Communication/Comunicação

In vitro antifungal activity of fatty acid methyl esters of the seeds of *Annona cornifolia* A.St.-Hil. (Annonaceae) against pathogenic fungus *Paracoccidioides brasiliensis*

Atividade antifúngica *in vitro* dos ésteres metílicos de ácidos graxos das sementes de *Annona cornifolia* A.St.-Hil. (Annonaceae) sobre o fungo patogênico *Paracoccidioides brasiliensis*

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ABSTRACT

Introduction: Fatty acids are abundant in vegetable oils. They are known to have antibacterial and antifungal properties. **Methods**: Antifungal susceptibility was evaluated by broth microdilution assay following CLSI (formerly the NCCLS) guidelines against 16 fungal strains of clinical interest. **Results**: In this work, fatty acid methyl esters (FAME) was able to inhibit 12 clinical strains of the pathogenic fungus *Paracoccidioides brasiliensis* and were also active in the bioautographic assay against *Cladosporium sphaerospermum*. **Conclusions**: FAME was a more potent antifungal than trimethoprim-sulfamethoxazole against *P. brasiliensis* under the experimental conditions tested.

Keywords: Fatty acid methyl esters. Antifungal activity. *Paracoccidioides brasiliensis.*

RESUMO

Introdução: Os ácidos graxos são abundantes em óleos vegetais. Eles são conhecidos por suas propriedades antibacterianas e antifúngicas. **Métodos:** A suscetibilidade a antifúngicos foi avaliada pelo ensaio de microdiluição em caldo de acordo com CLSI (anteriormente NCCLS) sobre 16 isolados de interesse clínico. **Resultados:** Nesse trabalho, os ésteres metílicos de ácidos graxos (FAME) inibiram doze isolados clínicos do fungo patogênico *Paracoccidioides brasiliensis*, e também foi muito ativo no ensaio de bioautografia sobre o fungo *Cladosporium sphaerospermum*. **Conclusões:** FAME foi um antifúngico mais potente do que sulfametoxazol-trimetoprim contra *P. brasiliensis*, nas condições utilizadas no presente trabalho.

Palavras-chaves: Ésteres metílicos de ácidos graxos. Atividade antifúngica. *Paracoccidioides brasiliensis.*

Fungal infections have increased significantly, contributing to the cause of morbidity and mortality. The increase in antimicrobial resistance and populations of patients at risk, in conjunction with the restricted number of commercially available antifungal drugs that still present many side effects, are the cause for this problem[1]. These limitations emphasize the need to develop new and more effective antifungal agents. Natural products are attractive prototypes for this purpose due to their broad spectrum of biological activities. Although most antibiotics in clinical use have been obtained from microorganisms, a renewed interest in plant antimicrobials has emerged in the last 20 years[2]. The use of extracts or isolation of their compounds is an important tool, especially when the mycosis is difficult to treat, such as paracoccidioidomycosis (PCM). PCM is a systemic endemic disease that affects at least 10 million people in Latin America and it is the 8th most common cause of death due to chronic/recurrent infections and parasitic diseases in Brazil[3].

Fatty acids are widely distributed in vegetable oils and they play an important role as nutritious substances and metabolites in living organisms. Many fatty acids are known to have antibacterial and antifungal properties[4]. However, little is known regarding the antifungal property of *A. cornifolia*. In this paper, our group presents data on the fatty acid methyl esters (FAME) of *A. cornifolia* seeds and their antifungal activity.

Fruits of *Annona cornifolia* A. St. -Hil. were collected in the town of Curvelo, State of Minas Gerais, Brazil, from January to March 1998. The species was identified by Dr. R. Mello-Silva and a voucher specimen (BHCB 68114) was deposited at the Herbarium of the Institute of Biological Sciences of the Federal University of Minas Gerais (*Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais*, ICB-UFMG), Belo Horizonte, MG, Brazil. The fruits were open, the seeds removed, washed, dried at 40°C, powdered (850g) and extracted exhaustively with hexane to achieve extract F01 (79.5g), which was then fractionated by column chromatography on silica gel using gradient systems of n-hexane/dichloromethane/ethyl acetate/methanol in increasing polarities; a fraction rich in fatty acids (F01-1, 53.2g) was isolated. Part of this fraction (500mg) was refluxed with 1.0mol/l methanolic sodium hydroxide solution for 30min and then extracted with ethyl ether. The aqueous phase was acidified with 1.0mol/l hydrochloric acid solution, extracted with ethyl ether. The organic phase was dissolved in hexane and then refluxed with 2% sulfuric acid methanolic solution v/v for 1h. The fraction rich in fatty acid methyl esters (FAME, 370mg) was obtained after extraction and solvent elimination.

