Experimental Parasitology 120 (2008) 295–299

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Anisakis simplex s.l.: Larvicidal activity of various monoterpenic derivatives of natural origin against L_3 larvae *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 31 January 2008
Received in revised form 23 July 2008
Accepted 23 July 2008
Available online 31 July 2008

Keywords:
Anisakis simplex s.l.
Larvicidal activity
Monoterpenic derivatives
α-Pinene
Ocimene

ABSTRACT

This paper describes the activity against *Anisakis simplex* s.l. L_3 larvae of six monoterpenic derivatives obtained from different essential oils, (α-pinene, β-pinene, ocimene, myrcene, geranyl acetate, and cineole). In *in vitro* assays, α-pinene, ocimene and cineole showed high activity at a concentration of 125 μg/mL (48 h) but only α-pinene and ocimene were active at 62.5 μg/mL. In *in vivo* assays, L_3 larvae and study compounds were simultaneously administered *per os* to Wistar rats. The most active compound was α-pinene, finding lesions in only 20% of treated rats versus 98% of controls. Further *in vivo* studies are required to investigate whether addition of these compounds to food could have a prophylactic effect, reducing the pathogenicity of *A. simplex* s.l. L_3 in humans, and to explore any possible synergy among compounds.

1. Introduction

The complex *Anisakis simplex* includes various sibling species that can produce human anisakidosis (Mattiucci and Nascetti, 2008).

Human anisakidosis is closely linked to the consumption of raw or undercooked fish. Man is an accidental host for *Anisakis* (Petithory and Marty, 1988), which dies a few weeks after its ingestion by humans, failing to reach adulthood. However, the presence of live *Anisakis* larvae can give rise to two differentiated diseases: infection (anisakidosis) of the digestive tract or occasionally other organs; and allergic reactions, with or without digestive symptoms (Ishikura, 1990; Daschner et al., 2000).

The lack of compounds for the effective treatment of anisakidosis, combined with reports on the biocidal effects of different products of natural origin (Koutsoumanis et al., 1999; Mejholm and Dalgaard, 2002; Harpaz et al., 2003; Burt, 2004), prompted research into the activity of various vegetable extracts and essential oils or their components against *A. simplex* third-stage larvae (L_3), including components of ginger (*Zingiber officinale*) (Goto et al., 1990) and (+)-ar-turmerone isolated from *Curcuma longa* (Suzuki et al., 1994). Observations of a lower prevalence of this parasitic disease in populations that used the aromatic plant *Perilla frutescens* (Lamiaceae) as a condiment for raw fish led Kasuya et al. (1988, 1990) to perform *in vitro* studies on compounds from *P. frutescens* leaf extract. They reported that larvicidal activity was exerted by perillaldehyde, oxidised monoterpene of essential oil from *P. frutescens*. Various essential oils have well-documented biocidal action (Megalla et al., 1980; Onawunmi et al., 1984; Uribe et al., 1985; Inouye et al., 1998; Farag et al., 1989; Aligiannis et al., 2001; Duru et al., 2003), and our group has demonstrated the activity of some essential oils against *A. simplex* (Hierro et al., 2004a, 2004b, 2006; Valero et al., 2006).

The objective of the present study was to investigate the possible activity against *A. simplex* L_3 of different components of essential oils structurally related to perillaldehyde, exploring the possible use of these compounds as food additives for prophylaxis against anisakiosis.

2. Materials and methods

2.1. Compounds tested

The following monoterpenes were tested against *A. simplex* s.l. L_3: α-pinene, β-pinene, ocimene, myrcene, cineole and geranyl acetate [provided by Sensient Fragrances (Granada, Spain)] (Fig. 1). The purity of these compounds, established by gas chromatography–mass spectrometry (GC: Hewlett-Packard 6890; MS: Hewlett-Packard 5973), was: α-pinene, 97%; β-pinene, 97%: ocimene, 98%; myrcene, 99%; cineole, 100%; and geranyl acetate, 95%.

