Artigo

Evaluation of the antifungal activity and mode of action of *Lafoensia pacari* A. St.-Hil., Lythraceae, stem-bark extracts, fractions and ellagic acid

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RESUMO: **"Avaliação da atividade antifúngica e modo de ação dos extratos da entrecasca, frações e ácido elágico de *Lafoensia pacari* A. St.-Hil., Lythraceae,".** Os extratos, frações e ácido elágico, isolados da entrecasca de *Lafoensia pacari* A. St.-Hil., Lythraceae, foram testados *in vitro* para atividade antifúngica, frente a um painel de leveduras, hialo-hifomicetos e dermatófitos, utilizando o método de microdiluição. O extrato EtOH, frações e ácido elágico exibiram atividade contra *Candida* spp. e *Saccharomyces cerevisiae* com valores de CIM entre 250-1000 μg/mL, porém não mostraram ação contra fungos filamentosos e dermatófitos (CIM>1000 μg/mL). Os extratos ativos foram avaliados nos ensaios de inibição do crescimento das hifas de *Neurospora crassa,* no teste do sorbitol, e pelo estudo do efeito do ergosterol na CIM do ácido elágico. Os extratos ativos, frações e ácido elágico mostraram zonas manchadas ao redor dos discos de papel e induziram malformações nas hifas. Além disso, a CIM do ácido elágico contra *Saccharomyces cerevisiae* passou de 62 para 250 μg/mL na presença do sorbitol 0,8 M, sugerindo que o ácido elágico provavelmente poderia exercer ação na parede celular fúngica. Esses resultados indicam que o ácido elágico pode ser o principal composto antifúngico de *Lafoensia pacari*, sugerindo que o modo de ação antifúngico desses extratos e ácido elágico poderia estar associado à inibição da parede celular fúngica.

Unitermos: *Lafoensia pacari,* mangava-brava, atividade antifúngica, extratos e frações, inibição da parede celular, ácido elágico.

ABSTRACT: Stem-bark extracts, fractions and the isolated constituent, ellagic acid of *Lafoensia pacari* St. Hil. (Lythraceae) were *in vitro* assayed for antifungal activity against a panel of yeasts, hialohyphomycetes as well as dermatophytes with the microbroth dilution method. The EtOH extract and its fractions and ellagic acid exhibited activity against *Candida* spp and *Saccharomyces cerevisiae* with MIC values between 250-1000 μg/mL, but they showed no action against filamentous fungi and dermatophytes (MIC>1000 μg/mL). Active extracts were evaluated in *Neurospora crassa* hyphal growth inhibition and sorbitol assays and then the effect of ergosterol on the MIC of ellagic acid was studied. The active extracts and its fractions and ellagic acid showed a blotchy zone around the paper disk and induced malformations of the hypha. Besides, MIC of the ellagic acid against the *Saccharomyces cerevisiae* was raised from 62 to 250 μg/mL in the presence of sorbitol 0.8 M, suggesting that the ellagic acid would probably exert its action on fungal cell wall. These results indicate that ellagic acid might be the main active antifungal compound of *Lafoensia pacari* and further suggest that the mode of antifungal action of these extracts and ellagic acid could be associated with the inhibition of fungal cell wall.

Keywords: *Lafoensia pacari,* mangava-brava, antifungal activity, extracts and fractions, inhibition of cell wall, ellagic acid.

INTRODUCTION

In the last twenty years, there has been a notable increase in fungal infections all throughout the world, more so among the growing number of immunocompromised patients (Quindos, 2002). Due to the ineffectiveness or toxicity of antifungal drugs currently available, treatment of human mycoses is very difficult (Barberán et al., 2008). The mode of action of most known antifungal drugs in use nowadays, is the inhibition of some of the steps of ergosterol biosynthesis, which is common to human cholesterol and sexual hormones biosynthesis too. As a consequence, many adverse effects have been reported for them (Zacchino et al., 2003; Kartsonis et al., 2003). Since fungal but not mammalian cells are encased in a wall, which serves as a protective barrier and is required for growth and viability of fungi, its inhibition represents an ideal mode of action of antifungal agents (Onishi et al., 2000).