Cladosporium sphaerospermum CCT 1740 was used in the bioautographic assay, which was performed using the procedure described by Homans and Fuchs[5].

Sixteen fungi strains, which included *Paracoccidioides brasiliensis* strains, Pb-01 (ATCC-MYA-826), Pb-18 (Fungi Collection of the

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São Paulo University School of Medicine, São Paulo, SP, Brazil), Pb-B339 (ATCC 32069), Pb-14 (clinical isolate from acute PCM, São Paulo, Brazil), Pb-3 and Pb-4 (clinic isolates from chronic PCM, São Paulo, Brazil-MHH Forjaz/TIE Svidzinski), Pb-2 (Epm 60), Pb-1578, Pb-ED01, Pb-11, Pb-9673 (clinic isolates from acute PCM, Paraná, Brazil, TIE Svidzinski) were used in the biological assays. *Candida albicans* ATCC 18804, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019 and *Cryptococcus gattii* ATCC 32608 were used in the biological assays. The strains of *P. brasiliensis* were maintained by continuous passages in YPD (yeast, peptone and dextrose) medium at 37ºC. The fungi were used after 7-10 days of growth. The species of *Candida* and *Cryptococcus* were maintained on Sabouraud dextrose agar (Oxoid, Basingstoke, UK) at 4ºC and transfers were performed at three-month intervals.

Suspensions from the cultures of the *P. brasiliensis, Candida* spp. and *C. gattii* were prepared according to both the CLSI M27-A2 document (formerly the NCCLS)[6] and modification suggested by Johann et al[7] to obtain a final suitable inoculum dilution for each strain. For *P. brasiliensis*, yeast cells in the exponential phase were collected aseptically with a platinum loop and resuspended in a tube containing 5ml of sterile saline. When large aggregates existed, they were allowed to settle for several minutes and the supernatants were collected. The transmittance of the suspension was measured at a wavelength of 530nm after homogenization by vortexing and adjusted to 70%. A 0.1-ml aliquot of this suspension was then added to 0.9-ml of RPMI medium (Sigma, St. Louis, MO, USA). The fungal cultures of *P. brasiliensis* had a final inoculum of 10^4 cells/ml, and the *Candida* spp. and *C. gattii* cultures had a final inoculum of $1.5 + 1.0 \times 10^3$ cells/ml (formerly the NCCLS)[6].

The minimal inhibitory concentrations (MIC) was obtained for broth microdilution testing performed in accordance with the guidelines described in the CLSI M27-A2 document (formerly the NCCLS)[6] and the assays with *P. brasiliensis* were performed in accordance with Johann et al[7]. Amphotericin B (Sigma, St Louis, USA) and trimethoprim-sulfamethoxazole (80mg/400mg) (Ducto, Goias, Brazil) were included as positive antifungal control, using stock solutions prepared in dimethysulfoxide and water, respectively. RPMI medium (Sigma, St. Louis, MO, USA) was used without compounds or solvents as a control for growth and sterility. Solvent DMSO at the same volumes used in the assay was used as control for toxicity. After inoculation of *Candida* spp. and *C. gattii* plates were incubated at 37°C for 48h for *Candida* spp. and 72h for *C. gattii*. For *P. brasiliensis* strains, the plates were incubated at 37°C for 10 days. The endpoints were determined visually by comparison with the drug-free growth control well. The MIC values were expressed in μg/ml and correspond to the lowest concentrations that did not permit detection of any visual fungal growth.

The minimal fungicidal concentration (MFC) values for pure compounds were determined as follows: from the microtiter plate used to determine the MIC values, the test wells that showed a) complete fungal growth inhibition (clear wells), b) growth similar to that of the no-drug control well, and c) growth control wells. The *in vitro* MFC of each compound tested was determined by streaking 10μl from each well that showed complete inhibition (100% inhibition or a clear well) and from the growth control well onto YPD plates for *P. brasiliensis* and Sabouraud dextrose agar (Oxoid, Basingstoke, UK) for *Candida* spp. and *C. gattii*. The plates were incubated at 37ºC for 10 days for *P. brasiliensis* and 48 and 72h

at 37ºC for *Candida* spp. and *C. gattii*, respectively. The MFC was determined as the lowest drug concentration at which fewer than three colonies were able to grow[8].

