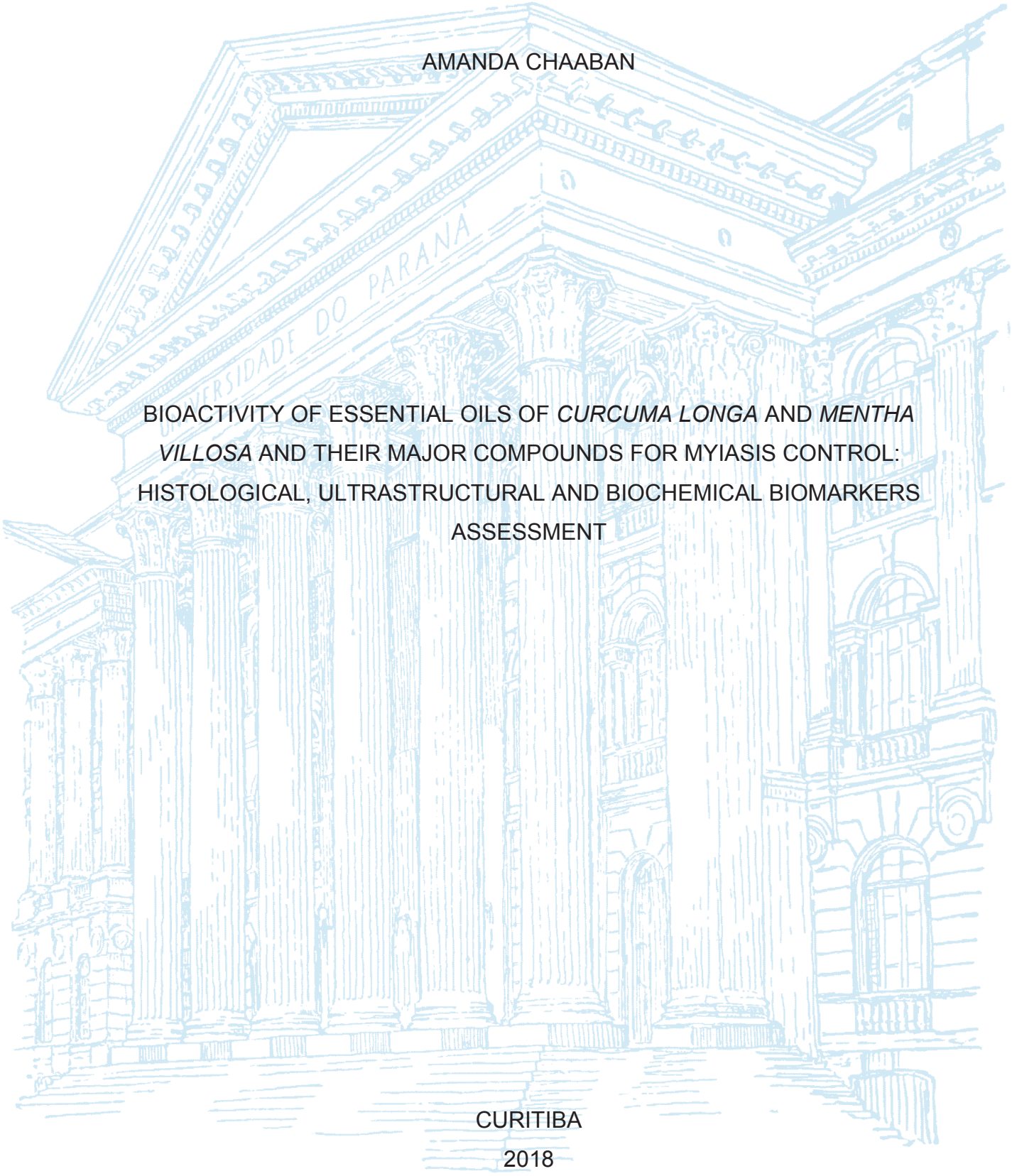
UNIVERSIDADE FEDERAL DO PARANÁ

AMANDA CHAABAN



BIOACTIVITY OF ESSENTIAL OILS OF *CURCUMA LONGA* AND *MENTHA VILLOSA* AND THEIR MAJOR COMPOUNDS FOR MYIASIS CONTROL: HISTOLOGICAL, ULTRASTRUCTURAL AND BIOCHEMICAL BIOMARKERS ASSESSMENT

CURITIBA

2018

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Orientador: Prof. Dr. Marcelo Beltrão Molento

Coorientador: Prof. Dr. Cícero Deschamps

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS VETERINÁRIAS da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **AMANDA CHAABAN** intitulada: **Bioactivity of essential oils of *Curcuma longa* and *Mentha villosa* and their major compounds for myiasis control: histological, ultrastructural and biochemical biomarkers assessment**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 16 de Outubro de 2018.

MARCELO BELTRÃO MOLENTO
Presidente da Banca Examinadora

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I would like to dedicate this work to my dear Professor Gonzalo Efrain Moya Borja for teaching me the first steps in the Word of Veterinary Entomology; to the generosity of the Mother Nature that provided the plants for developing this study; to the animals; and to the universe, that always conspired in my favor.

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Finally, I would like to thank the Federal University of Paraná for hosting our project and for giving the opportunity for the conclusion of my doctorate.

The mind that opens up to a new idea never returns to its original size.

(Albert Einstein)

RESUMO

A infestação do tecido vivo por larvas de dípteros (miíases) ainda é uma das principais ectoparasitoses que acometem animais de produção. Entretanto, o controle convencional através da aplicação de inseticidas sintéticos trouxe consequências negativas nas últimas décadas, tais como: aumento da seleção de ectoparasitos resistentes, riscos para a segurança alimentar através do acúmulo de resíduos em produtos de origem animal, contaminação ambiental e principalmente o comprometimento do bem-estar animal. Neste sentido, os metabólitos secundários produzidos por plantas, principalmente os óleos essenciais (OE), vêm ganhando destaque como uma nova alternativa de controle. Os OE são de origem natural, sustentáveis e de baixa toxicidade aos animais e ao meio ambiente. O objetivo deste estudo foi avaliar a bioatividade de dois OE e seus compostos majoritários sobre moscas causadoras de miíases, para selecionar novos candidatos a biopesticidas. Neste sentido, a presente tese está dividida em cinco capítulos, seguido de um material suplementar, apresentados em formato de Introdução, Manuscritos, Material suplementar e as Considerações Finais. O primeiro manuscrito acessou a atividade inseticida do OE extraído das folhas de *Curcuma longa* e de seu composto majoritário; α-felandreno sobre larvas de terceiro instar (L3) de *Lucilia cuprina*. O OE extraído das folhas de *C. longa* e α-felandreno inibiram a emergência de adultos em 96,22 e 100%, utilizando as doses de 1,59 e 1,47 μL/cm^2, respectivamente. Ainda, danos morfológicos em órgãos alvo foram observados através da observação macroscópica, ultraestrutural e histopatológica. O segundo manuscrito avaliou a atividade inseticida do OE extraído das folhas de *C. longa* e de seu composto majoritário; α-felandreno sobre L3 de *Cochliomyia macellaria* acessando também os biomarcadores de toxicidade larval. Valores de mortalidade de 96,66% foram observados utilizando a dose de 1,27 e 1,47 μL/cm^2 do OE de *C. longa* e α-felandreno, respectivamente. Além disso, alterações histológicas e ultraestruturais foram observadas. O objetivo do terceiro manuscrito seguiu a mesma abordagem, utilizando *C. macellaria* e *L. cuprina* como modelo biológico, acessando lesões histológicas. Os valores da concentração letal de 50% (CL_{50}) para o OE de *M. villosa* e carvone, 24 h após o contato, foi de 1,64 e 0,63μL/cm^2 para *C. macellaria*, e 1,1 and 0,3μL/cm^2 para *L. cuprina*, respectivamente. Citotoxicidade significativa também foi observada em órgãos alvo, como túbulos de Malpighi, trato digestivo, corpo gorduroso, músculo e cérebro das L3 tratadas ($p<0,05$). O objetivo do material suplementar foi avaliar bioquímicamente biomarcadores após contato com doses sub-lethais dos OE de *C. longa*, *M. villosa* e seus compostos majoritários, α-felandreno e carvone. O marcador de neurotoxicidade (AChE), a resposta de lipoperoxidação (LPO) e os marcadores de estresse oxidativo (CAT e SOD) foram mensurados após contato com doses sub-letais dos extratos utilizados após 6 h de exposição. Os resultados encontrados sugerem que os extratos avaliados induziram estresse oxidativo. De modo geral, resultados superiores foram observados utilizando o OE de *M. villosa* e seu composto majoritário carvone quando comparados aos resultados obtidos nos ensaios biológicos com o OE de *C. longa* e α-felandreno. Em conclusão, todos os extratos avaliados apresentaram significativa atividade inseticida em baixas doses, demonstrando seu potencial como candidatos a novos biopesticidas.

Palavras-chave: Biopesticidas, biomarcadores, óleo essencial, *Cochliomyia macellaria*, *Lucilia cuprina*, controle ecológico, subproduto.

ABSTRACT

The infestation of live tissue by dipterous larvae (myiasis) is still one of the main ectoparasitosis that affect animal production. However, the conventional control through the application of synthetic insecticides brought negative consequences in recent decades, such as: increased selection of resistant parasites, food security risks through the accumulation of residues in animal products, environmental contamination and mainly the commitment of animal welfare. In this sense, secondary metabolites produced by plants, especially essential oils (EO), have been gaining prominence, shedding light on new alternative controls for being natural and sustainable, having low toxic risk to animals and to the environment. The aim of this study was to evaluate the bioactivity of two EO and their major compounds on fly causing myiasis, to select new biopesticides candidates. In this sense, the present Thesis is divided in five Chapters, presented in the format of Introduction, Manuscripts, Supplementary Material and Final remarks. The first manuscript investigated the insecticidal activity of EO extracted from leaves of *Curcuma longa* and its major compound *α*-phellandrene against third instar larvae (L3) of *Lucilia cuprina*. EO and α-phellandrene inhibited adult emergence by 96.22 and 100%, using the dose of 1,59 and 1,47 μL/cm^2, respectively. Still, morphological and anatomical damages in target organs were observed through macroscopic, ultrastructural and histological assessment. The second manuscript evaluated the insecticidal activity of EO and *α*-phellandrene over L3 of *Cochliomyia macellaria*, accessing the biomarkers for larval toxicity. Mortality values of 96.66% were reported using the dose of 1.27 and 1.47 μL/cm^2 of EO and *α*-phellandrene, respectively. In addition, histological and ultrastructural changes were observed. The aim of the third manuscript followed the same approach of the previous, using *C. macellaria* and *L. cuprina* as biological models, with a thorough approach for histological lesions. The lethal concentration of 50% (LC_{50}) for EO and carvone 24 h after contact were 1.64 and 0.63μL/cm^2 to *C. macellaria*, whereas for *L. cuprina* the LC_{50} values were 1.1 and 0.3μL/cm^2, respectively. Significant cytotoxicity was also observed in target organs such as Malpighian tubules, digestive tract, fat body, muscle and brain of treated larvae. Finally, the objective of the supplementary material was to assess the biochemical biomarkers after contact with sub-lethal doses of the EO and their major compounds, *α*-phellandrene and carvone, respectively. The neurotoxicity marker (AChE), the lipoperoxidation response (LPO) and the oxidative stress markers (CAT and SOD) were measured in L3, 6 h after exposure. The results suggest that the extracts evaluated induced oxidative stress. In general, the best results of biological assays were observed using EO of *M. villosa* and carvone when compared to the results obtained with EO of *C. longa* and its main constituent, α-phellandrene. In conclusion, all the evaluated extracts presented significant insecticidal activity in low doses, demonstrating their potential as candidates for new biopesticides.

Keywords: Biopesticides, biomarkers, essential oil, *Cochliomyia macellaria*, *Lucilia cuprina*, ecofriendly control, by-product.

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MANUSCRIPT 2 - ESSENTIAL OIL FROM *Curcuma longa* LEAVES: CAN AN OVERLOOKED BY-PRODUCT FROM TURMERIC INDUSTRY BE EFFECTIVE FOR MYIASIS CONTROL?

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LIST OF ABBREVIATIONS

EO	- Essential oil
CLLEO	- *Curcuma longa* Leaves Essential oil
MVEO	- *Mentha villosa* Essential oil
GC/MS	- gas phase chromatography coupled to mass spectrometry
L3 -	- third instar larvae
LC	- lethal concentration
LM	- larvae mortality
PR	- pupation rate
EIR	- emergence inhibition rate
ADR	- adult deformity rate
SEM	- scanning electron microscopy
TEM	- transmission electron microscopy
CAS	- chemical Abstracts Service
IFC	- Catarinense Federal Institute
NIST	- National Institute of Standards and Technology, USA
MTs	- malpighian tubules
Bps	- botanical pesticides
AChE	- acetylcholinesterase
CAT	- catalase
SOD	- Superoxide dismutases
LPO	- lipid peroxidase
LC_C	- *Lucilia cuprina* solubilized in ethanol (control group)
LCPH40	- *L. cuprina* exposed to 40mg (0.59μL/cm^2) of *α*-phellandrene
LCPH20	- *L. cuprina* exposed to 40mg (0.30μL/cm^2) of *α*-phellandrene
LCCV20	- *L. cuprina* exposed to 20mg (0.30μL/cm^2) of carvone
LCCV10	- *L. cuprina* exposed to 10mg (0.15μL/cm^2) of carvone
CM_C	- *Cochliomyia macellaria* solubilized in ethanol (control group)
CMPH40	- *C. macellaria* exposed to 40mg (0.59μL/cm^2) of *α*-phellandrene
CMPH20	- *C. macellaria* exposed to 40mg (0.30μL/cm^2) of *α*-phellandrene
CMCV20	- *C. macellaria* exposed to 20mg (0.30μL/cm^2) of carvone
CMCV10	- *C. macellaria* exposed to 10mg (0.15μL/cm^2) of carvone
CMAC	- *C. macellaria* solubilized in acetone (control group)

CMCLAC2 - *C. macellaria* exposed to 16.68mg (0.31µL/cm^2) of CLLEO solubilized in acetone

CMCLAC4 - *C. macellaria* exposed to 33.37mg (0.63µL/cm^2) of CLLEO solubilized in acetone

CMMAC2 - *C. macellaria* exposed to 18.6mg (0.31µL/cm^2) of MVEO solubilized in acetone

CMMAC8 - *C. macellaria* exposed to 74.4mg (1.27µL/cm^2) of MVEO solubilized in acetone

CMET - *C. macellaria* solubilized in ethanol (control group)

CMCLET2 - *C. macellaria* exposed to 16.68mg (0.31µL/cm^2) of CLLEO solubilized in ethanol

CMCLET4 - *C. macellaria* exposed to 33.37mg (0.63µL/cm^2) of CLLEO solubilized in ethanol

CMMET2 - *C. macellaria* exposed to 18.6mg (0.31µL/cm^2) of MVEO solubilized in ethanol

CMMET8 - *C. macellaria* exposed to 74.4mg (1.27µL/cm^2) of MVEO solubilized in ethanol

LCAC - *L. cuprina* solubilized in acetone (control group)

LCCLAC2 - *L. cuprina* exposed to 16.68mg (0.31µL/cm^2) of CLLEO solubilized in acetone

LCCLAC4 - *L. cuprina* exposed to 33.37mg (0.63µL/cm^2) of CLLEO solubilized in acetone

LCMAC2 - *L. cuprina* exposed to 18.6mg (0.31µL/cm^2) of MVEO solubilized in acetone

LCMAC8 - *L. cuprina* exposed to 74.4mg (1.27µL/cm^2) of MVEO solubilized in acetone

LCET - *L. cuprina* solubilized in ethanol (control group)

LCCLET2 - *L. cuprina* exposed to 16.68mg (0.31µL/cm^2) of CLLEO solubilized in ethanol

LCCLET4 - *L. cuprina* exposed to 33.37mg (0.63µL/cm^2) of CLLEO solubilized in ethanol

LCMET2 - *L. cuprina* exposed to 18.6mg (0.31µL/cm^2) of MVEO solubilized in ethanol

LCMET8 - *L. cuprina* exposed to 74.4mg (1.27µL/cm^2) of MVEO solubilized in ethanol

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$\mu L/cm^2$ - microliter/square centimeter

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1 INTRODUCTION

Infections of live tissue by dipterous larvae have been described since the first missionaries arrived in 1587 in Latin America. Gabriel Soares de Souza described one of the first cases of myiasis caused by *Cochliomyia* sp. (GUIMARÃES and PRADO, 1982, PAPAVERO and COURI, 2012a), as well as the first reports of the use of natural products in the control of this disease in Brazil (PAPAVERO and COURI, 2012a, 2012b, CHAABAN et al., 2017).

This condition can impose a reduction in animal development capacity, creating stress and decrease of food intake, damage to the leather and in severe cases, can cause death (WALL, 2012, CHAABAN et al., 2017). Data obtained by the National Union of the Animal Health Products Industry showed that 55.3% of the sector's sales were directed to ruminants, and 27.2% of this figure were the sales of parasiticides (SINDAN, 2017). Still worth pointing out, the economic losses caused by flies, mainly *Cochliomyia hominivorax*, causing myiasis was estimated by Grisi et al., (2014) at US$ 336.6 million/year to the cattle industry in Brazil.

Indeed, two other species of the Calliphoridae family deserve attention, among them, *Lucilia cuprina* (Wiedemann, 1830) and *Cochliomyia macellaria (*Fabricius, 1775). *Lucilia cuprina*, in particular, presents great medical-sanitary and veterinary importance, due to the capacity of its larval forms to develop in decaying organic matter, besides being able to parasitise other vertebrates. Besides that, in Australia and New Zealand, this fly is the main cause of primary myiasis in sheep, and is responsible for millions of dollars of losses annually to the wool and meat industries (WALL, 2012, WINDSOR and LOMAX, 2013). *Cochliomyia macellaria*, as well as *L. cuprina* can be potential vectors of various human enteropathogenic diseases and may aggravate the already established primary myiasis with multiple oviposition (WALL, 2012, CHAABAN et al., 2017).

Currently, the conventional control of myiasis depends almost exclusively on synthetic insecticides (MOYA-BORJA, 2003, CHAABAN et al., 2017). Since 1940, the synthesis of long acting insecticides has led experts to hope for the control of various diseases caused or transmitted by arthropods or even eradicate harmful species (CHAABAN et al., 2017). However, the misuse of these compounds was the cause of the selection of insects resistant, environmental contamination and represents a risk to food safety (MOYA-BORJA, 2003, CHAABAN et al., 2017).

In this sense, natural products extracted from plants, especially essential oils (EO), are potential alternative sources in the control of flies causing myiasis (PAVELA and BENELLI, 2016, CHAABAN et al., 2018). The use of these complex substances (i.e. EO) for insect control may decrease resistance selection pressure, and represent a sustainable source of control when part of the plant is considered a byproduct. In addition, the use of native and endemic botanical species can stimulate regional development, reducing animal production costs (CHAABAN et al., 2017).

Thus, in an attempt to increase knowledge of the insecticidal activity against blowflies, we select four EO to be natural candidates biopesticides. *Curcuma longa*, *Mentha villosa* and its major compounds, *α*-phellandrene and carvone were selected due to their insecticidal properties and potential use in the myiasis control already reported in the literature (CHAABAN et al., 2017). This work also sought to describe morphological changes in target organs through macroscopic, ultrastructural and light microscopy observation to identify biomarkers for toxicity. Finally, biochemical biomarkers were also assessed to attempt to elucidate the mechanism of action of bioactive substances tested in this work.

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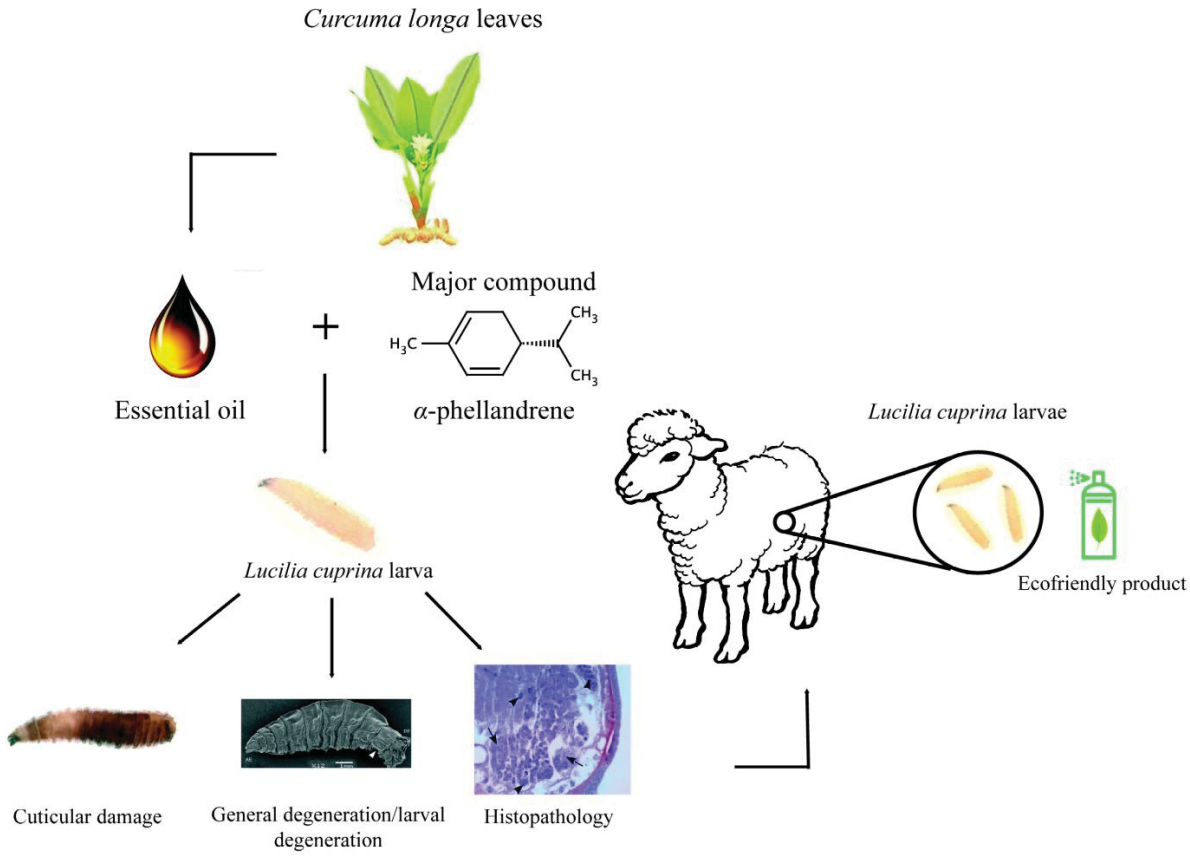
MANUSCRIPT 1 - INSECTICIDE ACTIVITY OF *Curcuma longa* (LEAVES) ESSENTIAL OIL AND ITS MAJOR COMPOUND *α*-phellandrene AGAINST *Lucilia cuprina* LARVAE (DIPTERA: CALLIPHORIDAE): HISTOLOGICAL AND ULTRASTRUCTURAL BIOMARKERS ASSESSMENT

ABSTRACT

Lucilia cuprina, known as the Australian blowfly, is of high medico-sanitary and veterinary importance due to its ability to induce myiasis. Synthetic products are the most frequent form of fly control, but their indiscriminate use has selected for resistant populations and accounted for high levels of residues in animal products. This study aimed to assess the insecticidal activity of essential oil from leaves of *Curcuma longa* (CLLEO), and its major compound *α*-phellandrene against *L. cuprina* L3. An additional goal was to determine the morphological alterations in target organs/tissues through ultrastructural assessment (SEM) and light microscopy, as well as macroscopic damage to cuticle induced by CLLEO and *α*-phellandrene. Groups of 20 L3 were placed on filter paper impregnated with increasing concentrations of CLLEO (0.15 to 2.86 µL/cm^2) and *α*-phellandrene (0.29 to 1.47 µL/ cm^2). Efficacy was determined by quantifying L3 mortality 6, 24 and 48 h after contact with extracts and by measuring the structural damage to L3. CLLEO and *α*-phellandrene inhibited adult emergence by 96.22 and 100%, respectively. Macroscopic cuticle damage, appeared as diffuse pigment and darkening of larval body, was caused by both extracts. The SEM revealed dryness on the cuticle surface, distortion of the sensorial structures and general degeneration in treated L3. Furthermore, alterations in target organs (digestive tract, fat body and brain) were noticed and shall be used as biomarkers in future attempts to elucidate the mechanism of action of these compounds. The vacuolar degeneration and pyknotic profiles observed in the brain tissue of treated larvae with both extracts and the decreased motility within ≤ 6h after treatment leads us to suggest a neurotoxic activity of the products. This work demonstrates the potential use of CLLEO and its major compound *α*-phellandrene as bioinsecticides to be used against *L. cuprina*, representing an ecofriendly alternative for myiasis control in humans and animals.

Keywords: Ecofriendly Products, Biopesticide, by-product, Myiasis, Blowflies, Toxicity.

GRAPHICAL ABSTRACT



MANUSCRITO 1 - ATIVIDADE INSETICIDA DO ÓLEO ESSENCIAL DE *Curcuma longa* (FOLHAS) E DE SEU COMPOSTO MAJORITÁRIO *α*-felandreno SOBRE LARVAS DE *Lucilia cuprina* (DIPTERA: CALLIPHORIDAE): AVALIAÇÃO DE BIOMARCADORES HISTOLÓGICOS E ULTRASTRUTURAL

RESUMO

Lucilia cuprina, conhecida como a mosca varejeira australiana, possui grande importância veterinária e médico-sanitária devido à sua capacidade de induzir miíases. Os pesticidas sintéticos são a forma mais frequente de controle deste inseto, entretanto, seu uso indiscriminado tem selecionado populações resistentes, além de determinar altos níveis de resíduos em produtos de origem animal. Neste sentido, este trabalho teve o objetivo de avaliar a atividade inseticida do OE extraído das folhas de *Curcuma longa* e de seu composto majoritário, α-felandreno, sobre larvas de terceiro ínstar (L3) de *L. cuprina*. Adicionalmente, objetivou-se determinar as alterações morfológicas em órgãos-alvo, através da avaliação ultraestrutural e histopatológica, bem como os danos macroscópicos observados a nível de cutícula induzidos pelo OE de *C. longa* e α-felandreno. Assim, grupos de 20 L3 foram colocados em papel de filtro impregnado com concentrações crescentes do OE de *C. longa* (0,15 a 2,86 μL/cm^2) e α-felandreno (0,29 a 1,47 μL/cm^2). A eficácia foi determinada através da quantificação da mortalidade das L3, 6, 24 e 48 h após o contato com os extratos e mensurando seu dano estrutural. O OE de *C. longa* e o α-fellandrene inibiram a emergência de adultos em 96,22 e 100%, respectivamente. Além disso, danos cuticulares macroscópicos como pigmento difuso e escurecimento do corpo larval, foi noticiado utilizando ambos os extratos. Ainda, alterações como ressecamento na superfície da cutícula, distorção das estruturas sensoriais e degeneração geral das larvas tratadas foram observadas através da MEV. Alterações nos órgãos-alvo (trato digestivo, corpo gorduroso e cérebro) foram noticiadas e poderão ser utilizadas como biomarcadores em futuras tentativas de elucidar o mecanismo de ação desses compostos. A diminuição da motilidade em menos de seis horas após o tratamento com ambos os extratos, bem como as alterações histológicas como degeneração vacuolar e cérebro com perfil picnótico, nos levam a sugerir uma atividade neurotóxica dos extratos avaliados. Este trabalho demonstra o potencial de uso do OE extraído das folhas de *C. longa* e seu composto

majoritário, α-felandreno, como bioinseticidas contra *L. cuprina*, representando uma alternativa ecológica para o controle de miíases em humanos e animais.

Palavras-chave: Produtos Ecológicos, Biopesticida, subproduto, Miíase, Moscas Varejeiras, Toxicidade.

1 INTRODUCTION

Lucilia cuprina is the cause of myiasis, a serious disease that affects humans and animals, and is considered an important ectoparasite of livestock, affecting productivity and animal welfare (GUIMARÃES et al., 1978; WINDSOR and LOMAX, 2013; SANDEMAN et al., 2014). The Calliphoridae family contains the major species with the capacity to develop skin lesions in farm animals (i.e. *Lucilia cuprina,* Wiedemann, 1830). These flies deserve attention for their ability to develop cutaneous myiasis in sheep and they are considered pest of significant economic importance for neotropical agriculture (WALL, 2012; GRISI et al., 2014; ANSTEAD et al., 2016). The economic loss caused by the infestation of living tissue by Diptera larvae was estimated at US$ 336.6 million/year from *Cochliomyia hominivorax* primary myiasis in Brazil (GRISI et al., 2014). Although there has been no study on the losses caused by *L. cuprina* in the country, this species has a cosmopolitan distribution and displays typical synanthropic behavior, being intimately associated with human habitation and the artificial infestation of sheep, as demonstrated by MOREIRA LIMA and MOYA BORJA (1997). However, chemical control is still the most used form of fly reduction, and its indiscriminate application is the cause of selection of resistant flies, and deposition of chemical residues in animal products (e.g. meat and milk) (LABBÉ et al., 2017; QIN et al., 2017). Several alternative methods of control of *L. cuprina* have been proposed, among them (1) parasitoids: *Nasonia vitripenis*, *Aphaereta aotea*, *Tachinaephagus zealandicus*; (2) gram-positive bacteria: *Bacillus thuringiensis*; (3) immunological control employing enzymes with histone deacetylase inhibitors (HDACi) as vaccine candidates; (4) mass rearing of sterile insects by radiation; and (5) development of transgenic technologies for pest insects (SANDEMAN et al., 2014). However, studies about biopesticides derived from plants, especially essential oils (EO) and their individual compounds against *L. cuprina* control, are still incipient. Potential candidates can be found within the family Zingiberaceae. This family consists of 53 genera and over 1,200 species native to tropical regions, especially Southern and Southeast Asia, occurring in tropical Africa and in Central and South America. Many of these species have economic value, providing food (starches rhizomes), perfumes and condiments with aromatic and medicinal properties (MOGHADAMTOUSI et al., 2014). *Curcuma longa* Linnaeus (Zingiberaceae) is a perennial herb, and its EO exhibits acaricidal and insecticidal

properties, having been extensively studied for biological activities conferred by its broad chemical composition (TAVARES et al., 2013; CHAGAS et al., 2016). However, not much attention has been given to the activity of the EO extracted from aerial parts (leaves) of the plant and their potential use as insecticide. The leaves of *C. longa* are considered residues during the rhizome harvest, and although few studies have addressed this part of the plant, its chemical composition is of interest in myiasis control. Previous reports have shown the presence of a cyclic monoterpene *α*-phellandrene in its chemical composition, showing good insecticidal activity (Evergetis et al., 2013). In Kerala, India, *α*-phellandrene (56.7%), 1,8-cineole (8.1%), *p*-cymene (7,5%) and *β*-pinene (5.3%) were the major constituents of EO from *C. longa* leaves (MCCARRON et al., 1995), while another study used GC-MS analysis to confirm the presence of β-sesquiphellandrene (22.8%), terpinolene (9.5%), aromatic curcumene (7.8%), 1,8-cineole (6.3%) and a minor amount of the monoterpene *α*-phellandrene (4.8%) (PRIYA et al., 2012). The leaf oil of *C. longa* from Bhutan, India has been reported to contain *α*-phellandrene (18.2%) and 1,8-cineole (14.6%), both constituents with insecticidal activity, evidencing the potential of *C. longa* as a sustainable alternative to chemical insecticides (SHARMA et al., 1997; ENAN, 2014; JACK and BUSCH, 2016). Although there are several studies on the chemical composition and biological activity of tumeric, there are no published reports of its larvicidal activity against *L. cuprina* using EO extracted from the aerial parts (leaves) of the plant or its monoterpene compound *α*-phellandrene. Morphological biomarkers through histopathological assessment and scanning electron microscopy, may also be used to determine changes in structure and intoxication of target cells. This study aimed to assess the effect of EO from the leaves of *C. longa* (CLLEO) and its major compound *α*-phellandrene against third stage larvae (L3) of *L. cuprina*. An additional goal was to show morphological biomarkers in target organs through ultrastructural assessment and light microscopy, as well as macroscopic damage on L3 cuticle, induced by CLLEO and its major compound.

2 MATERIALS AND METHODS

2.1 Plant Material

The leaves from *C. longa* used in this work were grown in the Unit of Medicinal Plants of the Catarinense Federal Institute, IFC, located at 26° 23' 33.6691" S and 48° 44' 18.3336" W, at 10.6 m above sea level, in the city of Araquari, Santa Catarina State, Southern Brazil. The cultivation was carried out in an agroecological system without the addition of chemicals. Leaves were collected from approximately 100 individuals in September 2016 (spring), 10 months after cultivation. A sample of the botanical species was deposited at the Herbarium of the Botanic Museum, located in the Botanical Garden of Curitiba, PR, under the number 358970.

2.2 Essential Oil Extraction and Chemical Characterization of *Curcuma longa* Leaf EO

Leaves from plants of the same cultivar were homogenized, and the EO was extracted from about 3 kg by hydrodistillation for 4 h in a Clevenger apparatus. The EO composition was analyzed by gas chromatography coupled with a mass spectrometric detector (GC/MS) (Shimadzu, Model 2010 Plus) (Kyoto, Japan) at the Department of Chemistry (UFPR, Brazil) using a HP-5MS capillary column (5% phenyl-/95% dimethylpolysilo-xane, 30 m x 0.25 mm x 0.25 µm) (Torrance, CA, USA). The injection temperature was 250 ^{o}C and the carrier (helium gas) flow was 1.0 mL/min^{-1}. The chromatograph oven was optimized with an initial temperature of 60 to 240 ºC and an incremental increase of 3 °C/min. The oil sample was diluted to 1% in hexane, followed by injection into the GC/MS. Quantification was determined by normalizing the area (%) of each chemical constituent peak, the total area being the sum of all areas of the chromatogram peaks (100%) using the chromatograph Agilent 7890A, with a similar capillary column to the one described above. For the quantification of hydrogen gas, the material was used with a carrier at a flow rate of 1.5 mL/min^{-1}. The retention indexes were calculated by the method of VAN DEN DOLL and KRATZ (1963) using the n-alkane standard solutions (relative to C7–C30 n-alkanes), in the same chromatographic conditions. The compounds were identified

by comparison of their GC mass and retention data with the available library (WILEY, 1994; NIST , 2013). The EO was analyzed in triplicate.

2.3 Dilution of extracts

The *α*-phellandrene (CAS: 99-83-2) was obtained from Sigma-Aldrich Brazil (São Paulo, Brazil) and had a purity of ≥ 99%. CLLEO was diluted in absolute ethanol or acetone as *α*-phellandrene was solubilized only in ethanol. The solvents have shown no toxicity to L3 of *L. cuprina* (CHAABAN et al., 2018; CHAABAN et al., submitted in 2017). The CLLEO concentrations used were 0.15, 0.31, 0.63, 0.79, 0.95, 1.11, 1.27, 1.43, 1.59, 2.07, 2.38 and 2.86 μL/cm^2. The following *α*-phellandrene concentrations were used: 0.29, 0.59, 0.88, 1.18 and 1.47 μL/cm^2. EO of *C. longa* leaves were solubilized in ethanol or acetone. A control group was established, in which L3 were exposed only to absolute ethanol or acetone.

2.4 Colony of Flies

Wild flies were collected manually at the IFC, using bait (shrimp bark kept at 27 ± 1 °C for 48 h of decomposition) and entomological net. The establishment of stock colonies, insect identification, maintenance, mass reproduction and the protocol for the biological tests were performed as described by CHAABAN et al. 2018. For this work, we used fresh, drug-free bovine meat (approx. 2 g/larvae) for larval development.

2.5 Larval Toxicity

The toxicity evaluation of CLLEO and *α*-phellandrene on L3 of *L. cuprina* was performed as described by CHAABAN et al. (2018). Groups of 20 mature L3 (1 day after they left the substrate) from the second generation were introduced into glass vials (9 × 4 cm diameter) containing a filter paper (12.56 cm^2) impregnated with 0.2 mL of *α*-phellandrene or EO solutions. After the application of CLLEO and *α*-phellandrene, the glass vials were closed with voile fabric to facilitate aeration, kept for 5 min in an exhaust hood and transferred to a climatic chamber at 27 °C and 70% relative humidity. All treatments were performed in triplicate (n = 60). Toxicity was

evaluated by observing L3 mortality at 6, 24 and 48 h after contact. Total L3 mortality (TLM) was calculated (CHAABAN et al., 2018; CHAABAN et al., 2017b; CHAABAN et al., 2017c; KUMAR et al., 2014) as follows:

TLM = (total dead larvae x 100)/total tested larvae

2.6 Analysis of Physiological Parameters

After CLLEO and *α*-phellandrene contact, L3 were kept under controlled conditions for recording of the following parameters: pupation rate (PR), emergence inhibition rate (EIR) and adult deformity (AD) (CHAABAN et al., 2018; CHAABAN et al., 2017b; CHAABAN et al., 2017c; SINGH et al., 2016; KUMAR et al., 2014):

PR = (total pupae x 100)/total tested larvae)
EIR = (total control adults − total treated adults x 100)/total control adults
AD = (total deformed adults x 100)/total emerged adults

2.7 Scanning Electron Microscopy (SEM)

For SEM, L3 treated with 1.59 μL/cm^2 of CLLEO and 1.47 μL/cm^2 of *α*-phellandrene were fixed in AFA solution (ethyl alcohol at 70%, buffered formalin at 37% and glacial acetic acid) in a ratio of 2.5:1:1.5, 6 h and 7 days after contact. Subsequently, the samples were submitted to a dehydration process using five alcohol baths. The larvae were placed in a support for electron microscopy (stub) and dehydrated in an oven using the protocol described by CANEPARO (2017), with modifications (37 °C for 6 h). The specimens were examined and photographed at a magnification ranging from 12X to 600X (JEOL JSM 6360-LV) at the Center for Electron Microscopy of UFPR.

2.8 Larval Histopathology

For larval histopathology, L3 treated with 1.59 μL/cm^2 of CLLEO and 1.47 μL/cm^2 of *α*-phellandrene and solubilized in ethanol were fixed in 10% buffered formalin, 6 and 24 h after contact with the solutions. For slide preparation, two longitudinal

sections were embedded in paraffin and L3 were serially sectioned (4 µm - thickness) and stained with hematoxylin-eosin (CHAABAN et al., submitted in 2017).

2.9 Statistical Analysis

Lethal concentrations (LC_{10}, LC_{50} and LC_{90}) were calculated using Probit analysis. L3 mortality, PR and EIR were analyzed for exposition time, concentrations, carriers and the interaction between concentrations and carriers through an analysis of variance (ANOVA) in generalized linear model, assuming a Poisson distribution. The averages were compared using the Tukey test. All analyses were performed using the statistical software SPSS (2013), considering the significance level of 5%. The values were corrected using the Abbott's formula (ABBOTT, 1925).

3 RESULTS

3.1 Chemical Characterization of *Curcuma longa* Leaf EO

Eighteen compounds were identified from CLLEO, representing 97.27% of the total chromatographic peaks (Table 1). The major compounds were *α*-phellandrene (41.99%), *ρ*-mentha-2,4(8)-diene (24.89%), and 1,8-cineole (7.82%), while *ρ*-cimene (2.79%), myrcene (2.63%) and *α*-pinene (2.52%) represented the smaller chromatographic area.

3.2 Larval Toxicity and Analysis of Physiological Parameters

Lethal concentrations of CLLEO and α-phellandrene are shown in Table 2. Dose- and time-dependent activities were demonstrated 6 h after exposure to CLLEO and *α*-phellandrene, with an LC_{50} of 1.34 and 1.17 µL/cm^2, respectively. The LC_{50} showed no significant variation between carriers (Figure 1). After 6 h of exposure, we observed that the LC_{10}, LC_{50} and LC_{90} (0.92, 1.17 and 1.48 µL/cm^2) for *α*-phellandrene (Table 2). Likewise, CLLEO/ET had a LC_{10}, LC_{50} and LC_{90} of 1.01, 1.34 and 1.78 µL/cm^2, while CLLEO/AC were 1.17, 3.28 and 9.19 µL/cm^2, respectively, showing statistically ($P<0.05$) higher values for LC_{50} and LC_{90} than CLLEO/ET (Table 2). Regarding the assessment of LM and physiological parameters

of *L. cuprina,* we observed LM of 85%, 48 h after contact with 10% CLLEO/ET (1.59 µL/cm^2) and only 46.66% LM when using CLLEO/AC (Table 3). *α*-phellandrene (1.47 µL/cm^2) had a statistically significantly different LM when compared to the other concentrations (Table 4). Furthermore, the same doses inhibited adult emergence by 96.22% and 100% using CLLEO and *α*-phellandrene, respectively. In addition, the pupation ratio had significantly different results when L3 were treated with CLLEO (1.27 µL/cm^2) and *α*-phellandrene (0.88 µL/cm^2), solubilized in ethanol, when compared to control groups. It can be observed that the higher the efficacy the greater is the activity over the other physiological measurements, reaching 100% for EIR, for both products (Matrix CLLEO and EIR r=0.8834; and Matrix *α*-phellandrene vs. EIR r=0.9763, respectively). The correlation of efficacy for LM and PR was r=0.91835 and r=-0.91838 for CLLEO, and r=0.98522 and r=-0.98521 for *α*-phellandrene, respectively.

TABLE 1 - CHEMICAL COMPOSITION OF *CURCUMA LONGA* LEAVES ESSENTIAL OIL.

Compounds	RT (min)	IRc	IRt	RA (%)	SD
α-tujene	4.013	925	924	0.11	0.0000
α-pinene	4.152	932	932	2.52	0.10214
sabinene	4.967	971	969	0.34	0.00577
β-pinene	5.048	975	974	5.65	0.12490
myrcene	5.36	991	988	2.63	0.03000
δ-2-carene	5.605	1002	1001	0.17	0.05774
α-phellandrene	5.742	1006	1002	41.99	0.46608
δ-3-carene	5.852	1010	1008	1.06	0.02082
α-terpinene	6.015	1016	1014	1.50	0.01000
ρ-cimene	6.228	1023	1020	2.79	0.08083
limonene	6.347	1027	1024	3.41	0.03464
1.8-cineole	6.413	1029	1026	7.82	0.37269
(E)-*β*-ocimene	6.902	1045	1044	0.42	0.00577
γ-terpinene	7.208	1056	1054	1.87	0.01528
ρ-mentha-2,4(8)-diene	8.172	1088	1085	24.89	0.18824
linalol	8.51	1100	1095	0.69	0.01528
terpinen-4-ol	11.185	1174	1174	0.40	0.01528
α-terpineol	11.695	1188	1186	0.39	0.03000
			Total identified compounds (%)	97.27	
			Other unidentified compounds (%)	1.36	

Note: RT = Retention time (min), IRc = Retention index calculated, IRt = Retention index tabulated (Adams, 2012), RA (%) = Relative area, SD = Standard deviation.

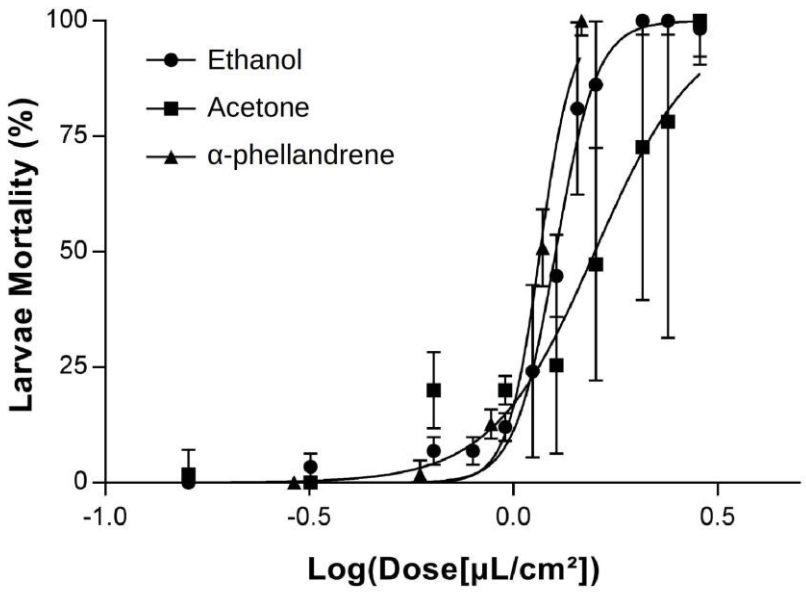
SOURCE: the author

TABLE 2 - LETHAL CONCENTRATION (µL/cm²) OF *Curcuma longa* LEAVES ESSENTIAL OIL (CLLEO) AND *α*-phellandrene AGAINST *Lucilia cuprina* THIRD STAGE LARVAE IN THE CONTACT ASSAY OVER TIME.

Extract	Evaluation time	*LC_{10} (LCI–UCI)	LC_{50} (LCI–UCI)	LC_{90} (LCI–UCI)	Chi-square ($\chi 2$)	Probability
CLLEO/ET	6h	1.01 (0.95-1.07)	1.34 (1.3-1.38)	1.78 (1.7-1.89)	8.68	0.37
	24h	1.02 (0.91-1.01)	1.35 (1.28-1.41)	1.78 (1.67-1.95)	14.19	0.07
	48h	0.98 (0.85-1.06)	1.28 (1.21-1.34)	1.68 (1.58-1.87)	10.59	0.1
CLLEO/AC	6h	1.17 (0.95-1.34)	3.28 (2.8-4.29)	9.19 (6.34-17.81)	6.17	0.19
	24h	1.05 (0.52-1.37)	2.44 (2.04-3.34)	5.64 (3.86-16.87)	9.56	0.05
	48h	0.94 (0.54-1.21)	1.67 (1.37-1.92)	2.96 (2.5-4.17)	9.52	0.05
α-phellandrene	6h	0.92 (0.48-1.07)	1.17 (0.95-1.33)	1.48 (1.31-2.39)	4.41	0.11
	24h	0.92 (0.48-1.07)	1.17 (0.95-1.33)	1.48 (1.31-2.39)	4.41	0.11
	48h	0.9 (0.41-1.05)	1.16 (0.93-1.36)	1.49 (1.3-2.83)	5.24	0.07

CLEO/ET: *Curcuma longa* Leaves essential oil solubilized in ethanol; CLLEO/AC: *Curcuma longa* Leaves essential oil solubilized in acetone. *α*-phellandrene was solubilized only in ethanol. *The lethal concentrations were calculated by the Probit analysis. LCI, lower limit of 95% confidence interval; UCI, upper limit of 95% confidence interval.

SOURCE: the author



SOURCE: the author

FIGURE. 1. AVERAGE AND STANDARD DEVIATION OF *Lucilia cuprina* AFTER EXPOSITION TO *Curcuma longa* LEAVES ESSENTIAL OIL (CLLEO) (log[µL/cm^2]) USING DIFFERENT CARRIERS (ETHANOL AND ACETONE) AND ITS MAJOR COMPOUND *α*-phellandrene USING ETHANOL.

TABLE 3 - LARVAE MORTALITY (LM), PUPARIATION RATE (PR), EMERGENCE INNIBITION RATE (EIR), SEX RATIO (MALE:FEMALE) AND ADULT DEFORMITY OF *Lucilia cuprina* TREATED WITH *Curcuma longa* LEAVES ESSENTIAL OIL.

C(µL/cm^2)(%)	*LM (%)	PR (%)	EIR (%)	SR (M:F)	AD (%)
Ethanol	3.33 (±1.67) c	96.66 (±1.67) a	0.0 (±0.00) c	29:24	0.0
0.15 (1%)	1.66 (±1.67) c	98.33 (±1.67) a	18.66 (±9.11) c	19:24	4.65
0.31 (2%)	5.0 (±0.0) c	95.0 (±0.0) a	16.98 (±16.6) c	25:19	4.54
0.63 (4%)	8.33 (±1.67) c	91.66 (±1.67) a	16.98 (±14.22) c	20:24	15.90
0.79 (5%)	8.33 (±1.67) c	91.66 (±1.67) a	20.75 (±13.92) c	21:21	7.14
0.95 (6%)	13.33 (±1.67) c	86.66 (±1.67) a	24.52 (±9.59) bc	18:22	7.50
1.11 (7%)	25.0 (±10.41) bc	75.0 (±10.41) ab	75.47 (±3.59) a	4:9	0.0
1.27 (8%)	45.0 (±5.00) b	55.0 (±5.00) b	69.81 (±5.26) ab	5:11	12.5
1.43 (9%)	80.0 (±10.41) a	20.0 (±10.41) c	83.01 (±7.18) a	3:6	0.0
1.59 (10%)	85.0 (±7.64) a	15.0 (±7.64) c	96.22 (±1.91) a	2:0	0.0
2.07 (13%)	98.33 (±1.67) a	1.66 (±1.67) c	98.11 (±1.96) a	0:1	0.0
2.38 (15%)	98.33 (±1.67) a	1.66 (±1.67) c	98.11 (±1.67) a	0:1	0.0
2.86 (18%)	96.66 (±3.33) a	3.33 (±3.33) c	100.0 (±0.0) a	0:0	0.0
Acetone	1.66 (±1.67) c	98.33 (±1.67) a	0.0 (±0.00) b	27:25	0.0
0.15 (1%)	5.0 (±2.89) c	95.0 (±2.89) a	17.30 (±8.23) b	29:14	0.0
0.31 (2%)	3.33 (±3.33) c	96.66 (±3.33) a	19.23 (±12.04) b	19:23	0.0
0.63 (4%)	21.66 (±4.41) bc	78.33 (±2.89) ab	25.0 (±10.6) ab	16:23	0.0
0.95 (6%)	21.66 (±1.67) bc	78.33 (±1.67) ab	28.84 (±18.77) ab	11:27	0.0
1.27 (8%)	26.66 (±10.14) bc	73.33 (±10.14) ab	57.69 (±29.6) ab	10:12	0.0
1.59 (10%)	46.66 (±13.33) abc	53.33 (±13.33) abc	71.15 (±23.66) ab	9:6	0.0
2.07 (13%)	70.0 (±17.56) ab	30.0 (±17.56) bc	86.53 (±16.66) ab	3:4	0.0
2.38 (15%)	75.0 (±25.0) ab	25.0 (±25.0) bc	100.0 (±0.0) a	0:0	0.0
2.86 (18%)	95.0 (±5.0) a	5.0 (±5.0) c	100.0±0.0 a	0:0	0.0

*48h of exposure
Absolute ethanol and acetone were used with control.
The letters display a significant difference (P <0.05) in the concentrations of the essential oils.

