Journal Pre-proof

Lethal and behavioural effects of a green insecticide against an invasive polyphagous fruit fly pest and its safety to mammals

Giovanni Benelli, Camilla Ceccarelli, Valeria Zeni, Roberto Rizzo, Gabriella Lo Verde, Milko Sinacori, Maria C. Boukouvala, Nickolas G. Kavalieratos, Massimo Ubaldi, Daniele Tomassoni, Federica Benvenuti, Proshanta Roy, Riccardo Petrelli, Loredana Cappellacci, Eleonora Spinozzi, Filippo Maggi, Angelo Canale

PII: S0045-6535(21)02561-3

DOI: https://doi.org/10.1016/j.chemosphere.2021.132089

Reference: CHEM 132089

To appear in: *ECSN*

Received Date: 5 July 2021

Revised Date: 23 August 2021

Accepted Date: 27 August 2021

Please cite this article as: Benelli, G., Ceccarelli, C., Zeni, V., Rizzo, R., Lo Verde, G., Sinacori, M., Boukouvala, M.C., Kavalieratos, N.G., Ubaldi, M., Tomassoni, D., Benvenuti, F., Roy, P., Petrelli, R., Cappellacci, L., Spinozzi, E., Maggi, F., Canale, A., Lethal and behavioural effects of a green insecticide against an invasive polyphagous fruit fly pest and its safety to mammals, *Chemosphere* (2021), doi: https://doi.org/10.1016/j.chemosphere.2021.132089.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2021 Published by Elsevier Ltd.

CRediT author statement

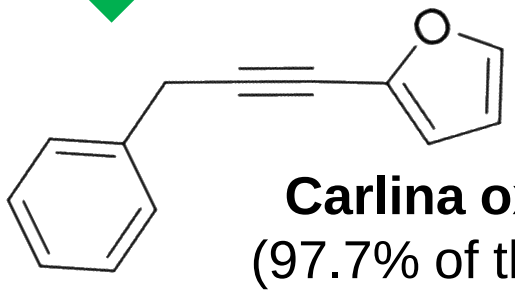
Giovanni Benelli: Conceptualization, Investigation, Methodology, Data curation, Project administration, Original draft preparation, Writing- Reviewing and Editing.
Camilla Ceccarelli: Investigation, Visualization, Data curation, Original draft preparation, Writing- Reviewing and Editing,
Valeria Zeni: Investigation, Methodology, Visualization, Data curation, Original draft preparation, Writing- Reviewing and Editing.
Roberto Rizzo: Investigation, Methodology, Visualization, Data curation, Original draft preparation, Writing- Reviewing and Editing.
Gabriella Lo Verde: Investigation, Methodology, Visualization, Data curation, Writing- Reviewing and Editing.
Milko Sinacori: Investigation, Visualization, Data curation, Writing- Reviewing and Editing,
Maria C. Boukouvala: Visualization, Data curation, Writing- Reviewing and Editing.
Nickolas G. Kavalieratos: Visualization, Data curation, Writing- Reviewing and Editing.
Massimo Ubaldi: Conceptualization, Methodology, Investigation, Visualization, Data curation, Original draft preparation, Writing- Reviewing and Editing.
Daniele Tomassoni: Investigation, Methodology, Visualization, Data curation, Writing- Reviewing and Editing.
Federica Benvenuti: Investigation, Methodology, Visualization, Data curation, Writing- Reviewing and Editing.
Proshanta Roy: Investigation, Methodology, Visualization, Data curation, Writing- Reviewing and Editing,
Riccardo Petrelli: Investigation, Visualization, Data curation, Writing- Reviewing and Editing.
Loredana Cappellacci: Investigation, Visualization, Data curation, Writing- Reviewing and Editing.
Eleonora Spinozzi: Investigation, Visualization, Data curation, Writing- Reviewing and Editing.
Filippo Maggi: Conceptualization, Investigation, Methodology, Visualization, Data curation, Project administration; Writing- Reviewing and Editing.
Angelo Canale: Methodology, Visualization, Data curation, Project administration; Writing- Reviewing and Editing.

Journal Pre-proof

Journal Pre-proof

Carlina acaulis

- Essential oil extraction
- Carlina oxide purification
- NMR analysis

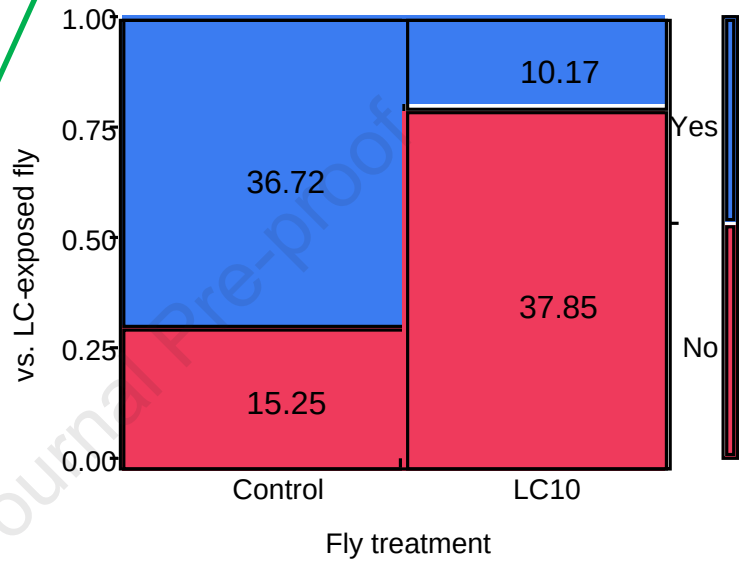


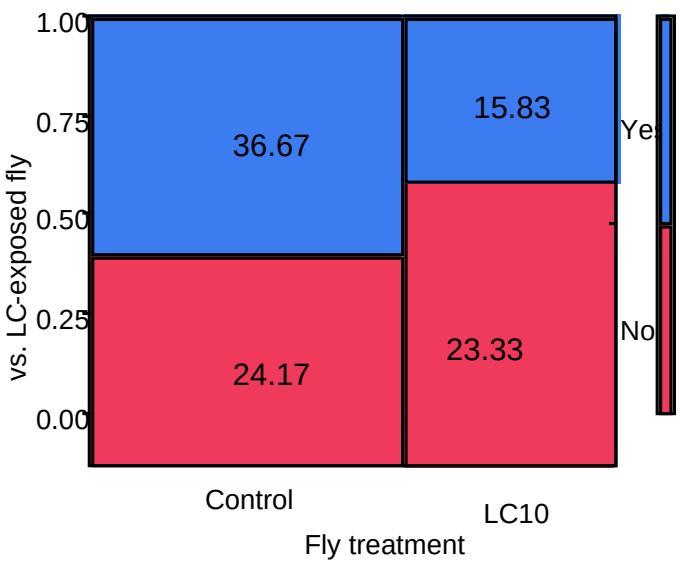
Carlina oxide
(97.7% of the oil)

Ingestion toxicity tests on adult medflies, *Ceratitis capitata*

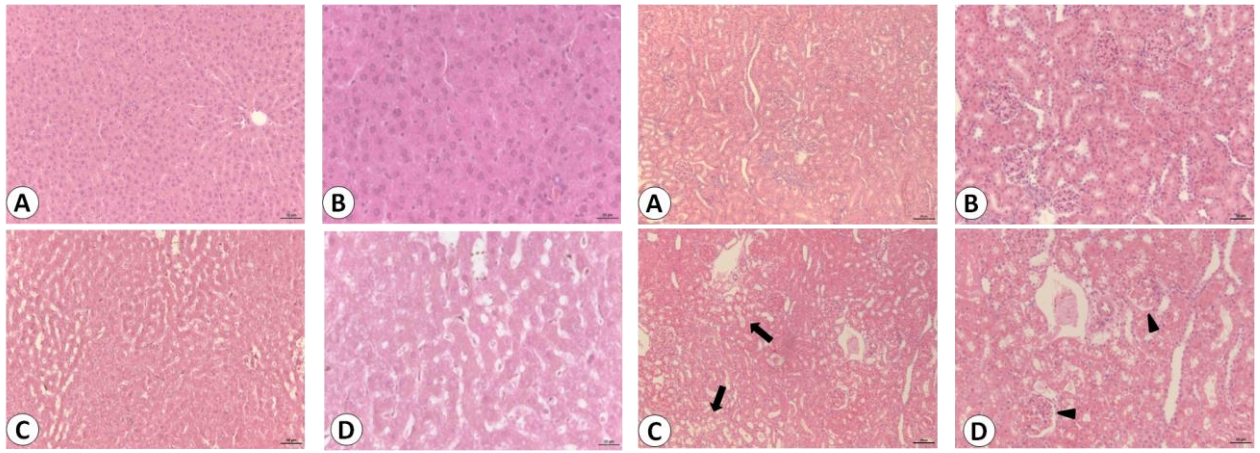
LC_{10}=555, LC_{30}=906, LC_{50}=1273, LC_{90}=2922 ppm

Impact on medfly aggressive interaction asymmetries





Impact on non-target mammals: histological insights



Journal Pre-proof

Lethal and behavioural effects of a green insecticide against an invasive polyphagous fruit fly pest and its safety to mammals

Giovanni Benelli [a,*], Camilla Ceccarelli [a], Valeria Zeni [a], Roberto Rizzo [b], Gabriella Lo Verde [c], Milko Sinacori [c], Maria C. Boukouvala [d], Nickolas G. Kavalieratos [d], Massimo Ubaldi [e], Daniele Tomassoni[f], Federica Benvenuti [e], Proshanta Roy [f], Riccardo Petrelli [e], Loredana Cappellacci [e], Eleonora Spinozzi [e], Filippo Maggi [e], Angelo Canale [a]

[a] *Department of Agriculture, Food and Environment, University of Pisa, via del Borghetto 80, 56124, Pisa, Italy*

[b] *CREA Research Centre for Plant Protection and Certification, S.S. 113 - km 245.500, 90011, Bagheria, PA, Italy*

[c] *Department of Agricultural, Food and Forest Sciences, University of Palermo, viale delle Scienze, Ed. 5, 90128 Palermo, Italy*

[d] *Laboratory of Agricultural Zoology and Entomology, Department of Crop Science, Agricultural University of Athens, 75 Iera Odos Str., 11855 Athens, Attica, Greece*

[e] *School of Pharmacy, University of Camerino, via Sant'Agostino 1, 62032, Camerino, Italy*

[f] *School of Biosciences and Veterinary Medicine, University of Camerino, via Gentile III da Varano, 62032, Camerino, Italy*

* Corresponding author. Tel.: +39-0502216141. E-mail address: giovanni.benelli@unipi.it (G. Benelli).

Journal Pre-proof

Abstract

Plant essential oil-based insecticides, with special reference to those that may be obtained from largely available biomasses, represent a valuable tool for Integrated Pest Management. However, the sublethal effects and the potential effects on aggressive insect traits of these green insecticides are understudied. Herein, the lethal and sub-lethal effects of the carlina oxide, constituting more than 97% of the whole *Carlina acaulis* (Asteraceae) root essential oil (EO), were determined against an invasive polyphagous tephritid pest, *Ceratitis capitata* (medfly). The carlina oxide was formulated in a mucilaginous solution containing carboxymethylcellulose sodium salt, sucrose, and hydrolysed proteins, showing high ingestion toxicity on medfly adults. The behavioural effects of carlina oxide at LC_{10} and LC_{30} were evaluated on the medfly aggressive traits, which are crucial for securing reproductive success in both sexes. Insecticide exposure affected the directionality of aggressive actions, but not the aggression escalation intensity and duration. The EO safety to mammals was investigated by studying its acute toxicity on the stomach, liver, and kidney of rats after oral administration. Only the highest dose (1000 mg/kg, slightly lower than the LD_{50} calculated on medflies) of the EO caused modest neurological signs and moderate effects on the stomach, liver, and kidney. The other doses, which are closer to the practical use of the EO when formulated in protein baits, did not cause side effects. Overall, *C. acaulis*-based products are effective and safe to non-target mammals, deserving further consideration for eco-friendly pesticide formulations.

Keywords: Aggressiveness; attract and kill; *Carlina acaulis*; carlina oxide; plant essential oil; Tephritidae

Journal Pre-proof

1. Introduction

Growing concerns about climate change, biodiversity, animal welfare, and food security have pushed agriculture towards a more sustainable approach (Bijani *et al.*, 2019; Karami *et al*., 2017; Valizadeh *and* Hayati, 2021). The overuse of synthetic insecticides affected human health (**S**ingh *et al*., 2018), the environment (Cachada *et al*., 2016; Capowiez *et al.*, 2005; Postigo *et al*., 2021; Senthil Rathi *et al.*, 2021), especially non-target organisms (Bozhgani *et al*., 2018; Forson *et* Storfer, 2006; Ricupero *et al*., 2020; Wu *et al*., 2018). In addition, synthetic insecticides are also responsible for the quick development of insecticide-resistant pest strains (Guillem-Amat *et al.*, 2020; Hsu *and* Feng, 2006; Nwankwo, 2021; Horowitz *et al*., 2020). Given all the side effects of using synthetic insecticides, (Postigo *et al.*, 2021; Singh *et al.*, 2018; Wu *et al.*, 2018;), botanical pesticides, such as essential oils (EOs) and their main compounds, represent a valid alternative for the control of pests of agricultural interest (Benelli *et al.*, 2021).

