

Article

Toxicity, Histopathological Alterations and Acetylcholinesterase Inhibition of *Illicium verum* Essential Oil in *Drosophila suzukii*

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Abstract: In recent years, studies on the potential use of essential oils (EOs) as pesticides have enormously increased owing to their remarkable biological activities and health benefits. However, given the scant knowledge on the mode(s) of action behind insecticidal activity of individual essential oils, as well as their mixtures, much more work has yet to be undertaken. Furthermore, the variable and complex mixtures of essential oils suggest that their biological activities are likely due to several mechanisms acting on different physiological processes. Here, we firstly assessed the toxicity of *Illicium verum*, *Myristica fragrans* and *Schinus molle* EOs on *Drosophila suzukii* adults. Then, their acetylcholinesterase inhibitory activity as a potential mode of action was investigated. Subsequently, we explored potential structural alterations caused by exposure to low concentrations (LC₂₀ and LC₅₀) of *I. verum* oil on the fat body, midgut and muscular tissues of female flies. The results showed that the three EOs had good insecticidal activity against *D. suzukii* flies and although *I. verum* oil was more toxic than the two others, its acetylcholinesterase inhibition was the lowest. However, exposure to *I. verum* EO promoted severe concentration-dependent histological and structural alterations in the carbohydrate contents, muscle fiber, midgut epithelium as well as fat droplets area of exposed females. Collectively, our findings revealed that the insect's internal organs are potential target sites of *I. verum* EO's acute toxicity. Further studies are needed to confirm *I. verum* as a promising insecticidal compound against insect pests, to better elucidate its physiological and molecular action sites as well as assess its toxicity to non-target organisms.

Keywords: essential oils; mode of action; spotted wing drosophila; internal morphology



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1. Introduction

In the agricultural sector, studies on plant-derived compounds have focused on obtaining new insecticides against novel molecular targets [1]. Attention has focused on the search for alternative control methods that are environmentally friendly, sustainable and effective [2]. In this sense, products of plant origin have gained prominence in research, especially essential oils, due to the availability of raw materials and the good cost-benefit ratio [3].

Essential oils (EOs) are complex mixtures of volatile organic compounds produced as secondary metabolites in plants. In recent years, the use of EOs and their constituents as low-risk insecticides, insect repellents, and oviposition deterrents has increased considerably owing to their popularity among organic growers and environmentally conscious consumers. EOs extracted from plant seeds, stems, leaves, and flowers have been widely tested for their fungicidal, bactericidal, and insecticidal properties [4]. They have yielded

promising results under laboratory conditions and are being considered an important alternative strategy for sustainable insect pest management [5–7]. These botanical insecticides have shown strong insecticidal, repellent and fumigant activity against many insect pest species [4,8–13].

The mechanisms of the toxic action of EOs have not been fully elucidated, to date. In many cases, visible symptoms of the insecticidal activity of EOs such as hyperactivity, convulsions, tremors and paralysis suggest a neurotoxic mode of action [14]. Such symptoms indicate that the potential target site of these EOs may be the insect's neural signaling, acting on the enzyme acetylcholinesterase, GABA ionotropic receptors and, more evidently, octopamine metabotropic receptors [15]. Moreover, as EOs are generally mixtures of many compounds, their mechanism of action is usually not specific to one target but includes a variety of actions. Thus, the intrinsic properties of EOs can interfere with basic metabolic, biochemical, and physiological functions of insect pests and their ability to reduce and suppress the activity of detoxifying enzymes have been frequently reported [3]. Besides metabolic and physiological alteration, the action of EOs may produce histological modifications. Previous studies have reported several histopathological changes in insects exposed to EOs from different plants. Such changes can help to elucidate mechanisms involved in the insecticidal action of EOs [16–20].

Drosophila suzukii (Diptera: Drosophilidae) is a species of fly that has great potential to cause economic damage [21,22]. These insects can infest a wide variety of hosts, such as cultivated and wild red-fruit species [23,24]. It is a native species from East Asia and was first reported in Brazil in the summer of 2012/2013 in the southern region of the country [25]. Males differ from females by the presence of a dark spot near the edge of each wing, a characteristic that gives rise to the popular name of spotted-wing fly [26]. Females have serrated ovipositor apparatus that allow them to infest immature and undamaged fruits before harvesting, a unique feature among members of the *Drosophila* genus [24,27]. The use of synthetic insecticides is an alternative to minimize the economic damage generated by this species despite their disadvantages such as toxicity to non-target organisms and relatively high cost [28]. In this sense, studies on EOs have shown to be promising for the management of different pest arthropods [29,30]. This fact can be very important for the management of *D. suzukii*, considered the primary pest of small fruits in Brazil [25]. However, the effect of EOs from plants of families such as Schisandraceae, Myristicaceae and Anacardiaceae has not yet been evaluated in *D. suzukii*. Furthermore, the effects of low concentrations of EOs on the internal morphology of these flies have not yet been explored.

The star anise *Illicium verum* belongs to the Schisandraceae family, native to tropical and subtropical regions of Asia, and it is used as a spice [31]. *Myristica fragrans* belongs to the Myristicaceae family, its ovoid seed is used as a condiment and it is popularly known as nutmeg [32]. *Schinus molle* belongs to the Anacardiaceae family, native to subtropical regions of South America, and is commonly called pink pepper. These plants stand out for their broad range of biological activities such as antibacterial [33], antifungal [34] and antioxidant [35]. Additionally, the insecticidal activity of these species has already been reported for several pest insects [36–38]. However, the effects of their essential oil on the control of *D. suzukii* have not yet been explored, to the best of our knowledge.

The present study aimed firstly to investigate the lethal effects of the EOs from *Illicium verum*, *Myristica fragrans* and *Schinus molle*, as well as their inhibitory activity of acetylcholinesterase enzyme on *D. suzukii* adults. Secondly, the histopathological alterations caused by the exposure to low concentrations of *I. verum* oil in the thoracic muscles, midgut and fat body of adult flies were assessed as potential alternative targets for this oil toxic action.

2. Material and Methods

2.1. Plant Material and Insect Population

The plant material used in the present study consisted of leaves of *S. molle* collected from trees growing on the Campus of the Federal University of Lavras (UFLA) (21°14'

latitude S, 45°00′ longitude W) in Minas Gerais state (Brazil). A voucher was deposited in the Herbarium of the Federal University of Alfenas, receiving the code HUNIFAL298. Seeds of *M. fragrans* (CHAMEL, Campo Largo—PR; Brazil) and fruits of *I. verum* (CHAMEL, Campo Largo—PR; Brazil) were obtained from the local market (21°24′ latitude S, 44°99′ longitude W). The plant materials were cut into smaller parts for subsequent extraction of the EO.

The *D. suzukii* adult flies used in the bioassays were obtained from a stock colony kept under controlled conditions (i.e., temperature: 25 ± 2 °C; relative humidity: $60 \pm 5\%$; photoperiod: 12 h L:12 h D) in the Molecular Entomology and Ecotoxicology Laboratory of Entomology Department (DEN) at UFLA. Rearing was carried out on an artificial diet and followed previously described methods [39–41].

