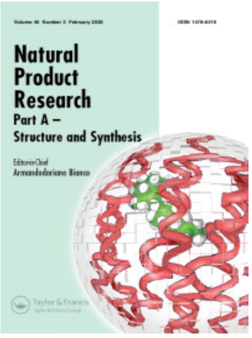

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Natural Product Research
Formerly Natural Product Letters

ISSN: 1478-6419 (Print) 1478-6427 (Online) Journal homepage: www.tandfonline.com/journals/gnpl20

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To cite this article: Gaetano Cammilleri , Luca Nalbone , Andrea Armani , Calogero Alfano , Vincenzo Sala , Francesco Giuseppe Galluzzo , Elisa Maria Domenica Messina , Licia Pantano , Maria Drussilla Buscemi , Vittorio Calabrese , Vincenzo Ferrantelli & Filippo Giarratana (05 Feb 2026): Natural extracts and bioactive compounds against L3 larvae of *Anisakis* spp: *in vitro* and *ex vivo* evaluation and potential application in marinated products, Natural Product Research, DOI: 10.1080/14786419.2026.2623206

To link to this article: https://doi.org/10.1080/14786419.2026.2623206

NATURAL PRODUCT RESEARCH
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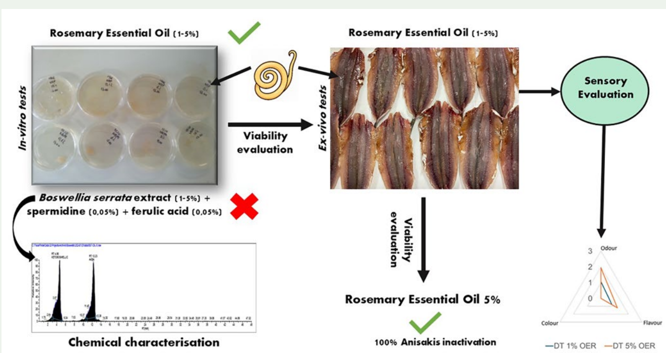
Natural extracts and bioactive compounds against L3 larvae of *Anisakis* spp: *in vitro* and *ex vivo* evaluation and potential application in marinated products

Gaetano Cammilleri[a], Luca Nalbone[b], Andrea Armani[c], Calogero Alfano[a], Vincenzo Sala[b], Francesco Giuseppe Galluzzo[a], Elisa Maria Domenica Messina[a], Licia Pantano[a], Maria Drussilla Buscemi[a], Vittorio Calabrese[d], Vincenzo Ferrantelli[a] and Filippo Giarratana[b]

[a]Istituto Zooprofilattico Sperimentale della Sicilia, Palermo, Italy; [b]Department of Veterinary Sciences, University of Messina, Messina, Italy; [c]FishLab, Department of Veterinary Sciences, University of Pisa, Pisa, Italy; [d]Dipartimento di scienze biomediche e biotecnologiche, Università degli studi di Catania, Catania, Italy

ABSTRACT

This work investigated the efficacy of rosemary essential oil (REO) and *Boswellia serrata* extracts (BSE) against *Anisakis* larvae for possible industrial applications. *In-vitro* assays exposed *Anisakis* type I larvae to various media containing REO (1-5%) or BSE (1-5%) with spermidine and ferulic acid at different temperatures and times. *Ex-vivo* trials in experimentally infested anchovy fillets were carried out only for REO. REO demonstrated significant anisakicidal activity, achieving the fastest efficacy in oil (15h at 4 °C with 5% REO). Lower concentrations (1%) showed limited effectiveness. BSE achieved a maximum 92% larval devitalisation at 5%. *Ex-vivo* experiments revealed initial devitalisation after 72h, reaching 100% by 168h in anchovy fillets with 5% REO-oil at 20 °C. REO exhibited substantial anisakicidal effects in marinated anchovies under refrigeration and lipid conditions. These findings highlight REO as a promising natural additive for enhancing marinated fish product safety, offering an alternative or complement to freezing for *Anisakis* risk management.