Polyacetylenes are a chemical class that includes all compounds with one or more carbon-carbon triple bond or alkynyl functional groups (Minto *et al.*, 2008). These compounds are widely distributed and occurred in several botanical families among which Asteraceae, Apiaceae, Campanulaceae, and Pittosporaceae are the most representative ones (Negri, 2015). Polyacetylenes may act as phytoalexins, i.e., insect-induced defence compounds (Yactayo-Chang *et al.*, 2020), and therefore they may represent an interesting prototype for the development of ecological insecticides. In particular, our research group recently focused on the insecticidal potential of carlina oxide (syn. 2-(3-phenylprop-1-ynyl)furan), an aromatic C_{13} polyacetylene bearing one carbon-carbon triple bond and a furan ring, which is the main component (> 95%) of *Carlina acaulis* L. (Asteraceae) EO oil as well as other *Carlina* species (Mejdoub *et al.*, 2020; Strzemski *et al.*, 2017), and *Carthamus caeruleus* L. (Mami *et al.*, 2020). The carlina oxide and its corresponding EOs, probably due to their phytotoxicity and ease of

penetration into the insect cuticle (Champagne *et al.*, 1986), are effective against a wide number of noxious insects, including mosquitoes (*Culex quinquefasciatus* Say), house flies (*Musca domestica* L.), tephritid flies (*Ceratitis capitata* (Wiedemann)), moths (*Lobesia botrana* (Denis & Schiffermüller)), and stored-product beetles (*Prostephanus truncatus* (Horn) and *Trogoderma granarium* Everts) (Benelli *et al.*, 2019, 2020, 2021; Kavallieratos *et al*., 2020; Pavela *et al*., 2020).

To develop a proper pest management protocol, it is also essential to gain knowledge about the impact of EOs applied at low doses. Sublethal doses of botanical pesticides may impact the insect life span, development, sex ratio, fertility, fecundity, and behavioural traits (Lee, 2000). The large majority of sublethal effect research is focused on synthetic pesticides (Fernandes et al 2016; Lin et al 2020), while a lower number of efforts has been directed to understand the impact of new plant-borne insecticides (Izakmehri *et al.*, 2013; Khosravi *et al.* 2010; Borzoui *et al.*, 2016; Nouri-Ganbalani *et* Borzoui, 2017). Concerning the EO of *C. acaulis*, the exposure of *M. domestica* adults to a sublethal dose (LD_{30}) led to significant reductions in female longevity, fecundity, and F_1 vitality (Pavela *et al.*, 2020). Recent studies investigated how exposure of *C. capitata* adults to *C. acaulis* essential oil also impacts insect intraspecific aggression dynamics (Benelli *et al.*, 2021).

As a continuation of our studies on this interesting plant species, herein we evaluated the acute toxicity of the main compound of *C. acualis* EO: carlina oxide (constituting > 90% of the whole *C. acaulis* EO) towards adult of *Ceratitis capitata* (Diptera: Tephritidae) in ingestion toxicity assays. Also known as medfly, *C. capitata* is a highly invasive polyphagous pest species causing both quantitative and qualitative damages to several crops (Schliserman *et al.*, 2014). Besides its importance as a fruit pest, the medfly is a model organism for behavioural research, due to its complex aggressive and mating behavior (Benelli *et al.*, 2015a; Benelli *and* Romano, 2018; Briceño *et al.*, 1999). Therefore, LC_{10} and LC_{30} of carlina oxide were evaluated for their

potential impact on aggressive traits of medfly adults, which are crucial for securing reproductive success in both sexes (Benelli *et al*., 2015b). Lastly, to give new insights into the mammal safety of *C. acaulis* based products, herein we evaluated acute toxicity of its EO, containing 97.7% of carlina oxide, on the stomach, liver, and kidney of rats after oral administration. These assays were performed with *C. acaulis* EO, for sake of practicality, yield, and costs related to carlina oxide purification. Our findings shed light on the possible utilization of the polyacetylenes carlina oxide as an active ingredient to substitute insecticides in "attract and kill" formulations, and at the same time, we provide new important information about mammal safety.

2. Materials and methods

2.1. *Carlina acaulis* EO extraction

Carlina acaulis dry roots were purchased from A. Minardi & Figli S.r.l. (Bagnacavallo, Ravenna, Italy, https://www.minardierbe.it). The roots were powdered with a grinder (Albrigi, Stallavena, Verona, Italy, mod. E0585) using a 1.5 mm sieve. One kg of the roots was soaked overnight in 6 L of distilled water into a 10 L Pyrex glass flask and hydrodistilled for 8 h with a Clevenger-type device, heated by a Falc MA mantle (Falc Instruments, Treviglio, Italy). The yellowish EO was isolated in 0.89% yield (w/w, dw), with a density of 1.063 g/mL.

2.2. Chemical analysis of *C. acaulis* EO and purification of carlina oxide

The chemical composition of the EO was investigated by an Agilent 6890 N gas chromatograph provided with a single quadrupole 5973 N mass spectrometer and an auto-sampler 7863 (Agilent, Wilmingotn, DE). The separation of EO chemical constituents was performed using

an HP-5MS capillary column (30 m length, 0.25 mm i.d., 0.1 µm film thickness; 5% phenylmethylpolysiloxane) from Agilent (Folsom, CA, USA). The column was allowed to reach a temperature of 60 °C for 5 min, then of 220 °C at 4 °C/min, and finally of 280 °C at 11 °C/min held for 15 min. Injector and detector were thermostated at 280 °C. The mobile phase was constituted of 99.9% He, with a flow of 1 mL min^{-1}. Before the analysis, the EO was diluted 1:100 in *n*-hexane and then 2 µL were injected in split mode (1:50). Electron impact (EI, 70 eV) mode in the range of 29-400 *m/z* was used for peak acquisition. The analysis of chromatograms was performed using the MSD ChemStation software (Agilent,Version G1701DA D.01.00), while data analysis was performed using the NIST Mass Spectral Search Program for the NIST/EPA/NIH EI and NIST Tandem Mass Spectral Library v. 2.3. The identification of the EO components was achieved by the combination of the temperature programmed retention indices (RIs) and mass spectra (MS) in comparison with those of ADAMS, NIST 17, and FFNSC2 libraries (Adams, 2007; NIST 17, 2017; FFNSC 2, 2012). The RI were calculated using a mix of *n*-alkanes (C_8-C_{30}, Supelco, Bellefonte, CA, USA), according to the Van den Dool and Kratz (1963) formula. The EO was mainly characterized by carlina oxide (97.7%) and two minor components such as benzaldehyde (1.14%) and *ar*-curcumene (0.29%). The total of identified compounds was 99.13%. The EO (1.41 g) was chromatographed by silica gel column chromatography (70–230 mesh, 60 Å, Merck) using 100% of *n*-hexane as mobile phase, yielding 1.34 g of pure carlina oxide. NMR analysis confirmed its chemical structure, through a Bruker Avance 400 Ultrashield spectrometer using tetramethyl silane (TMS) as an internal standard. The NMR spectrum was linear with the literature (Benelli *et al*., 2019).

2.3. Carlina oxide ingestion toxicity on medflies

C. capitata medflies were from a mass-rearing of the University of Pisa; they were reared as detailed by Canale *and* Benelli (2012) at 25 ± 1 °C and 45 % R.H., 16:8 (L:D) photoperiod. Following Benelli *et al*. (2012, 2021), ingestion toxicity was evaluated on 30 medfly adults (both males and females), placing them in a plastic container (600 mL) covered with a thin mesh. *C. capitata* adults were fed for 96 h with 2 mL of viscous formulation containing 0.0039, 0.0078, 0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1% of carlina oxide. The viscous formulation was obtained emulsifying carlina oxide with dimethyl sulfoxide (DMSO) (1:1), then adding 2% of carboxymethylcellulose Na salt, 12.5% of sucrose, and 1% of protein bait (NuBait, Biogard, Italy). The formulation was provided in a bakelite cup (Ø =30 mm). For the prevention of medfly drowning, a cotton disk (Ø =30 mm) was used to cover the cup. The negative control for each test was performed testing the viscous carrier without carlina oxide. During the experiments, medfly mortality was noted after 96 h. No less than 4 replicates were performed per carlina oxide concentration, over different days, under laboratory conditions (21 ± 1 °C, 45 ± 10 % R.H., 16:8 (L:D) photoperiod).

2.4. Analysis of medfly aggressive behaviour

Carlina oxide at the LC_{10} and LC_{30} estimated on adult medflies as described above was evaluated for its potential impact on *C. capitata* aggressive behaviour, following the methodology recently proposed by Benelli *et al.* (2021). Either the mucilage with carlina oxide or the negative control was prepared and administered as described in paragraph 2.3. Prior observation, medfly sorted by sex were fed on the viscous formulation for 96 h, and the potential impact of feeding on carlina oxide on the fly aggressiveness was observed till 4 days post-feeding.

The behavioural assays were performed inside an arena (i.e., a plastic container, volume: 600 mL) covered by a piece of glass, where a *Citrus limon* (L.) Osbeck twig or leaf (~15 cm, 1 leaf/twig) was placed. Trials on a given LC and fly sex were repeated on 30 groups composed

by 4 *C. capitata* adults, two earlier were fed on the mucilage containing carlina oxide, while the remaining were fed with the vicious control carrier. Each replicate lasted 30 min, with an initial adaptation phase of 10 min. At the end of each replica, the glass and the plastic container were washed with soap and hot water and new specimens and lemon twig were used. An aggression event occurred when a *C. capitata* adult (treated or control) approached the other and displayed an escalating aggressive behaviour as reported by Benelli *et al.* (2015a). The intensity of the aggression event was recorded in terms of aggression score, both during ♂-♂ and ♀-♀ aggressive interactions. The above-mentioned aggression score ranges from 0 to 12 and includes the following events: 1. Avoidance (one). 2. Wing waving (one). 3. Wing waving (both). 4. Chasing. 5. Fast head rocking. 6. Pouncing. 7. Labellar (one). 8. Labellar (both). 9. Wing strike. 10. Dive. 11. Boxing (one). 12. Boxing (both). Each event is defined in detail in Benelli *et al.* (2015a). We also noted which fly (i.e., control or treated) started attacking a conspecific, as well as the number of aggressive events per sex, and the length of each aggressive event (Benelli *et al*. 2021). Experiments were conducted from May to October 2020 in a room illuminated with fluorescent daylight tubes (Philips 30W/33) (16:8 (L:D) photoperiod).

2.5. Non-target toxicity on rats

To have enough amount of sample to perform *in vivo* toxicological assays, we decided to test the *C. acaulis* EO containing 97.7 % of carlina oxide instead of the purified compound (99%) which requires additional costs in terms of devices for chromatographic separations and consumption of organic solvent with relative waste disposal.

2.5.1. Animals

For the acute toxicity studies were employed female Wistar rats weighting 250-300 g. Each animal was single caged and kept in cycles of 12 h of the dark followed by 12 h of light at 20-

22 °C and R.H. 44-45%. Water and food were available ad libitum. Housing and experiments were carried out following the guidelines of the European Community Council Directive Care and Use of Laboratory Animals (Ministry of Health Authorization n° 1D580.22).

2.5.2. Acute toxicity procedures

The doses of the *C. acaulis* EO were constituted by the EO dissolved in 2% Tween 80 vehicle. The administration was performed by gavage. For the acute toxicity studies, the animals were divided into four groups, each composed of four individuals. The first group received the vehicle; the second group 250 mg of *C. acaulis* EO per kg by oral administration; the third group 500 mg/kg; the fourth group 1000 mg/kg. After the oral administration, possible signs of toxicity were observed in each animal for the first 30 min., then periodically, for the remaining 48 h until the harvesting of the tissues. When occurring, the time of death was registered. Signs of toxicity noted focused on central nervous and autonomic system activities and were convulsions, tremors, ataxia, straub, ptosis, coma, cyanosis, lacrimation, piloerection, and salivation. 48 h after the dosing, the weight of each rat was recorded, and the rats were sacrificed. The organs were surgically removed, and their weight and characteristics were observed. Finally, the organs were placed in a fixative solution or frozen at – 80°C.

