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A Rapid Primary Screening Method for Antitumor Using the Oomycete *Pythium aphanidermatum*

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Abstract

Investigating bioactive components from microbial metabolites can provide potential source for drug discovery. There are a number of traditional antitumor compounds screening assays already described. In this study, we have developed an efficient method to search for antitumor compounds using the Oomycete *Pythium aphanidermatum* as model on agar plates for high throughtput screening. Antioomycete compounds showed cytotoxic effects in human cancer cells lines due to the possible similarity between the lipid cell membrane of the *Pythium* species and cancerous cells, suggesting the compounds act through similar mechanisms of inhibition. In this assay, it is possible to evaluate hundreds of samples, with results in few hours. The *Pythium* assay was shown to be sensitive for several chemical substances and allowed the detection and isolation of antitumor compounds from microorganisms. This bioassay was shown to be sensitive, rapid, simple and reliable for antitumor activity. It takes just few hours to evaluate the properties of the extracts.

Keywords: Antitumoral screening; *Pythium aphanidermatum*; Antifungal assay; Secondary metabolites

Introduction

Cancer is one of the leading causes of death. According to global cancer statistics released by the American Cancer Society [1], the total number of deaths from cancer in 2007 was 7.6 million and by 2050, 27 million new cancer cases and 17.5 million cancer deaths are projected to occur worldwide. Some antifungal substances can inhibit the growth of some tumor cells. They can be used as test model in cytotoxicity assays. Morphological deformations observed on mycelia of certain fungi induced by secondary metabolites have been known and were applied to screening for these compounds. This method detects morphological deformations of mycelia by culture broth of marine fungi, and has positive correlation with anticancer activity [2-4].

Pythium belongs to the Phylum Oomycota, classified as false fungus due to taxonomic and physiological factors. One of the factors to be considered is hyphal walls which are composed of 80-90% polysaccharides, mainly β1-6 linked glucans and β1-3 and β1-4 [5]. It should be noted that *Pythium* spp. do not contain chitin or chitosan in the hyphal walls and cell membrane, but they do contain proteins and lipids at levels varying from 3-8% and from 1-3%, respectively and sterols (cholesterol, β-sitosterol, etc.) which resemble cancer cells [6-8]. Studies suggest that cholesterol and cancer are closely associated, where the lipid bilayer membrane of cancer cells has a high concentration of cholesterols [9,10]. Rapidly proliferating tumor cells presumably require cholesterol for new membrane synthesis. Cholesterol accumulation may be a more general property of cancer, and it has been especially well correlated with breast and prostate cancer progression [11]. For many years, evidences have been accumulated indicating a possible central role of endogenous cholesterol in the pathobiology of cancer. Alterations in the synthesis, uptake, and membrane content of cholesterol have been observed in a variety of experimental tumor models as well as in human neoplasms [12-14]. It can be seen that the existence of similarity between the lipid physiological cell wall of *Pythium* species and cancerous cells suggest that the compounds act through similar mechanisms of inhibition.

Considering the need for effective methods to screen natural products for potential antitumor activity, the continuous demand for bioassays and the huge biodiversity for novel drugs in a quick way, the present study was undertaken to develop and be applied to the primary screening for antitumor compounds from microorganisms using the plant pathogenic fungus *Pythium aphanidermatum*. Here, we present an approach of utilizing bioassay-based highly sensitive antifungal activity, which is capable of detecting mycelia inhibition growth to a test compound with the advantages of being a rapid, quantitative, non invasive and *in vitro* method.

Materials and Methods

Pythium aphanidermatum was used as prescreen for discovery of antitumor and antifungal compounds. Extracts and fractions of 415 endophytic fungi isolated from *Combretum leprosum*, according to Santos et al. [13], were used in all bioassays. Fungal isolates were donated by the Culture Collection of Agricultural and Environmental Importance of the Brazilian Agricultural Research Corporation (EMBRAPA), Jaguariúna, São Paulo State, Brazil.

Cultivation conditions, and the preparation of crude fungal extracts

Each of the endophytic fungi was grown in 1 liter Erlenmeyer flasks containing 500 mL Czapek broth (3% $NaNO_3$, 2% K_2HPO_4, 2% $MgSO_4$: $7H_2O$, 1% KCl, 30 mg glucose, 1 g Bacto yeast extract, 3 mg $FeSO_4 \cdot 7H_2O$, HiMedia®) and incubated at room temperature for 30 days under stationary conditions. Three pieces (0.5 × 0.5 cm^2) of mycelia agar plugs of 8-days-old culture was used as inoculum. The broth culture was filtered to separate the filtrate and mycelia. The extraction of the secondary metabolites was performed with dichloromethane (1:1 w/v). The solvent was then removed by flash evaporation at 45°C. The extracts were stored in a freezer at -20°C, until the evaluation of the bioactivity.

