

In vitro and *in vivo* activity of three sesquiterpenes against L₃ larvae of *Anisakis* type I

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ABSTRACT

In order to investigate the possible use of terpenic derivatives to treat anisakiasis caused by L₃ larvae of *Anisakis*, we studied the *in vitro* and *in vivo* larvicidal activity of three sesquiterpenes (nerolidol, farnesol and elemol). *In vitro* experiments included the histological study of larval damage and *in vivo* studies the measurement of myeloperoxidase activity in rat gastrointestinal tract after administration of the sesquiterpenes. In the *in vitro* assays, the most active compound against the L₃ larvae was nerolidol, followed by farnesol; both caused the death of all nematodes, which showed cuticle changes and intestinal wall rupture. In the *in vivo* assays, only 20% of infected rats treated with nerolidol or farnesol showed gastric wall lesions in comparison to 86.6% of control animals. According to these results, nerolidol and farnesol are good candidates for further research as biocidal agents against L₃ larvae of *Anisakis* type I.

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1. Introduction

Anisakiasis is a widely distributed zoonosis associated with fish consumption. Most cases have been attributed to infection by *A. simplex* s.l. Recent genetic molecular studies by Mattiucci et al. (2009) and other authors described two clades of the *Anisakis* genus. Clade I includes larvae of *Anisakis* type I (*sensu* Berland, 1961) with six species, three of which constitute the *A. simplex* s.l. complex (*Anisakis simplex* s.s., *A. pegreffii* and *A. simplex* C), which differ in their genetic structure, biological cycle and geographical distribution.

Anisakiasis is characterized by an eosinophilic infiltration which evolves to the formation of granuloma in the digestive tract. Endoscopic excision of larvae is the most widely reported treatment for acute cases of gastric, duodenum or colon anisakiasis (Oshima, 1972; Petithory and Marty, 1988; Kasuya et al., 1989; De Montigni et al., 1991; Plath et al., 2001; Repiso et al., 2003). In chronic gastric anisakiasis or in severe cases with endoscopically inaccessible larvae, the most frequent approach is surgery to excise the affected area (Deardorff et al., 1986; Kwee and Sautter, 1987; Petithory and Marty, 1988; Repiso et al., 2003). When abdominal symptoms are not severe, the treatment is usually conservative; a wide variety of drugs have been prescribed (Matsui et al., 1985; Verhamme and Ramboer, 1988; Moore et al., 2002; Ramos et al., 2005), but no comparative data are available on their effec-

tiveness. Some researchers, mainly Japanese, have investigated the action of traditional natural products against L₃ larvae of *Anisakis* (Kasuya et al., 1988,1990; Goto et al., 1990; Suzuki et al., 1994). Our group has established a research line on the *in vitro* and *in vivo* activity of different essential oils and their main components against L₃ larvae of *Anisakis* (Hierro et al., 2004a; Valero et al., 2006; Navarro et al., 2008). The present study analyses the larvicidal activity of three sesquiterpenes: nerolidol, farnesol and elemol, which have shown biocidal activity against different pathogens, including protozoa (Brehm-Stecher and Johnson, 2003; Arruda et al., 2005; Hoet et al., 2006).

2. Material and methods

2.1. Sesquiterpenes tested

Three concentrations of each sesquiterpene (nerolidol, farnesol and elemol) were prepared with 96% ethanol: 12.50, 6.25 and 3.12 mg/ml. Selection of these concentrations was based on the study by Kasuya et al. (1990).

2.2. *In vitro* larvicidal activity

L₃ of *Anisakis* type I were collected by dissecting the fish *Micromesistius poutassou* (blue whiting), fished in the Cantabric sea, and selecting only larvae with a length >2.0 cm. Larvae were axenised in antibiotic solution (Iglesias et al., 1997), introduced into polystyrene plate wells with 2 ml of sterile solution of 0.9%

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NaCl and 20 µl of each dilution of test compound, and then incubated at 36 °C in 5% CO₂ atmosphere. As controls, larvae were assayed without test compound under identical experimental conditions. Larvae were examined under stereoscopic microscope at 4, 8, 24 and 48 h to test the biocidal effect of the compound. Larvae with no mobility at all were considered dead. Each dilution was tested three times on larvae from fish captured on different days.

2.3. Histological study

Conventional histological methods were used to detect damage in the dead larvae, which were transferred from the wells into tubes with 10% formalin buffer, staining 1 µm thick sections with toluidine blue.