The stomach, the liver, and the kidneys were removed, separated into small pieces, and fixed in Bouin's solution for 6 hours. After fixation, samples were dehydrated in gradual ethanol from 70% to absolute and cleared in xylene for the paraffin embedding. 5 µm consecutive sections were stained with haematoxylin and eosin dye (H&E), observed under a light microscope Leica DMR (Germany) connected by a DS-R12 Nikon camera to the computer and estimated using a NIS Elements Nikon image analyser software. Sections were blindly analysed; at the level of the stomach, the presence of inflammatory aggregate, and the presence of ulcer and elements of necrosis were evaluated. In the sections of the liver, the following parameters were evaluated: inflammatory elements, degeneration of hepatocytes, vacuolization, presence of apoptotic cells

or hepatic necrosis, dilated sinusoids. Signs of infiltration, glomerular and tubular alterations were examined in the kidney.

2.6. Data analysis

In *C. capitata* ingestion toxicity assays, control mortality values ranging from 1 to 20% were adopted to accurate experimental mortality rates through Abbott's formula (Abbott, 1925). Ingestion LC_{10}, LC_{30}, LC_{50}, and LC_{90} with associated 95% confidence intervals (CI), χ^2 values, and *p*-values were assessed using probit analysis (Finney, 1978).

Following Benelli *et al*. (2021), differences in the abundance of *C. capitata* adults showing aggressive displays after feeding on a given carlina oxide LC were analysed using a likelihood chi-square test with Yates' correction (Rohlf *and* Sokal, 1981). Differences among the aggression duration values characterizing carlina oxide-exposed or control *C. capitata* adults were analysed through a weighted generalized linear model (GLZ) described by Benelli *et al*. (2015a, 2021), i.e., $y = X\beta + \varepsilon$, where y is the vector of the observation (i.e., aggression duration), X is the incidence matrix, β is the vector of the fixed effect (i.e., exposure to a given carlina oxide LC), and ε is the vector of the random residual effects ($\alpha = 0.05$). Differences among the aggression scores characterizing carlina oxide-exposed or control *C. capitata* adults were assessed through the Kruskal-Wallis test ($p = 0.05$). JMP® 9 (SAS) and Minitab Inc., State College, PA were used for these analyses.

In mammal safety experiments, the rat body weight data were analysed by one-way ANOVA as the main effects. When appropriate, Tukey's multiple tests was used as post-hoc test (α=0.05). GraphPad Prism 8 software (San Diego, CA) was used for analysing these data.

3. Results

3.1. Carlina oxide ingestion toxicity on medfly

The toxicity of carlina oxide obtained in *C. capitata* adults was estimated by probit analysis and evaluated at different concentrations. Dose-response bioassays showed that the LC values of the oxide were: LC_{10} = 555.086 ppm, LC_{30} = 906.731 ppm, LC_{50} = 1273.708 ppm, and LC_{90} = 2922.671 ppm. Overall, the carlina oxide was highly effective and proved to be a good candidate as an active ingredient in the "attract and kill" formulation tested adult medflies (Table 1).

3.2. Analysis of the medfly aggressive behaviour

3.2.1. Number of aggressive events

The potential impact of feeding on carlina oxide was firstly examined on *C. capitata* aggressive behaviour at a population level, i.e., impact on the overall abundance of medfly adults displaying aggressive behaviour, regardless of the intensity and length of the events. As a general trend, both male and female medflies fed on carlina oxide-based viscous formulations showed an aggressiveness comparable to control individuals, except for females exposed to LC_{10} (Figure 1). Indeed, LC_{10}-fed females showed a significant difference in number of aggressive events, if compared with the control ones (47 vs. 73 aggressive events, respectively, χ^2= 5.642, *d.f.* = 1, p = 0.017), while the number of aggressive events performed by LC_{10}-fed males (85 vs. 92 aggressive events, χ^2= 0.282, *d.f.* = 1, p = 0.598), LC_{30}-fed males (86 vs. 94 aggressive events, χ^2=0.361, *d.f.* =1, p = 0.551), and LC_{30}-fed females (55 vs. 70 aggressive events, χ^2 =1.808, *d.f.* = 1, p = 0.179) did not differ from the respective controls (Figure 1). Overall, control medflies performed a higher number of aggressive actions compared to medflies exposed to the carlina oxide, but a significant difference was found only between LC_{10}-fed females and control flies.

3.2.2. Asymmetries in aggressive interactions

Herein, we evaluated the directionality of the aggressions to discriminate between medflies carrying out the aggression and those suffering it. The tested LC significantly influenced the directionality of aggressive actions. Both medfly sexes fed on carlina oxide LC_{30} were more attacked by control flies (♂: $\chi^2 = 26.945$, *d.f.* = 1, $p < 0.0001$; ♀: $\chi^2 = 24.890$, *d.f.* = 1, $p < 0.0001$) (Figure 2). Similarly, medflies fed on carlina oxide LC_{10} were more attacked by control flies (♂: $\chi^2 = 43.427$, *d.f.* = 1, $p < 0.0001$; ♀: $\chi^2 = 4.517$, *d.f.* = 1, $p = 0.033$) (Figure 2). Overall, the exposure to both concentrations of the tested carlina oxide seemed to affect the willingness to receive attacks from control flies by the treated flies.

3.2.3. Sex of the fighting flies

Aggressive interactions might vary according to the sex of the involved fly. Contingency analysis carried out between the fly treatment, i.e, LC-fed or LC-unfed fly, and the sex showed no significant differences between sexes for both sub-lethal doses (LC_{10}: $\chi^2 = 2.272$, *d.f.* = 1, $p = 0.131$; LC_{30}: $\chi^2 = 0.424$, *d.f.* = 1, $p = 0.515$) (Figure 3), highlighting that there is no difference in term of aggressiveness between males and females of *C. capitata.*

3.2.4. Aggression score

Ceratitis capitata fights are escalating and highly ritualized. Relying to the aggression score described in the Materials and Methods section, herein we quantified the intensity of the aggressions carried out by medflies. As regards aggression scores, no differences were found between *C. capitata* fed on distinct carlina oxide concentrations and the control insects (males fed on LC_{10}: $\chi^2 = 1.670$, *d.f.* = 1, $p = 0.196$; males fed on LC_{30}: $\chi^2 = 0.585$, *d.f.* = 1, $p = 0.444$; females fed on LC_{10}: $\chi^2 = 0.097$, *d.f.* = 1, $p = 0.754$; female fed on LC_{30}: $\chi^2 = 1.670$, *d.f.* = 1, *p*

294 = 0.196) (Figure 4). The carlina oxide did not influence the aggression score of exposed
295 medflies.
296
297 3.2.5. Aggression duration
298 As for the aggression score, also the overall duration of the aggression was not affected by the
299 ingestion of the carlina oxide. Indeed, our results showed that there was no significant
300 difference in terms of aggression duration between *C. capitata* adults fed on carlina oxide and
301 control flies (males fed on LC_{10}: χ^2= 1.226, *d.f.* = 1, *p* = 0.268; males fed on LC_{30}: χ^2= 0.0076,
302 *d.f.* = 1, *p* = 0.930; females fed on LC_{10}: χ^2= 0.482, *d.f.* = 1, *p* = 0.487*;* females fed on LC_{30}: χ^2=
303 0.419, *d.f.* = 1, *p* = 0.517) (Figure 5). The duration of an individual aggressive event ranged
304 from a few seconds to a maximum of 30 s, i.e., wing waving.
305
306 **3.3. Non-target acute toxicity on rats**
307 Signs of toxicity consisting of tremors, sedation, ataxia, and ptosis were observed in the rat
308 group administered with the dose of 1000 mg/kg which was slightly lower than the LD_{50}
309 previously determined (Pavela *et al*., 2021). No evident signs of toxicity were instead observed
310 for the lower doses. No significant alterations in the body weight and the organ's weight were
311 evident after the acute administration of the different doses of the *C. acaulis* EO, even if a non-
312 statistically significant decrease in the body weight was noticeable in animals treated with the
313 highest dose (Table 2).
314 In the gross anatomy evaluation of the inner wall of the stomach, an area of hyperaemia with a
315 sign of necrosis was evident at the level of the fundus, in animals treated with the highest dose
316 of *C. acaulis* EO (Figure 6). No evident signs of damage were present in the wall of the stomach
317 of the animals of the other experimental groups (data not shown). Analysis of 5 μm sections of
318 the organs stained with H&E allowed for comparison of structures and evaluation of the

presence of damages resulting from the exposure to the EO. The stomachs of rats of groups "vehicle" and the rats treated with 250 and 500 mg/kg of *C. acaulis* EO did not show any significant damage (data not shown). The stomach of two out of four rats treated with 1000 mg/kg of *C. acaulis* EO showed mucosae necrosis (Figure 6), especially at the level of the fundus. The morphological analysis at the level of *gastric mucosae* of the body revealed no morphological alterations in the animal treated with vehicle or 250 mg/kg and 500 mg/kg of *C. acaulis* EO (data not shown). Ulcers with the presence of inflammatory elements were evident in the animals treated with *C. acaulis* EO at the highest dose (Figure 6). In the gross anatomical analysis of the liver, the hepatic parenchyma was not macroscopically affected by the different doses of *C. acaulis* EO we observed the occurrence of the normal capsule made of dense connective tissue (data not shown). The liver of the animals treated with an acute dose of 250 and 500 mg/kg of *C. acaulis* EO did not reveal damage in the parenchyma (data not shown). Differently in the liver of animal treated with the highest dose (1000 mg/kg) were evident signs of the dilated sinusoid, vacuolations in a perivascular central zone, without inflammatory aggregate, degenerated hepatic cord, and apoptotic cells (Figure 7).

Finally, the kidney was histologically investigated in micrometers sections stained with haematoxylin and eosin. The section of the renal cortex showed, even at low magnification, normal renal corpuscles, and convoluted tubules (Figure 8A and B). The images of kidney sections from an animal treated with 1000 mg/kg of *C. acaulis* EO showed that alterations of the renal interstitium were oedematous even with a lack of mononucleate cellular infiltrates such as macrophages and lymphocytes. Glomeruli were normal, but in some elements, capillary and Bowman's spaces were more dilated (arrowheads in Figure 8D). The *C. acaulis* EO at the dose of 500 and 1000 mg/kg induced an increase of the luminal diameter of renal tubules compared with control ones, which was more evident with the highest dose in all animals (arrow in Figure 8C).

4. Discussion

Several studies have been carried out to propose novel plant-borne insecticides effective against medfly adults, and various routes of applications of botanicals have been attempted, including contact, fumigation, and ingestion toxicity. For example, topical applications of *Melaleuca alternifolia* (L.) (Myrtaceae) EO were toxic to medfly, even at 0.117 µL/cm^2 (Benelli *et al*., 2013). *Baccharis spartioides* (Hook *et* Arn.) (Asteraceae) and *Schinus polygama* (Cav.) (Anacardiaceae) EOs also obtained a good level of toxicity when applied at 10-22 µg/fly (Barud *et al.*, 2014). *Baccharis spartioides* and *S. polygama* showed toxicity comparable to *Tagetes* species EOs (i.e., $LC_{50} \leq 20$ µg/fly) (Lopez *et al*., 2011), as well as *Baccharis darwinii* (L.) (Asteraceae) (i.e., $LC_{50} < 31$ µg/fly) (Kurdelas *et al*., 2012) and *Azorella cryptantha* (Clos) Reiche (Apiaceae) (i.e., $LC_{50} = 11$ µg/fly) (Lopez *et al*., 2012).

