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Artículo Original

Antimicrobial bioprospection among environmental bacteria in the amazon biome

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ABSTRACT

Introduction. The chemical synthesis and bioengineering of new antimicrobials is not enough to face the increasing resistance of infectious microorganisms to current antimicrobial agents. Biodiversity, however, is a rich source of natural products presenting a vast array of biological activity including antibiosis between species.

Objective. Environmental bacteria isolated from Brazilian Amazon were assayed for the production of antimicrobial compounds.

Materials and Methods. Microorganisms were isolated from soil samples, fruits and seeds of brazilian nuts collected in different regions of the Brazilian Amazon.

Results. The supernatant of *Burkholderia pseudomallei* and *Dermacoccus nishinomiyaenses* cultures, isolated from soil, was found to inhibit colony formation of *Staphylococcus aureus* ATCC 25923. The supernatant of Serratia rubidaea and *Pantoea* sp. cultures also inhibited colony growth of *Enteroccocus faecalis* ATCC 29212. The supernatant of *Proteus mirabilis* colonies was found to inhibit colony formation of *Candida albicans* FIOCRUZ 40006. The metabolites obtained from the brazilian nut microbiota, likewise Pseudomonas aeruginosa, *Proteus vulgaris*, *Kocuria varians*, *Granulicatella elegans*, *Staphylococcus lentus*, *Staphylococcus intermedius*, *Escherichia coli*, *Oligella ureolytica*, *Acinetobacter* sp., and *Bacillus cereus*, inhibited colony growth of *Staphylococcus aureus* ATCC 25923.

Conclusions. Therefore, the metabolites of 2 species of bacteria isolated from the Amazonian soil exhibited activity against pathogenic bacteria, while metabolites of 11 species of bacteria isolated from the Brazilian nut inhibited the colony growth of both pathogenic bacteria and yeasts. Potential antimicrobial compounds found here will be chemically characterized and assayed in the future for cytotoxicity, in order to carry out further studies for prospective biotechnological purposes.

Key words: Antimicrobials, environmental microorganisms, bacteria, soil, Amazon

RESUMEN

Bioprospección de antimicrobianos entre bacterias del ambiente en el bioma amazónico

Introducción. La síntesis de nuevos antimicrobianos no es suficiente para hacer frente al incremento de la farmacorresistencia de microorganismos infecciosos. La biodiversidad es rica fuente de productos que presentan amplias opciones de actividad biológica, incluyendo la antibiosis.

Objetivo. Bacterias aisladas del medio ambiente de la Amazonia brasileña fueron investigadas en

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cuanto a su potencial antimicrobiano.

Materiales y Métodos. Microorganismos fueron aislados de muestras de suelo, frutos y semillas de nueces brasileñas (*Berthollethia excelsa*), colectadas en diferentes regiones de la Amazonia brasileña.

Resultados. El sobrenadante del cultivo de *Burkholderia pseudomallei* y *Dermacoccus nishinomiyaensis*, aisladas del suelo, inhibieron la formación de colonias de *Staphylococcus aureus* igualmente, ATCC 25923; como también el sobrenadante del cultivo de *Serratia rubidaea* y *Pantoea* sp. inhibieron el desarrollo de colonias de *Enteroccocus faecalis* ATCC 29212. También, el sobrenadante del cultivo de *Proteus mirabilis* bloqueó el crecimiento de colonias de *Candida albicans* FIOCRUZ 40006. Los metabolitos obtenidos de la microbiota de las nueces brasileñas, tales como *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Kocuria varians*, *Granulicatella elegans*, *Staphylococcus lentus*, *Staphylococcus intermedius*, *Escherichia coli*, *Oligella ureolytica*, *Acinetobacter* sp. y *Bacillus cereus*, impidieron la formación de colonias de *Staphylococcus aureus* ATCC 25923.

