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RESEARCH ARTICLE

Larvicidal activity of the skin secretion of *Rhinella marina* and *Rhaebo guttatus* (Anura: Bufonidae) against the Brazilian malaria vector, *Anopheles darlingi* (Diptera: Culicidae)

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ABSTRACT

Malaria, a mosquito-borne disease, is caused by protozoa of the genus *Plasmodium* and constitutes a serious public health problem. Because current insecticides used to control malaria face resistance due to continuous use, new alternatives are prompted. Considering this context, and the insecticidal potential of vertebrate venoms/secretions, crude and methanolic extracts from two frog species were tested as larvicides against *Anopheles darlingi*. Skin secretions of *Rhinella marina* and *Rhaebo guttatus* were obtained by manual stimulation. Then, methanol was added to obtain steroidal fractions from both venoms. Mosquitos were captured in suburban areas of Porto Velho and *An. darlingi* females were later fed with blood and stimulated to oviposit. The larvae were fed with fish food until the 3rd and 4th instars. For the larvicidal assays, crude secretions and methanolic fractions of both frog species were evaluated, and larvae mortality was recorded after 48 hours. Crude extracts and steroidal fractions from both species had larvicidal effects, with an LC_{50} of 127.5 and 133 ppm for the crude extract and steroidal fraction of *R. marina*, and an LC_{50} of 37.5 and 35.8 ppm for the crude extract and steroidal secretion of *R. guttatus*, respectively. The present work reports for the first time the larvicidal effects of the skin secretions from bufonid species occurring in the western Amazon region. Further studies should be carried out to investigate the purified components responsible for the observed activity.

Keywords: Amazon biodiversity; Anura, *Rhinella*; skin secretion; bufodienolides.

INTRODUCTION

Mosquito-borne diseases, such as malaria, constitute major public health problems, particularly in tropical and sub-tropical regions, where they are transmitted to humans during the blood feeding of *Anopheles* mosquitoes.

Human malaria, an infectious disease caused by protozoans of the genus *Plasmodium* (i.e., *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plamodium ovale* and *Plasmodium knowlesi*), remains a major public health problem worldwide. In 2019, an estimated 229 million cases of malaria occurred worldwide (WHO, 2020), with more than 153,296 cases reported in Brazil and the Amazon region accounting for more than 80% of these cases (MS, 2020). The Brazilian anopheline fauna includes 54 species, but *Anopheles darlingi* is widely distributed in the northern region (Sinka *et al*., 2012) and is reported to be the major malaria vector (Tadei & Dutary-Thatcher, 2000).

Despite efficient treatment protocols for malaria-infected patients, in recent years, insecticides remain the most important tool to control *Anopheles.* Despite this, the resistance of *An. darlingi* to insecticides has been an obstacle for malaria prevention, given that the indiscriminate and inappropriate use of these chemicals has promoted the selection of multiresistant vectors (Hemingway *et al*., 2016). In this scenario, the importance of biodiversity as a source of new molecules for control and treatment of tropical diseases is constantly highlighted in the literature (Calderon *et al*., 2009; Da Silva *et al*., 2014; Trindade *et al*., 2014).

Bioinsecticides have been investigated as effective and safe alternatives to the chemical insecticides currently used, and various bioactive compounds from plants and animals have been listed as potential bioinsecticides (Carlini & Grosssi-de-Sá, 2002; Nicholson, 2007). Thus, the search for bioinsecticide molecules from synthetic analogs, or

alternative sources such as plants and animals, have been investigated by research groups around the world.

For a long time, plants have been the main source of bioactive compounds, but recently, studies based on natural products have intensified and their sources have expanded. Therefore, the investigation of bioactive compounds of animal origin has become increasingly common (Calderon *et al*., 2009), and insecticidal activity of animal venoms, such as those from spiders (Gimenez *et al*., 2014) and anurans (Trindade *et al*., 2014), against mosquitoes has already been shown.

Frogs' skin secretions are a promising source of biotechnological products based on a wide variety of toxic or noxious substances that repel or kill some predators and pathogens (Barra & Simmaco, 1995; Calderon *et al*., 2009; Calderon *et al*., 2011) and also display biological activity against bacteria, fungi, viruses and protozoa (Rinaldi, 2002; Calderon *et al*., 2011).

