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Short communication

19-Hydroxy-bufalin, a major bufadienolide isolated from the parotoid gland secretions of the Panamanian endemic toad *Rhinella centralis* (Bufonidae), inhibits the growth of *Trypanosoma cruzi*

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ABSTRACT

American trypanosomiasis is a parasitic neglected disease, responsible for the death of approximately 10,000 people every year. Amphibians are recognized for producing in their cutaneous glands substances with pharmacological potential against a variety of pathologies. Here we investigated the antiprotozoal activity against *Trypanosoma cruzi* of bufadienolides isolated from the parotoid glands secretions of the toad *Rhinella centralis* from Panama. NMR and mass spectrometry analysis led to the identification of the active compound 19-hydroxy-bufalin, for which its antitrypanosomal activity and occurrence in the genus *Rhinella* are reported for the first time. This compound showed low cytotoxicity and significant selectivity which confers to it a potential role for the treatment of Chagas disease.

American trypanosomiasis, also known as Chagas disease, is a zoonotic disease caused by the protozoan parasite *Trypanosoma cruzi*. Each year more than 10,000 people worldwide die of Chagas disease-related causes, and 8 million people are estimated to be infected by *T. cruzi* in Latin America (WHO, 2019). Currently, benznidazole and nifurtimox are the drugs available for the treatment of Chagas disease; however, these medications lead to the development of severe secondary effects and are effective only during the first weeks after infection (Castro et al., 2006).

Amphibians of the genus *Rhinella* contains in their skin secretions metabolites of the bufadienolides family, which are polyhydroxy steroids containing a 2-pyrone ring at position 17 of the steroidal core. These compounds are of interest due to their high chemical diversity and promising pharmacological activities against bacteria, fungi and trypanosomatid parasites (Barnhart et al., 2017; Cunha Filho et al., 2005; Tempone et al., 2008). The endemic Panamanian toad *Rhinella centralis* (Narvaes and Rodrigues, 2009), formerly known as *Bufo granulosus*, inhabits the lowlands of the Pacific slope of Panamá from Chiriquí to Darién provinces (Frost, 2019; IUCN, 2019). This relatively small species of toad is nocturnal and terrestrial, and breeds throughout the wet season particularly on rainy nights (Roberto Ibáñez et al., 1999). In this communication, we are reporting for the first time the occurrence in the genus *Rhinella* and the antitrypanosomal activity of 19-hydroxy-bufalin, a bufadienolide isolated from the parotoid gland secretions of the toad *R. centralis*.

Adult specimens of *R. centralis* were collected in Pacora, Panamá Province (Fig. 1-A) (collection permit SE/A-32-15). Parotoid glands secretion was obtained by electrical stimulation, after applying 5 V at 60 Hz for 30 s on the toads' dorsal region, using a homemade transcutaneous amphibian stimulator (Grant and Land, 2002). The secretions were placed in vials containing MeOH and stored at −20 °C until analysis. The methanolic soluble extract was loaded in Supelclean LC-18 solid phase extraction (SPE) cartridges (Supelco, Bellefonte, PA, USA), and fractionated using vacuum employing a three-step gradient

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composed of mixtures of 40, 80 and 100% of methanol in water at 10 mL min^{-1}. Methanol was removed by rotary evaporation under reduced pressure and the remaining water lyophilized obtaining three fractions F1–F3. The three fractions were evaluated against trypomastigotes of *T. cruzi*. Active fraction F2 was diluted in MeOH at 30 mg mL^{-1} and subjected to reversed-phase high performance liquid chromatography (RP-HPLC) employing a semi-preparative Polar C18 column (250 × 10 mm, 4 μm, 80 Å) (Phenomenex®, Torrance, CA, USA). Injections of 100 μL were carried out and elution was set at a flow rate of 3 mL min^{-1}. The solvent system consisted of water with 0.1% of trifluoroacetic acid (TFA) (Solvent A), and a 1/1 mixture of MeOH/CH_3CN acidified with TFA at 0.1% (Solvent B). Elution was carried out using a gradient from 20% to 100% of solvent B in solvent A in 60 min.

