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Short communication

Larvicidal activity of *Ramalina usnea* lichen against *Aedes aegypti*

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ABSTRACT

The larvicidal activity of the methanol extract, fractions and compounds 2-hydroxy-4-methoxy-6-propyl-methyl benzoate and (+)-usnic acid identified from the lichen *Ramalina usnea* (L.) R. Howe, Ramalinaceae, was tested against the third instar larvae of the *Aedes aegypti* mosquito. The methanol extract and three fractions showed activity, killing 100% and 96.6% of the larvae at a concentration of 150 μg/ml at 24 h. The isolated compounds, 2-hydroxy-4-methoxy-6-propyl-methyl benzoate and the (+)-usnic acid showed larvicidal activity, presenting LC_{50} values of 4.85 and 4.48 μg/ml, respectively. This is the first study of its kind reporting the larvicidal activity against the *A. aegypti* mosquito with compound (**1**).

Introduction

Mosquitoes are vectors that transmit many diseases, such as malaria, encephalitis, dengue fever among others. Dengue fever is a neglected tropical disease, currently ranked as the most important viral disease transmitted by mosquitoes worldwide (WHO, 2012). The mosquito *Aedes aegypti* transmits dengue and is also responsible for the transmission of other diseases, such as yellow fever, chikungunya fever (WHO, 2015), and the fever caused by zika virus (MS, 2015). Brazil is currently experiencing a dengue epidemic in several states and 1,485,397 suspected cases of dengue were reported in the country with about 19,380 confirmed cases and 761 deaths (Boletim Epidemiológico, 2015).

As there is no specific antiviral drug for the treatment of these diseases, vector control is the best strategy (WHO, 2015). To solve this problem, many insecticides may be used to control mosquitoes, but many of them are not selective and can harm beneficial insects (Cetin et al., 2012), increase the resistance of these insects (Benli et al., 2009), and produce environmental contamination (Santos et al., 2011). In the search for alternative control methods less or non-toxic to the population and the environment, several studies have surveyed plants as alternative control agents (Govindarajan and Sivakumar, 2014). Other natural sources include lichens (Cetin et al., 2012) and marine natural products (Samidurai and Saravanakumar, 2011), as these natural sources have a rich diversity of bioactive chemical compounds. New mosquito control methods have been tested in Brazil. The British biotech company Oxitec, through an innovative project involving genetic engineering, produces sterile mosquitoes, genetically modified, in order to suppress the population by the release of insects with dominant lethality. The result of 96% of suppression was observed in Jacobina, in the district of Bahia, where the tests have been done since 2013 (www.oxitec.com/).

Lichens consist of symbiosis between a fungus and a green alga or cyanobacteria. They contain a large number of secondary compounds (Huneck and Yoshimura, 1996). Consequently, lichens have been used as sources of many metabolites with different biological activities (Ingólfsdóttir, 2002) as antibiotic (Ingólfsdóttir, 2002), antiviral (Lai et al., 2013), and larvicidal (Cetin et al., 2012), among others. In the present study, we evaluated the larvicidal activity of two compounds isolated from the lichen *Ramalina usnea* (L.) R. Howe, Ramalinaceae, 2-hydroxy-4-methoxy-6-propyl-methyl beazoate (**1**) and (+)-usnic acid (**2**). This study is the first report in the literature of the larvicidal activity against *A. aegypti* of 2-hydroxy-4-methoxy-6-propyl-methyl benzoate **(1)** and MeOH-soluble extracts of the studied lichen.

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Table 1
Larval mortality (in percent) of the methanol extract of *Ramalina usnea* lichen, fractions and control against third-instar of *Aedes aegypti* after 24 h.

	Concentration (μg/ml)					
	150	30	15	10	5	1
MeOH extract	100	–	–	–	–	–
LM6	57	–	–	–	–	–
LM7	96.6	–	–	–	–	–
LM6-1	100	–	–	–	–	–
LM6-2	100	–	–	–	–	–
LM6-3	0	–	–	–	–	–
LM6-4	0	–	–	–	–	–
LM6-5	10	–	–	–	–	–
LM6-2-3 (**1**)	–	100	88.67	78	35.33	11.33
LM6-2-7 (**2**)	–	100	93.33	66.67	33.33	20
DMSO	0	0	0	0	0	0
DMSO/H_2O	0	0	0	0	0	0

–, not tested concentration.

