Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
22(1): 82-87, Jan./Feb. 2012

Phototoxic and modulatory effects of natural products from the skin of *Rhinella jimi* (Stevaux, 2002)

Samuel V. Brito,[1] Felipe S. Ferreira,[1] José P. Siqueira-Júnior,[1] José G. M. Costa,[2] Waltécio O. Almeida,[2] Henrique D. M. Coutinho*[,2]

[1]Centro de Ciências Exatas e da Natureza, Universidade Federal da Paraíba, Brazil,
[2]Departamento de Química Biológica, Universidade Regional do Cariri, Brazil.

Article

Received 26 Mar 2011
Accepted 1 Aug 2011
Available online 1 Nov 2011

Keywords:
biologically active compounds
modulatory activity
Phototoxic activity
Photoactivated antibacterial activity
Rhinella jimi
UV-A

ISSN 0102-695X
http://dx.doi.org/10.1590/S0102-695X2011005000198

Abstract: The skin of amphibians possesses a large diversity of biologically active compounds that are associated with the natural defenses of these animals against pathogens. Five different extracts and fractions were obtained from the skin of *Rhinella jimi*: methanol extract (ME), methanol fractions (MF), chloroform extract of methanol extract (CF), aqueous alkaloid fraction (AAF) and aqueous non-alkaloid fraction (ANAF). All fractions were evaluated with respect to their antibiotic modifying activity in standard bacterial strains and multiresistant clinical isolates. Antagonism was detected with kanamycin and gentamicin when combined with substances obtained from the skin of *R. jimi*. Phototoxic activity was observed in the methanol and chlorophorm fractions, as well as the aqueous non-alkaloid fraction. The antagonistic action was apparently associated with the protection afforded by the bacterial populations that inhabit the skin of this amphibian, preventing colonization by pathogenic fungi. The phototoxic activity demonstrated by natural products from the skin of *R. jimi* showed an interruption of the bacterial growth after UV exposure. This could indicate an antibacterial effect activated by the UV light, opening a path for carrying the attack by pathogenic fungi, causing the disease related with the amphibian decline.

Introduction

The skin of amphibians has multiple important functions associated with the survival of the animal (Mortari et al., 2004), and a number of biologically active substances with a great variety of biological and pharmacological properties have been demonstrated to be present on the skin (Daly et al., 2004). Despite the evidence of their skin possessing various protective compounds, amphibians are suffering from a marked decline in populations, where about a third of their populations are threaten by extinction (Mendelson III et al., 2006). Many investigators have searched for the causes of this decline in numbers, but these causes still cannot be defined with certainty. Meanwhile, various possibilities have been proposed, such as habitat loss, introduction of exotic species, indiscriminant use of agricultural pesticides, increased UV radiation and infectious diseases (Alford & Richards, 1999; Houlahan et al., 2002; Sparling et al., 2001). Chitridiomycosis is a disease caused by the fungus *Batrachochytrium dendrobatidis* and has been indicated by many investigators as the principal cause for the decline in amphibian populations. It is likely that anthropic factors and environmental alterations have caused the dispersion of this fungus (Picco & Collins, 2008). However, Harris et al. (2006) have demonstrated that some amphibians are not showing a decline in numbers due to the action of bacteria associated with their skin, which inhibit the growth of *B. dendrobatidis*.

Rhinella jimi belongs to the family Bufonidae which contains approximately 471 species and 33 other genera (Amphibiaweb, 2011). The genus *Rhinella* (previously classified as *Bufo*) contains more than 250 species present in the majority of the continents (Frost, 2004; Pramuk, 2006). In Brazil, *R. jimi* is widely distributed, where it is very common throughout the Northeast region (Stevaux, 2002). Despite their geographic distribution, this species has been studied little, where there are some preliminary data about endoparasitism (Anjos et al., 2009), about antileishmania and antitrypanosome activity of steroids extracted from their skin (Tempone et al., 2008) and antifungic activity (Santos et al., 2010). There are no

data in the scientific literature that indicate that this amphibian species is undergoing some type of decline in population.

