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Selective nematocidal effects of essential oils from two cultivated *Artemisia absinthium* populations

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Abstract: Essential oils (EOs) obtained from two crops and populations of thujone-free cultivated *Artemisia absinthium* were tested against two nematode models, the mammalian parasite *Trichinella spiralis*, and the plant parasitic root knot nematode *Meloidogyne javanica*. The EOs were characterized by the presence of (*Z*)-epoxyocimene and chrysanthenol as major components and showed time and population dependent quantitative and qualitative variations in composition. The EOs showed a strong *ex vivo* activity against the L1 larvae of the nematode *Trichinella spiralis* with a reduction of infectivity between 72 and 100% at a dose range of 0.5–1 mg/ml in absence of cytotoxicity against mammalian cells. Moreover, the *in vivo* activity of the EO against *T. spiralis* showed a 66% reduction of intestinal adults. However, these oils were not effective against *M. javanica*.

Keywords: *Artemisia absinthium*; essential oil; *Meloidogyne*; nematocidal; *Trichinella*.

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1 Introduction

Parasitic nematodes that infect humans, animals, and plants cause serious diseases that are deleterious to human health and agricultural productivity. Chemical and biological control methods have reduced the impact of these parasites. However, development of resistance to nematocidal and anthelmintic agents and the increasing reduction in chemical control due to new regulations pose significant obstacles for ongoing effective parasite control.

In this context, animal parasitic nematodes [1–5] and plant parasitic nematodes [6–9] have been increasingly targeted by studies on the effects of essential oils. However, most research on parasitic nematodes has been done either on animals or in plants because of the inherent differences in host species [10].

The species *Trichinella spiralis* is responsible for the disease trichinellosis with an extensive geographical distribution as well as a wide range of hosts [11]. The estimated global prevalence of trichinellosis is 11 million people [12], while an increased prevalence and incidence in animal cases has been detected in eastern European countries [13]. Benzimidazoles are broad spectrum drugs used to treat trichinellosis; however these compounds have low bioavailability [14], and resistance against them has been reported in various target nematodes [15].

Root-knot nematodes (RKNs) belong to the genus *Meloidogyne* and constitute a large group of ubiquitous plant parasites. The four major species (*M. incognita*, *M. javanica*, *M. arenaria* and the temperate species *M. hapla*), and a few emerging ones are generally considered responsible for the vast majority of crop damage, with a global estimate of multibillion dollar annual losses on a worldwide scale [16]. Root-knot nematodes are very difficult to manage because of their wide host range and high rate of reproduction. Because many of the most effective chemicals used for controlling *Meloidogyne* sp. are highly toxic and are being gradually phased out by European Union (EU) environmental restrictions, it has become necessary to develop new control techniques based upon

alternative substances safer for people and the environment. Thus, in the last few years much effort has focused on the study of the nematocidal activity of plant essential oils (EOs) and their constituents as potential sources of commercial products for management of RKNs [6].

Artemisia species have traditionally been used as anthelmintics [17] and different extracts and components (such as artemisinin from *A. annua*) have been reported as plant [18] and animal [3, 19] nematocidal agents. *Artemisia absinthium* L. (wormwood) is an aromatic and medicinal plant of ethnopharmacological interest [20] with reported antihelminthic effects [21]. The composition and biological effects of *A. absinthium* EO has been widely studied and found to have antimicrobial [22–24], antiprotozoal, and insect antifeedant effects [25, 26].

The composition of *A. absinthium* EO can vary depending on the plant origin [24]. One common component of *A. absinthium* EO is thujone, a GABA receptor antagonist [27], which is potentially toxic. Thujone-free Spanish populations of wormwood have been experimentally cultivated and the biological effects (insect antifeedant, antifungal, antioxidant, antiparasitic) and chemical composition of their different extracts (EO, organic, CO_2-supercritical) have been evaluated [25, 28, 29]. Based on these results, a long-term field cultivation of selected *A. absinthium* germplasm has been established for its domestication and valorization [30].

