Antifungal activity of *Eugenia uniflora* L. fractions against *Paracoccidioides brasiliensis* (Splendore) Almeida

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RESUMO: Atividade antifúngica de frações de *Eugenia uniflora* L. contra *Paracoccidioides brasiliensis*. O fracionamento biomonitorado do extrato bruto etanólico (EBE) das folhas de *Eugenia uniflora* foi conduzido frente ao fungo patogênico dimórfico *Paracoccidioides brasiliensis*. Os bioensaios, utilizando-se técnica de diluição em ágar, demonstraram atividade fungitóxica do EBE com uma concentração inibitória mínima (CIM) de 750 mg.mL^{-1}. Após fracionamento por partição com solventes, obtiveram-se as frações etérea, acetato de etila e aquosa. Dessas, apenas a fração etérea inibiu completamente o fungo (CIM de 187,5 mg.mL^{-1}). Essa fração foi refracionada por cromatografia em coluna sob vácuo fornecendo quatro subfrações. As subfrações F1 e F2 apresentaram valores de CIM de 375 mg.mL^{-1}, enquanto que F3 apresentou uma CIM de 500 mg.mL^{-1}. Análise por CG/EM de F1 e F2 indicou a presença de sesquiterpenos oxigenados. Cromatografia em camada delgada sugeriu que F3 contém terpenoides e um flavonóide, enquanto que F4 (inativa) contém apenas flavonóides. Pela composição química das subfrações sugere-se que os componentes ativos sejam terpenóides, principalmente sesquiterpenos oxigenados. Adicionalmente, observou-se uma diminuição da bioatividade da fração etérea em comparação às subfrações, sugerindo sinergismo entre as substâncias bioativas.

Palavras-Chave: *Eugenia uniflora*, pitangueira, *Paracoccidioides brasiliensis*, sesquiterpenos, plantas medicinais.

ABSTRACT: The Bioassay-directed fractionation has investigated the activity of *E. uniflora* leaves crude extract against the dimorphic fungus *Paracoccidioides brasiliensis* using the agar dilution method. The ethanolic crude extract inhibited the fungal growth at 750 mg.mL^{-1}. Fractionation by partition between solvents yielded three fractions. The ether fraction showed the best activity with minimal inhibitory concentration (MIC) at 187.5 mg.mL^{-1}. This fraction was refractionated by vacuum liquid chromatography, affording four sub-fractions. F1 and F2 achieved MIC at 375 mg.mL^{-1}, while F3 MIC at 500 mg.mL^{-1}. GC-MS analysis of F1 and F2 have indicated the presence of mainly oxygenated sesquiterpenes. TLC analysis of F3 and F4 (inactive), suggested the presence of terpenoids and flavonoids for the first and only flavonoids for the later. The chemical constituents of the ether fraction are terpenoids, mainly oxygenated sesquiterpenes, which probably are the responsible for the antifungal activity. Furthermore, the reduction in the antifungal activity indicated a synergism among the bioactive compounds.

Kew words: *Eugenia uniflora*, pitangueira, *Paracoccidioides brasiliensis*, sesquiterpenes, plants medicinal.

INTRODUCTION

Eugenia uniflora L. (Myrtaceae) is a shrubby tree from Brazil edible cherry-like fruits, in Brazil called "pitangueira" (Corrêa, 1984). The leaves has been used in folk medicine for treating diarrhoea (Almeida *et al.* 1995), inflammation (Schapoval *et al.*, 1994), hyperglycemia (Arai *et al.*, 1999) and hypertension (Consolini *et al.*, 1999). Phytochemical studies were focused, until the present moment, on the essential oil (Weyerstahl *et al.*, 1988) and polyphenolic constituents (Lee *et al.*, 1997; 2000) in the leaf. Antimicrobial activities were reported for essential oils and expressed juice, including bacterial (Fadeyi & Akpan, 1989), and dermatophyte isolates (Lima *et al.*, 1993).

The dimorphic fungus *Paracoccidioides brasiliensis* (Splendore) Almeida is the agent of human paracoccidioidomycosis (PCM), the main endemic systemic mycosis in Brazil (Costa *et al.*, 1995). PCM

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starts in the respiratory tract and can evolve to different clinical forms, which are associated with various degrees of suppressed cell-mediated immunity. Some antifungal drugs, such as polyene macrolides and azoles, are currently used in antifungal therapies with certain limitations due to side effects (Kullberg, 1997). Therefore, the development of more effective and less toxic antifungal agents is required for the treatment of patients with fungal infectious diseases (Janssen & Cauwenbergh, 1990).

As part of our work on the characterisation of antifungal compounds from Brazilian plants, we now report on the results obtained for antifungal activity-guided fractionation of the 96% ethanolic extract from *E. uniflora* leaves.

