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Laboratory assessment of divaricatic acid against Biomphalaria glabrata and Schistosoma mansoni cercariae

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ABSTRACT

In this study, the molluscicidal and antiparasitic activities of divaricatic acid was evaluated, targeting the mollusc *Biomphalaria glabrata* and cercariae of the helminth *Schistosoma mansoni*. In addition, the environmental toxicity of divaricatic acid was assessed by bioassay using the microcrustacean *Artemia salina*. Divaricatic acid showed high toxicity against both adult snails (5 μg/mL) and embryos (20 μg/mL after 6 h of exposure). Similar activity was observed in *Schistosoma mansoni* cercariae after only a short exposure time (10 μg/mL after 30 min of exposure). The divaricatic acid did not show toxicity in the acute test using *Artemia salina* at concentrations equal to or below 200 μg/mL. The divaricatic acid proved to be a promising substance for the elimination of the snail *Biomphalaria glabrata*, an intermediate host of schistosomiasis, as well as the cercariae of the pathogen, while being non-toxic to the *Artemia salina* at the same concentrations. This is the first experimental observation of the molluscicidal and cercaricide activity of divaricatic acid.

1. Introduction

Schistosomiasis is a parasitic disease caused by trematode worms of the genus *Schistosoma*. Its transmission in humans begins with penetration by cercariae, the larval stage of the parasite, following contact of the skin with contaminated water (Gryssels et al., 2006; World Health Organization, 2015). In order for the parasite to complete its life-cycle, the presence of certain species of freshwater snails, its intermediate hosts, is required. Species of snails in three different genera are considered the principal schistosomiasis vectors: *Biomphalaria*, *Bulinus* and *Oncomelania*. In the Americas and Africa, snails of the genus *Biomphalaria* are the intermediate hosts for *Schistosoma mansoni* (Colley et al., 2014; World Health Organization, 1988).

The World Health Organization (WHO) estimates that at least 261 million people require treatment for schistosomiasis. The 78 countries where the disease is endemic are distributed in Africa, Asia and South America (World Health Organization, 2015; Rapado et al., 2013). One of the strategies to combat schistosomiasis cited by the World Health Organization is the use of the synthetic molluscicide Niclosamide (Bayluscide, Bayer) (World Health Organization, 2002). However, the use of this substance has significant drawbacks, i.e: its high toxicity to non-target organisms and the high costs involved in its application (Oliveira-Filho et al., 2010; Andrews et al., 1982; Abreu et al., 2002). Thus, there is a need for an alternative compound and several studies have aimed at identifying molluscicides of natural origin to combat vector snails (Albuquerque et al., 2014; Rocha-Filho et al., 2015; Yadav and Jagannadham, 2008; Santos et al., 2010).

Lichens are symbiotic associations between mycobiont and photobiont organisms (Vatne et al., 2011; Turkes et al., 2012; Mitrović et al., 2011). They are widely distributed around the globe, colonizing a variety of habitats from arctic to desert regions (Taylor et al., 1995). The organisms produce a variety of secondary metabolites known as lichen substances, which are present inside the lichen thallus and sometimes form crystals on the surfaces of hyphae and algal cells. These substances may represent up to 30% of the dry weight of the thallus, in some species (Backorováet al., 2012; Ranković and Misić, 2014). These substances are produced by the mycobiont (Shukla et al., 2013), aiming not only to preserve the symbiotic structure, but also protect against pathogens and predators, intense UV radiation or oxidative stress (Oettl et al., 2014). Several studies have reported on the extensive biological

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and ecological potential of lichens and their secondary metabolites, such as antimicrobial, antioxidant, cytotoxic (Manojlović et al., 2012), antitumor (Russo et al., 2012), antifungal (Shahi et al., 2001) activities. In addition, lichens are considered excellent bioindicators of environmental pollution (Grangeon et al., 2012).

