Carlina oxide from *Carlina acaulis* root essential oil acts as a potent mosquito larvicide ~~through acetylcholinesterase inhibition~~

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Abstract

Developing effective larvicides for mosquito control is being challenging due to the quick development of resistance in targeted vectors. Botanical products can help, due to their multiple mechanisms of action coupled with eco-friendly features. *Carlina acaulis* (Asteraceae) is an Alpine perennial herb used as a traditional remedy and food. Its root essential oil (EO) has antimicrobial and antitrypanosomal properties, and is currently listed among botanicals employable in food supplements. Its insecticidal activity has not been explored. Here, after analysing the *C. acaulis* EO chemical composition by GC-MS and NMR, we detected extremely high larvicidal activity of this EO and its main constituent, carlina oxide, against *Culex quinquefasciatus* larvae by using the standard WHO protocol. LC_{50} were 1.31 and 1.39 µg mL^{-1}, respectively. 24-h exposure to both products triggered significant mortality rates for five days post-treatment. Larvicidal tests on a wider scale confirmed >95% larvicidal effectiveness of the EO and carlina oxide tested at 1.25 µg L^{-1}. Their non-target impact was evaluated through experiments on *Daphnia magna* adults. Both showed significantly lower toxicity if compared to cypermethrin. To shed light on the modes of action, carlina oxide was tested for anti-acetylcholinesterase activity by the Ellman method, with lower performances over galantamine. A moderate antioxidant potential was observed using DPPH and ABTS assays, since it has a role for preserving the shelf-life of herbal-based insecticides. Finally, moderate cytotoxicity on vertebrate cells was noted, testing carlina oxide on human dermis, HCT116 and MDA-MB231 cell lines by MTT assay. Overall, the outstanding toxicity of the tested products make them excellent candidates to develop novel mosquito larvicides for real-world applications.

Keywords: aquatic organisms; cypermethrin; *Daphnia magna*; filariasis; mosquito larvicidal activity; non-target effect

1. Introduction

Managing populations of insect pests and vectors in the era of global warming, increasing urbanization and boosted food production is a hard challenge (Benelli and Mehlhorn, 2016; Drieu and Rusch, 2017; Athanassiou et al., 2018; Deutsch et al., 2018). The widespread overuse of chemical pesticides leads to the quick development of resistance in targeted species (Ranson et al., 2002; Hemingway et al., 2004; Naqqash et al., 2016), as well as to serious non-target effects on the environment and human health (Desneux et al., 2007; Datta et al., 2016; Ullah et al., 2018). Therefore, reliable and eco-friendly tools to manage insect pests and vectors are urgently needed (Isman et al., 2011; Lucchi and Benelli, 2018; Vasantha-Srinivasan et al., 2018a).

In this context, plant-borne products represent an outstanding reservoir of molecules with proven efficacy against arthropod pests and vectors ~~and stored product pests (Afshar et al., 2017; Stevenson et al., 2017; Sut et al., 2018; Hashem et al., 2018), as well as and urban pests (Palacios et al., 2009; Pavela et al., 2017) as well as insects of medical and veterinary importance~~ (Isman and Grieneisen, 2014; ~~Benelli, 2015; Ali et al., 2016; Al-Shebly et al., 2017~~; Banumathi et al., 2017; Benelli and Pavela, 2018a,b; Pavela et al., 2018a, 2019a). In particular, it has been outlined that plant essential oils as well as selected compounds isolated from these mixtures can exert their activity on a wide number of insect species through multiple modes of action (Isman, 2006, 2015), such as binding to GABA receptors (e.g., thymol), inhibiting acetylcholinesterase (AChE) (e.g., fenchone, carvone and linalool), activating receptors for octopamine (e.g., eugenol and α-terpineol) and/or inhibiting P450 cytochromes (e.g., dillapiole and piperamides) (Pavela and Benelli, 2016; Jankowska et al., 2017; Vasantha-Srinivasan et al., 2018b).

The Asteraceae family is a source of essential oils and other bioactive secondary metabolites (Cecchini et al., 2012; Orsomando et al., 2016; Venditti et al., 2016; Sut et al., 2018), targeting several insects and pests of public and economical interest (e.g., Amer and Mehlhorn, 2006; Seo et al., 2014; Govindarajan and Benelli, 2016a; Govindarajan et al., 2018; Pavela et al., 2018a).

