COMPARISON OF IN VITRO ACTIVITIES OF AMPHOTERICIN, CLOTRIMAZOLE, ECONAZOLE, MICONAZOLE, AND NYSTATIN AGAINST *FUSARIUM OXYSPORUM*

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ABSTRACT

The inhibitory activity of amphotericin B, clotrimazole, econazole, miconazole and nystatin was compared against *Fusarium oxysporum f.sp. radicis-cucumerinum*. The most efficient antifungal agent against the growth of *Fusarium oxysporum* was econazole, followed by clotrimazole, miconazole, amphotericin and nystatin. The ED₅₀ and ED₉₀ values were 0.053 and 1.002 ppm for econazole, 0.088 and 1.100 ppm for clotrimazole, 0.173 and 3.210 ppm for miconazole, 0.713 and greater than 48 ppm for amphotericin and 3.860 and 16.702 ppm for nystatin. The ED₅₀ values of nystatin and amphotericin against spore germination of *Fusarium oxysporum* were determined at 3.1427 ppm and 8.3990 ppm respectively, nystatin was 2.76 times more effective than amphotericin, while no effect was observed after the addition of econazole, clotrimazole and miconazole. The tested azoles were more effective than amphotericin and nystatin on growth inhibition of *Fusarium oxysporum* but amphotericin and nystatin acted significantly better on spore germination of *Fusarium*.

*Key Words:* Amphotericin B; Clotrimazole; Econazole; Miconazole; Nystatin; *Fusarium oxysporum*.

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INTRODUCTION

Polyene antibiotics like nystatin and amphotericin B and azoles, like econazole, miconazole and clotrimazole are used for the treatment of fungal infection. Amphotericin B (C_{47}H_{73}NO_{17}) is a product of the fungus *Streptomyces nodocus*. It is active against a broad spectrum of fungi *in vitro* and *in vivo*, including *Aspergillus*, *Candida*, *Coccidioides* and *Cryptococcus* (1). It is very toxic and the common side effects of its intravenous administration are kidney damage, fever, shivering, headache, vomiting and anaemia. Nystatin (C_{47}H_{75}NO_{17}) is a polyene macrolide antibiotic, derived from *Streptomyces noursei* and *Streptomyces aureus*. It usually administered orally and topically and it is used for the treatment of superficial fungal infections of the skin and mucous membranes (2), (3).

Despite their long clinical history, the molecular mechanism of amphotericin and nystatin antifungal action is not well understood. According to the most widely accepted mechanism, their molecules interact with membrane sterols to form channels. Their chemotherapeutic use is based on the higher affinity of these antibiotics for ergosterol (principal sterol in fungal cells). However amphotericin and nystatin have a relatively high affinity for cholesterol too and this gives rise to the toxic effects that these compounds exert on the mammalian cells (4), (5).

Clotrimazole (C_{22}H_{18}ClN_{2}), miconazole (C_{18}H_{14}Cl_{4}N_{2}O) and econazole (C_{18}H_{15}Cl_{3}N_{2}O) belong to the group of azoles. Clotrimazole is administered orally and topically for the treatment of systemic infection or superficial fungal infection of the skin. The side effects of clotrimazole use are anorexia, nausea, vomiting and diarrhoea. Miconazole is less toxic and less effective than clotrimazole but it can be administered intravenously. The above-mentioned antifungals seem to react with the ergosterol of fungal membranes (6).

Patients who are immunocompromised due to cancer chemotherapy are predisposed to several fungal infections. Although *Candida* is the microorganism most often associated with serious fungal infections, other fungi such as *Aspergillus*, *Penicillium* and *Fusarium* as well as dimorphic fungi like *Blastomyces*, *Histoplasma* and *Sporothrix* have also been observed in these patients (7), (8). Amphotericin B, azoles and their derivatives are the drugs mainly used for the treatment of fungal infections.

The aim of the present work was to compare the *in vitro* activity of amphotericin, clotrimazole, econazole, miconazole and nystatin against the growth and spore germination of *Fusarium oxysporum* f.sp. *radicis-cucumerinum*. This study is part of our research in order to select candidate bioactives for new sustained release formulations (9), (10).

MATERIALS AND METHODS

Reagents and Chemicals

The organic solvents used (acetone, methanol, dimethylsulfoxide) were trade products of analytical grade. Amphotericin B (80%), econazole (nitrate salt), clo-
trimazole, miconazole (nitrate salt) and nystatin were provided by Sigma Chemical Co. Dextrose and agar were provided by Merck. Nystatin was found as a 1.000.000 unit pack from Sigma Chemical Co.

