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Toxicity of Usnic Acid from *Cladonia substellata* (Lichen) to embryos and adults of *Biomphalaria glabrata*

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ABSTRACT

This study reports the molluscicidal activity of usnic acid isolated from *Cladonia substellata* Vanio (lichen) on embryos at various stages of development and in adult mollusks of *Biomphalaria glabrata*. The toxicity of usnic acid was also evaluated through *Artemia salina* larvae mortality. Usnic acid was extracted with diethyl ether, isolated, purified, and its structure confirmed by analyzing the spectra of proton nuclear magnetic resonance. LC_{90} for 24 h of exposure were 1.62, 4.45, 5.36, and 4.49 μg mL^{-1} for blastula, gastrula, trocophore, and veliger embryonic stages, respectively, and 3.45 μg mL^{-1} for adult snails; LC_{50} of usnic acid against *A. salina* was 2.46 μg mL^{-1}. LC_{90} assessed 7 days after exposure was 2.56 μg mL^{-1} for adult mollusks. In conclusion, these findings demonstrate that under laboratory conditions usnic acid has teratogenic and molluscicide potential to control the aquatic snail *B. glabrata* and may prove to be a promising candidate in the search for new molluscicide agents, but further detailed studies on its molluscicidal effect and possible environmental effects are needed.

1. Introduction

Schistosomiasis is present in Africa, America, Asia, and the Eastern Mediterranean Region. Approximately 200 thousand deaths occur annually associated with the infections by the worms of the genus *Schistosoma* (World Health Organization, 2016). Control of schistosomiasis is challenging, mainly due to inadequate sanitation facilities and lack of effectiveness of snail control, which facilitate the continuous appearance of new cases of this disease (Asaolu and Ofoezie, 2003; Yi-Xin and Manderson, 2005; Gazzinelli et al., 2008; Mwanga and Lwambo, 2013; Mwakitalu et al., 2014).

Biomphalaria glabrata is the main vector of *Schistosoma mansoni* in Brazil. The species is distributed at the coastal strip covering the Northeast, Southeast and Northern regions (Pointier et al., 2005; Scholte et al., 2012). As a method of snail control, the World Health Organization Pesticide Evaluation Scheme (WHOPES) recommends the use of synthetic molluscicide niclosamide, as it is currently the only compound that meets the WHOPES requirements for toxicity testing of pesticides (World Health Organization, 2017). However, the use of this product is problematic due to its effect on other biota, most notably fish, high cost and development of resistance (King and Bertsch, 2015; Coelho and Caldeira, 2016).

Natural and easy accessible sources of compounds that can be used as a means of controlling the burden of schistosomiasis in the poorest areas of the world are important. Thus, the search for a molluscicide from a natural source gained a new prominence, in order to obtain an alternative product with low cost, biodegradable, safe and local availability to control the population of mollusks (Al-Zanbagi, 2013; Rapado et al., 2013). Previous studies have reported a molluscicidal activity of natural origin related to the presence of polyphenols and their interactions with molecules, including proteins and polysaccharides (Haslam, 1996; Gurib-Fakim, 2006; Singh et al., 2010). These polyphenols are compounds commonly found superior plants and organisms such as lichens, symbiotic beings found in tropical and subtropical regions (Ahti, 2000).

Among the natural substances derived from lichens secondary metabolism, the usnic acid (Fig. 1) is one of the most studied. Different biological activities are attributed to this dibenzofuran, such as cicatrizing (Bruno et al., 2013), antiviral (Sokolov et al., 2012), anti-inflammatory, antioxidant (Su et al., 2014), antimicrobial (Taresco et al., 2015), and anti-parasitic (Luz et al., 2015) activities. However, the molluscicidal activity was only reported for the potassium salt

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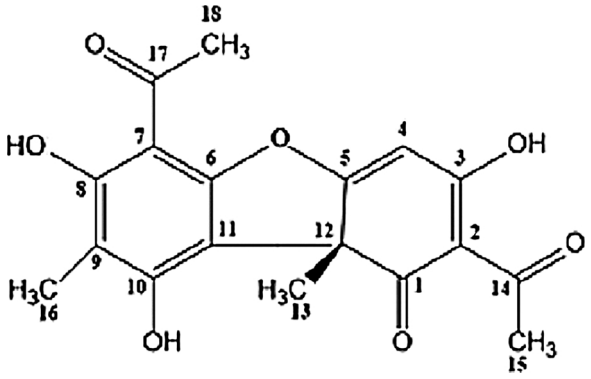

Fig. 1. Usnic acid chemical structure.

