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Screening of antibacterial extracts from plants native to the Brazilian Amazon Rain Forest and Atlantic Forest

I.B. Suffredini[1], H.S. Sader[2], A.G. Gonçalves[3], A.O. Reis[2], A.C. Gales[2], A.D. Varella[1] and R.N. Younes[1]

[1]Laboratório de Extração, Universidade Paulista, São Paulo, SP, Brasil
[2]Laboratório Especial de Microbiologia Clínica, Universidade Federal de São Paulo, São Paulo, SP, Brasil
[3]Centro de Ciências Biológicas e da Saúde, Universidade Federal do Maranhão, São Luís, MA, Brasil

Abstract

More than 20% of the world's biodiversity is located in Brazilian forests and only a few plant extracts have been evaluated for potential antibacterial activity. In the present study, 705 organic and aqueous extracts of plants obtained from different Amazon Rain Forest and Atlantic Forest plants were screened for antibacterial activity at 100 µg/ml, using a microdilution broth assay against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*. One extract, VO581, was active against *S. aureus* (minimum inhibitory concentration (MIC) = 140 µg/ml and minimal bactericidal concentration (MBC) = 160 µg/ml, organic extract obtained from stems) and two extracts were active against *E. faecalis*, SM053 (MIC = 80 µg/ml and MBC = 90 µg/ml, organic extract obtained from aerial parts), and MY841 (MIC = 30 µg/ml and MBC = 50 µg/ml, organic extract obtained from stems). The most active fractions are being fractionated to identify their active substances. Higher concentrations of other extracts are currently being evaluated against the same microorganisms.

Key words
- Plant extract
- Antibacterial activity
- Screening
- Amazon Rain Forest
- Atlantic Forest

Correspondence
I.B. Suffredini
Laboratório de Extração
Universidade Paulista
Av. Paulista, 900, 1º andar
01310-100 São Paulo, SP
Fax: +55-11-3170-3978
E-mail: extractlab@unip.br

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Introduction

The Brazilian Amazon Rain Forest is home to 17% of the biodiversity found within the country (1). The Atlantic Forest contains approximately 35% of the world's Angiospermae, and more than 8% of the Pteridophytae (2). In view of this wealth of species and that the Atlantic Forest is one of the world's foci for conservation (3), this biomas should be studied in terms of pharmacological or biological activity.

Approximately 20% of the plants found in the world have been submitted to pharmacological or biological test, and a substantial number of new antibiotics introduced on the market are obtained from natural or semisynthetic resources. It has been reported that between the years 1983 and 1994, of 93 new antibacterial agents submitted to analysis by the FDA six were natural products (teicoplanin, mupirocin, miokamycin, carumonam, isepamicin, and RV-11), 45 were semisynthetic products modeled on a natural product lead, and 7 antivirals were synthetic compounds based on natural product models (4). The systematic screening of antibacterial plant extracts represents a continuous effort to find new compounds with the potential to act against multi-resistant bacteria.

The principal aim of the present study was to screen organic and aqueous extracts obtained from the Brazilian Amazon Rain Forest and Atlantic Forest for antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Escherichia coli*.

Material and Methods

Plant collection and extract preparation

All plants were collected with the authorization of Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), permits No. 053/99 and 038/99. Plants were identified by Dr. Alexandre A. Oliveira, USP, Ribeirão Preto, SP, Brazil, and vouchers are deposited at the Herbarium UNIP (Universidade Paulista, São Paulo, SP, Brazil).

Seven hundred and five organic and aqueous extracts were obtained from plants native to the Amazon Rain Forest and Atlantic Forest. Plants were collected using a traditional (5) and chemosystematic (6) approach. Plant parts were collected according to the biomass availability of each individual or population, which varied according to the species and habitat of each one, i.e., trees, herbaceous plants, lianas, epiphytes or shrubs. Plant material was dried and ground before being submitted to 24-h maceration with methanol:dichloromethane (1:1) followed by 24-h maceration with water, resulting in two extracts from each plant material. Further information on the technique can be found elsewhere (7). The number of extracts of each family tested is given in the current text.

