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A look into *Calliphora vomitoria* after topical treatment with *Origanum vulgare* essential oil

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ABSTRACT

Blowflies like *Calliphora vomitoria* (Diptera: Calliphoridae) are a notorious nuisance and dangerous mechanical vectors of numerous foodborne pathogens. For these reasons, humans have the necessity to manage them in homes and places visited by people and animals, mainly with indoor and outdoor sprays acting by contact. To reduce the side-effects associated with the overuse of such formulations of synthetic origin, essential oils (EOs) are now proposed as safer and greener botanical alternatives. However, the exact mode(s) of penetration and action of an EO in an insect pest have not yet been exhaustively investigated. In the present work, we first ascertained the toxicity of an *Origanum vulgare* (Lamiaceae) carvacrol chemotype EO on *C. vomitoria* adults when applied topically to the thorax. Based on the median lethal dose by direct contact after 24 hours ($LD_{50} = 0.067 \mu\text{L EO blowfly}^{-1}$), we then decided to treat other specimens with $0.2 \mu\text{L EO blowfly}^{-1}$ to potentially observe thoracic structural and ultrastructural abnormalities through light and transmission electron microscopy (TEM) and possible acetylcholinesterase (AChE) inhibition by Ellman's colorimetric method performed *in vivo*. We verified that the EO, showing lipophilic properties, can penetrate the integument, thus causing morphological alterations at the level of the cuticle, epidermis and the underlying muscular and tracheal system, with vacuolization, disorganization and degeneration of the tissues compared to the untreated control. Instead, the AChE activity was not affected by our *O. vulgare* EO treatment, indicating that this enzyme is not involved in its insecticidal activity.

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1. Introduction

The blue bottle fly, *Calliphora vomitoria* (L.) (Diptera: Calliphoridae), is widespread in Europe and is present in some North American, African and Asian countries. Like other flies, it is connoted both as a beneficial and pest insect. Adult blowflies are considered good pollinators of fruit trees (Saeed et al. 2016), horticultural crops (Cook et al. 2020) and wild medicinal plants (Rashid et al. 2023). Furthermore, the presence of calliphorid larvae and their succession on corpses are keys to estimating the minimum post-mortem interval in forensic investigations (Benkenana et al. 2024). Despite their fundamental ecological role, maggots are myiasis-inducing in humans (Bugelli et al. 2023) and animals (Stevens 2003) and can likewise develop in raw and processed meat and fish (Aak et al. 2010; Niederegger et al. 2013), implicating their spoilage. Furthermore, adults are a notorious nuisance and dangerous mechanical vectors of numerous potentially pathogenic microorganisms (Fischer et al. 2004; Pava-Ripoll et al. 2012) encountered in the decomposing organic matter they frequent for nourishment and reproduction.

Especially in homes and places visited by people and animals, adult flies are managed with indoor and outdoor sprays (Zhu et al. 2016) that act by direct and indirect contact on the pests (Ruberti 2024). The main insecticide class to which such formulations rely on is pyrethroids that, despite the acclaimed affordability and vertebrate safety, are harmful to non-target aquatic (Antwi & Reddy 2015) and terrestrial organisms (He et al. 2023) and can lead to resistance when repeatedly used (Khan et al. 2017).

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While looking for safer and greener alternatives, the applicability of essential oils (EOs) and other active ingredients of botanical origin has been assessed under laboratory (Bedini et al. 2017, 2021; Farina et al. 2021, 2022) and operative conditions (Bedini et al. 2020). For instance, due to their lipophilic nature, EOs and their constituents interfere with the basic metabolic, biochemical, physiological and behavioural functions of arthropods, acting as neurotoxins, enzyme inhibitors, protein denaturants and growth regulators (Cossolin et al. 2019; Shahriari et al. 2019; Brügger et al. 2021; Chen et al. 2021; Serdeiro et al. 2023). Based on the administration modality, botanicals can penetrate the pest body through the integument, gustatory and olfactory sensilla, mouth, respiratory system or other openings and have access to the underlying tissues. The sites directly or indirectly affected can be evidenced through transmission electron microscopy (TEM) observations, which can highlight internal and external ultrastructural abnormalities in cells, tissues and organs (Massoud et al. 2005; Sharma et al. 2008; Sirisuda et al. 2008; Spochacz et al. 2021; Shah et al. 2023).

The exact mode of action of all the botanical active ingredients is currently unknown, but the research is progressing in this direction. For instance, it has been observed that azadirachtin from neem oil can reduce the ecdysteroids level (Andrade Coelho et al. 2006) and that thymol can bind to specific transmembrane receptors of the inhibitory neurotransmitter γ -aminobutyric acid (Waliwitiya et al. 2010). Mainly, the inhibitory effect of EOs, plant extracts and singular compounds has been verified on the enzyme acetylcholinesterase (AChE) through Ellman's (Ellman et al. 1961) colorimetric method both *in vitro* (Seo et al. 2015; Park et al. 2016; Bedini et al. 2017) and *in vivo* (Rizvi et al. 2018; Shah et al. 2023; Chaubey 2024) or with assay kits (Chen et al. 2021). The primary role of AChE is to catalyse the hydrolysis of acetylcholine to choline and acetate in the space between synapses so that the nerve signal can be correctly stopped after the transmission (Rajashekar et al. 2014). Exactly in *C. vomitoria*, an *in vitro* assay ascertained a concentration-dependent AChE enzyme reduction given by two *Artemisia* spp. (Asteraceae) EOs (Bedini et al. 2017).

In the present work, we decided to work with an *Origanum vulgare* L. (Lamiaceae) EO as having already proven insecticidal activity (Plata-Rueda et al. 2021, 2022; Jbilou et al. 2024; Mrabet et al. 2025) also on *C. vomitoria* (Bedini et al. 2021). We first ascertained the toxicity of this EO on adult *C. vomitoria* blowflies when applied topically to the thorax, thus imitating the action of a spray insecticide but having the certainty of the dose applied on each specimen. Based on the results, we then aimed to potentially observe, for the first time, (i) thoracic structural and ultrastructural abnormalities at the level of the integument, epidermis, muscles and tracheal system using light and transmission electron microscopy (TEM) and (ii) AChE inhibition by Ellman's colorimetric method performed *in vivo* in *C. vomitoria* adults treated by contact.

