Investigation on mechanism of antifungal activity of eugenol against *Trichophyton rubrum*

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*Trichophyton rubrum* is a worldwide agent responsible for chronic cases of dermatophytosis which have high rates of resistance to antifungal drugs. Attention has been drawn to the antimicrobial activity of aromatic compounds because of their promising biological properties. Therefore, we investigated the antifungal activity of eugenol against 14 strains of *T. rubrum* which involved determining its minimum inhibitory concentration (MIC) and effects on mycelial growth (dry weight), conidial germination and morphogenesis. The effects of eugenol on the cell wall (sorbitol protect effect) and the cell membrane (release of intracellular material, complex with ergosterol, ergosterol synthesis) were investigated. Eugenol inhibited the growth of 50% of *T. rubrum* strains employed in this study at an MIC = 256 μg/ml, as well as mycelial growth and conidia germination. It also caused abnormalities in the morphology of the dermatophyte in that we found wide, short, twisted hyphae and decreased conidiogenesis. The results of these studies on the mechanisms of action suggested that eugenol exerts antifungal effects on the cell wall and cell membrane of *T. rubrum*. Eugenol act on cell membrane by a mechanism that seems to involve the inhibition of ergosterol biosynthesis. The lower ergosterol content interferes with the integrity and functionality of the cell membrane. Finally, our studies support the potential use of the eugenol as an antifungal agent against *T. rubrum*.

**Keywords** dermatophytosis, *Trichophyton rubrum*, antifungal, eugenol, ergosterol

Introduction

The dermatophyoses are infections of the keratinized tissues caused by fungi known as dermatophytes. *Trichophyton rubrum* is one such dermatophyte responsible for chronic cases of *tinea unguim*, *tinea pedis*, *tinea manuum*, *tinea corporis* and *tinea capitis* [1]. While *T. rubrum* is found throughout the world, it is one of the most frequently isolated fungi in South (including Brazil) America, North America, and Northern and Central Europe with known resistance to the local therapy [2–6].

Antifungal agents such as griseofulvin, azole derivatives and allylamines may become of little use in the treatment of dermatophyoses as a result of the development of fungal resistance, prolonged duration of treatment and side-effects [7]. There exists a clear demand for additional antifungals with therapeutic potential. In this context, attention has focused on the antifungal activity of aromatic plants and their constituents due to their potential biological properties [8]. Eugenol (C\textsubscript{10}H\textsubscript{12}O\textsubscript{2}) is a phenylpropanoid component of aromatic plants, the biological properties of which are related to the presence of phenolic group. Eugenol is the main volatile compound of the buds and leaves of clove (*Eugenia caryophyllata* Thunb.). Plants of the genus *Myristica*, *Cinnamomum* and *Ocimum* are natural sources of this compound and can be detected as minor constituent of the essential oils of *Cymbopogon* species [9,10].

Eugenol has been extensively studied due to its broad range of pharmacological activities such as its anti-inflammatory, antioxidant, antimicrobial properties, etc. [11–14]. In recent years, insight into the mechanisms of
the antifungal actions of natural products has markedly increased. This study demonstrates the importance of investigations on their antifungal activity against *T. rubrum*, the main causative agent of dermatophytosis in man and the need to increase our knowledge of the possible modes of antifungal action of eugenol.

**Materials and methods**

**Chemicals**

Eugenol, ketoconazole and amphotericin B were purchased from Sigma-Aldrich, Brazil. The drugs were dissolved in DMSO (dimethylsulfoxide) and sterile distilled water was used to obtain solutions of 1,024 μg/ml for each of these antifungals. The concentration of DMSO did not exceed 0.5% in the assays.

**Microorganisms**

Dermatophytes used in antifungal assays were obtained from the archival collection of Laboratory of Mycology (LM), Federal University of Paraíba, Brazil. They included 13 clinical isolates of *T. rubrum* (LM) and one from the American Type Culture Collection (ATCC 1683). Stock inocula suspensions of *T. rubrum* strains were prepared from 10-day old potato dextrose agar (Difco Lab., USA) cultures grown at 28°C. Fungal colonies were covered with 5 ml of sterile saline solution (0.9%), the surface was gently scraped with a sterile loop and the saline solution with fungal elements was transferred to a sterile tube. These suspensions were shaken for 2 min using a vortexer, allowed to stand for 5 min to allow hyphal fragments to fall out of the suspensions so that the supernatant containing the conidia could be collected. Inocula were standardized at 0.5 tube of McFarland scale (10⁶ CFU/ml). Quantification was confirmed by inoculating 0.01 ml of a 1:100 dilution of each suspension onto Sabouraud dextrose agar. The plates were incubated at 28°C and examined daily for the development of fungal colonies [15].

