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ORIGINAL ARTICLE

In vitro antimicrobial activity of methanolic extracts from cutaneous secretions of Amazonian amphibians against phytopathogens of agricultural interest

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ABSTRACT

The biochemical defense mechanisms of amphibians involve cutaneous secretions of bioactive molecules with antimicrobial activity. This study evaluated the *in vitro* activity of methanolic extracts from cutaneous secretions of two amphibian species of the Bufonidae family, *Rhaebo guttatus* and *Rhinella marina*, in the control of the phytopathogens *Fusarium udum*, *Fusarium solani*, *Colletotrichum truncatum*, *Aspergillus flavus*, *Rhizoctonia solani*, *Macrophomina phaseolina*, and *Calonectria pseudometrosideri*. The *R. guttatus* extract decreased the mycelial growth of *F. udum*, *F. solani*, *A. flavus*, and *M. phaseolina* at some tested concentrations. The *R. marina* extract decreased the mycelial growth of *C. truncatum* at the concentration of 0.5 mg mL^{-1}, and inhibited the mycelial growth of *A. flavus* at concentrations of 0.1 and 0.5 mg mL^{-1}, which was similar to the inhibition by the positive control. The *R. marina* extract also decreased the microsclerotia production by *R. solani* at concentrations of 0.2 and 0.3 mg mL^{-1}. In addition, the extracts inhibited conidial sporulation and germination at varying degrees. The inhibition of appressoria formation in *C. truncatum* by the *R. guttatus* and *R. marina* extracts was 85–99% and 63–100%, respectively. Our results demonstrated that treatment with extracts from *R. guttatus* and *R. marina* cutaneous secretions showed antifungal activity against the studied phytopathogens.

KEYWORDS: inhibition; conidia; *Rhaebo guttatus*; *Rhinella marina*

Atividade antimicrobiana *in vitro* de extratos metanólicos de secreções cutâneas de anfíbios amazônicos sobre fitopatógenos de interesse agrícola

RESUMO

Os mecanismos de defesa bioquímica dos anfíbios envolvem secreções cutâneas de moléculas bioativas com atividade antimicrobiana. Este estudo avaliou a atividade *in vitro* de extratos metanólicos da secreção cutânea de duas espécies de anfíbios da família Bufonidae, *Rhaebo guttatus* e *Rhinella marina*, no controle dos patógenos *Fusarium udum*, *Fusarium solani*, *Colletotrichum truncatum*, *Aspergillus flavus*, *Rhizoctonia solani*, *Macrophomina phaseolina* e *Calonectria pseudometrosideri*. O extrato de *R. guttatus* inibiu o crescimento micelial de *F. udum*, *F. solani*, *A. flavus* e *M. phaseolina* em algumas concentrações testadas. O extrato de *R. marina* inibiu o crescimento micelial de *C. truncatum* na concentração de 0,5 mg mL^{-1}, e inibiu o crescimento micelial de *A. flavus* nas concentrações de 0,1 e 0,5 mg mL^{-1}, que foi semelhante à inibição pelo controle positivo. O extrato de *R. marina* também diminuiu a produção de microescleródios de *R. solani* nas concentrações de 0,2 e 0,3 mg mL^{-1}. Além disso, os extratos inibiram a esporulação e germinação de conídios em graus variados. A inibição da formação de apressórios em *C. truncatum* pelos extratos de *R. guttatus* e *R. marina* foi de 85%–99% e 63%–100%, respectivamente. Nossos resultados demonstraram que o tratamento com extratos da secreção cutânea de *R. guttatus* e *R. marina* apresentou atividade antifúngica sobre os fitopatógenos estudados.

PALAVRAS-CHAVE: inibição; conídios; *Rhaebo guttatus*; *Rhinella marina*

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INTRODUCTION

The biodiversity of Amazonia is a potential source of chemical compounds of biological and industrial interest (Skirycz *et al*. 2016). Natural chemotypes have biological activities which result in combinatorial chemistry which, in addition to generating stereo chemically complex structures with many functional groups, also produce compounds which specifically interact with biological targets (Cragg and Newman 2013; Loiseleur 2017).

Natural products have a long history as a source of and inspiration for novel agrochemicals (Sparks *et al*. 2017). For example, strobilurins are synthetic pyrethroids which are reconfigurations of the core structure isolated from the mycelium of *Strobilurus tenacellus* (Pers.) Singer (Loiseleur 2017; Sparks *et al*. 2017). Certain phytochemicals act on various types of diseases and can be applied in the food and agro-industry (Dileep *et al*. 2013). Compounds obtained from natural products have shown direct fungitoxic action by inhibiting spore germination and mycelial growth of phytopathogens (Stangarlin *et al*. 2011; Oliveira *et al*. 2014). In this context, the prospection for new active compounds is relevant to overcome pathogen resistance to existing fungicides, which results from the indiscriminate and excessive use of these substances, leading to a feedback cycle of ever higher concentrations of antifungals and a consequent increase in toxic residues in food products (Barros-Velazquez 2016).

Some animals produce structurally unique secondary metabolites which can be useful as new chemical templates for drug discovery (Cunha-Filho *et al*. 2010; Banfi *et al*. 2016). An example is the development of Captopril, a medication derived from small peptides isolated from the venom of a South American snake (*Bothrops jararaca* Wied-Neuwied), known to potentiate the action of bradykinin (Opie and Kowolik 1995; Harvey 2014).

Such secondary metabolites are also synthesized during metabolism in amphibians (Banfi *et al*. 2016) and involve defensive adaptations which help protect them against predators, parasites, and microorganisms (Ferreira *et al*. 2013; Huang *et al*. 2016). This chemical defense arsenal includes alkaloids and bufadienolides (Ferreira *et al*. 2013; Perera Córdova *et al*. 2016), which are secreted by cutaneous glands (Rodríguez *et al*. 2017), especially in frogs of the Bufonidae family (Schmeda-Hirschmann *et al*. 2016; Petroselli *et al*. 2018). Bufadienolides are an important group of steroid hormones with physiological and defense functions (Perera Córdova *et al*. 2016) that have vasoconstriction (Kamboj *et al*. 2013), antiviral (Sousa *et al*. 2017), antitumor (Yuan *et al*. 2016), cytotoxic (Ferreira *et al*. 2013; Li *et al*. 2015), antibacterial and antifungal properties (Rodríguez *et al*. 2017). Therefore, bufadienolides have potential for use to minimize damage and losses resulting from diseases caused by plant pathogenic fungi (Raasch-Fernandes *et al*. 2019).