ARTICLE HISTORY
Received 17 September 2025
Accepted 22 January 2026

KEYWORDS
Anisakis; marinated anchovy; natural antiparasitic; food safety

CONTACT Gaetano Cammilleri gaetano.cammilleri@izssicilia.it

Supplemental data for this article can be accessed online at https://doi.org/10.1080/14786419.2026.2623206.

1. Introduction

Anisakiasis is a fish-borne parasitic zoonosis caused by the nematode larvae of the genus *Anisakis* (family Anisakidae). Thousands of anisakiasis cases are diagnosed each year, particularly in Japan and in parts of Europe where anchovy consumption is common (Lăcătușu et al. 2019). At present, there are no specific pharmacological treatments for anisakiasis beyond symptomatic management, endoscopic removal of the larvae and, in some cases, laparotomy, making prevention the primary strategy for protecting consumers (Guardone et al. 2018).

In Italy, most anisakiasis cases are linked to marinated anchovies, particularly home-marinated or lightly cured products (Guardone et al. 2018). Cleaning/evisceration before marination can allow cross-contamination of fillets (Şen & Temelli 2003). Vinegar–salt marinades may not reliably inactivate Anisakis (BIOHAZ, 2024). Thus, EU Regulation EC 853/2004 mandates pre-freezing at −20 °C for 24 h (or equivalent), which is effective but can impair texture and add logistical burdens (Santos, 2018; Guardone et al. 2018). To avoid freezing, alternative or adjunct treatments consistent with "clean-label" trends e.g. natural antiparasitic compounds, are under investigation (Mali & Mehta, 2008). Recent studies have highlighted plant-derived substances, especially essential oils (EOs), as promising natural anisakicidal agents. Several EOs have demonstrated the ability to kill or immobilise *Anisakis* larvae *in vitro* or in experimentally infected fish fillets.

Rosemary (Rosmarinus officinalis L.) is widely used in Mediterranean cuisine, and its extracts/oil exhibit antimicrobial and antioxidant activity. Its extracts are approved food additives (EU E392). Trabelsi et al. (2019) reported *Anisakis* larval devitalisation after 7 days in anchovy fillets marinated in olive oil favoured with rosemary and other spices, motivating evaluation of direct rosemary essential oil as an anisakicidal treatment. In addition to essential oils, natural extracts from phytotherapeutic plants such as Indian olibanum (*Boswellia serrata*) contain terpenes, which are known to compromise the integrity of cell membranes, which may result in paralysis or necrotic damage to parasitic tissues (Stavropoulou et al. 2021). However, terpenes tend to exhibit lower acute toxicity to nematodes compared to phenolic compounds such as flavonoids which demonstrated significant nematicidal activity (Cao et al. 2019; Karakoti et al. 2025). At present, there is a lack of studies on the presence and characterisation of phenolic compounds in extracts of *B. serrata*, despite their potential contribution to the plant's pharmacological properties. In addition, other bioactive substances such as spermidine and ferulic acid, commonly found in several natural products, may act synergistically with other constituents to enhance the antihelmintic effect, suggesting a multifactorial mode of action (Mali and Mehta 2008; Saha et al. 2024).

This study evaluated the anisakicidal activity of rosemary essential oil (REO) and *Boswellia serrata* extract (BSE) against Anisakis spp. L3 uses *in vitro* assays. For REO, we performed *ex vivo* trials on artificially infested anchovy fillets processed under industrial-like marination and oil storage, and assessed sensory effects. BSE was chemically profiled by LC-HRMS to identify bioactive contributors. Together, these data appraise the efficacy and practical feasibility of natural interventions for marinated anchovies as potential complements or alternatives to freezing.

2. Results and discussion

2.1. In vitro anisakicidal activity

The *in vitro* trials for BSE and REO showed a clear time- and dose-dependent devitalisation of Anisakis larvae (Table S1).