Lichens produce a variety of metabolites, organized into different classes, such as depsides, depsidones, dibenzofurans, xanthones, benzyl esters, anthraquinones, among others (Honda and Vilegas, 1998; Bellio et al., 2015). Depsides are compounds consisting of two or more phenolic aromatic rings joined by an ester linkage. They are mainly found in lichens, but can be isolated from some higher plants species (Honda and Vilegas, 1998; Peng-Cheng et al., 2009). Biological activities such as enzyme inhibition (Umezawa et al., 1983), anti-proliferative, pro-apoptotic (Backorová et al., 2011) and antioxidant activities (Luo et al., 2009) have been observed in various depsides. Divaricatic acid belongs to the class of depsides and is commonly found in lichens of the genus *Ramalina*, yet is still rarely mentioned in the literature and its biological potential has remained unknown.

In this study, divaricatic acid, isolated from the lichen *Ramalina aspera* Räsänen, has been tested against *Biomphalaria glabrata,* a snail vector of schistosomiasis, in its embryonic and adult stages, as well as against *Schistosoma mansoni* cercariae. Concomitantly, its environmental toxicity was assessed using the microcrustacean *Artemia salina*.

2. Material and methods

2.1. Collection of lichen material, preparation of ether extract and isolation of divaricatic acid

Lichens of the species *Ramalina aspera* Räsänen were collected in the city of Limoeiro (Pernambuco, Brazil) and kept at room temperature (28 ± 3 °C) under dry conditions for further identification and preparation of the extracts. Thallus identification was performed and a voucher specimen was deposited at the UFP Herbarium, Department of Botany of the Universidade Federal de Pernambuco, Brazil (voucher n° 54299).

Divaricatic acid was isolated by the crystallization method as described by Asahina and Shibata (1954), with modifications. Ten grams of lichen samples were subjected to successive extractions with diethyl ether in Soxhlet apparatus at 40 °C. Subsequently, the solvent was evaporated at 55 °C and the extract was kept in a desiccator for subsequent isolation of divaricatic acid. The ether extract of *R. aspera* was dissolved in 50 mL of chloroform and refluxed in a water bath at 40 °C for 15 min. The concentrated material obtained was maintained at a temperature of ± 4 °C for 15 days until forming crystals, which were filtered out using a G4 porous bottom funnel.

2.2. Chemical and physicochemical analysis

2.2.1. Thin layer chromatography (TLC) and HighPerformance liquid chromatography (HPLC)

The crystals purified were subjected to chromatographic analysis (TLC), unidimensional, on silica gel 60 plates (Merk® $PF_{254+366}$) (Culberson, 1969). The elution system was performed in toluene/dioxane/acetic acid (45:12.5:2 − v/v/v) and revelation by spraying with 10% sulfuric acid and subsequent heating to 50 °C for 5 min. Bands were observed using UV radiation (254 and 366 nm). To determine the purity of the sample was perform the HPLC analysis, as described Huneck and Yoshimura (1996), in a Hitachi Chromatograph (655 A-11, Tokyo, Japan) coupled to a CG437-B UV detector set at 254 nm. For the separation, a C-18 reverse phase column MicroPack MCH-18 de 300 × 4 mm, Berlin, Germany (Merck® KGaA, Darmstadt, Germany) was used. The sample was injected at a concentration of 1.0 mg mL^{-1} in chloroform (Merk®). The mobile phase consisted of methanol/water/acetic acid (80:19.5:0.5 v/v/v) in isocratic system. Other analytical parameters were: volume of injection 20 mL, attenuation 0.16, pressure 87 atm, flow rate 1.0 mL min^{-1} at room temperature (28 ± 3 °C).

2.2.2. ^1H nuclear magnetic resonance (^1H NMR) and infrared (IR)

To confirm the molecular structure of divaricatic acid, ^1H NMR was performed using a Varian Unity Plus spectrometer at 300 MHz, 26 °C, with DMSO-D6 as the solvent. The IR analysis was performed using a Bruker spectrophotometer coupled to a Fourier transformer (IF model 566) using KBr pellets. The analysis was performed at the Fundamental Chemistry Department of the Universidade Federal de Pernambuco − UFPE, Brazil.