Rhinella marina is distributed across the eastern Andes of Peru, Brazil, Colombia and Venezuela, and is popularly known as the cane toad. Often, this species is involved in accidental poisoning of dogs when the toads are bitten, causing parotid secretion release (Stuart *et al*., 2008; Peterson & Roberts, 2013).

The *Rhaebo* genus contains nine different species, distributed in Central and South America. *Rhaebo guttatus,* popularly known as smooth-sided toad or spotted toad, is richly distributed in Brazil, especially in the Amazon, in the north of the country (Frost, 2021). This is the only species capable of voluntarily releasing jets of venom from its glands when threatened (Felipe Toledo *et al*., 2011). These toads produce a rich blend of bufodienolides, biogenic amines and proteins, which are very effective in protecting the animal against predators and microorganisms (Toledo & Jared, 1995; Cunha-Filho *et al*., 2010; Rash *et al*., 2011).

Most previous studies have focused on the antimicrobial, antiparasitic and anticancer properties of substances from frogs' skin secretions, but some investigations (Williams *et al*., 1998; Weldon *et al*., 2006; Williams *et al*., 2006) have also reported insecticidal and repellent effects of different species. Trindade *et al*. (2014) reported very low lethal concentrations of the skin secretions of *Leptodactylus knudseni* and *Phyllomedusa vaillantii* for adults and larvae of *An. darlingi,* suggesting that bioactive molecules from frog skin secretions of other anuran species might have similar activity. Therefore, this work evaluated the larvicidal activity of crude and steroidal fractions from the skin secretion of *R. marina* and *R. guttatus* against *An. darlingi,* an important vector of malaria in south America.

MATERIAL AND METHODS

Obtaining the crude venom and steroidal fraction

Rhinella marina and *R. guttatus* adults were collected in Porto Velho, Rondonia, Brazil. The skin secretion was obtained by manual stimulation of the parotoid glands, followed by washing of the glandular dorsal region of each individual with deionized water and collection in Becker. This solution was filtered through a "steriflip" filter, lyophilized and stored at -86°C.

For the fractionation, dried and lyophilized venom was placed in a 100 mL flask containing 50 mL of methanol and 10 glass pearls boiling and one magnetic bat. This solution was homogenized by stirring using a magnetic stirrer for 5 minutes. Afterwards, the flask was transferred to a heating mantle and mated to the flask to a ball type condenser (45 cm). The system was kept under reflux at 75°C for 1 hour and then placed on the magnetic stirrer for 5 minutes. The methanolic fraction was removed using a pipette and the remaining suspended material was refluxed with methanol again twice, before combining all methanolic fractions and placing them in a rotary evaporator at low pressure, at a temperature of about 70°C to obtain the dry methanol fraction. The Lieberman Burchard's reagent assay indicated the presence of steroidal compounds in methanolic fraction, named the steroidal fraction in this work.

Mosquito capture and breeding

Mosquito capture was performed at various sites, mostly periurban, in Porto Velho, Rondônia, using the modified BG-Sentinel® trap (Gama *et al*., 2013; Rodrigues *et al*., 2014). Then, adult females were allowed to feed on rabbit blood for 15 minutes (Siria *et al*., 2018) After 72 hours, oviposition was induced by wing removal. Larvae were kept under laboratory conditions (28ºC, 80% relative humidity and 12 h photoperiod) and fed with ground fish food (TetraMin Tropical Flakes) until the 3º and 4º instar.

Larvicidal bioassays

The crude secretion and methanol (MeOH) fractions of the venom were diluted in distillated water and evaluated as larvicides. A dose-response experiment was performed using five concentrations (10, 25, 50, 80, 100 mg/L), with four replicates and a control (distillated water), and 25 third-fourth instar larvae in each condition. The experiment was repeated three times. Larval mortality was recorded after 48 hours. Moribund larvae that were debilitated and unable to reach the water surface when disturbed were considered dead (WHO, 2005).

