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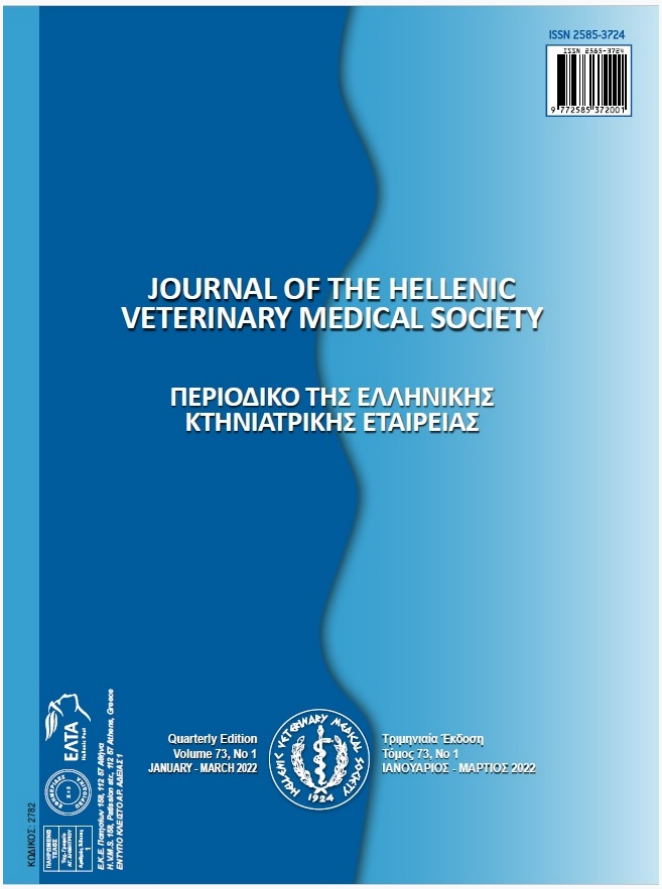

The biological potential of a product containing Pythium oligandrum against Uncinaria stenocephala (Railliet, 1884) larvae

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Research article
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The biological potential of a product containing *Pythium oligandrum* against *Uncinaria stenocephala* (Railliet, 1884) larvae

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ABSTRACT: *Pythium oligandrum* is an oomycete commonly used in the biological control of plant and vegetable pests. In veterinary medicine is used to treat dermatophytosis produced by *Microsporumcanis*. It acts as an antagonist, negatively influencing the development of other fungi. Through hydrolytic enzymes, such as kinase and cellulase, it destroys the cell wall and the internal cytoplasmic content. Many fungi are considered nematophagous. This potential has not been exploited for *Pythium oligandrum*, which is why in this study the potential larvicidal action against *Uncinaria stenocephala* larvae was investigated.

Ecosin® product, which contains *P. oligandrum* and other excipients, was used. A solution was prepared according to manufacturer. The *Uncinaria stenocephala* larvae were exposed to this substance and the action was investigated after 7 days. The results obtained showed 37.23% larval reduction.

Being an eco-friendly product, further studies are needed to improve the protocol for its use in cleaning spaces and surfaces in veterinary clinics, where various parasitic forms (larvae or eggs) are found.

Keywords: Pythium oligandrum, *Uncinaria stenocephala*, larvicide, dog parasites.

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INTRODUCTION

Pythium oligandrum is a common fungus used in agriculture due to the various actions that offer plants pest resistance. It can act through mycoparasitism, antibiosis and through the competitive mechanism regarding the nutrition reserves and space necessary for the development of other fungi (Bradshaw-Smith et al., 1991; Benhamou et al., 2012). The most frequent localization of *Pythium oligandrum*, under natural conditions, is at the root of the plant. Multiplication only occurs at the epidermal or at the most cortical level, without affecting the integrity of the morphological components of plants. Unlike other fungi, *Pythium oligandrum* multiplies in the hyphae and produces different hydrolytic enzymes (kinase, cellulase) that destroy the cell wall and internal cytoplasm (Picard et al., 2000). Through cellulolytic enzymes, *Pythium oligandrum* destroys other oomycetes or fungi. The mechanism of the antibiosis consists in the production of secondary toxic metabolites with antagonistic effect on other fungi (Gerbore et al., 2014).

The action of plant protection against various pathogens is conferred by *Pythium oligandrum* through some eliciting proteins, such as: oligandrine and elicitin (Takenaka and Tamagake, 2009). *Pythium oligandrum* is used in silviculture in the biological control of various pests attacking plants and vegetables. In veterinary medicine *Pythium oligandrum* is used against the dermatophyte *Microsporum canis* but has antagonistic action for about 50 fungi and oomycete species (Gabrielová et al., 2018). Through chemotropism, *P. oligandrum* develops and forms a network of hyphae around the targeted agent (fungus). Binding to the pathogenic fungus also occurs in the hyphae, through cell surface receptors. Penetration inside cells takes place after the hydrolytic enzyme release. Once inside, it continues to multiply and to form papillary structures. The multiplication process is favored by the need of carbon provided through the gates created after cell wall degradation. Where *P. oligandrum* develops, the cell contents are neutralized and only wall fragments remain (Benhamou et al., 2012; Horner et al., 2012).

