Phytomedicine 19 (2012) 520–523

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Activity of *Matricaria chamomilla* essential oil against anisakiasis

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ARTICLE INFO

Keywords:
Anisakis
Anisakiasis
Matricaria
Essential oil
α-Bisabolol

ABSTRACT

The increase in diagnosed cases of anisakiasis and the virtual absence of effective treatments have prompted the search for new active compounds against *Anisakis* L_3 larvae. The biocidal efficacy against different pathogens shown by various essential oils (EO) led us to study the *Matricaria chamomilla* EO and two of its main components (chamazulene and α-bisabolol) against the L_3 larvae of *Anisakis* type I. The activity of *M. chamomilla* EO, chamazulene and α-bisabolol was established by *in vitro* and *in vivo* experiments. The EO (125 μg/ml) caused the death of all nematodes, which showed cuticle changes and intestinal wall rupture. In the *in vivo* assays, only 2.2% ± 1.8 of infected rats treated with *M. chamomilla* EO showed gastric wall lesions in comparison to 93.3% ± 3.9 of control. Chamazulene was ineffective, while α-bisabolol showed a high activity to that of the EO *in vitro* tests but proved less active *in vivo*. These findings suggest that the larvicidal activity may result from the synergistic action of different compounds of *M. chamomilla* EO. Neither of the tested products induces irritative damage in the intestinal tissues. In conclusion, *M. chamomilla* EO is a good candidate for further investigation as a biocidal agent against *Anisakis* type I.

Introduction

Anisakiasis is an especially important health problem in countries with high fish consumption, and reported cases have increased exponentially since 1980. Sakanari and McKerrow (1989) gathered the main causes in their review, pointing to the growth in popularity of raw fish dishes such as sushi. More than a thousand cases are reported annually in Japan, representing 95% of cases worldwide Abe et al. (2005). Clinical symptoms and signs develop as a result of the inflammatory reaction caused by penetration of larvae into the digestive tract wall mucosa. *Anisakis* larva also has high allergenic potency and can induce manifestations of hypersensitivity ranging from urticaria or angioedema to anaphylactic shock as well as mixed gastrointestinal and allergic symptoms. Over the past 20 years, the application of molecular techniques has considerably improved our knowledge of the taxonomic position of the species of the *Anisakis* genre Mattiucci et al. (2009) and of diagnostic aspects. However, these advances have not been paralleled by the development of more effective treatments of anisakiasis, prompting the search for new molecules with possible anti-anisakis activity. Our research work group has established a research line on the action against the L_3 larvae of *A. simplex* exerted by various natural products, especially the essential oils (EO) of different aromatic plants and the main EO components (Hierro et al. 2004, 2006; Valero et al. 2006; Navarro et al. 2008). In this sense, taking into account the biocide activity of *Matricaria chamomilla* EO and its main components against various pathogenic agents (Aggag and Yousef 1972; Franke and Schilcher 2005; McKay and Blumberg 2006; Koch et al. 2008; Morales-Yuste et al. 2010; Tolouee et al. 2010; De Lucca et al. 2011), the objective of the present study was to examine the activity of this EO and two of its main components against L_3 larvae of type I *Anisakis*.

Materials and methods

The essential oil of *M. chamomilla*, collected in the flower period in the locality of Carboneras (Spain) (GDA 25225, Herbarium of Grenade University), was obtained by hydrodistillation in a Clevenger-type apparatus, following the method recommended by the Real Farmacopea Española (2002).

The yield was expressed as the volume of essential oil with respect to the weight of dried plant distilled (% v/w). The isolated oil was dried over anhydrous sodium sulphate and stored at 4 °C.

The composition of EO (essential oil) was analyzed by gas chromatography/mass spectrometry (GC/MS) using a Hewlett-Packard GC/MS 5973/6890 system. DB5 cross-linked 5%-PH-95%

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doi:10.1016/j.phymed.2012.02.005

ME siloxane (HP-5MS 30 m × 0.25 mm × 0.25 μm film thickness) and polyethylene glycol (HP-Innowax 30 m × 0.25 mm × 0.25 μm film thickness) fused capillary columns were used, with helium as carrier gas (flow rate = 1.2 ml/min). Oven temperature was kept at 65 °C for 3 min and raised to 210 °C at 3.5 °C/min; then kept at 210 °C for 30 min. Split ratio was adjusted to 1:70. Injector and detector were at 250 °C. Mass spectra were acquired in electron impact mode (70 eV). Components were identified by mass spectral fragmentation, by comparing Kovats-calculated (Dabrio 1973) retention indices with those of known constituents and by using a mass spectral matching library search system (Adams 2001).

