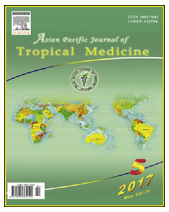
HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

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 1

Filippo Giarratana, Daniele Muscolino, Graziella Ziino✉, Alessandro Giuffrida, Stefania Maria Marotta, Vittorio Lo Presti, Vincenzo Chiofalo, Antonio Panebianco

Department of Veterinary Sciences, University of Messina, Polo Universitario dell'Annunziata, 98168 Messina, Italy

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, fiver, 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 smocked fish) [30–33]. *Anisakis, Contraceacum* 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

First author: Filippo Giarratana, Department of Veterinary Sciences, University of Messina, Polo Universitario dell'Annunziata, 98168 Messina, Italy.
✉Corresponding author: Graziella Ziino, Department of Veterinary Sciences, University of Messina, Polo Universitario della Annunziata, 98168 Messina, Italy.
Tel: +39(0)903503761
E-mail: gziino@unime.it
Peer review under responsibility of Hainan Medical University.

1995-7645/Copyright © 2017 Hainan Medical University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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 20 000 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 1.

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 μL 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 × 200 mm i.d. × 0.33 μm 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 β-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	*β-phellandrene*	2.96
2	*limonene*	26.91
3	*β-ocimene*	36.40
4	*allo-ocimene*	6.31
5	*(E)-tagetone*	2.54
6	*(Z)-tagetone*	16.86
7	*(Z)-tagetenone*	0.83
8	*(E)-tagetenone*	0.58
9	*caryophyllene*	4.50
10	*germacrene D*	2.10

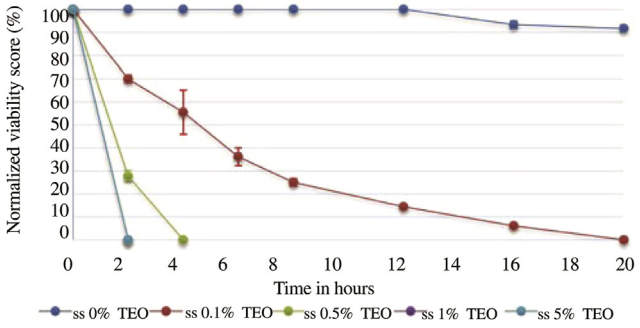


Figure 1. Viability score (%) of *Anisakis* larvae Type 1 in saline solution (SS) with 0% (Control), 0.1%, 0.5%, 1% and 5% of *T. minuta* essential oil.

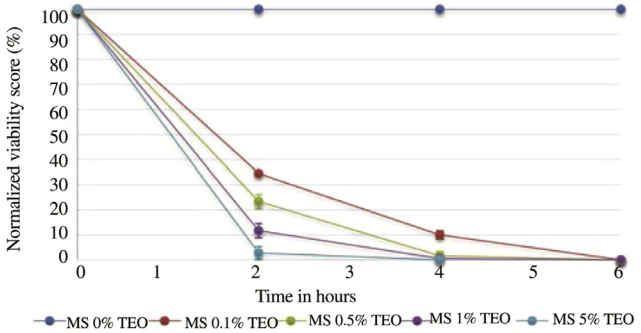


Figure 2. Viability score (%) of *Anisakis* larvae type 1 in marinate solution (MS) with 0% (Control), 0.1%, 0.5%, 1% and 5% of *T. minuta* essential oil.

3.2.2. Marinate 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. 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

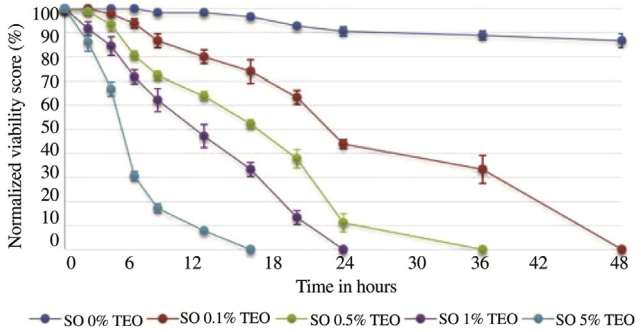


Figure 3. Viability score (%) of *Anisakis* larvae type 1 in sunflower seeds oil (SO) with 0% (Control), 0.1%, 0.5%, 1% and 5% of *T. minuta* essential oil.