74 *Carlina acaulis* L. (Compositae, Cardueae tribe), also known as carline thistle, stemless
75 carline thistle, dwarf carline thistle or silver thistle, is a perennial herb growing on dry and rocky
76 soils, up to 2000 m of altitude, in Alpine regions of central and southern Europe (Tutin et al., 1976).
77 The plant is endowed with leaves grouped in basal rosette, a shortened stem which rarely attains 15
78 cm of height, and a brownish, stout, upright rhizome, reaching 40 cm of length (Pignatti, 1982).
79 In the last decades, massive collections of aerial parts together with the introduction of trees
80 limiting the phenology cycle of this species threatened the *C. acaulis* wild populations and forced
81 local authorities to set up protective laws (Piękoś-Mirkowa and Mirek, 2003; Trejgell et al., 2009a).
82 Nonetheless, *in vitro* micropropagation techniques and adaptation of plants to the *ex vitro*
83 conditions allow to supply the bulk plant material from which bioactive compounds and food
84 supplements can be obtained (Trejgell et al., 2009a, 2009b).
85 *Carlina acaulis* is one of the most used traditional medicinal plants in the Alpine regions of
86 central and southern Europe. For instance, it is used to treat kidney and bladder ailments, pain and
87 colic, worms, wounds and skin infections and cancer (Jordanov et al., 1966; Kresanek and Krejca,
88 1988; Tylkowa, 1989; Strzemski et al., 2017; Kozlowska et al., 2018; Gilca et al., 2018). *Carlina*
89 *acaulis* has also been used as a food plant. Indeed, in Alpine regions, the inflorescence's receptacle
90 is consumed like that of artichoke (Abbet et al., 2014). The same receptacle is used to prepare
91 liqueurs and snacks (Armand, 1993; Pieroni and Giusti, 2009).
92 From the roots of *C. acaulis* is possible to obtain an essential oil (~1%) (EO) which has
93 already been found as a potent antimicrobial and antiprotozoan agent (Stojanović-Radić et al., 2012;
94 Herrmann et al., 2011). The major bioactive compound of this oil is the polyacetylene carlina oxide
95 (syn. 2-(3-phenylprop-1-ynyl)furan, MF: $C_{13}H_{10}O$, MW= 182). The latter can also be obtained from
96 the roots of *Carlina acathifolia* All. (Dorđević et al., 2005, 2007; Stojanović-Radić et al., 2012).
97 Besides, *C. acaulis* extracts and carlina oxide showed *in vivo* protective effects on *Caenorhabditis*
98 *elegans* Maupas (Rhabditida: Rhabditidae) through antioxidant effects (Link et al., 2016). Anti-

inflammatory and antiproliferative activities were also observed studying *C. acaulis* extracts (Dorđević et al., 2012; Strzemski et al., 2017).

To the best of our knowledge, no data are available on the insecticidal potential of *C. acaulis* EO and its main constituents. Therefore, in the present work, after analysing the chemical composition of *C. acaulis* root essential oil, of commercial origin, by GC-MS and NMR techniques, we then evaluated the toxicity of *C. acaulis* EO and its main constituent, i.e., carlina oxide, against *Culex quinquefasciatus* Say (Diptera: Culicidae), commonly known as the southern house mosquito. This species is widely recognized as a major lymphatic filariasis vector (Samy et al., 2016; Vadivalagan et al., 2017). In addition, it is an important arbovirus vector, transmitting St. Louis encephalitis, Western equine encephalitis and West Nile. Recently, it is under investigation as a potential Zika virus vector (Guo et al., 2016; Benelli and Romano, 2017; van den Hurk et al., 2017). The management of *C. quinquefasciatus* mainly rely to the employment of chemical insecticides, with severe non-target effects for human health and the environment, along with the quick development of pesticide resistance in targeted mosquitoes (Mariappan and Tyagi, 2018). In this framework, research aimed to propose eco-friendly control tools to manage *C. quinquefasciatus* is extremely important (Qualls et al., 2016; Tamilselvan et al., 2017).

To develop a novel and highly effective insecticide against this important vector, the *C. acaulis* EO and carlina oxide acute larvicidal activity as well as mortality over time for five days after 24 h of exposure to the toxics, were evaluated ~~assessed on 3rd instar larvae~~. Then, the larvicidal activity was validated in large water tanks tests. ~~Therefore, their insecticidal activity was validated at 1.25 µg L^{-1} in large-scale tests using 10 L water tanks and large mosquito populations (200 larvae) per replicate.~~ To shed light on the possible modes of action, we tested ~~the major *C. acaulis* EO component, i.e.~~ carlina oxide for its anti-acetylcholinesterase (AChE) activity ~~using the Ellman assay~~. The potential non-target impact of both larvicides ~~*C. acaulis* EO and carlina oxide~~ on aquatic organisms was assessed ~~through experiments~~ on the microcrustacean *Daphnia magna* Straus (Cladocera: Daphniidae). Besides, the antioxidant effects of *C. acaulis* EO, which may have a

relevant impact to increase the shelf-life of herbal-based insecticides developed for commercial purpose, were evaluated using the DPPH and ABTS assays. Finally, to evaluate its potential cytotoxicity on vertebrate cells, the major *C. acaulis* EO component, carlina oxide, was tested on human dermis (HuDe) and human tumour (HCT 116 and MDA-MB 231) cell lines by the MTT assay.