Fungus: The isolate *Fusarium oxysporum f.sp. radicis-cucumerinum* AFu-68 used in this study as a model biological material, was obtained from a cucumber plant with “root and stem rot” symptoms (11).

Stock Solutions

Ten mg of clotrimazole, econazole or miconazole were dissolved in 100 ml of acetone to give a final concentration 0.1 mg/ml. Five mg of nystatin were added to 5 ml methanol (C=1 mg/ml), while 5 mg amphotericin (80% w/w) were dissolved in 2 ml dimethylsulfoxide (DMSO), (C=2 mg/ml).

Culture Conditions

*Fusarium oxysporum f.sp. radicis-cucumerinum* was grown on autoclaved potato dextrose agar (PDA), which was prepared by adding the extract from 200 g of boiled potatoes to 600 ml of distilled water, also containing 20 g of dextrose and 18 g of agar until a final volume of 1 L. The pH of the growth medium was adjusted to pH=6.4 by adding phosphate buffer 1M Na₂HPO₄/KH₂PO₄. The fungal growth inhibitors were added to PDA at various quantities giving the desired concentrations. The final concentrations used respectively were, for nystatin 0, 0.5, 1, 2, 5, 10, 15, 20, 30 ppm, for amphotericin 0.1, 0.2, 0.5, 1, 2, 4, 8, 16, 32 ppm, for miconazole 0, 0, 0.02, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 ppm and for econazole and clotrimazole were 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 0.8 ppm. PDA without growth inhibitors but with the suitable amount of each organic solvent (acetone, methanol, DMSO) was used as a control.

The substrate was transferred into 9 cm in diameter sterile petri-dishes and let solidify. A 5mm in diameter mycelium plug was placed at the center of each dish. The dishes were incubated in the dark at 27°C for 6 days. Readings were taken daily, by measuring the diameter of each colony. The growth inhibition for each concentration was calculated from the diameter measurements as a mean of five replicate dishes compared with the control.

The effects of the above-mentioned antifungal agents on *F. oxysporum f.sp. radicis-cucumerinum* spore germination were also assessed. The fungus was grown in autoclaved potato dextrose broth (PDB), which was prepared as described previously but without agar. The medium was dispersed into several 100ml Erlenmeyer flasks, (50 ml PDB/flask). A 5 mm in diameter mycelium plug of *F. oxysporum f.sp. cucumerinum* was added to each flask. The flasks were incubated in a rotary shaker for 3 days at 19°C in the dark. Conidia were removed from PDB, filtered through a double layer of cheesecloth, the mycelial mat was washed with sterile distilled water and the suspension was centrifuged at 3000 g for 10
min. Spores were resuspended in sterile distilled water and the concentration was adjusted to 0.5*10^7 spores/ml. An aliquot of 0.1 ml of the above-mentioned spore suspension, 0.1 ml of 0.1% w/v glucose solution, 0.1 ml of 1 M tris-buffer, pH=7.0, 0.2 ml of sterile distilled water and 0.5 ml solution of each fungistatic agent were transferred to 1 ml eppendorf tubes to achieve the following concentrations: 0, 0.1, 0.2, 1, 2, 4, 8 ppm for amphotericin, 0, 0.25, 0.5, 1, 2, 5, 10 ppm for nystatin, 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 ppm for clotrimazole, 0, 0.005, 0.01, 0.02, 0.04, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 ppm for econazole and 0, 0.02, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 ppm for miconazole. The mixture was incubated for 10 hours at 28°C and the number of germinated conidia was counted.

**RESULTS**

The ED_{50} and ED_{90} values for amphotericin, clotrimazole, econazole, miconazole and nystatin against growth inhibition of *Fusarium oxysporum* are presented in Table 1. Econazole was the most effective agent with ED_{50}=0.053 ppm and ED_{90}=1.002 ppm followed by clotrimazole (ED_{50}=0.088 ppm, ED_{90}=1.100 ppm), miconazole (ED_{50}=0.173 ppm, ED_{90}=3.210 ppm), amphotericin (ED_{50}=0.713 ppm, ED_{90}>48 ppm) and nystatin (ED_{50}=3.860 ppm, ED_{90}=16.702 ppm) (Table 1). The antifungal agents belonging to the group of azoles were more effective than nystatin and amphotericin, which belong to the group of polyenes.

