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Short communication

Diphenyl diselenide *in vitro* and *in vivo* activity against the oomycete *Pythium insidiosum*

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ABSTRACT

This study evaluated the *in vitro* activity of diphenyl diselenide against 19 *Pythium insidiosum* isolates and the *in vivo* therapeutic response of rabbits with experimentally induced pythiosis. *In vitro*: susceptibility tests were performed using the broth macrodilution method in accordance with the CLSI document M38-A2. The criteria for interpretation were as follows: MIC-1 and MIC-2 (inhibition of 90% and 100% of mycelium growth, respectively) and the minimum fungicide concentration (MIC-3). *In vivo*: twenty rabbits were divided into four groups with five animals each and treated for 40 consecutive days: groups 1 and 2 (experimentally induced pythiosis) were treated with diphenyl diselenide (10 mg/kg/day) and canola oil (1 mL/kg/day), respectively; groups 3 and 4 (controls) were treated with canola oil (1 mL/kg/day) and diphenyl diselenide (10 mg/kg/day), respectively. Toxicity was evaluated using biochemical and haematological parameters. *In vitro* susceptibility tests showed that 89.4% of isolates had a MIC-1 $\leq$ 0.5 μg/mL, 84.2% of isolates had a MIC-2 $\leq$ 1.0 μg/mL and 94.7% of isolates had a MIC-3 $\leq$ 2.0 μg/mL. The *in vivo* assay suggested that this compound has a fungistatic activity, and the biochemical and haematological parameters indicated that there was no renal, hepatic or haematological toxicity. The comparison of the unsaturated iron binding capacity levels between animals with and without pythiosis suggested the involvement of iron metabolism in the pathogenesis of pythiosis. This study demonstrated the absence of detectable toxicity caused by diphenyl diselenide and the *in vitro* fungicidal and *in vivo* fungistatic activities of this drug, which makes it an option for future therapeutic approaches in the treatment of pythiosis.

1. Introduction

The oomycete *Pythium insidiosum* is a pathogen that causes a chronic pyogranulomatous disease in the subcutaneous tissue in humans and animals, especially horses, dogs and cats (Gaastra et al., 2010; Perez et al., 2005). The clinical presentations of pythiosis include cutaneous, gastrointestinal, vascular and systemic forms, depending on the species affected and the site of infection. The disease, which progresses rapidly, has been reported in tropical and subtropical areas, and if not treated in the early stages, the infected hosts often die within weeks. The recommendations for the treatment of this disease with antifungal agents remain contradictory, and the combination of surgical treatment with antifungal agents or immunotherapy is described in cases of therapeutic cure

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(Argenta et al., 2010; Cavalheiro et al., 2009b; Gaastra et al., 2010).

Diphenyl diselenide [$(PhSe)_2$] is a simple, stable and highly lipophilic organoselenium compound (OS) that is widely used as an intermediate in organic synthesis. Extensive studies have focused on the pharmacological and toxicological effects of $(PhSe)_2$ in different biological models, highlighting its antioxidant, antinociceptive, anti-inflammatory, antiulcer and hepatoprotective activities (Nogueira et al., 2004; Rosa et al., 2007).

Recently, we demonstrated that $(PhSe)_2$ has *in vitro* fungistatic and fungicidal activity against yeasts and filamentous fungi (Loreto et al., 2011b). However, the anti-oomycete activities of $(PhSe)_2$ against *P. insidiosum* were not described. This study aimed to evaluate the *in vitro* susceptibility of *P. insidiosum* to $(PhSe)_2$ using the Clinical and Laboratory Standards Institute (CLSI) standardised broth macrodilution test and the response of rabbits with experimentally induced pythiosis to *in vivo* therapy with $(PhSe)_2$.

2. Materials and methods

2.1. Microorganisms

Seventeen clinical *P. insidiosum* isolates (two from Rio Grande do Sul and thirteen from the Pantanal region, Brazil) from animals with pythiosis (sixteen horses and one sheep) and the ATCC 58.637 and CBS 101.555 reference strains were used.

