medical sciences forum

MDPI

Proceeding Paper

Anti-Nematodal Essential Oils with Activity against *Anisakis* †

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† Presented at the 1st International Electronic Conference on Molecular Sciences: Druggable Targets of Emerging Infectious Diseases (ECMS 2021), 1–14 September 2021; Available online: https://ecms2021.sciforum.net/.

Abstract: Anisakiasis is a human parasitic infection caused by the larvae of the *Anisakis* nematode through the consumption of raw or undercooked seafood, namely fish and cephalopods. To date, no effective drug has been uncovered and common anthelmintic treatments seem to have reduced activity against this parasite. Essential oils (EOs) are an unexplored source of natural products able to counteract *Anisakis*. The present work reviews the available literature on EOs tested *in vitro* against *Anisakis* nematodes and compiles the activity and composition of the most active EOs. Over a dozen plant species were used as sources of EOs, mainly from the Asteraceae, Lamiaceae, Apiaceae and Myrtaceae families. The lowest half maximal effective concentrations (EC_{50}) were reported for *Origanum syriacum* and *O. compactum* EOs, both rich in carvacrol (83% and 50%, respectively). The EOs extracted from *Tagetes minuta* and *Nepeta cataria* were reported as the fastest acting, with half maximal effective times (ET_{50}) under 4 h, and were rich in geraniol (55%) or β-ocimene (36%) and limonene (27%), respectively. Given their complex chemical composition, additive, synergistic and antagonistic interactions between EO compounds can be responsible for EO activity. A deeper analysis of the chemical structures that are active against *Anisakis*, and the nature of their interactions, can be unveiled with further studies on this parasitosis.

Keywords: anisakiasis; *Anisakis simplex*; Apiaceae; Asteraceae; essential oil; Lamiaceae; Myrtaceae; natural products

Citation: Faria, J.M.S.; Silva, I.V.d. Anti-Nematodal Essential Oils with Activity against *Anisakis* . *Med. Sci. Forum* **2021**, *7*, 7. https://doi.org/10.3390/ECMS2021-10827

Academic Editor: Claudiu T. Supuran

Published: 31 August 2021

1. Introduction

Anisakiasis, a gastrointestinal parasitosis, has become an emerging human health concern due to a rise in the worldwide number of cases. This increase was caused by a growing demand for raw or lightly cooked foods and as a result of the improvement in expertise and diagnostic tools [1]. Besides the repercussions of this parasitosis on human health, the negative effect on consumers' confidence and the consequent marketability of raw fish products also has high economic impacts. The majority of cases are reported in Japan (more than 90%), as a result of the dietary tradition based on raw fish; however, its occurrence in Europe is rising, particularly in Spain [2,3]. Anisakiasis is caused by members of the family Anisakidae, the most common being *Anisakis simplex*. The eggs of these gastrointestinal parasitic nematodes hatch freely in the ocean, where the free-living third stage (L3) larvae are generally consumed by crustaceans. Small crustaceans are the food basis for sea fish, cephalopods and, ultimately, marine mammals, transmitting the nematode to their gastrointestinal tract [4]. Although human physiology does not allow the progression of these nematode parasites' life cycle, humans can become accidental hosts

and be directly affected by debilitating diseases or by the initiation of a state of immune hypersensitivity [5].

The nematodes enter the human gastrointestinal tract through the ingestion of raw, smoked or undercooked fish contaminated with infective *Anisakis* L3 larvae. Within hours following ingestion, nematode larvae can induce an acute and transient infection (gastric anisakiasis) that may lead to abdominal pain, nausea, vomiting, abdominal distention, diarrhea, blood and mucus in the stool, and mild fever. The small intestine is less commonly affected; however, it expresses a more chronic form of the disease. After 1 to 2 weeks of infection, inflammatory mass formation, lumen thickening, severe eosinophilic granulomatous response and interloop ascites characterize intestinal *Anisakis* parasitosis, resembling Crohn's disease symptomatology, and making the diagnostic difficult. Allergic reactions, such as urticaria and anaphylaxis, are also common, triggering an immune response to the presence of the nematode, with a high production of immunoglobulin E (IgE) [5].