Materials and methods

^1H (500 MHz) and ^{13}C (125 MHz) NMR experiments were conducted on a Bruker spectrometer. IR analyses were made with a Shimadzu IRAffinity-1 instrument using KBr pellets. GC–MS analyzes were performed using a Shimadzu QP-5050 A equipment. Column chromatography was performed on Merck silica gel (0.063–0.2 mm) and Merck silica gel 60 F_{254} was used for TLC. The lichen *R. usnea* (H.) L. Howe, Ramalinaceae, was collected on the Restinga of Iquipari, coastline of Grussaí in the São João da Barra municipality, Rio de Janeiro state, Brazil. Voucher specimens were identified by Dr. Michel Navarro Benatti, herbarium curator of the Botanical Institute of São Paulo and deposited at the herbarium of the Universidade Estadual do Norte Fluminense Darcy Ribeiro, Biosciences and Biotechnology Center (identification number LI 0001).

The lichen stems was dried at room temperature and powdered. About 550 g of the dried material was macerated with 3 l of methanol for three times. After filtration, the solvent was removed under reduced pressure and it produced approximately 60 g of crude extract. A sample was taken to perform preliminary biological tests and 35 g were fractionated by column chromatography on ambient pressure using silica gel, and eluted with hexane:CH_2Cl_2 with a gradient of polarity (100:0, 95:5 to 0:100 v/v), and finalizing with CH_2Cl_2:MeOH (8:2 v/v) to obtain 8 fractions of approximately 80 ml each (LM1 to LM8). Fraction LM6 (2.82 g) was further fractionated by similar methods to those described above, and eluted with the same polarity gradient of hexane:CH_2Cl_2, obtaining six new subfractions (LM6-1 to LM6-6). Fraction LM6-2 (689.6 mg) was chromatographed through a similar process to that of LM6 producing ten new subfractions (LM6-2-1 a LM6-2-10). Fraction LM6-2-3 (89.5 mg) was analyzed by spectroscopic methods and identified as 2-hydroxy-4-methoxy-6-propyl-methyl benzoate (**1**). Fraction LM6-2-7 (149 mg) was subjected to preparative TLC eluted with hexane:CH_2Cl_2 (20:80 v/v, three times) and, after analysis by spectroscopic methods, usnic acid (**2**) was identified.

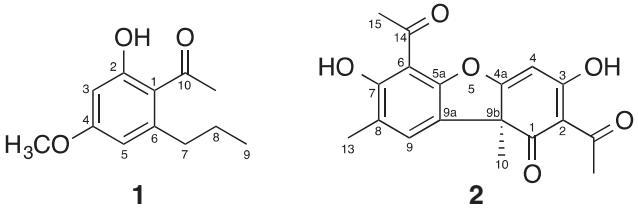
The mosquitoes *A. aegypti* (Diptera: Culicidae) were obtained from the insectary of the Biotechnology Laboratory, Universidade Estadual do Norte Fluminense Darcy Ribeiro. The larvicidal toxicity assays were developed according to standard methodology of World Health Organization (WHO, 2005). The concentration of the stock solution and the solutions tested were chosen based on preliminary tests. The compounds were solubilized in DMSO/H_2O (**1**) or DMSO (**2**). Fifteen third instar larvae were added to the pots containing distilled water and test solutions at concentrations that were between 1 and 30 μg/ml for a total of five concentrations, at room temperature. The tests were performed in triplicate and in two replicates. Negative controls were water, DMSO and a solution of DMSO/H_2O (2.5%). For positive control, imidacloprid was used at concentrations between 0.01 and 1.0 μg/ml. Assessment of mortality was made 24 h after exposure of the larvae to the test solutions. Data were analyzed using the analysis program Probit/EPA version 1.5 to calculate the 50% (LC_{50}) and 90% (LC_{90}) lethal concentrations as well as the confidence limit for each treatment in the confidence level of 95% (http://www.epa.gov/nerleerd).

Results and discussion

Preliminary bioassays with crude methanol extract and fractions were undertaken at a concentration of 150 μg/ml. The crude extract (LM) produced 100% of mortality in the larvae of the third instar of *A. aegypti* after 24 h, the fraction LM6 57%, LM7 96.6%, and 100% for all fractions LM6-1 (97.8 mg) and LM6-2 (689.6 mg). The other fractions of this column produced low mortality rates. However, subfractions LM6-2-3 (**1**) and LM6-2-7 (**2**) showed activity (Table 1). The mortality data at various concentrations allowed the determination of the LC_{50} and LC_{90} values (Table 2) for pure compounds **1** and **2** and showed that this activity occurred in a concentration dependent manner. 2-Hydroxy-4-methoxy-6-propyl-methyl benzoate (**1**) displayed values of 11.3, 35.3, 78.0, 88.7 and 100% of mortality, while for usnic acid (**2**) were obtained 20.0, 33.3, 66.7, 93.3 and 100%, at concentrations of 1.0, 5.0, 10.0, 15.0 and 30.0 μg/ml, respectively, after 24 h (Table 1). The values for LC_{50} were 4.85 and 4.48 for compounds **1** and **2**, respectively, while the LC_{90} were 17.5 and 20.7 (Table 2). Therefore, these compounds have a potential as new leads for larvicidal natural products.