2.4. *In vivo* larvicidal activity

Selection of the dose of each compound (46.9 mg) was based on earlier findings by our research group (Hierro et al., 2004a; Navarro et al., 2008); dilutions were in 0.5 ml olive oil (vehicle). Female Wistar rats weighing around 150 g were infested with six L₃ of *Anisakis* type I by gastric probe. Test compounds were simultaneously administered using the same procedure. Fifteen rats were used for each compound. A control test was performed in one group of 15 animals, infesting each with six L₃ of *Anisakis* type I in 0.5 ml olive oil and with no administration of test compound. Regulated necropsy of the rat was performed (Feldman and Seely, 1988), recording the locations of the larvae, whether they were alive or dead and the presence of any gastrointestinal lesions.

2.5. Measurement of tissue myeloperoxidase (MPO) activity

Because essential oils tend to irritate the mucosa, we investigated the presence of local inflammatory reaction in the gastrointestinal tract after administration of the test compound (46.9 mg), measuring MPO activity (marker of neutrophilic infiltration) by the method of Krawisz et al. (1984).

3. Results and discussion

3.1. *In vitro* assays

At the maximum concentration of 125 µg/ml, all three sesquiterpenes tested were effective *in vitro* against L₃ larvae of *Anisakis* type I, achieving 100% mortality at the first reading (4 h). Cuticular alterations were observed under stereoscopic microscope (Fig. 1). At concentrations of 62.5 µg/ml, the mortality at 4 h was 100% with nerolidol and elemol but 90% with farnesol. At concentrations of 31.2 µg/ml, the mortality at 4 h was 100% with nerolidol and 80% with farnesol obtained 80%, while elemol was ineffective at

this time. Because of the high mortality obtained by nerolidol and farnesol at this concentration, we tested them at half the dose (15.6 µg/ml), finding no significant reduction in the mortality rates (Fig. 2). According to these findings; the order of *in vitro* activity against the L₃ larvae is nerolidol > farnesol > elemol.

Histological studies revealed that nerolidol produced *in vitro* an alteration of the nematode cuticle and a rupture of the intestinal wall of the parasite, while farnesol and elemol only damaged the cuticle (Fig. 3). Inoue et al. (2004) found that the activity of nerolidol against *Staphylococcus aureus* is at least in part due to their action on its cell membrane. Both nerolidol and farnesol showed greater *in vitro* activity in comparison to the following monoterpenes investigated by us: cuminic aldehyde, carvacrol, citral, citronellol, eucalyptol, geraniol, myrcene, perillaldehyde, α and β pinenes, and ocimene (Hierro et al., 2004a, 2004b, 2006; Valero et al., 2006; Navarro et al., 2008).

3.2. *In vivo* assays

In the *in vivo* test, gastric wall lesions were observed in 20% of infected rats treated with nerolidol or farnesol, in 40% of those treated with elemol, and in 86.6% of those treated with vehicle (olive oil) alone. Lesions were smaller in all nerolidol and farnesol-treated animals than in the control group (Fig. 4), whereas they were similar between elemol-treated animals and controls. Under our experimental conditions, the order of their *in vivo* activity is nerolidol = farnesol > elemol.

According to the MPO values, farnesol and nerolidol do not induce irritative damage in stomach, small intestine or caecum and may exert some protective effect on these tissues. There was no difference in MPO findings between elemol-treated animals and with controls (Table 1).

The lower level of activity against *Anisakis* shown by elemol may be related to its different structure (monocyclic sesquiterpene), since both nerolidol and farnesol derive from an acyclic skeleton. This possibility is supported by the observation of Priestley et al. (2003) that small structural modifications can have a large effect on the activity of a molecule by changing its bioavailability and capacity to access action sites.

Our findings are consistent with reports on the biocidal activity of nerolidol against other pathogen organisms. Hoet et al. (2006) demonstrated that nerolidol, the main component of the essential oil of *Strychnos spinosa* (plant used traditionally in Africa for the treatment of African trypanosomiasis), has a powerful and selective effect against *Trypanosoma brucei*. The biocidal activity of nerolidol against amastigotes and promastigotes of different *Leishmania* species (*L. amazonensis*, *L. braziliensis* and *L. chagasi*) has also been demonstrated (Arruda et al., 2005); the authors related this action to its capacity to block the formation of ergosterol and dolichols 11 and 12 in both forms of *Leishmania* (amastigotes and promastigotes), thereby inhibiting one of the

