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ORIGINAL PAPER

Green drugs in the fight against *Anisakis simplex*—larvicidal activity and acetylcholinesterase inhibition of *Origanum compactum* essential oil

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Abstract

Anisakiasis is a fish-borne parasitic disease caused by the consumption of raw or undercooked fish, as well as cephalopods, contaminated by third instar larvae (L3) of species belonging to the genus *Anisakis* (Anisakidae). *Origanum compactum* is a small herbaceous aromatic plant endemic to Spain and Morocco. In Morocco, the plant is used under infusion to treat heart diseases and intestinal pains or as preservative for foodstuffs. This is the first time that the *O. compactum* essential oil is tested against the parasitic nematode *Anisakis simplex*. The phytochemical analysis by GC-MS revealed carvacrol (50.3%) and thymol (14.8%) as the major oil constituents. The essential oil and its major constituents carvacrol and thymol were tested against *A. simplex* L3 larvae isolated from blue whiting fish (*Micromesistius poutassou*). *A. simplex* mortality (%) after 24 and 48 h of treatment at 1 μl/ml was 100%, with a low LD_{50} compared with other essential oils and extracts, and the penetration in the agar assay was also reduced, if compared with control wells. The oil, as well as its major constituents, demonstrated a dose-dependent larvicidal activity. Inhibition of the enzyme acetylcholinesterase through a colorimetric assay in 96-well plates was used to elucidate the pharmacological mechanism as this enzyme plays a key role in nematodes neuromuscular function. Interestingly, *O. compactum* essential oil, carvacrol and thymol inhibited the enzyme, confirming that this could be one of the mechanisms involved in the anthelmintic activity. To the best of our knowledge, this is the first time that *O. compactum* essential oil is reported as a larvicidal agent against *A. simplex* L3 larvae.

Keywords Carvacrol · Thymol · Anisakis · Anthelmintic · Nematode · Waterborne parasitology

Introduction

Anisakiasis is a fish-borne parasitic disease; it is caused by the consumption of raw or undercooked fish, as well as cephalopods, contaminated by third instar larvae of species belonging to the genus *Anisakis* (Anisakidae), with special reference to *Anisakis simplex*, *Anisakis pegreffii* and *Pseudoterranova decipiens* (Grabda 1976; Mattiucci and Nascetti 2006; Audicana and Kennedy 2008; Pravettoni et al. 2012). After ingestion, the larvae penetrate the gastric and intestinal mucosa, causing the symptoms of anisakiasis (Broglia and Kapel 2011). Recently, it has been estimated that about 20,000 cases of anisakiasis are reported per year worldwide. The large majority of the cases is from Japan (> 90%), due to the traditional use of *sashimi* as food. Other countries characterized by a rather large number of anisakiasis cases are Spain, the Netherlands, Germany and Scandinavia (Bao et al. 2017).

Live larvae of *A. simplex* can cause a parasitic infection of the digestive tract. In addition, they can also affect other organs causing erosive and/or haemorrhagic lesions, ascites and perforations until granulomas and masses, if the larvae are not promptly removed. Furthermore, the presence of larvae of

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A. simplex can lead to allergic reactions, as anaphylaxis, acute/chronic urticaria and angioedema (Asturias et al. 2000; Nieuwenhuizen et al. 2003; Berger and Marr 2006; Choi et al. 2009; Pravettoni et al. 2012). Notably, the larvae of *A. simplex* can induce an immune adaptive response characterized by T-lymphocyte proliferation with polyclonal and monoclonal (which are responsible for allergic symptoms), IgE production, eosinophilia and mastocytosis (Park et al. 2009; Pravettoni et al. 2012).

Nowadays, the endoscopic removal of live larvae still represents the main effective treatment against anisakiasis (Sugita et al. 2008; Pravettoni et al. 2012). Furthermore, protection against anisakiasis can be enhanced educating consumers about the risks linked with eating raw or undercooked seafood. From a pharmacological point of view, antibiotics, anticholinergics and/or corticosteroids have been employed for the treatment of anisakiasis, showing patchy and limited efficacy (Matsui et al. 1985). However, a relevant exception is represented by albendazole (Dziekońska-Rynko et al. 2002; Romero et al. 2014), since it has been recently showed that this compound dose-dependently reduced (500 μg/ml led to 100% 48 h post-treatment) the survival of *A. simplex* third instar larvae (Arias-Diaz et al. 2006). However, it has been noted that acidic medium pH reduced its efficacy (Arias-Diaz et al. 2006). In this scenario, there is an urgent need to develop novel and effective tools and drugs for the treatment of *A. simplex* parasitic infections (Molina-Garcia and Sanz 2002; Brutti et al. 2010), including the use of natural products (Hierro et al. 2004, 2006; Lin et al. 2010; Gómez-Rincón et al. 2014; Valero et al. 2015).