(potassium usnate), which was obtained by modification of the isolated usnic acid from *Cladonia substellata* (Martins et al., 2014). In this previous research, we observed that the salt caused the death of *B. glabrata* embryos in the first stage of development. Although the usnic acid is not as hydrophilic as its salt, we hypothesize that it could be used as a potential prototype substance for control of mollusks.

Substances obtained from natural sources may also bring risks to the environment. For this reason, the WHOPES has recommended that toxicity tests should be carried out in order to ensure the use of substances in aquatic environments and, therefore, establishing tolerable and non-toxic concentrations to zoo- and phytoplankton (Canesi et al., 2015; Schiavo et al., 2017; Schiffer and Liber, 2017; World Health Organization, 2017). Among the organisms indicated as experimental models, *Artemia salina* has shown high sensitivity for ecotoxicological monitoring of novel agents (Lima et al., 2002; Albuquerque et al., 2014; Martins et al., 2014).

Therefore, due to the recognized great potential of biological substances obtained from animals, higher plants and/or lichens (El-Sherbini et al., 2009; Ribeiro et al., 2009; Miyasato et al., 2012; Al-Zanbagi, 2013; Martins et al., 2014; El-Beshbishi et al., 2015), this study aimed to investigate the effectiveness of a novel, simple and low-cost substance (usnic acid) to control *S. mansoni*'s vectors (*B. glabrata*) at different embryonic and adult stages.

2. Material and methods

2.1. Samples of Cladonia substellata

C. substellata Vainio samples were collected at the municipality of Mamanguape, Paraíba Brazil (6°42′1.5" S/35°8′3.3"W) in February 2015. A voucher specimen was deposited in the UFP herbarium Geraldo Mariz, Dept. of Botany of the Federal University of Pernambuco, Recife/PE, Brazil (voucher n° 77. 474).

2.2. Etheric extract preparation, isolation and purification of Usnic Acid (UA)

C. substellata samples (120 g) were cleaned, dried, and grinded to a powder which was subjected to successive extractions with diethyl ether (150 mL) in a Soxhlet apparatus at 40 °C until exhaustion of the thallus (7x). After each extraction, organic extracts were kept at 4 °C (24 h) and filtered. Then, the extracts were dried in a rota-evaporator coupled to a water bath at 37 °C (Asahina and Shibata, 1954).

The usnic acid was isolated and purified as described previously (Martins et al., 2014). Briefly, 243 mg of the ether extract was fractionated on a silica gel column (70–230 mesh), eluted in the solvent system chloroform-hexane (80:20 v/v), and evaporated until dryness. The obtained fractions were monitored by thin layer chromatography (TLC). Those samples that showed only one band were combined. The fractionation and monitoring processes were repeated until highly pure usnic acid (> 95%) was obtained, and the molecular structure analyzed by the spectra of proton nuclear magnetic resonance (^{1}H NMR) obtained at 300 MHz in $CDCl_3$ (Varian UNITY spectrometer) and infrared spectroscopy.

2.3. Bioassays

2.3.1. Biomphalaria glabrata (Say, 1818, mollusca, gastropoda, planorbidae) culture

B. glabrata pigmented adults (*S. mansoni* negative) were collected at the municipality of São Lourenço da Mata (Pernambuco – Brazil) and reared by successive generations at the Radiobiology Laboratory, at the Department of Biophysics and Radiobiology of the Federal University of Pernambuco, Recife/PE, Brazil. The mollusks were maintained in 20 L plastic tanks filled with filtered and dechlorinated water, pH 7.0 and temperature 25 ± 3 °C, and fed daily with organic *Lactuca sativa*. All cultures, dilutions and experiments were made in filtered and dechlorinated water.