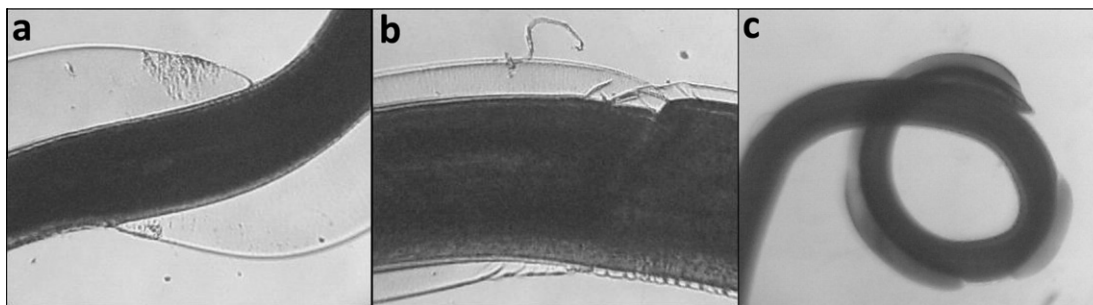


Fig. 1. Cuticular alteration, visible under stereoscopic microscope, in L₃ larvae treated *in vitro* with (a) nerolidol; (b) farnesol; (c) elemol.

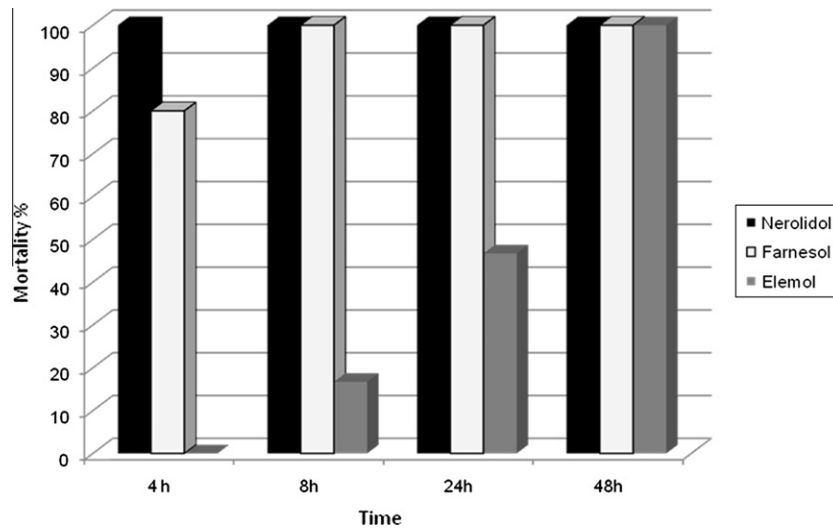


Fig. 2. *In vitro* activity of nerolidol, farnesol and elemol against L₃ larvae of *Anisakis simplex* type I at the lowest concentration tested (31.2 µg/ml). (1) Nerolidol; (2) farnesol; (3) elemol. Activity is expressed as mortality.

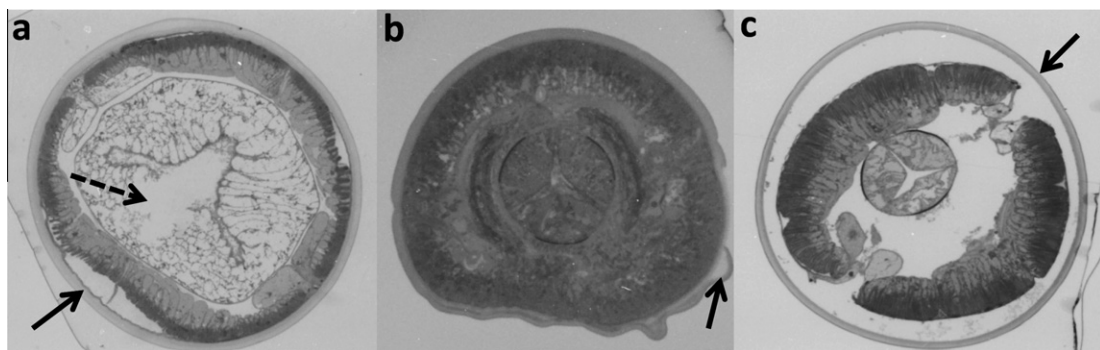


Fig. 3. Histological damage produced *in vitro* by nerolidol, farnesol and elemol in L₃ larvae of *Anisakis simplex* type I. Continuous arrows indicate cuticular alterations; discontinuous arrows indicate intestinal alterations. (a) Nerolidol; (b) farnesol; (c) elemol.

