

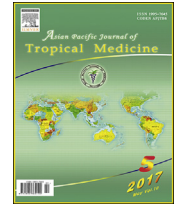
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## Asian Pacific Journal of Tropical Medicine

journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2017.05.005>Activity of *Tagetes minuta* Linnaeus (Asteraceae) essential oil against L3 *Anisakis* larvae type 1Filippo Giarratana, Daniele Muscolino, Graziella Ziino<sup>✉</sup>, Alessandro Giuffrida, Stefania Maria Marotta, Vittorio Lo Presti, Vincenzo Chiofalo, Antonio Panebianco

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## ARTICLE INFO

## Article history:

Received 28 Nov 2016

Received in revised form 10 Apr 2017

Accepted 21 Apr 2017

Available online 17 May 2017

## Keywords:

*Anisakis*

Essential oil

*Tagetes minuta*

Biocidal activity

Parasites

## ABSTRACT

**Objective:** To evaluate *in vitro* effects of *Tagetes minuta* L. essential oil (TEO) on L3 *Anisakis* larvae type 1.**Methods:** In order to evaluate the potential use of *Tagetes minuta* essential oil against L3 *Anisakis* larvae three different media were tested: 1) a saline solution (SS); 2) an industrial marinating solution (MS); 3) sunflower seeds oil (SO). For each media and concentrations of TEO (0.1%, 0.5%, 1.0% and 5.0% v/v), 20 parasites were introduced into plastic Petri dishes (diameter 90 mm) and maintained at room temperature. As controls, larvae were maintained without TEO under identical experimental conditions in SS, MS and SO. A total of 900 larvae were tested. The normalized mean viability, LT100, LT50 and the percentage of inactivation at 24 h were calculated.**Results:** *In vitro* tests revealed a complete inactivation of parasites in saline solution after 2 h with 5% and 1% of TEO. In marinating solution, a complete inactivation of parasites was observed after 4 h at all concentrations used. A slower activity for all TEO concentration was reported in SO.**Conclusions:** The results obtained, showing a strong activity against *Anisakis* larvae, confirm TEO as a larvicidal agent in the treatment of human anisakidosis and in the industrial marinating process.

## 1. Introduction

In the last years, there has been an increasing interest in phytomedicine. Several herbs and plants were used, in fact, not only for the preventive and therapeutic diseases treatment but also as natural additives in food [1–9]. Among natural compounds used in phytomedicine, the essential oils (EOs) gained great importance. Several authors have demonstrated, in fact, that EOs are effective against bacteria, yeasts, fungi, and parasites as well as are characterized by antioxidant, anti-inflammatory, anti-carcinogenic properties [10–17].

*Tagetes* species, belonging to family Asteraceae, are used as traditional medicines in many countries to treat colic, diarrhea, vomit, fever, skin diseases and hepatic disorders [18]. These plants possess, indeed, antimicrobial properties demonstrated

on gram-negative, gram-positive such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli*, *Salmonella enteritidis*, *Salmonella typhi*, *Shigella dysenteriae* and *Shigella flexneri* [19–23]. Phytochemical studies carried out on different species of *Tagetes* have revealed that the presence of flavonoids and terpenes displaying antibacterial, antifungal, nematocidal and insecticidal properties [19–22,24–26]. Essential oil from *Tagetes* species has not been explored yet for its biological effects on zoonotic parasites, however recently, a good efficacy was demonstrated against plant-parasitic nematodes and animal ticks [27–29]. Among zoonotic parasites, *Anisakis* is one of the most important fish-borne zoonotic parasites related to the consumption of raw or almost raw seafood products (such as marinated and cold smoked fish) [30–33]. *Anisakis*, *Contracaecum* and more rarely *Pseudoterranova* and *Hysterothylacium*, belonging to Anisakidae or Raphidascaridae families, are the genus associated to human anisakidosis [31,34]. These worms utilize aquatic mammals, reptiles, aquatic birds and fish as definitive hosts, and aquatic crustaceans (krill) as intermediate hosts. Fish and cephalopods also act as transport hosts, which carry the infective larvae (L3) [31,35]. The human disease, characterized

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Peer review under responsibility of Hainan Medical University.

by digestive disorders and/or allergies, so is consequentially to accidental ingestion of parasitized hosts by the third-stage larvae [36–39]. Of the approximately 20000 cases of anisakidosis reported to 2010 worldwide, over 90% are from Japan, with most of the rest from Spain, the Netherlands and Germany [31,36].

For these reasons, a great interest on the inactivation of L3 larvae of *Anisakis* has been raised, especially on the use of essential oils and their components [40–50]. The aim of our study was to evaluate *in vitro* effects of *Tagetes minuta* L. (*T. minuta*) essential oil (TEO) on L3 *Anisakis* larvae type I.