While the impact of EO fumigation efficacy against a frugivorous pest like the medfly is of limited interest and can be disregarded, replacing synthetic insecticides with eco-friendly molecules in "attract and kill" programs is a valuable goal for modern tephritid research (Scolari *et al*., 2021). In this framework, the insecticidal efficacy of *C. acaulis* EO on *C. capitata*, has been recently highlighted by Benelli *et al.* (2021). The study showed high ingestion toxicity in both sexes. The EO, formulated in protein baits, showed an LC_{50} of 1094 ppm, which is markedly lower if compared with the LC_{50} estimated for other EOs tested against the medfly, such as *Lavandula angustifolia* Mill. (Lamiaceae), *Hyptis suaveolens* (L.) Poit. (Lamiaceae), *Thuja occidentalis* L. (Cupressaceae), and *Rosmarinus officinalis L*. (Lamiaceae), all showing LC_{50} ranging from 3664 ppm (*R. officinalis*) to 13041 ppm (*H. suaveolens*) (Benelli *et al.*, 2012). The relevant insecticidal activity of *C. Acaulis* EO seems to be linked to carlina oxide (>97% of the EO). Of note, carlina oxide has been recently reported as a highly effective

insecticide on other key Diptera species, such as *C. quinquefasciatus* (LC_{50} =1.39 µg mL^{-1}), and its mode of action seems to be moderately related to acetylcholinesterase (AChE) inhibition (Benelli *et al.*, 2019). In the present study, carlina oxide showed a very promising LC_{50} (1273 ppm) against adult medflies, outlining that the overall toxicity of *C. acaulis* EO is mostly linked to the bioactivity of this major constituent. Polyacetylenes, as carlina oxide, have insecticide, fungicide, and nematicide properties (Gorman *et al.*, 1993). Their toxicity can be linked to several modes of action dictated by environmental conditions. Aromatic polyacetylenes, such as carlina oxide, can lead to phototoxicity in insects (Arnason *et* Bernards, 2010; Konovalov, 2014). In the absence of light, polyacetylenes are antifeedant to insects, while in the presence of light the toxicity may be caused by a photocatalytic cycle of single oxygen generation and another excited state molecule that leads to rapid lipid peroxidation and cell death (Haouas *et al*., 2011). Furthermore, several polyacetylenes are responsible for modulating the gamma-aminobutyric acid-A (GABA-A) receptors (Lin *et al.*, 2016). The compounds binding to GABA receptors related to chloride channels located on the membrane of postsynaptic neurons disrupt the functioning of the GABA synapse (Pavela *and* Benelli, 2016).

As reported by Benelli *et al.* (2019), the carlina oxide toxicity, is partially attributable to cholinergic system blockage by AChE inhibition, which mediates nerve transmission splitting acetylcholine into choline and acetic acid. The insect dies of acetylcholine accumulation in the synaptic space (Boison, 2007). Furthermore, the high lipophilicity of carlina oxide leads to an easy entrance into the insect body (Benelli *et al.*, 2019).

The aggressive behaviour of *C. capitata* plays a key role in routing the reproductive success of this species (Benelli *et al.*, 2014, 2015 a, b). The research carried out by Benelli *et al.* (2021) and this study, considered the influence of *C. acaulis* EO and carlina oxide, respectively, at low concentrations on the medfly aggressive behaviour dynamics. Our results showed a significant impact of feeding on the carlina oxide on the directionality of aggressive actions; at both tested

concentrations (LC_{10} and LC_{30}), medfly males and females have received more aggressions by control flies, at variance with the results obtained testing the *C. acaulis* EO (Benelli *et al.*, 2021). Surprisingly, our study displayed a substantial decrease of medfly aggressive interactions at the population level only for females fed on LC_{10} vs. control flies. However, by testing the whole EO an important reduction of medfly aggressive interaction at the population level was noted, along with a shorter time of aggressive events, in medflies treated with both EO LC_{10} and LC_{30} (Benelli *et al.*, 2021). The differences exposed above may be due to a synergic action of the other minor components of EO, with special reference to benzaldehyde (1.14 %) and *ar*-curcumene (0.29 %), as synergistic and antagonistic interactions between EO constituents have been reported in earlier studies (Benelli *et al.*, 2017a,b; Yuan *et al.*, 2019). The two minor constituents mentioned above have insecticidal activity and can also act as carrier agents for carlina oxide to penetrate better into the insect cuticle. Indeed, as reported by Alshebly *et al*. (2016), *Hedychium larsenii* M. Dan & C. Sathish Kumar (Zingiberaceae) EO and its main components, *ar*-curcumene and *epi-β*-bisabolol showed sublethal effects on *Anopheles stephensi* Liston (Diptera: Culicidae), *Aedes aegypti* L. (Diptera: Culicidae) and *C. quinquefasciatus.* Low concentrations of *ar*-curcumene and *epi-β*-bisabolol negatively influenced the oviposition of the three species tested. Another study, carried out by Nattudurai *et al*. (2012), showed how the exposition of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) to low concentration (i.e., LC_{10}, $LC_{20,}$ and LC_{30}) of benzaldehyde reduced the female fecundity (i.e., from 4.7 to 0.92 eggs/female). Further research on the bioactivity of these two molecules against adult medflies is required, with a special focus on synergistic toxicity tests.

Concerning mammal toxicity, changes in body and organs weight are valuable indicators of toxicity (Michael et al., 2007; OECD, 2008). Herein, we did not observe any change in the weights of the liver and kidney of rats due to the different dosages of *C. acaulis* EO. Similarly,

the total body weight was unaffected by the treatments. Thus, our results showed that the treatment with the EO does not affect the body weight. However, it should be considered that we carried out an acute administration, and the time of observation was limited. A trend in the reduction of body weight was observed after 48 h in the animals with the highest dosage compared with the vehicle and the lower doses. Symptoms of neurological toxicity were observed only after acute oral administration of the highest dose (1000 mg/kg), a little lower than the LD_{50}. Increasing signs of sedation, ataxia, ptosis, and tremors were detected over the 48 h of observation after the administration, indicating that the main component of the EO impacts the central nervous system. Neurotoxicity is not uncommon among the EOs that can pass the brain-blood barrier without difficulty due to their lipophilicity. For instance, it is well known that thujone can cause convulsion and excitation (PMID: 23201408). Similarly, 1,8-cineole and camphor, which are abundant compounds in the EO of eucalyptus and rosemary, are capable of inducing seizures (PMID: 19893077). These effects are probably due to the modulation of the GABAergic system. On the other hand, no signs of neurological toxicity were observed at the lower doses, suggesting a significant safe profile of *C. acaulis* EO since its dosage in the insecticidal formulations would be much lower than the ones tested here.

The assessment of histopathological alterations in organs represents one of the basic tests for the assessment safety of tested materials (Greaves 2011). No abnormality was observed on gross anatomy evaluations of organs examined in this study. The histopathological findings on the liver, the stomach, and the kidney indicated a moderately toxic effect of *C. acaulis* EO, at the dose of 1000 mg/kg. On the other hand, the dose of 500 mg/kg did not cause any toxicity in the rats. Based on our evidence, the *C. acaulis* EO seemed to be slightly toxic to rats, with LD_{50} overlapping those of other industrially important EO elements such as thymol and cinnamaldehyde (Pavela *and* Benelli, 2016). Therefore the *in vivo* toxicological study reveals that the EO has low oral toxicity. Nevertheless, it should be appropriate to avoid high dosages

444 to prevent possible harmful effects. The LD_{50} of *C. acaulis* EO was higher when compared with
445 that of plant extracts containing polyacetylenes as active compounds. Polyacetylenes are widely
446 distributed among the families Apiaceae, Araliaceae, and Asteraceae, and some of them showed
447 antibacterial, antifungal, anti-inflammatory, anticancer, and antiplatelet aggregation properties
448 (Christensen *et al.*, 2006; Hinds *et al.*, 2017; Zaini *et al.*, 2012). Some of these compounds have
449 been considered undesirable due to their toxic properties. A study on the toxicity of *Bupleurum*
450 *longiradiatum* (Apiaceae) displayed that the CH_2Cl_2 fraction and the ethanol extract exhibited
451 high toxicity, with LD_{50} values of 37.5 mg/kg (95% CI: 32.8–42.9 mg/kg) and 77.7 mg/kg (95%
452 CI: 67.7–89.3 mg/kg), respectively, and toxicity correlate to the amount of polyacetylenes in
453 this plant (You *et al.*, 2012).
454
455 **5. Conclusions**
456
457 The chief contribution of carlina oxide to the overall bioactivity of *C. acaulis* EO for the
458 development of "attract and kill" tools has been outlined in the present study. Concerning *C.*
459 *acaulis* EO and carlina oxide, marked differences have been found about their impact through
460 ingestion on medfly aggressive behaviour. Our results show an influence of carlina oxide on
461 aggression directionality, with the actions of control flies directed mostly to medflies previously
462 fed on carlina oxide concentrations. Further research is still needed to assess possible subtle
463 interactions among EO minor constituents, as well as to assess toxicity and potential
464 behavioural variations (e.g., impact on predation or parasitization activity) in invertebrates
465 acting as biocontrol agents of *C. capitata* treated with a low concentration of carlina oxide.
466 Although the cultivation of *Carlina acaulis* and the consequent extraction of the essential oil
467 and the oxide is a feasible process, certain limitations related to the physical and chemical
468 properties of the compounds, especially their photosensitivity, must be overcome. Indeed, from

an application point of view, it should be stressed that nanotechnologies can be particularly useful to improve the effectiveness and stability of *C. acaulis* EO and carlina oxide and enable long-term effectiveness in the field (Pavoni *et al.*, 2019). So, further studies are needed to analyse the lethal and sub-lethal effects of micro- or nano-emulsion of *C. acaulis* EO and carlina oxide. The *in vivo* toxicological assays displayed that the *C. acaulis* EO containing 97.7% of carlina oxide, can produce modest neurological signs and moderate effects on the stomach, liver, and kidney only at the highest dose (1000 mg/kg), slightly lower than the LD_{50}. The other tested doses, which are closer to the practical use of the EO when formulated in protein baits, did not cause side effects worthy of mention. Therefore, our study proved the safety of this natural product to be used in "attract and kill" approaches for controlling major agricultural pests.

Acknowledgements

The authors are grateful to Dr. Augusto Loni (University of Pisa) for his kind assistance in medfly mass-rearing.

Conflict of Interest

The authors declare no competing interests.

References

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265-267. https://doi.org/10.1093/jee/18.2.265a.

Adams, R., 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed. Allured Publishing Corp., Carol Stream, IL, USA.

Journal Pre-proof

493 Alshebly, M., AlQahtani, F., Govindarajan, M., Gopinath, K., Vijayan, P., Benelli, G., 2016.
494 Toxicity of *ar*-curcumene and epi-β-bisabolol from *Hedychium larsenii* (Zingiberaceae)
495 essential oil on malaria, chikungunya and Japanese encephalitis mosquito vectors.
496 Ecotoxicol. Environ. Saf. 137, 149-157. https://doi.org/10.1016/j.ecoenv.2016.11.028.
497 Arnason, J.T., Bernards, M.A., 2010. Impact of constitutive plant natural products on
498 herbivores and pathogens. Can. J. Zool. 88(7), 615-627. https://doi.org/10.1139/Z10-038.
499 Barud, F., López, S., Tapia, A., Feresin, G.E., López, M.L., 2014. Attractant, sexual
500 competitiveness enhancing and toxic activities of the essential oils from *Baccharis*
501 *spartioides* and *Schinus polygama* on *Ceratitis capitata* Wiedemann. Ind. Crops Prod. 62,
502 299–304. https://doi.org/10.1016/j.indcrop.2014.08.045.
503 Benelli, G., Romano, D., 2018. Does indirect mating trophallaxis boost male mating success
504 and female egg load in Mediterranean fruit flies? J. Pest Sci., 91 (1) (2018), 181-188
505 Benelli, G., Flamini, G., Canale, A., Cioni, P.L., Conti, B., 2012. Toxicity of some essential
506 oil formulations against the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Diptera
507 Tephritidae). Crop Prot. 42, 223–229. https://doi.org/10.1016/j.cropro.2012.05.024.
508 Benelli, G., Canale, A., Flamini, G., Cioni, P.L., Demi, F., Ceccarini, L., Macchia, M., Conti,
509 B., 2013. Biotoxicity of *Melaleuca alternifolia* (Myrtaceae) essential oil against the
510 Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae), and its parasitoid *Psyttalia*
511 *concolor (*Hymenoptera: Braconidae). Ind. Crops Prod. 50, 596–603.
512 https://doi.org/10.1016/j.indcrop.2013.08.006.
513 Benelli, G., Daane, K.M., Canale, A., Niu, C.Y., Messing, R.H., Vargas, R.I., 2014. Sexual
514 communication and related behaviours in Tephritidae: Current knowledge and potential
515 applications for Integrated Pest Management. J. Pest Sci. 87(3), 385–405.
516 https://doi.org/10.1007/s10340-014-0577-3.

