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Mechanisms of Action of Essential Oils on Enzymes Relevant to Insect and Nematode Development

MASTER DISSERTATION

Mariana Barreto Martins
MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA
A Nossa Universidade
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SUPERVISOR
Paula Cristina Machado Ferreira Castilho

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This dissertation was developed at the Organic Chemistry and Natural Products Laboratory (NatLab) of the Madeira Chemistry Research Centre (CQM), University of Madeira, under the supervision of Professor Dr. Paula Cristina Machado Ferreira Castilho. It was submitted to the University of Madeira as part of the requirements for obtaining a Master's degree in Applied Biochemistry.

Mariana Barreto Martins

September 2025

Funchal, Madeira Island – Portugal

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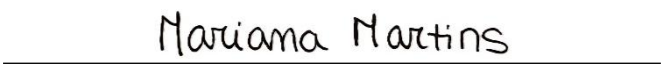
September 2025

Mariana Barreto Martins



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Abstract

Insects, such as the Mediterranean fruit fly (Medfly, *Ceratitis capitata*) and the armyworm (*Mynthimna unipuncta*), and nematodes, such as the root-knot nematode (RKN, *Meloidogyne incognita*) and the pine wood nematode (PWN, *Bursaphelenchus xylophilus*), are major agricultural pests. Botanical pesticides such as essential oils (EOs) have proven effective in controlling agricultural pests due to their repellent, anti-feeding properties, etc. Therefore, the objective of this study was to evaluate the potential of aromatic plants in controlling the pests *C. capitata*, *M. unipuncta*, *M. incognita*, and *B. xylophilus* by studying their mechanisms of action on the neural and digestive enzymes. This study focused on the EOs of *Origanum vulgare*, *Thymus vulgaris*, *Ocimum gratissimum*, *Mentha pulegium*, and *Cinnamomum cassia*, mainly composed of carvacrol, thymol, eugenol, pulegone, and *trans*-cinnamaldehyde, respectively, as several studies have mentioned their insecticidal and/or nematicidal effects. Their interactions with enzymes relevant to insect and nematode development, namely acetylcholinesterase (AChE), α- and β-glucosidase, were investigated using crude enzyme extracts obtained from the selected species. Enzyme activity and soluble protein content were quantified using standard assays, and SDS-PAGE was performed to attempt protein identification. *In vitro* inhibition assays with the EOs and their major components were then conducted to assess inhibitory activity and provide insights into potential mechanisms of action. The results revealed that all tested EOs inhibited AChE but had no effect on the digestive enzymes. Among the EOs studied, *M. pulegium* EO showed the strongest inhibition of AChE in both Medfly (IC_{50} = 0.022 mg/mL) and PWN (IC_{50} = 0.1209 mg/mL). *C. cassia* EO was particularly effective against armyworm (IC_{50} = 0.0020 mg/mL) and RKN (IC_{50} = 0.0197 mg/mL). These findings suggest that these EOs primarily exert their toxic effects through interference with the nervous system, supporting their potential as sustainable alternatives to synthetic pesticides in environmentally friendly pest management.

Keywords: Essential oils; Insects; Nematodes; Acetylcholinesterase; α-glucosidase; β-glucosidase.

Resumo

Insetos, como a mosca-da-fruta-mediterrânica (*Ceratitis capitata*) e a lagarta-das-pastagens (*Mynthimna unipuncta*), e nemátodos, como o nemátodo-das-galhas (*Meloidogyne incognita*) e o nemátodo-da-madeira-do-pinheiro (*Bursaphelenchus xylophilus*), constituem pragas agrícolas de elevada importância. Os pesticidas botânicos, como os óleos essenciais (OEs), têm demonstrado eficácia no controlo de pragas agrícolas devido às suas propriedades repelentes, anti-*feeding*, etc. Assim, o objetivo deste estudo foi avaliar o potencial de plantas aromáticas no controlo das pragas *C. capitata*, *M. unipuncta*, *M. incognita* e *B. xylophilus*, investigando os seus mecanismos de ação sobre enzimas neurais e digestivas. Este estudo centrou-se nos OEs de *Origanum vulgare*, *Thymus vulgaris*, *Ocimum gratissimum*, *Mentha pulegium* e *Cinnamomum cassia*, compostos maioritariamente por carvacrol, timol, eugenol, pulegona e *trans*-cinamaldeído, respetivamente, pois diversos estudos têm reportado os seus efeitos inseticidas e/ou nematicidas. Foram investigadas as interações destes OEs com enzimas relevantes para o desenvolvimento de insetos e nemátodos, nomeadamente acetilcolinesterase (AChE), α- e β-glucosidase, utilizando extratos enzimáticos brutos obtidos das espécies selecionadas. A atividade enzimática e o teor de proteínas solúveis foram quantificados através de ensaios padrão e realizou-se SDS-PAGE com o objetivo de identificar proteínas. Posteriormente, ensaios de inibição *in vitro* com os OEs permitiram avaliar a atividade inibitória e elucidar potenciais mecanismos de ação. Os resultados mostraram que todos os OEs testados inibiram a AChE, porém não afetaram as enzimas digestivas. Entre eles, o OE de *M. pulegium* apresentou a maior inibição da AChE tanto em adultos de *C. capitata* (IC_{50} = 0,022 mg/mL) quanto em *B. xylophilus* (IC_{50} = 0,1209 mg/mL), enquanto o OE de *C. cassia* foi particularmente eficaz contra a lagarta de *M. unipuncta* (IC_{50} = 0,0020 mg/mL) e *M. incognita* (IC_{50} = 0,0197 mg/mL). Estes resultados sugerem que os OEs estudados exercem a sua toxicidade sobretudo por interferência no sistema nervoso, reforçando o seu potencial como alternativas sustentáveis aos pesticidas sintéticos no controlo integrado de pragas.

Palavras-chave: Óleos essenciais; Insetos; Nemátodos; Acetilcolinesterase; α-glucosidase; β-glucosidase.

List of Communications

List of Oral Communications

Martins M, **Castilho P**. Evaluation of the Mechanisms of Action of Essential Oils on Enzymes Relevant to Animal Development (Insects and Nematodes). 11th CQM Annual Meeting; 2024 Oct 3-4; Funchal, Portugal.

Martins M, **Castilho P**. Elucidation of the Mechanisms of Action of Essential Oils on Enzymes Relevant to Insect and Nematode Development. 12th CQM Annual Meeting; 2025 Jul 25-26; Funchal, Portugal.

List of Poster Communications

Martins M, **Castilho P**. Mechanisms of Action of Essential Oils on Acetylcholinesterase from Insects: *In Vitro* and *In Silico* Approaches. PSE Meeting 2025 – Phytochemicals as Drugs, Foods and Biocommunicators; 2025 Oct 22-24; Funchal, Portugal.

Martins M, Ferreira R, **Castilho P**. Mechanisms of Action of *Mentha pulegium* and *Cinnamomum cassia* Essential Oils on Nematodes: *In Vitro* and *In Vivo* Studies. PSE Meeting 2025 – Phytochemicals as Drugs, Foods and Biocommunicators; 2025 Oct 22-24; Funchal, Portugal.

List of Publications

Ferreira R, **Martins M**, Santos V, Sardinha D, Tavares W, Sabina S, Espinel G, Barreto MC, Oliveira L, Cabrera R, **Castilho P**. *Origanum vulgare* L. Essential Oils: Chemotypes and Bioactivity as Antifungal, Antifeeding and Enzyme Inhibitors. Plants. 2025;14:3001.

Preamble

This work is a follow-up to the *in vivo* studies performed by Rui Ferreira during his PhD program. Therefore, only the aromatic plants that showed the most promising *in vivo* results were selected for further evaluation through *in vitro* assays to elucidate the possible mechanisms of action underlying this activity. It constitutes a deliverable of the project MACBIOPEST – Botanical Biopesticides from Macaronesia: Research and Popular Knowledge (MAC2/1.1a/289), carried out between 2019 and 2023.

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List of Abbreviations

1-DNJ	1-Deoxynojirimycin
ACh	Acetylcholine
AChE	Acetylcholinesterase
ATChI	Acetylthiocholine iodide
BSA	Bovine serum albumin
CcEO	*Cinnamomum cassia* essential oil
DMSO	Dimethyl sulfoxide
DTNB	Dithiobis-2-nitrobenzoic acid (Ellman's reagent)
EC_{50}	Median effective concentration
EO	Essential oil
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Hsp	Heat shock protein
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
Medfly	Mediterranean fruit fly
MpEO	*Mentha pulegium* essential oil
Mw	Molecular weight
OgEO	*Ocimum gratissimum* essential oil
OvEO	*Origanum vulgare* essential oil
PWD	Pine wilt disease
PWN	Pine wood nematode
RKN	Root-knot nematode
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TvEO	*Thymus vulgaris* essential oil

USA	United States of America
α-GLU	alpha-Glucosidase
α-pNPG	p-Nitrophenyl-α-D-glucopyranoside
β-GLU	beta-Glucosidase
β-pNPG	p-Nitrophenyl-β-D-glucopyranoside

I. Introduction

1. Agricultural Pests

The exponential growth of global trade, the increase in efficiency and availability of travel, and other factors have raised the number of agricultural pests that become established in new areas every year, thereby becoming a critically important ecological problem around the world [1]. Pests are undesirable organisms that damage agricultural, forestry, and storage activities. This category includes invasive and/or allelopathic plants, microorganisms such as fungi, bacteria, and viruses, as well as animals (e.g., insects and nematodes) [2]. Invasive plant species, unlike agricultural weeds, can successfully occupy and spread to new habitats without further assistance from humans, invading new areas already fully occupied by native vegetation and displacing many species [3]. Animals, in general, damage cultivated plants and crops by feeding on them and transmitting pathogens [2]. Bacteria and fungi represent significant threats to global agriculture, as they are responsible for a wide range of plant diseases that reduce crop yield and quality [2].

The losses in crops caused by pests are quite high both in developed and developing countries: in the United States of America (USA), the European Union, and Japan, losses are estimated to be in the range of 10 to 30%, while in the developing parts of the world, the values are substantially higher [4]. These low-loss values are observed in the developed countries due to the availability of advanced technologies for pest control, which are largely absent in developing countries. Therefore, the development of new pest control techniques and making them accessible in developing regions is essential, contributing to improved agricultural productivity and quality of life.

1.1. Insects

Insects represent the most diverse group of animals on the planet and are present in nearly all habitats. As a dominant component of animal biomass on Earth, insects occupy diverse trophic niches and perform a wide range of ecological functions within their natural ecosystems, including herbivory, carnivory, and detritus feeding [5]. Since insects are often perceived as pests or potential pests, their ecological importance is frequently overlooked. The main ecological functions of insects in ecosystems are: ecosystem cycling, by changing the quality, quantity, and timing of plant detrital inputs; pollination, since most wild flowering plant species in temperate regions need insects (bees, beetles, butterflies, and flies) for pollination; predation/parasitism, with predators and parasites, as higher-level consumers, regulate herbivore populations, preventing excessive damage; and decomposition of organic waste [5].

The majority of insects are relevant to humans and the environment; however, insect pests inflict damage on crops both by transmitting diseases and by feeding on fruits and leaves by piercing, sucking, or chewing. Insect pests in agricultural systems are one of the major causes of damage to crop production and storage, leading to negative impacts on agricultural production, market access, natural environment, and lifestyle [6]. Larvae often represent the main feeding stage, during which they can cause significant damage to crops, stored products, or forest resources. Adults, on the other hand, are usually responsible for reproduction and dispersal, playing a key role in colonization of new habitats, disease transmission, and the regulation of population size. Studying both stages provides valuable insights into life cycles, survival strategies, and ecological interactions, which are fundamental for the development of effective and sustainable pest management strategies. Bearing this in mind, this study will focus on two insect pests: adult Mediterranean fruit fly (*Ceratitis capitata*) and armyworm (*Mythimna unipuncta*).

1.1.1. Mediterranean Fruit Fly (*Ceratitis capitata*)

Ceratitis capitata (Wiedemann, 1824) (Diptera: Tephritidae), widely known as the Mediterranean fruit fly (Medfly), is one of the most destructive frugivorous pests worldwide due to its high polyphagy and invasive ability [7]. Medfly is native to Sub-Saharan Africa, but has dispersed to almost all continents and can feed on more than 300 host plants [8]. The adult females only attack fruit close to ripeness, biting it to lay their eggs. The Medfly eggs are small (about 1 mm), slim, curved in shape, and have a smooth whitish surface (Fig.1c). Once the fruit is bitten, the discoloration of the epidermis occurs, and a small soft spot appears, where the larva can be found. Medfly has three larval instars. Larvae are white colored, cylindrical, with a narrow anterior side and a flattened caudal part (Fig. 1d), and the dimensions vary depending on the instar [6,7]. All their development takes place inside the fruit, where they can feed, causing it to rot and be destroyed completely, which is reflected in its early fall. Generally, attacks by this pest close to harvest are not systematic in pear and apple trees, but they are very serious in citrus and peach trees. A ready-to-pupate third instar larva can measure from 7 to 9 mm, and the pupa is enclosed by a 4–4.3 mm long, dark red-brownish, cylindrical, barrel-shaped, and smooth-ended puparium (Fig. 1e) formed by the hardened exoskeleton of this last larval stage [8]. Finally, adults are typically 3-5 mm long and display the main characteristics common to all species within the *Ceratitis* genus: transparent wings marked with yellowish-brown to dark brown bands, consisting of a discal, an anterior apical and a subapical band, along with various streaks and spots near the wing base; a wing cell bcu with sinusoidal apex; the scutellum shows a pattern with three apical spots, which may be either separate or fused; and the abdomen bears bands on tergites 2 and 4 (Fig. 1a, b) [8].

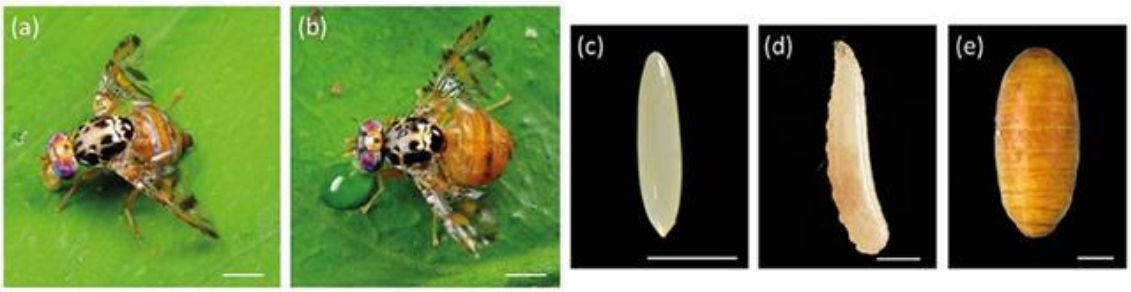


Figure 1 – Morphology of the developmental stages of *C. capitata*: (a) adult female, (b) adult male, (c) egg, (d) third instar larva, and (e) puparium. Scale bar: 1 mm (a, b, d, e) and 0.25 mm (c) (photo credit: P. Giannotti). Reproduced from Giunti et al. [8].

The duration of the Medfly cycle depends on temperature, with adults returning to their active state once temperatures rise above 14 ºC and lasting between 21 and 30 days under optimal conditions [10]. Their activity increases in spring and peaks during summer, with pupae remaining inactive during winter if weather conditions are unfavorable. Oviposition begins at the start of fruit ripening, with an average of 30-80 eggs per female fly, resulting in a total of 500 eggs per female [10]. A single copulation in the female's life cycle is sufficient to ensure continuous egg fertilization, as sperm is stored in the female's spermatophore [10]. Attracted by the fruit odor and color – particularly yellow and orange – fertilized females lay eggs in the fruit pulp. In the absence of suitable fruit, females may delay oviposition for extended periods, resuming egg laying when conditions become favorable, without requiring additional copulations [10].

Medfly activity is optimal in regions with temperatures between 16 and 32 ºC and relative humidity levels of 75-85% [10]. For these reasons, Medfly is one of the most damaging fruit pests in the Autonomous Region of Madeira, attacking more than 40 different species. The high populations of Medfly cause significant economic losses, estimated at around 2.5 million euros a year, and are the main obstacle to increasing fruit production on the island [11]. The application of insecticides to control this pest does not produce satisfactory results and causes environmental damage. The sterile insect technique (SIT) program was initiated in 1996 by the Regional Government of Madeira, supported by the European Union and

the International Atomic Energy Agency, in order to control Medfly, reducing infestation levels to less than 2% in 10 specific edible fruit species [11]. The overall strategy consisted of releasing sterile males in the areas of the island where flies spend the winter and thus where the pest is active year-round, and a genetic sexing strain was used so that few female medflies were released [11]. In addition, the Madeira Medfly program was among the first to use SIT to control, rather than eradicate, this pest. However, this program was discontinued for economic and governmental reasons. This pest can also be controlled by disrupting larval feeding, interfering with mating through pheromone-based sensory disruption, inhibiting adult movement, or impairing visual perception.

1.1.2. Armyworm (*Mythimna unipuncta*)

Mythimna unipuncta (Haworth, 1809) (Lepidoptera: Noctuidae), commonly known as armyworm, is considered a seasonal migrant and a polyphagous pest of crops in North America and in Europe [12]. This species has a cosmopolitan distribution and is well known in tropical, subtropical, and warm temperate zones [13]. *M. unipuncta* goes through four stages of development – egg (Fig. 2a), larva (Fig.2b, c, d, e, f, g), pupa (Fig. 2h), and adult (Fig. 2i, j) – where the larval stage is the most damaging, as the armyworm feeds intensely on leaves and other parts of the plant. Larvae of *M. unipuncta* prefer to feed on cultivated cereal and forage crops, including oat, wheat, barley, and corn [14]. The pest is typically detected by farmers and technicians only after significant pasture damage becomes visible, by which time the majority of the population has already reached the final two larval instars [13]. This occurs due to several factors: highly clumped distribution of young larvae, which causes the majority of the crops to remain uninfested until the larvae reach advanced development stages and increased mobility; a feeding preference for plants that are inedible to humans, only moving on to crops after the herbs been depleted; feeding preponderance in the last instar (around 80%); larvae's nocturnal behavior; and the gregarious, mobile behavior of 6th larvae, which tend to form large groups, thereby intensifying defoliation.

M. unipuncta is one of the most serious pests of the pastures in the Azores, where cattle breeding is a major industry [12]. High infestations by armyworms in summer and early autumn cause considerable financial damage, estimated at 8% of the annual vegetal production, valued at 5 million euros [12,13]. In the USA and Canada, sporadic outbreaks have resulted in 50-100% defoliation in corn with total estimated yield losses of approximately 2 million dollars [14].

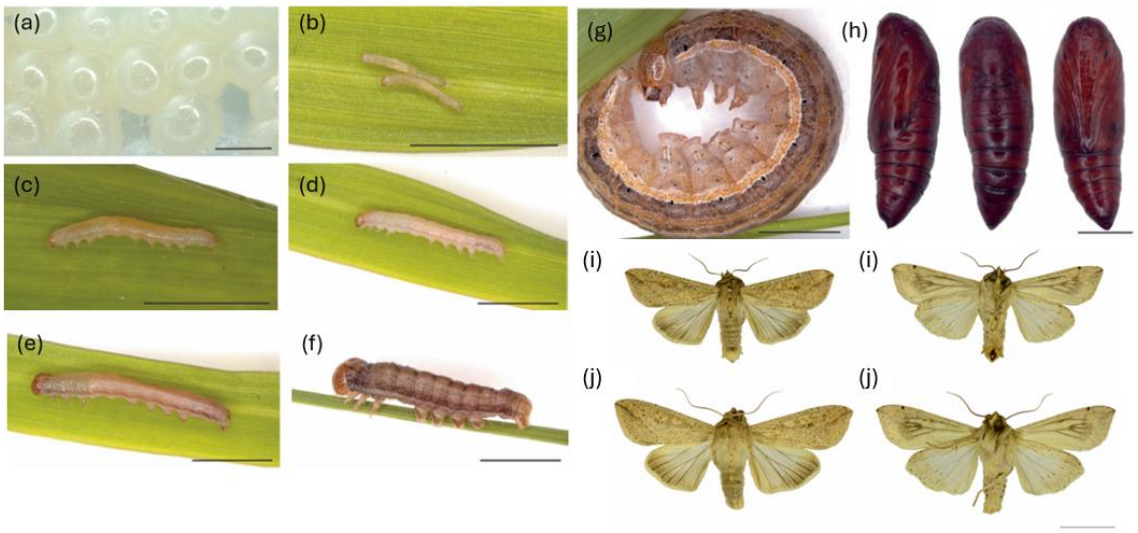


Figure 2 – Morphology of the developmental stages of *M. unipuncta*: (a) eggs (scale: 500 µm), (b, c, d, e, f, g) 1st - 6th instar larvae (scale: 5 mm), (h) pupa (scale: 5 mm), (i) adult male (scale: 1 cm), and (j) adult female (scale: 1 cm). Adapted from Madruga et al. [15].

1.2. Nematodes

Nematodes are microscopic worms belonging to the phylum Nematoda that are known to cause significant damage to crops (plant-parasitic nematodes). However, there are beneficial species as well, which are used in the biological control of pests (entomopathogenic nematodes). Nematodes are cosmopolitan, found in almost all habitats around the world, since they can adapt to diverse and extreme conditions [16].

The plant-parasitic nematodes are among the four most important groups of plant pathogens in terms of global economic losses caused in agriculture and horticulture (estimated at 157 billion dollars annually), the others being viruses,

fungi, and bacteria [17,18]. Based on their habitats and lifestyles, plant-parasitic nematodes are classified as either ectoparasites or endoparasites. Nematodes that remain on the plant surface and feed by inserting their stylet into cells are ectoparasites, while those that enter the host tissue and feed from within are endoparasites [16]. This study will focus on two plant-parasitic nematodes: root-knot nematode (*Meloidogyne incognita*) and pine wood nematode (*Bursaphelenchus xylophilus*).

1.2.1. Root-Knot Nematode (*Meloidogyne incognita*)

Meloidogyne incognita (Kofoid and White, 1919) (Tylenchida: Heteroderidae) is a well-known root-knot nematode (RKN). *M. incognita* is an obligatory sedentary endoparasite with a polyphagous feeding habit, infecting the roots of cultivated plants all over the world, making it perhaps the most damaging of all crop pathogens [16,18]. RKN *M. incognita* reproduces by mitotic parthenogenesis, and the mature female lays egg masses on the surface of the root. After the embryogenesis molts within the egg, the first juvenile stage (J1) becomes an infective juvenile of the second stage (J2), which hatches from the egg and penetrates the root of the host plant, migrating between cells to reach the plant vascular cylinder (Fig. 3) [16]. The stylet is used to pierce the plant cell wall to release esophageal secretions and take up nutrients [18]. Each J2 induces the dedifferentiation of five to seven root cells into multinucleate and hypertrophied feeding cells (giant cells, Fig. 3*), causing the nematode to become sedentary and go through three molts (J3, J4, and adult) (Fig. 3) [18]. The pear-shaped female produces eggs that are released on the root surface, and the cycle repeats itself. Roots damaged by RKN present galls or knots and lose their ability to absorb water and nutrients effectively, resulting in poor growth, reduced quality and yield, and also decrease the resistance of crops against drought and diseases [16]. Infection in mature plants only causes a decrease in yield; however, it may be lethal in the young ones. RKNs are considered one of the economically important pests that cause damage to plant growth and reduce yield, since there is an estimated loss of 100 billion dollars per year worldwide caused by *M. incognita* alone [19]. Diseases caused by RKN are not epidemic; nevertheless,

there is a slow decrease in production year after year due to the sedentary endoparasitic nature of this nematode, which makes its control difficult [16].

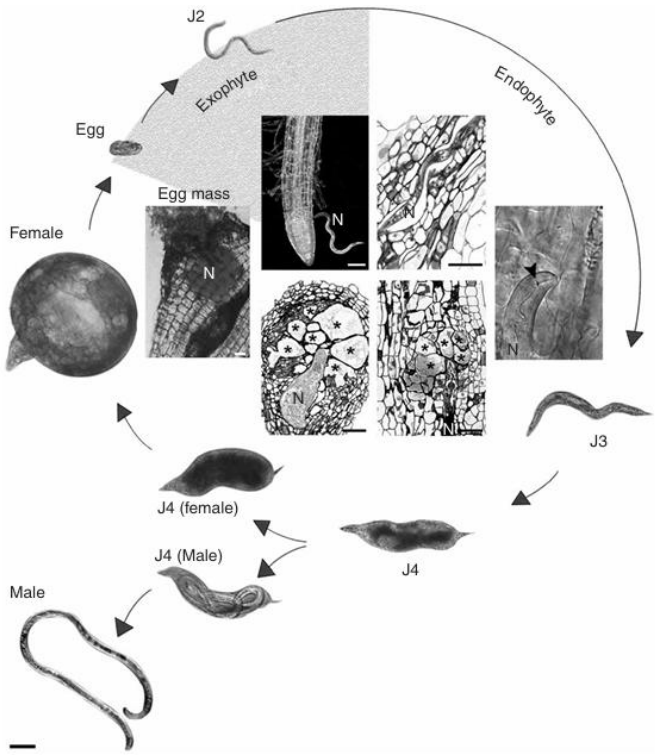


Figure 3 – The parasitic life of *M. incognita* (scale bars: 50 µm). Reproduced from Abad et. al [18]. Legend: J2, J3, J4 – second, third, and fourth juvenile stages, respectively; N – nematode; * - giant cells.

1.2.2. Pine Wood Nematode (*Bursaphelenchus xylophilus*)

Bursaphelenchus xylophilus (Steiner and Buhrer, 1934) (Tylenchida: Parasitaphelenchidae), commonly known as the pine wood nematode (PWN), is a lethal pest that infects pine trees – pine wilt disease (PWD), a slow progressive disease that results in economic losses worldwide. This pest originated in North America, namely the USA and Canada, but has caused extensive damage in pine forests of East Asian countries, in particular Japan (total financial loss estimated at 3.7 billion US dollars, assuming a market price of pine trees of 100 US dollars/m^3) and Korea [20]. PWN was introduced into Portugal in 1999 and later into Spain in 2008, causing severe forest damage with losses exceeding 80% [20,21].

