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IDENTIFICATION OF COMPOUNDS FROM WHITE FROG (*ANURA: HYLIDAE*) CUTANEOUS SECRETIONS WITH POTENTIAL TO BE USED IN BIOTECHNOLOGICAL PROCESSES

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Abstract

Amphibians are an interesting natural source of different metabolites and peptides with potential biological activity against insect pests as well human and phyto-pathogens. Recently, diverse molecules exhibiting this kind of activity have been described from anuran families. Among these, the Hylidae family is considered a good source of these valuable compounds. In this work we have collected skin secretions from white frog specie *Hypsiboas crepitans* in order to evaluate the antimicrobial and cytotoxic activities against human pathogens as well on the insect cell line *Spodoptera frugiperda*: IPLB-SF-21, respectively. The cell viability of SF21 insect line ranged from 14% to 45% and the lethal concentration 50 (LC_{50}) ranging from 4525 to 5768 ppm, showing a positive exposure-response relationship between concentration and time. Moreover all bacteria shows low inhibition when were tested with Kirby Bauer method; but it was possible to determine a more accurate inhibition from microdilution assays, these ranging from 11.06 to 100% inhibition at the first hour of bacteria cell culturing, and also showing at the exponential phase 19.08% inhibition to *Escherichia coli*, 35.11% to *Klebsiella pneumoniae*, 20.42% to *Staphylococcus aureus* wild type and 38.72% to *Staphylococcus aureus* methicillin resistant strain. Additionally, by using Liquid chromatography–mass spectrometry (LC-MS), Epibatidine, Eburnamonine and Makaluvamine P were found to be the main compounds from cutaneous secretions.

Keywords: Bioprospecting; Antimicrobial; Cytotoxic; *Hypsiboas crepitans;* White frog

Introduction

The nature exhibits great number of organisms that secrete substances through their skin as the first defense mechanism against pathogens, predators or competitors. The amphibians are known to possess many glands that produce compounds which are involved in those mechanisms [1, 2]. Although the cutaneous secretions of amphibians contain a wide number of biologically active substances, those with antimicrobial activity have particular interest because they could represent novel and useful molecules with potential to be used in several areas of the human development such as: industry, crop protection and medicine among others. Some Anuran families and genera from which dermal antimicrobial peptides have been isolated and structurally characterized include Hylidae family, although in this family it was not possible to identify cytolytic peptides [3]. However, all species of anurans coming from low-lands usually faces extreme climatic conditions due to water stress and high temperatures that are characteristic of this ecosystem; which could affect the secretion of substances from physiologic repertoire not only to protect them from drying, but also against typical pathogens in places like the ponds used in the reproduction [4, 5]. It is well known that *Hypsiboas crepitans* is an arboreal nocturnal hylid frog distributed from Panama to Northern Brazil between sea level and 2300 m altitude. This anphibian lives in a wide variety of natural habitats including humid tropical forests, semiarid environments, grasslands, plains and lower mountain forests. The main goal of the present study was to evaluate the biological activity of skin secretions from white frog (*Hypsiboas crepitans*) as a potential source of useful compounds to be used either in medicine or in agriculture.

Material and Methods

Biological Material

Samples were taken from four adults of the white frog (*Hypsiboas crepitans*) from a seasonal pond in Repelón (Atlantic's Department, Colombia); located to 10°34'59.7"N 75°07'35.8"W. secretions from these specimens were obtained by rubbing them the dorsal part of their bodies and also their heads with cotton swabs.

Then, the final extraction was prepared dissolving them with double distilled water (DDW) or Tris-HCl 50 mM buffer solution pH 7.0, followed by centrifugation at 4.000 g for 15 min and preserving them at – 80°C until be used for the antimicrobial and cytotoxicity bioassays. In the compounds identification using Liquid Chromatography Mass Spectrometry (LC-MS) two replicas (HB1 – HB2) were used; thus, the individuals were washed twice with DDW for 5 minutes, followed of submersion in Milli-Q water and mechanic stimulation during 10 min.