2. Materials and methods

2.1. Plant material

A commercial batch (code MP0136, no C-250518310518) of *C. acaulis* roots was purchased from A. Minardi & Figli Srl (Bagnacavallo, RA, Italy). They were obtained from an Albanian accession harvested in autumn 2017.

2.2. Isolation of the essential oil

One kg of *C. acaulis* crushed dry roots was inserted into a 10 L round flask filled with 6 L of distilled water and subjected to hydrodistillation using a Clevenger-type apparatus for 4 h. The heating apparatus consisted of a mantle system Falc MA (Falc Instruments, Treviglio, Italy). Once obtained, the root essential oil (EO), having a density higher than water, was decanted, separated from the aqueous layer and dehydrated using anhydrous Na_2SO_4; it was then inserted in vials sealed with PTFE/silicon caps which were maintained at 4°C until chemical analysis and biological assays. The *C. acaulis* root EO, of orangish colour, was weight and its yield (0.4%, w w^{-1}) was estimated on a dry weight basis (n=2).

2.3. GC-MS analysis

An Agilent 6890N gas chromatograph equipped with a single quadrupole 5973N mass spectrometer and an auto-sampler 7863 (Agilent, Wilmingotn, DE) was used for the analysis of *C. acaulis* EO. For separation, an HP-5 MS capillary column (30 m, 0.25 mm i.d., 0.1 μm film thickness; 5% phenylmethylpolysiloxane), supplied by Agilent (Folsom, CA, USA) was used. It was thermostatted at 60°C for 5 min, then raised up to 220°C at 4°C min^{-1}, finally up to 280°C at 11°C min^{-1} held for 15 min. The temperature of injector and detector was 280°C. The mobile phase consisted in 99.9% He with a flow of 1 mL min^{-1}. The EO samples were diluted 1:100 in *n*-hexane and 2 μL injected in split mode (1:50). Peaks were acquired in the electron impact (EI, 70 eV) mode in the range 29–400 $m\ z^{-1}$. Chromatograms were studied by using the MSD ChemStation software (Agilent, Version G1701DA D.01.00) and the NIST Mass Spectral Search Program for the NIST/EPA/NIH EI and NIST Tandem Mass Spectral Library v. 2.3 were used to analyze data. The identification of the EO components was firstly approached by the interactive combination of the temperature-programmed retention indices (RIs) and mass spectra (MS) with respect to those of ADAMS, NIST 17, FFNSC2 and MAGGI libraries (Adams, 2007; NIST 17, 2017; FFNSC2, 2012) (Table 1). RI was 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:

$$RI_x = 100_n + 100(t_x\text{-}t_n)/(t_{n+1} - t_n);$$

where *n* is the number of carbon atoms of the alkane eluting before the compound *x*, t_n and t_{n+1} are retention times of the reference alkanes eluting before and after compound *x*, and t_x is the retention time of the compound *x*. Relative peak area percentages were obtained by peak area normalization without using correction factors. Percentages values were the mean of two independent distillations.

2.4. NMR analysis of essential oil and carlina oxide purification

NMR spectra were acquired on a Bruker Avance 400 Ultrashield spectrometer. The chemical shift values are expressed in δ values (ppm), and coupling constants (J) are in hertz; tetramethylsilane (TMS) was used as an internal standard. Proton chemical data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, q = quartet, m = multiplet, brs = broad singlet) coupling constant (s), integration. The EO (20 mg) was diluted in deuterated chloroform and used for analysis. Standard Bruker library pulse programs were used for H, HSQC-DEPT, HMBC and COSY experiments.

Half mL of essential oil (approximatively 400 mg) was chromatographed on a silica gel (20 g) column (70−230 mesh, 60 Å, Merck) using hexane, followed by a stepwise gradient solvent system consisting of hexane/ethyl acetate 99:1 to 98:2. A total of 32 fractions was collected and monitored by thin-layer chromatography (TLC). Fractions 15-22 (called CA15-22) yielded 330 mg of a pure compound (99% by GC-MS), and the structure was identified as carlina oxide. The NMR data were comparable with data described in the literature (Djordjevic et al., 2005).

^{1}H NMR ($CDCl_3$-d_6): 3.85(s, 2H, CH_2), 6.37 (dd, 1H, *J* = 1.8, 3.4 Hz, C*H* furan), 6.59 (d, 1H, *J* = 3.4 Hz, C*H* furan), 7.15–7.43 (m, 6H, CH phenyl + CH furan).