Accordingly, PWN is legally listed as a quarantine pest in many countries, and protecting pines against it is recognized as an urgent problem for forestry [20].

The nematode life cycle has four propagative juvenile stages (Fig. 4). The first juvenile stage (L_1, Fig. 4) occurs inside the egg, which hatches into the second-stage juvenile (L_2, Fig. 4) [21]. Molting to the third juvenile stage (L_3, Fig. 4) is environmentally induced by deteriorating conditions in the pine tree, but molting to the fourth juvenile stage (L_4, Fig. 4) is dependent on the presence of an emerging *Monochamus* adult beetle [22]. The nematode grows rapidly under optimal conditions (approximately 25 ºC), completing its life cycle in just four to five days. *B. xylophilus* forms a particular and intimate mutualistic relationship with its vector, *Monochamus* beetle, and an investigation of the insects from withered or dead pine trees has revealed that *Monochamus alternatus* (Hope, 1842) (Coleoptera: Cerambycidae) plays a major role in the spread of pine nematode infections [22]. The beetle develops inside dead pine trees and later moves to healthy ones, where it gnaws on succulent branches, leaving wounds through which the nematode is transmitted [21]. PWN then migrates through the tree's vascular system and resin canals, feeding on epithelial and living parenchyma cells during its phytophagous phase, causing the tree's death [21]. The earliest sign of PWD is a reduced production of oleoresin in response to wounds. A decline follows this in leaf transpiration, which eventually ceases completely. The first visible external symptoms include needle yellowing and wilting, progressing to the drying and death of branches and twigs.

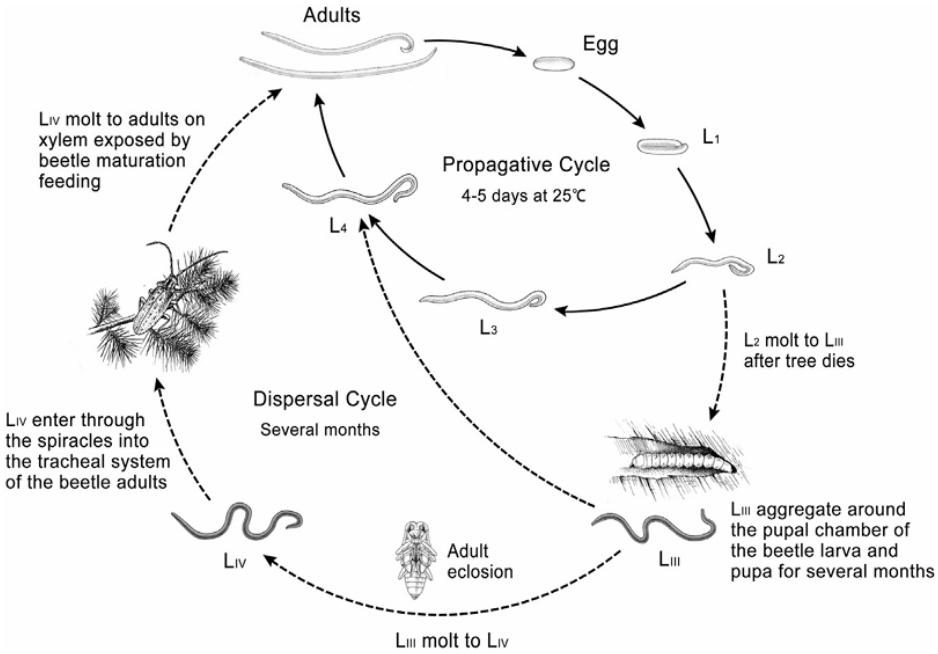


Figure 4 - Life Cycle of *B. xylophilus*. Reproduced from Zhao et. al [22]. Legend: L_1, L_2, $L_{3/III}$, $L_{4/IV}$ – first to fourth juvenile stages; solid lines – propagative developmental pathway; dashed lines – dispersal cycle.

1.3. Pest Control

Effective control of agricultural pests is essential to ensure food security, maintain food quality, promote environmental sustainability, and uphold the agricultural economy. However, pest control strategies should be selective – targeting harmful pests specifically while minimizing adverse effects on beneficial organisms such as entomopathogenic nematodes and pollinators, as well as on the environment and human health. Achieving this requires an understanding of the target pest's biology, ecology, and behavior. Therefore, the identification of key digestive, metabolic, or reproductive enzymes involved in the crop invasion process is crucial for developing precise and sustainable control methods.

2. Biopesticides

Synthetic pesticides continue to provide significant crop protection by controlling agricultural pests. However, their longer-term use is threatened by their well-documented adverse effects, including carcinogenicity, teratogenicity, high and acute residual toxicity, their ability to disrupt mammalian hormonal systems, their relatively long environmental persistence, and last but not least, the presence of residues in food, which has become an important issue for consumers [23]. Therefore, it is crucial to implement safer and more sustainable alternatives for effective pest management, such as biopesticides.

Biopesticides are pesticides derived from living organisms, such as microorganisms (bacteria and fungi), plants, and animals (e.g. nematodes) [23]. Successful biopesticides have been developed from compounds produced by the soil Actinomycete and from η-endotoxins isolated from *Bacillus thuringiensis* (Berliner, 1915) (Bacillales: Bacillaceae), both of which effectively control insect pests, plant diseases, and weeds [23]. For example, avermectins, emamectin, milbemectin, *B. thuringiensis* η-endotoxins, spinosad, and polynactins are effective in controlling insect pests. Microorganisms can also serve as biopesticides. Baculoviruses, which are arthropod-specific, are used to control lepidopteran pests in cotton, vegetable crops, forests, and ornamental plants [23]. Several fungi genus, including *Gliocladium*, *Trichoderma*, *Ampelomyces*, *Candida*, and *Coniothyrium,* are also effective against pests [24]. Bacterial biopesticides, however, are more widely used than viruses and fungi because they are less expensive to produce; among them, *B. thuringiensis* is the most widely applied biopesticide worldwide [23]. Plants, as stationary organisms, have evolved many chemicals to protect themselves against mobile herbivores and pathogens, some of which have been used in the past for pest management [25]. Most of the bioactive compounds derived from plants can be classified as phenylpropanoids, phenolics, terpenoids, steroids, alkaloids, and nitrogenated compounds [23]. A great diversity of plant secondary metabolites has been identified, and some of them are available on the market: nicotine [26], azadirachtin [27], matrine [28], rotenone [29], limonene [30], and pyrethrin [31] control insect pests; berberine [32], ethylicin [33], physcion [34],

and carvacrol [35] control plant diseases; brassinolide affects the growth and development of plants; and triptolide and curcumenol control rats [23]. Pheromones are chemicals emitted by living organisms used to communicate, which are categorized into sex, alarm, aggregation, and dispersal pheromones. They can cause a behavioral change in an individual of the same or different species. Insect sex pheromones are the most widely used pheromones for crop protection, some of which can now be synthesized and are used commercially for monitoring or pest control by mating disruption, mass trapping, and lure-and-kill systems [23].

Natural products and microorganisms have been widely used as biopesticides around the world. Since they are derived from the environment, they are generally safe for non-target organisms, including humans, have low persistence in the environment, and are potentially suitable for use in organic agriculture.

2.1. Botanical Biopesticides

All living organisms share certain chemicals and biochemical reactions that constitute their primary metabolism, such as nucleic acids, proteins, and specific carbohydrates. Beyond these primary metabolites, plants have evolved diverse secondary metabolic pathways that generate a wide range of novel compounds. Since most secondary metabolites are derived from universally present precursors, they are often categorized according to their biosynthetic pathways into nitrogen-containing compounds, phenolics, polyacetylenes, and terpenoids [25]. Pesticidal compounds exist within almost all classes of secondary metabolites. Botanical insecticides are generally complex mixtures of several secondary metabolites that may or may not have an important role in the toxicity of the mixture: they can exhibit internal interactions in the form of synergy or antagonism [25].

Plants have developed diverse groups of chemicals that act as major barriers to herbivory: some compounds directly affect the herbivore, while others attract organisms from other trophic levels. Commercially, the most important botanical pesticides are pyrethrum, neem, and essential oil-based products, with the latter

being the most diverse. Due to their versatility, they are used in a wide range of applications, from contact toxicants and fumigants to behavior-modifying products, such as attractants and repellents (Fig. 5) [25].

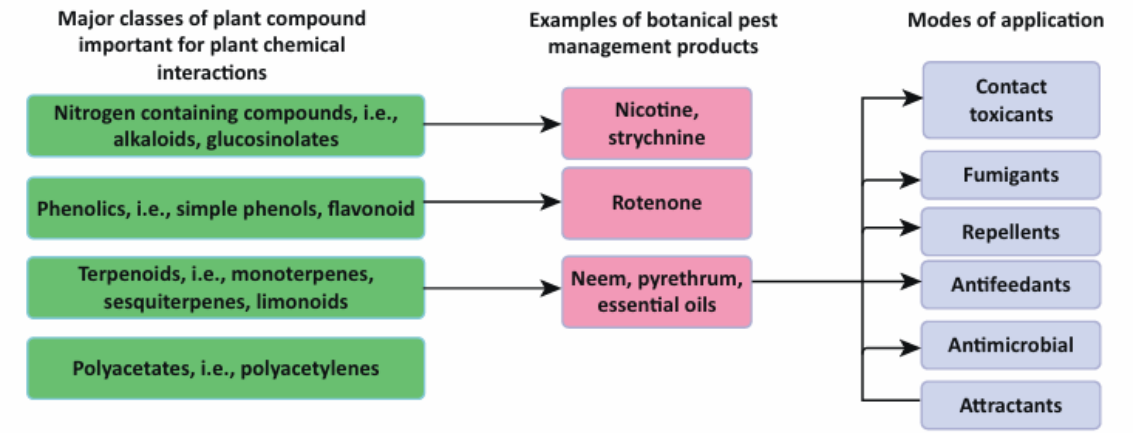


Figure 5 – Examples of botanical biopesticides based on major classes of plant compounds. Reproduced from Miresmailli and Isman [25].

2.1.1. Essential Oils

Essential oils (EOs) are a volatile, natural, and complex mixture of compounds that are produced by some plants as secondary metabolites [36]. They generally consist of several tens or hundreds of constituents of low molecular weight, of which the great majority possess an isoprenoid skeleton – monoterpenes, sesquiterpenes, and diterpenes [37]. EOs are characterized by a strong odor and can be extracted from various aromatic plants by the steam or hydrodistillation of dry vegetal material [36]. They are particularly abundant in plants from the Conifer, Rutaceae, Umbelliferae, Myrtaceae, and Labiatae families [37]. In nature, EOs play an important role in the protection of plants due to their antibacterial, antiviral, antifungal, insecticide, repellent, and food deterrence activities. In contrast, they also may attract some insects to favor the dispersion of pollen and seeds [36]. Therefore, EOs are an alternative for controlling phytopathogen agents and agricultural pests.

EOs can have a wide range of acute and sublethal effects on insects and related arthropods. These effects can be primarily behavioral (repellency,

attraction, and deterrence; mediated by peripheral chemosensory systems) or physiological (toxicity, growth inhibition, and developmental disruption). The toxic effects of EOs, in addition to the variability in phytochemical profiles, involve other factors such as the toxin entry point. They can typically be inhaled, ingested, or absorbed through the insect's cuticle. EOs exhibit a broad spectrum of activity against insects and phytopathogenic fungi, including insecticidal, antifeeding, repellent, oviposition-deterring, growth-regulating, and vector-inhibiting activities. Most monoterpenes are cytotoxic to plants and animal tissues, causing a drastic reduction in the number of intact mitochondria and Golgi bodies, impairing respiration and photosynthesis, and decreasing cell membrane permeability [38]. Table 1 describes the advantages and limitations of EO-based pesticide use.

EOs are gaining attention as biopesticides because they are natural, renewable, biodegradable, environmentally friendly, and safe for non-target organisms and humans. It has been demonstrated that EOs exhibit repellent [39], larvicidal [40], and insecticidal [41] properties against various pests; however, their practical application also faces some drawbacks due to their high volatility and low aqueous solubility. In this regard, nanoencapsulation can improve the efficacy and stability of EOs, avoiding rapid volatilization and degradation, and prolonging the biocidal effect against the pests [42].

EOs' pesticidal activity can be assessed using methods such as fumigation, in which EOs can be absorbed, ingested, or inhaled; or contact, in which the EO must penetrate the insects' cuticle. The fumigants act in gaseous or smoke form with high penetration power and are usually applied in an enclosure system [43]. EOs can also act as attractants in traps, helping to divert pests away from crops.

Table 1 - Advantages and limitations of EO-based pesticides. Adapted from Nollet and Rathore [38].

Advantages	Limitations
Broad-spectrum pesticides present insecticidal, antifeedant, repellent, oviposition-deterrent, growth regulatory, and vector-inhibiting activities	Inconsistency in raw material composition obtained from plants grown in different geographical, genetic, climatic, and seasonal areas
Useful in foodstuffs and stored foods	Lower efficacy than chemical pesticides
Reduced-risk pesticides are nontoxic to mammals and fish	Greater application rate and frequent reapplication are required
Widely used as flavoring agents in beverages and foodstuffs	Poor specificity due to the presence of several compounds
Commercialization is possible due to the abundant availability	Lack of sufficient supply, protection technology, and regulatory approval
Largely used against home and garden pests	Few pest control products are available on the market
Slow pest resistance due to complex mixtures of several compounds	Minimum involvement from low-budget companies
Unique impact on integrated pest management	Oil can harm the germination power of the treated seeds
Limited persistence	High volatility
No harm to predators, parasitoids, and pollinators	-

2.1.1.1. Mechanisms of Action

Elucidating the mechanisms of action of EOs is important because it provides rational information about the correct formulation, allows the study of the toxicity profile, and prevents unintended cytotoxicity in non-target organisms. EOs can affect the insects' nervous system, growth, development, and energy production, and may also inhibit cytochrome P450 enzymes and alter the function of the octopaminergic signaling pathway (Fig. 6) [43]. Furthermore, they can impede

the search for food and its absorption (antifeeding effect), oviposition, and mating (Fig. 6). Ideally, EOs should act through various mechanisms of action to minimize the risk of resistance development in pests.

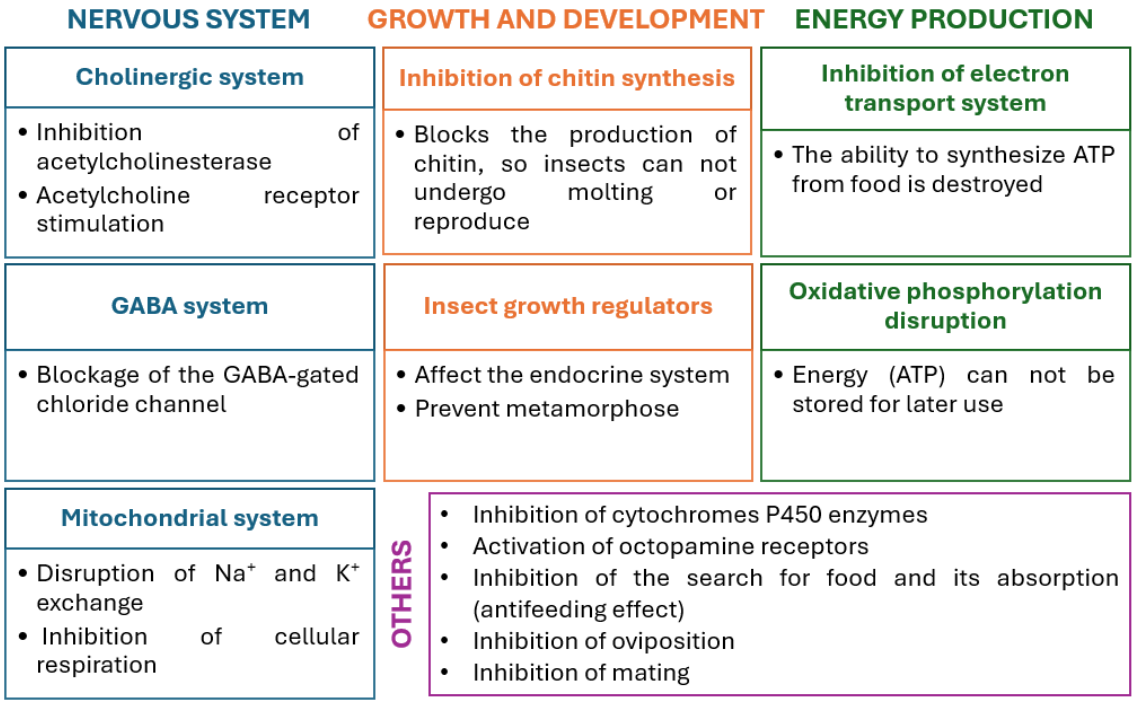


Figure 6 – Mechanisms of action and molecular targets of natural insecticides. Adapted from Singh et al. [43].

2.1.1.2. Essential Oils Against Insects and Nematodes

This study will focus on the EOs of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), clove basil (*Ocimum gratissimum*), pennyroyal (*Mentha pulegium*), and cinnamon (*Cinnamomum cassia*). These aromatic plants were selected for their promising results in the *in vivo* studies conducted in NatLab.

2.1.1.2.1. Oregano (*Origanum vulgare*)

Oregano (*O. vulgare*) (Lamiaceae) is an aromatic herb widely distributed across Asia, Europe, and northern Africa that is used to treat respiratory and urinary tract disorders, dyspepsia, menstrual pain, rheumatoid arthritis, and scrofulosis, in folk medicine [44]. The chemical profile of *O. vulgare* EO (OvEO) from diverse geographical locations has been characterized by several authors [44–48], with

thymol (Fig. 7a) and carvacrol (Fig. 7b) typically identified as the major components. Variations in the chemical composition of OvEO may be related to differences in environmental and climatic conditions, seasonal sampling periods, geographic origins, plant populations, growth stages, and extraction and quantification methods [44]. Oregano EO exhibits several biological properties, including antimicrobial, antibiofilm, and antioxidant activities [49]; however, this study will only focus on its ovicidal, larvicidal, insecticidal, repellent, and nematicidal effects.

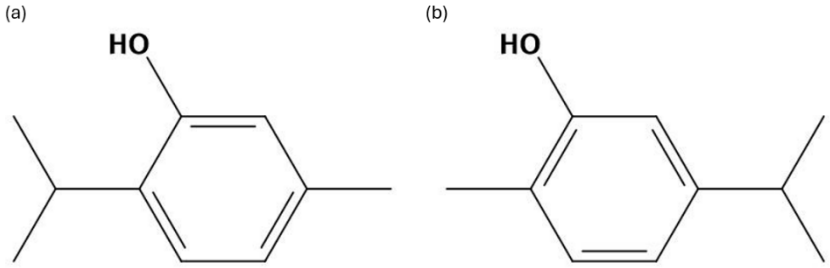


Figure 7 – Chemical structure of thymol (a) and carvacrol (b).

Gong and Ren [45] evaluated the larvicidal and ovicidal activities of OvEO and its major components – carvacrol (78.35%), p-cymene (6.85%), and γ-terpinene (3.70%) – against the cotton bollworm, *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae). In larvicidal activity assay, OvEO exhibited a median lethal concentration (LC_{50}) value of 265.51 µg/mL, while carvacrol, p-cymene, and γ-terpinene showed higher efficacy against *H. armigera*, with LC_{50} values of 51.53 µg/mL, 121.32 µg/mL, and 150.15 µg/mL, respectively; additionally, median effective concentration (EC_{50}) values of carvacrol, p-cymene, and γ-terpinene against *H. armigera* eggs were 33.48 µg/mL, 47.85 µg/mL, and 56.54 µg/mL, respectively [45].

Oliveira et al. [46] assessed the larvicidal, adulticidal, and repellent activities of OvEO – with terpinene-4-ol (17.4%), carvacrol (16.0%), and thymol (10.4%) as main constituents – against *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae). OvEO proved to be toxic to larvae (LC_{50} = 37.5 µg/mL) and adult mosquitoes (LC_{50} = 14.3 µg/mL); in addition, the EO showed a significant repellence effect with values ranging from 8.9% to 37.8% [46].

Amora et al. [47] evaluated the uptake of OvEO (77.0% carvacrol and 4.3% thymol) by tomato plants to assess its effect on the development of root-knot nematodes within the roots – *Meloidogyne javanica* (Treub, 1885) (Tylenchida: Heteroderidae). *In vitro* assays demonstrated that oregano EO has a strong nematicidal activity, causing over 99% mortality of *M. javanica* juveniles and reducing egg hatching to approximately 2%, regardless of the concentration used; however, in the *in vivo* assays, foliar application of the EO did not reduce the number of galls or *M. javanica* eggs, independently of the dosage. Several factors may have hindered the EO's effectiveness *in vivo*, including the volatilization of active compounds before plant absorption, metabolic degradation of toxic components by the plant, or transformation by soil microorganisms into less active or non-nematicidal substances [47]. Consequently, although oregano EO exhibited potent nematicidal activity *in vitro*, it unexpectedly promoted *M. javanica* development in the *in vivo* study.

Barbosa et al. [48] studied the nematicidal activity of OvEO against the PWN (*B. xylophilus*). To evaluate the toxic effects of the EO, nematodes were exposed to 2 mg/mL of oregano EO for 24 hours, resulting in 100% mortality (LC_{100} = 1.984 ± 0.008 mg/mL) [48].

2.1.1.2.2. Thyme (*Thymus vulgaris*)

Thyme (*T. vulgaris*) (Lamiaceae) is a well-known aromatic plant native to Southern Europe and Southern Italy [46]. *T. vulgaris* EO (TvEO) is an enriched source with a wide variety of aromatic bioactive components such as thymol (Fig. 7a) – usually the major component – and carvacrol (Fig. 7b). Several authors have described the chemical profile of TvEO, which depends on the growing conditions [46,47,50,51]. Even though thyme EO has a variety of biological properties, including antimicrobial, antibiofilm, and antioxidant activities [52], this study will focus on its insecticidal, nematicidal, and repellent effects.

Oliveira et al. [46] assessed the larvicidal, adulticidal, and repellent activities of TvEO – with thymol (40.0%), p-cymene (19.3%), and γ-terpinene (17.3%) as main

components – against *A. aegypti*. TvEO was toxic to larvae (LC_{50} = 38.9 µg/mL) and adult mosquitoes (LC_{50} = 11.7 µg/mL); in addition, the EO presented a significant repellency activity with values ranging from 4.4% to 68.9% [46].

Lazarević et al. [50] conducted a study that aimed to assess residual contact toxicity and effects of TvEO (43.52% thymol, 31.65% p-cymene, 5.38% linalool, and 5.11% carvacrol) on the longevity, oviposition, and adult emergence of the bean weevil – *Acanthoscelides obtectus* (Say, 1831) (Coleoptera: Chrysomelidae) – one of the main pests of stored bean seeds. Exposure to TvEO reduced adult survival and longevity, induced damage to lipids and proteins, and depleted protein and non-protein thiols in a concentration-dependent manner; sub-lethal concentrations of TvEO affected oxidative stress indices, prevented females from laying eggs, and strongly inhibited adult emergence [50].

Szczepanik et al. [51] evaluated the insecticidal activity of TvEO (57.44% thymol and 2.80% carvacrol), thymol, and carvacrol against various larval stages of the flour maggot – *Alphitobius diaperinus* (Panzer, 1797) (Coleoptera: Tenebrionidae). The insecticidal activity of TvEO and pure monoterpenes against *A. diaperinus* larvae depended on the dose and the age of the larvae – only the growth of younger larvae was significantly affected: the application of 2% acetone solution TvEO, thymol, and carvacrol caused mortality of 62.5%, 91.67%, and 97.5%, respectively [51].

Amora et al. [47] also evaluated the uptake of TvEO (56.0% thymol and 31.0% p-cymene) by tomato plants. *In vitro* assays demonstrated that TvEO has a strong nematicidal activity, causing over 99% mortality of *M. javanica* juveniles and reducing egg hatching to approximately 3%. So, TvEO proved to be highly toxic to nematodes *in vitro*.

Gamal et al. [53] assessed the nematicidal activity of TvEO against *B. xylophilus* nematodes. At a concentration of 500 ppm, TvEO caused 99.3%, 99.5%, and 100% mortality of PWN after 24, 48, and 72 hours, respectively.

2.1.1.2.3. Clove Basil (*Ocimum gratissimum*)

O. gratissimum (Lamiaceae) is an aromatic plant that is popularly known as clove basil and commonly found in Africa, Asia, and South America. Its pharmacological properties are associated with its EO, which is concentrated mainly in the stem, leaves, and flowers, and is composed mainly of eugenol (Fig. 8), linalool, camphor, and thymol (Fig. 7a) [54]. In addition to its various biological properties – antioxidant, anxiolytic, antinociceptive, neuroprotective, antimicrobial activities, among others [55] – *O. gratissimum* EO (OgEO) also exhibits repellent, insecticidal, and nematicidal properties.

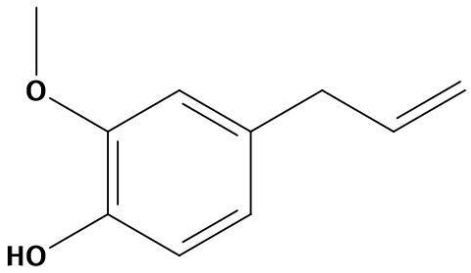


Figure 8 - Chemical structure of eugenol.