SOURCE: the author

TABLE 4 - LARVAE MORTALITY (LM), PUPARIATION RATE (PR), EMERGENCE INNIBITION RATE (EIR), SEX RATIO (MALE:FEMALE) AND ADULT DEFORMITY OF *Lucilia cuprina* TREATED WITH *α*-phellandrene.

C(µL/cm^2)	*LM (%)	PR (%)	EIR (%)	SR (M:F)	AD (%)
Control	0.0 (±0.0) d	100.0 (±0.0) a	0.0 (±0.00) d	29:30	0.0
0.29	0.0 (±0.0) d	100.0 (±0.0) a	0.0 (±2.96) d	35:24	0.0
0.59	1.66 (±1.67) d	98.33 (±1.67) a	3.38 (±3.33) d	30:27	0.0
0.88	11.66 (±1.67) c	88.33 (±1.67) b	23.72 (±5.83) c	26:19	2.22
1.18	46.66 (±4.41) b	53.33 (±4.41) c	83.05 (±4.28) b	3:7	10.0
1.47	91.66 (±1.67) a	8.33 (±1.67) d	100.0 (±0.0) a	0.0	0.0

*48h of exposure
Absolute ethanol was used with *α*-phellandrene control.
The letters display a significant difference (P <0.05) in the concentrations of the essential oils.

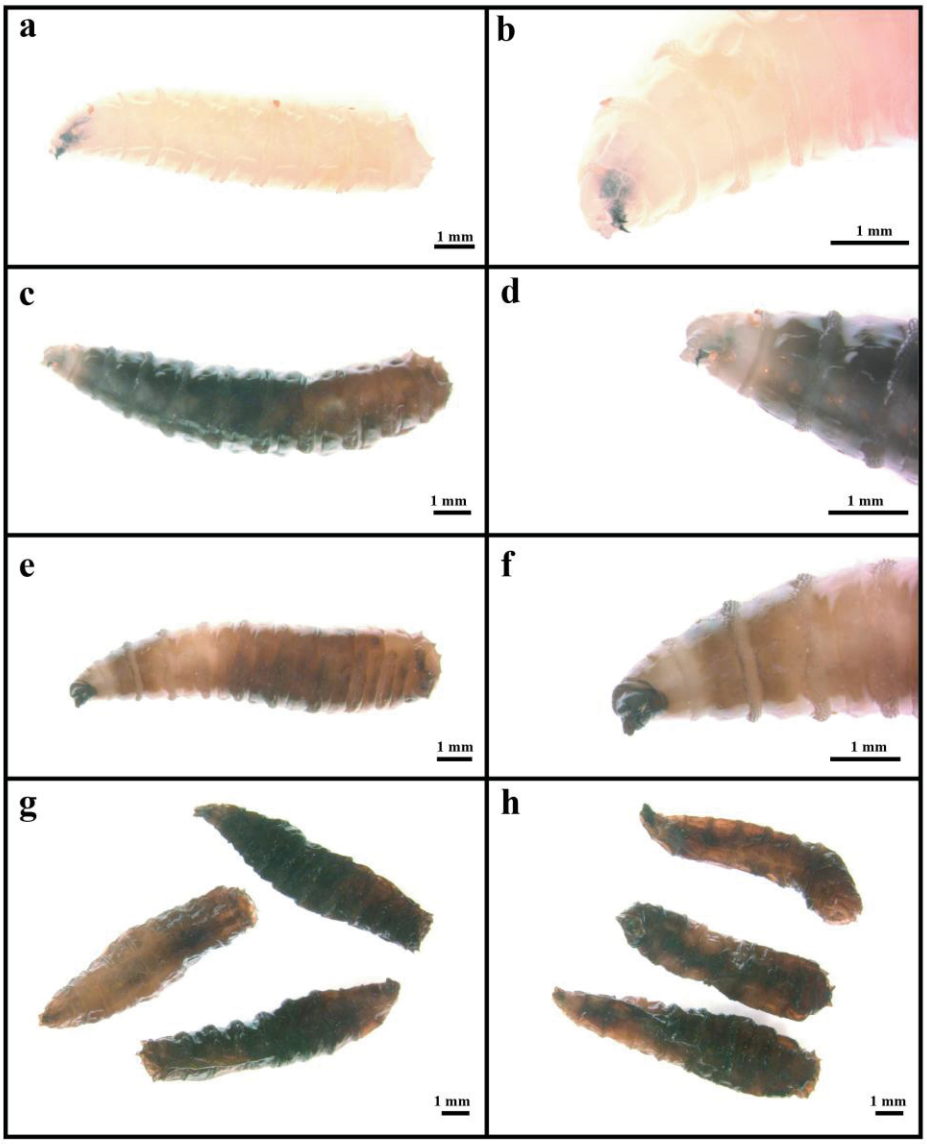
SOURCE: the author

3.3 Macroscopic Cuticle Damage

Macroscopic cuticle damage, such as diffuse pigment and darkening throughout the body and decreased motility after treatment with CLLEO and its major compound *α*-phellandrene, were observed after 6h of *L. cuprina* L3 exposure. Changes in color (darkening) throughout the L3 body with emphasis from the second through eighth segments using 1.59 μL/cm^2 of CLLEO were also reported starting at 6 h of contact (Figure 2c, d). Similar effects after the same exposure time to 1.47 μL/cm^2 to α-phellandrene were also observed (darkening of the anterior end of larva and diffuse pigment on the body.) but they were less pronounced (Figure 2e, f). Likewise, progressive lesions appearing as marked cuticle dryness were shown in dead L3, 7 days after exposure to both extracts (1.59 μL/cm^2 to CLLEO and 1.47 μL/cm^2 to *α*-phellandrene) (Figure 2g, h). Ethanol did not have any effect over L3, showing normal size and *sui generis* cuticle color (light yellow) (Figure 2a, b).

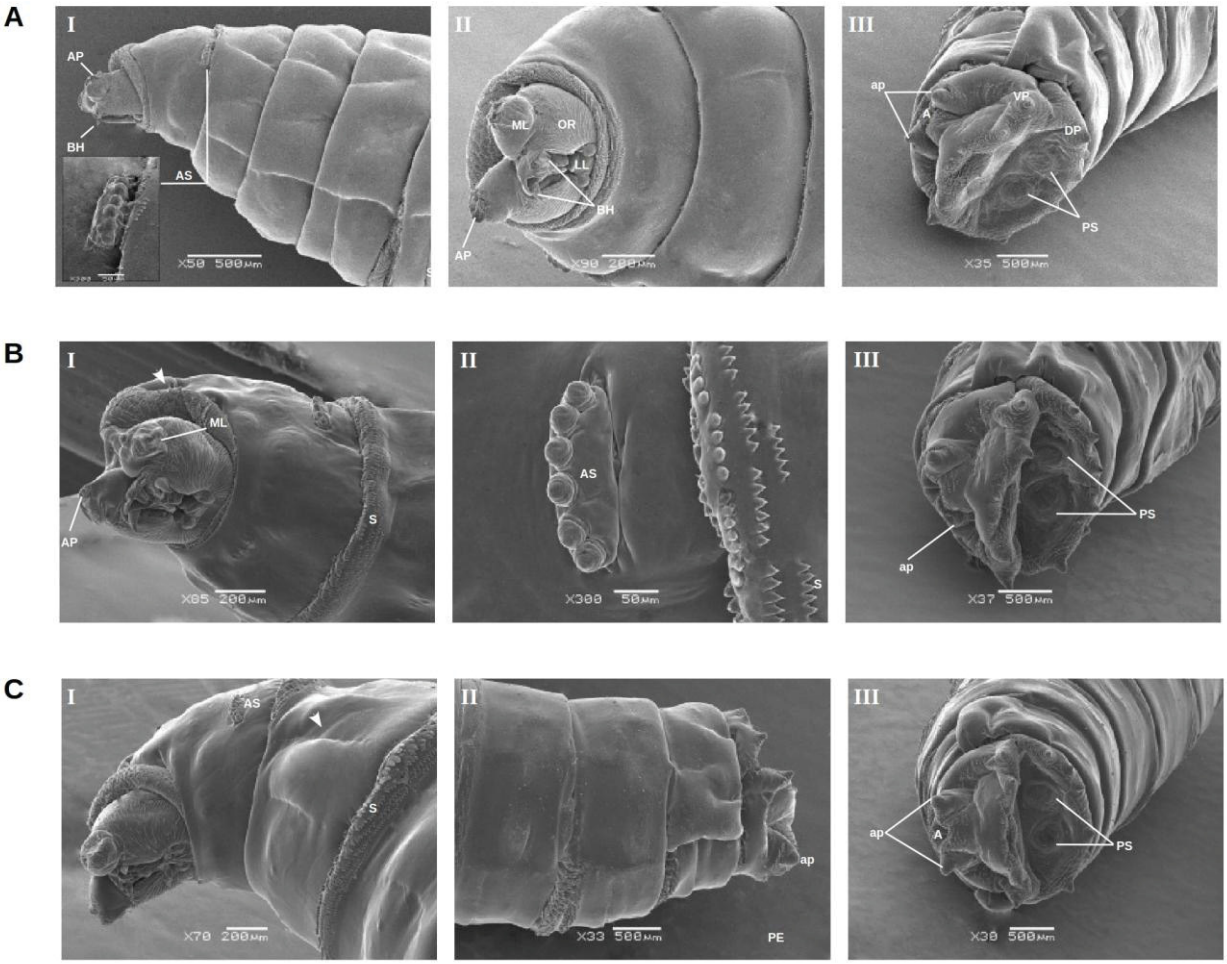
3.4 Scanning electron microscopy

The SEM of L3 of *L. cuprina* from the control group showed typical Calliphoridae morphology with smooth body tegument and preserved cuticle (Figure 3A, I-III). SEM of *L. cuprina* L3 6 h after treatment with 1.59 μL/cm^2 of CLLEO showed dryness on the cuticle surface, distortion of the sensory structures (antenna sensory papillae) and maxillary lobe, as well as slight distortion of the anal papillae and spiracle plate (Figure 3B, I-III). Likewise, we observed slight dryness on the cuticle surface and contraction of the cephalic segment 6 h after treatment with *α*-phellandrene (1.47 μL/cm^2) (Figure 3C, I-III). Notably, many changes were observed 7 days after treatment with both extracts, particularly extreme distortion and cuticle damage in all segments of larvae; degeneration of antenna sensory papillae, maxillary lobe, oral ridges, labial lobe and anterior spiracle and deformation on anal papillae, ventral spinules and posterior spiracles (Figure 4).



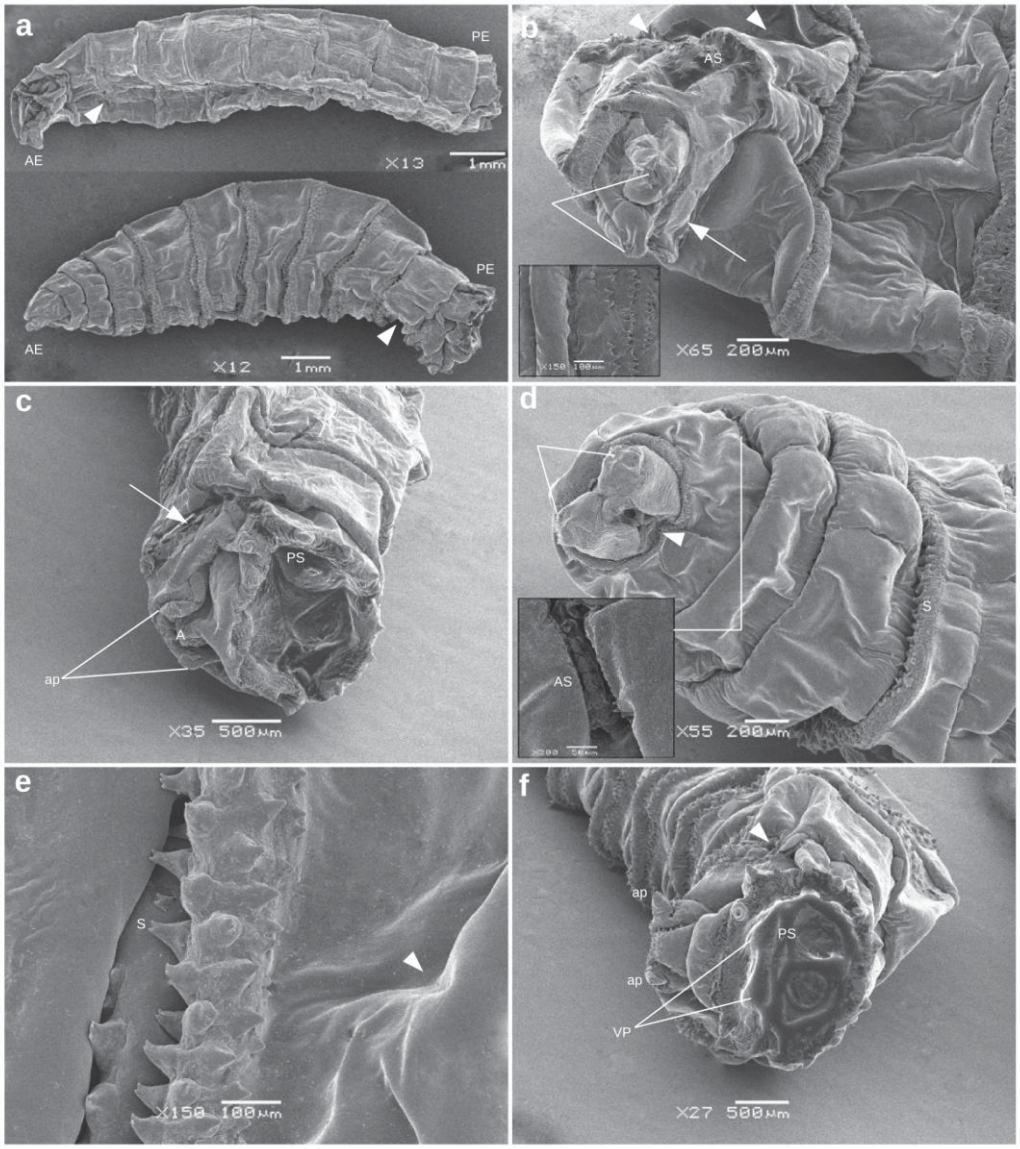
SOURCE: the author

FIGURE. 2. MACROSCOPIC CUTICULAR DAMAGE OF *Lucilia cuprina* L3 AFTER TREATMENT WITH *Curcuma longa* LEAVES ESSENTIAL OIL (CLLEO) AND ITS MAJOR COMPOUND *α*-phellandrene. a, b) NORMAL L3 6H AFTER TREATMENT (CONTROL GROUP TREATED WITH ETHANOL); c, d) L3 WITH CUTICLE DAMAGE 6H AFTER TREATMENT WITH 1.59 µL/cm^2 OF CLLEO; e, f) L3 WITH CUTICLE DAMAGE 6H AFTER TREATMENT WITH 1.47 µL/cm^2 OF *α*-phellandrene; g, h) L3, 7 DAYS AFTER TREATMENT WITH CLLEO (1.59 µL/cm^2) AND *α*-phellandrene (1.47 µL/cm^2), RESPECTIVELY.



SOURCE: the author

FIGURE 3. SCANNING ELECTRON PHOTOMICROGRAPHS OF *Lucilia cuprina* L3. A) CONTROL GROUP (ONLY ETHANOL). AI) ANTERIOR END OF LARVA WITH NORMAL BODY, DETAILS OF ANTERIOR SPIRACLES (AS) WITH 6-7 LOBES, BUCCAL HOOK (BH) AND ANTENNA SENSORY PAPILLAE (AP) PRESERVED. AII) CEPHALIC SEGMENT OF LARVA, NOTE THE PRESERVED STRUCTURES AND THE LARGE MOUTH HOOKS PROJECTING BEYOND THE ORAL CAVITY. AIII) POSTERIOR END OF LARVA, OBSERVE SPIRACULAR PLATE WITH THREE SPIRACULAR OPENINGS. B) *L. cuprina* L3, 6 H AFTER TREATMENT WITH 1.59 µL/cm^2 of CLLEO. BI) ANTERIOR END OF LARVA. NOTE THE DISTORTION OF THE SENSORIAL STRUCTURES, ANTENNA SENSORY PAPILLAE (AP) AND MAXILLARY LOBE (ML). DETAILS OF THE CUTICULAR SURFACE DRYNESS (ARROWHEAD) AND MARKED SPINULES (S) ON CEPHALIC SEGMENT. BII) ANTERIOR SPIRACLE (AS) AND SPINULES (S) PRESERVED. BIII) POSTERIOR END (PE) OF LARVA WITH SLIGHT DISTORTION OF ANAL PAPILLAE AND SPIRACLE PLATE. C) *L. cuprina* L3 6 H AFTER TREATMENT WITH 1.47 µL/cm^2 OF *α*-phellandrene. CI) ANTERIOR END OF LARVA, NOTE THE SLIGHT DRYNESS (ARROWHEAD) AND MARKED SPINULES (S). CII; III) POSTERIOR END OF LARVA. NOTE POSTERIOR SPIRACLES (PS), ANAL OPENING (A) AND ANAL PAPILLAE PRESERVED (AP). KEY: PE = POSTERIOR END; S = SPINULES; AP = ANTENNA SENSORY PAPILLAE; AS = ANTERIOR SPIRACLE; ML = MAXILLARY LOBE; OR = ORAL RIDGES; LL = LABIAL LOBE; BH = BUCAL HOOK; DP = DORSAL PAPILLAE; VP = VENTRAL PAPILLAE; A = ANUS; PS = POSTERIOR SPIRACLES; ap = ANAL PAPILLAE.

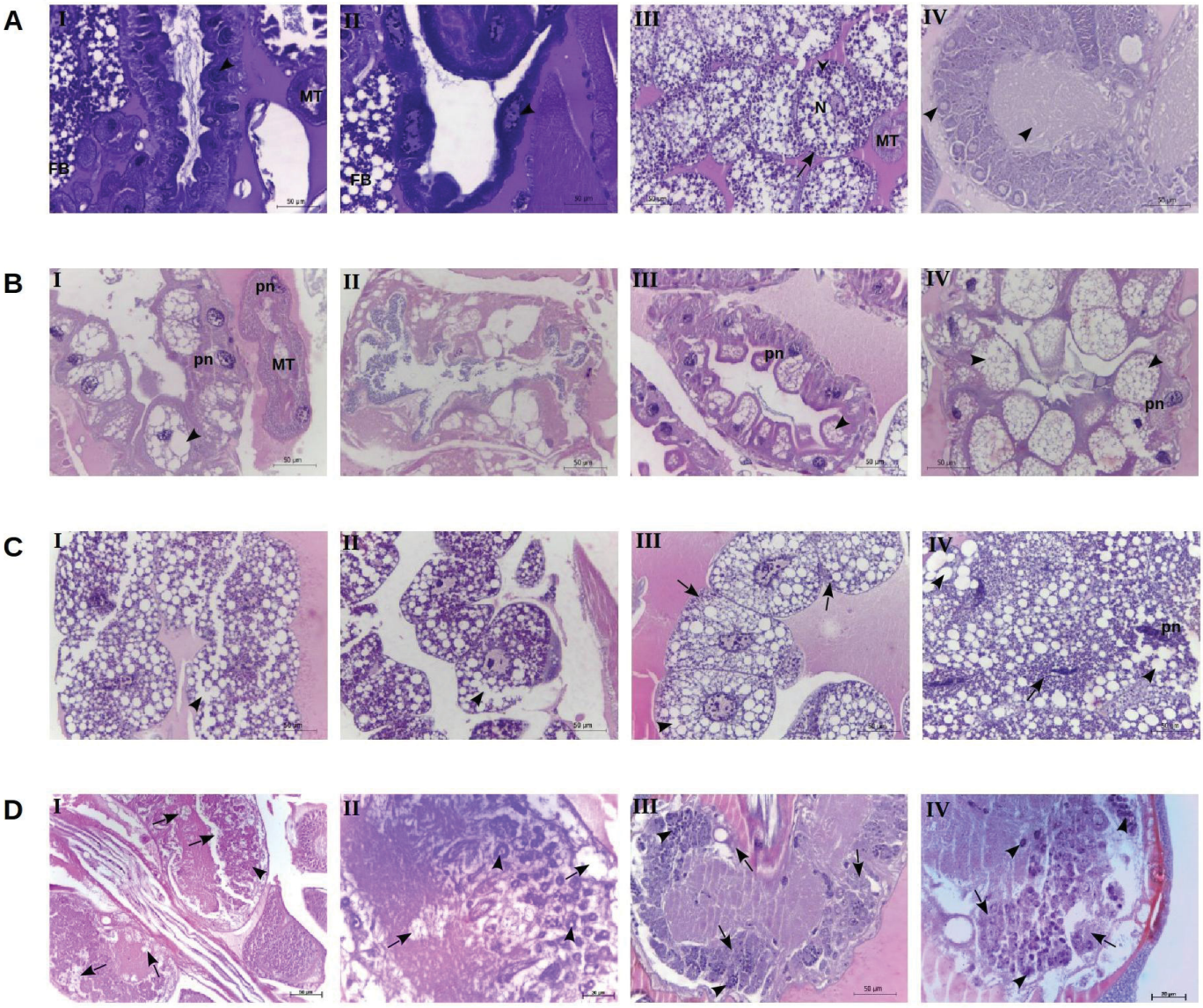


SOURCE: the author

FIGURE. 4. SCANNING ELECTRON PHOTOMICROGRAPHS OF *Lucilia cuprina* L3 7 DAYS AFTER TREATMENT WITH CLLEO (1.59 µL/cm^2) AND ITS MAJOR COMPOUND *α*-phellandrene (1.47 µL/ cm^2). a) EXTREME DISTORTION AND CUTICLE DAMAGE IN ALL SEGMENTS OF THE LARVA. L3 OF *L. cuprina* TREATED WITH CLLEO (ABOVE) AND *α*-phellandrene (BELOW). B) ANTERIOR END OF LARVA TREATED WITH CLLEO. NOTE: DEGENERATION OF ANTENNA SENSORY PAPILLAE, MAXILLARY LOBE, ORAL RIDGES, LABIAL LOBE AND ANTERIOR SPIRACLE. DETAILS OF THE DEFORMED AND WRINKLED CUTICULAR SURFACE (ARROWHEAD). C) POSTERIOR END WITH SEVERE DEGENERATION OF LARVA TREATED WITH CLLEO. OBSERVE THE ANAL PAPILLAE, POSTERIOR SPIRACLES AND DEFORMED VENTRAL SPINULES. D) ANTERIOR END OF LARVA TREATED WITH *α*-phellandrene. NOTE: EXTREME DISTORTION OF ANTENNA SENSORY PAPILLAE AND LABIAL LOBE. DETAILS OF ANTERIOR SPIRACLE WITH SEVERE DEGENERATION. E) SPINULES PROTRUDING OF LARVA TREATED WITH *α*-phellandrene. OBSERVE THE CUTICULAR SURFACE DRYNESS (ARROWHEAD). F) POSTERIOR END OF LARVA TREATED WITH *α*-phellandrene WITH EXTREME CUTICULAR DAMAGE. NOTE: DISTORTION OF ANAL PAPILLAE, SEVERE DEGENERATION IN VENTRAL PAPILLAE AND PROTRUDING SPINULES (ARROWHEAD). KEY: CLLEO = *CURCUMA LONGA* LEAVES ESSENTIAL OIL; AE = ANTERIOR END; PE = POSTERIOR END; AP = ANTENNA SENSORY PAPILLAE; AS = ANTERIOR SPIRACLE; S = SPINULES; A = ANUS; ap = ANAL PAPILLAE; VP = VENTRAL PAPILLAE; PS = POSTERIOR SPIRACLES.

3.5 Larval Histopathology

Histological sections of *L. cuprina* L3 showed different alterations after treatment with CLLEO and its major compound *α*-phellandrene. Vacuolization in the cytoplasm, pyknotic nuclei and necrosis of the digestive tract were seen after the use of both extracts (Figure 5B, I-IV). Similarly, we observed severe alterations in the fat body of treated larvae, such as cytoplasmic vacuolation and irregular morphology of trophocytes with pyknotic nuclei, as well as changes in protein granules (Figure 5C, I-IV). Histological sections of *L. cuprina* L3 brain showed vacuolar degeneration, pyknotic profiles and disorganized and condensed cells, characteristic of degeneration and necrosis after CLLEO and *α*-phellandrene exposure (Figure 5D, I-IV). Figure 5A (I-IV) show control larvae with no marked alterations.



SOURCE: the author

FIGURE 5. PHOTOMICROGRAPHS OF DIGESTIVE TRACT, FAT BODY AND BRAIN OF *Lucilia cuprina* L3. A) CONTROL GROUPS. AI, AII) INTACT DIGESTIVE TRACT WITH NUCLEUS PRESERVED (ARROWHEAD), FAT BODY (FB) AND MALPIGEAN TUBULES (MT) INTACT (40X). AIII) FAT BODY SHOWING THE TROPHOCYTES WITH A LARGE NUMBER OF VACUOLE (ARROWS) AND NUCLEI (N) SURROUNDED BY PROTEIN GRANULES (ARROWHEADS). AIV) LARVAE BRAIN SHOWING HOMOGENEOUS STRUCTURES AND PRESERVED (ARROWHEADS) (40X). BI) L3 OF *L. cuprina* 6 H AFTER TREATMENT WITH 1.59 μL/cm^2 OF CLLEO. NOTE THE INTENSE CYTOPLASMIC VACUOLATION (ARROWHEADS) (40X). BII) *L. cuprina* L3 24 H AFTER TREATMENT WITH 1.59 μL/cm^2 of CLLEO. DETAILS FOR MARKED NECROSIS OF THE INTESTINAL TRACT (40X). BIII) DIGESTIVE TRACT OF *L. cuprina* L3 6 h AFTER TREATMENT WITH 1.47 μL/cm^2 OF *α*-phellandrene. NOTE THE INTENSE CYTOPLASMIC VACUOLATION (ARROWHEADS) AND PYKNOTIC NUCLEI (pn) (40x). BIV) GUT CELLS OF *L. cuprina* L3 24 H AFTER TREATMENT WITH 1.47 μL/cm^2 OF *α*-phellandrene. OBSERVE THE INTENSE PROGRESSION VACUOLIZATION IN THE CYTOPLASM (ARROWHEADS) AND PYKNOTIC NUCLEI (pn) (40x). CI) *L. cuprina* L3 6 H AFTER TREATMENT WITH 1.59 μL/cm^2 OF CLLEO. OBSERVE THE TROPHOCYTES WITH SLIGHT VACUOLATION (ARROWHEADS). CII) *L. cuprina* L3 24 H AFTER TREATMENT WITH 1.59 μL/cm^2 OF CLLEO. NOTE SLIGHT VACUOLATION (ARROWHEADS) AND IRREGULAR MORPHOLOGY OF TROPHOCYTES. CIII) FAT BODY OF *L. cuprina* L3 6 H AFTER TREATMENT WITH 1.47 μL/cm^2 OF *α*-phellandrene. DETAILS TO VACUOLATION (ARROWHEADS) AND REDUCTION OF PROTEIN GRANULES WITH PERIPHERAL PROTEIN GRANULE (ARROWS). CIV) L3 24 H AFTER TREATED WITH 1.47 μL/cm^2 OF *α*-phellandrene. NOTE THE IRREGULAR TROPHOCYTES AND NUCLEUS, PYKNOTIC NUCLEI (pn), GREAT QUANTITY OF VESICLES (ARROWHEADS) AND GROUPING OF PROTEIN GRANULES NEAR OF THE NUCLEUS (ARROWS). DI, DII) BRAIN OF *L. cuprina* L3 24 H AFTER TREATMENT WITH 1.59μL/cm^2 OF CLLEO. OBSERVE VACUOLAR DEGENERATION SHOWING MEDIUM SIZED CLUSTERS OF VACUOLES, LIGHT IN THE NEUROPIL AND MILD IN THE CORTICAL LAYER (ARROW). THE TROPHOBLASTS ARE SHRUNKEN (ARROWHEADS) (40X, 100X). DIII, DIV) BRAIN OF *L. cuprina* L3 24 H AFTER TREATMENT WITH 1.47μL/cm^2 OF *α*-phellandrene. NOTE THE PYKNOTIC PROFILES (ARROWHEADS), DISORGANIZED AND CONDENSED CELLS CHARACTERISTICS OF DEGENERATION AND NECROSIS (ARROW) (40X, 100X). H & E, HEMATOXYLIN–EOSIN.

4 DISCUSSION

4.1 Chemical Characterization of *Curcuma longa* Leaf EO

Within the Zingiberaceae family, turmeric is one of the most investigated Curcuma species due to the medicinal properties of its rhizome (TAVARES et al., 2013; MOGHADAMTOUSI et al., 2014; CHAGAS et al., 2016). Interestingly, in contrast to observed in rhizomes, where the sesquiterpene ar-turmerone is frequently reported to be the major compound (EVERGETIS et al., 2013), in the EO from *C. longa* leaves, α-phellandrene has often been reported as a major constituent. In this way, the effects of environmental conditions were demonstrated by SANDEEP et al., (2016) regarding the quantity of secondary metabolite contents of *C. longa*, where the range of variation in *α*-phellandrene content in leaf EO ranged from 23.48 to 67.64% in nine different zones. In the present study, *α*-phellandrene represented 41.99% of the EO, shedding light on the potential use of *C. longa* leaf oil for myiasis control, since this monoterpene (*α*-phellandrene) is largely used for synergistic pest-control compositions (ENAN, 2014). The chemical profile and insecticidal activities of EO from *Anethum graveolens*, which has *α*-phellandrene as its majority constituent (59%) were determined by EVERGETIS et al., (2013). *In vitro* assays against L3 to L4 of *Culex pipiens* biotype molestus evidenced an LC_{50} value of 52.74 mg/L for *A. graveolens* EO, while the highest toxicity was found with isolated *α*-phellandrene, with an LC_{50} of 38.20 mg/L. Likewise, *α*-phellandrene exhibited strong activities in *in vitro* tests against *Aedes aegypti* and *A. albopictus* larvae, with LC_{50} of 16.6 and 39.9 µg/mL, respectively (CHENG et al., 2009). Notably, *α*-phellandrene was found to attenuate inflammatory responses through neutrophil migration inhibition and mast cell degranulation, highlighting the role of this monoterpene as an anti-inflammatory agent (SIQUEIRA et al., 2016). Although *α*-phellandrene was the major constituent found in our study, we observed the presence of other minor compounds. Constituents such as limonene (3.41%), ρ-cimene (2.79%), myrcene (2.63%) and α-pinene (2.52%), although present in small quantities in leaves, also have a varied of biological activities including insecticidal, antioxidant, anti-inflammatory and bactericidal activities; giving the ability to improve skin permeability and a synergistic effect when added to other compounds (LIM et al., 2006; CIFTCI et al., 2011; FREEHAUF et al., 2011; FALCONIERI et al., 2013; ZIELINSKI et al., 2013;

CAVALLARO, 2015; GOVINDARAJAN et al., 2016). Previous reports demonstrated several biological properties of *β*-pinene, such as antifungal, antimicrobial, insecticidal and insect repellent activities and enhancement of skin permeability (FALCONIERI et al., 2011; ENAN, 2014, CAVALLARO, 2015). Notwithstanding, the terpene 1,8-cineole known as eucalyptol may be used in preparations for the treatment of oxidative skin damage and may act in synergy with natural pesticides, in pharmaceutical transdermal preparations and for the treatment of inflammation in cattle (FREEHAUF et al., 2011; ZIELINSKI et al., 2013; JACK and BUSCH, 2016).

4.2 Larval Toxicity and Analysis of Physiological Parameters

Toxicity in the early hours after contact with EO against blowflies has recently been demonstrated. Investigating the insecticidal activity of *Baccharis dracunculifolia* EO, CHAABAN et al., (2017b) determined an LD_{50} of 2.63 μL/cm^2 6 h after contact with *C. macellaria* L3, using ethanol as the carrier. Likewise, similar effects were observed, using *Piper gaudichaudianum* EO against *L. cuprina* 6 h hours after contact with an LD_{50} of 3.71 μL/cm^2 where ethanol was more effective than acetone (CHAABAN et al., 2018). Our work shows better results when compared with previous reports for both CLLEO and *α*-phellandrene, demonstrating also a dose- and time-dependent activity. Previous reports assessed a transdermal therapeutic system to promote an increase in skin permeability using ethanol and the terpene d-limonene as carriers. D-limonene penetrated the skin when mixed with ethanol, causing changes in the barrier structure of the skin (TAKAYAMA and NAGAI, 1994). In this sense, we believe that the major affinity between CLLEO and ethanol could be associated with the presence of the monoterpene limonene in the chemical composition of EO and the interaction of the constituents with ethanol, increasing the penetration of the bioactive compounds through the insect tegument. Although some articles have reported insecticidal activity of several EO, this is the first study to demonstrate the effects of CLLEO and *α*-phellandrene on blowfly larvae. Although *α*-phellandrene has shown clear insecticidal activity, this compound could be modulated by other minor molecules present in CLLEO, such as 1,8-cineole, β-pinene, limonene and α-pinene (LIM et al., 2006; CIFTCI et al., 2011; CAVALLARO, 2015; GOVINDARAJAN et al., 2016). In addition, more scientific evidence has emerged that several minor constituents of the EO play a key role in improving skin

penetration and cellular distribution (i.e. limonene, 1,8-cineole) acting synergistically within molecules (LIM et al., 2006; BAKKALI et al., 2008). Previous reports also suggested that interactions between different combinations of monoterpene compounds (synergistic action), carriers and the lipid layer of the insect's cuticle may explain their enhanced penetration and increased activity (TAK and ISMAN, 2015). Moreover, some studies suggested differences in the effects of EO and individual compounds on the insect cuticle when using different routes of administration, obtaining better toxicity results when using topical administrations (contact assays) (SIQUEIRA et al., 2016). Suppression of insect development using EO as a natural control have been reported in several studies. KHATER et al. (2011) observed 100% suppression of adult emergence of *L. sericata* treated with 2% of *Lactuca sativa* and *Matricaria chamomilla* EO. In the same way, reports of adult emergence suppression after treatment of *L. sericata* larvae with 8% of *Brassica compestris* and 12% of *Raphanus sativus* were shown by KHATER and KHATER, (2009). Similarly, the adult emergence inhibition rate of *L. cuprina* exposed to 1.59 μL/cm^2 of *Piper gaudichaudianum* EO presented 94.44% inhibition, while the same dose of *T. minuta* showed 100% of inhibition (CHAABAN et al., 2018; CHAABAN et al., submitted in 2017).

4.3 Macroscopic Cuticle Damage

Recent reports of cuticle damage in L3 of blowflies have been conducted using biological assays. SHALABY et al. (2016) showed cuticular swelling and distortion in L3 of *L. sericata* treated with *Lavandula angustifolia* (lavender oil) and *Cinnamomum camphora* (camphor oil). Similarly, malformations of *L. sericata* larvae, such as small sized and damaged larvae with weak cuticles, after the contact with *Commiphora molmol* were reported by HODA et al., (2016). *C. macellaria* L3 showed decreased motility and cuticle abnormalities, 6 h after exposure to *B. dracunculifolia* (CHAABAN et al., 2017b). Likewise, biological assays using *P. gaudichaudianum* EO against the same biological model as in our work, also exhibited a decrease in motility and cuticle damage (CHAABAN et al., 2018). Thus, macroscopic observations of cuticle lesions associated with histological changes in target organs may be an important way to elucidate the mechanism of action of EO and their individual compounds (see section 3.5). Although most studies focused on *in vitro*

tests, the present results offer the possibility of using EO in the control of veterinary ectoparasites, as investigations of botanical pesticides have grown considerably in the last decade (PAVELA, 2015; PAVELA and BENELLI, 2016; CHAABAN et al., 2017a).

4.4 Scanning Electron Microscopy

Until now, few ultrastructural injury investigations have been carried out in contact experiments with biopesticides in blowflies, especially EO and isolated its compounds. SHALABY et al., (2016) have determined the larvicidal activity of *C. camphora* (camphor) and *L. angustifolia* (lavender) against *L. sericata*. The authors have observed distortion of sensorial structures, wrinkling of the cuticle surface and slight degeneration of the anterior spiracle in L3, treated with camphor oil, while L3 treated with lavender EO showed cuticle distortion. Comparably, changes such as cuticle swelling, degeneration of anterior spiracles, a wrinkled and shrunken cuticle and degeneration of papillae were also reported by HODA et al., (2006) using an alcoholic extract of *Balanites aegyptiaca* and the EO from *Commiphora molmol*, against different stages of *L. sericata*. Ultrastructural assessment of L3 may assist in elucidating the damage caused by biopesticides, improving the identification of the mechanism of action (MENDONÇA et al., 2014). In this sense, it is noteworthy that this is the first study assessing the damage caused by CLLEO and α-phellandrene to *L. cuprina* L3 by SEM. One of the first bioactives known to induce anomalies in different insects, azadirachtin extracted from the Indian neem tree *Azadirachta indica* A. Juss (Meliaceae) has been tested against different insects, including *L. cuprina*. This effect may occur due to the interference of neuroendocrine control of molting and ecdysis (SCHMUTTERER, 1990). Another study about the effects of azadirachtin on the morphology of *L. cuprina* larvae investigated morphological changes in the ultrastructure of the endocrine glands (the prothoracic gland, the *corpus allatum* and the *corpus cardiacum*) responsible for controlling molting and leant support to this theory (SCHMUTTERER, 1990; MEURANT et al., 1994). It is worth mentioning that, among natural products, azadirachtin has been approved for use in The United States since 1990, was subsequently approved in Germany and is currently approved in 10 other countries in the European Union, in addition to China, India and Canada (ISMAN, 2015).

4.5 Larval Histopathology

The results of this study demonstrate the neurotoxic effect of CLLEO and *α*-phellandrene. In addition, the appearance of small inclusions in the fat body of L3 may be due to L3 stress mechanism after treatment, signalling energy reserves in a process of drug detoxification. Supporting this argument, microscopic observations could be used to determine the ability of EO candidates to penetrate L3 cuticle, to elucidate the target cells for metabolism of EO and/or to determine the effect of biopesticides over time (CHAABAN et al., submitted in 2017). Some studies regarding pesticide action to the midgut, fat body and brain morphology of insects have provided new insights into the mode of action and damage by thiamethoxam, fipronil, deltamethrin, temephos, ivermectin and abamectin (ALVES et al., 2010; CRUZ et al., 2010; JACOB et al., 2015; TAVARES et al., 2015). Following pesticide treatment, several changes in specific organelles in the digestive tract of insects suggest that energy is necessary for vacuolation and vesicle release, possibly as an attempt to detoxify cells (ALVES et al., 2010; CRUZ et al., 2010). The insect fat body may be another important insect structure due to its prime location for intermediary metabolism and detoxification processes. Moreover, several studies have suggested that the fat body also plays a role in the immune response (ALVES et al., 2004; ALVES et al., 2010; ALMEIDA et al., 2014). We believe that the damage found on the fat body of larvae in our work, such as cytoplasmic vacuolation and irregular morphology of trophocytes with pyknotic nuclei, suggests an attempt by detoxicative metabolism to excrete toxicants (CLLEO and *α*-phellandrene). The brain is also another important target organ for pesticides that may also help to elucidate the mode of action of some EO. OLIVEIRA et al., (2014) reported side-effects of sub-lethal doses of thiamethoxam in the brain and the midgut of *Apis mellifera.* Morphological and histochemical alterations, such as cytoplasm vacuolization and condensed cells with more intense staining in the brain, have been demonstrated. Photomicrographs showing morphological alterations induced by boric acid and fipronil in the midgut of *A. mellifera* larvae revealed changes such as cytoplasmic vacuolisations with the absence of autophagy vacuoles, and chromatinic compaction of cells (CRUZ et al., 2010). JACOB et al., (2015) assessed the impact of sub-lethal doses of fipronil, a neurotoxic insecticide, on the brain mushroom bodies of the stingless bee *Scaptotrigona postica*. The authors showed morphological changes

(pyknotic profiles in brain) suggestive of cell death, apoptosis and necrosis. Histological alterations in organs such as fat body and brain of L3 of *L. cuprina* treated with natural compounds have not been demonstrated previously. The damages that were found in the brains of *L. cuprina* L3 (vacuolar degeneration, pyknotic profiles, disorganized and condensed cells), in our study support previous reports and reinforce the neurotoxic effect of CLLEO and *α*-phellandrene. However, recent work has shown that photomicrographs of *L. cuprina* exposed to 1.59 μL/cm^2 (10%) of *T. minuta* EO had significant degeneration of the digestive tract (CHAABAN et al., submitted in 2017). HODA et al., (2016) reported microscopic damage (destruction of gut epithelium) in *L. sericata* treated with an alcoholic extract and EO of *C. molmol* and *Balanites aegyptiaca*. Although this result corroborates with our data regarding the damage to the digestive tract after CLLEO and *α*-phellandrene exposure, additional studies are required in order to better elucidate the mechanism of action of both products (CLLEO and α-phellandrene).

5 CONCLUSION

We report that a single application of CLLEO or *α*-phellandrene was significantly toxic to L3 of *L. cuprina* and could be considered as a good ecofriendly product to control this pest. Damage to target organs such as the cuticle, brain, midgut and fat body of L3 was evident, even at low concentrations of both products. These evidences shall be used as future biomarkers to help elucidate the mechanism of action of these compounds. Thus, the vacuolar degeneration and pyknotic profiles observed in the brains of L3 treated with CLLEO and *α*-phellandrene, as well as the decreased motility observed within ≤ 6h after treatment suggest that this compound has neurotoxic activity. The potential of both extracts as bioinsecticides against *L. cuprina* L3 represent a sustainable alternative for myiasis control in humans and animals. Finally, this work leads the way to new investigations about the application of leaves from *C. longa*, as part of the plant is still considered a by-product of the turmeric harvest.

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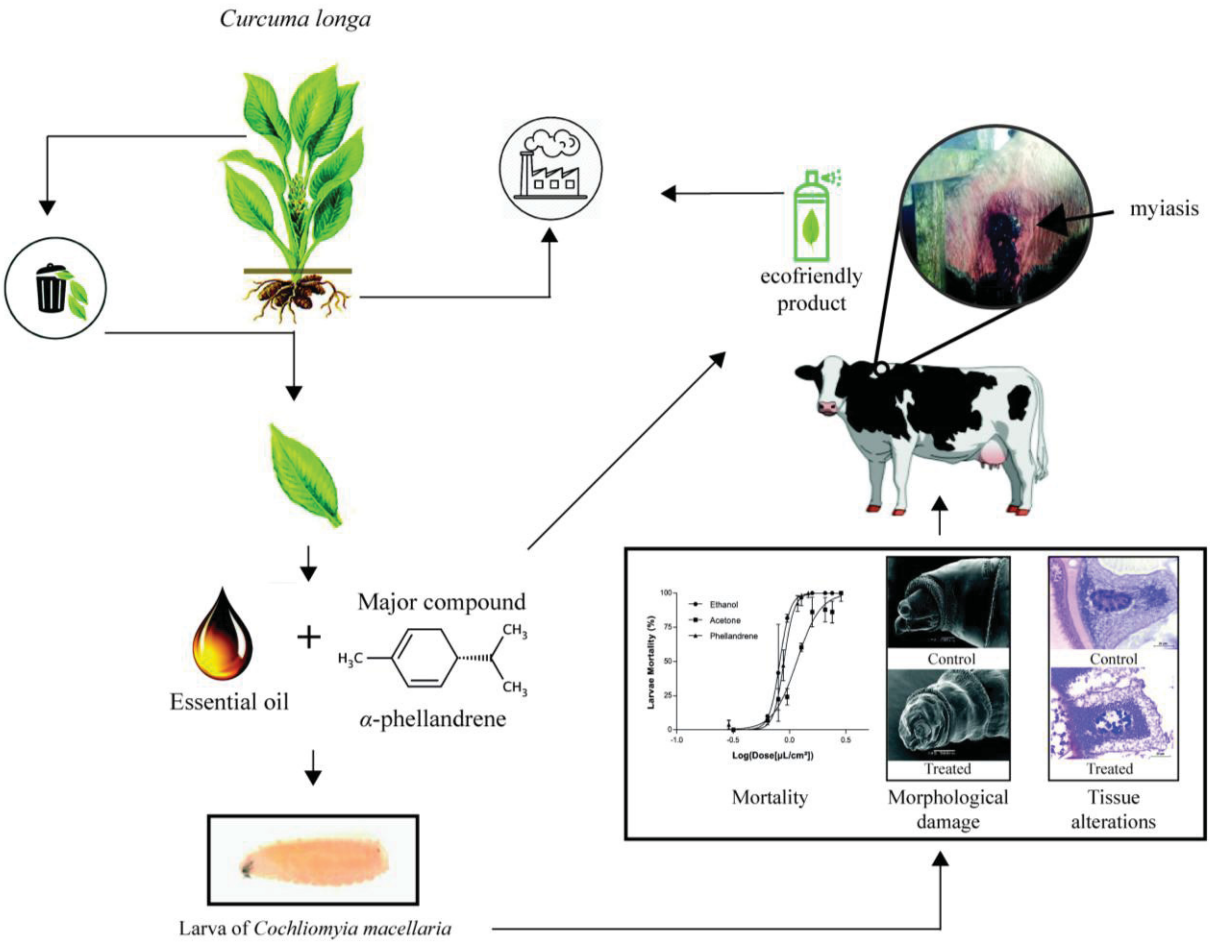
MANUSCRIPT 2 - ESSENTIAL OIL FROM *Curcuma longa* LEAVES: CAN AN OVERLOOKED BY-PRODUCT FROM TURMERIC INDUSTRY BE EFFECTIVE FOR MYIASIS CONTROL?

ABSTRACT

Curcuma longa L., popularly known as turmeric, is one of the most studied *Curcuma* species due to its rhizome, widely used as a dye, and its multipurpose uses in medicine, cosmetics, food flavoring and textile industries. However, the knowledge of essential oils (EO) from *C. longa* leaves is still scarce. In turmeric production, the stems and leaves above the ground are considered a waste product after harvesting. The present study was designed to assess the chemical composition of *C. longa* leaves EO (CLLEO) and investigate its bioactivity and of its major constituent *α*-phellandrene against third instar larvae (L3) of the fly *Cochliomyia macellaria*. In addition, we intended to demonstrate, through ultrastructural and histological assessment, the morphological damages of L3 *C. macellaria*. Groups of 20 L3 were placed on filter papers impregnated with increasing concentrations (0.31 to 2.86 µL/ cm^2) of CLLEO, quantifying L3 mortality after 6, 24 and 48 h of contact. The major compound of CLLEO, *α*-phellandrene, was used (0.29 to 1.47 µL/cm^2) with the same assay. *C. macellaria* L3 mortality 48 h after contact with 1.27 µL/cm^2 of CLLEO was 96.66% and 58.33% when using ethanol and acetone. The highest *α*-phellandrene dose assessed in this work (1.47 µL/cm^2) presented a similar rate of mortality as when using 1.27 µL/cm^2 of CLLEO solubilized in ethanol (96.66%). The dose of 0.79 µL/cm^2 of CLLEO solubilized in ethanol resulted in 11.11% of emerging adults with deformities, while a 10.00% alteration in this parameter was reported using acetone. The compound *α*-phellandrene represented 41.99% of the essential oil. Other compounds found were: *α*-pinene, *β*-*p*inene, myrcene, *ρ*-*c*ymene, limonene and 1,8-cineole. Structural analysis revealed lesion progression (cuticle surface dryness and severe degeneration on the spiracular plate) for both extracts. Histological sections of L3 showed marked necrosis of the intestinal tract and Malpighian tubules, fat body trophocytes with vacuolization and the brain had pyknotic profiles and vacuolar degeneration for both EO derivations. We confirm that CLLEO represents a sustainable bio-product to control livestock flies.