^{13}C NMR (DMSO-d_6): 25.7 (1C, CH_2), 72.9 (1C, C≡C), 91.9 (1C), 110.7 (1C, CH), 114.2 (1C, CH), 126.7 (1C, CH), 127.9 (2C, phenyl), 128.6 (2C, phenyl), 135.9 (1C), 137.3 (1C), 142.9 (1C, CH). (API-ESI): *m/z* 183.07 $[M+H]^+$. Anal. calcd. for ($C_{13}H_{10}O$) C, 85.69; H, 5.53; Found: C, 85.67; H, 5.50.

2.5. Larvicidal activity in the short term on Culex quinquefasciatus

Culex quinquefasciatus larvae were reared as reported by Benelli et al. (2018a). They were maintained and tested at 25±1 °C, 60±10% R.H. and 16:8 h (L:D). Third-instar larvae were tested in larvicidal assays diluting the *C. acaulis* root EO and carlina oxide in dimethyl sulfoxide (DMSO) in 500-mL glass bowl as detailed by Benelli et al. (2018b). The *C. acaulis* root EO and carlina oxide

were tested at 0.5, 0.75, 1.0, 1.25, 1.5, 1,75 and 2.0 µg mL^{-1} to estimate the $LC_{50(90)}$ values (for each concentration, 4 groups, each composed by 25 larvae, were tested) (Table 2). Final surface area was 125 cm^2. Distilled water plus DMSO (used to formulate the *C. acaulis* root EO and carlina oxide) was the negative control (4 groups of 25 larvae each were tested). Cypermethrin (Cyperkill Max®), a widely used commercial insecticide (Benelli et al., 2019), was the positive control (concentrations 0.01, 0.02, 0.03, 0.05, 0.07 and 0.1 µg mL^{-1}, each concentration was tested on 4 groups of 25 larvae each). Mortality was recorded after 24 h. The treated insects were placed in a growth chamber [25±1 °C; 16:9 (L:D)].

2.6. Larvicidal activity over time

To assess the larvicidal activity over time of *C. acaulis* root essential oil, and carlina oxide on *C. quinquefasciatus*, 3rd instar larvae were exposed to each product for 24 h. Then, they were transferred to clean water and provided with standard diet (Benelli et al., 2017a). The application methods are described in the paragraph above for short-term tests. Different concentrations (i.e., 0.5, 0.75, 1, 1.25, 1.50, 1.75 and 2 µg mL^{-1}) were tested. Larval mortality was evaluated daily for the subsequent 5 days, according to Benelli et al. (2017a). Four replicates were done for each concentration. The treated *C. quinquefasciatus* were placed in a growth chamber (25±1 °C; 16:9 (L:D)] during the whole experimental period.

2.7. Larvicidal activity in water tanks

To verify their larvicidal effectiveness on a larger scale, *C. acaulis* root EO and carlina oxide larvicidal efficacy were evaluated against *C. quinquefasciatus* in 10 L water tanks. Herein, 8 L of water was mixed with 10 µg of *C. acaulis* EO or carlina oxide (sublethal dose 1.25 µg L^{-1}). Then, 200 larvae (3rd instar) of *C. quinquefasciatus* were placed in the water tank (35 x 35 x 50 cm), and

mortality was evaluated for the first three days post-application [25±2 °C, R.H.=60±10%, 16:8 (L:D) h]. *Culex quinquefasciatus* mortality was expressed as mean values (%) ±SE.

2.8. AChE inhibition assay

The modified method of Ellman et al. (1961) was used to assess the AChE inhibition activity of carlina oxide. Electric eel acetylcholinesterase was used and acetyl thiocholine iodide (ATCI) was the substrate of the reaction. 5, 5-dithiobis (2-nitrobenzioc) acid (DTNB) was used for the measurement of AChE activity as previously reported (Benelli et al., 2018a). Experiments were performed in triplicates. Results were also presented as mg of galantamine equivalents (GE) g^{-1}, which were indicative of the galantamine-equivalent inhibition capacity (GEIC).

2.9. Antioxidant activity: prospects for insecticide shelf-life

The *in vitro* radical scavenging activity of carlina oxide was determined using the 2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. Results were expressed as trolox equivalent per g of compound (µmol TE g^{-1}) as previously reported (Benelli et al., 2018c). All experiments were performed in triplicate measurements.

