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Antibacterial activity of Apocynaceae extracts and MIC of *Tabernaemontana angulata* stem organic extract

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Thirty-eight aqueous and organic extracts obtained from 11 Amazonian Apocynaceae *species were submitted to an antimicrobial dilution in broth media screening for* Staphylococcus aureus, Pseudomonas aeruginosa *and* Candida albicans, *performed on microtiter plates. The organic extract obtained from the stem of* Tabernaemontana angulata *presented activity against Gram positive* Staphylococcus aureus. *The minimal inhibitory concentration of the organic crude extract was 2.50-1.25 mg/mL. Cloramphenicol was used as standard. Alkaloids and triterpenes were found in the active organic extract.*

Unitermos:
- Apocynaceae
- *Tabernaemontana angulata*
- Antimicrobiral activity
- Amazon rain forest
- Minimal Inhibitory Concentration (MIC)

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INTRODUCTION

The Amazon Rain Forest is home to 20% of the biodiversity found in the world. The Rio Negro Basin has unique features not found elsewhere, as it is the converging point of species richness endemic to distant locations, markedly the Lower Rio Negro, near the city of Manaus (Oliveira, Daly, 1999). The interest in finding new antimicrobial substances becomes evident and mechanisms of action related to bacterial resistance are elucidated (Moellering, 1998). Apocynaceae has been chosen as a group to be evaluated in this study because of its popular use (Duke, Vasquez, 1994), chemosystematic correlations (Cronquist, 1988) and pharmacognostic interest.

MATERIALS AND METHODS

Plant material

All plants were collected in the Amazon Rain Forest under authorization of Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis – IBAMA numbers 053/99 and 038/99. The selected species are listed in Table I, together with sites of collection, herbarium and collector's number, parts used and types of extract (organic or aqueous). Vouchers are deposited in the Herbarium UNIP and were authenticated by Dr. Alexandre A. de Oliveira.

Preparation of crude extracts

Plant parts were separated, dried in a stove at 40 °C, ground, labeled and stored in a freezer until use. Extractions were carried out in a glass percolator, and an organic (CH_2Cl_2: H_3COH, 1:1) and an aqueous 24 hour-macerate were obtained from each sample. Organic extracts were rotavaporated and the aqueous ones were lyophilized (Younes *et al*., 2000).

Antimicrobial assay

Staphylococcus aureus (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida*

TABLE I - Species of Apocynaceae used to obtain crude extracts to be screened against *S. aureus*, *P. aeruginosa* and *C. albicans* by the dilution in broth media test. Collector's and herbarium numbers, sites of collection and parts used are given, as well as extract numbers and information whether the extract is organic (O) or aqueous (A)

Species	Collector's number	Herbarium number	Place of collection	Parts used (extract number and status)
Aspidosperma pachypterum Müell.-Arg	AAO 3263	000364	Igapó Forest	Stem (1,O; 2,A); bark (3,O; 4,A); roots (5,O; 6,A); stem+leaves (7,O; 8,A)
Aspidosperma sp.	PS 360	000276	Igapó Forest	Stem+leaves (9,O; 10,A); stem (11,O; 12A)
Couma utilis (Mart.) Müell.-Arg	AAO 3336	000478	Terra firme Forest	Stem (13,O); leaves (14,O; 15A)
Himatanthus attenuatus (Benth.) Woodson	PS 150	000074	Igapó Forest	Leaves (16,O; 17A); stem (18,O; 19,A)
Macoubea sprucei.(Müell.-Arg) Markgr.	AAO 3373	000515	Campinarana[3]	Stem (20,O); fruit (21,O)
Malouetia tamaquarina (Aubl.) A. DC.	IBS 10	000444	Igapó[1] Forest	Aerial parts (22,O; 23,A); stem (24,O; 25,A)
Mandevilla rugosa (Benth.) Woodson	AAO 3354	000496	Igapó[1] Forest	Aerial parts (26,O)
Microplumeria anomala (Müell.-Arg) Markgr.	PS 136	000060	Igapó[1] Forest	Stem (27,O; 28,A); leaves (29,O; 30,A)
Odontadenia macrantha (Roem. & Schult.) Markgr.	PS 94	000018	Igapó[1] Forest	Stem+leaves (31,O; 32,A); stem (33,O; 34,A)
Tabernaemontana angulata (Mart. ex Müell.Arg)	PS 416	000321	Igapó[1]/ terra firme[2] forest	Stem (35,O; 36,A)
Tabernaemontana rupicola Benth	IBS 4	000438	Igapó Forest	Aerial parts (37,O; 38,A)

1. Floodplain forests flooded by blackwater rivers (Ferreira, 1997); **2**. Tropical lowland densed forest (Oliveira, Nelson, 2000); **3**. A low, relatively light forest with thin-stemmed trees (10-20 m) with large, broad-trunked individuals (Guilaumet, 1987).

albicans (ATCC 10231) were used and were grown in specific media (TSA, TSB, SDA and SDB, all from Difco). Preparation and standardization of microorganism suspensions were made according to techniques described elsewhere (Kubota, Ohara, 1998).