In this work we have tested the *in vitro* and *in vivo* nematocidal activity of EOs from two populations and crops of cultivated *A. absinthium* with different domestication levels against two parasitic nematode models, the animal parasite *Trichinella spiralis* and the RKN *Meloidogyne javanica*.

2 Materials and methods

2.1 Plant material and cultivation

The plants for field cultivation were obtained from seeds of two populations (Teruel, T1 and Sierra Nevada, SN0) and planted in 2008 to produce the domesticated populations T2 and SN1 [30]. The experimental field is located in Ejea de los Caballeros, Zaragoza, Spain. A detailed description of the field and the cultivation parameters has been reported [28]. Flowering plants were harvested yearly and processed for EO extraction. The EOs studied here corresponded to crops collected in 2009 and 2010 (T2-09, T2-10, SN1-09, SN1-10).

2.2 Essential oil extraction and analysis

Plant material was distilled in an industrial stainless steel vapor pressure extraction as previously described [30]. The extracted EOs (0.135–0.172% and 0.101–0.114% yield, plant fresh weight, for T-SN populations and year) were analyzed by GC-MS using an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 5973N mass detector (electron ionization, 70 eV) (Agilent) and equipped with a 25 m×0.20 mm i.d. capillary column (0.2 μm film thickness) HP-1 (methyl silicone bonded) (Hewlett-Packard, Palo Alto, CA, USA). Working conditions were as follows: split ratio (20:1), injector temperature, 260 °C; temperature of the transfer line connected to the mass spectrometer, 280 °C; initial column temperature 70 °C, then heated to 270 °C at 4 °C min^{-1}. Electron ionization mass spectra and retention data were used to assess the identity of compounds by comparing them with those of standards or found in the Wiley Mass Spectral Database (2001). Quantitative data were obtained from the TIC peak areas without the use of response factors.

2.3 Parasites

Trichinella spiralis MFEL/ES/S2 GM-1-ISS48 isolate (Trichinellosis Reference Center, Istituto Superiore di Sanitá, Roma, Italy) was used for *ex vivo* and *in vivo* assays. The parasites were maintained in the laboratory by periodical passage in outbred Swiss-CD-1 mice. The methods used for infection and muscle larvae and intestinal adult worm recovery have been previously described [31].

A field-selected *M. javanica* population from Barcelona (Spain) was maintained on *Solanum lycopersicum* L. plants (var. Marmande) in pot cultures at 25±1 °C, >70% relative humidity. Egg masses of *M. javanica* were handpicked from infected tomato roots 2 months after inoculation of the seedlings. Second-stage juveniles (J2) were obtained by incubating egg masses in a water suspension at 25 °C for 24 h.

2.4 Nematocidal effects

2.4.1 *Ex vivo* assays on *T. spiralis*: The larvicidal activity was evaluated by measuring the infective capacity of the *ex vivo*-treated larvae. As a control, untreated larvae were also included in all assays. Larvae of *T. spiralis* suspended in HBSS medium were seeded in 24 well plates, (300±50/well) and incubated at 37 °C and 5% CO_2 with the *A. absinthium* EOs at different concentrations (serial double dilutions from 1 to 0.03 mg/ml). After 24 h, the larvae from each well were collected and orally administered to the corresponding mice (6 per dosage). After 7 days, the infected animals were sacrificed by anesthesia and the adult worms present in the intestinal mucosa counted under an stereomicroscope. The experiment was repeated twice.