**Minimum inhibitory concentration (MIC)**

Broth microdilution assays were used to determine the MICs of eugenol and ketoconazole [16]. Sabouraud dextrose broth (SDB; Difco Lab., USA) was added to all wells of 96-well plates. Serial 2-fold dilutions of the two agents were prepared to obtain concentrations varying between 1 μg/ml and 1,024 μg/ml. Finally, 10 μl aliquots of the inoculum suspension were added to the wells and the plates incubated at 28°C for 5 days. Negative controls (without drugs) were used to confirm conidia viability and sensitivity controls to DMSO were also included in the studies. MIC was defined as the lowest concentration capable of visually inhibiting 100% the fungal growth. The results were expressed as geometric mean of three experiments.

**Effects on mycelial growth**

Analysis of the interference of eugenol and ketoconazole on mycelial growth was determined by assessing the dry mycelial weight of *T. rubrum* ATCC 1683 and LM 422, in triplicate [17]. SDB (4.5 ml) with the drugs (MIC and 2 × MIC) and 0.5 ml of the inoculum suspension were added to sterile tubes. Control studies involved the use of sterile distilled water in place of the drugs. The tubes were incubated at 28°C for 12 days, the mycelia filtered through sterile filter paper (retention of particles: 11 μm) and then dried at 60°C for 10 min. The filter paper containing dry mycelia was weighed and percent of mycelia produced was calculated, using the growth in the control tubes as being 100% of potential dry mycelia weight.

**Effects on conidial germination**

Eugenol and ketoconazole were tested to evaluate their effects on the germination of *T. rubrum* conidia. Strains ATCC 1683 and LM 422 were chosen for use in this test, along with negative controls. Double concentrated SDB (500 μl) containing the drugs (MIC and 2 × MIC) was added to sterile tubes, mixed with 500 μl of fungal suspension and then immediately incubated at 28°C. Samples were taken at 24 h to assess the number of germinated and ungerminated conidia through the use of a hemocytometer from which the percentage of germinated conidia was calculated. The test was performed in triplicate [18].

**Effects on morphogenesis**

Possible alterations in the morphogenesis induced by eugenol and ketoconazole in *T. rubrum* ATCC 1683 and LM 422 were analyzed by the slide culture technique. At first, a block of Sabouraud dextrose agar containing the drugs (half the MIC) was transferred to the center of a glass slide, in a moist chamber. After that, a sample of mycelia was taken and inoculated onto the agar medium block. The moist chamber plates were incubated at 28°C for 5 days and then the two slides of each test strain were fixed in lactophenol-cotton blue stain and 10 fields on each slide were examined with a light microscope at 400× to evaluate morphologic abnormalities. Control assay using agar blocks without drugs was performed. The experiment was performed in duplicate and 10 microscopy fields were viewed on each slide. The predominant structural changes
observed in the tests were recorded as strong (+++), intermediate (++), low (+) or absent (−) [19].

Release of intracellular material

Measurements of the release of material absorbing at 260 nm from *T. rubrum* ATCC 1683 were conducted using 2 ml aliquots of the fungal inocula after the addition of the eugenol and ketoconazole (MIC and 2 × MIC). At different time intervals (4 and 8 h), fungal cells were centrifuged at 3,000 rpm for 5 min, and the supernatant examined by measuring the absorbance at 260 nm with a UV-visible Spectrophotometer (Shimadzu). Control flask without drugs were tested similarly. Alcoholic potassium hydroxide solution (25% diluted with ethanol at 70%) was used as reference compound since it causes 100% cellular leakage. Rate of release of intracellular material absorbing at 260 nm, primarily nucleotides, was calculated by comparing the test values with the lysing agent which was considered to be 100%. The experiment was performed in triplicate [20].

Sorbitol, ergosterol and cholesterol effect assay

MIC values of eugenol and ketoconazole were determined with *T. rubrum* ATCC 1683 by the microdilution method, in the absence and presence of 0.8 mol/l of sorbitol (VETEC Química Fina Ltda Brazil), 400 μg/ml of ergosterol and 400 μg/ml of cholesterol (Sigma-Aldrich Brazil). Amphotericin B was used as a control drug for ergosterol tests. The MIC was determined after 5 days and the tests were performed in triplicate to calculate the geometric mean values [21].

