Paraconiothyrium sp. P83F4/1: Antioxidant and Antiproliferative Activities an Endophytic Fungus Associated with Rheedia brasiliensis Plant

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Abstract: The endophyte Paraconiothyrium sp. P83F4/1 was isolated from leaves of the Rheedia brasiliensis medicinal plant from a bioprospecting study. To the best of researcher’s knowledge, this is the first report of isolation of Paraconiothyrium in leaves of R. brasiliensis and the fermentation product obtained with this fungus in ethyl acetate was evaluated for their potential bioactive through antioxidant assay by DPPH (1,1-diphenyl-2-picrylhydrazyl) method and antiproliferative assays in human tumoral cells using the sulphorhodamine B technique for evaluating the cellular growth. According to the antioxidant assay, tested at 90 μg/mL (90 ppm), the fungal extract showed scavenging capacity of 58.92 %, being significant (p<0.05) compared to the standard commercial butyl hydroxy toluene: 39.52 %. In the antiproliferative assay, the extract was selective on human keratinocyte cells (HaCat) with GI₅₀ 0.95 μg/mL, whose mean log GI₅₀ = -0.02, according to National Cancer Institute (NCI-USA) criteria, it makes a powerful extract. HaCat Cells are involved directly in diseases such as psoriasis. These results indicate the antioxidant and antiproliferative potentials of the crude extract of fermentation of the endophytic fungus Paraconiothyrium sp. P83F4/1 isolated from the leaves of R. brasiliensis.

Keywords: Paraconiothyrium, Endophytic fungus, Psoriasis, DPPH, HaCat cells.

INTRODUCTION

A crucial aspect for obtaining new drugs from natural products is the selection of the source to be studied, because sources scarcely explored or with knowledge etnopharmacologic are frequently associated to a new chemical substance. Considering the great biodiversity of Brazilian flora and, according to claim researchers, it is estimated that each plant species has one or more microorganisms endophytic not yet discovered or described [1].

The plants of the genus Rheedia (family Clusiaceae) have chemical and pharmacological properties well described in the literature. In popular medicine the Rheedia brasiliensis leaves Planch. & Triana (Syn. Garcinia brasiliensis Mart.) are used for treatment of tumors, urinary tract inflammations, arthritis and for relieving pain [2].

Fungi and bacteria are still weakly studied about secondary metabolism. In this context, the endophytes, which live at least one stage of their life-cycles inside plant tissues and during that time, produce or induce production of primary and secondary metabolites with novel chemical diversity and molecules, which may confer several advantages to plant [3].

Study with endophytic fungi can contribute to preservation of plants, because for isolating these microorganisms it is just necessary a small portion of plant tissue, obtained in a non-predatory manner.

Paraconiothyrium sp. (Paraconiothyrium Verkley gen. nov.) is part of the genus Paraconiothyrium newly created and described by Verkley et al. [4]. In Brazil, the literature describes the isolation of Paraconiothyrium sp as endophytic just from the Coffea arabica fruit, and from sea sediments [4].

Thus, from bioprospecting leaves of R. brasiliensis and using screening criteria, the endophytic
Paraconiothyrium sp. P83F4/1 was isolated and selected for evaluation of its ethyl acetate extract for their ability to produce bioactive metabolites with biological activities of interest.

MATERIALS AND METHODS

Isolation of the Endophytic Fungi and Obtention of Bioactive Metabolites

Apparently healthy leaves of R. brasiliensis Planch. & Triana (Syn. Garcinia brasiliensis Mart.) were collected from trees grown under control from the herbarium of the Federal University of Viçosa (S 20º45’15” and W 42º52’55”), Minas Gerais, Brazil, where the voucher specimen is deposited under the number VIC2604. The process of isolation of Paraconiothyrium sp. endophytic fungi was conducted in accordance with guidelines methodology described by Petrini et al. [5], according to morphological characteristics.