Antimicrobial bioassays

Antibacterial activity bioassay were carried out by using Kirby–Bauer testing and microdilution protocol in broth, as described in the M7-A6 of the National Committee for Clinical Laboratory Standards (2012), against the bacteria *Staphylococcus aureus* methicillin resistant and *Klebsiella pneumoniae (donated by Instituto Nacional de Salud – Colombia), Escherichia coli* ATCC 25922 and *Staphylococcus aureus* wild type ATCC 25923.

All of them are considered the main cause of infections at hospital level. To estimate the percentage of inhibition of bacterial growth on nutrient agar plates, the area without bacteria around the Sensi-Disc (Halo) containing 5 µl of the extracts was measured by using ImageJ v1.48 software and compared with the halos of Gentamicin (20 $\mu g.ml^{-1}$) and Milli-Q Water as controls. Additionally, the inhibition tested following the microdilution method in liquid media (50% Bactotriptone + 25% yeast extract + 35% NaCl) was determined monitoring bacterial growth at the exponential phase, measuring the absorbance at 600 OD during 6 hours by spectrophotometry.

Cytotoxic assays: Cell viability

The tests of cytotoxic activity were developed on insect cell line *Spodoptera frugiperda*: IPLB-SF-21 maintained at 27˚C in TNM-FH medium, following the methodology described by Arboleda *et al*. (2011) [6]. Extracts from *H. crepitans* were tested at concentrations ranging 100 -10000 ppm on cultures at the early stationary phase with a density of 1×10^6 cells/ml. All experiments were evaluated with 3 repetitions including appropriate controls: DDW and Tris-HCl 50 mM buffer solution pH 7.0. Insect cells were seeded in 24-well microtitration plates and incubated for 24, 48 and 72 h at 25˚C ± 2˚C. Afterwards, the ratio of live to dead cells (dyed) (cell viability) was resolved to each treatment by using trypan blue protocol, mixing 100 µl of cell solution with dye (solution 0.4%) in a ratio of 1:1 and observed by standard light microscopy (40X).

Finally, the determination of lethal concentration that caused 50% mortality values (LC_{50}) for the extracts was carried out in triplicate.

Analysis of the extracts by LC-ESI-HRMS

Extracts were analyzed using a HPLC system (Shimadzu Corp, Japan) equipped with a LC20AD pump and coupled to a high resolution mass spectrometer (Bruker Corp., USA) with a micro ToF-QII analyzer and an electrospray ion source (ESI). A Synergi RP C18 (Phenomenex) (150 x 4.6 mm; 4 µm) was employed (1.0 mL/min flow rate; 30°C column oven temperature). 0.005% trifluoroacetic acid (TFA) in water and acetonitrile (ACN) were used as mobile phases A and B, respectively, in gradient elution as follows: 0 min, 15% B, 4 min, 40% B; 8 min, 70% B; 10 min, 80% B; 12 min, 100% B; 14 min, 15% B; 15 min, 15% B. Extracts were diluted in 1:20 ratio and then 2 µL were injected. ESI was operated in positive ion mode (scan 100 – 1200 m/z; 250 °C CDL temperature; 1.2 kV detector voltage; 1.5 $L.min^{-1}$ nebulizing gas flow rate; 9.0 $L.min^{-1}$ drying gas flow rate). Ten main compounds were tentatively identified according to the mass spectra, exact mass measurement (used for calculating the molecular formulae) and by comparison with literature data.

Statistical

For statistical analysis, one way variance analysis (ANOVA), Tukey's mean comparison test and Insect cell viability statistical analyses were performed with SPSS software (SPPS Inc., Chicago, Illinois). Differences were considered significant at $p \leq 0.05$. LC_{50} values and their lower and upper confidence limits (confidence 95%) were calculated with the Probit statistical program.