2.3.2. B. glabrata embriotoxicity assay

The embryotoxicity assay was performed according to the methodology described by Rapado et al. (2013). Briefly, colorless polyethylene pieces (10 × 10 cm) were placed on the water surface where the mollusks were cultured to allow subsequent egg collection. Egg masses selected and analyzed using a stereoscopic microscope (Wild M3B, Heerbrugg, Switzerland) according to Kawano et al. (1992) regarding to their viability (Fig. 2).

Embryo stages were identified after cleavage of the eggs as follows: first (blastula − 0–15 h, Fig. 2A), second (gastrula − 24–39 h, Fig. 2B), third (trocophore − 48–87 h, Fig. 2C) and fourth cleavage (veliger − 96–111 h, Fig. 2D).

Groups of 100 embryos from each stage (blastula, gastrula, trocophore and veliger) were selected and deposited in petri dishes with 10 mL of UA solutions (0.5% DMSO) at different concentrations (1; 1.5; 2; 2.5; 3; 3.5; 4; 4.5; 5; 5.5; and 6 μg mL^{-1}), water (negative control 1), 0.5% DMSO (negative control 2), and 1.0 μg mL^{-1} niclosamide (positive control, NCL). All groups were exposed for 24 h, then embryos were washed and placed in clean plates with filtered and dechlorinated water, and observed using microscope during 7 consecutive days, in order to check their positive (hatch) or negative (death or malformation) viability. Two independent experiments were performed in triplicate, so in total 600 embryos per concentration for each stage.

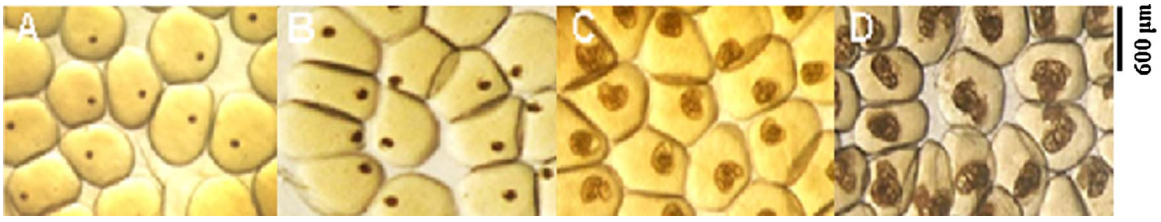

Fig. 2. *Biomphalaria glabrata* embryos in different embryonic stages. (A) Blastula, (B) Glastrula, (C) Trocophore, (D) Veliger.

2.3.3. B. glabrata toxicity assay

B. glabrata toxicity assay was performed according to World Health Organization (1965). Pigmented *B. glabrata* (10 and 14 mm) were placed in individual containers (180 mL water). Eight groups (10 snails in each) were used for the following treatments: Negative Controls − Filtered and dechlorinated water only (control 1), 0.5% DMSO Water (control 2); Positive Control (1.0 μg mL^{-1} niclosamide) and UA (1, 2, 2.5, 3 and 4 μg mL^{-1}) during 24 h. After exposure, the living mollusks were transferred to vessels containing 1000 mL of filtered and dechlorinated water, fed and monitored daily for 7 days. The mortality criteria were the cephalopodal mass retraction into the shell, loss of hemolymph, discoloration of shell color and absence of beats in the pericardial cavity. Two independent experiments were performed in triplicate, 60 snails per treatment.

2.3.4. Environmental toxicity test using Artemia salina

A. salina encysted eggs were placed in a beaker with 500 mL seawater (pH 8.0) and constant aeration at room temperature (25 ± 3 °C) for 48 h. After hatching, the larvae were collected and split in experimental groups (n = 10) with the help of a stereomicroscope (Wild M3B, Heerbrugg, Switzerland) as follows: Negative controls (sea water and 5% DMSO diluted sea water), positive (1.0 μg mL^{-1} niclosamide), and UA at 1, 1.5, 2, 2.5, 3, 3.5 and 4 μg mL^{-1}) for 24 h at 25 ± 3 °C according to the procedure described by Meyer et al. (1982). Two experiments were performed in quadruplicate with a total of 80 specimens per treatment, and assessments of mortality and survival of larvae were carried out by observation of mobility with the help of a stereomicroscope.