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0014-4894/$ - see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.exppara.2008.07.014

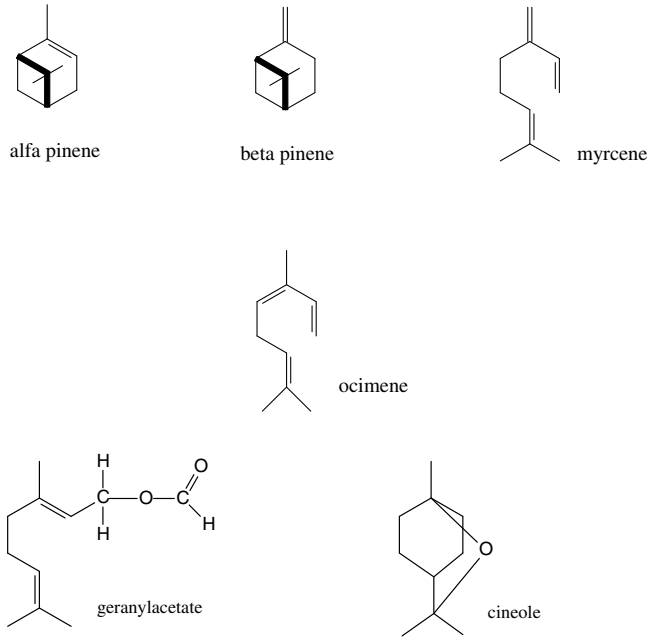

Fig. 1. Chemical structures of assayed monoterpenes.

2.2. In vitro larvicidal activity

Anisakis simplex L_3 larvae were collected by dissecting *Micromesistius poutassou* (blue whiting). Larvae were identified under optical microscope after removing debris adhering to the cuticle. L_3 larvae over 2.0 cm in length were selected for the experiments. A total of 2.0 mL of sterile 0.9% NaCl solution and 20 μL of the product (at indicated dilution) were deposited into each well of polystyrene plates. The final concentrations tested were 125, 62.5 and 31.25 μg/mL. The larvae, pre-treated with antibiotic solution (Iglesias et al., 1997), were placed in the wells and incubated at 36 °C in 5% CO_2-air atmosphere. Two controls with 0.9% NaCl solution (control 1) or 1% ethanol (control 2) were used. Larval motility was observed under stereoscopic microscope at 4, 8, 24 and 48 h. Each dilution of the natural products was tested three times on different days.

2.3. Histological study

Damage caused to *A. simplex* s.l. L_3 by the two most active components (α-pinene and ocimene) was assessed by optical microscopy study of Epon-embedded transverse sections (1- and 0.5-μm thick) stained with toluidine blue.

2.4. In vivo assays

Female Wistar rats with body weight of 150 g were infested by gastric probe with six *A. simplex* L_3 larvae and simultaneously administered with a dose of α-pinene or ocimene directly into the stomach. The dose was 46.9 mg/0.5 mL of olive oil, based on previous studies by our group (Valero et al., 2006). After necropsy (Feldman and Seely, 1988), performed at 4 h post-infestation, data were gathered on the localization of the larvae, the number alive and dead and the presence of gastrointestinal lesions. Fifteen rats were used for each assay. A parallel control test was also performed, administering six L_3 larvae with 0.5 mL of olive oil to each control animal ($n = 15$).

The viability of larvae obtained from the rats was determined by keeping the larvae (extracted from α-pinene, ocimene and control groups) in sterile saline solution at 36 °C in 5% CO_2-air atmosphere and observing the mortality daily for seven days.

3. Results and discussion

3.1. In vitro larvicidal activity

At the maximum concentration tested (125 μg/mL), two out of the six monoterpenic compounds, α-pinene and ocimene (both non-oxygenated monoterpenes), were 100% lethal at 4 h. At this concentration, the *in vitro* larvicidal activity of these two compounds was similar to that of the oxygenated monoterpenic derivatives carvacrol, thymol, citronellol and citral (Hierro et al., 2004a; Valero et al., 2006). At the same concentration (125 μg/mL), β-pinene (positional isomer of α-pinene) and myrcene (positional isomer of ocimene) were practically inactive until 48 h, when a mortality of 35.48% and 35.55%, respectively, was observed. The difference in larvicidal activity between myrcene and ocimene was similar to that between α-pinene and β-pinene. The change in double-binding site has a major effect on the larvicidal activity of myrcene, which is considerably lower than that of ocimene. Among the oxygenated monoterpenic derivatives, the maximum concentration (125 μg/mL) of geranyl acetate exerted larvicidal activity at 24 h, with a mortality of 20%, which rose to 90% at 48 h (Fig. 2). However, the mortality was lower with geranyl acetate than previously observed with geraniol (Valero et al., 2006), possibly due to acetylation of the hydroxyl group in the latter. Cineole, another oxidised monoterpene, was less effective than geranyl acetate at 24 h but larval mortality was 100% at 48 h. The activity of cineole against *A. simplex* s.l. L_3 was lower than that of thymol, carvacrol, geraniol, citronellol, citral, aldehyde perilla, cuminic aldehyde and (+)-ar-turmerone (Table 1) at 4 h, 8 h and 24 h but similar at 48 h (Fig. 2) (Suzuki et al., 1994; Hierro et al., 2004a, 2004b, 2006; Valero et al., 2006).