The effect of each organic solvent (methanol, acetone and dimethylsulfoxide) on the spore germination of *Fusarium oxysporum* was evaluated and was subtracted from the total ED_{50} values of amphotericin, clotrimazole, econazole, miconazole and nystatin. The ED_{50} values of nystatin and amphotericin against spore germination of *Fusarium oxysporum* were 3.1427 ppm and 8.3990 ppm respectively (Table 2) while no effects on spore germination were observed when econazole, clotrimazole and miconazole were added, at concentrations that resulted in 90% growth inhibition.

**Table 1.** The Effective Dose (ED_{50} and ED_{90} Values, in ppm) for Amphotericin B, Clotrimazole, Econazole, Miconazole, and Nystatin Against Growth Inhibition of *Fusarium oxysporum f.sp. radicis-cucumerinum*

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>ED_{50} (ppm)</th>
<th>SD*</th>
<th>CV(%)+</th>
<th>ED_{90} (ppm)</th>
<th>SD</th>
<th>CV(%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Econazole</td>
<td>0.053</td>
<td>±0.0035</td>
<td>6.604</td>
<td>1.002</td>
<td>±0.1133</td>
<td>11.307</td>
<td></td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>0.088</td>
<td>±0.0061</td>
<td>6.932</td>
<td>1.100</td>
<td>±0.0630</td>
<td>6.364</td>
<td></td>
</tr>
<tr>
<td>Miconazole</td>
<td>0.173</td>
<td>±0.0168</td>
<td>9.711</td>
<td>3.210</td>
<td>±0.1974</td>
<td>6.8804</td>
<td>0.0027</td>
</tr>
<tr>
<td>Amphotericin</td>
<td>0.713</td>
<td>±0.0814</td>
<td>11.416</td>
<td>&gt;48</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Nystatin</td>
<td>3.860</td>
<td>±0.3183</td>
<td>8.246</td>
<td>16.702</td>
<td>±3.1860</td>
<td>19.075</td>
<td></td>
</tr>
</tbody>
</table>

*SD: Standard deviation.  
CV: Coefficient of variation.
The statistical analysis of the results was performed by one way ANOVA (Excel) as described by Tzatzarakis (10). The ED$_{50}$ and ED$_{90}$ values obtained by all inhibitors were significantly different ($P<0.05$) while the % values of the coefficient of variation (CV%) were small ($<20\%$) confirming the good reproducibility of the experiments (Tables 1 and 2).

**DISCUSSION**

Both amphotericin and nystatin are potent antifungal agents widely used but they are insoluble in water and very toxic. The high inhibitory efficacy of amphotericin on fungal growth has been reported for *Aspergillus fumigatus* (MICs = 1 to 4 µg/ml), for *Aspergillus flavus* (MICs = 4 µg/ml), for *Aspergillus terreus* (MICs = 1 to 2 µg/ml) (12). Moore determined the mean MIC values (2.42 µg/ml) (range 1–64 µg/ml) for *Aspergillus fumigatus*, *A. flavus*, *A. terreus* and *A. niger* using the method of microdilution (13). Ramani determined the MIC values of amphotericin (0.03 to 2 µg/ml) and fluconazole (0.125 to 128 µg/ml) against 31 species of *Candida albicans* proving the higher activity of amphotericin (14). Exposure of *Aspergillus fumigatus* and *Candida albicans* to 10 µg/ml amphotericin resulted to the death of the fungi. On the other hand no killing of *Candida albicans* occurred in the presence of itraconazole and voriconazole at concentration of 10 µg/ml, (8).

Johnson (1) compared the *in vitro* activity of four liposomal formulations of amphotericin B with those of free nystatin and a liposomal formulation of nystatin against 200 isolates of *Aspergillus spp.*, *Candida spp.* and *Cryptococcus neoformans* by a broth microdilution method. It was shown that nystatin was less active than amphotericin and that liposomal nystatin was more active than free nystatin against most species but less active than some of amphotericin formulations.

As indicated in Figure 1, the activity of amphotericin was almost stable after the concentration of 4 ppm. The % growth inhibition of amphotericin against *Fusarium oxysporum* at concentration of 4 ppm was 72.9% while at concentration of 48 ppm was 87.6% (Figure 1). The same was observed for econazole and clotrimazole at concentration of 0.4 ppm and for miconazole at concentration of 1.6 ppm (Figure 2). On the contrary, the activity of nystatin against the growth of *Fusarium oxysporum* increased (constantly) until the concentration of 30 ppm

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>ED$_{50}$ (ppm)</th>
<th>SD</th>
<th>CV(%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nystatin</td>
<td>3.1427 ± 0.139</td>
<td>4.423</td>
<td>0.0362</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>8.3990 ± 1.402</td>
<td>16.692</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
where 100% growth inhibition of \textit{Fusarium oxysporum} was observed. Thus, nystatin appeared more effective than amphotericin at concentrations greater than 12.4 ppm (Figure 1).

