Colombian frogs: promising source of new antibiotics

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Research

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Abstract

Background: In middle Magdalena of the Antioquia region, Colombia frog secretions have been used as antibacterial agents, the purpose of this study is to assess the antibacterial activity of six frog species secretions. **Methods**: the Kirby-Bauer and the microdilution methods were used to evaluate antibacterial activity of the frogs secretions against *S. aureus* and *E. coli*, using two positive controls, ampicillin and ciprofloxacin. **Results**: secretions of all six families showed inhibition zones, the concentration at which this zone was bigger was assayed later by the microdilution method and compared to ampicillin and ciprofloxacin. Only the secretion from the *Phyllomedusidae* exhibited a comparable effect to that one of control antibiotics. **Conclusions**: in here we provide evidence that secretions from local frogs have an antibacterial effect against two strains of bacteria, further studies are needed to identify the peptides in the secretions and a wider range of safe concentrations for human use.

1. Introduction

The emergence of bacterial resistance to commonly used antibiotics According to The Review on Antimicrobial Resistance, in 2014 infectious diseases caused 5,2% of deaths worldwide and it is expected that by 2050 this number increases up to 44,6% (1). Bacterial resistance is escalating due to the irrational use of antibiotics in human and animal health and a highly adaptive response to challenges in the bacteria (2).

The search for new antibiotics has led to investigate organisms that live in hostile conditions and that have not been evaluated previously, becoming a target for pharmaceuticals (3). Amphibians are among this selected group, they are continuously exposed to several pathogens and their secretions have shown to protect them, these contain peptides and toxins, the earlier are a known defense mechanism against microbes(4,5). Colombia has a high amphibian diversity, over 813 species belonging to 21 families and three orders, despite this fact few have been investigated (6).

The aim of this work is to present preliminary results on the antibacterial activity of some amphibian secretions that have not been studied before either nationally or internationally and contribute to the novel development of new antibiotic therapeutic arsenal.

2. Material And Methods

2.1. Collection and zoological material preparation

Seventeen secretions were evaluated, belonging to eight families of Anura from a subregion in middle Magdalena of the Antioquia province. These species live in the open field and early weeds of two localities, vereda of San Cipriano from Maceo municipality, and the district of Jerusalem from Sonsón municipality. Samples were obtained from frogs washed with sterile water, then introduced into a clean

plastic bag with a slider, then the bag was cleansed for five minutes with 5 mL of sterile water. Secretions were collected with a sterile syringe placed into a 1.5 mL mL conical tube, wrapped onto tin foil and stored at –21°C, as a stock solution

2.2. Bacterial strains

Gram-negative and gram-positive bacteria used in the assays*: Escherichia coli* (ATCC:8739) and *Staphylococcus aureus* (ATCC:6538), were obtained from Instituto Colombiano de Medicina Tropical (ICMT), both strains were sensitive to clinically used antibiotics. They were cultured in nutrient agar at 37°C for 24h and stored at 4°C until used. Amoxicillin and ciprofloxacin were a kind gift from CORPAUL and Genfar pharmaceutical SA.

2.3 Antimicrobial activity assays

2.3.1 Kirby-Bauer antibiogram

To determine antimicrobial activity from all samples a preliminary test was performed, the Kirby Bauer test, the strains were grown in a Mueller Hinton culture (MH) Merck® in Petri dishes with Brain Heart Infusion (BHI) agar. All tests were done at 37°C for 24h. Sterile disks from paper towel (6mm), were impregnated with 30 µL of each one of the frog's secretions or control antibiotics. The inhibition zone mas measured, all tests were performed in triplicate, and the samples that exhibited a higher antibiotic activity were selected for a MIC determination.

2.3.2 Minimum inhibitory concentration (MIC) and drug action modulation

Microdilution test: secretions from all six families with highest antibiotic activity in the Kirby-Bauer test, were analyzed using the microdilution assay according to CLSI-M07A9 (7). Ampicillin (Genfar 2g/mL) and ciprofloxacin (Corpaul 2mg /mL).