## 2. Materials and method

### 2.1. Chemical composition

*T. minuta* EO used in this study was supplied by Silky Scents (Corona, California, USA) and extracted with steam distillation of flowering herb. Oil sample was analyzed using GC–MS on an Agilent 6860 N GC system coupled directly to a 5973 MS. 0.5  $\mu$ L of sample were injected into the GC with a 200:1 split ratio and an inlet temperature of 250 °C. Separation of components was done on an HP-5 MS Agilent J&W capillary column (50 mm  $\times$  200 mm i.d.  $\times$  0.33  $\mu$ m film thickness). Oven temperature was 60 °C, rising to 220 °C at a rate of 4 °C/min; after holding for 10 min the temperature was raised to 240 °C at a rate of 1 °C/minutes. Helium was used as carrier gas at a flow rate of 1.2 mL/min. Scanning was performed from 35 to 450 *m/z* with electron impact of 70 eV. Chromatogram peak areas were acquired and calculated using Chemstation software (Agilent Technologies). Concentration of individual components was expressed as % of total identified components.

### 2.2. Anisakid nematode collection

For the present study, Anisakidae larvae were collected from several specimens of *Lepidopus caudatus* (*L. caudatus*) (silver scabbardfish) harvested within 4 h from the sampling. *L. caudatus* is well known because often parasitized by nematodes of genus *Anisakis* with high prevalence and intensity of infestation [51]. The visceral cavity, digestive tract, liver, gonads and mesenteries from each fish were examined for the detection of *Anisakis* larvae. All the collected nematodes, selected for viability, were rinsed three times with sterile saline solution (NaCl 0.9%). All these larvae were examined under stereoscopic microscope (Leica M 205 C) for the belonging to the *Anisakis* genus according to guidelines proposed by Murata *et al.* [52]. Actively moving parasites without any injury were maintained in sterile saline solution at room temperature until use. All Anisakidae larvae used for the study were identified as L3 larvae of *Anisakis* type I.

### 2.3. In vitro larvicidal activity

In order to evaluate the potential use of *T. minuta* essential oil against L3 *Anisakis* larvae three different media were tested: 1) a saline solution (SS) (NaCl 0.9%) chosen to evaluate TEO effectiveness in the treatment of human anisakidosis; 2) a typical industrial marinating solution (MS) (water and vinegar 1:1, with 3% NaCl and 1% citric acid), selected to estimate the nematocidal activity during anchovy marinating process; 3) sunflower

seeds oil (SO), in order to test *Anisakis* inactivation during the normal packaging and storage of marinated anchovy.

The TEO used was characterized by a good miscibility in marinating solution, while in saline solution the miscibility was obtained by a magnetic stirrer. For each media and concentrations of TEO (0.1, 0.5, 1.0 and 5.0% v/v), 20 parasites were introduced into plastic Petri dishes (diameter 90 mm) and maintained at room temperature.

As controls, larvae were maintained without TEO under identical experimental conditions in SS, MS and SO. All experiments were carried for three times in separate occasion. A total of 900 larvae were tested.

Larvae were examined under stereoscopic microscope (Leica M 205 C) at regular intervals up to 48 h to test the biocidal effect of the compound. During the experimental treatments, at each fixed time interval, the viability were checked according to previous study, assessing the following score: 3 (viable), 2 (reduction of mobility), 1 (mobility only after stimulation) and 0 (death) [42]. Larvae were considered dead, when no mobility was observed under stereoscopic microscope in a new saline solution (0.9% NaCl). The normalized mean viability according to the method of Giarratana *et al.* [53], LT100 (Lethal time: time required to kill 100% parasites), LT50 (Time required to kill 50% parasites) and the percentage of inactivation at 24 h were calculated. Scanning electron microscopy (SEM) observations of dead parasites were done with a Phenom SEM (Phenom-World BV, Eindhoven, The Netherlands).

## 3. Results

### 3.1. Chemical composition of *T. minuta* essential oil

Ten compounds were detected in the *T. minuta* essential oil analyzed by GC–MS (Table 1). The main component of the TEO was  $\beta$ -ocimene (36.4%) followed by limonene (26.9%) and then (*Z*)-tagetone (16.8%). The remaining seven compounds ranged from 0.58% to 6.31%.