Essoung et al. [56] evaluated the repellency and smoke toxicity of OgEO (39.5% methyl eugenol and 29.6% eugenol) against adult stages of the tomato moth – *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae). OgEO demonstrated significant repellent activity and toxicity, probably due to its eugenol content (LC_{50} = 0.24 µL/mL; 83.3% mortality at 1 µL/mL after 24 hours; RI_{50} = 0.15), highlighting its potential in the integrated control of tomato pests [56]. Ogendo et al [57] also investigated the fumigant toxicity and repellent effects of OgEO and its constituents, β-(Z)-ocimene and eugenol, against adults of five pests that attack stored food products – *Sitophilus oryzae* (Linnaeus, 1763) (Coleoptera: Curculionidae); *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae); *Oryzaephilus surinamensis* (Linnaeus, 1758) (Coleoptera: Silvanidae); *Rhyzopertha dominica* (Fabricius, 1792) (Coleoptera: Bostrichidae); and *Callosobruchus chinensis* (Linnaeus, 1758) (Coleoptera: Chrysomelidae) – and concluded that OgEO and its constituents are potential alternatives to synthetic fumigants in the treatment of

durable agricultural products. Except for *T. castaneum*, which was more tolerant, LC_{50} values for tested insects ranged from 0.20 to 14 mL/L, 0.01 to 17 mL/L, and 0.80 to 23 mL/L air 24 hours after treatment for OgEO, eugenol, and β-(Z)-ocimene, respectively [57]. More recently, Liu et al. [58] assessed the insecticidal activity of OgEO and eugenol (63.04%), its major compound, against two storage pests: *T. castaneum* and *Liposcelis bostrychophila* (Badonnel, 1931) (Psocodea: Liposcelididae). Fumigation toxicity tests showed that OgEO has limited effectiveness against *T. castaneum* but great toxicity toward *L. bostrychophila*; contact toxicity assays revealed that eugenol alone was more effective than OgEO; and repellency tests indicated that OgEO repelled both pests to varying degrees, depending on the concentration and exposure time [58].

Ferreira et al. [59] evaluated the nematicidal and nematostatic activities of EOs derived from plant species native to the Macaronesia region or commonly used as culinary herbs in Mediterranean cuisine, by assessing their effects through direct contact and hatching bioassays on RKN *M. javanica*, root-lesion nematode *Pratylenchus penetrans* (Cobb, 1917) (Tylenchida: Pratylenchidae), and PWN *B. xylophilus*. Eugenol-rich OgEO (94.56%) induced more than 90% mortality in *M. javanica* but exhibited low activity against *P. penetrans* and *B. xylophilus*, with mortality rates lower than 30%, while also showing an attractive response [59].

2.1.1.2.4. Pennyroyal (*Mentha pulegium*)

M. pulegium (Lamiaceae), commonly known as pennyroyal, is an aromatic herb native to the Middle East, Europe, and North Africa [60]. Extracts obtained from this plant, including the EO, have traditionally been used in the last centuries in food preparation and also as antiseptic, antispasmodic, carminative, anti-inflammatory, antimicrobial, aromatic stimulant, analgesic, abortifacient, and as a traditional insecticide and insect repellent [60]. The *M. pulegium* EO (MpEO) is characterized by a high content of oxygenated monoterpenes, and the different chemotypes are defined by the predominant secondary metabolite – the most common and characteristic is the pulegone (Fig. 9a) chemotype; however, the less frequent piperitone, menthol (Fig. 9b), menthone, and piperitone oxide chemotypes also

exist [60]. This study will only focus on the pulegone chemotype of MpEO and on its repellent, insecticidal, and nematicidal effects.

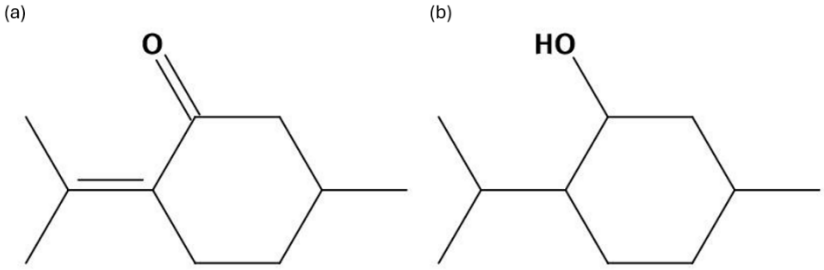


Figure 9 – Chemical structure of pulegone (a) and menthol (b).

Miguel et al. [61] studied the effects of a MpEO, mainly constituted by menthol, menthone, isomenthone, and pulegone, on *C. capitata* adults. Soon after exposure, the Medflies showed obvious signs of loss of motor coordination and difficulty in flying, and after 48 hours, over 90% of mortality was achieved, suggesting that MpEO produces a potent depressive effect on the fly's nervous system [61].

Ntalli et al. [62] evaluated the nematicidal activity of MpEO and its main constituent, pulegone (51.5%), against *M. incognita* during three immersion periods (24, 48, and 96 hours). MpEO exhibited high nematicidal activity against *M. incognita* (EC_{50} (96 h) = 3.15 µL/mL); however, pulegone, tested individually, was more active than as a component in EO (EC_{50} (24 h) = 150 µg/mL), implementing antagonistic action [62].

Ferreira et al. [59] also assessed the nematicidal and nematostatic activities of MpEO by evaluating its effects through direct contact and hatching bioassays on *M. javanica*, *P. penetrans*, and *B. xylophilus*. MpEO, containing primarily pulegone (54.26%) and menthol (31.90%), displayed strong nematicidal activity against *M. javanica* (induced more than 90% mortality) but only moderate effects against *P. penetrans* and *B. xylophilus*, and was classified as repellent for *M. javanica* J2 [59]. In addition, MpEO was the most effective EO in hindering egg hatching (64.78% ± 4% after 216 hours), due to the abundance of pulegone and menthol [59].

2.1.1.2.5. Cinnamon (*Cinnamomum cassia*)

The genus *Cinnamomum* (Lauraceae), with 250 species of trees and shrubs distributed in Southeast Asia, China, and Australia, includes well-known species such as the camphor tree (*Cinnamomum camphora*), the true cinnamon (*Cinnamomum verum*, synonym *Cinnamomum zeylanicum*), and the Chinese cassia (*Cinnamomum cassia*) [63]. This study will only focus on *C. cassia* since it is the most common and available type of cinnamon in international trade. The chemical profile of *C. cassia* EO (CcEO) is mainly constituted by *trans*-cinnamaldehyde (Fig. 10), which content can vary according to factors as pedoclimatic conditions, plant age, and method of EO extraction [63]. Along with insecticidal and nematicidal activities, this EO is very well studied for other applications, such as antimicrobial and antioxidant effects [64].

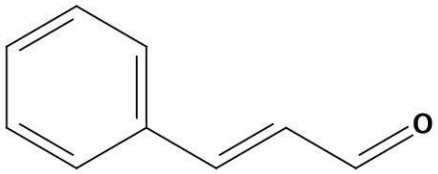


Figure 10 – Chemical structure of *trans*-cinnamaldehyde.

Liu et al. [65] investigated the insecticidal activity of CcEO and its main constituent compound, *trans*-cinnamaldehyde (49.33%), against the booklice, *L. bostrychophila*. CcEO displayed contact and fumigant toxicity against adult *L. bostrychophila* with LC_{50} values of 55.68 μg/cm^2 and 1.33 mg/L air, respectively; additionally, *trans*-cinnamaldehyde exhibited strong contact and fumigant toxicity with LC_{50} values of 43.40 μg/cm^2 and 1.29 mg/L air, respectively [65]. Minozzo et al. [66] also evaluated the insecticidal efficacy of CcEO (86.4% *trans*-cinnamaldehyde) against *Sitophilus zeamais* (Motschulsky, 1855) (Coleoptera: Curculionidae) in maize: median lethal dose (LD_{50}) values of 8.90 μL/cm^3 for free EO and 24.76 mg/cm^3 for microencapsulated EO.

Jardim et al. [67] studied CcEO and its main component, *trans*-cinnamaldehyde (83.3% of the oil), as a potential natural nematicide against *M.*

incognita. Emulsions of CcEO (500 µg/mL) and *trans*-cinnamaldehyde (416 µg/mL) reduced *M. incognita* galls and eggs in soybean plants to levels comparable to those treated with the chemical nematicide carbofuran (416 µg/mL); furthermore, vapors of CcEO and *trans*-cinnamaldehyde were also as active as the fumigant nematicide Basamid against *M. incognita* [67]. D'Addabbo et al. [63] also investigated the activity of CcEO and *trans*-cinnamaldehyde (93.74% of the oil) on J2 and eggs of *M. incognita*. A 24 hour exposure to a 12.5 µg/mL solution of *trans*-cinnamaldehyde resulted in more than 68% mortality of *M. incognita* J2, while a poor mortality occurred at the same concentration of the whole EO; egg hatch was reduced by more than 90% after exposing *M. incognita* egg masses to 800 µg/mL of both EO and *trans*-cinnamaldehyde for 24 hours; soil treatments with both products significantly reduced *M. incognita* infestation in tomato plants, with *trans*-cinnamaldehyde generally proving more effective than the whole EO [63]. Kong et al. [68] evaluated the nematicidal activity of CcEO and its main component, *trans*-cinnamaldehyde (80.2%), against *B. xylophilus* adults using a direct contact bioassay. After 24 hours, the obtained LC_{50} values confirmed that CcEO (LC_{50} = 0.084 mg/mL) was toxic to the nematodes, with *trans*-cinnamaldehyde (LC_{50} = 0.057 mg/mL) showing the highest nematicidal activity [68]. These findings indicate that *trans*-cinnamaldehyde is the principal nematicidal agent in CcEO and that both products are potential environment-friendly nematicides.

3. Inhibitory Effects of Essential Oils on Enzymes Relevant to Insect and Nematode Development

EOs and their components have demonstrated potent insecticidal and/or nematicidal effects against various agricultural and household pests, as described above. However, the mechanisms of action of these EOs remain unclear, and their understanding is crucial for the effective selection of potent insecticidal and/or nematicidal agents. Therefore, it is important to study the interactions between EOs and enzymes relevant to insect and nematode development, such as neurochemical and digestive enzymes. In the present work, the effects of the EOs

and their main components on acetylcholinesterase (AChE), α-glucosidase (α-GLU), and β-glucosidase (β-GLU) activity in the target insects and nematodes were studied.

3.1. Acetylcholinesterase

AChE (E.C. 3.1.1.7) is a serine hydrolase that terminates impulse transmission at cholinergic synapses via rapid hydrolysis of the neurotransmitter acetylcholine (ACh) into acetic acid and choline (Equation 1), regulating ACh levels in mammals, fish, birds, insects, and other species with cholinergic nerves [69]. Since AChE inhibition elevates the level of ACh in the synaptic cleft and consequently prevents or interrupts neurotransmission depending on the mode of inhibition, AChE is a target for neuroactive insecticides that rapidly incapacitate insect pests [69]. AChE is one of the most important enzymes in neuro-neuronal and neuromuscular junctions in both insects and mammals [70]. Many studies have shown that EOs can inhibit its activity in various plants [71–74]. Since the insect AChE differs from the mammalian one by a single residue, known as the insect-specific cysteine residue, AChE can be an insect-selective target for the newly developed insecticides, safe for non-target vertebrates [70]. The inhibition of AChE activity by EOs is shown in Figure 11.

$$ACh + AChE \rightarrow AChE - Acetyl + Choline \rightarrow AChE + Acetic\ Acid$$

Equation 1 - Mechanism of action of AChE.

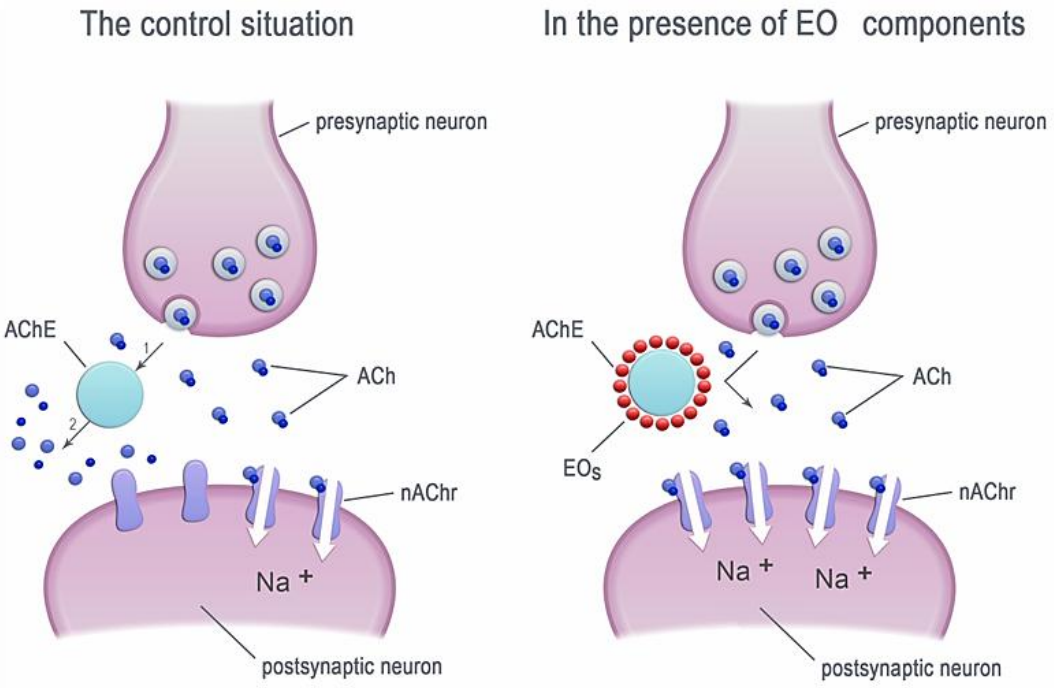


Figure 11 – Inhibition of AChE activity by EOs. Reproduced from Jankowska et al. [70]. Legend: AChE – acetylcholinesterase; ACh – acetylcholine; nAChr – nicotinic acetylcholine receptors; EOs – essential oil components.

Understanding the effectiveness of EOs in AChE inhibition requires the identification of their exact mode of action and type of inhibition. It is challenging to elucidate the mechanism of action of EOs because their activity as complex compounds differs from that of their individual components, which can function as competitive inhibitors (for example, pulegone and menthol) or as uncompetitive inhibitors [70]. Competitive inhibitors bind to the active site of AChE, blocking the access of ACh without affecting the enzyme's maximal activity. On the other hand, uncompetitive inhibitors bind to allosteric sites of AChE, preferentially interacting with the enzyme-substrate complex, which alters the enzyme's conformation, prevents product formation, and reduces the maximum activity of the enzyme [70]. These distinct modes of inhibition of EOs suggest the existence of multiple binding sites within the AChE molecule. Several authors have evaluated the anti-AChE activity of the EOs under study (Table 2) and their main components (Table 3). Analysis of Tables 2 and 3 indicates that the literature on insect-specific AChE is scarce, and even more so regarding nematode AChE.

Table 2 – AChE inhibitory values of *M. pulegium*, *O. vulgare,* and *T. vulgaris* EOs. Adapted from Asnaashari et al. [75].

EO	AChE source	AChE inhibitory values (IC_{50} or % inhibition)	Main components (%)	References
MpEO	*Tribolium castaneum*	*In vitro* AChE inhibition of EO: 85%	Menthone: 23.64 Pulegone: 21.70 Piperitenone: 12.09 Piperitone: 8.92	[71]
OvEO	*Electrophorus electricus*	*In vitro* AChE inhibition of EO at 1 mg/mL: 97.9% ± 1.56%	Not specified	[72]
TvEO	Not specified	IC_{50}: 4.13 mg/mL	Thymol: 41.70 p-Cymene: 35.70 Carvacrol: 7.10	[73]
	Electrophorus electricus	IC_{50}: 0.54 mg/mL	Thymol: 35.30 % p-Cymene: 34.70% Terpinene: 8.40% Carvacrol: 5.8%	[74]

Table 3 - AChE inhibitory values of EO components: carvacrol, *trans*-cinnamaldehyde, eugenol, menthol, and thymol. Adapted from Jankowska et al. [70] and Asnaashari et al. [75].

EO components	AChE source	AChE inhibitory values	Ref.
Carvacrol	*Aedes aegypti*	IC_{50}: 0.0012 mM	[76]
	Aedes albopictus	52.9% inhibition rate at 1 mg/mL	[77]
	Aedes albopictus	IC_{50}: 0.057 mg/mL	[78]
	Drosophila melanogaster	IC_{50}: 0.175 mM	[79]
	Drosophila suzukii	20% inhibition rate at 0.5 mg/mL	[80]
	Bursaphelenchus xylophilus	≈ 10% inhibition rate at 1 mg/mL	[81]

(Continues on next page)

Table 3 (cont.) - AChE inhibitory values of EO components: carvacrol, *trans*-cinnamaldehyde, eugenol, menthol, and thymol. Adapted from Jankowska et al. [70] and Asnaashari et al. [75].

EO components	AChE source	AChE inhibitory values	Ref.
Carvacrol	*Electrophorus electricus*	70.3% ± 1.24% inhibition rate at 1 mg/mL	[72]
		IC_{50}: 0.063 mg/mL	[74]
	Musca domestica	IC_{50}: 0.0012 mM	[76]
	Periplaneta americana	IC_{50}: 0.0004 mM	[76]
trans-Cinnamaldehyde	*Bursaphelenchus xylophilus*	> 20% inhibition rate at 1 mg/mL	[81]
Eugenol	*Bursaphelenchus xylophilus*	< 5% inhibition rate at 1 mg/mL	[81]
	Electrophorus electricus	IC_{50}: 0.48 mg/mL	[82]
	Blatella germanica	21% inhibition rate at 1 mg/mL	[83]
Menthol	*Bursaphelenchus xylophilus*	< 10% inhibition rate at 1 mg/mL	[81]
	Drosophila suzukii	< 10% inhibition rate at 0.5 mg/mL	[80]
	Sitophilus oryzae	< 10% inhibition rate at 1 mg/mL	[84]
Thymol	*Aedes albopictus*	46.7% inhibition rate at 1 mg/mL	[77]
	Drosophila melanogaster	IC_{50}: 25 mM	[79]
	Drosophila suzukii	42.9% and 34.5% inhibition rate for males and females at 0.5 mg/mL	[80]
	Bursaphelenchus xylophilus	≈ 10% inhibition rate at 1 mg/mL	[81]
	Electrophorus electricus	28.4% ± 0.64% inhibition rate at 1 mg/mL	[72]
		IC_{50}: 0.740 mg/mL	[74]
	Ephestia kuehniella	IC_{50}: 0.137 µg/mL	[85]

3.2. α- and β-Glucosidases

The insect gut is the major interface between them and their environment; therefore, the study of insect digestive enzymes and their interaction with their substrates is crucial for developing effective insect control methods [86]. The study of insects' carbohydrates, especially in herbivorous species, enhances the knowledge of digestive biochemistry and also supports the development of pest management strategies [86]. Several studies have demonstrated that EOs can prevent insect feeding (antifeedant activity) [87–89]. This effect may result from the inhibition of enzymes that play a key role in digestion, such as α- and β-glucosidases; however, these enzymes remain largely underexplored as potential targets for insecticide development.

α-Glucosidase (α-GLU), E.C. 3.2.1.20, is an exohydrolase enzyme that catalyzes the hydrolysis of 1,4-α-glucosidic linkages, releasing α-glucose (Fig. 12); in other words, α-GLU hydrolyzes terminal non-reducing 1,4-linked α-glucose residues to release a single α-glucose molecule [86].

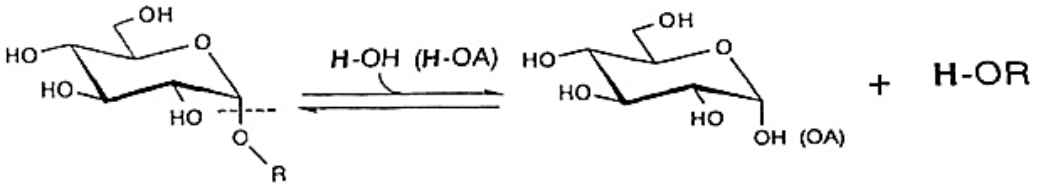


Figure 12 – Mechanism of action of α-GLU. Reproduced from Seddigh and Darabi [86].

Cellulose, a linear polymer consisting of β-1,4-linked D-glucopyranosyl units, is synthesized by all higher plants and represents the most abundant structural compound of their cell walls [90]. Cellulase is a general term for cellulolytic enzymes, of which three classes are recognized based on the mode of enzymatic actions and the substrate specificities: endoglucanases (E.C. 3.2.1.4, cleave amorphous sites of cellulose chains at random), exoglucanases (E.C. 3.2.1.74 and 3.2.1.91, act on the nonreducing or reducing termini of cellulose fibers to release either cellobiose or glucose), and β-glucosidases (β-GLU, E.C. 3.2.1.21, hydrolyze

cellobiose or cello-oligomers to glucose – Equation 2) (Fig. 13) [90]. The β-GLU role in insect digestion is to hydrolyze the terminal, nonreducing β-D-glucosyl residues by hydrolysis of the β-1,4 glycosidic bond of various glycoconjugates, including glucosides, oligosaccharides, and 1-O-glucosyl esters, to form glucose [91].

$$Cellobiose + H_2O \rightarrow 2\ Glucose\ \boldsymbol{or}\ Oligosaccharide + H_2O \rightarrow Glucose$$

Equation 2 – Mechanism of action of β-GLU.

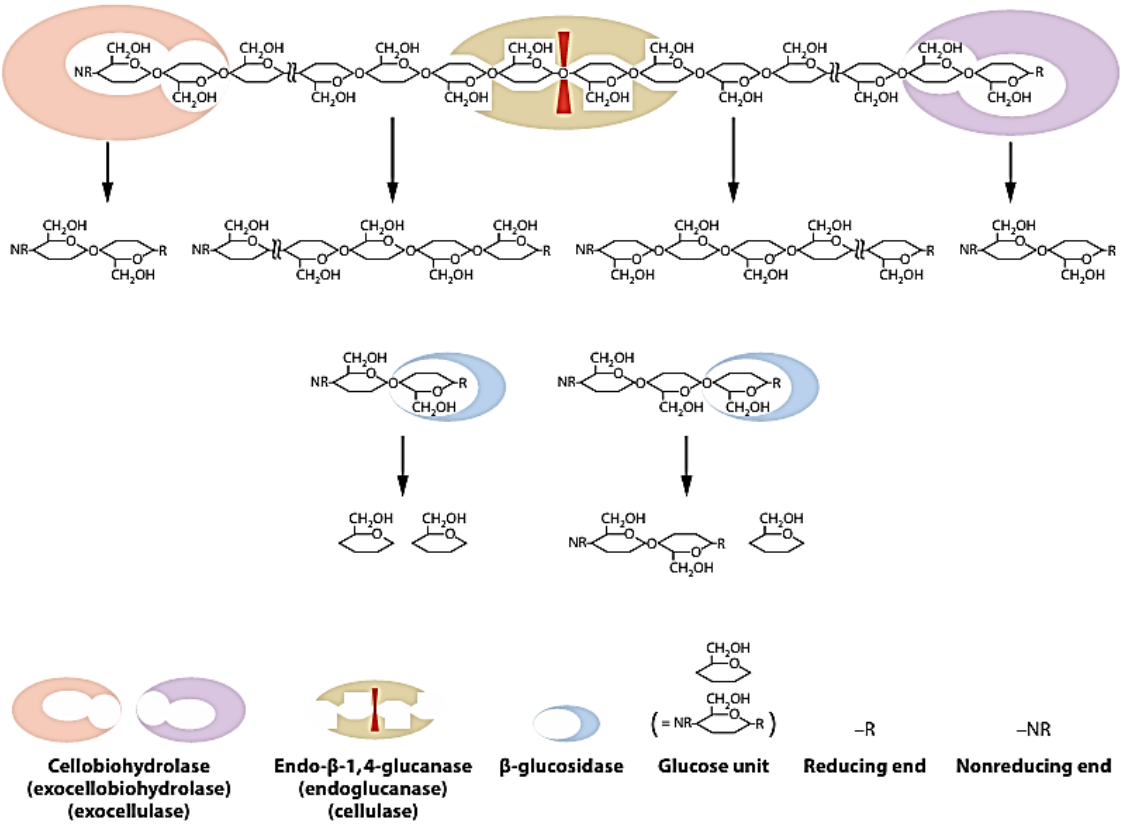


Figure 13 – Schematic view of the biodegradation of cellulose. Reproduced from Watanabe and Tokuda [90].

The inhibitory potential of EOs on these digestive enzymes has been minimally explored. Sarikurkcu et al. [92] reported that OvEO inhibited 6.04 ± 0.91 mmol ACEs/g (acarbose equivalents per gram) of α-GLU. Ali et al. [93] reported that TvEO strongly inhibited α-GLU, showing an IC_{50} value of 125.1 ± 4.25 µg/mL.

Magierowicz et al. [94] proved that the treatment with pure carvacrol induced α- and β-GLU activity in tissues of *Acrobasis advenella* (Zincken, 1818) (Lepidoptera: Pyralidae), indicating that the toxicity mechanism of carvacrol is associated with the disruption of carbon metabolism and/or osmoregulation in the insect body. Since α-GLU can catalyze the production of monosaccharides and the synthesis of glucose-dominated oligosaccharides, the direct consequences of an increase in its activity can vary [94]. Carvacrol significantly affected β-GLU activity in the insect homogenates, resulting in an increase in its activity to 214.67% compared to the control, which could also impact sugar metabolism and osmoregulation in *A. advenella* tissues, as β-GLU can hydrolyze certain oligosaccharides [94].

4. Objectives of the Dissertation

This dissertation aimed to investigate the interactions between EOs from selected aromatic plants (*O. vulgare*, *T. vulgaris*, *O. gratissimum*, *M. pulegium*, and *C. cassia*) and enzymes relevant to insect and nematode development, namely AChE, α-GLU, and β-GLU. The study was conducted using crude enzyme extracts, without purification, obtained from the chosen insects (*C. capitata* and *M. unipuncta*) and nematodes (*M. incognita* and *B. xylophilus*). Enzyme activity and soluble protein content were quantified using standard assays, and SDS-PAGE was performed to attempt protein characterization. The extracts and standard enzymes were then subjected to inhibition assays to evaluate the inhibitory effects of the EOs and their major components, thus contributing to the elucidation of their mechanisms of action on key developmental enzymes.

II. Experimental Procedure

1. Materials and Sample Collection

1.1. Reagents, Standards, Enzymes, and Equipment

The list of reagents and standards, enzymes, and equipment used, along with their relevant information, is described in the Supplementary Information Section Table S1, S2, and S3, respectively.

