Chemical Constituents and Antimicrobial Activity of Soil Fungus

*Aspergillus* sp. FRIZ12

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Constituintes Químicos e Atividade Antimicrobiana do Fungo de Solo

*Aspergillus* sp. FRIZ12

Resumo: O presente trabalho relata o isolamento de nove compostos da biomassa do fungo de solo *Aspergillus* sp. FRIZ12. Os compostos 2-benzil-4H-piran-4-ons (1), flavasperona (2), 5- (hidroximetil) furfural (3), uridina (4), ergosterol (5), peróxido de ergosterol (6), cerivisterol (7), estigmasterol (8) e sitosterol (9) foram isolados por métodos cromatográficos, e as estruturas foram identificadas por RMN, EM e comparação com dados da literatura. O composto 1 está sendo relatado pela primeira vez no gênero *Aspergillus* e pela segunda vez como um produto natural. Os compostos 1 e 2 mostraram moderada atividade antimicrobiana.

Palavras-chave: *Aspergillus*; metabolitos secundários; fungos de solo; atividade antimicrobiana.

Abstract

The present work reports the isolation of nine biomass compounds of soil fungus *Aspergillus* sp. FRIZ12. The compounds 2-benzil-4H-piran-4-one (1), flavasperone (2), 5-(hydroxymethyl) furfural (3), uridin (4), ergosterol (5), ergosterol peroxide (6), cerivisterol (7), stigmasterol (8) and sitosterol (9) were isolated by chromatographic methods, and the structures were identified by NMR, MS, and comparison with literature data. Compound 1 is being reported for the first time in the genus *Aspergillus* and for the second time as a natural product. Compounds 1 and 2 showed moderate antimicrobial activity.

Keywords: *Aspergillus*; secondary metabolites; soil fungi; antimicrobial activity.

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Chemical Constituents and Antimicrobial Activity of Soil Fungus *Aspergillus* sp. FRIZ12

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1. Introduction

The soil is an ecological niche highly explored by microorganisms producing biologically active natural products, including antibiotics. Most antibiotics are isolated from *Streptomyces* and fungi, commonly found living in the soil. Current researches have emphasized the need to explore new niches, such as tropical forests, marine sponges, mangroves, and endophytes to discovery pharmacologically active compounds.¹ ²

Fungi of the genus *Aspergillus* (Phylum Ascomycota, Family Trichocomaceae) can be isolated from soil, water, vegetation, decomposition material, and air. In general, *Aspergillus* fungi are frequent in regions with tropical and subtropical climate, and many species produce compounds with antimicrobial activity.³

The World Health Organization has considered the bacterial resistance to antibiotics a serious threat to public health. The bacteria *Escherichia coli* (Phylum Proteobacteria, Family Enterobacteriaceae), *Pseudomonas aeruginosa* (Phylum
Proteobacteria, Family Pseudomonadaceae), *Bacillus subtilis* (Phylum Firmicutes, Family Bacillaceae), *Staphylococcus aureus* (Phylum Firmicutes, Family Staphylococcaceae) and *Salmonella typhimurium* (Phylum Proteobacteria, Family Enterobacteriaceae), are frequently associated with disease in humans and animals. The increasing phenomenon of bacterial resistance to antibiotics has led to the need to discover new antimicrobial agents and methods.

Thus, the present work aimed at obtaining compounds with antimicrobial activity from the fungus *Aspergillus* sp. FRIZ12, in addition to studying their chemistry. Nine compounds were isolated from the biomass extracts: 2-benzyl-4H-pyran-4-one (1), flavasperone (2), 5-(hydroxymethyl) furfural (3), uridin (4), ergosterol (5), ergosterol peroxide (6), cerivisterol (7), sitosterol (8), and stigmasterol (9) (Figure 1). Compound 1 is reported for the first time in the genus *Aspergillus* and for the second time as a natural product.

![Figure 1. Compounds isolated from Aspergillus sp. FRIZ12](image)

### 2. Experimental

#### 2.1. General Procedures

The \(^1\)H and \(^{13}\)C NMR experiments were recorded in an NMR spectrometer (Mercury 300, Varian, Oxford, Oxford shire, UK) with CDCl\(_3\) (Cambridge\textsuperscript{*}) as solvent and standard. MS spectra were carried out in the a mass spectrometer (Acquity TQD, Waters, Milford, MA, USA) using electrospray ionization in positive ion mode ESI(+). Chemical shifts were given in delta (\(\delta\)) and constants coupling (\(J\)) are given in Hertz (Hz).

#### 2.2. Microorganism isolation

For the isolation of the fungus *Aspergillus* sp. FRIZ12, the serial suspension technique was used. 1 g of soil, previously sieved in a 2 mm mesh, was transferred to a test tube containing 9 mL of sterilized distilled water and agitated in a Vortex (100 rpm) for 10 minutes. From this solution was carried out serial dilutions until 1:1000 dilution. Then, 0.1 mL was added in Petri dishes containing BDA culture medium and incubated for seven days. After the growth of the colonies the fungi were isolated by successive samplings. The fungus FRIZ12 was identified by
morphological analysis on an optical microscope as being of the genus *Aspergillus*.