### 3.2. In vitro larvicidal activity

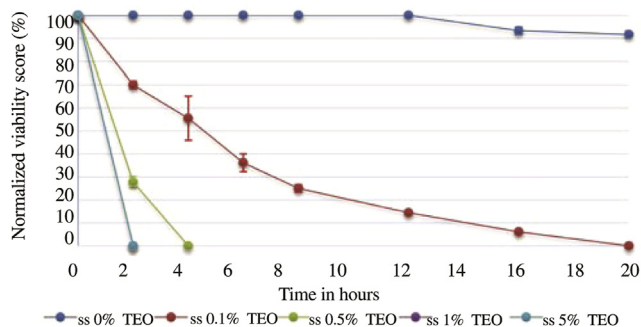
#### 3.2.1. Saline solution

*In vitro* tests revealed strong activity of TEO in SS against *Anisakis* larvae at all tested concentrations (Figure 1). In particular, at 5% and 1% concentrations, the complete inactivation (LT100) of parasites was observed just after 2 h of treatment, while LT50 was 1 h for both concentrations. At 0.5% and 0.1% of TEO, LT100 was reached at 4 and 20 h, respectively (Table 2).

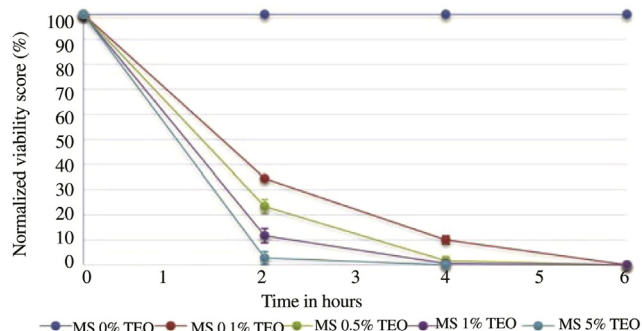
**Table 1**

Chemical composition of *T. minuta* essential oil.

No.	Component	%
1	$\beta$ -phellandrene	2.96
2	limonene	26.91
3	$\beta$ -ocimene	36.40
4	allo-ocimene	6.31
5	( <i>E</i> )-tagetone	2.54
6	( <i>Z</i> )-tagetone	16.86
7	( <i>Z</i> )-tagetenone	0.83
8	( <i>E</i> )-tagetenone	0.58
9	caryophyllene	4.50
10	germacrene D	2.10



**Figure 1.** Viability score (%) of *Anisakis* larvae Type 1 in saline solution (SS) with 0% (Control), 0.1%, 0.5%, 1% and 5% of *T. minuta* essential oil.



**Figure 2.** Viability score (%) of *Anisakis* larvae type 1 in marinate solution (MS) with 0% (Control), 0.1%, 0.5%, 1% and 5% of *T. minuta* essential oil.

### 3.2.2. Marinade solution

In MS a LT100 was observed after 4 h for the highest concentration (5%TEO) with a LT50 of 1.7 h (Figure 2). The remaining concentration reached LT100 in 6 h, while LT50 ranged from 2.5 to 3.2 h (Table 2).

### 3.2.3. Sunflower seeds oil

A slower activity for all TEO concentration was reported in SO (Figure 3). LT100 in SO ranged from 16 h (5% TEO) to 48 h (0.1% TEO), while LT50 from 8.6 h (5% TEO) to 42.3 h (0.1% TEO). The complete parasite inactivation at 24 h from treatment was reached only for 5% and 1% concentrations of TEO (Table 2).

### 3.3. Microscopic observation

Microscopic analysis (stereo microscopy and SEM) showed injuries of parasites cuticle and digestive tract, in all media tested. *Anisakis* digestive tract showed interruptions in a number ranging from 1 to 4 for each parasite (Figure 4).

## 4. Discussion

Concerning the chemical composition of TEO, the compounds obtained and their abundance were consistent with the chemical characterization provided by several authors, especially considering volatile substances such as terpenes [54–58]. Due to the chemical complexity of essential oils, it should be noted that many of their properties are related to the synergistic or complementary effects of their components.

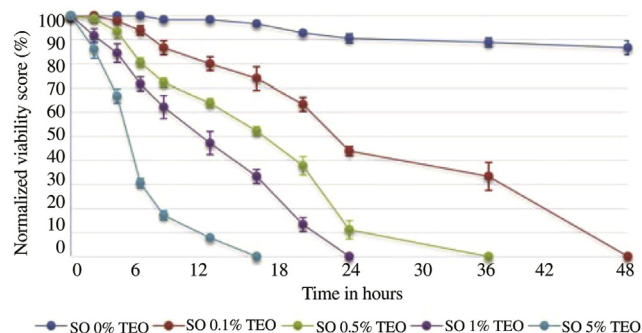
**Table 2**

LT100, LT50 and percentage of larvae inactivated in 24 h in saline solution (SS), marinate solution (MS) and in sunflower seeds oil (SO) with different concentration of TEO.