Structural elucidation was carried out by spectroscopic and spectrometric means. Nuclear magnetic resonance (NMR) experiments were carried out at 25 °C on an Eclipse+ 400 Fourier transform spectrometer (JEOL, Peabody, MA, USA). For NMR analysis, the samples were dissolved in deuterated methanol, and the spectra referenced based on the non-deuterated methanol residual signal (3.31 for δ_H and 49.0 for δ_C). High resolution mass spectrometry analysis was carried out on an UltrafleXtreme MALDI-TOF/TOF (Bruker Daltonics, Billerica, MA, USA). For MALDI-TOF-TOF analysis, 2 μL of the sample (19-hydroxy-bufalin) dissolved in methanol, were transferred to a MTP plate (Bruker Daltonics, Billerica, MA, USA), and mixed with 1 μL of the matrix (α-cyano-4-hydroxy-cinammic acid, Sigma Aldrich). All experiments were carried out in reflectron positive mode. Peptide calibration standard II (Bruker, Daltonics, MA) was used as external calibrant.

Antitrypanosomal bioassay was carried out on recombinant Tulahuen clone C4 of *T. cruzi* trypomastigotes (American Type Culture Collection, ATCC), Manassas, VA, USA), which expresses the β-galactosidase enzyme that serves as a reporter of viability (Buckner et al., 1996). Transfected parasites were maintained at 37 °C under a 5% CO_2 atmosphere in RPMI-1640 culture medium with L-glutamine, 4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid buffer, $NaHCO_3$, 10% FBS and 1% penicillin/streptomycin as supplements.

Vero cells (ATCC) were grown for 24 h, and one day prior to the experiment the cells were infected with trypomastigotes. Samples were dissolved in DMSO and added to the medium at 10.0, 2.0, 0.4 and 0.1 μg/mL and incubated for five days. To determine the antitrypanosomal activity, chlorophenol-red-β-D-galactopyranoside (Roche Applied Science) was added to each well and allowed to react with the β-galactosidase of the remnant living parasites for 4.5 h. Benznidazole was used as positive control (IC_{50} = 0.75 μg/mL). Absorbance was measured at 570 nm using a color plate reader (Sinergy HT, BioTek Instruments Inc, Winooski, VT, USA).

For cytotoxicity assays, Vero cells were cultivated in 96-well plates at 37 °C under a 5% CO_2 atmosphere, using RPMI-1640 medium (Sigma-Aldrich, USA) sterilized with 1% penicillin/streptomycin and supplemented with 10% FBS (fetal bovine serum; Gibco, Invitrogen, Carlsbad, CA, USA). The cells were allowed to adhere for 24 h prior to incubation for five days with the samples. During the treatment, DMSO was used as negative control, and samples were assayed at the same concentrations used in the anti-trypanosomal assay. After incubation, MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-di-phenyl-tetra-zolium bromide) was added to each well and absorbance was determined 4 h later at 570 nm, using a color plate reader. Cytotoxicity was evaluated colorimetrically by calculating the ability of the remnant living Vero cells to reduce the pale yellow MTT into the black purple formazan product, as described earlier (Mosmann, 1983). All bioassays were performed in duplicate and the IC_{50} values were determined employing the Data Analysis complement