2.10. Impact on non-target microcrustaceans

Daphnia magna microcrustaceans was reared as reported by Pavela (2014). Then, we assessed the impact of *C. acaulis* root EO, carlina oxide, and cypermethrin on the non-target aquatic species *D. magna*, following Pavela (2014). In the tests, adults (2-5 days old) of *D. magna* were exposed to LC_{90} estimated on *C. quinquefasciatus* larvae. In detail, twenty adults of *D. magna* were transferred to plastic dishes containing 100 mL of water plus the proper concentration of *C. acaulis* root EO,

254 carlina oxide or cypermethrin (1.8, 1.9 and 1.0 µg mL^{-1}, respectively). Four replicates were done for
255 each concentration. Mortality was determined after 24 and 48 h.
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257 *2.11. Cytotoxicity assays*
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259 Human dermis (HuDe) and human breast adenocarcinoma (MDA-MB 231) cell lines were cultured
260 in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU mL^{-1} penicillin,
261 100 µg mL^{-1} streptomycin, and supplemented with 10% heat-inactivated fetal bovine serum (HI
262 FBS). Human colon carcinoma (HCT116) cell line was cultured in RPMI1640 medium with 2 mM
263 L-glutamine, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and supplemented with 10% HI-
264 FBS. Cells were cultured in a humidified atmosphere at 37°C in presence of 5% CO_2. The cytotoxic
265 effects of carlina oxide on the above-mentioned human cell lines were assayed by the MTT assay,
266 as described by Quassinti et al. (2013). Briefly, cells were seeded at the density of 2×10^4 cells mL^{-1}.
267 After 24 h, cells were exposed to different concentrations of carlina oxide (0.78-200 µM). After 72
268 h of incubation, each well received 10 µL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-
269 tetrazoliumbromide) solution (5 mg mL^{-1} in phosphate-buffered saline, PBS) and the plates were
270 incubated for 4 h at 37°C. Cisplatin was used as the positive control (0.5-40 µM). The extent of
271 MTT reduction was measured spectrophotometrically at 540 nm using a Titertek Multiscan
272 microElisa (Labsystems, FI-Helsinki). The cell survival curves were calculated after comparing
273 with the vehicle (Et-OH). Experiments were conducted in triplicate.
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275 *2.12. Statistical analysis*
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277 In *C. quinquefasciatus* larvicidal tests, if control mortality ranged from 1 to 20%, Abbott's formula
278 was used to correct experimental mortality; if control mortality was >20% experiments were

discharged and repeated (Abbott, 1925). Therefore, $LC_{50(90)}$ were estimated by probit analysis (Finney, 1971).

Moreover, *C. quinquefasciatus* mortality data (%) over five days, mortality data (%) from large larvicidal tests in water tanks, non-target *D. magna* mortality data (%) as well as AChE inhibition data (%) were transformed by arcsine√ and analysed using ANOVA followed by Tukey's HSD test ($P \leq 0.05$). Furthermore, concerning AChE inhibition data, we also calculated the IC_{50} values (i.e., the carlina oxide and galantamine concentration inhibiting AChE by 50%) from dose-response curves.

The ability to scavenge the DPPH and ABTS radicals was evaluated using the equation: Radical scavenging effect (%) = $[A_0 - A_1/A_0] \times 100$, where A_0 and A_1 are the absorbance of the control and sample respectively, after 30 min of incubation. By fit of experimental data the antiradical activity (IC_{50}) was expressed as the concentration of antioxidant needed to decrease the initial radical scavenger concentration by 50%. In cytotoxicity assays, the IC_{50} values were determined with GraphPad Prism 4 computer program (GraphPad Software, S. Diego, CA, USA).

3. Results

3.1. Chemical analysis of the Carlina acaulis essential oil

The chemical constituents of the EO obtained from roots of *C. acaulis*, as determined by GC-MS and NMR analyses, are reported in Table 1. Seven compounds were characterized in the EO, accounting for 98.3% of the total composition. Carlina oxide the main component, accounting for 94.6% of the total peak areas (Figure 1). Among the minor components, the aromatic benzaldehyde (3.1%) was the most representative compound followed by *ar*-curcumene (0.4%). Acetophenone, benzyl methyl ketone, camphor and carvone were the remaining compounds, all occurring at trace levels.