Essential oils, which are complex mixtures of volatile components such as monoterpenoids and sesquiterpenoids (Pavela and Benelli 2016), can represent a new strategy to combat several parasites including L3 larvae of *A. simplex* (Giarratana et al. 2014; Hierro et al. 2004; Romero et al. 2012). In this regard, *Origanum* essential oils have been shown to possess in vivo efficacy against larvae of *Anisakis* (Pérez et al. 2016; Abattouy et al. 2010). On this basis, we focused our attention on the essential oil obtained from *Origanum compactum* Benth., belonging to the Lamiaceae family.

O. compactum is a small herbaceous aromatic plant endemic to Spain and Morocco (Emberger and Maire 1941) where it is particularly appreciated in cuisine to enhance the flavour of foods and as natural food preservative (Ghanmi et al. 2015; Sbayou et al. 2014). *O. compactum* enjoys a long-standing use in the traditional medicine as well as a good reputation and economic importance in the trade of medicinal and aromatic plants (Briguiche and Zidane 2016; Bouiamrine et al. 2017). In Morocco, the plant, locally known as *Zaetar*, is used under infusion to treat heart and intestinal pains or as preservative for foodstuffs (El-Hilaly et al. 2003). It is also a famous remedy for the treatment of wounds, diabetes, hypertension and cardiac diseases, digestive and respiratory problems and gastrointestinal and gingival cancers (Ziyyat et al. 1997; Jouad et al. 2001; Kabbaj et al. 2012; Eddouks et al. 2002; Bouhdid et al. 2009; Jamila and Mostafa 2014). *O. compactum* has also been used as vermifuge, aphrodisiac, antispasmodic, antiulcer, laxative and antidiarrheal agent (Hmamouchi et al. 2000; Jamila and Mostafa 2014).

In the search of scalable products to use as treatments of anisakiasis, we here evaluated the activity of *O. compactum* essential oil as well as its major constituents carvacrol and thymol on *A. simplex* L3 larvae. The pharmacological mechanism was studied through the inhibitory activity of the acetylcholinesterase enzyme, which plays a crucial role in the nematode neuromuscular function.

Materials and methods

Origanum compactum essential oil

The essential oil of *O. compactum* (obtained from flowering aerial parts) was kindly supplied by Pranarôm International (http://www.pranarom.com). Its analysis was achieved on an Agilent 6890 N gas chromatograph equipped with a 5973-N mass spectrometer (MS). The settings for the MS were as follows: EI mode, 70 eV, and mass to charge ratio (*m/z*) scan between 35 and 400. A HP-5 MS capillary column (30 m × ID 0.25 mm × 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA) using helium gas flow (1.0 ml/min) was used for separation. The GC temperature program was as follows: initial 50 °C for 5 min, then increasing with 20 °C/min to 300 °C. The injector temperature was 150 °C. Quali-quantitative analysis of the essential oil was performed according to the work of Benelli et al. (2017). Thymol and carvacrol were purchased from Sigma-Aldrich (Madrid, Spain).

Anisakis simplex from blue whiting

Anisakis simplex L3 larvae were isolated from the intermediary host *Micromesistius poutassou* (Risso) (blue whiting) purchased from the fishmonger in Villanueva de Gállego (Zaragoza, Spain). The worms were washed several times on sterile solution of 0.9% NaCl and identified under light microscope according to morphological features. Only larvae with length > 2.0 cm were used for the larvicidal assays.

Anthelmintic activity against *Anisakis simplex* L3 larvae

This assay was performed in 6 well plates. Ten larvae were introduced in each well of polystyrene plates with a final volume of 2 ml sterile saline solution containing different concentrations of the test solution (Gómez-Rincón et al. 2014). *O. compactum* essential oil was tested against *Anisakis* in the range of 0–1 µl/ml. The major constituents carvacrol and thymol were tested in the same range of concentrations. Appropriate control wells without treatments were also carried out in each experiment. The parasites were incubated at 37 °C for 24 and 48 h. Levamisole was used as the reference antiparasitic drug. Larvae were examined at 24 and 48 h under microscope, and immobile L3 was considered dead.