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

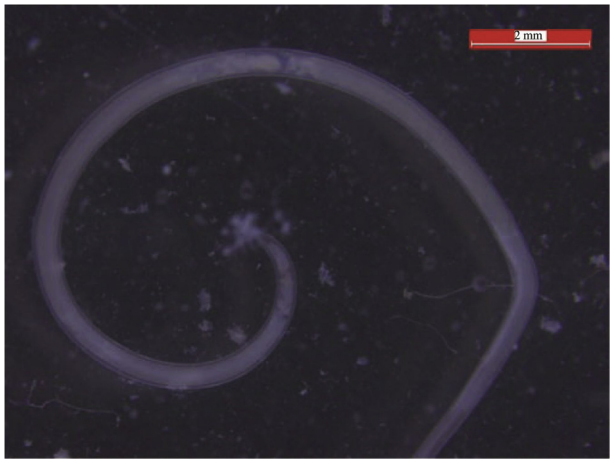


Figure 4. L3 larvae of *Anisakis* type 1 treated with *T. minuta* essential oil: interruption on the digestive tract (Stereoscopic microscope).

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.

References

[1] Amuka O, Okemo P, Machocho A, Njagi P, Mbugua ENM, Nyamache A. Intercession of phytomedicine in the challenges of emerging, re-emerging diseases; and pathogens resistance to antibiotics. *Int J Herb Med* 2013; **1**(4): 102-106.

[2] Caceres A, Cano O, Samayoa B, Aguilar L. Plants used in guatemala for the treatment of gastrointestinal disorders. 1. Screening of 84 plants against enterobacteria. *J Ethnopharmacol* 1990; **30**: 55-73.

[3] Caceres A, Alvarez A, Ovando A, Samayoa B. Plants used in Guatemala for the treatment of respiratory diseases. 1. Screening of 68 plants against gram-positive bacteria. *J Ethnopharmacol* 1991; **31**: 193-208.

[4] Giarratana F, Muscolino D, Beninati C, Ziino G, Giuffrida A, Panebianco A. Activity of R(+) limonene on the maximum growth rate of fish spoilage organisms and related effects on shelf-life prolongation of fresh gilthead sea bream fillets. *Int J Food Microbiol* 2016; **237**: 109-113.

[5] Giarratana F, Muscolino D, Ragonese C, Beninati C, Sciarrone D, Ziino G, et al. Antimicrobial activity of combined thyme and rosemary essential oils against Listeria monocytogenes in Italian mortadella packaged in modified atmosphere: thyme & Rosemary EOs vs L. monocytogenes. *J Essent Oil Res* 2016; **28**(6): 467-474.

[6] Hirota R, Roger NN, Nakamura H, Song HS, Sawamura M, Suganuma N. Anti-inflammatory effects of limonene from Yuzu (*Citrus junos* Tanaka) essential oil on eosinophils. *J Food Sci* 2010; **75**(3): 87-92.

[7] Lu XG, Zhan LB, Feng BA, Qu MY, Yu LH, Xie JH. Inhibition of growth and metastasis of human gastric cancer implanted in nude mice by d-limonene. *World J Gastroenterol* 2004; **10**(14): 2140-2144.

[8] Muscolino D, Giarratana F, Beninati C, Ziino G, Giuffrida A, Panebianco A. Effects of allyl isothiocyanate on the shelf-life of gilthead sea bream (*Sparus aurata*) fillets. *Czech J Food Sci* 2016; **34**(2): 160-165.

[9] Rachuonyo H, Ogola P, Arika W, Wambani J, Gatheri G. Combined effect of crude leaf extracts of selected medicinal plants against selected enteric bacterial pathogens and *Candida albicans*. *J Antimicro* 2016; **2**(110): 2.