2.2. Essential Oils Extraction and Chemical Characterization

Extraction of the EOs was performed by hydrodistillation in a modified Clevenger apparatus and was carried out at the Laboratory of Organic Chemistry and Essential Oils of the Chemistry Department (DQI) at UFLA. For this purpose, 630 g of *I. verum*, 143 g of *M. fragrans* and 901 g of *S. molle* were added to a 5 L round-bottom flask half-filled with water. After 2 h of gentle boiling, the hydrolate was collected and subjected to centrifugation at $965 \times g$ for 15 min. Subsequently, the EO was pipetted and placed in a suitable container in the absence of light and under refrigeration (<5 °C) [42] until used in the experiments. Extractions were performed in triplicate and extraction yield was calculated by the difference in moisture-free base (%w/w BLU) [43].

The identification of chemical constituents was performed by gas chromatography coupled to mass spectrometry (GC-MS), using a model QP 2010 Plus equipment (Shimadzu Corporation, Kyoto, Japan) operating with a capillary column of fused silica (30 m \times 0.25 mm) with a DB-5 bound phase (film thickness, 0.25 μ m). Helium was used as carrier gas at a flow of 1.0 mL min⁻¹. Injector and detector temperatures were 220 and 240 °C, respectively. The sample injection volume was 0.5 μ L, diluted in hexane (1%) (Sigma-Aldrich®, St. Louis, MO, USA) and injection volume partition ratio (split) of 1:100. Temperature ramp started at 60 °C, with an increase at a rate of 3 °C min⁻¹ to 240 °C, followed by an increase at 10 °C min⁻¹ until reaching 300 °C, with the final temperature maintained for 7 min. Column pressure was around 71.0 kPa. The mass spectrometer was operated at ionization potential of 70 eV and ion source temperature of 200 °C. The mass analysis was performed in full-scan mode, ranging from 45 to 500 Da, with a scan speed of 1000 Da s⁻¹ and a scanning interval of 0.5 fragments s⁻¹. Data were obtained and processed using the Lab Solutions LC/GC Workstation 2.72 software (Shimadzu, Kyoto, Japan). The retention index of the compounds was calculated in relation to a homologous series of n-alkanes (nC₉–nC₁₈), using the equation of Van den Dool and Kratz [44]. The identification of compounds was performed by comparing the calculated retention indices with those described in the literature [45]. Comparisons of the mass spectra obtained with those existing in the FFNSC 1.2, NIST107 and NIST21 libraries were also performed.

Quantitative analysis was performed by gas chromatography with flame ionization detector (GC-FID), using model GC-2010 equipment (Shimadzu Corporation, Kyoto, Japan), with experimental conditions identical to those used in the qualitative analysis, except the detector temperature, which was 300 °C. The relative percentages of each constituent were obtained by the area normalization method.

2.3. Toxicity Assessment of Essential Oils against *Drosophila suzukii*

Concentration–mortality bioassays were conducted to determine contact and ingestion lethal activity of the three EOs to *D. suzukii* adults. The exposure procedure was based on the IRAC (Insecticide Resistance Action Committee) protocol number 026 [46], recommended for bioassays with adults of *Musca domestica* L. (Diptera: Muscidae), with minor modifications. Briefly, dental cotton (2 cm) treated with 1.9 mL of the solution composed of dimethyl sulfoxide (DMSO) (3% v/v), aqueous sucrose solution (20% w/v) and the EO in

serial concentrations was deposited in a glass vial (200 mL). For *I. verum* EO, concentrations between 0.1–100 $\mu\text{L mL}^{-1}$ were used, for *M. fragrans* EO the range was 5–50 $\mu\text{L mL}^{-1}$ and *S. molle* 10–150 $\mu\text{L mL}^{-1}$. As a negative control, a 2% *v/v* DMSO solution was used. For each repetition, twenty-five non-sexed, same-age insects were introduced into the glass bottles, and the bottles were closed with foam plugs and kept in a BOD incubator at 24 ± 2 °C and 50% RH, with a 12 h L:12 h D photoperiod. After 24 h, mortality was assessed visually. Flies that showed no movement even after stimulation with a fine brush were considered dead.

2.4. Acetylcholinesterase Inhibitory Activity of Essential Oils

The AChE inhibitory activity of the three EOs was assessed using a modified version of Ellman's method [47]. The method used consists of monitoring the rate of formation of the chromophore compound 5-thio-2-nitrobenzoate produced from the reaction of Ellman's Reagent (5,5-dithiobis-2-nitrobenzoic acid or its ionized form) with thiocholine. Thiocholine is obtained by the hydrolysis of acetylthiocholine (substrate) catalyzed by the enzyme acetylcholinesterase (AChE) (0.04 U mL^{-1} ; type VI-S) from *Electrophorus electricus*.

In a test tube, 2970 μL of the Tris-HCl buffer (50 mmol L^{-1}), pH 8, was firstly mixed with 254 μL of the acetylthiocholine solution (1000 U mL^{-1}) and incubated at 37 °C for 15 min. Then, 25 μL of the EO diluted in ethanol at concentrations of 0.25; 0.50; 1.0; 5; 10; 50 and 100 mg mL^{-1} was added to the mixture followed by the addition of 100 μL of 10 mmol L^{-1} Ellman's reagent and 80 μL of the 0.02 mol L^{-1} substrate solution. The mixture was incubated again at 37 °C for 15 min, and the absorbance was measured on a spectrophotometer at 412 nm. As a blank, 3.2 mL of Tris-HCl buffer was used.

For comparison purposes, carvacrol was used as a positive control at the same concentrations used for EOs. To consider the spontaneous hydrolysis of acetylthiocholine, non-enzymatic controls were performed for each concentration of oil tested, replacing the enzyme with a Tris-HCl buffer. The negative control contained all reagents except the EO, which was replaced by ethanol. The tests were performed in three repetitions and the percentage of enzyme inhibition was calculated according to the following equation:

$$I(\%) = \left(\frac{A_T - A_C}{A_O} \right) \cdot 100$$

where $I(\%)$ = percent of enzyme inhibition; A_T = absorbance of the treatment containing the essential oil/positive control; A_C = non-enzymatic control absorbance; and A_O = negative control absorbance.

2.5. Evaluation of the Effects of Exposure to Low Doses of *Illicium verum* Essential Oil on Females' Histological Structures

Based on the results of toxicity and enzyme inhibition bioassays (see Section 3), the EO of *I. verum* was chosen to assess potential structural alterations caused by exposure to low concentrations on the fat body, midgut and muscular tissues of female flies.

2.5.1. Histopathological Analysis

For histopathological analysis, *D. suzukii* pupae were exposed to *I. verum* EO solutions (LC_{20} and LC_{50}), determined in the toxicology experiment, and control (water and DMSO aqueous solution at 3% *v/v*) by submersion for 30 s. Afterward, they were dried with absorbent paper and stored in a container sealed with voile tissue. After emergence, which took approximately five days, five adult females were collected for each treatment and fixed in a 4% paraformaldehyde solution for 72 h and transferred to 70% ethanol solution. Females were chosen for this analysis due to their importance for the propagation of the species and possible greater resistance to insecticidal treatment compared to males [48,49]. The insects were, then, dehydrated using serial concentrations of ethanol (70, 80, 90 and 95%) and soaked in *Leica* historesin for 24 h at 4 °C before being transferred to plastic molds for inclusion. Subsequently, slides of 4 μm thickness were prepared using a microtome (Luptec MRP09) and stained with Hematoxylin and Eosin [50]. A minimum of

two histological glasses containing at least six sections were obtained for each sample. Finally, the midgut, thoracic muscle tissue and fat body of the insects were evaluated under conventional light microscopy to verify possible histopathological changes. We decided to evaluate these tissues due to their abundance in the female's body as well as their physiological importance.