2.3. Bioassays

2.3.1. Source and maintenaince of Biomphalaria glabrata

Pigmented adult snails of *B. glabrata* were used, measuring 10–14 mm of diameter, from São Lourenço da Mata, Pernambuco, and maintained for successive generations in the Radiobiology Laboratory of the Department of Biophysics and Radiobiology of UFPE. The snails were kept in plastic tanks of approximately 20L filtered and dechlorinated water, pH 7.0 and a temperature of about 25 ± 3 °C. The snails were fed daily with fresh organic lettuce.

2.3.2. Embryotoxicity test in Biomphalaria glabrata

The assay was performed according to the methodology described by Oliveira-Filho and Paumgartten (2000). Egg masses were collected from breeding tanks, placed in Petri dishes (10 mL) and classified by embryonic stage with the aid of a stereomicroscope (Wild M3B, Heerbrugg, Switzerland). Five egg masses containing 100 embryos (n = 100) in the blastula stage were selected. The embryos were exposed to divaricatic acid in concentrations ranging from 7.5 to 20 µg/mL. Divaricatic acid was solubilized in 0.5% DMSO. The embryos were incubated for 6, 12, 18 and 24 h (25 °C ± 3) and subsequently washed with filtered and dechlorinated water (pH 7.0). Eight days after exposure, the embryos were analyzed for inviability (malformed embryos or dead). The negative control was formed by two groups of embryos exposed to filtered and dechlorinated water (Control 1) and 0.5% DMSO solution (Control 2). Niclosamide (Bayluscide, Bayer) was used for the positive control, at a concentration of 1 µg/mL. Assays were performed in triplicate for each concentration.

2.3.3. Lethality test in Biomphalaria glabrata

Adult snails of *B. glabrata* were pre-selected as to their sexual maturity through the deposition of egg masses. Sexually mature snails were separated into groups of 10 specimens and kept in containers containing 60 mL of divaricatic acid solution dissolved in 0.5% DMSO at concentrations of 2.5, 3.5, 4.5 and 5.5 µg/mL. The snails were incubated for 24 h (25 °C ± 3) and subsequently washed with filtered and dechlorinated water (pH 7.0). Eight days after exposure, they were analyzed for lethality (absence of body movement, deep retraction into the shell, loss of hemolymph and absence of heartbeat). The negative control was formed by two groups of snails exposed to filtered and dechlorinated water (Control 1) and 0.5% DMSO solution (Control 2). Niclosamide (Bayluscide, Bayer) was used for the positive control, at a concentration of 1 µg/mL. The assay was performed in triplicate.

2.3.4. Lethality test on Schistosoma mansoni cercariae

B. glabrata infected with *S. mansoni* (Belo Horizonte strain) were exposed to artificial light for release of cercariae. A suspension of approximately 100 cercariae was kept in a glass container and exposed to divaricatic acid at concentrations of 0.5, 1, 5, 10 and 100 µg/mL (28 °C ± 3). The divaricatic acid was solubilized in 0.5% DMSO. The negative control was formed by two groups exposed to filtered and dechlorinated water (pH 7.0) (Control 1) and 0.5% DMSO solution (Control 2). Niclosamide (Bayluscide, Bayer) was used for the positive control, at a concentration of 1 µg/mL. The assay was performed in triplicate. The lethality of cercariae was observed 15, 30, 60 and

120 min after exposure to divaricatic acid with the aid of a stereomicroscope (Wild M3B, Heerbrugg, Switzerland). The following criteria were used: 100% of death (+ + +), higher than 50% of cercariae death (+ +), less than 50% of cercariae death (+) and absence of death (−).