The aim of this work was to determine the chemical character of the natural products in the skin of *R. jimi,* phototoxic activity and their effect on the growth of bacterial populations foreign to this species.

Material and Methods

Strains

The experiments were performed with clinical isolates of *Escherichia coli* (EC27), resistant to neomycin and gentamicin (low level) and to tobramycin, amikacin and kanamycin, and of *Staphylococcus aureus* 358 (SA358), resistant to several aminoglycosides. The EC-ATCC10536 strain of *Escherichia coli* and SA- 25923 strain of *Staphylococcus aureus* were used as positive controls. All strains were maintained in heart infusion agar slants (HIA, Difco), and prior to assay, the cells were grown overnight at 37 ºC in brain heart infusion broth (BHI, Difco).

Preparation of extracts and fractions of Rhinella jimi *and chemical prospection*

The skins of 37 specimens were removed and the specimens were deposited in the Zoological Collection of the Universidade Regional do Cariri with the following identification numbers: LZ-URCA 0469-500, 0503, 0504, 0506-0508. These specimens were collected in caatinga areas in the region of Cariri, Ceará (IPECE, 2005). A quantity of 295 g of skins was dried at room temperature and powdered. The powdered material was extracted by maceration using 1 L of methanol as solvent at room temperature, and the homogenate was allowed to stand for 72 h at room temperature (yield of 5.5%). The extracts were then filtered and concentrated under vacuum in a rotary evaporator (Brasileiro et al., 2006). Chemical prospecting of the methanol extract of the skin of *R. jimi* followed the method suggested by Matos (1998). The extract was submitted to Sephadex LH20 column chromatography, where the column was packed and equilibrated with methanol. After analysis of the different fractions by thin-layer chromatography, using Dragendorff's reagent, the fractions were separated based on their chromatographic profile and their respective retention factors (R_f). The following fractions were obtained: MF: methanol fraction; CF chloroform fraction; AAF: aqueous alkaloid fraction, and ANAF: aqueous non-alkaloid fraction. For the tests, the dry extract material was dissolved in DMSO. The crude methanol extract was analyzed by IR spectroscopy, utilizing a Perkin-Elmer model FT-IR spectrophotometer, spectrum 1000, with KBr.

Drugs

Gentamicin, kanamycin, amikacin and neomycin were obtained from Sigma Chemical Co. All drugs were dissolved in sterile water.

Antibacterial and modulatory activity

The minimum inhibitory concentration (MIC) of the extract and fractions of *R. jimi* skin and antibiotics were determined in BHI by the microdilution assay using suspensions of 10^5 CFU/mL and a concentration range of 1024 to 1 µg/mL (twofold serial dilutions) (Javadpour et al., 1996). MIC was defined as the lowest concentration at which no growth was observed. For the evaluation of extracts and fractions as modulators of antibiotic activity, MIC of the antibiotics were determined in the presence of each extract and fraction (64 µg/mL) at sub-inhibitory concentrations, and the plates were incubated for 24 h at 37 ºC.

Phototoxic activity

8-MOP was obtained from the Sigma Chemical Co., St. Louis, MO, USA, and norfloxacin disks were obtained from Laborclin, Brazil. Antibacterial assays were performed according to (Lopez et al., 2001). Norfloxacin disks were used as antibiotic standards (positive controls), while 8-methoxypsoralen (8-MOP - 10 mg/mL) in a sterile aqueous solution was used as a positive control of any light activation effects. Two replicate experiments were carried out to monitor light-activated antimicrobial activity: one replicate plate was exposed to ultraviolet (UV) light (5 Wy/m^2, 320-400 nm from four Sylvania F20T12-BLB lamps, maximum emission at 350 nm) for 2 h, while the other plate was kept in the dark. These plates were then incubated at 37 ºC overnight; the inhibition zones were determined.

Results

Bioprospecting of the methanol extract of *R. jimi* by IR spectroscopy revealed a predominance of alkaloids, as well as terpenes, steroids and saponins. Spectophotometric analyses of the extract demonstrated a strong absorption at 3434 cm-1, indicating the presence of NH and/or OH groups, characteristic of amines and alcohols. Based on earlier reports, we believe these compounds to be biogenic amines, since these are found to be very abundant in the skin of amphibians. Thin Layer Chromatography (TLC) analyses confirmed the presence of alkaloids in the fractions obtained.