1.2. Aromatic Plants

The selected aromatic plants were obtained from various sources: *O. vulgare* and *T. vulgaris* were acquired from the local market of Funchal, Madeira Island, Portugal; *O. gratissimum* seeds were collected in *Jardim das Plantas Aromáticas e Medicinais* – Funchal, Madeira Island, Portugal, and cultivated at the facilities of *Centro de Floricultura do Governo Regional da Madeira*; *M. pulegium* was obtained from the experimental fields of *Escola Superior Agrária de Elvas*, Portalegre, Portugal; and *C. cassia* is a commercial sample, bought in a local supermarket. *O. gratissimum* leaves were dried for approximately 48 h in a ventilated food dehydrator at 40 ºC, whereas the remaining samples were obtained already in the dried form.

1.3. Insects and Nematodes

Both armyworm (*M. unipuncta* larvae) and Medfly (*C. capitata* adults) were obtained from established colonies of the Biotechnology Centre of Azores (University of Azores, São Miguel Island, Portugal). Nematodes (*M. incognita* J2 and *B. xylophilus*) were obtained from established colonies of NEMATO-lab (Centre for Functional Ecology, University of Coimbra, Portugal). No separation according to sex was performed. Both insects and nematodes were sent to NatLab in the lyophilized form.

2. Essential Oil Extraction and Characterization

The EO extraction and characterization by Gas Chromatography – Flame Ionization Detector (GC-FID) was carried out according to the procedure established in NatLab for previous studies [59].

2.1. Essential Oil Extraction

The dried plant material was ground into a fine powder using a mechanical grinder. EOs were obtained by hydrodistillation for 4 h at a ratio of 1:20 (w/v) using a Clevenger apparatus, dried with magnesium sulphate, and stored at -20 ºC [59].

2.2. Gas Chromatography – Flame Ionization Detector (GC-FID) Conditions

The composition of the EOs was determined by GC-FID, following the procedure described by Ferreira et al. [59]. Samples were prepared by dissolving 20 mg of each EO in 1 mL of n-hexane and subsequently analyzed using a GC-FID method, performed on an Agilent 7890A GC System equipped with an Agilent 7693 autosampler, a SPB™-FA fused silica capillary column (30 m × 0.25 mm × 0.2 µm), and an FID detector. Helium served as the carrier gas at a constant flow rate of 800 µL/min; the oven temperature was initially set at 60 ºC for 2 minutes, then increased to 220 ºC at a rate of 2 ºC/min, and held at this final temperature for 20 minutes, resulting in a total runtime of 102 minutes; both the injector and FID detector temperatures were maintained at 250 ºC [59]. A 1 µL aliquot of the EO–hexane mixture was injected in split mode with a 60:1 ratio and a 4-minute delay; the FID detector received air and hydrogen at flow rates of 400 mL/min and 40 mL/min, respectively [59]. All measurements were performed in duplicate. Data acquisition and analysis were carried out using proprietary software version A.01.04. The identification of the EO compounds was performed by injecting several standards and comparing their retention times with those observed in the EO phytochemical profile.

3. Biochemical Analysis of Insect and Nematode Protein Extracts

3.1. Extraction of Protein-Rich Extract

The extraction procedure was adapted from Brogan et al. [95]. Freeze-dried insect and nematode samples were ground separately into a fine powder using a mortar. For insect protein extraction, the powdered material was weighed and suspended in 0.1 M sodium phosphate buffer (pH 7.0) to obtain a final concentration of 2 mg/mL. For nematode protein extraction, the suspension was prepared at a final concentration of 0.1 mg/mL in the same buffer. Extraction was performed at room temperature for 30 minutes under constant stirring. The mixture was then centrifuged at 10,000 × g for 10 minutes at room temperature, and the resulting supernatant was collected, aliquoted, and stored at -80 ºC for further analyses. The supernatant was used as an enzyme source.

3.2. Protein Quantification

The Bradford dye-binding method [96] was used to determine the concentration of soluble protein in insect extracts. For this purpose, 1.5 mL of Bradford reagent was added to 50 µL of insect extract and, after 10 minutes of incubation, the absorbance of the solution was measured at 595 nm. Each extract was analyzed in triplicate. A calibration curve was prepared using bovine serum albumin (BSA) standard solutions (serially diluted in 0.1 M sodium phosphate buffer, pH 7.0), following the protocol described above. The BSA calibration line ($y = 1.169x + 0.038$, $R^2 = 0.984$) is displayed in the Supplementary Information Section, Figure S2.

The Bradford method did not allow quantification of soluble protein content in the nematode extracts, so protein concentration was estimated by measuring absorbance at 280 nm using a Nanodrop spectrophotometer, assuming that an absorbance unit corresponds to 1 mg/mL of protein.

3.3. Enzymatic Activity Assays

3.3.1. Acetylcholinesterase Activity Assay

This protocol is based on the work of Ellman et al. [97] and bioactive compound – enzyme interaction and was adapted from Barreto and Simões [98]. The activity of AChE is measured using a thiol analogue of the natural substrate – acetylthiocholine iodide (ATChI). Enzymatic catalysis of ATChI results in thiocholine, which reacts with Ellman's reagent (dithiobis-2-nitrobenzoic acid, DTNB), resulting in 5-thio-2-nitrobenzoic acid (TNB), which is strongly yellow and can be quantified by measuring the absorbance at 405 nm (Equation 3) [98].

$$ATChI \rightarrow Acetate + Thiocholine$$

$$Thiocholine + DTNB \rightarrow TNB\ (yellow)$$

Equation 3 – Mechanism of action of the AChE activity assay.

In a 96-well microplate, 20 µL of protein extract (concentration of 1 mg/mL for insect extract, 0.025 mg/mL for RKN extract, and 0.0125 mg/mL for PWN extract) and 20 µL of substrate solution were added to 230 µL of 0.1 M sodium phosphate buffer (pH 8.0). The substrate solution consists of a mixture of equal parts of 3 mM DTNB in 0.1 M sodium phosphate buffer (pH 8.0) and 75 mM ATChI in 0.1 M sodium phosphate buffer (pH 8.0). Using a microplate reader, the mixture was stirred and incubated at 37 ºC, and the absorbance was measured at 405 nm at 0 and 20 minutes. The sample blank was prepared by replacing the substrate solution with 0.1 M sodium phosphate buffer (pH 8.0). Each sample was analyzed in triplicate. The AChE activity was determined according to Equation 4.

$$EA\ (U) = \frac{\Delta A \times V_{total}}{\varepsilon \times l \times V_{enz} \times t} = \frac{\Delta A \times 0.27}{14.15 \times 0.61 \times 0.02 \times 20}$$

Equation 4 – Calculation of AChE activity. Legend: EA – enzymatic activity; U – enzyme unit; ΔA – mean absorbance variation; V_{total} – total volume (mL); ℰ - molar extinction coefficient of the product ($M^{-1}\ cm^{-1}$); l - optical path (cm); V_{enz} – enzyme volume (mL); t – incubation time (min).

3.3.2. α-Glucosidase Activity Assay

The α-GLU activity was determined by measuring the amount of p-nitrophenyl hydrolyzed by the enzyme from p-nitrophenyl-α-D-glucopyranoside (α-pNPG) [99]. This assay was performed using a previous methodology [99] with some adaptations [100].

In a 96-well microplate, 50 µL of protein extract (concentration of 1 mg/mL for insect extract, 0.025 mg/mL for PKN extract, and 0.0125 mg/mL for PWN extract), 50 µL of 5 mM α-pNPG solution, and 50 µL of 0.1 M sodium phosphate buffer (pH 7.0) were mixed [100]. Using a microplate reader, the mixture was stirred and incubated at 37 ºC, and the absorbance was measured at 405 nm at 0 and 20 minutes. The sample blank was prepared by replacing the substrate solution with 0.1 M sodium phosphate buffer (pH 7.0). Each sample was analyzed in triplicate. The α-GLU activity was determined according to Equation 5.

$$EA\ (U) = \frac{\Delta A \times V_{total}}{\varepsilon \times l \times V_{enz} \times t} = \frac{\Delta A \times 0.15}{18 \times 0.61 \times 0.05 \times 20}$$

Equation 5 – Calculation of α- and β-GLU. Legend: EA – enzymatic activity; U – enzyme unit; ΔA – mean absorbance variation; V_{total} – total volume (mL); ε - molar extinction coefficient of the product ($M^{-1}\ cm^{-1}$); l - optical path (cm); V_{enz} – enzyme volume (mL); t – incubation time (min).

3.3.3. β-Glucosidase Activity Assay

The β-GLU activity was estimated by measuring the amount of p-nitrophenyl released from p-nitrophenyl-β-D-glucopyranoside (β-pNPG) by the enzyme. This assay was carried out as above (section 3.3.2) using β-pNPG as substrate [101]. The β-GLU activity was determined according to Equation 5.

3.4. SDS-PAGE

The electrophoretic pattern of protein in the insect protein extracts was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [95]. A new extraction of insect protein was carried out at a concentration of

8 mg/mL, following the procedure described in section 3.1. The soluble protein present in the extract was quantified using the Bradford method, and its enzymatic activity (AChE, α- and β-GLU) was calculated. The concentration (5%) and separation (12%) gels were then prepared, and the electrophoresis system was set up. To prepare the samples for loading, 50 µL of Laemmli Buffer 4x was added to 150 µL of the sample, and the mixture was heated to 90 ºC for 5 minutes [95]. To evaluate optimal band resolution, 10 µg, 15 µg, and 20 µg of protein were loaded into separate wells, and the molecular weight markers – PageRuler™ Prestained Protein Ladder (180 to 10 kDa) and Novex® Sharp Pre-Stained Protein Standard (250 to 3.5 kDa) – were applied to the tips of the gel. A voltage of 57 V was applied for 10 minutes, then continued at 200 V for 1 hour without interruption. The gel obtained was stained for 15 minutes under constant agitation with a staining solution containing Coomassie Brilliant Blue G-250 and then bleached until the background was colorless. Finally, the bands formed were photographed and analyzed using GelAnalyzer software [102].

4. *In vitro* Inhibition Assays

4.1. Acetylcholinesterase Inhibition Assay

The AChE inhibition assay was performed as described by Barreto and Simões [98], with slight modifications. The mechanism of action of this assay was described above (Equation 3).

Stock solutions of EOs and standards were prepared in ethanol at concentrations of 50 mg/mL and 25 mg/mL, respectively [98]. In an Eppendorf, 12 µL of stock solution was added to 1988 µL of 0.1 M sodium phosphate buffer (pH 8.0) to prevent any solvent-related inhibition of the enzyme. In a 96-well microplate, 270 µL of 0.1 M sodium phosphate buffer (pH 8.0) was added to the wells of column 1 (blank), 240 µL of EO/standard solution was pipetted into the column 2-wells, and 120 µL of 0.1 M sodium phosphate buffer (pH 8.0) was added to the remaining wells (columns 3-12) [98]. Using a multichannel micropipette, 120 µL was transferred from column 2-wells to column 3-wells. After homogenization, the procedure was

repeated until column 11 (Fig. 14), followed by the addition of 110 µL of 0.1 M sodium phosphate buffer and 20 µL of AChE solution. The number of AChE units used in the assay varied according to the enzyme source: 0.25 U/mL for *Electrophorus electricus* (Lineu, 1766) (Gymnotiformes; Gymnotidae); 0.43 U/mL and 0.36 U/mL for *C. capitata* and *M. unipuncta*, respectively; and 0.13 U/mL and 0.07 U/mL for *M. incognita* and *B. xylophilus*, respectively. After a 5-minute incubation at 37 ºC, 20 µL of substrate mixture (equal parts of 3 mM DTNB and 75 mM ATChI, prepared in 0.1 M sodium phosphate buffer, pH 8.0) was pipetted into wells of columns 2-12 and the absorbance was read at 405 nm at 0 s, 150 s, 300 s, and 450 s using a microplate reader [98]. The control (column 12-wells) represented 100% enzyme activity. Galantamine hydrobromide was used as a positive control. Each assay was conducted in duplicate. Reaction rates (v, ΔAbs_{405nm}/min) were determined by measuring the variation in absorbance over time for each well [98], and the percentage inhibition was calculated as demonstrated in Equation 6. The inhibitory activity was expressed as the IC_{50} value (mg/mL), determined from the least-squares regression of the logarithmic concentrations plotted against the percent inhibition [100]. This value reflects the concentration of EO/standard needed to achieve a 50% reduction in enzyme activity compared to the control.

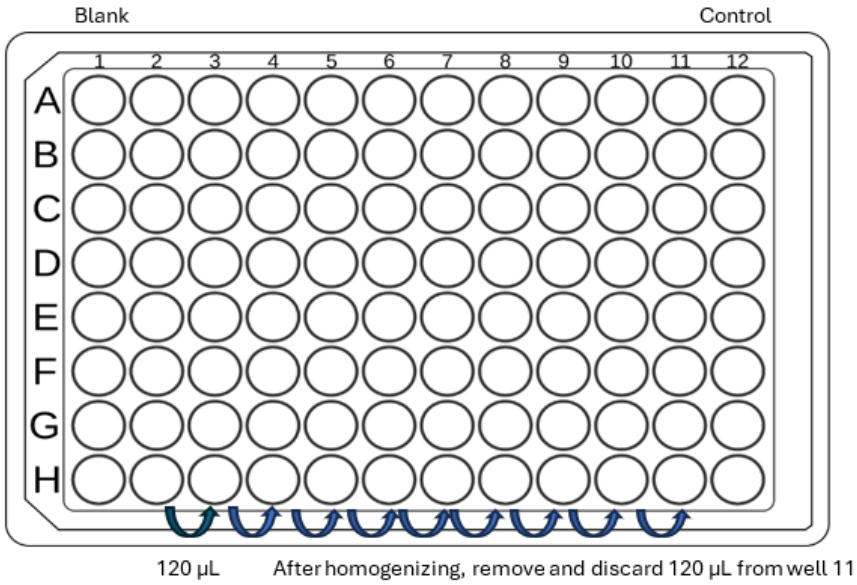


Figure 14 - Serial dilution from column 2-11. Column 1-wells are left for blanks, and column 12-wells for the control reaction without inhibitor. Reproduced from Barreto e Simões [98].

$$\% \, Inhibition = 100 - \frac{v_{sample}}{v_{control}} \times 100$$

Equation 6 – Calculation of the percentage of inhibition of AChE. Reproduced from Barreto e Simões [98]. Legend: v_{sample} – reaction rate of the sample; $v_{control}$ – reaction rate of the control.

4.2. α-Glucosidase Inhibition Assay

The inhibition of α-GLU activity was evaluated by measuring the amount of p-nitrophenyl produced from the enzymatic hydrolysis of α-pNPG. This assay was performed based on a previously established method [99] with some adaptations [100].

Stock solutions of EOs and standards were prepared in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL. In an Eppendorf, 12 µL of stock solution was added to 1988 µL of 0.1 M sodium phosphate buffer (pH 7.0) to prevent any solvent-related inhibition of the enzyme, and a serial dilution was prepared (Fig. 15). In a 96-well microplate, 50 µL of extract solution, 50 µL of 5 mM α-pNPG solution, and 50 µL of enzyme solution were mixed [100]. The concentration of the enzyme solution was adjusted based on the α-GLU source: 0.01 mg/mL for *Saccharomyces cerevisiae* (Hansen, 1888) (Saccharomycetales: Saccharomycetaceae), and 1 mg/mL for *C. capitata* (0.04 U/mL) and *M. unipuncta* (0.09 U/mL). After a 20-minute incubation at 37 ºC, 100 µL of sodium carbonate aqueous solution (0.1 M) was added, and the absorbance was read at 405 nm using a microplate reader [100]. Enzyme control (C) represented 100% enzyme activity (inhibitor replaced by buffer); appropriate sample blanks (SB) were included for each sample to correct background absorbance (substrate replaced by buffer); and control blank (CB) consisted of buffer and enzyme solution [101]. 1-deoxynojirimycin (1-DNJ) was used as a positive control. Each assay was conducted in triplicate. The percentage of inhibition was calculated using Equation 7. The inhibitory activity was expressed as the IC_{50} value (mg/mL), determined from the least-squares regression of the logarithmic concentrations plotted against the percent inhibition [100]. This value reflects the concentration of EO/standard needed to achieve a 50% reduction in enzyme activity compared to the control.

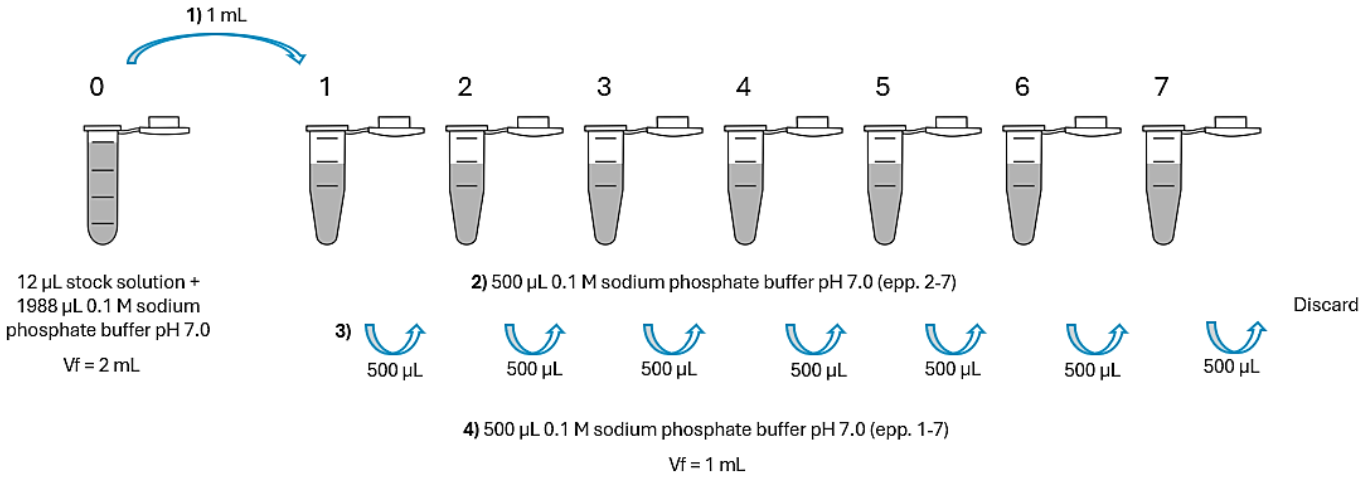


Figure 15 – Preparation step-by-step of a serial dilution.

$$\%\ Inhibition = \frac{(A_C - A_{CB}) - (A_S - A_{SB})}{(A_C - A_{CB})} \times 100$$

Equation 7 – Calculation of the percentage of inhibition of α- and β-GLU. Reproduced from Spínola [101]. Legend: A_C – absorbance of control; A_{CB} – absorbance of control blank; A_S – absorbance of sample; A_{SB} - absorbance of sample blank.

4.3. β-Glucosidase Inhibition Assay

The inhibition of β-GLU activity was assessed by measuring the amount of p-nitrophenyl produced from enzymatic hydrolysis of β-pNPG. This assay was carried out as above (section 4.2.), using β-pNPG as substrate [101]. The concentration of the enzyme solution varied according to the β-GLU source: 0.1 mg/mL for β-GLU from almonds, and 1 mg/mL for *M. unipuncta* (0.004 U/mL).

5. Molecular Docking

5.1. Preparation of Receptors

The crystal structures of AChE from *E. electricus* (code: 1C2O) with resolution 4.20 Å [103] and *Drosophila melanogaster* (Meigen, 1830) (Diptera: Drosophilidae) (code: 6XYS) with resolution 2.46 Å [104] were obtained through RCSB Protein Data Bank (PDB) (https://www.rcsb.org/) (Fig. 16). ChimeraX version 1.7.1 [105] was used to verify and remove non-standard residues from the receptor structures. Subsequently, AutoDockTools version 1.5.6 (ADT) [106] was utilized to convert the files from the PBD format to the PDBQT format, which includes partial charges and atom type specifications.

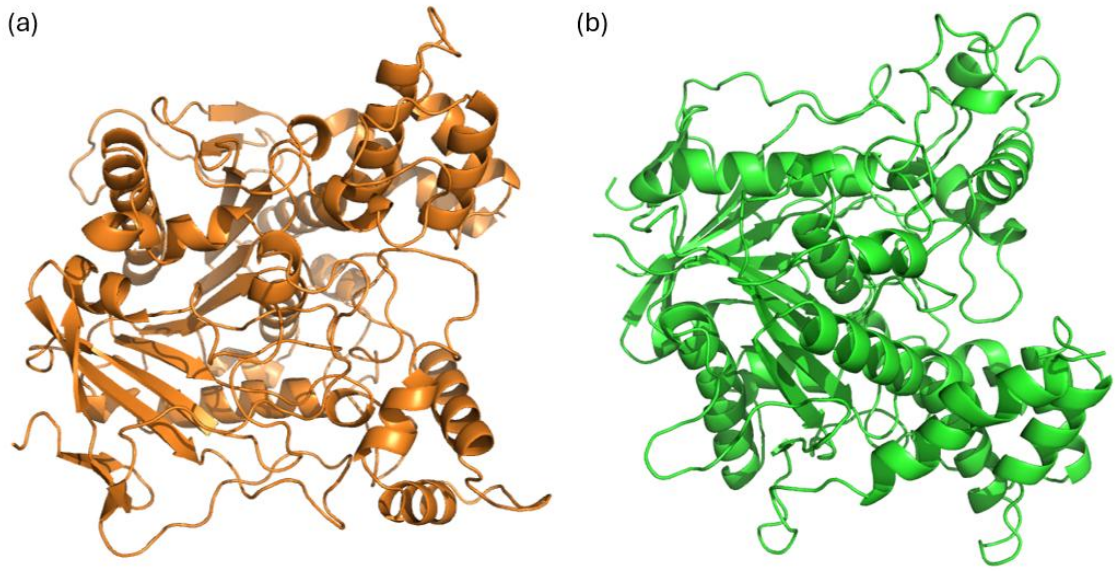


Figure 16 – Crystal structures of AChE from *E. electricus* (code: 1C2O) (a) and *D. melanogaster* (code: 6XYS) (b). The image was generated by Pymol version 3.0 [107].

5.2. Preparation of Inhibitors

Carvacrol (Fig. 7b), eugenol (Fig. 8), pulegone (Fig. 9a), *trans*-cinnamaldehyde (Fig. 10), and galantamine (Fig. S1, in the Supplementary Information Section) were selected for further investigation with molecular docking studies. The molecules were drawn in Avogadro version 1.2.0 [108], and their

geometry was optimized using the general AMBER force field (GAFF). ADT [106] was used to convert the files from the PBD format to the PDBQT format.

5.3. Molecular Docking Simulation

Docking simulations were performed on two receptor structures (1C2O and 6XYS) to analyze their interactions with five inhibitors: carvacrol, eugenol, pulegone, *trans*-cinnamaldehyde, and galantamine. A rigid docking approach was performed using AutoDock Vina [109,110] to evaluate ligand affinity and interaction profiles. Although this approach does not account for protein flexibility, it is widely accepted for screening purposes and was suitable given the static nature of the active site residues observed in the crystallographic structure. The grid boxes were configured to encompass the entire binding site. The configuration files were created to provide commands to AutoDock Vina [109,110], and this software was used to generate detailed ligand-receptor interactions, with binding affinity values (ΔG) expressed in kcal/mol. The results were analyzed using ChimeraX version 1.7.1 [105] and Pymol version 3.0 [107]. All software used in the current study is open-source.

III. Results and Discussion

1. Extraction Yield and Essential Oils Phytochemical Profile

Extraction yield values ranged from 1.0 to 2.0% among the EOs, with 1.0% for OvEO, 2.0% for TvEO, 1.9% for OgEO, 1.5% for MpEO, and 1.0% for CcEO. The yields were calculated as the ratio between the mass of EO obtained and the mass of dried plant material. Overall, the obtained yields are within the typical range reported for these species, indicating efficient extraction under the applied hydrodistillation conditions. The chemical composition of each EO (n = 2) is summarized in Table 4.

Table 4 – Chemical composition of each EO, including the percentage of each compound (n = 2).

Compound		OvEO (%)	TvEO (%)	OgEO (%)	MpEO (%)	CcEO (%)
(-) - Isopulegol	I.	-	-	0.28	1.38	-
	II.	-	-	0.35	1.72	-
(-) - Pulegone	I.	-	-	-	**90.75**	-
	II.	-	-	-	**91.62**	-
(-) - α - Pinene	I.	1.29	2.02	-	0.24	0.48
	II.	1.59	2.04	-	0.20	0.49
(-) - β - Caryophyllene	I.	6.35	1.74	0.93	1.45	0.32
	II.	6.96	2.29	1.29	1.38	0.47
(+) - Isomenthone	I.	0.20	-	-	4.58	-
	II.	0.26	-	-	3.37	-
(±) - Linalool	I.	-	-	0.24	0.96	-
	II.	-	-	0.15	1.00	-
Δ3 - Carene	I.	-	0.05	-	-	-
	II.	-	0.12	-	-	-
Carvacrol	I.	**44.19**	-	-	-	-
	II.	**43.14**	-	-	-	-

(Continues on next page)

Table 4 (cont.) – Chemical composition of each EO, including the percentage of each compound (n = 2).