Conclusiones. De esta forma, metabolitos de 2 aislados bacterianos del suelo amazónico presentaron actividad contra patógenos bacterianos; mientras que de la microbiota de la nuez brasileña, metabolitos de 11 aislados bacterianos contrarrest. aron la formación de colonias de bacterias y levaduras patógenas. Compuestos con potencial actividad antimicrobiana aquí encontrados serán caracterizados químicamente y ensayados en cuanto a la actividad citotóxica, en estudios futuros con perspectivas de aplicaciones biotecnológicas.

Palabras clave: antimicrobianos, microorganismos ambientales, bacteria, suelo, Amazonia

INTRODUCTION

The Amazon biome is exceptionally rich, and as a consequence is also under intense pressure from human activities. The number of disappearing living organisms remains undetermined, along with the increasing numbers of species risking extinction. Negative effects on microorganisms are also unknown (1,2).

Antibiosis mechanisms are the fundamentals to unravel new antimicrobials, even though modern bioinformatics approaches contribute heavily to introducing new antibiotics into the world's markets. Usually, antibiotics have been derived from natural compounds produced by competing metabolic microorganism that compete for nutrients and environmental niches (3).

Soil microorganisms can play a crucial roles in plant development, producing growth factors and protections from harmful microorganisms (4,5). Similarly, skin and mucosae saprophyte microorganisms can protect vertebrate hosts against pathogenic bacteria and fungi (6). Natural sources of antibiotics include soil and plant-associated environments, or endophytic bacteria (7-9), and also lactic acid bacteria (probiotics) found in crude milk, and natural compounds found in honey (10-12). Among microorganisms producing antibiotics, *Actinomycetes* represent the most important supplier of antibiotics used in industry (13). The members of the *Bacillus* gender are known as producers of a number of antifungal compounds against phytopathogenic and human pathogenic fungi (14), and gram-positive and gram-negative bacteria (15). Another gender, the *Pseudomonas*, have been described as antibiotic synthesizing microorganisms (8, 16), as *Pseudomonas batumici* and *Pseudomonas fluorescens* Pf0-1. As a matter of fact, any fungi or bacteria can be a potential producer of antimicrobial compounds. The most successful microorganisms in the natural environment fabricate effective compounds capable of inhibiting or killing other microorganisms, in order to take advantage of nutrients, and to dominate desirable niches (17).

The chemical nature of antimicrobial compounds ranges from polyketides derivatives, small peptides, lipopeptides, 3-phenyllactic acid as also other organic acids from glucose fermentation likewise lactic and acetic acid, the main end products of probiotic metabolism, and quinolones

(18-20). Besides antibiosis, other biological activities are displayed by microorganisms' secondary metabolites, as antineoplastic and modulators of inflammation (20,21).

The soil surface in the tropical forests, particularly in the Amazon biome, is extremely fragile, requiring constant renewal of organic compounds furnished by the rich and diverse biota, which are then washed out by intense rainfalls (22-23). Therefore, soil microbiota is heavily influenced by the organic matter that enriches the litter on the forest floor. Among the numerous botanical species of economic and ecological relevance in the Amazon, the *Bertholettia excelsa*, well-known for the production of brazil nuts, had the fruits and seeds analyzed for the microbial content in the natural environment, hanging on the tree, fallen in the soil, and stocked in warehouses. Preliminary results of microbiological analysis described the presence of a rich and diverse biota, sometimes corresponding to microorganisms found in the soil (23-24).

In the present study, we investigated the antimicrobial activity of metabolites of microorganisms isolated from soil, and brazil nuts (*Berthollethia excelsa*), collected in different areas of Amapa and Amazon state respectively, in the Amazon region of Brazil.

MATERIALS AND METHODS

Soil samples. A total of 36 soil samples were collected in different areas of the Brazilian Amazon region in the Amapa state, in May 2010, from the uppermost layer of the vegetation floor, in shaded areas, in the forest floor litter, up to a maximum depth of 15 cm, with each sample weighing 1 kg, kept in clean zipped polystyrene bags at 4° C until use.