Anisakis larvae can survive some traditional food treatments (i.e., smoking and pickling) but are devitalized by salting [6], and destroyed by cooking at temperatures above 63 °C or freezing below −20 °C for 7 days (or −35 °C for more than 15 h) [4]. Several anthelmintics have been proposed for *Anisakis* parasitosis therapy, namely mebendazole, thiabendazole and albendazole. Although these anthelmintics are successfully active against other gastrointestinal nematodes, there is no clear evidence of their effectiveness against *Anisakis* L3 larvae [7]. Thus, there is still no effective drug on the market to treat this digestive parasitosis.

Natural products isolated from plants and microbes are known as excellent sources of pharmaceutical drugs, and many are the basis of the most active pharmaceuticals used today. An antiparasitic activity of plant-derived natural products effective against *Anisakis* is thought to occur, given the lower prevalence of the disease in populations that use the aromatic plant *Perilla frutescens* as a condiment in their raw fish diet [8].

Essential oils (EOs) are composed of several active phytochemicals that have a wide range of biological activities including antimicrobial, fungicidal, insecticidal and insect repellent, herbicidal, acaricidal and nematicidal [9]. These complex mixtures of volatiles are exclusively obtained from plant material obtained by hydro-, steam- or dry-distillation, or in the case of *Citrus* fruits, mechanically without heating [10]. EOs are generally composed of highly active chemical classes of compounds, namely terpenes (mono-, sesqui-, and di-terpenes) and phenylpropanoids, and are usually dominated by one to three major components at relatively high amounts [11]. The study of the activity of EOs against gastrointestinal nematodes has focused mainly on *Haemonchus contortus*, a parasitic nematode of small ruminants (goats and sheep), showing promising results. In the present work, the available literature was reviewed on EOs tested *in vitro* against Anisakis nematodes, and the activity and composition of the most active EOs were compiled.

A comprehensive study of these parameters can contribute to an activity guided screening of natural products that are active against these parasites and provide a deeper analysis of the most active chemical structures.

2. Available Literature

Research was performed, using the Web of Science search engine, on published works reporting on the activity of EOs against *Anisakis* nematodes, using the topics "*Anisakis*" and "essential oil". Identification of the EO source plant, qualitative and quantitative chemical composition and the antinematode activity was retrieved when available.

Eight publications reported on assays using EOs against *Anisakis* L3 larvae, from 2012 to 2019 [12–19]. These works were published in journals mainly covering areas of parasitology (38%), tropical medicine (38%), and public environmental and occupational health (25%). These reports were cited 135 times (113, excluding those reports on this list) by a total of 85 reports (78, excluding those on this list), with an average of 17 citations per work. Citing articles were reported by journals publishing on the research areas of food

science technology, biochemistry, molecular biology, pharmacology, and pharmacy. The cumulative number of citations increased from 2012 to 2018 but has since become stable.

3. Essential Oils and Respective Toxicological Parameters

The reported EOs were extracted from plants that belong to the Asteraceae, Lamiaceae, Apiaceae, and Myrtaceae families. Publications reported on the activity of EOs extracted from *Cuminum cyminum*, *Lavandula angustifolia*, *L. stoechas*, *Matricaria chamomilla*, *Melaleuca alternifolia*, *Nepeta cataria*, *Origanum compactum*, *O. majorana*, *O. syriacum*, *O. vulgare*, *Rosmarinus officinalis*, *Tagetes minuta* and *Thymus vulgaris*. Except for 2 EOs obtained by hydrodistillation, all of the other EOs were acquired from commercial sources. EO activity was expressed through one of three types of parameters, namely nematode mortality, half maximal effective concentrations (EC_{50}) and half maximal effective times (LT_{50}).