2. Materials and methods

2.1. *Origanum vulgare* essential oil purchase and chemical characterization

Origanum vulgare EO obtained through steam distillation of the aerial parts was purchased from Herbal Products Italia S.r.l. (Sant'Elena, Italy).

It was chemically characterized in triplicate ($n = 3$) at the Department of Pharmacy of the University of Pisa. The EO was diluted to 5% in HPLC-grade *n*-hexane and then injected into a GC–MS apparatus. Gas chromatography–electron impact mass spectrometry (GC–EIMS) analyses were performed with an Agilent 7890B gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an Agilent HP-5 MS (Agilent Technologies Inc.) capillary column (30 m \times 0.25 mm; coating thickness 0.25 μ m) and an Agilent 5977B single quadrupole mass detector (Agilent Technologies Inc.). Analytical conditions were as follows: injector and transfer line temperatures 220 and 240°C, respectively; oven temperature programmed from 60 to 240°C at 3°C min⁻¹; carrier gas helium at 1 mL min⁻¹; injection of 1 μ L (5% HPLC grade *n*-hexane solution); split ratio 1:25. Acquisition parameters were as follows: full scan; scan range: 30–300 *m/z*; scan time: 1.0 sec.

The identification of the constituents was based on the comparison of their retention times with those of the authentic samples (when available), comparing their linear retention indices relative to the series of *n*-hydrocarbons (C6–C25). Computer matching was also used against a commercial (National Institute of Standards and Technology 2014) and a laboratory-developed mass spectra library built up from pure substances and components of commercial EOs of known composition and MS literature data (Adams 2007).

2.2. *Calliphora vomitoria* rearing and treatment by direct contact with the *Origanum vulgare* essential oil

Calliphora vomitoria larvae were purchased from the live fishing baits retailer Nonno Ippai (Vittoria Apuana, Italy) and reared until the adult emergence, as seen in Farina et al. (2021). Larvae were housed in a polypropylene box (27 × 21 × 12 cm) with a netted closure for ventilation and kept under laboratory conditions (temperature 23°C, RH 60–70%, natural photoperiod). They were fed raw beef mince *ad libitum*, until they voluntarily left the substrate to pupate. Pupae were moved into a 75.0 × 75.0 × 115.0 cm polyester and knitted mesh rearing tent (BugDorm-2400, MegaView Science Co., Ltd., Taichung, Taiwan). The then emerged adults were provided with a solid diet made of sucrose:yeast extract (4:1 *w/w*) and water *ad libitum*. Species identification was performed on 20 randomly picked adults by using the specific keys (Szpila 2012).

The toxicity exerted by the purchased *O. vulgare* EO on *C. vomitoria* adults was ascertained by opting for a direct contact assay to imitate the action of a spray insecticide. In detail, on the thorax of 10–20-day-old blowflies of undetermined sex, we applied a 2.0 µL drop of seven different ethanol (EtOH) solutions of the EO (from 0.5 to 15%), corresponding to doses from 0.01 to 0.3 µL EO blowfly⁻¹. For the control samples, adults were topically treated with a 2.0 µL drop of just EtOH on the thorax. The drop application was performed on previously anaesthetized flies (by cold treatment at –18°C for 3 min maximum) through a hand micro-applicator (Burkard Scientific Ltd., Uxbridge, UK), as seen in Farina et al. (2021). Blowflies, divided according to treatment, were then housed in polypropylene cages with a netted closure and provided with a sucrose: water solution (1:4 *w/v*) as *ad libitum* nourishment. Mortality was checked 24 hours after the drop application. For each EO dose and control, we tested ten blowflies at a time and replicated the procedure four times (*n* = 4).

Based on the results obtained from the toxicity trial, in order to successively characterize the mode of penetration of the EO and ascertain its potential effect on AChE, we treated the *C. vomitoria* blowflies with a 2.0 µL drop of a 10% EtOH solution of the *O. vulgare* EO, corresponding to the dose of 0.2 µL EO blowfly⁻¹. The dose chosen is specifically higher than the lethal dose calculated for 95% of the tested blowflies (see paragraph 3.2), in order to result toxic to all the specimens subjected to it. The control samples were, as seen before, topically treated with a 2.0 µL drop of just EtOH.

2.3. *Calliphora vomitoria* sample preparation for light and transmission electron microscopy (TEM)

After the application of the 2.0 µL drop of the 10% *O. vulgare* EO solution in EtOH, the EO-treated specimens that started manifesting signs of intoxication but were still moving (i.e. after approximately 30 min) were anesthetized at –18°C and dissected. The control specimens, who received just EtOH, were processed analogously (but they never showed signs of distress). The treated upper part of the thorax was placed in a fixative buffer (2% glutaraldehyde in a 0.1 M sodium phosphate buffer, pH 7.4) for 24 hours and then stored in a 0.1 M sodium phosphate buffer until further processing. Before microscopy observations, the thorax pieces were fixed overnight (24 hours) in 2% glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.4). After washing in 0.1 M sodium phosphate buffer, the specimens were post-fixed in a 2% osmium tetroxide solution. Then, they were rinsed in the same buffer, dehydrated in a graded acetone series and embedded in Araldite. Semi-thin (1 µm thick) and ultrathin sections (70–80 nm thick) were cut with an LKB Ultratome 2088 V. Semi-thin sections were stained with 1% Toluidine blue in distilled water and observed under a light microscope (Nikon Eclipse Ti2-U inverted research microscope). Ultrathin sections were contrasted with a solution of UranylLess (Electron Microscopy Sciences, Hatfield, PA, USA), followed by lead citrate solution (Electron Microscopy Sciences), and observed under a Philips CM10 transmission electron microscope (TEM).

2.4. *Calliphora vomitoria* sample preparation for acetylcholinesterase (AChE) and protein assay

After the application of the 2.0 µL drop of the 10% *O. vulgare* EO solution in EtOH, the EO-treated specimens that started manifesting signs of intoxication but were still moving (i.e. after approximately 30 min) were deprived of legs and antennae and homogenized in 1 mL of ice-cold 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5% TritonX-100. The samples were then filtered and centrifuged at 10,000 rpm for 5 min at

0°C. The supernatants were used as an enzyme source. The specimens that received just EtOH (named Ethanol) were processed analogously (but they never showed signs of distress). Furthermore, blowflies without any application (untreated) were processed in the same way and regarded as Controls.