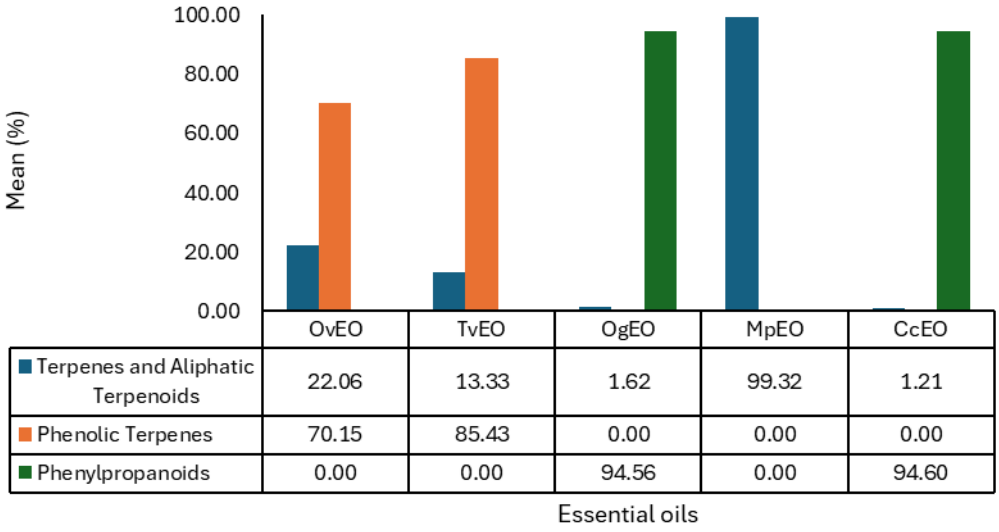
Compound		OvEO (%)	TvEO (%)	OgEO (%)	MpEO (%)	CcEO (%)
Carvacrol methyl ether	I.	2.37	-	-	-	-
	II.	1.95	-	-	-	-
Eugenol	I.	-	-	**95.28**	-	3.26
	II.	-	-	**93.83**	-	2.46
Thymol	I.	**26.54**	**87.53**	-	-	-
	II.	**26.42**	**83.32**	-	-	-
trans - Cinnamaldehyde	I.	-	-	-	-	**91.88**
	II.	-	-	-	-	**91.60**
α - Terpinene	I.	2.45	0.70	-	-	0.32
	II.	2.21	0.91	-	-	0.33
α - Phellandrene	I.	0.23	0.21	-	-	-
	II.	0.34	0.27	-	-	-
γ - Terpinene	I.	11.20	6.70	-	-	-
	II.	11.06	9.42	-	-	-
Total identified compounds	I.	94.82	98.95	96.73	99.36	96.26
	II.	93.93	98.57	95.62	99.29	95.35

Legend: (-) – not present.

The three main classes of compounds that contributed to the phytochemical profile of the EOs are terpenes and aliphatic terpenoids, phenolic terpenes, and phenylpropanoids (Table 4). OvEO and TvEO are mainly composed of phenolic terpenes (carvacrol and thymol); OgEO and CcEO are rich in phenylpropanoids – namely eugenol and *trans*-cinnamaldehyde; and MpEO only consists of terpenes and terpenoids (Fig. 17).

Nine compounds were identified in OvEO, with carvacrol (on average, 43.67%) and thymol (on average, 26.48%) being the most abundant, as previously reported in several studies [44–47]. Regarding TvEO, seven different compounds

were identified, and thymol (85.43%) was the main component as expected [46,47,50,51,59]. Only four compounds were identified in OgEO, with eugenol (on average 94.56%) being the most abundant by far. This was expected because the abundance of eugenol has been described in several studies [56,58,59,111]. With regard to MpEO, six compounds were identified, with pulegone (on average 91.18%) as the main component, as described before by other authors [59,61,62]. Finally, five compounds were identified in CcEO, a *trans*-cinnamaldehyde-rich oil (on average 91.74%). The abundance of this phenylpropanoid is well reported in several studies [63,65–68].



	OvEO	TvEO	OgEO	MpEO	CcEO
Terpenes and Aliphatic Terpenoids	22.06	13.33	1.62	99.32	1.21
Phenolic Terpenes	70.15	85.43	0.00	0.00	0.00
Phenylpropanoids	0.00	0.00	94.56	0.00	94.60

Figure 17 – Bar chart showing the relative distribution (%) of compound classes – terpenes and terpenoids, phenolic compounds, and phenylpropanoids – in the different EOs (OvEO, TvEO, OgEO, MpEO, and CcEO).

2. Biochemical Analysis of Insect and Nematode Protein Extracts

2.1. Protein and Enzymatic Activity Quantification

The insect and nematode protein extracts were evaluated in terms of soluble protein concentration and enzymatic activities, namely AChE, α- and β-GLU. The

results of the quantification of protein and enzymatic activity of insect and nematode protein extracts are presented in Tables 5 and 6.

Table 5 – Quantification of protein and enzymatic activity of protein extracts of insects and nematodes: extract concentration, soluble protein concentration and percentage, and AChE activity.

Insect/ Nematode	Extract concentration (mg/mL)	Soluble protein concentration (mg/mL)	Soluble protein percentage (%)	AChE activity (U/mL)	Specific activity (U/mg)
C. capitata	1.0000	0.1140 [1]	11.4	0.4315	3.7851
M. unipuncta	1.0000	0.1220 [1]	12.2	0.3558	2.9164
M. incognita	0.0250	0.0013 [2]	5.2	0.1290	99.2308
B. xylophilus	0.0125	0.0011 [2]	8.8	0.0683	62.0909

Legend: [1] using the Bradford Method; [2] using Nanodrop spectrophotometer (280 nm), assuming that an absorbance unit corresponds to 1 mg/mL of protein.

Table 6 – Quantification of protein and enzymatic activity of insect protein extracts: extract concentration, soluble protein concentration and percentage, and α- and β-GLU activity.

Insect	Extract concentration (mg/mL)	Soluble protein concentration (mg/mL)	Soluble protein percentage (%)	α-GLU activity (U/mL)	β-GLU activity (U/mL)	Specific activity (U/mg)
C. capitata	1.0000	0.1380 [1]	13.8	0.0413	-	0.2993
M. unipuncta	1.0000	0.1030 [1]	10.3	0.0860	-	0.8350
		0.1040 [1]	10.4	-	0.0041	0.0394

Legend: [1] using the Bradford Method.

The soluble protein concentration of insect extracts (concentration of 1 mg/mL), determined by the Bradford method, was 0.114 mg/mL and 0.138 mg/mL for *C. capitata* extracts, and 0.122 mg/mL, 0.103 mg/mL, and 0.104 mg/mL for *M.*

unipuncta extracts. The soluble protein percentages were 11.4% and 13.8% for Medfly extracts, and 12.2%, 10.3%, and 10.4% for armyworm extracts, respectively. Brogan et al. [95] studied the protein solubility of insect powders as a function of pH by the Bradford method. At pH 7.0, the protein solubility, expressed as percent soluble protein of crude protein in insect powders, was 18% for *Acheta domesticus* (Linnaeus, 1758) (Orthoptera: Gryllidae), 17% for *Locusta migratoria* (Linnaeus, 1758) (Orthoptera: Acrididae), and 10% for *Bombyx mori* (Lineu, 1758) (Lepidoptera: Bombycidae) [95]. Thus, the protein solubility profile (at pH 7.0) of these insects is similar to that here obtained for *C. capitata* and *M. unipuncta* (approximately 10-14%), supporting the reliability of the extraction and quantification approach. Zaguri et al. [112] quantified the soluble protein content of extracts of *Schistocerca gregaria* (Forsskål, 1775) (Orthoptera: Acrididae) (~25%), *Apis mellifera* (Linnaeus, 1758) (Hymenoptera: Apidae) (~35%), and *D. melanogaster* (~40%) using the Bradford method. These results are quite different from those obtained here for *C. capitata* and *M. unipuncta,* probably due to differences in the pH of the extraction solvents, since pH is a key factor in protein extraction – the higher the pH, the greater the protein solubility – as shown before [95]. Since the present study aimed to assess enzyme activities rather than quantify insect protein, the pH was kept at 7. The extracts of *C. capitata* and *M. unipuncta* (without purification) showed an AChE activity of 3.7851 U/mg protein and 2.9164 U/mg protein, respectively. Day and Scott [113] determined the AChE activity of *Hydropsyche* spp. (0.0969 U/mg protein), *Claassenia* sp. (0.0785 U/mg protein), and *Ephemerella* sp. (0.0259 U/mg protein). These results differ from those here obtained for *C. capitata* and *M. unipuncta*, probably because the authors did not grind the insect material into a fine powder before extraction, a step that enhances the surface area exposed to the extraction buffer and promotes the release of intracellular proteins into the solution, and due to interspecies variation. Pandey et al. [114] measured AChE activity in the head tissue of honeybees collected from Nepal during the pre-winter season: 1.81 U/g head tissue for *Apis cerana* (Fabricius, 1793) (Hymenoptera: Apidae); 1.82 U/g head tissue for *Apis dorsata* (Fabricius, 1793) (Hymenoptera: Apidae); and 1.67 U/g head tissue for *A. mellifera*. The differences between these results and those obtained for Medfly and armyworm may be attributed to interspecies variation, including

differences in protein content, the use of the whole insect body rather than only heads, and the constituents of the extraction solvent. Concerning the digestive enzymes activity, in this study, extracts of *C. capitata* and *M. unipuncta* (without purification) showed an α-GLU activity of 0.0413 U/mL and 0.0860 U/mL, respectively, and the *M. unipuncta* extract presented a β-GLU activity of 0.0041 U/mL. Mehrabadi et al. [115] quantified the α- and β-GLU activity in the third ventriculus of *Eurygaster integriceps* (Puton, 1881) (Hemiptera: Scutelleridae) midgut, and the results were 0.020 U/mL and 0.022 U/mL, respectively. The higher α-GLU activity values observed for extracts of *C. capitata* and *M. unipuncta*, as well as the lower β-GLU activity value detected in *M. unipuncta* extract, may be explained by interspecies variation, such as differences in protein content, the use of the whole insect body rather than a specific midgut section, and the constituents of the extraction solvent. This suggests that these insects possess highly active α-GLU, as elevated activity was observed even when using the whole-body extracts rather than only the gut, where the enzyme is primarily located.

Extracts of *M. incognita* and *B. xylophilus* were prepared at concentrations of 0.0250 mg/mL and 0.0125 mg/mL, respectively. The soluble protein content, determined using a Nanodrop spectrophotometer, was 0.0013 mg/mL (5.2%) for *M. incognita* and 0.0011 mg/mL (8.8%) for *B. xylophilus*. Extracts of *M. incognita* and *B. xylophilus* (without purification) presented an AChE activity of 0.1290 U/mL and 0.0683 U/mL, respectively. The specific activity was also calculated: 99.2308 U/mg protein and 62.0909 U/mg protein for *M. incognita* and *B. xylophilus* extracts, respectively. The high specific activity values indicate that a larger proportion of AChE was extracted from nematodes compared to insects, suggesting that this enzyme is either more abundant or more efficiently extracted in nematodes.

To the best of my knowledge, this is the first study to investigate the extraction of protein-rich extracts from the target organisms – *C. capitata*, *M. unipuncta*, *M. incognita*, and *B. xylophilus* – and to quantify the soluble protein content and enzymatic activity in each extract.

2.2. SDS-PAGE Analysis

The electrophoretic pattern of proteins in insect extracts was determined by SDS-PAGE, a qualitative analysis that allows determination of whether certain protein fractions are present or not [95]. The SDS-PAGE gel revealed thirteen bands for *C. capitata* extract and six bands for *M. unipuncta* extract (Fig. 18). Their molecular weights (Mw) were estimated at 14, 17, 19-21, 24, 27, 31, 35, 37, 42, 45-46, 56, 81, 106, 162, and 176 kDa (Tables 7 and 8) by comparison with the molecular size market (M1) – PageRuler™ Prestained Protein Ladder, 10-180 kDa (Table S4 and Fig. S3, in the Supplementary Information Section). The protein fraction with Mw = 81 kDa was the most prominent in both cases.

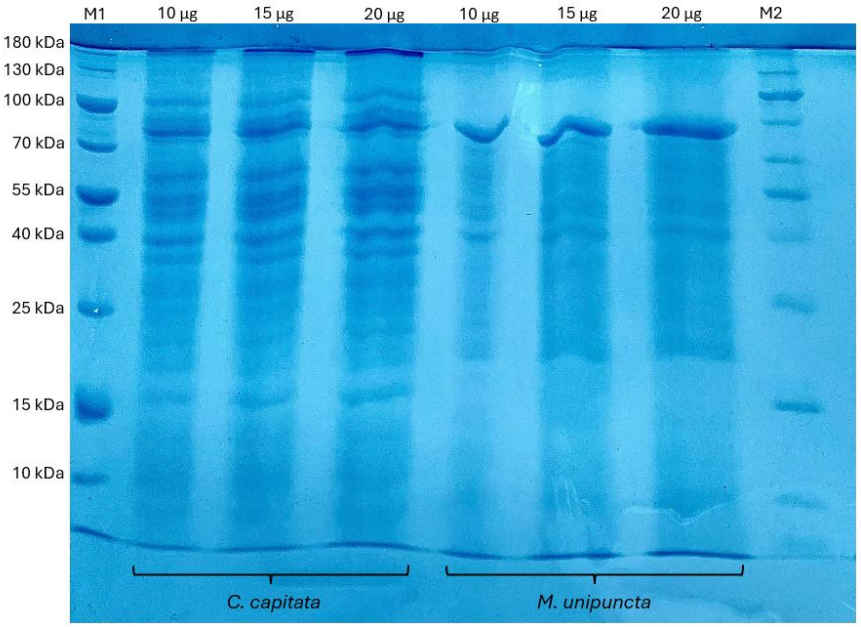


Figure 18 – 5-12% gradient SDS-PAGE obtained gel. Legend: M – molecular size marker.

Table 7 – Relative mobility, raw volume, molecular weight (Mw, in kDa), and identity of *C. capitata* proteins.

Band #	Rf	Raw volume	Mw (kDa)	Protein Identity
1	0.005	612	176	Myosin
2	0.111	355	106	Paramyosin
3	0.170	774	81	Hsp70/Hsp90 or Hexamerin
4	0.255	133	56	Miniparamyosin
5	0.303	167	46	Actin
6	0.326	135	42	Arginine kinase
7	0.382	272	35	GAPDH
8	0.420	134	31	Cuticle proteins
9	0.468	69	27	
10	0.512	46	24	
11	0.574	88	21	
12	0.630	144	19	
13	0.687	215	17	

Table 8 – Relative mobility, raw volume, molecular weight (Mw, in kDa), and identity of *M. unipuncta* proteins.

Band #	Rf	Raw volume	Mw (kDa)	Protein Identity
1	0.022	99	162	Hexamerin dimer
2	0.169	1219	81	Hexamerin
3	0.309	21	45	Actin
4	0.364	76	37	GAPDH
5	0.587	323	20	Cuticle proteins
6	0.885	368	14	

According to Andersen et al. [116], the Mw of insect cuticle proteins ranges from 14 to 32 kDa. Therefore, the protein fractions with Mw of 14, 17, 19-21, 24, 27, and 31 kDa appear to represent cuticle proteins (Tables 7 and 8). Cuticle proteins are structural materials that interact with chitin and form the insect exoskeleton [95]. Harris and Waters [117] reported that the Mw of GAPDH is 36 kDa. The protein

fractions with Mw of 35 kDa (Table 7) and 37 kDa (Table 8) likely correspond to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis that plays a crucial role in insects. Zhao et al. [118] demonstrated that GAPDH also plays an important role in maintaining growth and promoting normal metamorphosis of *H. armigera* larvae. The protein fraction at 42 kDa in *C. capitata* extract matches arginine kinase (Table 7), a transferase enzyme common in insects and crustaceans [119]. Arginine kinase has a Mw of approximately 40 kDa and has been identified in other insects: *Blattella germanica* (Linnaeus, 1767) (Blattodea: Blattellidae) [120], *Plodia interpunctella* (Hünber, 1813) (Lepidoptera: Pyralidae) [121], *B. mori* [95,122], *A. domesticus* [95], and *L. migratoria* [95]. The protein fractions with a Mw of 45-46 kDa are actin (Tables 8 and 9), and those with a Mw of 56, 106, and 176 kDa are *C. capitata* miniparamyosin, paramyosin, and myosin, respectively (Table 7). Actin filaments, usually associated with myosin, are responsible for cell movements [123]. Actin and myosin are considered muscle proteins that allow muscle contraction [123]. It is well established that thick myofilaments of invertebrate muscle cells consist mainly of myosin (~200 kDa) and paramyosin (~100 kDa) [124]. Miniparamyosin is a specific isoform of paramyosin, weighing 55-60 kDa [124]. Royuela et al. [124] performed a Western blot analysis of myosin, paramyosin, and miniparamyosin in *D. melanogaster'*s striated muscle, observing single bands at 200 kDa for myosin, 100 kDa for paramyosin, and 55-60 kDa for miniparamyosin. Brogan et al. [95] identified actin (42 kDa) in insect powders of *B. mori*, *A. domesticus*, and *L. migratoria*, while myosin (220 kDa) was found exclusively in *L. migratoria* powder. The protein fraction at 81 kDa in *C. capitata* extract could correspond to a heat shock protein (Hsp) or a hexamerin (Table 7). Additionally, the protein fractions with a Mw of 81 and 162 kDa in *M. unipuncta* extract correspond to a hexamerin and a hexamerin dimer, respectively (Table 8). Hsp is highly conserved and induced by stress factors such as extreme temperatures, heavy metals, and hunger, helping proteins fold properly, degrading denatured proteins, and functioning as molecular chaperones to protect against adverse environments [125]. Hsp70 (72–80 kDa) and Hsp90 (81–99 kDa) are the most studied Hsp families in insects, acting as molecular chaperones that assist in proper protein folding and prevent the aggregation of denatured proteins [125].

Hsp70 enables thermal resistance in *D. melanogaster* adults [126], while Hsp90 functions as part of a buffering mechanism in *Drosophila* [127]. In insects, significant quantities of amino acids are stored in storage proteins within the hemolymph and fat body [128]. Several protein classes serve storage functions; among them, the most common are hexamerins, which are hemocyanin-related proteins with a native Mw around 500 kDa, composed of six homologous subunits typically weighing 70-85 kDa each [129]. In holometabolous insects, hexamerins accumulate during larval stages and are crucial for providing amino acids needed for metamorphosis during the non-feeding pupal stage [130]. In both holo- and hemimetabolous insects, these proteins also serve roles such as providing amino acids for egg production and tissue rebuilding after adult diapause [128]. Short et al. [131] investigated whether larval serum protein 2, a specific hexamerin, regulates reproductive behavior in male Caribbean fruit fly (*Anastrepha suspensa* (Loew, 1862) (Diptera: Tephritidae)), a species known for its nutrient-demanding sexual displays, and found that the larval serum protein 2 signaling may accelerate and increase reproductive activity. Four major larval serum polypeptides, a subtype of hexamerins with Mw between 81 and 87 kDa, have been identified in the Medfly (*C. capitata*) [132].

SDS-PAGE analysis did not allow the visualization of the enzymes under study, likely due to their high molecular weight. This approach represents only a preliminary attempt to identify the proteins observed in the SDS-PAGE gel. To confirm their identity, more specific techniques, such as Western blotting with target-specific antibodies or mass spectrometry analysis (e.g., Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF)) are required.

3. *In vitro* Inhibition Assays

In vitro inhibition assays were conducted to elucidate the mechanisms of action of the EOs and their main compounds, and to determine whether their insecticidal/nematicidal effects were associated with interference in the nervous or gastrointestinal systems.

3.1. Acetylcholinesterase Inhibition Assays

3.1.1. Electric eel (*Electrophorus electricus*)

In vitro inhibition assays were performed using a standard AChE from the electric eel (*E. electricus*). The results obtained are summarized in the Supplementary Information Section, Table S5, and the best data are summarized in Table 9.

Table 9 – AChE inhibition assay: IC_{50} mean and SD values (mg/mL) for pure compounds and EOs against AChE from electric eel (*E. electricus*) (n = 3) (best data).

	IC_{50} (mean ± SD) (mg/mL)
Carvacrol	0.0269 ± 0.0001
Thymol	2.5309 ± 0.5145
TvEO	0.9258 ± 0.1349
Galantamine hydrobromide	0.0018 ± 0.0001

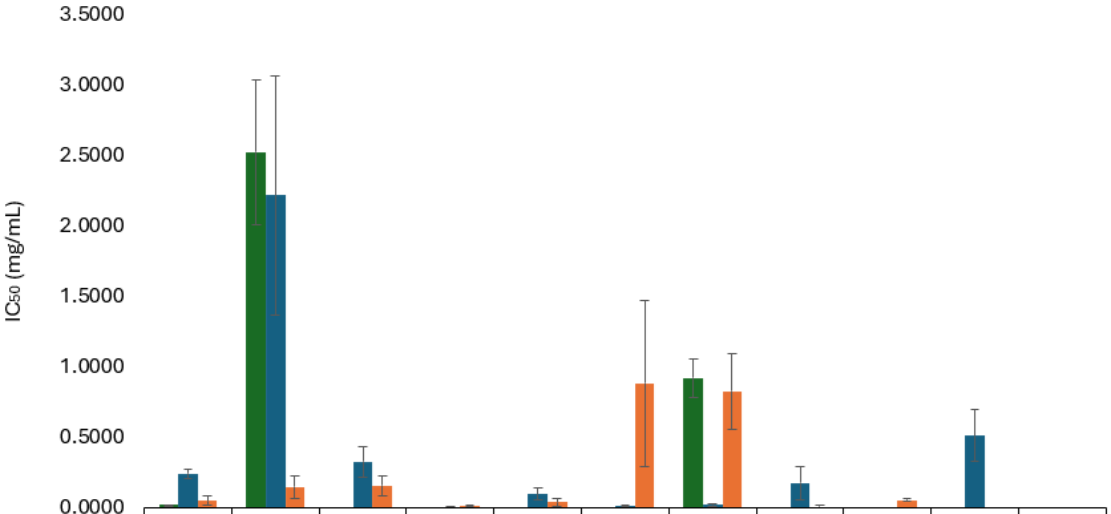
Legend: IC_{50} – concentration of extract that inhibits 50% of AChE.

AChE was only inhibited by carvacrol (IC_{50} = 0.0269 ± 0.0001 mg/mL), thymol (IC_{50} = 2.5309 ± 0.5145 mg/mL), and TvEO (IC_{50} = 0.9258 ± 0.1349 mg/mL). Jukic et al. [74] reported that the AChE inhibitory potential decreased in the following order: carvacrol > TvEO (35.30% thymol, IC_{50} = 0.5400 mg/mL) > thymol. The study also reported that the AChE inhibitory effect exerted by carvacrol (IC_{50} = 0.0630 mg/mL) was ten times stronger than that exerted by its isomer thymol (IC_{50} = 0.7400 mg/mL), despite thymol and carvacrol having a very similar structure [74]. The results presented in this study are in accordance with those reported; however, the AChE inhibitory effect exerted by carvacrol was one hundred times stronger than that exerted by thymol. This discrepancy between the results may be attributed to variations in experimental conditions, such as pH and buffer composition, as well as to the solvent used for compound solubilization and the compound's final concentration, which can influence their stability and bioavailability. Orhan et al.

[72] reported that 1 mg/mL solutions of carvacrol and thymol caused an AChE inhibition rate of 70.30% ± 1.24% and 28.40% ± 0.64%, respectively.

3.1.2. Insects

In vitro inhibition assays were performed using AChE from adults of the Medfly (*C. capitata*) and from the armyworm (*M. unipuncta*). The results obtained are summarized in the Supplementary Information Section, Tables S6 and S7, respectively. Figure 19 shows a bar graph presenting the mean and SD IC_{50} values obtained for pure compounds and EOs in the standard (*E. electricus*) and insect AChE inhibition assays (based on data from Tables S5, S6, and S7, in the Supplementary Information Section).



	C	T	E	P	TC	OvEO	TvEO	OgEO	MpEO	CcEO	GH
Electric eel	0.0269	2.5309	n.i.	n.i.	n.i.	n.i.	0.9258	n.i.	n.i.	n.i.	0.0018
Medfly	0.2480	2.2253	0.3327	0.0110	0.1015	0.0222	0.0234	0.1793	0.0022	0.5163	0.0035
Armyworm	0.0552	0.1507	0.1600	0.0222	0.0444	0.8896	0.8304	0.0105	0.0587	0.0020	0.0003

Figure 19 – AChE inhibition assays: IC_{50} mean and SD values (mg/mL) for pure compounds and EOs against AChE from the electric eel (*E. electricus*), the Medfly (*C. capitata*), and the armyworm (*M. unipuncta*) (n = 3). Legend: C – carvacrol; T – thymol; E – eugenol; P – pulegone; TC – *trans*-cinnamaldehyde; GH – galantamine hydrobromide; n.i. – no inhibition of AChE.

Insect AChE was significantly inhibited by all EOs. The results obtained in the inhibition assays using electric eel and insect AChE revealed marked differences (Fig. 19), suggesting structural divergences between them.

3.1.2.1. Mediterranean Fruit Fly (Ceratitis capitata)

Medfly AChE was significantly inhibited by all EOs (Fig. 19 and Table S6, in the Supplementary Information Section).

OvEO, which contains on average 43.67% carvacrol (IC_{50} = 0.2480 ± 0.0347 mg/mL) and 26.48% thymol (IC_{50} = 2.2253 ± 0.8503 mg/mL), exhibited a significantly lower IC_{50} value of 0.0222 ± 0.0025 mg/mL, indicating a synergistic effect between these two major compounds. As mentioned before, Oliveira et al. [46] assessed the adulticidal and repellent activities of OvEO (17.4% terpinene-4-ol, 16.0% carvacrol, and 10.4% thymol) against *A. aegypti* and proved that OvEO is toxic to adult mosquitoes (LC_{50} = 14.3 µg/mL). The insecticidal effect may be attributed to the low IC_{50} values observed for both carvacrol and OvEO, suggesting that OvEO exerts its toxic effect through interference with the insect AChE. Other studies have investigated the inhibitory effects of carvacrol on insect AChE, particularly *A. aegypti* [76], *A. albopictus* [78], *D. melanogaster* [79], and *M. domestica* [76], reporting low IC_{50} values.

TvEO, mainly composed of thymol, also showed a low IC_{50} value of 0.0234 ± 0.0076 mg/mL. Since thymol alone exhibited a higher IC_{50} value (2.2253 mg/mL), the strong AChE inhibitory activity of TvEO may result from a synergistic interaction among its components. Lazarević et al. [50] reported that TvEO (containing 43.52% thymol and 31.65% p-cymene) reduced the survival and longevity of *A. obtectus* adults in a concentration-dependent manner. The study also showed that TvEO induced lipid and protein damage, depleted protein and non-protein thiols levels, affected oxidative stress indices, and suppressed oviposition and adult emergence [50]. These impacts may explain the observed reduction in survival and are likely linked to the inhibition of AChE by TvEO.

Regarding OgEO, which contains primarily eugenol (IC_{50} = 0.3327 ± 0.1099 mg/mL), the obtained IC_{50} value was 0.1793 ± 0.1147 mg/mL. Some studies have mentioned the insecticidal activity of eugenol and OgEO against various insect pests [56–58]. The toxicity of OgEO is likely associated with its high eugenol content [56]. Both eugenol and OgEO exhibit AChE inhibition activity, which may contribute to their insecticidal activity.