Journal Pre-proof

517 Benelli, G., Romano, D., Desneux, N., Messing, R.H., Canale, A., 2015a. Sex differences in
518 fighting-induced hyperaggression in a fly. Anim. Behav. 104, 165–174.
519 https://doi.org/10.1016/j.anbehav.2015.02.026.
520 Benelli, G., Romano, D., Messing, R.H., Canale, A., 2015b. Population-level lateralized
521 aggressive and courtship displays make better fighters not lovers: evidence from a fly.
522 Behav. Process., 115, 163-168. https://doi.org/10.1016/j.beproc.2015.04.005.
523 Benelli, G., Pavela, R., Iannarelli, R., Petrelli, R., Cappellacci, L., Cianfaglione, K., Heshmati
524 Afshar, F., Nicoletti, M., Canale, A., Maggi, F., 2017a. Synergized mixtures of Apiaceae
525 essential oils and related plant-borne compounds: larvicidal effectiveness on the filariasis
526 vector *Culex quinquefasciatus* Say. Ind. Crops Prod. 96, 186-195.
527 https://doi.org/10.1016/j.indcrop.2016.11.059.
528 Benelli, G., Pavela, R., Canale, A., Cianfaglione, K., Ciaschetti, G., Conti, F., Nicoletti, M.,
529 Senthil-Nathan, S., Mehlhorn, H., Maggi, F., 2017b. Acute larvicidal toxicity of five
530 essential oils (*Pinus nigra*, *Hyssopus officinalis*, *Satureja montana*, *Aloysia citrodora* and
531 *Pelargonium graveolens*) against the filariasis vector *Culex quinquefasciatus*: Synergistic
532 and antagonistic effects. Parasitol. Int. 66(2), 166-171.
533 https://doi.org/10.1016/j.parint.2017.01.012.
534 Benelli, G., Pavela, R., Petrelli, R., Nzekoue, F.K., Cappellacci, L., Lupidi, G., Quassinti, L.,
535 Bramucci, M., Sut, S., Dall'Acqua, S., Canale, A., Maggi, F., 2019. Carlina oxide from
536 *Carlina acaulis* root essential oil acts as a potent mosquito larvicide. Ind. Crops Prod. 137,
537 356–366. https://doi.org/10.1016/j.indcrop.2019.05.037.
538 Benelli, G., Pavela, R., Drenaggi, E., Desneux, N., Maggi, F. 2020. Phytol, (*E*)-nerolidol and
539 spathulenol from *Stevia rebaudiana* leaf essential oil as effective and eco-friendly botanical
540 insecticides against *Metopolophium dirhodum*. *Ind. Crops Prod*. 155, 112844.

Benelli, G., Rizzo, R., Zeni, V., Govigli, A., Samková, A., Sinacori, M., Lo Verde, G., Pavela, R., Cappellacci, L., Petrelli, R., Spinozzi, E., Morshedloo, M.R., Maggi, F., Canale, A., 2021. *Carlina acaulis* and *Trachyspermum ammi* essential oils formulated in protein baits are highly toxic and reduce aggressiveness in the medfly, *Ceratitis capitata.* Ind. Crops Prod. 161, 113191. https://doi.org/10.1016/j.indcrop.2020.113191.

Bijani, M., Ghazani, E., Valizadeh, N., Haghighi, N.F., 2019. Predicting and understanding farmers' soil conservation behavior in mazandaran province, Iran. J. Agric. Sci. Technol. 21 (7) (2019), pp. 1705-1719

Boison D., 2007. Acetylcholinesterase. XPharm: The Comprehensive Pharmacology Reference, 1–8.

Borzoui, E., Naseri, B., Abedi, Z., Karimi-Pormehr, M.S., 2016. Lethal and Sublethal Effects of Essential Oils from *Artemisia khorassanica* and *Vitex pseudo-negundo* Against *Plodia interpunctella* (Lepidoptera: Pyralidae). Environ. Entomol. 45(5), 1220–1226. https://doi.org/10.1093/ee/nvw100.

Bozhgani, N.S.S, Talebi A.A., 2018. The effects of spirotetramat on the demographic parameters of *Neoseiulus californicus* (Phytoseiidae). Syst. Appl. Acarol. 23(10), 1952-1964.

Briceño, R., Ramos, D., Eberhard, W., 1999. Aggressive behavior in medflies (*Ceratitis capitata*) and its modification by mass rearing (Diptera: Tephritidae). J. Kansas Entomol. Soc., 72 (1), 17-27

Cachada, A., Silva, E.F.D., Duarte, A.C., Pereirabd R., 2016. Risk assessment of urban soils contamination: the particular case of polycyclic aromatic hydrocarbons. Sci. Total Environ. 551-552 (5), 271-284. https://doi.org/10.1016/j.scitotenv.2016.02.012.

Canale, A., Benelli, G., 2012. Impact of mass-rearing on the host seeking behaviour and parasitism by the fruit fly parasitoid *Psyttalia concolor* (Szépligeti) (Hymenoptera:

Braconidae). J. Pest Sci. 85, 65–74. https://doi.org/10.1007/s10340-011-0382-1.

Capowiez, Y., Rault, M., Costagliola, G., Mazzia, C., 2005. Lethal and sublethal effects of imidacloprid on two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*). Biol. Fertil. Soils 41, 135-143. https://doi.org/10.1007/s00374-004-0829-0.

Champagne, D.E., Arnason, J.T., Philogene, B.J.R., Morand, P., Lam, J., 1986. Light-mediated allelochemical effects of naturally occurring polyacetylenes and thiophenes from Asteraceae on herbivorous insects. J. Chem. Ecol. 12(4), 835-858. https://doi.org/10.1007/BF01020255.

Christensen, L.P., Brandt, K., 2006. Bioactive polyacetylenes in food plants of the Apiaceae family: occurrence, bioactivity and analysis. J Pharm Biomed Anal. 41(3), 683-93. https://doi.org/10.1016/j.jpba.2006.01.057.

Fernandes, M.E.S., Alves, F.M., Pereira, R.C., Aquino, L.A., Fernandes, F.L., Zanuncio, J.C., 2016. Lethal and sublethal effects of seven insecticides on three beneficial insects in laboratory assays and field trials. Chemosphere, 156, 45-55, https://doi.org/10.1016/j.chemosphere.2016.04.115.

FFNSC, 2012. Flavors and Fragrances of Natural and Synthetic Compounds. MassSpectral Database. 2. Shimadzu Corps, Kyoto.

Finney D.J., 1978. Statistical Method in Biological Assay, third ed. Charles Griffin & Co., London.

Forson, D.D., Storfer, A., 2006. Atrazine increases Ranavirus susceptibility in the tiger salamander (*Ambystoma tigrinum*). Ecol. Appl. 16, 2325–2332. https://doi.org/10.1890/1051-0761(2006)016[2325:airsit]2.0.co;2.

Gorman, C.B., Ginsburg, E.J., Grubbs, R.H., 1993. Soluble, Highly Conjugated Derivatives of Polyacetylene from the Ring-Opening Metathesis Polymerization of Monosubstituted Cyclooctratetraenes: Synthesis and the Relationship between Polymer Structure and Physical Properties. J. Am. Chem. Soc. 115 (4), 1397–1409.

https://doi.org/10.1021/ja00057a024.

Greaves, P., 2011. Histopathology of preclinical toxicity studies: interpretation and relevance in drug safety evaluation, fourth ed. Academic Press, Boston, USA.

Guillem-Amat, A., Ureña, E., López-Errasquín, E., Navarro-Llopis, V., Batterham, P., Sánchez, L., Perry, T., Hernández-Crespo, P., Ortego, F., 2020. Functional characterization and fitness cost of spinosad-resistant alleles in *Ceratitis capitata*. J. Pest Sci. 93(3), 1043–1058. https://doi.org/10.1007/s10340-020-01205-x.

Haouas, D., Flamini, G., Monia, B.H.K., Habib, B.H.M., 2011. Identification of an insecticidal polyacetylene derivative from *Chrysanthemum macrotum* leaves. Ind. Crops Prod. 34(1), 1128–1134. https://doi.org/10.1016/j.indcrop.2011.03.032.

Hinds, L., Kenny, O., Hossain, M.B., Walsh, D., Sheehy, E., Evans, P., Gaffney, M., Rai, D.K., 2017. Evaluating the Antibacterial Properties of Polyacetylene and Glucosinolate Compounds with Further Identification of Their Presence within Various Carrot (*Daucus carota*) and Broccoli (*Brassica oleracea*) Cultivars Using High-Performance Liquid Chromatography with a Diode Array Detector and Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry Analyses. J. Agric. Food Chem. 65(33), 7186-7191. https://doi.org/10.1021/acs.jafc.7b02029.

Horowitz, A.R., Ghanim, M., Roditakis, E., Nauen, R., Ishaaya, I., 2020. Insecticide resistance and its management in *Bemisia tabaci* species. J. Pest Sci., 93, 893–910. https://doi.org/10.1007/s10340-020-01210-0

Hsu, J.C., Feng, H.T., 2006. Development of resistance to spinosad in oriental fruit fly (Diptera: Tephritidae) in laboratory selection and cross-resistance. J. Econ. Entomol. 99(3), 931–936. https://doi.org/10.1093/jee/99.3.931.

Izakmehri, K., Saber, M., Mehrvar, A., Bagher, M., 2013. Lethal and sublethal effects of essential oils from *Eucalyptus camaldulensis* and *Heracleum persicum* against the adults of *Callosobruchus maculatus*. J. Insect Sci. 13, 1–10. https://doi.org/10.1673/031.013.15201.

Karami, S., Karami, E., Buys, L., Drogemuller, R., 2017. System dynamic simulation: a new method in social impact assessment (SIA). Environ. Impact Assess. Rev. 62, 25-34. https://doi.org/10.1016/j.eiar.2016.07.009.

Kavallieratos, N.G., Boukouvala, M.C., Ntalli, N., Skourti, A., Karagianni, E.S., Nika, E.P., Kontodimas, D.C., Cappellacci, L., Petrelli, R., Cianfaglione, K., Morshedloo, M.R., Tapondjou, L.A., Rakotosaona, R., Maggi, F., Benelli, G., 2020. Effectiveness of eight essential oils against two key stored-product beetles, *Prostephanus truncatus* (Horn) and *Trogoderma granarium* Everts. Food Chem. Toxicol. 139, 111255. https://doi.org/10.1016/j.fct.2020.111255.

Khosravi, R., Sendi, J.J., Ghadamyari, M., 2010. Effect of *Artemisia Annua* L. on deterrence and nutritional efficiency of lesser mulberry pyralid (*Glyphodes Pylolais* Walker) (lepidoptera: Pyralidae). J. Plant Prot. Res. 50(4). https://doi.org/10.2478/v10045-010-0071-8.

Konovalov, D.A., 2014. Polyacetylene Compounds of Plants of the Asteraceae Family (Review). Pharm. Chem. J. 48(9), 613–631. https://doi.org/10.1007/s11094-014-1159-7.

Kurdelas, R.R., López, S., Lima, B., Feresin, G.E., Zygadlo, J., Zacchino, S., López, M.L., Tapia, A. Freile, M.L., 2012. Chemical composition, anti-insect and antimicrobial activity of *Baccharis darwinii* essential oil from Argentina, Patagonia. Ind. Crops Prod. 40(1), 261–267. https://doi.org/10.1016/j.indcrop.2012.03.024.

Lee, C., 2000. Sublethal Effects of Insecticides on Longevity, Fecundity and Behaviour of Insect Pests*:* a Review. J. Biosci. 11(1), 107-112. https://doi.org/10.5772/66461.

638 Lin, M., Zhang, W., Su, J., 2016. Toxic polyacetylenes in the genus *Bupleurum* (Apiaceae) –
639 Distribution, toxicity, molecular mechanism and analysis. J. Ethnopharmacol. 193, 566–573.
640 https://doi.org/10.1016/j.jep.2016.09.052.
641 Lin, R., He, D., Men, X., Zheng, L., Cheng, S., Tao, L., Yu, C., 2020. Sublethal and
642 transgenerational effects of acetamiprid and imidacloprid on the predatory bug *Orius sauteri*
643 (Poppius) (Hemiptera: Anthocoridae). Chemosphere, 255, 126778,
644 https://doi.org/10.1016/j.chemosphere.2020.126778.
645 López, S.B., López, M.L., Aragón, L.M., Tereschuk, M.L., Slanis, A.C., Feresin, G.E.,
646 Zygadlo, J.A., Tapia, A.A., 2011. Composition and anti-insect activity of essential oils from
647 *Tagetes* L species (Asteraceae, Helenieae) on *Ceratitis capitata* Wiedemann and Triatoma
648 infestans Klug. J. Agric. Food Chem. 59(10), 5286–5292.
649 https://doi.org/10.1021/jf104966b.
650 López, S., Lima, B., Aragón, L., Espinar, L.A., Tapia, A., Zacchino, S., Zygadlo, J., Feresin,
651 G.E., López, M.L., 2012. Essential oil of *Azorella cryptantha* collected in two different
652 locations from San Juan Province, Argentina: Chemical variability and anti-insect and
653 Antimicrobial Activities. Chem. Biodivers. 9(8), 1452–1464.
654 https://doi.org/10.1002/cbdv.201100319.
655 Mami, I.R., Belabbes, R., Amine Dib, M.E., Tabti, B., Costa, J., Muselli, A., 2020. Biological
656 activities of carlina oxide isolated from the roots of *Carthamus caeruleus*. Nat. Prod. J. 10(2),
657 145-152. https://doi.org/10.2174/2210315509666190117152740.
658 Mejdoub, K., Mami, I.R., Belabbes, R., Dib, M.E.A., DJabou, N., Tabti, B., Nassira, G.B.,
659 Costa, J., Muselli, A., 2020. Chemical variability of *Atractylis gummifera* essential oils at
660 three developmental stages and investigation of their antioxidant, antifungal and insecticidal
661 activities. Curr. Bioact. Compd. 16(4), 489-497.
662 https://doi.org/10.2174/1573407215666190126152112.

