

## Activity of Catmint (*Nepeta cataria*) essential oil against *Anisakis* larvae

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**Abstract.** *Nepeta cataria* L., commonly known as Catnip, is an aromatic plant belonging to the mint family, Limiaceae. Nematicidal activity of Catnip essential oil (CEO), was assayed *in vitro* against L3 larvae of *Anisakis* type 1. Anisakidosis is one of the most important fish-borne zoonotic diseases related to the ingestion of nematode larvae belonging to the genus *Anisakis*, *Contraceacum* and more rarely *Pseudoterranova* and *Hysterothylacium*. *In vitro* tests revealed a complete inactivation of parasites after 6 and 12 h of treatment, at 10 and 5% respectively, in saline solution. In marinating solution a complete inactivation of parasites was observed after 12 and 18 hours at 10 and 5% concentrations respectively. The data obtained showing a significant activity against *Anisakis* larvae and suggest further investigations on CEO as a larvicidal agent.

### INTRODUCTION

Anisakidosis is one of the most important fish-borne zoonotic diseases caused by parasites belonging to Anisakidae or Raphidascaridae families. *Anisakis*, *Contraceacum* and more rarely *Pseudoterranova* and *Hysterothylacium*, are the genus associated to anisakidosis (Lima dos Santos & Howgate, 2011). As well known, the human disease is related to the consumption of raw or almost raw seafood products since several fish and cephalopods, commonly parasite hosts (Chai *et al.*, 2005). Digestive disorders and/or allergies in human may occur as a consequence of accidental ingestion of fish products parasitized by third-stage larvae (Audicana *et al.*, 2002; Audicana & Kennedy, 2008). Recently, a great interest on the effects of natural products against the L3 larvae of *Anisakis* has been raised, especially the essential oils and their components (Hierro *et al.*, 2006; Romero Mdel *et al.*, 2012; Giarratana *et al.*, 2014;

Gomez-Rincon *et al.*, 2014; Giarratana *et al.*, 2015a; Giarratana *et al.*, 2015b; Anastasio *et al.*, 2015; Valero *et al.*, 2015).

Several herbs used in folklore for the treatment of diseases are being screened for their activity against parasites and the results obtained from these scientific studies so far have rationalized the tradomedical use of many plants and plant parts. Among natural compounds, essential oils derived from aromatic plants, have been explored and gained prominence. Several authors have been demonstrated that essential oils (EO) were effective against bacteria, yeasts, fungi, and parasites as well as possess anti-oxidant, anti-inflammatory, anti-carcinogenic properties (Aggarwal *et al.*, 2002; Lu *et al.*, 2004; Sun, 2007; Viuda-Martos *et al.*, 2008; Hirota *et al.*, 2010; Giarratana *et al.*, 2013; Giarratana *et al.*, 2016).

*Nepeta cataria* L., commonly known as Catnip, is an aromatic plant belonging to the mint family, Limiaceae. *Nepeta* species, including *Nepeta cataria*, are used as

traditional medicines in many countries in gastrointestinal and respiratory hyperactive disorders such as colic, diarrhea, cough, asthma and bronchitis (Bandh *et al.*, 2011; Zomorodian *et al.*, 2015). Catnip possess also antimicrobial properties demonstrated on spoilage microorganisms, foodborne and postharvest pathogens, including gram-negative, gram-positive bacteria and mould (Bandh *et al.*, 2011; Edewor & Usman, 2011; Zomorodian *et al.*, 2015). Recently nepetalactones, one of the principal component from *Nepeta cataria* essential oil, was reported as a stable fly feeding and oviposition repellent against *Stomoxys calcitrans* (L.) (Zhu, 2012; Zhu *et al.*, 2014). Catnip essential oil (CEO) has not been explored yet for its biological effects on parasites, however recently, a good efficacy was demonstrated against *Haemonchus contortus* (Alam *et al.*, 2015). Therefore, the activity of CEO against parasites is not well known notably in food parasites such as *Anisakis simplex*. The aim of our study was to evaluate *in vitro* effects of *Nepeta cataria* EO on *Anisakis* larvae.