Statistical analysis

Lethal concentrations (LC_{50} and LC_{90}) were estimated using the logit model. In this case, the model could be expressed as:

$$y_i \sim bin(p_i, n_i)$$
$$logit(p_i) = \beta_0 + \beta_1 * concentration$$

So, a given LC could be defined as:

$$LC = (log(p/(1 - p)) - \beta_0)/ \beta_1$$

where p is the desired probability (in our case 50 and 90).

This model was fitted using a Bayesian approach. In this case, the values and their credible intervals were obtained from a posterior. To compute the posterior, 50000 samples were drawn using the NUTS algorithm and two chains. The first 5000 samples were used to tune the algorithm. Then, we discarded the first 1000 samples and collected samples from the chains at every five cycles. Chain convergence was checked by visual inspection and Rhat. The model was fitted using python 3.7, using pymc3 to fit the Bayesian model. Figures were constructed using matplotlib, and the libraries pandas and numpy were used to perform data manipulation.

RESULTS

During the experiments, a total of 4800 larvae were used. In general, mortality was lower when we used secretions originating from *R. marina*. In this case, the overall mortality for the crude secretion was about 23% (standard deviation =

11%) and 19% (sd = 3%) for the steroidal fraction (Figure 1). Secretions from *R. guttatus* led to a seven-fold higher overall mortality, irrespective of the fraction (crude secretion: 80%, sd = 2.5%; steroidal: 92.2%, sd = 2.8%) (Figure 2).

According to our model, an increase in the concentration of the secretions leads to an increase in the mortality. Thus, an increase of 1 mg/L of crude secretion from *R. guttatus* leads to a mean 1.04-fold increase in the odds of *An. darlingi* larvae mortality (Credible Interval: 1.03–1.04), whereas, in the steroidal fraction, this leads to a mean 1.06-fold increase in mortality (CrI: 1.05–1.07). A similar pattern was observed for *R. marina*; however, for fractions from this species, the mean increase in the odds was slightly lower, 1.02 (CrI: 1.02–1.03) for the crude fraction and 1.02 (CrI: 1.02–1.03) for the steroidal fraction.

Estimated lethal concentrations (LC) varied between the different anuran species and the fraction tested. Both the crude fraction and steroidal fraction LCs from secretions of *R. marina* were higher than those of the secretions of *R. guttatus* (LC_{50}: 70.6 and 80.6%; and LC_{90}: 59.4 and 73.2%, respectively). Moreover, the LC_{50} and LC_{90} of the steroidal fraction were lower for *R. guttatus*, but similar for *R. marina* (Table 1).

DISCUSSION

Animal venoms share common characteristics and are typically characterized by complex combinations of proteins and peptides with great structural diversity. Important biochemical, physiological and pathological tools for the development of new drugs have arisen due to research on animal venoms (Calderon *et al*., 2009; Calderon *et al*., 2014). In this context, the toxins purified from the animal venom or skin secretion have a high potential for pharmacological and biochemical study, and may have biomolecules with great therapeutic and biotechnological applicability (Beleboni *et al*., 2004; King & Hardy, 2012; Da Silva *et al*., 2014; Gimenez *et al*., 2014).

There is little data available regarding the toxic activity of anuran secretions on insects, and none are from true toads (Bufonidae). Williams *et al*. (1998) reported that a 1% skin secretion solution from *Litoria caerulea* (Anura: Hylidae) caused 57% mortality in larvae (topically applied) and 100% in adults (orally delivered) of flies (*Lucilia cuprina* and *Calliphora stygia*). Here, the tested concentration was 100 times higher than those used as larvicides in the present study (Figure 1 and 2). On the other hand, crude secretions from the skin of poison frogs (Anura: Dendrobatidae) kill larvae of *An. darlingi* and *Aedes aegypti* (Trindade *et al*., 2014) at much lower concentrations than those reported in the present work. Additionally, isolated compounds, such as pumiliotoxins seems to deter landing of *Ae. aegypti* on treated membranes (Weldon *et al*., 2006), supporting their role in the protection against predatory and ectoparasitic arthropods.