MpEO, which is mainly composed of pulegone (IC_{50} = 0.0110 ± 0.0026 mg/mL), exhibited the lowest IC_{50} value among the tested EOs: 0.0022 ± 0.0013 mg/mL. A study by Miguel et al. [61] suggested that MpEO induces a potent depressive effect on the nervous system of *C. capitata*. However, despite its known neurotoxic properties, no studies to date have explored the AChE inhibition activity of MpEO in insects. The results obtained indicate that MpEO is a potent AChE inhibitor, likely due to its high pulegone content.

Finally, CcEO, which is mainly composed of *trans*-cinnamaldehyde (IC_{50} = 0.1015 ± 0.0444 mg/mL), obtained the highest IC_{50} value among the tested EOs: 0.5163 ± 0.1842 mg/mL; yet CcEO exhibited a great AChE inhibition activity. Previous studies have reported the insecticidal properties of CcEO and *trans*-cinnamaldehyde [65,66]. To the best of my knowledge, no studies have been published on the effect of CcEO or *trans*-cinnamaldehyde on insect AChE. The results obtained suggest that CcEO and its main component are AChE inhibitors.

The results from *in vitro* inhibition assays suggest that the insecticidal activity of the EOs studied may be due to the inhibition of AChE, which disrupts the insect's nervous system, affecting mobility, orientation, and mating ability, ultimately causing death. To the best of my knowledge, this is the first study to examine the interactions between the selected EOs – OvEO, TvEO, OgEO, MpEO, and CcEO – and their main components with Medfly AChE, highlighting the need for further research to clarify their potential mechanism of action.

3.2.1.2. Armyworm (Mynthimna unipuncta)

All EOs notably inhibited armyworm AChE (Fig. 19 and Table S7, in the Supplementary Information Section).

OvEO, mainly composed of carvacrol (IC_{50} = 0.0552 ± 0.0341 mg/mL) and thymol (IC_{50} = 0.1507 ± 0.0808 mg/mL), showed a significantly higher IC_{50} value of 0.8896 ± 0.5888 mg/mL, indicating an antagonistic effect between these two compounds. Gong and Ren [45] evaluated the larvicidal activity of OvEO and its major component, carvacrol (78.35%), against *H. armigera*: OvEO and carvacrol exhibited LC_{50} values of 265.51 µg/mL and 51.53 µg/mL, respectively. Oliveira et al. [46] also assessed the larvicidal activity of OvEO (17.4% terpinene-4-ol, 16.0% carvacrol, and 10.4% thymol) against *A. aegypti,* and it proved to be toxic to larvae (LC_{50} = 37.5 µg/mL). Regarding TvEO, mainly composed of thymol, the obtained IC_{50} value was 0.8304 ± 0.2675 mg/mL, indicating an antagonistic effect between its components. Oliveira et al. [46] also reported that TvEO (40.0% thymol, 19.3% p-cymene, and 17.3% γ-terpinene) was toxic against *A. aegypti* larvae (LC_{50} = 38.9 µg/mL). Szczepanik et al. [51] evaluated the larvicidal activity of TvEO (57.44% thymol and 2.80% carvacrol), thymol, and carvacrol against *A. diaperinus* larvae, and reported that the growth was significantly affected, causing mortalities of 62.5%, 91.67%, and 97.5%, respectively. These effects can be explained by the AChE inhibition activity of OvEO, TvEO, and their components.

OgEO, which is primarily composed of eugenol (IC_{50} = 0.1600 ± 0.0753 mg/mL), exhibited a low IC_{50} value of 0.0105 ± 0.0094 mg/mL. The larvicidal activity of eugenol is well documented [133–135]. Osanloo et al. [133] reported that clove EO (*Syzygium aromaticum*, 67% eugenol) was more effective against *Anopheles stephensi* (Liston, 1901) (Diptera: Culicidae) larvae than pure eugenol, with LC_{50} values of 57.49 ppm and 86.96 ppm, respectively. This larvicidal effect may be attributed to the AChE inhibition activity of both OgEO and eugenol, with OgEO once again exhibiting greater potency.

MpEO, mainly composed of pulegone (IC_{50} = 0.0222 ± 0.0023 mg/mL), showed a low IC_{50} value of 0.0587 ± 0.0091 mg/mL. Therefore, pulegone is responsible for the high AChE inhibition activity of MpEO. Michaelakis et al. [136]

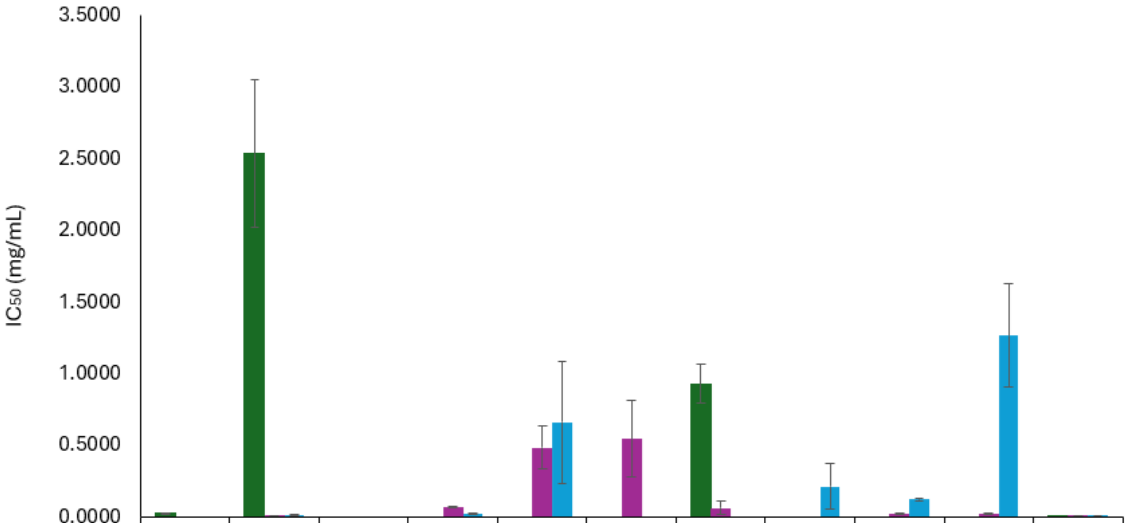
evaluated the larvicidal activity of MpEO and its main component, pulegone (61.1%). The LC_{50} values obtained for MpEO and pulegone were 46.97 mg/L and 27.23 mg/L, respectively [136]. These results indicate that pulegone is more potent than MpEO in terms of larvicidal activity, which may be associated with its high capacity to inhibit AChE. To the best of my knowledge, the effects of MpEO and pulegone on AChE from insect larvae have not been reported.

Finally, CcEO, primarily composed of *trans*-cinnamaldehyde (IC_{50} = 0.0444 ± 0.0268 mg/mL), exhibited the lowest IC_{50} value of 0.0020 ± 0.0004 mg/mL among the EOs under study. Cheng et al. [137] reported that a cinnamon EO and its main component, *trans*-cinnamaldehyde, had an excellent inhibitory effect against the fourth-instar larvae of *A. aegypti*, with LC_{50} values in 24 hours of 36 ppm and 29 ppm, respectively. The results obtained in this study suggest that the strong larvicidal activity of CcEO may be linked to its potent AChE inhibition activity. To the best of my knowledge, the effects of CcEO and *trans*-cinnamaldehyde on AChE from insect larvae have not been reported.

The results obtained from the *in vitro* inhibition assays suggest that the larvicidal activity of the EOs under study may be associated with AChE inhibition, leading to nervous system dysfunction in the larvae and ultimately causing their death. To the best of my knowledge, this is the first study to investigate the interactions between the selected EOs – OvEO, TvEO, OgEO, MpEO, and CcEO – and their major components with the armyworm AChE, highlighting the need for additional research to elucidate their mode of action.

3.1.3. Nematodes

In vitro inhibition assays were performed using AChE from the J2 RKN (*M. incognita*) and the PWN (*B. xylophilus*). The results obtained are summarized in the Supplementary Information Section, Tables S8 and S9. Figure 20 shows a bar graph presenting the mean and SD IC_{50} values obtained for pure compounds and EOs in the standard (*E. electricus*) and nematode AChE inhibition assays (based on data from Tables S5, S8, and S9, in the Supplementary Information Section).



	C	T	E	P	TC	OvEO	TvEO	OgEO	MpEO	CcEO	GH
Electric eel	0.0269	2.5309	n.i.	n.i.	n.i.	n.i.	0.9258	n.i.	n.i.	n.i.	0.0018
RKN	n.i.	0.0055	n.i.	0.0680	0.4848	0.5444	0.0633	n.i.	0.0222	0.0197	0.0036
PWN	n.i.	0.0131	n.i.	0.0251	0.6610	n.i.	n.i.	0.2100	0.1209	1.2627	0.0084

Figure 20 – AChE inhibition assays: IC_{50} mean and SD values (mg/mL) for pure compounds and EOs against AChE from the electric eel (*E. electricus*), the RKN (*M. incognita*), and the PWN (*B. xylophilus*) (n = 3). Legend: C – carvacrol; T – thymol; E – eugenol; P – pulegone; TC – *trans*-cinnamaldehyde; GH – galantamine hydrobromide; n.i. – no inhibition of AChE.

Nematode AChE was significantly inhibited by MpEO and its main component, pulegone. Electric eel and nematode AChE inhibition assays revealed strong differences (Fig. 20), suggesting structural divergences between them.

3.1.3.1. Root-Knot Nematode (Meloidogyne incognita)

All EOs, except OgEO, inhibited AChE (Fig. 20 and Table S8, in the Supplementary Information Section).

OvEO, predominantly composed of carvacrol (inactive against AChE) and thymol (IC_{50} = 0.0055 ± 0.0003 mg/mL), showed a relatively low IC_{50} value of 0.5444 ± 0.2645 mg/mL, suggesting a possible synergistic interaction between these two compounds. TvEO, which is mainly composed of thymol, the pure compound that demonstrated the lowest IC_{50} value, showed a slightly higher IC_{50} value of 0.0633 ± 0.0480 mg/mL, suggesting an antagonistic effect between the EO components.

Amora et al. [47] demonstrated that OvEO and TvEO exhibit a strong nematicidal activity, resulting in over 99% mortality of *M. javanica* juveniles. This effect may be associated with the AChE inhibition activity of thymol and these two EOs.

Ferreira et al. [59] evaluated the nematicidal activity of eugenol-rich OgEO and reported more than 90% mortality in the RKN (*M. javanica*). However, AChE inhibition assays revealed that neither eugenol nor OgEO inhibited AChE from the RKN (*M. incognita*), indicating that its nematicidal action does not involve AChE inhibition.

MpEO, mainly composed of pulegone (IC_{50} = 0.0680 ± 0.0058 mg/mL), exhibited a low IC_{50} value of 0.0222 ± 0.0056 mg/mL. Pulegone-rich MpEO has demonstrated a strong nematicidal activity against *M. incognita* [62] and *M. javanica* [59]. The low IC_{50} values obtained in the AChE inhibition assays suggest that the mechanism of action of MpEO likely involves inhibition of this neurochemical enzyme.

Finally, CcEO, mainly composed of *trans*-cinnamaldehyde (IC_{50} = 0.4848 ± 0.1524 mg/mL), showed a significantly lower IC_{50} value of 0.0197 ± 0.0063 mg/mL. Some studies have mentioned the strong nematicidal activity of CcEO and *trans*-cinnamaldehyde against *M. incognita* [63,67]. The nematicidal activity of CcEO is likely associated with its potent AChE inhibitory effect, as evidenced by the low IC_{50} values obtained in the inhibition assays.

To the best of my knowledge, no studies have evaluated the interaction between AChE from RKN (*M. incognita*) and EOs and pure compounds under study, highlighting the need for further research to elucidate its potential mechanism of action at the molecular level. The findings of this study suggest that the nematicidal activity of these EOs may be mediated through AChE inhibition, disrupting the nematode's nervous system and leading to its death.

3.1.3.2. Pine Wood Nematode (Bursaphelenchus xylophilus)

From the five EOs under study, only OgEO, MpEO, and CcEO inhibited AChE (Fig. 20 and Table S9, in the Supplementary Information Section).

OvEO, mainly composed of carvacrol (inactive against AChE) and thymol (IC_{50} = 0.0131 ± 0.0056 mg/mL), did not inhibit AChE from the PWN. Barbosa et al. [48] reported that OvEO (35.7% thymol and 23.5% γ-terpinene) exhibits strong nematicidal activity against *B. xylophilus*. The OvEO tested showed no inhibitory activity against PWN AChE, probably due to its high carvacrol content and a possible antagonistic interaction among its constituents. In addition, Kang et al. [81] evaluated the inhibition of PWN AChE by carvacrol and thymol, which showed only about 10% inhibition at a concentration of 1 mg/mL in both cases. These results are not in full agreement with those obtained in the inhibition assays carried out with the pure compounds, as thymol was the most potent AChE inhibitor. This discrepancy may be attributed to variations in experimental conditions, including pH and buffer composition, as well as the solvent and final concentration used for compound solubilization, which can affect compound stability and bioavailability. Adbel-Rahman et al. [138] reported that thymol showed an impressive nematicidal action against *B. xylophilus*. The strong nematicidal activity of thymol may be related to its ability to inhibit AChE. Although TvEO contains a high percentage of thymol, it is inactive against PWN AChE. This result suggests the presence of antagonistic interactions among its components. However, Elbadri et al. [53] reported a strong nematicidal activity of TvEO against *B. xylophilus*. Therefore, the nematicidal effect of TvEO is not related to the inhibition of PWN AChE.

OgEO, primarily composed of eugenol (inactive against AChE), showed a relatively low IC_{50} value of 0.2100 ± 0.1593 mg/mL, suggesting a potential synergistic effect among its components. Kang et al. [81] reported that eugenol inhibited less than 5% of PWN AChE activity at a concentration of 1 mg/mL, which is consistent with the results obtained in the *in vitro* inhibition assays. In addition, Ferreira et al. [59] reported that eugenol-rich OgEO exhibited low activity against *B. xylophilus*, with mortality rates lower than 30%. The present AChE inhibition assays indicate

that OgEO can inhibit PWN AChE; however, such inhibition may not be sufficient to induce nematode mortality.

MpEO, mainly composed of pulegone (IC_{50} = 0.0251 ± 0.0024 mg/mL), exhibited the lowest IC_{50} value among the EOs tested (IC_{50} = 0.1209 ± 0.0136 mg/mL). Ferreira et al. [59] reported that MpEO, containing 54.26% pulegone and 31.90% menthol, showed only moderate nematicidal activity against *B. xylophilus*. This moderate effect may be attributed to the lower pulegone content and the higher percentage of menthol, which is inactive against AChE (in the Supplementary Information Section, Table S9). Supporting this, Kang et al. [81] observed that menthol inhibited less than 5% of PWN AChE activity at a concentration of 1 mg/mL. Given that pulegone is the most potent PWN AChE inhibitor identified, and MpEO is pulegone-rich while exhibiting strong AChE inhibition activity, the results suggest that its high pulegone content is directly associated with enhanced AChE inhibition. However, to the best of my knowledge, no previous studies have investigated the interaction between AChE from *B. xylophilus* and either MpEO or pulegone. The present findings suggest that the nematicidal activity of MpEO may be related to its pulegone content and mediated through AChE inhibition.

Finally, CcEO, primarily composed of *trans*-cinnamaldehyde (IC_{50} = 0.6610 ± 0.4237 mg/mL), showed a slightly higher IC_{50} value of 1.2627 ± 0.3591 mg/mL. Some studies have noted the strong nematicidal activity of *trans*-cinnamaldehyde-rich CcEO [59,68]. According to Kang et al. [81], *trans*-cinnamaldehyde inhibited about 30% of PWN AChE activity at a concentration of 1 mg/mL. The results obtained in the present study suggest that CcEO and *trans*-cinnamaldehyde do not exert their strong nematicidal effects against *B. xylophilus* via inhibition of AChE.

To the best of my knowledge, this is the first study to evaluate the interactions between AChE from PWN (*B. xylophilus*) and these EOs, emphasizing the need for further research to clarify its potential mechanism of action at the molecular level.

3.2. α- and β-Glucosidase Inhibition Assays

3.2.1. Standard Enzymes

In vitro inhibition assays were performed using standard digestive enzymes: α-GLU from *S. cerevisiae* and β-GLU from almonds. The results obtained for standard α- and β-GLU are summarized in the Supplementary Information Section, Tables S10 and S11, respectively. α-GLU was only inhibited by thymol (IC_{50} = 0.3043 ± 0.0254 mg/mL) and TvEO (IC_{50} = 2.4080 ± 0.0971 mg/mL). On the other hand, β-GLU was not inhibited by the tested EOs. β-GLU from plants may play a particular role in processing plant defense glycosides, typically considered protoxins, non-toxic, glycosylated precursors that are brought into contact with compartmentalized plant glycosidases upon tissue damage to yield toxic aglycones [139]. Therefore, EOs do not affect the plant defense against herbivorous insects. To the best of my knowledge, no studies have been published on the effect of selected EOs and standards on these standard digestive enzymes.

3.2.2. Mediterranean Fruit Fly (*Ceratitis capitata*)

In vitro inhibition assays were performed using α-GLU from the Medfly (*C. capitata*). The results obtained are summarized in the Supplementary Information Section, Table S12. Neither the EOs nor the standards inhibited this enzyme. To the best of my knowledge, the effects of these EOs and standards on Medfly's α-GLU have not been reported. However, Magierowicz et al. [94] proved that the toxicity mechanism of carvacrol is associated with the disruption of carbon metabolism and/or osmoregulation in the insect body through the induction of α-GLU activity in *A. advenella* tissues.

3.2.3. Armyworm (*Mynthimna unipuncta*)

In vitro inhibition assays were performed using α- and β-GLU from the armyworm (*M. unipuncta*). The results obtained are summarized in the Supplementary Information Section, Tables S13 and S14, respectively. These digestive enzymes were not inhibited by EOs nor standards. To the best of my

knowledge, the effects of these EOs and standards on α- and β-GLU from *M. unipuncta* have not been reported. However, some studies mention the antifeeding or feeding deterrence effects of these EOs against other insect larvae. Ribeiro et al. [140] reported that TvEO exhibited a moderate feeding deterrence at sublethal concentrations to *Anticarsia gemmatalis* (Hübner, 1818) (Lepidoptera: Noctuidae) larvae. Other studies reported the antifeeding effects of thymol against 6th instar *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae) larvae [141] and the feeding deterrence of thymol, carvacrol, and eugenol to 5th instar *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae) larvae [142]. Some studies have reported the antifeeding and feeding deterrence effects of MpEO against 6th instar *S. littoralis* larvae [143], 2nd and 3rd instar *H. armigera* larvae [144], and 3rd instar *P. interpunctella* larvae [145]; its main component, pulegone, also exhibited antifeeding and feeding deterrence properties against 6th instar *S. littoralis* larvae [143]. *In vitro* assay results suggest that the antifeeding and deterrence effects of the EOs and standards are unrelated to the inhibition of larval α- and β-GLU.

4. Molecular Docking

A molecular docking study was performed to evaluate the molecular-level interactions between AChE – from the electric eel (*E. electricus*) and the fruit fly (*D. melanogaster*) – and five ligands: carvacrol, eugenol, pulegone, *trans*-cinnamaldehyde, and galantamine (a known AChE inhibitor). These compounds were selected due to their opposite inhibitory effects on eel and insect AChE.

A rigid docking approach was used to evaluate ligand affinity and interaction profiles. Although this approach does not account for protein flexibility, it is widely accepted for screening purposes and was suitable given the static nature of the active site residues observed in the crystallographic structure.

4.1. Acetylcholinesterase from *Electrophorus electricus*

The crystal structure of AChE from electric eel (*E. electricus*) with resolution 4.20 Å (code: 1C2O) [103] was submitted to a molecular docking study to elucidate the interactions between this enzyme and the selected ligands at a molecular level. The results obtained in the *in silico* assays are summarized in Table 10.

Table 10 – *In silico* assays with AChE from *E. electricus* (code:1C2O): affinity values (kcal/mol), number of hydrogen bonds formed between the enzyme and the ligand, and the amino acids involved in these hydrogen bonds (best data).

	Affinity (kcal/mol) [1]	**Hydrogen bonds** [2]	**Amino acids involved in hydrogen bonds** [2]
Carvacrol	-6.8	1	-
Eugenol	-6.4	2	Tyr337
Pulegone	-6.8	0	-
trans-Cinnamaldehyde	-6.4	1	Tyr124
Galantamine	-7.7	1	Tyr72

Legend: [1] The results were obtained via AutoDock Vina [109,110]; [2] The results were obtained via ChimeraX version 1.7.1 [105].

The active site of AChE from *E. electricus* (code: 1C2O) is composed of: an esteratic site containing the catalytic triad (Ser203, Glu334, and His447); an oxyanion hole (Gly121), that stabilizes the tetrahedral intermediate binding of the carbamate carbonyl group; an acyl binding site (Ala204, Trp236, Phe295, Phe297, and Phe338), which binds the acetyl group of ACh or possibly the alkyl moiety of diverse inhibitors; and a peripheral anionic binding site (Tyr72, Trp86, Ser125, Gly126, Trp286, and Tyr337) at the entrance of this active site gorge [146].

The affinity values obtained for the components of the EOs (-6.4 kcal/mol and -6.8 kcal/mol) were more positive than those obtained for the known AChE inhibitor, galantamine (-7.7 kcal/mol). The more negative the affinity value obtained (in kcal/mol), the greater the stability between the ligand and the receptor, and

therefore the better the molecular docking result. The best result was obtained for carvacrol, with an affinity value of -6.8 kcal/mol and one hydrogen bond between the hydroxyl group of carvacrol and the enzyme (Table 10). This result was expected, as carvacrol was the pure compound with the lowest IC_{50} value in the *in vitro* AChE inhibition assays using electric eel AChE (Table S5, in the Supplementary Information Section). Based on the analysis of the results using ChimeraX version 1.7.1 [105] and Pymol version 3.0 [107], it was concluded that carvacrol binds to the acyl binding site, as evidenced by the surrounding amino acid residues (Fig. 21). Regarding eugenol, the binding affinity was -6.4 kcal/mol, and two hydrogen bonds with the enzyme were identified, involving the amino acid Tyr337 (Table 10). Pulegone showed a slightly higher affinity (-6.8 kcal/mol) but did not form any hydrogen bonds (Table 10). Finally, *trans*-cinnamaldehyde exhibited a binding affinity of -6.4 kcal/mol, forming one hydrogen bond with Tyr124 (Table 10). These results, along with the positioning of these compounds, suggest that eugenol and pulegone are located at the peripheral anionic site, whereas *trans*-cinnamaldehyde is positioned deeper within the active site gorge. The positions of eugenol, pulegone, and *trans*-cinnamaldehyde in the active site of the enzyme are shown in the Supplementary Information Section, Figures S4, S5, and S6, respectively. Despite these interactions, the *in silico* results do not align with the *in vitro* inhibition assays, as none of these three compounds inhibited AChE from *E. electricus*. To the best of my knowledge, no studies to date have evaluated the *in silico* interactions between these compounds and electric eel AChE.

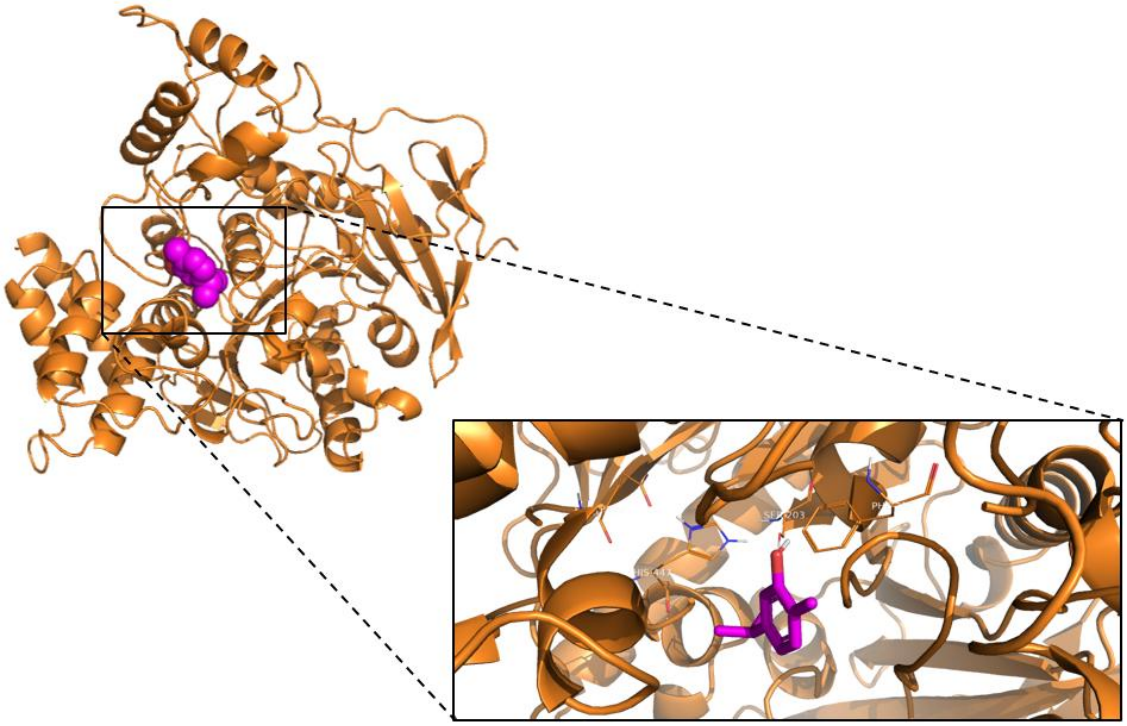


Figure 21 – Carvacrol in complex with AChE from *E. electricus* (code: 1C2O). The image was generated by Pymol version 3.0 [107].