## MATERIALS AND METHODS

### Chemical composition

*Nepeta cataria* EO was supplied by Fitomedical (Binasco, Italy). The analyses were carried out by GC-MS, an Agilent 6890N Gas Chromatograph equipped with a HP-5MS capillary column (50 m x 0.200 mm i.d., film thickness 0.33 µm). The GC oven temperature was programmed to increase from 60 to 250°C at a rate of 4°C/min and finally held for 15 min. The transfer line temperature was 250°C. Helium was used as the carrier gas at a flow rate of 1.1 mL/min with a split ratio equal to 1/100. The quadrupole mass spectrometer was scanned over the 35–465 amu with an ionizing voltage of 70eV and an ionization current of 150mA. The percentage composition of oils was computed from GC peak areas without correction factors. Qualitative analysis was based on a comparison of mass spectra with corresponding data in the computer mass spectra libraries (NIST MS SEARCH 2.0).

### Anisakid nematode collection

For the present study, Anisakidae larvae were collected from 10 specimens of *Lepidopus caudatus* (silver scabbard fish) purchased from several fish markets in Messina (Sicily, Italy) harvested within 4 hours from the sampling. The visceral cavity, digestive tract, liver, gonads and mesenteries from each fish were examined for the detection of *Anisakis* larvae. All the collected nematodes were washed several times on sterile saline solution (NaCl 0.9%) and examined under stereoscopic microscope (Leica M 205 C) for the viability and the belonging to the *Anisakis* genus according to guidelines proposed by Murata *et al.* (2011). Actively moving parasites without any injury were maintained in sterile saline solution (NaCl 0.9%) at room temperature until use. All the 1620 anisakidae larvae used for the study were identified as L3 larvae of *Anisakis* type I.

### *In vitro* larvicidal activity

Parasites were then introduced into glass Petri dishes with 20 ml of different concentrations of test compound, and then leaved at room temperature (21±1°C). In particular, the study was carried out in two different steps. The first one concerned tests where 540 larvae were introduced in saline solution (SS) with different concentration of CEO, in order to evaluate its effectiveness against larvae.

The second experimental step, was carried out on 1080 larvae in two different liquid media in order to evaluate the use of CEO in fish marinating process: i) a marinating solution (MS) of water/vinegar with ratio 1:1, 3% NaCl and 1% citric acid with CEO at different concentration, reproducing the solution used by several industrial producers; ii) in oil sunflower seeds (SSO) with the same concentrations of CEO to simulate the normal post-marinating packaging and storage.

For each media (SS, MS and SSO) the concentrations of CEO were: 10% (100 µl/ml), 5% (50 µl/ml), 1% (10 µl/ml), 0.5% (5 µl/ml), 0.1% (1 µl/ml) and 0% as control. For each media and concentration, 30 *Anisakis* larvae

were tested. All experiments were performed in three replicates.

Larvae were examined under stereoscopic microscope (Leica M 205 C) at different interval from 0 to 120 hours 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)(Giarratana *et al.*, 2015). Larvae were considered dead, when no mobility was observed under stereoscopic microscope in saline solution. The normalized mean viability according to Giarratana *et al.* (2012), LT50 and the percentage of inactivation at 24 hours were calculated. Scanning electron microscopy (SEM) observations of parasites dead were done with a Phenom SEM (Phenom-World BV, Eindhoven, The Netherlands).

## RESULTS AND DISCUSSION

### Composition of *Nepeta cataria* essential oil

Fifteen compounds were detected in the *Nepeta cataria* essential oil analyzed by

GC-MS (Table 1). The compounds obtained and their abundance confirm the extremely variability in composition of CEO as reported by Zomorodian *et al.* (2012). The low quantity of nepetalacotene (4.394%) founded in our CEO, is consistent with the chemical characterization provided by several authors (Baranauskiene *et al.*, 2003; Klimek & Modnicki 2005; Gilati *et al.*, 2008; Saeidnia *et al.*, 2008) and in disagree with the quantity (until 85%) reported by Zhu (2012), Zomorodian *et al.* (2012) and Morteza-Semnani & Saeedi (2004). The main component of our CEO is geraniol (Table 1), as reported by Klimek & Modnicki (2005) for *Nepeta cataria* L. var. *citriodora*.