3.1. Anisakis Mortality Assays

The mortality percentages were reported for the EOs of *C. cyminum*, *L. angustifolia*, *L. stoechas*, *M. chamomilla*, *O. majorana*, *O. vulgare*, *R. officinalis* and *T. vulgaris*. Only *M. chamomilla* EO showed 100% mortality at 0.125 mg/mL. This EO was composed of bisaboloxide A (49%), α-bisaboloxide B (8%), (−)-α-bisabolol (6%), *trans*-β-farnesene (5%) and chamazulene (2%) (Figure 1).

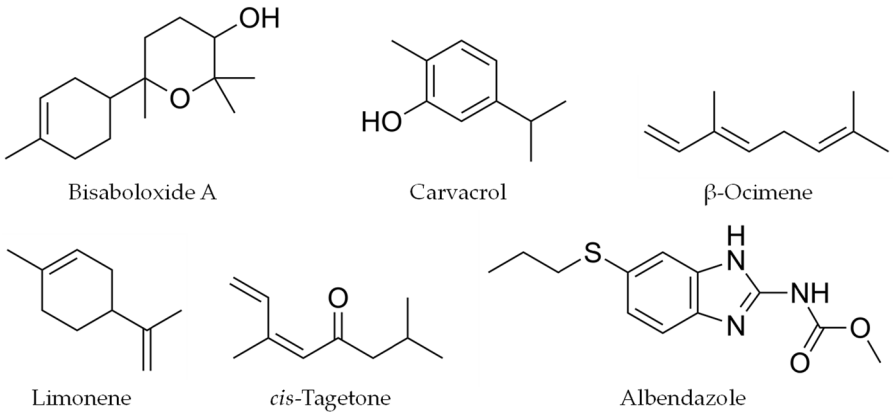

Figure 1. Chemical structure of the main compounds of the most effective essential oils (EOs) and of the commonly used anthelmintic albendazole.

A previous work, published in 2015 [7], reviewed reports on EOs with activity against Anisakis nematodes, but some references were currently not available. In this work, activities are additionally described for the EOs of *Cymbopogon citratus*, *C. martini*, *C. winterianus*, *Litsea cubeba*, *Mentha piperita*, *Myristica fragrans*, *Pelargonium graveolens* and *Piper nigrum*. In these EOs, 100% mortality was obtained at 0.125 mg/mL, except for *M. piperita*, that required a concentration of 0.250 mg/mL to induce complete mortality.

3.2. Half Maximal Effective Concentration (EC_{50})

EC_{50} values are a measure of toxicity for a given substance and correspond to the concentration required to kill half of a tested population, after a specified duration. EC_{50} values were reported for the EOs of *M. alternifolia*, *O. compactum* and *O. syriacum* (Table 1).

Table 1. Half maximal effective concentrations (EC_{50}) reported for the essential oils (EOs) of *Melaleuca alternifolia*, *Origanum compactum* and *O. syriacum* against *Anisakis simplex* after 24 or 48 h of exposure. The EO main composition is reported for compounds above 5%.

EOs	Time of Exposure (h)	EC_{50}	Main Composition (≥5%)
M. alternifolia	24 48	4.53 [1] 4.27 [1]	Terpinene-4-ol, 47% γ-Terpinene, 23% α-Terpinene, 10% *p*-Cymene, 6%
O. syriacum	24 48	0.087 [2] 0.067 [2]	Carvacrol, 83% γ-Terpinene, 65%
O. compactum	24 48	0.429 [2] 0.344 [2]	Carvacrol, 50% Thymol, 15% γ-Terpinene, 14% *p*-Cymene, 8%

[1] μL/mL; [2] mg/mL.

The lowest values were obtained for the EO of *O. syriacum*, which showed a very high relative amount of carvacrol (Figure 1). In the EO of *O. compactum,* the activities decreased exponentially, probably due to its lower relative amount of carvacrol.

3.3. Half Maximal Effective Times (LT_{50})

LT_{50} values correspond to the time required to kill half of a tested population, after exposure to a specified substance concentration. The LT_{50} values were reported for the EOs of *N. cataria* and *T. minuta* (Table 2).

Table 2. Half maximal effective times (ET_{50}) reported for the essential oils (EOs) of *Nepeta cataria* and *Tagetes minuta* against *Anisakis* L3 larvae after exposure to 10, 5, 1, 0.5 and 0.1% of EO. The EO main composition is reported for compounds above 5%.