663 Michael, B., Yano, B., Sellers, R.S., Perry, R., Morton, D., Roome, N., Johnson, J.K., Schafer,
664 K., Pitsch, S., 2007. Evaluation of organ weights for rodent and non-rodent toxicity studies:
665 a review of regulatory guidelines and a survey of current practices. Toxicol. Pathol. 35, 742-
666 50, doi: 10.1080/01926230701595292
667 Minto, R.E., Blacklock, B.J., 2008 Biosynthesis and function of polyacetylenes and allied
668 natural products. Progr. Lipid Res., *47*(4), 233–306.
669 https://doi.org/10.1016/j.plipres.2008.02.002
670 Nattudurai, G., Paulraj, M.G., Ignacimuthu, S., 2012. Fumigant toxicity of volatile synthetic
671 compounds and natural oils against red flour beetle *Tribolium castaneum* (Herbst)
672 (Coleopetera: Tenebrionidae). J. King Saud Univ. Sci. 24(2), 153–159.
673 https://doi.org/10.1016/j.jksus.2010.11.002.
674 Negri, R., 2015. Polyacetylenes from terrestrial plants and fungi: recent phytochemical and
675 biological advances. Fitoterapia, 106, 92-109. https://doi.org/10.1016/j.fitote.2015.08.011.
676 NIST, 2017. Mass Spectral Library (NIST/EPA/NIH). 17. National Institute of Standards and
677 Technology, Gaithersburg, USA.
678 Nouri-Ganbalani, G., Borzoui, E., 2017. Acute toxicity and sublethal effects of *Artemisia*
679 *sieberi* Besser on digestive physiology, cold tolerance and reproduction of *Trogoderma*
680 *granarium* Everts (Col.: Dermestidae). J. Asia Pac. Entomol. 20(1), 285–292.
681 https://doi.org/10.1016/j.aspen.2017.01.002.
682 Nwankwo, A., 2021. Quantifying the impact of insecticide resistance in the transmission
683 dynamics of malaria. Chaos, Solitons and Fractals 142, 110481.
684 https://doi.org/10.1016/j.chaos.2020.110481.
685 OECD. 2008. Guidelines for testing of chemical. Acute oral toxicity – up-and-down procedure.
686 OECD, Paris, France.

Journal Pre-proof

687 Pavela, R., Benelli G., 2016. Essential Oils as Ecofriendly Biopesticides? Challenges and
688 Constraints. Trends Plant Sci. 21(12), 1000–1007.
689 https://doi.org/10.1016/j.tplants.2016.10.005.
690 Pavela, R., Maggi, F., Petrelli, R., Cappellacci, L., Buccioni, M., Palmieri, A., Canale, A.,
691 Benelli G., 2020. Outstanding insecticidal activity and sublethal effects of *Carlina acaulis*
692 root essential oil on the housefly, *Musca domestica*, with insights on its toxicity on human
693 cells. Food Chem. Toxicol. 136, 111037. https://doi.org/10.1016/j.fct.2019.111037.
694 Pavela, R., Pavoni, L., Bonacucina, G., Cespi, M., Cappellacci, L., Petrelli, R., Spinozzi, E.,
695 Aguzzi, C., Zeppa, L., Ubaldi, M., Desneux, N., Canale, A., Maggi, F., Benelli, G., 2021.
696 Encapsulation of *Carlina acaulis* essential oil and carlina oxide to develop long-lasting
697 mosquito larvicides: Microemulsions versus nanoemulsions. Journal of Pest Science 94,
698 899–915. https://doi.org/10.1007/s10340-020-01327-2.
699 Pavoni, L., Pavela, R., Cespi, M., Bonacucina, G., Maggi, F., Zeni, V.,Canale, A., Lucchi, A.,
700 Bruschi, F., Benelli, G., 2019. Green micro-and nanoemulsions for managing parasites,
701 vectors and pests. Nanomaterials, 9(9), 1285. https://doi.org/10.3390/nano9091285.
702 Postigo, C., Ginebreda, A., Barbieri, M.V., Barceló, D., Martín-Alonso, J., de la Cal, A.,
703 Boleda, M.R., Otero, N., Carrey, R., Solà, V., Queralt, E., Isla, E., Casanovas, A., Frances,
704 G., Lòpez de Alda, M., 2021. Investigative monitoring of pesticide and nitrogen pollution
705 sources in a complex multi-stressed catchment: The lower Llobregat River basin case study
706 (Barcelona, Spain). Sci. Total Environ., 755, 142377.
707 https://doi.org/10.1016/j.scitotenv.2020.142377.
708 Ricupero, M., Desneux, N., Zappalà, L., Biondi, A., 2020. Target and non-target impact of
709 systemic insecticides on a polyphagous aphid pest and its parasitoid. Chemosphere, 247,
710 125728 https://doi.org/10.1016/j.chemosphere.2019.125728.
711 Rohlf, F.J., Sokal, R.R., 1981. Biometry: The Principles and Practice of Statistics in Biological

712 Research. Freeman, New York.
713 Schliserman, P., Aluja, M., Rull, J., Ovruski, S.M., 2014. Habitat degradation and introduction
714 of exotic plants favor persistence of invasive species and population growth of native
715 polyphagous fruit fly pests in a Northwestern Argentinean mosaic. Biol. Invasions, 16, 2599-
716 2613.
717 Scolari, F., Valerio, F., Benelli, G., Papadopoulos, N.T., Vaníčková, L., 2021. Tephritid Fruit
718 Fly Semiochemicals: Current Knowledge and Future Perspectives. Insects, 12, 408.
719 https://doi.org/10.3390/insects12050408.
720 Senthil Rathi, B., Senthil Kumar, P., Show, P.-L., 2021. A review on effective removal of
721 emerging contaminants from aquatic systems: Current trends and scope for further research.
722 J. Hazard. Mater., 409, 124413, https://doi.org/10.1016/j.jhazmat.2020.124413.
723 Singh, N.S., Sharma, R., Parween, T., Patanjali, P.K., 2018. Pesticide Contamination and
724 Human Health Risk Factor, in: Oves, M., Zain Khan, M., M.I. Ismail, I. (eds), Modern Age
725 Environmental Problems and their Remediation. Springer, Cham.
726 https://doi.org/10.1007/978-3-319-64501-8_3.
727 Strzemski, M., Wójciak-Kosior, M., Sowa, I., Agacka-Mołdoch, M., Drączkowski, P.,
728 Matosiuk, D., Kurak, L., Kocjan, R., Dresler, S., 2017. Application of Raman spectroscopy
729 for direct analysis of *Carlina acanthifolia* subsp. *utzka* root essential oil. Talanta 174, 633-
730 637. https://doi.org/10.1016/j.talanta.2017.06.070.
731 Valizadeh, N., Hayati, D., 2021. Development and validation of an index to measure
732 agricultural sustainability. J. Clean. Prod. 280, 123797.
733 https://doi.org/10.1016/j.jclepro.2020.123797.
734 Van den Dool, H., Kratz, P.D., 1963. A generalization of the retention index system including
735 linear temperature programmed gas—liquid partition chromatography. J. Chromatogr. A 11,
736 463–471.

737 Wu, H., Rao, Q., Zheng, J., Mao, C., Sun, Y., Gu, D., Wang, M., Liu, X., 2018. Biochemical
738 and histological alterations in adult zebrafish（*Danio rerio*）ovary following exposure to
739 the tetronic acid insecticide spirotetramat. Ecotoxicol. Environ. Saf. 164, 149-154.
740 https://doi.org/10.1016/j.ecoenv.2018.08.020.
741 Yactayo-Chang, J.P., Tang, H.V., Mendoza, J., Christensen, S.A., Block, A.K., 2020. Plant
742 Defense Chemicals against Insect Pests. Agronomy, 10(8), 1156.
743 https://doi.org/10.3390/agronomy10081156
744 You, Y.J., Lee, I.S., Yong, K., Bae, K.H., Ahn, B.Z., 2002. Antiangiogenic activity of
745 *Bupleurum longiradiatum* on human umbilical venous endothelial cells. Arch. Pharm. Res.
746 25, 640−642. https://doi.org/10.1007/BF02976936.
747 Yuan, L., Yang, X., Yu, X., Wu, Y., Jiang, D., 2019. Resistance to insecticides and synergistic
748 and antagonistic effects of essential oils on dimefluthrin toxicity in a field population of
749 *Culex quinquefasciatus* Say. Ecotoxicol Environ. Safe. 169, 928-936.
750 https://doi.org/10.1016/j.ecoenv.2018.11.115.
751 Zaini, R.G., Brandt, K., Clench, M.R., Le Maitre, C.L., 2012. Effects of Bioactive Compounds
752 from Carrotts (*Dacus carota* L.), Polyacetylenes, Beta-Carotene and Lutein on Human
753 Lymphoid Leukaemia Cells. Anti-Cancer Agents Med. Chem., 12 (6).
754 https://10.2174/187152012800617704
755

Journal Pre-proof

Table 1. Ingestion toxicity of carlina oxide in proteic baits on *Ceratitis capitata* adults.

Tested product	**LC_{10} (ppm) (95% CI)**	**LC_{30} (ppm) (95% CI)**	**LC_{50} (ppm) (95% CI)**	**LC_{90} (ppm) (95% CI)**	***χ^2*, (*df*), *p-value***
Carlina oxide	555 (394-698)	906 (725-1071)	1273 (1078-1480)	2922 (2413-3839)	5.860, (7), $p = 0.556$ ns

LC = lethal concentration.

95 % CI = lower and upper limits of the 95 % confidence interval.

ns = not significant ($p > 0.05$).

Journal Pre-proof

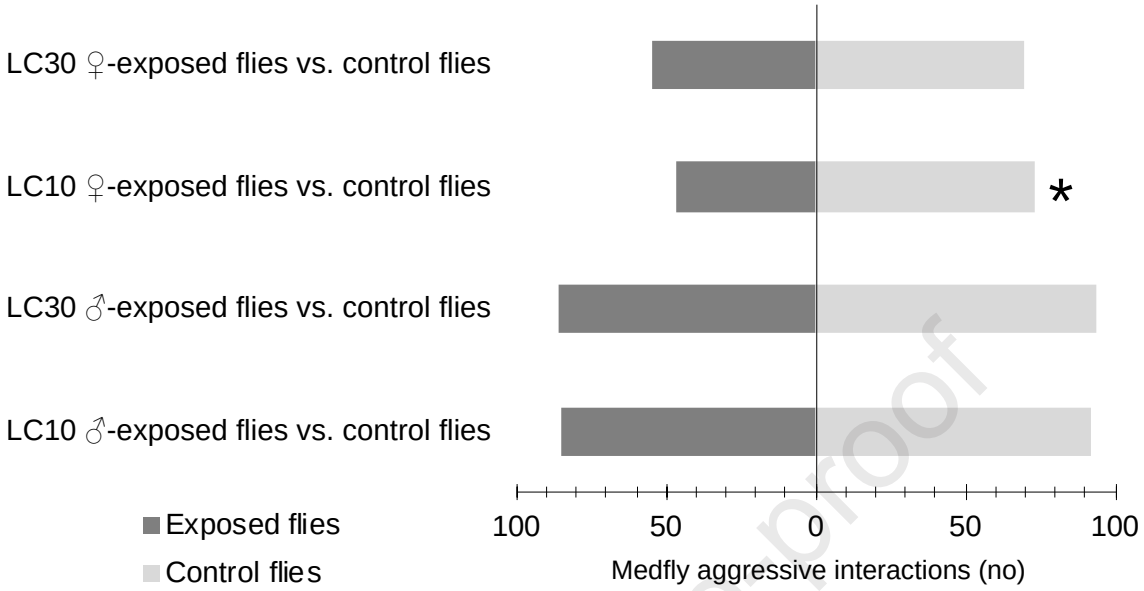
Table 2. Non-target toxicity of *Carlina acaulis* essential oil on rats: body and organs weight in the different experimental groups.