At a concentration of 62.5 μg/mL, α-pinene and ocimene exerted a very modest larvicidal activity and only at 48 h (Fig. 3). No activity was detected for either compound at the lowest concentration tested (31.25 μg/mL). The other tested monoterpenic derivatives showed weak larvicidal activity at both 62.5 μg/mL and 31.25 μg/mL. The lower activity found in the present compounds, compared with the oxidised monoterpenes previously studied by our group, may be generally attributed to the lack of oxygenated groups in all of the present test compounds, with the exception of 1.8-cineol and geranyl acetate.

The ranking of study compounds in descending order of activity against *A. simplex* s.l. L_3, was ocimene > α-pinene > geranyl acetate > cineole > β-pinene > myrcene.

In relation to the histological damage, two different types of lesion were produced by the most active compounds, α-pinene and ocimene. The former affected the lateral chords of the larvae, as found with other monoterpenic derivatives, e.g., carvacrol, whereas ocimene damaged the intestinal wall, as observed with both thymol and citral (Valero et al., 2006) (Fig. 4). These findings may indicate that the complete integrity of the parasite is critical to its viability. This hypothesis is supported by results obtained by our group with other terpenic derivatives (Hierro et al., 2004b), in which the viability of *A. simplex* s.l. L_3 was affected by modifications to the cuticle, i.e., different from the lesion site observed after α-pinene or ocimene treatment. The structural changes in L_3 larvae produced by both monoterpenic derivatives may derive from the ability of these apolar compounds to penetrate the phospholipid bi-layer, which can produce major changes in the permeability and integrity of membranes, changing their functionality by affecting the proteins they contain (Sikkema et al., 1995; Carson et al., 2002).

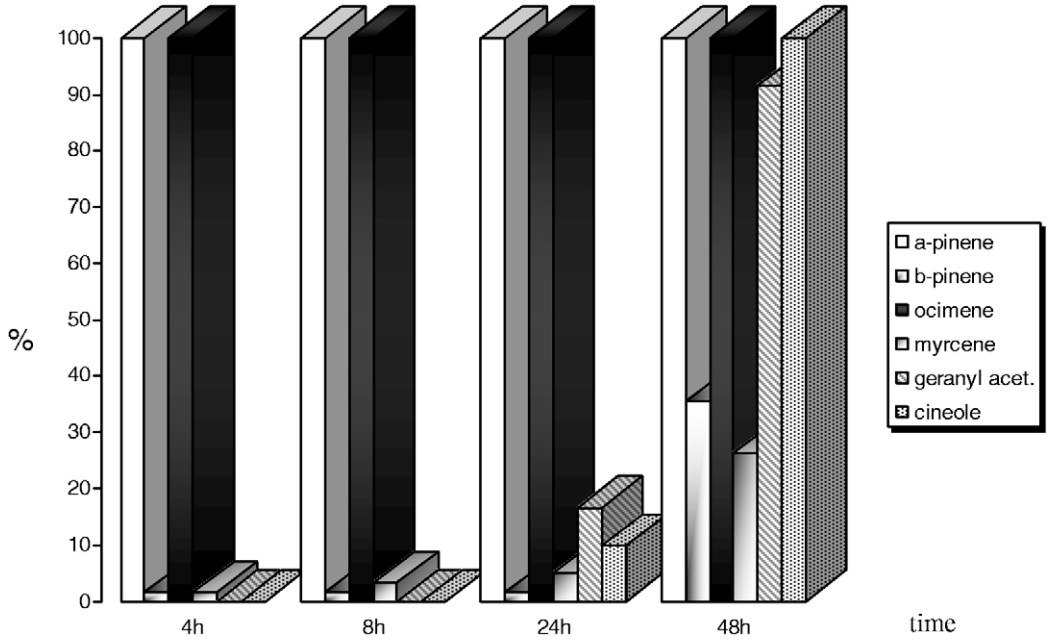

Fig. 2. *In vitro* larvicidal activity (%) of tested monoterpenes against L_3 larvae of *Anisakis simplex* s.l. (125 μg/mL).