When tested in strains of *Escherichia*

coli ATCC10536 and *S. aureus* ATCC25923, and multiresistant strains of *E. coli* (EC27) and S. aureus (SA358), the extracts did not show any appreciable antibacterial activity. The only extracts and fractions that showed antimicrobial activity were CF, ME and ANAF, which yielded MIC values of about 512 µg/mL when tested against EC27; this result was not considered significant from a technical point of view (Table 1).

In the strains *Staphylococcus aureus* ATCC25923 and SA358, the extracts and fractions did not show an inhibitory effect on bacterial growth, where all minimal inhibitory concentrations (MIC) were ≥1024 µg/mL, with exception of MF which inhibited bacterial growth at about 512 µg/mL, and this too did not represent technically a relevant antimicrobial effect (Table 1).

Table 1. MIC values of extract and fractions of Rhinella jimi skin (µg/mL).

	[f]EC10536	[f]EC 27	[g]SA25923	[g]SA358
ME[a]	≥1024	512	≥1024	≥1024
MF[b]	≥1024	≥1024	≥1024	512
CF[c]	≥1024	512	≥1024	≥1024
AAF[d]	≥1024	≥1024	≥1024	≥1024
ANAF[e]	≥1024	512	≥1024	≥1024

[a]ME: methanol extract; [b]MF: methanol fraction; [c]CF: chlorophorm fraction; [d]AAF: aquous alkaloid fraction; [e]ANAF: aquous non-alkaloid fraction; [f]EC: *Escherichia coli*; [g]SA: *Staphylococcus aureus*.

In relation to the modifying effect of the methanol extract and fractions on antibiotic activity, antagonism was observed with MF and kanamycin in strain EC27, while no effect of the natural products was

Table 2. MIC values of aminoglycosides in the absence and presence of 64 µg/mL of extract and fractions of the *Rhinella jimi* skin (values in µg/mL).

Ant[a]	[b]EC27				[c]SA358			
	MIC alone	+ MF[d]	+ CF[e]	+ ME[f]	MIC alone	+ MF	+ CF	+ ME
MF	512	-	-	-	≥1024	-	-	-
CF	512	-	-	-	≥1024	-	-	-
ME	≥1024	-	-	-	512	-	-	-
Amik[g]	32	32	32	32	64	64	64	64
Kana[h]	32	128	32	32	32	32	32	32
Neo[i]	256	256	256	256	64	64	64	64
Gent[j]	64	64	64	64	256	≥1024	≥1024	≥1024

[a]Ant: antibiotics; [b]EC: *Escherichia coli*; [c]SA *Staphylococcus aureus*; [d]MF: methanol fraction; [e]CF: chlorophorm fraction; [f]ME: methanol extract; [g]Amik: amikacin; [h]Kana: kanamycin; [i]Neo: neomycin; [j]Gent: gentamicin.

Table 3. MIC values of aminoglycosides in the absence and presence of 64 µg/mL of aquous fractions of the *Rhinella jimi* skin (values in µg/mL).

Ant[a]	[b]EC27			[c]SA358		
	MIC alone	+AAF[d]	+ANAF[e]	MIC alone	+AAF	+ANAF
AAF	≥ 1024	-	-	≥ 1024	-	-
ANAF	512	-	-	≥ 1024	-	-
Amik[f]	32	32	32	64	64	64
Kana[g]	32	32	32	32	32	32
Neo[h]	256	256	256	64	64	64
Gent[i]	64	64	64	256	256	256

[a]Ant: antibiotics; [b]EC: *Escherichia coli*; [c]SA: *Staphylococcus aureus*; [d]AAF: aquous alkaloid fraction; [e]NAF: aquous non-alkaloid fraction; [f]Amik: amikacin; [g]Kana: kanamycin; [h]Neo: neomycin; [i]Gent: gentamicin.

Table 4. Light-mediated antimicrobial activity of methanol extract and fractions from the skin of *Rhinella jimi* (mm).