2.4. Statistical analysis

The lethal concentrations (LC) required to kill 10%, 50% and 90% of *B. glabrata* (embryos and adult snails) and *A. salina* larvae were calculated by the Probit analysis with a confidence interval of 95% using the StatPlus® 2009 software (Soft Analyst, Vancouver, BC, Canada).

3. Results and discussion

The molecular structure of UA (Fig. 1) isolated from the lichen *C. substellata* was highly pure as revealed by ^1H NMR spectroscopy. The lichen used to obtain UA was collected from a region endemic for schistosomiasis mansoni, and along with the good availability in the region, UA showed good activity against *B. glabrata* embryos (Table 1). One possible simple method to accomplish disease control is to eliminate the vector to human infection (Lima et al., 2002; El-Beshbishi et al., 2015). However, concerning to *S. mansoni*, niclosamide, the actual available compound has been criticized over the years for different reasons, such as water flow rate, vegetation, temperature, effect on other biota and development of resistance (King and Bertsch, 2015; Coelho and Caldeira, 2016). Other candidates as molluscicidal have been reported, but the bottleneck in compound development remains as the high cost, low availability and environmental toxicity related to affected regions (Al-Zanbagi, 2013; Albuquerque et al., 2014).

Table 1
Lethal concentration to all embryonic stages and adult mollusks of *Biomphalaria glabrata* exposed to usnic acid during 24 h.

Embryonic stages and adult mollusks	Lethal Concentration (μg mL^{-1})		
	LC_{10}	LC_{50}	LC_{90}
Blastula	1.13 [1.11–1.14]	1.38 [1.36–1.39]	1.62 [1.6–1.63]
Gastrula	1.35 [1.24–1.46]	3.47 [3.36–3.58]	4.45 [4.34–4.56]
Trocophore	2.54 [2.43–2.64]	5.11 [5.0–5.21]	5.36 [5.25–5.46]
Veliger	1.36 [1.3–1.42]	2.93 [2.87–2.99]	4.49 [4.43–4.55]
Adult mollusk	0.80 [0.54–1.07]	2.12 [1.86–2.39]	3.45 [3.19–3.72]
Adult mollusk (7days)*	0.61 [0.39–0.84]	1.58 [1.36–1.81]	2.56 [2.34–2.79]

[] 95% confidence interval.
* Adult mollusks observed during 7 days after a 24-h exposition to usnic acid.

In our study, blastula and gastrula embryonic stages were the most sensitive to UA, presenting 100% mortality when exposed to 2 and 4.5 μg mL^{-1} UA, respectively. Piplartine amide from *Piper tuberculatum* (Rapado et al., 2013) and CH_2Cl_2 fraction of *Liagora farinosa* extract (Miyasato et al., 2012) also showed increased efficacy to the same *B. glabrata* stages. Such increased susceptibility to UA can be attributed to the strong cell proliferation observed at the beginning of these stages. At those stages, organisms seem to be more prone to undergo teratogenic side effects due to chemical treatments, even at low concentrations and during short periods (Tallarico et al., 2014; Burić et al., 2015; Gombeau et al., 2017).

As demonstrated in Fig. 3B–E, at lower concentrations not all embryos were dead. We observed that as the embryo matures the concentration needed to cause 100% malformation increases. Interestingly, the trocophore stage demonstrated to be more resistant to UA action than the other stages, as shown in Table 1. Trochophore (Fig. 2C) is characterized by having a double cell layer and the whole embryo is covered by cilia, unlike any other embryonic stage (Kawano et al., 1992). This feature may have hindered the absorption of UA and, therefore, justify the higher levels of UA needed to cause effective embryotoxicity to this stage. CL_{10}, CL_{50}, and CL_{90} in the different embryonic stages and adult snails exposed to UA for 24 h and monitored for 7 days are also described in Table 1.