Paraconiothyrium sp. was inoculated on potato dextrose agar medium (HIMEDIA, Mumbai, Índia) and incubated in BOD incubator (TE-391, TECNAL, Piracicaba, Brazil) at 25°C for 10 days. The fresh mycelium grown on potato dextrose agar medium at 25°C for 20 days, under static conditions, was aseptically inoculated to 500 mL erlenmeyer flasks containing 200 mL czapek broth. This medium has the following composition g/L: Glucose: 30,0; NaNO3: 2,0; K2HPO4: 1,0; MgSO4.7H2O: 0,5; KCl: 0,5; FeSO4.7H2O: 0,01; Yeast Extract: 1,0 [6]. After incubation, the mycelium of the culture was separated by vacuum filtration. The liquid filtrate obtained was extracted four times with ethyl acetate 1:1 (v/v) (VETEC, Duque de Caxias-RJ, Brazil). The organic phase was collected and the solvent was removed using a rotary vacuum evaporator (801, FISATOM, Perdizes-SP, Brazil). As a negative control, un-inoculated czapek broth extract, prepared using the same methodology was applied.

Antioxidant Activity

Four mL of crude wheat bran and fungus extracts (90 ppm) were added to 1.0 mL of 0.5 mM DPPH (1,1-diphenyl-2-picryl-hydrazyl radical) (ALDRICH, Alemanha) in ethanol (SYNTH, Diadema-SP, Brazil). Mixtures were vigorously shaken and left to stand at room temperature for 30 minutes in the dark, according to the methodology described by Yen, Chang and Duh [7] with few modifications. Absorbance at 517 nm (Spectrophotometer B295 II- model 456-E, MICRONAL, Brazil) was measured using ethanol as blank. Standard samples (ascorbic acid (ISOFAR, Jacaré-RJ, Brazil) and butyl hydroxytoluene (ISOFAR, Duque de Caxias-RJ, Brazil) were used in the same concentration. DPPH degradation was evaluated by comparison with a negative control solution (4.0 mL ethanol and 1.0 mL of DPPH solution). Triplicate tests were conducted for each sample. The DPPH radical-scavenging capacity was calculated by the formula (I):

\[
\% \text{SC} = \left( \frac{\text{Abs negative control} - \text{Abs sample}}{\text{Abs negative control}} \right) \times 100
\]

Where: Abs = Absorbance

\% SC = % scavenging capacity

Anti-oxidant results were analysed by ANOVA test (variance analysis) that statistically compared (with p<0.05) to the extracts (fungal and Czapek broth) together with the positive controls BHT and ascorbic acid, using MatLab 7.0 program.

Antiproliferative Activity

Cell lines [U251 (glioma), K-562 (chronic myeloid leukemia), MCF-7 (breast adenocarcinoma), PC-3 (prostate adenocarcinoma), HT-29 (colon adenocarcinoma), 786-0 (kidney adenocarcinoma), NCI-ADR/RES (ovarian adenocarcinoma, multidrug resistant phenotype) and HaCat cells (human keratinocytes, immortalized cell)] were kindly provided by the National Cancer Institute (NCI-USA). Stock cultures were grown in a medium containing 5 mL of RPMI 1640 (GIBCO BRL, EUA) supplemented with 5% of fetal bovine serum (GIBCO, EUA). Penicillin: Streptomycin (1000 UI/mL: 1000 µg/mL, 1 mL/L) was added to experimental cultures. Cells in 96 well plates (100 µL cells/well) were exposed for sample concentrations in DMSO/ RPMI (0.25, 2.5, 25 and 250 µg/mL) at 37 ºC, 5% of CO2 in the air for 48 h. Final DMSO concentration did not affect cell viability. Doxorubicine (Eurofarma, Brazil) (0.025, 0.25, 2.5 and 25 µg/mL) was used as positive control. Afterwards, cells were fixed with 50 % trichloroacetic acid (MERCK, EUA). Penicillin: Streptomycin (1000 UI/mL: 1000 µg/mL, 1 mL/L) was added to experimental cultures. Cells in 96 well plates (100 µL cells/well) were exposed for sample concentrations in DMSO/ RPMI (0.25, 2.5, 25 and 250 µg/mL) at 37 ºC, 5% of CO2 in the air for 48 h. Final DMSO concentration did not affect cell viability. Doxorubicine (Eurofarma, Brazil) (0.025, 0.25, 2.5 and 25 µg/mL) was used as positive control. Afterwards, cells were fixed with 50 % trichloroacetic acid (MERCK, EUA) and cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content (VersaMax ELISA Microplate Reader, EUA), employing sulforhodamine B (SIGMA, EUA) assay [8]. Using a concentration–response curve for each cell line, GI50 (concentration that produces 50 % growth inhibition) was determined through non-linear regression analysis, utilizing software ORIGIN 7.5 (OriginLab Corporation).
Determination of the Chemical Composition of the Extracts by GC-MS