The activity of AChE was determined according to the method described by Ellman et al. (1961) using acetylthiocholine iodide (ATChI) as a substrate. The reaction mixture contained 2.8 mL of 0.1 M potassium phosphate buffer (pH 7.5), 0.2 mL of homogenate, 0.1 mL of 0.01 M 5,5'-dithiobis (2-nitrobenzoic acid (DTNB) prepared in 0.1 M potassium phosphate buffer (pH 7.5) and 20 mL of 0.075 M ATChI. The change in absorbance was monitored at 412 nm for 7 min in a UV-visible double-beam spectrometer (model UV-2600i, Shimadzu, Kyoto, Japan). All readings were corrected from the blank reaction containing phosphate buffer, DTNB and ATChI but not the enzyme protein. Specific activity was calculated using $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ as the extinction coefficient. One unit of AChE activity was expressed as nanomoles of ATChI hydrolysed $\text{min}^{-1} \text{ mg}^{-1}$ protein under experimental conditions.

The total protein contents of enzyme preparations were determined according to the Bradford (1976) protein assay by using bovine serum albumin as the quantitative standard. Absorbance data were monitored at 595 nm.

For EO-treated, Ethanol and Control samples, we tested six blowflies at a time and replicated the procedure three times ($n = 3$).

2.5. Statistical analysis

The median lethal dose (LD_{50}) and the lethal dose for 95% of the blowflies (LD_{95}) treated by direct contact with the *O. vulgare* EO were calculated by the probit model regression (Finney 1971).

The AChE enzymatic activity values of the EO-treated, Ethanol and Control blowflies were elaborated through one-way ANOVA after checking for normality (Shapiro-Wilk test, $p = 0.103$) and homogeneity (Levene's test, $p = 0.222$) of data distribution.

All the analyses were run using the JMP Pro Software v 17.2 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Chemical composition of the *Origanum vulgare* essential oil

The chemical analysis by GC–EIMS of the *O. vulgare* EO employed in the trials is given in Table 1. A total of 24 compounds were identified, covering 100% of the composition. Nine of the constituents belong to the chemical class of oxygenated monoterpenes (OMs – total $82.2 \pm 1.31\%$), with the predominance of carvacrol ($70.2 \pm 1.6\%$). The second most abundant compound detected, *p*-cymene ($6.2 \pm 0.57\%$), is a monoterpene hydrocarbon (MH – total $12.5 \pm 1.36\%$).

3.2. Toxicity by direct contact of the *Origanum vulgare* essential oil on *Calliphora vomitoria*

The toxicity exerted by the *O. vulgare* EO on *C. vomitoria* adults when administered by contact on the thorax showed a dose-dependent trend, as represented in Figure 1. Mortality grew from $12.5 \pm 5.0\%$ (mean value \pm SD with $n = 4$) at the dose of $0.01 \mu\text{L EO blowfly}^{-1}$ to $92.5 \pm 5.0\%$ at $0.2 \mu\text{L EO blowfly}^{-1}$ and 100% at $0.3 \mu\text{L EO blowfly}^{-1}$. Conversely, the application of EtOH as a control caused no mortality (0%) in any of the four replicates performed.

After 24 hours from the EO-containing drop application, the LD_{50} was calculated by Probit regression as $0.067 \mu\text{L EO blowfly}^{-1}$ (confidence intervals = $0.0552\text{--}0.0811 \mu\text{L EO blowfly}^{-1}$) and the LD_{95} as $0.180 \mu\text{L EO blowfly}^{-1}$ (confidence intervals = $0.152\text{--}0.224 \mu\text{L EO blowfly}^{-1}$) [intercept = -0.99 ± 0.142 ; model slope = 14.65 ± 1.90 ; Pearson goodness-of-fit test, $\chi^2 = 23.083$, degrees of freedom = 26, $p = 0.628$].

3.3. Morphological observation of control and essential oil-treated samples

The control (with just EtOH) and EO-treated samples highlighted morphological differences, both at the level of the integumentary system, exocuticle, endocuticle and epidermis and at the level of the underlying area, in which it was possible to observe the muscles and the tracheal system.

Table 1. Chemical composition (compounds > 0.1%) of the *Origanum vulgare* essential oil.

Compounds	Chemical class	l.r.i.	Relative abundance (%) \pm SD
α -thujene	MH	931	0.1 \pm 0.03
α -pinene	MH	941	0.4 \pm 0.08
camphene	MH	955	0.1 \pm 0.02
β -pinene	MH	982	0.6 \pm 0.11
3-octanone	NTD	987	0.1 \pm 0.00
myrcene	MH	993	0.4 \pm 0.05
α -terpinene	MH	1018	0.5 \pm 0.06
<i>p</i> -cymene	MH	1027	6.2 \pm 0.57
limonene	MH	1032	0.5 \pm 0.06
1,8-cineole	OM	1034	0.9 \pm 0.06
γ -terpinene	MH	1062	3.7 \pm 0.39
linalool	OM	1101	2.9 \pm 0.13
camphor	OM	1143	0.8 \pm 0.01
borneol	OM	1165	1.1 \pm 0.03
4-terpineol	OM	1178	0.6 \pm 0.01
α -terpineol	OM	1189	0.7 \pm 0.01
methyl carvacrol	OM	1239	0.3 \pm 0.02
thymol	OM	1292	4.8 \pm 0.03
carvacrol	OM	1298	70.2 \pm 1.6
eugenol	PP	1358	0.2 \pm 0.00
β -caryophyllene	SH	1420	3.5 \pm 0.02
α -humulene	SH	1456	0.6 \pm 0.01
β -bisabolene	SH	1509	0.2 \pm 0.02
caryophyllene oxide	OS	1581	0.8 \pm 0.01
Oxygenated monoterpenes (OMs)			82.2 \pm 1.31
Monoterpene hydrocarbons (MHs)			12.5 \pm 1.36
Sesquiterpene hydrocarbons (SHs)			4.3 \pm 0.06
Oxygenated sesquiterpenes (OSs)			0.8 \pm 0.01
Phenylpropanoids (PP)			0.2 \pm 0.00
Non-terpene derivatives (NTDs)			0.1 \pm 0.0
Total identified (%)			100 \pm 0.01

l.r.i. = linear retention index on an HP-5MS capillary column; SD = std dev ($n = 3$). Chemical classes: OM = oxygenated monoterpene; MH = monoterpene hydrocarbon; SH = sesquiterpene hydrocarbon; OS = oxygenated sesquiterpene; PP = phenylpropanoid; NTD = non-terpene derivative.