Fruits and seeds of *Bertollethia excelsa*. In the municipality of Manicoré, in the Amazon state, in November 2010, fruits were collected in the forest soil, and seeds were obtained from a warehouse for brazil nut (*Bertollethia excelsa*) production (Manicoré Agricultural Cooperative/COVEMA) .

ATCC Pathogenic bacteria and Fiocruz Foundation

Collection of microorganisms. For assays of antibacterial activity, the following ATCC (American Type Culture Collection) pathogenic strains; *Escherichia coli* 25922, 35218, *Staphylococcus aureus* 25923, 564, *Salmonella typhimurium* 5190, *Pseudomonas aeruginosa* 27853 and *Enterococcus faecalis* 29212 were utilized. Also, microorganisms obtained from the collection of Oswaldo Cruz Institute Foundation (FIOCRUZ), *Escherichia coli* 00219; *Salmonella typhi* 0029; *Acinetobacter baumannii* 00143; *Klebsiella ozaenae* 0075; *Enterococcus faecalis* 00531 and the fungi *Candida albicans* 40006 were assayed as challenge models. The microorganisms were maintained in nutrient agar at 4°C until the assays were carried out. All samples were kindly donated by Dr. Ernesto Hofer from Oswaldo Cruz Institute Foundation, Rio de Janeiro, Brazil.

Isolation of bacterial organisms from soil samples. Fifty mg of each soil sample from a total of 36 samples were diluted in 9 mL of sterile distilled water and spread out in 90x15mm plates with Luria-Bertani agar, and incubated at 37 °C for 24 hours. Isolated grown colonies were transferred to 4 mL of Luria-Bertani broth and incubated under the same conditions previously stated. In order to confirm the purity of each colony, a sample aliquot of growth broth was spread out in Luria-Bertani agar plate. The supernatant samples of isolated bacteria were centrifuged for 3 min at 15,000 rpm and sterilized through a 0.22 nm syringe filter. Filtered supernatants were utilized for the biological assays. Alternatively, 1 g of each soil sample was cultured in 25 mL of thioglicolate broth in Falcon tubes for 72 hours at 37 °C, and the crude supernatants of these grown broths were utilized for the biological assays.

Isolation of bacterial organisms from fruits and

seeds of *Bertollethia excelsa***.** The fruits and seeds of *Bertollethia excelsa* were separately cultured in thioglycolate broth in the same conditions as carried out for soil samples. The crude supernatants of grown organisms were utilized for the biological assays.

Biological assays in 96 well flat bottom microtitration plates. Preliminary screening for antibacterial activity was carried out utilizing filtered supernatants of isolated bacteria from soil samples. Also, crude supernatants of soil growth, fruits and seeds growth of Bertollethia excelsa were screened for antibacterial activity. Briefly, 7 ATCC bacterial strains were isolatedly inoculated in Luria-Bertani broth, (26x103 UFC/mL). Each horizontal row, with 12 wells each, was divided in 3 groups (4 wells per group), composed of 2 controls, a positive and negative one, and a test sample. The first group contained 10 µL of ATCC strain + 90µL de Luria-Bertani broth. The second one, 40 µL of filtered bacterial supernatant + 50 µL of Luria-Bertani broth + 10 µL of ATCC strain, and the third one (negative control), 50 µL of Luria-Bertani broth + 50 µL of filtered bacterial supernatant. The plates were incubated for 24 hs at 37°C. In order to detect antibacterial activity against ATCC strains, the plates were read in a spectrophotometer at 630 nm (THERMO PLATE, ELISA Tp Reader).

Disc diffusion test in agar for bacterial supernatants. Ten microliters of filtered supernatant, of each bacterial growth from 90 isolated soil bacterial organisms, was dropped onto a 6 mm Whatman filter disc and allowed to dry. Sequentially, the discs were evenly placed over a 140x15mm petri dish containing 100 mL Müeller-Hinton agar previously inoculated with the ATCC bacterial strains individually (1x10-3 UFC/mL), utilizing glass pearls to spread the bacterial inoculum homogeneously. All procedures were carried out in triplicate, under aseptic conditions in a laminar flow prep area. The antibacterial activity was checked out by the development of an inhibition halo around the discs and controlled by filter disc containing ciprofloxacin and distilled water.