2.4.2 *In vivo* assays on *T. spiralis*: Groups of 10 mice maintained in a temperature and light/dark controlled environment with water and food *ad libitum* were used for each experiment. All animal husbandry and experimental conditions were carried out according to the Directive 2010/63/EU of the European Parliament and the Council of the European Union and controlled in Spain by Royal Decree 53/2013 of 1 February, on the care and use of laboratory animals. Eight groups of male mice (6–8 weeks) were orally infected with 300±50 L1 larvae. Twenty-four hours later, the mice were treated with the oils at 50, 100, 150, 200, 250 and 500 mg/kg body weight (BW). Three

additional groups were included as controls for infection, placebo, and treatment (albendazole 5 mg/kg BW dissolved in hydroxypropyl-β-cyclodextrin). Seven days after infection, the mice were sacrificed, their small intestine removed, opened lengthwise, and incubated in saline solution during 3 h at 37 °C to obtain adult worms from the mucosa. Worm counting was performed under a binocular lens, and the experiment was repeated twice.

2.4.3 *In vitro* assays on *M. javanica*: Essential oil solutions in dimethyl sulfoxide (DMSO) with 0.6% Tween 20 (20 µg/µl), were placed (5 µl) in each well of 96-well plates to give a final concentration of 1 mg/ml. An inoculum of 100–150 J2 in 95 µl of water was added to each microwell and incubated at 25 °C. Percentages of dead J2 were recorded after 72 h. All treatments were replicated four times [6]. The data are presented as J2 mortality corrected according to Schneider–Orelli's formula [32].

2.5 Cytotoxicity

J774 murine macrophages were seeded (70,000 cells/well) in flat-bottom 96-well microplates (Costar, NY, USA) with 100 µl of RPMI 1640 (Sigma-Aldrich) supplemented with 10% of fetal bovine serum and antibiotics. The cells were attached to the flat-bottom plates for 24 h at 37 °C and 5% CO_2 atmosphere and then 100 µl of the oil diluted in the macrophage medium at the same concentrations as described for the *in vitro* assays were added to the wells. After 24 h, 20 µl of 1 mM resazurin were added to the plates and the cultures incubated for 3 h. The reduction of the redox dye was measured in a plate fluorometer at 535 nm and 590 nm of excitation and emission wavelengths, respectively (Infinite 200, TECAN, Männedorf, Switzerland).

2.6 Statistical analysis

Statistical analysis was performed using the SPSS v.20 (IBM, Armonk, NY, USA) software. The results of the *T. spiralis* assays were expressed as mean±standard error. The 50% inhibitory infectivity (II_{50}) were determined by Probit analysis. The nematocidal *in vivo* results were statistically analyzed by using the non-parametric test (Kruskal–Wallis test), and by the Mann–Whitney U-test.

3 Results and discussion

3.1 Composition of the essential oils

Table 1 shows the composition of the EOs. (*Z*)-Epoxyocimene was the major component of all the EOs analyzed (26–43%), followed by chrysanthenol (13–30%). Linalool was also present in all samples, ranging between 3.4 and 4.3%. Among the sesquiterpenes, (*E*)-caryophyllene was found in all samples except for SN1-09 (2.5–5.4%). (*5Z*)-2,6-dimethylocta-5,7-diene-2,3-diol was found in T2-10 in relatively large amounts (20.2%), ranging between 1.9 and 1.7% for the other samples. An unknown compound of $[M]^+152$ was also present in all samples (0.5–2%). None of the EOs analyzed contained thujone.

Chemical variations have been described for *A. absinthium* EOs from plants cultivated *in vitro*, in the

Table 1: Composition of *A. absinthium* EOs from two cultivated populations (T2 and SN1) collected in 2009 and 2010.

Compound	Abundance (% of total)			
	T2		SN1	
	2009	2010	2009	2010
α-Pinene	2.70			1.62
Camphene	1.07			
β-Myrcene	0.68		1.15	1.13
β-Ocimene			1.54	1.27
1,8-Cineole	2.43			
Linalool	4.28	3.42	3.49	3.54
$[M]^+152$ (**81** 68-79-41-53)	1.25	0.54	1.94	0.49
(*Z*)Epoxyocimene	38.92	25.89	42.72	38.77
(*E*)-Epoxyocimene	7.74	0.99	2.45	2.16
Chrysanthenol	12.71	30.32	23.48	22.49
(*E*)-3-Hexenyl butyrate	1.26	1.34	1.30	1.64
Chrysanthenyl acetate	7.35		0.71	0.46
(5*Z*)-2,6-Dimethylocta-5,7-diene-2,3-diol	1.72	20.21	1.72	1.90
β-Elemene		0.98	1.46	2.57
(*E*)-Caryophyllene	4.20	2.47		5.43
Germacrene D	1.36	1.15	5.99	1.86
Sesquiterpene alcohol	0.51	1.79		0.74
7-Ethyl-1,4-dimethylazulene	1.09	0.87		