Different authors suggest that this monoterpene is responsible, at least in part, for the larvicidal activity against *Contracaecum* and *Anisakis simplex* larvae (Hierro *et al.*, 2006; Barros *et al.*, 2009). Several monoterpenes detected in CEO posses larvicidal activity such as citral, citranellol and netylacetate (Hierro *et al.*, 2006; Kim *et al.*, 2008; Barros *et al.*, 2009). Furthermore, due to the chemical complexity of essential oils, it should be noted that many of their properties are due to the synergistic or complementary effects of several components.

Table 1. Peak identification relative to *Nepeta cataria* essential oil

	Library/ID	R.T.	Area	Area %
1	Limonene	19.835	9092014	1.073
2	(Beta Citronellol)6-Octen-1-ol, 3,7-dimethyl-, (R)-	26.987	51114209	6.030
3	(cis Geraniol)2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	27.997	70578157	8.326
4	(piperitone)2-Cyclohexen-1-one, 3-methyl-6-(1methylethyl)	28.554	2387278	0.282
5	(Citral)2,6-Octadienal, 3,7-dimethyl-	28.694	6451554	0.761
6	(Geranyl formate)2,6-Octadien-1-ol, 3,7-dimethyl-, formate, (E)-	29.677	2810471	0.332
7	(Citronellyl acetate)6-Octen-1-ol, 3,7-dimethyl-, acetate	30.963	3291225	0.388
8	2,6-Octadiene, 2,6-dimethyl-	31.346	1.73E+08	20.374
9	(Nerylacetate)2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	31.724	10329600	1.219
10	(cis Geraniol)2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	32.432	3.98E+08	46.938
11	nepetalactone	33.572	37249697	4.394
12	Caryophyllene	34.625	52879286	6.238
13	Caryophylleneoxide	39.86	17966303	2.120
14	Farnesol isomer a	56.865	7148378	0.843
15	(Geranyl acetate)2,6-Octadien-1-ol, 3,7-dimethyl-, acetate	61.808	5782848	0.682

R.T. = retention time.

### ***In vitro* larvicidal activity**

*In vitro* tests revealed significant activity (LT50, LT100 and % inactivation at 24h) of CEO in saline solution against *Anisakis* larvae at all concentration tested (Table 2). In particular, at 10% (100 µl/ml) and 5% (50 µl/ml) concentrations, a complete inactivation of parasites was observed after 6 and 12 h of treatment respectively. At 1% (10 µl/ml), treatment produced the inactivation of 100% of larvae at 24 h whereas, at 0.5% and 0.1%, the complete inactivation was reached after 30 h (Figure 1). LT50 (6.9 hours) and the percentage of

inactivation (100%) at 24 hours at 5% CEO are in agreement with the value reported for several EO in saline solution (Valero *et al.*, 2015).

In marinating solution a complete inactivation (LT100) of parasites was observed after 12h and 18h at 10 and 5% concentrations respectively. LT100 of 1% in MS was observed at 36 hours. The remain concentrations tested (0.5 and 0.1 %) caused inactivation of 100% (LT100) of larvae after 48 h (Figure 2). LT50 of 10 and 5% in MS was reported at 6.2 and 9.6 hours respectively (Table 2).

Table 2. Effects of *Nepeta cataria* essential oil on *Anisakis* type 1

CEO	LT 50			LT 100			% inactivation at 24h		
	SS	MS	SSO	SS	MS	SSO	SS	MS	SSO
10%	3.9	6.2	31.7	6.0	12.0	48.0	100%	100%	23.3±14.4%
5%	6.6	9.6	74.1	12.0	18.0	135*	100%	100%	1.67±2.9%
1%	14.9	17.0	168.2*	24.0	36.0	307.6*	100%	80±5%	0%
0.5%	17.7	26.1	332.7*	30.0	48.0	646.9*	85±5%	48.3±5.8%	0%
0.1%	20.2	31.1	1000.9*	30.0	48.0	1967.5*	70±5%	36.7±2.9%	0%

SS: Saline solution; MS: marinating solution; SSO: sunflower seeds oil; \* presuntive value.