In the present study, we evaluated the *in vitro* activity of the methanolic extract of the cutaneous secretion of two amphibian species of the Bufonidae family, *Rhaebo guttatus* (Schneider) and *Rhinella marina* (Linnaeus), in the control of seven phytopathogenic fungi.

MATERIAL AND METHODS

Cutaneous secretion extracts and phytopathogen isolates

The cutaneous secretion was obtained from six individuals of *R. guttatus* and nine of *R. marina* collected in 2015–2016 in Colniza (9°13'46.71"S, 60°17'41.75"W) in the state of Mato Grosso, Brazil (collection license no. 30034-1 issued to D.J. Rodrigues by Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais - IBAMA/SISBIO). The procedure for collecting the cutaneous secretion of the animals is non invasive and does not cause harm to ther animals. All sampled animals were returned to nature after the procedure. Voucher specimens were deposited in the Biologic Collection of Meridional Amazonia - ABAM/UFMT/Sinop (*R. guttatus* - ABAM-H 1538 and *R. marina* - ABAM-H 1262) in the context of another study (Oliveira *et al*. 2019). The secretion was extruded by manual compression of the parotoid macrogland. The collected material was dried in silica gel, extracted three times in methanol, and evaporated on a rotary evaporator. The methanolic extracts were weighed, diluted in sterile water, and filtered through a Millipore membrane (0.22 µm) (Ferreira *et al*. 2013). These procedures were carried out in the Laboratory of Chemical Research (LiPeQ) of UFMT/Sinop.

We obtained the isolates of seven phytopathogenic fungi from the mycoteque of UFMT: *Fusarium udum* E.J. Butler, Mem. and *Fusarium solani* (Mart.) Sacc. (causing agents of Fusarium wilts), *Colletotrichum truncatum* (Schwein.) Andrus and W.D. Moore (anthracnose), *Aspergillus flavus* Link (Aspergillus rot), *Rhizoctonia solani* J.G. Kühn (root rot), *Macrophomina phaseolina* (Tassi) Goid (gray root rot) and *Calonectria pseudometrosideri* R.F. Alfenas, L. Lombard, Crous and A.C. Alfenas (leaf spot). All these phytopathogens are found in agricultural crops in northern Mato Grosso state. The pathogens were grown in Petri dishes containing potato dextrose agar (PDA) culture medium, which was prepared using 39 g of the commercial product (Acumedia) and incubated in a BOD chamber at 25 °C for approximately 10 days. For *C. pseudometrosideri*, 20 g L^{-1} of agar-agar (Micromed) was added to the prepared PDA medium for culturing and testing.

Antimicrobial activity assays

All assays were conducted in the Laboratory of Phytopathology and Microbiology of UFMT/Sinop. To test the effect of the extracts on *F. udum*, *F. solani*, *C. truncatum*, *A. flavus*, *R. solani*, and *M. phaseolina*, we used concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mg mL^{-1} of the methanolic extracts of *R. guttatus*

and *R. marina* cutaneous secretions (Raasch-Fernandes *et al.* 2019), sterile water as negative control, and a fungicide mixture of metconazole (80 mg mL^{-1}) + pyraclostrobin (130 mg mL^{-1}) (Opera Ultra EC; BASF Corporation) as positive control. To test the effect on *C. pseudometrosideri*, we used the same extract concentrations and negative control, and azoxystrobin (200 mg mL^{-1}) + cyproconazole (80 mg mL^{-1}) (Priori Xtra; Syngenta Crop Protection AG) as positive control. The fungicides were applied at the recommended dosage rate: metconazole + pyraclostrobin (0.65 + 0.40 mg mL^{-1}) and azoxystrobin + cyproconazole (0.50 + 0.20 mg mL^{-1}).

The antifungal activity of the methanolic extracts was evaluated by determining the mycelial growth using the disc-diffusion test in PDA medium (Bauer *et al.* 1966). Four sterilized filter-paper discs with a 5-mm diameter were soaked in the treatment solution and arranged equidistant on a 9-cm petri dish, and a 5-mm mycelial disc was deposited on the center of each petri dish. Five replicates were used for each treatment and each control. Each petri dish constituted one replicate. The petri dishes were sealed and incubated in a BOD incubator at 25 °C in the dark. Evaluations were performed at the same time every day by measuring colony diameter along two orthogonal axes (average of two diametrically opposed measurements) starting 24 h after dish preparation.

The mycelial growth (MG) and the mycelial growth rate index (MGRI) were evaluated until one of the treatments reached two thirds of the surface of the petri dish in all replicates (Costa and Carvalho *et al.* 2013; Andrade *et al.* 2018). The mycelial growth rate index was subsequently calculated from the mycelial growth data:

$$\text{FMGRI} = \frac{\Sigma\,(D - Da)}{N}$$

where MGRI = mycelial growth rate index, d = current average diameter of the colony; da = average diameter of the colony from the previous day and, n = number of days after inoculation (Dias *et al.* 2005; Maia *et al.* 2011).

The mycelial growth inhibition percentage (MGI%) was determined according to the methodology proposed by Edgington *et al.* (1971).

The effects of the extracts on the sporulation of *F. udum*, *F. solani*, *C. truncatum*, *A. flavus*, and *C. pseudometrosideri* were analyzed after completing the mycelial growth assay (covering two thirds of the petri dish). After this period, 10 mL of sterile water were added to each petri dish, and then each fungal colony was scraped. The resulting suspension was filtered through a gauze and the number of conidia was counted in a Neubauer chamber under an optical microscope. The obtained data were converted into sporulation inhibition percentage (SI%) for each treatment relative to the negative control (Fernandes *et al.* 2015).

The effect of the extracts on the production of *R. solani* microsclerotia was determined after completing the mycelial growth assay (21 days of incubation of the mycelial disc). Each petri dish was divided into four equal quadrants starting at the central mycelial disc. A scale score was used for the visual quantification of the number of microsclerotia as follows: ++++ indicates a large number, +++ a moderate number, ++ a small number, + a very small number, and – the absence of microsclerotia. The sum of the scores was converted to a numerical scale from 0 to 4 for statistical analysis.

The effect of the extracts on the conidial germination of *F. udum*, *C. truncatum*, and *C. pseudometrosideri* was evaluated according to the methodology proposed by Stangarlin *et al.* (1999). Colonies of the fungal pathogens cultured in PDA medium for approximately 10 days were used to obtain the conidial suspension. Sterile water (10 mL) was added to the fungal cultures, and the cultures were scraped with a Drigalski loop and then filtered through a sterile gauze. After filtration, the suspension was adjusted to the following concentrations using a Neubauer chamber: 1×10^4, 1×10^5, and 5×10^4 conidia mL^{-1} for *C. pseudometrosideri*, *C. truncatum*, and *F. udum*, respectively. Conidial germination was evaluated under an optical microscope (400x magnification) using 100 conidia per repetition. Germination was deemed to have occurred when the germ tube emerged.

