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

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**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 jimī*: 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. jimī*. 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. jimī* 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.

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

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**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 threatened 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, indiscriminate 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 jimī* 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. jimī* 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 $R. \textit{jimi}$, 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 \textit{Escherichia coli} (EC27), resistant to neomycin and gentamicin (low level) and to tobramycin, amikacin and kanamycin, and of \textit{Staphylococcus aureus} 358 (SA358), resistant to several aminoglycosides. The EC-ATCC10536 strain of \textit{Escherichia coli} and SA-25923 strain of \textit{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 \textit{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 \textit{R. jimi} followed the method suggested by Matos (1998). The methanol extract of \textit{R. jimi} 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 \textit{R. jimi} skin and antibiotics were determined in BHI by the microdilution assay using suspensions of $10^9$ 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 W/m², 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 \textit{R. jimi} by IR spectroscopy revealed a predominance of alkaloids, as well as terpenes, steroids and saponins. Spectrophotometric 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 \textit{Escherichia}}
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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 jimī* skin (μg/mL). |
|---------------------------------|---------------------|-----------------|-----------------|-----------------|
|                                  | **EC10536** | **EC27** | **SA25923** | **SA358** |
| MEa                             | ≥1024       | 512      | ≥1024         | ≥1024         |
| MFb                             | ≥1024       | ≥1024    | ≥1024         | 512           |
| CFc                             | ≥1024       | 512      | ≥1024         | ≥1024         |
| AAFd                            | ≥1024       | ≥1024    | ≥1024         | ≥1024         |
| ANAFe                           | ≥1024       | ≥1024    | ≥1024         | ≥1024         |

*ME*: methanol extract; *MF*: methanol fraction; *CF*: chloroform fraction; *AAF*: aqueous alkaloid fraction; *ANAF*: aqueous non-alkaloid fraction; *EC*: *Escherichia coli*; *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 observed with amikacin in strain SA358.

| Table 2. MIC values of aminoglycosides in the absence and presence of 64 μg/mL of extract and fractions of the *Rhinella jimī* skin (values in μg/mL). |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | **EC27** | **SA358** |
|                                  | MIC alone | + MF | + CF | + ME | MIC alone | + MF | + CF | + ME |
| MF                              | 512      | -    | -    | -    | ≥1024    | -    | -    | -    |
| CF                              | 512      | -    | -    | -    | ≥1024    | -    | -    | -    |
| ME                              | ≥1024    | -    | -    | -    | 512      | -    | -    | -    |
| Amika                           | 32       | 32    | 32    | 32    | 64       | 64    | 64    | 64    |
| Kanab                           | 32       | 128   | 32    | 32    | 32       | 32    | 32    | 32    |
| Neoa                            | 256      | 256   | 256   | 256   | 64       | 64    | 64    | 64    |
| Gentb                           | 64       | 64    | 64    | 64    | 64       | 64    | 64    | 64    |

*Ant*: antibiotics; *EC*: *Escherichia coli*; *SA*: *Staphylococcus aureus*; *MF*: methanol fraction; *CF*: chloroform fraction; *ME*: methanol extract; *Amik*: amikacin; *Kana*: kanamycin; *Neo*: neomycin; *Gent*: gentamicin.

| Table 3. MIC values of aminoglycosides in the absence and presence of 64 μg/mL of aqueous fractions of the *Rhinella jimī* skin (values in μg/mL). |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | **EC27** | **SA358** |
|                                  | MIC alone | + AAF | + ANAF | MIC alone | + AAF | + ANAF |
| AAF                             | ≥1024    | -    | -    | ≥1024    | -    | -    |
| ANAF                            | 512      | -    | -    | ≥1024    | -    | -    |
| Amikc                           | 32       | 32    | 32    | 64       | 64    | 64    |
| Kanaa                           | 32       | 32    | 32    | 32       | 32    | 32    |
| Neoab                           | 256      | 256   | 256   | 64       | 64    | 64    |
| Gentf                           | 64       | 64    | 64    | 64       | 64    | 64    |

*Ant*: antibiotics; *EC*: *Escherichia coli*; *SA*: *Staphylococcus aureus*; *AAF*: aqueous alkaloid fraction; *ANAF*: aqueous non-alkaloid fraction; *Amik*: amikacin; *Kana*: kanamycin; *Neo*: neomycin; *Gent*: gentamicin.

| Table 4. Light-mediated antimicrobial activity of methanol extract and fractions from the skin of *Rhinella jimī* (mm). |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                  | **EC-ATCC25923** | **EC-ATCC10536** |
|                                  | MEa | MFb | CFc | AAFd | ANAFe | NORg | 8MOPh | ME | MF | CF | AAF | ANAF | NOR | 8MOP |
| UV+                             | -   | -   | -   | -    | -    | -    | -    | 11 | 10 | -  | 11  | 40   | 18  |
| UV-                             | -   | -   | -   | -    | -    | -    | -    | -  | -  | -  | -   | -    | -   |

*SA*: *Staphylococcus aureus*; *EC*: *Escherichia coli*; *ME*: methanol extract; *MF*: methanol fraction; *CF*: chloroform fraction; *AAF*: aqueous alkaloid fraction; *ANAF*: aqueous non-alkaloid fraction; *NOR*: norfloxacin (10 μg/disk); *8MOP*: methoxyl-psoralen (10 mg/mL); 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 antibiotic resistance 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 chytridiomycosis. 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.

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References


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


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