The DNA sequences of the partial cytochrome oxidase II (COX II) gene and a fragment of the internal transcribed spacer regions (ITS) of all isolates tested were amplified by PCR and sequenced, confirming the molecular identity of the isolates as *P. insidiosum* (unpublished data).

2.2. Diphenyl diselenide

The synthesis, chemical purity (99.9%) and analytical data for $(PhSe)_2$ were provided and determined by the Laboratory of Synthesis, Reactivity, Toxicological and Pharmacological Evaluation of Organochalcogens, located at the Federal University of Santa Maria, Brazil.

2.3. In vitro susceptibility test

The susceptibility was evaluated with the broth macrodilution method, according to the CLSI M38-A2 protocol (Clinical and Laboratory Standards Institute, 2008). The inocula, diluted in RPMI 1640 broth, pH 7.0, contained 2–3×10^3 zoospores/mL and were obtained by the zoosporogenesis induction technique as previously described (Santurio et al., 2003). The minimum inhibitory concentrations (MICs) were determined by visual observation of the presence or absence of mycelium growth after 24 h of incubation at 37 °C. MIC-1 and MIC-2 represent inhibition of 90% and 100% of mycelium growth, respectively. The minimum fungicidal concentration (MIC-3) was obtained by determining the concentration at which subcultured samples showed no visual evidence of mycelia growth in Sabouraud dextrose broth for up to 96 h at 37 °C. The geometric mean (GM) was calculated for the values of MIC-1, MIC-2 and MIC-3. The tests were performed in duplicate.

2.4. Experimentally induced pythiosis and treatment with diphenyl diselenide

Twenty *New Zealand* rabbits of three months of age, including males and females, were divided into four groups of five animals each:

Group 1, experimentally induced pythiosis, treated with $(PhSe)_2$ (10 mg/kg/day).
Group 2, experimentally induced pythiosis (disease control), treated with canola oil (1 mL/kg/day).
Group 3, control without pythiosis, treated with canola oil (1 mL/kg/day).
Group 4, treatment control (without pythiosis), treated with $(PhSe)_2$ (10 mg/kg/day).

The vehicle (canola oil) was chosen due to the high lipophilicity of $(PhSe)_2$. The rabbits in groups 1 and 2 were infected subcutaneously with 20,000 zoospores/mL of *P. insidiosum*, according to the methodology proposed by Santurio et al. (2003). The treatment with oral $(PhSe)_2$ (gavage) began 30 days after inoculation, approximately 1 week after the observation of measurable subcutaneous lesions. The lesions' subcutaneous nodular areas (cm^2) in groups 1 and 2 were determined at intervals of 4 days after the beginning of the treatment with $(PhSe)_2$ using a calliper and measuring the transverse and longitudinal lengths of the lesions. All animals were treated for 40 days. The procedure was approved by the Animal Welfare Committee of the Federal University of Santa Maria.

2.5. Biochemical and haematological parameters

At the end of the experiment, the animals were euthanised through deepening of anaesthesia using thiopental (20 mg/kg), and serum and plasma samples were collected and separated to carry out biochemical and haematological tests. Biochemical parameters were evaluated using commercial kits (Labtest®), and an electrolyte analyser (AVL 9140®, Roche). Haematological parameters were determined by an automated haematology analyser (Pentra® Horiba ABX) and by preparing stained haematological slides for microscopy observation of the cells. The biochemical and haematological parameters evaluated are shown in Table 2.

2.6. Statistical analysis

The results are presented as the mean ± standard deviation. Statistical analysis was performed using a two-way analysis of variance (ANOVA) followed by Duncan's test, using a significance level of 5%.

3. Results

3.1. In vitro susceptibility test

The results of the test of the *in vitro* susceptibility of 19 *P. insidiosum* isolates to $(PhSe)_2$ are described in Table 1.