Sterile 96-well microplates were used to evaluate conidial germination (Almeida *et al.* 2009). In this study, an aliquot of 40 µL of the suspension of the evaluated phytopathogens was deposited in each well. A 40-µL aliquot of the treatments was subsequently added to each well. For the control, 40 µL sterile water were added instead of the treatments. The conidial germination assays were performed with eight replications for each treatment. The plates were incubated at 25 °C for 3, 6, and 24 h for *C. pseudometrosideri*, *C. truncatum*, and *F. udum*, respectively. After the incubation period, germination was interrupted with 20 µL of blue cotton and lactophenol. This period was determined with preliminary germination tests of the conidia of each phytopathogen in sterile water.

A Drigalski-loop scattering method was used to evaluate the conidial germination of *A. flavus*. This method was used because the conidia of *A. flavus* did not germinate when using microplates in preliminary tests. Petri dishes of 9-cm diameter were used, where each plate was considered one replicate, with eight replicates per treatment level (5) or control (2) and two treatments (*R. guttatus* and *R. marina* extracts). The plates were filled with agar–water medium and 100 µL of the test solution was distributed on the medium after solidification using a sterile Drigalski loop. After 2 h, 100 µL of conidial suspension (2.6×10^6 conidia mL^{-1}) of *A. flavus* were added and spread using a sterile Drigalski loop. Plates were incubated at 25 °C in the dark and evaluated after 48 h, when germination was interrupted with 20 µL of blue cotton and lactophenol. The

results were expressed as conidial germination inhibition percentage (CGI%) for each treatment level compared to the negative control (Fernandes *et al.* 2015). The germination of *F. solani* was not evaluated because there was no conidial production for this isolate.

For *C. truncatum*, we evaluated the emergence of the appressoria in conidial germination assays (Bonaldo *et al.* 2004) and expressed this variable as the appressorial formation inhibition percentage (AFI%) relative to the negative control. A total of 100 randomly selected germinated conidia per repetition were examined for number of appressoria, totaling 800 conidia per treatment. Post-germination appressoria development was averaged using an optical microscope.

Statistical analysis

The assays were conducted in a completely randomized design and the data were analyzed using the SISVAR version 4.3 program (Ferreira 2008). The results of each extract were subjected to analysis of variance (ANOVA) using the F-test, and the means were compared by the Scott–Knott test ($p < 0.05$). The original SI% data did not meet the requirements of normality and homogeneity and were therefore transformed to $(x + 1)^{0,5}$.

RESULTS

The *R. guttatus* extract significantly decreased MG and MGRI of *F. udum*, *F. solani*, *A. flavus* and *M. phaseolina* (Table 1; Figure 1). MG and MGRI of *F. udum* were significantly lower for the 0.5-mg mL^{-1} concentration relative to the negative control. MG was lower in the concentration of 0.5 mg mL^{-1} (5.21 cm) in relation to the negative control (6.60 cm). This concentration also showed reduced MGRI (0.74 cm day^{-1}) in relation to the negative control (0.94 cm day^{-1}). MG and MGRI of *F. solani* were significantly lower at all *R. guttatus*-extract concentrations compared with the negative control, with MG values of 5.71, 5.96, 5.48, 5.62, and 5.84 cm for 0.1, 0.2, 0.3, 0.4 and 0.5 mg mL^{-1}, respectively (negative control =7.18 cm), and corresponding MGRI values of 0.71, 0.75, 0.69, 0.70, and 0.73 cm day^{-1}, respectively (negative control average = 0.90 cm day^{-1}). This suggests that the *R. guttatus* extract has properties which reduce *in vitro* development of *F. solani* at the tested concentrations.

MG and MGRI of *A. flavus* were significantly lower than the negative control at 0.1 and 0.5 mg mL^{-1} (MG = 4.20 and 4.13 cm, respectively, and negative control =6.78 cm). The MGRI was lower at 0.1 mg mL^{-1} (0.47 cm) and 0.5 mg mL^{-1} (0.46 cm) relative to the negative control (0.75 cm). MG of *M. phaseolina* was significantly lower at 0.1, 0.2 and 0.3 mg mL^{-1}, with mean values of 6.88, 7.53, and 7.76 cm, respectively, relative to the negative control (7.94 cm). MG was lower at 0.1 mg mL^{-1}, differing from the other concentrations. The MGRI of *M. phaseolina* was significantly lower than the negative control (2.65 cm) at 0.1 mg mL^{-1} (2.29 cm).

MG and MGRI of *C. pseudometrosideri* indicated colony development promotion at 0.1 and 0.2 mg mL^{-1} compared

