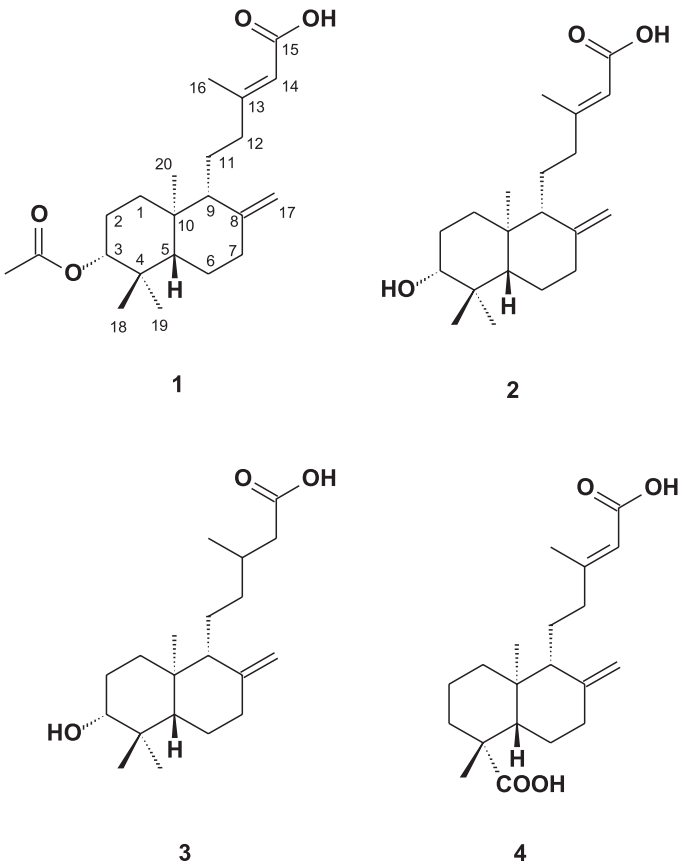

Fig. 1 - Structures of diterpenoids **1-4**. Diterpenoids **1** [(-)-3β-acetoxylabdan-8(17)-13-dien-15-oic acid]; **2** {alepterolic acid [(-)-3β-hydroxilabdan-8(17)-13-dien-15-oic acid]}; **3** [(-)-3β-hydroxilabdan-8(17)-en-15-oic acid] and **4** (*ent*-agatic acid).

Statistical analysis: The lethal concentrations (LC50 and LC90) and their respective confidence intervals were calculated through Probit analyses, using the Statistics Analyses System (SAEG, version 9.1 - 2007).

RESULTS

Bioassay-guided fractionation of this extract led to the isolation of diterpenoids **1-4**. Structures of these compounds (Fig. 1) were proposed from their spectral data (^{1}H NMR, IR and MS) and comparison with those reported in literature[4,5,15,31].

The toxicity of these compounds against *Ae. aegypti* was studied and lethal concentrations (LC_{50} and LC_{90}) are showed in Table 1. The results indicated that diterpenoid **1** was the most toxic compound against 3rd instar of *Ae. aegypti* exhibiting a LC_{50} and LC_{90} of 0.8 and 8.2 ppm, respectively, followed by diterpenoid **2** that showed a moderate larvicidal effect (LC_{50} of 87.3 ppm and LC_{90} of 128.8 ppm). There was not observed any mortality in groups treated with diterpenoids **3** and **4**. No mortality was detected in untreated group.

Table 1
Larvicidal activity of diterpenoids **1-4**, isolated from *Copaifera reticulata,* against *3rd* instar of *Aedes aegypti*

Compounds	LC_{50} (ppm) 95%CI	LC_{90} (ppm) 95% CI
1	0.8 (0.1 - 1.9)	8.2 (6.5 - 11.3)
2	87.3 (41.5 - 146.2)	128.8 (81.4 - 265.8)
3	No activity	No activity
4	No activity	No activity

95% CI - Confidence interval at 95% probability. There was no mortality in the control group.
Diterpenoids **1** [(-)-3β-acetoxylabdan-8(17)-13-dien-15-oic acid]; **2** {alepterolic acid [(-)-3β-hydroxilabdan-8(17)-13-dien-15-oic acid]}; **3** [(-)-3β-hydroxilabdan-8(17)-en-15-oic acid] and **4** (*ent*-agatic acid).

