Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **28** (4): 580-4 (2009)

Short Communication

Received: January 20, 2009
Accepted: March 15, 2009

Larvicidal Activity of *Cladonia substellata* Extract and Usnic Acid against *Aedes aegypti* and *Artemia salina*

Rangel R. BOMFIM [1], Adriano A.S. ARAÚJO [1]*, Sara CUADROS-ORELLANA [2], Marcélia G.D. MELO [1], Lucindo J. QUINTANS-JÚNIOR [1] & Sócrates C.H. CAVALCANTI [1]

[1] *Laboratório de Ensaios Farmacêuticos e Toxicidade, Departamento de Fisiologia, Universidade Federal de Sergipe, São Cristóvão-SE-Brazil.*

[2] *Laboratório de Engenharia de Bioprocessos, Instituto de Tecnologia e Pesquisa-ITP, Aracaju-SE-Brazil.*

[3] *Laboratório de Química Farmacêutica, Departamento de Fisiologia, Universidade Federal de Sergipe, São Cristóvão-SE-Brazil.*

SUMMARY. **The chloroform extract of *Cladonia substellata* Vainio was assayed against larvae of *Aedes aegypti,* the mosquito vector of Dengue fever and *Artemia salina.* The extract was tested at concentrations ranging from 1 to 15 ppm in an aqueous medium for 24 h. LC_{50} and LC_{90} were evaluated. Since the chloroform extract proved to be lethal for third to fourth instar larvae, downstream processing was undertaken to purify the active agents in the extract. The major compound in the chloroform extract was purified by crystallization followed by column chromatography to yield yellow crystals. Furthermore, usnic acid (UA) was evaluated for its larvicidal potential. The major compound in the chloroform extract, UA, exhibited LC_{50} of 6.6 ppm (6.1 to 7.0 ppm). Therefore, UA is most likely the active principle in *C. substellata.* UA showed to be toxic to *A. salina,* a reference organism in assays to evaluate the potential toxicity hazard to invertebrates in ecosystems.**

INTRODUCTION

Dengue is a viral disease caused by a Flavivirus transmitted by the mosquito *Aedes aegypti.* Its symptoms vary from mild fever, to life threatening dengue hemorrhagic fever and dengue shock syndrome [1,2]. The propagation of Dengue is currently a public health threat, particularly in tropical and subtropical countries [3]. Since there are no effective treatment for this disease, the most effective way to control the virus outbreak is to avoid vector spreading. The control of mosquito larvae worldwide depends primarily on continued applications of organophosphates such as temephos, fenthion and insect growth regulators such as diflubenzuron and methoprene [4]. However, resistance to pesticides has guided research to find new methods intended to control *Aedes aegypti* propagation [5]. Additionally, the synthetic insecticides are toxic and adversely affect the environment by contaminating soil, water and air.

The necessity for continued research has been even more apparent in the late years, aiming to find new methods to control the vector and reduce the incidence of dengue. Recent research has focused on natural product alternatives, such as lichens, for pest control in developing countries [6].

Lichens are symbiotic associations between fungal (mycobiont) and algal or cyanobacterial (photobiont) partners [7]. They are able to synthesize several metabolites, comprising aliphatic, cycloaliphatic, aromatic and terpenic compounds [8]. Usnic acid (UA) is uniquely found in lichens, and is especially abundant in genera such as *Alectoria, Cladonia, Usnea, Lecanora, Ramalina* and *Evernia.* Many lichens and extracts containing UA have been utilized for medicinal, perfumery, cosmetic as well as ecological applications [9].

Usnic acid, [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzo-furandione; $C_{18}H_{16}O_7$], is the most common and abundant yellow cortical pigment lichen metabolite (Fig.

KEY WORDS: Aedes aegypti, Artemia salina, Cladonia substellata, Larvicidal activity, Usnic acid.

* Author to whom correspondence should be addressed. *E-mail address:* adriasa2001@yahoo.com.br

ISSN 0326-2383

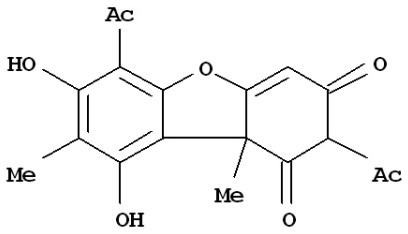

Figure 1. Chemical structure of usnic acid (UA).