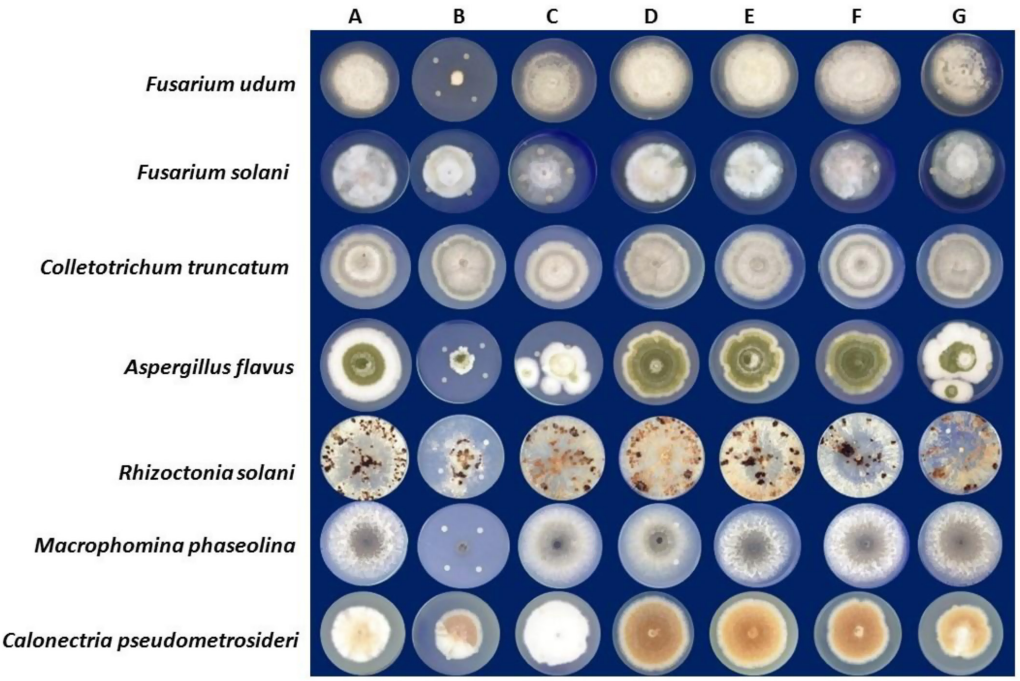

Figure 1. Growth of mycelial colonies of the pathogens *Fusarium udum, Fusarium solani, Colletotrichum truncatum, Aspergillus flavus, Rhizoctonia solani, Macrophomina phaseolina*, and *Calonectria pseudometrosideri* treated with the methanolic extract of *Rhaebo guttatus* cutaneous secretion. A – negative control (sterile water); B – positive control (fungicide); C – extract concentration 0.1 mg mL^{-1}; D – 0.2 mg mL^{-1}; E – 0.3 mg mL^{-1}; F – 0.4 mg mL^{-1}; G – 0.5 mg mL^{-1}. This figure is in color in the electronic version.

Table 1. Mycelial growth (MG) and mycelial growth rate index (MGRI) of fungal pathogens treated with different concentrations of the methanolic extract of *Rhaebo guttatus* (Rg) and *Rhinella marina* (Rm) cutaneous secretions. C- = negative control (sterile water); C+ = positive control [fungicides pyraclostrobin + metconazole (*F. udum*, *F. solani*, *C. truncatum*, *A. flavus*, *R. solani* and *M. phaseolina*) or azoxystrobin + ciproconazole (*C. pseudometrosideri*)].

Extract (mg mL^{-1})	*Fusarium udum*		*Fusarium solani*		*Colletotrichum truncatum*		*Aspergillus flavus*		*Rhizoctonia solani*		*Macrophomina phaseolina*		*Calonectria pseudometrosideri*	
	MG (cm)	MGRI (cm day^{-1})	MG (cm)	MGRI (cm day^{-1})	MG (cm)	MGRI (cm day^{-1})	MG (cm)	MGRI (cm day^{-1})	MG (cm)	MGRI (cm day^{-1})	MG (cm)	MGRI (cm day^{-1})	MG (cm)	MGRI (cm day^{-1})
Rhaebo guttatus														
C-	6.60 ± 0.22 c	0.94 ± 0.03 c	7.18 ± 0.52 c	0.90 ± 0.06 c	6.30 ± 0.77 a	0.53 ± 0.06 a	6.78 ± 0.49 c	0.75 ± 0.05 c	6.48 ± 2.83 b	2.16 ± 0.94 b	7.94 ± 0.22 d	2.65 ± 0.07 c	5.88 ± 0.98 a	0.42 ± 0.07 a
C+	1.37 ± 0.22 a	0.20 ± 0.03 a	3.56 ± 0.33 a	0.45 ± 0.04 a	6.52 ± 0.29 a	0.54 ± 0.02 a	2.53 ± 0.47 a	0.28 ± 0.05 a	2.48 ± 0.32 a	0.83 ± 0.11 a	1.07 ± 0.17 a	0.36 ± 0.06 a	5.00 ± 1.37 a	0.36 ± 0.10 a
0.1	6.62 ± 0.33c	0.95 ± 0.05 c	5.71 ± 0.62 b	0.71 ± 0.08 b	6.33 ± 0.40 a	0.53 ± 0.03 a	4.20 ± 0.70 b	0.47 ± 0.08 b	7.73 ± 1.08 b	2.58 ± 0.36 b	6.88 ± 0.39 b	2.29 ± 0.13 b	6.64 ± 1.39 b	0.47 ± 0.10 b
0.2	6.74 ± 0.42c	0.96 ± 0.06 c	5.96 ± 0.57 b	0.75 ± 0.07 b	6.60 ± 0.31 a	0.55 ± 0.03 a	5.99 ± 1.46 c	0.67 ± 0.16 c	8.06 ± 0.83 b	2.69 ± 0.28 b	7.53 ± 0.36 c	2.51 ± 0.12 c	7.36 ± 0.97 b	0.53 ± 0.07 b
0.3	6.82 ± 0.35 c	0.97 ± 0.05 c	5.48 ± 0.77 b	0.69 ± 0.10 b	6.37 ± 0.57 a	0.53 ± 0.05 a	6.02 ± 0.88 c	0.67 ± 0.10 c	7.64 ± 1.49 b	2.55 ± 0.50 b	7.76 ± 0.20 c	2.59 ± 0.07 c	5.04 ± 0.47 a	0.36 ± 0.03 a
0.4	6.84 ± 0.47 c	0.98 ± 0.07 c	5.62 ± 0.54 b	0.70 ± 0.07 b	6.74 ± 0.38 a	0.56 ± 0.03 a	5.67 ± 1.23 c	0.63 ± 0.14 c	8.03 ± 0.70 b	2.68 ± 0.23 b	7.92 ± 0.26 d	2.64 ± 0.09 c	5.23 ± 1.48 a	0.37 ± 0.11 a
0.5	5.21 ± 1.32 b	0.74 ± 0.19 b	5.84 ± 0.82 b	0.73 ± 0.10 b	6.41 ± 0.66 a	0.53 ± 0.05 a	4.13 ± 2.13 b	0.46 ± 0.24 b	8.19 ± 0.46 b	2.58 ± 0.31 b	8.10 ± 0.22 d	2.70 ± 0.07 c	4.92 ± 0.39 a	0.35 ± 0.03 a
Rhinella marina														
C-	4.90 ± 1.78 b	0.61 ± 0.22 b	6.44 ± 0.32 b	0.81 ± 0.04 b	6.48 ± 0.37 a	0.54 ± 0.03 b	6.93 ± 1.47 b	0.63 ± 0.13 b	6.68 ± 0.38 b	3.34 ± 0.19 b	8.32 ± 0.33 b	2.77 ± 0.11 b	5.87 ± 1.28 a	0.31 ± 0.07 a
C+	2.70 ± 0.31 a	0.34 ± 0.04 a	3.26 ± 0.26 a	0.41 ± 0.03 a	5.94 ± 0.60 a	0.50 ± 0.05 a	3.87 ± 0.43 a	0.35 ± 0.04 a	2.23 ± 0.50 a	1.12 ± 0.25 a	1.60 ± 0.09 a	0.53 ± 0.03 a	6.83 ± 1.86 a	0.36 ± 0.10 a
0.1	5.73 ± 1.91 b	0.72 ± 0.24 b	5.93 ± 1.09 b	0.74 ± 0.14 b	6.82 ± 0.38 a	0.57 ± 0.03 b	7.06 ± 1.00 b	0.64 ± 0.09 b	6.66 ± 0.43 b	3.33 ± 0.22 b	8.10 ± 0.35 b	2.70 ± 0.12 b	7.21 ± 1.24 a	0.38 ± 0.07 a
0.2	6.77 ± 0.18 b	0.85 ± 0.02 b	5.66 ± 1.18 b	0.71 ± 0.15 b	6.74 ± 0.37 a	0.56 ± 0.03 b	6.74 ± 0.34 b	0.61 ± 0.03 b	7.08 ± 0.22 b	3.54 ± 0.11 b	8.44 ± 0.08 b	2.81 ± 0.03 b	6.14 ± 0.48 a	0.32 ± 0.03 a
0.3	6.10 ± 0.43 b	0.76 ± 0.05 b	6.01 ± 1.35 b	0.75 ± 0.17 b	6.76 ± 0.41 a	0.56 ± 0.03 b	7.31 ± 0.77 b	0.66 ± 0.07 b	6.62 ± 0.56 b	3.31 ± 0.28 b	8.13 ± 0.54 b	2.71 ± 0.18 b	7.01 ± 1.47 a	0.37 ± 0.08 a
0.4	6.41 ± 0.77 b	0.80 ± 0.10 b	5.03 ± 0.26 b	0.63 ± 0.03 b	6.86 ± 0.46 a	0.57 ± 0.04b	6.76 ± 1.08 b	0.62 ± 0.07 b	6.87 ± 0.13 b	3.44 ± 0.06 b	8.37 ± 0.14 b	2.79 ± 0.05 b	6.86 ± 0.86 a	0.36 ± 0.05 a
0.5	5.58 ± 1.75 b	0.70 ± 0.22 b	5.68 ± 0.42 b	0.71 ± 0.05 b	5.63 ± 0.81 a	0.47 ± 0.07 a	6.80 ± 0.87 b	0.62 ± 0.10 b	7.03 ± 0.27 b	3.52 ± 0.14 b	8.33 ± 0.18 b	2.78 ± 0.06 b	7.28 ± 1.39 a	0.38 ± 0.07 a