Antifungal activity with *Pythium aphanidermatum* as screening model

Procedures for the antifungal assay against *P. aphanidermatum* were performed using the agar cup disc diffusion method with few

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adaptations [14], described below. Fungal discs from overnight cultures were placed into Petri dishes with potato dextrose agar (PDA) and incubated for 24 h at 28°C. All extracts and fractions were dissolved in Dimethyl Sulphoxide P.A. (Sigma-Aldrich, Saint Louis) solvent, 5 mm filter paper discs (Whatman nº1) were impregnated with 10 µL of the extracts with 250 $\mu g \cdot mL^{-1}$ of concentration. The plates were prepared according to the following intructions: an agar plug with plenty growth of *P. aphanidermatum* was positioned in the center of the plates. The paper disc was impregnated with the extracts and positioned equidistantly 2 cm of the center. All plates were incubated at 28°C for 17 hours, when the evaluation of the bioactivity was recorded. Combretastatin (50 $\mu g \cdot mL^{-1}$) and Pirarubicin (30 $\mu g \cdot mL^{-1}$) were used as control. All tests were performed in triplicate.

Cytotoxicity assay

Analysis of cytotoxic effects of the fractions were carried out against the following tumor cell lines: ECV304 (bladder carcinoma), B16F10 (mouse melanoma), J774 (histiocytic sarcoma), and k562 (Chronic myelogenous leukemia). Solution with different concentrations (1.10^4 $\mu g \cdot mL^{-1}$, 1.10^3 $\mu g \cdot mL^{-1}$ 1.10^2 $\mu g \cdot mL^{-1}$ 10 $\mu g \cdot mL^{-1}$, 1 $\mu g \cdot mL^{-1}$) were obtained by diluting the extracts with RPMI1640 medium and dimethyl sulfoxide (9:1). All tumor cell lines used in the present study were obtained from the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. The colorimetric methodology for the respiratory route of mitochondria MTT (3-(4,5-dimethylthiazol-2-y1)-2,5- diphenyl tetrazolium bromide) was used, as describe by some authors [13,15].

All experiments were performed in quintuplicate. Commercial chemotherapeutic pirarubicin was considered as standard drug. The inhibition of cell growth by samples tested was calculated using the following formula: percentage of anticancer activity (Ac As/ Ac) × 100%. Ac and as refer to the absorbance of control and the sample, respectively. The IC_{50} (i.e., the dose of extracts response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The IC_{50} value was determined by linear regression from individual experiments using GraphPad software (GraphPad software, San Diego, CA, USA) and was used for statistical analysis.

Effect on the morphology of transformed tEnd.1 cells

To assess the effects of the compounds on the morphology of endothelial cells, we used tEnd.1 cells line, which is derived from the murine thymic (tEnd.1) endothelioma cells. Strains were grown in RPMI1640 containing 2mM L-glutamine, 10% fetal bovine serum and 100 $\mu g \cdot mL^{-1}$ streptomycin (37°C, 5% CO_2). Exponentially growing tEnd.1 cells were plated onto 24 well plates at 2.5×10^4/500 µL/well. Twenty-four hour after plating, the medium was aspirated, and 300 µL of medium containing the test compound solubilized in RPMI1640 and DMSO (1%) was added to the well containing the wells (in triplicate) in 1.10^3 $\mu g \cdot mL^{-1}$ dilutions and incubated for 2 h at 37°C and 5% CO_2 [13]. After the 2 h incubation period, digital photographs were taken of representative center areas of each well at a magnification of 100X. Pirarubicin and combretastatin CA4 were included in the experiments as chemotherapic standards.

Results and Discussion

In this investigation, we have efficiently proved the use of the plant pathogenic fungus *Pythium aphanidermatum* is a reliable and reproducible procedure for rapidly testing great quantities of samples. In this assay, we can evaluate hundreds of samples, being possible to have all results in this period of time as the fungal growth reaches the whole plate in 24 hours. Dichloromethane extracts of 415 endophytic fungal strains were tested against *P. aphanidermatum* and 28% of all dichloromethane extracts showed strong inhibition (3+) followed by medium inhibition of mycelial growth (2+) and moderate (+) as showed in Figure 1.