An inoculum of each strain was prepared through colony suspension in MH medium, after a 24h incubation period, absorbance of inoculum was measured to adjust it later to an absorbance of 0,08 a 625nm, which is equal to 0,5 in the McFarland scale

Sample preparation: Bradford assay was used for protein determination in frogs secretions, all were sterilized through filtration, cellulose acetate filters with 0,22µm pores; later, the samples were once again filtered (amoxicillin and ciprofloxacin were also filtered independently) and diluted in sterile water into 50%, 10%, 5% y 1% concentrations. Flat bottom, 96-well plates were used. The complete protein assay reagent contained a higher amount of cyclodextrins (up to approximately 90 µL of the concentrated

cyclodextrin solution per mL of Bradford reagent; 250 µL of this reagent was pipetted into each well, and 5 or 10 µL of the protein sample (in water, SDS buffer or RIPA buffer) was then added (8). The contents of each well were then mixed. After 5 minutes at room temperature, the absorbance was read at 595 nm. An empty plate was used as control. The protein assays were always performed in triplicate for verification result.

Bacteriostatic assays were performed in 96 well plates, each well contained MH medium 50 50 µL; inoculum 100 µL, and samples or reference antibiotics 50 µL, a negative control was used, instead of the antibiotic, 50 µL of sterile water was added. The total volume of each well was 200 µL.

Cell determination using optical density was carried out in Biotek® SYNERGY HTX spectrophotometer, after a 24h incubation period, at a 625 wavelength.

2.4. Statistical analysis

The linear range of concentrations for Bradford method was calculated via linear regression analysis. All calculations and analyses were done using GraphPad Prism v. 10.0 (GraphPad Software, San Diego, CA, USA).

For bacteriostatic assays, data were analyzed to obtain the inhibition constant (IC50) and where applicable, one way analysis of variance (ANOVA) was used to determine statistical significance, followed by the Tukey test considering $p < 0.05$. All tests were accomplished in triplicate, and the results are expressed as the mean + standard error.

3. Results

The present research began evaluating the antibacterial activity of raw secretions of 16 species belonging to eleven families, the selection was done through a Kirby-Bauer antibiogram with fresh collected samples, a greater antibacterial activity was observed in six species, belonging to six families: Bufonidae, Hylidae, Leptodactylidae, Phyllomedusidae, Dendrobatidae y Microhylidae; which were then selected for the microdilution test, in which they also were filtered for sterilization purposes.

The samples that exhibited a more potent inhibition halo were used in the continuing experiments, using the microdilution technique (figures 1 and 2), an antibacterial effect can be observed on *S aureus* y *E. coli* even at a low concentration. However, the only sample that had an equivalent effect to that one of control antibiotics (ciprofloxacin and ampicillin) was that of the Phyllomedusidae family. In the case of *S. aureus,* the sample from Phyllomedusidae, shows and even greater effect than that of ciprofloxacin (figures 1 and 2)

For protein concentration, the Bradford assay exhibited that protein content in frog's secretions was not proportional to the individual size, some small size species such as Microhylidae, had equal protein concentration to those of bigger species, Leptodactylidae.

4. Discussion

Amphibians skin features an extraordinary exocrine system with several granular and mucous glands, located at strategic places in the individuals, that are able to secrete highly concentrated poisons and other defense related substances in each species (9–11). Being part of protection strategies, the granular glands are specialized in producing diverse compounds such as steroids, alkaloids and peptides (4,9,12). From these secretions the first AMP molecules were isolated more than 45 years ago, to date more than 500 structures have been reported for having biological activity, besides these substances can be modified by interactions with the microbiota found in the skin, working synergically (13).

The peptides contained in these secretions are marked by intrinsic characteristics to each family that secretes them, for instance AMP is found mainly in Bufonidae, Hylidae, Leptodactylidae, Phyllomedusidae y Ranidaefamilies (12). The present research began evaluating the antibacterial activity of raw secretions of 16 species belonging to eleven families, the selection was done through a Kirby-Bauer antibiogram with fresh collected samples, where a greater antibacterial activity was observed in six species, belonging to six families: Bufonidae, Hylidae, Leptodactylidae, Phyllomedusidae, Dendrobatidae y Microhylidae; which were then selected for the microdilution test, in which thet also were filtered for sterilization purposes.