To explore the possibility of assessment of drug interactions, the following processes were performed: eight serial dilutions of FAME (300-2.34μg/ml) and amphotericin B (1-0.008μg/ml) were prepared with the same solvents and medium (Synthetic RPMI/Sigma, St. Louis, MO, USA) with L-glutamine buffered to pH 7.0 with 0.165 morpholine propanesulfonic acid (MOPS, Sigma) used in the MIC test. Fifty microliter aliquots of each dilution of FAME were added to the wells of 96-well plates in vertical orientation and 50 microliters of each amphotericin B dilutions were added in horizontal orientation so that the plate contained various concentration combinations of the two compounds (amphotericin B and FAME). A 100μl suspension of Pb-18, same used in MIC test, was added to each well and cultured for 14 days. The drug combination in which the growth was completely inhibited was taken as effective MIC for the combination. Fractional inhibitory concentration (FIC) of amphotericin B was calculated as the MIC of amphotericin B in the presence of FAME divided by the MIC of amphotericin B alone. The FIC of FAME was calculated in the same way for amphotericin B. The fractional inhibitory concentration index (FICI) was calculated by adding both FICs and was interpreted as a synergism effect when it was ≤ 0.5, as additive or no interaction when it was ≤0.5 to 2.0 and as antagonistic when it was >2.0. This experiment was also performed to determine the effect of the combination FAME with trimethoprim-sulfamethoxazole (dilutions 600-4.6μg/ml)[9].

Gas chromatography analysis of the fraction rich in methyl esters from seeds of *Annona cornifolia* revealed a higher percentage of unsaturated fatty acids (71.4%). Among the latter, oleic acid (51.5%) and linoleic acid (19.1%) showed the highest quantities. Among the saturated fatty acids, palmitic acid was the most abundant (16.9%), followed by stearic acid (5.6%) (**Table 1**).

TABLE 1 - Fatty acid composition and percentages of oils from the fraction rich in methyl esters of *Annona cornifolia*.

Peak (n)	Fatty acids in methyl esters form	Relative percentage
1	methyl myristate	0.2
2	methyl palmitate	16.9
3	methyl stearate	5.6
4	methyl oleate	51.5
5	methyl linoleate	19.1
6	methyl linolenate	0.8
Total		**94.1**

FAME: fatty acid methyl esters

The antifungal activity of FAME obtained from the seeds of *Annona cornifolia* was evaluated against 12 clinical isolates of *P. brasiliensis*, *C. albicans, C. tropicalis, C. parapsilosis* and *C. gattii*. Analysis of the results showed that *C. albicans, C. tropicalis, C. parapsilosis* and *C. gattii* strains were not affected by FAME (**Table 2**). For *C. sphaerospermum*, the MIC value was 12.5μg/ml. *P. brasiliensis* isolates were more susceptible to FAME than trimethoprim-sulfamethoxazole, with MIC values that varied from 3.4 to 55.5μg/ml. The isolates Pb-2, Pb-8 and Pb-B339 were more sensitive to FAME compared with other isolates of *P. brasiliensis* (MIC 3.4, 6.9 and 6.9μg/ml, respectively). Pb-01 was the most resistant isolate to FAME (55.5μg/ml). Pb-18 and Pb-18 virulent showed the same results for the MIC test,

TABLE 2 - Minimal inhibitory concentration and minimal fungicidal concentration of FAME from *Annona cornifolia* against 16 clinical important fungi.

	FAME		Amphotericin B		Trimethoprim-sulfamethoxazole	
	MIC	MFC	MIC	MFC	MIC	MFC
Fungi	(µg/ml)	(µg/ml)	(µg/ml)	(µg/ml)	(µg/ml)	(µg/ml)
Candida albicans	≥ 500	-	1.0	1.0	-	-
Candida tropicalis	≥ 500	-	0.25	0.25	-	-
Candida parapsilosis	≥ 500	-	0.5	0.5	-	-
Cryptococcus gattii	≥ 500	-	1.2	1.2	-	-
Paracoccidioides brasiliensis Pb-18	27.7	27.7	0.250	0.250	300	300
Paracoccidioides brasiliensis ED01	13.8	13.8	0.031	0.031	75	75
Paracoccidioides brasiliensis 2	3.4	3.4	0.031	0.031	150	150
Paracoccidioides brasiliensis 11	13.8	13.8	0.031	0.031	150	150
Paracoccidioides brasiliensis Pb-01	55.5	55.5	0.125	0.125	300	300
Paracoccidioides brasiliensis B339	6.9	6.9	0.062	0.062	75	75
Paracoccidioides brasiliensis 3	13.8	13.8	0.015	0.015	300	300
Paracoccidioides brasiliensis 14	27.7	27.7	0.125	0.125	75	75
Paracoccidioides brasiliensis 8	6.9	6.9	0.062	0.062	300	300
Paracoccidioides brasiliensis 1578	13.8	13.8	0.062	0.062	75	75
Paracoccidioides brasiliensis 4	27.7	27.7	0.062	0.062	150	150
Paracoccidioides brasiliensis Pb-18 virulent	27.7	27.7	0.062	0.062	300	300

FAME: fatty acid methyl esters, MIC: minimal inhibitory concentration, MFC: minimal fungicidal concentration.

showing that FAME presents activity against virulent isolates. FAME showed MFC results equivalent to MIC results (**Table 2**). Activity against *P. brasiliensis* and not *Candida* spp. and *Cryptococcus* could be explain by the fact that *Candida* (Saccharomycetales), *Cryptococcus* (Tremelalles) and *P. brasiliensis* (Onygenalles) are phylogenetically quite distant, presenting many morphological and physiological differences. The specific activity against *P. brasiliensis* also suggests the selectivity of natural products.