Before GC-MS (gas chromatography/mass spectrometry) analysis, both crude extracts VersaMax Molecular Devices (fungi and broth) were submitted to a TMS derivation, according to Fernàndez et al. [10] with modifications. A Shimadzu gas chromatograph, model 2010 GC, coupled mass spectrometer Shimadzu, model QP 2010 Plus, were employed for all analyses.

RESULTS AND DISCUSSION

Isolation and Selection of the Endophytic Fungi

This study was focused on bioprospecting analyzed 245 fragments from *R. brasiliensis* leaves and isolated 154 fungi (62.86% isolation frequency). To the best of our knowledge, this is the first report of isolation of bioactive metabolites from the leaves of *R. brasiliensis*.

Some of the works in literature presented new bioactive compounds from the *Paraconiothyrium* fermentation, such as the isolation of three new epoxypholamines (3-5), whose cytotoxic activities, particularly on the prostate and bladder, are mentioned for epoxyphomaline 4 [10]. New tricyclic sesquiterpenes denominated brasiliamides A-D (1-4) were isolated in the culture of *P. brasiliensis* Verkley [11].

Extract of the endophytic fungus *Paraconiothyrium* sp. P83F4/1 in this study was selected for further study based on its highly bioactive potency compared to other isolated strains.

Chemical Profile

Chemical profile, according to GC-MS analysis, establish that fungi extract is mainly composed by saturated fatty acids butanodioic, with relative intensity of 22.80 %. Identified other saturated fatty acids such as butanoic acid (0.73 %), as well as phenolic compounds (benzoic acid (0.54 %), benzenepropanoic acid (9.78 %)), sugars and carbohydrates (Table 1).

Antioxidant Activity

Table 2 summarize the antioxidant activity of the extracts tested at 90 µg/mL, assayed by the DPPH method.

Table 1: Retention Times, % Area of Each Component, and Important Ions Present in the Mass Spectra of Silylated Compounds of Ethyl Acetate Crude Extract of *Paraconiothyrium* sp. P83F4/1 by GC-MS