305 The EO of *C. acaulis* was analysed by H-NMR and 2D NMR experiments (HSQC-DEPT,
306 HMBC, COSY) allowing the identification of the main constituent, i.e. carlina oxide (Figure 2).
307 The characteristic signals are the singlet at δ 3.85 (δC- 25.7) ascribable to the CH_2, the doublet at δ
308 6.59 (δ 110.7), the doublet of doublets at 6.41 (δ 114.2) and the doublet at δ 7.24 (δ 142.8)
309 ascribable to the furan moiety as well as to the aromatic proton signals in the region δ 7.29-7.48 (δ
310 C 128-137) ascribable to benzene ring. HMBC correlations from the singlet at δ 3.85 revealed the
311 quaternary carbons at 91.9 and 72.9 ascribable to the triple bond as well as the correlation with the
312 benzene ring carbons. Minor signals can be ascribable to aromatic aldehyde (benzaldehyde as
313 reported above) as well as other sp^2 containing compounds are present, minor signals are also
314 detectable in the aliphatic region of the spectrum as singlets or multiplets.
315
316 *3.2. Culex quinquefasciatus larvicidal activity*
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318 Both the *C. acaulis* EO and its main constituent carlina oxide were extremely toxic to *C.*
319 *quinquefasciatus* 3rd instar larvae in acute toxicity assays (Table 2), showing LC_{50} values of 1.31
320 and 1.39 µg mL^{-1}, while LC_{90} values were 1.83 and 1.94 µg mL^{-1}, respectively. Overlapping 95%
321 CI were detected when estimating LC_{50} and LC_{90} values of *C. acaulis* EO and carlina oxide,
322 highlighting no significant differences between the LC values of the two products (Table 2).
323 Concerning the positive control, α-cypermethrin, $LC_{50(90)}$ values on *C. quinquefasciatus* larvae were
324 0.03 and 0.06 µg mL^{-1} (Table 2).
325 Furthermore, the mortality of *C. quinquefasciatus* larvae exposed for 24 h to *C. acaulis* EO
326 (Table 3) and carlina oxide (Table 4), both tested at concentrations ranging from 0.5 to 2 µg mL^{-1},
327 was monitored daily for seven days, showing a significant decrease (P<0.05) of larval survival over
328 time for all the seven concentrations tested. Interestingly, even though the larvae were transferred to
329 clean water after 24 h of exposure, there was further significant mortality during the next five days.
330 Testing the concentrations ≥1.25 µg mL^{-1} we observed 100% mortality of *C. quinquefasciatus*

larvae. At 1 µg mL^{-1} the larval mortality was higher than 90% testing both the essential oil and carlina oxide.

Lastly, larvicidal tests on a wider scale, i.e., in 10 L water tanks with 200 *C. quinquefasciatus* larvae per replicate, confirmed the larvicidal effectiveness of the *C. acaulis* EO and carlina oxide, both tested at 1.25 µg L^{-1} of water (Table 5). The effect of these treatments over the negative control was highly significant after 24 h ($F_{2,6}$=660.2; P<0.0001), 48 h ($F_{2,6}$=666.4; P<0.0001) and 72 h ($F_{2,6}$=3248.3; P<0.0001). Both products showed comparable performances; no significant differences were found between the mortality rates achieved by *C. acaulis* EO and carlina oxide (Table 5).

3.3. AChE inhibition

Acetylcholinesterase inhibition rates obtained testing increasing concentrations of carlina oxide, as well as the positive control galantamine, are detailed in Figure 3. For both compounds, a significant effect of the tested concentration on AChE inhibition was observed (carlina oxide: $F_{7,17}$=5.436; P=0.002; galantamine: $F_{7,8}$=195.941; P<0.0001). Concerning IC_{50} values, the AChE inhibitory activity of carlina oxide and galantamine are summarized in Table 6. The IC_{50} of 0.6 mg mL^{-1} was indicative of good enzyme inhibition by carlina oxide, even if resulting 54-fold lower if compared to that of galantamine (Table 6).

3.4. Antioxidant activity: prospects for insecticide shelf-life

The antioxidant activity of carlina oxide, tested by the (DPPH•) and ABTS+ assays (Table 7), resulted in a half maximal effective concentration (IC_{50}) of 320.4 and 662.5 µg/mL, respectively. These values were about 125 and 157 times lower with respect to those of Trolox in the same experimental conditions (Table 7).

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358 *3.5. Impact on non-target microcrustaceans*

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360 The acute toxicity of *C. acaulis* EO and carlina oxide was evaluated against *D. magna* adults (Table
361 8). Both products were tested on microcrustaceans at the LC_{90} calculated on *C. quinquefasciatus* 3rd
362 instar larvae (i.e., 1.8 and 1.9 μg mL^{-1}, respectively, see Table 2). Results showed that both the EO
363 and carlina oxide exerted relevant mortality rates on *D. magna* adults, i.e. 38.5±4.2 and 44.4±4.1 %,
364 respectively (after 24 h of exposure), and 44.4±4.1 and 51.9±2.4 %, respectively (after 48 h of
365 exposure). However, the observed mortality was significantly lower if compared to the positive
366 control cypermethrin, which always achieved 100% mortality when tested at a lower concentration,
367 i.e., 1 μg mL^{-1} (Table 8).

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369 *3.6. Cytotoxic activity*

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371 We analysed the cytotoxic activity of the major compound of the EO, carlina oxide, on human
372 dermis fibroblasts (HuDe), as well as on human tumour cell lines (HCT116 and MDA-MB 231). As
373 reported in Table 9, IC_{50} values ranged from 21 μM (3.83 μg mL^{-1}) on the HuDe cells to 37 μM
374 (6.74 μg mL^{-1}) on MDA-MB 231 cell line. The cytotoxic activity of carlina oxide results lower than
375 that of cisplatin that is about 4 times higher on the same cell lines tested. Furthermore, the
376 compound did not show selectivity between the tumour and non-tumour cells.