Results and Discussion

The *In vitro* test performed with extracts of cutaneous secretions from different parts of the frog *H. crepitans,* have showed antimicrobial activity and significant statistical differences in all assessments when compared with the respective controls using Gentamicin throughout Kirby–Bauer testing. The Antimicrobial activity ranged from 11.06 to 100% inhibition at the first hour of bacterial growth by using the broth micro-dilution method described previously. In the exponential phase the percentage inhibition values were 19.08% to *Escherichia coli*, 35.11% to *Klebsiella pneumoniae*, 20.42% to *Staphylococcus aureus* wild type and 38.72% to *Staphylococcus aureus* methicillin resistant.

Amphibians are important sources of molecules with potential applications in different areas. For example, peptides from the brown Russian frog, *Rana temporaria* have demonstrated similar inhibitory effect than antibiotic compounds acting on gram-negative and gram-positive bacteria [7]. Since the first antimicrobial peptides were described in *Xenopus laevis,* hundreds of this kind of peptides has been reported in several families. Likewise, the toxin batrachotoxin obtained from frogs of the family Dendrobatidae was reported as an interestingly compound that exhibits biological activity against some predators [8]. However, anuran defense peptides frequently display potent cytolytic activities against a broad range of pathogenic bacteria and against important fungi, which is consistent with the hypothesis that they play an important role in host defense [3].

It was observed that a minimum concentration of aqueous extract (1:25 v/v) was required to induce membrane rupture. The cell viability of control ranged from 91% ± 1 to 40% ± 3, at 24 and 72 h of exposition respectively; showing a positive exposure-response relationship between concentration and time (Figure 1). Although, these extracts did not decrease the cell viability significantly, different cytotoxic effects were observed such as: vacuolization, stress, decreased in growth rate and deformation of the insect cell line Sf-21 (Figure 2). In addition, Probit statistical analysis show significant differences between treatments and control for CL_{50} values for this extract, which were 5768.37 ppm at 24h, 4846,51 at 48 h and 4525.18 at 72 h. Different authors worldwide have previously demonstrated the presence of molecules from frogs with potential to be used in many biotechnological applications [1, 7]. Moreover, these results confirm the initial hypothesis of the presence of antimicrobial substances in the cutaneous secretions of the white frog, which are of interest in pharmaceutical or agrochemical industry. The chromatographic profile of *H. crepitans*-derived extracts is showed in the Figure 3. A very similar composition was observed between extracts replicates. This profile exhibited eleven compounds as main constituents of the extracts. The table 1 summarized the identified compounds from *H. crepitans* extract. In contrast, with other species of this genus, *Hypsiboas pulchellus* have no presence of antimicrobial peptides or alkaloid [9]. Previously in *H. boans* and *H. lanciformis* was reported the presence of antimicrobial peptides but without cytotoxicity on different vertebrate cell lines [10]. Likewise, in *H. crepitans* it was proposed the presence of mechanism of innate defenses or immune system adaptive against *Batrachochytrium dendrobatidis* (known as Bd) or the amphibian chytrid fungus. Conditions as temperature and humidity tolerance of

this specie allow *H. crepitans* be considered as an interestingly source of compounds with antimicrobial activity, because particularly the specie in repeated diagnostics by using real-time polymerase chain reaction (rt–PCR) assays of postmetamorphic individuals at several stages after exposure to *Batrachochytrium dendroabatidis* (Bd) demonstrated that is able to clear infection within a few weeks at 23°C [11]. These results confirm the presence of antimicrobial substances (peptides and alkaloids) probably involved in this mechanism (Figure 3). In this way, Epibatidine, Eburnamonine and Makaluvamine P were found as main compounds and could be considered as the responsible compounds for the antimicrobial and cytotoxic activity, since these compounds have toxic properties [12, 13]. However, *Pipa carvalhoi* skin secretion does not possess peptides as toxins and its main constitutive kynurenic acid does not act as protective agent against microorganisms [14].