Compound development relies on the lowest concentration enough to provoke the best efficiency. So, in our context, it was important to verify not only embryos but also adults of *B. glabrata*, in order to cover all possible steps of the mollusk life cycle. The UA proved to be efficient against *B. glabrata* adults, and we observed that concentrations of 3 and 4 μg mL^{-1} UA caused 87% and 100% mortality, respectively, in the first 24 h ($p \leq 0.001$). In addition, when mollusks were checked 7 days after the UA treatment 3.0 μg mL^{-1} was able to reach 100% mortality, and LC_{90} was observed with 2.56 μg mL^{-1}, in laboratory trials.

The World Health Organization (1983) published methodological specifications classifying natural substances as inactive when the percentage of mortality of mollusks represents from 0 to 30%; partially active when they cause mortality 40–60% and active when 70–100% of them are eliminated after 24 h exposure. However, according to this publication, substances of natural origin will be considered active when it gets 90% mortality at 20 μg mL^{-1} for single molecules and 100 μg mL^{-1} for crude vegetables. It was shown that the usnic acid had high molluscicidal action against *B. glabrata*, even when used approximately 1/7 of the concentration recommended by the World Health Organization (1983). Therefore, in the present work, UA fits World Health Organization specifications and demonstrated to be a promising molluscicide of natural origin. Previously, we reported that molluscicidal activity of the potassium salt derived from UA caused 100% mortality to *B. glabrata* at a concentration of 1 μg mL^{-1}, thereby such UA molecule modification in salt form that may facilitate its spread in intracellular medium, increasing their molluscicide action (Martins et al., 2014).

The research of molluscicide substances that act upon egg masses and adults are as important as analyzing their mechanism of action. Some authors suggest that the performance of the UA occurs because of its heterocyclic structure, formed by conjugated dienes and polar OH groups (lipophilic molecule) that promptly diffuses through cell membranes (Ingólfsdóttir, 2002; Joseph et al., 2009). Another possible mechanism of UA action is the influence on the inner mitochondrial