MATERIAL AND METHOD

Chemicals

Amphotericin B (Bristol-Myers Squibb, USA) and Itraconazole (Johnson & Johnson, USA) were suspended in sterile physiological Tris buffer (pH 7.4, 0.05 M) and included in assay as positive controls. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, USA).

Plant material

E. uniflora leaves were collected in Anápolis city (16°20'12.8"S, 48°56'19.3"W), Goiás State, Brazil, in April 1998, authenticated by Professor Heleno D. Ferreira, Departamento de Biologia Geral, Universidade Federal de Goias. Voucher specimens are deposited at the Herbarium of the Universidade Federal de Goias (UFG), with code number 25477.

Extraction procedure and fractionation

Air-dried and powdered leaves (150g) were exhaustively extracted at room temperature with 96% ethanol. Evaporation under reduced pressure at 35°C furnished the crude extract (52g). A part of the extract (10g) was dissolved in water and subjected to fractionation by partition with ether and then with ethyl acetate. Further fractionation by vacuum liquid chromatography on silica-gel of ether fraction (2g) was carried out using gradient of hexane:ethyl acetate as eluent, four subfractions were obtained: F1 (0.17g), F2 (0.56g), F3 (0.18g) and F4 (0.12g).

Chromatography analysis

TLC on silica gel PF_{254} layers (Merck) eluted with acetone:toluene:formic acid 3:3:1 for polar fractions and hexane:ethyl acetate (7:3) for no polar fractions. Plates were sprayed with vanillin/sulphuric acid and heated to 120°C or with $FeCl_3$/HCl.

GC-MS analysis of no polar fractions

Samples analysis were performed on a GC-MS Shimadzu QP5050A instrument employing the following conditions: Column: CBP-5 (Shimadzu) fused silica capillary column (30 m long ´ 0.25 mm i.d. ´ 0.25 mm film thickness composed of 5% phenylmethylpolysiloxane) connected to a mass detector operating in EI mode at 70 eV; carrier gas: He (1 $ml.min^{-1}$); injector and ion-source temperatures were 220 and 250°C, respectively, and a split ratio of 1:5. Injection volume was 0.5 ml (10% in CH_2Cl_2) and the oven temperature was programmed from 60°C (isothermal for 2 min), with an increase of $3°C.min^{-1}$, to 240°C, then $10°C.min^{-1}$ to 270°C, ending with a 5 min at 270°C. Individual components were identified by comparing their kovats index and mass spectra with those of literature (Adams, 1995), and with a computerized MS-data base using NIST libraries.

Microorganism

P. brasiliensis (*Pb*01 strain) was isolated from patient with pulmonary lesions in the Hospital of Tropical Diseases, Goias State, Brazil, and kindly provided by Dr Maria Rosario R. Silva, Departamento de Microbiologia, Universidade Federal de Goias. Recently, this strain was deposited in the American Type Collection Culture (MYA 826).

Antifungal Testing

Antifungal activity was measured using a dilution in agar technique (Alves & Cury, 1992). Extract and fractions dissolved in DMSO (1 mL) and serially two-fold diluted in Sabouraud molten medium (*ca.* 45°C) to obtain a concentration range of 1000-62.5 $mg.mL^{-1}$. Into each Petri dish (88 mm in diameter), 15 mL of the medium was distributed. DMSO devoid of the extracts was appropriately incorporated into the medium to serve as a control. Circular colonies of *P. brasiliensis*, yeast-form, from stock culture (4 mm in diameter, grown on Sabouraud medium) were punched using a sterile cork-borer and were centrally placed onto the medium incorporated with the extracts in the Petri dish, and incubated at 37°C for 15 days. Growth in diameter (mm) of each colony was measured at 48 h intervals. The minimal inhibitory concentration (MIC) was defined as the minimal concentration of the crude extract or fractions which completely inhibited the visible growth of the fungus. The percentage of growth inhibition was calculated using the formula: % inhibition = [(1 - growth in treated/growth in control) ´ 100]. The sensitivity of *P. brasiliensis* to antifungal agents Amphotericin B and Itraconazole, included as positive controls, were tested using the same technique. Duplicates were maintained for each concentration.

RESULT AND DISCUSSION

Paracoccidioides brasiliensis yeast cells were used to evaluate the antifungal activity of *Eugenia uniflora* leaves. Initially, the crude ethanolic extract

showed total growth inhibition of *P. brasiliensis* at concentration of 750 mg.mL^{-1}. A part of the crude extract was then subjected to fractionation by partition between solvents, water/ether and water/ethyl acetate. This process yielded three fractions with different polarities, which were antifungal assayed in the range of 500-31.25 mg.mL^{-1}. The yeast colonies were only inhibited completely in their growth by the ether fraction at 187.5 mg.mL^{-1}. The ethyl acetate and water fraction showed no minimal inhibitory concentration (MIC) at the range used in the assay (Table 1).