No data has been found on its potential action against various parasitic forms in carnivores or other animals.

The present study follows the action of Ecosin®, containing *Phytium oligandrum*, on *Uncinaria stenocephala* larvae. The purpose of this study is to investigate the biological potential of *P. oligandrum*, so that it can be used as an agent in cleaning various surfaces in current veterinary practice.

MATERIALS AND METHODS

The source of fungus

In this experiment the Ecosin® (Galenicka laborator Ostrava, Obrancumiru 234/41, 703 00 Ostrava - Vitkovice, Czech Republic) product was used, whose components are: citric acid, sodium bicarbonate, sorbitol, silicon dioxide, *Panicum miliaceum, Pythium oligandrum*, polyethylene glycol (PEG 6000) and sodium carbonate. This product comes in the form of tablets weighing 3 g. The indicated therapeutic dose was chosen to see the effect that may be observed on *U. stenocephala* larvae: one tablet was dissolved in 2 l of hot water (approximately 34°C).

The source of larvae

Fresh feces were collected from several adult dogs from the public shelter. Each sample was initially examined using the Willis method to identify possible eggs from the *Ancylostomatidae* family. From each positive sample, larval cultures were made using the Harada-Mori method.

Identification of larval species (*Uncinaria stenocephala*)

The PCR reaction was performed according to the technique described by Silva et al. (2006) and Gasser et al. (1996), with some minor changes. The actual amplification was performed by classical PCR and was based on the creation of several copies of an ITS gene sequence, 8850-bp in size, for *Ancylostoma* ITS region.

The primers used were: NC5 (forward 5′-GTAGGTGAACCTGCGGAAGGATCATT-3) and NC2 (reverse 5′-TTAGTTTCTTTTCCTCCGCT-3′) for the first PCR; NC1 (forward 5′- ACGTCTGGTTCAGGGTTGTT-3′) and NC2 (reverse 5′-TTAGTTTCTTTTCCTCCGCT-3′) for the second PCR.

The amplification was done according to the protocol described in the article, modified according to mixture requirements. For the PCR reaction a MyTaqTM Red Mix Master Mix (BIOLINE®) was used. The final volume used in the PCR reaction was 25 µl: 12.5 µlMyTaqTM Red Mix (BIOLINE®), 1 µL primer 1F, 1 µl primer 2R (diluted to 10 pmol / µl accord-

ing to protocol described by the manufacturer), DNA extracted from the test sample and ultrapure water.

The amplification program was performed using a fast My Cycler (BioRad®) thermocycler. The program used for the first and second PCR, included: the DNA denaturation steps at 95 °C for 1 minute; 32 denaturation cycles at 95 °C, for 30 seconds; a hybridization at 50 °C for 30 seconds and extension at 72 °C, for 30 seconds; followed by incubation at 4 °C.

The analysis and amplicons control were done using horizontal electrophoresis in a submerged system of 1.5% agarose gel electrophoresis, adding Midori Green (Nippon Genetics® Europe) fluorescent staining, at 120 V and 90 mA, for 60 minutes.

The 100 bp Ladder DNA marker was used in the first well in the gel.

After the sample migration in agarose gel, the image with the migrated DNA fragments was captured using a UV photo documentation system (UVP®).

To identify the species, the PCR products were sequenced and compared with those available in the GenBank database, using BLAST alignment. *Uncinaria stenocephala* (Railliet, 1884) has been identified.

Exposure of *Uncinaria stenocephala* larvae to *Pythium oligandrum* fungus

Thirty Petri dishes with 9 cm diameter were prepared, in which *Uncinaria stenocephala* larvae were placed (about 100 larvae in each plate), together with 3 ml of physiological serum. In 15 of the Petri dishes (considered the treated group), 7 ml of Ecosin® solution were added. In the control samples (n = 15) another 7 ml of physiological serum were added. According to the manufacturer, after 2 days of exposure, 5 ml of Ecosin® solution were removed and re-added. The same operation was performed after another 2 days to ensure that all the indicated therapeutic protocol steps were followed. The solution was put on media several times, due to its inactivation after 24 hours. The samples were sputtered daily to provide oxygen. They were examined daily and after 7 days of exposure, the percentage of larval reduction in the treated samples was evaluated, according to the following equation:

$$\% \text{ Reduction} = \frac{\text{(average of recovered L3 from control-average of recovered L3 from treated)}}{\text{average of recovered L3 from control}} \times 100$$

Statistical analysis

The average and standard deviation for treated and control group were determined. The statistical interpretation of the results was made in GraphPad Prism program, QuickCalcs, using two-tailed Fischer's exact test to obtain the P-value.