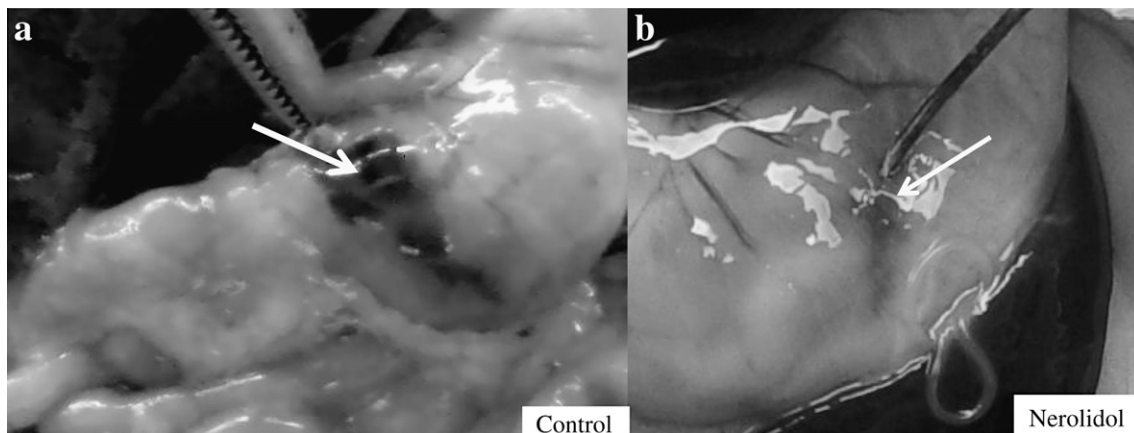


Fig. 4. Lesions caused in the stomach by L₃ larvae of *A. simplex* type I. (a) Control group; (b) group treated with nerolidol.

enzymes responsible for the formation of farnesyl pyrophosphate (FPP) precursors. Other authors, in a study on *Plasmodium falciparum*, attributed the action of nerolidol to the elongation of isoprene chains of dolichols and ubiquinone (Rodrigues Goulart et al., 2004). Hence, nerolidol may act *via* more than one pathway on isoprenoid

derivatives in eukaryotic cells. Lopes et al. (1999) proposed that nerolidol acts on the trophozoite stage of *P. falciparum* via competitive inhibition of the biosynthesis of isoprene derivatives (e.g., dolichols), resulting in the inhibition of protein glycosylation. Inoue et al. (2004) and Brehm-Stecher and Johnson (2003) reported that

Table 1

MPO values in stomach, small intestine and caecum of rats treated with nerolidol, farnesol and elemol. Vehicle: olive oil.

	Stomach (mU/g prot.)	Small bowel (mU/g prot.)	Caecum (mU/g prot.)
Control (0.5 ml olive oil)	8228.9	3640.9	4543.4
Nerolidol (46.9 mg/0.5 ml)	1126.48 <i>p</i> < 0.001	1219.88 <i>p</i> < 0.001	1257.83 <i>p</i> < 0.001
Farnesol (46.9 mg/0.5 ml)	2356.56 <i>p</i> < 0.001	2035.26 <i>p</i> = 0.019	1293.32 <i>p</i> < 0.001
Elemol (46.9 mg/0.5 ml)	6672.8 <i>p</i> = 0.173	5140.65 <i>p</i> = 0.171	3070.08 <i>p</i> = 0.027

nerolidol and farnesol acted against *Staphylococcus aureus* by increasing its membrane permeability and hence its susceptibility to different antibiotics.

Based on the above data, two mechanisms can be proposed for the action of nerolidol and farnesol on L₃ larvae of *Anisakis* type I: inhibition of the biosynthesis of isoprene compounds indispensable for protein glycosylation in eukaryotic cells; and an increase in the permeability of the membrane.

In comparison to the monoterpenic derivatives previously studied by us (Hierro et al., 2004a,2004b,2006; Valero et al., 2006; Navarro et al., 2008), nerolidol and farnesol are less active against L₃ larvae of *Anisakis* type I *in vivo* in comparison to citral, citronellol and perillyl aldehyde but are more active than the other terpenic derivatives tested.

Given these findings on the efficacy and low toxicity of nerolidol and farnesol, further investigation is warranted into their use as biocidal agents against L₃ larvae of *Anisakis* type I.

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