Identification of microorganisms exhibiting antimicrobial activity. Crude supernatants obtained from mixed bacterial growth exhibiting antimicrobial activity, also showed small colonies in the border of the cavity (surrounded by inhibition halo). These bacterial colonies were picked up and cultured in broth medium, and the filtered supernatant was again tested for antimicrobial activity. Pure bacterial colonies were identified by manual and automated methods (VITEK 2 automated identification system) utilizing the Biomerieux phenotypic identification kit.

RESULTS

Fruits and seeds of *Berthollethia excelsa*, the brazil nut, plus the following isolated microorganisms; *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Escherichia coli*, *Kocuria varians*, *Granulicatella elegans*, *Staphylococcus lentus*, *Staphylococcus intermedius*, *Oligella ureolytica*, *Bacillus cereus* and *Acinetobacter* sp., produced metabolites presenting inhibitory activity against *Staphylococcus aureus* ATCC 25923 (**Figure 1a** and **1d**).

Of 36 soil samples collected in different geographical locations in the Amapa state, Brazil, *Burkholderia pseudomallei*, *Dermacoccus nishinomiyaenses*, *Proteus mirabilis*, *Pantoea* sp. and *Serratia rubidae* were isolated. The metabolites produced by *Burkholderia pseudomallei* and *Dermacoccus nishinomiyaenses* inhibited the growth of *Staphylococcus aureus* ATCC strain 25923 (**Figure 1b**). The supernatant of *Proteus mirabilis* cultures exhibited antimicrobial activity against *Candida albicans* colony growth (figure not shown), while growth supernatant of *Serratia rubidae* and *Pantoea* sp. inhibited colony growth of *Enterococcus faecalis* ATCC 29212 (Figure 1c). The supernatant of *Pantoea* sp. cultures inhibited colony formation of *Enterococcus faecalis* ATCC 29212 (**Figure 1c**).

The initial assays here, did not utilize microorganisms growing in groups, but came from isolated Whatman paper discs impregnated with filtered supernatant of isolated microorganisms that were preliminarily utilized for antimicrobial screening. Through this approach, antimicrobial activity was not detected, unless the unfiltered supernatant applied to the cavities in the agar plate were of mixed culture of different bacteria. It is remarkably clear that different microorganisms sharing the same microenvironment will compete for nutrients and space in order to survive and reproduce. In this way, secondary metabolites would be secreted by competing microorganisms inhibiting the growth of other microorganisms.

The antimicrobial activity demonstrated by the crude growth supernatant of mixed bacterial populations, and also of growth supernatant of isolated bacteria, in agar plates, visually inspected for halo inhibition development, was confirmed by turbidity reduction, in 96 well plate, measured using a ELISA reader spectrophotometer at 630 nm. The optical density values were usually reduced twofold in bacterial growth of pathogenic microorganisms exposed to filtered supernatants of mixed and isolated microorganisms of soil and seeds/fruits of brazilian nut (**Table 1**).