In the course of our screening program to look for new antifungal Brazilian plant extracts from the rich biodiversity of Cerrado region (Figure 1), whose mode of action was the inhibition of fungal cell wall, we tested *Lafoensia pacari* A. St. Hil., Lythraceae, a tree commonly known as "mangava-brava" or "pacari". The macerate prepared from the stem bark is a popular remedy for the treatment of wound healing, fever, gastric ulcer, gastritis, utero inflammation and upheavals of biliary vesicle (Guarim Neto & Moraes, 2003). Previous works have shown that *Lafoensia pacari* stem bark ethanol and methanol extracts possess anti-ulcer (Tamashiro-Filho,

1999), anti-inflammatory (Albuquerque, 1996a, Rogerio et al., 2008a), immunostimulating (Albuquerque, 1996b), free radicals scavenging (Solon et al., 2000), antiviral (Müller et al., 2007), anti-eosinophilic (Rogerio et al., 2008b), analgesic and anti-oedematous (Rogerio et al., 2006) and inhibition of interleukin (IL)-5 production (Rogerio et al., 2003) activities. According to Solon (1999), this plant is rich in ellagic acid, which presents gastric anti-secretory (Murakami et al., 1991) and antibacterial effects (Chung, 1998; Lima et al., 2006) and a recent clinical study carried out with *Lafoensia pacari* extract revealed positive effects on the symptomatology of *Helicobacter pylori* infected patients (Mota Menezes et al., 2006), but to the best of our knowledge, there was no published report on the plant's antifungal properties.

We present here the antifungal properties of the different extracts, fractions and ellagic acid of *L. pacari*. At first, we tested them using the microbroth dilution method against a panel of standardized yeasts, filamentous fungi as well as dermatophytes. Then, to gain insight into the mode of action, the whole-cell *Neurospora crassa* hyphal growth inhibition assay, was performed on them. This method is useful to detect agents whose mode of action is associated with inhibition of the fungal cell wall (Fukuda et al., 1991). In addition, since fungal cells shows changes in morphological features after treatment with drugs that interfere with the biosynthesis of fungal wall (Gunji et al., 1983; Fukushima et al., 1993), the microscopical appearance of fungal hypha treated with extracts of *L. pacari* is also described.

Figure 1. Cerrado region within the State of Mato Grosso where *Lafoensia pacari* A. St. Hil. were collected. Source: Embrapa, Cenargen, 1999.

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MATERIAL AND METHODS

Plant material and phytochemical analysis

Lafoensia pacari A. St.-Hil., Lythraceae, stem bark was collected (675 g) in Várzea Grande Municipality of Mato Grosso State, Brazil, and its botanical identity was confirmed by Harri Lorenzi of the Plantarum Institute, in Nova Odessa-SP, Brazil. A voucher specimen (Nº. 35577) was deposited at the Central Herbarium of Federal University of Mato Grosso. The collection was authorized by the Brazilian Institute of Environment and Renewable Natural Resources.

The stem bark was dried at 40 ºC for three days to constant weight (520 g) and later triturated. The dried powdered material (200 g) was macerated successively with n-hexane, dichloromethane, ethyl acetate and ethanol 75% (3:1 w/v) at room temperature for seven days to yields residues of 115.80; 377.70; 758.00 mg and 6.52 g, respectively. Each macerate was separated by filtration and concentrated under reduced pressure. Active ethyl acetate extract was fractionated with methanol (6.4 g) and acetone (436 mg). The fractions of methanol and acetone had been obtained by chromatography in column (CC), using silica Gel 60 (70-230 mesh) (Cechinel Filho & Yunes, 1998), while the phytochemical analysis was performed according to literature procedure, such as colorations and precipitations tests (Matos, 1988).