The microdilution test helped identify that some families such as Leptodactylidae and Bufonidae, showed a marked antibiotic activity and this diminished after filtration, which suggests that this is a critical step in handling the samples and that it is also necessary to check what microorganisms are present in these secretions that power the antibacterial activity of the raw secretions.

The sample with greater biological activity, was that taken from the Phyllomedusidae family, this species have not been investigated previously, the antibacterial effect was similar to those of control antibiotics, an extended spectrum beta lactam: ampicillin and a quinolone: ciprofloxacin, but this bactericidal activity was achieved at lower protein concentrations (fig 1) when compared to other secretions and to those normally used for the reference antibiotics, this can be explained by antimicrobial peptides described formerly in other organisms belonging to this family (14). These peptides also showed activity against the same microorganisms used in this research but in other investigations, where they have also been active against resistant strains of *S. aureus* and *E. coli (*15).

The Phyllomedusidae species is distributed from tropical Mexico to Argentina, of these, two families of AMP with great antibacterial activity have been identified, dermaseptin, which has been widely studied (12,14) and, recently, medusin is described (16), this new family consists of three peptides and each one has demonstrated the ability to inhibit the growth of gram positive bacteria (*S. aureus)* and gram negative bacteria (*E. coli)* besides yeast (*C. albicans),*, these microorganisms were also sensitive to the tests carried out by (14).

Other researches (17) describe at least seven AMP families in secretions of the Phyllomedusidae family, taking into account the results of the raw extract assay, it is reasonable to corelate the antibiotic activity

to the presence of peptides, and as can be seen in figures 1 and 2, the higher the protein concentration the higher the antibiotic activity, at 47 and 94 g/ mL concentration.

Finally it is worth noting that the concentration of proteins obtained using the new method “under stress”, frogs in bags, it is considerably higher than that reported in the literature, even using the conventional methods such as intravenous administration of norepinephrine; (18) reported that after using this novel method, the animals were returned to the aquarium. Other methods (19) sacrificed the individual for skin removal and later proceeded to extract the secretion through solvents, altogether these methods managed to obtained proteins in a concentration range in between 0,1- 1000 µg/mL. Whereas using the frog in bag method obtained a protein concentration range of 380–1380 µg/mL, demonstrating that this new technique to obtain secretions from anurans is effective and its negative impact for the subjects is minimal, avoiding the sacrifice step, which might also contribute to preserve the species and make research sustainable, although further investigations are needed to clarify the mechanism of action and the time or concentration action on bacteria.

5. Conclusions

Since the 80’s there has been a dramatic reduction (81%) in the production of new antibiotics (20) this helps explain the low availability and diversity of anti-infective compounds, as resistance spreads rapidly, pharmaceuticals have opted by modifying the basic pharmacophore, thus not creating a truly novel antibiotic, that is why research onto new antibiotic molecules with an also new mechanism of action is a must (21). The identification of cutaneous secretions from frogs with antibiotic activity might lead investigation onto the discovery of new anti-bacterials, with a new mode of action thus offering a wider anti-infective therapeutic arsenal, all obtained from richest Colombian biodiversity.

6. Declaration

6.1 Ethics approval and consent to participate

All experiments were approved by Universidad CES biodiversity committee, code N° 006. All samples were obtained by a new bag extraction methodology, which allows to collect the samples in enough quantities from the different families without the need to sacrifice de frogs or harm them.

6.2 Consent for publication

Not applicable

6.3 Availability of data and materials

Please contact author for data requests

6.4 Funding

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6.5 Competing interests

The authors declare that they have no competing interests.

6.6 Authors' contributions

EA Conceived the study, collected data, carried out experiments, contributed data and analysis tools, performed analysis and drafted the manuscript. LM carried out experiments. MCN carried out experiments. JB conceived, designed and performed analysis. DGP contributed data and analysis tools, and drafted the manuscript. PZ conceived and designed analysis, contributed data and analysis tools, performed analysis.