2.3.5. Evaluation of environmental toxicity using the bioassay with Artemia salina

Artemia salina cysts were placed in a beaker containing 500 mL of filtered, natural seawater (pH 8.0) under constant aeration at a temperature of 25 °C ± 3. After 48 h, hatching of the cysts and release of larvae were observed. Larvae were placed in tubes assay containing 5 mL of divaricatic acid solutions at concentrations of 25, 50, 100, 200 and 400 µg/mL, in groups of 10 specimens. The negative control comprised two groups of *A. salina* exposed to filtered, natural seawater (Control 1) and a solution of 0.5% DMSO in seawater (Control 2). Exposure of *A. salina* to divaricatic acid was performed for 24 h and, thereafter, mortality was assessed using a stereomicroscope.

2.4. Statistical analysis

The results are expressed as mean and standard deviation (SD) or percentage (%) and the estimated lethal concentrations for 10, 50 and 90% of the specimens (LC_{10}, $_{50}$ and $_{90}$) were performed by Probit analysis with a 95% confidence limit, using the StatPlus® 2009 Professional software (AnalystSoft, Canada).

3. Results

3.1. Chemical analysis

The TLC analysis of the sample after the purification process showed the presence of a single band with a retention factor of 0.39, a result consistent with the findings of Huneck and Yoshimura (1996). Confirmation of substance isolation was performed using HPLC, where the crystals showed 97.45% purity and a retention factor of 12.70. The ^{1}H NMR and IR analyses confirmed the chemical structure of the purified divaricatic acid (Fig. 1), resulting in the following data: ^{1}H NMR (DMSO − d_6, 300 MH_Z) δ: 0.88, 0.91 (2 × 3H, 2 x T, J = 8.1H_Z, Me-3″, Me-3‴); 1.51–1.62 (2 × 2H, m, −CH_2-2″, −CH_2-2‴); 2.59, 2.63 (2 × 2H, 2 x T, J = 8.0H_Z, −CH_2-1″, −CH_2-1‴); 3.74 (1 × 3H, s, MeO-4); 6.35, 6.36 (2 × 1H, 2 x d, J = 2.2H_Z, H-3′, H-5′); 6.50, 6.58 (2 × 1H, 2 x d, J = 2.4H_Z, H-3, H-5); 10.23 (2 × 1H, s, −C−OH-2′, −C−OH-2). IR (KBr): 2955, 2872, 1670, 1646, 1609, 1545, 1305, 1284, 1242, 1202, 1155, 1139 cm^{-1}.

3.2. Toxicity in embryos and adult snails of Biomphalaria glabrata exposed to divaricatic acid

The findings of embryotoxic activity, at different exposure times, are described in Table 1. All exposure intervals (6, 12, 18 and 24 h) showed values of inviability. Exposure for 24 h showed the highest inviability, reaching 100% at the 15 µg/mL concentration. At the other exposure times, 100% of inviability was achieved at a concentration of 20 µg/mL. The LC_{50} for *B. glabrata* embryos at all tested time intervals were 13.34 (13.19–13.50), 11.60 (11.44–11.76), 10.54 (10.36–10.72), and 10.29 (9.51–11.07) µg/mL at exposures of 6, 12, 18 and 24 h, respectively, where a decrease in LC_{50} is observed as the exposure time is increased.

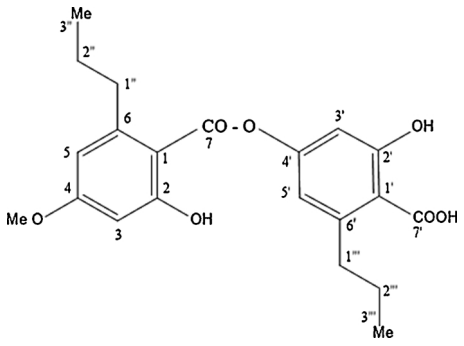

Fig. 1. Chemical structure of divaricatic acid.

Table 1
Lethal concentration (LC) for adult snails and *Biomphalaria glabrata* embryos exposed to divaricatic acid.