EOs	Concentration (%) [1]	ET_{50} (h)	Main Composition (≥5%)
N. cataria	10 5 1 0.5 0.1	3.9 6.6 14.9 17.7 20.2	Geraniol, 55% 2,6-Dimethylocta-2,6-diene, 20% β-Citronellol, 6% *trans*-β-Caryophyllene, 6%
T. minuta	5 1 0.5 0.1	1.0 1.0 2.3 11.2	β-Ocimene, 36% Limonene, 27% *cis*-Tagetone, 17% allo-Ocimene, 6% *trans*-β-Caryophyllene, 5%

[1] % of EO in a saline solution of 0.9 % NaCl.

The EO of *T. minuta* showed the highest nematicidal efficacy, killing half of the test population in 1 hour, in concentrations as low as 1%. This EO was mainly composed (80%) of the hydrocarbon monoterpenes β-ocimene and limonene and the monoterpene ketone *cis*-tagetone (Figure 1).

4. Active Essential Oil Components

The activity of isolated EO components was also determined in some reports. The activities of carvacrol and thymol, two oxygen-containing isomers, were higher than *O. compactum* EO, where they can be found in high amounts [13]. A higher efficiency was reported for carvacrol (EC_{50} values of 0.176 mg/mL at 24 h and 0.178 mg/mL at 48 h) than for thymol (EC_{50} values of 0.291 mg/mL at 24 h and 0.214 mg/mL at 48 h), which indicates the specificity of molecule isomerism in the activity against the *Anisakis* nematode. Nematicidal activity was further linked to the inhibition of the enzyme acetylcholinesterase. The

EO of *M. chamomilla* and one of its main components α-bisabolol showed high activities, leading to changes in the cuticle and digestive tract of *Anisakis* L3 larvae [19]. Chamazulene, another *M. chamomilla* EO main component, showed no activity, which suggests that oxygen-containing molecules may be more successful in controlling this parasite. Nevertheless, given their complex composition, EO activity cannot be solely attributed to its main components. Although the EO of *M. alternifolia* induced mortality in *Anisakis* L3 larvae and inhibited acetylcholinesterase, the component terpinene-4-ol, which 47% of this EO is comprised of, showed no activity [14]. This suggests that the activity can be caused by lesser dominant compounds or by the presence of synergistic compound relations.

Monoterpenoids seem to be a particularly successful source of active EO components against Anisakis L3 larvae. Promising *in vitro* and *in vivo* activities have been attributed to the aldehyde citral, a mixture of the enantiomers geranial and neral, and the alcohol citronellol, but also to the hydrocarbons α-pinene and ocimene, which are capable of decreasing 80% of the disease symptomatology [8,20]. Nematicidal activity is associated with damage to the L3 larvae cuticle and intestinal walls [8,18].

The use of EOs in food products is a promising technology, e.g., for increasing the shelf lives of cereal products or for increasing the quality of food safety. Research must tackle the challenge of selecting the most adequate EOs and/or EO components by finding a compromise between the required bioactivity, the suitability for human consumption, maintaining a desirable aroma and flavor, and eliminating any adverse effects on the food products [21].

5. Conclusions

Plant extracts and EOs seem to be successful in killing *Anisakis* nematode larvae. The use of EOs and their active components can provide an alternative treatment to this parasitosis or can function as possible additives in raw food preparation for prophylaxis against anisakiasis. Further studies and screening of additional EOs and their active compounds can lead to the discovery of chemical structures with improved activity and security to be used in food products.

Author Contributions: Conceptualization, J.M.S.F.; investigation, J.M.S.F. and I.V.d.S.; writing—original draft preparation, J.M.S.F.; writing—review and editing, J.M.S.F. and I.V.d.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the findings of this study are available from the corresponding author (Jorge M. S. Faria) upon reasonable request.

Acknowledgments: The authors wish to thank Ana M. Rodrigues from Plant Metabolomics Laboratory at ITQB NOVA for her assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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