This is the first report of cytotoxic activity on insect cell line with the *H. crepitans* skin secretions (Figures 1 and 2). The cytotoxicity was probably caused by the presence of alkaloids like Hydroxy saxitoxin (H-STX) and Chiriquitoxin (CqTX) (Figure 3), both of them analogues to Tetrodotoxin (TTX) [15]. It is well known that these compounds block the sodium channel and unrelated to such an action, CqTX also shows the activation of the fast potassium current in ~ 40% of the muscle skeletal fiber of frogs [15]; it was firstly isolated from the bufonid frog *Atelopus chiriquiensis* [16]. Different alkaloids produced by frogs exhibiting potential applications in biotechnological processes [17, 18], in this case we found Epibatidine, a trace alkaloid previously reported from the Ecuadorian poison frog *Epipedobates tricolor* with potent analgetic activity that represent a different class of amphibian alkaloids [18, 19].

Finally, several low lands correspond to “biodiversity hotspots”, which are currently being threatened and should be studied because they offer a natural source of important molecules with a great potential to be used in different biotechnological approaches. For this reason, it is necessary a systematic bioprospecting of promising compounds from Anuran families involving aspects as purification, assisted identification and complete characterization of novel molecules.

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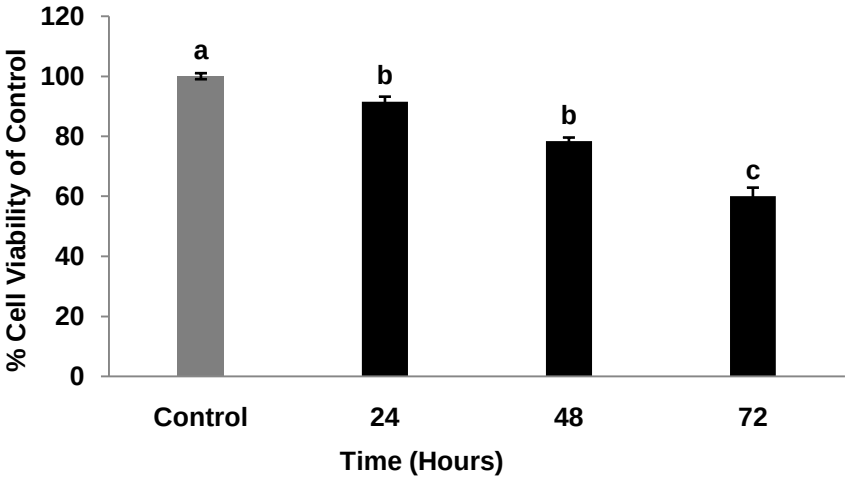

Figure 1. Viability response of SF21 insect cells exposed to extracts from *H. crepitans*, analyzed at 24, 48 and 72 h. Negative control SF21 cells at 72 h. Vertical bars represent standard deviation. Each assay was performed in triplicate. To each time of evaluation significant differences are indicated by different letters Tukey ($p \leq 0.05$)

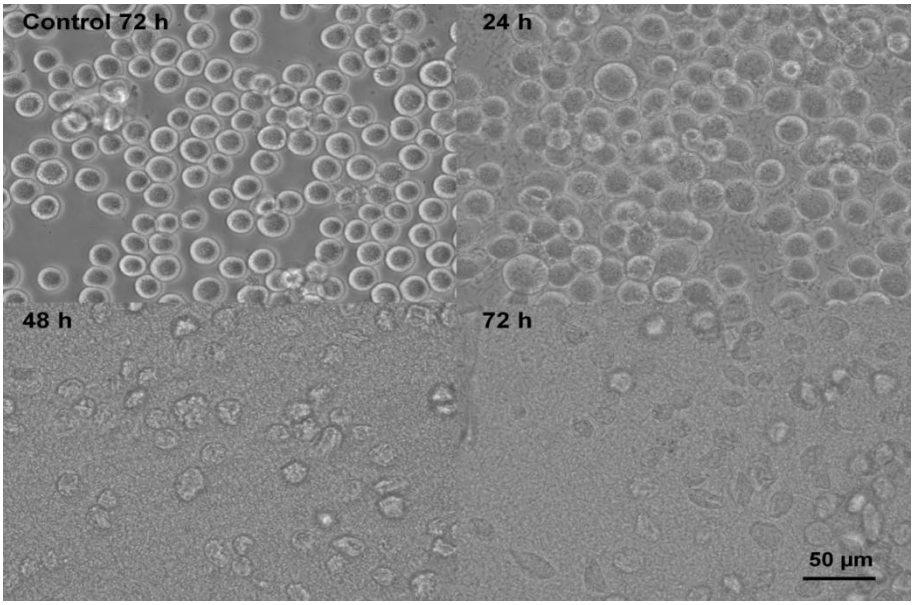

Figure 2. Cytotoxic activity of extracts from *Hypsiboas crepitans* (dorsal secretion) on SF21 insect cells. Microphotographs (40X) control at 72 hours and cells exposed to extracts at 24, 48 and 72 hours.