2.5.2. Histochemical Analysis

To assess changes in factors such as the presence, frequency and distribution of proteins and polysaccharides in the female flies emerging from exposed and unexposed pupae, Periodic Acid-Schiff (polysaccharides) and Bromophenol Blue Stain (proteins) techniques were used. Briefly, for polysaccharide detection, slides containing six sections for each treatment were immersed for 30 min in 0.5% periodic acid, washed with distilled water for 20 min and stained with Schiff's reagent for 30 min in the dark before being submerged in water for 30 min and dried at room temperature. After drying, the slides were mounted in Entellan® [50]. For protein detection, slides containing six sections for each treatment were stained with bromophenol blue for 2 h at room temperature. Then washed with 0.5% acetic acid for five minutes and rinsed in distilled water for 15 min, quickly immersed in tertiary butyl alcohol, left to dry at room temperature and mounted in Entellan® [51].

2.5.3. Morphometric Analysis

For morphometric analysis, histological sections were photographed using a trinocular image capture system (Olympus Optical Ltd. Brazil, São Paulo, SP, Brazil) and camera (SCOS Color for light microscopy). Measurements of thoracic muscular structures, midgut thickness and fat body area were performed using Image J (NIH) software (National Institutes of Health, Bethesda, MD, USA). For thorax muscles, longitudinal histological sections in which it was possible to visualize the junction of the insect's head with the thorax were selected. In these sections, for each insect, ten measurements of the thickness of the muscle fibers arranged horizontally and of the space between each fiber were taken. Five insects were used for each treatment. Three measurements of the horizontal thoracic diameter were also performed on each insect. To assess the midgut thickness, ten measurements of the portion identified as the midgut were taken per insect and five insects were used for each treatment. In addition, finally, to determine the average area of fat cells (trophocytes) of the fat body, 50 measurements were performed per insect, five insects for each treatment, of histological sections of the abdominal portion.

2.6. Statistical Analysis

Dose-mortality data were subjected to probit analysis (SAS Institute, Cary, NC, USA), and 95% confidence intervals for toxicity ratios were estimated following Robertsen et al. [52]; the values were considered significant if the range did not include the value 1. In enzyme inhibition test, the values obtained for IC₅₀ of EOs and the pattern of carvacrol were compared to each other by analysis of variance by the Scott–Knott test at the level of 5% probability, using the Sisvar software (UFLA, Lavras, Brazil). Morphometric data were submitted to the Shapiro–Wilk normality test. Then, they were compared by analysis of variance (ANOVA), followed by Tukey's post-hoc test ($\alpha < 0.05$) using the Graph Pad Prism 7.00 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Chemical Characterization of Extracted Essential Oils

The chemical characterization showed that ten and fourteen different constituents were found in *M. fragrans* and *S. molle* EOs, respectively. The phenylpropanoid (*E*)-anethole was the major (99.61% of the mix) compound identified in the *I. verum* oil (Table 1 and Supplementary Figure S1). In the oil of *M. fragrans*, the four major constituents belonged to monoterpenic hydrocarbons and were sabinene (27.39%), limonene (25.51%), β -pinene (17.95%) and α -pinene (17.77%). In the EO of *S. molle*, two major components were

monoterpenic hydrocarbons (limonene: 25.55% and sabinene: 19.66%) and the two others were sesquiterpenic hydrocarbons (bicyclogermacrene: 22.93% and *trans*- β -caryophyllene: 12.74%).

Table 1. Moisture content, yield and chemical composition of essential oils of *Illicium verum*, *Myristica fragrans* and *Schinus molle*.

		<i>Illicium verum</i>	<i>Myristica fragrans</i>	<i>Schinus molle</i>
Mass (g)		630	143	901
Humidity (%)		4.00	3.55	56.86
Yield (%)		2.49 \pm 0.15	5.14 \pm 0.11	1.08 \pm 0.13
Retention Time (min)	RI _{exp}	RI _{tab}	Constituents	Percentage (%)
6.223	925	924	α -thujene	-
6.466	933	932	α-pinene	17.77
7.610	972	969	sabinene	19.66
7.806	979	974	β-pinene	17.95
8.061	987	988	myrcene	0.61
8.815	1010	1002	α -phelandrene	0.54
9.079	1017	1014	α -terpinene	1.13
9.346	1024	1020	<i>p</i> -cymene	0.65
9.533	1029	1024	limonene	0.36
10.612	1057	1054	γ -terpinene	-
11.702	1085	1086	terpinolene	0.09
15.702	1180	1174	terpinen-4-ol	5.81
18.964	1255	1247	<i>p</i> -anisaldehyde	0.04
20.358	1287	1282	(E)-anethole	99.60
20.444	1289	1285	safrole	-
26.022	1419	1417	<i>trans</i>-β-caryophyllene	0.12
28.539	1480	1484	germacrene D	-
29.159	1495	1500	bicyclogermacrene	-
30.102	1519	1517	myristicin	0.23
32.354	1576	1577	spathulenol	-
Total identified		100	100	100
Monoterpenic hydrocarbons		0.36	93.84	56.45
Sesquiterpenic hydrocarbons		-	-	40.70
Oxygenated monoterpenes		-	5.81	-
Oxygenated sesquiterpenes		-	-	2.85
Phenylpropanoids		99.64	0.35	-

Where: RI_{exp} is the experimental retention index and RI_{tab} is the index tabulated in the literature. In bold are the components considered to be majors.

3.2. Insecticidal Activity

The mortality levels obtained in the dose-mortality bioassays were satisfactorily described by the probit model (goodness-of-fit tests exhibiting low χ^2 -values (<3) and high *p*-values (>0.05)). The toxicity ratios (TR) were estimated relative to the *I. verum* EO as it presented the lowest LC₅₀ (1.9 μ L mL⁻¹). The toxicities of the *M. fragrans* (LC₅₀ = 26.5 μ L mL⁻¹) and *S. molle* (LC₅₀ = 58.7 μ L mL⁻¹) EOs were similar and were, respectively, 14- and 31-fold less lethal to the *D. suzukii* flies compared to the *I. verum* EO (Table 2).

3.3. Acetylcholinesterase Inhibitory Activity of Essential Oils

The three EOs presented enzyme inhibitory activity that was concentration-dependent (Supplementary Figure S2). The *S. molle* EO promoted considerable enzyme inhibition at lower concentrations, when compared to the other oils and to carvacrol used as a comparison standard (Table 3). The *I. verum* oil presented a higher value, (IC₅₀ = 0.117 mg mL⁻¹), differing statistically (F = 16.04; df = 3; *p* = 0.0009) from the others and, thus, showing the

least enzyme inhibitory activity among the tested EOs. There was no statistical difference among the IC_{50} values for carvacrol ($IC_{50} = 0.029 \text{ mg mL}^{-1}$) and the oils of *M. fragrans* ($IC_{50} = 0.057 \text{ mg mL}^{-1}$) and *S. molle* ($IC_{50} = 0.047 \text{ mg mL}^{-1}$).