The Bufonidae family secrete compounds from four main classes: i) alkaloids; ii) biogenic amines; iii) steroids, such as bufodienolids or bufotoxins; and iv) proteins (Zelnik *et al*., 1964; Toledo & Jared, 1995; Perry, 2000). Unlike the peptide-rich skin secretions of Dendrobatidae frogs, Bufonidae frogs' skin secretions usually contain a high concentration of steroids such as bufodienolides (Chen & Kovarikova, 1967; Filho *et al*., 2005; Conlon *et al*., 2009) and a low concentration of peptides (Rash *et al*., 2011), or even none at all (Medeiros *et al*., 2019).

Among different compounds in the crude secretion, bufodienolides extracted from *R. marina* and *R. guttatus* present several biological activities (Krenn & Kropp, 1998; Cunha-Filho *et al*., 2010). Plant-derived bufodienolides, such as bryophyllin A and C, showed insecticidal properties when ingested by third instar larvae of *Bombyx mori* (Supratman *et al*., 2000), but no data for the insecticidal effect of skin secretions from bufonids was found.

Despite both anuran species studied being true toads (Bufonidae), the crude and steroidal fractions of the skin secretion of *R. guttatus* caused higher mortality and resulted in lower LC values for *An. darlingi* larvae compared to those obtained from *Rhinella marina* (Figures 1, 2 and Table 1). However, Ferreira *et al.* (2013) reported that all of the fractions obtained from *R. marina* presented much higher cytotoxicity in different evaluated tumor cells compared to *R. guttatus,* arguing that this might have resulted from synergistic effects due to the higher number of bufodienolids present in the former species. Conversely, Oliveira *et al*. (2019) argued that *R. guttatus* extract had a higher inhibitory effect on splenic cells compared to the effect of *R. marina* extract.

Interestingly, Ferreira *et al*. (2013) reported that the secretion of *R. marina* contained four bufadienolides (telocinobufagin, marinobufagin, bufalin and resibufogenin) and the *R. guttatus* secretion contained only one (marinobufagin), while Kerfhoff *et al*. (2016) reported three bufadienolides (telocinobufagin, marinobufagin and bufalin) and Medeiros *et al*. (2019) reported only two bufadienolides (marinobufagin and desacetylcinobufagin) in the secretions from *R. marina*. However, the authors used different solvents during fractionation of crude secretions, for example: $CHCl_3$ /MeOH (8:2) (Ferreira *et al*., 2013); ethyl acetate (100%), ethyl acetate/methanol (80:20 v/v), ethyl acetate/methanol (50:50 v/v), ethyl acetate/methanol (20:80 v/v) and methanol (100%) (Kerfhoff *et al.* 2016); and only methanol (100%) (Medeiros *et al*., 2019).

Interestingly, Kerfhoff *et al*. (2016) argued that ethyl acetate (100%) extraction resulted in greater amounts of bufadienolides. Therefore, the use of methanol, a more polar solvent, and a less diverse source of bufadienolides in the specimens used (Medeiros *et al*., 2019) in this work may have affected the synergistic effects of bufadienolides from *R. marina,* as argued by Ferreira *et al*. (2013). This is also

Table 1. Lethal concentrations (LC_{50} and LC_{90}) for the crude extract and methanolic fraction of *Rhinella marina* and *Rhaebo guttatus* secretions tested as larvicides on *Anopheles darlingi*

Anura Species	Substance	LC_{50} (mg/L) (CI 95%)	LC_{90} (mg/L) (CI 95%)
Rhinella marina	Crude extract	127.54 (113.20–143.5)	224.4 (191.5–260.4)
	Steroidal fraction	133 (117.7–149)	220.2 (188.2–254.8)
Rhaebo guttatus	Crude extract	37.5 (34.2–41)	91.2 (84.1–98.2)
	Steroidal fraction	25.8 (23.4–28.2)	59.1 (54.4–63.8)

LC_{50} and LC_{90} = concentrations necessary to kill 50% and 90%, respectively, of the larvae during assays; mg/L = milligram per liter; CI = upper and lower confidence intervals.