Inhibition of acetylcholinesterase

The inhibition of acetylcholinesterase (AChE) was determined in 96 microplates using the method by Ellman et al. (1961) with some modifications. Each well contained 25 µl of 15 mM ATCI in Millipore water, 125 µl of 3 mM DTNB in buffer C (50 mM Tris–HCl, pH 8, 0.1 M NaCl, 0.02 M MgCl2 6 H2O), 50 µl buffer B (50 mM Tris–HCl, pH 8, 0.1% bovine serum), and 25 µl of test compound or extract. Every concentration was tested in triplicates. Then, 25 µl and 0.22 U/ml AChE were added and the absorbance was measured eight times every 13 s at 405 nm. Galantamine was used as reference.

Penetration assays

Agar block plates were prepared in 12-well plates to study the capacity of the living larvae to penetrate. The agar solution was made with the following reagents: 0.75% agar in RPMI 1640 medium solution (pH 4, Sigma, USA) with 20% FBS (Lonza, USA). One millilitre of the solution was poured into each well. Then, 100 µl of supernatant, RPMI-1640 (RPMI-1640, 20% FBS, 1% commercial pepsin, pH 4.0), was placed into each well. *A. simplex* L3 larvae were incubated with a sub-lethal concentration (0.125 mg/ml) of *O. compactum* essential oil for 24 h. Five larvae were placed on each control or simple well. The plates were incubated for 24 h, and the number of L3 larvae that penetrated the solid agar block was counted after that period.

Statistical analysis

All experiments were performed in triplicates in different weeks using new *A. simplex* larvae. LD_{50} (median lethal dose) and IC_{50} (half maximal inhibitory concentration) values for larvicidal and mechanistic assays were calculated using non-linear regression (GraphPad Prism 5).

Results

Essential oil composition

O. compactum essential oil was characterized by eight chemical compounds accounting for 93.6% of the total composition. The major component was the monoterpene phenol carvacrol accounting for half essential oil composition (50.32%), followed by its isomer thymol (14.8%) and by γ-terpinene (13.6%) and *p*-cymene (8.40%). Minor components were (*E*)-caryophyllene (2.1%), α-terpinene (1.6%), myrcene (1.5%) and linalool (1.3%).

Anthelmintic activity

O. compactum essential oil showed a dose dependent larvicidal activity at 24 and 48 h of treatments. All larvae were killed at doses of 1 µl/ml after 24 h showing a paralysis that indicates mortality. Although the efficacy of the treatment can be considered similar at 24 and 48 h because the mortality was 100% (Fig. 1), the LD_{50} of essential oil was lower after 48 h.

The effects of carvacrol and thymol on the larvae were similar to those of the oil (Fig. 2); however, carvacrol exhibited a stronger activity than *O. compactum* and thymol, indicating that this compound might be responsible for the larvicidal effects. Levamisole induced 100% mortality at 0.1 mg/ml.

Inhibition of acetylcholinesterase

O. compactum essential oil, carvacrol and thymol acted as AChE inhibitors, indicating that this could be one of the mechanisms involved in the larvicidal activity (Fig. 3). The activity profile of carvacrol on the enzyme was very similar to that of the essential oil. However, higher doses of thymol were needed to inhibit the AChE enzyme at the same level. The AChE inhibitory potential decreased in the following order: carvacrol > *O. compactum* > thymol. Galantamine at 0.1 mg/ml induced 100% of AChE inhibition.

Penetration assays

The penetration ability of *A. simplex* L3 larvae was evaluated after exposure to *O. compactum* essential oil and compared with untreated larvae. The penetration rate of control wells was constant at 100% while it decreased 25% for treated larvae with sub-lethal doses of the oil (the agar was penetrated by 75% of the larvae).