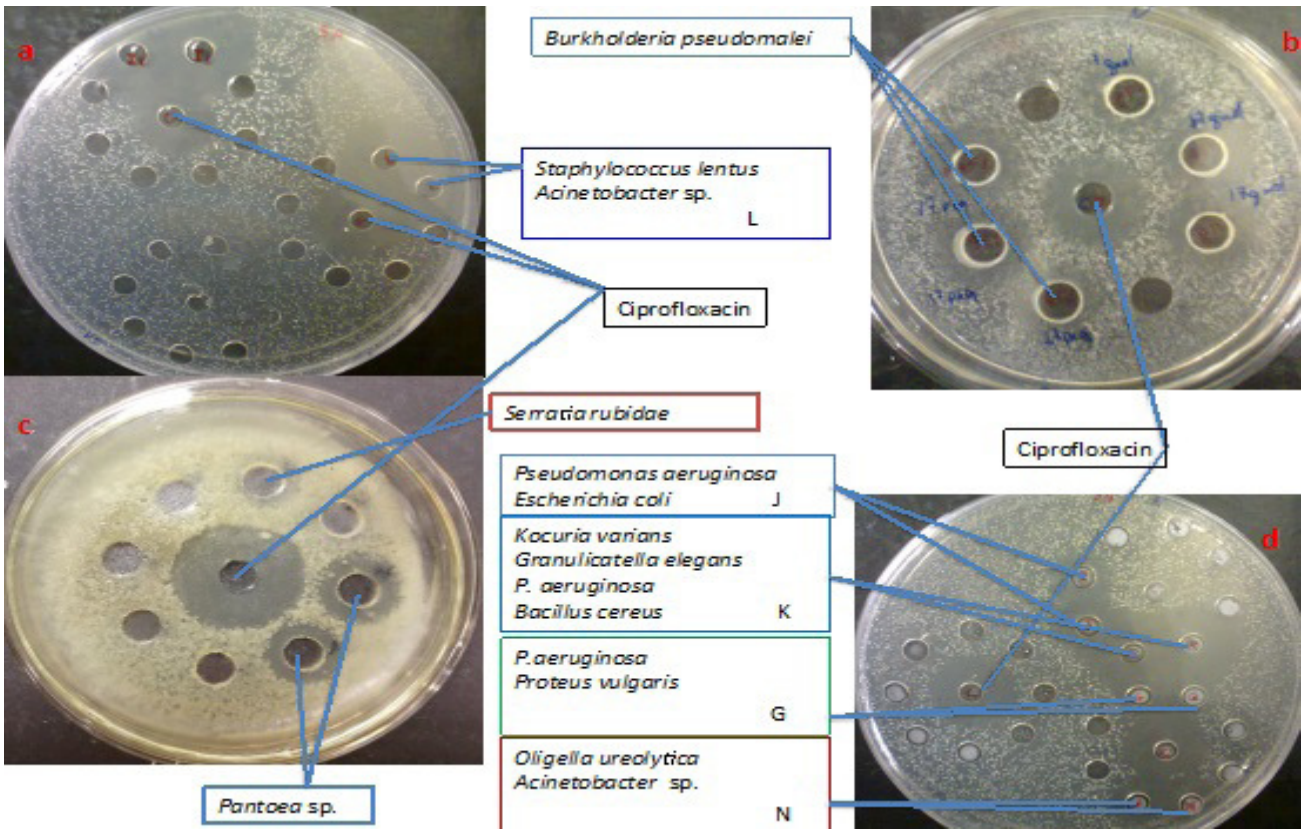

Figure 1. (a) Crude mixed filtered supernatant of bacterial growth (*Staphylococcus lentus* & *Acinetobacter* sp.) exhibiting antimicrobial activity against *Staphylococcus aureus* ATCC 25923. (b) *Staphylococcus aureus* ATCC 25923 colony growth inhibition by filtered growth supernatant of *Burkholderia pseudomallei*. (c) Filtered growth supernatant of *Serratia rubidaea* and *Pantoea* sp. exhibiting antimicrobial activity against *Enterococcus faecalis* ATCC 29212 colony growth. (d) Crude growth supernatant of bacterial isolates (*Pseudomonas aeruginosa*, *Escherichia coli*, *Kocuria varians*, *Granulicatella elegans*, *Bacillus cereus*, *Proteus vulgaris*, *Oligella ureolytica* and *Acinetobacter* sp.) from seeds and fruits of brazilian nut exhibiting activity against *Staphylococcus aureus* ATCC 25923 colony growth.