The extract of *B. serrata* with the addition of spermidine and ferulic acid, showed Anisakicidal efficacy only at +20 °C in marination brine after 96 h of treatment, however without determining the devitalisation of all the larvae tested (92%, 84%, and 80% of larvae inactivated for BSE at 5%, 2% and 1%, respectively). The LC-HRMS analysis of BSE showed highest contents of acetil-11-keto-β-boswellic acid (AKBA; 50.3 µg/mg) followed by 11-keto-boswellic acid (KBA 8.4 µg/mg), constituting the representative compounds of this extract. The analysis of phenolic compounds showed the presence of flavonoid compounds such as naringenin (81.52 µg/Kg) and myricetin (47.10 µg/Kg), and phenolic acids such as chlorogenic acid (66.38 µg/Kg). The *in vitro* results obtained for the *B. serrata* extract clearly revealed an anisakicidal potential of this compound when combined with spermidine and ferulic acid; however, this outcome is not sufficient to ensure complete safety for consumption. The LC-HRMS analyses revealed a high content of terpenes (AKBA and KBA) and phenolic compounds, including flavonoids. Flavonoids isolated from *Leucosceptrum canum* demonstrated significant nematicidal activity against the parasitic nematode *Meloidogyne incognita*. These flavonoids exhibited strong to moderate acetylcholinesterase (AChE) inhibitory activity, suggesting a neuromuscular mechanism of action (Karakoti et al. 2025). Moreover, polyphenols have been shown to interact with various enzymes, including those involved in neurodegenerative disorders, such as butyrylcholinesterase, in other contexts. Although this study was not conducted on nematodes, it supports the broader potential of polyphenols to inhibit neuromuscular enzymes. Even terpenes, highly represented in the *B. serrata* extract, have demonstrated nematicidal activity against different nematode species (Stavropoulou et al. 2021). These terpenes act by penetrating the nematode cuticle, causing cuticular lesions and altering locomotion mechanisms (André et al. 2018). Complementarily to the action of terpenes, ferulic acid and spermidine have shown strong anthelmintic activity against parasites *in vitro* (Mali et al. 2004; Saha et al. 2024). The irreversible damage to the tegument and the rapid onset of paralysis suggest that these substances could represent a promising natural alternative to current anthelmintic drugs. Its mechanism of action appears to involve the alteration of tegumental permeability, leading to nutrient loss and increased exposure of the parasite to the host's hostile environment. Despite the partial anisakicidal efficacy of this mixture of substances, the results are promising and warrant further studies aimed at optimising the concentrations of the compounds under investigation. However, in interpreting the anisakicidal findings for the possible application in marinated anchovies, the regulatory status of the candidate co-formulants diverges sharply. Ferulic acid (4-hydroxy-3-methoxycinnamic acid; FL-no 08.089) is a plant-derived phenolic that appears on the EU Union List of flavouring substances and, as such, may be used as a flavouring ingredient in foods under Regulation (EC) No 1334/2008 and good manufacturing practice (EFSA 2011; EU Lists of Flavourings).

By contrast, spermidine, as a standalone substance, is neither an authorised flavouring nor a food additive in the EU; the authorised route is the novel food "spermidine-rich wheat germ extract (*Triticum aestivum*)," whose use is restricted to food supplements for adults at an intake equivalent to a maximum of 6 mg/day spermidine, with a defined name for labelling conditions that do not encompass general foods such as marinades (EU Regulation 2017/2470).

Finally, extracts from *Boswellia serrata* are recognised to flavour food in the EU context (Burdock 2016; EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) et al. 2022), placing them within the framework for flavouring preparations under Regulation (EC) No 1334/2008; however, these extracts are intrinsically aromatic rather than organoleptically neutral, so measurable sensory impact at efficacious levels should be anticipated and managed in formulation.

Collectively, these considerations indicate that ferulic acid can be positioned as a compliant, plant-derived co-formulant in the marination process, whereas translating spermidine's anisakicidal activity into a consumer-ready marinade would require additional regulatory clearance.