Antimicrobial assay

Broth microdilution method was used to screen the 705 plant extracts. The inoculum was prepared at the concentration of 10^{-2} CFU/ml, starting from a 0.5 McFarland (or 10^8 CFU/ml) prepared from fresh colonies of bacteria as described below (8).

S. aureus ATCC 29213 (Sau), *E. coli* ATCC 25922 (Ecol), *E. faecalis* ATCC 29212 (Efae) and *P. aeruginosa* ATCC 27853 (Psa) were the bacterial strains tested. The bacterial inoculum of each ATCC strain was obtained from fresh colonies grown on Müeller Hinton agar plates. Each strain was inoculated into 5 ml of Müeller-Hinton broth in order to obtain a concentration of 1.5 x 10^8 CFU/ml (0.5 MacFarland). The inoculum was then diluted to 1.5 x 10^2 CFU/ml. One hundred and ninety microliters of this suspension was transferred to each microplate well. Ten microliters of each extract solution was added to the microplate wells and incubated at 35ºC for 18 to 20 h. Extracts were prepared to 20 times the desired test concentration (2 mg/ml) in water or 50% DMSO solution. Results were analyzed visually and classified according to the following patterns: + = bacterium colonies deposited in the bottom of the well, ++ = turbidity with bacterium colonies being deposited, +++ = light turbidity, and ++++ = total growth inhibition.

The extracts were screened at a concentration of 100 µg/ml. Extracts that showed inhibitory activity at this concentration were submitted to a subculture of the broth media in Müeller Hinton agar (Oxoid®, Basingstoke, Hampshire, England) in order to evaluate bacterial growth (8).

Determination of minimal inhibitory concentration and minimal bactericidal concentration

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined for the extracts that showed total growth inhibition using the protocol described above. Extract concentrations of 10 to 100 µg/ml in steps of 10 µg were evaluated. The concentration at which there was no visually detectable bacterial growth was taken as the MIC, and the concentration at which there was no bacterial

growth after inoculation in Müeller-Hinton agar (Oxoid®) was taken as the MBC.

Results

Results were visually analyzed and bacterial growth inhibition was confirmed after inoculation in Müeller-Hinton agar. Many extracts showed some degree of inhibition of bacterial growth at concentrations of 100 µg/ml, represented by "++". The MIC and MBC of these extracts are currently being obtained. The families of the plants from which the extracts were obtained, the total number of extracts obtained for the current family, and the number of extracts whose antibacterial activities were classified as "++" against the respective bacteria tested are reported in Table 1.

Table 2 lists the seven extracts tested at the concentration of 100 µg/ml and classified as "+++" against *E. faecalis*. Table 3 lists the three plant extracts that showed antibacterial activity against *S. aureus* and *E. faecalis*, and their respective MIC and MBC.

Table 1. Antibacterial activity of extracts prepared from plants obtained in the Amazon Rain Forest and the Atlantic Forest.