Isolation and identification of ellagic acid

Ellagic acid was detected by thin layer chromatography (TLC) in both fractions (acetone and methanol), but in more concentration on methanol fraction. A part of a last (500 mg) was chromatographed several times over Sephadex LH-20, eluted with MeOH:H$_2$O gradient, giving 25 mg of pure ellagic acid. It was identified by usual spectroscopic methods (IR, RMN ^{1}H and ^{13}C) and direct comparison with an authentic sample.

Microorganisms

Antifungal activity was studied using microorganisms from the American Type Culture Collection (ATCC, Rockville, MD, USA) or clinical isolates provided by the Centro de Referencia en Micología (C, Ceremic), Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531, (2000) Rosario, Argentina and the Institute Adolfo Lutz, São Paulo, Brazil (IAL): *Candida albicans* ATCC 10231, *Candida krusei* ATCC 6258, *Candida tropicalis* ATCC 750, *Candida parapsilosis* ATCC 22019, *Candida glabrata* ATCC 90030, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, *Neurospora crassa* ATCC 9279, *Aspergillus niger* ATCC 16404, *Candida lusitanea*e C131, *Candida kefyr* C123 and *Candida colliculosa* C122, *Aspergillus flavus* IAL

552, *Aspergillus fumigatus* IAL 640, *Microsporum canis* IAL 578, *Microsporum gypseum* IAL 579, *Trichophyton rubrum* IAL 612, *Trichophyton mentagrophytes* IAL 581, *Trichophyton tonsurans* IAL 592 and *Epidermophyton floccosum* IAL 577. The strains were maintained on slopes of Sabouraud-dextrose agar (Oxoid) and subcultured every fifteen days to prevent pleomorphic transformations.

Antifungal assays

Agar diffusion assay

The agar diffusion assay described for Clinical and Laboratory Standards Institute (CLSI), formerly National Committee for Clinical and Laboratory Standards (NCCLS) (CLSI M44-A, 2002) was adopted. Petri dishes has been prepared with Müller-Hinton agar (Oxoid) supplemented with 2% of glucose (Oxoid) and inoculated in its surface 10^4-10^6 yeast-cells or spores/mL. The compound tests had been dissolved in DMSO 1% to prepare stock solution. Records of barren paper had been impregnated with the test solution to reach final concentration up to 100 μg of extract/disk, being 30 μg for ketoconazol and 100 μg for amphotericin B, used as standards. Dimethyl sulfoxide (DMSO) 15 μL served as negative control. The assays had been carried through in duplicate and the results expressed in mm, using arithmetic mean of the diameter of growth inhibition zones formed around the disks. This assay was carried out in duplicate.

Minimal inhibitory concentration (MIC)

MIC values were determined using microplates of 96 wells following the guidelines of CLSI, M27-A2 for yeasts and M38-A for filamentous fungi. All guidelines were followed with the exception of the incubation temperature, which was recommended as 35 ºC by CLSI for both type of fungi but we used 30 ºC. This change was made in order to allow the growth of all type of fungi, since not all dermatophytes grow at 35 ºC. Stock solutions of extracts, fractions and ellagic acid diluted in DMSO were added to each medium to give serial two-fold dilutions, resulting in concentrations ranging from 1000 to 8 μg/mL for extract, fractions and ellagic acid. Inocula of 100 μL (10^3-10^4 CFU/mL) were added to RPMI-1640 broth medium (Sigma). Amphotericin B (Sigma) and terbinafine were used as positive controls. Plates were incubated for 24, 48 or 72 h at 30 ºC (according to the control fungus growth) up to fifteen days for dermatophyte strains. MIC was defined as the lowest concentration of extracts and fractions capable to the total inhibition of the growth of fungi. All antifungal assays were tested in duplicate.