Dose	**Body weight**		**Liver weight**	**Kidney weight**
	0 h	**48 h**	**48 h**	**48 h**
1000 mg/kg	223.25 ± 6.22	211.75 ± 8.68	10.58 ± 0.51	0.96 ± 0.04
500 mg/kg	212.50 ± 9.00	219.75 ± 11.02	11.70 ± 0.94	0.93 ± 0.05
250 mg/kg	237.50 ± 13.17	241.5 ± 15.19	13.52 ± 1.04	0.93 ± 0.06
Vehicle	223.75 ± 6.91	231.5 ± 10.07	10.13 ± 0.76	0.87 ± 0.02
		0 h *vs.* 48 h		
		$F_{7,24}$=1.096	$F_{3,12}$= 3.244	$F_{3,12}$=0.7463
		p=0.3966	p=0.0602	p=0.5450

Time 0: weight at the time of essential oil administration. Time 48 h: weight at the moment of the sacrifice. Data are the mean ± SE. No significant differences between experimental groups were noted (p>0.05).

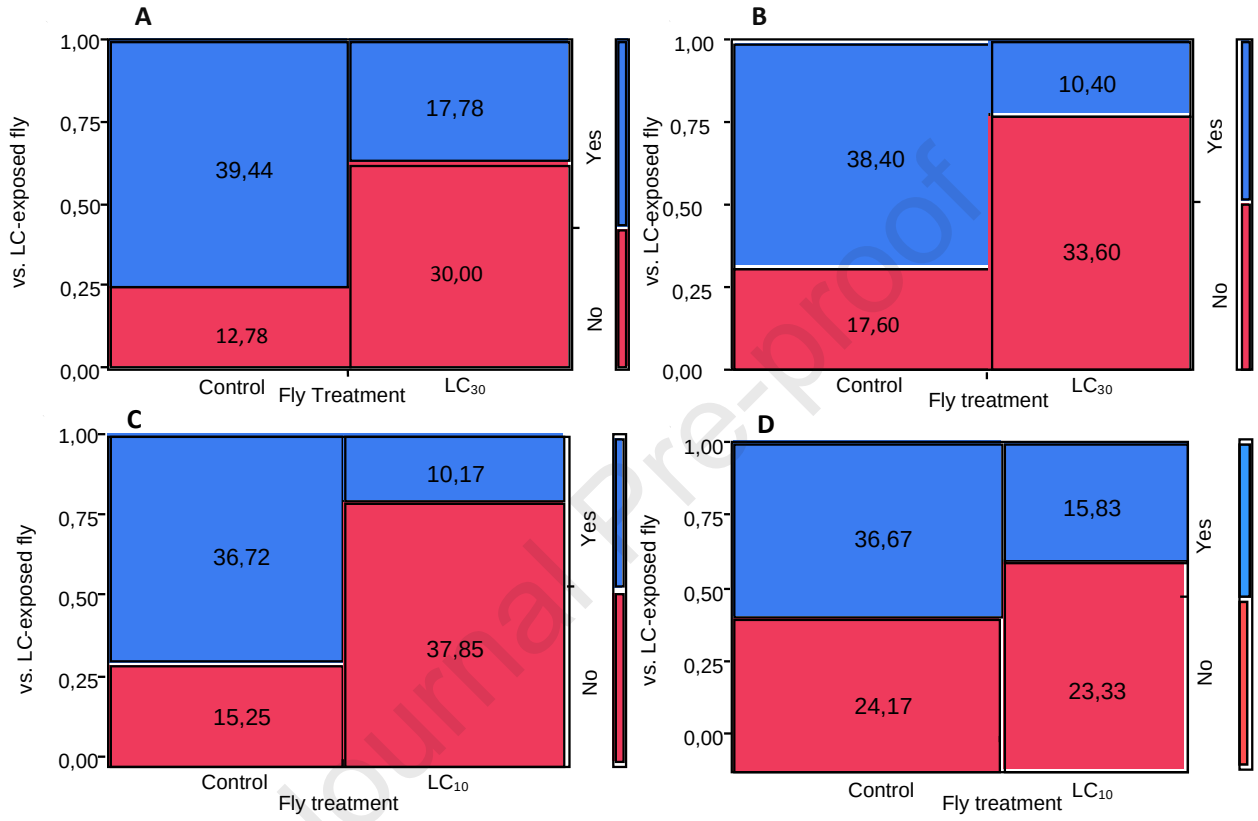
Journal Pre-proof

Figure 1. Overall abundance of aggressive events performed by *Ceratitis capitata* adults fed on LC_{10} and LC_{30} of carlina oxide *vs*. control flies. The asterisk shows a significant difference over the control (χ^2 test with Yates' correction, $p<0.05$).



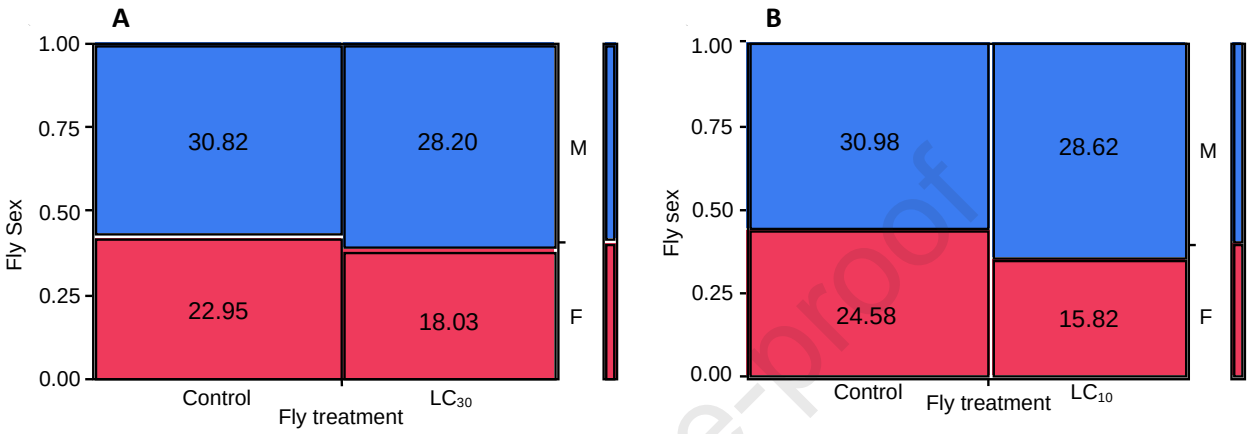
Journal Pre-proof

Figure 2. Mosaic diagram of the aggressions in *Ceratitis capitata* adults after being fed on carlina oxide. The bar on the right (yes/no) shows the directionality of the action, i.e., whether it has been directed or not towards the target subject, namely: **(A)** males fed on carlina oxide LC_{30}; **(B)** females fed on carlina oxide LC_{30}; **(C)** males fed on carlina oxide LC_{10}; **(D)** females fed on carlina oxide LC_{10}. The value within each mosaic tile specifies the percentage of aggressions. The size of each various mosaic tile varies according to the number of individuals who have shown aggressive behaviour.



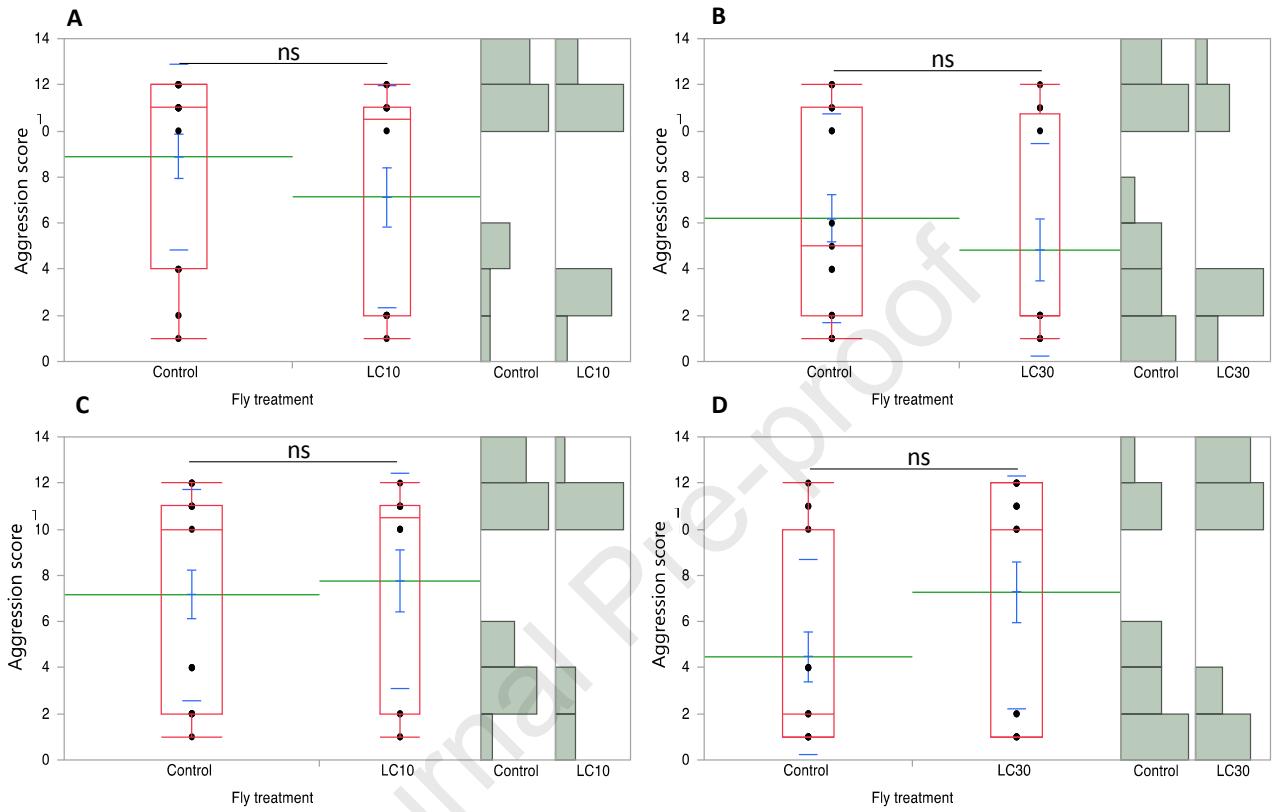
Journal Pre-proof

Figure 3. Mosaic diagram of the aggressions in *Ceratitis capitata* adult males and females feeding on carlina oxide: **(A)** adults fed on LC_{30} of carlina oxide **(B)** adults fed on LC_{10} of carlina oxide. The bars on the right denote the percentage of males and females out of the total number of individuals tested. The numbers inside each box show the percentage of aggressions based on gender (red = female; blue = male). The size of each mosaic tile varies according to the number of adults that have shown aggressive behaviour.



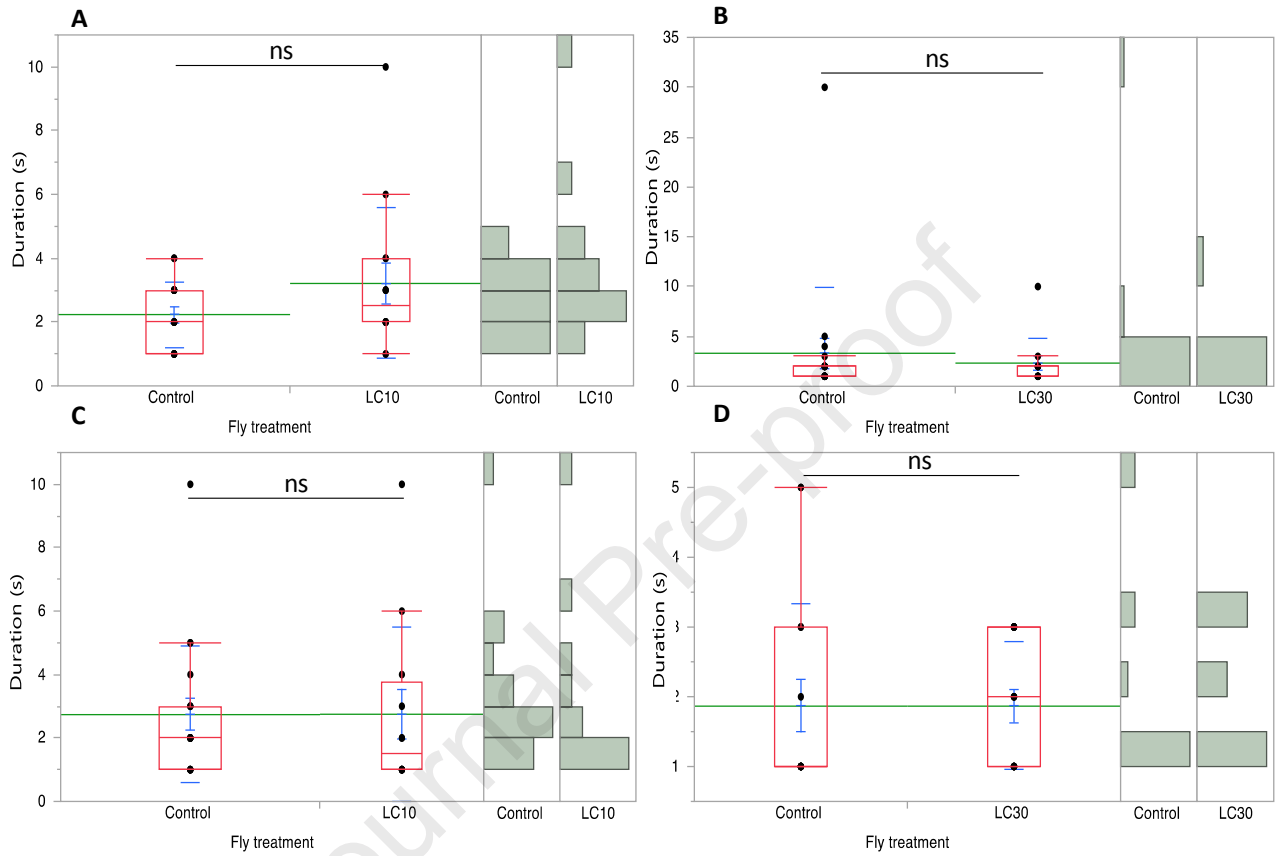
Journal Pre-proof

Figure 4. Aggression score of *Ceratitis capitata* males and females fed or not to LC_{10} and LC_{30} of carlina oxide **(A)** male fed on LC_{10}, **(B)** male *f*ed on LC_{30}, **(C)** female fed on LC_{10}, **(D)** female fed on LC_{30}. Each box plot indicates the median (red line) and its dispersion range (lower, upper quartile and extreme values, outliers). The mean is indicated by a green line, the standard error is a blue T-bar; ns = not significant (Kruskal-Wallis test, $p>0.05$).