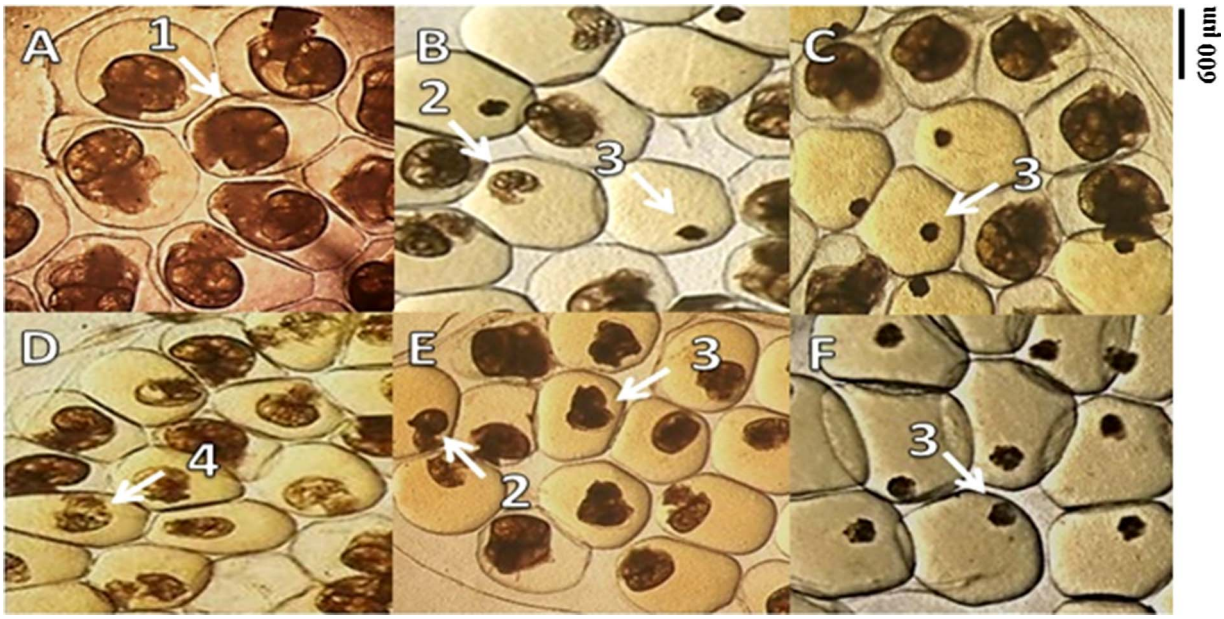

Fig. 3. *Biomphalaria glabrata* embryos in different embryonic stages exposed to usnic acid. (A) Water (Negative Control), (B) Blastula stage (1.5 µg mL^{-1} UA), (C) Gastrula stage (3 µg mL^{-1} UA), (D) Trocophore stage (5.5 µg mL^{-1} UA), (E) Veliger stage (4 µg mL^{-1} UA), (F) Blastula stage (1 µg mL^{-1} niclosamide, as positive control). 1, Healthy embryo. 2, Developmental delay. 3, Dead embryo. 4, Shell malformation.

membrane polarity, by it causes an electron transport chain decoupling and provokes proton leakage. Therefore triggering important biochemical and physiological changes to cell homeostasis, which can lead to cell apoptosis (Joseph et al., 2009).

Although UA has demonstrated to be an effective molluscicide compound, its development as a possible schistosomiasis control method through *B. glabrata* elimination must evaluate the extend of the possible environmental side effects, as the death of the macrofauna, food chain baseline in rivers for other bodies of water (Li et al., 2015; Lechuga et al., 2016). An important component of this macrofauna is the species *A. salina*, whose survival after contact with the tested agent is frequently used as a measure of environmental toxic effects (Oliveira-Filho and Paumgartten, 2000; Oliveira et al., 2011; Gambardella et al., 2015). The UA LC_{50} found for *A. salina* was 2.46 µg mL^{-1}, so we evidenced from this result that the use of AU for the control of schistosomiasis requires special attention, since the CL_{90} for adult snails was 2.56 µg mL^{-1} (Table 1). In addition, a comparison of the effect of niclosamide with that of UA demonstrated that in concentrations lower than 3 µg mL^{-1} UA causes significant less mortality of *A. salina* than Niclosamide ($p < 0.001$); at the concentration of 1 µg mL^{-1} niclosamide it was observed 100% mortality of *A. salina*. Therefore, the possible use of the molluscicide should be focused, restricted and controlled in order to evaluate the efficacy in the field, also and the effect of this substance on other non-target organisms present in the aquatic environment (Oliveira-Filho and Paumgartten, 2000; Li et al., 2015; Schiffer and Liber, 2017).

4. Conclusion

The isolated molecule of usnic acid showed molluscicidal activity to all embryonic stages and during the adult life of *B. glabrata*. Moreover, toxicity of usnic acid evaluated against *A. salina* was found to be lower than niclosamide the molluscicide adopted by the World Health Organization. In conclusion, the teratogenic and molluscicide potential of the usnic acid to control aquatic snail *B. glabrata* demonstrated that this compound may be a promising candidate in the search for new molluscicide agents, as a natural product of importance in the fight against schistosomiasis mansoni transmission.

Competing interest

The authors have no conflict of interest.

Author contributions

H.D.A. Araújo and V.L.M. Lima designed the study protocol; H.D.A. Araújo, L.R.S. Silva, W.N. Siqueira, N.H. Silva, M.C.B. Martins, V.L.M. Lima and A.M.M.A. Melo carried out the assays and were involved in analysis and interpretation of all the data; C.S.M. Fonseca and H.D.A. Araújo did statistical analysis; H.D.A. Araújo, A.M.M.A. Melo, M.C.B. Martins, C.S.M. Fonseca and V.L.M. Lima contributed to drafting the manuscript and/or critically revising the paper and intellectual content. All authors read and approved the final manuscript.

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