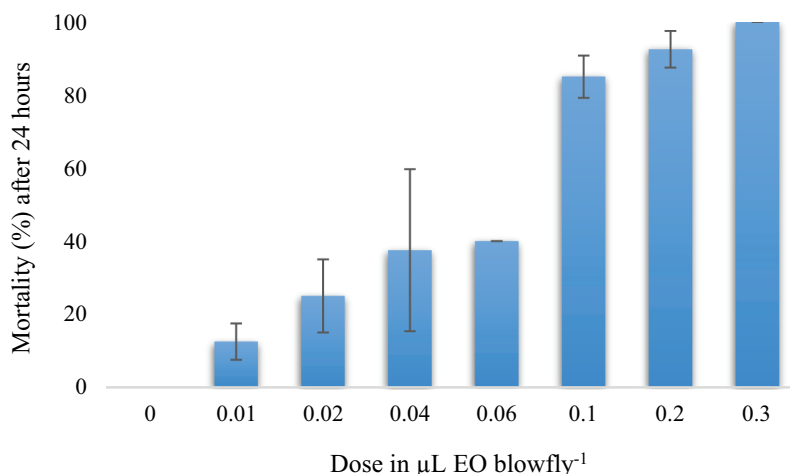


Figure 1. Mortality (%) of *Calliphora vomitoria* adults 24 hours after the treatment by direct contact with different doses (expressed in $\mu\text{L EO blowfly}^{-1}$) of the *Origanum vulgare* EO. Histograms represent mean values \pm SD ($n = 4$, with 10 blowflies each).

In the control samples, the exocuticle is formed by chitin fibres perfectly organized to form a compact and completely electron-dense layer ($5 \mu\text{m}$ thick, Figure 2(A, B)). The endocuticle, on the other hand, is made up of many lamellar layers parallel to each other (thickness at least $10 \mu\text{m}$), and each of them is made up of three zones with different electron densities: one with low, one with medium and one with high electron density (Figure 2(E, F)). The latter zone is made up of structures (asterisks) like small denticles, which attach to the upper layer (Figure 2(F)). In the lamellar layers, it is possible to notice the canal pores and one layer thicker than the others (Figure 2(E, F)). This layer is characterized only by the zone with medium electron density. The

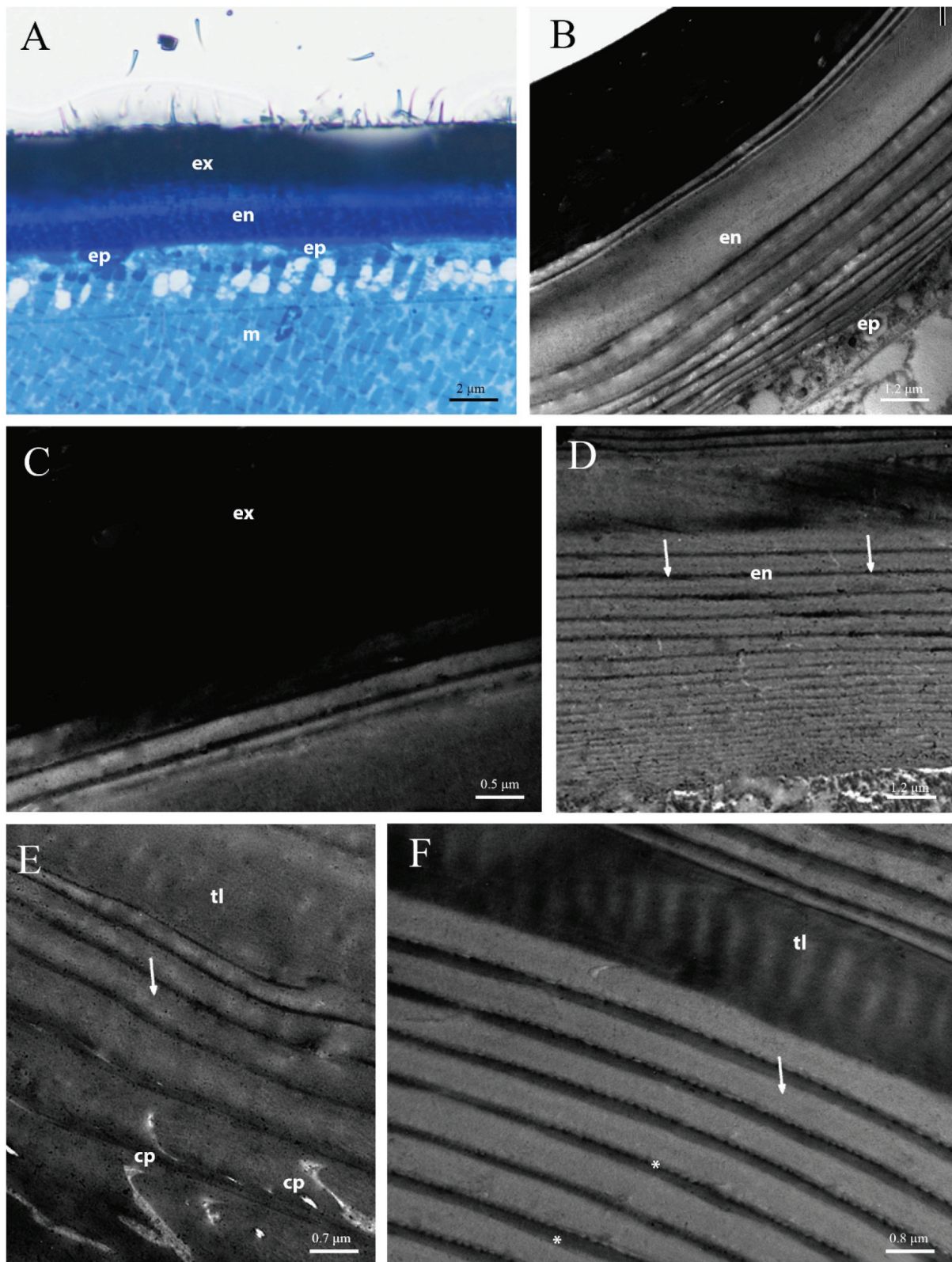


Figure 2. Control specimens of *Calliphora vomitoria*. (A) Light microscopy image showing the exocuticle (ex), the endocuticle (en), the epidermis (ep) and the muscular fibres (m). (B, C, D) Transmission electron microscopy micrographs showing the completely electron-dense compact exocuticular layer (ex) and the endocuticle (en) formed by lamellar layers (arrows) parallel to each other. The monolayered epidermis (ep) is well preserved. (E) Detail of the endocuticular lamellar layers: the canal pores (cp) and the thicker lamellar layer (tl) are visible. (F) Enlargement of the lamellar layers, each of which (arrow) is characterized by three distinct zones: one with low, one with medium and one with high electron density. The latter zone is made up of structures like small denticles (asterisks). The thicker lamellar layer (tl) is characterized only by medium electron density.