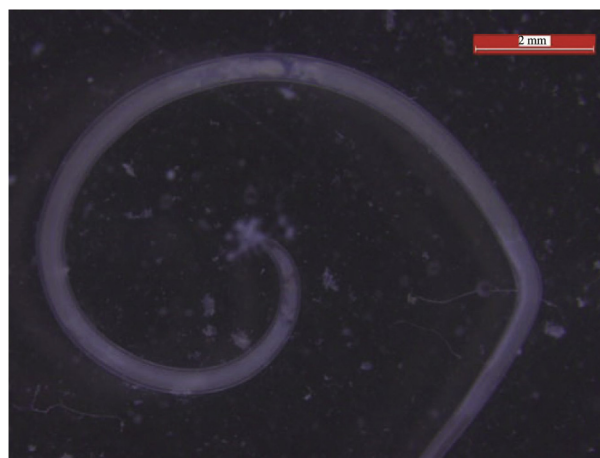
Treatment	LT100	LT50	Inactivation in 24 h (%)
SS 5% TEO	2 h	1.0 h	100.00 ± 0.00
SS 1% TEO	2 h	1.0 h	100.00 ± 0.00
SS 0.5% TEO	4 h	2.3 h	100.00 ± 0.00
SS 0.1% TEO	20 h	11.2 h	100.00 ± 0.00
MS 5% TEO	4 h	1.7 h	100.00 ± 0.00
MS 1% TEO	6 h	2.5 h	100.00 ± 0.00
MS 0.5% TEO	6 h	2.8 h	100.00 ± 0.00
MS 0.1% TEO	6 h	3.2 h	100.00 ± 0.00
SO 5% TEO	16 h	8.6 h	100.00 ± 0.00
SO 1% TEO	24 h	18.1 h	100.00 ± 0.00
SO 0.5% TEO	36 h	25.0 h	66.67 ± 11.55
SO 0.1% TEO	48 h	42.3 h	3.33 ± 5.77

Different authors suggested, indeed, that monoterpenes are responsible for essential oils larvicidal activity against nematodes such as *Anisakis simplex* and *Contracaecum* sp. [47].

Larvicidal action is performed by the rupture of internal wall and the broadening of the intestinal lumens, while the cuticle of the parasite showed roughness, deformations or projections. These results confirmed, as reported by several studies, that the effectiveness of EOs against parasites, is related to the cuticle and digestive tract damages [42,45,50]. The lipophilia of these compounds seems play an important role in the cellular damage exerted by already mentioned terpenic components of EOs. It was reported on bacteria that essential oils produce cellular damage and structural changes of the cellular membrane, modifying the permeability of the membrane and



**Figure 3.** Viability score (%) of *Anisakis* larvae type 1 in sunflower seeds oil (SO) with 0% (Control), 0.1%, 0.5%, 1% and 5% of *T. minuta* essential oil.



**Figure 4.** L3 larvae of *Anisakis* type 1 treated with *T. minuta* essential oil: interruption on the digestive tract (Stereoscopic microscope).



causing the leakage of ions and other cell contents [59]. Observed lesions on larval cuticle and digestive tract could be related to this kind of activity.

With regard to the used media as vehicle of TEO diffusion, they resulted effective in parasites inactivation, thus, with important differences on the lethal times. TEO action in SS resulted stronger and faster for the higher concentrations of 5.0% and 1.0%, determining a total inactivation in just two hours, against 4/6 h and 16/24 h in MS and SO, respectively. On the other side, TEO in MS at the lowest concentration of 0.1% revealed a quicker activity than in the other media. Death of all parasites was, indeed, registered after 6 h, against the 20 h in MS and 48 h in SO. This evidence could be explained by the different media composition. In MS, vinegar and citric acid probably potentiate the action of volatile active substances, even after their partial expiration, resulting in a rapid parasite inactivation. Furthermore, in MS could be employed a low concentration (0.1%) of TEO, which is also less flavored and, therefore, better tolerated by the consumer. Finally, SO resulted the media with the higher inactivation time at all TEO concentrations. However, TEO resulted certainly more efficient and faster in parasites inactivation than other EOs employed in previous studies such as *Thyme vulgaris* and *Nepeta cataria* essential oils. For Thyme and Nepeta EOs, inactivation time was up to 14 h and 12 h, respectively, at 5% concentration in the most efficient media [42,45]. The 0.1% TEO showed lethal effects against *Anisakis* larvae similar to those reported for several EOs [41,50].

Finally, this work is the first study concerning *in vitro* effect of *T. minuta* essential oil against *Anisakis* larvae. According to our results, TEO showed a remarkable *in vitro* nematocidal effect at all concentrations and media tested. The results revealed that the larvicidal activity appears proportional to the EO concentration and is probably explicated by the damages found in the parasite digestive tract. Due the increase of worldwide cases of anisakidosis and the lack of effective pharmacological treatments, the effectiveness of *T. minuta* EOs against *Anisakis* larvae could be applied in food marinating process and for the treatment of human anisakidosis.

### Conflict of interest statement

We declare that we have no conflict of interest.

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