RESULTS

After 7 days, mobile and immobile larvae were identified in the treated samples. There were no morphological changes at the cuticular level due to the multiplication of the oomycete *Pythium oligandrum*. The percentage of larval reduction was 37.23%, the result being encouraging.

Extremely statistically significant differences were observed between the treated and control samples ($P < 0.0001$). The average number of L3 recovered in the treated samples was 52.8 (±12.89) compared to 84.13 (±8.97) found in the control samples (Table 1).

Table 1. The number of viable larvae recovered from control and treated samples

Treated samples	Recovered L3/ Total	Control samples	Recovered L3/ Total
1	56/100	**1**	73/100
2	48/100	**2**	82/100
3	70/100	**3**	90/100
4	62/100	**4**	90/100
5	32/100	**5**	75/100
6	70/100	**6**	77/100
7	47/100	**7**	82/100
8	70/100	**8**	82/100
9	44/100	**9**	90/100
10	54/100	**10**	90/100
11	56/100	**11**	100/100
12	47/100	**12**	100/100
13	49/100	**13**	78/100
14	60/100	**14**	71/100
15	27/100	**15**	82/100

DISCUSSION

Being the first study on the biological potential of Ecosin® in controlling the viability of *U. stenocephala* larvae, no other results have been identified in the literature to be compared.

About 99, 000 fungi species have been described (Kirk et al, 2008). Many of these are found in soil and are saprophytic but can become predatory agents in contact with various parasitic forms (eggs or larvae). In general, fungi are not resistant in the external environment and require a nutrient substrate to form spores or to develop mycelium (forms of resistance). *Duddingtonia flagrans*, *Monacrosporium* spp. and *Arthrobotrys* spp. have larvicidal action in hookworms and roundworms (Saumell et al., 2016). Satisfactory results were observed in experiments using *Duddingtonia flagrans*. Chlamydospores determined a percentage of larval reduction (*Ancylostoma* spp.) between 57 - 79.4% (Maciel et al., 2010) or 4.5 - 63% (De Mello et al., 2014). The percentages varied depending on chlamydospore number to which the larvae were exposed. The best effect was observed using 25.000 *Duddingtonia* chlamydospores. Thus it can be a limiting agent in the development of infesting larvae from *Ancylostomatidae* family (De Mello et al., 2014).Other studies suggest that *Duddingtonia flagrans* has no larvicidal effect against *Muellerius capillaris* (first-stage larvae), in small ruminants (goats) (Paraud et al., 2005).*Duddingtonia* can also be used to control the viability of L1 *Angiostrongylus vasorum*. The raw extract of *D. flagrans* determined 53.5% (after 24h) and 71.3% (after 48h) larval reduction (Braga et al., 2009). Similar results were obtained after using raw extract of *Monacrosporium thaumasium*. Conidia used in coprocultures determined 40% larval reduction.Higher percentages were observed in water-agar media, 74.5% respectively (Soares et al., 2015).

Other authors observed a high larvicide action (73.84%) using *Arthrobotrys robusta* (isolated I-31) preserved on silica gel against L3 of *Haemonchus contortus* in ruminants (Braga et al., 2014).Good results were also observed after *Drechmeria coniospora* (Santos and Charles, 1995) or *Harposporium anguillulae*use (Charles et al., 1996).

The fungi action used in the treatment of various diseases is also due to its secondary metabolites. The ones with medical use are as following: alkaloids, quinones, peptides, terpenes, polyketides, coumarins and sterols (Costa et al., 2016). Among the alkaloids, diketopiperazine alkaloids produced by various fungi showed antileishmanic action (Metwaly et al., 2015). The quinones (cercosporin) have antiparasitic action in the case of *L. donovani* and *T. cruzi* (Martínez-Luis et al., 2011). The peptides can be used in parasitism with *T. gondii*, *N. caninum* and *Cryptosporidium parvum* due to the inhibitory action reflected on histone diacetylase, thus intervening in DNA transcription and affecting the reproduction of the parasite (Darkin-Rattray et al., 1996). The sesquiterpene molecules produced by different fungi have antiprotozoal action (*Leishmania* spp., *T. gondii* and *N. caninum*) (Loo et al., 2017). Triterpenoids produced by basidiomycete fungi are also good agents in controlling certain parasites (Nyongbela et al., 2013). In case of malaria, coumarin products, such as dihydroisocoumarins, have worked effectively (Kongsaeree et al., 2003).

The advantage of identifying fungi that can be used in the control of various parasites is related to their less devastating effect on the ecosystem. Thus, it is desired to use natural biotic agents, with localization and development in the external environment, to be reused in the same context, only as a protection against harmful agents to animals, plants, etc.

CONCLUSIONS

The results obtained in this study indicate a potential larvicidal action of Ecosin®, containing *P. oligandrum* (37.23% larval reduction). However, further studies are needed to improve the protocol for the use of Ecosin® product as a potential biological agent in the control of parasites.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of paper.

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