Table 2: *Ex vivo* activity of different *A. absinthium* EOs (T2 and SN1 populations, 2009 and 2010 harvest) against *T. spiralis* L1 muscle larvae.

EO (mg/ml)	T2-09		T2-10		SN1-09		SN1-10	
	AW	LRa %[a]	AW	LRa %	AW	LRa %	AW	LRa %
0.03	97.13±9.96	13.18	100.99±7.4	9.83	81.63±7.87	27.04	81.23±6.37	27.37
0.06	90.88±6.38	18.77	80.13±7.72	28.38	74.38±6.65	33.52	76.38±8.96	31.73
0.12	71.00±11.48	36.54	60.50±4.87	45.92	67.75±8.31	39.44	65.00±7.88	41.90
0.25	58.88±8.32	47.37	57.00±6.05	49.05	46.13±10.08	58.77	59.00±11.88	47.26
0.50	27.13±4.58	75.75	31.00±6.29	72.29	18.88±5.84	83.13	19.13±6.69	82.91
1	1.64±0.71	98.54	0.09±0.36	99.92	0.73±0.11	99.35	0.00±0.00	100
Control	111.9±8.35							

Results are expressed as mean± standard error (SE) of adult worms (AW) recovered from intestine of mice infected with *ex vivo* treated larvae (n=12). [a]Larval reduction rate (p<0.05).

greenhouse, and in the field [26]. Variable concentrations of these compounds were also found in EOs and supercritical extracts of the cultivated Spanish *A. absinthium* populations from which the plants used here originated [26] and have already been described in the EOs of wild populations [33, 34].

Two chemotypes have been described from the Iberian Peninsula: the (*Z*)-epoxyocimene type (with more than 50% of this compound) which was predominant in all populations, and a (*Z*)-epoxyocimene+chrysanthenyl acetate type (with 25–65% of (*Z*)-epoxyocimene and 15–50% of chrysanthenyl acetate). Wormwood EOs from other locations had (*Z*)-chrysanthenol as the main component [33], (*Z*)-epoxyocimene and β-thujone/(*Z*)-chrysanthenyl acetate among other compositions [35]. The EOs of cultivated Spanish wormwood used here contained both the (*Z*)-epoxyocimene+(*Z*)-chrysanthenol chemotype.

3.2 Effects on parasitic nematodes (*T. spiralis* and *M. javanica*)

Table 2 shows the *ex vivo* activity obtained for the different EO concentrations tested against *T. spiralis*. A linear correlation between oil concentration and adult nematodes recovered from the intestinal mucosa was observed. All the EOs showed a high nematocidal activity (II_{50} values of 206 and 205 μg/ml for SN1 and 291 and 265 μg/ml for T2, respectively). The small variation observed in the nematocidal effects of the EOs correlated with their limited variation in composition.

When T2-10 and SN1-10 EOs were tested *in vivo*, larval reduction values ≥50% were observed (49–66%) at doses between 100 and 500 mg/kg (Table 3) vs. 66% obtained with the reference drug albendazole (5 mg/kg).

Table 3: *In vivo* activity of *A. absinthium* EOs (T2 and SN1 populations, 2009 and 2010 harvest) against intestinal infection by *T. spiralis*.