4.2. Acetylcholinesterase from *Drosophila melanogaster*

In the absence of data on AChE from the insect species analyzed in the present study, *D. melanogaster* AChE was selected for the *in silico* analysis, as this fruit fly is a well-established model organism. The crystal structure of AChE from the fruit fly (*D. melanogaster*) with resolution 2.46 Å (code: 6XYS) [104] was submitted to a molecular docking study to elucidate the interactions between this enzyme and the selected ligands at a molecular level. The results obtained in the *in silico* assays are summarized in Table 11.

Table 11 – *In silico* assays with AChE from *D. melanogaster* (code: 6XYS): affinity values (kcal/mol), number of hydrogen bonds formed between the enzyme and the ligand, and the amino acids involved in these hydrogen bonds (best data).

	Affinity (kcal/mol) [1]	**Hydrogen bonds [2]**	**Amino acids involved in hydrogen bonds [2]**
Carvacrol	-6.5	0	-
Eugenol	-6.9	1	Tyr370
Pulegone	-7.5	1	Tyr370
trans-Cinnamaldehyde	-6.5	1	Ser238
Galantamine	-6.8	0	-

Legend: [1] The results were obtained via AutoDock Vina [109,110]; [2] The results were obtained via ChimeraX version 1.7.1 [105].

The active site gorge of AChE from *D. melanogaster* (code: 6XYS) is composed of a catalytic triad (Ser238, Glu367, and His480), an oxyanion hole (Gly150, Gly151, and Ala239), a key residue of the peripheral site (Trp321), an acyl-binding pocket (Trp271), and a choline-binding pocket (Trp83) [104]. The residues that form the active site are: Trp83, Gly150, Gly151, Ser238, Ala239, Glu367, Tyr370, and His480 [104].

Carvacrol and *trans*-cinnamaldehyde showed similar affinity values (-6.5 kcal/mol), although slightly less negative than that of the reference AChE inhibitor, galantamine (-6.8 kcal/mol) (Table 11). However, eugenol (-6.9 kcal/mol) and pulegone (-7.5 kcal/mol) exhibited more negative affinity values than galantamine (Table 11), suggesting that these two compounds may act as strong inhibitors of fruit fly AChE. The results obtained in the *in silico* assays agree with those obtained in the *in vitro* inhibition assays, which suggests that AChE from the fruit fly (*D. melanogaster*) and the Medfly (*C. capitata*) are structurally similar.

Carvacrol, with an affinity value of -6.5 kcal/mol and no hydrogen bonds with the receptor, highly inhibited AChE from *C. capitata* in the *in vitro* inhibition assays (in the Supplementary Information Section, Table S6). Eltalawy et al. [147] also calculated the binding affinity between carvacrol and AChE from *T. castaneum* and

obtained a value of -7.1 kcal/mol. These findings suggest that carvacrol is a strong inhibitor of insect AChE. Eugenol (-6.9 kcal/mol), which inhibited Medfly AChE in the *in vitro* inhibition assays (in the Supplementary Information Section, Table S6), formed a hydrogen bond with the residue Tyr370 of the receptor. Regarding *trans*-cinnamaldehyde (-6.5 kcal/mol), this ligand formed a hydrogen bond with the residue Ser238, which is part of the catalytic triad of the receptor. In the *in vitro* inhibition assays, *trans*-cinnamaldehyde strongly inhibited AChE from *C. capitata* (in the Supplementary Information Section, Table S6). Pulegone, with the best binding affinity value in the *in silico* assays (-7.5 kcal/mol) and the lowest IC_{50} value in the *in vitro* inhibition assays (IC_{50} = 0.0110 ± 0.0026 mg/mL, Table S6, in the Supplementary Information Section), was expectedly the strongest AChE inhibitor and formed a hydrogen bond with the residue Tyr370 of the receptor. The positions of carvacrol, eugenol, pulegone, and *trans*-cinnamaldehyde in the active site of AChE from *D. melanogaster* are shown in Figures S7 (in the Supplementary Information Section), S8 (in the Supplementary Information Section), 22, and S9 (in the Supplementary Information Section), respectively. To the best of my knowledge, no studies to date have evaluated the *in silico* interactions between these compounds and AChE from *D. melanogaster*.

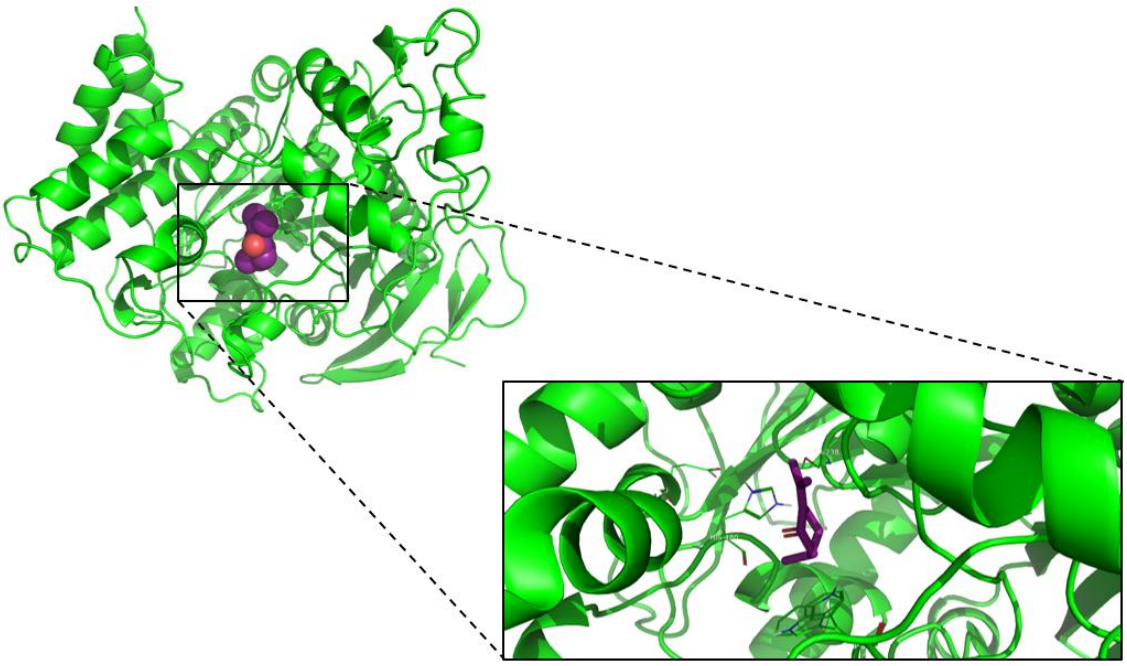


Figure 22 – Pulegone in complex with AChE from *D. melanogaster* (code: 6XYS). The image was generated by Pymol version 3.0 [107].

The *in silico* and *in vitro* inhibition results for AChE from both the electric eel and the fruit fly reveal that the selected pure compounds interact differently with this neurochemical enzyme, likely due to species-specific structural differences. The EO-derived compounds selectively and strongly inhibit the insect AChE, while showing no significant effect on the electric eel AChE. These findings highlight the potential of EOs as biopesticides.

IV. Conclusions and Future Work

The present dissertation elucidates the inhibitory effects of EOs derived from selected aromatic plants (*O. vulgare, T. vulgaris, O. gratissimum, M. pulegium,* and *C. cassia*) on enzymes that are crucial for the development and survival of insect and nematode pests. The experimental design, based on crude enzyme extracts obtained from *C. capitata*, *M. unipuncta*, *M. incognita*, and *B. xylophilus*, allowed for the assessment of EO activity under physiologically relevant conditions, thereby providing insights into their potential mechanisms of action.

The results showed that AChE, rather than α- and β-GLU, is the main enzymatic target of the tested EOs. MpEO had the lowest IC_{50} values against AChE from *C. capitata* adults (0.0022 ± 0.0013 mg/mL) and *B. xylophilus* (0.1209 ± 0.0136 mg/mL), while CcEO had the lowest IC_{50} values against AChE from *M. unipuncta* larvae (0.0020 ± 0.0004 mg/mL) and *M. incognita* J2 (0.0197 ± 0.0063 mg/mL). The strong AChE inhibitory activity observed for MpEO and CcEO across both insect and nematode species emphasizes the role of terpenes, terpenoids, and phenylpropanoids in disrupting cholinergic neurotransmission, which affects mobility, orientation, and mating, ultimately leading to death.

The combination of *in vitro* and *in silico* approaches provided a robust framework for interpreting the results. The molecular docking studies supported the experimental findings by revealing distinct interaction patterns between the EO compounds and insect AChE, compared to those with electric eel AChE, likely due to species-specific structural differences. Selectivity is highly desirable in pest management strategies, as it minimizes off-target effects on non-pest organisms.

In summary, this dissertation strengthens the evidence supporting the role of EOs as promising biopesticides. By selectively inhibiting pest AChE, MpEO and CcEO, in particular, demonstrate strong potential to disrupt pest physiology while reducing risks to non-target organisms. These insights enhance our understanding of the mechanisms of action of EOs and support the rational development of safer, more sustainable pest management strategies.

For future work, it would be interesting to: investigate the antagonistic or synergic interactions among EO compounds; accurately identify the proteins observed in the SDS-PAGE analysis through Western blotting with target-specific antibodies or MALDI-TOF; focus on enzyme purification and kinetic characterization, as crude extracts may introduce variability in enzyme composition and activity; and validate the findings under *in vivo* and field conditions.

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VI. Supplementary Information

Table S1 – List of reagents and standards and their relevant information: molecular formula, CAS number, manufacturer, and purity.

Reagents and Standards	Molecular formula	CAS number	Manufacturer (country)	Purity
(-) - Menthol	$C_{10}H_{20}O$	89-78-1	TCI Chemicals (Japan)	> 99.0 %
(-) - Isopulegol	$C_{10}H_{18}O$	89-79-2	Fluka (Japan)	99+% (GC)
(-) - Pulegone	$C_{10}H_{16}O$	89-82-7	Fluka (Switzerland)	≥ 98.0% (GC, sum of enantiomers)
(-) - α - Pinene	$C_{10}H_{16}$	80-56-8	Fluka (Spain)	98+%
(-) - β - Caryophyllene	$C_{15}H_{24}$	87-44-5	Unknown	Unknown
(+) - Isomenthone	$C_{10}H_{18}O$	491-07-6	Unknown	Unknown
(±) - Linalool	$C_{10}H_{18}O$	78-70-6	Unknown	Unknown
Δ3 - Carene	$C_{10}H_{16}$	13466-78-9	Unknown	Unknown
1 - Deoxynojirimycin	$C_6H_{13}NO_4$	19130-96-2	Biopurify Phytochemicals Ltd. (China)	> 98%
4 - nitrophenyl - α - D - glucopyranoside	$C_{12}H_{15}NO_8$	3767-28-0	Sigma-Aldrich (Switzerland)	≥ 99.0%
4 - nitrophenyl - β - D - glucopyranoside	$C_{12}H_{15}NO_8$	2492-87-7	Sigma-Aldrich (Switzerland)	≥ 98.0% (TLC)
5,5' - Dithiobis (2-nitrobenzoic acid)	$C_{14}H_8N_2O_8S_2$	69-78-3	Thermo Scientific (USA)	99.0%
Acetic acid (glacial) 100% anhydrous	$C_2H_4O_2$	64-19-7	Merck (Germany)	GR for analysis
Acetylthiocholine iodide	$C_7H_{16}NOSI$	1866-15-5	TCI Chemicals (Japan)	> 98.0%

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Table S1 (cont.) – List of reagents and standards and their relevant information: molecular formula, CAS number, manufacturer, and purity.

Reagents and Standards	Molecular formula	CAS number	Manufacturer (country)	Purity
Acrylamide	C_3H_5NO	79-06-1	Acros Organics (India)	99.0+%
Ammonium persulfate	$(NH_4)_2S_2O_8$	7727-54-0	PlusOne (Sweden)	98%
BSA	-	9048-46-8	Fisher BioReagents (USA)	Standard grade
Bromophenol blue for analysis	$C_{19}H_{10}Br_4O_5S$	115-39-9	PanReac AppliChem (Germany)	ACS reagent
Carvacrol	$C_{10}H_{14}O$	499-75-2	Sigma-Aldrich (Israel)	98.0%
Carvacrol methyl ether	$C_{11}H_{16}O$	6379-73-3	Unknown	Unknown
Coomassie Brilliant Blue G-250	$C_{47}H_{48}N_3NaO_7S_2$	6104-58-1	Panreac AppliChem (Germany)	Unknown
Dimethyl sulfoxide	C_2H_6OS	67-68-5	Fisher Scientific (United Kingdom)	Analytical reagent grade ≥ 99.9%
Disodium phosphate dihydrate	$Na_2HPO_4.2H_2O$	10028-24-7	Riedel-de Haën (Germany)	≥ 98.5%
Ethanol	C_2H_5OH	64-17-5	CQM (Portugal)	96.0%
Eugenol	$C_{10}H_{12}O_2$	97-53-0	Sigma-Aldrich (Germany)	99.0%
Galantamine hydrobromide	$C_{17}H_{22}BrNO_3$	1953-04-4	Merck (USA)	Unknown

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Table S1 (cont.) – List of reagents and standards and their relevant information: molecular formula, CAS number, manufacturer, and purity.

Reagents and Standards	Molecular formula	CAS number	Manufacturer (country)	Purity
Glycerol	$C_3H_8O_3$	56-81-5	Fisher Scientific (USA)	Unknown
Glycine	$C_2H_5NO_2$	56-40-6	Sigma-Aldrich (USA)	≥ 99.0% (HPLC)
Hydrochloric acid	HCl	7647-01-0	Honeywell (Austria)	≥ 37.0%
Methanol	CH_3OH	67-56-1	Fisher Scientific (United Kingdom)	Analytical reagent grade 99.9%
N, N, N', N' - Tetramethylethylenediamine	$C_6H_{16}N_2$	110-18-9	Sigma-Aldrich (China)	~ 99.0%
N, N' – Methylenebis (acrylamide)	$C_7H_{10}N_2O_2$	110-26-9	Sigma-Aldrich (USA)	99.0%
n-Hexane	C_6H_{14}	110-54-3	PanReac AppliChem (Germany)	95% for UV, IR, HPLC, ACS
Novex™ Sharp Pre-stained Protein Standard	-	-	Invitrogen (USA)	Unknown
Orthophosphoric acid	H_3PO_4	7664-38-2	Fisher Scientific (United Kingdom)	85.3%
PageRuler™ Prestained Protein Ladder, 10 to 180 kDa	-	-	Thermo Scientific (Lithuania)	Unknown
SDS for molecular biology	$C_{12}H_{25}NaO_4S$	151-21-3	PanReac AppliChem (Germany)	Unknown
Sodium carbonate	Na_2CO_3	497-19-8	Sigma-Aldrich (Germany)	ACS reagent ≥ 99.8%

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Table S1 (cont.) – List of reagents and standards and their relevant information: molecular formula, CAS number, manufacturer, and purity.

Reagents and Standards	Molecular formula	CAS number	Manufacturer (country)	Purity
Sodium hydroxide	NaOH	1310-73-2	Fisher Chemical (Germany)	Unknown
Sodium phosphate monobasic monohydrate	$NaH_2PO_4.H_2O$	10049-21-5	Merck (Germany)	≥ 99.0%
Thymol	$C_{10}H_{14}O$	89-83-8	Fluka (Germany)	≥ 99.0% (GC)
trans - Cinnamaldehyde	C_9H_8O	14371-10-9	Thermo Scientific (USA)	98+%
Tris(hydroxymethyl)aminomethane	$C_4H_{11}NO_3$	77-86-1	Pronalab (Portugal)	≥ 99.8%
α - Phellandrene	$C_{10}H_{16}$	99-83-2	Unknown	Unknown
α - Terpinene	$C_{10}H_{16}$	99-86-5	Fluka (Switzerland)	≥ 95.0% (GC)
β-Mercaptoethanol for molecular biology	C_2H_6OS	60-24-2	PanReac AppliChem (Germany)	Unknown
γ - Terpinene	$C_{10}H_{16}$	99-85-4	Fluka (Switzerland)	≥ 98.5% (GC)

Table S2 – List of enzymes and their relevant information: CAS number, manufacturer, and purity.

Enzymes	CAS number	Manufacturer (country)	Purity
Acetylcholinesterase from *Electrophorus electricus* (electric eel)	9000-81-1	Sigma-Aldrich (USA)	Unknown
α-Glucosidase from *Saccharomyces cerevisiae*	9001-42-7	Sigma-Aldrich (Israel)	Unknown
β-Glucosidase from almonds	9001-22-3	Sigma-Aldrich (Israel)	Unknown

Table S3 – List of equipment and their model and brand.

Equipment	Model, ***Brand***
Analytical Balance	M124AI, *BEL Engineering*
GC-FID	7890A GC System, *Agilent Technologies*
	7693 Autosampler, *Agilent Technologies*
Heating and Stirring Plates	Isotemp, *Fisher Scientific*
	MR 2002, *Heidolph*
	MS-H-S, *I@LAB*
Microplate Reader	Multiskan FC, *Thermo Scientific*
Mini Vertical Protein Electrophoresis Unit	SE260, *Hoefer*
pH Meter	744 pH Meter, *Metrohm*
Power Supply	PS300-B, *Hoefer*
Refrigerated Centrifuge	3-30K, *Sigma*
Shaking Incubator	NB-T205, *N-Biotek*
UV-Visible Spectrophotometer	Genesys 180, *Thermo Scientific*
	NanoDrop One, *Thermo Scientific*

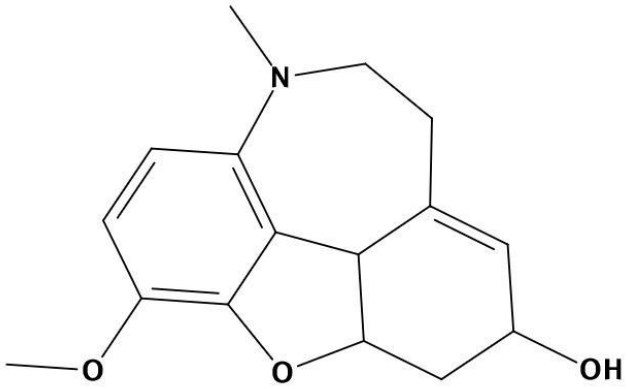

Figure S1 – Chemical structure of galantamine.

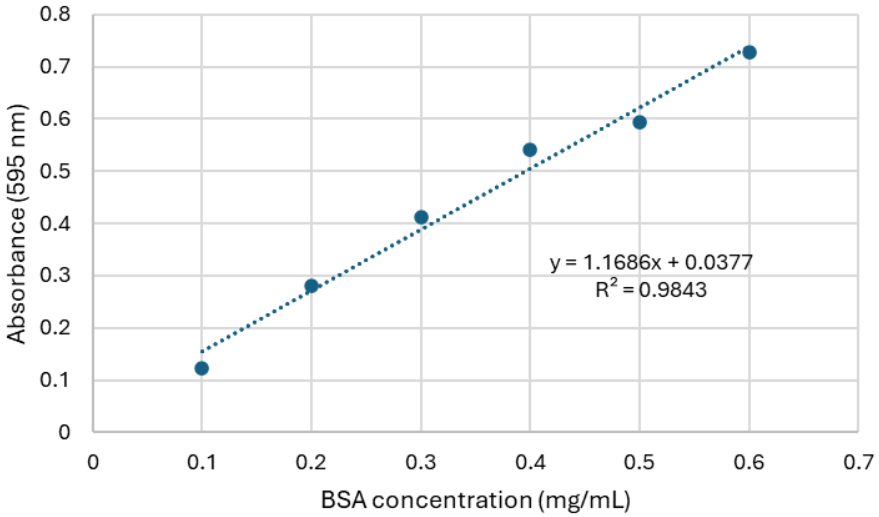

Figure S2 – BSA calibration line.

Table S4 – Relative mobility, raw volume, and molecular weight (Mw, in kDa) of the molecular size market – PageRuler™ Prestained Protein Ladder, 10-180 kDa.

Band #	Rf	Raw volume	Mw (kDa)
1	0.015	144	180
2	0.046	214	130
3	0.118	1377	100
4	0.198	775	70
5	0.298	2032	55
6	0.371	1375	40
7	0.510	1002	25
8	0.713	2488	15
9	0.849	779	10

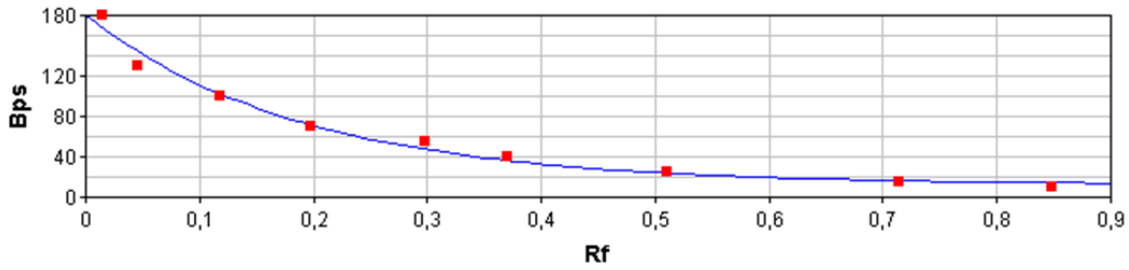

Figure S3 – Calibration curve (relative mobility vs. Mw) of the molecular size market – PageRuler™ Prestained Protein Ladder, 10-180 kDa.

Table S5 – AChE inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against AChE from electric eel (*E. electricus*) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
Pure compounds	Carvacrol	0.0269	0.0001	y = 46.56x + 123.14	0.981
	Thymol	2.5309	0.5145	y = 17.99x + 38.58	0.968
	Eugenol	-	-	y = 4.74x + 6.29	0.913
	Pulegone	-	-	y = 9.24x + 6.11	0.972
	Menthol	-	-	y = 3.38x - 1.73	0.939
	trans-Cinnamaldehyde	-	-	y = 8.43x + 15.15	0.893
EOs	*O. vulgaris*	-	-	y = 4.62x - 1.67	0.824
	T. vulgare	0.9258	0.1349	y = 33.56x + 53.04	0.889
	O. gratissimum	-	-	y = 6.42x + 8.79	0.954
	M. pulegium	-	-	y = 14.50x + 22.82	0.968
	C. cassia	-	-	y = 5.72x + 17.87	0.874
Galantamine hydrobromide		0.0018	0.0001	y = 35.33x + 146.62	0.984

Pure compounds serial concentrations: 0.0780-0.0002 mg/mL; EOs serial concentrations: 0.1565-0.0003 mg/mL; Galantamine hydrobromide serial concentrations: 0.0300-0.0001 mg/mL. **Legend:** IC_{50} – concentration of extract that inhibits 50% of AChE; (-) – no inhibition of AChE.

Table S6 – AChE inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against AChE from the Medfly (*C. capitata*) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
Pure compounds	Carvacrol	0.2480	0.0347	y = 17.93x + 62.14	0.976
	Thymol	2.2253	0.8503	y = 10.42x + 48.99	0.941
	Eugenol	0.3327	0.1099	y = 15.49x + 60.17	0.962
	Pulegone	0.0110	0.0026	y = 52.44x + 158.40	0.956
	Menthol	1.7084	0.8445	y = 5.86x + 37.71	0.954
	trans-Cinnamaldehyde	0.1015	0.0444	y = 12.83x + 61.07	0.875
EOs	*O. vulgaris*	0.0222	0.0025	y = 32.84x + 105.34	0.956
	T. vulgare	0.0234	0.0076	y = 22.41x + 90.65	0.996
	O. gratissimum	0.1793	0.1147	y = 35.77x + 75.57	0.998
	M. pulegium	0.0022	0.0013	y = 24.26x + 116.23	0.995
	C. cassia	0.5163	0.1842	y = 7.47x + 53.61	0.842
Galantamine hydrobromide		0.0035	0.0008	y = 30.19x + 124.52	0.972

Pure compounds serial concentrations: 0.0780-0.0002 mg/mL; EOs serial concentrations: 0.1565-0.0003 mg/mL; Galantamine hydrobromide serial concentrations: 0.0300-0.0001 mg/mL. **Legend:** IC_{50} – concentration of extract that inhibits 50% of AChE.

Table S7 – AChE inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against AChE from the armyworm (*M. unipuncta*) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	± SD		
Pure compounds	Carvacrol	0.0552	0.0341	y = 9.31x + 61.67	0.848
	Thymol	0.1507	0.0808	y = 12.90x + 65.63	0.915
	Eugenol	0.1600	0.0753	y = 17.51x + 67.33	0.899
	Pulegone	0.0222	0.0023	y = 28.29x + 97.51	0.927
	Menthol	0.4313	0.2028	y = 9.44x + 56.61	0.797
	trans-Cinnamaldehyde	0.0444	0.0268	y = 19.24x + 72.46	0.991

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Table S7 (cont.) – AChE inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against AChE from the armyworm (*M. unipuncta*) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	± SD		
EOs	*O. vulgaris*	0.8896	0.5888	y = 17.78x + 46.72	0.956
	T. vulgare	0.8304	0.2675	y = 6.26x + 49.80	0.899
	O. gratissimum	0.0105	0.0094	y = 15.94x + 83.85	0.790
	M. pulegium	0.0587	0.0091	y = 17.19x + 71.87	0.947
	C. cassia	0.0020	0.0004	y = 13.17x + 84.94	0.776
Galantamine hydrobromide		0.0003	0.0002	y = 11.33x + 87.43	0.911

Pure compounds serial concentrations: 0.0780-0.0002 mg/mL; EOs serial concentrations: 0.1565-0.0003 mg/mL; Galantamine hydrobromide serial concentrations: 0.0300-0.0001 mg/mL. **Legend:** IC_{50} – concentration of extract that inhibits 50% of AChE.