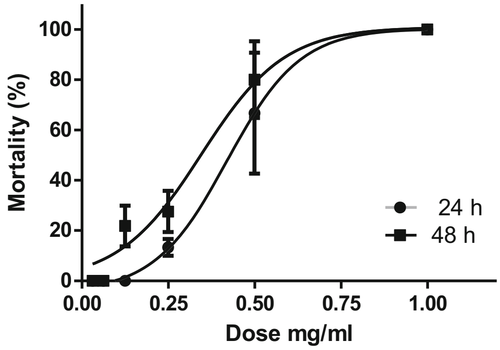

Fig. 1 Larvicidal activity of *Origanum compactum* essential oil against *Anisakis simplex* L3 larvae after 24 and 48 h (LD_{50} 0.429 mg/ml at 24 h and 0.344 mg/ml at 48 h)

Discussion

In the present work, the essential oil from *O. compactum* was assayed for the first time against L3 larvae of *A. simplex*. For the purpose, a commercial oil was used and analysed showing a chemical composition fully consistent with those reported in literature concerning Moroccan oregano accessions, with phenolic compounds such as carvacrol and thymol as the most abundant components, followed by their biogenetic precursors γ-terpinene and *p*-cymene (Bakhy et al. 2014)

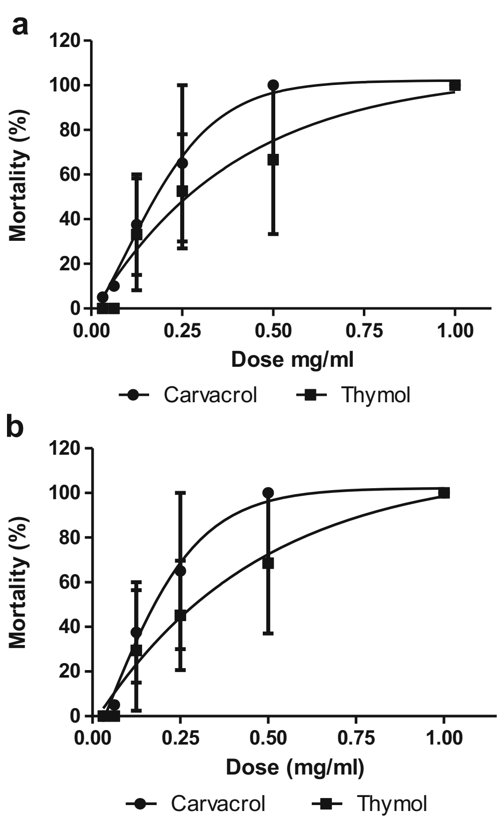

Fig. 2 Larvicidal activity of carvacrol and thymol against *Anisakis simplex* L3 larvae at 24 (**a**) and 48 h (**b**). LD_{50} values for carvacrol were 0.176 mg/ml at 24 h and 0.178 mg/ml at 48 h. LD_{50} values for thymol were 0.291 mg/ml at 24 h and 0.214 mg/ml at 48 h

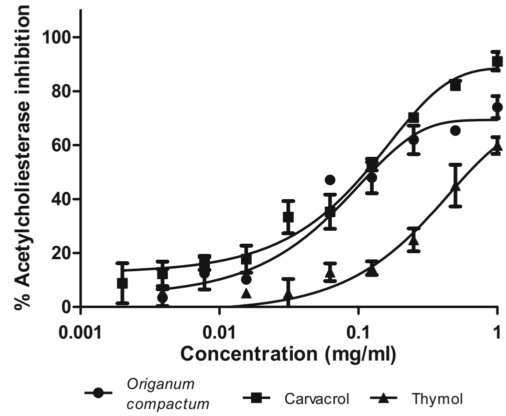

Fig. 3 Acetylcholinesterase inhibition induced by *Origanum compactum* essential oil (IC_{50} 0.124 mg/ml), carvacrol (IC_{50} 0.113 mg/ml) and thymol (IC_{50} 0.625 mg/ml)

(Table 1). Only quantitative differences have hitherto noticed among the main essential oil constituents. Notably, the monoterpene phenols thymol and carvacrol may be detected in comparable amounts (Ghanmi et al. 2015; Kloucek et al. 2012; Sbayou et al. 2014; Bouhdid et al. 2008; Mezzoug et al. 2007) or with the latter more abundant than the former (Bouchra et al. 2003; Bakhy et al. 2014; Pavela 2008; Lamiri et al. 2001) as in our case. Thus, our sample of *O. compactum* can be defined as a carvacrol-rich chemotype (Bakhy et al. 2014).

In our experiments, *O. compactum* essential oil showed anthelmintic effects against *A. simplex* L3 larvae as well as inhibitory activity of the acetylcholinesterase enzyme. Levamisole is used as an antiparasitic agent in veterinary, so we tried the concentration of 0.1 mg/ml as a control substance. One microlitre per millilitre of *O. compactum* essential oil is equivalent to 1 mg/ml approximately, so levamisole was more potent. However, this treatment is not available for humans and is used only in veterinary.