Keywords: *α*-phellandrene, *Cochliomyia macellaria*, Ecofriendly products, Sustainability, Bioinsecticide, Blowflies, Biomarkers.

GRAPHICAL ABSTRACT



MANUSCRITO 2 – ÓLEO ESSENCIAL DAS FOLHAS DE *Curcuma longa*: PODE UM SUBPRODUTO NEGLIGENCIADO NA PRODUÇÃO DO AÇAFRÃO SER EFICAZ PARA O CONTROLE DAS MIÍASES?

RESUMO

Curcuma longa L., popularmente conhecida como açafrão da terra, é uma das espécies do gênero *Curcuma* mais estudadas, devido ao seu rizoma ser amplamente utilizado como um corante, cosméticos, aromatizantes de alimentos e indústrias têxteis e seus usos polivalentes na medicina. Entretanto, pesquisas utilizando o óleo essencial (OE) extraído das folhas de *C. longa* ainda é escasso. Na produção do açafrão, os caules e folhas acima do solo são considerados um produto residual após a colheita. Neste sentido, o presente estudo foi desenvolvido para avaliar a composição química do OE das folhas de *C. longa* e investigar sua bioatividade e de seu principal constituinte α-felandreno contra larvas de terceiro ínstar (L3) da mosca *Cochliomyia macellaria*. Além disso, pretendemos demonstrar, através de avaliação ultraestrutural e histológica, os danos morfológicos de L3 *C. macellaria*. Assim, grupos de 20 L3 foram colocados em papel filtro impregnados com concentrações crescentes (0,31 a 2,86 μL/cm^2) do OE de *C. longa*, quantificando a mortalidade das L3 após 6, 24 e 48 h de contato. O constituinte majoritário, α-felandreno, foi utilizado em concentrações crescentes (0,29 a 1,47 μL/cm^2) com o mesmo ensaio. A mortalidade de *C. macellaria* L3 48 h após o contato com 1,27 μL/cm^2 do OE de *C. longa* foi de 96,66% e 58,33%, utilizando etanol e acetona, respectivamente. A maior dose de α-felandreno avaliada neste trabalho (1,47 μL/cm^2) apresentou uma taxa de mortalidade semelhante a dose de 1.27 μL/cm^2 de CLLEO solubilizado em etanol (96,66%). A dose de 0,79 μL/cm^2 do OE de *C. longa* solubilizado em etanol resultou em 11,11% de adultos emergentes com deformidades, enquanto uma alteração de 10,00% neste parâmetro foi relatada usando acetona como carreador. α-Felandreno representou 41,99% do óleo essencial. Outros compostos encontrados foram: α-pineno, β-pineno, mirceno, ρ-cimeno, limoneno e 1,8-cineol. A análise ultraestrutural revelou lesão progressiva (ressecamento da superfície da cutícula e degeneração severa na placa espiracular) utilizando ambos os extratos. Cortes histológicos de L3 mostraram acentuada necrose no trato intestinal e nos túbulos de Malpighi, corpo gordurosos com vacuolização e cérebro com perfil picnótico e degeneração vacuolar, utilizando

ambos os extratos. Confirmamos que o OE de *C. longa* representa um subproduto sustentável para controlar as moscas causadoras de miíases em animais de produção.

Palavras-chave: α-fellandrene, *Cochliomyia macellaria*, produtos ecológicos, Sustentabilidade, Bioinseticida, Moscas varejeiras, Biomarcadores.

1 INTRODUCTION

Curcuma longa L., popularly known as turmeric, is a perennial rhizomatous plant belonging to the Zingiberaceae family (GUPTA et al., 2015). Turmeric is one of the most investigated *Curcuma* species due to its rhizome, widely used as dye and with multipurpose uses in medicine, cosmetics and textile industries (SANDEEP et al., 2016; ZHANG et al., 2017). Turmeric is cultivated in many warm regions of the world, being mostly cultivated in India, which is the largest producer, consumer and exporter. In India, turmeric is grown in about 180,000 ha, producing about 25 million tons/year. The plant is exported to countries such as the USA, United Kingdom, Japan, Iran, United Arab Emirates, Saudi Arabia, the Netherlands, South Africa and Singapore due to commercialization of whole dry rhizome, turmeric powder, oleoresin, essential oil, curry powder and curcumin (MISHRA et al., 2015; ILANGOVAN et al., 2018). Currently, turmeric represents one of the most targeted plant species for pharmacological research due to the antioxidant properties of curcuminoids, including curcumin, which are abundant in the rhizomes (Leong-Škorničková et al., 2008). Despite their importance, curcuminoids are not the only compounds with pharmacological importance in *C. longa*, where at least 235 compounds have been identified. Oleoresin consists of a heavy fraction containing curcuminoids and a light fraction containing essential oils (EO) (ZHANG et al., 2017). Several studies were carried out recently describing the use of *C. longa* rhizomes EO compositions and related bioactivities. The antifungal activity on *Aspergillus flavus*, for example, was related to disruption of fungal plasma membrane caused by turmeric essential oil, which was also effective in down-regulating aflatoxin gene expression (HU et al., 2017). The activity against *Fusarium graminearum*, in turn, was attributed to reactive oxygen species induced apoptotic cell death (Kumar et al., 2016). Other properties described to *C. longa* rhizome EO include antioxidant, antimicrobial, anti-inflammatory (ZHANG et al., 2017), anti-angiogenic (YUE et al., 2015), anticonvulsant (ORELLANA-PAUCAR et al., 2012), acaricide (CHAGAS et al., 2016), insect repellant (DAS et al., 2015) and insecticide (TAVARES et al., 2013). The repellent effect over insects is strongly associated with the EO of the major compound or the rhizome, ar-turmerone (DAMALAS, 2011). In contrast to the abundance of recent studies with rhizomes, knowledge about EO from *C. longa* leaves are still scarce, and mainly regard biological activities. In the turmeric

production chain, the stems and leaves above the ground are considered a waste product (KIM et al., 2016), since compounds such as curcumin only form a minor part of the crop in terms of produced biomass. ILANGOVAN et al. (2018) have reported that it is highly convenient to use other parts of the plant (by-products), which can add value to the crop. BABU et al. (2007) reported a 0.28% EO yield in *C. longa* leaves when extracted by conventional distillation. The oil, predominantly composed by monoterpenes, has shown antifungal activity. Turmeric leaf EO was also capable of retarding oxidation reactions and free radical damage, showing a potent antioxidant effect (PRIYA et al., 2012). The insecticide activity was reported in both contact and fumigant assays for *C. longa* leaf oil, with myrcene (40.19%), p-cymene (23.05%) and 1,8-cineole (13.16%) as major compounds (TRIPATHI et al., 2002). In addition, several studies on *C. longa* leaf EO have shown the cyclic monoterpene *α*-phellandrene as the major compound (OGUNTIMEIN et al., 1990; MCCARRON et al., 1995; SHARMA et al., 1997; RAINA et al., 2002). This molecule is frequently indicated as a great candidate for use in insecticidal compositions (EVERGETIS et al., 2013; CHAABAN et al., 2017a). This background provides rationale for investigation of the effects of *C. longa* leaf EO on veterinary parasites for future development of natural pesticides. Traumatic cutaneous myiasis caused by *Cochliomyia macellaria*, known as secondary screwworm, is a parasitic infestation that involves the development of Diptera larvae in animal or human tissues. Flystrike may cause a reduction in animal development, create stress, decrease food intake, damage the skin and can significantly compromise the commercial value of the products (GUIMARÃES et al., 1983; WALL, 2012). Heavy larvae infestations may also cause animal deathes (KAUFMAN et al., 1989). Blowflies are conventionally controlled by using different insecticide classes, primarily pyrethroids and macrocyclic lactones. However, their misuse can have undesirable results, i.e. affect non-target organisms and select for resistant populations (POLYRAKIS 2009; ZINSSTAG et al., 2011). Thus, EO are an ecofriendly alternative based on exploiting the toxicity of aromatic hydrocarbons contained in the oils. Active substances present in EO have shown several biological activities and are able to inhibit growth, food intake and oviposition in numerous important pests with potential use for myiasis control (PAVELA and BENELLI, 2016; CHAABAN et al., 2017a). The present study was designed to assess the chemical composition of *C. longa* leaves essential oil (CLLEO), a by-product from turmeric production, and investigate it, as well as, its

major constituent *α*-phellandrene, against third instar larvae (L3) of *C. macellaria*. Additionally, we intended to demonstrate, through ultrastructural assessment, the morphological damages in L3 of *C. macellaria* (used as biomarkers for toxicity) after CLLEO and *α*-phellandrene treatments. Ultimately, this study aimed to highlight the potential use of *C. longa* leaves as a sustainable natural product based insecticide for myiasis control in veterinary science.

2 MATERIALS AND METHODS

2.1 Plant Material

The botanical species used in this work were grown in the Medical Plants Unit at the Catarinense Federal Institute (IFC), located at 26° 23' 33.6691" S and 48° 44' 18.3336" W at 10.6 m above sea level in the city of Araquari, Santa Catarina State, South of Brazil. Plants propagated from rhizome segments were cultivated in an agroecological system without synthetic chemical applications. Leaves were collected at 10 a.m. from about 100 individuals in September 2016 (Brazilian spring), ten months after planting of rhizome segments. A voucher specimen of the botanical material was deposited at the Herbarium of the Municipal Botanical Museum, located in the Botanical Garden of Curitiba, PR; with the number 358970.

2.2 Essential Oil (EO) Extraction and Chemical Characterization

Leaves of the plants of the same cultivar were homogenized and the EO was extracted from about 3 kg by hydrodistillation for 4 h in a Clevenger apparatus. The yield (%) of the essential oil was calculated by the average of three distillations of 100g/dry matter. The EO composition was analyzed by gas chromatography coupled with a mass spectrometric detector (GC/MS) using the same methodology described by CHAABAN et al. (2018). The injection temperature was 250°C and the carrier (helium gas) flow was 1.0 mL/min^{-1}. The chromatograph oven was optimized with an initial temperature of 60 to 240ºC with a rate of increase of 3°C/min. The oil sample was diluted at 1% in hexane, followed by injection into the GC/MS. Quantification was determined by normalizing the area (%) of each chemical constituent peak, the total area being the sum of all areas of the chromatogram peaks (100%) using the

chromatograph Agilent 7890A with a similar capillary column as described above. For the quantification of hydrogen gas, the material was used with a carrier at a flow of 1.5 mL/min^{-1}. Retention index were calculated by the method of Van den Doll and Kratz (1963) using n-alkane standard solutions (relative to C7–C30 n-alkanes), under the same chromatographic conditions. The identification of the compounds was made by comparison of their GC mass and retention data with the available library (WILEY, 1994, NIST, 2013). The EO was analyzed in triplicates.

2.3 Dilution of Extracts

α-Phellandrene (CAS: 99-83-2) was acquired commercially and certified as having purity of ≥ 99% (Sigma-Aldrich, Brazil). The EO and the purified *α*-phellandrene were diluted in absolute ethanol or acetone as these solvents were safe for L3 in the contact tests of *C. macellaria* (CHAABAN et al., 2017b, c). The following *α*-phellandrene concentrations were used: 0,29, 0.59, 0.88, 1.18, and 1.47 µL/cm^2, with each concentration representing one treatment, and were diluted in absolute ethanol. The following CLLEO concentrations were used: 0.31, 0.63, 0.79, 0.95, 1.27, 1.59, 2.07, 2.38 and 2.86 µL/cm^2, solubilized in ethanol or acetone. A control group was established, in which L3 were exposed only to the solvents, absolute ethanol or acetone.

2.4 Establishment of *Cochliomyia macellaria* Colonies

Wild flies were collected manually at the IFC, using bait and insect nets. The establishment of stock colonies, insect identification, maintenance, mass reproduction and the protocol for the biological tests were performed as described by CHAABAN et al. (2017b, c). For this work, fresh, drug-free bovine meat (approx. 2 g/larvae) was used for larval development. The mature L3 used in the assay left the substrate spontaneously.

2.5 Larval Toxicity

The evaluation of CLLEO and *α*-phellandrene against L3 of *C. macellaria* was performed as described by CHAABAN et al. (2018). Groups of 20 one-day old mature L3, from the second generation, were introduced into glass vials (9 × 4 cm diameter) containing a filter paper (12.56 cm^2) impregnated with 0.2 mL of *α*-phellandrene or CLLEO solutions. After the application of the products, the glass vials were closed with voile fabric to facilitate aeration, kept for 5 min in an exhaust hood and finally transferred to a climatic chamber at 27°C and 70% relative humidity. All treatments were performed in triplicate (n = 60) using a total of 1.560 larvae. Toxicity was evaluated by observing L3 mortality at 6, 24 and 48 h after contact. Total L3 mortality (*TLM)* was calculated (KUMAR et al., 2014; CHAABAN et al., 2017b) as follows:

TLM = (total dead larvae x 100) / total tested larvae

2.6 Analysis of the Physiological Parameters

After the contact of the L3 with CLLEO and *α*-phellandrene, they were kept under controlled conditions and the physiological parameters of pupation rate (*PR*), emergence inhibition rate (*EIR*) and adult deformity (*AD*) were recorded and calculated (KUMAR et al., 2014; SINGH and KAUR 2016; CHAABAN et al., 2017b) as follows:

PR = (total pupae x 100) / total tested larvae
EIR = (total control adults - total treated adults x 100) / total control adults
AD = (total deformed adults x 100) / total emerged adults

2.7 Larval Histopathology

For larval histopathology, L3 treated with 1.59 µL/cm^2 of CLLEO and 1.47 µL/ cm^2 of *α*-phellandrene, solubilized in ethanol, were fixed in 10% buffered formalin 6 and 24h after contact. Two longitudinal sections were embedded in paraffin and the L3 were serially sectioned (4 µm - thickness) and stained with hematoxylin-eosin.

2.8 Scanning Electron Microscopy

For scanning electron microscopy, L3 treated with 1.59 µL/cm^2 of CLLEO or 1.47 µL/cm^2 of *α*-phellandrene were fixed in AFA solution (ethyl alcohol at 70%, buffered formalin at 37% and glacial acetic acid – in the ratio of 2.5:1:1.5), 6h and 7 days after contact with solutions. Then, the samples were submitted to a dehydration process using five alcohol baths. The larvae were placed in support for electron microscopy (stub) and dehydrated in an oven at 37°C for 6 h using the protocol described by CANEPARO (2017) with modifications (CHAABAN et al., 2018). The specimens were examined and photographed with a scanning electron microscope at magnifications ranging from 12 to 600X (JEOL JSM 6360-LV) at the Center of Electron Microscopy of UFPR.

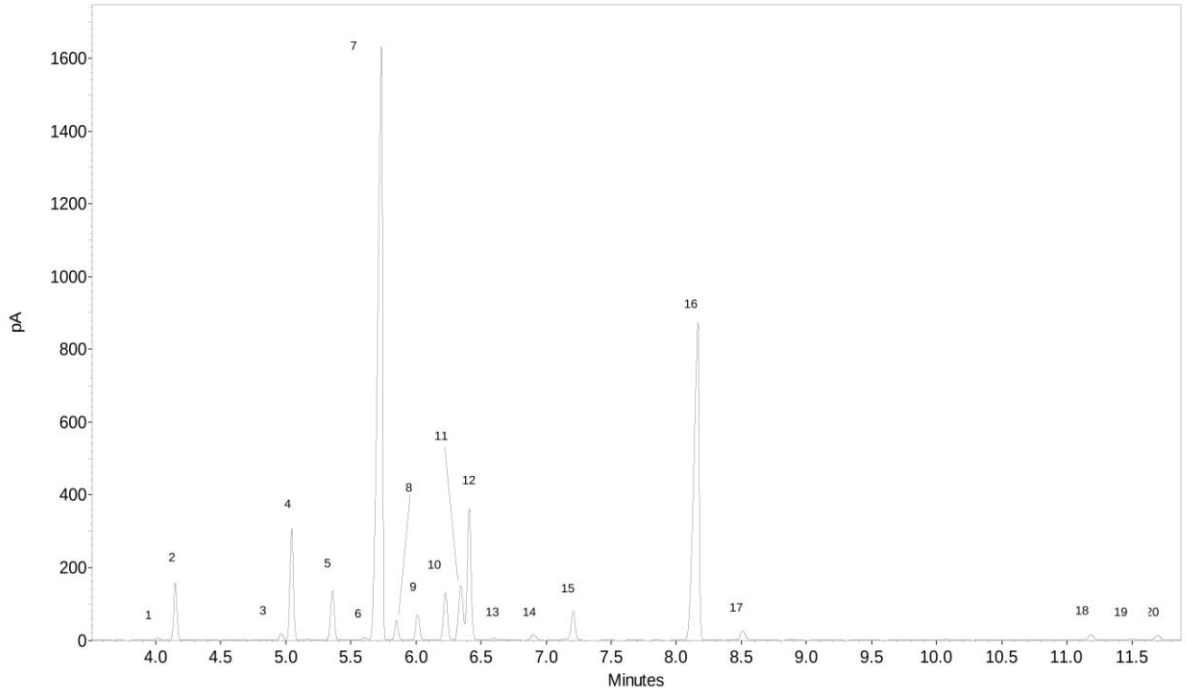
2.9 Statistical Analysis

Lethal concentrations (LC_{10}, LC_{50} and LC_{90}) were calculated using Probit analysis. L3 mortality, PR and EIR were analyzed for exposition time, concentrations, carriers and the interaction between concentrations and carriers through an analysis of variance (ANOVA) in generalized linear model, assuming a Poisson distribution. The averages were compared using the Tukey test. All analyses were performed using the statistical software SPSS 23.0 (2013), considering the significance level of 5%. The values was corrected using the Abbott's formula (ABBOTT, 1925).

3 RESULTS

3.1 Chemical Composition of *Curcuma Longa* Leaves Essential Oil

Eighteen compounds were identified from CLLEO (see in CHAABAN et al., 2018), comprising 98.65% of the chromatographic peaks (Figure 1). The vast majority of the compounds were monoterpene hydrocarbons (97.17%). Only three compounds, linalool, terpinen-4-ol and *α*-terpineol, that together represented 1.48% of the EO, were classified as oxygenated monoterpenes. α-Phellandrene (41.99%), ρ-mentha-2,4(8)-diene (24.89%) and 1,8-cineole were the major constituents of CLLEO. The yield of CLLEO was 0.85%.



SOURCE: the author

FIGURE 1 – CG/MS CHROMATOGRAM OF *Curcuma longa* LEAVES ESSENTIAL OIL: α-tujene **1**, α-pinene **2**, sabinene **3**, β-pinene **4**, mircene **5**, δ-2-carene **6**, α-phellandrene **7**, δ-3-carene **8**, α-terpinene **9**, ρ-cimene **10**, limonene **11**, 1.8-cineole **12**, ni **13**, (E)-β-ocimene **14**, γ-terpinene **15**, ρ-mentha-2,4(8)-diene **16**, linalol **17**, terpinen-4-ol **18**, ni **19**, α-terpineol **20**.

3.2 Larval Toxicity and Physiological Parameter Changes

Cochliomyia macellaria L3 emergence inhibition rates were 98.30% and 83.92% after contact with 0.79 μL/cm^2 (5%) of CLLEO when solubilized in acetone and ethanol, respectively. The dose of 0.88 μL/cm^2 of α-phellandrene presented a 92.85% reduction in adult emergence. The values of *C. macellaria* L3 mortality 48 h after contact with 1.27 μL/cm^2 of CLLEO (8%) were 96.66% and 58.33% for ethanol and acetone, respectively. The highest *α*-phellandrene dose assessed in this work (1.47μL/cm^2) presented the same value of mortality when using ethanol as a carrier (96.66%) (Table 1, Table 2). Regarding adult deformity, the dose of 0.79 μL/cm^2 of CLLEO solubilized in ethanol showed 11.11% of emergency of adults with deformities, while 10.00% alterations (same parameter) were observed using acetone (Table 1, Table 2). Moreover, incomplete emergency, adults with small size, deformity of wings, legs and abdomen, as well as progressive dryness and cuticle darkening in L3 were observed 7 days after treatment (Figure 2). No deformity of *C. macellaria* adults was observed using *α*-phellandrene. Lethal concentrations of CLLEO and α-phellandrene are shown in Table 3. Dose- and time-dependent activities were demonstrated 6 h after exposure to CLLEO and *α*-phellandrene, with LC_{50}s of 0.84 and 0.91 μL/cm^2, respectively. The LC_{50} varied for the different carriers (Figure 3). After 6 h of exposure, we observed the highest values of LC_{10}, LC_{50} and LC_{90} (0.67, 0.91 and 1.24 μL/cm^2) for *α*-phellandrene (Table 3). Likewise, CLLEO showed LC_{10}, LC_{50} and LC_{90} of 0.70, 1.37 and 2.70 μL/cm^2 for acetone, while ethanol had the lowest values for LC_{10}, LC_{50} and LC_{90} of 0.66, 0.84 and 1.08 μL/cm^2, respectively (Table 3).

TABLE 1 - LARVAL MORTALITY (LM), PUPARIATION RATE (PR), EMERGENCE INNIBITION RATE (EIR), SEX RATIO (MALE:FEMALE) AND ADULT DEFORMITY OF *Cochliomyia macellaria* TREATED WITH *Curcuma longa* LEAVES ESSENTIAL OIL.

C(µL/cm^2)	*LM (%)	PR (%)	EIR (%)	SR (M:F)	AD (%)
Ethanol	0.0 (±0.0) cA	100.0 (±0.0) aA	0.0 (±0.0) c	30:26	0.0
0.31(2%)	0.0 (±0.0) cA	100.0 (±0.0) aA	0.0 (±0.0) c	33:23	0.0
0.63 (4%)	10.0 (±0.0) bcA	90.0 (±1.6) aA	51.78 (±4.1) b	11:16	0.0
0.79 (5%)	41.66 (±20.5) bA	58.33 (±20.5) bA	83.92 (±12.1) a	7:2	11.11
0.95 (6%)	81.66 (±1.6) aA	18.33 (±1.6) cB	100.0 (±6.7) a	2:1	0.0
1.27 (8%)	96.66 (±3.3) aA	3.33 (±3.3) cB	100.0 (±1.8) a	0:0	0.0
1.59 (10%)	100.0 (±0.0) aA	0.0 (±0.0) cA	100.0 (±1.8) a	0:0	0.0
2.07 (13%)	100.0 (±0.0) aA	0.0 (±0.0) cA	100.0 (±1.8) a	0:0	0.0
2.38 (15%)	100.0 (±0.0) aA	0.0 (±0.0) cA	100.0 (±1.8) a	0:0	0.0
2.86 (18%)	100.0 (±0.0) aA	0.0 (±0.0) cA	100.0 (±1.8) a	0:0	0.0
Acetone	0.0 (±0.0) dA	100.0 (±0.0) aA	0.0 (±0.0) c	37:22	5.08
0.31(2%)	0.0 (±0.0) dA	100.0 (±0.0) aA	1.69 (±1.6) c	26:32	5.17
0.63 (4%)	6.66 (±1.6) cdA	93.33 (±1.6) abA	66.10 (±15.7) b	9:11	10.0
0.79 (5%)	21.66 (±4.4) cB	78.33 (±4.4) bA	98.30 (±1.6) a	1:0	0.0
0.95 (6%)	23.33 (±3.3) cB	76.66 (±3.3) bA	93.22 (±4.4) ab	2:2	0.0
1.27 (8%)	58.33 (±1.6) bB	41.66 (±1.6) cA	100.0 (±0.0) a	0:0	0.0
1.59 (10%)	83.33 (±6.0) aB	16.66 (±6.0) dA	100.0 (±0.0) a	0:0	0.0
2.07 (13%)	85.0 (±5.0) aA	15.0 (±5.0) dA	100.0 (±0.0) a	0:0	0.0
2.38 (15%)	83.33 (±4.4) aB	16.66 (±4.4) dA	100.0 (±0.0) a	0:0	0.0
2.86 (18%)	96.66 (±3.3) aA	3.33 (±3.3) dA	100.0 (±0.0) a	0:0	0.0

*48 of exposure.
Absolute ethanol and acetone were used with control.
The letters display a significant difference (P <0.05). Capital letter in concentrations between carriers and lowercase letters differences in concentrations within carriers.

SOURCE: the author

TABLE 2 - LARVAL MORTALITY (LM), PUPARIATION RATE (PR), EMERGENCE INNIBITION RATE (EIR), SEX RATIO (MALE:FEMALE) AND ADULT DEFORMITY OF *Cochliomyia macellaria* TREATED WITH *α*-phellandrene.

C(µL/cm^2)	*LM (%)	PR (%)	EIR (%)	SR (M:F)	AD (%)
Control	0.0 (±0.0) c	100.0 (±0.0) a	0.0 (±0.0) b	26:30	0.0
0.29	11.66 (±1.67) c	88.34 (±1.67) a	5.35 (±6.08) b	35:18	0.0
0.59	8.33 (±3.33) c	91.67 (±5.0) a	12.5 (±1.66) b	20:29	0.0
0.88	50.0 (±5.77) b	50.0 (±5.77) b	92.85 (±1.95) a	3:1	0.0
1.18	90.0 (±2.89) a	10.0 (±2.87) c	100.0 (±0.0) a	0:0	0.0
1.47	96.66 (±1.67) a	3.34 (±1.67) c	100.0 (±0.0) a	0:0	0.0

*48h of exposure
Absolute ethanol was used with α-phellandrene control.
The letters display a significant difference (P<0.05) in the concentrations of the essential oils.

SOURCE: the author

TABLE 3. LETHAL CONCENTRATION (µL/cm^2) OF *Curcuma longa* LEAVES ESSENTIAL OIL AND IT IS MAJOR COMPOUND *α*-phellandrene ON *Cochliomya macellaria* LARVAE IN THE CONTACT ASSAY OVER TIME.

Extract	Evaluation time	*LC_{10} (LCI–UCI)	LC_{50} (LCI–UCI)	LC_{90} (LCI–UCI)	Chi-square ($\chi 2$)	Probability
CLLEO/ET	6h	0.66 (0.56-0.72)	0.84 (0.78-0.89)	1.08 (1.04-1.15)	6.73	0.35
	24h	0.66 (0.56-0.72)	0.84 (0.78-0.89)	1.08 (1.04-1.15)	6.73	0.35
	48h	0.63 (0.55-0.69)	0.82 (0.77-0.87)	1.07 (1.02-1.14)	3.34	0.77
CLLEO/AC	6h	0.75 (0,64-0,84)	1.28 (1.18-1.39)	2.18 (1.94-2.57)	11,65	0.07
	24h	0.75 (0.5-0.93)	1.22 (1.01-1.38)	2.02 (1.8-2.38)	10.91	0.05
	48h	0.68 (0.58-0.78)	1.18 (1.09-1.28)	2.28 (1.97-2.82)	11.45	0.07
α-phellandrene	6h	0.67 (0.61-0.73)	0.91 (0.87-0.96)	1.24 (1.18-1.33)	2.98	0.22
	24h	0.67 (0.61-0.73)	0.91 (0.87-0.96)	1.24 (1.18-1.33)	2.98	0.22
	48h	0.68 (0.61-0.74)	0.91 (0.86-0.96)	1.23 (1.16-1.31)	3.77	0.15

CLLEO/ET: *Curcuma longa* Leaves essential oil solubilized in ethanol; CLLEO/AC: *Curcuma longa* Leaves essential oil solubilized in acetone. *α*-phellandrene was solubilized only at etanol.
*The lethal concentrations were calculated by the Probit analysis. LCI, lower limit of 95% confidence interval; UCI, upper limit of 95% confidence interval.

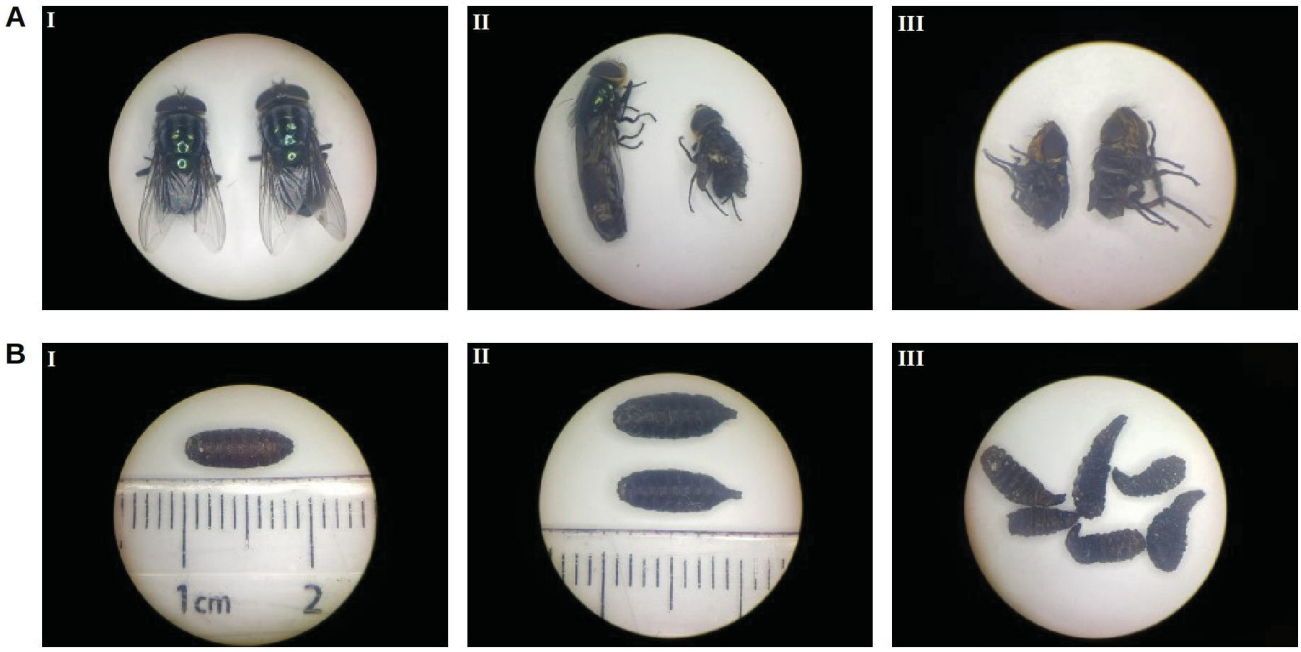


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FIGURE 2 – MALFORMATIONS OF *Cochliomyia macellaria* AFTER TREATMENT WITH *Curcuma longa* LEAVES ESSENTIAL OIL (CLLEO) AND ITS MAJOR COMPOUND *α*-phellandrene. AI) NORMAL ADULT. AII) INCOMPLETE EMERGENCY (LEFT). NOTE ADULT DEFORMITY WITH WINGS AND SMALL SIZE (RIGHT). AIII) ADULTS OF *C. macellaria* DEFORMED. OBSERVE THE SMALL SIZE AND DEFORMITY OF WINGS, LEGS AND ABDOMEN. BI) NORMAL PUPAE. BII) PUPAE OF *C. macellaria* TREATED WITH CLLEO (0.95 µL/cm^2). DETAIL FOR THE LARVIFORM ASPECT, CHARACTERISTIC OF INCOMPLETE MOLTING. BIII) LARVAE *C. macellaria* 7 DAYS AFTER TREATMENT WITH *α*-phellandrene (1.18 µL/cm^2). NOTE THE PROGRESSIVE DRYNESS AND CUTICLE DARKENING.

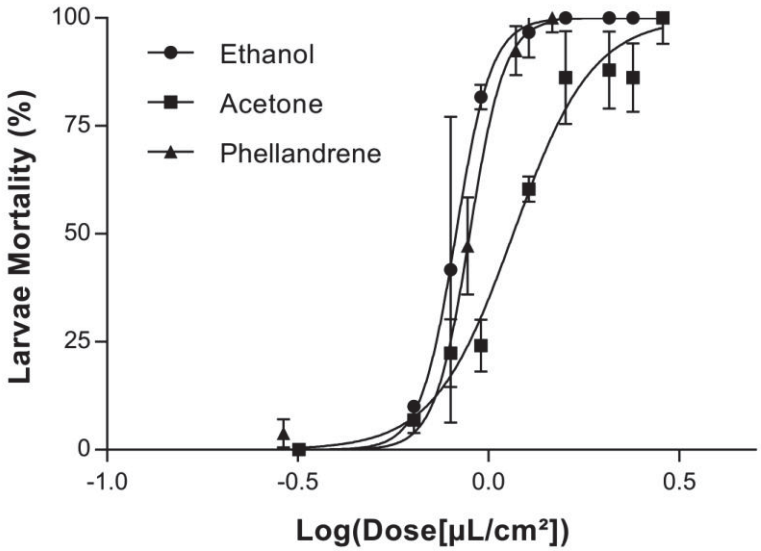

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FIGURE 3 - LARVAE TOXICITY (µL/cm^2) OF *Cochliomyia macellaria* AFTER EXPOSURE TO *Curcuma longa* LEAVES ESSENTIAL OIL USING DIFFERENT CARRIERS (ETHANOL AND ACETONE) AND ITS MAJOR COMPOUND *α*-phellandrene USING ETHANOL WITH SOLVENT.

3.3 Morphological Damage - Biomarkers of Toxicity in *C. macellaria*

3.3.1 Macroscopic Cuticle Damage

Larvae of *C. macellaria* from the control group showed cuticles without any macroscopic damage or color change (Figure 4a, 4d, 4g). Motility decreased 6 h after exposure and cuticle damage with diffuse darkening on the *C. macellaria* body was observed 48 h after exposure using 1.27 μL/cm^2 of CLLEO or 1.18μL/cm^2 of *α*-phellandrene. In addition, an accentuated change in color (darkening) throughout the body (1st to 6th segment) and diffuse dark pigment was observed using higher concentrations of CLLEO and *α*-phellandrene (Figure 4b, 4c, 4e, 4f, 4h).

3.3.2 Scanning Electron Microscopy

The scanning electron microscope of the control group showed typical Calliphoridae morphology. Larval body was composed of 12 segments (one cephalic, three thoracic, seven abdominal and one anal) with no alterations of intersegmental spines around each segment (cephalic segment to body medium). The cephalic segment contained antenna sensory papillae, maxillary lobe, oral ridges, labial lobe and buccal hook, and all remained visibly preserved (Figure 5AI). The anterior spiracle was also undamaged (Figure 5AII). The posterior end was preserved, showing the anal segment with spiracular plate containing posterior spiracle, marked ventral spicules, anal papillae and the ventral and dorsal papillae (Figure 5AIII). The structure of L3 of *C. macellaria* 6 h after CLLEO showed dryness on the cuticular surface with evidence of intersegmental spines (Figure 5BII). Likewise, we observed distortion of the sensorial structures such as antenna sensory papillae and maxillary lobe, marked dorsal spicules and anterior spiracle suggesting cuticle drying (Figure 5BI). The CLLEO treatment caused a slight distortion of ventral papillae, cuticle dryness and distortion of dorsal papillae to the posterior end (Figure 5BIII). Similarly, *α*-phellandrene showed distortion of L3 body and structures (Figure 5C), such as marked dryness of the cuticle surface, distortion of antenna and slight distortion of ventral papillae (Figure 5CI). Finally, lesion progression (cuticle surface dryness, severe degeneration of the spiracular plate, dorsal papillae and posterior spiracle) were observed 4 days after treatment for both extracts (Figure 6).

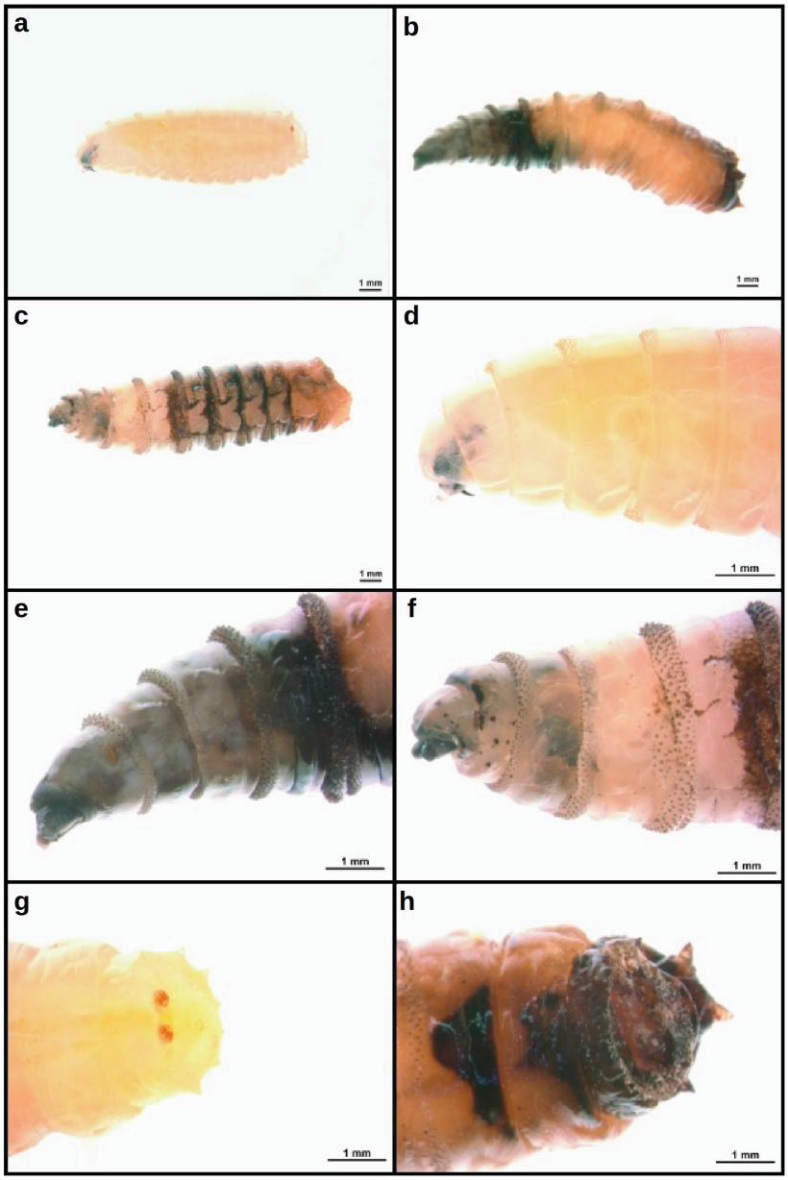

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FIGURE 4 – MACROSCOPIC CUTICULAR DAMAGE OF *Cochliomyia macellaria* L3 AFTER TREATMENT WITH *Curcuma longa* LEAVES ESSENTIAL OIL (CLLEO) AND ITS MAJOR COMPOUND *α*-phellandrene. A) NORMAL L3 6H AFTER TREATMENT (CONTROL GROUP): NOTE THE NORMAL SIZE AND NO CHANGE IN CUTICLE COLOR (8X). B) L3 WITH CUTICLE DAMAGE 6H AFTER TREATMENT WITH 1.59 µL/cm^2 OF CLLEO: OBSERVE THE CHANGE IN COLOR (DARKENING) THROUGHOUT THE BODY, ACCENTUATED AT 1ST TO 6TH SEGMENT AND POSTERIOR END (8X). C) L3 WITH CUTICLE DAMAGE 6H AFTER TREATMENT WITH 1.47 µL/ cm^2 OF *α*-phellandrene: NOTE THE DIFFUSE PIGMENT ON THE BODY (8X). D) NORMAL L3 6H AFTER TREATMENT (CONTROL GROUP): DETAILS FOR NORMAL SIZE AND ABSENCE OF PIGMENT (22X). E) L3 WITH CUTICLE DAMAGE 6H AFTER TREATMENT WITH 1.59 µL/cm^2 OF CLLEO (22X). F) L3 WITH CUTICLE DAMAGE 6H AFTER TREATMENT WITH 1.47 ML/CM^2 OF *α*-phellandrene (22X). G) POSTERIOR END OF *C. macellaria* L3 (CONTROL GROUP): OBSERVE THE NORMAL COLOR OF BODY AND SPIRACLES (22X). H) POSTERIOR END OF *C. macellaria* 6H AFTER TREATMENT WITH 1.59 µL/cm^2 OF CLLEO: NOTE THE DIFUSE DARK PIGMENT (22X).

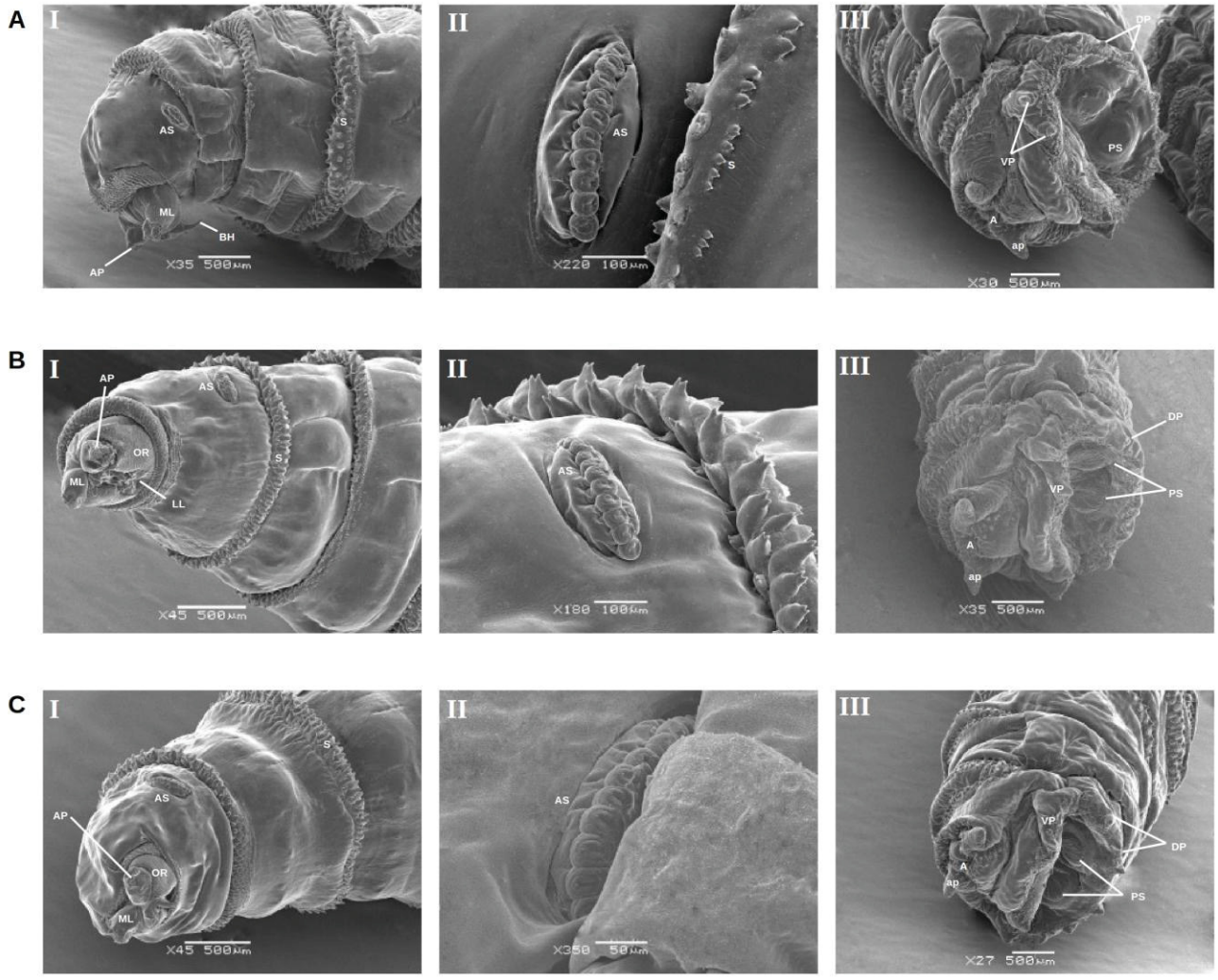

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FIGURE 5 – SCANNING ELECTRON PHOTOMICROGRAPHS OF *Cochliomyia macellaria* L3. A) CONTROL GROUP (ONLY ETHANOL). I) ANTERIOR END OF LARVA WITH NORMAL BODY, DETAILS OF ANTERIOR SPIRACLES, SPINULES, BUCAL HOOK (BH) AND ANTENNA SENSORY PAPILLAE (AP) PRESERVED. II) DETAILS OF ANTERIOR SPIRACLE WITH 10-11 LOBES AND LATERAL SPINULES (SIDE VIEW). III) POSTERIOR END OF LARVA. DETAILS TO ANAL SEGMENT WITH SPIRACULAR PLATE CONTAINING POSTERIOR SPIRACLE, DORSAL, VENTRAL AND ANAL PAPILLAE. B) *C. macellaria* L3, 6 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF CLLEO. I) ANTERIOR END OF LARVA. NOTE THE DISTORTION OF THE SENSORIAL STRUCTURES (ANTENNA SENSORY PAPILLAE), LABIAL LOBE AND OF THE CUTICULAR SURFACE DRYNESS WITH EVIDENCE OF SPINULES. II) DORSAL SPINULES AND ANTERIOR SPIRACLE EVIDENCED SUGGESTING CUTICLE DRYING. III) POSTERIOR END OF LARVA. NOTE THE SLIGHT DISTORTION OF VENTRAL AND DORSAL PAPILLAE AND CUTICLE DRYENESS. C) *C. macellaria* L3 6 H AFTER TREATMENT WITH 1.47 µL/cm^2 OF *α*-phellandrene. I) ANTERIOR END OF LARVA. OBSERVE THE MARKED DRYNESS IN CEPHALIC SEGMENT, DISTORTION OF THE ANTENNA SENSORY PAPILLAE AND SPINULES EVIDENCED. II) DETAILS OF ANTERIOR SPIRACLE WITH SLIGHT DEGENERATION. III) POSTERIOR END OF LARVA. OBSERVE THE SLIGHT DISTORTION OF VENTRAL AND DORSAL PAPILLAE AND CUTICLE DRYENESS. ABBREVIATIONS: S, SPINULES; AP, ANTENNA SENSORY PAPILLAE; ML, MAXILLARY LOBE; AS, ANTERIOR SPIRACLE; OR, ORAL RIDGES; LL, LABIAL LOBE; BH, BUCAL HOOK; A, ANUS; DP, DORSAL PAPILLAE; VP, VENTRAL PAPILLAE PS, POSTERIOR SPIRACLE; ap, ANAL PAPILLAE.