Preparation of antibiotic solutions

Dilutions at 40, 20, 10, 5, 3, 2, 1 and 0.5 μg/ mL were prepared with cloramphenicol. Dilutions at 80, 60 and 40 μg/mL were obtained for amphotericin B. In order to establish the minimal inhibitory concentration against the tested microorganisms, 10 ml of all dilutions were transferred to the appropriate wells filled with 190 μL of inoculated broth media (diluted 1: 20).

Dilution of the extracts

The aqueous extracts 2, 4, 6, 8, 10, 12, 15, 19, 23, 25, 28, 30, 32, 34, 36 and 38 (Table I) were prepared at the

initial concentration of 50 mg/mL; extract 17 (Table I) was diluted to 86.4 mg/mL; and organic extracts 1, 3, 5, 7, 9, 11, 13, 14, 16, 18, 20, 21, 22, 24, 26, 27, 29, 31, 33, 35 and 37 (Table I) were prepared at the concentration of 50 mg/mL. All dilutions were 20 times higher than the desired final concentration, so as to be diluted to the proper concentration after the addition to the microtiter plates. A mixture of water and DMSO was used as a solvent to dilute the organic extracts (1: 1) (Kubota, Ohara, 1998).

Determination of antimicrobial activity in 96-well microtiter plates

Inoculated broth seeded with 1 x 10^2 CFU/mL was used to the test and MIC determination, according to Mazzanti *et al*. (2000). Each extract was analyzed in octuplicates, and the technique was extracted and adapted from NCCLS protocols (2000). The results were visually evaluated according to the legend of table 2.

Calculation of MIC

MIC tests were carried out using the extracts, chloramphenicol and amphotericin B, according to a modification of the procedures of NCCLS (2000) and Mazzanti *et al*. (2000). For the evaluation of the active extract, three dilutions at 50, 25 and 12.5 mg/mL were prepared. Thus the final concentrations were 2.5, 1.25 and 0.625 mg/mL (1/20 of the original dilutions).

Phytochemical analysis

Phytochemical analysis were performed in order to verify the presence of tanins, flavonoids (flavanon and flavanonols), alkaloids, saponins, anthraquinones and triterpenes (Costa, 1994a 1994b). The extract was diluted to 1,67 mg/mL to be tested for saponins and tanins, to 2mg/mL to be tested for flavonoids, triterpenes and anthraquinones, and to 3 mg/mL to be tested for alkaloids.

RESULTS AND DISCUSSION

One out of the 38 extracts evaluated was considered active against *S. aureus* (organic extract obtained from stem of *T. angulata)*, and 10 extracts were slightly active against *S. aureus* (aqueous extract from stem+leaves of *Aspidosperma pachypterum* # 8, aqueous extract from leaves of *Himatanthus attenuatus* # 25, aqueous extract and from leaves of *Microplumeria anomala* # 30), *P. aeruginosa* (organic extract from leaves of *Himatanthus attenuatus* # 16) and *C. albicans* (organic extract from stem + leaves of *Aspidosperma* sp. # 9, organic extract from stem of *Macoubea sprucei* # 20, aqueous and organic extracts from stem of *Malouetia tamaquarina* # 24 and 25, organic extract from stem of *Microplumeria anomala* # 27 and organic extract from stem of *T. angulata* # 35).

The MIC obtained for the active extract lays ranges from 2.5 to 1.25 mg/mL, and that obtained for standard cloramphenicol was 2 μg/mL. Thus the activity of the selected extract cannot be considered high (Table II). We believe that the active substance is highly diluted in the crude extract. If bioguided fractionation is carried out, it will be possible to verify whether the outcome is loss or gain of antibacterial activity.

We have observed that some extracts were slightly active against the microorganisms tested, which could be related to growth inhibition; extracts with such activity were not, however, considered for further MIC determination (Table II).