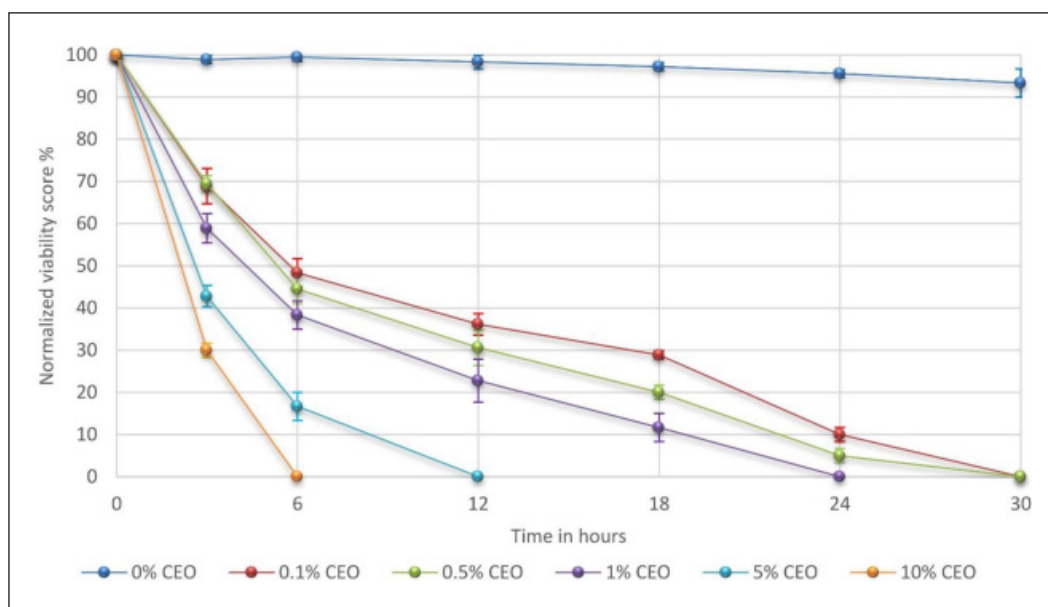


Figure 1. Normalized viability score of *Anisakis* type 1 in saline solution with 0% (Control), 0.1%, 0.5%, 1%, 5% and 10% of *Nepeta cataria* essential oil.

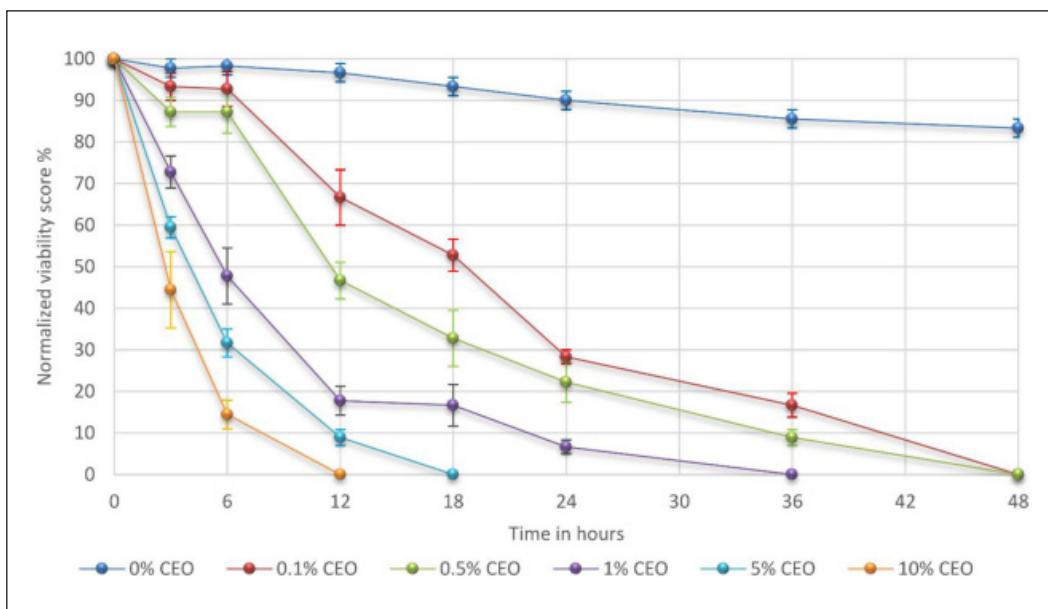


Figure 2. Normalized viability score of *Anisakis* type 1 in marinating solution with 0% (Control), 0.1%, 0.5%, 1%, 5% and 10% of *Nepeta cataria* essential oil.