377

378 **4. Discussion**

379

380 *4.1. Chemical composition of Carlina acaulis essential oil*

381

GC-MS and NMR analyses showed that the *C. acaulis* EO was mostly composed of carlina oxide (94.6%), with minor contributions by other components (e.g. benzaldehyde and *ar*-curcumene). The EO chemical composition of this commercial batch of *C. acaulis* was rather consistent to those previously reported in literature and referring to both cultivated and wild populations (Stojanović-Radić et al., 2012; Chalchat et al., 1996). Also, similarity in the EO chemical profile was found respect to that of C. *acanthifolia* which is often used to adulterate the commercial *C. acaulis* herbal drug (Dorđević et al., 2005, 2007).

4.2. Culex quinquefasciatus larvicidal activity

Developing highly effective insecticides from botanicals is an interesting route to attempt tackling fast-growing resistance to synthetic compounds (Pavela, 2015a; Govindarajan and Benelli, 2016b; Stevenson et al., 2017). This work represents the first investigation of the insecticidal potential of *C. acaulis* EO. In our insecticidal assays, the *C. acaulis* EO showed an outstanding efficacy (LC_{50}=1.31 µg mL^{-1} and LC_{90}=1.83 µg mL^{-1}) comparable with some synthetic larvicides used to manage *C. quinquefasciatus* worldwide (Beketov, 2004; Rodríguez et al., 2005; Sunday et al., 2016).

Actually, mosquito larvae can be significantly more sensitive to synthetic pyrethroids than natural products. However, they can very quickly develop resistance to pyrethroids, as reported, for example, with deltamethrin applied against the larvae of *C. quinquefasciatus* (Sarkar et al., 2009). On the other hand, EOs containing more than one active substance usually have more mechanisms of action, and the substances are often synergistic (Pavela, 2015b; Benelli et al., 2017a). This phenomenon avoids the development of insecticide resistance and they are, therefore, now considered to be promising active substances to develop eco-friendly and effective pesticides (Lourenco et al., 2018).

Moreover, concerning botanical products, a recent review showed that – despite hundreds of studies on EOs as mosquito larvicides - only seven of them (i.e., *Blumea densiflora* D.C., *Auxemma glazioviana* Taub., *Callitris glaucophylla* Joy Thomps. & L.P. Johnson, *Cinnamomum microphyllum* Ridl., *Cinnamomum mollissimum* Hook. F., *Cinnamomum rhyncophyllum* (Miq.), and *Zanthoxylum oxyphyllum* Edgew.) had LC_{50} lower than 10 ppm (Pavela 2015a). However, of them, only two EOs, i.e, the ones from *C. glaucophylla* (LC_{50}=0.7 and 0.2 ppm on *Aedes aegypti* L. and *Culex annulirostris* Skuse, respectively) (Shaalan et al. 2006) and *A. glazioviana* (LC_{50}=3 ppm on *A. aegypti*) (Costa et al. 2004), showed an effectiveness comparable to the *C. acaulis* EO reported in the present work. Overall, our evidences pointed out that *C. acaulis* can be currently recognized as the most effective botanical larvicide against the important filariasis and Zika virus vector *C. quinquefasciatus* (Guo et al., 2016).

According to our experimental hypothesis, the insecticidal capacity exhibited by the *C. acaulis* EO can be mainly explained by its major compound, i.e. carlina oxide, an aromatic acetylene compound containing a carbon-carbon triple bond. Till now, no insecticidal data are available on this compound. Our experiments testing this molecule showed that the toxicity of carlina oxide against *C. quinquefasciatus* larvae (LC_{50}=1.90 µg mL^{-1} and LC_{90}=2.72 µg mL^{-1}) did not significantly differ from that of *C. acaulis* EO, since both LC_{50} and LC_{90} values showed overlapping 95% CI. Therefore, the toxicity of this EO can be directly linked with that of carlina oxide, which far exceed a wide number of EOs already tested as mosquito larvicides (Pavela, 2015a) as well as the most effective isolated constituents (e.g., Govindarajan and Benelli, 2016b,c; Al-Shebly et al., 2017).