3.2. In vivo assays

The two compounds that showed highest activity in the *in vitro* tests, α-pinene and ocimene, were tested for their *in vivo* larvicidal activity. Post-mortem gastric injuries were observed in 3 out of 15 rats administered with α-pinene and L_3 (lesion in pyloric region in one case and stomach in two cases). We highlight the absence of fixed larvae in the gastric or peritoneal cavity, despite the presence of lesions at these sites (Fig. 5). This suggests that the larvae were released from the gastric mucus after producing the injury, probably remaining located at other intestinal sites. This phenomenon may have positive effects, since patients are known to recover after endoscopic removal of larvae (Ikeda et al., 1989; Castán et al., 2002). Furthermore, 40% of the larvae were dead and the remainder showed little motility. Recovered live larvae kept in saline solution at 36 °C had a viability of 20% at one week, compared with 92% in the control group, indicating that larvae from α-pinene-treated animals underwent some form of modification that reduced their

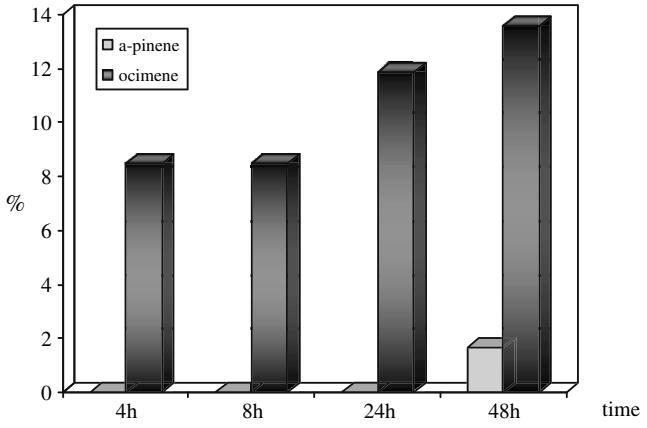

Fig. 3. *In vitro* larvicidal activity (%) of α-pinene and ocimene at 62.5 μg/mL against L_3 larvae of *Anisakis simplex* s.l.

Table 1
In vitro activity of different natural compounds against L_3 of *A. simplex*

Compound	Concentration (μg/mL)	Dead L_3 (% larvae)[a] (%)	Reference
[6] Shogaol	62.5	100	Kasuya et al. (1988), Goto et al. (1990)
[6] Gingerol	250	100	Kasuya et al. (1988), Goto et al. (1990)
Perillaldehyde	125	100	Kasuya et al. (1988, 1990), Hierro et al. (2004a, 2006)
Perillylalcohol	250	100	Kasuya et al. (1988,1990)
(+)-ar-turmerone	25	100	Suzuki et al. (1994)
Geraniol	125	100	Hierro et al. (2004a), Valero et al. (2006)
Citronellol	125	100	Hierro et al. (2004a)
Citral	125	100	Hierro et al. (2004a)
Carvacrol	125	100	Hierro et al. (2004a)
Cuminic aldehyde	125	100	Hierro et al. (2004a)
Thymol	125	100	Hierro et al. (2004b)
α-Pinene[b]	125	100	
β-Pinene[b]	125	0	
Ocimene[b]	125	100	
Myrcene[b]	125	4.92	
Geranyl acetate[b]	125	16.66	
Cineole[b]	125	9.84	

[a] Percentage of dead larvae after 24 h of treatment with the tested compounds.
[b] Tested in the present study.