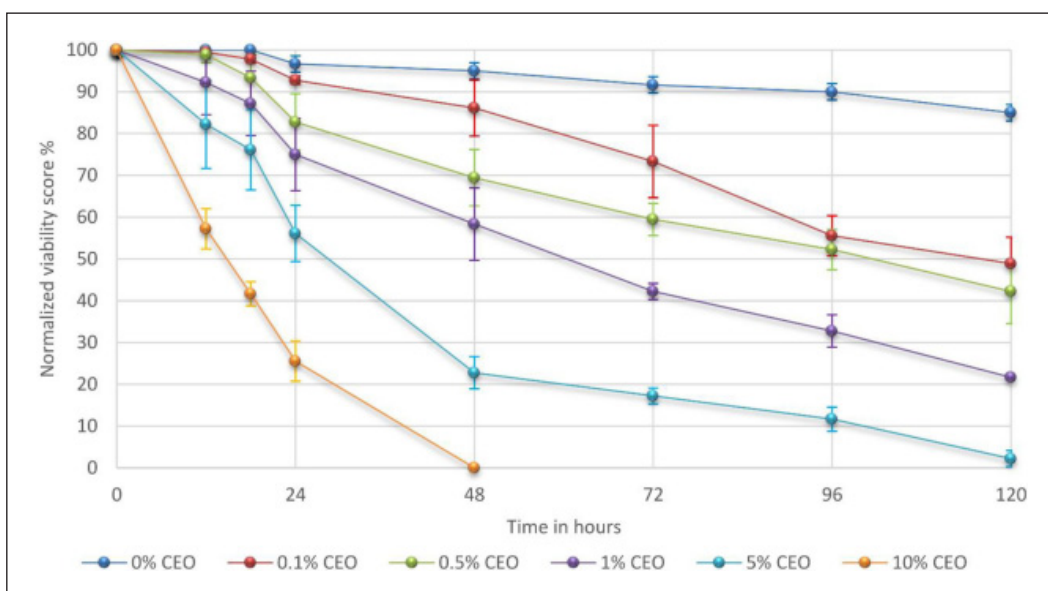


Figure 3. Normalized viability score of *Anisakis* type 1 in sunflower seeds oil with 0% (Control), 0.1%, 0.5%, 1%, 5% and 10% of *Nepeta cataria* essential oil.

Poor efficacy was detected for larvae treated in SSO, where the complete inactivation of parasites was observed at major concentration (10%) and after 48h (Figure 3). The value of LT50 and LT100 of remaining concentrations are reported in Table 2.

Microscopic analysis (stereo microscopy and SEM) showed damages of digestive tract of parasites treated in saline and marinating solutions (Figure 4 and 5). No effect was observed in *Anisakis* larvae subjected to treatments with catnip in SSO at all concentrations tested. These results