6.7 Acknowlegments

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Figures

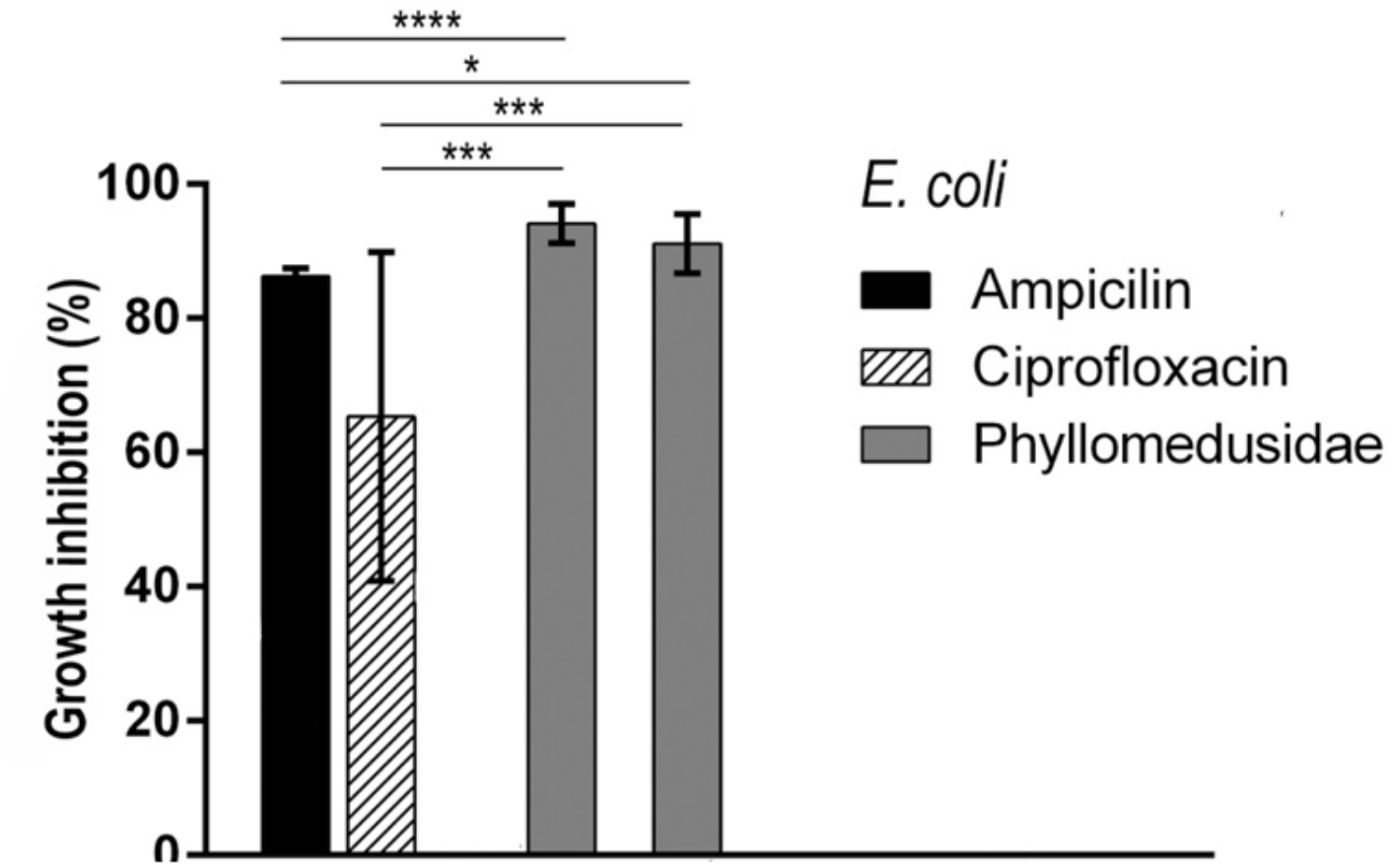

Figure 1