Alkaloids were detected together with triterpenes, and the presence of alkaloids may be the responsible for the antibacterial activity observed in the crude organic extract from *T. angulata*. According to van Beek *et al*. (1984a), who studied a large number of *Tabernaemontana* sp. ethanolic extracts against bacteria, fungi, amoeba and virus, two out of 19 *Tbernaemontana* extracts showed activity against *S. aureus* (*T. undulata* and *T. verticosa*). In a continuous screening work, van Beek *et al.* (1985) have isolated forty five indol alkaloids from *T. chipii*, some of them presented significant activity against Gram-positive bacteria.

van Beek *et al.* (1984b) related a chemotaxonomy review on *Tabernaemontana* sp. showing the classification of indol alkaloids occurring in the genus, according to their biogenesis: vincosan, corynanthean, vallesiachotaman, strychnan, aspidospermatan, plumeran, eburnan, ibogan, tacaman, *bis*-indole and miscellaneous. In their work, the authors listed forty six out of seventy five *Tabernaemontana* species and their traditional use related to antimicrobial activity. Crude extracts as well as some indol alkaloids isolated from these species had been pharmacologically studied against bacteria. Verpoorte *et al.* (1983) studied antimicrobially active indol alkaloids belonging to Apocynaceae. Those manuscripts reinforce our aim of isolating indol alkaloids from *T. angulata* in order to correlate them with the primary antibacterial activity.

Apocynacee is also known to contain cardioactive glycosides, as in *Nerium* (Evans, 1996, Langford, Boor, 1996), *Adenium* (Yamaguchi, Abe, 1990) and *Trachomitum* (Waclaw-Rozkrutowa, 1975), and flavonoid glycosides and allies, as found in *Parameria laevigata*

TABLE II - Results of the antimicrobial evaluation of the aqueous and organic extrats obtained from Brazilian Amazon Apocynaceae species and minimal inhibitory concentration of amphotericin B, cloramphenicol and the active extract 35 (organic extract obtained from stem of *Tabernaemontana angulata*). See Table 1 for meaning of extract numbers

	Microorganism			
Exract number	*Candida albicans*	*Staphyllococus aureus*	*Pseudomonas aeruginosa*	MIC/microorganism
1	++	++	+++	
2	+++	++	+++	
3	++	++	+++	
4	+++	++	+++	
5	+++	++	+++	
6	+++	+++	+++	
7	+++	++	+++	
8	+++	+	+++	
9	+	+++	+++	
10	+++	++	+++	
11	++	++	+++	
12	*	*	*	
13	+++	++	+++	
14	++	++	+++	
15	+++	+++	+++	
16	++	++	+	
17	+++	+++	+++	
18	++	++	+++	
19	+++	++	+++	
20	+	++	+++	
21	++	++	+++	
22	++	++	+++	
23	+++	+++	+++	
24	+	++	+++	
25	+	+	++	
26	++	++	+++	
27	+	++	+++	
28	+++	++	+++	
29	+++	++	+++	
30	+++	+	+++	
31	++	++	+++	
32	+++	+++	+++	
33	++	++	++	
34	+++	++	+++	
35	+	-	+++	2,5-1,25 mg/mL/ *S. aureus*
36	+++	++	+++	
37	++	++	+++	
38	+++	++	+++	
Cloramphenicol	-	-	-	2,0 µg/mL/*S. aureus* > 2,0 µg/mL/*P. aeruginosa*
Amphotericin B	-	-	-	< 2,0 µg/mL/*C. albicans*

(-) no growth; (+) colony growth and no broth turbidity; (++) growth with slight broth turbidity; (+++) growth and massive broth turbidity; (*) extract not tested; MIC = minimal inhibitory concentration

(Kamiya *et al*., 2001), *Apocynum venetum* (Sakushima *et al*., 1978), *Cerbera mangas* (Sakushima *et al*., 1976), *Vinca minor* (Szostak, Kowalewski, 1975), and *Thevetia peruviana* (Sticher, 1971). These two groups of compounds also may be found to cause the observed antimicrobial activity related to the stem organic extract of *T. angulata* Tannins, saponins, anthraquinones and flavonoids which are not detected by Shinoda's reagent were not found in the crude extract.

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RESUMO

Atividade antibacteriana de extratos deApocynaceae e CIM de extrato orgânico de caule de *Tabernaemontana angulata*

Trinta e oito extratos orgânicos e aquosos obtidos de 11 espécies de Apocynaceae *foram submetidos a triagem utilizando o método da microdiluição em caldo contra* Staphylococcus aureus*,* Pseudomonas aeruginosa *e* Candida albicans*. O extrato orgânico obtido do caule de* Tabernaemontana angulata *apresentou atividade contra a bactéria Gram positiva* S. aureus*. A concentração inibitória mínima verificada para esse extrato variou de 2,50 a 1,25 mg/mL. Cloranfenicol foi utilizado como antimicrobiano padrão. A análise fitoquímica indicou a presença de triterpenos e alcalóides no extrato ativo.*

UNITERMOS: Apocynaceae. Tabernaemontana angulata. Atividade antibacteriana. Floresta Amazônica. CIM.

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