Analysis of the fractions by thin layer chromatography (TLC) showed the presence of galloyl esters and flavonol glycosides in the ethyl acetate fraction and ellagitannins in the aqueous fraction, as described by Lee *et al.* (1997). The less polar compounds, mainly terpenoids, were observed in the ether fraction. Subsequently, the ether fraction was refractionated by vacuum liquid chromatography on silica-gel, with gradient of hexane:ethyl acetate as eluent. Four subfractions were obtained and bio-assayed in the range of 500-31.25 mg.mL^{-1}. The first two subfractions, F1 and F2, showed higher inhibition of *P. brasiliensis* growth with MIC at 375 mg.mL^{-1}, while the F3 inhibited the fungal growth with MIC at 500 mg.mL^{-1}. The last sub-fraction was inactive in the concentration range used. The sensitivity of the same fungal isolate to commonly used antifungal agents as Amphotericin B and Itraconazole afforded MIC values at 5 and 2.5 mg.mL^{-1}, respectively (Table 1).

The most active subfractions, F1 and F2, were totally soluble in hexane, which allowed the GC-MS analysis. F1 was a mixture with 4 compounds, 3 of which were completely identified. The components with their percentages and relative retention times on a CBP-5 column are listed on Table 2. GC-MS analysis

TABLE 1 - Antifungal activity of *Eugenia uniflora* extracts and therapeutic drugs against *P. brasiliensis* yeast.

Plant material	Concentration (mg.mL^{-1})	Growth inhibition (%)
Crude extract	750	100
Ether fraction	187.5	100
subfraction F1	375	100
subfraction F2	375	100
subfraction F3	500	100
subfraction F4	500	0
Ethyl acetate fraction	500	46.4
Aqueous fraction	500	42.4
Amphotericin B	5.0	100
Itraconazole	2.5	100

TABLE 2 - Chemical constituents of *E. uniflora* subfraction F1.

Compound	RRT[a]	RI[b]	%
Isofuranegermacrene	31.03	1492	65.9
Germacreme B	33.55	1554	25.1
Atractylone	37.48	1653	1.6
Atractylone[c]	38.84	1689	7.4

[a]Relative Retention Time (min); [b]Retention index; [c]Isomer not identified

Table 3 - Chemical constituents of *E. uniflora* subfraction F2.

Compound	RRT[a]	RI[b]	%
Trimethylbenzene[c]	7.67	955	8.6
Trimethylbenzene[c]	7.91	962	2.6
2,4,6-Trimethylbenzene	8.81	988	7.8
g-Eudesmol	36.62	1630	2.2
7-*epi*-a-Eudesmol	37.70	1658	3.9
Unknown[d]	38.29	1673	18.0
Germacrone	39.02	1692	38.2
Unknown[d]	39.20	1697	3.6
Guaiol acetate	40.42	1730	11.8
Unknown[d]	45.31	1865	3.4

[a]Relative Retention Time (min); [b]Retention index; [c]Isomer not identified; [d]Oxygenated Sesquiterpene

indicated that it consists of sesquiterpenoids (74.9% oxygenated sesquiterpenes and 25.1% sesquiterpene hydrocarbon). Isofuranegermacrene (65.9%) was the major oxygenated sesquiterpene and germacrene B was the sesquiterpene hydrocarbon. These compounds were also present in the essential oil of *E. uniflora* leaves (Weyerstahl *et al.*, 1988). The subfraction F2 was a more complex mixture with 10 compounds, 5 of which were totally identified. Percentages and relative retention time of the components are listed in table 3. Oxygenated sesquiterpenes (81%) were the major constituents followed by a mixture of three trimethylbenzene isomers (19%). Germacrone (38.2%), also reported for the essential oil of *E. uniflora* (Weyerstahl *et al.*, 1988), and guaiol acetate (11.8%) were the major oxygenated sesquiterpenes. TLC analysis of the sub-fractions F3 and F4 suggested the presence of terpenoids and flavonoids for the first and only flavonoids for the later.

Bioassay-directed fractionation of *E. uniflora* leaves showed initially an increase of the antifungal activity from the crude extract (MIC at 750 $mg.mL^{-1}$) to the ether fraction (MIC at 187.5 $mg.mL^{-1}$). However, after the refractionation of the ether fraction, the activity decreased to MIC values at 375 and at 500 $mg.mL^{-1}$. These facts allowed us to conclude that there is a synergism among the bioactive compounds, as oxygenated sesquiterpenes, which probably are responsible for the antifungal activity.

More studies must be undertaken to correlate the production of sesquiterpenes by this specie and environmental factors, such as humidity, sunlight intensity, temperature, water, soil and herbivore pressure. Those factors might change the amounts of secondary metabolites, and consequently, increase or decrease the biological response.

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