E. coli growth inhibition by Phyllomedusiae

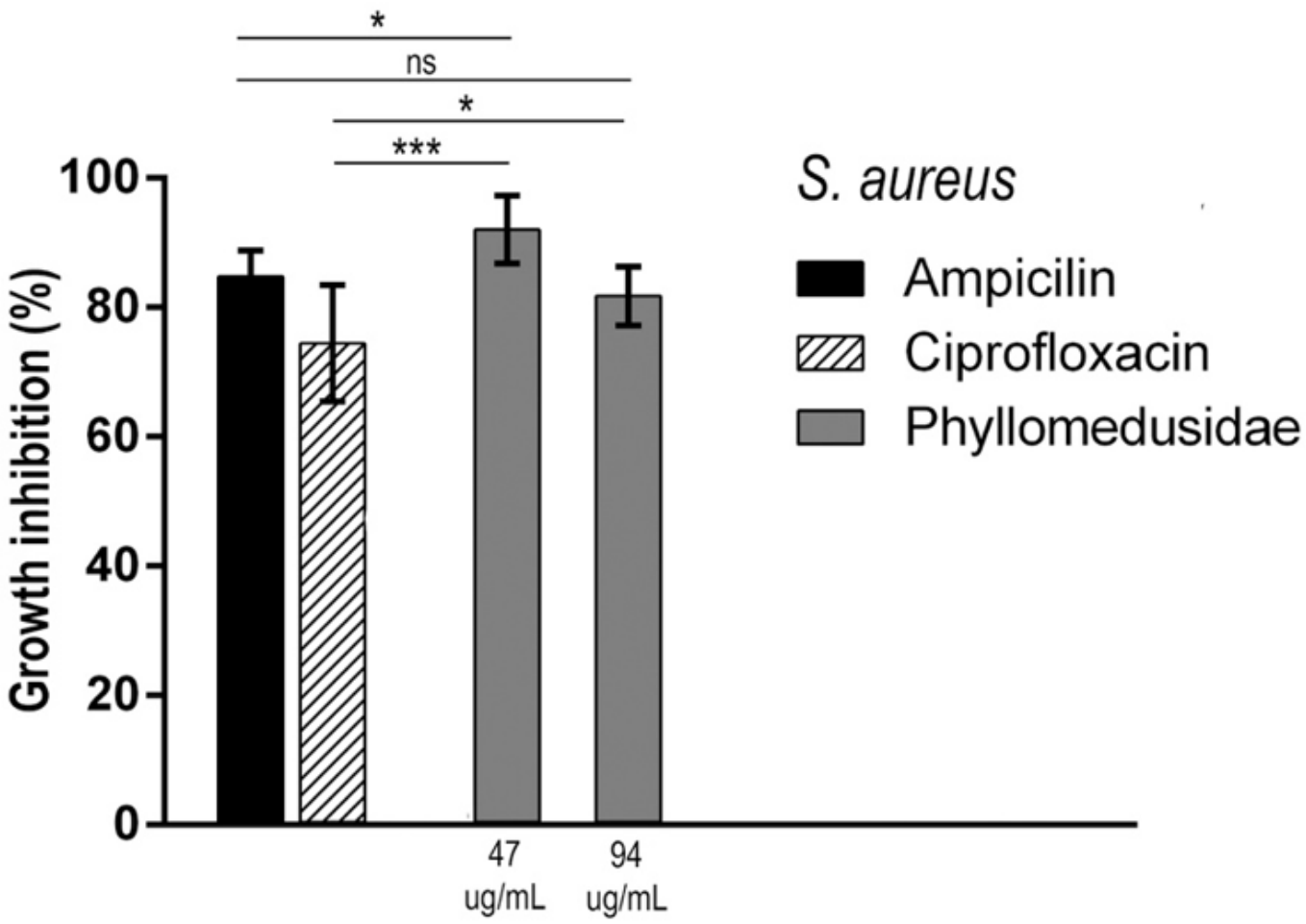

Figure 3

S. aureus growth inhibition by Phyllomedusiae