Exposure Time (h)	Concentration (µg/mL)		
	$LC_{10(95\%CL)}$	$LC_{50(95\%CL)}$	$LC_{90(95\%CL)}$
Embryos			
06	10.29 (10.14 − 10.45)	13.34 (13.19 − 13.50)	16.39 (16.24 − 16.55)
12	8.23 (8.07 − 8.39)	11.60 (11.44 − 11.76)	14.96 (14.80 − 15.12)
18	6.95 (6.77 − 7.13)	10.54 (10.36 − 10.72)	14.12 (13.94 − 14.30)
24	6.02 (5.24 − 6.80)	10.29 (9.51 − 11.07)	13.29 (12.51 − 14.07)
Adult Snails			
24	1.68 (1.25 − 2.13)	3.58 (3.15 − 4.03)	5.49 (5.06 − 5.94)

In Fig. 2, embryonic alterations caused by divaricatic acid can be seen. In addition to the observed lethality (Fig. 2c and e), the presence of abnormalities was found (shell malformations, hydropic embryos and nonspecific malformations) as well as a delay in embryonic development, with several embryos not breaking out (Fig. 2b–d).

The molluscicidal evaluation on adult snails shows different values of mortality at all tested concentrations. The results were 26.66%, 40%, 76.66% and 100% for the concentrations of 2.5, 3.5, 4.5 and 5.5 µg/mL, respectively. The lethal concentrations obtained were 1.68, 3.58 and 5.49 µg/mL for the LC_{10}, LC_{50} and LC_{90}, respectively.

3.3. Activity of divaricatic acid on Schistosoma mansoni cercariae

Table 2 shows the mortality of cercariae exposed to divaricatic acid in relation to exposure time. Lethal effects started after 60 min of exposure at a concentration of 0.5 µg/mL, reaching 100% of mortality at 15 and 30 min at concentrations of 100 and 10 µg/mL, respectively. At these concentrations, it was possible to observe separation of tail and body of cercariae, as shown in Fig. 3(b and c). Also, changes in cercariae motility were observed at all concentrations, with atypical movement, such as slow rotation and vibration, rotation on its own axis, a type of creeping dislocation and contraction of the cercariae body with varying intensity. These observations were dependent on exposure time and concentration. The cercariae of control groups 1 and 2 had normal movements of swimming by rotation and vibration, with preservation of the body and tail (Fig. 3a). Cercariae exposed to Niclosamide showed 100% lethality after just one minute of exposure (Fig. 3d).

The cercariae were counted at the end of experiment (120 min of exposure) to obtain the final percentage of death and calculation of the LC_{50}. It was possible to observed that all concentrations showed mortality compared with control, with values of 23.33%, 56%, 70%, 100% and 100% at the concentrations of 0.5, 1, 5, 10 and 100 µg/mL, respectively, exhibiting an LC_{50} of 0.89 µg/mL.

3.4. Environmental toxicity of divaricatic acid

Toxicity tests of divaricatic acid on *A. salina* (Table 3) showed low toxicity at all concentrations tested. Among which, only the