Family	No. of extracts evaluated	No. of extracts with ++ activity	Family	No. of extracts evaluated	No. of extracts with ++ activity
Anacardiaceae	1	1 Sau; 1 Efae	Melastomataceae	23	13 Sau; 8 Efae
Annonaceae	34	28 Sau; 5 Efae	Meliaceae	9	5 Sau; 3 Efae
Apocynaceae	54	20 Sau; 6 Efae	Memecilaceae	4	3 Sau; 2 Efae
Aquifoliaceae	3	3 Sau; 2 Efae	Monimiaceae	1	1 Sau; 1 Efae
Balanophoraceae	1	1 Efae	Moraceae	3	3 Sau;
Bignoniaceae	16	10 Sau; 1 Efae	Myristicaceae	6	6 Sau; 2 Efae
Bombacaceae	2	2 Sau; 2 Efae	Myrsinaceae	6	6 Sau; 3 Efae
Boraginaceae	5	4 Sau; 1 Psa	Myrtaceae	17	17 Sau; 11 Efae
Burseraceae	10	6 Sau; 1 Efae	Ochnaceae	3	2 Sau; 2 Efae
Capparidaceae	8	2 Sau	Olacaceae	6	5 Sau; 3 Efae
Caryocaraceae	5	4 Sau; 3 Efae	Orchidaceae	1	1 Sau
Chrysobalanaceae	15	12 Sau; 1 Efae	Passifloraceae	1	1 Sau
Clusiaceae	43	34 Sau; 18 Efae; 1 Ecol	Polygalaceae	1	1 Sau
Combretaceae	8	8 Sau; 5 Efae	Polygonaceae	14	11 Sau; 5 Efae
Commelinaceae	1	1 Sau	Proteaceae	2	2 Efae
Connaraceae	10	9 Sau; 4 Efae	Rabdodendraceae	7	4 Sau; 2 Efae
Convolvulaceae	1	1 Sau	Rhyzophoraceae	3	2 Sau; 2 Efae
Dilleniaceae	4	3 Sau; 2 Efae	Rubiaceae	51	35 Sau; 10 Efae
Ebenaceae	5	4 Sau; 2 Efae	Rutaceae	4	4 Sau; 2 Efae
Erythroxylaceae	2	1 Sau	Sapindaceae	12	7 Sau; 5 Efae
Euphorbiaceae	33	21 Sau; 14 Efae	Sapotaceae	4	4 Sau; 1 Efae
Flacourtiaceae	18	15 Sau; 6 Efae	Simaroubaceae	9	6 Sau; 3 Efae
Gentianaceae	5	2 Sau	Smilacaceae	5	4 Sau; 3 Efae; 1 Psa
Heliconiaceae	2	2 Sau; 1 Efae	Solanaceae	2	2 Sau
Hippocrateaceae	6	5 Sau; 3 Efae	Styracaceae	2	2 Sau; 1 Efae
Humiriaceae	2	2 Sau; 2 Efae	Theaceae	6	2 Sau
Lacistemataceae	2	1 Sau; 1 Efae	Teophrastraceae	1	1 Sau
Lauraceae	19	17 Sau; 6 Efae	Trigoniaceae	3	1 Sau
Lecythidaceae	6	6 Sau; 2 Efae	Trimeriaceae	1	1 Sau; 1 Efae
Leguminosae	119	95 Sau; 53 Efae	Verbenaceae	1	1 Sau
Linaceae	2	2 Efae	Violaceae	7	4 Sau; 4 Efae
Loranthaceae	5	5 Sau; 4 Efae	Vochysiaceae	5	4 Sau; 5 Efae
Malpighiaceae	21	17 Sau; 11 Efae			

Activity was measured by the microdilution broth assay at 100 µg/ml. "++" indicates that the extracts caused turbidity without culture flocks and that they will be evaluated at higher concentrations. Ecol = *Escherichia coli* ATCC 25922; Efae = *Enterococcus faecalis* ATCC 29212; Psa = *Pseudomonas aeruginosa* ATCC 27853; Sau = *Staphylococcus aureus* ATCC 29213.

Discussion

Plant extracts have been studied against bacteria for years, but in a more intensified way in the last three decades. During this period, a lot of antimicrobial screening evaluations have been published based on the traditional use of Chinese, African, and Asian plant drugs (9-15). During the late 1990's, a large number of manuscripts describing methodologies of screening took part and resulted in more than 30 articles representing antibacterial extracts obtained from Asian and African native plants, but only a few studies relating antibacterial activity of Brazilian plant extracts were found (16-21). Brazil is home to more than 20% of the world's biodiversity (1) and only a few species have been submitted to any sort of large-scale biological screening. We collected a substantial amount of information about the antimicrobial activity of 705 Brazilian plant extracts which have been collected randomly.