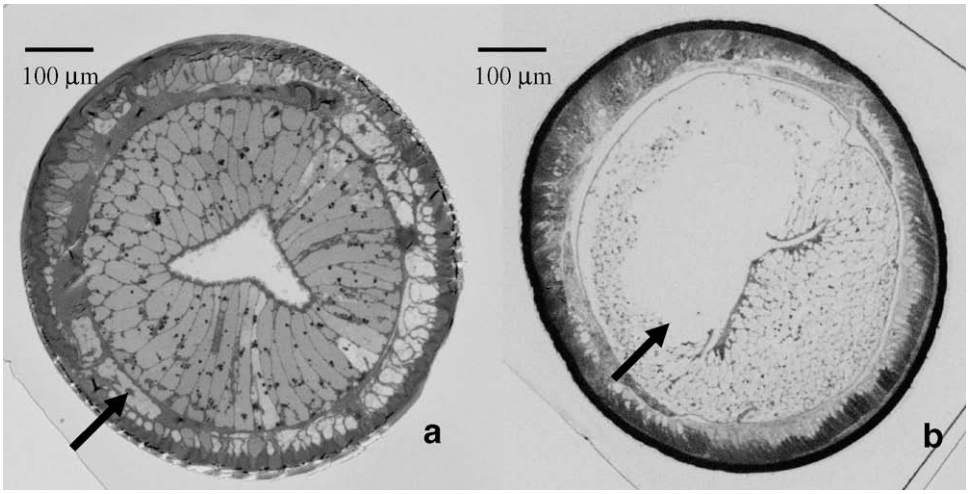

Fig. 4. Damage produced to L_3 of *A. simplex* s.l. by *in vitro* treatment with α-pinene and ocimene. (a) α-Pinene: alteration of lateral chords. (b) Ocimene: gut damage.

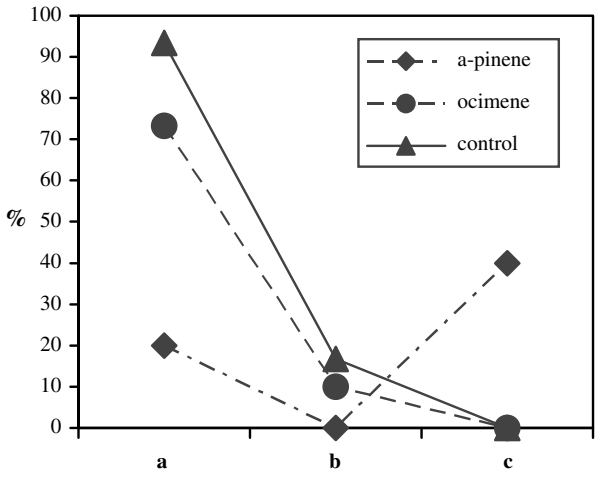

Fig. 5. *In vivo* activity of α-pinene and ocimene (46.9 mg). (a) Percentage of rats with lesions. (b) Percentage of rats with larvae in body cavity. (c) Percentage of rats with dead larvae in digestive tract.

viability. Thus, histology study of the dead larvae revealed a slight alteration of the lateral chords, as observed in the *in vitro* assay, In addition, studies by Sikkema et al. (1995) on the action of α-pinene on different pathogenic agents demonstrated that monoterpene can compromise cell integrity and inhibit mitochondrial respiratory activity.

Although ocimene showed higher activity than α-pinene in *in vitro* tests, its *in vivo* larvicidal activity was lower. Thus, 11 out of the 15 rats administered with ocimene and L_3 showed gastric lesions and none of the larvae were dead, whereas 3 of the 15 rats administered with α-pinene had gastric lesions and 38% of the larvae in the gastric cavity were dead. Moreover, 10% larvae in the ocimene group were detected in various locations of the peritoneal cavity (pancreas, liver, etc), whereas no migration of larvae to the peritoneal cavity was observed in the α-pinene group. No dead larvae were found at any location besides the gastric cavity in either α-pinene- or ocimene-treated rats (Fig. 5).

This difference in anti-L_3 activity between the two components *in vivo* cannot be attributed to variations in their degree of absorption, since the damage to larvae was observed in the gastric cavity, before absorption of the product. It is possible that the medium in the gastric cavity may influence their action capacity, since the structure of α-pinene is cyclic whereas that of ocimene is open, theoretically favouring reduction of its double-binding. Histological study of the dead larvae recovered from the ocimene-treated group showed no changes in the intestinal wall, unlike the *in vitro* observations. The distinct effect of both monoterpenes on the larvae in the *in vivo* versus *in vitro* assays may at least in part explain the lower *in vivo* larvicidal effect observed for ocimene (Fig. 6). The viability at one week of the live larvae extracted from