2.3. Culture of *Aspergillus* sp. FRIZ12 in rice and isolation of the chemical constituents

Fifteen Erlenmeyer flasks (1000 mL), containing 200 g of rice (“Uncle Ben’s®”) and 75 mL of water each, were autoclaved for 45 min at 121 °C. Small pieces of PDA containing mycelium of *Aspergillus* sp. FRIZ12 were added to 13 Erlenmeyer flasks under sterile conditions, and then the Erlenmeyer flasks were incubated at 25°C for 25 days for colony growth, two Erlenmeyer flasks were used as control. Biomass was macerated with hexane, ethyl acetate, and methanol, in which the hexane (13.2 g), ethyl acetate (8.3 g), and MeOH (150.7 g) extracts were obtained after evaporation of the resulting solutions in a rotary evaporator. The ethyl acetate extract was fractioned on silica gel column chromatography using as mobile phase hexane, hexane/ethyl acetate (7:3, 1:1, 3:7), ethyl acetate, ethyl acetate/methanol (1:1) and methanol resulting in 7 fractions. The fraction ethyl acetate after chromatographed on silica gel CC by using hexane, ethyl acetate, and methanol as mobile phase in a gradient of polarity, monitored by TLC the following chemical constituents were isolated: 2-benzyl-4H-pyran-4-one (1), flavasperone (2), 5-(hydroxymethyl) furfural (3), uridin (4), ergosterol (5), ergosterol peroxide (6), cerivisterol (7), sitosterol (8), and stigmasterol (9). Compound 1 was isolated as a yellow crystalline solid, soluble in dichloromethane. The mass spectrum was observed at m/z 187 [M+H]⁺ which together with the NMR data, made possible to propose the molecular formula C₁₂H₁₀O₂. In the ¹H NMR spectrum, a multiplete signal was observed at δ_H 7.24 - δ_H 7.33, attributed to the hydrogens of the compounds 1 and 2 were determined by microbroth dilution assay as recommend by the Clinical and Laboratory Standards Institute (CLSI). The assay was carried out on 96 well plates with 100 μL of Mueller Hinton Broth (MHB), 100 μL of test compounds, and 5 μL of test bacteria at 1.0 x 10⁶ CFU.mL⁻¹, followed by incubation at 37 °C (24h). Compounds 1 and 2 were dissolved in dimethyl sulfoxide at the initial concentration of 500 μg.mL⁻¹. The microorganisms used in the antimicrobial assay were *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), and *Salmonella typhimurium* (ATCC 14028), obtained from the Instituto Evandro Chagas, Belém – Brazil. Bioactivity was observed by absence of red coloration in the wells after addition of 10 μL of 2,3,5-triphenyltetrazolium chloride. Penicillin, vancomycin, and tetracycline (25 μg.mL⁻¹) were used as positive control. The culture medium (MHB only) was used as negative control.

3. Results and Discussion

3.1. Isolated compounds

In the present work, nine compounds were isolated from the ethyl acetate extract of the *Aspergillus* sp. FRIZ12 biomass, namely: 2-benzyl-4H-pyran-4-one (1), flavasperone (2), 5-(hydroxymethyl) furfural (3), uridin (4), ergosterol (5), ergosterol peroxide (6), cerivisterol (7), sitosterol (8), and stigmasterol (9). Compound 1 is reported for the first time in the genus *Aspergillus* and for the second time as a natural product.

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monosubstituted aromatic ring. The aromatic ring was confirmed by HETCOR correlation of the $\delta_H$ 7.24 - $\delta_H$ 7.33 signals with signals of the aromatic carbons $\delta_C$ 127.6, 128.9, 129.1, and 134.4. The signal in the $^1$H NMR spectrum at $\delta_H$ 3.83 (s, 2H, H-1') was attributed to benzylic hydrogen. Hydrogen H-1' showed HMBC correlation with aromatic carbons C-3' and C-7', and olefinic carbon C-2; moreover, the benzylic hydrogen H-1' showed COSY correlation with H-3 ($\delta_H$ 6.21, $dl$, 1H, $J=2.4$ Hz). Through the HMBC and COSY correlation to H-3 (Figure 2), it was possible to propose a pyran ring to compound 1. Through the HMBC correlation observed to H-6 with the signal at $\delta_C$ 179.3, it was possible to attribute the position of $\alpha$, $\beta$-unsaturated carbonyl group on C-4. Through the HMBC correlation to H-3 with benzylic carbon C-1' ($\delta_C$ 40.0), it was possible to determine the exact place of the link between benzylic and pyran rings. Compound 1 was identified as the gamma-pyran 2-benzyl-4H-pyran-4-one. This compound was previously isolated from an endophytic fungus from mangrove plants and is being reported for the first time in the genus Aspergillus and for the second time as a natural product. Table 1 shows the $^1$H and $^{13}$C NMR data to compound 1.

