# Activity of R(+) limonene against *Anisakis* larvae

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## Abstract

The aim of this work is to evaluate the activity of R(+) limonene of against *Anisakidae* larvae. Its effectiveness was tested *in vitro*. The results obtained showed a significant activity of the compound against *Anisakis* larvae, suggesting further investigation on its potential use in the industrial marinating process. In this regard, the use of R(+) limonene in seafood products could be interesting, also due the sensory attributes resulting from its use and its relatively safe status.

## Introduction

R(+) limonene (LMN) is the major aromatic compound in essential oils obtained from oranges, grapefruits, and lemons. It is used as a flavouring agent in perfumes, creams, soaps, household cleaning products as well as in several foods such as fruit beverages and confectionary products (Espina *et al.*, 2011). The addition of LMN as flavouring substances in foods is authorised by the Regulation EC N° 872/2012 (European Commission, 2012). The LMN possess also antioxidant, antimicrobial, antifungal, anti-inflammatory, anti-carcinogenic properties (Aggarwal *et al.*, 2002; Hirota *et al.*, 2010; Lu *et al.*, 2004; Sun, 2007; Viuda-Martos *et al.*, 2008). However, it is well known that the LMN often have activity against a variety of parasites including nematodes, but its effect against food parasites such as *Anisakis* larvae has not yet been reported (Arruda *et al.*, 2009; Kanojiya *et al.*, 2014; Macedo *et al.*, 2010; Urban *et al.*, 2014). Anisakiosis is one of the most important fish-borne zoonotic diseases caused by parasites belonging to Anisakis and Pseudoterranova genera. 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 in human may occur as a consequence of accidental ingestion fish products parasitized by third-stage larvae (Audicana *et al.*, 2002; Audicana and Kennedy, 2008). For these reasons, the European Regulation EC No 853/2004 (European Commission, 2004) establishes a freezing treatment at -20°C for 24 h for fishery products *to be consumed raw or almost raw* as well as *marinated and/or salted fishery products, if the processing is insufficient to destroy nematode larvae*. Recently, several authors had demonstrated a significant action against the L3 larvae of *Anisakis simplex* exerted by various natural products, especially the essential oils and their components (Giarratana *et al.*, 2014, 2015; Gomez-Rincon *et al.*, 2014; Hierro *et al.*, 2006; Romero Mdel *et al.*, 2012). In this regard, the use of LMN in seafood products could be interesting, also due the sensory attributes resulting from its use (pleasant smell and like-lemon taste) and its relatively safe status. However, the aim of this work is to evaluate the effect of LMN against *Anisakis* larvae and its potential use during the anchovy marinating process.

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## Materials and Methods

The present study was carried out *in vitro* in order to assess the effectiveness of LMN against *Anisakis* larvae. The compound was supplied by Sigma Aldrich (Milan, Italy). Several concentrations (0.1, 0.5, 1 and 5%) in three different liquid media were tested. For each test were used at least 50 *Anisakis* larvae sampled from 8 specimens of *Lepidopus caudatus* harvested within 6 hours from the sampling. Larvae were, previously, microscopically evaluated for viability and to assess that they belong to the *Anisakis* genus type I. Tests *in vitro* were done by using the following liquid media: i) a solution 1:1 (vol/vol) of distilled water and vinegar (6% acetic acid), 3% NaCl and 1% citric acid in order to reproduce the solution of marinating used by several producers (Colavita, 2012); ii) seeds oil; iii) physiological solution (0.9% NaCl). Each liquid media was used to prepare four solutions with different LMN concentrations. The addition of LMN was made at 0.1, 0.5, 1 and 5% concentrations. Furthermore, a solution for each liquid media was prepared without the addition LMN, as control. *Anisakis* larvae were introduced into plate dishes with 20 ml of each solution, maintained at 20°C and checked for the viability at 0, 8, 16, 24, 32, 40 and 48 hours. During the experimental treatments, at each fixed time interval, the viability was microscopically checked according to the criteria of Hirasa and Takemasa (1998) assessing the following score: 3 (viable), 2 (reduction of mobility), 1 (mobility only after stimulation) and 0 (death). Larvae were considered dead, when no mobility was observed under stereoscopic microscope in saline solution (0.9% NaCl). The normalized mean score were then used in order to assess the inactivation rate (IR=percentage viability reduction in a minute, under fixed treatment conditions), according to Giarratana *et al.* (2012).

## Results and Discussion

*In vitro* tests revealed significant activity of LMN against *Anisakis* larvae. In particular in saline solution, at 5% LMN, a complete inactivation of parasites was observed after 24 hours of treatment, while after 48h at 1% and 0.5% (Figure 1). In marinating solution a complete inactivation of parasites was observed after 16 h and 24 h at 5% and 1% concentrations respectively (Figure 2).

Poor efficacy was detected for larvae treated in seeds oil, where a complete inactivation of parasites was never observed at all concentrations tested after 7 days of treatment (Figure 3).

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 (del Carmen Romero *et al.*, 2012; Giarratana *et al.*, 2014; Navarro *et al.*, 2008; Romero Mdel *et al.*, 2012).