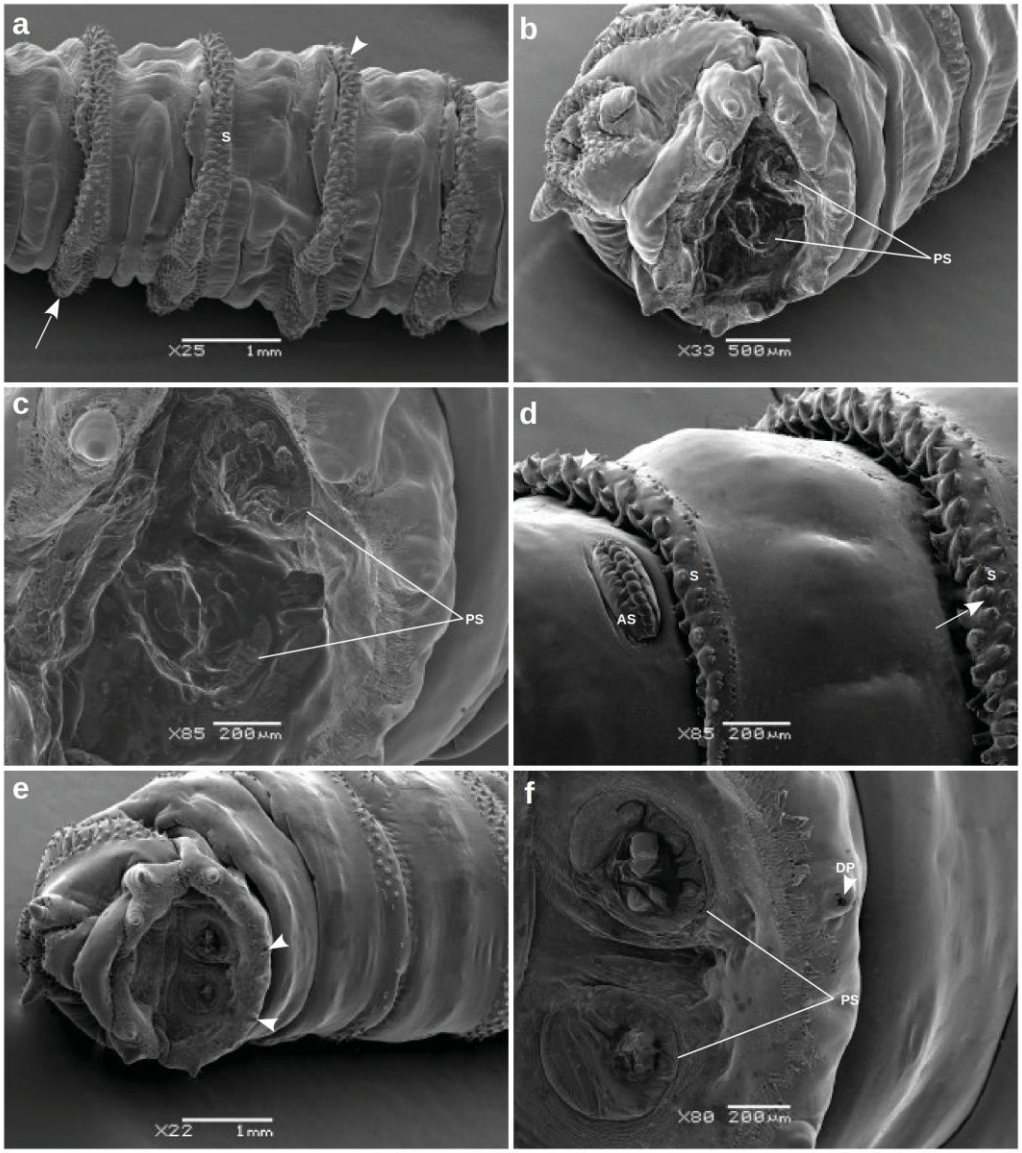

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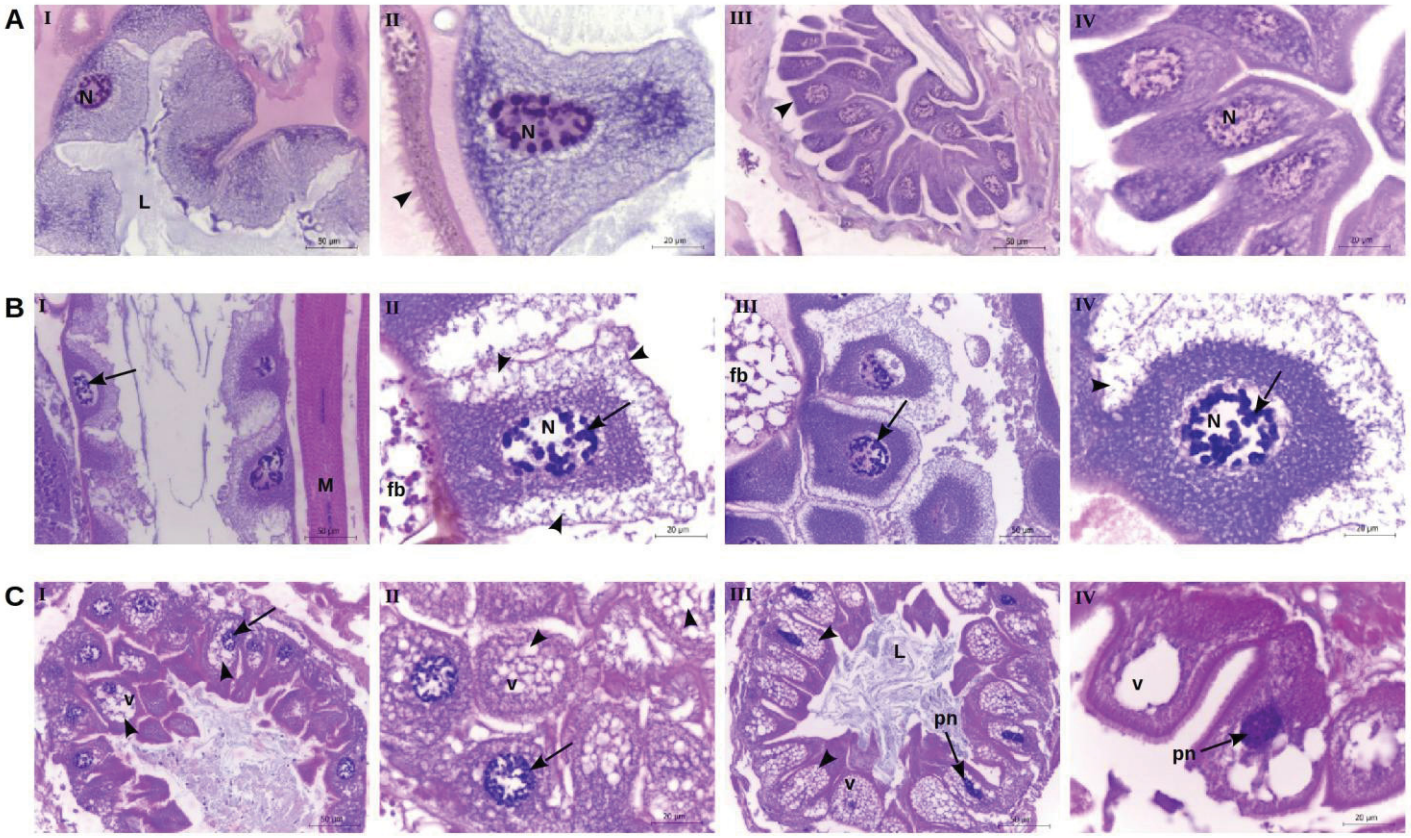
FIGURE 6 – SCANNING ELECTRON PHOTOMICROGRAPHS OF *Cochliomyia macellaria* L3 4 DAYS AFTER TREATMENT WITH CLLEO (1.59 µL/cm^2) AND ITS MAJOR COMPOUND *α*-phellandrene (1.47 µL/cm^2). A) MEDIUM BODY OF *C. macellaria* L3 TREATED WITH CLLEO. OBSERVE THE CUTICULAR SURFACE DRYNESS WITH VENTRAL SPINULES (ARROW) AND DORSAL SPINULES (ARROWHEAD) PROTRUDING. B) POSTERIOR END OF *C. macellaria* L3 TREATED WITH CLLEO. NOTE THE EXTREME SEVERE DEGENERATION ON THE SPIRACULAR PLATE AND POSTERIOR SPIRACLE (PS). C) ANAL SEGMENT OF *C. macellaria* L3 TREATED WITH CLLEO. DETAIL TO POSTERIOR SPIRACLE WITH SEVERE DEGENERATION. D) *C. macellaria* L3 AFTER TREATMENT WITH *α*-phellandrene. DETAILS OF PRONOUNCED DORSAL SPINULES (ARROWHEAD) AND LATERAL SPINULES (ARROW). E) POSTERIOR END OF *C. macellaria* L3 TREATED WITH *α*-phellandrene. DETAILS OF ANAL SEGMENT WITH DISTORTION OF SPIRACLE PLATE AND DORSAL PAPILE. F) SPIRACLE PLATE OF *C. macellaria* L3 AFTER TREATMENT WITH *α*-phellandrene. NOTE THE SEVERE DEGENERATION OF POSTERIOR SPIRACLE AND DORSAL PAPILE. H & E, HEMATOXYLIN–EOSIN. ABBREVIATIONS: S, SPINULES; PS, POSTERIOR SPIRACLES, AS, ANTERIOR SPIRACLES; DP, DORSAL PAPILLAE.

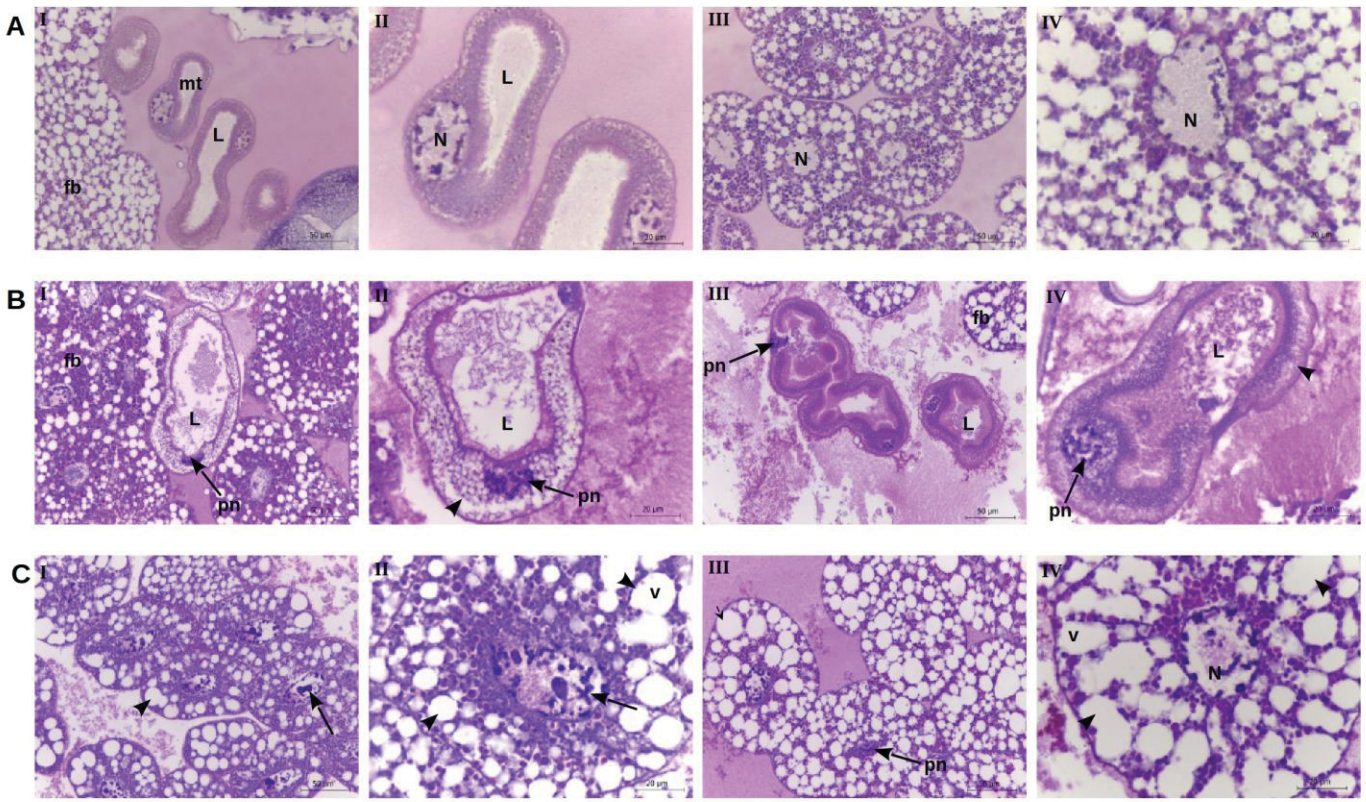
3.3.3 Larval Histopathology

Histological sections of *C. macellaria* L3 showed several alterations 6 h after CLLEO and *α*-phellandrene treatment in distinct target cells. Intense secretion in the lumen of the anterior digestive tract was observed after contact with CLLEO (Figure 7BI; BII), while condensation of nuclear chromatin was reported for *α*-phellandrene (Figure 7BIII; BIV). The posterior digestive tract of *C. macellaria* L3 showed marked necrosis of the intestinal tract, characterized by intense cytoplasmic vacuolization, condensation of nuclear chromatin and pyknotic nuclei (Figure 7C). Other important target structures as biomarkers are the Malpighian tubules (MTs), fat body and brain. Malpighian tubules as intense degeneration with presence of a part of cell disposed in the lumen, intense vacuolization, condensation of nuclear chromatin and pyknotic nuclei were noticed (Figure 8B). In the same way, trophocytes with vacuolization, presence of vesicles, condensation of nuclear chromatin and pyknotic nuclei were seen on photomicrographs of L3 fat body after CLLEO or *α*-phellandrene treatments (Figure 8C). Finally, histological sections of *C. macellaria* L3 brain showed relevant alteration such as pyknotic profiles, vacuolar degeneration and cytoplasmic granulation (Figure 9).



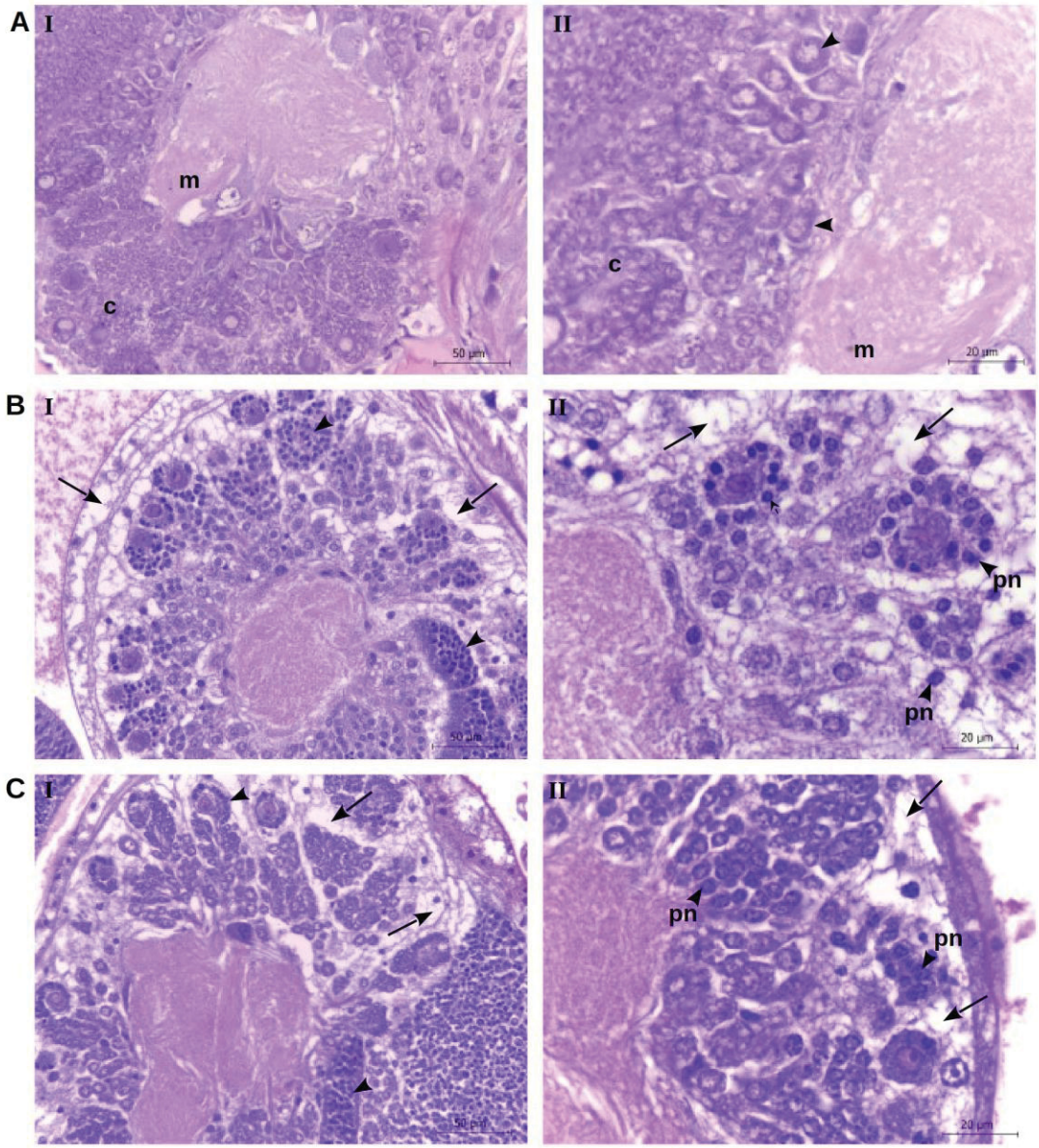
SOURCE: the author

FIGURE 7 - PHOTOMICROGRAPHS OF ANTERIOR AND POSTERIOR DIGESTIVE TRACT OF *Cochliomyia macellaria* L3. A) CONTROL GROUPS WITH INTACT ANTERIOR (I, II) AND POSTERIOR (III, IV) DIGESTIVE TRACT (EPITHELIAL CELLS WITH NUCLEUS AND BRUSH BORDER). NOTE THE NORMAL MORPHOLOGY OF CELLS (ARROWHEAD) (40, 100X). B) ANTERIOR DIGESTIVE TRACT OF *C. macellaria* L3 6 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF CLLEO (I, II) OR 1.47 µL/cm^2 (III, IV) OF *α*-phellandrene (40, 100X). I, II) NOTE THE INTENSE SECRETION IN THE LUMEN OF ANTERIOR DIGESTIVE TRACT (ARROWHEAD) AND CONDENSATION OF NUCLEAR CHROMATIN (HYPERCHROMATOSIS) (ARROW). III, IV) OBSERVE THE SECRETION IN LUMEN OF ANTERIOR DIGESTIVE TRACT AND CONDENSATION OF NUCLEAR CHROMATIN (HYPERCHROMATOSIS) (ARROW) AND SECRETION IN THE LUMEN OF ANTERIOR DIGESTIVE TRACT (ARROWHEAD). C) POSTERIOR DIGESTIVE TRACT OF *C. macellaria* L3 6H AFTER TREATMENT WITH 1.59 µL/cm^2 OF CLLEO (I, II) OR 1.47 µL/cm^2 (III, IV) OF *α*-phellandrene (40, 100X). I, II) NOTE THE INTENSE VACUOLIZATION (ARROWHEAD) AND CONDENSATION OF NUCLEAR CHROMATIN (HYPERCHROMATOSIS) (ARROW). III, IV) OBSERVE THE SECRETION IN THE LUMEN OF DIGESTIVE TRACT, MARKED VACUOLIZATION (ARROWHEAD) AND PYKNOTIC NUCLEI (ARROW). H & E, HEMATOXYLIN–EOSIN. ABBREVIATIONS: N, NUCLEI; pn, PYKNOTIC NUCLEI; L, LUMEN; v, VACUOLE; fb, FAT BODY; M, MUSCLE.



SOURCE: the author

FIGURE 8 – PHOTOMICROGRAPHS OF MALPIGHIAN TUBULES AND FAT BODY OF *Cochliomyia macellaria* L3. A) NORMAL CONTROL GROUPS 6H AFTER TREATMENT (ONLY ETHANOL) (40, 100X). I, II) OBSERVE THE MTs SHOWING TYPICAL MORPHOLOGY. III, IV) NOTE THE NORMAL FAT BODY TROPHOCYTES. B) MTs OF *C. macellaria* L3 6H AFTER TREATMENT WITH 1.59 µL/cm^2 OF CLLEO (I, II) OR 1.47 µL/cm^2 OF *α*-phellandrene (III, IV) (40, 100X). I, II) OBSERVE THE INTENSE DEGENERATION OF MTs WITH PRESENCE OF A PART OF CELL DISPOSED IN THE LUMEN, INTENSE VACUOLIZATION (ARROWHEAD) AND CONDENSATION OF NUCLEAR CHROMATIN (PYKNOTIC NUCLEI) (ARROW). III, IV) NOTE THE PYKNOTIC NUCLEI (ARROW), VACUOLIZATION (ARROWHEADS) AND LUMEN CONTENT PART OF CELL. C) FB OF *C. macellaria* L3 6H AFTER TREATMENT WITH 1.59 µL/cm^2 OF CLLEO (I, II) OR 1.47 µL/cm^2 OF *α*-phellandrene (III, IV) (40, 100X). I, II) DETAILS TO THE FB TROPHOCYTES WITH CONDENSATION OF NUCLEAR CHROMATIN (ARROW) AND VACUOLIZATION (ARROWHEAD). III, IV) NOTE THE PYKNOTIC NUCLEI (ARROW) AND MARKED VACUOLATION IN THE CELL (ARROWHEAD). H & E, HEMATOXYLIN–EOSIN. ABBREVIATIONS: N, NUCLEI; L, LUMEN; fb, FAT BODY TROPHOCYTES; pn, PYKNOTIC NUCLEI; mt, MALPIGHIAN TUBULES; v, VACUOLE.



SOURCE: the author

FIGURE 9 – PHOTOMICROGRAPHS OF BRAIN OF *C. macellaria* L3. A) NORMAL CONTROL GROUPS (ONLY ETHANOL) 6H AFTER TREATMENT (40, 100X). I, II) OBSERVE THE NORMAL NEUROPIL (M) AND CORTICAL LAYER (C) WITH PRESENCE OF GLIAL CELLS (ARROWHEAD). B) BRAIN OF *C. macellaria* L3 6 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF CLLEO (40, 100X). I) OBSERVE THE MARKED VACUOLAR DEGENERATION (ARROW) AND CYTOPLASMIC GRANULATION (ARROWHEAD). II) DETAILS TO VACUOLIZATION (ARROW) AND NUCLEI WITH PYKNOTIC PROFILE (ARROWHEAD). C) BRAIN OF *C. macellaria* L3 6H AFTER TREATMENT WITH 1.47 µL/cm^2 OF *α*-phellandrene (40, 100X). I) NOTE THE EVIDENCED VACUOLIZATION (ARROW), CYTOPLASMIC GRANULATION (ARROWHEAD). II) DETAILS TO INTENSE VACUOLIZATION IN CORTICAL LAYER (ARROW) AND NUCLEI WITH PYKNOTIC PROFILE (ARROWHEAD). H & E, HEMATOXYLIN–EOSIN. ABBREVIATIONS: m, MEDULAR LAYER; c, CORTICAL LAYER.

4 DISCUSSION

4.1 Chemical Composition of *Curcuma Longa* Leaves Essential Oil

In contrast to that observed in rhizomes, where the sesquiterpene ar-turmerone is frequently reported as the major compound (FERREIRA et al., 2013; TAVARES et al., 2013; SANDEEP et al., 2016), in the EO from *C. longa* leaves neither ar-turmerone nor other sesquiterpenes were identified. These results are in agreement with OGUNTIMEIN et al. (1990), who stated that EO from turmeric leaves are mainly composed of monoterpenes. MCCARRON et al. (1995) compared the yield and composition of *C. longa* EO from leaves and rhizomes. The authors reported yields of 0.41% (w/w) in leaves and 0.29% in rhizomes, containing 92.9% and 16.3% monoterpenes, respectively. In the monoterpene fraction of both EO, *α*-phellandrene was the major compound. Similarly, evaluating nine agroclimatic regions in India for turmeric cultivation, SANDEEP et al. (2016) reported ar-turmerone as the major compound in rhizomes EO for all studied regions and *α*-phellandrene for leaf EO in seven of them. Our results, and the data from the literature, suggest that despite the differences in environmental conditions, the predominance of monoterpenes and the presence of *α*-phellandrene as the major compound are found in EO from turmeric leaves. In the present study, *α*-phellandrene represented 41.99% of the EO (Figure 1), suggesting the potential use of *C. longa* leaf oil for diverse pharmacologic applications, since *α*-phellandrene is largely used for synergistic pest-control compositions (CHAABAN et al., 2017a). Other compounds found in this study were previously reported in CLLEO. *α*-Pinene, *β*-pinene, myrcene, *ρ*-cymene, limonene and 1,8-cineole are the most frequently cited (OGUNTIMEIN et al.,1990; MCCARRON et al., 1995; GARG et al., 2002; RAINA et al., 2002; BABU et al., 2007; PRIYA et al., 2012; SANDEEP et al., 2016). Such compounds were also considered potential candidates for use in insecticide, antimicrobial, antioxidant and anti-inflammatory formulations. The second most abundant compound identified in our study, however, was never reported in this species before, neither in rhizomes nor leaves EO. The cyclic monoterpene *ρ*-mentha-2,4(8)-diene, also known as isoterpinolene, represented 24.98% of the EO in this study. Although reported as a component in some species (TSOUKATOU et al., 2001; ALALI and AL-LAFI, 2003; TAHERPOUR et al., 2010; HASIMI et al., 2015), no

information of the isolated activity of this compound was found in the literature. Alali and AL-LAFI (2003) reported that the EO from *Salvadora persica* L., containing *ρ*-mentha-2,4(8)-diene, had a considerable antibacterial effect and suggested that the product be used as a natural tool for teeth cleaning and as a natural analgesic for toothaches. It is convenient that *ρ*-mentha-2,4(8)-diene is structurally similar to *α*-phellandrene, which suggests that they are directly related to a biosynthesis pathway of the plant. It is not possible to affirm, though, that they have the same or similar activities, since it is well known that slight changes in a molecule spatial conformation can modify its whole spectrum of bioactivity (SANTOS et al., 2011). Therefore, both the isolated *ρ*-mentha-2,4(8)-diene and its interactions with other compounds in *C. longa* leaves EO represent an unexploited potential for research and development.

4.2 Larval Toxicity and Physiological Parameter Changes

PARK et al. (2003) investigated the insecticidal activity of *α*-phellandrene against adults of *Callosobruchus chinensis* and *Sitophilus oryzae* and determined mortalities of 97% and 80%, respectively. Another study conducted by CHENG et al. (2009) found LC_{50}s of 16.6 and 39.9 µg/mL for larvicidal effect on the 4th instar of *Aedes aegypti* and *A. albopictus*, respectively, 24 h after contact. More recently, this monoterpene was evaluated against 3rd to 4th instar larvae of *Culex pipiens* biotype molestus, showing LD50 of 38.20 mg/L (EVERGETIS et al., 2013). Similarly, it is worth mentioning that recent reports showed insecticidal activity of CLLEO and *α*-phellandrene against 3rd instar *Lucilia cuprina*, the ovine blowfly. Dose- and time-dependent activities were demonstrated 6 h after exposure to CLLEO and *α*-phellandrene with LC_{50}s of 1.35 and 1.15 µL/cm^2, respectively (CHAABAN et al., 2018). Although *L. cuprina* and *C. macellaria* belong to the same family, the results observed in this work showed more toxicity, for both extracts, among *C. macellaria* L3. Besides that, the rapid effects observed in both flies with CLLEO and *α*-phellandrene suggest a neurotoxic action. These effects were reported to decrease motility and increase larval death a few hours after contact with the extracts (see results in sections 3.4.1 and 3.4.3). Insecticidal EO have been reported to interact with octopaminergic receptors in the insect nervous system, as agonists and/or antagonists, being able to interfere with basic physiological, behavioral, metabolic and biochemical functions (EVANS and ROBB, 1993; OHTA and OZOE, 2014). This

fact can be explained by the action of octopamine (octopamine 1 and octopamine 2), a biogenic amine found in insects acting as a neurotransmitter. Thus, EO can rapidly act against some pests, suggesting a neurotoxic mode of action. When observing the results of larval mortality, pupation rate and emergence inhibition rate in this work using 0.79 µL/cm^2 of CLLEO, this dose differed significantly to control groups for both parameters (using both carriers: ethanol and acetone). Similarly, values of 0.88 µL/cm^2 of *α*-phellandrene showed significant difference when compared with the control group (ethanol). In general, these concentrations (0.79 µL/cm^2 for CLLEO and 0.88 µL/cm^2 for *α*-phellandrene) showed promising results against L3. A few other EO were recently evaluated against *C. macellaria* with reported larvicidal activity, reduction in adult emergence and increased adult deformities. Investigating the insecticidal activity of *Baccharis dracunculifolia* EO, CHAABAN et al. (2017b) found 62% emergence inhibition using 0.79 µL/cm^2 and 10.63% deformities in adults at dose of 1.27 µL/cm^2. Likewise, 87.27% and 0% were determined for emergence inhibition using ethanol and acetone as carriers, respectively, for the dose of 0.79 µL/cm^2 in contact test with *T. minuta* EO (CHAABAN et al., 2017c). *Tagetes minuta* EO showed 73.33% and 1.66% larval mortality using ethanol and acetone, respectively. *B. dracunculifolia* EO caused no larval mortality at this concentration (0.79 µL/cm^2). Comparing our results with previously reports, we can observe a high potential of CLLEO, as well as *α*-phellandrene for fly control. Among the EO with insecticidal activities, CLLEO and *α*-phellandrene have advantages that make them suitable for myiasis management. Other advantages may include: rapid effects in contact tests, promising results using different carriers, visible macroscopic damage to larvae, population control of chemical-resistant blowflies, and sustainable use of by-product (leaves) of *C. longa* industry.

4.3 Morphological Damage - Biomarkers of Toxicity in *C. macellaria*

4.3.1 Macroscopic Cuticle Damage

Activity of EO and the assessment of macroscopic damage can help to select biopesticides for Calliphoridae control, which include vector species of pathogenic microorganisms and myiasis caused by *C. macellaria* (GUIMARÃES et al., 1983). CHAABAN et al. (2018) reported insecticidal activity and macroscopic damage using CLLEO and *α*-phellandrene against *Lucilia cuprina*, the blowfly parasite of sheep.

The authors noticed similar body injuries when using 1.59 and 1.47 μL/cm^2 of CLLEO and *α*-phellandrene, respectively. In addition, marked cuticle dryness was observed after treatment of *L. cuprina* L3 7 days after contact with these extracts. Likewise, decreased motility a few hours after exposure using *B. dracunculifolia* and *Piper gaudichaudianum* EO was reported in *C. macellaria* and *L. cuprina* L3 (CHAABAN et al., 2017b; CHAABAN et al., 2018). These results suggest a neurotoxic mechanism of action of the EO, which is in agreement with results observed in our work, with both CLLEO and *α*-phellandrene. Besides that, the histological lesions of the brain using CLLEO or *α*-phellandrene against *L. cuprina* together with the results shown in the present work reinforce the description of the mechanism of action (see section 3.3.3).

4.3.2 Scanning Electron Microscopy

Ultrastructure assessment of L3 can assist to elucidate the damages made by biopesticides, improving the identification of the mechanism of action (MENDONÇA et al., 2014). In this sense, it is noteworthy that this was the first study assessing the damage caused by CLLEO and α-phellandrene to *C. macellaria* L3 using SEM. SHALABY et al. (2015) assessed the larvicidal activity of *Cinnamomum camphora* (camphor) and *Lavandula angustifolia* (lavender) in dipping tests against L3 of *L. sericata*. Cuticle damage, distortion of the sensorial structures and slight degeneration of the anterior spiracle were reported 24 h post treatment using 32% camphor. The lavender EO showed larva cuticle distortion, blebbing of the cuticle surface and extreme cuticle damage on the posterior end of L3. HODA et al. (2016) reported *L. sericata* cuticle damage (degeneration of dorsal and ventral papillae and of anterior spiracles) using ingestion assays with *Commiphora molmol* EO.

4.3.3 Larval Histopathology

The study of biomarkers at the cellular level can be used to assess the damage in target cells after contact with EO, and mainly to elucidate the mechanism of action of new biopesticides. In this sense, histopathological damage has been shown in some studies regarding pesticide actions such as on the midgut, MT, fat body and brain morphology of the insects (ROSSI et al., 2013a; TAVARES et al.,

2015; GREGORC et al., 2016; RIBEIRO-NETO et al., 2017). The assessment of ultrastructural changes in gut cells of insects can contribute to the development of new strategies for controlling pests. CRUZ et al. (2010) reported the morphological alterations (cytoplasmic vacuolization, absence of autophagy vacuoles and chromatic compacting) induced by boric acid and fipronil in the midgut of worker honeybee, *Apis mellifera* larvae by transmission electron microscopy. Likewise, morphological and histochemical alterations in midgut (cytoplasm vacuolization, increased apocrine secretion and cell elimination) were reported as a side-effect of sublethal doses of thiamethoxam in *A. mellifera* midgut (OLIVEIRA et al., 2014). Lavarías et al. (2017) also observed secretion activity and vacuolization on the midgut epithelium of L3 *Chironomus calligraphus* exposed to cypermethrin. More recently, histological and ultrastructural changes in the cells of midgut of *Anticarsia gemmatalis* induced by squamocin were assessed by FIAZ et al. (2018). Histological sections showed apocrine secretions released in lumen and the brush border, as well as vacuolization in the digestive cells, goblet cells, nucleus and longitudinal muscles. Another organ where the insecticide comes in contact during the course of the metabolism of its compounds are the MTs. In insects, the MTs are responsible for the excretion of substances in the body, defense system and especially for chemical detoxification processes (ROSSI et al., 2013a; NOCELLI et al., 2016). Notably, morphological data of the MTs from flies treated with CLLEO or *α*-phellandrene has not been previously examined. Previous reports showed the effects of chronic exposure to sublethal doses of imidacloprid in MTs of Africanized *Apis mellifera*. ROSSI et al. (2013a) reported an increase in the number of cells with pyknotic nuclei, loss of part of the cell into the lumen and the presence of cytoplasmic vacuolization in the tubules of treated bees. FERREIRA et al. (2013) also evaluated the MT morphology of worker bees (*Scaptotrigona postica*) exposed to fipronil and boric acid. Treatment with continuous doses of boric acid or 0.1 ng/bee fipronil showed brush border evidence, presence of cellular content in the lumen, a few nuclei with condensed chromatin and vacuolization alterations. More recently, cytotoxic effects of thiamethoxam on MTs were analyzed in *A. mellifera*. The damage was measured on the 5^{th} day of observation, revealing disruption of the cytoplasm and the basal labyrinth and loss of cytoplasmic organelles of MTs. Likewise, on the 8^{th} day of exposure the insects presented a near complete loss of the basal labyrinth of the MTs and nuclei with condensed chromatin (CATAE et al., 2014). The insect fat body is another important

structure as it is the prime location for intermediary metabolism and detoxification of pesticides, equivalent to the vertebrate' liver. Histological assessment of *C. quinquefasciatus* fat body after exposure to the natural insecticide, i.e., ascaridole-enriched fraction – EF4-5, were assessed by CASTRO et al. (2016) and showed the cells of the fat body with larger protein granules. Likewise, alterations as smaller granules demonstrating the strongest reaction for the presence of protein in the fat body of *Culex quinquefasciatus* larvae following exposure to ivermectin were reported by ALVES et al. (2010). In the same way, morphological changes as pyknotic profiles in the brain mushroom, suggesting cell death, apoptosis or necrosis were noticed using sublethal doses of fipronil against the stingless bee *Scaptotrigona postica* (JACOB et al., 2015). On the other hand, histological alterations in the brain were reported by ROSSI et al. (2013b) after exposure to imidacloprid for 10 days.

5 CONCLUSIONS

Coming back to the question: Is this industrial by-product of interest as an insecticide for control of myiasis? We consider the answer to be yes, as we have demonstrated the remarkable insecticidal activity of CLLEO, a bio-product of turmeric, and its major compound *α*-phellandrene. Cytotoxicity was observed in the digestive tract, MTs, fat body and the brain of L3, as well as cuticle damage was demonstrated through microscopic and ultrastructural analysis. Even, the biomarkers as pyknotic nuclei and condensation of nuclear chromatin were noticed in brain, fat body and digestive tract. Finally, Malpighian tubules with intense degeneration, condensation of nuclear chromatin and pyknotic nuclei also were observed in *C. macellaria* L3 suggesting neurotoxic activity, detoxicative metabolism and failure to excretion of CLLEO and *α*-phellandrene. Lastly, new research about inhibitory activities of detoxifying enzymes can better clarify the mechanism of action of *C. longa* leaves EO and its majority compound, *α*-phellandrene.

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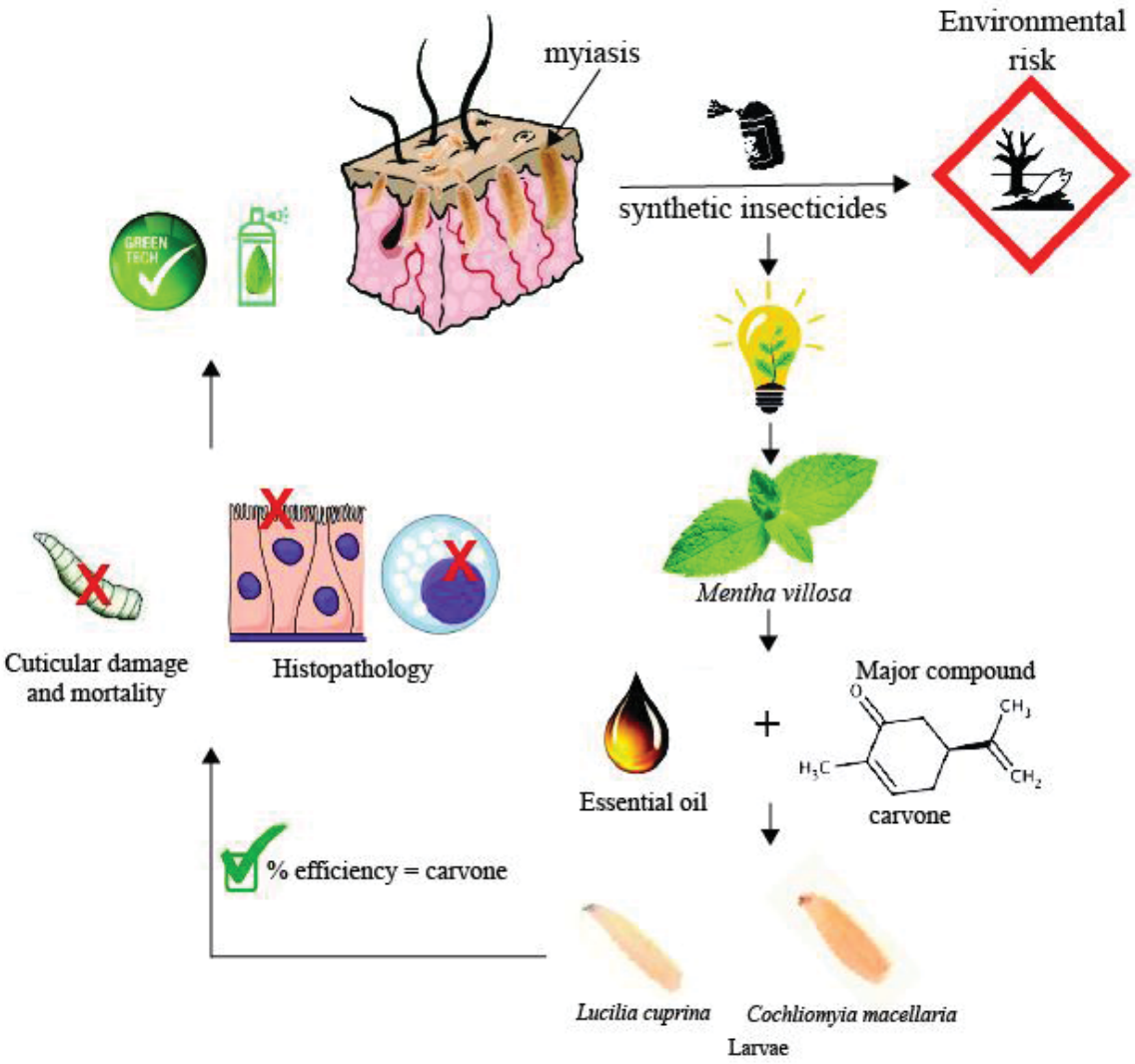
MANUSCRIPT 3 - INSECTICIDAL ACTIVITY, ULTRASTRUCTURAL AND HISTOLOGICAL DAMAGE CAUSED BY *Mentha villosa* ESSENTIAL OIL AND carvone AGAINST BLOWFLIES

ABSTRACT

Mentha villosa essential oil (MVEO) and its major constituent, carvone were investigated to assess biomarkers of insecticidal activity through ultrastructural and histological analysis on blowflies. Blowfly (i.e. *Cochliomyia macellaria* and *Lucilia cuprina*) are considered important ectoparasites that infest the living tissues of farm animals. Treatment comprises synthetic insecticides that can constitute a hazard to human health, as well as foment environmental contamination, toxicity to non-target organisms and the development of drug resistance. The objective of our work was to determine the insecticidal activity of MVEO and carvone against third instar larvae (L3) of *C. macellaria* and *L. cuprina,* using contact assays. Groups of 20 L3 were placed on filter paper impregnated with increasing concentrations of MVEO (0.079 to 3.18µL/cm^2) and carvone (0.038 to 0.764 µL/cm^2). Light microscopy and ultrastructural analyses with treated L3 were undertaken. Efficacy was determined by quantifying L3 mortality 6, 24 and 48 h after contact with MVEO and carvone using Probit analysis. The lethal concentration of 50% (LC_{50}) for MVEO and carvone 24 h following contact were 1.64 and 0.63µL/cm^2 to *C. macellaria*, whereas for *L. cuprina* the LC_{50} values were 1.1 and 0.3µL/cm^2, respectively. Carvone showed a better efficacy against L3 when compared to MVEO in all assays. Morphological damage in the Malpighian tubules (MTs), digestive tract, fat body, muscle and brain were identified. Significant alterations such as the presence of cell residue in the lumen, vacuolar degeneration and pyknotic nuclei were observed in the MTs. In addition, fat body trophocytes with pyknotic nuclei, loss of digestive cell architecture, substantial mineral accumulation in the larval muscle and pyknotic profile in the brain were also noted. Finally, marked changes including damage to sensorial structures, posterior spiracle, dryness on the cuticle surface, as well as extreme degeneration of L3 were observed via scanning electron microscopy. The extracts had the potential to induce cytotoxic effects on target organs. The results demonstrate that MVEO and carvone can be considered efficient biopesticides, even at low doses.

Keywords: *Lucilia cuprina, Cochliomyia macellaria*, Ecofriendly products, Sustainability, Biopesticide, Biomarkers.

GRAPHICAL ABSTRACT



MANUSCRITO 3 - ATIVIDADE INSETICIDA, DANOS ULTRASTRUTURAIS E HISTOLÓGICOS CAUSADOS PELO ÓLEO ESSENCIAL DE *Mentha villosa* E CARVONA CONTRA BLOWFLIES

RESUMO

O OE de *Mentha villosa* e seu principal constituinte, carvona, foram investigados para avaliar biomarcadores da atividade inseticida, através da análise ultraestrutural e histológica em varejeiras. As moscas-varejeiras (ou seja, *Cochliomyia macellaria* e *Lucilia cuprina*) são consideradas importantes ectoparasitas, que causam infestação em tecidos vivos dos animais de produção. O tratamento convencional consiste no uso de inseticidas sintéticos, podendo constituir um perigo para a saúde humana, além de fomentar a contaminação ambiental, a toxicidade para organismos não-alvo e o desenvolvimento de resistência a drogas. Neste sentido, o objetivo de nosso trabalho foi determinar a atividade inseticida do OE de *M. villosa* e seu constituinte majoritário, carvona, contra larvas de terceiro estádio (L3) de *C. macellaria* e *L. cuprina*, através de ensaios de contato. Assim, grupos de 20 L3 foram colocados em papel de filtro impregnados com concentrações crescentes do OE de *M. villosa* (0,079 a 3,18μL/cm^2) e carvona (0,038 a 0,764μL/cm^2). Microscopia de luz e análises ultraestruturais com L3 tratadas foram realizadas. A eficácia foi determinada pela quantificação da mortalidade L3 6, 24 e 48 h após o contato com o OE de *M. villosa* e carvona usando a análise Probit. A concentração letal de 50% (CL50) para o OE de *M. villosa* e a carvona, 24 h após o contato foi de 1,64 e 0,63μL/cm^2 para *C. macellaria*, enquanto que para *L. cuprina* os valores de CL50 foram 1,1 e 0,3μL/cm^2, respectivamente. Carvona demonstrou uma melhor eficácia contra L3 quando comparado ao OE de *M. villosa* em todos os ensaios. Danos morfológicos nos túbulos de Malpighi, trato digestivo, corpo gorduroso, músculo e cérebro foram identificados. Alterações significativas como a presença de resíduos celulares no lúmen, degeneração vacuolar e núcleos picnóticos foram observadas nos túbulos de Malpighi. Além disso, foram observados trofócitos do corpo gorduroso com núcleos picnóticos, perda da arquitetura celular digestiva, acúmulo mineral substancial no músculo larval e perfil picnótico no cérebro. Finalmente, mudanças marcantes, incluindo danos às estruturas sensoriais, espiráculos posteriores, ressecamento na

superfície da cutícula, bem como degeneração em L3 foram observadas através de microscopia eletrônica de varredura. Os extratos demontraram potencial de induzir efeitos citotóxicos nos órgãos alvo. Os resultados demonstram que o OE de *M. villosa* e a carvona podem ser considerados biopesticidas eficientes, mesmo em baixas doses.

Palavras chaves: *Lucilia cuprina, Cochliomyia macellaria*, Produtos ecológicos, Sustentabilidade, Biopesticida, Biomarcadores.

1 INTRODUCTION

Blowfly larvae are considered one of the primary ectoparasites that affect livestock. Infestation (myiasis) treatment comprises the use of synthetic insecticides. One example are the macrocyclic lactone derivatives administered in different routes (subcutaneous, intramuscular and pour-on) or in various topical combinations that largely contain organophosphates and pyrethroids. However, the misuse of chemical pesticides can constitute a hazard to human health owing to the possibility of exceeding the maximum residue limit (MRL) in foodstuffs of animal origin. It can also instigate environmental contamination and toxicity to non-target organisms, as well as to facilitate the development of pest resistance (MOYA-BORJA, 2003; QIN et al., 2017; CAMPOS et al., 2018). Interestingly, although some countries have their own regulatory standards in order to control ectoparasiticide residues, different levels of tolerance continue to be observed. For instance, in Brazil, MRL levels of macrocyclic lactone derivatives such as abamectin and eprinomectin at 100 and 2,000µg/kg, respectively, are considered tolerable in the liver. In contrast, the European Union (EU) defined through the Commission Regulation N° 37/2010 the MRL for the liver as being 20µg/kg for abamectin and 1,500µg/kg for eprinomectin. Regulation discrepancies can also be observed in terms of the quantity in target tissues. In Brazil, liver analysis alone is sufficient by the National Plan for Residues and Contaminants Control (PNCRC) for abamectin, and for eprinomectin. The regulation also allows the analysis of milk samples. In contrast, the EU regulation allows the analyses of abamectin in the liver and fat, and for eprinomectin an even larger range of target tissues (muscle, fat, liver, kidney and milk) has been examined. Similarly, clear differences in tolerance levels can be observed in the active substances conventionally used in topical applications to control livestock ectoparasites, including blowflies. The same limits of amitraz are tolerated in honey by the Brazilian and EU regulation authorities. However, whereas in Brazil amitraz analysis have only focused in honey, the EU target tissues, fat, liver, kidneys and milk, of all ruminants have been investigated. A similar situation was observed for cypermethrin and deltamethrin where tissues such as muscles, fat, liver, kidneys and milk were analyzed by the EU, whereas in Brazil only the muscle is included in the PNCRC. Moreover, the highest tolerance levels of both active substances in the muscle are

much lower in the EU (20µg/kg cypermethrin and 10µg/kg deltamethrin, compared to 50 and 30µg/kg, respectively, in Brazil).

After these arguments, the questions is: Why are there so many discrepancies in the tolerance levels of active substances used in topical applications in order to control ectoparasites? Considering that infestation by ectoparasites is clearly important, especially in the tropics, why has the analysis of residues in the main target organs including the skin did not receive a greater attention? We think that this issue should be of a major concern. In this sense, botanical pesticides (BPs) produced from plant metabolites may represent ecofriendly alternatives, as they may leave low levels of residues in foodstuffs of animal origin or cause little harm to the environment. Given that the majority of BPs are biodegradable, interest in the use of either plant extracts or essential oils (EO) was revised by ISMAN (2015). BPs in the form of isolated substances or complex mixtures, as EO exhibit a range of biological activities, acting as repellents, insecticides, fungicides, nematicides, and bactericides (CHAABAN et al., 2017a). The genus Mentha, an important member of the Lamiaceae family, is represented by approximately 18 species and 11 natural hybrids. They are a fast-growing and an invasive perennial plant that generally tolerate a wide range of agro-climatic conditions, with a wide distribution (Europe, Africa, Asia, Australia, and America) (LAWRENCE, 2006). *Mentha x villosa* Huds. is a hybrid of *M. spicata* and *M. suaveolens,* of which two important varieties have been described in the literature: *M. x villosa* var. *villosa*, that is rich in piperitone oxide, and *M. x villosa* var. *alopecuroides*, rich in carvone (GOBERT et al., 2002; LAWRENCE 2006). Carvone is a monocyclic monoterpene ketone that exists both as R- and S-enantiomers in natural products and represents an important chemical constituent of the EO of many Mentha species (*M. spicata, M. viridis, M. longifolia, M piperita, M. suaveolens and M. x villosa*) (LAWRENCE, 1978; KOKKINI and PAPAGEORGIOU, 1987; KAROUSOU et al., 1998; POOTER and SCHAMP, 1987; CARVALHO and FONSECA 2006; KUMA et al., 2011; PAVELA et al., 2014). The following forms of antimicrobial, antifungal, acaricidal, repellent and insecticidal activity have been reported in the literature with regard of carvone, and their safe usage has been reported in crop protection and agro-food storage (PEIXOTO et al., 2015; MORCIA et al., 2016; CHAABAN et al., 2017a). However, carvone has also been identified as acting synergistically with synthetic insecticides such as carbofuran, carbaryl,

parathion, and dichlorodiphenyltrichloroethane, improving their insecticidal activity (LICHTENSTEIN et al., 1974).

For these, *M. villosa* EO and its major constituent, carvone, may represent a sustainable alternative to control myiasis. Recently, the insecticidal activity and damage to target organs (i.e., cuticle, digestive tract, body fat, Malpighian tubules – MTs – and brain) of some EO and individual compounds were described and suggested as biomarkers of biological activity (CHAABAN et al., submitted in 2017; CHAABAN et al., 2018). This study aimed to assess the potential of *Mentha villosa* EO and its major compound, carvone, as a botanical insecticide against blowflies (*Lucilia cuprina* and *Cochliomyia macellaria* L3), with attention to morphological alterations in the target organs through ultrastructural assessment and light microscopy.

2 MATERIALS AND METHODS

2.1 Plant material

The botanical species used in this work were grown in the Medical Plants Unit at the IFC, located at 26° 23' 33.6691" S and 48° 44' 18.3336" W at 10.6 m above sea level in the city of Araquari, Santa Catarina State, Southern Brazil. Plants were cultivated in an agroecological system, without the application of agrochemical products. Leaves and stems were collected at 11 am from approximately 100 individuals in February 2017 (Brazilian summer). A voucher specimen of the botanical material was deposed at the Botanical Museum Herbarium, located in the Botanic Garden of Curitiba, PR, with the number 358966.