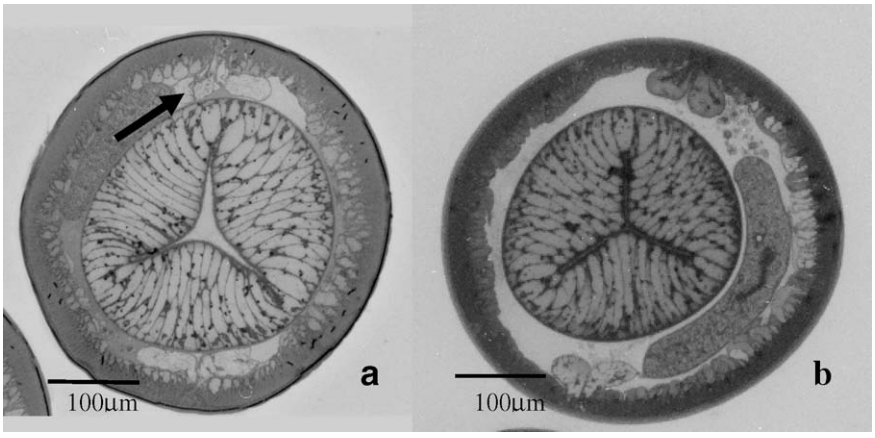

Fig. 6. Damage produced to L_3 of *A. simplex* s.l. by *in vivo* treatment with α-pinene. (a) α-Pinene: alteration of lateral chords. (b) Control.

Table 2
In vivo activity of different natural compounds against L_3 of *A. simplex*

Compound	Concentration	Lesions (% rats)[a] (%)	Reference
Perillaldehyde	46.9 mg/mL	0	Hierro et al. (2006)
Geraniol	46.9 mg/mL	0	Valero et al. (2006)
Citronellol	46.9 mg/mL	0	Hierro et al. (2006)
Citral	46.9 mg/mL	0	Hierro et al. (2006)
Carvacrol	9.5 mg/mL	28.6	Hierro et al. (2006)
Cuminic aldehyde	26.0 mg/mL	7.7	Hierro et al. (2006)
Thymol	17.3 g/mL	0	Hierro et al. (2004b)
α-Pinene[b]	46.9 mg/mL	20.0	
Ocimene[b]	46.9 mg/mL	73.3	

[a] Percentage of lesions in rats infected with *A. simplex* L_3 larvae and treated with the tested compounds.
[b] Tested in the present study.

ocimene-treated rats was 35%, indicating that the parasite was more severely affected by α-pinene (20%).

According to these results, the order of larvicidal activity *in vivo* differs from that observed in the *in vitro* experiments, with a higher *in vivo* activity for α-pinene than for ocimene. The *in vivo* larvicidal activity of both compounds was lower than that reported for almost all other oxygenized monoterpenic derivatives, e.g., thymol, geraniol, citronellol, citral, cuminic aldehyde and perillaldehyde (Hierro et al., 2004a, 2004b, 2006; Valero et al., 2006); which may be due to the presence of oxygenated functional groups in the structure of these compounds, as in the *in vitro* experiments. The frequency of gastric lesions was higher (28.57%) after treatment with carvacrol (Hierro et al., 2006) than after α-pinene treatment (20%) (Table 2).

In the control group, all larvae were recovered alive in stomach and intestine at the end of the assay, and 93.33% of the animals had gastrointestinal lesions (Fig. 5), a much higher percentage than observed in the α-pinene- and ocimene-treated groups. Moreover, the lesions mostly consisted of gastric haemorrhagic areas, ulcers and, when larvae were in gastric folds, the enlargement of these folds with adenomatous areas.

We can conclude from the above results that treatment with the monoterpenic derivates α-pinene and ocimene inhibits in some way the fixation and/or penetration capacity of simultaneously administered *A. simplex* s.l. L_3 larvae, but to a lesser degree in comparison to other monoterpenes (thymol, geraniol, citral, citronelol) studied by our group (Hierro et al., 2004a,b, 2006). Nevertheless, further *in vivo* studies are required to investigate whether the addition of these compounds to food could have a prophylactic effect, reducing the pathogenicity of *A. simplex* s.l. L_3 in humans, and to explore any possible synergy among compounds.

Acknowledgments

This work was partly funded by the Research Groups grant from the Junta de Andalucía, Spain. The English translation was done by Mr. Richard Davies, MA. The experiments complied with current Spanish legislation.

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