Sterol quantitation assay

Aliquots of the *T. rubrum* (ATCC 1683) inocula (1 ml) were combined in sterile tubes with 9 ml of SDB containing either eugenol or ketoconazole (MIC and 2 × MIC) and incubated for 5 days at 28°C. The fungal cells were then centrifuged at 3,000 rpm for 5 min, washed once with sterile distilled water and the wet weight of the cell pellet determined. Three milliliters of lysing agent were added to each pellet and vortexed for 1 min. Cell suspensions were incubated in 85°C water bath for 1 h and then allowed to cool at room temperature. Sterols were then extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of n-heptane followed by vigorous vortexing for 3 min. The heptane layer was transferred to Eppendorf tubes and stored under refrigeration for 24 h. Aliquots of sterol extracts were examined by measuring the absorbance at 281.5 nm and 230 nm with a UV-visible spectrophotometer (Shimadzu). This assay was performed in triplicate. Ergosterol content was calculated as percent of the wet weight of the cell as reported by Arthington-Skaggs [22].

Data analysis

The results were expressed in mean ± SE. Statistical analyses were performed with *t*-test. *P* < 0.05 was considered significant.

Results

Studies of antifungal activity

MIC values of eugenol against *T. rubrum* strains varied from 64–512 μg/ml, with 50% of the strains tested inhibited at 256 μg/ml and 90% at 512 μg/ml. MICs of ketoconazole ranged from 16–128 μg/ml in that 50% of the isolates were inhibited at 16 μg/ml and the MIC for 90% of the strains was 32 μg/ml. DMSO did not inhibit the growth of the controls and all strains were found to grow in the absence of drugs, confirming the viability of the fungi. Two *T. rubrum* strains (ATCC 1683 and LM 422) were employed to explore the effects of eugenol and ketoconazole on mycelial growth, conidial germination and morphogenesis as both isolates had MIC values of 256 μg/ml (eugenol) and 16 μg/ml (ketoconazole).

The effect of MIC and 2 × MIC of the drugs on the mycelial growth was determined by measuring the dry mycelia weight of *T. rubrum* strains (Fig. 1). As seen in Figure 1A, all tested concentrations, especially at 2 × MIC, inhibited the mycelial growth of *T. rubrum* LM 422 (*P* < 0.05) as compared with the control (100% mycelia yield). Similar effects were noted with ATCC 1683 in that the drugs effectively inhibited the mycelial growth (Fig. 1B).

The percentage of germinated conidia of *T. rubrum* LM 422 and ATCC 1683 are recorded in Figure 2A. At their MICs, the drugs significantly inhibited conidial germination (*P* < 0.05), with the effects most evident at 2 × MIC.

The effects of eugenol and ketoconazole on morphogenesis of *T. rubrum* ATCC 1683 and LM 422 were analyzed (Table 1). In the absence of drugs, we observed long, narrow, septate, hyaline hyphae, with tear-drop shaped microconidia arranged laterally on the conidiophores (macroconidia were not observed). Eugenol (128 μg/ml) and ketoconazole (8 μg/ml) induced similar morphological changes in both strains of *T. rubrum*. While there was a decreased level of conidio genesis in the presence of eugenol, ketoconazole completely inhibited conidial formation. Normal hyphal formation of *T. rubrum* was visibly affected in the presence of both drugs, with the formation of wide, short and crooked hyphae, which
are not characteristic of the species. Furthermore, chlamydoconidia production by *T. rubrum* did occur in the presence of ketoconazole, but not eugenol.

**Studies on mechanisms of action**

The results of the sorbitol protection assay are presented in Table 2. The eugenol MIC was increased by 4-fold with sorbitol as compared to tests without it. Meanwhile, an 8-fold increase was observed in the ketoconazole MIC. Fungal growth was noted in the presence of sorbitol and absence of drugs.

The effect of MIC and 2×MIC of the drugs on cell membrane was initially determined by measurement of the release of 260 nm absorbing intracellular material from *T. rubrum* ATCC 1683 (Fig. 3). Eugenol caused significant leakage of intracellular material compared to control (no drugs), after 4 h interaction (*P < 0.05*).