In contrast to BSE, REO added to sunflower oil at 5% was able to inactivate of all the larvae within 15 h at +4 °C, and within 24 h at +20 °C. Even at 1% in oil, complete larval devitalisation was achieved by 24 h at +4 °C and by 48 h at +20 °C. Conversely, the marination in brine with the REO acted more slowly. The experiments with 5% REO in marination brine required 48 h at +4 °C to inactivate 100% of larvae, and 96 h at +20 °C to reach full devitalisation. At 1% in brine, REO was only partially effective within the 4-day test span: at +4 °C, showing 100% mortality in 96 h; however, at +20 °C, a 50% of larvae showed viability at 96 h. None of the larvae tested with 1% REO in brine at +20 °C showed motility cessation before the experiment ended (96 h). These results demonstrate the influence of the medium and the temperature on the Anisakicidal efficacy of REO. REO is considerably more effective in oil than in aqueous solution. Comparing 5% treatments, the time to inactivate 100% of the larvae at +20 °C was 24 h in oil vs 96 h in brine. Even at +4 °C, REO 1% in oil was able to inactivate all the larvae by 24 h whereas 1% in brine needed 96 h. The devitalisation was faster at +4 °C than at +20 °C with the same REO concentration, suggesting that the combination of cold stress and EO exposure accelerated larval death.

All the Anisakis larvae, during the control test remained active, with vigorous wriggling movement and no visible damage throughout the 96 h. Conversely, in REO-treated samples, larvae exhibited a progression: at 1–2 h their movement became sluggish compared to controls, then they started showing periods of immobility punctuated by occasional twitching, and finally they ceased all movement. In oil with REO, 45% of larvae coiled tightly or became curved and rigid prior to death, whereas in brine with REO, larvae often became immotile in an extended, relaxed state. Furthermore, the larvae subjected to the REO treatments (especially in oil) showed subtle structural changes when examined under magnification; these included a cloudy or opaque appearance (loss of translucency), suggesting internal tissue damage confirmed by a loss of integrity of the cuticle.

2.2. *Ex vivo* anisakicidal activity of REO

Considering that 100% Anisakicidal efficacy was observed only when REO was used, *ex vivo* efficacy trials were performed only using this compound. The addition of REO to the preserving oil at 5% resulted in a significant reduction in larval viability over time. At the first 24 h sampling, fillets in 5% REO-oil still showed 100% live larvae. At 72 h of storage in 5% REO-oil, 17% of larvae were found dead, in contrast to what was found in the control group. At 96 h, ~17% of larvae remained alive. A total devitalisation of *Anisakis* larvae in the 5% REO fillets was found at 168 h. The progressive lethal effect of the 5% REO treatment was reproducible across the three trial replicates. Regarding the anchovy fillets stored in oil with 1% REO, the Anisakicidal efficacy was slower and only partial. Up to 72 h, the product treated with 1% EO treatment showed 100% alive larvae. At 96 h of treatment, about 11% of larvae were inactivated, in contrast to 100% survival verified in the control groups. Finally, at 168 h the survival of *Anisakis* larvae stabilised around 41%, in contrast to what was found during the treatment with 5% REO, indicating that 1% REO was not sufficient to completely eliminate *Anisakis* within the timeframe of typical product storage and highlighting a dose-dependent efficacy.