underlying epidermis has the appearance of a monolayer in which cells and cellular organelles appear well preserved (Figure 2(A, B)). In the area beneath the body wall, the tracheae, the muscular cells and the mitochondria are visible (Figures 2(A) and 3(A-C)). The tubular tracheae have sclerotized rings (the tenidia) well defined and arranged (Figure 3(A-C)). The muscular fibres are formed by fibrils embedded in the sarcoplasm; all filaments are aligned so that the whole fibres appear transversely striated (Figure 3(A-C)). The mitochondria appear as oval or spherical organelles, always in close contact with fibrils; their double membrane and the ridges immersed in the matrix appear well-defined (Figure 3(A-C)).

In the *O. vulgaris* EO-treated samples, the exocuticle appears broken in many points and the chitin fibres are organized in such a way as to form a less compact and less electron-dense structure (4 μm thick; Figure 4 (A, B)). The endocuticle (6 μm thick) is made up of a smaller number of layers and each layer does not show the three zones with different electron densities observed in the control samples; furthermore, the tendency of the single layers to merge with each other and lose their individuality is evident (Figure 4(C, D)). The epidermis is made up of cells in which numerous vacuolizations are observed and the still-recognizable organelles are in an evident degenerative state (Figure 4(A, C, D)). Observations of the area beneath the integument show the muscular fibres detached from the epidermis and that numerous swollen mitochondria appear in irregular shapes or disintegrated (Figures 4(A) and 5(A)). In addition, the tracheae are in such a great state of disorganization that the tracheal tubules are no longer recognizable (Figure 5B, C).

3.4. Acetylcholinesterase activity in control and essential oil-treated samples

The catalytic activity of AChE was assessed in homogenates obtained from Control, Ethanol and EO-treated *C. vomitoria* samples. The results of the biochemical test for AChE activity were found to be 32.25 ± 2.42 $\text{nmol min}^{-1} \text{mg}^{-1}$ of protein (mean value \pm SE with $n = 3$) for the untreated Control, 19.81 ± 5.84 $\text{nmol min}^{-1} \text{mg}^{-1}$ for the Ethanol samples and 28.06 ± 4.41 $\text{nmol min}^{-1} \text{mg}^{-1}$ for the *O. vulgare* EO-treated *C. vomitoria* adults. Although the enzyme activity level is lower in the blowflies that received EtOH, no statistically significant differences among the samples were highlighted by the one-way ANOVA ($F_{2,6} = 2.018$; $p = 0.213$), as represented in Figure 6.

4. Discussion

As commonly reported in Lamiaceae (Rodríguez-Solana et al. 2014), *O. vulgare* can reveal different chemotypes based on plant intrinsic factors and the influence of the growth environment (e.g. location, climate, temperature variations, soil composition, precipitation). Overall, five main chemotypes of *O. vulgare* EOs have been identified based on the compounds with the highest abundance (Raal et al. 2024). One of these is the carvacrol chemotype, the same we characterized here, as testified by the presence of $70.2 \pm 1.6\%$ of this

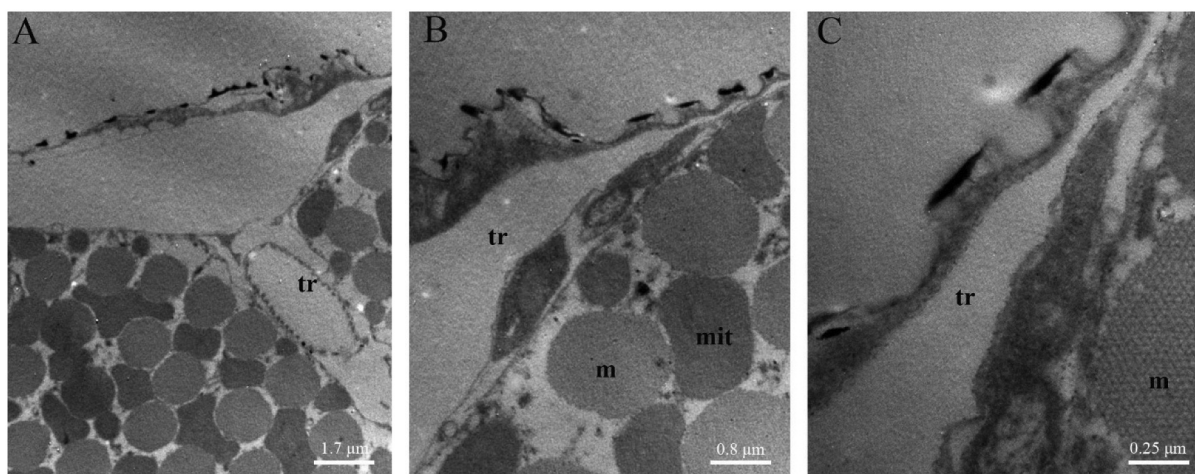


Figure 3. Control specimens of *Calliphora vomitoria*. (A, B, C) Area beneath the body wall showing the tracheae (tr), muscular fibres (m) and numerous mitochondria (mit), respectively, with well-defined and preserved ridges.