In vitro larvicidal activity

Three concentrations of EO and two of its main components [chamazulene (Extrasynthese, >90% chromatographic purity grade) and (−)-α-bisabolol (Sensient Fragrances, 96.2% chromatographic purity grade)] were prepared with 96% ethanol: 12.50 mg/ml; 6.50 mg/ml, or 3.25 mg/ml.

L_3 larvae of *Anisakis* type I were collected by dissecting *Micromesistius poutassou* (blue whiting), 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% NaCl and 20 μl of each dilution of test compound, and then incubated at 36 °C in 5% CO_2 atmosphere. As controls, larvae were assayed without test compound under identical experimental conditions. The final concentrations of the tested compounds were: 125 μg/ml; 65 μg/ml, and 32.5 μg/ml. Each dilution was tested three times on larvae from fish captured on different days.

Larvae were examined under stereoscopic microscope at 4 h, 8 h, 24 h and 48 h to test the biocidal effect of the compound. Larvae with no mobility at all were considered dead. Mortality of these larvae was verified by absence of infectivity after administration by gastric probe to animals (Wistar rats)

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 (m thick sections with toluidine blue.

In vivo larvicidal activity

Selection of the dose (46.9 mg) was based on earlier findings by our research group (Hierro et al. 2004, 2006; Valero et al. 2006; Navarro et al. 2008); dilutions were in 0.5 ml olive oil (vehicle). Female Wistar rats weighing around 150 g were infested with six L_3 of *Anisakis* type I by gastric probe. Test compounds were simultaneously administered using the same procedure. Three groups of fifteen rats were used for each product (EO and (−)-α-bisabolol, one of the four stereoisomers of this compound). A control test was performed in three groups of 15 animals, infesting each with six L_3 of *Anisakis* type I in 0.5 ml olive oil but without administering a test product. Regulated necropsy of the rats 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.

Measurement of tissue myeloperoxidase (MPO) activity

Because EO 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), using the method of Krawisz et al. (1984) to measure the MPO activity (marker of neutrophilic infiltration).

Statistics

For the measurements of *in vitro* and *in vivo* larvicidal activity, the results are presented as mean percentages ± SEM of three independent experiments. In the case of MPO, a logistic regression analysis was used to test for differences between groups. Differences were considered statistically significant when $p < 0.05$. SPSS 15.0 (SPSS, Chicago, IL) was used for the data analyses.

Results

The EO yield was 0.4%, v/w (mean of four determinations). We detected 130 compounds in *Matricaria chamomilla* EO; the main components were: E-β-farnesene (4.68%), α-bisaboloxide B (8.44%), (−)-α-bisabolol (5.90%), chamazulene (1.85%) and bisaboloxide A (49.02%). Only two of the main components (α-bisabolol and chamazulene) of *M. chamomilla* EO were assayed in the *in vitro* tests, because the other major components of this EO were not available. At 125 μg/ml, the EO was effective *in vitro*

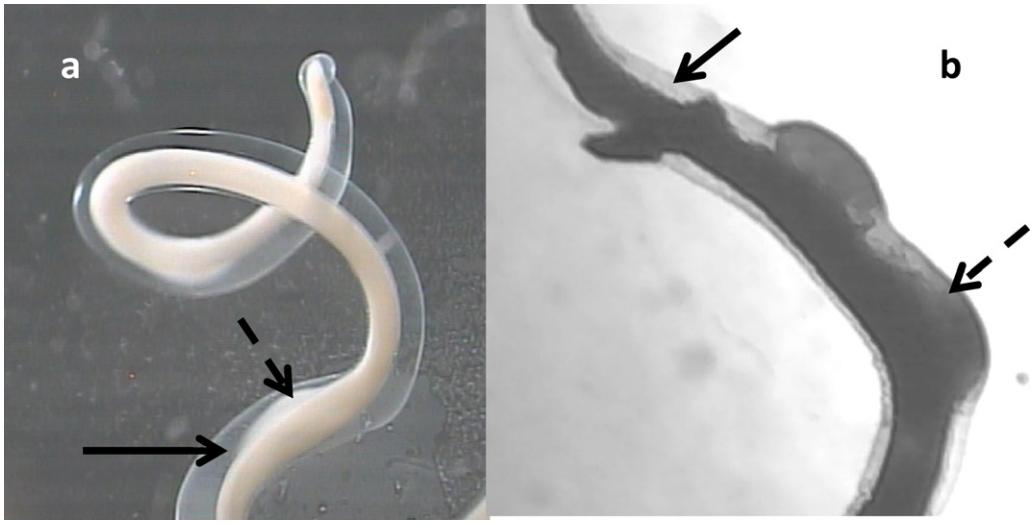

Fig. 1. Stereoscopic microscope images of L_3 larvae of *A. simplex* treated with (a) *Matricaria chamomilla* EO and (b) α-bisabolol. Solid arrows indicate the damage on the cuticle and the broken arrows alterations in the digestive tract of the nematode.