Few works reporting the biological activity of natural products against the pathogenic fungus *P. brasiliensis* were available in the literature. The natural product (*R*)-goniothalamin and its synthetic enantiomer were evaluated against a panel of microorganisms that included three strains of *P. brasiliensis* (Pb-01, Pb-18, and Pb-B339). Their MIC values ranged between 7 and 22µg/ml on *P. brasiliensis*[10]. In this study, FAME from *A. cornifolia* showed activity similar to enantiomers goniothalamin against *P. brasiliensis* isolates. In another experiment, Campos et al[11] tested the *in vitro* susceptibility of 11 clinical *P. brasiliensis* isolates to trichothecenes from *Fusarium* sp. The results of the study show that all the isolates were susceptible to T2-toxin and a mixture of 8-n-butyryl-neosolaniol and 8-isobutyryl-neosolaniol.

Silva et al[12] verified the susceptibilities of virulent *P. brasiliensis* (Pb-18) for amphotericin B, fluconazole, itraconazole, sulfamethoxazole and ketoconazole. The MIC value for amphotericin B was higher (0.5µg/ml) than that determined in the present work (0.062µg/ml) for virulent Pb-18. However, the value observed for the nonvirulent Pb-18 strain (0.25µg/ml) was much closer to the value obtained by Silva et al[12]. Nakai et al[13] also tested amphotericin B against seven isolates of *P. brasiliensis* and the MIC values ranged between 0.0078 and 0.25µg/ml, while in the present work, results for MIC values ranged between 0.015 and 0.25µg/ml. This can be explained by the fact that different isolates of *P. brasiliensis* were used.

For sulfamethoxazole, Silva et al[12] showed a MIC value of 300µg/ml. Despite the obtaining equivalent results in the present work (300µg/ml), comparison was not possible because our group used

sulfamethoxazole combined with trimethoprim and a different culture medium. Using MVM medium, Stevens & Phuoc[14] determined MIC values for sulfamethoxazole-trimethoprim (5:1) that were lower than that determined in the present work using RPMI medium. In another study[15] involving determination of MIC for sulfamethoxazole-trimethoprim (5:1) using the MVM medium, the values obtained were very similar with those reported in the present work (320µg/ml). Stevens & Phuoc[14] used four different isolates of *P. brasiliensis* and observed great variation in the MIC values (ranging between 0.9 and 46.9µg/ml) for sulfamethoxazole-trimethoprim (5:1). Stevens & Phuoc[14] also observed variation among the isolates treated only with sulfamethoxazole, with MIC values of 0.97µg/ml for isolate 262 and ≥ 2.000µg/ml for isolates 268, 263 and 264. These results show that great variation occurs among different isolates of *P. brasiliensis*. The *P. brasiliensis* isolates used by Stevens & Phuoc[14] were different from the isolates used in the present work and this could explain the differences observed in the MIC values between these studies. In addition the authors cited above used only a few isolates of *P. brasiliensis*.

To explore the possibility of developing a more powerful combination therapy of FAME with amphotericin B and with trimethoprim-sulfamethoxazole, the checkerboard microtiter test was performed with combined samples. **Table 3** demonstrates the occurrence of an additive effect of FAME with amphotericin B. This

TABLE 3 - Fractional inhibiting concentration and fractional inhibitory concentration index of fatty acid methyl esters from *Annona cornifolia* against *Paracoccidioides brasiliensis* isolate Pb-18.

	MIC in combination		
Compounds	(µg/ml)	FIC	FICI
1. FAME	27.7	1	1.5
2. Amphotericin B	0.125	0.5	
1. FAME	0.86	0.03	2.03
2. Trimethoprim-sulfamethoxazole	600	2	

MIC: minimal inhibitory concentration, FIC: fractional inhibiting concentration, FICI: fractional inhibitory concentration index, FAME: fatty acid methyl esters.

is an important observation, as amphotericin B is known for its significant nephrotoxicity, indicating that following further assays with FAME, this drug could be used in combination in order to reduce the amount of amphotericin B administered.

Many natural products are useful as drugs or as biochemical tools to investigate disease processes. The FAME obtained from *Annona cornifolia* were tested for the first time against the pathogenic fungus *P. brasiliensis*. The results showed that this compound is active against several clinical strains of this fungus and opens the possibility for discovery of new compounds to treat paracoccidioidomycosis.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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