[10] Aggarwal KK, Khanuja SPS, Ahmad A, Santha Kumar TR, Gupta VK, Kumar S. Antimicrobial activity profiles of the two enantiomers of limonene and carvone isolated from the oils of *Mentha spicata* and *Anethum sowa*. *Flav Frag J* 2002; **17**(1): 59-63.

[11] Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils – a review. *Food Chem Toxicol* 2008; **46**(2): 446-475.

[12] Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int J Food Microbiol* 2004; **94**(3): 223-253.

[13] Giarratana F, Muscolino D, Beninati C, Ziino G, Giuffrida A, Panebianco A. Effects of thyme and rosemary essential oils on the microbiology and shelf life of Italian Mortadella. *Fleischwirtschaft* 2013; **93**(4): 183-187.

[14] Giarratana F, Crinò C, Muscolino D, Beninati C, Ziino G, Giuffrida A, et al. Preliminary investigation on the use of allyl isothiocyanate to increase the shelf-life of gilthead sea bream (*Sparus aurata*) fillets. *It J Food Saf* 2015; **4**(3): 172-174.

[15] Klein G, Rüben C, Upmann M. Antimicrobial activity of essential oil components against potential food spoilage microorganisms. *Curr Microbiol* 2013; **67**(2): 200-208.

[16] Silva-Angulo AB, Zanini SF, Rosenthal A, Rodrigo D, Klein G, Martínez A. Comparative study of the effects of citral on the growth and injury of *Listeria innocua* and *Listeria monocytogenes* cells. *PloS One* 2015; **10**(2): e0114026.

[17] Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J, Pérez-Álvarez J. Antifungal activity of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L.) and orange (*Citrus sinensis* L.) essential oils. *Food Control* 2008; **19**(12): 1130-1138.

[18] Jain R, Katare N, Kumar V, Amit Kumar S, Swati G, Shrotri CK. *In vitro* anti bacterial potential of different extracts of *Tagetes erecta* and *Tagetes patula*. *J Nat Sci Res* 2012; **2**(5): 84-90.

[19] Faizi S, Siddiqi H, Bano S, Naz AL, Mazhar K, Nasim S, et al. Antibacterial and antifungal activities of different parts of *Tagetes patula*: preparation of patuletin derivatives. *Pharm Biol* 2008; **46**(5): 309-320.

[20] Gakuubi MM, Wanzala W, Wagacha JM, Dossaji SF. Bioactive properties of *Tagetes minuta* L. (Asteraceae) essential oils: a review. *A J EO Natl Prod* 2016; **4**(2): 27-36.

[21] Rondón M, Velasco J, Hernández J, Pecheneda M, Rojas J, Morales A, et al. Chemical composition and antibacterial activity of the essential oil of *Tagetes patula* L. (Asteraceae) collected from the Venezuela Andes. *Rev Latinoam Quím* 2006; **34**: 1-3.

[22] Tereschuck M, Riera M, Castro G, Abdala L. Antimicrobial activity of flavonoids from leaves of *Tagetes minuta*. *J Ethnopharmacol* 1997; **56**: 227-232.

[23] Tereschuk M, Baigorí M, Abdala L. Antibacterial activity of *Tagetes terniflora*. *Fitoterapia* 2003; **74**: 404-406.

[24] Perich M, Wells C, Bertsch W, Tredway K. Isolation of the insecticidal components of *Tagetes minuta* (Compositae) against mosquito larvae and adults. *J Am Mosq Control Assoc* 1995; **11**: 307-310.