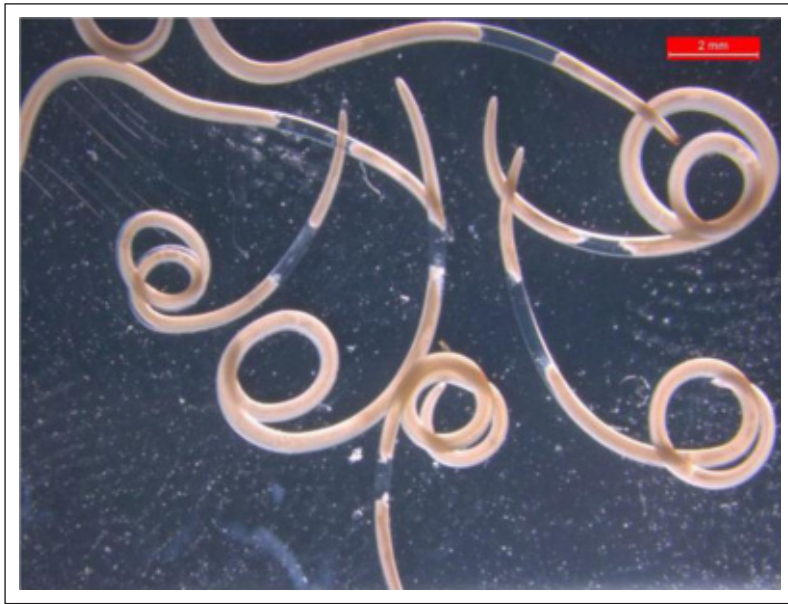


Figure 4. L3 larvae of *Anisakis* type 1 treated with CEO: interruption on the digestive tract (Stereoscopic microscope).



Figure 5. Aspect of interruption on the digestive tract of *Anisakis* larvae 1 treated with CEO, observed with Scanning electron microscope (SEM).

confirm data of several authors that attribute the effectiveness of EOs to the damage caused to cuticle and digestive apparatus of the parasite (del Carmen Romero *et al.*, 2012; Giarratana *et al.*, 2014). In this regard, there are not specific studies in order to explain the mechanisms of action of EOs against parasites. However, the lipophilicity of these compounds seems play an important role in the cellular damage exerted by terpenic components of EOs. In bacteria, EOs produces cellular damage and structural changes of the cellular membrane and causes the leakage of ions and other cell contents (Burt, 2004). These interaction between the EOs components and bacterial membranes, produces structural changes (swelling, shrivelling, vacuolations, leakage) modifying the permeability of the membrane and causing the leakage of ions and other cell content (Bakkali *et al.*, 2008). Observed lesions on larval cuticle and digestive tract could be related to this kind of activity.

In recent years, the nematocidal activity against *Anisakis* L3 of several essential oils such as *Matricaria chamomilla*, *Thyme vulgaris* and *Melaleuca alternifolia*, and some of its components has been demonstrated (Navarro *et al.*, 2008; del Carmen Romero *et al.*, 2012; Romero Mdel *et al.*, 2012; Giarratana *et al.*, 2014; Giarratana *et al.*, 2015a; Valero *et al.*, 2015). This has an interesting practical implication since marinated seafood product, where the addition of essential oils could represent, according to our results, an alternative method for the inactivation of Anisakidae larvae as well as an innovative method to prevent human anisakidosis.

The use of the high concentration (more than 1%), in a fish marinating production are not economically viable and the taste of fish could not be acceptable. The combination of several EOs could be used to reduce the minimum effective dose of EO thus minimizing the loss in sensory quality caused by a higher concentration of EO when added alone. In this regards the combined use of EOs at lower concentrations could allow the achieving of a balance between sensory acceptability, health benefit and nematocidal efficacy.

Moreover essential oils and their components possess a relatively safe status and potential functional and technological properties. In this regard, several authors have demonstrated the antioxidant, antimicrobial, antiviral, anti-inflammatory, anti-ulcerous, anti-carcinogenic properties of aromatic plant essential oils. Many of these properties are related to some of their components, such as terpenes, monoterpenes and sesquiterpenes (Bakkali *et al.*, 2008; del Carmen Romero *et al.*, 2012).

## CONCLUSIONS

This work is the first study concerning *in vitro* effect of catnip essential oil against *Anisakis* larvae. According to our results *Nepeta cataria* essential oil showed a remarkable *in vitro* nematocidal effect at concentrations of 5 and 10% in saline and marinating solution. The results revealed larvicidal activity that appears proportional to the EO concentration. The larvicidal activity probably is related to the damage found in the parasite digestive tract. The effectiveness of *Nepeta cataria* EOs against *Anisakis* larvae demonstrated in these experiments justifies further investigations to evaluate the potential use of catnip EO for treatment of human anisakidosis and in fish marinating process.

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