As can be observed from Figures 1 and 2, the curves of antifungal action of amphotericin, clotrimazole, econazole, miconazole and nystatin against concentration are not linear. A suitable conversion of the $x$ (concentration) and $y$ values

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Inhibitory action of nystatin and amphotericin B on mycelium growth of \textit{Fusarium oxysporum} f.sp. \textit{radicis-cucumerinum} in pure cultures at 27°C, in the dark.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Inhibitory action of clotrimazole, econazole and miconazole on mycelium growth of \textit{Fusarium oxysporum} f.sp. \textit{radicis-cucumerinum} in pure cultures at 27°C, in the dark.}
\end{figure}
(\% inhibition) will give linear biological activity curves. The concentration values of the x-axis are converted into logarithms while the \% inhibition values of growth on the y-axis undergo a special transformation (probit). The curves provided in this way are linear and are presented in Figures 3 and 4. The slope (a) of the curve is indicative to how effective a drug is and is used as a means of comparison between two drugs. As indicated in Table 3 (data derived from Figures 3 and 4), the slope of the curves of econazole (0.9867) and clotrimazole (1.1373) have almost similar values, suggesting that these two drugs possess similar potency against *Fusarium*

### Table 3. Linear Equations, R-squared, and Significance Value of Amphotericin B, Clotrimazole, Econazole, Miconazole and Nystatin Against Growth Inhibition of *Fusarium oxysporum* f.sp. *radicis-cucumerinum*

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>y = ax + b</th>
<th>R²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Econazole</td>
<td>y = 0.9867x + 1.3300</td>
<td>0.9907</td>
<td></td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>y = 1.1373x + 1.3145</td>
<td>0.9864</td>
<td></td>
</tr>
<tr>
<td>Miconazole</td>
<td>y = 0.7795x + 0.7210</td>
<td>0.9681</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>y = 0.6660x + 0.0749</td>
<td>0.9499</td>
<td></td>
</tr>
<tr>
<td>Nystatin</td>
<td>y = 1.6298x – 0.8815</td>
<td>0.9915</td>
<td></td>
</tr>
</tbody>
</table>

*Figure 3.* Linear activity curves of amphotericin and nystatin against growth inhibition of *Fusarium oxysporum* f.sp. *radicis-cucumerinum* in pure cultures at 27°C, in the dark.
oxysporum. On the other hand, the slope of the activity curve of miconazole is much smaller (0.7795), something that can be seen by looking at the ED$_{50}$ and ED$_{90}$ values (Table 1). The slope of nystatin activity curve (1.6298) is greater than that of amphotericin (0.6660) and this is attributed to the better fungicidal action of nystatin when compared to amphotericin, at concentrations greater than 12 ppm.

Our results indicate that econazole, clotrimazole and miconazole are more active than nystatin and amphotericin against growth inhibition of *Fusarium oxysporum* in vitro. Econazole is slightly more effective than clotrimazole and about 3.3 time more effective than miconazole. The ED$_{50}$ value of amphotericin is 4.4 higher than the ED$_{50}$ value of nystatin but the ED$_{90}$ value of nystatin is lower than that of amphotericin (Table 1). Nystatin exhibited fungicidal action against *Fusarium oxysporum* at the concentration of 30 ppm.

Addition of econazole, clotrimazole and miconazole had no effects on the spore germination of *Fusarium oxysporum*. Nystatin was about 2.67 times more effective than amphotericin on spore germination (Table 2). The maximum % inhibitory activity of nystatin on spore germination was 72% and was achieved at concentration of 5 ppm, while for amphotericin the maximum % inhibitory activity was 49% at concentration of 8 ppm (Figure 5).

**CONCLUSIONS**

The inhibitory activity of amphotericin B, econazole, clotrimazole, miconazole and nystatin was compared against *Fusarium oxysporum* f.sp. radicis-
The most efficient antifungal agent against the growth of *Fusarium oxysporum* was econazole, followed by clotrimazole, miconazole, amphotericin and nystatin. Econazole, clotrimazole and miconazole demonstrated higher activity in vitro than amphotericin and nystatin against growth of *Fusarium oxysporum* f.sp. *cucumerinum* but they were not effective against spore germination of *Fusarium oxysporum*. Although amphotericin showed lower ED$_{50}$ value than nystatin against growth of *Fusarium oxysporum*, nystatin exhibited better fungicide action and was more effective on spore germination inhibition.

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