2.2 Essential oil (EO) extraction and chemical characterization

Leaves and stems were homogenized and the EO was extracted by hydrodistillation for 4 h in a Clevenger apparatus, according to Wasicky (1963). The oil was dried over anhydrous sodium sulfate, and stored in amber vials at 4°C until testing. The EO was analyzed by gas chromatography coupled with a mass spectrometric detector (GC/MS) (Shimadzu, Model GCMS - QP2010) (Kyoto, Japan) using a ZB-5MS capillary column (30 m × 0.25 mm x 0.25 µm) (Torrance, CA, USA).

The injection temperature was 25^{o}C and the carrier (helium gas) flow was 1.0 ml/min. The chromatograph oven was optimized with an initial temperature of 60°C for 4 min up to 210ºC for 6 min, in a 35-min run. The oil sample was diluted 200 times in hexane, followed by injection into the GC/MS. The obtained mass spectra were compared with the database of the NIST Library GC/MS. The retention index was calculated using n-alkane standard solutions (relative to C 10 – C 21n-alkanes) in the same chromatographic conditions. The identification of the compounds was undertaken by comparing their GC mass and retention data with those in the NIST-05 Library (ADAMS, 2007). The yield (%) of the essential oil was calculated by the average of three distillations of 100g/dry matter.

2.3 Dilution of extracts

Carvone (CAS: 6485-40-1) was acquired commercially and certified as having a purity of ≥98%. The product was obtained from Sigma-Aldrich (São Paulo, SP, Brazil). The substances, MVEO and carvone, were diluted in absolute ethanol or acetone, as these solvents are not toxic to L3 (contact tests) of *L. cuprina* and *C. macellaria* (CHAABAN et al., 2017b, c). Carvone was used from 0.038 to 0.764 µL/ cm^2, and each dose represented one treatment, diluted in absolute ethanol. The following MVEO concentrations were from 0.079 to 3.18µL/cm^2 solubilized in ethanol or acetone. A control group was established, in which L3 were only exposed to the solvent used (absolute ethanol and acetone).

2.4 Establishment of *Lucilia cuprina* and *Cochliomyia macellaria* colonies

Wild flies were collected at the IFC using bait and insect nets. The establishment of stock colonies, insects' identification, maintenance, mass reproduction and the protocol for the biological tests were performed as described by CHAABAN et al., (2017b, c). For this work we used fresh, drug-free bovine meat (approx. 2g/ larvae) for larval development. Mature L3 used in this assay left the substrate spontaneously.

2.5 Larval toxicity

The toxicity evaluation of MVEO and carvone against L3 of *L. cuprina* and *C. macellaria* was performed as described by CHAABAN et al., (2017b, c). Groups of 20 mature L3 (one day old after leaving the substrate) from the second generation were introduced into glass vials (9 × 4 cm diameter) containing filter paper (12,56 cm^2) impregnated with 0.2 mL of carvone or MVEO solutions. Following the applications, the glass vials were closed with voile fabric to facilitate aeration. They flasks were kept for 5 min in an exhaust hood, and finally transferred to a climatic chamber under 27°C and 70% relative humidity. All treatments were performed in triplicate (n=60) using a total of 1.620 larvae to *C. macellaria* and 1.560 to *L. cuprina*. The toxicity was evaluated by observing L3 mortality at 6, 24 and 48 h after contact. Total L3 mortality (*TLM)* was calculated (CHAABAN et al., 2017b, c, 2018; KUMAR et al., 2014) as follows:

TLM = (total dead larvae x 100) / total tested larvae

2.6 Analysis of the physiological parameters

Following the contact of the L3 with MVEO and carvone, the L3 were retained under controlled conditions and physiological parameters such as the pupation rate (*PR*), emergence inhibition rate (*EIR*) and adult deformity (*AD*) were recorded and calculated (KUMAR et al., 2014; SINGH and KAUR 2016; CHAABAN et al., 2017b, c, 2018) as follows:

PR = (total pupae x 100) / total tested larvae)
EIR = (total control adults - total treated adults x 100) / total control adults
AD = (total deformed adults x 100) / total emerged adults

2.7 Larval histopathology

For larval histopathology, three L3 of *C. macellaria* were treated with 1.59 µL/ cm^2 of MVEO and 0.76 µL/cm^2 of carvone, whereas a dose of 0.95µL/cm^2 of MVEO and 0.30µL/cm^2 of carvone was used for *L. cuprina*. The doses were selected in

agreement with the criteria published by the Working Party about the Efficacy of Veterinary Medicines (EUROPEAN COMMISSION III/3682/92-EN). The Guide indicates that the efficacy of insecticides for Diptera species should be between 80 and 100%, preferably greater than 90%. The extracts were solubilized in ethanol and the treated larvae were fixed in 10% buffered formalin, 24 h after contact. Two longitudinal sections were embedded in paraffin. L3 were serially sectioned (4 µm) and stained with hematoxylin-eosin.

2.8 Scanning electron microscopy

For scanning electron microscopy (SEM), L3 treated with the same doses as described in section 2.7 were used and fixed in AFA solution (ethyl alcohol at 70%, buffered formalin at 37% and glacial acetic acid in the ratio 2.5: 1: 1.5), 48, 72 h and 4 days after solutions contact. The samples were subsequently submitted to a dehydration process using five alcohol baths. The larvae were placed in support for SEM (stub) and dehydrated in an oven at 37°C for 6 h using the protocol described by CANEPARO (2017), with modifications by CHAABAN et al. (submitted in 2018). The specimens were examined and photographed with a SEM at a magnification ranging from 12 to 600X (JEOL JSM 6360-LV) at the Center of Electron Microscopy of the Federal University of Paraná, PR, Brazil.

2.9 Statistical analysis

Lethal concentrations (LC_{10}, LC_{50} and LC_{90}) were calculated using Probit analysis. L3 mortality data were analyzed with concentrations, carriers and their interactions. The average LM, PR and EIR were compared among treatments using the Tukey HSD test. Statistical analyses were performed using the statistical software SPSS version 23 (2013), where a significance level of 5% was considered. Pearson analysis was use to establish correlation (r) values between matrix values.

3. RESULTS

3.1 Chemical composition of Mentha villosa essential oil

Fourteen compounds were identified from MVEO, representing 99.98% of the total chromatographic peaks (Table 1). The major compounds of MVEO were, carvone (52.52%), dihydrocarveol (15.39%), dihydrocarveol acetate (12.07%) and d-limonene (7.6%), whereas cis-carvyl acetate (3.68%), beta-caryophyllene (2.31%) and trans-carveol (1.76%) represented the smaller chromatographic area. The yield of MVEO was 0.74%.

TABLE 1 – LIST OF CHEMICAL COMPONENTS OF *Mentha villosa* ESSENTIAL OIL.

Compounds	RT (min)	IRc	IRt	RA (%)
alfa-pinene	6,22	932	939	0,37
L-beta-pinene	7,53	978	981	0,53
D-limonene	8,97	1029	1031	7,6
L-4-terpineol	13,12	1180	1182	0,35
Dihydrocarveol	13,57	1197	1192	15,39
trans-carveol	14,08	1217	1217	1,76
cis-carveol	14,41	1231	1229	0,68
carvone	14,72	1243	1242	52,52
Dihydrocarveol acetate	16,62	1322	1305	12,07
cis-carvyl acetate	17,40	1356	1362	3,68
beta-bourbonene	18,04	1384	1384	1,41
beta-caryophyllene	18,84	1420	1418	2,31
(+)-Epi-bicyclosesquiphellandrene	19,75	1463	1471	0,46
gamma-muurolene	20,15	1481	1477	0,85
		Total identified compounds (%)		99.98
		Other unidentified compounds (%)		0.02

Note: RT = Retention time (min), IRc = Retention index calculated, IRt = Retention index tabulated (Adams, 2007), RA (%) = Relative area.

SOURCE: the author

3.2 Larval toxicity and changes in physiological parameters

Lethal concentrations of MVEO and carvone against *C. macellaria* and *L. cuprina* are displayed in Tables 2 and 3. Dose- and time-dependent activities were demonstrated 24 h after exposure to both extracts, with an LC_{50} of 1.64 and 0.63µL/ cm^2 to *C. macellaria*, whereas for *L. cuprina* the values to LC_{50} were 1.1 and 0.3µL/ cm^2 to MVEO and carvone using ethanol with carrier, respectively. The LC_{50} varied between carriers and demonstrated the greatest toxicity to *L. cuprina* when acetone was used with the carrier at a LC_{50} value of 0.52µL/cm^2 (Figure 1, Figure 2, Table 1, Table 2). Interestingly, carvone had the highest level of toxicity to both fly species when comparing the complex mixture of oil containing 52.52% of carvone in its chemical composition. Regarding the assessment of mortality of *C. macellaria*, we noticed values of 81.66%, using 0.95 µL/cm^2 and 73.33%, using 0.61 µL/cm^2 of MVEO and carvone, respectively. Accordingly, the same doses indicated an adult emergence inhibition of 94.33 and 100% to MVEO and carvone against *C. macellaria* (Table 4). *Lucilia cuprina* showed the greatest sensibility to both extracts, with L3 mortality and adult emergence inhibition values of 80.0 and 94.64%, using 0.31 µL/ cm^2 of MVEO, whereas, values of 98.33 and 100% were reported using 0.30 µL/cm^2 of carvone (Table 5).

TABLE 2 - LETHAL CONCENTRATION (µL/cm^2) OF *Mentha villosa* ESSENTIAL OIL AND IT IS MAJOR COMPOUND carvone ON *Cochliomyia macellaria* LARVAE IN THE CONTACT ASSAY OVER TIME.

Extract	Evaluation time	*LC_{10} (LCI–UCI)	LC_{50} (LCI–UCI)	LC_{90} (LCI–UCI)	Chi-square ($\chi 2$)	Probability
MVEO/ET	6h	-	-	-	-	-
	24h	0.69 (0.57-0.86)	1.64 (1.44-1.83)	3.85 (3.44-4.45)	7.55	0.18
	48h	0.64 (0.47-0.79)	1.14 (0.96-1.29)	2.02 (1.88-2.2)	6.85	0.23
MVEO/AC	6h	-	-	-	-	-
	24h	1.37 (1.17-1.52)	2.26 (2.13-2.4)	3.73 (3.36-4.34)	7.05	0.28
	48h	1.21 (1.07-1.32)	1.67 (1.56-1.76)	2.29 (2.16-2.47)	4.31	0.5
carvone	6h	-	-	-	-	-
	24h	0.36 (0.3-0.41)	0.63 (0.58-0.7)	1.1 (0.94-1.45)	4.81	0.31
	48h	0.34 (0.28-0.37)	0.51 (0.48-0.55)	0.78 (0.71-0.89)	2.47	0.65

MVEO/ET: *Mentha villosa* essential oil solubilized in ethanol; MVEO/AC: *Mentha villosa* oil solubilized in acetone. Carvone was solubilized only at etanol.
*The lethal concentrations were calculated by the Probit analysis. LCI, lower limit of 95% confidence interval; UCI, upper limit of 95% confidence interval.

SOURCE: the author

TABLE 3 - LETHAL CONCENTRATION (µL/cm^2) OF *Mentha villosa* ESSENTIAL OIL AND IT IS MAJOR COMPOUND carvone ON *Lucilia cuprina* LARVAE IN THE CONTACT ASSAY OVER TIME.

Extract	Evaluation time	*LC_{10} (LCI–UCI)	LC_{50} (LCI–UCI)	LC_{90} (LCI–UCI)	Chi-square ($\chi 2$)	Probability
MVEO/ET	6h	-	-	-	-	-
	24h	0.56 (0.23-0.8)	1.1 (0.74-1.38)	2.16 (1.7-3.39)	38.68	0.001
	48h	0.17 (0.0-0.52)	0.54 (0.05-1.19)	1.64 (0.58-4.01)	49.11	0.001
MVEO/AC	6h	-	-	-	-	-
	24h	0.34 (0.19-0.43)	0.52 (0.41-0.59)	0.8 (0.73-0.93)	0.65	0.96
	48h	0.19 (0.17-0.22)	0.26 (0.24-0.27)	0.34 (0.32-0.37)	1.14	0.89
carvone	6h	-	-	-	-	-
	24h	0.18 (0.06-0.24)	0.3 (0.21-0.39)	0.5 (0.38-1.23)	7.69	0.053
	48h	0.14 (0.13-0.15)	0.19 (0.18-0.21)	0.25 (0.23-0.3)	0.004	0.99

MVEO/ET: *Mentha villosa* essential oil solubilized in ethanol; MVEO/AC: *Mentha villosa* oil solubilized in acetone. Carvone was solubilized only at etanol.
*The lethal concentrations were calculated by the Probit analysis. LCI, lower limit of 95% confidence interval; UCI, upper limit of 95% confidence interval.

SOURCE: the author

TABLE 4 - LARVAL MORTALITY (LM), PUPARIATION RATE (PR), EMERGENCE INHIBITION RATE (EIR), SEX RATIO (MALE:FEMALE) AND ADULT DEFORMITY OF *Cochliomyia macellaria* TREATED WITH *Mentha villosa* ESSENTIAL OIL AND carvone IT IS THE MAJOR COMPOUND.

	C(µL/cm^2)	*LM (%)	PR (%)	EIR (%)	SR (M:F)	AD (%)
Mentha villosa						
	Control group (ethanol)	0.0 Ac	100.0 Aa	0.0 Ad	28:25	0:0
	0.31 (2%)	0.0 Ac	100.0 Aa	0.0 Ad	30:23	0:0
	0.63 (4%)	10.0 (±2.9) Ac	90.0 (±2.9) Aa	20.75 (±5.1) Ac	14:28	0:0
	0.95 (6%)	81.66 (±6.0) a	18.33 (±6.0) c	94.33 (±5.3) ab	2:1	0:0
	1.27 (8%)	55.0 (±2.9) Ab	45.0 (±2.9) Bb	73.58 (±2.7) Ab	5:9	14.28
	1.59 (10%)	83.33 (±4.4) Aa	16.66 (±4.4) Bc	94.33 (±0.2) Aab	1:2	0:0
	2.07 (13%)	90.0 (±5.0) Aa	10.0 (±5.0) Ac	100.0 Aa	0:0	0:0
	2.38 (15%)	91.66 (±6.0) Aa	8.33 (±6.0) Ac	100.0 Aa	0:0	0:0
	2.86 (18%)	98.33 (±2.9) Aa	1.66 (±2.9) Ac	100.0 Aa	0:0	0:0
	3.18 (20%)	100.0 Aa	0.0 Ac	100.0 Aa	0:0	0:0
Mentha villosa						
	Control group (acetone)	0.0 Ac	100.0 a	0.0 Ad	26:25	0:0
	0.31 (2%)	5.0 (±5.0) Ac	95.0 (±5.0) Aa	0.0 Ad	24:27	5.88
	0.63 (4%)	10.0 (±5.8) Ac	90.0 (±5.8) Aa	9.80 (±7.8) Ad	20:26	2.17
	1.27 (8%)	18.33 (±1.7) Bc	81.67 (±1.7) Aa	54.90 (±12.1) Ac	8:15	4.34
	1.59 (10%)	48.33 (±6.0) Bb	51.67 (±6.0) Ab	74.50 (±2.1) Ab	5:8	7.69
	2.07 (13%)	81.66 (±3.3) Aa	18.34 (±3.3) Ac	98.03 (±2.1) Aa	0:1	0:0
	2.38 (15%)	93.33 (±4.4) Aa	6.67 (±4.4) Ac	100.0 Aa	0:0	0:0
	2.86 (18%)	100.0 Aa	0.0 Ac	100.0 Aa	0:0	0:0
	3.18 (20%)	98.33 (±1.7) Aa	1.67 (±1.7) Ac	100.0 Aa	0:0	0:0
carvone						
	Control group (ethanol)	0.0 c	100.0 a	0.0 d	26:30	0:0
	0.038	1.66±1.7 c	98.34 (±1.7) a	0.0 d	25:31	0:0
	0.076	3.33±3.3 c	96.67 (±3.3) a	2.45 (±12.4) d	28:26	0:0
	0.1528	0.0 c	100.0 a	2.92 (±7.3) d	28:26	3.70
	0.3057	8.33±1.7 c	91.67 (±1.7) a	25.0 (±0.8) c	24:18	0:0
	0.4585	35.0±5.8 b	65.0 (±5.8) b	48.21 (±8.9) b	18:11	0:0
	0.611	73.33±11.7 a	26.67 (±11.7) c	100.0 a	0:0	0:0
	0.764	88.33±7.3 a	11.67 (±7.3) c	100.0 a	0:0	0:0

*48 of exposure.
Absolute ethanol and acetone were used with control and only ethanol was used with carvone.
The letters display a significant difference (P<0.05). Capital letter in concentrations between carriers and lowercase letters differences in concentrations within carriers.

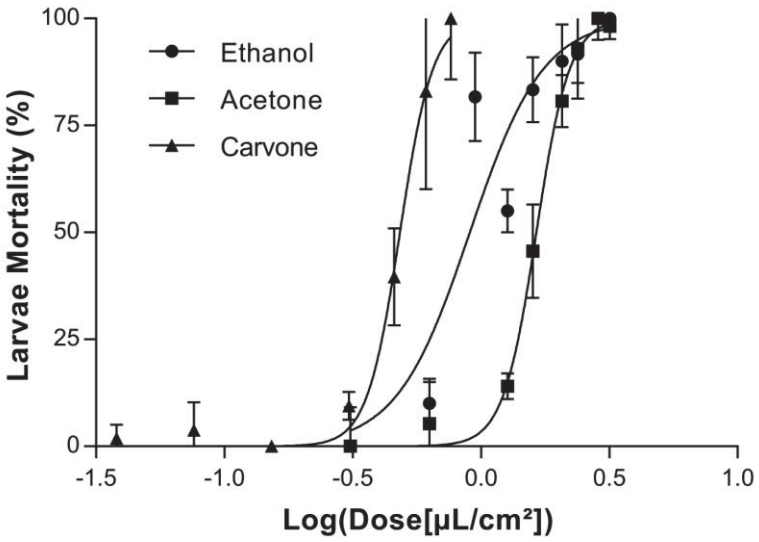
SOURCE: the author

TABLE 5 - LARVAL MORTALITY (LM), PUPARIATION RATE (PR), EMERGENCE INHIBITION RATE (EIR), SEX RATIO (MALE:FEMALE) AND ADULT DEFORMITY OF *Lucilia cuprina* TREATED WITH *Mentha villosa* ESSENTIAL OIL AND carvone, IT IS THE MAJOR COMPOUND.

	C(µL/cm^2)	*LM (%)	PR (%)	EIR (%)	SR (M:F)	AD (%)
Mentha villosa						
	Control group (ethanol)	0.0 Ad	100.0 Aa	0.0 Ad	30:27	0.0
	0.31 (2%)	5.0 (±2.9) Bd	95.0 (±2.9) Aa	17.54 (±11.2) Bc	21:26	0.0
	0.47 (3%)	60.0 (±5.0) Ac	40.0 (±5.0) Ab	71.92 (±4.4) b	7:9	0.0
	0.63 (4%)	63.33 (±10.1) Bbc	36.67 (±10.1) Abc	64.91 (±2.9) Bb	9:11	0.0
	0.95 (6%)	91.66 (±4.4) a	8.34 (±4.4) d	96.49 (±3.9) a	2:0	0.0
	1.27 (8%)	86.66 (±3.3) Aabc	13.34 (±3.3) Abcd	85.96 (±4.6) Aab	2:6	0.0
	1.59 (10%)	78.33 (±10.1) Aabc	21.67 (±10.1) Abcd	75.43 (±12.1) Bb	7:7	0.0
	2.07 (13%)	96.66 (±1.7) Aa	3.34 (±1.7) Ad	92.98 (±7.8) Aa	1:3	0.0
	2.38 (15%)	88.33 (±3.3) ab	11.67 (±3.3) cd	89.47 (±5.5) a	0:6	0.0
	2.86 (18%)	95.0 (±2.9) Aa	5.0 (±2.9) d	100.0 a	0:0	0.0
	3.18 (20%)	100.0 a	0.0 d	100.0 a	0:0	0.0
Mentha villosa						
	Control group (acetone)	0.0 Ac	100.0 Aa	0.0 Ad	26:30	0.0
	0.07 (0.5%)	0.0 c	100.0 a	0.0 d	27:29	0.0
	0.15 (1%)	0.0 c	100.0 a	12.18 (±12.7)c	18:31	0.0
	0.31 (2%)	80.0 (±10.4) Ab	20.0 (±10.4) Bb	94.64 (±3.2) Aab	2:1	0.0
	0.47 (3%)	73.33 (±10.9) Ab	26.67 (±10.9) Ab	82.14 (±7.2) b	1:7	0.0
	0.63 (4%)	100.0 Aa	0.0 Bc	100.0 Ba	0:0	0.0
	1.27 (8%)	100.0 Aa	0.0 Ac	100.0 Aa	0:0	0.0
	1.59 (10%)	100.0 Aa	0.0 Ac	100.0 Aa	0:0	0.0
	2.07 (13%)	100.0 Aa	0.0 Ac	100.0 Aa	0:0	0.0
carvone						
	Control group (ethanol)	0.0 c	100.0 a	0.0 d	29:30	0.0
	0.038	0.0 c	100.0 a	0.0 d	32:27	0.0
	0.076	0.0 c	100.0 a	5.08 (±0.1) c	25:31	0.0
	0.1528	16.66 (±7.3) b	83.34±7.3 b	69.49 (±4.8) b	10:8	0.0
	0.3057	98.33 (±1.7) a	1.67±1.7 c	100.0 a	0:0	0.0
	0.4585	100.0 a	0.0 c	100.0 a	0:0	0.0

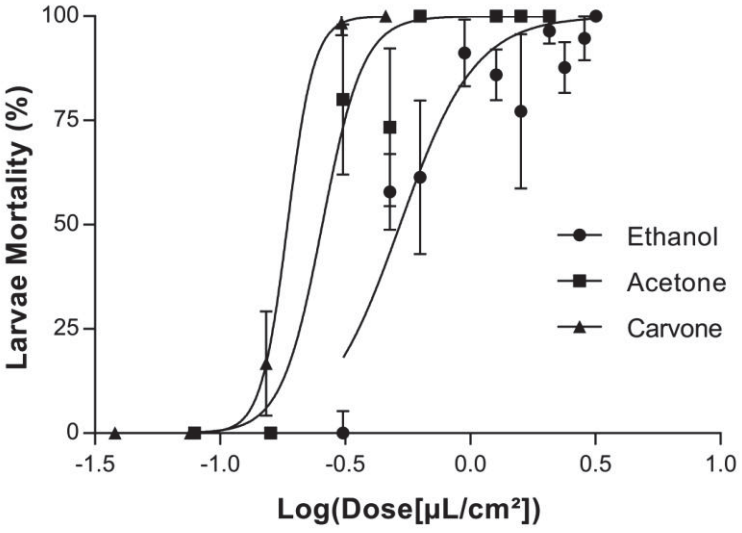
*48 of exposure.
Absolute ethanol and acetone were used with control to MVEO and only ethanol was used by carvone.
The letters display a significant difference (P <0.05). Capital letter in concentrations between carriers and lowercase letters differences in concentrations within carriers.

SOURCE: the author



SOURCE: the author

FIGURE 1 - LARVAE TOXICITY (µL/cm^2) OF *Cochliomyia macellaria* AFTER EXPOSURE TO *Mentha villosa* ESSENTIAL OIL USING DIFFERENT CARRIERS (ETHANOL AND ACETONE) AND ITS MAJOR COMPOUND carvone USING ETHANOL WITH SOLVENT.



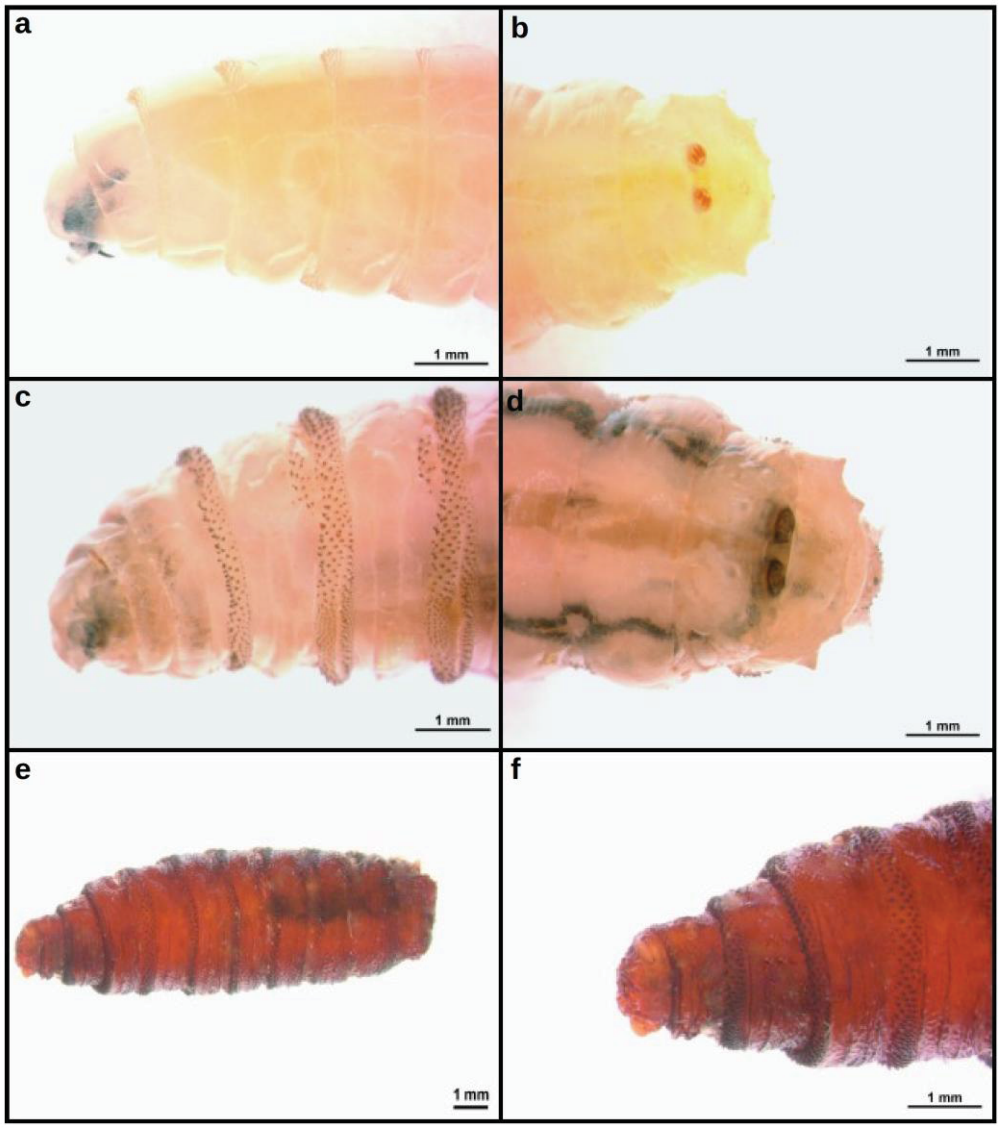
SOURCE: the author

FIGURE 2 - LARVAE TOXICITY (µL/cm^2) OF *Lucilia cuprina* AFTER EXPOSURE TO *Mentha villosa* ESSENTIAL OIL USING DIFFERENT CARRIERS (ETHANOL AND ACETONE) AND ITS MAJOR COMPOUND carvone USING ETHANOL WITH SOLVENT.

3.3 Morphological damage - Biomarkers toxicity in *Cochliomyia macellaria* and *Lucilia cuprina*

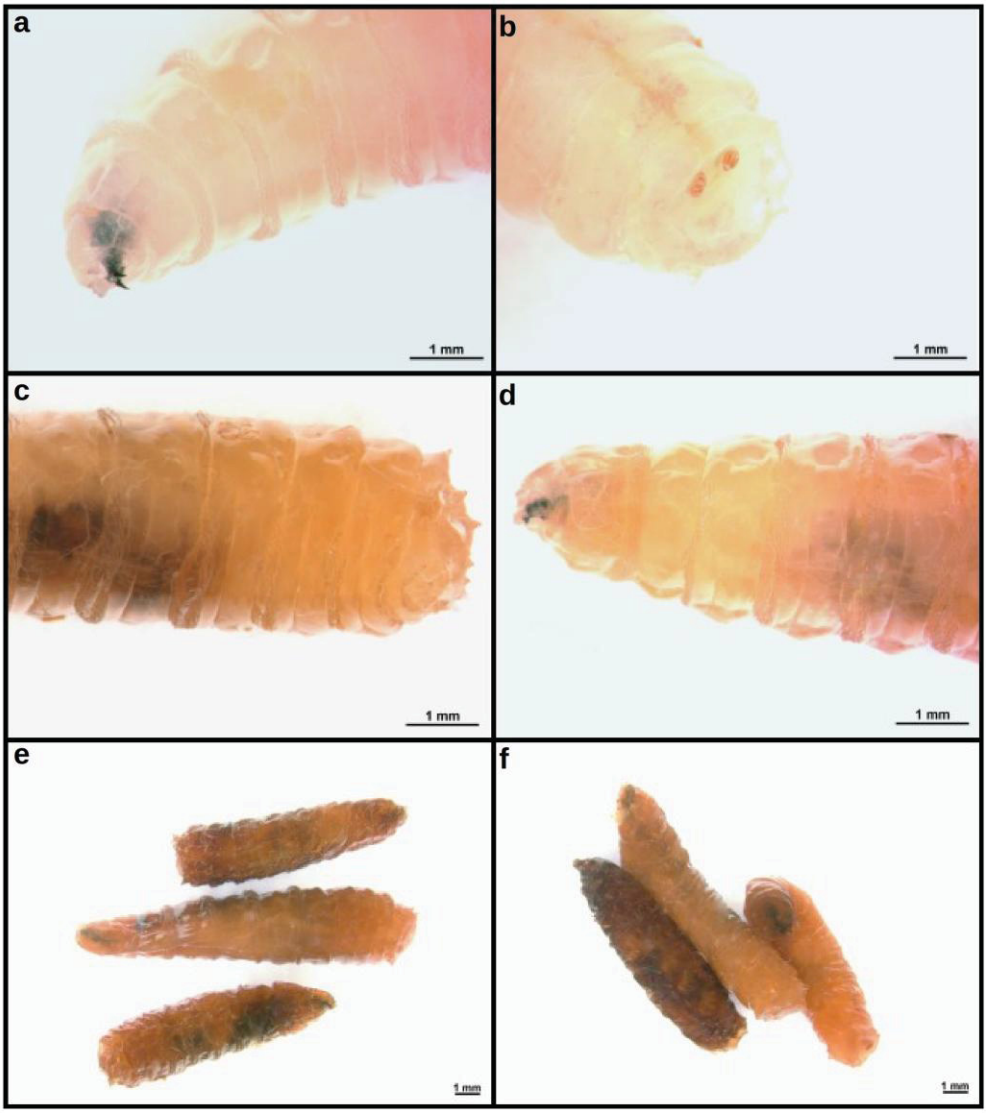
3.3.1 Macroscopic cuticle damage

The larvae of *L. cuprina* and *C. macellaria* from the control group demonstrated cuticles without any macroscopic damage or color change (Figure 3a, b). Changes in cuticle color on the larval body of flies treated with MVEO and carvone, were observed. *C. macellaria* displayed alterations in the cuticle using 1.59μL/cm^2 of MVEO and 0.76μL/cm^2 of carvone, with marked darkening at segmental spinules, tracheal branches and the respiratory spiracle, as well as dryness on the larval body (Figure 3c, d, e, f). *Lucilia cuprina* exhibited the greatest sensibility, with changes in cuticle color, such as accentuated darkening at the ventral medium body, using 0.95μL/cm^2 of MVEO and 0.30μL/cm^2 of carvone, 48 h after exposure (Figure 4 c, d). In addition, darkening and accentuated dryness on the *L. cuprina* larval body were observed 7 days after treatment (Figure 4 e, f). Diminished motility with larval crowding was reported 48h after exposure to MVEO and carvone.



SOURCE: the author

FIGURE 3 - MACROSCOPIC CUTICULAR DAMAGE OF *Cochliomyia macellaria* L3 AFTER TREATMENT WITH *Mentha villosa* ESSENTIAL OIL (MVEO) AND ITS MAJOR COMPOUND carvone. A, B) ANTERIOR AND POSTERIOR END OF NORMAL L3 48 H AFTER TREATMENT (CONTROL GROUP – ONLY ETHANOL): NOTE THE NORMAL SIZE AND NO CHANGE IN CUTICLE COLOR (22X). C) ANTERIOR END OF L3 WITH CUTICLE DAMAGE 48 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO: OBSERVE THE ALTERATION IN COLOR THROUGHOUT THE BODY AND MARKED DARKENING AT SEGMENTAL SPINULES (22X). D) POSTERIOR END OF L3 WITH CUTICLE DAMAGE 48 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO: NOTE THE ACCENTUATED DARKENING IN TRACHEAL BRANCHES AND RESPIRATORY SPIRACLE (22X). E, F) L3 WITH CUTICLE DAMAGE 48 H AFTER TREATMENT WITH 0.76 µL/cm^2 OF carvone: NOTE THE DARKENING AND DRYNESS ON THE LARVAE BODY (8X, 22X).

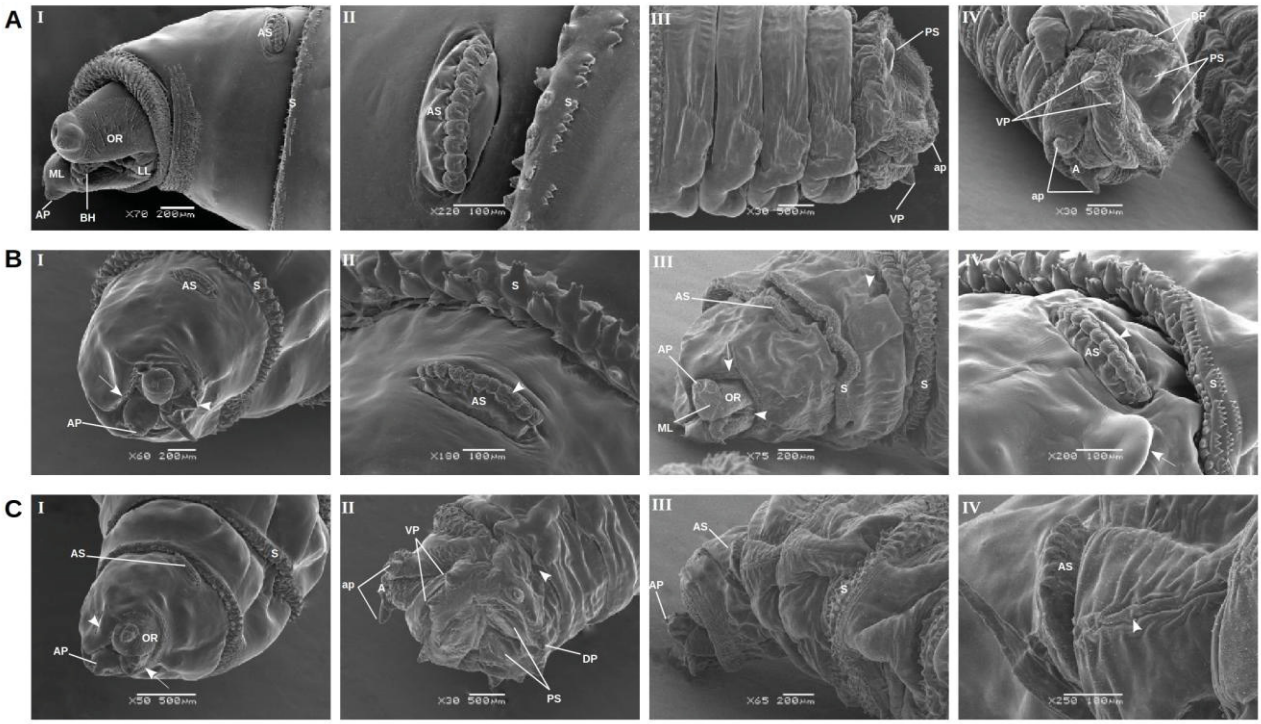


SOURCE: the author

FIGURE 4 - MACROSCOPIC CUTICULAR DAMAGE OF *Lucilia cuprina* L3 AFTER TREATMENT WITH *Mentha villosa* ESSENTIAL OIL (MVEO) AND ITS MAJOR COMPOUND carvone. A, B) ANTERIOR AND POSTERIOR END OF NORMAL L3 48 H AFTER TREATMENT (CONTROL GROUP – ONLY ETHANOL): NOTE THE NORMAL SIZE AND NO CHANGE IN CUTICLE COLOR (22X). C, D) L3 WITH CUTICLE DAMAGE 48 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO AND 0.30 µL/cm^2 OF carvone, RESPECTIVELY: OBSERVE THE CHANGE IN CUTICLE COLOR AND ACCENTUATED DARKENING AT VENTRAL MEDIUM BODY OF LARVA (22X). E, F) L3 WITH CUTICLE DAMAGE 7 DAYS AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO AND 0.30 µL/cm^2 OF carvone, RESPECTIVELY: NOTE THE DARKENING AND DRYNESS ON THE LARVAE BODY (8X).

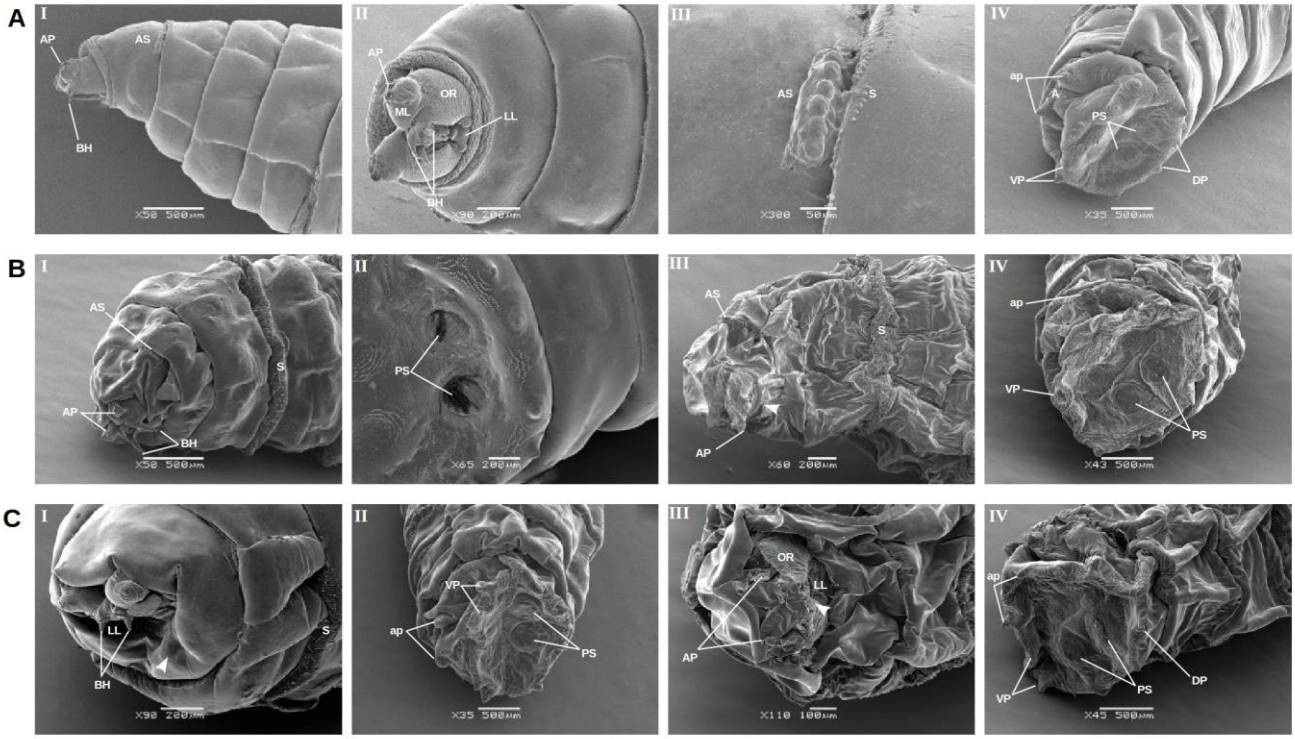
3.3.2 Scanning electron microscopy

The SEM of normal L3 of *C. macellaria* and *L. cuprina* exhibited typical Calliphoridae morphology. The control group had a smooth body tegument with preserved intersegmental and all other structures (Figure 5A, I-IV). Scanning electron micrographs of *C. macellaria* L3 48 h after treatment with 1.59 μL/cm^2 of MVEO showed damage to the anterior spiracle and cephalic segment in terms of shrinkage on the spinules, dryness of the cuticle and degeneration of the bucal hook and labial lobe (Figure 5B, I-IV). Similarly, we observed damage to the structures of the cephalic segment and marked dryness of L3 48 h after treatment with 0.76 μL/cm^2 of carvone in *C. macellaria* L3 (Figure 5C, I-IV). Notably, the damage was more accentuated 72 h after treatment in both flies (Figure 5B, 6B,C-III-IV). A contact test using MVEO and carvone on *L. cuprina* L3 also displayed significant changes following treatment with 0.95 μL/cm^2 to MVEO and 0.30 μL/cm^2 to carvone (Figure 6B, 6C, I-IV). Marked changes in terms of damage to sensorial structures, posterior spiracle, dryness on the cuticle surface, as well as extreme degeneration of L3 were also observed, and were more pronounced after 72 h (Figure 6B, 6C, III-IV).



SOURCE: the author

FIGURE 5 - ULTRASTRUCTURAL MICROGRAPHS OF *Cochliomyia macellaria* L3. A) CONTROL GROUP (ONLY ETHANOL). I) CEPHALIC SEGMENT OF LARVA WITH ANTENNA SENSORY PAPILLAE, MAXILLARY LOBE, ORAL RIDGES, LABIAL LOBE, BUCAL HOOK, MARKED SPINULES AND ANTERIOR SPIRACLE. NOTE THE PRESERVED STRUCTURES. II) ANTERIOR SPIRACLE WITH 10-11 LOBES AND LATERAL SPINULES PRESERVED (SIDE VIEW). III) POSTERIOR END OF LARVA. DETAILS OF VENTRAL SPINULES, DORSAL PAPILLAE, VENTRAL PAPILLAE AND ANAL PAPILLAE. IV) ANAL SEGMENT WITH ANAL PAPILLAE, ANUS, VENTRAL PAPILLAE AND DORSAL PAPILLAE. DETAILS TO POSTERIOR SPIRACLES WITH THREE SPIRACULAR OPENING. B) *C. macellaria* AFTER TREATMENT WITH MVEO. I) CEPHALIC SEGMENT OF L3 48 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO: OBSERVE THE DAMAGE AS SHRINKAGE OF CEPHALIC SPINULES (ARROW), DRYNESS OF CUTICLE AND DEGENERATION OF BUCAL HOOK (ARROWHEAD) AND LABIAL LOBE. II) ANTERIOR SPIRACLE AND SPINULES OF L3 48 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO (SIDE VIEW): DETAILS TO MAKED SPINULES AND DAMAGE ON THE ANTERIOR SPIRACLE (ARROWHEAD). III) CEPHALIC SEGMENT OF L3 72 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO: NOTE THE DRYNESS ON THE CUTICLE SURFACE (ARROWHEAD), DAMAGE ON THE BUCAL HOOK, LABIAL LOBE (ARROWHEAD), MAXILLARY LOBE AND ANTENNA SENSORY PAPILLAE AND STILL MARKED SHRINKAGE OF CEPHALIC SPINULES (ARROW). IV) ANTERIOR SPIRACLE OF L3 72 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO: DETAILS TO MARKED DAMAGE ON THE CUTICLE SURFACE (ARROW) AND ANTERIOR SPIRACLE (ARROWHEAD). C) *C. macellaria* AFTER TREATMENT WITH carvone. I) CEPHALIC SEGMENT OF L3 48H AFTER TREATMENT WITH 0.76 µL/cm^2 OF carvone: NOTE THE DRYNESS ON THE CUTICLE SURFACE (ARROWHEAD), DAMAGE ON THE BUCAL HOOK, LABIAL LOBE (ARROW), MAXILLARY LOBE AND ANTENNA SENSORY PAPILLAE AND STILL MARKED SHRINKAGE OF CEPHALIC SPINULES. II) POSTERIOR END OF L3 48 H AFTER TREATMENT WITH 0.76 µL/cm^2 OF carvone: NOTE THE DRYNESS AND DAMAGE ON CUTICLE SURFACE (ARROWHEAD) AND SPINULES ACCENTUATED. III) CEPHALIC SEGMENT AND ANTERIOR BODY OF L3 72 H AFTER TREATMENT WITH 0.76 µL/cm^2 OF carvone: NOTE THE ACCENTUATED CUTICLE DRYNESS, DAMAGE ON THE ANTENNA SENSORY PAPILLAE AND ANTERIOR SPIRACLE. IV) ANTERIOR SPIRACLE OF L3 72 H AFTER TREATMENT WITH 0.76 µL/cm^2 OF carvone: DETAILS TO MARKED DAMAGE ON THE ANTERIOR SPIRACLE AND DRYNESS ON LARVA CUTICLE (ARROWHEAD). ABBREVIATIONS: AE, ANTERIOR END; S, SPINULES; AP, ANTENNA SENSORY PAPILLAE; ML, MAXILLARY LOBE; AS, ANTERIOR SPIRACLE; OR, ORAL RIDGES; LL, LABIAL LOBE; BH, BUCAL HOOK; A, ANUS; DP, DORSAL PAPILLAE; VP, VENTRAL PAPILLAE; PS, POSTERIOR SPIRACLES; AP, ANAL PAPILLAE

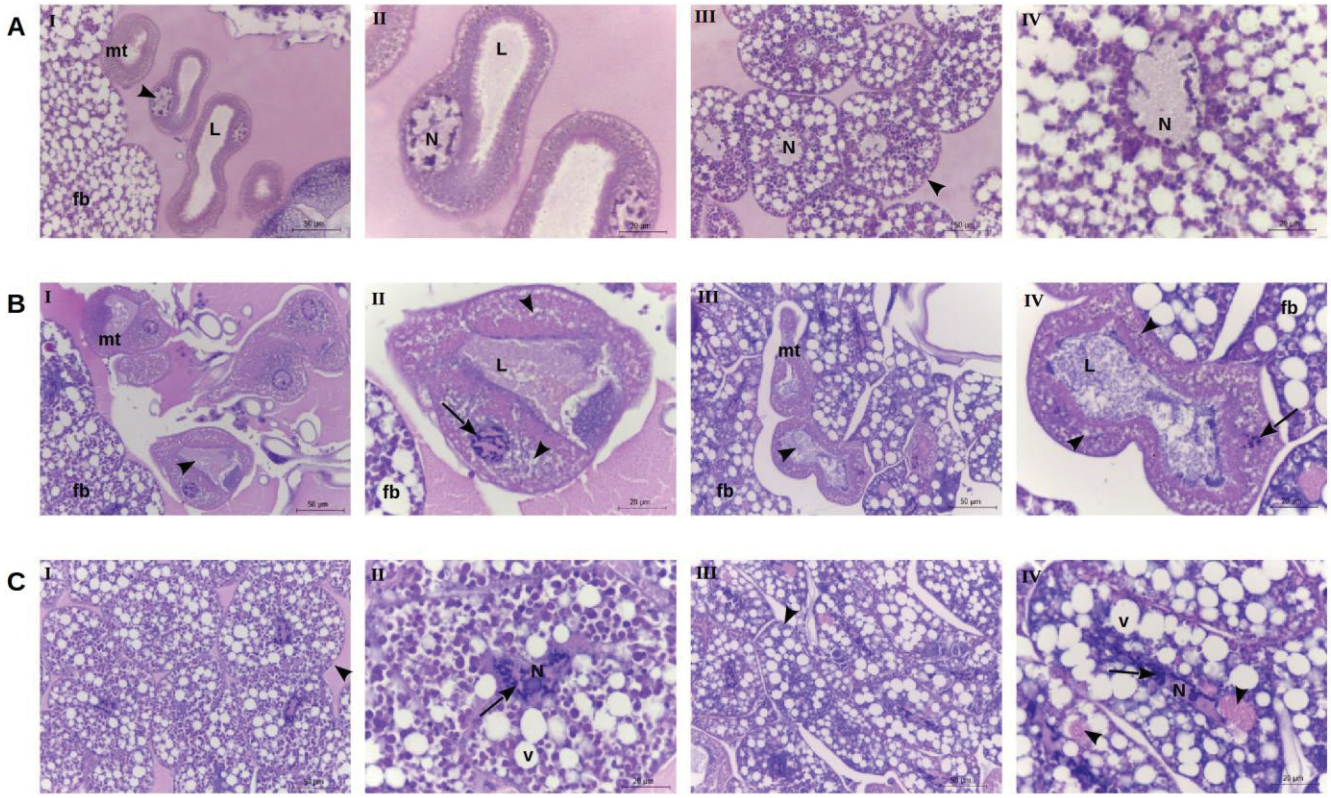


SOURCE: the author

FIGURE 6 - ULTRASTRUCTURAL MICROGRAPHS OF *Lucilia cuprina* L3. A) CONTROL GROUP (ONLY ETHANOL). I) NORMAL BODY SHAPE OF *L. cuprina* WITH SMOOTH CUTICLE WITH INTERSEGMENTAL SPINES PROJECTED BACKWARD. II) CEPHALIC SEGMENT OF LARVA WITH ANTENNA SENSORY PAPILLAE, MAXILLARY LOBE, ORAL RIDGES, LABIAL LOBE, MARKED SPINULES AND BUCAL HOOK PROJECTING BEYOND THE ORAL CAVITY. OBSERVE THE PRESERVED STRUCTURES. III) DETAILS OF ANTERIOR SPIRACLES WITH 6-7 LOBES (SIDE VIEW) AND SPINULES PRESERVED. IV) POSTERIOR END OF LARVA. DETAILS OF SPIRACULAR PLATE WITH THREE SPIRACULAR OPENINGS, ANAL SEGMENT WITH ANAL PAPILLAE, ANUS, VENTRAL PAPILLAE AND DORSAL PAPILLAE. B) *L. cuprina* L3 AFTER TREATMENT WITH MVEO. I) CEPHALIC SEGMENT OF L3 48 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO: OBSERVE THE SLIGHT DRYNESS ON CUTICLE SURFACE, ANTERIOR SPIRACLE EVIDENCED AND DAMAGE ON ANTENNA SENSORY PAPILLAE. II) ANAL SEGMENT OF L3 48 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO: DETAILS TO THE DAMAGE IN SPIRACLE OPENING. III) ANTERIOR END OF L3 72 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO: NOTE THE EXTREME CUTICLE DRYNESS, ACCENTUATED DAMAGE IN ORAL (ARROWHEAD) AND SENSORIAL STRUCTURES AND MARKED SHRINKAGE OF CEPHALIC SEGMENT. IV) POSTERIOR END OF L3 72 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO: DETAILS TO MARKED DAMAGE ON POSTERIOR SPIRACLE, LARVA CUTICLE, VENTRAL AND ANAL PAPILLAE. C) *L. cuprina* L3 AFTER TREATMENT WITH carvone. I) CEPHALIC SEGMENT OF L3 48 H AFTER TREATMENT WITH 0.30 µL/cm^2 OF carvone: DETAILS TO PRONUNCIATED CUTICLE DRYNESS (ARROWHEAD), ACCENTUATED DAMAGE ON ORAL RIDGES, ANTENNA SENSORY PAPILLAE AND LABIAL LOBE. II) POSTERIOR END OF L3 48 H AFTER TREATMENT WITH 0.30 µL/cm^2 OF carvone: OBSERVE THE CUTICLE DRYNESS AND DAMAGE ON VENTRAL AND ANAL PAPILLAE. III) CEPHALIC SEGMENT OF L3 72 H AFTER TREATMENT WITH 0.30 µL/cm^2 OF carvone: NOTE THE EXTREME DEGENERATION IN ORAL STRUCTURES (ARROWHEAD), ACCENTUATED DAMAGE AND CUTICLE DRYNESS. IV) POSTERIOR END OF L3 72 H AFTER TREATMENT WITH 0.30 µL/cm^2 OF carvone: OBSERVE THE DEGENERATION ON CUTICLE SURFACE AND POSTERIOR SPIRACLE, DAMAGE ON THE ANAL, VENTRAL AND DORSAL PAPILLAE. ABBREVIATIONS: AE, ANTERIOR END; PE, POSTERIOR END; S, SPINULES; AP, ANTENNA SENSORY PAPILLAE; AS, ANTERIOR SPIRACLE; ML, MAXILLARY LOBE; OR, ORAL RIDGES; LL, LABIAL LOBE; BH, BUCAL HOOK; DP, DORSAL PAPILLAE; VP, VENTRAL PAPILLAE; A, ANUS; PS, POSTERIOR SPIRACLES; ap, ANAL PAPILLAE.