Means followed by the same letter in each column did not differ significantly by the Scott–Knott test ($p < 0.05$). Values are expressed as the mean ± standard deviation.

with the negative control. MG mean values were 6.64 and 7.36 cm at 0.1 and 0.2 mg mL^{-1}, respectively, while the negative control showed 5.88 cm. MGRI was higher at 0.1 and 0.2 mg mL^{-1}, with mean values of 0.47 and 0.53 cm day^{-1}, respectively, relative to the negative control (0.4 cm day^{-1}). These data suggest that lower concentrations showed no beneficial effects in the mycelial growth reduction of *C. pseudometrosideri*.

The *R. marina* extract did not decrease the mycelial growth relative to the negative control (Table 1). The positive control significantly inhibited mycelial growth in *F. udum*, *F. solani*, *A. flavus*, *R. solani*, and *M. phaseolina* (Table 1; Figure 2). The MGRI of *C. truncatum* was reduced at 0.5 mg mL^{-1} (0.47 cm day^{-1}) when compared with the negative control (0.54 cm day^{-1}), and did not differ significantly from the positive control (0.50 cm day^{-1}).

The *R. guttatus* extract inhibited the MGI% of *F. udum*, *A. flavus* and *C. pseudometrosideri* (Table 2). MGI% of *F. udum* increased by 21.3% at 0.5 mg mL^{-1} and also increased significantly in *A. flavus* at 0.1 mg mL^{-1} (38.0%) and 0.5 mg mL^{-1} (40.6%), thus showing fungitoxic effect of the extract at these concentrations. The MGI% of *C. pseudometrosideri* at 0.3, 0.4 and 0.5 mg mL^{-1} did not differ significantly from the positive control. The *R. marina* extract increased the MGI% of *C. truncatum* at 0.5 mg mL^{-1} to a similar level to the positive control (Table 2).

The *R. guttatus* extract significantly affected the sporulation inhibition (SI%) in *A. flavus* at 0.1 and 0.5 mg mL^{-1} (93.1 and 83.6%, respectively), showing similar toxic effect to the positive control (95.9%) (Table 3). The SI% of *A. flavus* for the *R. marina* extract at 0.4 mg mL^{-1} (54.9%) showed similar results to the positive control (85.7%).

Microsclerotia production in *R. solani* was significantly reduced at 0.2 and 0.3 mg mL^{-1} of the *R. marina* extract when compared with the negative and positive control (Table 4).

The conidial germination of *F. udum* was significantly inhibited at 0.3 mg mL^{-1} (55.5%) of the *R. guttatus* extract, which did not differ significantly from the positive control (83.9%). The CGI% values for *C. pseudometrosideri* at 0.3, 0.4 and 0.5 mg mL^{-1} showed germination inhibition of 50.9, 55.4 and 50.9%, respectively (Table 4). Appressoria formation of *C. truncatum* was inhibited by both extracts. The *R. guttatus* extract inhibited AFI% at 0.2, 0.3 and 0.4 mg mL^{-1} (99.2, 95.6 and 98.3%, respectively), which did not differ significantly from the positive control (100%). The *R. marina* extract at 0.1, 0.2, 0.3 and 0.4 mg mL^{-1} did not differ significantly from the positive control (100%), with mean AFI% values of 100, 98.2, 95.1 and 98.2%, respectively. These results suggest that higher concentrations showed no beneficial effects in the appressoria formation of *C. truncatum*.

