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Byrsonima crassa Niedenzu (IK): antimicrobial activity and chemical study

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ABSTRACT

The methanolic extract of leaves from *Byrsonima crassa*, a Brazilian medicinal plant, was analyzed by CC and HPLC. Four constituents were isolated and identified as quercetin, methyl gallate, (-)-epigallocatechin gallate and quercetin-3-*O*-(2"-galloyl)-a-L-arabinopyranoside. The methanolic and hydromethanolic extract, as well as fractions, were evaluated regarding their possible antimicrobial activity using *in vitro* methods. Results showed that both extracts and fractions exhibited significant antimicrobial activity against all tested strains.
Keywords: Byrsonima crassa, antimicrobial activity, Malpighiaceae.

RESUMO

Byrsonima crassa Niedenzu (IK):
atividade antimicrobiana e estudo químico

O extrato metanólico de *Byrsonima crassa*, uma planta medicinal brasileira, foi analisado por CC e HPLC. Foram isolados e identificados quatro constituintes como quercetina, galato de metila, (-)-epigalocatequina galato e quercetina-3-*O*-(2"-galoil)-a-L-arabinopiranosídeo. Os extratos metanólico e hidrometanólico e frações foram submetidos a ensaios para avaliação da possível atividade antimicrobiana *in vitro* usando métodos de difusão e diluição. Todos os extratos e frações analisados exibiram uma significante atividade antimicrobiana frente a todas as linhagens testadas.
Palavras-chave: Byrsonima crassa, atividade antimicrobiana, Malpighiaceae.

INTRODUCTION

Byrsonima crassa Niedenzu (IK) is a tree native to savannah-like areas in Brazil, which is popularly known as 'murici-cascudo' or 'murici-vermelho'. The bark and leaves are used in Brazilian popular medicine to treat diarrhea, ulcers, gastritis and even cutaneous problems (Silva et al., 2001; Lopez et al., 2001).

A small number of *Byrsonima* species have been evaluated chemically. Such phytochemical studies have led to the isolation of flavonoids, triterpenes, steroids, non-protein amino acids, sulphonoglycolipids and proanthocyanidins (Rastrelli et al., 1997; Bejar et al., 1995; Geiss et al., 1995; Amarquaye et al., 1994; Gottlieb et al., 1975; Mendes et al., 1999). Phytochemical investigation of the MeOH extract from the leaves of *Byrsonima crassa* have led to the isolation of catechins and flavonoid glycosides (Sannomiya et al., 2004; 2005).

Antimicrobial tests performed *in vitro* have shown that the extract *B. verbascifolia* was able to inhibit the growth of *Streptococcus faecalis*, *Mycobacterium phlei*, *Staphylococcus aureus* and *Bacillus subtilis* (Martínez-Vásquez et al., 1999; Lopez et al., 2001). Martínez-Vásquez et al. (1999) reported the antibacterial activities of *B. crassifolia* extracts against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *Staphylococcus aureus*, *S. epididermis*, *Streptococcus pneumoniae* and *Micrococcus luteus*.

Despite its popular use as a medicinal plant, there are no data about the antimicrobial effect of leaf extract of this species. The present study was conducted to evaluate the antimicrobial activity of *B. crassa* leaf extracts by the disc-diffusion method. We also proceeded to the isolation and identification of some minor compounds in the leaf extract.

MATERIAL AND METHODS

Microorganisms: Eight microbial species were used and were taken from international collections. They included the bacteria: *Bacillus subtilis* (ATCC 9372), *Bacillus cereus* (ATCC 14579), *Shigella* sp. (IAL 1578), *Staphylococcus epidermidis* (ATCC 12226), *Proteus mirabilis* (CDC 305),

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Salmonella sp. (ATCC 19196), *Enterococcus faecalis* (ATCC 29212), and the yeast *Candida albicans* (ATCC 10231).

Plant material: Leaves of *Byrsonima crassa* Niedenzu (IK) were collected at Porto Nacional, Tocantins State, Brazil and authenticated by Eduardo Ribeiro dos Santos. A *voucher* specimen (n° 3377) was deposited at the Herbarium of Tocantins University.