	[a]SA-ATCC25923							[b]EC-ATCC10536						
	ME[c]	MF[d]	CF[e]	AAF[f]	ANAF[g]	NOR[h]	8MOP[i]	ME	MF	CF	AAF	ANAF	NOR	8MOP
[j]UV+	-	-	-	-	-	34	16	-	11	10	-	11	40	18
UV-	-	-	-	-	-	31	-	-	-	-	-	-	32	-

[a]SA: *Staphylococcus aureus*; [b]EC: *Escherichia coli*; [c]ME: methanol extract; [d]MF: methanol fraction; [e]CF: chlorophorm fraction; [f]AAF: aquous alkaloid fraction; [g]ANAF: aquous non-alkaloid fraction; [h]NOR: norfloxacin (10 µg/disk); [i]8MOP-8: methoxyl-psoralen (10 mg/mL); [j]UV: ultraviolet light.

seen with the other antibiotics (Table 2). With strain SA358, all natural products showed antagonism against gentamicin (Table 3). The aqueous alkaloid (AAF) and non alkaloid (ANAF) fractions did not show any type of antimicrobial activity (Table 3). Exposure to UV-A light elicited phototoxic activity against *E. coli* in three skin extracts (MF, CF and ANAF) (Table 4).

Discussion

Rhinella jimi does not produce compounds with antibacterial activity, perhaps as in other amphibians, allowing bacteria to play an essential role in their epidermis, constituting their associated microbial populations, which can be important in the protection of the amphibian against attack by pathogens (Brucker et al., 2008; Harris et al., 2006; 2009).

The antagonism observed between the natural products of *R. jimi* and the aminoglycosides was not expected, since compounds present in the skin of amphibians, an organ that appears to have as one of their principal functions protection against pathogens, impairs the action of antibiotics and protects bacterial populations. It is possible that this protection for bacteria against the action of antibiotics can represent a strategy of *R. jimi* against fungal pathogens, due to the fact that one of the main mechanisms utilized by fungi to colonize environments is antibiosis against pre-existing bacterial populations. As pointed out by Harris et al. (2006) mutualistic bacteria associated with the skin of two species of amphibians were capable of inhibiting the growth of various species of fungi including *B. dendrobatidis*. According to Harris et al. (2009), the current mortality of amphibians may be due principally to environmental factors that alter the microbiota profile of the skin of amphibians, favoring species that do not produce antifungal substances, thereby increasing the vulnerability of amphibians to chitridiomycosis. Brucker et al. (2008) affirms that besides the production of antifungal substances, the possibility of inhibition by competition cannot be discarded.

The antagonism observed between the antibiotic and the compounds of *R. jimi* can be associated with factors such as chelation of the antibiotic or binding of the compounds to specific sites of the antibiotics, thereby reducing their spectrum of activity. This antagonism is apparently not related to the action of any alkaloid, very common substances in the skin of amphibians (Table 3). This indicates that the antagonism is due to a "synergistic multi-target effect," indicating that constituents of an extract may affect the activity of these antibiotics interfering with several targets, cooperating in an antagonistic-synergistic way (Wagner & Ulrich-Merzenich, 2009).The antagonistic action observed is apparently associated with the protection afforded by the bacterial populations that inhabit the skin of this amphibian, preventing colonization by pathogenic fungi. Therefore, mutualistic bacteria could be a principal line of defense for *R. jimi* against *B. dendrobatidis.*

The compounds present in the skin of *R. jimi* showed phototoxic activity (Table 4), becoming toxic to the bacteria. Many substances become activated and exhibit phototoxicity when exposed to ultraviolet light. These substances are found distributed in many plant and fungal families and probably have defensive roles against herbivores (Berenbaum, 1995; Cheeptham & Towers, 2002; Coutinho et al., 2009, 2010; Kang et al., 2007; Matias et al., 2010; Taylor et al., 1995; Towers et al., 1997).