To further investigate the action of the drugs on the fungal cell membrane, we evaluated the ability of the test compounds to form complexes with ergosterol (ergosterol effect assay). We used exogenous cholesterol in another experimental group, as it is the steroid component of human cell membranes. Results showed (Table 2) that the MIC values of eugenol and ketoconazole remained unchanged, suggesting that the drugs did not act by binding to membrane ergosterol. Regarding amphotericin B, 8×MIC and 32×MIC were observed in the presence of ergosterol and cholesterol, respectively.

**Fig. 1** Percentage of dry mycelial weight produced by *Trichophyton rubrum* in the absence (control) and presence of eugenol (MIC: 256 μg/ml; 2×MIC: 512 μg/ml) and ketoconazole (MIC: 16 μg/ml; 2×MIC: 32 μg/ml). (A) LM 422; (B) ATCC 1683. Control produced 100% of dry mycelial weight. *P < 0.05* compared to control.

**Fig. 2** Percentage of conidial germination of *Trichophyton rubrum* LM 422 (A) and ATCC 1683 (B) in the absence (control) and presence of eugenol (MIC: 256 μg/ml; 2×MIC: 512 μg/ml) and ketoconazole (MIC: 16 μg/ml; 2×MIC: 32 μg/ml). *P < 0.05* compared to control.
Eugenol inhibits *Trichophyton rubrum* growth

**Table 1** Observations detected in microculture of *Trichophyton rubrum* in the absence (control) and presence of eugenol and ketoconazole (half of MIC).

<table>
<thead>
<tr>
<th>Observations</th>
<th>Control</th>
<th>Eugenol</th>
<th>Ketoconazole</th>
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<tbody>
<tr>
<td>Conidiogenesis</td>
<td>+++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Normal arrangement of conidia</td>
<td>+++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Macroconidia</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Chlamydoconidia</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>Wide hyphae</td>
<td>–</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Short and winding hyphae</td>
<td>–</td>
<td>++</td>
<td>+++</td>
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+++; strong; ++, intermediate; +, low; –, absent.

We also explored whether eugenol disrupts the synthesis of ergosterol by *T. rubrum* ATCC 1683. For this, the sterol contents produced by the strain in the presence of different concentrations (MIC and 2 × MIC) of eugenol and ketoconazole were quantified. Eugenol and ketoconazole inhibited the production of ergosterol by fungal cells (*P* < 0.05) as compared to control (Fig. 4). Ketoconazole was used as control in this experiment since it interferes with the biosynthetic pathway of ergosterol. It is worth noting that eugenol was more effective than ketoconazole at 2 × MIC (*P* < 0.05).

**Discussion**

According to criteria proposed by Sartoratto *et al.* [23], eugenol and ketoconazole showed strong antifungal activity against *T. rubrum* as the MIC values of eugenol were lower than 500 µg/ml. Ketoconazole was used as a positive control because it is one of the commonly used antifungal drugs for the treatment of infections caused by *Trichophyton* spp. [24]. In the literature, eugenol has been found to be active against yeast and filamentous fungal species [10]. In this study, eugenol disrupted mycelia growth as indicated in the dry mycelium mass test which reflects the production of fungal cell material. In dermatophyto genesis, hyphal production is important because they penetrate into the deeper layers of the epidermis. This is of particular importance since the outer tissue layers are constantly being lost [25].

*T. rubrum* conidia are considered the primary means of establishing dermatophytosis in human and animals. They adhere to epithelial cells of the keratinized tissues of the host where they germinate and initiate invasion of the stratum corneum. Hyphae can grow in multiple directions and form mycelium [24,26]. Given the importance of *T. rubrum* conidia, the findings of this study are of particular significance because eugenol, as well as ketoconazole, inhibited conidial germination of *T. rubrum*.

Eugenol also induced relevant morphological abnormalities of *T. rubrum* which are of great importance in preventing its growth, viability and virulence [17,19]. Chlamydoconidia were observed in the presence of ketoconazole. These structures are produced by many fungi, in response to environmental stimuli under adverse conditions, such as osmolarity, light, pH, temperature and presence of antifungal drugs [26]. The literature indicates that *T. rubrum* is responsible for most cases of chronic and recurrent dermatophytosis. This can be related to its ability to produce chlamydoconidia as an important defense mechanism against toxic agents, as observed in the results of this study [27]. There is some evidence that eugenol also affects the morphogenesis of *C. albicans* and *T. mentagrophytes* [28,29].