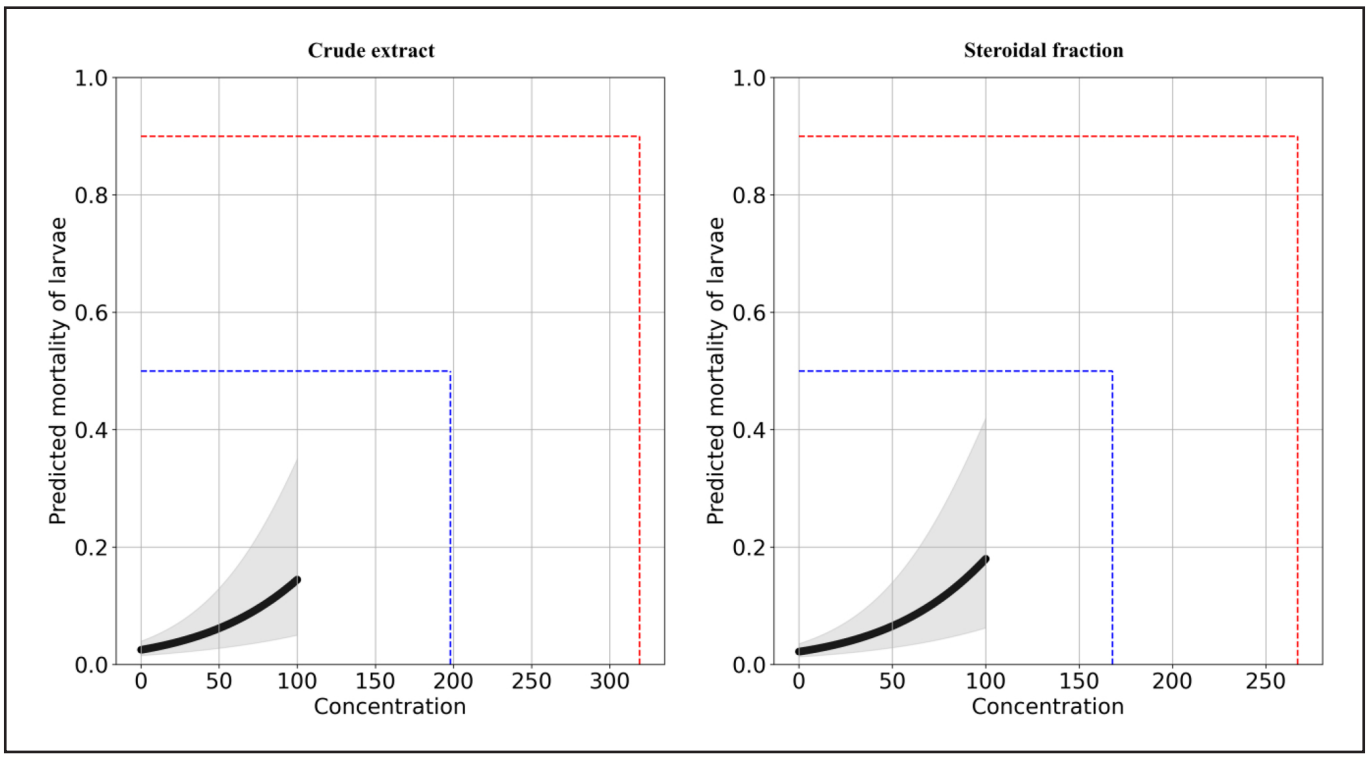

Figure 1. Larvicidal activity of *Rhinella marina* (Anura: Bufonidae) crude skin secretion and steroidal fraction against *Anopheles darlingi* at different concentrations and time points. Blue and red dotted lines indicate the estimated concentrations need to kill 50% and 90% of the larvae, respectively.