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>Retention time</th>
<th>% Area</th>
<th>Ion (m/z, abundance in parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Propanoic Acid, 2-Methyl-3-[(Trimethylsilyl)Oxy]-Trimethylsilyl Ester</td>
<td>8.46</td>
<td>2.98</td>
<td>233 (20) 177 (14) 147 (100) 103 (15) 73 (71) 66 (14) 45 (15)</td>
</tr>
<tr>
<td>2</td>
<td>Butanoic Acid, 3-Methyl-3-[(Trimethylsilyl)Oxy]-Trimethylsilyl Ester</td>
<td>9.66</td>
<td>0.73</td>
<td>247 (22) 205 (8) 147 (86) 131 (100) 75 (25) 73 (88)</td>
</tr>
<tr>
<td>3</td>
<td>2-Phenylethyl Trimethylsilyl Ether</td>
<td>10.06</td>
<td>1.51</td>
<td>194 (1) 179 (65) 105 (24) 103 (79) 75 (33) 73 (100)</td>
</tr>
<tr>
<td>4</td>
<td>Pentanoic Acid, 4-Methyl-2-[(Trimethylsilyl)Oxy]-Trimethylsilyl Ester</td>
<td>10.35</td>
<td>0.29</td>
<td>233 (6) 159 (87) 147 (53) 103 (71) 73 (100) 45 (16)</td>
</tr>
<tr>
<td>5</td>
<td>Butanedioic Acid, Bis(Trimethylsilyl) Ester</td>
<td>12.32</td>
<td>22.80</td>
<td>262 (1) 247 (21) 147 (100) 75 (18) 73 (56) 55 (8)</td>
</tr>
<tr>
<td>6</td>
<td>2-Butenedioic Acid (E)-Bis(Trimethylsilyl) Ester</td>
<td>13.09</td>
<td>0.44</td>
<td>246 (21) 245 (100) 147 (58) 75 (40) 73 (99) 45 (27)</td>
</tr>
<tr>
<td>7</td>
<td>2-Pentenedioic Acid, 3-Methyl-Bis(Trimethylsilyl) Ester, (E)-</td>
<td>15.46</td>
<td>0.53</td>
<td>273 (4) 182 (40) 183 (27) 170 (25) 147 (100) 109 (28) 73 (97)</td>
</tr>
<tr>
<td>8</td>
<td>Trimetilsilil-2-[(trimetilsili)oxi]ciclohexanoxcarboxilado</td>
<td>15.74</td>
<td>1.47</td>
<td>288 (5) 273 (64) 183 (30) 170 (28) 147 (54) 129 (83) 81 (83) 73 (100)</td>
</tr>
<tr>
<td>9</td>
<td>Unknow 9</td>
<td>18.56</td>
<td>4.93</td>
<td>282 (2) 267 (20) 193 (13) 179 (100) 103 (9) 73 (37)</td>
</tr>
<tr>
<td>10</td>
<td>Benzenepropanoic Acid, Alpha-[(Trimethylsilyl)Oxy]-Trimethylsilyl Ester</td>
<td>18.99</td>
<td>9.78</td>
<td>295 (22) 267 (17) 220 (43) 194 (40) 193 (100) 73 (79)</td>
</tr>
<tr>
<td>11</td>
<td>Silane,[1,2,3-Benzenetriyltris(Oxy)]Tri[Trimethyl-</td>
<td>19.39</td>
<td>4.13</td>
<td>343 (20) 342 (58) 327 (8) 239 (100) 133 (8) 73 (95)</td>
</tr>
<tr>
<td>12</td>
<td>Benzoic Acid, 4-[(Trimethylsilyl)Oxy]-Trimethylsilyl Ester</td>
<td>19.82</td>
<td>0.57</td>
<td>282 (26) 267 (90) 223 (59) 193 (60) 126 (16) 73 (100)</td>
</tr>
<tr>
<td>13</td>
<td>Unknow 13</td>
<td>24.79</td>
<td>1.93</td>
<td>204 (100) 73 (97) 205 (20) 226 (59) 239 (37) 147 (21) 246 (19) 267 (10)</td>
</tr>
</tbody>
</table>
Table 2: Determination of the Sequestrant Capacity (%) of the *Paraconiothyrium* sp P83F4/1 and Czapek Broth Extracts by the DPPH, Antioxidant Assay Using Ethanol as a Solvent

<table>
<thead>
<tr>
<th>Sample</th>
<th>SC (%) ± σ (90 ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paraconiothyrium</em> sp P83F4/1</td>
<td>58.92±0.024</td>
</tr>
<tr>
<td>Czapek Broth</td>
<td>0±0.013</td>
</tr>
<tr>
<td>Ascorbic acid (standard)</td>
<td>96.61±0.002</td>
</tr>
<tr>
<td>BHT (standard)</td>
<td>39.52±0.095</td>
</tr>
</tbody>
</table>

SC (%): scavenging capacity; standard deviation. Values marked by the different letter are significantly different (p<0.05).

It is important to note that how is a crude extract, *Paraconiothyrium* sp P83F4/1 becomes a potential sample for future studies, emphasizing its better performance (p<0.05) in relation to the BHT commercial standard, though with capacity smaller than demonstrated by vitamin C (ascorbic acid).

Liu et al. [12] also observed greater antioxidant activity in the *Xylaria* sp YX-28 extract than ascorbic acid and BHT standards. The authors attributed this activity to the presence of phenolic compounds, ferruginol, besides flavonoids. Similarly, Huang et al. [13] correlated the antioxidant capacity of cultures of endophytic fungi to their total phenolic content.