To evaluate the antitumor potential and verify our assertions, fractions from partitioning extract with the highest inhibitory activity

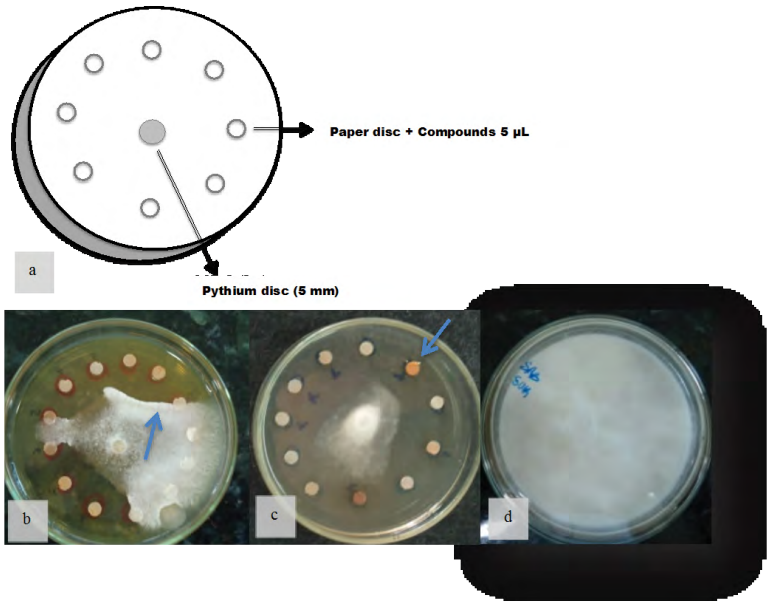

Figure 1: Schematic model of the disk diffusion test Kirby-Bauer adapted to Anti-oomycete activity against *Pythium aphanidermatum* of the endophytic fungi extracts; **a)** the center of the plate a plug with mycelium of *Pythium* grown 24 hours (5 cm), impregnated paper disc with extracts prepared test 2 cm from the center uniformly. The picture b) and c) are the results of Anti-oomycety activity with inhibitions of the *Pythium* mycelium growth, as indicated in arrows and as compared as control plate (d) normal growth of *Pythium aphanidermantum*.

were subjected to bioguided assay for cytotoxicity. The FR05 sample showed significant cytotoxic effects against lines causing no solids tumors: J774 with IC_{50} of 0.80 and K562 with IC_{50} of 1.47, Figure 2. Among the solid tumors, ECV304 showed inhibition with IC_{50} of 2.97 and B16F10 with IC_{50} of 5. The fraction FR05 as well as drug patterns showed cytotoxic activity in these cells, and the pirarubicin showed the highest cytotoxicity followed by pirarubicin and combretastatin A4. The fraction Fr03 and Fr08 did not show antitumor activity, in the same way, absence of inhibition of *P. aphanidermatum.*

Most often strategies have been developed to target tumor vasculature, combining therapies with cytotoxic agents that prevents the formation of new vessels in tumors as the anti-angiogenesis approach. Compounds that selectively target the preexisting tumor vasculature and cause damage to the endothelial cell layer are able stop the tumor blood supply [16]. However, in spite of this, we evaluated the action of the fractions in the transformation of tEnd.1 cells morphology, with observation of star-shaped cells and adhered to the bottom of plates, Figure 3. When treated with CA4, morphological changes were also observed leading to complete loss of adhesion, possibly preventing