2-Hydroxy-4-methoxy-6-propyl-methyl benzoate (**1**): dark yellow amorphous solid; mp 122.5–123.8 °C; IR (KBr, cm^{-1}): 3435, 2924, 2852, 1726, 1624, 1373, 1192, 960; ^1H NMR ($CDCl_3$, 500 MHz, TMS), δ 6.34 (1H, s, H-4), 6.29 (1H, S, H-6), 2.82 (2H, t, J = 7.0 Hz, CH_2-7), 1.54 (2H, m, CH_2-8), 0.95 (3H, t, J = 7.0, CH_3-9), 3.80 (3H, s, C5-OCH_3), 3.92 (3H, s, C10-OCH_3), 11.76 (1H, s, C3-OH); ^{13}C NMR ($CDCl_3$, 125 MHz, TMS), δ_C (ppm): 104.6 (C-1), 163.8 (C-2), 98.7 (C-3), 165.5 (C-4), 110.7 (C-5), 55.2 (OCH_3-4), 51.8 (OCH_3-10). NMR data and melting point are in agreement with those reported in the literature (Huneck and Yoshimura, 1996).

Usnic acid (**2**): yellow crystalline solid; $[\alpha]_D$ +494.2 (25 °C, $CHCl_3$, *c* 1.00), mp 202.5–204 °C; IR (KBr, cm^{-1}): 3662, 3088, 1695, 1452, 1373, 1286, 1190, 1118, 1037, 956, 817; ^1H NMR ($CDCl_3$, 500 MHz, TMS), δ 6.05 (1H, s, H-4), 1.79 (3H, s, CH_3-10), 2.68 (3H, s, CH_3-12), 2.08 (3H, s, CH_3-13), 2.71 (3H, s, CH_3-15), 13.34 (1H, s, C7-OH), 11.06 (1H, s, C9-OH). ^{13}C NMR ($CDCl_3$, 125 MHz), δ 198.1 (C-1), 105.2 (C-2), 191.8 (C-3), 179.3 (C-4a), 155.2 (C-5a), 101.5 (C-6), 163.9 (C-7), 109.3 (C-8), 157.5) (C-9), 104 (C-9a), 59.1 (C-9b), 31.9 (C-10). NMR data and melting point are in agreement with those reported in the literature (Rashid et al., 1999).



1 **2**

The literature on larvicidal activity of lichen compounds is scarce, especially for *A. aegypti*. No reports of larvicidal activity for compound **1** were found in the literature, while for compound (**2**) only the activity for some isomers was found with other insects. Cetin et al. (2012) have obtained excellent results with lichen metabolites, among them the (+)-usnic acid (**2**) against mosquito larvae of *Culiseta longiareolata* Macquart (Dipterous: Culicidae) that showed high larvicidal activity with values of 0.48 μg/ml.

Table 2
The LC_{50} and LC_{90} values (μg/ml) compounds 2-hydroxy-4-methoxy-6-propyl-methyl benzoate (**1**), usnic acid (**2**) and control Imidaclopride against third-instar of *Aedes aegypti* mosquito larvae after 24 h.

Compound	LC_{50} (limits)	LC_{90} (limits)	Slope (±SE) (limits)
1	4.85 (2.93–6.96)	17.5 (11.7–36.3)	2.30 (0.46) (1.4–3.2)
2	4.48 (2.51–6.73)	20.7 (12.8–51.5)	1.9 (0.4) (1.1–2.7)
Control	0.0412 (0.02–0.062)	0.095 (0.06–0.26)	0.036 (0.011) (0.014–0.058)

Conclusion

From this study, the larvicidal activity of the compounds 2-hydroxy-4-methoxy-6-propyl-methyl benzoate (**1**) and usnic acid (**2**) was demonstrated. These compounds could be leads for the development of new synthetic molecules with larvicidal activity for the control of *A. aegypti*.

Authors' contributions

ASNM, ROSF and FJAL contributed to the experimental work and wrote the manuscript. RBF and IJCV contributed to the NMR spectral analysis.

Conflicts of interest

The authors declare no conflicts of interest

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