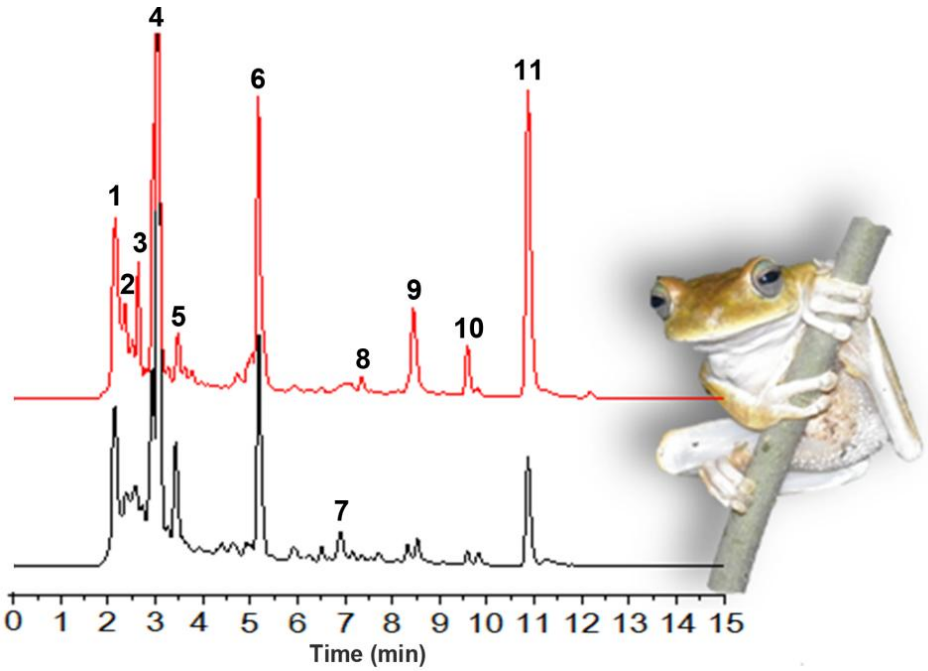

Figure 3. LC-MS-derived total ion chromatogram from *H. crepitans* extracts

Table 1. Identified compounds from *H. crepitans* extracts by LC-MS. n.i: unidentified

Peak No	t_R(min)	Name	Molecular Formula	$[M+H]^+$ (m/z)	Required (m/z)
1	2.14	Hydroxysaxitoxin	$C_{10}H_{17}N_7O_5$	316.1358	316.1369
2	2.38	n.i.	-	-	
3	2.63	Chiriquitoxin	$C_{13}H_{20}N_4O_{10}$	393.1245	393.1258
4	3.05	Epibatidine	$C_{11}H_{13}ClN_2$	209.0840	209.0846
5	3.43	Kynurenic acid	$C_{10}H_7NO_3$	190.0512	190.0504
6	5.17	Eburnamonine	$C_{19}H_{22}N_2O$	295.1810	295.1810
7	6.90	Lycogarubin B	$C_{24}H_{19}N_3O_5$	430.1409	430.1403
8	7.71	Roquefortine E	$C_{27}H_{31}N_5O_2$	458.2535	458.2556
9	8.73	Anisodorin 4	$C_{25}H_{42}O_6$	439.3071	439.3060
10	9.58	Calycanthine	$C_{22}H_{26}N_4$	347.2241	347.2236
11	10.86	Makaluvamine P	$C_{20}H_{22}N_3O_2$	337.1799	337.1790