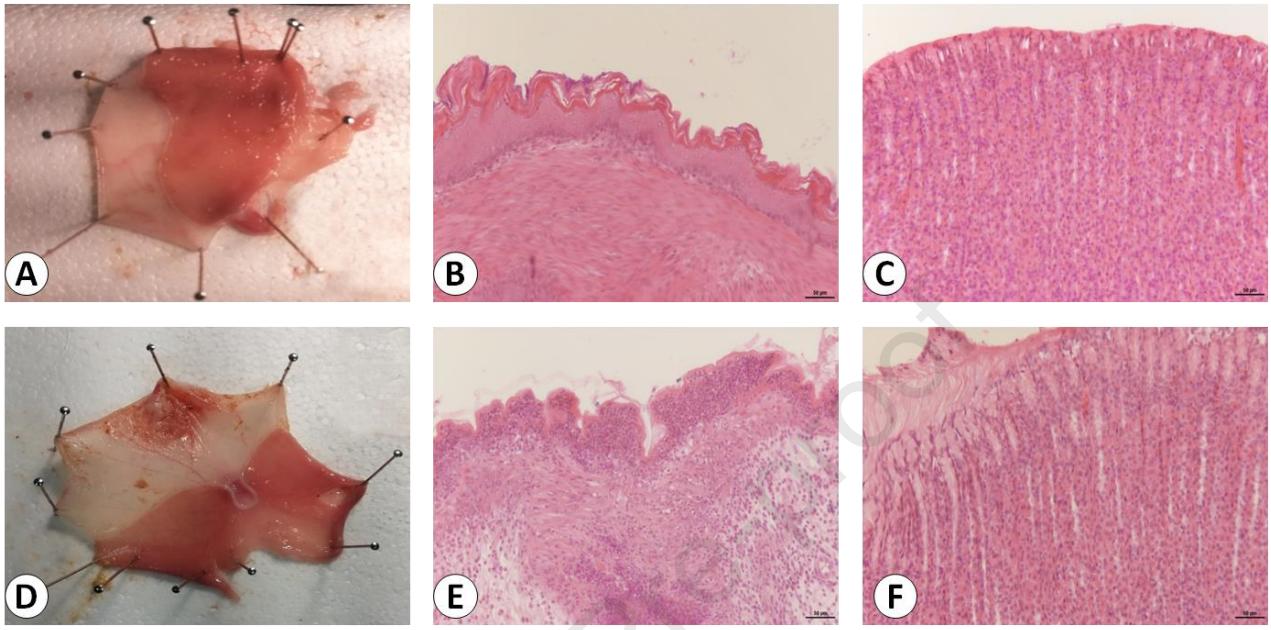
Journal Pre-proof

Figure 5. Aggression duration in *Ceratitis capitata* males and females fed or not to LC_{10} and LC_{30} of carlina oxide: **(A)** males fed on LC_{10}, **(B)** males fed on LC_{30}, **(C)** females fed on LC_{10}, **(D)** females fed on LC_{30}. Each box plot indicates the median (red line) and its dispersion range (lower, upper quartile and extreme values, outliers). The mean is indicated with a green line, the standard error is a blue T-bar. ns = not significant (Kruskal-Wallis test, $p>0.05$).



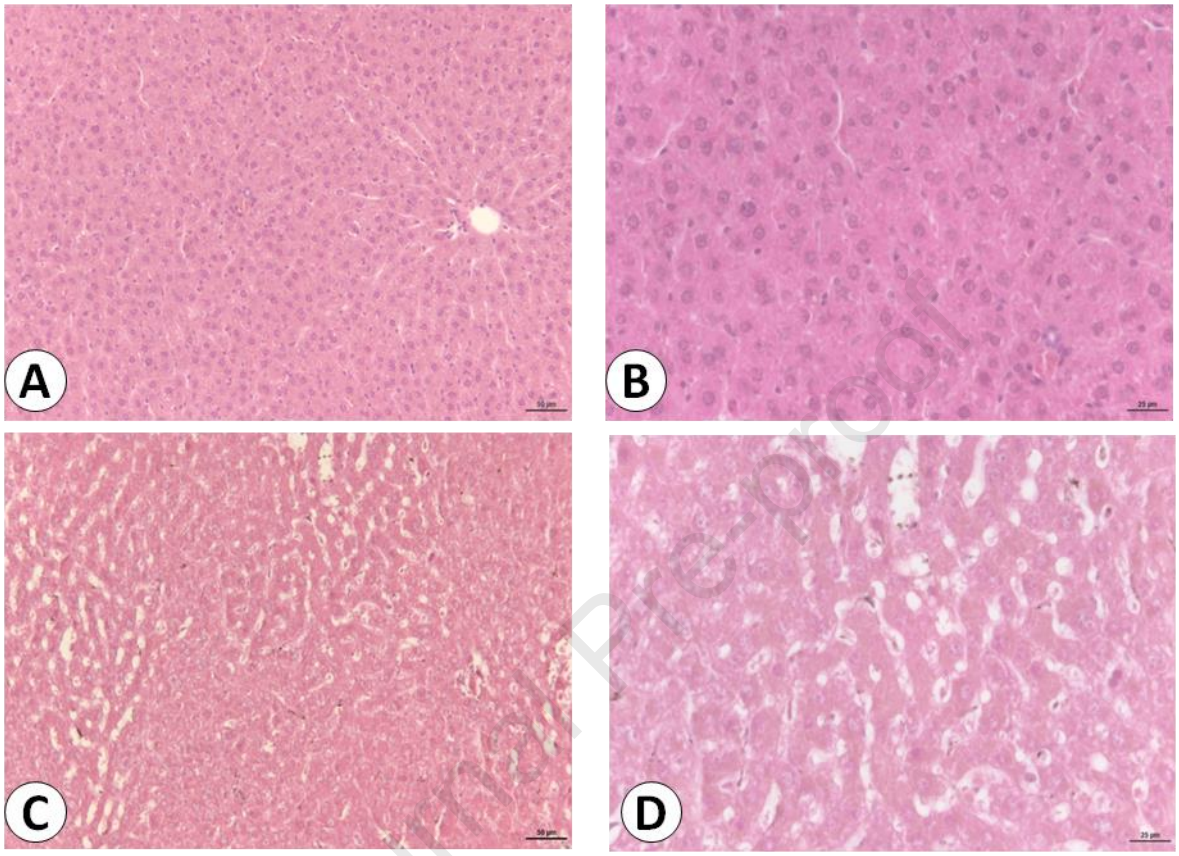
Journal Pre-proof

Figure 6. Inner wall of the stomach (A, D) and sections staining with haematoxilin and eosin at the levels of the fundus (B, E) and body (C, F) of animal treated with vehicle (A-C) and *Carlina acaulis* essential oil (D, E, F) at the dose of 1000 mg/kg. B, C, E, F: calibration bar 50 μm (magnification 20X).



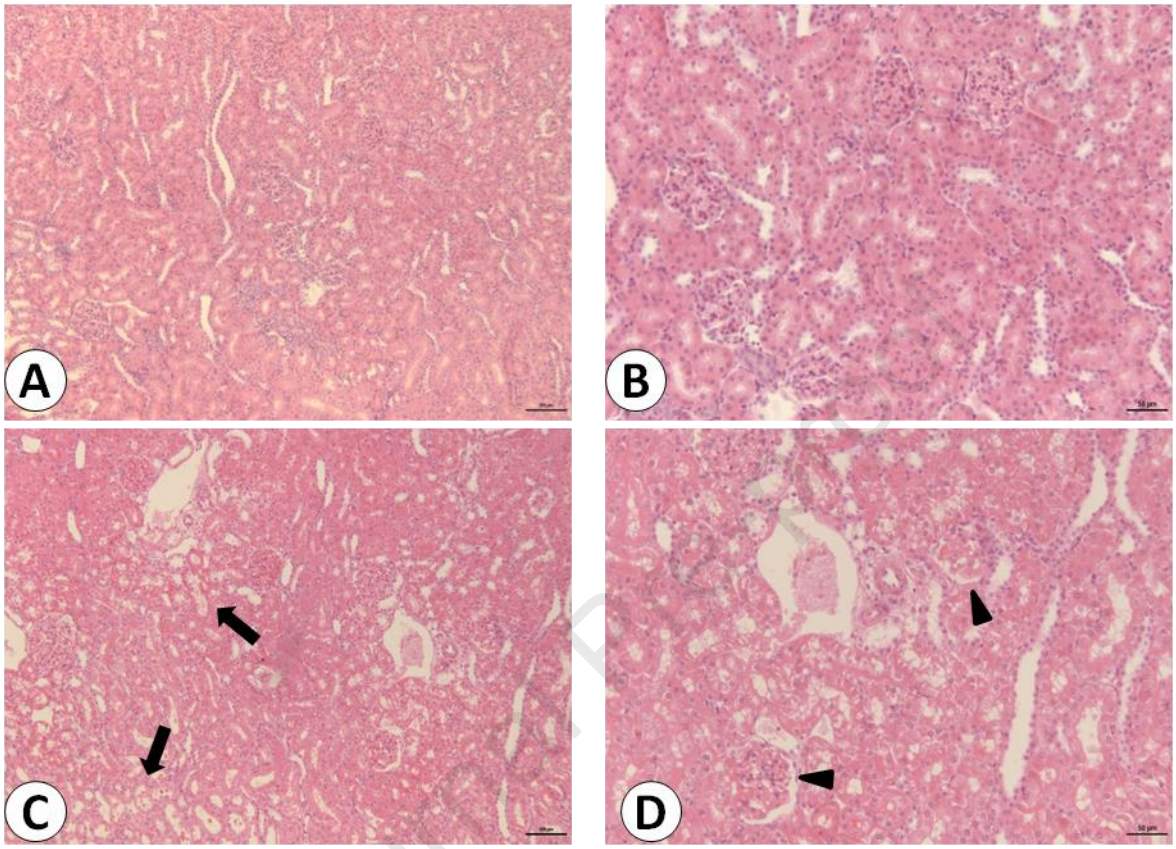
Journal Pre-proof

Figure 7. Sections of the liver stained with haematoxylin and eosin of animal treated with vehicle (A, B) and 1000 mg/kg of *Carlina acaulis* essential oil (C,D). A, C: calibration bar 50 µm (magnification 20X). B, D: calibration bar 25 µm (magnification 40X).



Journal Pre-proof

Figure 8. Sections of the cortical layer of kidney stained with haematoxylin and eosin of animal treated with vehicle (A, B) and 1000 mg/kg of *Carlina acaulis* essential oil (C, D). A, C: calibration bar 100 µm (magnification 10X). B, D: calibration bar 50 µm (magnification 20X).



Journal Pre-proof

Journal Pre-proof

Highlights

- *Carlina acaulis* essential oil represents green insecticides for fruit fly control
- Carlina oxide is the main constituent of the essential oil (>97%)
- Carlina oxide influences the aggression directionality of *C. capitata* adults
- A “lure & kill” formulation based on carlina oxide has been developed against *C. capitata*
- Oral toxicity tests on rats showed that the *C. acaulis* essential oil is safe for mammals

Journal Pre-proof

Journal Pre-proof

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Journal Pre-proof