3.3.3 Larval histopathology

Histological sections of *C. macellaria* and *L. cuprina* L3 displayed several alterations 24 h after MVEO and carvone treatment in distinct target cells using a dose of 1.59 μL/cm^2 of MVEO or 0.76 μL/cm^2 of carvone to *C. macellaria* and 0.95μL/cm^2 of MVEO or 0.30μL/cm^2 of carvone to *L. cuprina*. Regarding the histological alterations to the MTs, intense necrosis with the lumen content part of the cell (cell residue), vacuolar degeneration, hyperchromatosis and the presence of cytoplasmic granules were observed in this target organ (Figure 7, Figure 8). Fat body constituted a further cell target with significant changes to morphological structure including trophocytes with hyperchromatosis, vacuolization in the cell and accumulation of eosinophilic material, suggestive of protein globules (Figure 8, Figure 9). Similarly, alterations in terms of the loss of digestive cell architecture, presence of cell residue in the lumen, intense necrosis, presence of vacuoles in the cell, hyperchromatosis were noticed in the anterior and posterior digestive tracts of both fly species (Figure 9, Figure 10). Thus, pyknotic nuclei were observed in digestive tract of *C. macellaria* larvae (Figure 9). Interestingly, alterations suggestive of mineral accumulation and intense vacuolation were observed in the treated larval muscle (Figure 11). Finally, histological sections of the larvae of both flies' brains exhibited intense vacuolar degeneration and nuclei with pyknotic profiles (Figure 12).



SOURCE: the author

FIGURE 7 - PHOTOMICROGRAPHS OF MALPIGHIAN TUBULES AND FAT BODY OF *Cochliomyia macellaria* L3. A) NORMAL CONTROL GROUPS 24H AFTER TREATMENT (ONLY ETHANOL) (40, 100X). I, II) OBSERVE THE MTs SHOWING TYPICAL MORPHOLOGY. III, IV) NOTE THE NORMAL FB TROPHOCYTES. B) MTs OF *C. macellaria* L3 24 H AFTER TREATMENT WITH 1.59 ML/CM^2 OF MVEO (I, II) OR 0.76 µL/cm^2 OF carvone (III, IV) (40, 100X). I) DETAILS TO MTs WITH PRESENCE OF CELL RESIDUE IN THE LUMEN (ARROWHEAD). II) NOTE THE HYPERCHROMATOSIS (ARROW), VACUOLIZATION (ARROWHEADS) AND LUMEN CONTENT CELL RESIDUE. III) OBSERVE THE INTENSE NECROSIS OF MTs WITH LUMEN CONTENT PART OF CELL (ARROWHEAD). IV) DETAILS TO VACUOLIZATION OF THE CELL (ARROWHEAD), PRESENCE OF CELL RESIDUE DISPOSED IN THE LUMEN, HYPERCHROMATOSIS (ARROW) AND PRESENCE OF CYTOPLASMIC GRANULES (ARROWHEAD). C) FB OF *C. macellaria* L3 24 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO (I, II) OR 0.76 µL/cm^2 OF carvone (III, IV) (40, 100X). I, II) OBSERVE THE FB TROPHOCYTES (ARROWHEAD) WITH HYPERCHROMATOSIS (ARROW) AND VACUOLIZATION. III) NOTE THE CHANGE IN FB MORPHOLOGY (ARROWHEAD). IV) DETAILS TO NUCLEI WITH HYPERCHROMATOSIS (ARROW), VACUOLIZATION IN THE CELL AND ACCUMULATION OF EOSINOPHILIC MATERIAL, SUGGESTIVE OF PROTEIN GLOBULES (ARROWHEAD). HEMATOXYLIN-EOSIN (40, 100X). ABBREVIATIONS: v, VACUOLIZATION; N, NUCLEI; L, LUMEN; fb, FAT BODY TROPHOCYTES; mt, MALPIGHIAN TUBULES.

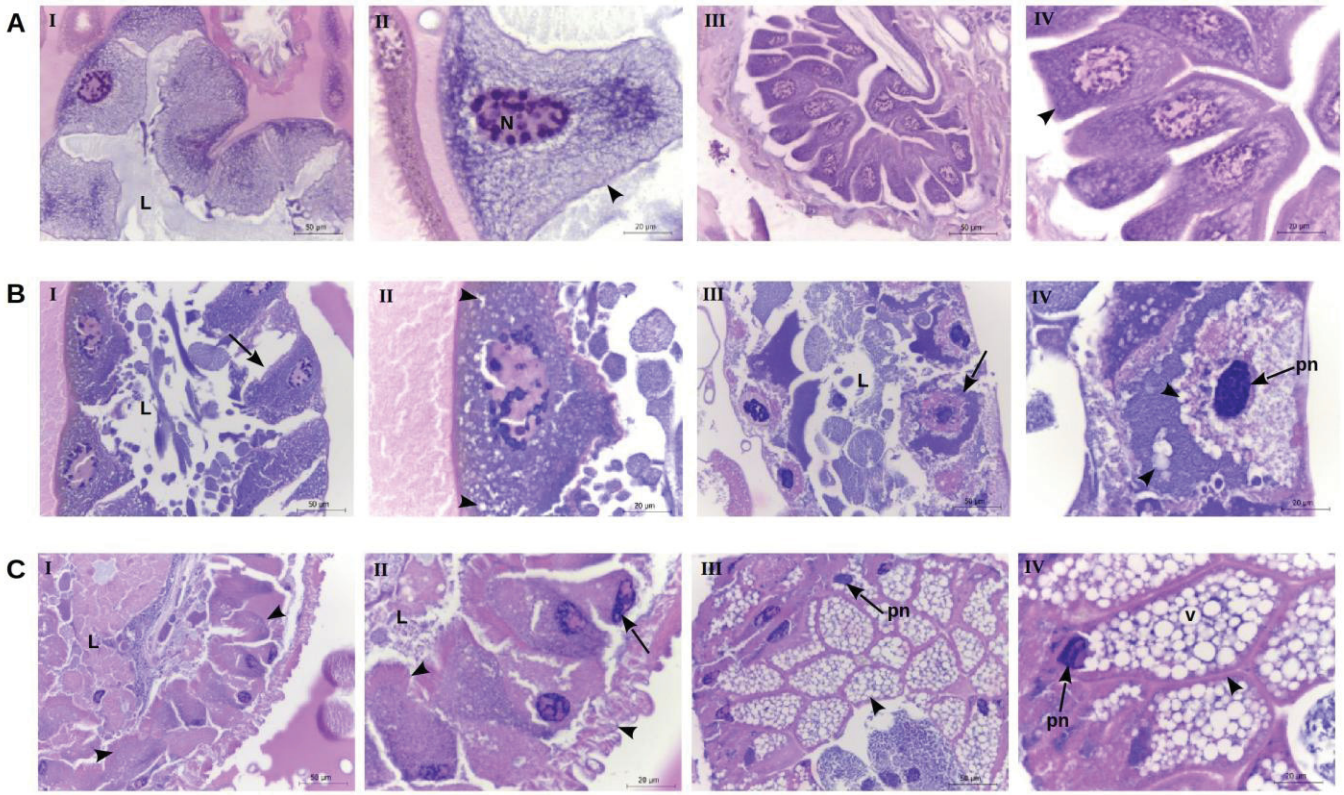


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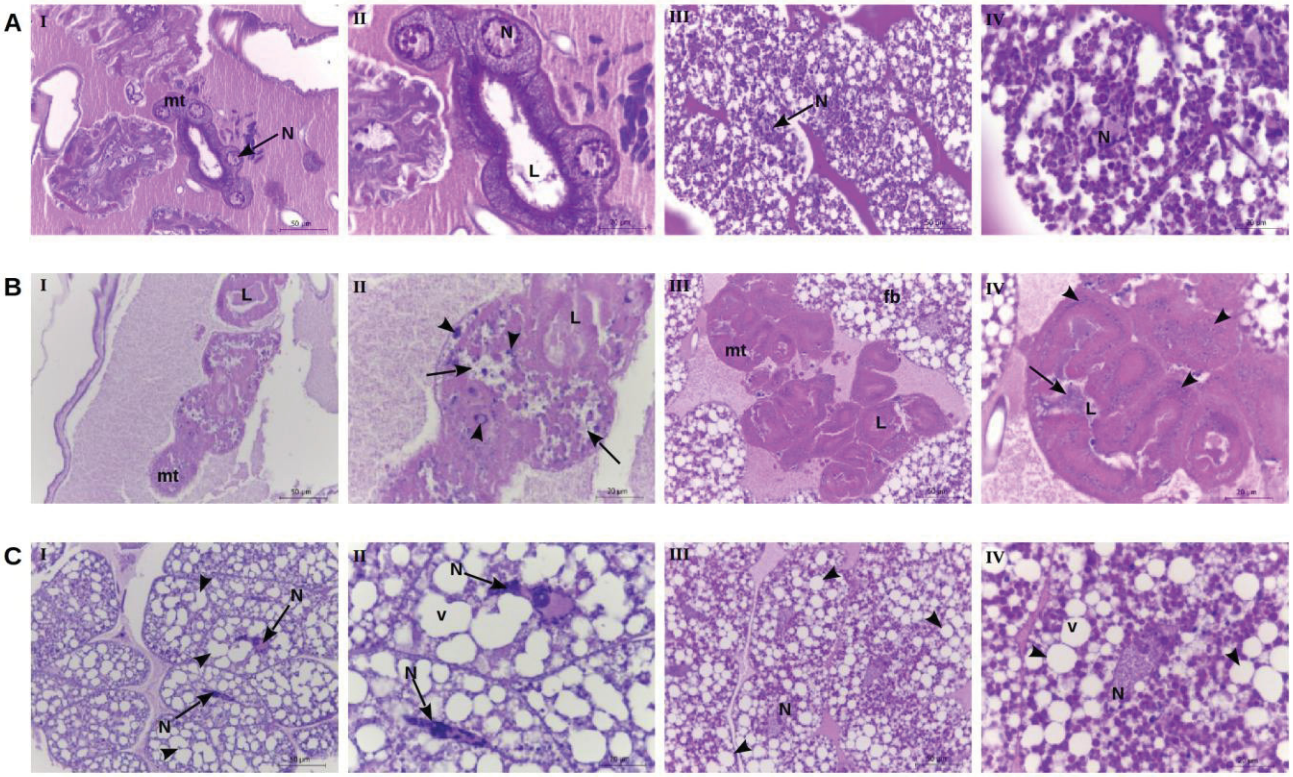
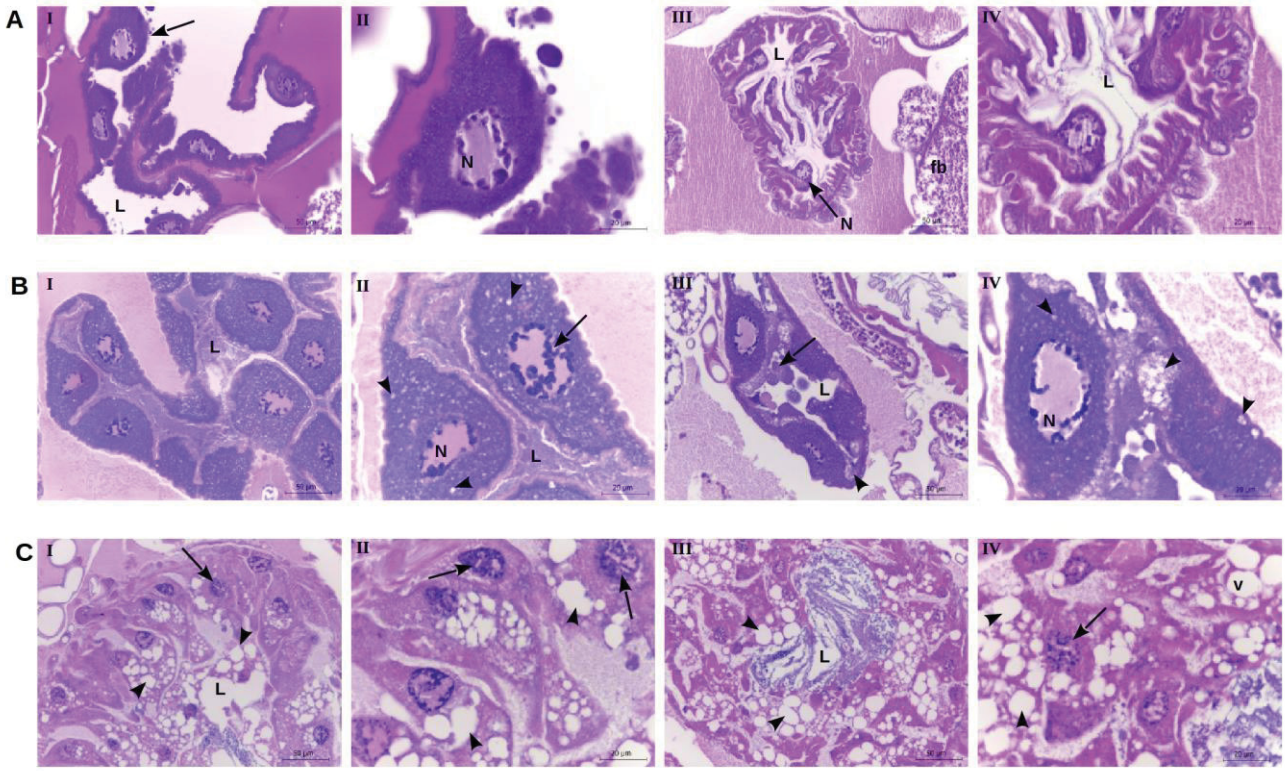
FIGURE 8 - PHOTOMICROGRAPHS OF ANTERIOR AND POSTERIOR DIGESTIVE TRACT OF *Cochliomyia macellaria* L3. A) CONTROL GROUPS WITH INTACT ANTERIOR (I, II) AND POSTERIOR (III, IV) DIGESTIVE TRACT (EPITHELIAL CELLS WITH NUCLEUS AND BRUSH BORDER). NOTE THE NORMAL MORPHOLOGY OF CELLS (ARROWHEAD) (40, 100X). B) ANTERIOR DIGESTIVE TRACT OF OF *C. macellaria* L3 24 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO (I, II) OR 0.76 µL/cm^2 OF carvone (III, IV) (40, 100X). I, II) NOTE THE LOSS OF DIGESTIVE CELL ARCHITECTURE (ARROW), CELL RESIDUE IN THE LUMEN OF DIGESTIVE TRACT AND THE PRESENCE OF VACUOLES IN CELL (ARROWHEAD). III, IV) DETAILS TO INTENSE NECROSIS OF THE DIGESTIVE CELL (ARROW), PRESENCE OF CELL RESIDUE IN THE LUMEN, MARKED VACUOLIZATION IN CELL (ARROWHEAD) AND PYKNOTIC NUCLEI (ARROW). C) POSTERIOR DIGESTIVE TRACT OF *C. macellaria* L3 24 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO (I, II) OR 0.76 µL/cm^2 OF carvone (III, IV) (40, 100X). I, II) OBSERVE THE NECROSIS WITH CHANGE OF DIGESTIVE CELL ARCHITECTURE (ARROWHEAD), PRESENCE OF A PART OF CELL DISPOSED IN THE LUMEN, ALTERATIONS IN THE BRUSH BORDER MORPHOLOGY AND HYPERCHROMATOSIS (ARROW). III, IV) NOTE THE MARKED AND INTENSE VACUOLIZATION OF CELL WITH NUCLEI DISPLACEMENT TO CELL BORDER AND PYKNOTIC NUCLEI (ARROW). DETAILS TO PRESERVED CELL MEMBRANE (ARROWHEAD). H & E, HEMATOXYLIN–EOSIN. ABBREVIATIONS: N, NUCLEI; pn, PYKNOTIC NUCLEI; L, LUMEN; v, VACUOLE.



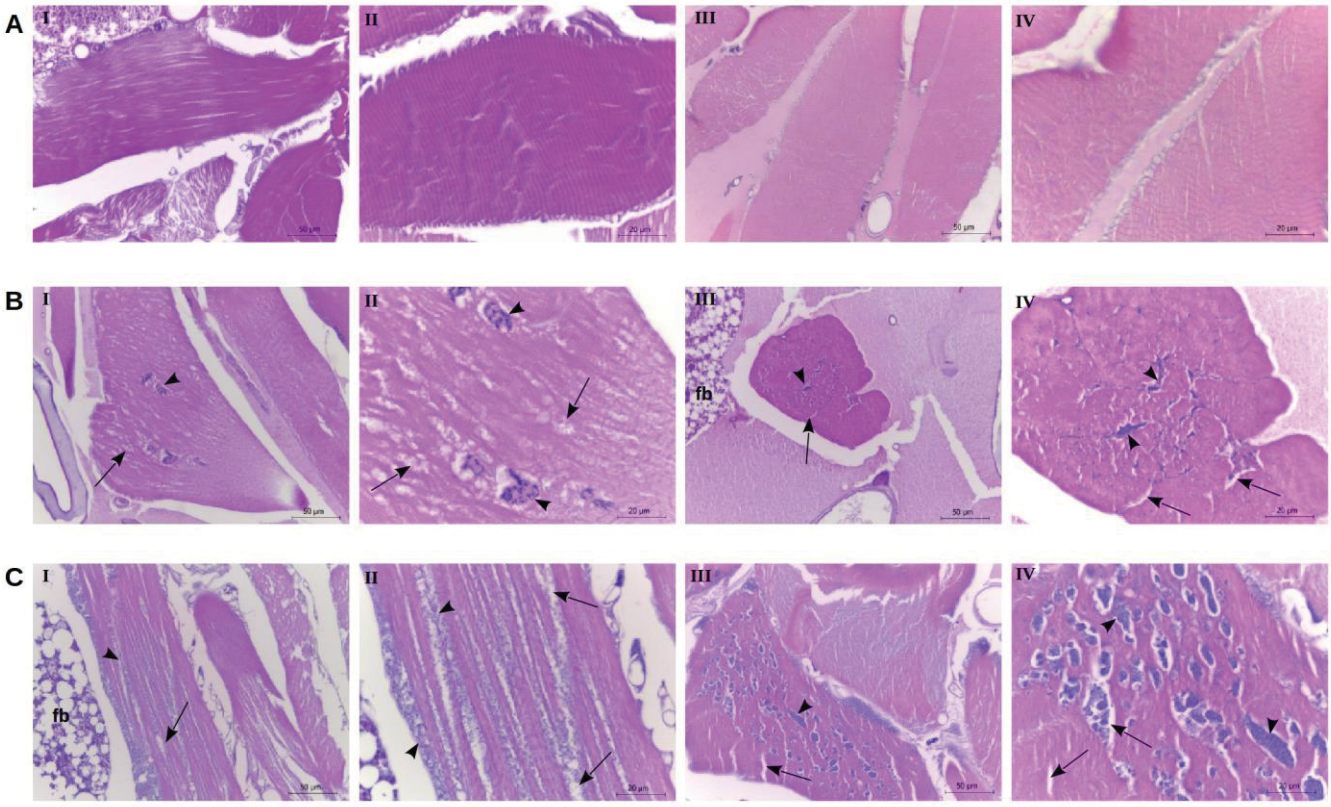
SOURCE: the author

FIGURE 9 - PHOTOMICROGRAPHS OF MALPIGHIAN TUBULES AND FAT BODY OF *Lucilia cuprina* L3. A) NORMAL CONTROL GROUPS 24H AFTER TREATMENT (ONLY ETHANOL) (40, 100X). I, II) OBSERVE THE Mts SHOWING TYPICAL MORPHOLOGY. III, IV) NOTE THE NORMAL FB TROPHOCYTES. B) MTs OF *L. cuprina* L3 24 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO (I, II) OR 0.30 µL/cm^2 OF carvone (III, IV) (40, 100X). I) NOTE THE INTENSE NECROSIS OF MTs WITH PRESENCE CELL RESIDUE DISPOSED IN THE LUMEN. II) OBSERVE THE MARKED VACUOLIZATION (ARROW) AND CYTOPLASMIC GRANULES (ARROWHEAD). III) DETAILS TO NECROSIS OF MTs WITH LUMEN CELL RESIDUE. IV) NOTE THE PRESENCE CELL RESIDUE DISPOSED IN THE LUMEN (ARROW), NECROSIS OF MTs AND CYTOPLASMIC GRANULES (ARROWHEAD). C) FB OF *L. cuprina* L3 24 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO (I, II) OR 0.30 µL/cm^2 OF carvone (III, IV) (40, 100X). I, II) OBSERVE THE FB TROPHOCYTES WITH INTENSE VACUOLIZATION (ARROWHEAD) AND PRESENCE OF HYPERCHROMATOSIS (ARROW). III, IV) NOTE THE MARKED VACUOLIZATION IN FB (ARROWHEAD). HEMATOXYLIN-EOSIN (40, 100X). ABBREVIATIONS: N, NUCLEI; L, LUMEN; fb, FAT BODY TROPHOCYTES; mt, MALPIGHIAN TUBULES; v, VACUOLE.



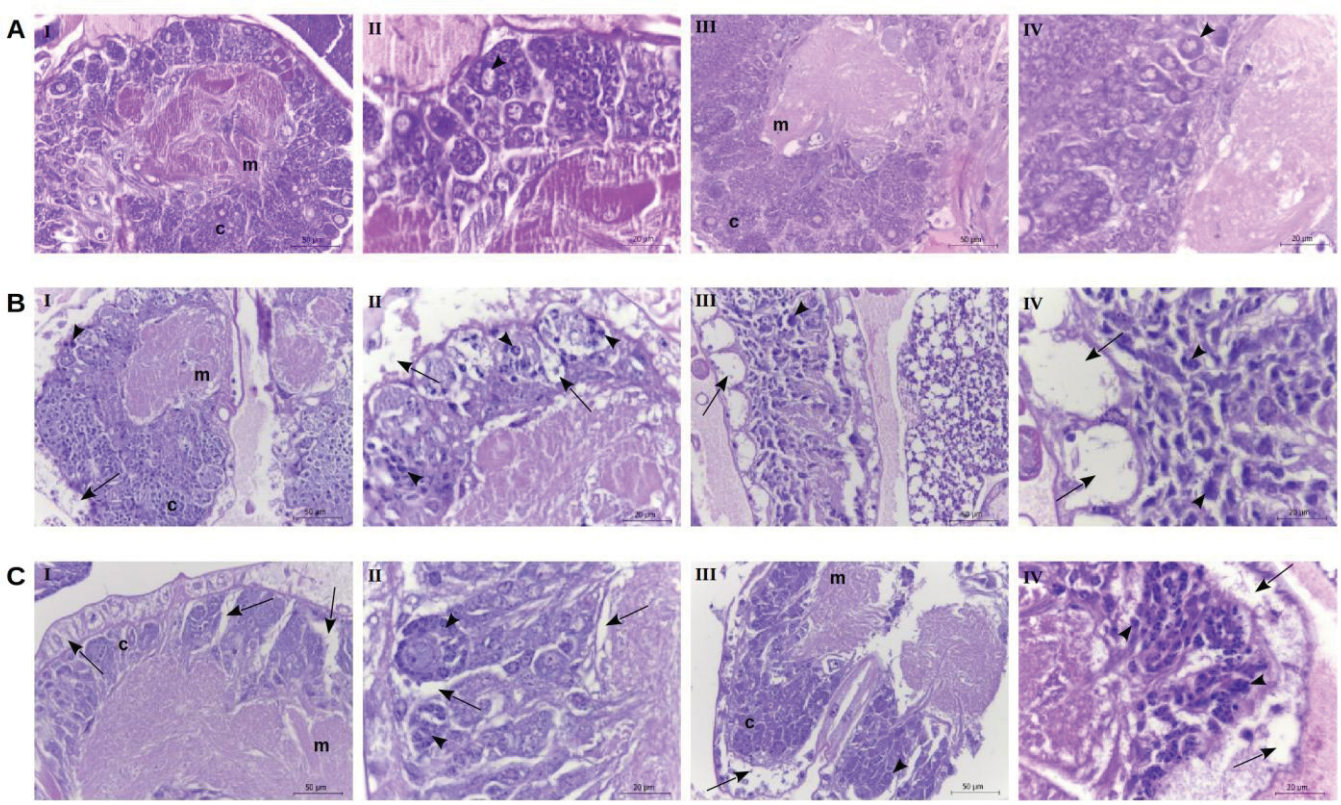
SOURCE: the author

FIGURE 10 - PHOTOMICROGRAPHS OF ANTERIOR AND POSTERIOR DIGESTIVE TRACT OF *Lucilia cuprina* L3. A) CONTROL GROUPS WITH INTACT ANTERIOR (I, II) AND POSTERIOR (III, IV) DIGESTIVE TRACT (EPITHELIAL CELLS WITH NUCLEUS AND BRUSH BORDER). NOTE THE NORMAL MORPHOLOGY OF CELLS (ARROW) (40, 100X). B) ANTERIOR DIGESTIVE TRACT OF *L. cuprina* L3 24 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO (I, II) OR 0.30 µL/cm^2 OF carvone (III, IV) (40, 100X). I, II) NOTE THE CELL RESIDUE IN THE LUMEN OF DIGESTIVE TRACT, PRESENCE OF VACUOLES IN CELL (ARROWHEAD) AND HYPERCHROMATOSIS (ARROW). III, IV) OBSERVE THE CELL RESIDUE IN THE LUMEN (ARROW) AND MARKED VACUOLIZATION IN CELL (ARROWHEAD). C) POSTERIOR DIGESTIVE TRACT OF *L. cuprina* L3 24 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO (I, II) OR 0.30 µL/cm^2 OF carvone (III, IV) (40, 100X). I, II) OBSERVE THE MARKED NECROSIS WITH LOSS OF DIGESTIVE CELL ARCHITECTURE, INTENSE VACUOLIZATION AND PRESENCE OF HYPERCHROMATOSIS (ARROW). III, IV) NOTE THE ALTERATIONS AS INTENSE NECROSIS, CELL RESIDUE IN THE LUMEN, MARKED VACUOLIZATION OF CELL (ARROWHEAD) AND HYPERCHROMATOSIS (ARROW). H & E, HEMATOXYLIN–EOSIN. ABBREVIATIONS: N, NUCLEI; L, LUMEN; v, VACUOLE; FB, FAT BODY.



SOURCE: the author

FIGURE 11 - PHOTOMICROGRAPHS OF MUSCLE OF *Lucilia cuprina* AND *Cochliomyia macellaria* L3. A) NORMAL CONTROL GROUPS 24H AFTER TREATMENT (ONLY ETHANOL) (40, 100X). I, II) MUSCLE OF *L. cuprina* L3. NOTE THE NORMAL MORPHOLOGY OF CELLS (40, 100X). III, IV) MUSCLE OF *C. macellaria* L3. OBSERVE THE NORMAL MORPHOLOGY OF CELLS (40, 100X). B) MUSCLE OF *L. cuprina* L3 24 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO (I, II) OR 0.30 µL/cm^2 OF carvone (III, IV) (40, 100X). DETAILS TO VACUOLATION (ARROW) AND ALTERATIONS SUGGESTIVE OF MINERAL ACCUMULATION (ARROWHEAD). C) MUSCLE OF *C. macellaria* L3 24 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO (I, II) OR 0.76 µL/cm^2 OF carvone (III, IV) (40, 100X). NOTE THE INTENSE VACUOLATION (ARROW) AND MARKED ALTERATIONS SUGGESTIVE OF MINERAL ACCUMULATION (ARROWHEAD). H & E, HEMATOXYLIN–EOSIN. ABBREVIATIONS: fb, FAT BODY.



SOURCE: the author

FIGURE 12 – PHOTOMICROGRAPHS OF BRAIN OF *Lucilia cuprina* AND *C. macellaria* L3. A) NORMAL CONTROL GROUPS 24H AFTER TREATMENT (ONLY ETHANOL) (40, 100X). BRAIN OF *L. cuprina* L3 (I, II) AND *C. macellaria* L3 (III, IV). NOTE THE NORMAL NEUROPIL (M) AND CORTICAL LAYER (C) WITH PRESENCE OF GLIAL CELLS (ARROWHEAD). B) BRAIN OF *L. cuprina* L3 24 H AFTER TREATMENT WITH 0.95 µL/cm^2 OF MVEO (I, II) OR 0.30 µL/cm^2 OF carvone (III, IV) (40, 100X). I, II) OBSERVE THE INTENSE VACUOLAR DEGENERATION SHOWING CLUSTERS OF VACUOLES (ARROW) AND NUCLEI WITH PYKNOTIC PROFILE. III, IV) NOTE THE INTENSE VACUOLAR DEGENERATION (ARROW) AND NUCLEI PYKNOTIC (ARROWHEAD). C) BRAIN OF *C. macellaria* L3 24 H AFTER TREATMENT WITH 1.59 µL/cm^2 OF MVEO (I, II) OR 0.76 µL/cm^2 OF carvone (III, IV)(40, 100X). I, II) DETAILS TO VACUOLIZATION IN CORTICAL LAYER (ARROW) AND NUCLEI PYKNOTIC (ARROWHEAD). III, IV) OBSERVE THE INTENSE VACUOLAR DEGENERATION (ARROW) AND NUCLEI PYKNOTIC (ARROWHEAD). H & E, HEMATOXYLIN–EOSIN. ABBREVIATIONS: m, MEDULAR LAYER; c, CORTICAL LAYER.

4 DISCUSSION

4.1 Chemical composition of *Mentha villosa* essential oil

To date, few investigations of the chemical composition of *M. villosa* essential oil have been undertaken. In a Brazilian context, it is important to note that this is the first report of this hybrid with essential oil rich in carvone (52.52%) in plants grown in the southern part of the country. In contrast, another study evaluated from the Brazilian Northeast found that the rotundifolone known as piperitenone oxide (70.96%) comprised the majority compound found in *M. villosa* essential oil, extracted from fresh leaves in June (winter) (MATOS-ROCHA et al., 2013). Similarly, these results are in agreement with GUERRA et al. (2015), who reported the value of 70.2% of monoterpene ketone (rotundifolone) in *M. villosa* EO in the same region. Other studies in Brazil's Northeast have also identified piperitenone oxide as a major compound in *M. villosa* essential oil, representing 95.87% in Ceará State and 64.4 to 72.50% in three different cities of Bahia State (LAHLOU et al., 2002; TELES et al., 2013).

LAWRENCE (2006) highlighted how similarities and differences between oil compositions found either within or between a species or hybrid shed light on the widespread occurrence of infraspecific chemical differences and the fact that composition cannot solely be used to characterize a species or hybrid. These variations can be explained by differences in climate, soil, plant age and harvest season between the regions of Brazil (Northeast and Southern), thus illustrating the potential for commercial production in Southern Brazil, as *M. villosa* cultivated in this region is rich in carvone.

Among natural products, it is important to acknowledge that monoterpene carvone is currently approved in Europe and the United States of America (USA) as active substances, with specific conclusions highlighted in this evaluation: 1) good plant protection practice and no harmful effects to human or animal health; 2) acceptable exposure scenarios for operators, workers and bystanders; 3) no unacceptable effects on the environment (US ENVIRONMENTAL PROTECTION AGENCY DATABASE, 2009; EU PESTICIDES DATABASE, 2018). In addition,

carvone can be used as an active substance in antimicrobial composition (BUCK et al., 2008), acting synergistically with antiparasitic and pest-control compositions (ENAN, 2011, 2014), and notably, this terpene had the acaricidal and insecticidal activity reported (SATO and HATA, 1993; WILLIS et al., 2014). Nevertheless, previous reports have demonstrated the capacity of carvone may be used as a potentiator to synthetic pesticide to be used in agriculture and veterinary applications (SHAH and HORSLER, 2012), and has been recommended for environmentally friendly parasiticides and insecticides (FRANZIOS et al., 1997).

Interestingly, *Mentha villosa* var. *alopecuroides* has been described as being rich in carvone in previous reports. Pooter and Schamp (1987) reported the carvone as majority compound (65.4 to 74.7%) followed of limonene (4.6 to 12.7%) and dihydrocarvone (2.4 to 4.0%), collected in three different locations in Belgium. On the other hand, KAKKINI and PAPAGEORGIOU (1987) have studied the chemical composition of *M. villosa-nervata* from Greece and highlighted two chemotypes in the same wild population: the chemotype 1 content 65.9% of piperitenone oxide and another chemotype rich in carvone (42.8%) and dihydrocarvone (15.9%). In our study, other constituents with important biological properties that have been previously described in the literature were identified. Dihydrocarveol, representing 15.39% of the chromatographic area of *M. villosa* in this work, has been described in terms of antimicrobial activity (CORNMELL et al., 2014). The terpene d-limonene (7.6%), for instance, has insecticide activity related and can also be used in pesticidal compositions to augment the penetration of other active substances (MUEHLEBACH et al., 2011; SHAH and HORSLER, 2012; CREEMER et al., 2013).

Accordingly, minor molecules such as cis-carvyl acetate (3.68%), beta-caryophyllene (2.31%) and trans-carveol (1.76%) have been described as active substances in antimicrobial, repellant and ectoparasiticidal composition (EINI and TAMARKIN, 1995; GANS et al., 2003; ARNDT, 2014; CORNMELL et al., 2014; WILLIS et al., 2014). In addition to the innumerable biological properties of carvone described, the other constituents of *M. villosa* EO can improve insecticidal activity as well as the lesions caused by myiasis.

4.2 Larval toxicity and physiological parameter changes

The guide published by the Working Party about the Efficacy of Veterinary Medicines (EUROPEAN COMMISSION III/3682/92-EN) indicated that the efficacy of insecticides for Diptera species should be between 80 and 100%, and preferably greater than 90%. The efficacy of the application of MVEO and its major compound, carvone, met these criteria in contact assays against *C. macellaria* and *L. cuprina*. Even, results of efficacy showed in this work are higher than previous reports of contact assays on blowflies L3 using some EO from Brazil, including *B. dracunculifolia*, *Tagetes minuta*, *Piper gaudichaudianum* and *C. longa* (CHAABAN et al., 2017a, b, c; CHAABAN et al., 2018).

Some botanical species with chemical compositions rich in carvone have been reported regarding insecticidal activity in different taxonomic groups of insects. In this sense, previous reports have indicated that botanical species rich in carvone have considerable insecticidal activity in low doses, and act in terms of physiological parameters such as adult emergence inhibition, as clearly demonstrated in our work. BENELLI et al. (2018) investigated the acute and sub-lethal toxicity of *Mentha spicata* (58.2% of carvone and 22.5% of limonene) and *Lippia alba* (35.2% of carvone, 32% of limonene and 14.8% of germacrene D) essential oil, both rich in carvone, against fourth instar larvae of *Culex quinquefasciatus* and adults of the housefly *Musca domestica*, 24 hours after application. The exposure of mosquito larvae demonstrated lethal doses of LD_{50} 59.6 µl L^{-1} to *L. alba*, while a LD_{50} of 88.2 µl L^{-1} was reported when using *M. spicata* essential oil. In contrast, contact assays on adult houseflies *M. spicata* EO demonstrated greater toxicity than *L. alba* with a LD_{50} of 88.2 and 115 µg $adult^{-1}$, respectively (BENELLI et al., 2018). Similarly, the larvicidal activity of essential oils from Mentha species rich in carvone such as *M. spicata* (57.4%), *M. piperita* (56.8%) and *M. villosa* (67.9%) on the third instar larvae of *C. quiquefasciatus* was reported by PAVELA et al. (2014), exhibiting LC_{50} of 111, 141 and 134 mg/l, respectively.

In this way, the efficacy of *M. spicata* EO containing 59.6% of carvone against the insect pest *Callosobruchus chinensis* was demonstrated in fumigation tests (dose of 0.100µl/ml), acting in physiological parameters with results including 98.46% oviposition deterrence, 100% ovicidal activity, 88.84% larvicidal activity (third

to fourth instar larvae) and 72.91% pupaecidal activity (KEDIA et al., 2014). Reports have also evaluated the toxicity of essential oils from different *L. alba* genotypes (carvone chemotypes LA-13 and LA-57) against insect pests of stored grains *Sitophilus zeamais* and *Tribolium castaneum* as well as the tick *Rhipicephalus microplus* (Peixoto et al., 2015a, b). *Lippia alba* chemotype LA-13 (52.94% of carvone) and chemotype LA-57 (63.47% of carvone) showed values of LC_{50} of 15.2 and 16.7μL mL^{-1} of *S. zeamais*, 28.7 and 19.5μL mL^{-1} of *T. castaneum*, and values of 16.8 and 27.0 mg/mL via larval packet test (LPT) for *R. microplus* in chemotypes LA-13 and LA-57, respectively (PEIXOTO et al., 2015a, b). Interestingly, previous studies of insecticidal activity of carvone provided results indicating the superior efficacy of this monocyclic monoterpene ketone than of a complex mixture (EO) containing carvone as the majority compound, corroborating the results of this paper. The fumigant toxicity of (+)-carvone, (−)-carvone and dihydrocarvone was assessed against adults of *S. oryzae* with LC_{50} values of 0.61, 0.84, 2.92 mg/L air, respectively (KIM et al., 2013). Similar results were reported in another assay of contact toxicity after 24 h against *S. zeamais* adults, indicating LC_{50} values of 17.6, 28.1 and 30.5 μL/L air to (R)-(−)-carvone, (S)-(+)-carvone and dihydrocarvone, respectively (Herrera et al., 2015). Carvone was active against fourth instar larvae of *C. pipiens* with values of LC_{50} of 132mg/L and showed a mortality rate of 96.7% 48h after treatment in contact tests against adults using 50mg/filter paper/L. Furthermore, when tested at sub-lethal doses, (R)-carvone, (S)-limonene reduced hatchability, pupation and adult emergence and induced high larval mortality (ZAHRAN and ABDELGALEIL, 2011).

The present study also indicated the higher level of toxicity of carvone than MVEO to both blowflies, *C. macellaria* and *L. cuprina*, and a greater toxicity to ovine flystrike *L. cuprina* L3. In this sense, it is worth noting that carvone was tested for acute toxicity in rats, demonstrating LD50 of >2000 and >4000 mg/kg in oral and dermal evaluation, respectively, and thus its safety in application to mammals via the two administration routes (EUROPEAN COMMISSION, 2008, 2015). Thus, this compound, as well as botanical species rich in this terpene ketone can be used to safely control myiasis in topical application. This study has reported the doses to control blowflies as being 0.3057 and 0.611 μL/cm^2 to *L. cuprina* and *C. macellaria*, respectively, having 100% emergence inhibition.

4.3. Morphological damage - Biomarkers of toxicity in *Cochliomyia macellaria* and *Lucilia cuprina*

4.3.1. Macroscopic cuticle damage

The effects of EO on the cuticles of blowfly larvae have been previously reported following the use of different botanical species in biological *in vitro* assays. Macroscopic cuticle damage including cuticle softening and color changes, reduced motility, progressive darkening and dryness of the larval cuticle have been identified using *Tagetes minuta* EO, and were reported in contact assays against *C. macellaria* and *L. cuprina* L3 (CHAABAN et al., 2017; CHAABAN et al., submitted in 2017). In the same way, black pigmentation on the anterior end and cuticle abnormalities in *L. cuprina* and *C. macellaria* L3 were observed few hours after contact with *Piper gaudichaudianum* and *Baccharis dracunculifolia* EO, respectively (CHAABAN et al., 2017, 2018). Similarly, KHATER (2011) has studied the insecticidal activity of Egptyan EO against *L. sericata* and has reported larval abnormalities such as diminished size, weak cuticle with ulceration and diffuse brown pigment. In another study using *Commiphora molmol* and *Balanites aegyptiaca* EO against *L. sericata*, larvae also exhibited morphological abnormalities (HODA et al., 2016). These results support the alterations observed in our work, regarding L3 of both species of flies treated with MVEO and carvone, such as marked darkening at segmental spinules, tracheal branches and the respiratory spiracle, as well as dryness on the larval body.

4.3.2 Scanning electron microscopy

Damage caused in specific larval structures can be assessed through SEM, displaying the target organs of biopesticides. Until now, few investigations of insecticidal activity and blowfly larvae have been undertaken that use ultrastructure assessment following treatment with EO and individual compounds. We consider that this paper is the first to report of ultrastructural damage in L3 of blowflies after the toxic contact of MVEO and carvone. Notably, in recent reports some alterations such as dryness on the cuticle surface, distortion of the sensory structures (antenna sensory papillae) and maxillary lobe, as well as a slight distortion of the anal papillae

and spiracle plate were demonstrated in *in vitro* assays against *C. macellaria* and *L. cuprina* in contact tests using *C. longa* EO and *α*-phellandrene (CHAABAN et al., submitted in 2018). Similarly, cuticle damage (distortion of the sensorial structures and slight degeneration of the anterior spiracle) was reported using 32% of camphor oil in dipping assays against *L. sericata* L3 (SHALABY et al., 2015). Similarly, the ultrastructural assessment showed degeneration in the anterior spiracles, ventral and dorsal papillae of *L. sericata* L3 using ingestion assays with *Commiphora molmol* EO (HODA et al., 2016). Although few studies were conducted using SEM, our results shed light on the use of MVEO and carvone in terms of marked damage to sensorial structures, dryness on the cuticle surface and extreme degeneration of the larvae.

4.3.3 Larval histopathology

Cytotoxicity in target cells of insects such as MTs, the digestive tract, fat body and brain can be used as biomarkers, to determine of chemical detoxification, in ecotoxicology studies as well as to assist in the elucidation the mode of action of biopesticides (NOCELI et al., 2016; CHAABAN et al., 2018). For example, MTs act in the transportation of organic substances, the body's defense system, and chemical detoxification processes, and are considered analogous to the nephridia of annelids or the kidneys of vertebrates (NOCELLI et al., 2016). In this sense, they can be considered key organs as biomarkers to clarify the effect of new biopesticide candidates at the cellular level. Notably, this is the first study with morphological data regarding the MTs of blowflies (*C. macellaria* and *L. cuprina*) treated with MVEO and the monoterpene ketone carvone. Similar results regarding cellular damage in MTs have been found in previous reports, including the pyknotic nuclei, the loss of part of the cell in the lumen, and the presence of cytoplasmic vacuolization in the MTs of Africanized bees (*Apis mellifera*) when exposed to sub-lethal doses of imidacloprid (ROSSI et al., 2013a). In the same way, evidence in brush border of MTs, presence of cellular content in the lumen, a few nuclei with condensed chromatin and vacuolization were observed following treatment with continuous doses of boric acid and fipronil in worker bees (*Scaptotrigona postica*) (FERREIRA et al., 2013). MTs with cytoplasmic vacuolation were also observed in adult workers of *Atta sexdens rubropilosa* treated with different doses of boric acid (0.2 and 0.5%) (SUMIDA et al.,

2010). Likewise, progressive damage including the disruption of the cytoplasm and the basal labyrinth, and loss of cytoplasmic organelles of MTs were reported as cytotoxic effects of thiamethoxam in *A. mellifera* (CATAE et al., 2014). In another study, the toxicological and histopathological effects of hydramethylnon on *Atta sexdens rubropilosa* (workers) were observed using 200 mg/mL of chemical compound (hydramethylnon) and revealed histological alterations on MTs such as nuclei with pyknosis and vacuolated cytoplasmic regions (DECIO et al., 2013). Insect fat body represents another organ involved in the detoxification, storage and neutralization of non-utilized substances for the insect, being compared to the liver of vertebrates (ROMA et al., 2010). A recent report has assessed the histological alteration of the fat body of Africanized honeybees exposed to thiamethoxam at a concentration of 0.001 ng a.i./µL. Damage including trophocytes with more branched nuclei, oenocytes with atypical morphology, and the presence of cytoplasmic vacuolization were noted (DOMINGUES et al., 2017). Other alterations including protein-positive granules demonstrating the strongest reaction to the presence of protein in the fat body cells of *Culex quinquefasciatus* larvae were observed following exposure to ivermectin (ALVES et al., 2010). In this work, vacuolization in the cell and the accumulation of eosinophilic material, suggestive of protein globules, were also observed in the treated larval fat body of both types of blowflies, implying that these changes are a common response to cellular intoxication. Similarly, some alterations observed in the digestive tract in this paper including the loss of digestive cell architecture, presence of cell residue in the lumen, intense necrosis, presence of vacuoles in cell and pyknotic nuclei have been reported in other studies against *L. cuprina* and *C. macellaria* L3 (CHAABAN et al., 2018; CHAABAN et al., submitted in 2018). Morphological alterations such as cytoplasmic vacuolization, absence of autophagy vacuoles, and chromatic compacting induced by 7.5 mg/g boric acid were observed in the midgut of worker honeybee larvae (Cruz et al., 2010). In the same way, OLIVEIRA et al. (2014) reported some cytotoxic changes including cytoplasm vacuolization, increased apocrine secretion and cell elimination in *A. mellifera* midgut following treatment with sub-lethal doses of thiamethoxam. Similar effects were shown by LAVARÍAS et al. (2017) in the anterior midgut epithelium of *Chironomus calligraphus* larvae treated with 0.037 µg/L and 0.075 µg/L of cypermethrin. Changes such as the vacuolization of the cytoplasm and a slight disorganization were noticed, while a degenerative vacuolar hypertrophy and complete disorganization of the

epithelium were observed using the higher dose of 0.3 µg/L. Morphological damage in the digestive tract induced by natural biopesticide candidates have also been reported in different insect species. Third instar larvae of *Anticarsia gemmatalis* fed on diets containing 500 ppm of Neem extracts displayed morphological changes in the midgut including the complete destruction of cells in some regions, swollen and detached from the basal membrane, epithelium midgut atrophy and narrowed lumen (ALMEIDA et al., 2014). Likewise, histological changes in the midgut such as irregular epithelium with high vacuolation in the cytoplasm and secretory vesicles expelled from the epithelium to the lumen were found after feeding *Anticarsia gemmatalis* larvae with squamocin, a natural product (FIAZ et al., 2018). More recently, cytotoxicity in the digestive tract following contact with essential oils was reported in blowflies L3. Significant changes such as a loss of the architecture of the digestive tract using *T. minuta* EO on *L. cuprina* were noticed (CHAABAN et al., submitted in 2017). Moreover, vacuolization in the cytoplasm, pyknotic nuclei and necrosis of the digestive tract were seen in the digestive tract following the use of *C. longa* EO and *α*-phellandrene in the same fly (CHAABAN et al., 2018). Some authors have emphasized that analysis of muscle layers cannot be overlooked in the evaluation of cytopathological effects on the midgut of the insect as a critical component of the functioning of this organ (SCUDELER et al., 2017; FIAZ et al., 2018). In this sense, a recent report has demonstrated that natural products such as Neem oil cause alterations at the cellular level (visceral muscle layer). Such results also indicate that Neem oil intake by *Ceraeochrysa claveri* is cytotoxic to muscle fibers of the midgut, which compromises the functioning of this organ (SCUDELER et al., 2017). Nevertheless, alterations to the muscle such as loss of muscle tone and a reduction in gut motility were observed after azadirachtin treatment, the predominant component of Neem oil (MORDUE et al., 1985; MORDUE and NISBET, 2000). These studies highlight the importance of observing signs of injury in this organ (muscle) caused by plant-derived treatments including intense vacuolation and marked mineral accumulation. Interestingly, the brain of blowflies L3 represented another target organ reported, with significant alterations following contact with MVEO and carvone such as intense vacuolar degeneration and nuclei with pyknotic profile. Similar results suggesting the neurotoxicity of sublethal doses of some pesticides has been demonstrated in previous reports. JACOB et al. (2015) have highlighted histological damage on the mushroom bodies of the brains of the

stingless bee *Scaptotrigona postica* following the use of sublethal doses of fipronil through oral and topical treatments. Results such as Kenyon cells with pyknotic profiles, suggesting cell death, were observed, and the differences in the number of pyknotic profiles noted were dose- and time-dependent. The side effects of sublethal doses of thiamethoxam to the brain of *Apis mellifera* have also been reported by previous authors. The presence of condensed cells in the brain (OLIVEIRA et al., 2013), condensed cells and early cell death in the optic lobes in acute and subchronic exposure to thiamethoxam have also been identified (TAVARES et al., 2015). In the same way, the exposure of *A. mellifera* to sublethal doses of imidacloprid indicated the presence of strongly stained nuclei visualized during histochemical and morphological analysis, implying the occurrence of cell death in Kenyon cells of exposed bees (ROSSI et al., 2013b). Finally, histological analysis using green pesticide candidates (*Curcuma longa* EO and *α*-phellandrene) displayed relevant alterations in *C. macellaria* and *L. cuprina* L3 brains, as condensation of nuclear chromatin, pyknotic profiles and vacuolar degeneration (CHAABAN et al., 2018; CHAABAN et al., submitted in 2018). Thus, these results stimulate us to believe that MVEO and carvone have neurotoxic activity when using lower doses than in previous reports of blowflies.