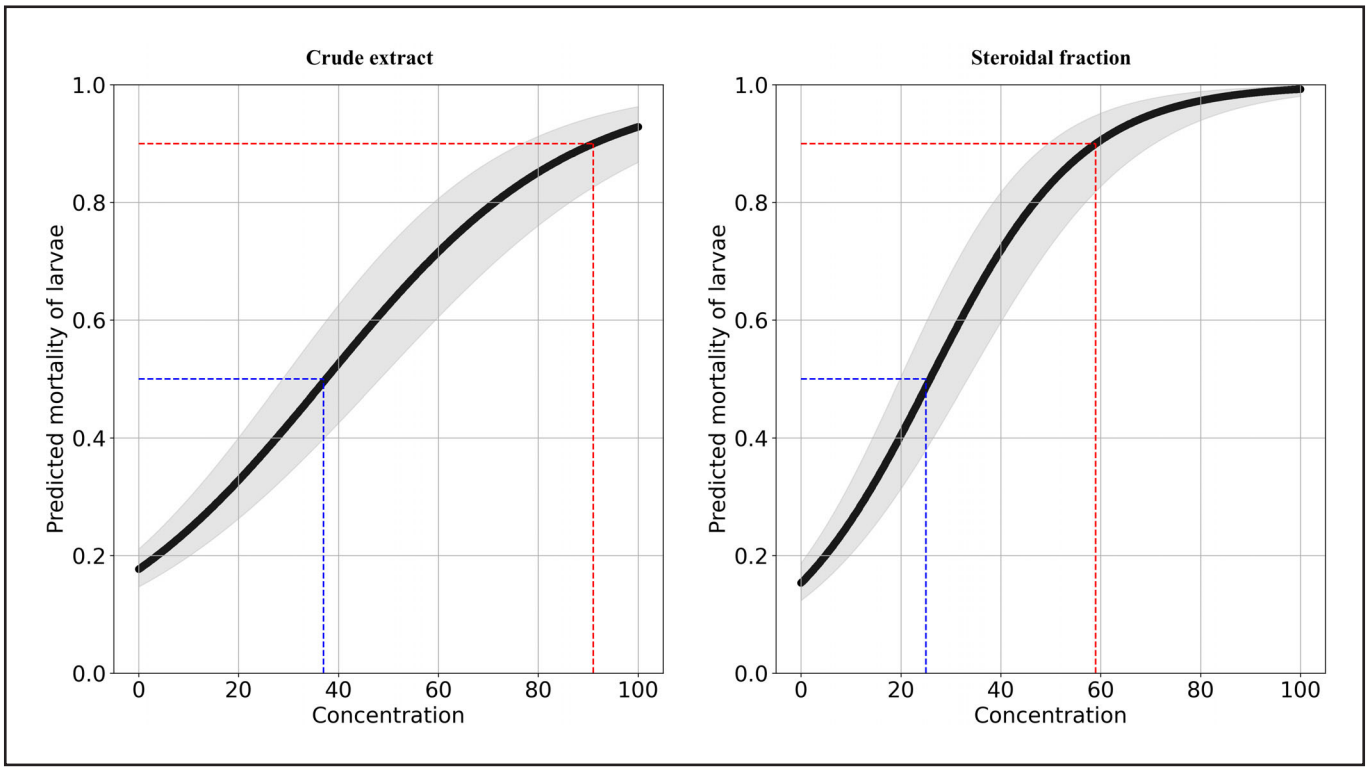

Figure 2. Larvicidal activity of *Rhaebo guttatus* (Anura: Bufonidae) crude skin secretion and steroidal fraction against *Anopheles darlingi* at different concentrations and time points. Blue and red dotted lines indicate the estimated concentrations need to kill 50% and 90% of the larvae, respectively.

supported by the lack of an important bufadienolide usually present in the secretion of *R. marina*, telocinobufagin, which may have been responsible for the large reduction in parasitemia of the *P. falciparum* W2 strain caused by the $CHCl_3$/MeOH extract produced by Banfi *et al*. (2016).

Moreover, the effect of different fractions of the secretion of *R. marina* seems also to depend on the organism tested. For example, the crude extract had the lowest IC_{50} against *P. falciparum,* while the methanolic fraction presented the lowest IC_{50} against *Leishmania guyanensis* (Medeiros *et al*., 2019). Additionally, an analysis of the skin secretion of *R. marina* showed that there was considerable variation (0.13 to 1.4 µmol/mg) in concentrations of venom components during the toad ontogeny (Hayes *et al*., 2009), and that other factors such as diet and environmental factors may affect the composition and concentration of components (Gao *et al*., 2010).

CONCLUSION

The present work reports for the first time the larvicidal effects of the skin secretions of bufonid species occurring in the western Amazon region. Future studies with fractions from crude skin using other solvents and isolated compounds will improve our understanding of the insecticidal effects of these anuran species against mosquitoes.

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Conflict of interest

The authors declare that they have no conflict of interests.

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