[25] Romagnoli C, Bruni R, Andreotti E, Rai MK, Vicentini CB, Mares D. Chemical characterization and antifungal activity of essential oil of capitula from wild Indian *Tagetes patula* L. *Protoplasma* 2005; **225**: 57-65.
[26] Zydalgo J, Guzman C, Grosso N. Antifungal properties of the leaf oils of *Tagetes minuta* L and *T. filifolia* Lag. *J Essent Oil Res* 1994; **6**: 617-621.
[27] Garcia MV, Matias J, Cavalcante Barros J, de Lima DP, da Silva Lopes R, Andreotti R. Chemical identification of *Tagetes minuta* Linnaeus (Asteraceae) essential oil and its acaricidal effect on ticks. *Rev Bras Parasitol Vet* 2012; **21**(4): 405-411.
[28] Mansoor A, Siddiquanid M, Mashkoor A. Control of plant parasitic nematodes by *Tagetes tenuifolia*. *Rev Nématol* 1988; **3**: 369-370.
[29] Wang KH, Hooks C, Ploeg A. Protecting crops from nematode pests: using marigold as an alternative to chemical nematicides. *UH–CTAHR* 2007; **35**: 1-6.
[30] Chai JY, Darwin MK, Lymbery AJ. Fish-borne parasitic zoonoses: status and issues. *Int J Parasitol* 2005; **35**(11–12): 1233-1254.
[31] European Food Safety Authority (EFSA). Scientific Opinion on risk assessment of parasites in fishery products. *EFSA J* 2010; **8**(4): 1543.
[32] Muscolino D, Giarratana F, Beninati C, Tornambene A, Panebianco A, Ziino G. Hygienic-sanitary evaluation of sushi and sashimi sold in Messina and Catania, Italy. *Int J Food Saf* 2014; **3**(2): 134-136.
[33] Bucca M, Brianti E, Giuffrida A, Ziino G, Cicciari S, Panebianco A. Prevalence and distribution of *Sarcocystis* spp. cysts in several muscles of cattle slaughtered in Sicily, Southern Italy. *Food Control* 2011; **22**(1): 105-108.
[34] Lima dos Santos CAM, Howgate P. Fishborne zoonotic parasites and aquaculture: a review. *Aquaculture* 2011; **318**(3–4): 253-261.
[35] Giuffrida A, Ziino G, Pennisi L. Larva *Anisakis* in *Sepia officinalis* del Mediterraneo. *Ind Aliment Italy* 2002; **41**(418): 1086-1088.
[36] Audicana MT, Fernandez de Corres L, Munoz D, Fernandez E, Navarro JA, del Pozo MD. Recurrent anaphylaxis caused by *Anisakis simplex* parasitizing fish. *J Allergy Clin Immunol* 1995; **96**: 558-560.
[37] Audicana MT, Ansotegui IJ, de Corres LF, Kennedy MW. Anisakis simplex: dangerous–dead and alive? *Trends Parasitol* 2002; **18**(1): 20-25.
[38] Audicana MT, Kennedy MW. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. *Clin Microbiol Rev* 2008; **21**(2): 360-379.
[39] Kasuya S, Hamano H, Izumi S. Mackerel-induced urticaria and *Anisakis*. *Lancet* 1990; **335**: 665.
[40] Anastasio A, Smaldone G, Cacace D, Marrone R, Lo Voi A, Santoro M, et al. Inactivation of *Anisakis pegreffii* larvae in anchovies (*Engraulis encrasicolus*) by salting and quality assessment of finished product. *Food Control* 2015; **64**: 115-119.
[41] del Carmen Romero M, Valero A, Martín-Sánchez J, Navarro-Moll MC. Activity of Matricaria chamomilla essential oil against anisakiasis. *Phytomedicine Int J Phytot Phytop* 2012; **19**(6): 520-523.
[42] Giarratana F, Muscolino D, Beninati C, Giuffrida A, Panebianco A. Activity of Thymus vulgaris essential oil against *Anisakis* larvae. *Exp Parasitol* 2014; **142**: 7-10.
[43] Giarratana F, Panebianco F, Muscolino D, Beninati C, Ziino G, Giuffrida A. Effect of allyl isothiocyanate against *Anisakis* larvae during the anchovy marinating process. *J Food Prot* 2015; **78**(4): 767-771.