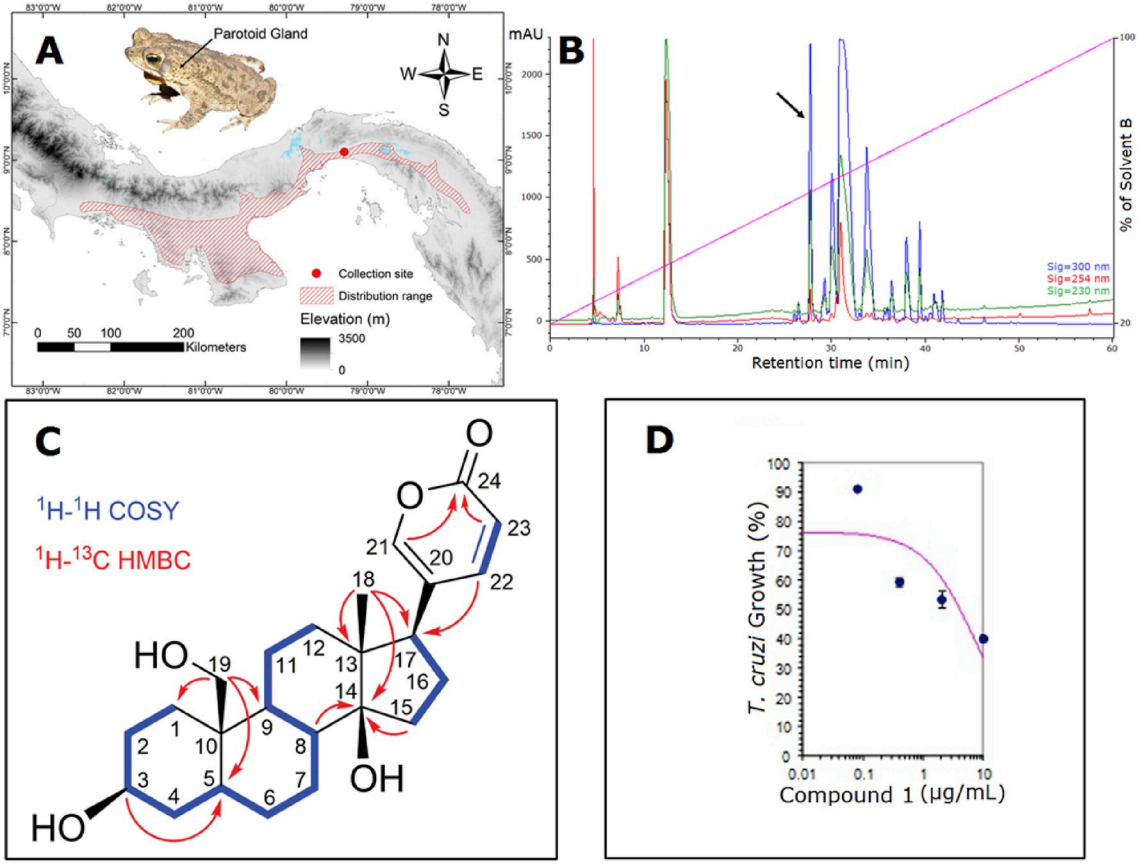

Fig. 1. (A) Distribution range, collection site and adult of *Rhinella centralis*. Its distribution was partly based on IUCN. (2019). (B) RP-HPLC Chromatogram profile of the fraction F2 obtained by VLC (80% MeOH in H_2O) from parotoid glands secretion of *R. centralis*. Black arrow indicates 19-hydroxy-bufalin (**1**). (C) Key COSY and HMBC correlations of 19-hydroxy-bufalin (**1**). (D) Effect of 19-hydroxy-bufalin (**1**) against trypomastigotes of *T. cruzi*.

Wizard of Excel (2000) (Microsoft, Seatle, WA, USA).

The skin secretion of *R. centralis* (1093 mg) subjected to SPE fractionation resulted in three fractions F1–F3. Antitrypanosomal activity was found in fraction F2 (320 mg) which was subjected to semi-preparative RP-HPLC purification leading to the isolation of compound **1** (17.6 mg), eluted at 27.7 min, active against *T. cruzi* trypomastigotes (Fig. 1-B). The structural elucidation of compound **1** was carried out by spectroscopic analyses and comparison with data from the literature (Verpoorte and Svendsen, 1979).

The molecular formula of compound **1** was determined as $C_{24}H_{34}O_5$ based on HR-MALDI-TOF-TOF MS data which showed a sodiated adduct of the molecular ion at *m/z* 425.2295 $[M + Na]^+$(calcd for $C_{24}H_{34}O_5Na$, 425.2298). Eight degrees of unsaturation were calculated from the molecular formula: three accounted for a carbonyl and two double bonds, hence compound **1** possess five rings.

In general, analysis of the ^1H and ^{13}C NMR data (Table 1) revealed the typical spectroscopic features of a bufadienolide including resonances for the 2-pyrone ring at δ_C 125.1 (C-20), 150.5 (C-21), 149.5 (C-22), 115.56 (C-23) and 164.9 (C-24), (Verpoorte et al., 1980). ^1H NMR showed signals for two diastereotopic protons mutually coupled, consistent with an oxomethylene at δ_H 3.83 (d, $J = 11.2$ Hz) and 3.41 (d, $J = 11.2$ Hz), attached to carbon C-19 (δ_C 66.17) using HSQC data. The steroidal nucleus was assembled using COSY and $J^{2,3}$ HMBC correlations (Fig. 1-C) being consistent with the structure of 19-hydroxy-bufalin (**1**).