1). It has been previously shown that UA has antibiotic [9], antiviral [10], antiprotozoal [11], antitumoral [12,13], anti-inflammatory, antipyretic, analgesic [14,15], gastroprotective, antioxidant [16], and hepatotoxic activities [17]. However, no report was found on the larvicidal activity of *C. substellata* extract and UA against *Aedes aegypti*. In order to determine the toxicity of *C. substellata* extract and UA towards non-target aquatic species, brine shrimp *(Artemia salina)* was employed as a model assay system since it provides a convenient in-house pre-screening for general toxicity and has been recently reviewed with regard to its applicability in modern ecotoxicology testing [18,19].

MATERIAL AND METHODS

Lichen material

C. substellata Vainio was collected in march, 2006, in the Itabaiana county, Sergipe State, northeastern Brazil (10° 44' S, 37° 23' W). *C. substellata* was identified by M.P. Marcelli (Botanical Institute of São Paulo-SP, Brazil).

Chloroform extract

The air-dried parts (100 g) of *C. substellata* were extracted with 150 mL of chloroform using a Soxhlet apparatus. The extract was filtered and concentrated to dryness under reduced pressure in a rotary evaporator to yield 2.8 g, 2.8 % of the chloroform extract. The dried chloroform extract was used in the lethality assays.

Extraction, purification and characterization of usnic acid

UA was isolated from the crude extract of the lichen C. substellata. The air-dried parts (100 g) of *C. substellata* were extracted with 150 mL of chloroform using a Soxhlet apparatus to isolate UA. The crude extract was filtered and stored at 4 °C for 24 h to precipitate UA. The UA precipitates were collected and subjected to silica gel (70–230 mesh) column chromatography (CC) eluting with chloroform:hexanes (80:20) as the solvent system. At the end of this process, 840 mg of UA was obtained with a 0.84 % (w/w) yield [16].

The obtained UA was characterized by thermal analysis (DSC and TG/DTG). DSC curves were obtained in a DSC-50 cell (Shimadzu) using aluminum crucibles with about 2 mg of samples, under dynamic nitrogen atmosphere (50 $mL.min^{-1}$) and heating rate of 10 °$C.min^{-1}$ in the temperature range from 25 to 600 °C. The DSC cell was calibrated with indium (m.p. 156.6 °C; $\Delta H_{fus.}$ = 28.54 $J.g^{-1}$) and zinc (m.p. 419.6 °C). TG/DTG curves were obtained with a thermobalance model TGA 50 (Shimadzu) in the temperature range of 25–900 °C, using platinum crucibles with ~3 mg of samples, under dynamic nitrogen atmosphere (50 $mL.min^{-1}$) and heating rate of 10 °$C.min^{-1}$.

The carbon, hydrogen and nitrogen contents were determined by usual microanalytical procedures using a Pekin-Elmer analyzer (Model 2400). The nuclear magnetic resonance (NMR) spectra of atranorin was taken on a Bruker DRX500 (1H: 500 MHz; 13C: 125 MHz) spectrometer.

Aedes aegypti hatching

Eggs of *A. aegypti* were provided by the Federal University of Sergipe insectary, attached to paper strips. The paper strips were placed in a rectangular polyethylene container containing natural mineral water. Rat ration (100 mg) was added to allow larvae development. The container was kept at room temperature for hatching and monitoring of larvae development for about five days.

Larvicidal activity

Testing extract or UA (20 mg) were dissolved 1 mL (0.1 mL Tween 80, 0.3 mL DMSO, and 0.6 mL water) to make a standard solution (20.000 ppm). The standard solution was used to make five 20 mL solutions ranging from 5.5 to 11 ppm (chloroform extract) and 5.5 to 9.5 ppm (UA). Twenty larvae were added to the solution. A mortality count was conducted every hour for 12 h and a final count was performed 24 h after treatment. A control solution using ethanol (0.1 mL) and water (19.9 mL) did not show larvicidal activity. Three replicates, with 20 larvae in each, were taken for each solution and the control. Positive control with the organophosphorate Temephos (O,O'-(thio-di-4,1-phenylene)Bis (O,O-dimethylphosphorothioate), a commonly used insecticide for larvae control, was used under the same conditions as used by health pro-

grams in Brazil (1 ppm). Probit analysis was conducted on mortality data collected after 24 h exposure to different concentration of testing solutions using to determine the lethal concentration for 50% and 90% mortality (LC_{50} and LC_{90}) values. 95% confidence intervals are also reported [19].