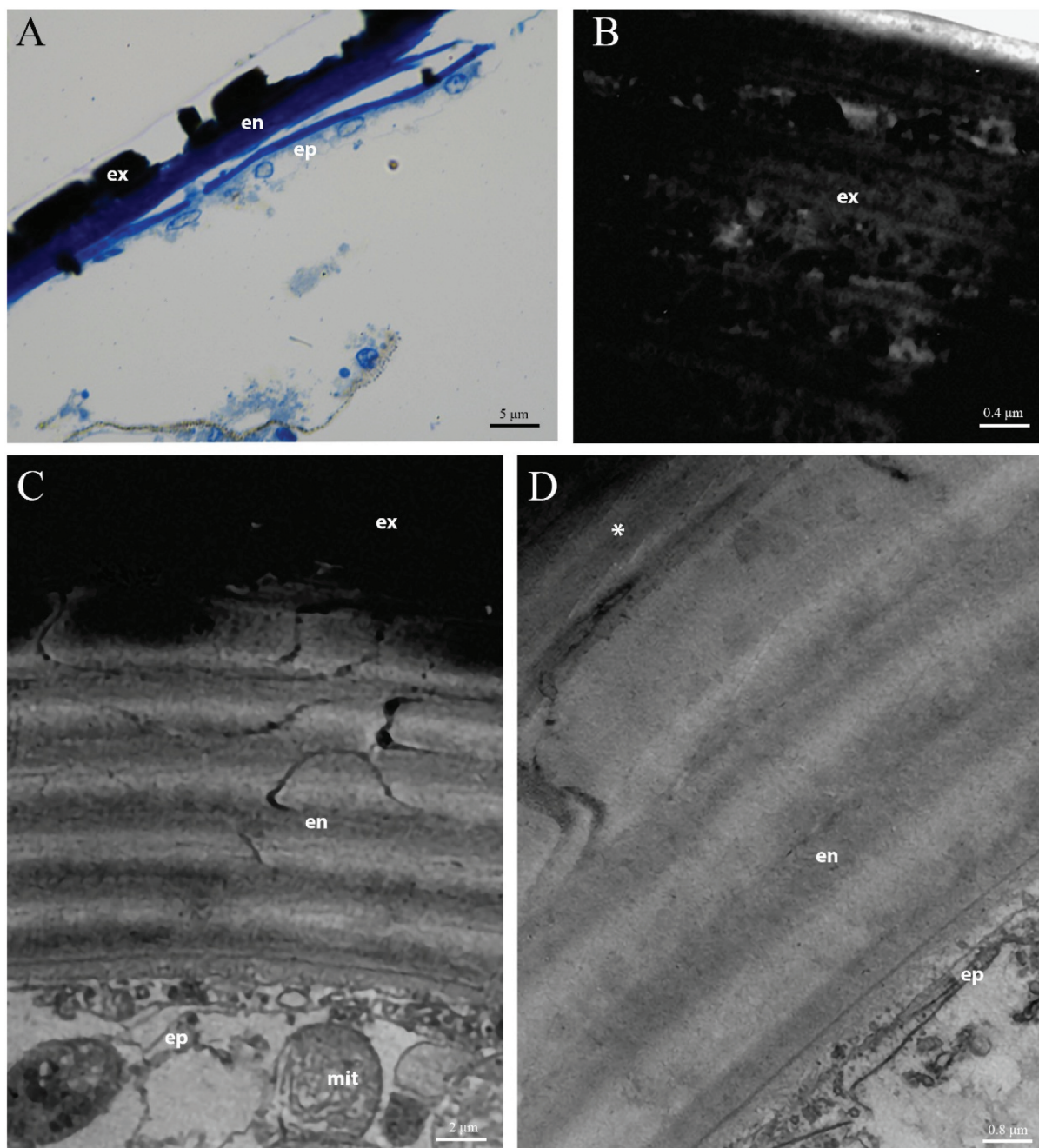


Figure 4. *Origanum vulgare* essential oil-treated specimens of *Calliphora vomitoria*. (A) Light microscopy image showing the exocuticle (ex), the endocuticle (en) and the epidermis (ep). (B) Transmission electron microscopy micrograph that documents the low compactness of the exocuticle (ex). (C, D) Exocuticle (ex), endocuticle (en) and epidermis (ep) are clearly visible. Note that the endocuticle is made up of a few morphologically identical layers. The single lamellar layers lose their individuality, as well as the three zones of different electron densities highlighted in the layers of the controls. The epidermis (ep) appears very vacuolated, and mitochondria (mit) are swollen and with non-intact cristae. The layers (asterisk) located immediately below the exocuticle appear thinner than the others.

monoterpenoid. For instance, a similar carvacrol content was recently reported by Moura et al. (2024) (76.8%) and Mrabet et al. (2025) (81%).

Several investigations have proven the toxic effects of *O. vulgare* EOs on a plethora of plant pathogens, nematodes, bacteria, mites (Catani et al. 2022, 2023; Jbilou et al. 2024) and insects like *C. vomitoria* itself (Bedini et al. 2021) and various stored foodstuff pests (Plata-Rueda et al. 2021, 2022). The *O. vulgare* EO can reduce the survival of the different developmental stages, respiration rates and behavioural responses in

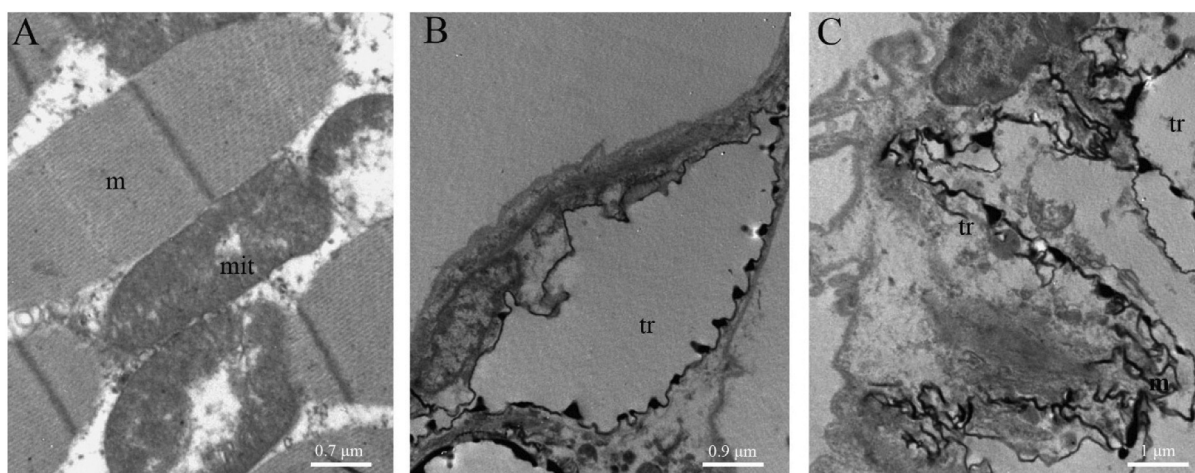


Figure 5. *Origanum vulgare* essential oil-treated specimens of *Calliphora vomitoria*. (A) Area under the body wall showing muscular fibres (m) and damaged mitochondria (mit). (B, C) Tracheae (tr) in a clear lack of organization.

