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SHORT COMMUNICATION/COURTE COMMUNICATION

In vitro activity of carvacrol and thymol combined with antifungals or antibacterials against *Pythium insidiosum*

Activités in vitro d'associations de carvacrol et de thymol avec des antifongiques ou des antibiotiques contre Pythium insidiosum

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Summary We describe the in vitro activities of the combinations of carvacrol and thymol with antibiotics (azithromycin, clarithromycin, minocycline and tigecycline) and antifungal agents (amphotericin B, caspofungin, itraconazole and terbinafine) against 23 isolates of the oomycete *Pythium insidiosum*. The assays were based on the M38-A2 technique and checkerboard microdilution. Based on the mean FICI values, the main synergies observed were combinations of carvacrol + itraconazole and thymol + itraconazole (96%), thymol + clarithromycin (92%), carvacrol + clarithromycin (88%), thymol + minocycline (84%), carvacrol + minocycline (80%), carvacrol + azithromycin (76%), thymol + azithromycin (68%), carvacrol + tigecycline (64%) and thymol + tigecycline (60%). In conclusion, we found that combinations of carvacrol or thymol with these antimicrobial agents might provide effective alternative treatments for cutaneous pythiosis due to their synergistic interactions. Future in vivo experiments are needed to elucidate the safety and therapeutic potential of these combinations.

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Carvacrol ;
Thymol

Résumé Au cours de cette étude, des activités antimicrobiennes ont été évaluées in vitro en combinant le carvacrol et le thymol avec des antibiotiques (azithromycine, clarithromycine, minocycline et tigécycline) ou des antifongiques (amphotéricine B, caspofungine, itraconazole et terbinafine) contre 23 isolats de l'oomycète *Pythium insidiosum*. Les essais ont été réalisés en microdilution selon les techniques standardisées M38-A2 et en damier. Après calcul des valeurs moyennes d'indice de concentration inhibitrice fractionnaire (FICI), il ressort que les principales activités synergiques anti-*Pythium* ont été observées pour les combinaisons d'itraconazole avec le carvacrol ou le thymol (96 %), de clarithromycine avec le thymol (92 %) ou le carvacrol (88 %), de minocycline avec le thymol (84 %) ou le carvacrol (80 %), d'azithromycine avec le carvacrol (76 %) ou le thymol (68 %), de tigécycline avec le carvacrol (64 %) ou le thymol (60 %). En conclusion, les combinaisons de carvacrol ou de thymol s'avèrent intéressantes grâce à leurs interactions synergiques avec des composés antimicrobiens dans l'élaboration de nouveaux traitements efficaces contre la pythiose cutanée. De futures expériences in vivo sont nécessaires pour confirmer en thérapie humaine le potentiel de ces combinaisons synergiques et s'assurer de leur innocuité.

Introduction

Similar to other species of its genus, *Pythium insidiosum* is a species of oomycete that causes severe disease in plants [24]. *P. insidiosum*, however, also has the unusual ability to cause severe disease in humans and other mammals [12,32]. *P. insidiosum* is considered a pseudofungus because it produces hyaline hyphae similar to those of true fungi. Because of this, many attempts have been made to treat pythiosis using antifungal drugs. However, most of these treatments have been unsuccessful [17,31] because this species is unable to synthesize ergosterol, the main target of most antifungal drugs. As a result, there is currently no gold standard treatment for pythiosis.

The pythiosis cases are usually from regions of tropical and subtropical climate and are associated with human or animal contact with areas where there is accumulation of water (lakes and wetlands) at environmental temperature between 30 and 40 °C and there are zoospores of *P. insidiosum*. The biological cycle proposed for the *P. insidiosum* is based on the colonization of aquatic plants, which serve as a substrate for mycelial vegetative growth and asexual formation of biflagellate zoospores, which in the presence of a mammalian host, can cause the disease. The hyphae of *P. insidiosum* does not penetrate the intact skin, suggesting that the macro- or microscopic lesions are necessary for the infection to occur. There are no reports of transmission between mammalian hosts [12,22].

Surgery (aggressive surgical excision and amputation) is the most common treatment of the pythiosis. Unfortunately, complete surgical excision, often, is impossible due to the anatomical site of the lesions, resulting in a high rate of local recurrence. Because of this, surgical excision is frequently associated with antifungal therapy and immunotherapy, and the therapeutic success depends on rapid diagnosis and proper identification of the pathogen [12,17].

The antifungal activity of carvacrol and thymol, as well as their synergistic potential with antibiotics, has been described in a number of studies [1,6,15,18,26,28,29,37]. However, the in vitro susceptibility of *P. insidiosum* to carvacrol and thymol, alone or with antibiotics, has not been evaluated. Clinical pythiosis can vary depending on the host. Cutaneous, gastrointestinal, vascular and systemic forms have been described [12]. Due to the limitations on oral or systemic administration of essential oils and derivatives [38], they may have a greater therapeutic potential against cutaneous and subcutaneous forms of pythiosis. In this context, this study aimed to assess the in vitro activity of carvacrol and thymol in combination with antibiotics or antifungal drugs.

Material and methods

We evaluated 23 Brazilian isolates of *P. insidiosum* from equine pythiosis cases, as well as the ATCC58637 and CBS 101555 reference strains. The identities of the isolates were confirmed using PCR [8]. Carvacrol (CRV, Sigma Aldrich®, St. Louis, USA) and thymol (THY, Sigma Aldrich®, St. Louis, USA) were diluted in ethanol (3.2×10^4 µg/mL stock solution) or distilled RPMI 1640® (working solution). Azithromycin (AZT, Pharma Nostra®, Rio de Janeiro, Brazil), clarithromycin (CLT, Genix®, Anápolis, Brazil), minocycline (MIN, Pharma Nostra®, Rio de Janeiro, Brazil), tigecycline (TIG, Pfizer®, New York, USA), amphotericin B (AMB, Sigma Aldrich®, St. Louis, USA), caspofungin (CAS, Merck®, Darmstadt, Germany), itraconazole (ITZ, Janssen Pharmaceutica®, São Paulo, Brazil), and terbinafine (TRB, Novartis®, Basel, Switzerland) were diluted in dimethyl sulfoxide or distilled water to generate stock solutions.

Susceptibility tests were performed according to the CLSI M38-A2 microdilution protocol [11], adapted according to Pereira et al. [30]. The interactions between carvacrol or thymol and antifungal agents or antibiotics were evaluated using a checkerboard test [13,27]. Synergy test results after 24 h of incubation at 37 °C were interpreted according to (a) the lowest fractional inhibitory concentration index (FICI) and (b) the mean FICI values for all of the non-turbid (clear) wells along the turbid/non-turbid growth interface. FICI values were defined as synergism when ≤ 0.5, indifference when between > 0.5 and 4 and antagonism when > 4. Each test was performed in duplicate. The high off-scale MICs were converted to the maximum concentration when needed.