Table 2. Toxicological performance of essential oils on *Drosophila suzukii*.

Essential Oil	Number of Insects	LC ₂₀ (95% IF)	LC ₅₀ (95% IF) ($\mu\text{L mL}^{-1}$)	LC ₉₅ (95% IF)	χ^2	<i>p</i>	TR LC ₅₀ (95% LC) ($\mu\text{L mL}^{-1}$)
<i>Illicium verum</i>	600	0.8 (0.6–1.0)	1.9 (1.6–2.2)	9.2 (7.3–12.4)	0.3569	0.9859	*
<i>Myristica fragrans</i>	500	16.9 (15.1–18.6)	26.5 (24.6–28.6)	63.4 (54.5–78.1)	2.5922	0.4589	13.9 (12.9–15.0)
<i>Schinus molle</i>	500	19.3 (13.3–25.1)	58.7 (49.3–68.8)	518.5 (360.5–882.4)	0.8475	0.8381	30.9 (25.9–36.2)

Where LC₂₀ is the lethal concentration for 20% of the individuals, LC₅₀ is the lethal concentration for 50% of the individuals; (95% IF) represents the 95% fiducial range; χ^2 is the chi-square for lack of fit to the probit model; *p* is the probability associated with the chi-square statistic; TR: toxicity ratio = LC₅₀ of the oil over the LC₅₀ of the oil with lowest LC₅₀ value, with a 95% confidence limit; * oil used as a reference for calculating the TR LC₅₀.

Table 3. Estimated IC_{50} values for acetylcholinesterase inhibition.

Essencial Oil	IC_{50} (mg mL^{-1})
Carvacrol	0.029 ± 0.004^a
<i>Illicium verum</i>	0.117 ± 0.002^b
<i>Myristica fragrans</i>	0.057 ± 0.004^a
<i>Schinus molle</i>	0.047 ± 0.002^a

Means followed by the same lowercase letters do not differ from each other by the Scott–Knott Test at the 5% level of significance.

3.4. Histology of Thoracic Muscles, Midgut and Fat Body of Unexposed Adult Female of *Drosophila suzukii*

Figure 1A presents a longitudinal histological section of an unexposed (control) adult female of *D. suzukii* subjected to the periodic acid–Schiff technique. Figure 1B shows details of histological sections of thoracic muscle (I, II and III), midgut (IV, V and VI) and fat body (VII, VIII and IX) stained with Hematoxylin and Eosin (HE) (I, IV and VII), Bromophenol blue (BB) (II, V and VIII) and the periodic acid–Schiff technique (PAS) (III, VI and IX).

In sections allowing to visualize the junction between the insect's head and thorax, on average, six muscle fibers arranged longitudinally were observed above the esophagus. Fibers were connected from one end of the exoskeleton to the other. Differences in thickness between fibers were verified, but the thickness was constant in the same fiber. Small spacings between fibers characterized by straight regions not reactive to hematoxylin and eosin were verified. In each muscle fiber, an average of five to eight myofibrils were observed, arranged in a straight line. In sections where the junction between the head and the thorax was not visualized, the fibers normally occupied the entire thoracic region and were arranged in different directions. In addition, many fibers in these regions were attached to one end of the thorax and extend to the center of that compartment, where a small spacing was found connecting one fiber to another. For bromophenol blue staining, proteins were evidenced by an intense blue stain, homogeneously arranged in the tissue. In the PAS technique, the tissue is weakly and homogeneously stained. Strongly reactive points (glycogen) distributed in a spaced fashion at the end of the muscle fibers were observed.

In the abdominal section, the midgut is evidenced as a tube that extends to approximately half of the abdomen followed by a thinner tube representing the hindgut. The tube could be viewed both transversely and longitudinally, depending on the position of the histological section. The midgut has columnar cells with nuclei positioned at approximately equal heights and all cells appeared to adhere to the basal membrane. The nucleus of the cells was not very evident, and the nucleolus was not observed. The thickness of the midgut epithelium was practically constant. The apical part of the epithelium has small spaces between the cells, which extend to the middle of the epithelium. The staining with

bromophenol blue allowed to observe uniform distribution of proteins in the intestine, being evidenced by a medium-intensity blue stain. The PAS technique strongly stained the intestinal contents as well as the apical part of the epithelial cells. In addition, thin extracellular rectilinear regions reactive to PAS were observed. These regions were evenly distributed in the midgut and connect the apical part to the basal pole of the cells.