[44] Giarratana F, Muscolino D, Panebianco F, Patania A, Benianti C, Ziino G, et al. Activity of R(+) limonene against *Anisakis* larvae. *Int J Food Saf* 2015; **4**(4): 209-211.
[45] Giarratana F, Muscolino D, Ziino G, Lo Presti V, Rao R, Chiofalo V, et al. Activity of catmint (*Nepeta cataria*) essential oil against *Anisakis* Larvae. *Trop Biomed* 2017; **34**(1): 22-31.
[46] Gomez-Rincon C, Langa E, Murillo P, Valero MS, Berzosa C, Lopez V. Activity of tea tree (Melaleuca alternifolia) essential oil against L3 larvae of Anisakis simplex. *BioMed Res Int* 2014; **2014**. 549510.
[47] Hierro I, Valero A, Navarro MC. *In vivo* larvicidal activity of monoterpenic derivatives from aromatic plants against L3 larvae of *Anisakis simplex* s.l. *Phytomedicine* 2006; **13**(7): 527-531.
[48] Navarro M, Noguera M, Romero M, Montilla M, de Selgas JG, Valero A. *Anisakis simplex* sl: larvicidal activity of various monoterpenic derivatives of natural origin against L 3 larvae *in vitro* and *in vivo*. *Exp Parasitol* 2008; **120**(4): 295-299.
[49] Panebianco A, Giuffrida A, Minniti A, Ziino G, Calabrò P, Triglia F. Effetto di alcuni gas sul disincistamento delle larve *Anisakis* in *Lepidopus caudatus*. *Ind Aliment-Italy* 2000; **39**(391): 467-470.
[50] Valero A, Romero MC, Gomez-Mateos M, Hierro I, Navarro MC. Natural products: perspectives in the pharmacological treatment of gastrointestinal anisakiasis. *Asian Pac J Trop Med* 2015; **8**(8): 612-617.
[51] Giarratana F, Muscolino D, Beninati C, Ziino G, Giuffrida A, Trapani M, et al. *Gymnorhynchus gigas* in *Lepidopus caudatus* (Actinopterygii: perciformes: Trichiuridae): prevalence and related effects on fish quality. *Czech J Food Sci* 2014; **32**(4): 320-325.
[52] Murata R, Suzuki J, Sadamasu K, Kai A. Morphological and molecular characterization of *Anisakis* larvae (Nematoda: Anisakidae) in *Beryx splendens* from Japanese waters. *Parasitol Int* 2011; **60**(2): 193-198.
[53] Giarratana F, Giuffrida A, Gallo F, Ziino G, Panebianco A. Study of the resistance variability of *Anisakis* larvae to some technological stressors. In: Pugliese A, Gaiti A, Boiti C, editors. *Veterinary science*. Berlin Heidelberg: Springer-Verlag; 2012, p. 155-159.
[54] Chamorro ER, Ballerini G, Sequeira AF, Velasco GA, Zalazar MF. Chemical composition of essential oil from *Tagetes minuta* L. leaves and flowers. *J Argent Chem Soc* 2008; **96**(1–2): 80-86.
[55] Ramaroson-Raonizafinimanana B, Ramanoelina PAR, Rasoarahona JRE, Gaydou EM. Chemical compositions of aerial part of *Tagetes minuta* L. chemotype essential oils from Madagascar. *J Essent Oil Res* 2009; **21**: 390-392.
[56] Shirazi MT, Gholami H, Kavoosi G, Rowshan V, Tafsiry A. Chemical composition, antioxidant, antimicrobial and cytotoxic activities of *Tagetes minuta* and *Ocimum basilicum* essential oils. *Food Sci Nutr* 2014; **2**(2): 146-155.
[57] Tankeu SY, Vermaak I, Viljoen AM, Sandasi M, Kamatou GP. Essential oil variation of *Tagetes minuta* in South Africa – a chemometric approach. *Biochem Syst Ecol* 2013; **51**: 320-327.
[58] Patel A, Patra DD. Influence of heavy metal rich tannery sludge on soil enzymes vis-à-vis growth of *Tagetes minuta*, an essential oil bearing crop. *Chemosphere* 2014; **112**: 323-332.
[59] Marotta SM, Giarratana F, Parco A, Neri D, Ziino G, Giuffrida A, et al. Evaluation of the antibacterial activity of bergamot essential oils on different *Listeria monocytogenes* strains. *Ital J Food Saf* 2016; **5**(5): 210-213.