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Short Communication

In vitro susceptibility of *Pythium insidiosum* to garlic extract

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***Pythium insidiosum* infections are difficult to treat with conventional antifungal agents because of the lack of ergosterol in the cytoplasmic membrane of this aquatic oomycete. The effectiveness of the garlic compound allicin has been demonstrated against a range of bacteria, fungi and plant oomycetes. Therefore, we evaluated the *in vitro* activity of *Allium sativum* extract against 17 clinical isolates of *P. insidiosum*. The assays were performed according to M38-A2 Clinical Laboratory and Standards Institute documents. Minimum inhibitory concentrations varied from <0.39 to 6.25 mg ml^{-1}. The results showed that garlic extract has strong inhibitory activity toward *P. insidiosum* isolates *in vitro*. By understanding the cons of extrapolating *in vitro* data to clinical studies, *A. sativum* seems to offer a promising alternative in the treatment of pythiosis infections in humans and animals**.

Key words: *Pythium insidiosum*, *Allium sativum* extract and broth microdilution.

INTRODUCTION

Pythiosis is a life-threatening disease of mammalians including humans in the tropical, subtropical and temperate regions of the world. It is caused by the oomycete *Pythium insidiosum*, a water-dwelling organism of the kingdom Straminipila (Mendoza and Newton, 2005). Pythiosis progresses rapidly, most often leading to death if untreated or if treatment fails. Antifungal therapy is scarcely effective because of the special oomycete's characteristic cell wall composition and the lack of ergosterol in the cytoplasmic membrane (Pereira et al., 2007).

Garlic (*Allium sativum*) has been reported to possess medicinal, insecticidal, antimicrobial, antiprotozoal and antitumour properties (Ghannoum, 1988). In addition, garlic extract shows *in vitro* inhibitory activity against a large number of filamentous and yeast-like fungi, including oomycetes (Caporaso et al., 1983; Ghannoum, 1988; Hughes and Lawson, 1991; Curtis et al., 2004). Allicin was shown to be the major antimicrobial substance in garlic and the antifungal properties of its metabolite ajoene have been observed since 1951 when it was shown that aqueous extracts of garlic inhibited growth of fungi recovered from soil samples (Thomaz et al., 2008).

The purpose of this study was to investigate the *in vitro* activity of garlic extract against 17 strains of *P. insidiosum* isolated from equines with pythiosis in Brazil.

MATERIALS AND METHODS

The identities of the isolates were confirmed by a PCR-based assay (Grooters and Gee, 2002). The inoculum consisted of *P. insidiosum* zoospores obtained through the process of zoosporogenesis and adjusted as proposed by Pereira et al. (2007) from protocol M38-A2 (CLSI, 2008). Garlic extract was obtained according to Abdalla et al. (2010), with minor modifications, that is, each 5 ml of the

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Table 1. *In vitro* activity of garlic extract against *P. insidiosum* isolates (n = 17) (mg ml^{-1}).

Isolates	MICs
LAPEMI 125	0.78
LAPEMI 126	0.78
LAPEMI 135	6.25
LAPEMI 137	1.56
LAPEMI 143	0.78
LAPEMI 148	<0.39
LAPEMI 156	0.78
LAPEMI 178	1.25
LAPEMI 198	0.78
LAPEMI 210	1.56
LAPEMI 223	0.78
LAPEMI 227	3.12
LAPEMI 232	6.25
LAPEMI 247	1.56
LAPEMI 253	1.56
LAPEMI 254	0.78
LAPEMI 260	1.56
MIC-range	<0.39-6.25
Geometric mean	1.41

Minimum inhibitory concentrations (MICs).

aqueous alcoholic garlic extract obtained was lyophilized in individual glass vials and stored in dark room at 20 to 30°C. Just before the assay, the extract was diluted in RPMI 1640 broth containing L-glutamine and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid.

The concentrations for use in the microdilution assay ranged from 0.39 to 50 mg ml^{-1} of crude extract. For each strain tested, a positive (inoculum diluted) and a negative control (RPMI broth only) were performed. The minimal inhibitory concentrations (MICs) were read after 24 h incubation at 37°C. The reading was v isual and assessed the growth or absence of growth of hyphae. MICs were defined as the lowest extract concentration at which there was 100% inhibition of fungal growth. The tests were carried out in duplicate on the same day; whenever the values obtained were not coincident, the assay was repeated. The experimental procedure from the lyophilization process until the last strain assayed lasts four months.

RESULTS AND DISCUSSION

All the isolates produced detectable growth at 24 h. Table 1 shows the *in vitro* activity of garlic extract against *P. insidiosum*. All strains were inhibited by the garlic extract used. Virtually all conventional antifungal agents are ineffective for the treatment of human and animal pythiosis. Successful management of pythiosis requires early recognition and appropriate treatment, which includes radical surgery and immunotherapy. However, there was one report of successful treatment of invasive facial pythiosis with the association of terbinafine and itraconazole (Shenep et al., 1998). Recently, Hummel et al. (2011) reported a case of canine gastrointestinal pythiosis in which lesions were resolved through the association of both of these antifungals and the agricultural fungicide mefenoxam.

Even while several authors have only shown broad-spectrum activity of garlic extracts against fungi *in vitro*, topical antifungal properties of the garlic compound ajoene have been demonstrated against *Tinea pedis* and chromoblastomycosis (Ledezma et al., 1996; Thomaz et al., 2008; Perez-Blanco et al., 2003). This would be interesting in cutaneous or subcutaneous pythiosis infections in animals and humans, as well as in corneal infections, which result in complete visual loss of the affected eye in approximately 80% of the cases (Tanhehco et al., 2011). Moreover, ajoene treated mice developed Th1-type cytokine responses producing higher levels of IFN-γ (Thomaz et al., 2008), which resembles the mechanism by which immunotherapy against *P. insidiosum* works, a switch in the host's immune response from a Th2 (during infection) to a Th1 immune response (after immunotherapy) (Mendoza and Newton, 2005).

We are unaware of any other active inhibitory natural compound against *P. insidiosum*. Pure allicin was found to have a high anticandidal activity with a MIC of 7 µg ml^{-1}. Interestingly, allicin is unstable (Caporaso et al., 1983), and its antimicrobial activity slowly decreases over a period of 10 days in dark room (Curtis et al., 2004). However, there was no detectable loss of activity over our 4-month study period. The possibility that the lyophilization process had stabilized the garlic extract

deserves further investigations. Commercially prepared garlic capsules were initially considered ineffective (Caporaso et al., 1983), although Slusarenko et al. (2008) reported promising results using alginate and other formulations to encapsulate garlic juice.

In conclusion, garlic extract showed great *in vitro* activity against *P. insidiosum* animal isolates. It is reason-able, therefore, to perform susceptibility tests using pure preparations of allicin or ajoene. Combination antifungal therapy, the current state of the art in *P. insidiosum* studies (Cavalheiro et al., 2009), using garlic compounds can also be an interesting approach, as a synergistic effect of allicin against *Mycobacterium tuberculosis* has been found with antibiotics (Gupta and Viswanathan, 1955).

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