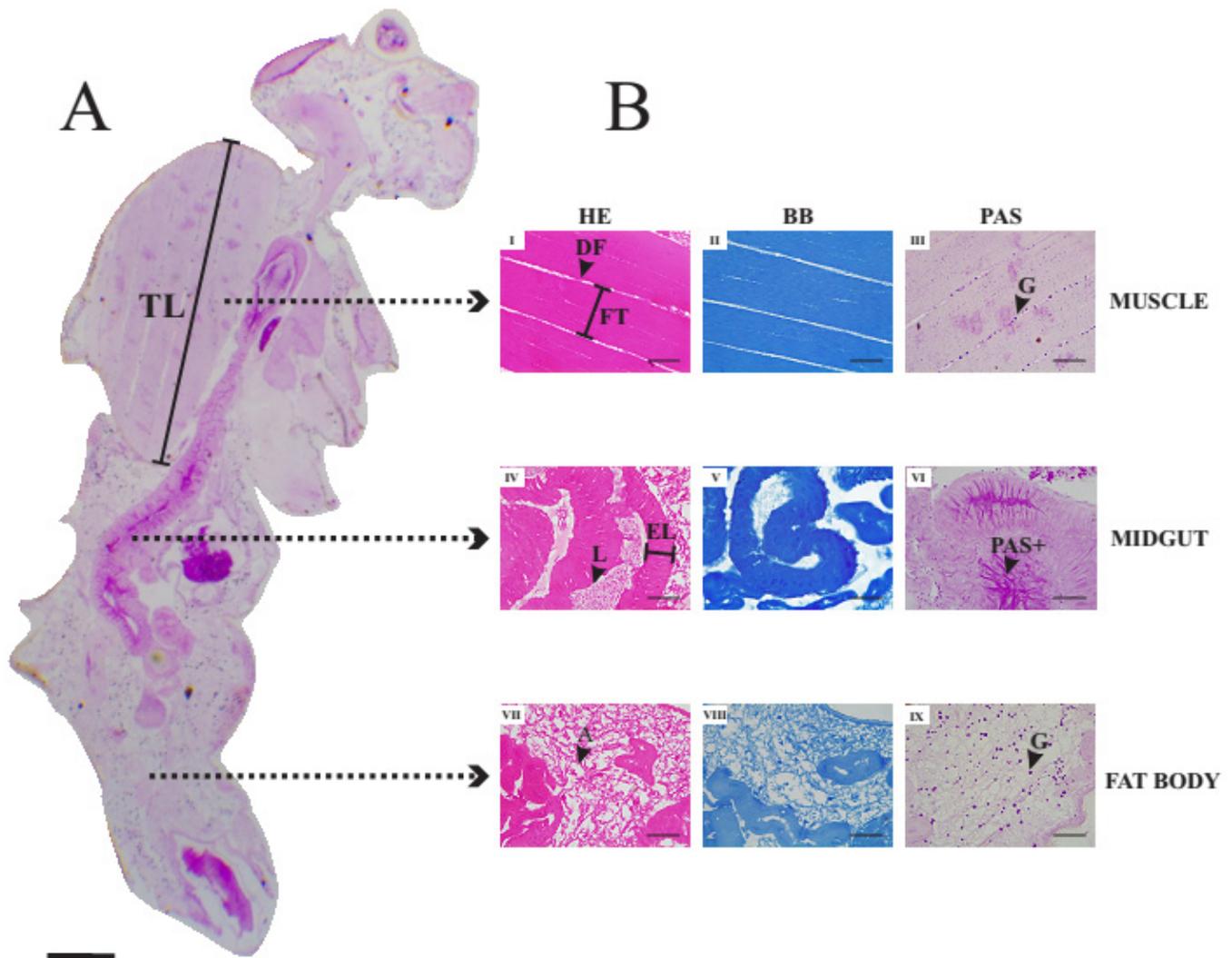


Figure 1. (A)—Longitudinal histological section of adult *Drosophila suzukii* female of the Control treatment subjected to the periodic acid–Schiff technique. Scale bar: 200 μm . (B)—Histological sections of muscle (I, II and III), midgut (IV, V and VI) and fat body (VII, VIII and IX) stained with Hematoxylin and Eosin (HE) (I, IV and VII), Bromophenol blue (BB) (II, V and VIII) and periodic acid–Schiff technique (PAS) (III, VI and IX) of adult *D. suzukii* female belonging to Control treatment. Scale bars: 50 μm . Legends: A—adipocytes; DF—distance between muscle fibers; EL—epithelium length; FT—muscle fiber thickness; G—glycogen granules; L—midgut lumen; PAS⁺—PAS positive region; TL—thorax length.

The fat body represents about 20 to 30% of the constitution of the abdomen of the adult female fly. This tissue is mainly present at the ends of the abdomen, particularly in the final portion of this compartment and characterized by the presence of adipose cells evidenced by regions not stained by hematoxylin and eosin (HE). The droplets have a shape ranging from circular to oval, with small variations in area. The droplets were surrounded by eosinophilic matrix, which is also homogeneously stained by bromophenol blue. Strongly reactive points stained in purple were observed to be uniformly distributed throughout the

fat body. The PAS-stained dots were about twenty times smaller than the lipid droplets and were also present in smaller numbers compared to the unstained droplets.

3.5. Histopathological Alterations Caused by Exposure to *Illicium verum* Essential Oil

The exposure of pupae to LC₂₀ and LC₅₀ of *I. verum* EO caused clear histological and morphological alterations in the structures analyzed (i.e., midgut, fat body and thoracic muscle fibers). These alterations were concentration-dependent.

3.5.1. Histological Alterations in the Midgut

The midgut of the LC₂₀-treated individuals presented a greater space between cells (Figure 2D–F). The intestinal lumen was strongly stained with PAS but epithelial cells appeared weakly stained at their apical poles, being non-reactive in their interior. No changes were revealed using bromophenol blue staining. Similar but more severe alterations in the midgut were encountered for the LC₅₀-treated individuals (Figure 2G–I). The cells appeared to be more elongated and spaced farther apart. The epithelium appeared thicker with hyperplasia of the cells in the intestinal wall. Although the PAS technique revealed strongly stained intestinal contents, the apical part of the cells was stained purple very faintly. No changes in intestinal protein content were seen by staining with bromophenol blue.