Table S8 – AChE inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against AChE from the RKN (*M. incognita*, J2) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
Pure compounds	Carvacrol	-	-	y = 5.69x + 30.97	0.983
	Thymol	0.0055	0.0003	y = 20.23x + 96.23	0.926
	Eugenol	-	-	y = 6.18x + 28.04	0.970
	Pulegone	0.0680	0.0058	y = 27.29x + 80.85	0.905
	Menthol	0.1324	0.0327	y = 7.33x + 56.55	0.920
	trans-Cinnamaldehyde	0.4848	0.1524	y = 9.98x + 53.41	0.960

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Table S8 (cont.) – AChE inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against AChE from the RKN (*M. incognita*, J2) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
EOs	*O. vulgaris*	0.5444	0.2645	y = 12.04x + 55.03	0.900
	T. vulgare	0.0633	0.0480	y = 10.17x + 59.43	0.976
	O. gratissimum	-	-	y = 14.10x + 24.82	0.980
	M. pulegium	0.0222	0.0056	y = 29.29x + 102.17	0.940
	C. cassia	0.0197	0.0063	y = 7.63x + 64.26	0.920
Galantamine hydrobromide		0.0036	0.0006	y = 10.46x + 75.79	0.980

Pure compounds serial concentrations: 0.0780-0.0002 mg/mL; EOs serial concentrations: 0.1565-0.0003 mg/mL; Galantamine hydrobromide serial concentrations: 0.0300-0.0001 mg/mL. **Legend:** IC_{50} – concentration of extract that inhibits 50% of AChE; (-) – no inhibition of AChE.

Table S9 – AChE inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against AChE from the PWN (*B. xylophilus*) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
Pure compounds	Carvacrol	-	-	y = 6.18x + 37.19	0.831
	Thymol	0.0131	0.0056	y = 16.32x + 83.72	0.943
	Eugenol	-	-	y = 5.36x + 20.53	0.962
	Pulegone	0.0251	0.0024	y = 39.39x + 112.69	0.903
	Menthol	-	-	y = 5.28x + 18.73	0.963
	trans-Cinnamaldehyde	0.6610	0.4237	y = 10.41x + 50.57	0.956
EOs	*O. vulgaris*	-	-	y = 5.11x + 28.05	0.930
	T. vulgare	-	-	y = 11.35x + 24.26	0.955
	O. gratissimum	0.2100	0.1593	y = 5.36x + 52.17	0.933
	M. pulegium	0.1209	0.0136	y = 36.48x + 85.68	0.995
	C. cassia	1.2627	0.3591	y = 9.53x + 47.91	0.964
Galantamine hydrobromide		0.0084	0.0027	y = 13.47x + 79.64	0.843

Pure compounds serial concentrations: 0.0780-0.0002 mg/mL; EOs serial concentrations: 0.1565-0.0003 mg/mL; Galantamine hydrobromide serial concentrations: 0.0300-0.0001 mg/mL. **Legend:** IC_{50} – concentration of extract that inhibits 50% of AChE; (-) – no inhibition of AChE.

Table S10 – α-GLU inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against α-GLU from *S. cerevisiae* (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
Pure compounds	Carvacrol	-	-	y = 12.43x + 23.89	0.954
	Thymol	0.3043	0.0254	y = 38.36x + 70.47	0.973
	Eugenol	-	-	y = 3.73x + 7.57	0.976
	Pulegone	-	-	y = 1.63x + 6.00	0.942
	Menthol	-	-	y = 1.44x + 4.30	0.953
	trans-Cinnamaldehyde	-	-	y = 6.81x + 17.27	0.986
EOs	*O. vulgaris*	-	-	y = 9.74x + 16.30	0.927
	T. vulgare	2.4080	0.0971	y = 22.77x + 41.51	0.933
	O. gratissimum	-	-	y = 2.41x + 6.09	0.950
	M. pulegium	-	-	y = 2.10x + 6.98	0.984
	C. cassia	-	-	y = 8.79x + 7.02	0.938
1-DNJ		0.2309	0.0031	y = 34.11x + 71.96	0.955

Pure compounds, EOs, and 1-DNJ serial concentrations: 0.3000-0.0023 mg/mL. **Legend:** IC_{50} – concentration of extract that inhibits 50% of α-GLU; (-) – no inhibition of α-GLU.

Table S11 – β-GLU inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against β-GLU from almonds (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
Pure compounds	Carvacrol	-	-	y = 9.86x + 23.04	0.966
	Thymol	-	-	y = 2.70x + 8.10	0.979
	Eugenol	-	-	y = 9.78x + 16.09	0.927
	Pulegone	-	-	y = 5.88x + 11.63	0.963
	Menthol	-	-	y = 6.81x + 12.56	0.986
	trans-Cinnamaldehyde	-	-	y = 1.77x + 4.58	0.979

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Table S11 (cont.) – β-GLU inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against β-GLU from almonds (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
EOs	*O. vulgaris*	-	-	y = 2.35x + 9.23	0.916
	T. vulgare	-	-	y = 3.08x + 6.75	0.996
	O. gratissimum	-	-	y = 3.78x + 10.44	0.980
	M. pulegium	-	-	y = 7.25x + 18.26	0.977
	C. cassia	-	-	y = 2.93x + 4.11	0.985
1-DNJ		0.0363	0.0008	y = 34.11x + 71.96	0.984

Pure compounds, EOs, and 1-DNJ serial concentrations: 0.3000-0.0023 mg/mL. **Legend:** IC_{50} – concentration of extract that inhibits 50% of β-GLU; (-) – no inhibition of β-GLU.

Table S12 – α-GLU inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against α-GLU from Medfly (*C. capitata*) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
Pure compounds	Carvacrol	-	-	y = 5.79x + 1.41	0.962
	Thymol	-	-	y = 2.73x + 11.28	0.956
	Eugenol	-	-	y = 2.72x + 8.64	0.915
	Pulegone	-	-	y = 2.46x + 5.94	0.903
	Menthol	-	-	y = 5.96x + 15.29	0.962
	trans-Cinnamaldehyde	-	-	y = 3.62x + 8.68	0.968
EOs	*O. vulgaris*	-	-	y = 3.76x + 5.68	0.965
	T. vulgare	-	-	y = 5.99x + 15.78	0.986
	O. gratissimum	-	-	y = 4.86x + 9.41	0.927
	M. pulegium	-	-	y = 3.07x + 7.64	0.927
	C. cassia	-	-	y = 5.26x + 8.41	0.949
1-DNJ		0.0072	0.0018	y = 27.70x + 112.91	0.967

Pure compounds, EOs, and 1-DNJ serial concentrations: 0.3000-0.0023 mg/mL. **Legend:** IC_{50} – concentration of extract that inhibits 50% of α-GLU; (-) – no inhibition of α-GLU.

Table S13 – α-GLU inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against α-GLU from armyworm (*M. unipuncta*) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
Pure compounds	Carvacrol	-	-	y = 3.31x + 8.28	0.887
	Thymol	-	-	y = 5.11x + 10.99	0.942
	Eugenol	-	-	y = 2.67x + 6.53	0.947
	Pulegone	-	-	y = 2.57x + 7.18	0.926
	Menthol	-	-	y = 2.99x + 7.68	0.935
	trans-Cinnamaldehyde	-	-	y = 6.58x + 7.30	0.909
EOs	*O. vulgaris*	-	-	y = 4.68x + 13.85	0.937
	T. vulgare	-	-	y = 2.27x + 8.38	0.974
	O. gratissimum	-	-	y = 4.89x + 7.38	0.961
	M. pulegium	-	-	y = 4.03x + 7.97	0.937
	C. cassia	-	-	y = 2.47x + 5.01	0.929
1-DNJ		0.0015	0.0002	y = 24.52x + 116.15	0.922

Pure compounds, EOs, and 1-DNJ serial concentrations: 0.3000-0.0023 mg/mL. **Legend:** IC_{50} – concentration of extract that inhibits 50% of α-GLU; (-) – no inhibition of α-GLU.

Table S14 – β-GLU inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against β-GLU from armyworm (*M. unipuncta*) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
Pure compounds	Carvacrol	-	-	y = 8.13x + 10.16	0.973
	Thymol	-	-	y = 7.74x + 12.71	0.978
	Eugenol	-	-	y = 4.37x + 8.41	0.955
	Pulegone	-	-	y = 15.04x + 18.01	0.997
	Menthol	-	-	y = 8.89x + 13.36	0.985
	trans-Cinnamaldehyde	-	-	y = 5.72x + 15.89	0.947

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Table S14 (cont.) – β-GLU inhibition assay: IC_{50} mean and SD values (mg/mL), regression equations, and R^2 values for pure compounds and EOs against β-GLU from armyworm (*M. unipuncta*) (n = 3).

		IC_{50} (mg/mL)		**Regression equation**	**R^2**
		Mean	SD		
EOs	*O. vulgaris*	-	-	y = 5.09x + 5.83	0.899
	T. vulgare	-	-	y = 8.33x + 12.95	0.913
	O. gratissimum	-	-	y = 4.12x + 10.04	0.920
	M. pulegium	-	-	y = 3.63x + 10.18	0.917
	C. cassia	-	-	y = 6.55x + 16.95	0.982
1-DNJ		0.4182	0.0576	y = 19.84x + 56.41	0.985

Pure compounds, EOs, and 1-DNJ serial concentrations: 0.3000-0.0023 mg/mL. **Legend:** IC_{50} – concentration of extract that inhibits 50% of β-GLU; (-) – no inhibition of β-GLU.

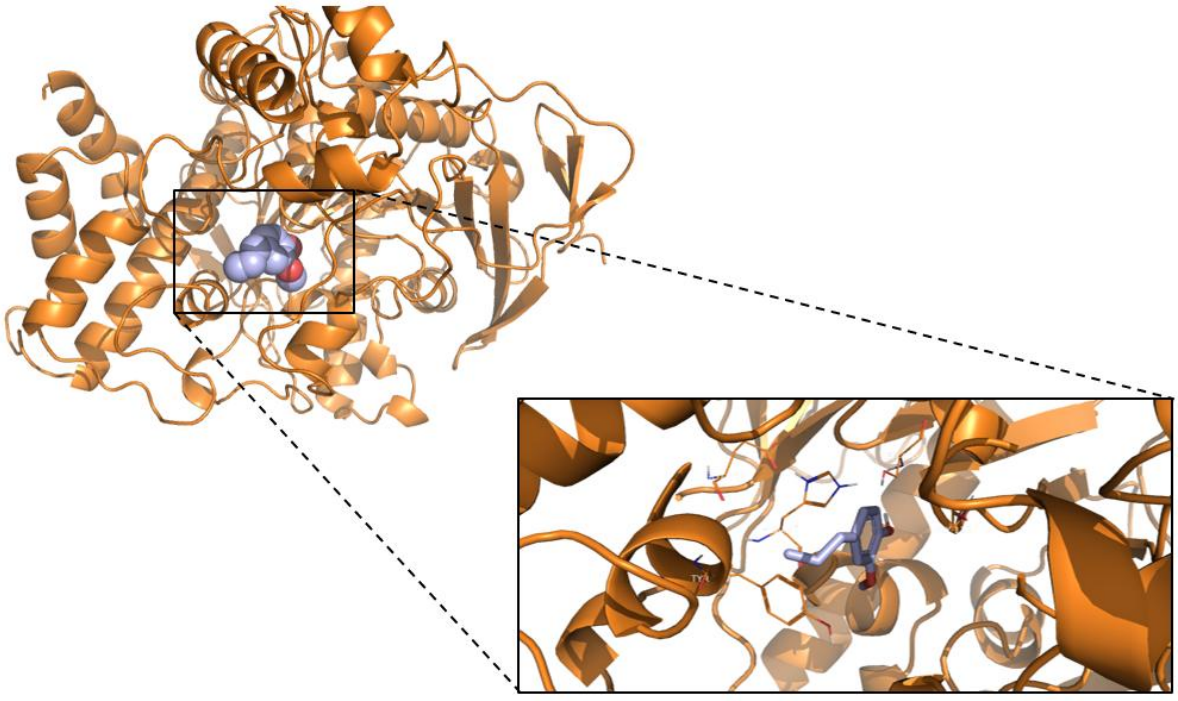

Figure S4 – Eugenol in complex with AChE from *E. electricus* (code: 1C2O). The image was generated by Pymol version 3.0 [107].

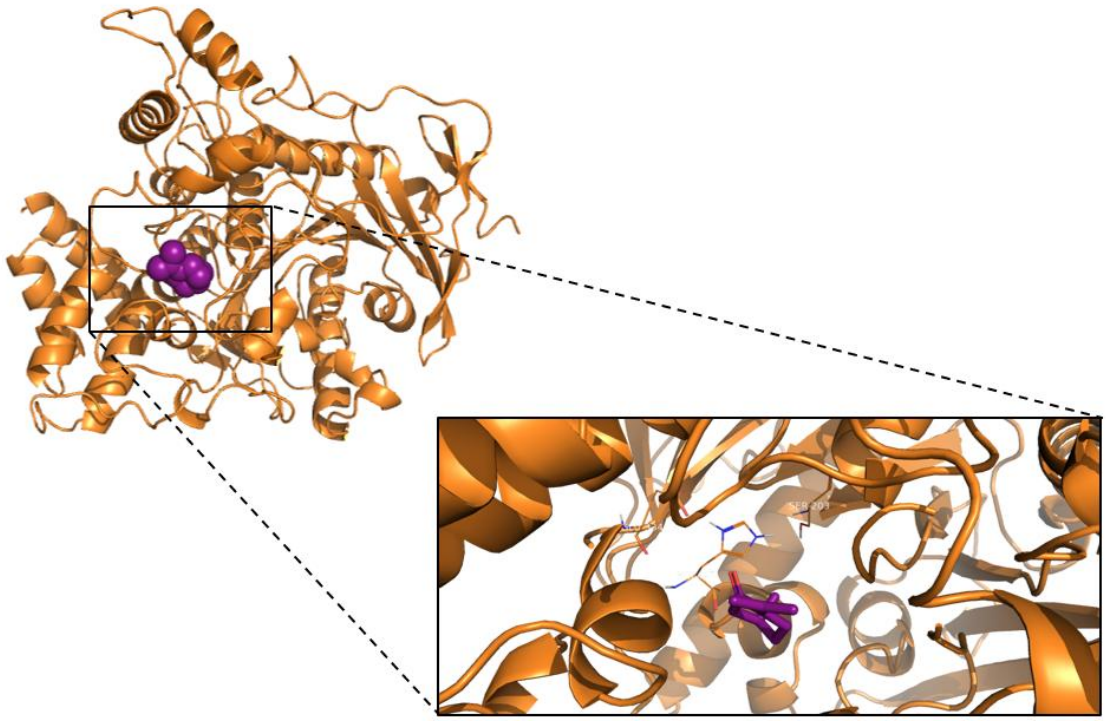

Figure S5 – Pulegone in complex with AChE from *E. electricus* (code: 1C2O). The image was generated by Pymol version 3.0 [107].

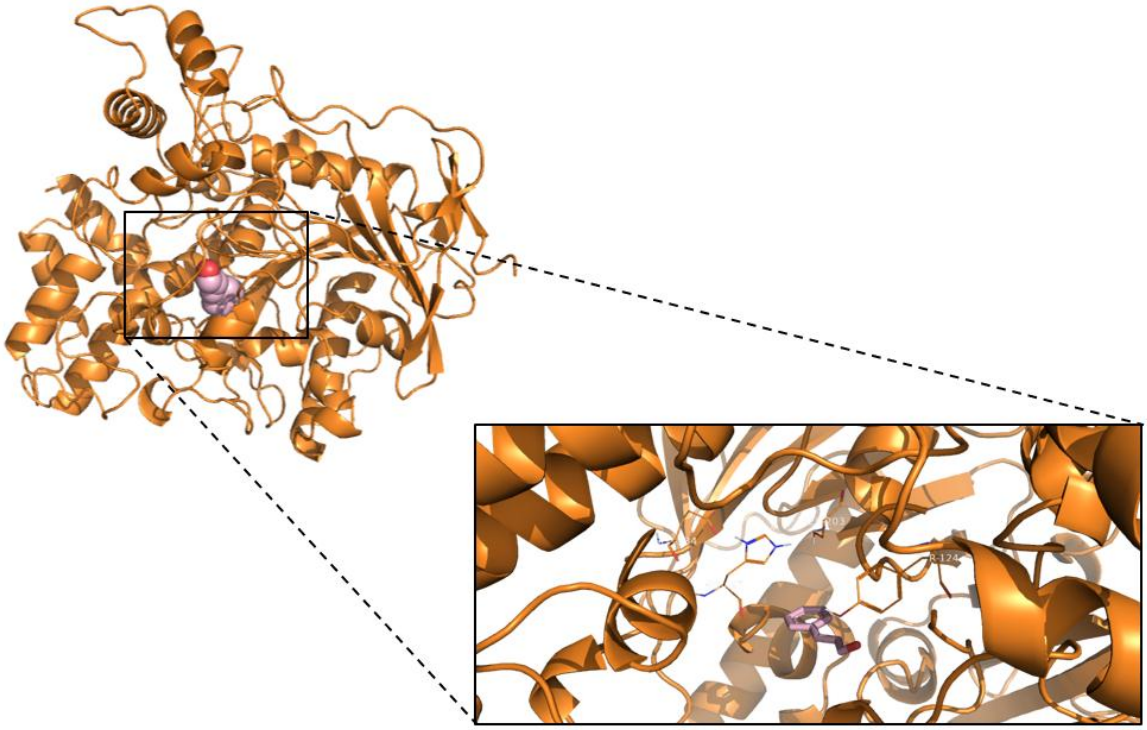

Figure S6 – *trans*-Cinnamaldehyde in complex with AChE from *E. electricus* (code: 1C2O). The image was generated by Pymol version 3.0 [107].

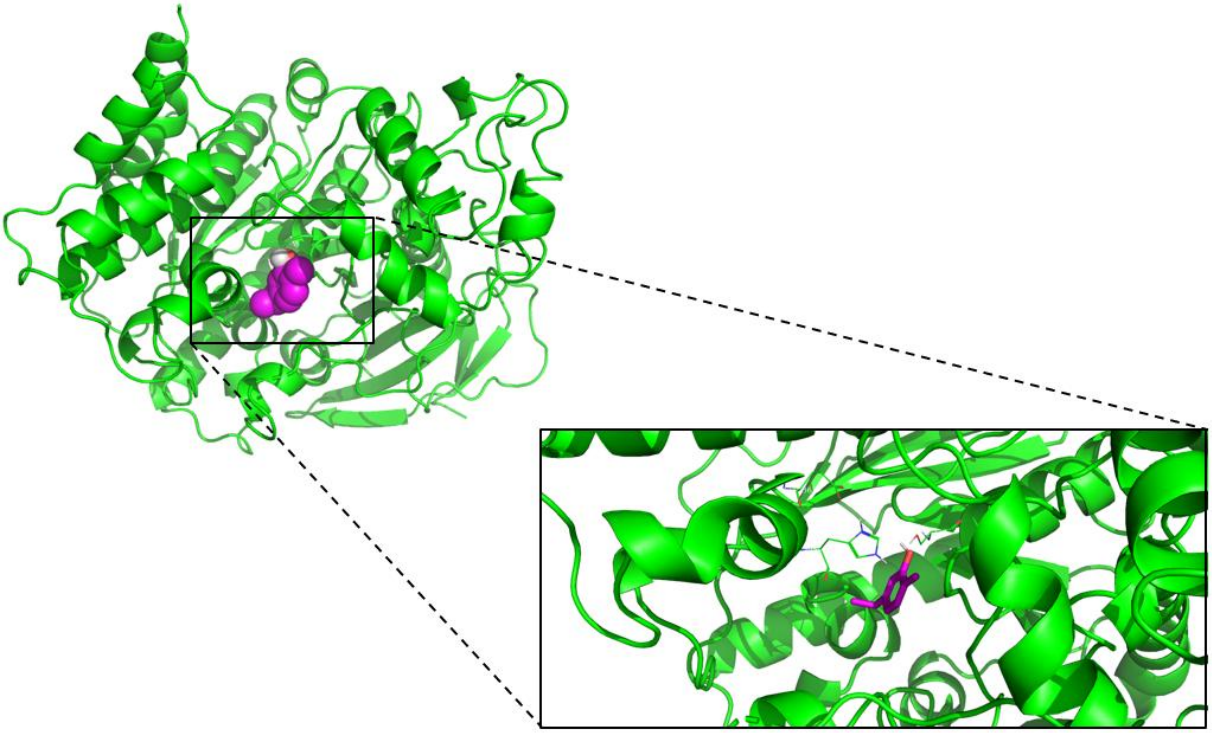

Figure S7 – Carvacrol in complex with AChE from *D. melanogaster* (code: 6XYS). The image was generated by Pymol version 3.0 [107].

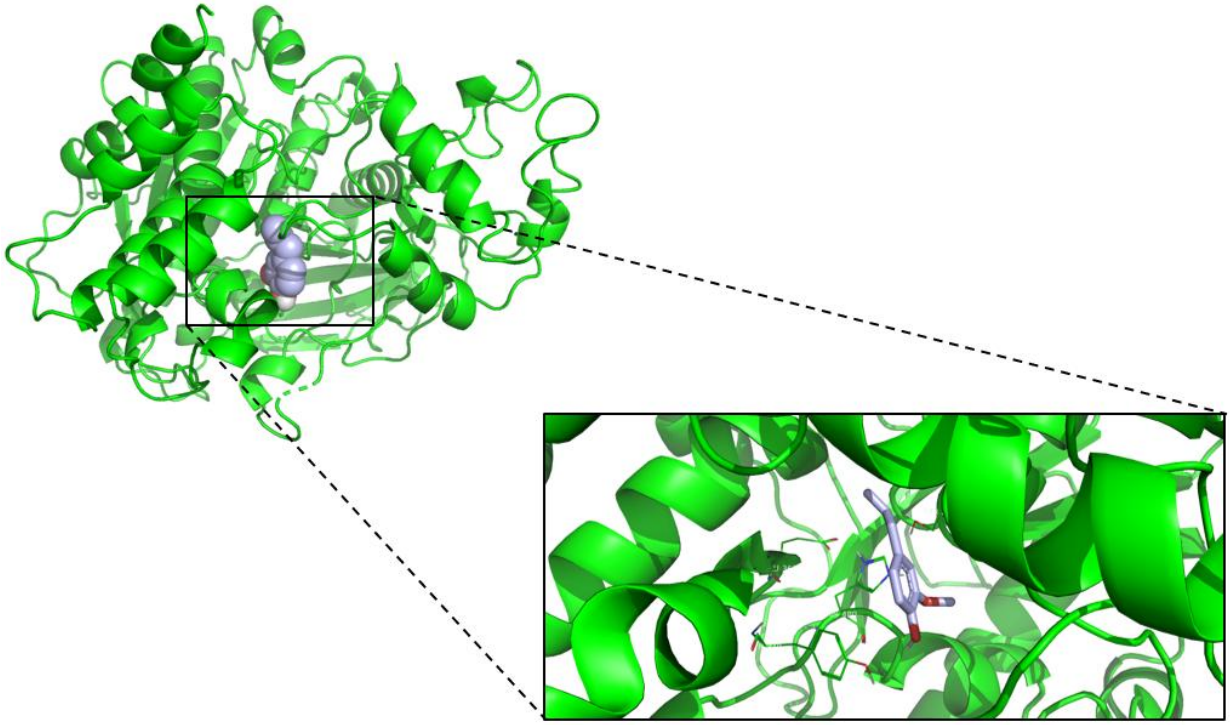

Figure S8 – Eugenol in complex with AChE from *D. melanogaster* (code: 6XYS). The image was generated by Pymol version 3.0 [107].

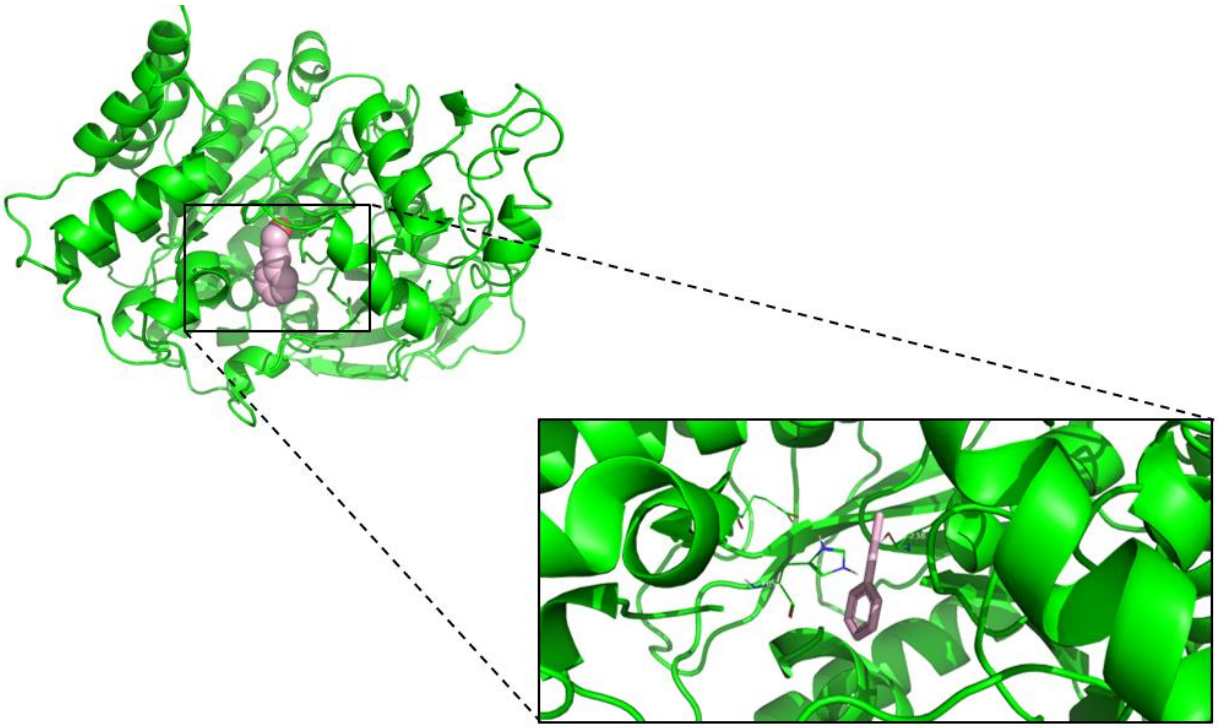

Figure S9 – *trans*-Cinnamaldehyde in complex with AChE from *D. melanogaster* (code: 6XYS). The image was generated by Pymol version 3.0 [107].


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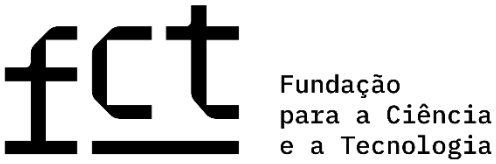
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