Eugenol and Isoeugenol In Association with Antifungal Against Cryptococcus neoformans

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Received: 14th March, 17; Revised 30th March, 17, Accepted: 16th April, 17; Available Online:25th April, 2017

ABSTRACT
The antifungal therapy combined is used in clinical practice of several mycoses as it may increase the efficacy of the treatment. The use of natural products (phytochemicals) in combination with conventional antifungal drugs has been related to beneficial effects, mainly synergistic effects. The aim of this study was to evaluate the effect of the combined use of eugenol / isoeugenol, compounds with recognized antimicrobial activity, in association with antifungal amphotericin B against strains of Cryptococcus neoformans. The combined antifungal effect were determined from the Fraction Inhibitory Concentration index - checkerboard technique. The results obtained in this study showed that eugenol in combination with amphotericin B had antagonistic effect against the strains of C. neoformans, LM 615 and INCQS 40221 (FIC index 6.0 and 4.0), respectively. The combination of the isoeugenol and amphotericin B also showed antagonistic effects for both the LM 615 strain and INCQS 40221 (FIC index 6.0 and 5.0), respectively. This study contributed to the understanding of the antifungal effects of the association of phenylpropanoids (eugenol / isoeugenol) with amphotericin B. Further studies are needed to evaluate and compare the effects of the association of these phytochemicals with other conventional antifungal drugs used against C. neoformans.

Keywords: association, eugenol, isoeugenol, amphotericin B, Cryptococcus neoformans, antagonistic.

INTRODUCTION
The use of antifungal drugs in combination is a common practice in the treatment of severe fungal infections. In cryptococcosis, the use of amphotericin B and 5-FC produces synergistic effects against C. neoformans, but presents serious challenges in terms of toxicity and acquired resistance. Studies have indicated that the increasing impact of this infection has motivated the search for alternative drugs from natural products and the combination therapy with synthetic antifungals, in an attempt to improve the success of the therapeutic response.

Essential oils and their products obtained, when used in combination with conventional antifungal drugs, may alter the activity of these antimicrobials, improving their performance, thereby reducing side effects and resistance.

Several structural compounds originate from the phenylpropanoid route. The combination of phenolic phenylpropanoids (exhibiting strong antimicrobial activity) with other components has been related to the increase in the biological activity of these mixtures.

Researches have demonstrated that the phenylpropanoids eugenol and isoeugenol when used in combination with pure substances (plant isolates) or with antifungal drugs exhibited synergistic activity in vitro against yeasts. The eugenol and its isoeugenol isomer are used in scientific research for various purposes, such as the production of organic compounds, medicinal products, carbohydrates and natural products. They are constituents in various aromatic plants and high antimicrobial activity has been reported to these compounds against a large variety of microorganisms, including yeasts.

Based on the above, the main objective of this study was observing the effects of combinations of eugenol and isoeugenol with amphotericin B used in clinical practice against yeasts encapsulated C. neoformans.

MATERIAL AND METHODS
Chemicals
Eugenol, isoeugenol and amphotericin B were purchased from Sigma-Aldrich® (Brazil). The substances were solubilized at the time of the tests dissolving them in Tween 80 (2%) and 5% dimethyl sulfoxide (DMSO) with sterile distilled water to obtain the desired concentrations.

The tubes were mixed for 5 min using a Vortex (Fanem).
Table 1: Minimum Inhibitory Concentration (MIC) of eugenol / isoeugenol and effect of combination with antifungal against C. neoformans (LM 615 and INCQS 40221).

<table>
<thead>
<tr>
<th>Eugenol / Isoeugenol + Antifungal</th>
<th>C. neoformans LM 615</th>
<th>C. neoformans INCQS 40221</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (μg/mL)</td>
<td>Type of interaction</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Eugenol</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Isoeugenol</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>ANFB</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>FIC* Index</td>
<td>6.0</td>
<td>Antagonistic</td>
</tr>
<tr>
<td>EU/ANFB</td>
<td></td>
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</tr>
<tr>
<td>FIC* Index</td>
<td>6.0</td>
<td>Antagonistic</td>
</tr>
<tr>
<td>ISO/ANFB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*FIC: Fractional Inhibitory Concentration; EU: eugenol; ISO: isoeugenol; ANFB: amphotericin B.

Strain of C. neoformans (LM 615) was provided by Professor Dr. Edeltrudes de Oliveira Lima; which belongs to the archival collection of Laboratory of Mycology, Federal University of Paraíba – Brazil. The strain INCQS 40221 was acquired by the National Institute for Quality Control in Health, Oswaldo Cruz Foundation, Rio de Janeiro - Brazil. In the preparation of the suspension to be used, each strain was seeded in Sabouraud dextrose agar, incubated at 35 °C for 24-72 h and after this period, colonies of this culture were suspended in sterile 0.85% NaCl to obtain the inoculum 10^6 count forming unit per mL (1 x 10^6 CFU mL^-1) corresponding to tube 0.5 of McFarland scale.