The fungal cell wall is a dynamic structure that protects fungal protoplasts from external osmotic shocks and defines fungal morphogenesis. Thus, changes in the organization or functional disruption of the cell wall induced by antifungal agents are involved in fungal death [23,30]. The sorbitol protection assay was performed to further explore the mode of action of eugenol on the integrity of the fungal cell wall. Drugs that act on the cell wall cause lysis of fungal cells in the absence of an osmotic stabilizer (sorbitol), but their growth can continue in the presence of sorbitol [21]. According to Frost *et al.* [31] this assay is generic in nature and is of use in the search of compounds that directly inhibit the synthesis of cell wall constituents such as glycans, mannans, chitin or the regulatory mechanisms as found in these studies of the effects of eugenol on cell walls. In contrast to eugenol results found in our study, Carrasco *et al.* [32] confirmed that antifungal activity of the eugenol derivative 4-allyl-2-methoxy-5-nitrophenol against *Cryptococcus neoformans* and *C. albicans* was not reversed in the presence of an osmotic support. This would suggest that inhibiting fungal cell wall synthesis or assembly is not altered when the chemical structure of eugenol is maintained.

It is reported that the antimicrobial mechanism of cyclic hydrocarbons, such as eugenol, is related to its lipophilic character in that they increase the fluidity and permeability of the cell membrane of microorganisms. In fact, these compounds interfere with ion transport, unbalancing osmotic conditions in the membrane and making its associated...
proteins inefficient. In any case, this can lead to inhibition of microbial growth, and death or cell lysis [33].

Because of these effects on microbial membranes, we investigated whether eugenol damages cell membrane of *T. rubrum* by the detection of intracellular components released into the external environment. These cellular components represent a class of compounds – primarily nucleotides – that show a strong absorption at 260 nm [22]. Our results suggest that eugenol interferes with the integrity of the cell membrane producing damage to the structure. Based on this, it became imperative to determine which factors present in the membrane are involved in this activity.

The fungal cell membrane is a dynamic structure composed of a lipid bilayer where enzymes and transport proteins are embedded. Ergosterol – lipid steroid of fungal cell membrane – functions as an important regulator of membrane fluidity. Therefore, in the search for new antifungals, their effects on ergosterol are investigated [34]. The ability of eugenol to form complexes with ergosterol was evaluated from the perspective of investigating its action on the fungal cell membrane (ergosterol effect assay). The drug will bind to exogenous ergosterol, avoiding its ergosterol-membrane binding to the membrane. In this assay, amphotericin B was used as positive control. The exogenous cholesterol was used in another experimental set since it is the steroid component present in human cell membranes. Our results suggest that eugenol did not act by binding to ergosterol or cholesterol because its MIC values remained unchanged.

Considering ergosterol as an important lipid of fungal cell membrane, changes in its biosynthetic pathway may also cause damage to the fungal cell [35]. In order to further clarify this possibility, we explored the absorption at 230 nm and 281.5 nm of sterols extracted from fungal cultures in the presence of eugenol (MIC and 2 × MIC). Both ergosterol and a late intermediate in the sterol pathway – 24(28)-dehydroergosterol (DEH) – absorb at 281.5 nm, whereas only DEH shows intense absorption at 230 nm. Changes in absorption patterns are indicative of interference with ergosterol synthesis route [22]. Similarly, it was reported that eugenol also exerted its antifungal activity on *Candida* strains by inhibiting sterol biosynthesis [36].

Since sterols inserted between phospholipids moderate its fluidity, the reduction in the content of ergosterol interferes with the integrity and functionality of the cell membrane. As a result, the functioning of membrane-bound enzymes such as those involved in cell wall synthesis may be impaired. It may cause damage to the cell wall, as observed in the morphogenesis and sorbitol assays [34,37].

This study support the view that eugenol exerts its antifungal activity on the cell wall and cell membrane of *T. rubrum* by disrupting ergosterol biosynthesis. Thus, our results may serve as a guide for future *in vivo* studies of clinical use of eugenol in treating dermatophyte infections.
Eugenol inhibits *Trichophyton rubrum* growth

### Acknowledgments

The authors thank Wellington Lima Navarro for support with UV spectrophotometer analysis.

### Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

### References


This paper was first published online on Early Online on 26 November 2012.