To the best of our knowledge, these results are new and here published for the first time. On the other hand, the antimicrobial activities of this oil were already known and previously reported. *O. compactum* essential oil is considered as a strong bactericidal and fungicidal, being capable to inhibit the growth of several pathogenic strains such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Listeria* spp., *Alternaria alternata* and *Aspergillus niger* (Bouhdid et al. 2009; Kloucek et al. 2012). The antimicrobial effects are related to the presence of monoterpene phenols thymol and carvacrol which can alter permeability of cell membrane and destabilize respiratory and enzymatic activities (Bouyahya et al. 2017; Bakkali et al. 2008). *O. compactum* essential oil has also shown toxic effects on *Botrytis cinerea* (Bouchra et al. 2003) as well as on wood decay fungi (Ghanmi et al. 2015), nematicidal activity against *Ditylenchus dipsaci* (Zouhar et al. 2009) and insecticidal effects against larvae of *Spodoptera littoralis* (Pavela 2005) and adults of *Musca domestica* and

Table 1 Chemical composition of the *Origanum compactum* essential oil tested against *Anisakis simplex* L3 larvae

Number	Component[a]	RI Exp.[b]	RI ADAMS[c]	Percent[d]	ID[e]
1	Myrcene	983	988	1.5 ± 0.2%	RI, MS
2	α-Terpinene	1010	1014	1.6 ± 0.2%	RI, MS
3	*p*-Cymene	1017	1020	8.4 ± 1.1%	RI, MS
4	γ-Terpinene	1051	1054	13.6 ± 2.1%	RI, MS
5	Linalool	1099	1095	1.3 ± 0.2%	RI, MS
6	Thymol	1294	1289	14.8 ± 2.8%	Std, RI, MS
7	Carvacrol	1299	1298	50.3 ± 3.9%	Std, RI, MS
8	(*E*)-caryophyllene	1410	1417	2.1 ± 0.4%	
	Total identified (%)			93.6%	
	Grouped components (%)				
	Monoterpene hydrocarbons			25.1	
	Oxygenated monoterpenes			66.4	
	Sesquiterpene hydrocarbons			2.1	

[a] Compounds are listed in order of their elution from a HP-5MS column

[b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{30} alkanes

[c] Linear retention index taken from Adams (2007)

[d] Percentage values are means of three independent analyses ± SD

[e] Identification methods: Std., based on comparison with authentic compounds; MS, based on comparison with ADAMS, FFNSC 2 (2012) and NIST 08 (2008) MS databases; RI, based on comparison of RI with those reported in ADAMS, FFNSC 2 (2012) and NIST 08 (2008)

Mayetiola destructor (Pavela 2008; Lamiri et al. 2001). The ethyl acetate extract was highly active against the schistosomiasis-transmitting snail *Bulinus truncatus* (Hmamouchi et al. 2000).

With the aim of elucidating which compounds of *O. compactum* essential oil were responsible for the anti-*Anisakis* activities, the two main monoterpenes, carvacrol and thymol, were also assayed. According to our data, carvacrol might be one of the active principles as it exhibited a higher larvicidal activity than thymol and the oil, revealing its potential as an anthelmintic drug. This is not the first time that carvacrol has been evaluated as a nematicidal compound (Trailović et al. 2015; Andre et al. 2016); in fact, the larvicidal activity of this monoterpene has also been evaluated on *A. simplex* (Hierro et al. 2004). The inhibitory activity of carvacrol and thymol on the acetylcholinesterase enzyme has also been tested (Jukic et al. 2007). Carvacrol and thymol are isomeric phenolic monoterpenes present in several aromatic medicinal and culinary plants. The different position of the hydroxyl group in the phenyl ring provides better anthelmintic and anti-cholinesterase activities for carvacrol in our case, which agrees with results of other authors (Jukic et al. 2007; Aazza et al. 2011; Seo et al. 2015).

Overall, the results of the present study pointed out the importance of the traditional use of *O. compactum* as a food preservative in Morocco and Spain. Its demonstrated anthelmintic properties, together with its well-known antibacterial and fungicidal effects, make it an ideal candidate as preservative agent for the prevention and treatment of food-borne diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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