Determination of Minimum Inhibitory Concentration (MIC)
The determination of the MIC of eugenol and isoeugenol, as well as of the antifungal amphotericin B (positive control), was carried out using the broth microdilution technique using sterile 96-well plates (Alamar, Diadema, SP, Brazil)\(^{12-14}\). Initially, 100 μL of doubly concentrated Sabouraud Dextrose Broth (SDB; Diêco Lab., USA) was then added to the wells. Then 100 μL of the suspension of each product being tested at the initial concentration of 2048 μg/mL (also doubly concentrated) in the first row of the plate in order to obtain by means of a serial dilution of two different concentrations of 1024 to 1 μg/mL. Finally, 10 μL of each suspension of the tested microorganism prepared on the McFarland 0.5 scale (1 x 10^6 CFU mL^-1) were also be added. In parallel, a negative control (without drugs) was made to confirm yeast viability and sensitivity to DMSO. Tween 80 was also included in the studies. It was performed a duplicate test, and the plates were incubated at 37°C for 72 hours.

The MIC for the products was determined by visual observation, since the growth of the microorganisms in the wells of the microdilution plate occurs through the formation of so-called growth buttons (cell agglomeration). In this way, the lowest concentration capable of causing a total visual inhibition of the growth of the strain tested at the end of the incubation period was considered as MIC\(^{15,16}\).

Association study using checkerboard method
The combined effect of tested phytochemicals (eugenol and isoeugenol) with standard antifungal agent (amphotericin B) were determined from the Fraction Inhibitory Concentration (FIC) index checkerboard technique. Solutions of the tested products were used in concentrations determined from their respective MICs. Initially, 100 μL of Saboraud dextrose broth was added to the wells of the 96-well sterile microplate (Alamar, Diadema, SP, Brazil). Then, 50 μL of each product tested at various concentrations (MIC ÷ 16, MIC ÷ 8, MIC ÷ 4, MIC ÷ 2, MIC, MICx2, MICx4, MICx8) were added vertically (standard antifungal) and horizontal (eugenol or isoeugenol) of the microplate. Finally, 10 μL of the fungal suspension was added. The assay was performed in duplicate, with the microplates incubated at 37 °C for 72 hours\(^{17,18}\).

The FIC was calculated by adding the FICA + FICB, where A represents the phytochemicals tested and B the standard antifungal. The FICA, in turn, was calculated through the combined MICA / MICA ratio alone, while the FICB = combined MICB / MICB alone. This index is interpreted as follows: synergism, antagonism, additive and indifference\(^19\).

RESULTS
In order to evaluate the possible combined use of the phenylpropanoids eugenol / isoeugenol with the standard antifungal in therapy of the cryptococcosis, the effect of the association of these phenolic compounds with amphotericin B against strains of C. neoformans was evaluated using the checkerboard technique for derivation of FIC Index, which classifies interactions as: synergic (≤0.5), additive (> 0.5 and <1), indifferent (≥1 and <4) or antagonistic (≥4.0)\(^19\).

The results of the analysis of the checkerboard assay are shown in Table 1. The interactions found for the LM 615 and INCQS 40221 strains were antagonistic both for eugenol and isoeugenol. The combination eugenol - amphotericin B showed FIC = 6.0 for strain LM 615 and FIC = 4.0 for strain INCQS 40221, while the interaction isoeugenol - amphotericin B showed FIC = 6.0 for strain LM 615 and FIC = 5.0 for strain INCQS 40221.

DISCUSSION
Compounds derived from plants have already been proven through several studies as efficient antifungal. Studies of association between compounds obtained from the plants and antifungal used in clinical practice are still considered scarce, although some results are positive\(^20\).

The use of combined drugs in low concentration may result in potentiation and greater efficacy of each drug, which can lead to better tolerability and safety, lower antifungal...
resistance, toxicity, lower dose and a higher possibility of cure.

Studies using the combination of natural compound, pedalitin, isolated from the plant *Pterogyne nitens*, when associated with amphotericin B, showed additive and synergistic effects against strains of *C. neoformans*. Suzano also reported who natural substances isolated from *Serjania erecta* and *Eclipta alba*, when combined with amphotericin B, presented synergistic activity for the resistant strains of *C. neoformans*.

Hamilton and Heliot reported that the results of antifungal association between amphotericin B and fluocytosine may present synergistic and antagonistic responses against strains of *C. neoformans*. These in vitro antifungal effects varying between synergism and antagonism, by the combined use of amphotericin B and fluocytosine, may occur depending on the species used and the strains.

Our results showed antagonistic effects for the combination of the studied phenylpropanoids (eugenol / isoeugenol) with amphotericin B, which does not make this association advantageous for growth control of *C. neoformans*.

The antifungal effect antagonistic of the combination of the phenylpropanoids used with amphotericin B can be explained by the fact that possibly these natural compounds may be acting via mechanism of action along the plasma membrane, at the same site or at another site that may interfere with amphotericin B activity.

CONCLUSION
This study provides important information about the effects of the association between eugenol / isoeugenol and amphotericin B against strains of *C. neoformans*. Since the antagonistic effects found need to be evaluated, if they may also be present in association of the phenylpropanoids with other antifungal agents conventionally used in cryptococcosis therapy.

ACKNOWLEDGEMENTS
The authors thank UFPB, CAPES, CNPQ.

DECLARACION OF INTEREST
The authors declare that do not have any conflict of interest.

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