Compound 2 was isolated as a yellow crystalline solid, soluble in dichloromethane. The mass spectrum peak at $m/z$ 287 [M+H]$^+$ allowed to calculate the molecular formula C$_{16}$H$_{14}$O$_5$. Through analysis of the NMR data, compound 2 was identified as the gamma-pyran flavasperone. The compound flavasperone had been isolated from Aspergillus species and recent works had showed the cytotoxic activities and inhibition of the ACAT2 isoenzyme by compound.

### Table 1. $^1$H and $^{13}$C NMR data to compound 1 (CDCl$_3$, 300 MHz).

<table>
<thead>
<tr>
<th>n</th>
<th>$^1$H ($\delta$, mult., $J$ in Hz)</th>
<th>$^{13}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-</td>
<td>168.5</td>
</tr>
<tr>
<td>3</td>
<td>6.21 ($dl$, 1H; $J=2.4$ Hz; H-3)</td>
<td>115.5</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>179.3</td>
</tr>
<tr>
<td>5</td>
<td>6.34 ($dd$, 1H; $J=5.7$ and 2.4 Hz; H-5)</td>
<td>116.6</td>
</tr>
<tr>
<td>6</td>
<td>7.70 ($d$, 1H; $J=5.7$ Hz; H-6)</td>
<td>155.4</td>
</tr>
<tr>
<td>1'</td>
<td>3.83 (s, 2H, H-1')</td>
<td>40.0</td>
</tr>
<tr>
<td>2'</td>
<td>-</td>
<td>134.4</td>
</tr>
<tr>
<td>3'</td>
<td>7.24 (m)</td>
<td>128.9</td>
</tr>
<tr>
<td>4'</td>
<td>7.33 (m)</td>
<td>129.1</td>
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<tr>
<td>5'</td>
<td>7.33 (m)</td>
<td>127.6</td>
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<tr>
<td>6'</td>
<td>7.33 (m)</td>
<td>129.1</td>
</tr>
<tr>
<td>7'</td>
<td>7.24 (m)</td>
<td>128.9</td>
</tr>
</tbody>
</table>

Compound 3 was identified as 5-(hydroxymethyl) furfural, it is one of the major components of honey quality measurement, and studies have shown that this compound has cytotoxic, genotoxic, mutagenic, and carcinogenic activities. In turn, compound 4 has been identified as nucleoside uridine; previous works have shown that uridine has antiviral activity.

The sterols ergosterol (5), ergosterol peroxide (6), cerivisterol (7), sitosterol (8), and stigmasterol (9) were isolated by chromatographic procedures and identified by NMR and with literature data.
Analysis of the control extracts by MS showed the absence of the isolated compounds in the culture medium, confirming that the isolated compounds are secondary metabolites produced by the fungus *Aspergillus* FRIZ12 and not from the culture medium.

3.2. Antimicrobial bioassay

Bacterial resistances to antibiotics have increased to dangerously high levels in all parts of the World. In this context, it is necessary to search for new antimicrobial agents, which agrees with the chemistry of fungi that are great producers of bioactive secondary metabolites. Several studies have led to the isolation of compounds with antimicrobial activities. Thus, in the present work, antimicrobial bioassays were performed with isolated compounds. The compounds 2-benzyl-4H-pyran-4-one (1) and flavasperone (2) showed bacteriostatic activities up to the lowest concentration tested (7.81 µg.mL⁻¹) against the bacteria *B. subtilis, E. coli*, and *P. aeruginosa*. As for *S. aureus*, both compounds 1 and 2 showed bacteriostatic activities up to the concentrations of 31.25 µg.mL⁻¹ and 15.62 µg.mL⁻¹, respectively. Against the bacterium *S. typhimurium*, compounds 1 and 2 were active up to the concentrations of 62.5 µg.mL⁻¹ and 15.6 µg.mL⁻¹.

Barrow and MuCulloch carried out a review on the biological importance of gamma-pyrans, which showed that they have a wide occurrence, in addition to a large range of biological activities, such as antimicrobial, antiviral, insecticide, and antiestrogen. Choque et al., also carried out a review and suggest that gamma-pyran compounds are of large industrial interest due to their biological activities, which can be useful to pharmaceutical, cosmetics, and food industries.

The data obtained in this work agree with the literature for the activities of gamma-pyrans. However, none of these studies reported the antimicrobial activity of the compound flavasperone, this being the first report on the antimicrobial activity of this gamma-pyran against the bacteria *B. subtilis, E. coli, P. aeruginosa, S. aureus*, and *S. typhimurium*.

4. Conclusion

The chemical study of soil fungus *Aspergillus* sp. FRIZ12 led to the isolation of the nine compounds, with 2-benzyl-4H-pyran-4-one (1) being reported for the first time in the genus *Aspergillus* and for the second time as a natural product. The compounds flavasperone (2) and 2-benzyl-4H-pyran-4-one (1) were tested against the bacteria *B. subtilis, E. coli, P. aeruginosa, S. aureus*, and *S. typhimurium*, showing good activity. Thus, it can be evidenced that *Aspergillus* sp. FRIZ12 has potential to produce antimicrobial compounds, especially regarding the production of gamma-pyrans.

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