Extract preparation: The air-dried leaves of *B. crassa* (2.0 kg) were grounded and extracted three times with each solvent in the each solvent in the series chloroform, methanol and finally methanol/water 80:20 (v/v), at room temperature (48 h per solvent). The extracts for each solvent were combined and evaporated *in vacuo* to yield three fractions, designated $ECHCl_3$ (53.8 g), EMeOH (158.3 g) and EMeOH 80% extracts (95.7 g), respectively. A portion (10.0 g) of EMeOH was suspended in water and extracted with ethyl acetate (EtOAc) to produce the EtOAc (4.5 g) and aqueous (4.1 g) fractions.

The EtOAc fraction (4.0 g) was submitted to Gel Permeation Chromatography (GPC) on a Sephadex LH-20 (Pharmacia) column (100 x 5 cm) was eluted with MeOH. Fractions (8 mL) were collected and checked by Thin Layer Chromatography (TLC) on silica gel plates eluted with a mixture of $CHCl_3$/MeOH/H_2O (80:18:2, v/v) and revealed either with NP/PEG reagent or with anisaldehyde/sulfuric acid solution (Wagner et al., 1984). Fractions 43-47 (336 mg) were purified by repeated on column chromatography (CC) with silica gel (Merck) eluted with $CHCl_3$/MeOH (75:25, v/v) furnishing methyl gallate (1) (70.0 mg) and epigallocatechin gallate (2) (3.4 mg). Fractions 82-85 (135.0 mg) were purified by the same procedure, yielding quercetin (3) (5.0 mg). Fractions 78-81 (140.0 mg) were purified by HPLC, using MeOH/H_2O (45:55, v/v) as eluent, yielding quercetin-3-*O*-(2”-galloyl)-a-L-arabinopyranoside (4) (12.0 mg). Their structures were characterized mainly by ^{1}H NMR, ^{13}C NMR, COSY and TOCSY experiments.

The NMR spectra were obtained in DMSO-d_6 solution, using a Varian INOVA 500 spectrometer, operating at 500 MHz for ^{1}H and 150 MHz for ^{13}C. Chemical shifts are given as d (ppm) using TMS as an internal standard. TLC analyses were performed on silica gel Si F254 (Merck) plates eluted with $CHCl_3$/MeOH (80:20, v/v). The plates were visualized using UV light (254 and 365 nm).

Disc diffusion method: The dried plant extracts were dissolved in DMSO to a final concentration of 30 mg/mL and sterilized by filtration through 0.45 mm Millipore filters. Antimicrobial tests were carried out by the disc diffusion method (Bauer et al., 1966).

All the microorganism inoculum cultures were grown in Brain Heart Infusion liquid medium at 37 ºC. After 6 h of growth, 100mL of inoculum, at a concentration of 10^6 cells/mL, was spread on the surface of Mueller-Hinton agar plates. After drying, filter paper discs (6 mm in diameter) saturated with extracts (20 mL) were placed on surface of each inoculated plate. The plates were incubated at 35 ºC for 24 h for bacteria and for 48 h for *C. albicans*. After this period, the zones of growth inhibition around the discs were measured. Overall, cultured microorganisms with diameter of inhibition zone equal to or greater than 7 mm (Nascimento et al., 2000) were considered susceptible to the tested extract. DMSO was used to dissolve the extracts.

The negative control was the solvent used and the positive control was ciprofloxacin (5 mg/disc) for bacteria and ketoconazole (40 mg/disc) for *C. albicans*. All determinations were made in duplicate.

Minimum inhibitory concentration: The minimum inhibitory concentration (MIC) was determined by the dilution method, as specified by the National Committee for Clinical Laboratory Standards (NCCLS, 2003). The bacteria were grown in nutrient broth (5 mL) for 6 h at 37 °C. After the incubation, 20 mL of this culture at 10^6 cells/mL were inoculated into tubes of nutrient broth supplemented with different volumes (25-800 mL) of the extracts of 30 mg/mL. After 24 h at 37 ºC, the MIC of each sample was found by measuring of the optical density (OD) in a spectrophotometer (620 nm) and comparing the sample OD with that of sterile nutrient broth (Nascimento et al., 2000). All determinations were made in duplicate.

RESULTS

A total of eight microorganisms, 7 bacteria and one yeast, were tested.