Table S2 summarises the survival of *Anisakis* larvae at the determined time intervals during the *ex vivo* experiment. The statistical analysis at each time point confirmed that Anisakicidal efficacy of 5% REO was significantly higher than in 1% REO ($p < 0.01$), and both treatments were significantly different from control ($p < 0.001$). The control group showed 100% viability of *Anisakis* larvae throughout the 7 days of storage. Larvae extracted from control fillets at each time point (24 h, 72 h, … 168 h) were all motile, confirming that the process of marination (24 h in vinegar/salt at +4 °C) followed by storage in oil at room temperature did not permit the devitalisation of *Anisakis* larvae. The qualitative observation from the *ex vivo* study showed that the dead larvae recovered from REO-treated fillets exhibited physical lesions of the cuticle and a disruption or partial extrusion of the digestive tract. Figure S1 illustrates examples of larval damage in the 5% REO group, compared to an intact larva from a control fillet. These damages confirm that the essential oil's bioactive components permeated the larval tissues and caused structural disintegration. In contrast, larvae from the control fillets appeared transparent, smooth, and undamaged internally. The results on both the *in vitro* and *ex vivo* tests for REO suggest that the anisakicidal efficacy is influenced by the medium, temperature, and concentration. REO was significantly more effective when applied in oil compared to in aqueous vinegar solution, which is consistent with the hydrophobic nature of essential oil constituents. The oil matrix likely facilitates intimate contact of the lipophilic bioactive compounds with the parasite's cuticle, whereas in the water-based marinade the EO may not disperse as well and can form droplets or evaporate, reducing its bioavailability to the larvae. Indeed, at room temperature, some of the volatile components of rosemary EO might evaporate from the brine before they can act on the larvae, whereas in oil they can remain dissolved and in contact with the parasites. In contrast, we also observed that larvae devitalisation was faster at +4 C than at +20 °C, a counterintuitive result since chemical reaction rates generally slow with cooling. However, these findings can be explained by a combination of factors: (i) *Anisakis* larvae become less active and possibly more physiologically stressed at refrigeration temperatures, rendering them more susceptible to EO's toxic effects, and (ii) the volatility of the REO is reduced at +4 °C, which prolongs the persistence of active compounds in the medium (Tuan Anh et al. 2019). The relatively moderate speed of action for rosemary oil may be attributed to its chemical profile: rosemary EO is rich in monoterpenes such as 1,8-cineole, α-pinene, and camphor, which have known antimicrobial and insecticidal effects but are generally less immediately toxic to nematodes than phenolic compounds found in thyme or oregano oils.

This suggests that the full spectrum of constituents in the rosemary oil (which includes limonene among other terpenes) may have a synergistic effect resulting in a greater overall efficacy than the single compound alone. Future studies could explore whether intermediate concentrations (e.g. 2–3% EO) or combinations of essential oils might achieve complete kill in shorter times or at lower flavour impact. Larvae that died in the presence of REO and BSE often exhibited severe structural damage, including cuticle disruption and degeneration or extrusion of internal tissues. These observations are consistent with what was reported for other essential oils acting on *Anisakis* and related parasites (Nalbone et al. 2022). The hypothesis is that small lipophilic molecules (terpenes) from the EO diffuse through the parasite's cuticle and inflict damage to vital organs, or interfere with essential metabolic pathways.

2.3. Sensory evaluation of anchovy fillets

The sensory analysis showed no spoilage or unacceptable odour in any anchovy fillets treated with REO; all the samples treated, including controls, retained the typical appearance and smell of fresh marinated anchovies. This suggests that REO did not promote any spoilage reactions; rather, their known antimicrobial properties may have helped preserve the product quality (Tavassoli et al. 2011). The sensory panel evaluation provided systematic data on how the REO affected odour, flavour, and colour (Table S3 and Figure S2). Control fillets received median scores of 0 for all attributes. Fillets treated with 1% REO were also scored 0 on colour by all panellists, indicating no visible difference in appearance compared to control. For odour, the 1% REO samples had a median score of 1. In contrast, fillets with 5% REO showed a more pronounced sensory impact, chiefly in aroma. The intensity of rosemary odour determined a median odour score of 2. For flavour, panellists noted a mild rosemary taste in the 5% REO samples but it was generally subtle, with a score range 1-2 (median 1). Notably, colour was virtually unaffected in 5% REO fillets, observing that the fillets retained the typical greyish colour of marinated anchovy flesh. Statistical analysis of the sensory data confirmed that odour scores were significantly higher for 5% REO fillets compared to both 1% and control (Friedman test $p < 0.01$, with Wilcoxon pairwise $p < 0.01$ for 5% vs control, and $p < 0.05$ for 5% vs 1%). Flavour scores showed a trend of 5% REO > 1% REO > control, but the differences were not statistically significant ($p > 0.05$). All panellists stated they would find the rosemary-favoured anchovies acceptable to eat, though one noted that the herbal scent was "unusual for anchovies" since traditionally they are not spiced with herbs. No off-flavors (such as bitterness or soapiness) were attributed to the essential oil. This suggests that incorporating rosemary EO at the effective antiparasitic concentration (5%) is organoleptically feasible: it significantly alters the aroma profile, but in a way that is likely acceptable to many consumers (especially if marketed accordingly). Given the small trained panel size ($n = 6$), consumer acceptance should be confirmed in larger studies, especially at 5% REO where odour differences are evident.