Brine shrimp lethality test

A 24 hour lethality bioassay method was carried out to determine the acute toxicity of UA and *C. substellata* extract in the brine shrimp *Artemia salina* (Meyer *et al.* [20], with modifications). UA was dissolved initially in 5 mL DMSO (1000 ppm) and ten aritmetic dilutions (2.5 to 10 ppm) were prepared in reconstituted seawater (34 $g.L^{-1}$). The *C. substellata* extract was dissolved initially in 5 mL DMSO (1000 ppm) and ten aritmetic dilutions (2.0 to 15 ppm) were prepared in reconstituted seawater (34 $g.L^{-1}$). Pure seawater and seawater containing 10 ppm DMSO were used as controls. All assays were done in triplicate, in glass tubes containing 5 mL test solution, and ten larvae (nauplii in instar III/IV) were exposed to each test condition. All tests were conducted at room temperature (28 °C), under a continuous light regime. Mortality was recorded after 24 h incubation, and the average lethal concentrations (LC_{50} and LC_{90} – 95% confidence intervals) per test compound were calculated. Larvae were considered dead if they did not exhibit any movement during several seconds of observation.

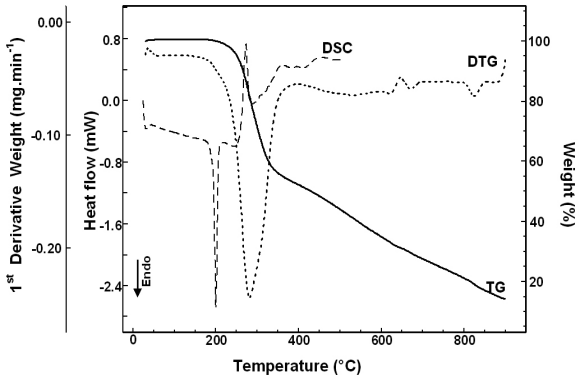

Figure 2. DSC and TG/DTG curves of UA obtained in dynamic nitrogen atmosphere (50 mL/min) and rate heating 10 °C/min.

RESULTS AND DISCUSSION

Characterization of usnic acid

DSC curve for pure UA showed a sharp endothermic transition at 195 °C (on-set temperature) confirming the melting point of the compound (Fig. 2). An observed second event corresponds to the decomposition of the material, by means of an exothermic reaction at 237 °C. The TG/DTG curves indicate that the thermal decomposition process of UA occurs in two stages in the following temperature ranges (weight loss): 210–350 °C (Δm = 46%) and 350-900 °C (Δm = 40%, Fig. 2). In the first stage partial thermal decomposition of UA occur with elemental carbon formation due to sample carbonization. Between 350 and 900 °C the elemental carbon is released slowly.

C/H $\delta c(CH_X)$ *,**	$\delta c(CH_X)$	$\delta_H (n_H , m)$ *,**	$\delta_H (n_H , m)$
C1 (198.2)	198.06		
C2 (179.4)	179.38		
C3 (155.1)	155.22	18.84 (1H,s, C-8-OH)	18.85
C4 (98.3)	98.34		
C5 (101.7)	101.54		
C6 (99.8)	98.34		
C7 (109.5)	109.33		
C8 (157.6)	157.51	13.31 (1H,s, C-8-OH)	13.31
C9 (104.2)	103.96		
C10 (164.1)	163.89	11.02 (1H,s,C-10-OH)	11.02
C11 (105.4)	105.24		
C12 (59.2)	59.09		
C13 (27.4)	27.90	1.75 (3H,s, Me-13)	1.76
C14 (200.1)	200.34		
C15 (32.0)	32.13	2.66 (3H,s, Me-15)	2.66
C16 (7.7)	7.55	2.10 (3H,s Me-16)	2.10
C17 (201.3)	201.79		
C18 (30.9)	31.29	2.67 (3H,s, Me-18)	2.68
		5.92 (1H,s,H-4)	5.98

Table 1. ^1H (500 MHz) and ^{13}C (100 MHz) NMR spectral data of usnic acid, in $CDCl_3$. Assignments based on *Huneck & Yoshimura [21] and **Ingólfsdóttir *et al.* [9].

Analytical data of C, H, N and percentages (found/calculated) for usnic acid are C: 62.28/62.79; H: 4.71/4.65; N: 0.01/0.00. These data are consistent with the general formula $C_{18}H_{16}O_7$ (344.32 g/mol).