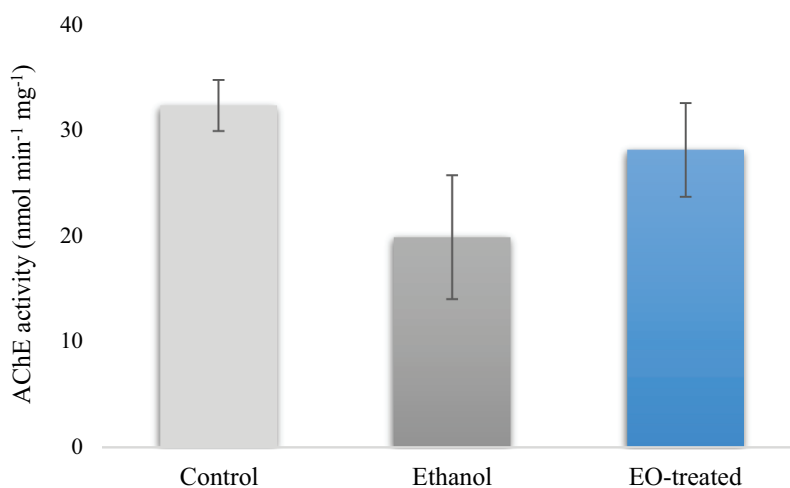


Figure 6. Acetylcholinesterase (AChE) activity (expressed in nmol min⁻¹ mg⁻¹ of protein) in *Calliphora vomitoria* adults untreated (control), treated with a 2.0 μ L drop of just ethanol (EtOH) on the thorax (Ethanol) or treated with a 2.0 μ L drop of a 10% *Origanum vulgare* essential oil (EO) solution in EtOH (EO-treated). Histograms represent mean values \pm SE ($n = 3$, with 6 blowflies each).

terms of walking distance, resting time and feeding activity (Plata-Rueda et al. 2021, 2022; Mrabet et al. 2025). In *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), the EO toxicity was shown to be due to its terpenoids (including carvacrol) acting on the insect integument or respiratory system (Plata-Rueda et al. 2021). In light of this, the toxicity shown when applied topically on our blowflies was not surprising and confirmed the validity of the subsequent investigation steps carried out in this work. The LD₅₀ here calculated 24 hours after the topical application of the EO, corresponding to 0.067 μ L EO blowfly⁻¹, was markedly lower than those reported for other *O. vulgare* EOs having different compositions (from 0.141 to 0.240 μ L blowfly⁻¹) (Bedini et al. 2021), *Artemisia* spp. (Asteraceae) EOs (0.49 and 0.79 μ L blowfly⁻¹) (Bedini et al. 2017) and EOs from culinary spices (from 0.44 to 1.97 μ L blowfly⁻¹) (Bedini et al. 2020).

The present study demonstrated that the *O. vulgare* EO, when topically applied on the thorax of *C. vomitoria* adult flies, as showing lipophilic properties, acts directly on the body wall, thus causing morphological alterations at the level of the cuticle and epidermis. Furthermore, we have documented that the action of this EO also extends to the underlying muscular and tracheal system, where it generates significant damage in their ultrastructure. In detail, the morphological modifications visible in the EO-treated specimens through the TEM observation are: (i) the exocuticle is broken in many points and formed by chitin

fibres organized to form a loose and thin layer; (ii) the endocuticle is thinned and formed by a low number of layers; the individual layers do not have the three electron-dense zones and tend to merge, losing their individuality; (iii) the epidermal cells present numerous vacuolization and the organelles are in an evident degenerative state; (iv) the muscular fibres are detached from the epidermis and the mitochondria between them show irregular shapes or become disintegrated; and (v) the tracheal system is in a great state of disorganization.

To the best of our knowledge, there are no similar studies to compare our results with. The detrimental effects of some botanicals on cells, tissues and organs of arthropods and nematodes have been observed through TEM (Massoud et al. 2005; Sirisuda et al. 2008; Spochacz et al. 2021), but any other researcher has yet worked on topical applications to adult Diptera. Sirisuda et al. (2008) employed two flies, but they examined larvae and pupae of *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) and *Musca domestica* L. (Diptera: Muscidae) after a single or multiple dippings in a 0.2% neem oil extract. Under TEM, larvae of both species exhibited increasing swelling in the integument and intersegmental spines, corresponding to intense thickness in the epicuticle and procuticle, with increasing repetitions of the dipping procedure. To a lesser extent, puparia integument grew thicker with multiple applications and showed blebbing and cracking.

Limiting our feeble discussion to EOs and singular secondary metabolites, one EO obtained from *Acorus calamus* L. (Acoraceae) rhizomes was orally administered to *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) sixth-instar larvae at concentrations of 500 and 1000 ppm. The TEM observation of the hemolymph from treated larvae evidenced intense cytoplasmic vacuolization and organelle degeneration in granular hemocytes and, especially, plasmatocytes, thus implying the weakening of cellular defense reactions (Sharma et al. 2008). Diallyl trisulfide, characteristic of EOs from garlic cloves, was used at a concentration of $0.01 \mu\text{L L}^{-1}$ air to fumigate *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) males. Testes from the treated specimens, observed through TEM, revealed malformed and defective mitochondrial derivatives of both eupyrene and apyrene spermatozoa, likely leading to male infertility (Shah et al. 2023). Females of the ovine gastrointestinal nematode *Haemonchus contortus*, when incubated in concentrations from 0.75 to 2.0 mg mL^{-1} of a *Eucalyptus citriodora* Hook. (Myrtaceae) EO and its major constituent, citronellal, exhibited internal ultrastructural modifications. In detail, TEM images showed marked disorganization in the muscle layer with vacuolization and degradation of the muscular fibrils and mitochondrial profile, causing the consequent loss of homeostasis and, probably, motility (de Araújo-Filho et al. 2019).