This has an interesting practical implication since marinated seafood products, 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 anisakiasis. Moreover essential oils

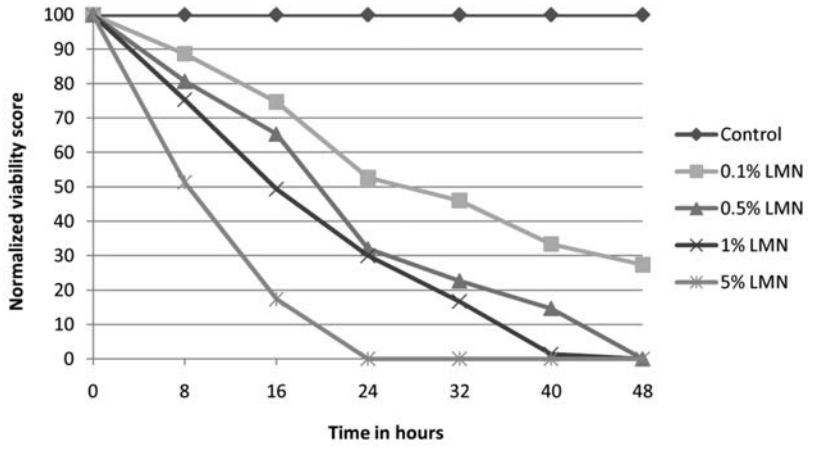


**Figure 1. Inactivation rate of larvae in saline solution with 0, 0.1, 0.5, 1 and 5% R(+) limonene.**

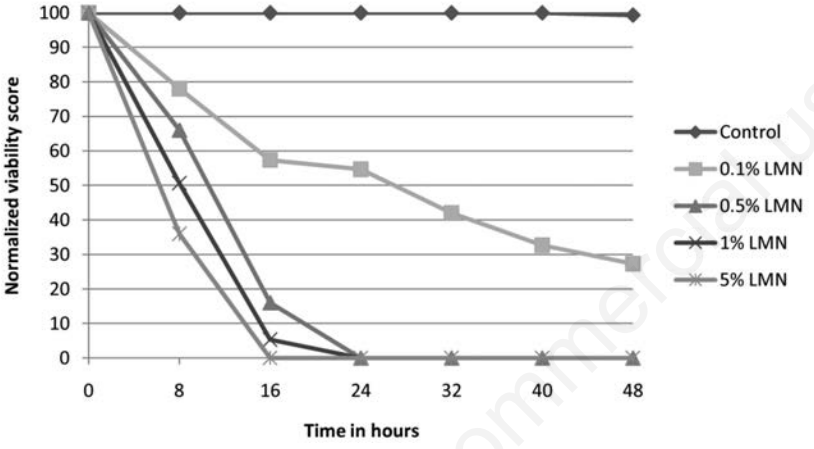


**Figure 2. Inactivation rate of larvae in marinating solution with 0, 0.1, 0.5, 1 and 5% R(+) limonene.**

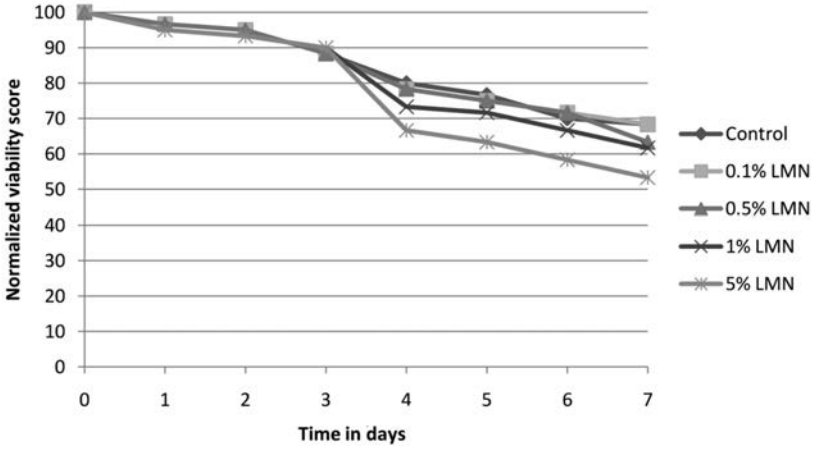


**Figure 3. Inactivation rate of larvae in seeds oil with 0, 0.1, 0.5, 1 and 5% R(+) limonene.**

and their components possess a relatively safe status and potential functional and technological properties (Bakkali *et al.*, 2008; del Carmen Romero *et al.*, 2012).

Microscopic analysis showed damages of the cuticle and digestive tract of parasites treated in saline and marinating solutions. These results confirm data several authors that linked the effectiveness of natural compounds 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, the lipophilia of these compounds seems play an important role in the cellular damage (Bakkali *et al.*, 2008; Burt, 2004). Observed lesions on larval cuticle and digestive tract could be related to this kind of activity.

## Conclusions

This work is the first study concerning *in vitro* effect of LMN against *Anisakis* larvae. According to our results LMN showed a remarkable *in vitro* nematocidal effect at higher concentrations used. The larvicidal activity probably is related to the damage found in the parasite digestive tract. The effectiveness of LMN against *Anisakis* larvae demonstrated in these experiments justifies further investigations to evaluate the potential use LMN for during industrial marinating process.

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