DISCUSSION

Species of genus *Copaifera* are well known for production of oil-resin exhibiting therapeutic activities, from which monoterpenes, sesquiterpenes and diterpenes (kaurane, labdane and clerodane skeleton) are the majority compounds[8,16,17,19].

Diterpenoids **1**, **2** and **4** have been isolated previously from commercial copaiba oil, the moisture of oil resins extracted from many species of *Copaifera*[8,15]. However, they were not related before in the oil obtained exclusively from *C. reticulata*. Moreover, the diterpenoid **3** is being described for the first time in the genus *Copaifera*, although it has been produced from compound **2** by Pd/C catalytic hydrogenation method[5].

A comparison of larvicidal potency between diterpenoid **1** and others terpenoids reported in literature would demonstrate **1** as a promissory natural product that could become an alternative method of control for vector *Ae. aegypti.* SIMAS *et al.*[24] described the lethal concentrations for the sesquiterpenes ε-nerolidol, farnesol and nerolidol, showing LC_{50} of 17.0, 13.0, and 17.0 ppm, respectively. Comparative analysis between **1** and ε-nerolidol showed that the former is 75 times more active than the later. Another diterpenoid isolated from *Melantheria albinervia* showed LC_{100} 62.5 ppm[25].

The most known natural product used as insecticide is the limonoid (tetranortriterpenoid) azadirachtin[29] that has been sold in commercial shops as Align, Azatin & Turplex (http://extoxnet.orst.edu/pips/azadirac.htm, accessed in 03/01/07). However, simple monoterpenes also perform protection against insects in the plants, demonstrating good insecticidal activity[13,28]. Moreover, other terpenoid compounds (diterpenes, nortriterpenes and sesquiterpenes) are reported as phagoinhibitors and growth inhibitors for many types of insects[13].

In conclusion, these preliminary assays indicated that diterpenoid **1** remains an interesting source for new larvicidal metabolite to be exploited. Further toxicological tests should be made in order to evaluate the impact of this compound on non-target species and if it may be used as an insecticidal ingredient in the formulations for the control of dengue vector.

RESUMO

Diterpenos de *Copaifera reticulata* Ducke com atividade larvicida contra *Aedes aegypti* (L.) (Diptera, Culicidae)

O objetivo deste trabalho foi avaliar a atividade larvicida de diterpenos isolados do óleo-resina de *Copaifera reticulata* sobre *Aedes aegypti*, principal vetor de dengue e febre amarela urbana. Quatro diterpenóides foram obtidos a partir da extração do óleo-resina com solventes orgânicos e, subseqüentes procedimentos cromatográficos e espectroscópicos permitiram o isolamento e a identificação desses compostos como ácido 3-β-acetoxylabdan-8(17)-13-dien-15-óico (**1**), ácido alepterólico (**2**), ácido 3-β-hidroxylabdan-8(17)-en-15-óico (**3**) e ácido *ent*-agático (**4**). Cada um desses compostos foi previamente solubilizado em dimetilsulfóxido, acrescentando-se água, até se obterem as concentrações desejadas. Em cada bioensaio foram utilizadas 20 larvas de 3° estádio de *Ae. aegypti* colocadas em 25 mL da solução-teste. Foram feitas cinco repetições, e a mortalidade avaliada 48 h após a exposição, indicada pela ausência de movimentos e escurecimento da cápsula cefálica. Os dados obtidos da mortalidade x concentração (ppm) foram analisados, em gráfico de Probit para avaliar as concentrações letais (CL_{50} e CL_{90}). Este estudo revelou que os diterpenóides **1** e **2** mostraram atividade larvicida com CL_{50} de 0,8 e 87,3 ppm, respectivamente, sendo o diterpeno **1** o composto mais promissor a ser usado como larvicida para o controle de *Ae. aegypti*.

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