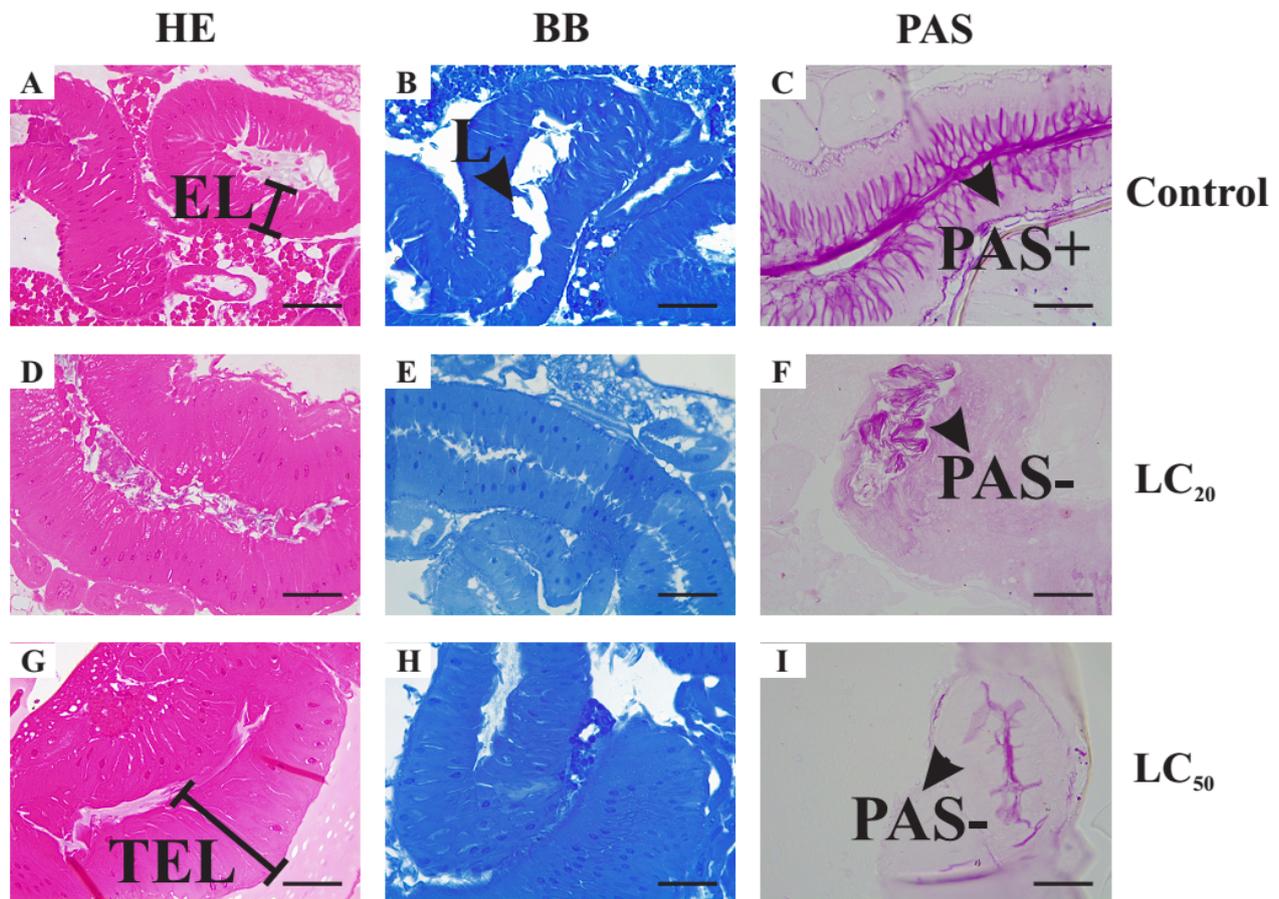


Figure 2. Histological sections of midgut of adult *Drosophila suzukii* females stained with Hematoxylin and Eosin (HE) (A–G), Bromophenol blue (BB) (B,E,H) and periodic acid–Schiff technique (PAS) (C,F,I). Control Group II (C II) (A–C)—3% DMSO solution. Treatment group I (D–F)—1 $\mu\text{L mL}^{-1}$ (LC₂₀) of the essential oil of *Illicium verum* diluted in 3% DMSO. Treatment group II (G–I)—2 $\mu\text{L mL}^{-1}$ (LC₅₀) of the essential oil of *I. verum* diluted in 3% DMSO. Scale bars: 50 μm . Legends: EL—epithelium length; L—midgut lumen; PAS[−]—PAS negative region (please note that the intestinal content is always PAS-positive, but the enterocytes are PAS negative in treatment groups); PAS⁺—PAS-positive region; TEL—thicker epithelium length.

3.5.2. Histological Alterations in the Fat Body

The proportion of fat body in the abdomen of EO-treated insects was smaller. It represented about 20% of the LC₂₀-treated insects' abdomen and about 10% of LC₅₀-treated ones. The fat body had apparently smaller trophocytes (Figure 3D–I) when compared to control (Figure 3A–C). The staining with bromophenol blue showed that the color of matrix surrounding the lipid droplets is non-homogeneous.

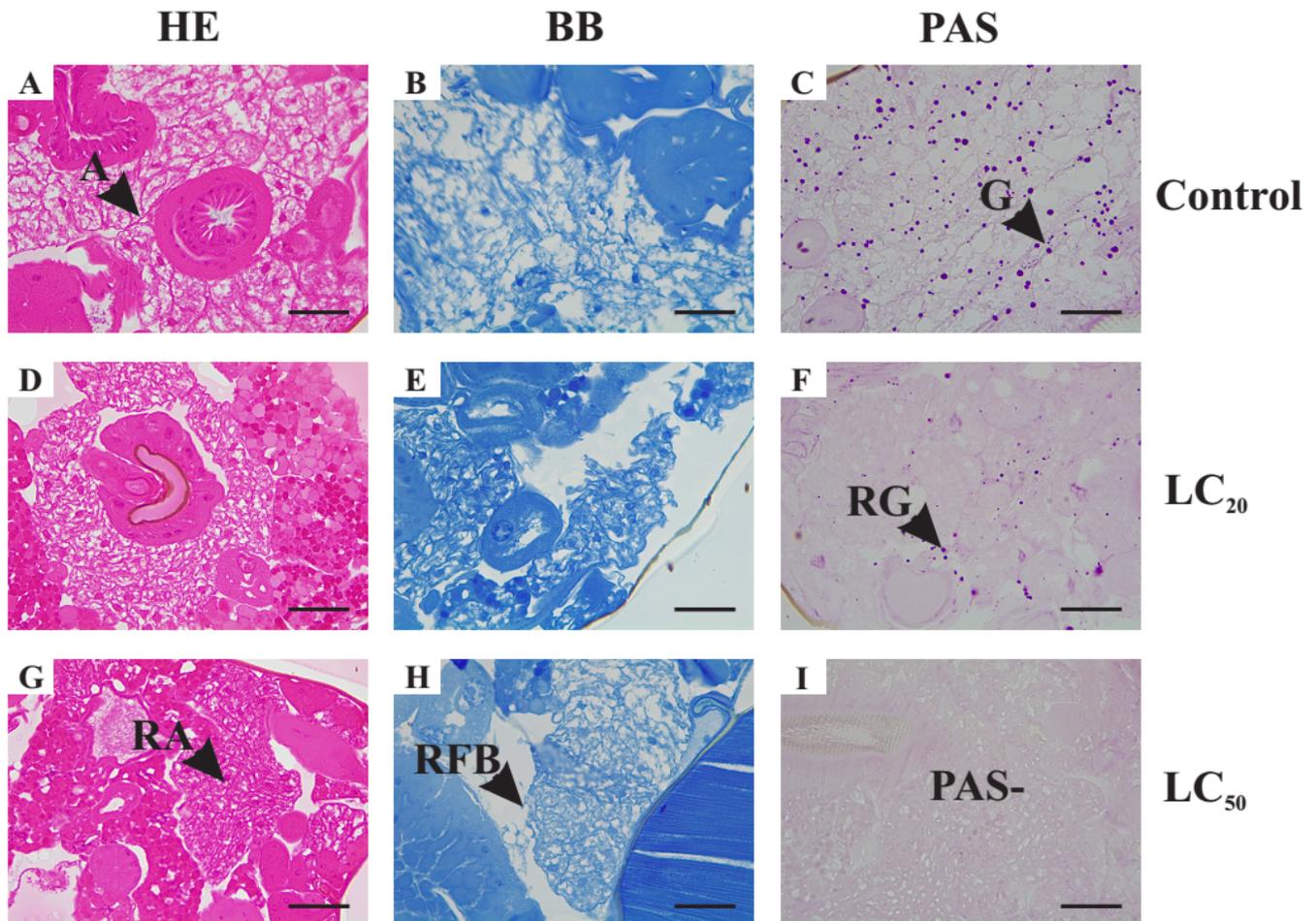


Figure 3. Histological sections of fat body of adult *Drosophila suzukii* females stained with Hematoxylin and Eosin (HE) (A,D,G), Bromophenol blue (BB) (B,E,H) and periodic acid–Schiff technique (PAS) (C,F,I). Control Group II (C II) (A–C)—3% DMSO solution. Treatment group I (D–F)—1 $\mu\text{L mL}^{-1}$ (LC₂₀) of the essential oil of *Illicium verum* diluted in 3% DMSO. Treatment group II (G–I)—2 $\mu\text{L mL}^{-1}$ (LC₅₀) of the essential oil of *I. verum* diluted in 3% DMSO. Scale bars: 50 μm . Legends: A—adipocytes; G—glycogen granules; PAS[−]—PAS negative region; RA—reduced adipocytes area; RFB—reduced fat-body area; RG—reduced number of glycogen granules.

Regarding the PAS technique, 60% of LC₂₀ treated insects have a non-reactive fat body with only 40% of visible pink-stained spots (Figure 3F), representing less than 50% of the total number observed in the controls (Figure 3C). The fat body of none of the LC₅₀-treated insects was reactive to PAS (Figure 3I). Finally, large spaces between organs, such as intestine and ovary, were observed in regions that, in the control groups, were filled by the fat body. Such regions do not show reactivity to any of the dyes used.

3.5.3. Histological Alterations in the Thoracic Muscle Fibers

The treated insects showed considerable morphological changes in their muscle fibers (Figure 4D–I) compared to the control (Figure 4A–C). Intense variations in fiber thickness,

as well as greater spacing between them, are observed. In 40% of LC₂₀-treated insects, myofibrils were also more clearly spaced and about 10% of these myofibrils had a torn appearance. In LC₅₀-treated insects, about half of the fibers seem not to be attached to the exoskeleton, with an eosinophilic acellular matrix being observed in this region. Tears in muscle fibers were also observed, with unstained regions being evidenced. Non-homogeneous color with bromophenol blue was seen in both treatments. In addition, glycogen granules were drastically reduced in LC₂₀-treated insects, while no glycogen reserves were observed in any of the LC₅₀-treated ones.