^1H (500 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR are shows in Table 1. Its 1H NMR spectrum showed the presence of singlet signals at δH 1.76, 2.10, 2.66 and 2.68 corresponding to four methyl groups bonded to sp^2 carbons, respectively. This spectrum also revealed six deshielded singlet signals corresponding to 1H,s,H-4 (δH 5.98) and three quelated hydroxyl groups (δH 11.02, 13.31 and 18.85). The ^{13}C NMR spectral data are summarized in Table 1. These data are similar with the related by Huneck & Yoshimura [21] and Ingólfsdóttir *et al.* [9].

Biological assays

Results on percent mortality of larvae of *A. aegypti* with increase in chloroform extract or UA are shown in Table 2. C. substellata chloroform extract induced 100% mortality of *A. aegypti* larvae after 24 h at 11 ppm. Positive control Temephos exhibited 100% mortality after 24 h. *C. substellata* chloroform extract exhibited LC_{50} of 7.70 ppm (7.26 to 8.28 ppm).

In order to find out which of the components present in *C. substellata* is responsible for the larvicidal activity the chloroform extract was submitted to crystallization followed by column chromatography to obtain yellow crystals. Furthermore, the purified compound was characterized as UA and its larvicidal potential was evaluated. The major compound in the chloroform extract of *C. substellata,* UA exhibited LC50 of 6.61 (6.16 to 7.06 ppm). Thus, UA is probably the active principle responsible for *C. substellata* larvicidal action, causing 100% larval mortality at 10 ppm.

The crustacean *A. salina* (brine shrimp) is an invertebrate model that have been widely used for studies of ecotoxicology, as well as of general toxicology of chemicals, including mycotoxins. Invertebrates are already used in tests that are required by some regulatory authorities for the environmental risk assessment of pesticides, chemicals and pollutants. The results of the bioassays performed in the A. salina model with chloroform extract and pure UA metabolites

Concentration (ppm)	Mortality (%)			
	A. aegypti		*A. salina*	
	Chloroform extract	UA	Chloroform extract	UA
1.0	-	0.0	-	-
2.0	-	-	-	0.0
3.5	0.0	-	0.0	-
5.0	-	-	-	26.6
5.5	-	20.0	-	-
6.0	-	-	23.3	-
6.5	15.0	40.0	-	-
7.0	-	-	-	50.0
7.5	45.0	75.0	-	-
9.5	81,6	-	-	73.3
10.0	-	100.0	50.0	100.0
11.0	100.0	-	76.6	-
13.0	-	-	-	-
15.0	-	-	100.00	-

Table 2. Percentage mortality of *Cladonia substellata* chloroform extract and UA.

	A. aegypti		*A. salina*	
	Chloroform extract	UA	Chloroform extract	UA
LC_{50}	7.77 (7.26-8.28)	6.61 (6.16-7.06)	8.6 (6.93-10.17)	6.58 (5.06-7.76)
LC_{90}	9.75 (9.02-11.20)	8.36 (7.67-10.02)	13.59 (11.25-21.16)	10.48 (8.68-18.16)

Table 3. Lethal concentrations (LC) of *Cladonia substellata* chloroform extract and UA to of *A. aegypti* and *A. salina* larvae after 24 h.

produced by *C. substellata* are shown in Tables 2 and 3. Similarly to *A. aegypti* larvae, the extract exhibited significant activity in the brine shrimp assay (i.e. LC50 value of 8.6 (6.93-10.17 ppm), whilst the UA showed the highest activity (LC_{50} value of 6.58 (5.06-7.76 ppm) after 24 h.

CONCLUSIONS

The larvae of *A. aegypti* are susceptible to the composition of *C. substellata* chloroform extract herein evaluated. UA, the major compound in *C. substellata,* was found to be the active principle responsible for the larvicidal action.

UA possessed efficacy against *A. aegypti*. However, it was toxic to brine shrimps, a reference organism in assays to evaluate the potential toxicity hazard to invertebrates in ecosystems. Our results indicate UA is not environmentally safe for use as larvicide. Our research group is currently working on synthetic derivatives of UA with the goal to achieve selectivity for *A. aegypti* larvae.

Acknowledgments. The authors acknowledge to Fundação de Amparo à Pesquisa do Estado de Sergipe (FAPITEC-SE) and CNPq for the financial support.

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