EO	T2-10		SN1-10	
Dose (mg/kg BW)	AW	LRa %[a]	AW	LRa %
50	146.20±10.11	−4.85	139.10±9.49	0.22
100	71.47±5.12	48.74	76.90±5.66	44.83
200	64.39±4.61	53.82	65.20±4.33	53.26
250	53.50±5.43	61.64	57.50±4.64	58.75
500	47.30±1.98	66.07	48.60±4.08	65.16
Albendazole[b]	47.44±5.62	65.97		
Control	139.44±5.17			

[a]Larval reduction rate (p<0.05). [b]Tested at 5 mg/kg.

Aqueous and ethanolic *A. absinthium* extracts have been shown to reduce the number of *Haemonchus contortus* adult worms *in vitro* and the faecal egg output in sheep infected with *H. contortus*, *Trichuris ovis*, *Chabertia ovina*, *Bunostomum trigonocephalum* and *Oesophagostomum columbianum* [19]. An ethanolic extract of *A. absinthium* showed *in vitro* trematocidal effects against *Schistosoma mansoni*, *Echinostoma caproni*, and *Fasciola hepatica* [36]. Furthermore, methanolic extracts of *Artemisia*, including *A. absinthium*, inhibited trichinellosis in rats [37]. However, this is the first report on trichinellosis inhibition by *A. absinthium* EO. None of the main components of the oils described here have been previously reported as nematocidals.

The oils were not cytotoxic to macrophage cells (growth inhibition <20%) at the highest concentration tested (200 μg/ml; data not shown). Therefore, (*Z*)-epoxyocimene+(*Z*)-chrysanthenol chemotype *A. absinthium* EOs might be considered for further development as anthelmintic agents.

Table 4: Effects of *A. absinthium* EOs (1 µg/ml) on mortality of second stage juveniles (J2) of *Meloidogyne javanica*.

EO	J2 mortality (%)[a] at 72 h
T2-09	5.44±1.83
T2-10	11.56±2.57
SN1-09	8.51±1.72
SN1-10	8.09±0.43

[a]Mean and standard error values corrected according to Schneider–Orelli's formula.

In contrast, the *A. absinthium* EOs tested here were not nematocidal against the plant parasitic nematode *M. javanica* (Table 4). Previous reports have shown that aqueous extracts of *A. absinthium* roots and aerial parts inhibited *M. incognita* hatching and caused J2 mortality [38]. However, *A. absinthium* EOs tested against the pine wood nematode *B. xylophilus* [39] and the stem nematode *D. dipsaci* [40] also gave negative results.

Plant and animal endoparasitic nematodes have some similarities in their adapted structures to parasitism such as the cuticle [41]. Products secreted from the cuticle of animal parasitic nematodes share cross-reactive epitopes with those of plant parasitic nematodes [42]. Specifically, cross-reactive cuticle protein epitopes were identified in *T. spiralis* and *M. incognita* [43, 44]. However, *A. absinthium* EOs were highly effective against animal (*T. spiralis*), but not plant parasitic nematodes (*M. javanica*). This difference could be related to their lack of close phylogenetic relationship [45] and their different host-parasite interactions [10]. *Trichinella spiralis* L1 larvae induce the nurse cell [46] within the vertebrate striated skeletal muscles to feed and protect the parasite from host attack. *Meloidogyne javanica* J2 induce giant cells in the roots of the parasitized plants [47]. Furthermore, parasites have coevolved with their hosts towards compatible life histories, and therefore *M. javanica* is possibly more tolerant to plant products than *T. spiralis*.

4 Conclusion

The EOs of cultivated Spanish wormwood with a (*Z*)-epoxyocimene+(*Z*)-chrysanthenol chemotype showed a high nematocidal activity against *T. spiralis* without being cytotoxic to macrophage cells, while they were not active against the plant parasitic nematode *M. javanica*. The reason for this contrasting effect could be related to the partial tolerance of root knot nematodes to some plant derived compounds. The (*Z*)-epoxyocimene+(*Z*)-chrysanthenol chemotype EOs from cultivated *A. absinthium* may be considered potential candidates for the development of new drugs against helminth parasites of man and domestic animals.

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