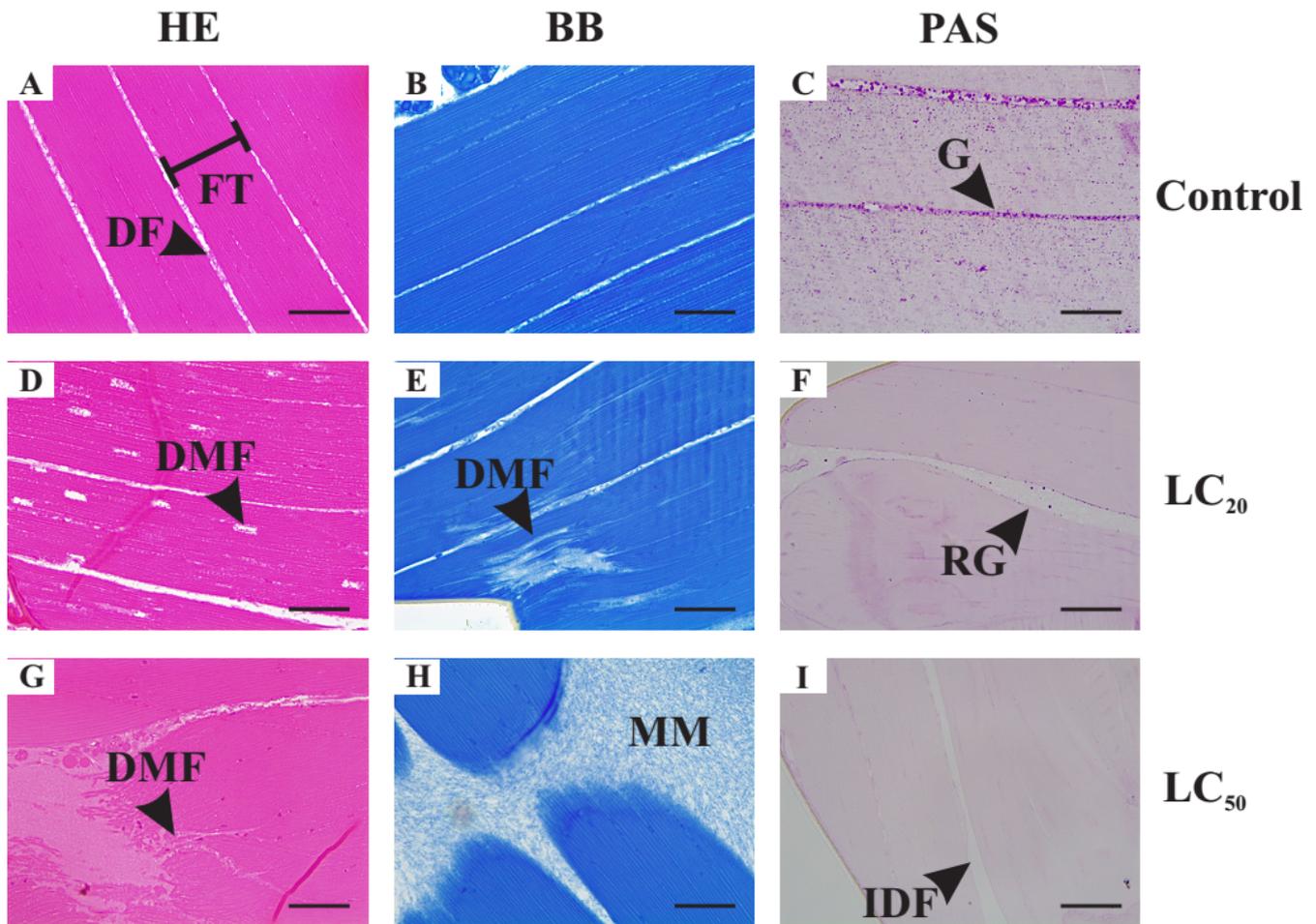


Figure 4. Histological sections of muscle of adult *Drosophila suzukii* females stained with Hematoxylin and Eosin (HE) (A,D,G), Bromophenol blue (BB) (B,E,H) and periodic acid-Schiff technique (PAS) (C,F,I). Control Group II (C II) (A–C)—3% DMSO solution. Treatment group I (D–F)—1 $\mu\text{L mL}^{-1}$ (LC₂₀) of the essential oil of *Illicium verum* diluted in 3% DMSO. Treatment group II (G–I)—2 $\mu\text{L mL}^{-1}$ (LC₅₀) of the essential oil of *I. verum* diluted in 3% DMSO. Scale bars: 50 μm . Legends: DF—distance between muscle fibers; DMF—disruption of muscle fibers; FT—muscle fiber thickness; G—glycogen granules; IDF—increased distance between muscle fibers; MM—extracellular muscle matrix; RG—reduced amount of glycogen granules.

3.5.4. Morphometric Analysis of the Histological Alterations

The alterations found in the histological analysis were confirmed by the results of the morphometric analysis of the thickness and distance between thorax muscle fibers, thorax diameter, thickness of the midgut epithelium and the average area of lipid droplets of fat body (Table 4). Significant differences between the measured parameters for LC₂₀- and LC₅₀-treated insects and the control were observed except for the thorax diameter (Table 4). The thickness of thorax muscle fibers as well as the average area of lipid droplets

of fat body decreased with increase in the concentrations used. Conversely, the distance between thorax muscle fibers and thickness of the midgut epithelium increases with the concentrations.

Table 4. Morphometric measurements of thorax, midgut and fat body structures of *Drosophila suzukii* unexposed (water + DMSO) and exposed to LC₂₀ and LC₅₀ of *Illicium verum* essential oil.

Structures	Water + DMSO	LC ₂₀	LC ₅₀
Thickness of thoracic muscle fibers (μm)	57.70 ± 5.65 ^a	43.08 ± 2.16 ^b	42.14 ± 0.94 ^b
Distance between thorax muscle fibers (μm)	3.54 ± 0.27 ^a	5.15 ± 0.13 ^b	6.51 ± 0.12 ^c
Thorax diameter (μm)	760.80 ± 33.74 ^a	737.92 ± 47.54 ^a	718.18 ± 103.31 ^a
Thickness of the midgut epithelium (μm)	37.05 ± 2.44 ^a	43.34 ± 1.03 ^b	47.16 ± 0.88 ^b
Average area of lipid droplets of fat body (μm ²)	42.54 ± 8.21 ^a	23.25 ± 3.61 ^b	16.15 ± 1.54 ^c

The normality of data was verified by the Shapiro–Wilk test. Means in the same line followed by equal letters do not differ from each other by analysis of variance (ANOVA), followed by Tukey’s post-hoc test ($p > 0.05$). LC = lethal concentration.

4. Discussion

Here, we demonstrated that *I. verum* EO acts on insect’ internal morphology, such as the thorax, midgut and fat-body tissues, which can contribute to its toxic efficacy against insect pests. Our findings revealed that in addition to its satisfactory toxicological performance against *D. suzukii*, the exposure to low concentrations of *I. verum* EO promoted drastic concentration-dependent histological and structural alterations in the female adults of this fly.