The preliminary biological screening indicated that EMeOH and EMeOH 80%, including both the EtOAc and aqueous fractions of EMeOH, showed antimicrobial activity against *Staphylococcus epidermidis*, *Bacillus cereus*, *B. subtilis*, *Salmonella* sp., *Proteus mirabilis*, *Enterococcus faecalis*, *Shigella* sp. and *Candida albicans*.

The results of the antimicrobial activity test, shown in Table 1, revealed that the growth of all microorganisms evaluated were affected by all the extracts and fractions by forming clear inhibition zones between 8 to 14 mm of diameter. The blind control (dimethyl sulphoxide) did not inhibit any of the microorganisms tested. MIC values of 1.5-12 mg/mL were obtained for the extracts and fractions in the tests (Table 2).

The chemical profiles of EtOAc and aqueous fractions of EMeOH were evaluated by TLC analysis. Plates revealed with NP/PEG reagent produced intense orange and yellow spots characteristic of flavonoids and plates revealed with anisaldehyde/sulfuric acid solution also produced reddish spots, suggesting the presence of catechin derivatives (Wagner et al., 1984). The EtOAc fraction showed the presence of flavonoids. The aqueous fraction showed exclusively tannins.

Since the EMeOH showed antimicrobial activity, we performed a phytochemical investigation on this

Table 1 - Antimicrobial activities of EMeOH, EMeOH 80% and fractions of *Byrsonima crassa,* ciprofloxacin and ketoconazole.

Dose (mg/mL)	*S.e.*	*B.c.*	*B.s.*	*Sal.*	*P.m.*	*E.f.*	*Shi.*	*C.a.*
			EMeOH					
50	11	9	9	11	9	12	10	11
75	12	10	10	11	10	12	11	11
100	12	11	11	12	10	13	11	13
EMeOH 80%								
50	10	11	10	10	10	12	9	12
75	11	12	10	10	11	12	10	14
100	11	12	10	11	11	13	10	14
EtOAc fraction								
50	10	10	9	11	10	10	9	11
75	11	10	9	11	10	10	10	11
100	11	11	9	11	10	11	11	12
Aqueous fraction								
50	8	8	8	9	9	9	9	10
75	9	9	8	9	9	10	10	10
100	11	9	8	9	9	10	10	10
Ciprofloxacin	25	20	20	22	22	22	22	NT
Ketoconazole	NT	NT	NT	NT	NT	NT	NT	16

Diameter of zone (mm), *S.e.*, *Staphylococcus epidermidis*, *B.c.*, *Bacillus cereus*, *B.s.*, *Bacillus subtilis*, *Sal.*, *Salmonella*, *P.m.*, *Proteus mirabilis*, *E.f.*, *Enterococcus faecalis*, *Shi.*, *Shigella*, *C.a.*, *Candida albicans,* NT, not tested.

Table 2 - Minimum inhibitory concentration (MIC) exhibited by *Byrsonima cra*ssa extracts.

Extract (mg/mL)	*S.e.*	*B.c.*	*B.s.*	*Sal.*	*P.m.*	*E.f.*	*Shi.*	*C.a.*
EMeOH	1.5	6.0	6.0	9.0	12.0	6.0	3.0	3.0
EMeOH 80%	9.0	6.0	9.0	6.0	7.5	7.5	7.5	3.0
EtOAc fraction	6.0	3.0	6.0	7.5	9.0	6.0	3.0	7.5
Aqueous fraction	7.5	6.0	6.0	6.0	9.0	6.0	7.5	6.0

S.e., *Staphylococcus epidermidis*, *B.c.*, *Bacillus cereus*, *B.s.*, *Bacillus subtilis*, *Sal.*, *Salmonella* sp., *P.m.*, *Proteus mirabilis*, *E.f.*, *Enterococcus faecalis*, *Shi.*, *Shigella* sp., *C.a.*, *Candida albicans.*

exctract. Fractionation of an aliquot of the EtOAc fraction from the EMeOH by GPC followed by purification by several chromatographic procedures led to the isolation of methyl gallate (**1**), quercetin (**3**), (-)-epigallocatechin gallate (**2**) and quercetin-3-*O*-(2"-galloyl)-a-L-arabinopyranoside (**4**) (Figure 1), identified by comparison of their spectroscopic data to those reported in the literature (Binutu & Cordell, 2000; Harborne, 1996; Agrawal, 1989).