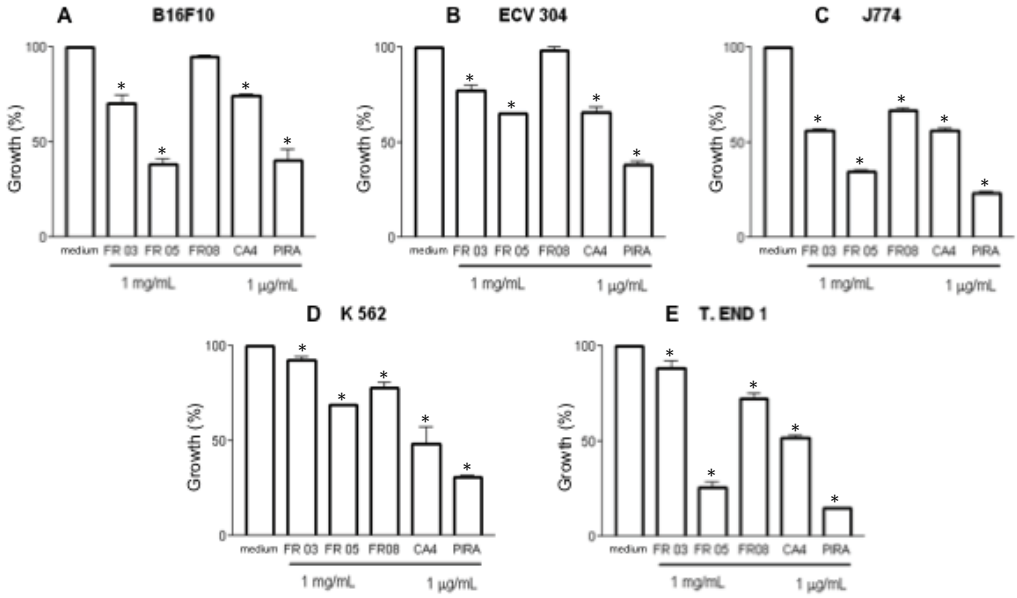

Figure 2: Cell growth measurements using conventional cytotoxicity MTT assay: Graphs showed the results of cytotoxicity of the fractions (Fr03, Fr05 and Fr08) in cancer human cells: **A)** B16F10 (Mouse melanoma), **B**) ECV304 (Bladder carcinoma), **C**) J774 (Histiocytic sarcoma), **D**) K562 (Chronic myelogenous leukemia) and **E**) T.end 1 (Endothelial cells) at a concentration 1 mg·mL^{-1} each fraction. The standard drugs used was Combretastatin (CA4) and Pirarubicin (PIRA) at concentration of 1 µg·mL^{-1}; Negative control was solution of medium RPMI and Tween (20%, Sigma, Saint. Lous) (t-test, *P<0.05, n=5 compared to the control, medium).

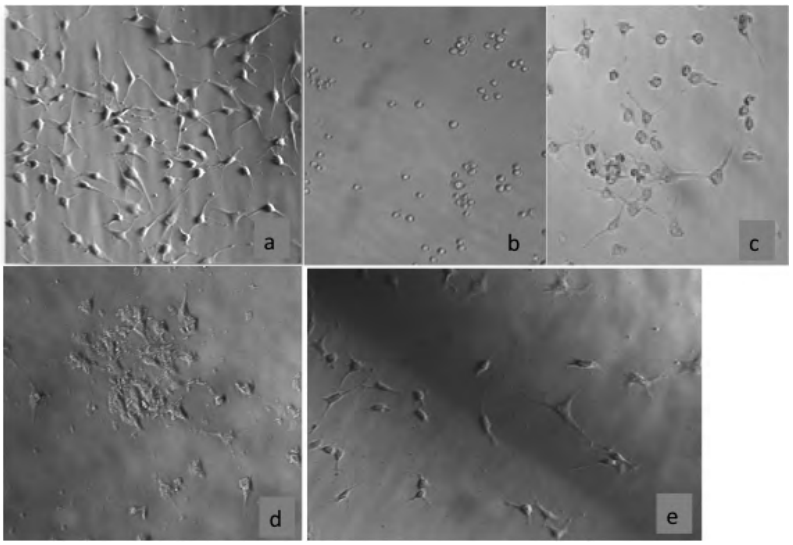

Figure 3: Image-based cell morphological effects. Endothelial cells (tEnd.1) were treated with Fr05 and incubated for 2 h at 1.10^{3} µg·mL^{-1} concentration. Representative photographs shown were recorded at a magnification of 360X **a)** culture media with cells tEnd.1; **b)** Combretastatin CA4 treatment; **c)** Pirarrubicin treatment; **d)** and **e)** effect of the fraction Fr05 from secundary metabolites from microorganisms (Zess: Axiovert inverted).

the formation of future blood vessels in tumors. The pirarubicin and fraction test (Fr05) caused rounding of the cells and decreased the starry shape, possibly preventing the formation of tumor vessels, but in different proportions to CA4. Tumor neovascularization occurs primarily through the sprouting of established vessels, a process that includes the migration of endothelial cells (EC) out of an existing vessel into the surrounding extracellular matrix (ECM) and their organization and morphogenesis into tube-like structures. Agents that interfere in these events potentially have antiangiogenic activity [16,17].

In this investigation, we have efficiently proved that the use of the plant pathogenic fungus is a reliable and reproducible procedure for rapidly testing great quantities of samples. In this assay, we could evaluate 415 samples, being possible to have all results in this period of time as the fungal growth reaches the whole plate in 24 hours.

Therefore, the results showed a positive correlation between the antifungal activity and antitumor tests using *P. aphanidermatum* model line as a prescreening agent for targeting potential antitumor compounds.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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