In order to establish the extract concentrations to be used during the screening, we observed that extract concentrations spanning from 25 µg/ml (22) to 2 mg/ml (23) or even 40 mg/ml (18) have been used. We screened the crude extracts using a concentration of 100 µg/ml (24). Such concentration is nowadays considered the proper concentration an antimicrobial extract should present. The dilution test is a very precise technique that permits us to work with such a low concentration.

Only 3 of 705 extracts showed bactericidal activity: extract VO581 against *S. aureus* and MY841 and SM053 against *E. faecalis*, with the subcultures showing no germ growth at concentrations ≥160 µg/ml. Several crude extracts apparently inhibited bacterial growth but when the bacteria were subcultured in agar there was growth, as shown above. Among them, we are currently determining the MIC and MBC for all 510 crude extracts (72%) that showed "++" results against *S. aureus*, for all the 240 crude extracts (34%) that showed "++" results against *E. faecalis*, for the 2 extracts (0.3%) that showed "++" results against *P. aeruginosa*, for the extract (0.14%) that showed the same result against *E. coli*, and for all the 7 extracts that showed "+++" results against *E. faecalis*.

Extract MY841 (MIC = 30 µg/ml; MBC = 50 µg/ml), obtained from the stem of a

Table 2. Antibacterial activity against *Enterococcus faecalis* of extracts prepared from plants obtained in the Amazon Rain Forest and the Atlantic Forest.

Family	Number of extracts evaluated	Number of extracts classified as +++ against *Enterococcus faecalis* ATCC 29212
Asteraceae	9	1
Connaraceae	10	1
Flacourtiaceae	18	1
Lauraceae	19	1
Melastomataceae	23	1
Myrsinaceae	6	1
Rabdodendraceae	7	1

Activity was measured by the microdilution broth assay at 100 µg/ml. "+++" indicates that the extracts caused light turbidity and that they will be evaluated at higher concentrations.

Table 3. Antimicrobial activity of plant extracts from the Amazon Rain Forest that showed strong activity, and their corresponding minimal inhibitory and minimal bactericidal concentrations.

Family	Number of extracts evaluated	Extract classified as ++++[a] against *Enterococcus faecalis* ATCC 29212	Extract classified as ++++[a] against *Staphylococcus aureus* ATCC 29213	Minimal inhibitory concentration (µg/ml)	Minimal bactericidal concentration (µg/ml)
Myrsinaceae	6	MY841	0	140	160
Smilacaceae	5	SM053	0	80	90
Vochysiaceae	5	0	VO581	30	50

[a]Activity was measured by the microdilution broth assay. "++++" indicates that the extracts caused total growth inhibition.

Myrsinaceae plant, showed antibacterial activity against *E. faecalis*, as also did extract SM053 (MIC = 80 µg/ml; MBC = 90 µg/ml), obtained from the aerial parts of a Smilacaceae plant. Extract VO581 (MIC = 140 µg/ml; MBC = 160 µg/ml), obtained from the stem of a Vochysiaceae plant, showed activity against *S. aureus*. The three active extracts are going to be bioguide fractionated in order to identify their active substances as well as the remaining extracts, whose MICs were ≤500 µg/ml. Vochysiaceae species have been studied phytochemically and biologically, and beta-sitosterol, betulinic acid and sericic acid have been isolated from their stem bark extracts (25). These substances have shown antibacterial activity against *S. aureus*. Polysaccharides were also isolated from the cell walls of 57 species of this family (26). Myrsinaceae have been found to contain saponins (27), terpenoids (28), and benzoquinone derivatives (29,30), whereas no phytochemical or biological activity has been reported for Smilacaceae.

It is a matter of major national interest to study the potential of Brazilian forests in offering new lead antibacterial compounds that can be further synthesized and have their activity improved. Thus, we strongly hope to contribute to the conservation and protection of the biodiversity of our forests and to the development of the Brazilian community as a whole.

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