Neurospora crassa assay

Thirty mL of a medium containing 0.5% proteose

peptone (Difco), 0.5% yeast extract (Merck), 4% sucrose (Merck) and 1.5% agar (Merck) was autoclaved (121 ºC, 15 min), inoculated with 25 µL of spore inoculum of *Neurospora crassa* (ATCC 9279) 1x 10^7 free of hypha at 40 ºC and then layered on Petri dish (diameter, 9 cm). After the medium has solidified, paper disks containing 100 µg of extract/disk were applied into media. Ketoconazol (30 µg - CECON, São Paulo, Brazil) was used as positive control to produce a clear zone. Zones of inhibition were examined macroscopically for hazy or mottled appearance following incubation of the plates at room temperature for 24 h with light. In those cases where hazy zones were observed, the microscopic appearance of hypha were analysed.

Sorbitol assay

The MIC of ellagic acid was determined with *Saccharomyces cerevisiae* ATCC 9763 using the broth microdilution method (CLSI M27-A2). Sorbitol was added to the culture medium to give a final concentration of 0.8 M. Following incubation at 25 ºC, the plates were read at 48 h and after seven days (Frost et al., 1995). This assay was carried out in duplicate.

Effect of ergosterol in MIC of ellagic acid

The MIC of the ellagic acid against *Saccharomyces cerevisiae* ATCC 9763 was determined by microdilution method (CLSI M27-A2), without or with the addition of ergosterol (Sigma) at concentrations of 50, 100 and 200 µg/mL This assay was carried out in duplicate.

RESULTS AND DISCUSSION

The antifungal property of extracts, fractions and ellagic acid was evaluated with the broth dilution method following the guidelines of the Clinical and Laboratory Standards Institute for yeasts (CLSI M27-A2) and filamentous fungi (CLSI M38-A) against a panel of yeast, hyalohyphomycetes as well as dermatophytes. MIC values ≤ 1000 µg/mL were considered active (Table 1).

Disks containing 100 µg of compound tests had been used for the agar diffusion assay. Zones of inhibition ≥ 10 mm were considered active. The results of disk-diffusion had evidenced antifungal activity only for the yeast-cells, thus showing selectivity for yeasts. The *n*-hexane and

Table 1. Antifungal activity of extracts, fractions and ellagic acid from *Lafoensia pacari* against yeasts, filamentous fungi and dermatophytes in broth dilution assays.

Strains	Amphotericin B	Terbinafine	Ellagic acid and extracts				Fractions from TOH	
			Ellagic acid	Hexane	DCM	EtOH	MeOH	Acetone
C. albicans	0.78[a]	_	500	> 1000	> 1000	> 1000	1000	1000
C. krusei	1.0	_	125	> 1000	> 1000	1000	250	500
C. parapsilosis	0.25	_	125	> 1000	> 1000	500	500	250
C. tropicalis	1.56	_	125	> 1000	> 1000	> 1000	500	1000
C. glabrata	0.5	_	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
C. lusitaneae	1.0	_	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
C. kerfyr	0.5	_	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
C. colliculosa	1.0	_	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
S. cerevisiae	0.78	_	62	> 1000	> 1000	500	250	250
C. neoformans	0.78	_	> 1000	> 1000	> 1000	1000	> 1000	> 1000
A. niger	0.78	_	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
A. flavus	0.78	_	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
A. fumigatus	3.12	_	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
M. canis	_	0.01	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
M. gypseum	_	0.04	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
T. rubrum	_	0.01	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
T. mentagrophytes	_	0.04	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
T. tonsurans	_	0.02	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
E. floccosum	_	0.004	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000

[a]Results are presented as MIC (µg/mL). DCM: dicloromethane; EtOH: ethanol; EtOAc: ethyl acetate; MeOH: methanol.

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dichloromethane extracts were not active against the other members of the fungal panel (Table 2). Results of broth dilution assays (Table 1) showed that only the EtOH extract and ellagic acid possesses antifungal activity. They inhibit the growth of yeasts *Candida krusei, Candida parapsilosis* and *Saccharomyces cerevisiae* with MIC values between 500-1000 µg/mL, but showed no efficacy against filamentous fungi and dermatophytes (MIC>1000 µg/mL). The active extract was fractionated between acetone and MeOH and the fractions and ellagic acid were tested in the panel of fungi. Both fractions and ellagic acid showed better activity than EtOH extract against *Candida krusei, Candida parapsilosis* and *Saccharomyces cerevisiae* (MIC = 250-500 µg/mL) and in addition they were also found to be active against *Candida albicans* (MIC = 1000 µg/mL) and *Candida tropicalis* (MIC = 500-1000 µg/mL).