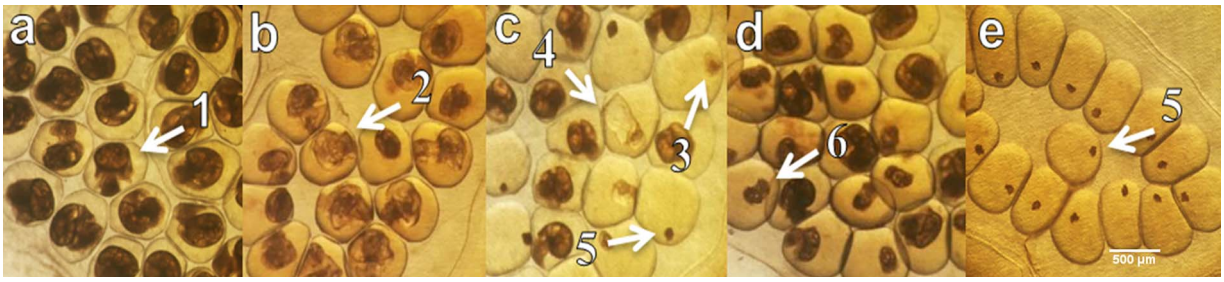

Fig. 2. Abnormalities resulting from exposure of *Biomphalaria glabrata* embryos to divaricatic acid. a-1 – Normal Embryo (Control 1 – filtered and dechlorinated water). b-2 – shell malformation (11.5 µg/mL/18 h). c-3–nonspecific anomaly; 4–hydropic embryo; 5 – dead embryo (9.5 µg/mL/24 h). d-6 – embryonic development delay (12 µg/mL/6 h). e-5 – dead embryo (Niclosamide – 1 µg/mL/6 h).

Table 2
Cercariae mortality after exposure to divaricatic acid in relation to exposure time.

Experimental groups (µg/mL)	Exposure time (minutes)			
	15	30	60	120
Control 1	–	–	–	–
Control 2	–	–	–	–
Niclosamide	+++	+++	+++	+++
Divaricatic acid				
0.5	–	–	+	+
1.0	–	–	+	++
5	–	+	+	++
10	+	+++	+++	+++
100	+++	+++	+++	+++

Control 1: filtered and dechlorinated water. Control 2: 0.5% DMSO in filtered and dechlorinated water. Niclosamide at a concentration of 1 µg/mL. Total elimination of cercariae (+++), elimination higher than 50% of cercariae (++), less than 50% elimination of cercariae (+) and absence of lethality (−).

Table 3
Lethality of divaticatic acid on *Artemia salina*.

Experimental groups (µg/mL)	(%)
Control 1	0
Control 2	2.5
25	0
50	5
100	7.5
200	15
400	30[a]

Control 1 (C1): filtered seawater. Control 2 (C2): 0.5% DMSO in seawater.

concentration of 400 µg/mL showed statistically significant results in relation to control 2 ($p < 0.001$).

4. Discussion

According to the World Health Organization (1983), molluscicidal activity of an isolated plant compound is recognized when it causes 90% mortality at concentrations equal to or below 20 µg/mL in 24 h of exposure. The results in Table 1 show that divaricatic acid falls within the parameters established by the WHO, showing embryotoxic activity above 90% against *B. glabrata* embryos from a concentration of 14.96 µg/mL at exposures of 24, 18 and 12 h and from 16.39 µg/mL at 6 h of exposure. Equivalent results were observed with lichen salt, potassium usnate, which presented embryotoxic effects, showing 100% lethality against *B. glabrata* embryos at a concentration of 10 µg/mL (Martins et al., 2014). Other natural compounds such as *Microgramma vacciniifolia* lectin and the CH_2Cl_2 fraction of *Liagora farinosa* extract had low activity compared to other compounds (Albuquerque et al., 2014; Miyasato et al., 2012). The malformations shown in Fig. 2 have also been observed in *B. glabrata* embryos in other, similar studies (Albuquerque et al., 2014; Tallarico et al., 2014; Oliveira-Filho et al., 2010).

The evaluation of molluscicidal activity of divaricatic acid on adult *B. glabrata* snails revealed significant results with 100% mortality of specimens at low concentration (5.5 µg/mL) when compared to other studies reported in the literature (Santos et al., 2010; Rocha-Filho et al., 2015). Mechanisms of action of various molluscicides are not well established, but some studies have reported on changes in the digestive system of snails, with possible changes in the dimensions of cells, apical membrane rupture, vacuolization, cytoplasmic fragmentation and degeneration of tissues (Rizk et al., 2012; Yousef and El-Kassas, 2013). Changes in the reproductive system (Rizk et al., 2012; Yousef and El-Kassas, 2013) and in the tegument (El-Beshbishi et al., 2015) have also been reported.