2.4. Food safety and industry perspective

From a food safety and industry perspective, the present findings on REO highlight a potential natural alternative to freezing for anisakiasis risk mitigation. Currently, food regulations (EU Regulation 1276/2011 and 853/2004) mandate freezing of fish intended for raw or marinated consumption to ensure parasite devitalisation. Freezing is highly effective but can be costly and may affect product texture (Weiqing et al. 2021). Our data suggest that at 5% REO and +4 °C, the required storage time for 100% devitalisation might be on the order of only 2–3 days (given that even at +20 °C it was 7 days, and lethality is enhanced at +4 °C). This could be an acceptable timeframe for manufacturers, as marinated products are often kept for a few days before distribution. The sensory impact of 5% REO, as found in our study, is a moderate herbal note that could be marketed as a positive attribute. Nonetheless, further works are necessary to confirm these results on a larger scale and to ensure uniform distribution of the essential oil in an industrial setting. On this basis, this work could fill a gap in the research for "green" solutions to Anisakiasis prevention. While previous studies have investigated various essential oils, REO and BSE had not been fully evaluated *ex vivo* in a fish product matrix. Our findings place rosemary among the candidates for non-thermal parasite control in seafood. Furthermore, the use of REO could impart additional benefits, such as antioxidant properties that extend the shelf-life of the marinated fish (since rosemary extracts are known to slow lipid oxidation). This dual functionality, acting as both a preservative and a parasiticide, is highly attractive for clean-label food production.

3. Experimental

See Supplementary Material.

4. Conclusions

This study shows that rosemary essential oil (REO) inactivates Anisakis larvae both *in vitro* and in marinated anchovy fillets within production-compatible times. Efficacy increases in oil media, likely *via* improved solubility and contact, and decreases in acidic aqueous conditions. Sensory testing found a noticeable but acceptable herbal note without adverse effects on taste or appearance. The BSE–ferulic acid–spermidine mixture was not fully effective, warranting optimisation. Overall, REO is a plausible natural adjunct or alternative to freezing for parasite control in lightly processed fish, though commercial use requires further validation; more broadly, natural products offer a "green" route to safer marinated seafood.

Acknowledgments

The authors declare that there is no conflict of interest regarding the publication of this article. This work was financed by the Italian Ministry of Health (Progetto di Ricerca corrente RC IZS SI 02/22).

Author contributions

CRediT: **Gaetano Cammilleri**: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – original draft; **Luca Nalbone**: Data curation, Formal analysis, Resources;

Andrea Armani: Supervision, Validation, Visualization, Writing – review & editing; **Calogero Alfano**: Data curation, Formal analysis, Investigation, Methodology, Software; **Vincenzo Sala**: Data curation, Formal analysis, Resources, Software; **Francesco Giuseppe Galluzzo**: Investigation, Software, Visualization; **Elisa Maria Domenica Messina**: Investigation, Methodology; **Licia Pantano**: Formal analysis, Methodology, Validation; **Maria Drussilla Buscemi**: Data curation, Formal analysis, Investigation, Visualization; **Vittorio Calabrese**: Conceptualization, Investigation, Resources, Supervision, Validation, Writing – review & editing; **Vincenzo Ferrantelli**: Funding acquisition, Project administration, Resources, Supervision, Validation; **Filippo Giarratana**: Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was financed by the Italian Ministry of Health (Progetto di Ricerca corrente RC IZS SI 02/22).

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