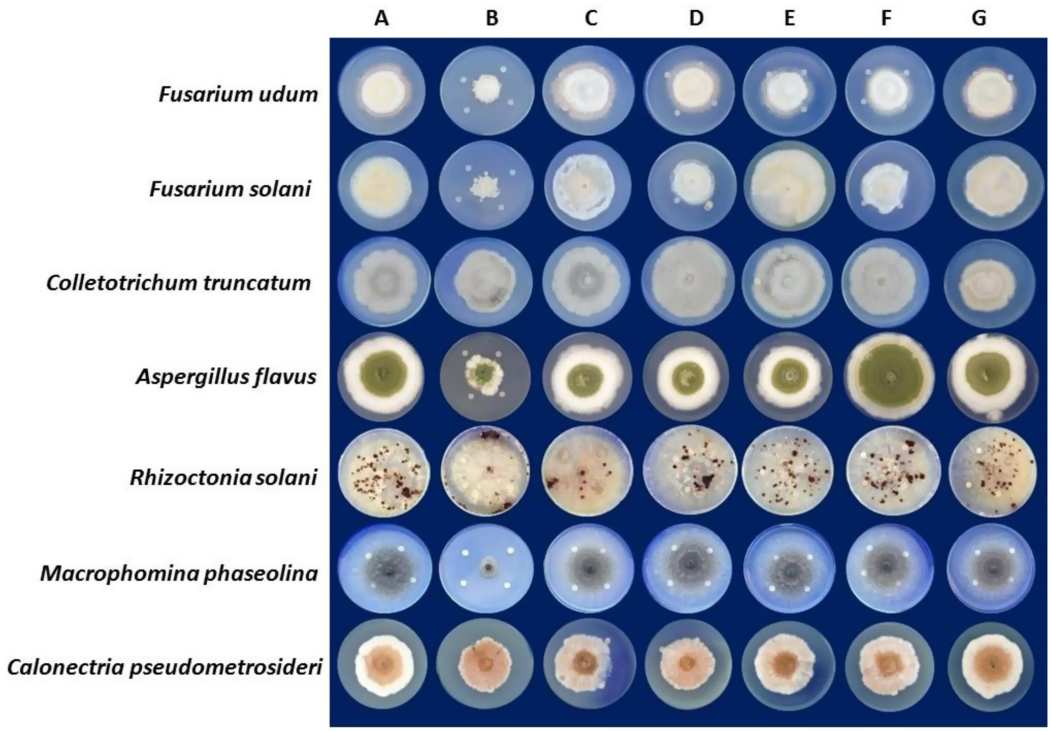

Figure 2. Growth of the mycelial colonies of the pathogens *Fusarium udum, Fusarium solani, Colletotrichum truncatum, Aspergillus flavus, Rhizoctonia solani, Macrophomina phaseolina*, and *Calonectria pseudometrosideri* treated with the methanolic extract of *Rhinella marina* cutaneous secretion. A – negative control (sterile water); B – positive control (fungicide); C – extract concentration 0.1 mg mL^{-1}; D – 0.2 mg mL^{-1}; E – 0.3 mg mL^{-1}; F – 0.4 mg mL^{-1}; G – 0.5 mg mL^{-1}. This figure is in color in the electronic version.

Table 2. Percentage of mycelial growth inhibition (%MGI) of fungal pathogens treated with methanolic extracts of *Rhaebo guttatus* (Rg) and *Rhinella marina* (Rm) cutaneous secretions. C+ = positive control [fungicides pyraclostrobin + metconazole (*F. udum, F. solani, C. truncatum, A. flavus, R. solani* and *M. phaseolina*) or azoxystrobin + ciproconazole (*C. pseudometrosideri*)].

Extract (mg mL^{-1})	*Fusarium udum*		*Fusarium solani*		*Colletotrichum truncatum*		*Aspergillus flavus*		*Rhizoctonia solani*		*Macrophomyia phaseolina*		*Calonectria pseudometrosideri*	
	Rg	Rm	Rg	Rm	Rg	Rm	Rg	Rm	Rg	Rm	Rg	Rm	Rg	Rm
C+	79.2 ± 3.1 a	38.2 ± 24.2 a	50.2 ± 5.9 a	49.2 ± 4.6 a	2.1 ± 3.1 a	9.4 ± 9.3 a	62.7 ± 6.1 a	41.1 ± 18.0 a	50.8 ± 33.3 a	66.5 ± 7.8 a	86.5 ± 2.1 a	80.7 ± 1.5 a	19.6 ± 19.7 a	4.2 ± 7.4 a
0.1	1.9 ± 3.0 c	12.1 ± 27.2 b	20.0 ± 11.2 b	10.9 ± 15.3 b	4.4 ± 5.9 a	0.6 ± 1.3 b	38.0 ± 9.7 a	10.6 ± 11.2 b	8.0 ± 13.3 b	1.7 ± 2.5 b	13.3 ± 4.8 b	2.6 ± 2.4 b	7.4 ± 16.6 b	0.5 ± 1.1 a
0.2	2.3 ± 5.1 c	1.4 ± 2.5 b	16.8 ± 12.4 b	13.8 ± 13.3 b	2.3 ± 5.2 a	0.0 ± 0.0 b	13.3 ± 18.6 b	8.1 ± 10.8 b	5.1 ± 9.7 b	0.0 ± 0.0 b	5.1 ± 5.0 c	0.4 ± 0.8 b	2.6 ± 5.9 b	4.6 ± 10.3 a
0.3	1.4 ± 2.8 c	6.5 ± 9.0 b	23.3 ± 12.9 b	11.9 ± 17.2 b	5.3 ± 7.7 a	2.2 ± 5.1 b	11.3 ± 12.4 b	5.6 ± 10.1 b	8.1 ± 18.2 b	4.9 ± 6.9 b	2.9 ± 2.7 c	2.3 ± 3.2 b	15.1 ± 9.5 a	0.8 ± 1.8 a
0.4	1.8 ± 3.7 c	5.6 ± 8.4 b	21.7 ± 3.4 b	21.8 ± 4.4 b	1.6 ± 3.3 a	0.3 ± 0.7 b	16.9 ± 13.8 b	11.7 ± 16.1 b	3.6 ± 8.2 b	1.1 ± 1.8 b	1.5 ± 3.5 d	1.1 ± 1.2 b	16.9 ± 18.6 a	4.7 ± 10.6 a
0.5	21.3 ± 18.2 b	3.6 ± 5.5 b	19.1 ± 13.3 b	11.6 ± 7.3 b	2.7 ± 4.9 a	13.1 ± 13.5 a	40.6 ± 27.9 a	7.2 ± 9.3 b	0.3 ± 0.8 b	0.0 ± 0.0 b	0.2 ± 0.5 d	0.8 ± 1.1 b	17.9 ± 13.7 a	1.5 ± 3.3 a

Means followed by the same letter in each column did not differ significantly by the Scott–Knott test ($p < 0.05$). Values are expressed as the mean ± standard deviation.

Table 3. Percentage of sporulation inhibition (%SI) of fungal pathogens treated with methanolic extracts of *Rhaebo guttatus* (Rg) and *Rhinella marina* (Rm) cutaneous secretions. C+ = positive control [fungicides pyraclostrobin + metconazole (*F. udum, F. solani, C. truncatum, A. flavus, R. solani* and *M. phaseolina*) or azoxystrobin + ciproconazole (*C. pseudometrosideri*)].