With regard to the mode of action of EOs, the classic method of Ellman (1961) is used to visualize, through spectrophotometry, the level of AChE activity by measuring the production of thiocholine derived from acetylthiocholine iodide (ATChI) enzymatic hydrolysis. In detail, it is based on the reaction between thiocholine and the chromogen 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) under the formation of 5-thio-2-nitrobenzoate (TNB^-), a product with an intense yellow colour detectable at 412 nm. When the method is applied to arthropods, the crude AChE protein can be extracted from control specimens and then mixed with growing concentrations of the test substance dissolved in acetone (Seo et al. 2015; Park et al. 2016; Bedini et al. 2017) or directly from specimens previously treated with the test substance (Rizvi et al. 2018; Shah et al. 2023; Chaubey 2024). The former is considered an *in vitro* evaluation of the AChE activity isolated from the target arthropod and under laboratory conditions; the latter is an *in vivo* approach, where the enzyme is still affected by physiological factors depending on the tested living organism. Despite the *in vitro* approach being highly specific towards the enzyme and reproducible under standardized and controlled conditions, the *in vivo* one has greater physiological relevance, as it considers the whole biological system with its metabolism, biochemical pathways, possible detoxification processes, etc.

The *in vivo* assay here performed was based on *C. vomitoria* adult flies previously treated by contact with the *O. vulgare* EO solution at 10% in EtOH. Our results show that the AChE activity is not affected by our EO-containing treatment ($28.06 \pm 4.41 \text{ nmol min}^{-1} \text{ mg}^{-1}$) and its values are not statistically different from those obtained from the ethanol-treated ($19.81 \pm 5.84 \text{ nmol min}^{-1} \text{ mg}^{-1}$) and control ($32.25 \pm 2.42 \text{ nmol min}^{-1} \text{ mg}^{-1}$) blowflies. The only previous assessment of AChE inhibition in *C. vomitoria* blowflies when using EOs was reported by Bedini et al. (2017). The authors, through an *in vitro* procedure, tested two *Artemisia* spp. EOs at concentrations ranging from 2 to 500 mg L^{-1} dissolved in acetone and observed a concentration-dependent enzyme reduction with median inhibitory concentrations (IC_{50}) of 202.6 and 472.4 mg L^{-1} .

Some similar *in vitro* assessments have focused on the AChE inhibition of several arthropods and nematodes when using carvacrol and carvacrol-containing EOs. For instance, the carvacrol inhibition rate of AChE was 78.8%

at 1000 mg L⁻¹ in third-instar larvae of the mosquito *Aedes albopictus* (Skuse) (Diptera: Culicidae) with an IC₅₀ of 57 mg L⁻¹ (Seo et al. 2015) and about 20% in *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) males and females at the concentration of 500 mg L⁻¹ (Park et al. 2016). On L3 larvae of the marine parasitic nematode *Anisakis simplex*, the *in vitro* test using an *Origanum compactum* Benth. (Lamiaceae) EO and its main constituent, carvacrol (50.32%), resulted in comparable IC₅₀ values of 124 and 113 mg L⁻¹, respectively (López et al. 2018).

Chaubey (2024) proposed an *in vivo* assay like the one we performed in this work. The author worked with *Callosobruchus chinensis* (L.) (Coleoptera: Chrysomelidae) adults after 24 hours of fumigation with carvacrol at 65.6 and 131.2 µL L⁻¹ air (representing 40 and 80% of the LC₅₀ at 24 hours). He obtained AChE activity reduction by about 30% and 50%, respectively, compared to the control beetles. Similarly, Rizvi et al. (2018) worked with AChE extracted from the head of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) adults that survived 24 hours after the topical application of carvacrol at the LD₃₀. The psyllid AChE activity was significantly reduced compared to the controls.

Our results indicate the absence of AChE inhibition under the specific conditions we set, in agreement with others reported for a fly relatively close to *C. vomitoria*. Carvacrol at the markedly high concentration of approximately 4500 mg L⁻¹ only halved the AChE activity *in vitro* in *M. domestica* adults (Anderson & Coats 2012). Anderson and Coats (2012) also underlined the species-specific sensitivity of AChE to carvacrol, but with a generally low effect on some selected model organisms (the housefly, a mosquito, a cockroach and a tick) compared to the positive control carbaryl. They concluded that AChE inhibition is likely not the primary mode of action of carvacrol as an insecticide. Moreover, as we worked with a complex EO containing 24 constituents, it is plausible that antagonistic reactions among the different metabolites could have interfered with the potential inhibition that pure carvacrol could give on the AChE activity (Savelev et al. 2003).

5. Conclusion

Our observations on the topical application of an *O. vulgare* EO on the blowfly *C. vomitoria* revealed a significant number of morphological alterations induced by its action on the integumentary system and also at the level of the underlying area. At the 10% concentration in EtOH here used (corresponding to the dose of 0.2 µL EO blowfly⁻¹), the EO penetrated through the integument, showing lipophilic properties that allowed it to interfere with the chitinous exoskeleton by contact. However, the marked toxicity, highlighted by an LD₅₀ lower than others reported for different EOs and corresponding to 0.067 µL EO blowfly⁻¹, was not associated with an inhibition of the AChE enzyme. Based on Rajashekar et al. (2014), we feel confident in ruling out the *in vitro* approach for additional AChE investigations. In fact, the authors, working with *M. domestica* and a botanical active ingredient, showed that the results of a double *in vitro* + *in vivo* investigation are correlated. Therefore, we would very likely again obtain a lack of influence of the *O. vulgare* EO on the *C. vomitoria* AChE inhibition from a potential *in vitro* evaluation.

This research provides promising data about *O. vulgare* EO as a natural insecticide and suggests its possible use as a sustainable tool for the effective management of *C. vomitoria* adults. However, it is important to remember that any EO used as a spray insecticide, whether indoors or outdoors, must be properly formulated to limit its volatility and instability, and efficacy tests must be carried out under real operating conditions to determine the correct dosage needed. Further assessments must also involve evaluating non-target effects and consumer acceptability in terms of smell pleasantness.

Disclosure statement

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Author contributions

Loretta Guidi: Conceptualization, Funding acquisition, Formal analysis, Supervision, Writing – original draft, Writing – Review & Editing; **Eleonora Grassi:** Formal analysis, Investigation, Writing – Review & Editing; **Antonella Petri:** Formal analysis, Investigation, Writing – Review & Editing; **Roberta Ascrizzi:** Formal analysis, Investigation, Writing – Review & Editing; **Federica Semprucci:** Conceptualization, Funding acquisition, Writing – Review & Editing; **Chiara Rosati:** Investigation; **Priscilla Farina:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – Review & Editing, Visualization; **Barbara Conti:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing – Review & Editing.

Data availability statement

Data are available from the corresponding author upon reasonable request.

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