19-hydroxy-bufalin (**1**) has been isolated from toads of the genera *Bufo*, *Bufotes* and *Duttaphrynus*, although not known in the genus *Rhinella* (Gao et al., 2010; Gella et al., 1995; Verpoorte et al., 1980). In our study, 19-hydroxy-bufalin (**1**) led to a growth inhibition in a dose-dependent manner with an IC_{50} of 7.81 μg/mL (Fig. 1-D) when assayed against *T. cruzi* trypomastigotes. Moreover, the cytotoxicity evaluation on epithelial kidney Vero cells exhibited a low inhibitory concentration with an IC_{50} of 71.58 μg/mL, resulting in a significant selectivity index (SI = IC_{50} Vero cells/IC_{50} *T. cruzi*) of 9.16. The selectivity index is a good reference of the pharmacological activity and safety of a drug. For instance, the higher the SI, the more effective and safe would be a compound during *in vivo* antiprotozoal treatment (Nogueira et al., 2009).

Only one bufadienolide, hellebrigenin, isolated from the parotoid macroglands of *Rhinella jimi*, has shown activity against *T. cruzi*, with a reported IC_{50} of 91.75 μg/mL (Tempone et al., 2008). To the best of our knowledge, in this study we report for the first time the antitrypanosomal activity of 19-hydroxy-bufalin and its occurrence in the genus *Rhinella*. The therapeutic window of 19-hydroxy-bufalin against American trypanosomiasis points out the need for research on the mechanism by which this natural product acts. This communication highlights the importance of amphibians as a source for the discovery of natural products with potential for the treatment of neglected human diseases.

Table 1
^1H and ^{13}C NMR data (400 MHz) of 19-hydroxy-bufalin (**1**) in CD_3OD, chemical shifts (δ) in ppm.

No.	δ_C, type[a]	δ_H, mult.[b,c,d] (*J* in Hz)
1	24.3, CH_2	1.88, m
		1.39, m
2	33.1, CH_2	2.13, m
		1.72, m
3	67.6, CH	4.04, m
4	28.4, CH_2	1.58, m
		n.d.
5	29.8, CH	2.21, m
6	27.6, CH_2	1.88, m
		1.22, m
7	22.8, CH_2	1.49, m
		1.22, m
8	43.0, CH	1.68, m
9	36.6, CH	1.80, m
10	40.6, C	
11	22.3, CH_2	1.80, m
		1.35, m
12	42.4, CH_2	1.49, m
		1.39, m
13	49.9, C	
14	86.4, C	
15	34.2, CH_2	1.85, m
		1.39, m
16	29.9, CH_2	2.13, m
		1.72, m
17	52.4, CH	2.54, m
18	17.4, CH_3	0.70, s
19	66.1, CH_2	3.83, d (11.24)
		3.41, d (11.24)
20	125.1, C	
21	150.5, CH	7.42, m
22	149.5, CH	7.98, dd (2.44, 9.28)
23	115.5, CH	6.27, d (9.76)
24	164.9, C	

[a] Type of carbon was determined by DEPT spectra (90° and 135°).
[b] Assignments were made by analysis of COSY, HSQC and HMBC spectra.
[c] Multiplets are identified as s (singlet), d (doublet), dd (doublet of doublets) or m (when multiplicity is not clear).
[d] Overlapped signals are reported without multiplicity. n.d. = not detected.

Ethical statement

Marcelino Gutiérrez and the coauthors state that this work submitted here has not been submitted for publication elsewhere. All work was conducted to the ethical standards outlined in the Publishing Ethics Resource Kit associated found on the journal webpage.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Candelario Rodriguez: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Roberto Ibáñez:** Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Writing - original draft, Writing - review & editing. **Michelle Ng:** Data curation, Formal analysis, Investigation, Methodology. **Carmenza Spadafora:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing - original draft, Writing - review & editing. **Armando A. Durant-Archibold:** Conceptualization, Data curation, Formal analysis, Investigation, Resources, Supervision, Methodology, Writing - original draft, Writing - review & editing. **Marcelino Gutiérrez:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Methodology, Data curation, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2020.02.009.

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