Extract (mg mL^{-1})	*Fusarium udum*		*Fusarium solani*		*Colletotrichum truncatum*		*Aspergillus flavus*		*Calonectria pseudometrosideri*	
	Rg	Rm	Rg	Rm	Rg	Rm	Rg	Rm	Rg	Rm
C+	6.6 ± 14.9 a	84.6 ± 29.9 a	18.5 ± 40.7 a	53.1 ± 49.2 a	20.0 ± 44.7 a	40.0 ± 54.8 a	95.9 ± 2.2 a	85.7 ± 9.7 a	60.0 ± 55.8 a	39.1 ± 44.4 a
0.1	0.0 ± 0.0 a	77.3 ± 43.4 a	58.2 ± 41.7 a	35.2 ± 49.0 a	0.0 ± 0.0 a	20.0 ± 44.7 a	93.1 ± 7.0 a	35.8 ± 28.4 b	12.5 ± 28.0 a	49.9 ± 46.1 a
0.2	20.0 ± 44.7 a	71.8 ± 41.5 a	55.5 ± 50.8 a	17.4 ± 38.9 a	40.0 ± 54.7 a	27.6 ± 43.7 a	18.3 ± 31.9 b	17.5 ± 25.1 b	18.7 ± 41.9 a	31.7 ± 45.9 a
0.3	20.0 ± 44.7 a	79.0 ± 44.2 a	27.8 ± 34.2 a	29.7 ± 29.0 a	40.0 ± 54.7 a	40.0 ± 54.8 a	29.1 ± 41.4 b	11.1 ± 11.9 b	52.5 ± 50.3 a	26.8 ± 41.0 a
0.4	20.0 ± 44.7 a	72.1 ± 40.9 a	64.6 ± 36.2 a	15.6 ± 34.9 a	20.0 ± 44.7 a	18.4 ± 41.3 a	45.4 ± 35.7 b	54.9 ± 35.3 a	38.7 ± 53.1 a	40.5 ± 46.9 a
0.5	6.6 ± 14.9 a	66.3 ± 30.9 a	25.2 ± 34.7 a	32.4 ± 33.2 a	20.0 ± 44.7 a	40.0 ± 54.8 a	83.6 ± 26.3 a	42.1 ± 41.4 b	58.7 ± 53.7 a	38.3 ± 52.6 a

Means followed by the same letter in each column did not differ significantly by the Scott–Knott test ($p < 0.05$). Values are expressed as the mean ± standard deviation. Data transformed into $(x + 1)^{0.5}$.

Table 4. Effect of different concentrations of the methanolic extracts of cutaneous secretions of *Rhaebo guttatus* (Rg) and *Rhinella marina* (Rm) on fungal pathogens measured by microsclerotia of *Rhizoctonia solani*, percentage of inhibition of appressorial formation (%AFI) in *Colletotrichum truncatum*, and percentage of inhibition of conidial germination (%CGI) of *Fusarium udum*, *C. truncatum*, *Aspergillus flavus* and *Calonectria pseudometrosideri*. C- = negative control (sterile water); C+ = positive control [fungicides pyraclostrobin + metconazole (*R. solani*, *F. udum*, *C. truncatum* and *A. flavus*) or azoxystrobin + ciproconazole (*C. pseudometrosideri*)].

Extract (mg mL^{-1})	*Rhizoctonia solani*		*Fusarium udum*		*Colletotrichum truncatum*				*Aspergillus flavus*		*Calonectria pseudometrosideri*	
	Microsclerotia		%CGI		%CGI		%AFI		%CGI		%CGI	
	Rg	Rm	Rg	Rm	Rg	Rm	Rg	Rm	Rg	Rm	Rg	Rm
C-	4.0 ± 0.0 a	3.8 ± 0.4 b	-	-	-	-	-	-	-	-	-	-
C+	4.0 ± 0.0 a	3.2 ± 1.7 b	83.9 ± 16.0 a	93.5 ± 8.2 a	95.6 ± 4.1 a	95.4 ± 4.7 a	100.0 ± 0.0 a	100.0 ± 0.0 a	66.6 ± 4.8 a	72.0 ± 9.4 a	95.7 ± 4.9 a	91.6 ± 6.1 a
0.1	4.0 ± 0.0 a	4.0 ± 0.0 b	0.0 ± 0.0 c	67.7 ± 10.6 b	41.4 ± 2.2 b	55.9 ± 8.2 b	85.3 ± 15.3 b	100.0 ± 0.0 a	22.9 ± 6.9 b	8.0 ± 5.4 b	38.3 ± 3.5 c	18.1 ± 10.5 b
0.2	4.0 ± 0.0 a	2.4 ± 2.1 a	31.6 ± 24.4 b	66.0 ± 11.4 b	52.8 ± 12.1 b	42.6 ± 10.9 b	99.2 ± 2.2 a	98.2 ± 5.0 a	24.2 ± 15.7 b	11.0 ± 9.3 b	37.6 ± 2.8 c	28.9 ± 13.8 b
0.3	4.0 ± 0.0 a	1.0 ± 1.7 a	55.5 ± 19.4 a	59.3 ± 11.6 b	43.5 ± 9.3 b	41.5 ± 14.7 b	95.6 ± 6.1 a	95.1 ± 11.6 a	20.3 ± 23.1 b	7.1 ± 7.0 b	50.9 ± 3.2 b	27.5 ± 9.0 b
0.4	3.2 ± 1.7 a	4.0 ± 0.0 b	33.9 ± 25.6 b	57.1 ± 12.9 b	39.3 ± 16.0 b	42.4 ± 18.1 b	98.3 ± 3.2 a	98.2 ± 3.3 a	12.6 ± 5.5 b	6.4 ± 5.44 b	55.4 ± 2.4 b	27.0 ± 8.4 b
0.5	3.8 ± 0.4 a	4.0 ± 0.0 b	33.0 ± 25.5 b	53.0 ± 14.1 b	26.9 ± 21.0 c	21.6 ± 19.9 c	85.9 ± 13.1 b	63.1 ± 36.1 b	18.5 ± 7.7 b	11.1 ± 11.2 b	50.9 ± 2.2 b	30.6 ± 12.7 b

Values are expressed as the mean ± standard deviation. Means followed by the same letter in each column did not differ significantly by the Scott–Knott test ($p < 0.05$).

DISCUSSION

The reduction in MG, MGRI, MGI% and CGI% by the *R. guttatus* extract at 0.5 mg mL^{-1} suggests antifungal activity against *F. udum*. The *R. guttatus* extract also had a fungitoxic effect on the development of *F. solani*, as all concentrations inhibited MG, even at the lowest concentration.

There is no information in the literature on the fungitoxic effect of secondary metabolites in extracts originating from animals against *Fusarium* Link ex Grey, 1821. Antifungal activity on MG of *F. solani* had been reported for the crude aqueous extracts of the floral buds of clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry) (Venturoso *et al*. 2011), and essential oil of thyme (*Thymus vulgaris* L.), which resulted in complete inhibition of mycelium development even at lower concentrations of the extracts (Krzyśko-Łupicka *et al*. 2016).