**Antiproliferative Activity**

For analysis and interpretation of results of GI50, shown in Table 3, was considered the criterion of the NCI (National Cancer Institute - USA) [14], which classifies the cytostatic effect of the extracts as inactive (average> 1.5), weak (1.1 <mean <1.5), moderate (1.1 > mean >0) and potent (mean <0) as a result of the average log GI50.

Analyzing the behavior of the fungal extract face the whole tested cellular panel, it may observe the extreme values of GI50, ranging from 0.95 to > 250 μg/mL. It is highlighted this extract in its action on the HaCat cell (human keratinocyte, immortalized cell), whose average value of log GI50 becomes indicative of the high toxicity on this lineage, considered important in diseases such as psoriasis.

Psoriasis is a chronic inflammatory skin disease mediated by T cells, characterized by erythematous lesions with formation of reddish plaques scaling white or silver, which is due to increased proliferation of keratinocytes, which accumulate on surface of skin involves a process local inflammatory and immune [15].

The causes are still unknown, but a genetic predisposition coupled with various factors such as diet and lifestyle (alcohol consumption and smoking), directly interfere with the disease and are a plausible etiologic explanation [16].

Other study conducted by Briganti and Picardo [17] demonstrated that individuals with this disorder showed impaired antioxidant status, with increased malonilaldeído, a marker of lipid peroxidation and decreased plasma levels of β-carotene, α-tocopherol and serum levels selenium. Wolters [18] emphasizes that increase in oxidative stress and free radicals are associated with inflammation of the skin in psoriasis and other cellular components.

Considering the character of the antioxidant extract *Paraconiothyrium* sp. P83F4/1 and observations of the literature, this statement probably would help in treating this disease by reducing the concentration of free radicals generated and consequently, the flags of this inflammatory process, contributing positively to the improvement of patient.

As the pathological condition of psoriasis is only subject to treatment and control, with no cure, the fungal extract, although crude, proved a more important possibility to control the proliferation of keratinocytes, stimulating further studies on its selectivity to line HaCat.

Other isolates of *Paraconiothyrium* genus has highlighted in the production of compounds with cytotoxic activities. In a recent study with *Paraconiothyrium* (MY-42), Shiono et al. [19] isolated

Table 3: Partial Inhibition of Cellular Growth (mean of GI50), in μg/mL, of Tumor Cells Against the Ethyl Acetate Extract of *Paraconiothyrium* sp. P83F4/1 (Crude Extract) and Czapek Broth (CZP)

<table>
<thead>
<tr>
<th>Cell panel tested</th>
<th>Mean logGI50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td><strong>Doxorubicin</strong></td>
<td>0.029</td>
</tr>
<tr>
<td><em>Paraconiothyrium</em> sp P83F4/1</td>
<td>110.9</td>
</tr>
<tr>
<td><strong>CZP</strong></td>
<td>&gt;250</td>
</tr>
</tbody>
</table>

2 = U251 (glioma, SNC); m = MCF-7 (breast); a = NCI-ADR/RES (ovary, with phenotype of multiple drug resistance); 7 = 786-O (renal); p = PC-3 (prostate); h = HT-29 (colorectal); q = HaCat (human keratinocyte, normal cell immortalized); k = K562 (leukemia); α = OVCAR-3 (ovary); 4 = NCI-H460 (lung); Doxorubicin: positive control.
six diterpenes and the structures were elucidated. Three compounds (1, 2 and 3) were mild with cytotoxic activity against the cell line HL60 human promyelocytic leukemia. These authors also evaluated induction of apoptosis in the formation of the DNA strand of HL60 cells and found that digestion of genomic DNA was dose-dependent.

CONCLUSION

Potentiality of the crude extract of Paraconiothyrium sp P83F4/1 is highlighted for their ability to sequester free radicals and a selectively inhibit cell growth of human keratinocytes immortalized (HaCat cells), may be promising in the fight against psoriasis by reducing free radicals involved in this pathology.

The plant Rheedia brasilensis has presented as a promising source of endophytic fungi capable of producing bioactive compounds of industrial interest, encouraging the search for deeper knowledge on the action mechanisms, as well as on the elucidation of the compound(s) involved in the reported biologic activities.

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