As for the major constituents, the *I. verum* EO was found to present a lesser chemical diversity of constituents compared to the two other EOs (i.e., *M. fragrans* and *S. molle*). The oil of *I. verum* is majorly constituted by (*E*)-anethole (99.61%). In other studies, (*E*)-anethole was also found as the major compound for this species, but it was quantified in different proportions [37,53]. For *M. fragrans* EO, the major ones were sabinene (27.39%), limonene (25.51%), β -pinene (17.95%) and α -pinene (17.77%). Kapoor et al. (2013) identified sabinene (29.4%), β -pinene (10.6%), α -pinene (10.2%) and terpinen-4-ol (9.6%) as the majority [54]. For *S. molle* EO, the major compounds were limonene (25.55%), bicyclogermacrene (22.93%), sabinene (19.66%) and trans- β -caryophyllene (12.74%). Do Prado et al. (2019) identified β -pinene (25.33%), epi- α -cadinol (21.29%), α -pinene (18.72%), and myrcene (11.54%) as major compounds [55]. The variations observed in the chemical composition of these oils depend on several factors, including the geographic locations where the plants are growing, seasons, availability of nutrients and water, pathogen- and herbivore-attacks pressure, pollution, solar radiation and genetic factors [56,57].

The EO of *I. verum* exhibited the best toxicological action on *D. suzukii*, with the lowest LC₅₀ (1.9 μL mL⁻¹) being 4 and 31-fold more lethal to the *D. suzukii* flies compared to *M. fragrans* and *S. molle* EOs, respectively. Such differential insecticidal activity shown by these EOs can be explained by their chemical diversity of constituents, as it can mirror different mechanisms of action [58–60].

However, the toxicological results are in contrast with the inhibitory activity of the acetylcholinesterase (AChE) enzyme of these oils considering that the inhibition of the AChE enzyme is one of the mechanisms widely recognized to be involved in the insecticidal activity of EOs [58]. Indeed, the star anise oil presented an enzymatic inhibition concentration (IC₅₀ = 0.117 mg mL⁻¹) twice as high as the other oils. The higher IC₅₀ values jointly with the lower LC₅₀ found for the *I. verum* EO compared to the two others suggest that the AChE inhibition is not the main mechanism involved in *I. verum* EO toxicity to *D. suzukii* flies. The oil of *I. verum* is basically constituted of (*E*)-anethole (99.61%), allowing to infer that the insecticidal activity of this oil is due to the action of this phenylpropanoid, and the occurrence of synergism with the other constituents is unlikely. The compound (*E*)-anethole is derived from *p*-coumaric acid, a phenolic compound, which undergoes

reduction and has a methyl group replacing the hydroxyl hydrogen [61]. The replacement of hydroxyl by a methoxyl gives the compound greater stability against oxidation [62].

It is noteworthy that the insects were exposed to EOs through direct contact, fumigation and, mainly, ingestion, allowing the toxic action of EO to affect different target organs and causing damage to other tissues of the exposed flies. In fact, the histological and histochemical analyses verified alterations in the epithelial thickness and in the distribution of carbohydrates in the midgut of *D. suzukii* exposed to low concentrations of *I. verum* EO. Additionally, in the fat body, there was a drastic reduction in the area of fat cells as well as carbohydrate reserves. In the thorax, muscle fibers became thinner, more spaced apart and prone to ruptures in some cases, and glycogen reserves were absent. Through the analysis of these changes arising from exposure to sublethal concentrations, it is possible to infer that an alternative mechanism of toxic action of *I. verum* oil may be happening.

Recently, similar morphological changes in insects' digestive system caused by EOs were reported by Hashem et al. (2018), who found that sublethal doses of the nanoemulsified EO of *Pimpinella anisum*, with (*E*)-anethole content equal to 81.2%, caused alterations in the integument and midgut of *Tribolium castaneum* [63]. Our results with the PAS technique demonstrate that the intestine of insects exposed to anise oil was severely damaged, especially in the treatment with LC₅₀, suggesting that carbohydrate absorption in the flies' intestine may be compromised. Carbohydrate metabolism is essential for the maintenance of energy balance in animals, and in insects the main energy sources are trehalose sugars and glycogen [64,65]. Trehalose is the primary carbohydrate in insects, playing a fundamental role in several physiological processes. This sugar is synthesized by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphatase, and is rapidly hydrolyzed by trehalase (TRE) to glucose for energy [66].

Alternatively, insects use lipids as a secondary source of energy. In this sense, the fat body of insects, where triglycerides, glycogen and proteins are stored, is characterized as the main energy reserve structure. In addition to storing biomolecules that provide energy, the fat body of insects acts in the biosynthesis of several proteins and other metabolites, and participates in the detoxification of xenobiotics [67–70]. Here, the positive PAS results in the control (water and DMSO exposed flies) confirmed the occurrence of glycogen in the fat body of *D. suzukii* flies. However, in LC₂₀, the carbohydrate content decreased and in LC₅₀ was inexistent. Simultaneously, a concentration-dependent reduction in the area of lipid droplets was observed in LC₂₀ and LC₅₀ when compared to control (water- and DMSO-exposed) flies. Thus, it can be inferred that when subjected to *I. verum* EO, the probable difficulty in absorbing carbohydrates from the diet may have led the animals to consume their glycogen and lipid reserves.

Moreover, when stored carbohydrate is consumed, there may also be protein degradation for energy [71,72]. Our findings showed clearly that the individuals in the control groups had glycogen deposits in the thoracic muscle regions. In contrast, stored glycogen was absent in individuals exposed to LC₅₀ and some fragmented muscle fibers were observed. Furthermore, in both LC₂₀ and LC₅₀ flies, a significant reduction in the thickness of muscle fibers and greater spacing between them were also observed. Such damage can strongly impact the insects' ability to move and fly, implying difficulty in obtaining food, mating and finding substrate for oviposition, and so effectively reducing the ability of this pest to prosper and generate damage.

The use of low concentrations of *I. verum* EO drastically affected the morphophysiology of *D. suzukii*. Probably, this oil does not act by inhibiting the acetylcholinesterase enzyme, since the results obtained suggest a possible inhibition of digestive enzymes or cell-membrane receptors that caused the non-absorption of carbohydrates from the food by the insect's intestine. Bromophenol blue staining indicated a constant protein content in the midgut for controls and exposed flies but with possible inhibition of digestive enzymes, insects cannot obtain energy from the ingested food. Such changes can be credited to (*E*)-anethole, the predominant compound in star anise EO. Thus, *I. verum* oil appears as an alternative for the long-term control of *D. suzukii*, as it has a high lethality rate for

this insect and causes severe damage to those who survive the exposure. However, more studies are warranted to assess its toxicity to non-target organisms and develop products for field application.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture12101667/s1>. Supplementary Figure S1: Chromatograms obtained by chemical characterization of essential oils of *Illicium verum*, *Myristica fragrans* and *Schinus molle*. Supplementary Figure S2: Acetylcholinesterase inhibition by essential oils of *Illicium verum*, *Myristica fragrans*, *Schinus molle* and *carvacrol*.

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