The *in vitro* response of the MG, MGRI and MGI% of *A. flavus* to the *R. guttatus* extract is of particular importance as this fungus is of major concern in agriculture. It produces highly carcinogenic toxins called aflatoxins in the seeds of a number of crops both before and after harvest which are a health hazard to animals (Adeyeye 2016), causing negative impact on the nutritional value of plants and considerable yield losses (Klich 2007; Htoon *et al*. 2019). The *R. guttatus* extract showed similar MG inhibition of *A. flavus* as the positive control agent in all tests, which makes it a candidate for future prospection against this phytopathogen.

The *R. guttatus* extract presented fungitoxic effect on *M. phaseolina* only in the lower concentrations. The action mechanisms involved in antifungal activities in phytopathogens by bioprospecting secondary metabolites of animals are still unknown. However, a higher inhibition rate at a lower concentration (50 µg mL^{-1}) was observed using crude aqueous extracts of ginger (*Zinziber officinalis* Roscoe) on banana fibers infected with *Helminthosporium* sp. Link as compared to higher concentrations (100, 200, and 400 µg mL^{-1}) (Rodrigues *et al*. 2006).

The antifungal effect of lower concentrations of *R. guttatus* extract on *M. phaseolina*, as well as its effect on the promotion of MG in *C. pseudometrosideri*, may be related to the interaction among chemical constituents of the extract. An extract can present compounds that stimulate pathogen growth (Venturoso *et al*. 2011). Thus, the relative amount of stimulating compounds and their half-lives, as well as the interaction between them, determines the action on the phytopathogen.

The *R. marina* extract reduced the MGRI of *C. truncatum.* This reduction is promising because the fungicide used as positive control was not very effective in controlling this pathogen. This response may be related to constant applications of the same fungicide over time. Constantly exposing the pathogen population to high rates of a fungicide may add undue selection pressure on the population, which in

turn may lead to resistance or tolerance (Deising *et al*. 2008). Thus, a change in the pathogen at this point can render the fungicide less effective or ineffective.

The sporulation inhibition of *A. flavus* by both extracts may be an important result, as this pathogen produces large quantities of asexual spores (conidia) which promote fungal dissemination (Mah and Yu 2006). Pimentel *et al*. (2010) also observed a significant reduction in sporulation of *A. flavus* with the essential oil from fresh leaves of *Tanaecium nocturnum* (Barb. Rodr.) Bureau & K.Schum using the contact and fumigation techniques. Likewise, the microsclerotia production inhibition observed in the treatment with the *R. marina* extract may be relevant, because these resistance structures ensure pathogen survival in adverse conditions, even in the absence of the host and in unfavorable climate conditions (Ritchie *et al*. 2013). Microsclerotia can remain in the soil and in organic matter after plant infection and serve as inocula for subsequent cultures (Chawla *et al*. 2012), with potential for more severe and frequent epidemics (Ma *et al*. 2015).

The inhibition of appressoria formation by both *R. guttatus* and *R. marina* extracts is also relevant, because pathogens with these structures can directly penetrate the intact surface of the host and remain at this point of contact, dissolve the tissue, and form a small hole in the tissue (Bergamin Filho *et al*. 1995). Consequently, inhibiting the emergence of this structure can contribute to controlling the disease caused by *C. truncatum* because its appressoria act as specialized infection structures formed from a hypha or germ tube and are important for fungal penetration through the leaf surface of the host (Khan and Hsiang 2003).

The difference in the antifungal activity of the *R. guttatus* and *R. marina* extracts observed in our study could be attributed to factors such as the existence of many receptors of the extract which select phytochemicals with different affinities in the receptors of the phytopathogens (Ahuja *et al*. 2012; Fesel and Zucca 2016), or the insufficient amount of active compound in the test concentrations (Oliveira *et al*. 2017). Also, the active principle may be present in the test concentrations, but other constituents are exerting antagonistic effects on the bioactive compounds (Caesar and Cech 2019). Although the bufadienolides are the main active components in the cutaneous secretion of *R. marina* and *R. guttatus*, there are interspecific differences in the number and type of constituents (Ferreira *et al*. 2013). Four bufadienolides are found in the *R. marina* cutaneous secretion (telocinobufagin, marinobufagin, bufalin and resibufogenin) and one in the secretion of *R. guttatus* (marinobufagin) (Banfi *et al*. 2016).

We used crude extracts containing several active compounds which have similar or synergistic activities that inhibit or promote pathogen growth. The advantage of using such extracts as antimicrobial agents is the lower risk of microbial resistance, because their high complexity limits microbial adaptability (Daferera *et al*. 2003). A crude (untreated) extract from any source of natural products typically contains novel, structurally diverse chemical compounds (Lahlou 2013), as well as active, partially active, or many inactive compounds (Cragg and Newman 2013). Therefore, it is necessary to study the application of these extracts through isolation processes, to evaluate more precisely the potential of the isolated compounds and understand their effective action in controlling the phytopathogens tested.

Our results present a potentially novel source of fungitoxic substances. The *R. guttatus* extract had a beneficial effect on reducing mycelial growth, and also lower concentrations of the extract showed antifungal activity. Our study provides a basis for further studies examining the antifungal activity of secondary metabolites produced by amphibians on phytopathogens of agricultural interest.

CONCLUSIONS

The methanolic extract of the cutaneous secretion of *Rhaebo guttatus* caused mycelial growth inhibition of *Fusarium udum*, *Fusarium solani*, *Aspergillus flavus* and *Macrophomina phaseolina* at a range of concentrations. Furthermore, all extract concentrations inhibited mycelial growth of *Fusarium solani*. The extract also inhibited *Aspergillus flavus* sporulation and conidia germination of *Fusarium udum* similarly to the positive control fungicide. The *Rhinella marina* extract inhibited the mycelial growth of *Colletotrichum truncatum* and microsclerotia production of *Rhizoctonia solani*. Both extracts decreased appressoria formation of *Colletotrichum truncatum* to the same level of the positive control. The results suggest that the *R. guttatus* extract has higher antifungal activity against the analyzed phytopathogens, but that both extracts represent a potential alternative for inhibiting appressoria formation and the consequent penetration through the leaf surface by phytopathogenic fungi. Further studies are needed to determine the bioactive compounds responsible for the antifungal activity of the extracts. The *Rhaebo guttatus* and *Rhinella marina* extracts should be tested in other phytopathogen fungi which affect the agricultural crops in the region. Our results provided relevant information for assays with components derived from secondary metabolites of amphibian species on the growth of phytopathogens.

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