A single miracidium after penetrating a snail can lead through asexual reproduction to the formation of a many sporocysts, containing thousands of cercariae (Colley et al., 2014), which are released into the water under specific endogenous and environmental conditions (Coelho and Bezerra, 2006). Thus, it is interesting to note the relationship between the concentrations that presented molluscicidal activity against adult *B. glabrata* snails and cercaricide of *S. mansoni* in this work, emphasizing the importance of molluscicides that have biological activity concomitantly against these two species. Divaricatic acid, as shown in

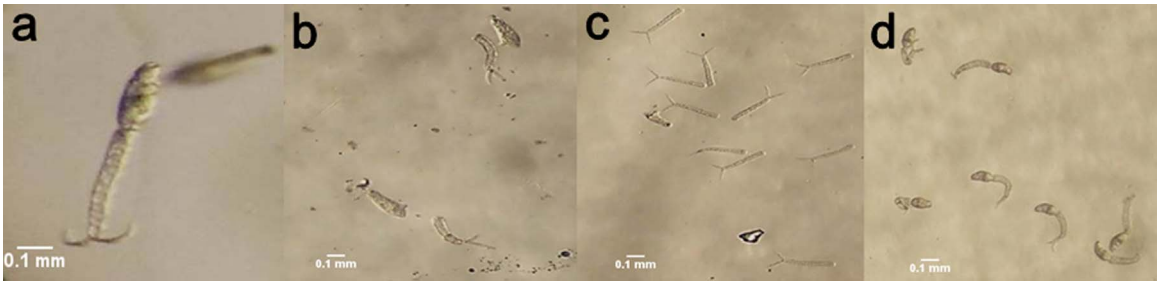

Fig. 3. *Schistosoma mansoni* cercariae exposed to divaricatic acid. In a, exposed to 0.5% DMSO in filtered and dechlorinated water showing the preservation of the body and tail. In b, exposed for 15 min at 100 µg/mL of divaricatic acid, showing the separation of body and tail. In c, exposed for 30 min at 10 µg/mL divaricatic acid. In d, dead cercariae exposed to 1 µg/mL of Niclosamide, presenting a contorted appearance.

Table 2, is lethal to 100% of cercariae at a concentration of 10 μg/mL, in 30 min, contributing to the elimination of the vector snail and parasite that causes schistosomiasis at the same concentration. Plant products have shown similar activity (Lima et al., 2002), where molluscicidal and cercaricidal activity of lapachol and isolapachol salt were effective at low concentrations. However, the *in vitro* efficacy of the essential oil extracted from *Piper cubeba* L. against *S. mansoni* cercariae showed activity at higher concentrations, reaching 200 μg/mL (Magalhães et al., 2012), showing that the data for divaricatic acid obtained in this work indicated more efficient cercaricide activity.

Environmental toxicity tests are commonly performed using a model organism such as fish and invertebrates. Among these organisms, *A. salina* stands out, with its use reported in several studies (Favilla et al., 2006; Pretti et al., 2014; Costa et al., 2015). The toxicity of extracts of *R. farinacea* on *A. salina* were evaluated, and low LC_{50} values were observed for ethanol, chloroform and *n*-hexane extracts, which ranged from 6.0 to 11.3 μg/mL. Only the aqueous extract of *R. farinacea* had a high LC_{50}, at 206.9 μg/mL (Esimone and Adikwu, 1999). Martins et al. (2014), when conducting a bioassay with *A. salina* to test the lichen salt, potassium usnate, obtained significant values of mortality at concentrations between 5 and 10 μg/mL, showing potassium usnate is safe to use at low concentrations. The data suggest that divaricatic acid had low toxicity on *A. salina* (Table 3), and can be considered a promising molecule in future environmental tests.

The results of this study are relevant for the control of schistosomiasis, as divaricatic acid has been shown to be a biological tool of high potential in combating the parasite vector in its embryonic and adult stages, as well as being effective against the cercarial form of the causative agent of the disease, while it did not demonstrate toxicity through the acute test using *Artemia salina*. In this sense, future studies should be conducted in order to evaluate the courses of action for this lichen substance and the use of testing in the field.

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