To gain insight into the mode of action of active extracts and fractions, they were evaluated for their capacity to interfere with fungal cell wall. Amongst methods that deal with this mode of action, we have chosen the *Neurospora crassa* assay, an agar diffusion method which allows us to detect macroscopically drugs that interfere with the biosynthesis or assembly of the fungal cell wall. *Neurospora crassa* usually grows as long hypha in a diffuse or branched way. When it grows in the presence of certain inhibitors of the cell wall, the hyphal growth is inhibited, and fungi grow as protoplasts. Macroscopically they can be seen as a blotchy or hazy appearance around the paper disk. The microscopic observation of this hazy zone showed morphological changes (Fukushima et al., 1993) such as shortened, anomalously ramified and zig-zag hypha, suggesting that the possible mode of antifungal action of these extracts, fractions and ellagic acid could be associated with the inhibition of fungal cell wall. Results obtained in the *Neurospora crassa* assay for active extracts, fractions and ellagic acid of *Lafoensia pacari* are shown on Table 3 and Figure 2.

The sorbitol assay consists of determining MIC in the presence and absence of sorbitol 0.8 M, which has an osmoprotector function and is essential for fungal growth. Fungal cells that lack cell wall or with impaired cell wall cannot grow in the absence of sorbitol. However, its growth will still be possible if sorbitol is supplemented. The inhibitors of the fungal cell wall could be identified when the MIC values obtained with sorbitol are higher than the ones in its absence after seven days of incubation (Frost et al., 1995). In the present assays, MIC of the ellagic acid against *Saccharomyces cerevisiae* was raised from 62 to 250 µg/mL in the presence of sorbitol 0.8 M, suggesting that the ellagic acid would act modifying the fungal cell wall, while the MIC of the amphotericin B was not modified, as expected.

To verify whether or not ellagic acid acts as an antifungal agent by acting on ergosterol, one observed the values of the MIC of same in the presence of increasing concentrations of ergosterol. If this mechanism operates, the MIC of the test compounds will be much higher in the presence of ergosterol than that in its absence (Lee et al., 1999). The results had shown that MIC of amphotericin B against *Saccharomyces cerevisiae* increased with the change to addition of ergosterol, while that of the ellagic acid remained unaltered, suggesting a different mode of action.

Extracts, fractions and ellagic acid of *Lafoensia pacari* evidenced good antifungal activity against *Saccharomyces cerevisiae* and *Candida* spp. and within this genus, mainly against *Candida* no-*albicans* such as *Candida krusei* and *Candida parapsilosis.* As it is well-known, *Candida* spp., are among the leading cause of nosocomial blood stream infections worldwide. In the past, *Candida albicans* was the usual species associated with invasive mycoses. At present, there is an epidemiological shift towards *Candida krusei* and other *Candida* no-*albicans* infections, which have increased in many medical centers (Fromtling, 1999). In aggravation, they are resistant to most available antifungal drugs (Grau Cerrato et al., 1996).

Phytochemical investigation with the methanol stem bark extract of *Lafoensia pacari* revealed the presence of tannins, steroids, triterpenes and saponins, which

Table 2. Antifungal activity of extracts, fractions and ellagic acid of *Lafoensia pacari* against yeasts in agar diffusion assay.