DISCUSSION

There is a large number of plant extracts which present antimicrobial activity. *Phyllanthus amarus* extracts possessed antibacterial activity against *Bacillus subtilis, Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella paratyphi* and *Staphylococcus aureus* (Srinivasan et al., 2001). Extracts of *Azadirachta indica*, *Cinnamomum cassia*, *Rumex nervosus*, *Ruta graveolens*, *Thymus serpyllum* and *Zingiber officinale* were active against *Bacillus cereus*, with MIC of 0.16 to 0.66 mg/mL (Alzoreky & Nakahara, 2003). *Hamamelis virginiana* showed antimicrobial activity on *Morganella morganii* with

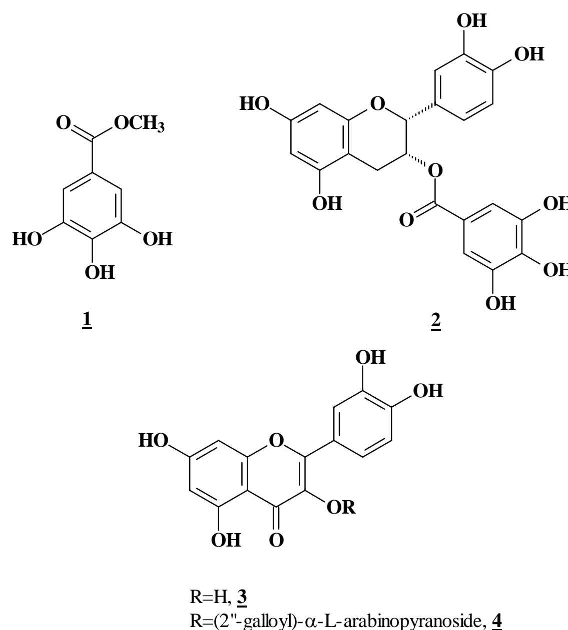

FIGURE 1 - Compounds isolated from the EMeOH of *B. crassa* leaves

MIC 180 µg/mL and the equal values of *H. virginiana* on *S. aureus* 105 µg/mL (Djipa et al., 2000). Martínez-Vasquez et al. (1999) had described the antimicrobial activity of *B. crassifolia*. In this study, we report the antibacterial activity of *B. crassa* leaf extracts. The EMeOH, EMeOH 80%, EtOAc and aqueous fractions were active, showing antibacterial activity against all the tested bacteria and antifungal activity against the yeast (Tables 1 and 2). MIC values are between 1.5 and 12 mg/mL, which are similar to those of plants extracts found in the literature. In some cases we observed that when the disc dosage level increases, the inhibitory effect also increased (Table 1).

Previously, we have reported the isolation of amentoflavone, quercetin-3-*O*-b-D-galactopyranoside, quercetin-3-*O*-a-L-arabinopyranoside, (+)-catechin and (-)-epicatechin from the *B. crassa* leaves (Sannomiya et al., 2004; 2005). Here we are adding the isolation of the minor compounds **1** – **4** of the EtOAc fraction from the EMeOH (Figure 1). In fact, Penna et al. (2001) reported the antimicrobial activity of methyl gallate against *Staphylococcus aureus* with MIC 128 mg/mL. Quercetin showed antibacterial activity against *Staphylococcus aureus, Proteus vulgaris, Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi* and *Enterobacter aerogens* (Basile et al., 2000). Epicatechin and quercetin-3-*O*-b-D-galactopyranoside were active in the inhibition the growth of *Moraxella catarrhalis, Streptococcus pneumoniae* and *Haemophilus influenza* (Pretorius et al., 2003). Thus, the isolated compounds can account to the antibacterial activity *B. crassa* leaves.

Further phytochemical studies are required to establish whelther these compounds are responsible for the bioactivity of this medicinal plant and evaluate the ethnobotanical approach for the screening of plants as potential sources of bioactive substances (Lopez et al., 2001). However, the aforementioned results may suggest that *B. crassa* possess compounds with antibacterial and antifungal properties which can be used as antimicrobial agents in new drugs for therapy of infectious diseases.

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