Several studies have demonstrated that UV radiation can cause serious physiological damage in amphibians, and this damage may vary from specie to specie as well as at different stages of life cycle in many amphibians (Bancroft et al., 2008; Blaustein et al., 1997; 2003; 2005). Faced with this data, we argue that increased UV radiation may be related with the change in microbial fauna of the skin of amphibians, since as phototoxic activity data demonstrated that the compounds from the skin of *R. jimi* showed no microbial activity, but when subjected to the presence of UV light were shown to be toxic to bacteria, from then on that bacteria may be associated with defense against other microorganisms. In the moment that these bacteria have their growth compromised, it could open a path for carrying the attack by pathogenic fungi, but further studies are needed to a more generalized evaluation in order to get data between the different groups of amphibians.

Acknowledgements

The authors would like to thanks to the brazilian research agencies CNPq and FUNCAP by the scholarships and grants of authors.

References

Alford RA, Richards SJ 1999. Global amphibian declines: A problem in applied ecology. *Ann Rev Ecol Syst 30*: 133-165.

Amphibiaweb 2011. *Information on amphibian biology and conservation.* http://amphibiaweb.org, accessed Apr 2011.

Anjos LA, Silva LEM, Almeida WO, Costa JGM, Vasconcellos A 2009. *Chaunus jimi* (NCN) endoparasites. *Herpetol Rev 39*: 337-338.

Bancroft BA, Baker NJ, Blaustein AJ 2008 A meta analysis of the effects of ultraviolet B radiation and its synergistic interactions with pH, contaminants, and disease on amphibian survival. *Conserv Biol 22*: 987-996.

Berenbaum M 1995. Phototoxicity of plant secondary metabolites: insect and mammalian perspectives. *Arch Insect Biochem Physiol 29*: 119-134.

Blaustein AR, Kiesecker JM, Chivers DP, Anthony RG 1997. Ambient UV-B radiation causes deformities in amphibian embryos. *Proc Natl Acad Sci USA 94*: 13735-13737.

Blaustein AR, Romansic JM, Kiesecker JM, Hatch AC 2003. Ultraviolet radiation, toxic chemicals and amphibian populations declines. *Divers Distrib 9*: 123-140.

Blaustein AR, Romansic JM, Scheessele EA, Han BA, Pessier AP, Longcore JE 2005. Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. *Conserv Biol 19*: 13735-13738.

Brasileiro BG, Pizziolo VR, Raslan DS, Jamal CM, Silveira D 2006. Antimicrobial and cytotoxic activities screening of some Brazilian medicinal plants used in Governador Valadares district. *Rev Bras Cienc Farm 42*: 195-202.

Brucker RM, Baylor CM, Walters RL, Lauer A, Harris RN, Minbiole KPC 2008. The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *J Chem Ecol 34*: 39-43.

Cheeptham N, Towers GHN 2002: Light - mediated activities of some Thai medicinal plant teas. *Fitoterapia 73*: 651-662.

Coutinho HDM, Costa JGM, Lima EO, Siqueira-Júnior JP 2009. *In Vitro* Phototoxic Activity of *Eugenia jambolana* L. and *Hyptis martiusii* Benth. *J Photochem Photobiol B 96*: 63-65.

Coutinho HD, Costa JGM, Siqueira-Jr JP, Lima EO 2010. In vitro screening by phototoxic properties of *Eugenia uniflora* L., *Momordica charantia* L., *Mentha arvensis* L. and *Turnera ulmifolia* L. *Braz J Biosci 8*: 299-301.

Daly JW, Noimai N, Kongkathip B, Kongkathip N, Wilham JM, Garrafo HM, Kaneko T, Spande TF, Nimit Y, Nabhitabhata J, Chan-Ard T 2004. Biologically active substances from amphibians: preliminary studies on anurans from twenty-one genera of Thailand. *Toxicon 44*: 805-815.

Frost DL 2004. *Amphibian Species of the World: an online reference*. version 3.0. http://research.amnh.org/herpetology/amphibian/index.html, accesses in Aug 2010.

Harris RN, James TY, Lauer A, Simon MA, Patel A 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth 3*: 53-56.

Harris RN, Lauer A, Simon MA, Banning JL, Alford RA 2009. Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis Aquat Organ 83*: 11-16.