5 CONCLUSIONS

This work demonstrates that MVEO and carvone have a remarkable insecticidal activity in low doses, and can be applied to control blowflies. In addition, the cytotoxic effects for target organs such as the MTs, fat body, digestive tract, muscle and brain of blowflies, can be used as biomarkers of toxicity in the future. The damage observed to the target organs indicates evidence of cytotoxicity in larvae exposed to both extracts evaluated. Finally, we emphasize that this work might influence new investigations regarding the seasonality of *M. villosa* (rich in carvone) and studies of biochemical biomarkers to elucidate the mode of action of these ecofriendly insecticides.

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5 - SUPPLEMENTARY MATERIAL

BIOCHEMICAL BIOMARKERS ASSESSMENT OF BLOWFLIES EXPOSED TO SUB-LETHAL DOSES OF *Curcuma longa* AND *Mentha villosa* ESSENTIAL OILS AND THEIR MAJOR COMPOUNDS

ABSTRACT

Contact assays of sub-lethal doses of CLLEO, MVEO and their major compounds (α-phellandrene and carvone) were performed in *Cochliomyia macellaria* and *Lucilia cuprina* third instar larvae aiming to biochemical biomarkers assessment. The neurotoxicity marker (AChE), lipoperoxidation response and the oxidative stress markers (CAT and SOD) were measured in L3 after 6 h of exposure. Essential oils and their major compounds exhibited significant impairment ($P<0.05$) cellular oxidative stress marker (SOD) activity. Significant differences with increase of level of LPO were observed in larvae of *L. cuprina* exposed to CLLEO. Decrease in catalase activity were noticed when *C. macellaria* larvae were exposure to MVEO. An increase in AchE values suggesting larval stress after contact with the all extracts. The data from the biochemical biomarkers suggest that all extracts induced some level of oxidative stress over the L3. These data may be used better describe the mechanism of action of the extracts.

RESUMO

Ensaios de contato utilizando doses subletais de CLLEO, MVEO e seus principais compostos (α-felandreno e carvone) foram realizados em larvas de terceiro estádio de *Cochliomyia macellaria* e *Lucilia cuprina* visando à avaliação bioquímica de biomarcadores. O marcador de neurotoxicidade (AChE), a resposta de lipoperoxidação e os marcadores de estresse oxidativo (CAT e SOD) foram mensurados em L3 após 6 h de exposição. Os óleos essenciais e seus principais compostos exibiram comprometimento significativo($P<0.05$) na SOD celular. Diferenças significativas com o aumento do nível de LPO foram observadas em larvas de *L. cuprina* expostas ao CLLEO. Diminuição na atividade de catalase foi observada quando as larvas de *C. macellaria* foram expostas ao MVEO. Houve um aumento nos valores da enzima AchE que sugere estresse larval após contato com

os extratos. Os resultados dos biomarcadores bioquímicos também sugerem que os extratos avaliados induziram estresse oxidativo 6 horas após a exposição. Estes dados poderão ser utilizados para melhor descrever o mecanismo de ação dos extratos avaliados.

Keywords: Acetylcholinesterase, Calalase, Lipid Lipoperoxidation, superoxide dismutase.

1 EXPERIMENTAL

1.1 Plant material, essential oil extraction, chemical characterization and origin of the flies

This items were described in material and methods section of chapters 1, 2 and 3.

1.2 Effect of sub-lethal of *EOs and its major compounds on biochemical responses in blowflies*

1.2.1 Dilution of extracts and contact applications

Contact assays of sub-lethal doses of CLLEO (0.31 and 0.63μL/cm^2), MVEO (0.31 and 1.27μL/cm^2), *α*-phellandrene (0.30 and 0.59μL/cm^2) and carvone (0.15 and 0.30μL/cm^2) were performed in *Cochliomyia macellaria* and *Lucilia cuprina* third instar larvae (L3). CLLEO and MVEO were diluted in absolute ethanol or acetone as well as *α*-phellandrene and carvone were solubilized only in ethanol. Ethanol and acetone were used with control group. All treatments were performed in triplicate (n = 10). The neurotoxicity marker (AChE), lipoperoxidation response and the oxidative stress markers (CAT and SOD) were measured in the *C. macellaria* and *L. cuprina* (L3) exposed to sub-lethal doses of extracts after 6 h of exposure.

1.3 Preparation of enzymes extract

The samples were homogenized in Milli-Q water, and centrifuged at 12,000 x g for 1 minute at 4°C. The aliquots to each biomarker were stored at -80°C freezer. Biochemical assays were performed in triplicate and measured in a microplate

spectrophotometer. Total proteins were quantified following BRADFORD, (1976) with 10 µl of sample and 250 µl of Bradford's reactive diluted in Mili-Q water. The assay was measured at 620 nm.

1.3.1 Lipid peroxidase activity (LPO)

Lipoperoxidation analysis (LPO) shows damage at cellular membrane the detection followed JIANG et al., (1992). Samples were diluted in methanol (1:1 v/v), centrifuged at 5000 x g for five minutes at 4°C. Supernatant (100 µl) plus 900 µl of Fox solution (orange xylenol 0.1 mM, BHT 4 mM (butylated hydroxytoluene), ferrous ammonium sulfate 2.5 mM and sulfuric acid 25 mM diluted in methanol) were incubated for 30 minutes (dark) and measured at 570 nm.

1.3.2 Acetylcholinesterase activity (AchE)

Acetylcholinesterase activity (AChE) was measured to evaluate neurotoxic effects. The protocol followed ELLMAN et al., (1961), modified to microplates by SILVA DE ASSIS, (1998). The assay was carried out with 25 µl of sample, 200 µl of DTNB (5,5-Dithio-bis-2nitrobenzoic acid/Na phosphate, 0.75 mM) and 50 µl of ATC (acetylthiocholine iodide, 10 mM). The reaction was incubated for 30 min and the kinetic was read for five minutes with a read at each minute at 405 nm.

1.4 Measurement of oxidative stress markers

1.4.1 Catalase activity (CAT)

CAT assay was performed following AEBI, (1984) using 5 µl of sample, 295 µl of reaction solution 80 mM (Tris base buffer 1 M/EDTA (Ethylenediamine tetraacetic acid) 5 mM pH 8.0, hydrogen peroxide 30% and Milli-Q water). The measure was recorded each 15 seconds for five minutes at 240 nm.

1.4.2 Superoxide dismutase activity (SOD)

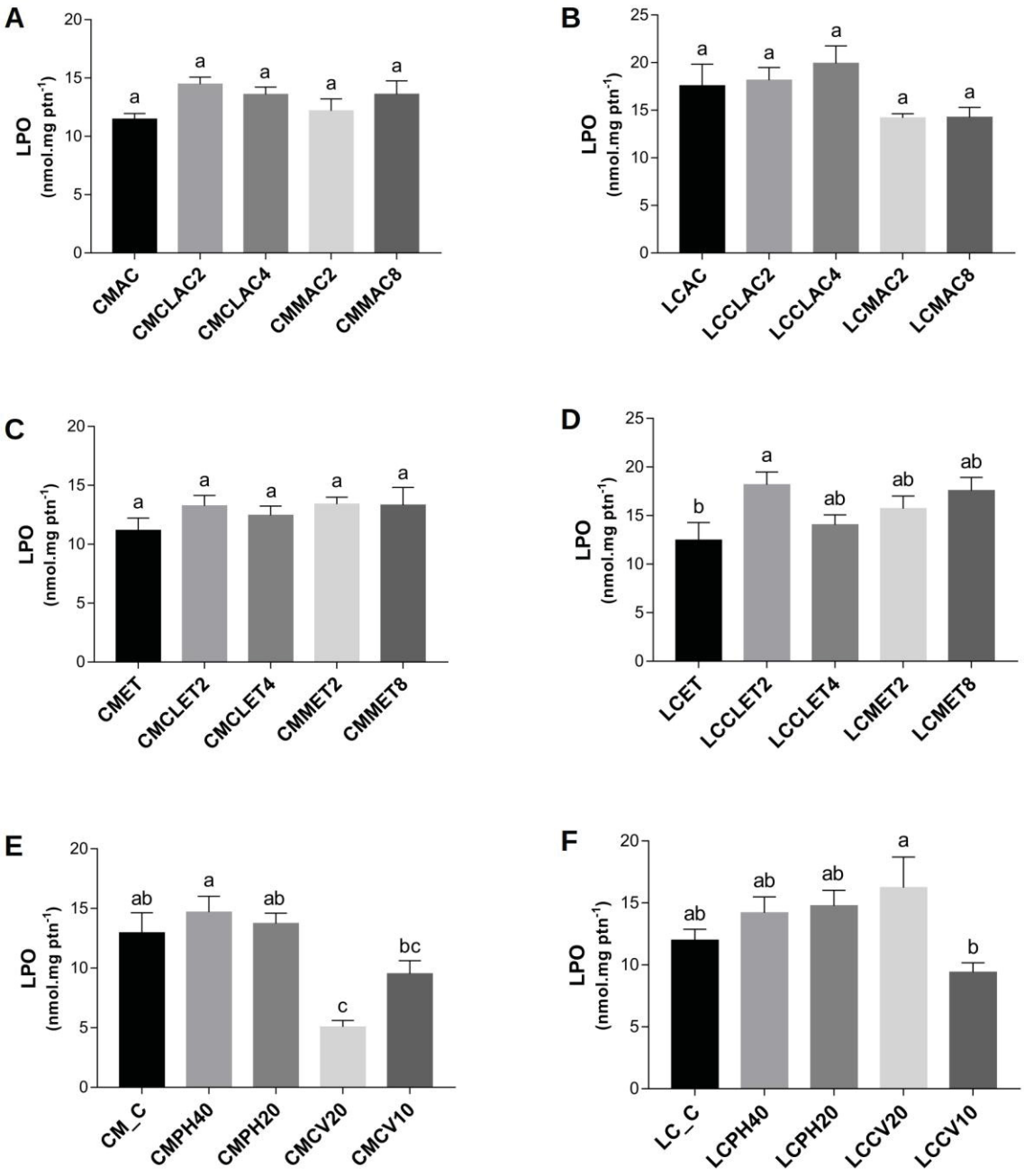
SOD activity assay followed GAO et al., (1998), 40 µl of samples and 885 µl of buffer (Tris base buffer 1 M/EDTA (Ethylenediamine tetraacetic acid). After mixing, 50 µl of pyrogallol were added and incubated for 30 minutes (dark). It was added 25 µl of HCl 1 N to interrupt the reaction. For each samples a control (without incubation) was carried out. Measurements were performed at 440 nm.

1.5 Statistical Analysis

The analysis of variance (ANOVA) in generalized linear model, assuming a Poisson distribution. The averages were compared using the Tukey test. All analyses were performed using the statistical software SPSS (2013), considering the significance level of 5%.

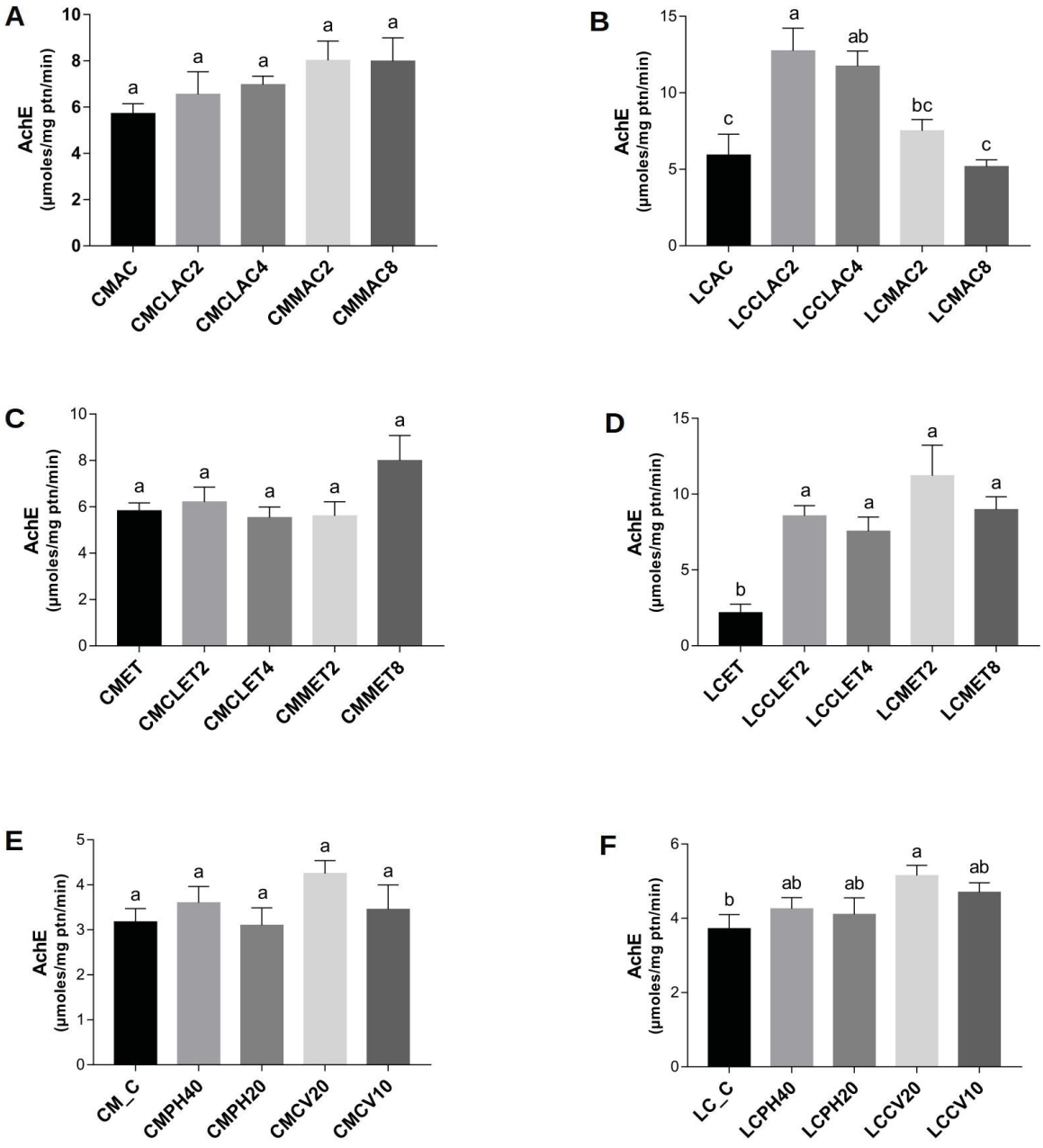
RESULTS

Essential oils (CLLEO and MVEO) and their major compounds exhibited significant impairment in cellular oxidative stress marker (LPO) level (Figure 1). A significant increase in LPO levels compared to control were observed in *C. macellaria* L3 using MVEO (1.27µL/cm^2) and carvone (0.15 µL/cm^2). Likewise, the use of both doses of *α*-phellandrene (0.30 and 0.59 µL/cm^2) also exhibited increase in LPO levels (Figure 1 – A, C, E). In contrast, *L. cuprina* showed significant increase in LPO levels only using the highest dose of carvone (0.35 µL/cm^2) (Figure 1 – F). Although no significant differences were observed in AchE activity, increase at enzime activity was noticed in sub-lethal doses of 0.31 µL/cm^2 and 0.63µL/cm^2 of CLLEO (Figure 2). Similary, MVEO at 0.31 µL/cm^2 and 1.27 µL/cm^2 showed increase on AchE activity, when compared with control groups, suggesting larval stress after contact with the extracts evaluated. No significant differences were observed in CAT and SOD activity in the *C. macellaria* and *L. cuprina* (L3) exposed to sub-lethal doses in both extracts after 6 h of exposure (short-exposure) (Figure 3 and 4).



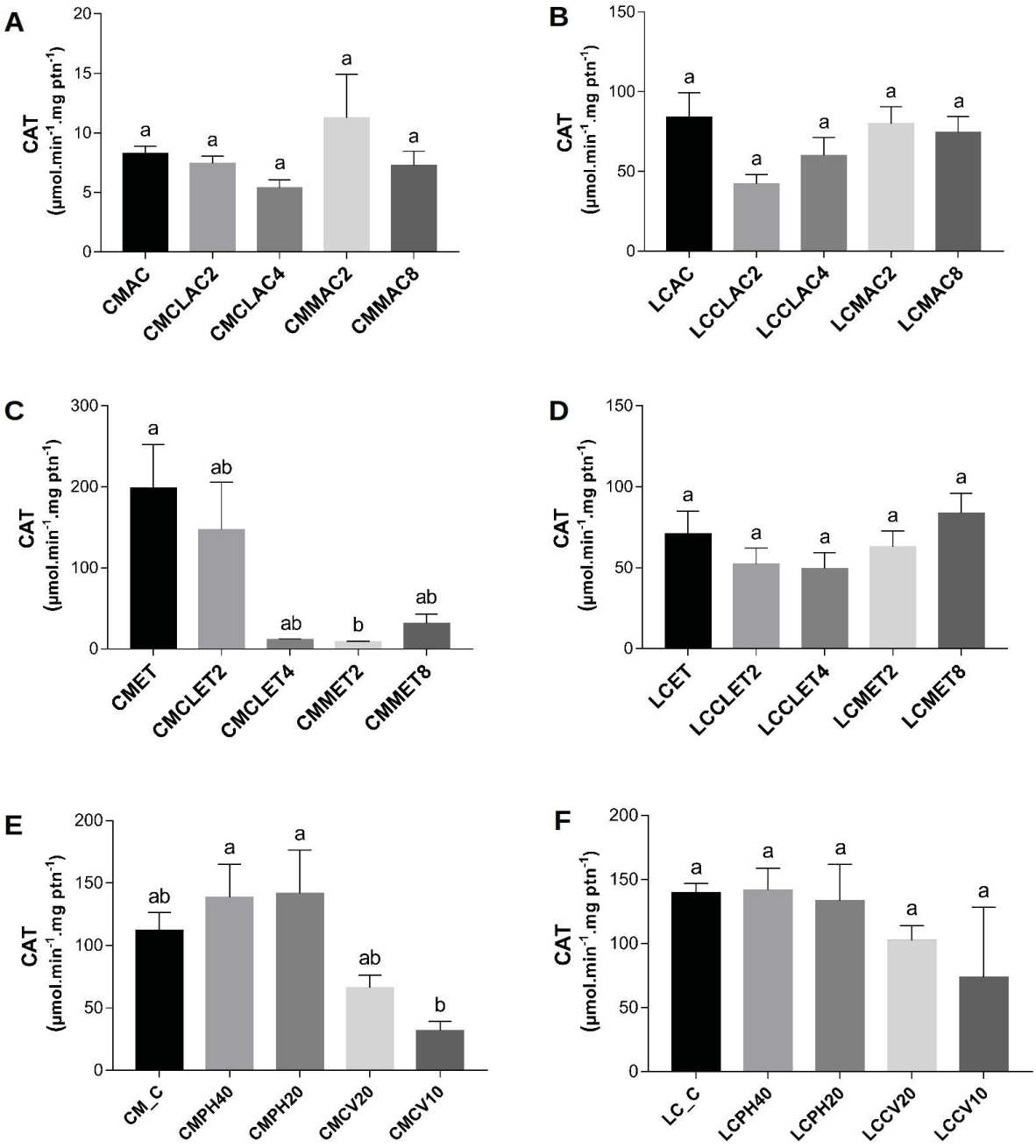
SOURCE: the author

FIGURE 1 - EFFECT OF DIFFERENT CONCENTRATIONS OF CLLEO AND MVEO AND ITS MAJOR COMPOUNDS, *α*-phellandrene AND CARVONE OF LPO lEVEL ON *Cochliomyia macellaria* (A, C, E) AND *Lucilia cuprina* (B, D, F) L3. THE MEAN FOLLOWED BY SAME LETTER IN THE BAR DIAGRAM ARE NOT SIGNIFICANT ACCORDING ANOVA AND TUKEY'S MULTIPLE COMPARISION TESTS.



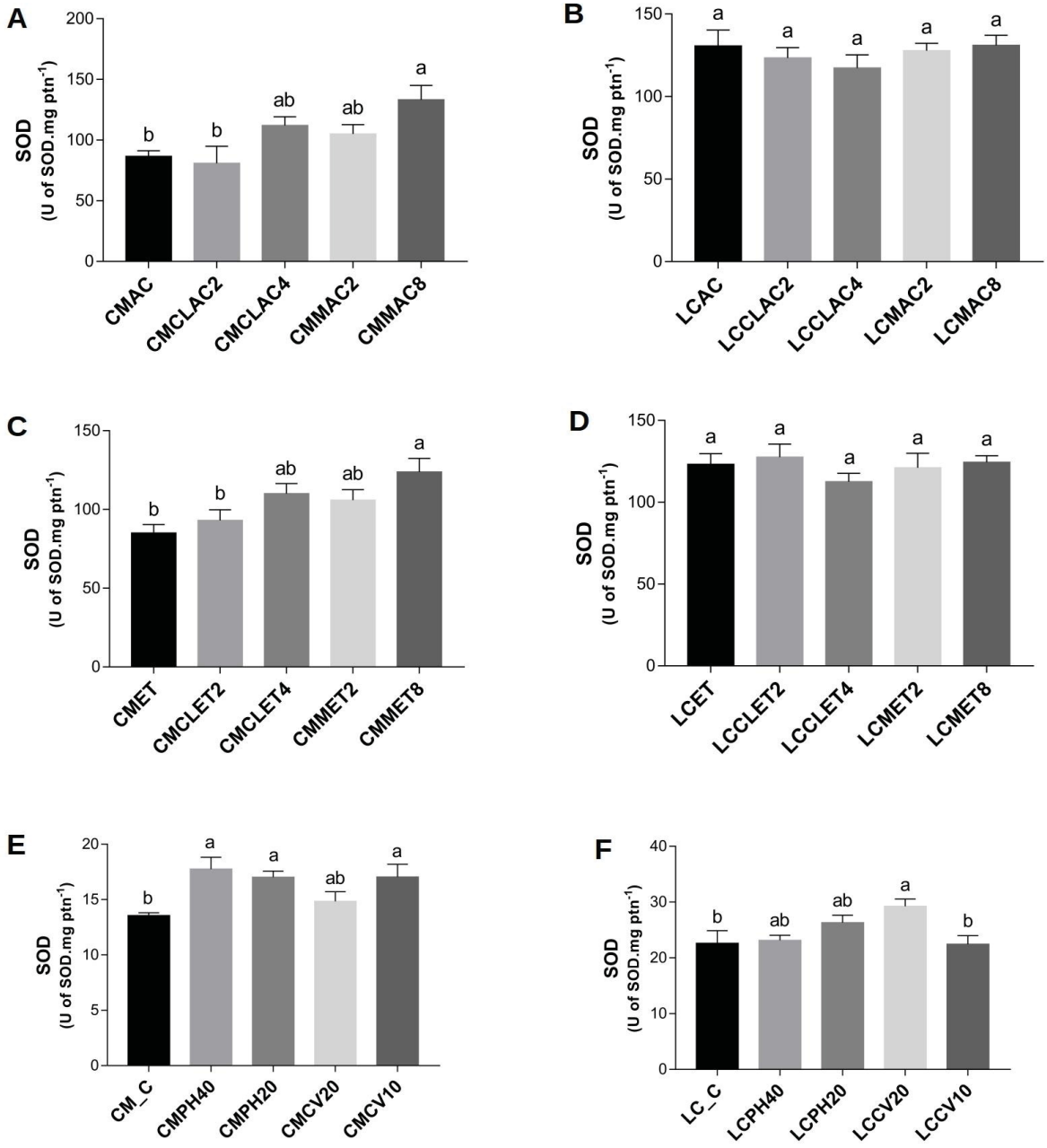
SOURCE: the author

FIGURE 2 - EFFECT OF DIFFERENT CONCENTRATIONS OF CLLEO AND MVEO AND ITS MAJOR COMPOUNDS, *α*-phellandrene AND carvone OF AChE ACTIVITY ON *Cochliomyia macellaria* (A, C, E) AND *Lucilia cuprina* (B, D, F) L3. THE MEAN FOLLOWED BY SAME LETTER IN THE BAR DIAGRAM ARE NOT SIGNIFICANT ACCORDING ANOVA AND TUKEY'S MULTIPLE COMPARISION TESTS.



SOURCE: the author

FIGURE 3 - EFFECT OF DIFFERENT CONCENTRATIONS OF CLLEO AND MVEO AND ITS MAJOR COMPOUNDS, *α*-phellandrene AND carvone OF CAT ACTIVITY ON *Cochliomyia macellaria* (A, C, E) AND *Lucilia cuprina* (B, D, F) L3. THE MEAN FOLLOWED BY SAME LETTER IN THE BAR DIAGRAM ARE NOT SIGNIFICANT ACCORDING ANOVA AND TUKEY'S MULTIPLE COMPARISION TESTS.



SOURCE: the author

FIGURE 4 - EFFECT OF DIFFERENT CONCENTRATIONS OF CLLEO AND MVEO AND ITS MAJOR COMPOUNDS, *α*-phellandrene AND carvone OF SOD ACTIVITY ON *Cochliomyia macellaria* (A, C, E) AND *Lucilia cuprina* (B, D, F) L3. THE MEAN FOLLOWED BY SAME LETTER IN THE BAR DIAGRAM ARE NOT SIGNIFICANT ACCORDING ANOVA AND TUKEY'S MULTIPLE COMPARISION TESTS.

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6 FINAL REMARKS

Currently, the search for food security through the consumption of food originated from agroecological and/or organic systems that aim animal welfare, has been growing exponentially. In this sense, sustainable natural alternatives to “one health”, human, animal and environmental balance are necessary in order to minimize impacts on these three important pillars. Unfortunately, myiasis is still a neglected pathology, sometimes by producers who use synthetic insecticides without any criterion, sometimes even by the regulatory agencies of these products, neglecting inspection.

The need for new control alternatives is present and studies related to new biopesticides have been gaining strength in recent decades. This thesis advances in this direction. We report here, for the first time, the insecticidal activity of CLLEO, MVEO and its major compounds on flies causing myiasis and accessed the biochemical, ultrastructural, cytotoxicity and macroscopic biomarkers on treated insects.

However, much remains to be done. Some challenges must be considered in researches in order to move towards ecofriendly insecticide products, such as: 1. biochemical assessment of sub-lethal doses in different time of exposition; 2. *in vivo* assays; 3. seasonality investigation of the compounds *α*-phellandrene and carvone in CLLEO and MVEO to atempt the best growth and harvest timing; 4. effects on fly reproduction and longevity; 5. synergistic investigation for new biopesticides formulations; 5. nanotecnology.

The road to sustainability and regional development are to toward to use of native plants with spontaneous growth since, for the most part “the plant you need is born in your yard”. The Mother Nature gives us all tools, we just have to open our eyes to see. The goal of this thesis is to make way in this direction.

Our greatest challenge is still within ourselves, in our consciousness, in our perception of the world in which we live in and in the exercise of putting ourselves in the animal's place. With this simple practice, we may one day get into balance with all beings around us.

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VITA

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Insecticide activity of *Curcuma longa* (leaves) essential oil and its major compound *α*-phellandrene against *Lucilia cuprina* larvae (Diptera: Calliphoridae): Histological and ultrastructural biomarkers assessment

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ABSTRACT

Lucilia cuprina, known as the Australian blowfly, is of high medico-sanitary and veterinary importance due to its ability to induce myiasis. Synthetic products are the most frequent form of fly control, but their indiscriminate use has selected for resistant populations and accounted for high levels of residues in animal products. This study aimed to assess the effect of essential oil from leaves of *Curcuma longa* (CLLEO), and its major compound *α*-phellandrene against L. *cuprina* L3. An additional goal was to determine the morphological alterations in target organs/tissues through ultrastructural assessment (SEM) and light microscopy, as well as macroscopic damage to cuticle induced by CLLEO. Groups of 20 L3 were placed on filter paper impregnated with increasing concentrations of CLLEO (0.15 to 2.86 μL/cm^2) and *α*-phellandrene (0.29 to 1.47 μL/cm^2). Efficacy was determined by quantifying L3 mortality 6, 24 and 48 h after contact with CLLEO and by measuring the structural damage to L3. CLLEO and *α*-phellandrene inhibited adult emergence by 96.22 and 100%, respectively. Macroscopic cuticle damage, appeared as diffuse pigment and darkening of larval body, was caused by both extracts. The SEM revealed dryness on the cuticle surface, distortion of the sensorial structures and general degeneration in treated L3. Furthermore, alterations in target organs (digestive tract, fat body and brain) were noticed and shall be used as biomarkers in future attempts to elucidate the mechanism of action of these compounds. The vacuolar degeneration and pyknotic profiles observed in the brain tissue of treated larvae with both extracts and the decreased motility within < 6 h after treatment leads us to suggest a neurotoxic activity of the products. This work demonstrates the potential use of CLLEO and *α*-phellandrene as bioinsecticides to be used against L. *cuprina*, representing an ecofriendly alternative for myiasis control in humans and animals.

1. Introduction

Lucilia cuprina is the cause of myiasis, a serious diseases that affects humans and animals, and is considered an important ectoparasite of livestock, affecting productivity and animal welfare (Guimarães et al., 1978; Windsor and Lomax, 2013; Sandeman et al., 2014). The Calliphoridae family contains the major species with the capacity to develop skin lesions in farm animals (i.e. *Lucilia cuprina,* Wiedemann, 1830). These flies deserve attention for their ability to develop cutaneous myiasis in sheep and they are considered pest of significant economic importance for neotropical agriculture (Sandeman et al., 2014; Wall, 2012; Anstead et al., 2016). The economic loss caused by the infestation of living tissue by Diptera larvae was estimated at US$336.6 million/year from *Cochliomyia hominivorax* primary myiasis in Brazil (Grisi

Abbreviations: EO, Essential oil; GC/MS, gas phase chromatography coupled to mass spectrometry; CLLEO, *Curcuma longa* leaves essential oil; L3, third instar larvae; IFC, Catarinense Federal Institute; LM, larvae mortality; PR, pupation rate; EIR, emergence inhibition rate; ADR, adult deformity rate; SEM, Scanning electron microscopy; CAS, Chemical Abstracts Service

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et al., 2014). Although there has been no study on the losses caused by L. *cuprina* in the country, this species has a cosmopolitan distribution and displays typical synanthropic behavior, being intimately associated with human habitation and the artificial infestation of sheep, as demonstrated by Moreira Lima and Moya Borja (Moreira-Lima and Moya-Borja, 1997). However, chemical control is still the most used form of fly reduction, and its indiscriminate application is the cause of selection of resistant flies, and deposition of chemical residues in animal products (e.g. meat and milk) (Labbé et al., 2017; Qin et al., 2017). Several alternative methods of control of L. *cuprina* have been proposed, among them (1) parasitoids: *Nasonia vitripenis*, *Aphaereta aotea*, *Tachinaephagus zealandicus*; (2) gram-positive bacteria: *Bacillus thuringiensis*; (3) immunological control employing enzymes with histone deacetylase inhibitors (HDACi) as vaccine candidates; (4) mass rearing of sterile insects by radiation; and (5) development of transgenic technologies for pest insects (Sandeman et al., 2014). However, studies about biopesticides derived from plants, especially essential oils (EO) and their individual compounds against L. *cuprina* control, are still incipient. Potential candidates can be found within the family Zingiberaceae. This family consists of 53 genera and over 1200 species native to tropical regions, especially Southern and Southeast Asia, occurring in tropical Africa and in Central and South America. Many of these species have economic value, providing food (starches rhizomes), perfumes and condiments with aromatic and medicinal properties (Moghadamtousi et al., 2014). *Curcuma longa* Linnaeus (Zingiberaceae) is a perennial herb, and its EO exhibits acaricidal and insecticidal properties, having been extensively studied for biological activities conferred by its broad chemical composition (Tavares et al., 2013; Chagas et al., 2016). However, not much attention has been given to the activity of the EO extracted from aerial parts (leaves) of the plant and their potential use as insecticide. The leaves of *C. longa* are considered residues during the rhizome harvest, and although few studies have addressed this part of the plant, its chemical composition is of interest in myiasis control. Previous reports have shown the presence of a cyclic monoterpene α-phellandrene in its chemical composition, showing good insecticidal activity (Evergetis et al., 2013). In Kerala, India, α-phellandrene (56.7%), 1,8-cineole (8.1%), *p*-cymene (7,5%) and β-pinene (5.3%) were the major constituents of EO from *C. longa* leaves (McCarron et al., 1995), while another study used GC–MS analysis to confirm the presence of β-sesquiphellandrene (22.8%), terpinolene (9.5%), aromatic curcumene (7.8%), 1,8-cineole (6.3%) and a minor amount of the monoterpene α-phellandrene (4.8%) (Priya et al., 2012). The leaf oil of *C. longa* from Bhutan, India has been reported to contain α-phellandrene (18.2%) and 1,8-cineole (14.6%), both constituents with insecticidal activity, evidencing the potential of *C. longa* as a sustainable alternative to chemical insecticides (Sharma et al., 1997; Enan, 2014; Jack and Busch, 2016). Although there are several studies on the chemical composition and biological activity of tumeric, there are no published reports of its larvicidal activity against L. *cuprina* using EO extracted from the aerial parts (leaves) of the plant or its monoterpene compound α-phellandrene. Morphological biomarkers, through histopathological assessment and scanning electron microscopy, may also be used to determine changes in structure and intoxication of target cells. This study aimed to assess the effect of EO from the leaves of *C. longa* (CLLEO) and its major compound α-phellandrene against third stage larvae (L3) of L. *cuprina*. An additional goal was to show morphological biomarkers in target organs through ultrastructural assessment and light microscopy, as well as macroscopic damage on L3 cuticle, induced by CLLEO and its major compound.

2. Materials and methods

2.1. Plant material

The leaves from *C. longa* used in this work were grown in the Unit of Medicinal Plants of the Catarinense Federal Institute, IFC, located at 26° 23′ 33.6691“ S and 48° 44’ 18.3336” W, at 10.6 m above sea level, in the city of Araquari, Santa Catarina State, Southern Brazil. The cultivation was carried out in an agroecological system without the addition of chemicals. Leaves were collected from approximately 100 individuals in September 2016 (spring), 10 months after cultivation. A sample of the botanical species was deposited at the Herbarium of the Botanic Museum, located in the Botanical Garden of Curitiba, PR, under the number 358970.

2.2. Essential oil extraction and chemical characterization of Curcuma longa leaf EO

Leaves from plants of the same cultivar were homogenized, and the EO was extracted from about 3 kg by hydrodistillation for 4 h in a Clevenger apparatus. The EO composition was analyzed by gas chromatography coupled with a mass spectrometric detector (GC/MS) (Shimadzu, Model 2010 Plus) (Kyoto, Japan) at the Department of Chemistry (UFPR, Brazil) using a HP-5MS capillary column (5% phenyl−/95% dimethylpolysilo-xane, 30 m × 0.25 mm × 0.25 μm) (Torrance, CA, USA). The injection temperature was 250 °C and the carrier (helium gas) flow was 1.0 mL/min^{-1}. The chromatograph oven was optimized with an initial temperature of 60 to 240 °C and an incremental increase of 3 °C/min. The oil sample was diluted to 1% in hexane, followed by injection into the GC/MS. Quantification was determined by normalizing the area (%) of each chemical constituent peak, the total area being the sum of all areas of the chromatogram peaks (100%) using the chromatograph Agilent 7890A, with a similar capillary column to the one described above. For the quantification of hydrogen gas, the material was used with a carrier at a flow rate of 1.5 mL min^{-1}. The retention indexes were calculated by the method of Van den Doll and Kratz (Van Den Doll and Kratz, 1963) using the n-alkane standard solutions (relative to C7–C30 n-alkanes), in the same chromatographic conditions. The compounds were identified by comparison of their GC mass and retention data with the available library (*Wiley Registry of Mass Spectral Data*, 1994; NIST Chemistry Webbook, P. J. Linstrom and W. G. Mallard, n.d.). The EO was analyzed in triplicate.

2.3. Dilution of extracts

The α-phellandrene (CAS: 99–83-2) was obtained from Sigma-Aldrich Brazil (São Paulo, Brazil) and had a purity of ≥99%. CLLEO was diluted in absolute ethanol or acetone as α-phellandrene was solubilized only in ethanol. The solvents have shown no toxicity to L3 of L. *cuprina* (Chaaban et al., 2018; Chaaban et al., n.d.). The CLLEO concentrations used were 0.15, 0.31, 0.63, 0.79, 0.95, 1.11, 1.27, 1.43, 1.59, 2.07, 2.38 and 2.86 μL/cm^2. The following α-phellandrene concentrations were used: 0.29, 0.59, 0.88, 0.18 and 1.47 μL/cm^2. EO of *C. longa* leaves were solubilized in ethanol or acetone. A control group was established, in which L3 were exposed only to absolute ethanol or acetone.

2.4. Colony of flies

Wild flies were collected manually at the IFC, using bait (shrimp bark kept at 27 ± 1 °C for 48 h of decomposition) and entomological net. The establishment of stock colonies, insect identification, maintenance, mass reproduction and the protocol for the biological tests were performed as described by Chaaban et al. (Chaaban et al., 2018; Chaaban et al., n.d.). For this work, we used fresh, drug-free bovine meat (approx. 2 g/larvae) for larval development.

2.5. Larval toxicity

The toxicity evaluation of CLLEO and α-phellandrene on L3 of L. *cuprina* was performed as described by Chaaban et al. (Chaaban et al.,

2018; Chaaban et al., n.d.). Groups of 20 mature L3 (1 day after they left the substrate) from the second generation were introduced into glass vials (9 × 4 cm diameter) containing a filter paper (12.56 cm^2) impregnated with 0.2 mL of α-phellandrene or EO solutions. After the application of CLLEO and α-phellandrene, the glass vials were closed with voile fabric to facilitate aeration, kept for 5 min in an exhaust hood and transferred to a climatic chamber at 27 °C and 70% relative humidity. All treatments were performed in triplicate (n = 60). Toxicity was evaluated by observing L3 mortality at 6, 24 and 48 h after contact. Total L3 mortality (TLM) was calculated (Chaaban et al., 2018; Kumar et al., 2014; Chaaban et al., 2017a; Chaaban et al., 2017b) as follows:

TLM = (total dead larvae×100)/total tested larvae.

2.6. Analysis of physiological parameters

After CLLEO and α-phellandrene contact, L3 were kept under controlled conditions for recording of the following parameters: pupation rate (PR), emergence inhibition rate (EIR) and adult deformity (AD) (Chaaban et al., 2018; Kumar et al., 2014; Chaaban et al., 2017a; Chaaban et al., 2017b; Singh and Kaur, 2016):

PR = (total pupae×100)/total tested larvae)

EIR = (total control adults−total treated adults × 100)/total control adults

AD = (total deformed adults×100)/total emerged adults

2.7. Scanning electron microscopy (SEM)

For SEM, L3 treated with 1.59 µL/cm^2 of CLLEO and 1.47 µL/cm^2 of α-phellandrene were fixed in AFA solution (ethyl alcohol at 70%, buffered formalin at 37% and glacial acetic acid) in a ratio of 2.5:1:1.5, 6 h and 7 days after contact. Subsequently, the samples were submitted to a dehydration process using five alcohol baths. The larvae were placed in a support for electron microscopy (stub) and dehydrated in an oven using the protocol described by Caneparo (Caneparo, 2017), with modifications (37 °C for 6 h). The specimens were examined and photographed at a magnification ranging from 12× to 600× (JEOL JSM 6360-LV) at the Center for Electron Microscopy of UFPR.

2.8. Larval histopathology

For larval histopathology, L3 treated with 1.59 µL/cm^2 of CLLEO and 1.47 µL/cm^2 of α-phellandrene and solubilized in ethanol were fixed in 10% buffered formalin, 6 and 24 h after contact with the solutions. For slide preparation, two longitudinal sections were embedded in paraffin and L3 were serially sectioned (4 µm) thickness and stained with hematoxylin-eosin (Chaaban et al., n.d.).

2.9. Statistical analysis

Lethal concentrations (LC_{10}, LC_{50} and LC_{90}) were calculated using Probit analysis. L3 mortality, PR and EIR were analyzed for exposition time, concentrations, carriers and the interaction between concentrations and carriers through an analysis of variance (ANOVA) in generalized linear model, assuming a Poisson distribution. The averages were compared using the Tukey test. All analyses were performed using the statistical software SPSS (IBM Corp, 2013), considering the significance level of 5%. The values were corrected using the Abbott's formula (Abbott, 1925).

3. Results

3.1. Chemical characterization of Curcuma longa leaf EO

Eighteen compounds were identified from CLLEO, representing 97.27% of the total chromatographic peaks (Table 1). The major compounds were α-phellandrene (41.99%), ρ-mentha-2,4(8)-diene (24.89%), and 1,8-cineole (7.82%), while ρ-cimene (2.79%), myrcene (2.63%) and α-pinene (2.52%) represented the smaller chromatographic area.

Table 1
Chemical composition of *Curcuma longa* Leaves essential oil.

Compounds	RT (min)	IRc	IRt	RA (%)	SD
α-tujene	4.013	925	924	0.11	0.0000
α-pinene	4.152	932	932	2.52	0.10214
sabinene	4.967	971	969	0.34	0.00577
β-pinene	5.048	975	974	5.65	0.12490
myrcene	5.36	991	988	2.63	0.03000
δ-2-carene	5.605	1002	1001	0.17	0.05774
α-phellandrene	5.742	1006	1002	41.99	0.46608
δ-3-carene	5.852	1010	1008	1.06	0.02082
α-terpinene	6.015	1016	1014	1.50	0.01000
ρ-cimene	6.228	1023	1020	2.79	0.08083
limonene	6.347	1027	1024	3.41	0.03464
1.8-cineole	6.413	1029	1026	7.82	0.37269
(*E*)-β-ocimene	6.902	1045	1044	0.42	0.00577
γ-terpinene	7.208	1056	1054	1.87	0.01528
ρ-mentha-2,4(8)-diene	8.172	1088	1085	24.89	0.18824
linalol	8.51	1100	1095	0.69	0.01528
terpinen-4-ol	11.185	1174	1174	0.40	0.01528
α-terpineol	11.695	1188	1186	0.39	0.03000
Total identified compounds (%) 97.27					
Other unidentified compounds (%) 1.36					

Note: RT = Retention time (min), IRc = Retention index calculated, IRt = Retention index tabulated (Adams, 2012), RA (%) = Relative area, SD = Standard deviation.

3.2. Larval toxicity and analysis of physiological parameters

Lethal concentrations of CLLEO and α-phellandrene are shown in Table 2. Dose- and time-dependent activities were demonstrated 6 h after exposure to CLLEO and α-phellandrene, with an LC_{50} of 1.34 and 1.17 µL/cm^2, respectively. The LC_{50} showed no significant variation between carriers (Fig. 1). After 6 h of exposure, we observed that the LC_{10}, LC_{50} and LC_{90} (0.92, 1.17 and 1.48 µL/cm^2) for α-phellandrene (Table 2). Likewise, CLLEO/ET had a LC_{10}, LC_{50} and LC_{90} of 1.01, 1.34 and 1.78 µL/cm^2, while CLLEO/AC were 1.17, 3.28 and 9.19 µL/cm^2, respectively, showing statistically ($P < 0.05$) higher values for LC_{50} and LC_{90} than CLLEO/ET (Table 2). Regarding the assessment of LM and physiological parameters of L. *cuprina,* we observed LM of 85%, 48 h after contact with 10% CLLEO/ET (1.59 µL/cm^2) and only 46.66% LM when using CLLEO/AC (Table 3). α-phellandrene (1.47 µL/cm^2) had a statistically significantly different LM when compared to the other concentrations (Table 4). Furthermore, the same doses inhibited adult emergence by 96.22% and 100% using CLLEO and α-phellandrene, respectively. In addition, the pupation ratio had significantly different results when L3 were treated with CLLEO (1.27 µL/cm^2) and α-phellandrene (0.88 µL/cm^2), solubilized in ethanol, when compared to control groups. It can be observed that the higher the efficacy the greater is the activity over the other physiological measurements, reaching 100% for EIR, for both products (Matrix CLLEO and EIR r = 0.8834; and Matrix α-phellandrene vs. EIR r = 0.9763, respectively). The correlation of efficacy for LM and PR was 0.91835 and − 0.91838 for CLLEO, and 0.98522 and − 0.98521 for α-phellandrene, respectively.

3.3. Macroscopic cuticle damage

Macroscopic cuticle damage, such as diffuse pigment and darkening throughout the body and decreased motility after treatment with CLLEO and its major compound α-phellandrene, were observed after 6 h of L. *cuprina* L3 exposure (Data in Brief - videos 1,2). Changes in color

Table 2
Lethal concentration (μL/cm^2) of *Curcuma longa* Leaves essential oil (CLLEO) and *α*-phellandrene against *Lucilia cuprina* third stage larvae in the contact assay over time.

Extract	Evaluation time	LC_{10} (LCI–UCI)[a]	LC_{50} (LCI–UCI)	LC_{90} (LCI–UCI)	Chi-square ($\chi 2$)	Probability
CLLEO/ET	6 h	1.01 (0.95–1.07)	1.34 (1.3–1.38)	1.78 (1.7–1.89)	8.68	0.37
	24 h	1.02 (0.91–1.01)	1.35 (1.28–1.41)	1.78 (1.67–1.95)	14.19	0.07
	48 h	0.98 (0.85–1.06)	1.28 (1.21–1.34)	1.68 (1.58–1.87)	10.59	0.1
CLLEO/AC	6 h	1.17 (0.95–1.34)	3.28 (2.8–4.29)	9.19 (6.34–17.81)	6.17	0.19
	24 h	1.05 (0.52–1.37)	2.44 (2.04–3.34)	5.64 (3.86–16.87)	9.56	0.05
	48 h	0.94 (0.54–1.21)	1.67 (1.37–1.92)	2.96 (2.5–4.17)	9.52	0.05
α-phellandrene	6 h	0.92 (0.48–1.07)	1.17 (0.95–1.33)	1.48 (1.31–2.39)	4.41	0.11
	24 h	0.92 (0.48–1.07)	1.17 (0.95–1.33)	1.48 (1.31–2.39)	4.41	0.11
	48 h	0.9 (0.41–1.05)	1.16 (0.93–1.36)	1.49 (1.3–2.83)	5.24	0.07

CLEO/ET: *Curcuma longa* Leaves essential oil solubilized in ethanol; CLLEO/AC: *Curcuma longa* Leaves essential oil solubilized in acetone. *α*-phellandrene was solubilized only in ethanol.