Plant deriveds and standard	µg/disk	Inhibition halos (mm)					
		Strain					
		Ca	Ck	Cp	Ct	Sc	Cn
Ethanol extract	100	---	12 [a]	10	---	09	10
Ethyl acetate extract	100	---	10	10	---	08	12
Methanol fraction	100	10	11	11	11	15	---
Acetone fraction	100	10	13	16	12	15	---
Ellagic acid	100	13	15	20	13	17	15
Amphotericin B	100	25	11	30	20	20	20

[a] Inhibition halos (mm); *C. albicans*; Ck: *C. krusei*; Cp: *C. parapsilosis*; Ct: *C. tropicalis*; Sc: *S. cerevisiae*; Cn: *C. neoformans*; ---: not observed.

contains several secondary metabolites well-known for their antimicrobial activity (Solon et al., 2000). Solon et al. (2000), using HPLC analysis and analytical TLC showed that ellagic acid is the main constituent of *Lafoensia pacari*. Similar results were evidenced in our study, which showed that ellagic acid is responsible, at least in part, for the antifungal activity of *Lafoensia pacari*.

The antifungal activity of extracts, fractions and an active compound (ellagic acid) from *Lafoensia pacari* towards *Candida* spp., and the probable selective target of action (fungal cell wall) evidenced from the results obtained in *Neurospora crassa* assay make this plant an attractive Brazilian species for more advanced antifungal targeted tests.

Therefore, the correlation of data obtained with traditional indication of *Lafoensia pacari* stem bark preparations can contribute for the treatment of the different diseases caused by fungi.

Table 3. *Neurospora crassa* assay of extracts, fractions and ellagic acid from *Lafoensia pacari* stem bark.

Plant deriveds and standard	*Neurospora crassa* assay[a]
Ethyl acetate extract	Hazy
Ethanol extract	Hazy
Methanol fraction	Hazy
Acetone fraction	Hazy
Ellagic acid	Hazy
Ketoconazol	Clear

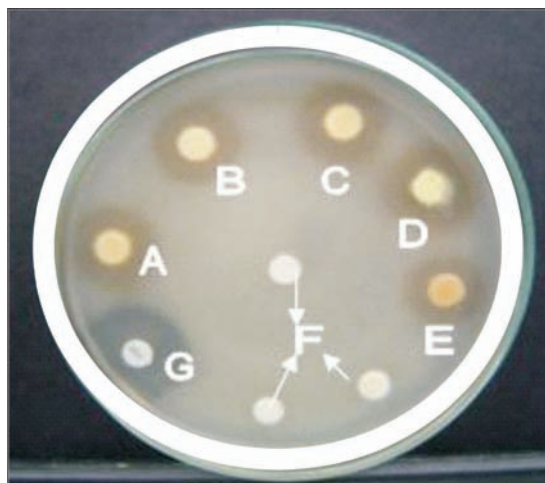[a]Aspect of the inhibition zone

Figure 2. Mottled halos showed by extracts and fractions and ellagic acid from *Lafoensia pacari* and clear halos obtained with ketoconazol in the *Neurospora crassa* assay. (A and B) EtOH extract, (C) acetone fraction, (D) MeOH fraction, (E) Ellagic acid, (F) DMSO and (G) ketoconazol.
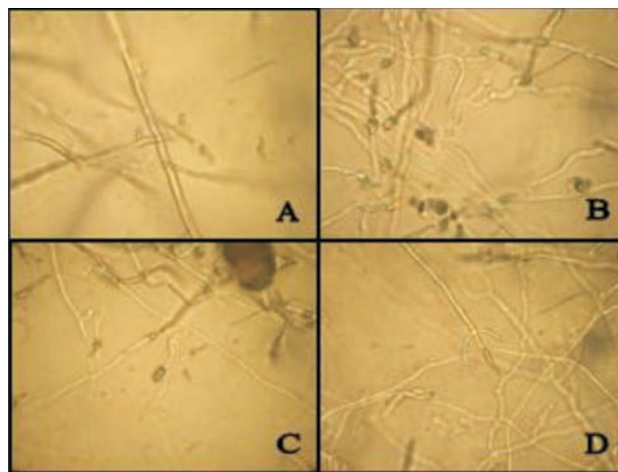

Figure 3. Effect of ellagic acid of *Lafoensia pacari* on the morphology of *Neurospora crassa.* (A) normal growth, (B) hypha in zig-zag, (C) short hypha, (D) anomalous ramified hypha.

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