DISCUSSION

Members of the Enterobacteriaceae family

Table 1
Median optical density values of growth culture medium of pathogenic bacteria challenged with filtered supernatant of bacterial isolates from soil, seeds and fruits of *Berthollethia excelsa*

Bacterial filtered growth supernatant of *B. excelsa* **and soil**	**Optical Density (630 nm)**	
	Control Group	Test Group (filteredd bacterial supernatant)
G. Group	618.5±64.9[a]	367.75±39.9[b]
Isolate G1	589.25±110.7[a]	203.5±18.4[b]
IsolateI1	584.75±160.3[a]	218.5±8.7[b]
J Group	548±189.1[a]	382.75±59.6[a]
K Group	571.5±162.3[a]	229±27.6[a]
L Group	619.5±183.6[a]	269.75±16.2[a]
N Group	610.75±179.9[a]	381.25±64.2[a]
Isolate 17	687.5=111.3[a]	412.75=10.5[a]

have also been reported by other authors producing colicins, pyocins and proticins by *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris* respectively (25, 26, 27). Many other bacteriocins have been reported, as variacin produced by *Kocuria varians* and arginine deaminase like enzyme synthesized by *Granulicatella elegans* (28, 29). The gram-positive cocci, *Staphylococcus lentus* and *Staphylococcus intermedius*, constitute the physiologic skin microbiota of many mammals including ruminants, carnivores and birds (30), despite the pathogenic potential of these microorganisms (31,32). There are no reports in the literature concerning the production of antimicrobials by *Staphylococcus lentus* and *Staphylococcus intermedius*; nevertheless the polyketide synthase gene was detected in *Staphylococcus lentus* isolated from South China Sea sponges (33).

Despite the *Burkholderia* genus's known propensity for producing agents that are pathogenic to plants, animals and humans, there are also beneficial species in this group likewise *B. vietnamiensis*, *B. unamae*, and *B. tropica*, associated to plants, carrying out nitrogen fixation and promoting plant growth. Here, the antibacterial activity of *Burkholderia pseudomallei* against *S. aureus* could be explained by the exceptional versatility and capacity of the *Burkholderia* gender to produce diverse antimicrobial secondary metabolites, besides toxins, like extracellular enzymes and sidephores as reviewed by Vial *et al.* (34).

Proteus mirabilis, a gram-negative bacteria, member of the Enterobacteriaceae family, is the second commonest cause, after *Escherichia coli*, of urinary tract infections (35). This organism is found in the human and animals' intestine, and also in polluted soils and water. Probably, the growth inhibition of *Candida albicans* by the growth supernatant of *Proteus mirabilis* occurred due to ammonia production by this microorganism that alkalinize the microenvironment (25).

Pantoea species can be isolated from environmental sources, in soil, water and vertebrates, including man. The extensive number of this gender makes the identification cumbersome (36). There are reports, in the literature, about *Pantoea aglomerans* involved in esophageal reflux, in man (37). Nevertheless, other members of this gender render beneficial effects in biotechnology. It was observed the inhibitory activity of *Pantoea agglomerans* over the phytopathogenic fungi *Fusarium graminearum* (38).

The opportunistic pathogen *Serratia rubidae* is rarely isolated from human tissues, but it has been reported in the respiratory tract, wounds, fecal samples, bile and blood, usually in immunocompromised patients (39).

Therefore, the metabolites of 2 species of bacteria isolated from the Amazonian soil exhibited activity against pathogenic bacteria, while metabolites of 11 species of bacteria isolated from the Brazilian nut inhibited the colony growth of pathogenic bacteria and yeast.

The results obtained here warrant further studies to identify and characterize the compounds yielding antimicrobial activity. The biotechnological interest in these substances,

probably secondary metabolites of peptidic nature, is justified by the increased demand of new antimicrobial drugs, in order to treat human and animal diseases of microbial etiology. This interest is emphasized by the crescent multiresistance to the available antibiotics in use. Also, besides the development of new drugs to combat infectious diseases, agricultural application of competing microorganisms is a natural way to overcome phytopathogenic aggression to plants utilized by man and animals, for food production. It is worthwhile to mention the economic impact of new antimicrobials in the market.

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