[a] The lethal concentrations were calculated by the Probit analysis. LCI, lower limit of 95% confidence interval; UCI, upper limit of 95% confidence interval.

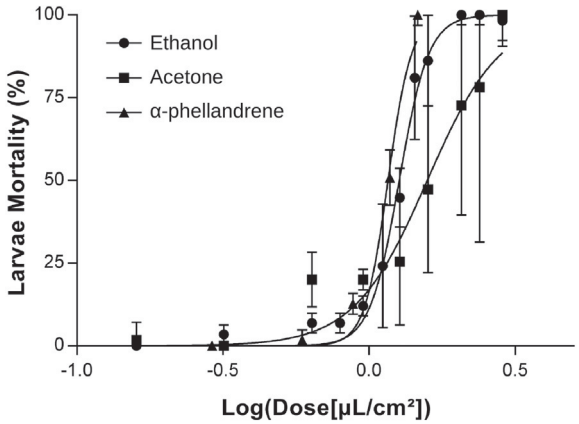

Fig. 1. Average and standard deviation of *Lucilia cuprina* after exposition to *Curcuma longa* leaves essential oil (CLLEO) (log[μL/cm^2]) using different carriers (ethanol and acetone) and its major compound *α*-phellandrene using ethanol.

(darkening) throughout the L3 body with emphasis from the second through eighth segments using 1.59 μL/cm^2 of CLLEO were also reported starting at 6 h of contact (Fig. 2c, d). Similar effects after the same exposure time to 1.47 μL/cm^2 to α-phellandrene were also observed (darkening of the anterior end of larva and diffuse pigment on the body.) but they were less pronounced (Fig. 2e, f). Likewise, progressive lesions appearing as marked cuticle dryness were shown in dead L3, 7 days after exposure to both extracts (1.59 μL/cm^2 to CLLEO and 1.47 μL/cm^2 to *α*-phellandrene) (Fig. 2g, h). Ethanol did not have any effect over L3, showing normal size and *sui generis* cuticle color (light yellow)(Fig. 2a, b).

3.4. Scanning electron microscopy

The SEM of L3 of L. *cuprina* from the control group showed typical Calliphoridae morphology with smooth body tegument and preserved cuticle (Fig. 3A, I-III). SEM of L. *cuprina* L3 6 h after treatment with 1.59 μL/cm^2 of CLLEO showed dryness on the cuticle surface, distortion of the sensory structures (antenna sensory papillae) and maxillary lobe, as well as slight distortion of the anal papillae and spiracle plate

Table 3
Larvae mortality (LM), Pupariation rate (PR), Emergence innibition rate (EIR), Sex ratio (Male:Female) and Adult deformity of *Lucilia cuprina* treated with *Curcuma longa* Leaves essential oil.

C(μL/cm^2)(%)	LM (%)*	PR (%)	EIR (%)	SR (M:F)	AD (%)
Ethanol	3.33 ± 1.67 c	96.66 ± 1.67 a	0.0 ± 0.00 c	29:24	0.0
0.15 (1%)	1.66 ± 1.67 c	98.33 ± 1.67 a	18.66 ± 9.11 c	19:24	4.65
0.31 (2%)	5.0 ± 0.0 c	95.0 ± 0.0 a	16.98 ± 16.6 c	25:19	4.54
0.63 (4%)	8.33 ± 1.67 c	91.66 ± 1.67 a	16.98 ± 14.22 c	20:24	15.90
0.79 (5%)	8.33 ± 1.67 c	91.66 ± 1.67 a	20.75 ± 13.92 c	21:21	7.14
0.95 (6%)	13.33 ± 1.67 c	86.66 ± 1.67 a	24.52 ± 9.59 bc	18:22	7.50
1.11 (7%)	25.0 ± 10.41 bc	75.0 ± 10.41 ab	75.47 ± 3.59 a	4:9	0.0
1.27 (8%)	45.0 ± 5.00 b	55.0 ± 5.00 b	69.81 ± 5.26 ab	5:11	12.5
1.43 (9%)	80.0 ± 10.41 a	20.0 ± 10.41 c	83.01 ± 7.18 a	3:6	0.0
1.59 (10%)	85.0 ± 7.64 a	15.0 ± 7.64 c	96.22 ± 1.91 a	2:0	0.0
2.07 (13%)	98.33 ± 1.67 a	1.66 ± 1.67 c	98.11 ± 1.96 a	0:1	0.0
2.38 (15%)	98.33 ± 1.67 a	1.66 ± 1.67 c	98.11 ± 1.67 a	0:1	0.0
2.86 (18%)	96.66 ± 3.33 a	3.33 ± 3.33 c	100.0 ± 0.0 a	0:0	0.0
Acetone	1.66 ± 1.67 c	98.33 ± 1.67 a	0.0 ± 0.00 b	27:25	0.0
0.15 (1%)	5.0 ± 2.89 c	95.0 ± 2.89 a	17.30 ± 8.23 b	29:14	0.0
0.31 (2%)	3.33 ± 3.33 c	96.66 ± 3.33 a	19.23 ± 12.04 b	19:23	0.0
0.63 (4%)	21.66 ± 4.41 bc	78.33 ± 2.89 ab	25.0 ± 10.6 ab	16:23	0.0
0.95 (6%)	21.66 ± 1.67 bc	78.33 ± 1.67 ab	28.84 ± 18.77 ab	11:27	0.0
1.27 (8%)	26.66 ± 10.14 bc	73.33 ± 10.14 ab	57.69 ± 29.6 ab	10:12	0.0
1.59 (10%)	46.66 ± 13.33 abc	53.33 ± 13.33 abc	71.15 ± 23.66 ab	9:6	0.0
2.07 (13%)	70.0 ± 17.56 ab	30.0 ± 17.56 bc	86.53 ± 16.66 ab	3:4	0.0
2.38 (15%)	75.0 ± 25.0 ab	25.0 ± 25.0 bc	100.0 ± 0.0 a	0:0	0.0
2.86 (18%)	95.0 ± 5.0 a	5.0 ± 5.0 c	100.0 ± 0.0 a	0:0	0.0

Absolute ethanol and acetone were used with control.
The letters display a significant difference ($P < 0.05$) in the concentrations of the essential oils.
* 48 h of exposure.

Table 4
Larvae mortality (LM), Pupariation rate (PR), Emergence innibition rate (EIR), Sex ratio (Male:Female) and Adult deformity of *Lucilia cuprina* treated with α-Phellandrene.

C(μL/cm^2)	LM (%)*	PR (%)	EIR (%)	SR (M:F)	AD (%)
Control	0.0 ± 0.0 d	100.0 ± 0.0 a	0.0 ± 0.00 d	29:30	0.0
0.29	0.0 ± 0.0 d	100.0 ± 0.0 a	0.0 ± 2.96 d	35:24	0.0
0.59	1.66 ± 1.67 d	98.33 ± 1.67 a	3.38 ± 3.33 d	30:27	0.0
0.88	11.66 ± 1.67 c	88.33 ± 1.67 b	23.72 ± 5.83 c	26:19	2.22
1.18	46.66 ± 4.41 b	53.33 ± 4.41 c	83.05 ± 4.28 b	3:7	10.0
1.47	91.66 ± 1.67 a	8.33 ± 1.67 d	100.0 ± 0.0 a	0.0	0.0

Absolute ethanol was used with α-Phellandrene control.
The letters display a significant difference ($P < 0.05$) in the concentrations of the essential oils.
* 48 h of exposure.

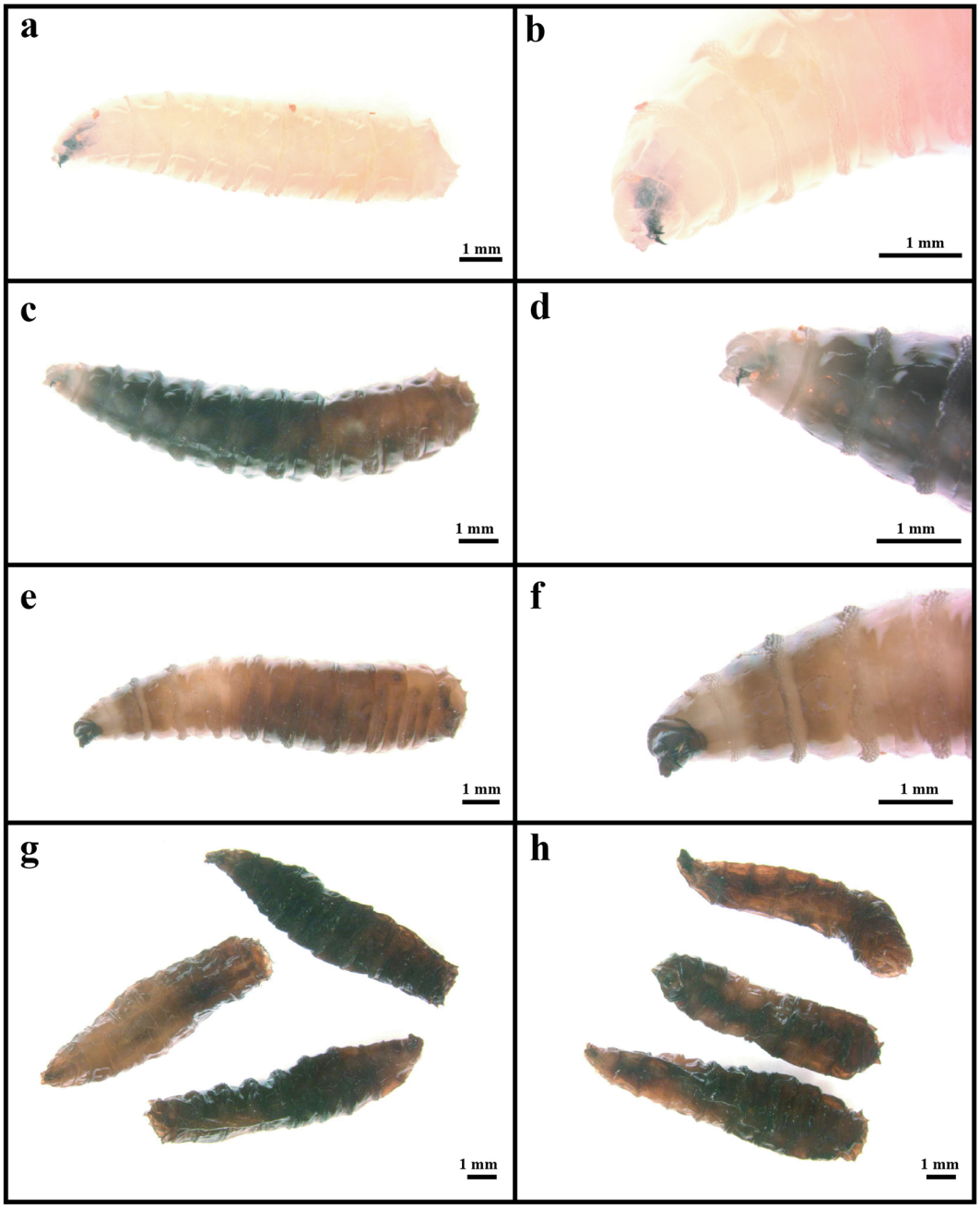

Fig. 2. Macroscopic cuticular damage of *Lucilia cuprina* L3 after treatment with *Curcuma longa* leaves essential oil (CLLEO) and its major compound α-phellandrene. a, b) Normal L3 6 h after treatment (control group treated with ethanol); c, d) L3 with cuticle damage 6 h after treatment with 1.59 μL/cm^2 of CLLEO; e, f) L3 with cuticle damage 6 h after treatment with 1.47 μL/cm^2 of α-phellandrene; g, h) L3, 7 days after treatment with CLLEO (1.59 μL/cm^2) and α-phellandrene (1.47 μL/cm^2), respectively.

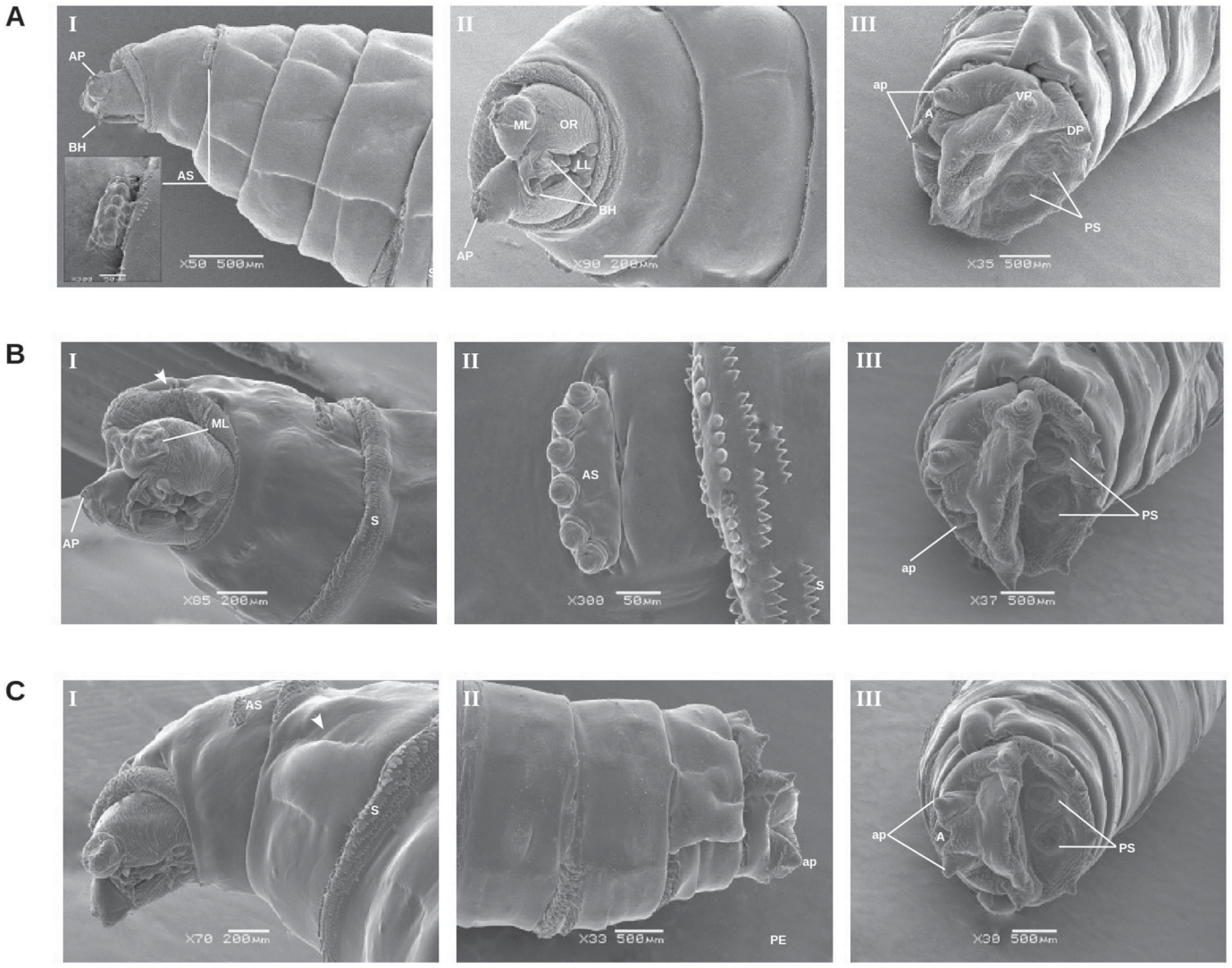

Fig. 3. Scanning electron photomicrographs of *Lucilia cuprina* L3. A) Control group (only ethanol). AI) Anterior end of larva with normal body, details of anterior spiracles (AS) with 6–7 lobes, buccal hook (BH) and antenna sensory papillae (AP) preserved. AII) Cephalic segment of larva, note the preserved structures and the large mouth hooks projecting beyond the oral cavity. AIII) Posterior end of larva, observe spiracular plate with three spiracular openings. B) *L. cuprina* L3, 6 h after treatment with 1.59 µL/cm^2 of CLLEO. BI) Anterior end of larva. Note the distortion of the sensorial structures, antenna sensory papillae (AP) and maxillary lobe (ML). Details of the cuticular surface dryness (arrowhead) and marked spinules (S) on cephalic segment. BII) Anterior spiracle (AS) and spinules (S) preserved. BIII) Posterior end (PE) of larva with slight distortion of anal papillae and spiracle plate. C) *L. cuprina* L3 6 h after treatment with 1.47 µL/cm^2 of *α*-phellandrene. CI) Anterior end of larva, note the slight dryness (arrowhead) and marked spinules (S). CII; III) Posterior end of larva. Note posterior spiracles (PS), anal opening (A) and anal papillae preserved (ap). Key: PE = Posterior end; S = spinules; AP = antenna sensory papillae; AS = anterior spiracle; ML = maxillary lobe; OR = oral ridges; LL = labial lobe; BH = bucal hook; DP = dorsal papillae; VP = ventral papillae; A = anus; PS = posterior spiracles; ap = anal papillae.

(Fig. 3B, I-III). Likewise, we observed slight dryness on the cuticle surface and contraction of the cephalic segment 6 h after treatment with *α*-phellandrene (1.47 µL/cm^2) (Fig. 3C, I-III). Notably, many changes were observed 7 days after treatment with both extracts, particularly extreme distortion and cuticle damage in all segments of larvae; degeneration of antenna sensory papillae, maxillary lobe, oral ridges, labial lobe and anterior spiracle and deformation on anal papillae, ventral spinules and posterior spiracles (Fig. 4).

3.5. Larval histopathology

Histological sections of L. *cuprina* L3 showed different alterations after treatment with CLLEO and its major compound *α*-phellandrene. Vacuolization in the cytoplasm, pyknotic nuclei and necrosis of the digestive tract were seen after the use of both extracts (Fig. 5B, I-IV). Similarly, we observed severe alterations in the fat body of treated larvae, such as cytoplasmic vacuolation and irregular morphology of trophocytes with pyknotic nuclei, as well as changes in protein granules (Fig. 5C, I-IV). Histological sections of *L. cuprina* L3 brain showed vacuolar degeneration, pyknotic profiles and disorganized and condensed cells, characteristic of degeneration and necrosis after CLLEO and *α*-phellandrene exposure (Fig. 5D, I-IV). Fig. 5A (I-IV) show control larvae with no marked alterations.

4. Discussion

4.1. Chemical characterization of Curcuma longa leaf EO

Within the Zingiberaceae family, turmeric is one of the most investigated Curcuma species due to the medicinal properties of its rhizome (Moghadamtousi et al., 2014; Tavares et al., 2013; Chagas et al., 2016). Interestingly, in contrast to observed in rhizomes, where the sesquiterpene ar-turmerone is frequently reported to be the major compound (Evergetis et al., 2013), in the EO from *C. longa* leaves, α-

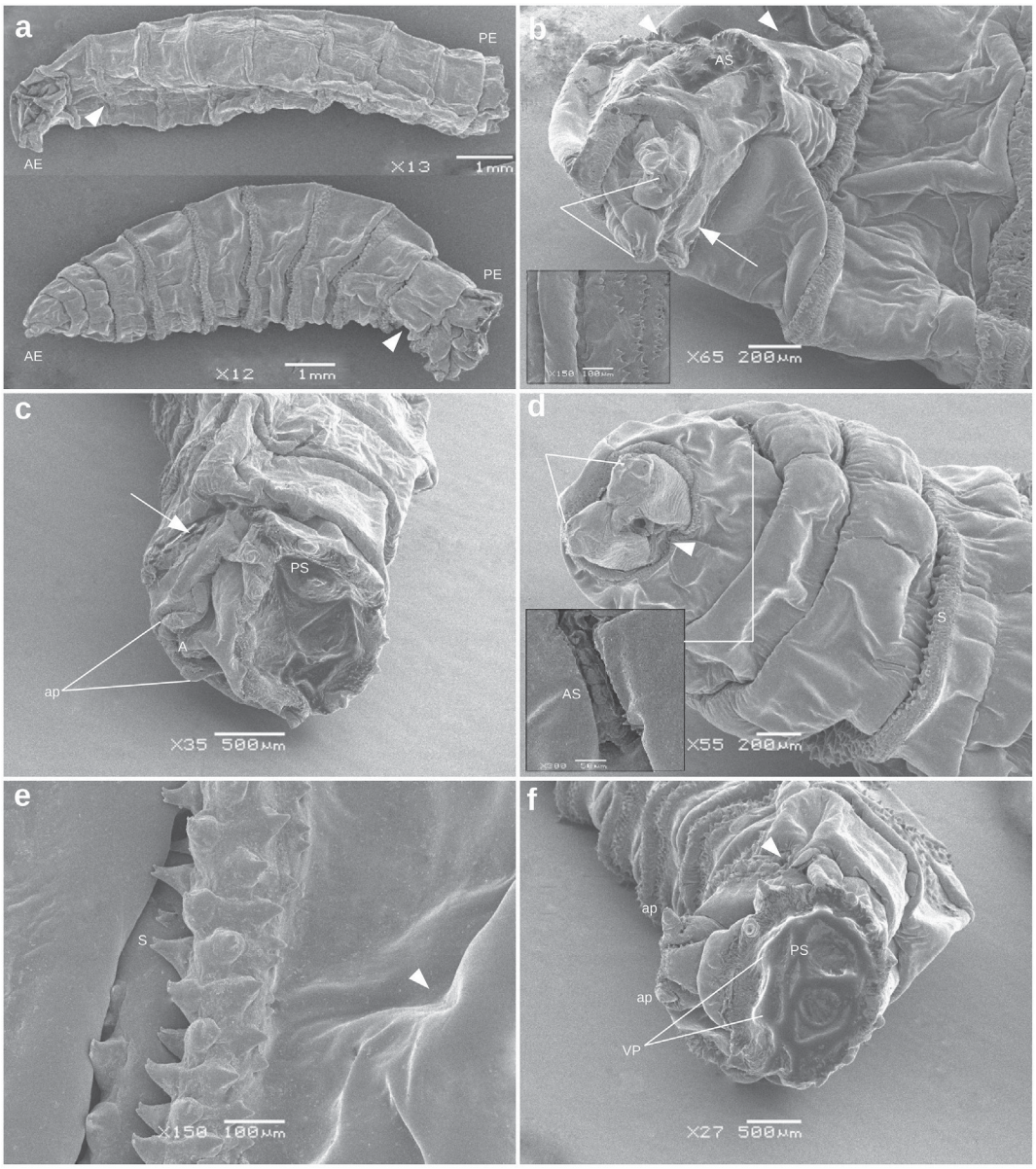

Fig. 4. Scanning electron photomicrographs of *Lucilia cuprina* L3 7 days after treatment with CLLEO (1.59 µL/cm^2) and its major compound *α*-phellandrene (1.47 µL/cm^2). a) Extreme distortion and cuticle damage in all segments of the larva. L3 of L. *cuprina* treated with CLLEO (above) and *α*-phellandrene (below). b) Anterior end of larva treated with CLLEO. Note: degeneration of antenna sensory papillae, maxillary lobe, oral ridges, labial lobe and anterior spiracle. Details of the deformed and wrinkled cuticular surface (arrowhead). c) Posterior end with severe degeneration of larva treated with CLLEO. Observe the anal papillae, posterior spiracles and deformed ventral spinules. d) Anterior end of larva treated with *α*-phellandrene. Note: extreme distortion of antenna sensory papillae and labial lobe. Details of anterior spiracle with severe degeneration. e) Spinules protruding of larva treated with *α*-phellandrene. Observe the cuticular surface dryness (arrowhead). f) Posterior end of larva treated with *α*-phellandrene with extreme cuticular damage. Note: distortion of anal papillae, severe degeneration in ventral papillae and protruding spinules (arrowhead). Key: CLLEO = *Curcuma longa* leaves essential oil; AE = anterior end; PE = Posterior end; AP = antenna sensory papillae; AS = anterior spiracle; S = spinules; A = anus; ap = anal papillae; VP = ventral papillae; PS = posterior spiracles.

phellandrene has often been reported as a major constituent. In this way, the effects of environmental conditions were demonstrated by Sandeep et al. (Evergetis et al., 2013) regarding the quantity of secondary metabolite contents of *C. longa*, where the range of variation in *α*-phellandrene content in leaf EO ranged from 23.48 to 67.64% in nine different zones. In the present study, *α*-phellandrene represented 41.99% of the EO, shedding light on the potential use of *C. longa* leaf oil for myiasis control, since this monoterpene (*α*-phellandrene) is largely used for synergistic pest-control compositions (Priya et al., 2012). The chemical profile and insecticidal activities of EO from *Anethum graveolens*, which has *α*-phellandrene as its majority constituent (59%) were determined by Evergetis et al. (Evergetis et al., 2013). In vitro assays against L3 to L4 of *Culex pipiens* biotype molestus evidenced an LC_{50} value of 52.74 mg/L for *A. graveolens* EO, while the highest toxicity was found with isolated *α*-phellandrene, with an LC_{50} of 38.20 mg/L. Likewise, *α*-phellandrene exhibited strong activities in in vitro tests against *Aedes aegypti* and *A. albopictus* larvae, with LC_{50} of 16.6 and 39.9 µg/mL, respectively (Cheng et al., 2009). Notably, *α*-phellandrene was found to attenuate inflammatory responses through neutrophil migration inhibition and mast cell degranulation, highlighting the role of this monoterpene as an anti-inflammatory agent (Siqueira et al., 2016). Although *α*-phellandrene was the major constituent found in our study, we observed the presence of other minor compounds. Constituents such as limonene (3.41%), ρ-cimene (2.79%), myrcene (2.63%) and α-

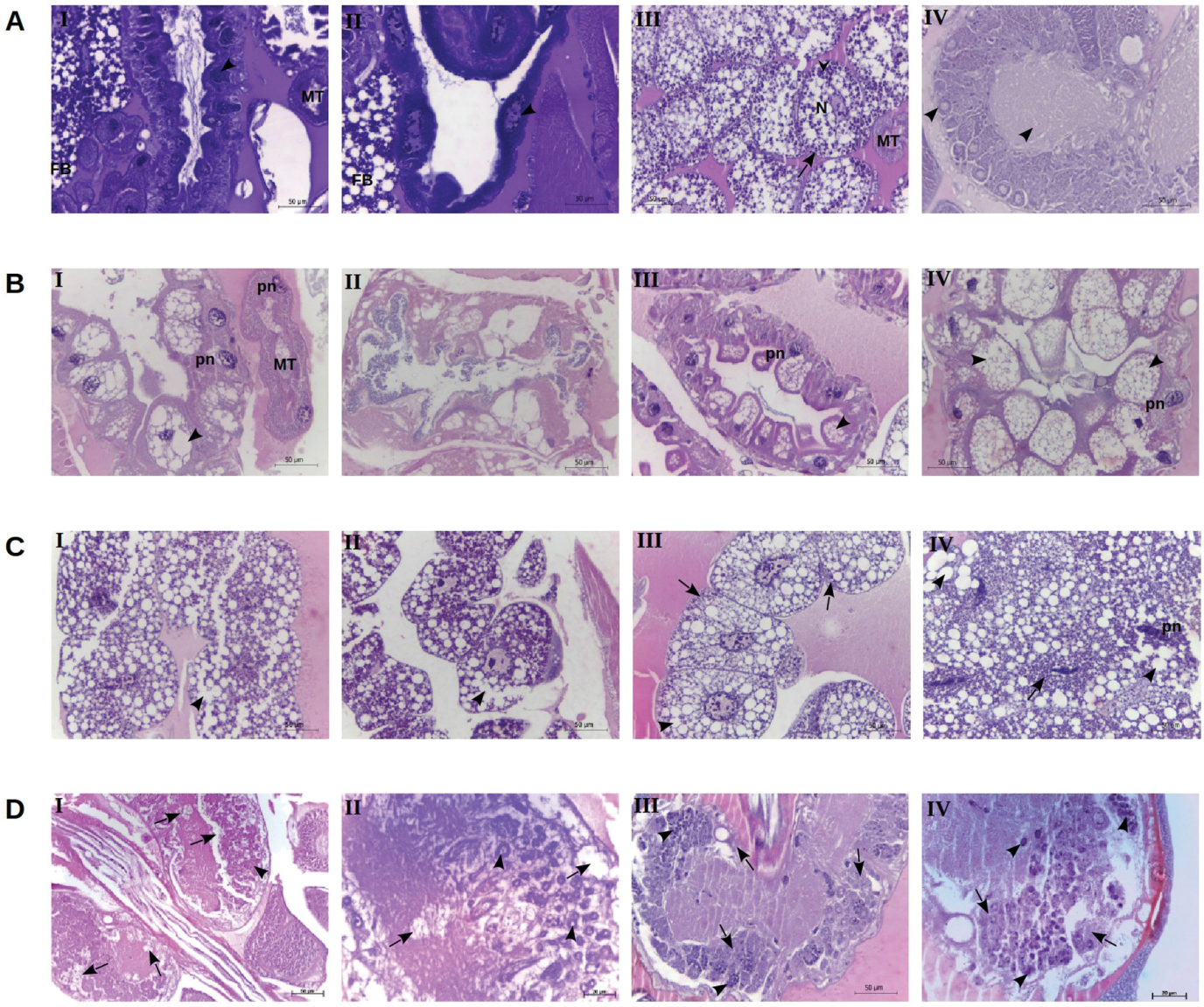

Fig. 5. Photomicrophotographs of digestive tract, fat body and brain of *Lucilia cuprina* L3. A) Control groups. AI, AII) Intact digestive tract with nucleus preserved (arrowhead), fat body (FB) and malpigean tubules (MT) intact (40×). AII) Fat body showing the trophocytes with a large number of vacuole (arrows) and nuclei (N) surrounded by protein granules (arrowheads). AIV) Larvae brain showing homogeneous structures and preserved (arrowheads) (40×). BI) L3 of L. *cuprina* 6 h after treatment with 1.59 μL/cm^2 of CLLEO. Note the intense cytoplasmic vacuolation (arrowheads) (40×). BII) *L. cuprina* L3 24 h after treatment with 1.59 μL/cm^2 of CLLEO. Details for marked necrosis of the intestinal tract (40×). BIII) Digestive tract of L. *cuprina* L3 6 h after treatment with 1.47 μL/cm^2 of *α*-phellandrene. Note the intense cytoplasmic vacuolation (arrowheads) and pyknotic nuclei (pn) (40×). BIV) Gut cells of L. *cuprina* L3 24 h after treatment with 1.47 μL/cm^2 of *α*-phellandrene. Observe the intense progression vacuolization in the cytoplasm (arrowheads) and pyknotic nuclei (pn) (40×). CI) *L. cuprina* L3 6 h after treatment with 1.59 μL/cm^2 of CLLEO. Observe the trophocytes with slight vacuolation (arrowheads). CII) *L. cuprina* L3 24 h after treatment with 1.59 μL/cm^2 of CLLEO. Note slight vacuolation (arrowheads) and irregular morphology of trophocytes. CIII) Fat body of L. *cuprina* L3 6 h after treatment with 1.47 μL/cm^2 of *α*-phellandrene. Details to vacuolation (arrowheads) and reduction of protein granules with peripheral protein granule (arrows). CIV)L3 24 h after treated with 1.47 μL/cm^2 of *α*-phellandrene. Note the irregular trophocytes and nucleus, pyknotic nuclei (pn), great quantity of vesicles (arrowheads) and grouping of protein granules near of the nucleus (arrows). DI, DII) Brain of L. *cuprina* L3 24 h after treatment with 1.59 μL/cm^2 of CLLEO. Observe vacuolar degeneration showing medium sized clusters of vacuoles, light in the neuropil and mild in the cortical layer (arrow). The trophoblasts are shrunken (arrowheads) (40×, 100×). DIII, DIV) Brain of *L. cuprina* L3 24 h after treatment with 1.47 μL/cm^2 of *α*-phellandrene. Note the pyknotic profiles (arrowheads), disorganized and condensed cells characteristics of degeneration and necrosis (arrow) (40×, 100×). H & E, hematoxylin–eosin.

pinene (2.52%), although present in small quantities in leaves, also have a varied of biological activities including insecticidal, antioxidant, anti-inflammatory and bactericidal activities; giving the ability to improve skin permeability and a synergistic effect when added to other compounds (Falconieri et al., 2011; Cavallaro, 2015; Freehauf et al., 2013; Zielinski et al., 2016; Lim et al., 2006; Ciftci et al., 2011; Govindarajan et al., 2016). Previous reports demonstrated several biological properties of *β*-pinene, such as antifungal, antimicrobial, insecticidal and insect repellent activities and enhancement of skin permeability (Enan, 2014; Falconieri et al., 2011; Cavallaro, 2015). Notwithstanding, the terpene 1,8-cineole known as eucalyptol may be used in preparations for the treatment of oxidative skin damage and may act in synergy with natural pesticides, in pharmaceutical transdermal preparations and for the treatment of inflammation in cattle (Jack and Busch, 2016; Freehauf et al., 2013; Zielinski et al., 2016).

4.2. Larval toxicity and analysis of physiological parameters

Toxicity in the early hours after contact with EO against blowflies has recently been demonstrated. Investigating the insecticidal activity of *Baccharis dracunculifolia* EO, Chaaban et al. (Chaaban et al., 2017b) determined an LD_{50} of 2.63 μL/cm^2 6 h after contact with *C. macellaria* L3, using ethanol as the carrier. Likewise, similar effects were observed, using *Piper gaudichaudianum* EO against L. *cuprina* 6 h hours after contact with an LD_{50} of 3.71 μL/cm^2 where ethanol was more effective than acetone (Chaaban et al., 2018). Our work shows better results when compared with previous reports for both CLLEO and α-phellandrene, demonstrating also a dose- and time-dependent activity. Previous reports assessed a transdermal therapeutic system to promote an increase in skin permeability using ethanol and the terpene D-limonene as carriers. D-limonene penetrated the skin when mixed with ethanol, causing changes in the barrier structure of the skin (Takayama and Nagai, 1994). In this sense, we believe that the major affinity between CLLEO and ethanol could be associated with the presence of the monoterpene limonene in the chemical composition of EO and the interaction of the constituents with ethanol, increasing the penetration of the bioactive compounds through the insect tegument. Although some articles have reported insecticidal activity of several EO, this is the first study to demonstrate the effects of CLLEO and α-phellandrene on blowfly larvae. Although α-phellandrene has shown clear insecticidal activity, this compound could be modulated by other minor molecules present in CLLEO, such as 1,8-cineole, β-pinene, limonene and α-pinene (Cavallaro, 2015; Lim et al., 2006; Ciftci et al., 2011; Govindarajan et al., 2016). In addition, more scientific evidence has emerged that several minor constituents of the EO play a key role in improving skin penetration and cellular distribution (i.e. limonene, 1,8-cineole) acting synergistically within molecules (Lim et al., 2006; Bakkali et al., 2008). Previous reports also suggested that interactions between different combinations of monoterpene compounds (synergistic action), carriers and the lipid layer of the insect's cuticle may explain their enhanced penetration and increased activity (Tak and Isman, 2015). Moreover, some studies suggested differences in the effects of EO and individual compounds on the insect cuticle when using different routes of administration, obtaining better toxicity results when using topical administrations (contact assays) (Siqueira et al., 2016). Suppression of insect development using EO as a natural control have been reported in several studies. Khater et al. (Khater et al., 2011) observed 100% suppression of adult emergence of L. *sericata* treated with 2% of *Lactuca sativa* and *Matricaria chamomilla* EO. In the same way, reports of adult emergence suppression after treatment of L. *sericata* larvae with 8% of *Brassica compestris* and 12% of *Raphanus sativus* were shown by Khater and Khater (Khater and Khater, 2009). Similarly, the adult emergence inhibition rate of L. *cuprina* exposed to 1.59 μL/cm^2 of *Piper gaudichaudianum* EO presented 94.44% inhibition, while the same dose of *T. minuta* showed 100% of inhibition (Chaaban et al., 2018; Chaaban et al., n.d.).

4.3. Macroscopic cuticle damage

Recent reports of cuticle damage in L3 of blowflies have been conducted using biological assays. Shalaby et al. (Shalaby et al., 2016) showed cuticular swelling and distortion in L3 of *L. sericata* treated with *Lavandula angustifolia* (lavender oil) and *Cinnamomum camphora* (camphor oil). Similarly, malformations of L. *sericata* larvae, such as small sized and damaged larvae with weak cuticles, after the contact with *Commiphora molmol* were reported by Hoda et al. (Hoda et al., 2016). *C. macellaria* L3 showed decreased motility and cuticle abnormalities, 6 h after exposure to *B. dracunculifolia* (Chaaban et al., 2017b). Likewise, biological assays using *P. gaudichaudianum* EO against the same biological model as in our work, also exhibited a decrease in motility and cuticle damage (Chaaban et al., 2018). Thus, macroscopic observations of cuticle lesions associated with histological changes in target organs may be an important way to elucidate the mechanism of action of EO and their individual compounds (see Section 3.5). Although most studies focused on in vitro tests, the present results offer the possibility of using EO in the control of veterinary ectoparasites, as investigations of botanical pesticides have grown considerably in the last decade (Pavela, 2015; Pavela and Benelli, 2016; Chaaban et al., 2017).

4.4. Scanning electron microscopy

Until now, few ultrastructural injury investigations have been carried out in contact experiments with biopesticides in blowflies, especially EO and isolated its compounds. Shalaby et al. (Shalaby et al., 2016) have determined the larvicidal activity of *C. camphora* (camphor) and L. *angustifolia* (lavender) against L. *sericata*. The authors have observed distortion of sensorial structures, wrinkling of the cuticle surface and slight degeneration of the anterior spiracle in L3, treated with camphor oil, while L3 treated with lavender EO showed cuticle distortion. Comparably, changes such as cuticle swelling, degeneration of anterior spiracles, a wrinkled and shrunken cuticle and degeneration of papillae were also reported by Hoda et al. (Hoda et al., 2016) using an alcoholic extract of *Balanites aegyptiaca* and the EO from *Commiphora molmol*, against different stages of L. *sericata*. Ultrastructural assessment of L3 may assist in elucidating the damage caused by biopesticides, improving the identification of the mechanism of action (Mendonça et al., 2014). In this sense, it is noteworthy that this is the first study assessing the damage caused by CLLEO and α-phellandrene to L. *cuprina* L3 by SEM. One of the first bioactives known to induce anomalies in different insects, azadirachtin extracted from the Indian neem tree *Azadirachta indica* A. Juss (Meliaceae) has been tested against different insects, including L. *cuprina*. This effect may occur due to the interference of neuroendocrine control of molting and ecdysis (Schmutterer, 1990). Another study about the effects of azadirachtin on the morphology of L. *cuprina* larvae investigated morphological changes in the ultrastructure of the endocrine glands (the prothoracic gland, the *corpus allatum* and the *corpus cardiacum*) responsible for controlling molting and leant support to this theory (Schmutterer, 1990; Meurant et al., 1994). It is worth mentioning that, among natural products, azadirachtin has been approved for use in The United States since 1990, was subsequently approved in Germany and is currently approved in 10 other countries in the European Union, in addition to China, India and Canada (Isman, 2015).

4.5. Larval histopathology

The results of this study demonstrate the neurotoxic effect of CLLEO and α-phellandrene. In addition, the appearance of small inclusions in the fat body of L3 may be due to L3 stress mechanism after treatment, signalling energy reserves in a process of drug detoxification. Supporting this argument, microscopic observations could be used to determine the ability of EO candidates to penetrate L3 cuticle, to elucidate the target cells for metabolism of EO and/or to determine the effect of biopesticides over time (Chaaban et al., n.d.). Some studies regarding pesticide action to the midgut, fat body and brain morphology of insects have provided new insights into the mode of action and damage by thiamethoxam, fipronil, deltamethrin, temephos, ivermectin and abamectin (Alves et al., 2010; Cruz et al., 2010; Jacob et al., 2015; Tavares et al., 2015). Following pesticide treatment, several changes in specific organelles in the digestive tract of insects suggest that energy is necessary for vacuolation and vesicle release, possibly as an attempt to detoxify cells (Alves et al., 2010; Cruz et al., 2010). The insect fat body may be another important insect structure due to its prime location for intermediary metabolism and detoxification processes. Moreover, several studies have suggested that the fat body also plays a role in the immune response (Alves et al., 2010; Alves et al., 2004; Almeida et al., 2014). We believe that the damage found on the fat body of larvae in our work, such as cytoplasmic vacuolation and

irregular morphology of trophocytes with pyknotic nuclei, suggests an attempt by detoxicative metabolism to excrete toxicants (CLLEO and α-phellandrene). The brain is also another important target organ for pesticides that may also help to elucidate the mode of action of some EO. Oliveira et al. (Oliveira et al., 2014) reported side-effects of sub-lethal doses of thiamethoxam in the brain and the midgut of *Apis mellifera*. Morphological and histochemical alterations, such as cytoplasm vacuolization and condensed cells with more intense staining in the brain, have been demonstrated. Photomicrographs showing morphological alterations induced by boric acid and fipronil in the midgut of *A. mellifera* larvae revealed changes such as cytoplasmic vacuolisations with the absence of autophagy vacuoles, and chromatinic compaction of cells (Cruz et al., 2010). Jacob et al. (Jacob et al., 2015) assessed the impact of sub-lethal doses of fipronil, a neurotoxic insecticide, on the brain mushroom bodies of the stingless bee *Scaptotrigona postica*. The authors showed morphological changes (pyknotic profiles in brain) suggestive of cell death, apoptosis and necrosis. Histological alterations in organs such as fat body and brain of L3 of L. *cuprina* treated with natural compounds have not been demonstrated previously. The damages that were found in the brains of L. *cuprina* L3 (vacuolar degeneration, pyknotic profiles, disorganized and condensed cells), in our study support previous reports and reinforce the neurotoxic effect of CLLEO and α-phellandrene. However, recent work has shown that photomicrographs of L. *cuprina* exposed to 1.59 μL/cm^2 (10%) of *T. minuta* EO had significant degeneration of the digestive tract (Chaaban et al., n.d.). Hoda et al. (Hoda et al., 2016) reported microscopic damage (destruction of gut epithelium) in L. *sericata* treated with an alcoholic extract and EO of *C. molmol* and *Balanites aegyptiaca*. Although this result corroborates with our data regarding the damage to the digestive tract after CLLEO and α-phellandrene exposure, additional studies are required in order to better elucidate the mechanism of action of both products (CLLEO and α-phellandrene).

5. Conclusion

We report that a single application of CLLEO or α-phellandrene was significantly toxic to L3 of *L. cuprina* and could be considered as a good ecofriendly product to control this pest. Damage to target organs such as the cuticle, brain, midgut and fat body of L3 was evident, even at low concentrations of both products. These evidences shall be used as future biomarkers to help elucidate the mechanism of action of these compounds. Thus, the vacuolar degeneration and pyknotic profiles observed in the brains of L3 treated with CLLEO and α-phellandrene, as well as the decreased motility observed within < 6 h after treatment suggest that this compound has neurotoxic activity. The potential of both extracts as bioinsecticides against L. *cuprina* L3 represent a sustainable alternative for myiasis control in humans and animals. Finally, this work leads the way to new investigations about the application of leaves from *C. longa*, as part of the plant is still considered a by-product of the turmeric harvest.

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Data Article

Cuticular damage of *Lucilia cuprina* larvae exposed to *Curcuma longa* leaves essential oil and its major compound α-phellandrene

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ABSTRACT

Morphological biomarkers as the histopathological assessment and scanning electron microscopy can be used to establish a diagnosis of structure damage and intoxication of target cells by new biopesticide candidate. In this sense, cuticle damage caused by active substances in larvae exposed to biopesticides can help to elucidate the mode action. Thus, insecticide activity analysis of essential oil of *Curcuma longa* leaves and its major compound α-phellandrene have proven to be a new biopesticide candidate against third instar larvae (L3) of the Australian blowfly *Lucilia cuprina*. In this way, groups of 20 L3 were placed on filter paper, impregnated with ranging concentrations (from 0.15 to 2.86 μL/cm^2) of *C. longa* leaves EO and (0.29–1.47 μL/cm^2) to

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α-phellandrene. The extracts were solubilized in ethanol. Progressive darkening in the body of L3, marked reduction of movement, color changes in larval cuticle and dead were observed 6 and 24 h after contact with both extracts.

Specifications table

Subject area	*Parasitology*
More specific subject area	*Entomology*
Type of data	*Videos*
How data were acquired	*Microscope stereoscopy*
Data format	*Raw data collection and analysis*
Experimental factors	*Fresh aerial parts of Curcuma longa (leaves) and its major compound α-phellandrene were assessed for insecticidal activity using biological assays on Lucilia cuprina performed as described by Chaaban et al., 2018* [1].
Experimental features	*Essential oil extraction and chemical characterization. Establishment of Lucilia cuprina colonies; and biological assays on laboratory conditions* (27 ± 2 °C and 70% *relative humidity*). *Contact tests using filter paper impregnated with Curcuma longa leaves essential oil and its major compound α-phellandrene. Cuticular damage and larvae motility were reported.*
Data source location	*Araquari, Santa Catarina, Brazil; 26°23′ 33.6691″ S and 48° 44′ 18.3336″ W.*
Data accessibility	*Data are displayed within this article.*
Related research article	*This Data in Brief article is submitted as a companion paper to: Chaaban, A., Richardi, V.S., Carrer, Brum, J.S., Cipriano, R.R.,Martins, C.E.N., Silva, M.A.N., Deschamps, C., Molento, M.B. (in press).* Insecticide activity of Curcuma longa (leaves) essential oil and its major compound α-phellandrene against Lucilia cuprina larvae (Diptera: Calliphoridae): Histological and ultrastructural biomarkers assessment. *Pesticide Biochemistry and Physiology* [1]

Value of the data

- Potential use of *Curcuma longa* and α-phellandrene as bioinsecticide against *Lucilia cuprina*.
- Contact activity of *C. longa* leaves essential oil and its major compound α-phellandrene over *L. cuprina* larvae.
- Determination of time-dependent damage of *L. cuprina* larvae exposed to *C. longa* leaves essential oil and α-phellandrene.

1. Data

The results of this study involve the experimental data from the cuticle damage of *L. cuprina* third instar larvae, exposed to *C. longa* leaves essential oil and its major compound α-phellandrene [1]. The

larvae of the control group, using ethanol as solvent, showed no cuticle alterations after 6 and 24 h of contact (Video 1a, 1b; Video 2a, 2b). The insecticide effects of *C. longa* leaves EO and α-phellandrene can be observed $\leq$6 h after contact with the tested solutions (Video 1c; Video 2c). Moreover, progressive darkening in the body of L3, marked reduction of movement, color changes in larval cuticle and dead were observed in both extracts 24 h after exposure (Video 1d; Video 2d).

Supplementary material related to this data article can be found online at https://doi.org/10.1016/j.dib.2018.11.001.

2. Experimental design, materials and methods

2.1. Plant material, essential oil extraction and chemical characterization

C. longa leaves used in this work were grown in the Medical Plants Unit of the Catarinense Federal Institute (IFC), located at 26° 23′ 33.6691″ S and 48° 44′ 18.3336″ W at 10.6 m above the sea level in the city of Araquari, Santa Catarina State, South of Brazil. The plant cultivation, essential oil extraction and chemical characterization were carried as described in the companion paper [1]. The α-phellandrene (CAS: 99-83-2) studied was acquired commercially and certified as having purity of $\geq$99%, from Sigma-Aldrich Brazil Ltda (São Paulo, SP, Brazil).

2.2. Establishment of L. cuprina colonies and larval toxicity

Wild flies were collected manually at the IFC, using bait and insect nets. The establishment of stock colonies, insects' identification, maintenance, mass reproduction and the protocol for the biological tests were performed as described by Chaaban et al. [2]. The toxicity of *C. longa* leaves EO and α-phellandrene to *L. cuprina* larvae was performed using groups of 20 L3, placed on filter paper, impregnated with a range of concentrations (0.15–2.86 μL/cm^2) of *C. longa* leaves EO and (0.29–1.47 μL/cm^2) to α-phellandrene. The L3 were put into glass vials containing a filter paper (12.56 cm^2) impregnated with 0.2 mL of solutions with EO, that were solubilized in ethanol. The toxicity was evaluated by observing L3 mortality at 6, 24 and 48 h after contact [1,2]. Total larval mortality (LM) was calculated [1–3] as follows:

$$LM = (total\ died\ larvae \times 100)/total\ tested\ larvae$$

For reported of the cuticle damage through the videos, L3 exposed to 1.59 μL/cm^2 of *C. longa* leaves EO and 1.47 μL/cm^2 of α-phellandrene solubilized in ethanol were used as described in the companion paper [1].

Transparency document. Supporting information

Transparency data associated with this article can be found in the online version at https://doi.org/10.1016/j.dib.2018.11.001.

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