Houhalan JE, Findlay CS, Schmidt AHM, Kuzmin SL 2002. Quantitative evidence for global amphibian population decline. *Nature 404*: 752-755.

Instituto de Pesquisa Estratégica Econômica do Ceará-IPECE 2005. *Perfil Básico Municipal*. Fortaleza: Governo do Estado do Ceará, Secretária do Planejamento e Coordenação.

Javadpour MM, Juban MM, Lo WC, Bishop SM, Alberty JB, Cowell SM, Becker CL, Mclaughlin ML 1996. De novo antimicrobial peptides with low mammalian cell toxicity. *J Med Chem 39*: 3107-3113.

Kang SJ, Kim SH, Liu P, Jovel E, Towers GHN 2007. Antibacterial activities of some mosses including *Hylocomium splendens* from South Western British Columbia, *Fitoterapia 78*: 373-376.

Lopez A, Hudson B, Towers GHN 2001. Antiviral and antimicrobial activities of Colombian medicinal plants. *J Ethnopharmacol 77*: 189-196.

Matias EFF, Santos KKA, Costa JGM, Coutinho HDM 2010: Light-enhanced antibiotic activity of Brazilian medical plants (*Croton campestris* A, *Ocimum gratissimum* L and *Cordia verbenaceae* DC). *Asian Biomed 4*: 183-186.

Matos FJA 1998. *Introdução à fitoquímica experimental*. Fortaleza: ED-UFC.

Mendelson III JR, Lips KR, Gagliardo RW, Rabb GB, Collins JP, Diffendorfer JE 2006. Confronting amphibian declines and extinctions. *Science 313*: 48.

Mortari MR, Schwartz ENF, Schwartz CA, Pires Jr OR, Santos MM, Bloch Jr C, Sebbena A 2004. Main alkaloids from the Brazilian dendrobatidae frog *Epipedobates flavopictus*: pumiliotoxin 251D, histrionicotoxin and decahydroquinolines. *Toxicon 43*: 303-310.

Picco AM, Collins JP 2008. Amphibian commerce as a likely source of pathogen pollution. *Conserv Biol 22*: 1582-1589.

Pramuk JB 2006. Phylogeny of South American Bufo (Anura:Bufonidae) inferred from combined evidence. *Zool J Linn Soc 146*: 407-452.

Santos KKA, Matias EFF, Almeida TS, Costa JGM, Coutinho HDM 2010. Antifungal activity of animal and plant extracts from the region of Cariri. *Cad Cult Ci URCA 1*: 53-65.

Sparling DW, Fellers GM, McConnel LL 2001. Pesticides and amphibian population declines in California, USA. *Environ Toxicol Chem 20*: 1591-1595.

Stevaux MN 2002. The new species of Bufo (Anura, Bufonidae) in Northeastern Brazil. *Rev Bras Zool 19*: 235-242.

Taylor RS, Manadhar NP, Towers GHN 1995. Screening of selected medicinal plants of Nepal for antimicrobial activities. *J Ethnopharmacol 46*: 153-159.

Tempone AG, Pimenta DC, Lebrun I, Sartorelli P, Taniwaki NN, Andrade-Júnior HF, Antiniazzi MM, Jared C 2008. Antileishmanial and antitrypanosomal activity of bufadienolides isolated from the toad *Rhinella jimi*

parotid macrogland secretion. *Toxicon 52*: 13-21.

Towers GHN, Page J, Hudson JB 1997. Light-mediated biological activities of natural products from plants and fungi. *Curr Org Chem 1*: 395-414.

Wagner H, Ulrich-Merzenich G 2009. Synergy research: approaching a new generation of phytopharmaceuticals. *Phytomedicine 16*: 97-110.

***Correspondence**

H. D. M. Coutinho
Laboratório de Microbiologia e Biologia Molecular, Departamento de Ciências Biológicas, Universidade Regional do Cariri
Rua Cel. Antonio Luis 1161, Pimenta, 63105-000, Crato-CE, Brazil
hdmcoutinho@urca.br
Tel.: +55 88 3102 1212
Fax: +55 88 3102 1291