In addition, our results pointed out a marked impact of 24 h exposure to low doses (<2 µg mL^{-1}) of the *C. acaulis* root EO and carlina oxide on larval survival in the following days, reaching mortality rates higher than 90% when exposed for 24 h to 1 µg mL^{-1} of root EO or carlina oxide and then monitored in clean water till 144 h. It has already been found that even short-term exposure of insects to sub-lethal doses of certain EOs can significantly reduce their longevity, vitality, fecundity

433 and fertility (Pavela, 2007; Benelli et al., 2017, 2018b), while studies of pure selected compounds
434 are limited. We believe that it is important to study this phenomenon thoroughly. Indeed, thanks to
435 this knowledge economically acceptable and yet sufficiently effective doses or concentrations of
436 botanical insecticides could be applied that do not cause short term mortality, nevertheless there
437 will still be a significant reduction in population density or occurrence of the target organisms up to
438 the threshold of their economic damage.

439 Furthermore, to verify the *C. acaulis* EO and carlina oxide effectiveness observed in
440 larvicidal tests, we tested both of them at the concentration of 1.25 $\mu g\ L^{-1}$ in larger containers with 8
441 L of water. Thereby, we confirmed that even on a larger scale, this EO rich in carlina oxide and its
442 main constituent have a noteworthy efficacy, as we observed 98% mortality 72 h after application.
443 Further tests to evaluate the efficacy of both products in the field are ongoing.

444

445 *4.3. Modes of action*

446

447 Concerning the mode of action, our work provided the first evidence of carlina oxide as an effective
448 AChE inhibitor (Murata et al., 2017). Thus, results of this work indicate that the insecticidal activity
449 observed for *C. acaulis* EO could correlate with the ability of carlina oxide to inhibit AChE.
450 Although the inhibition of AChE by plant EOs has been reported as a main mode of action on
451 insects, other still unexplored mechanisms (e.g., inhibition of $GABA_A$ and octopamine receptors)
452 may be involved in the *C. acaulis* insect toxicity (Enan, 2001; Yeom et al., 2012; Seo et al., 2014;
453 Yeom et al., 2015). Thus, further research on this issue is needed.

454 Polyacetylenes are marker compounds of the Asteraceae family where they play an
455 important role in the plant defence system (Konovalov, 2014; Seigler, 1998). Notably, aromatic
456 polyacetylenes, as carlina oxide, are known to cause phototoxicity in insects (Konovalov, 2014).
457 The high reactivity of these compounds often causes their quick oxidation and degradation,
458 depending on the UV light exposure and pH of the medium (Christensen, 1998). Notably, carlina

459 oxide is endowed with high lipophilicity that allows it to easily enter the insect body where it is
460 converted into a stable carbocation reacting with amino groups and SH of biomolecules (Wink
461 2012). Thus, their interaction with glutathione S-transferase and esterase activities could also be
462 investigated (Vasantha-Srinivasan et al., 2018b). Another possible mechanism of action causing
463 neurotoxicity in insects can be the ability of polyacetylenes to modulate the $GABA_A$ receptors
464 (Czyzewska et al., 2014).

465 A possible contribution to the insecticidal activity of the *C. acaulis* EO may also be given by
466 minor components such as benzaldehyde. The latter has been recently reported as highly effective
467 against *Galleria mellonella* L. larvae thus having the potential of use in the manufacturing of novel
468 botanical insecticides (Ullah et al., 2015). Benzaldehyde was also toxic to larvae of the Asian tiger
469 mosquito, *Aedes albopictus* Skuse, showing a LC_{50} of 47 ppm (Cheng et al., 2009).

470

471 *4.4. Antioxidant activity: prospects for insecticide shelf-life*

472

473 Compared to the present results, earlier studies reported a lower antioxidant activity for extracts of
474 *C. acaulis*, *C. acanthifolia* and *C. vulgaris* L. (Đordevic et al., 2012; Link et al., 2016; Strzemski et
475 al., 2017). On the other hand, a higher antioxidant effect was reported for the EO of *C. acanthifolia*
476 roots (Đordevic et al., 2007) suggesting that the active antioxidant compound is rather lipophilic. In
477 this framework, our data confirmed the moderate antioxidant activity of the main essential oil
478 constituent carline oxide suggesting its relatively important preservative properties within a possible
479 insecticide formulation (Đordevic et al., 2007).

480

481 *4.5. Non-target impact*

482

483 Among synthetic insecticides, cypermethrin is currently one of the most widely used molecules
484 worldwide (Ullah et al., 2018), due to its low mammalian toxicity coupled with low persistence in

485 the environment. However, researchers pointed out its high toxicity on non-target fish species [e.g.,
486 *Oryzias latipes* (Temminck & Schlegel, 1846)] as well as on *D. magna* neonates (Kim et al., 2008),
487 outlining the need to develop more eco-friendly pesticides. *Daphnia* microcrustaceans are among
488 the most sensitive representatives of aquatic plankton that are relatively tolerant to some EOs
489 (Barata et al., 2004, 2006; Pavela, 2014; Bandeira et al., 2017). In our non-target tests, even if both
490 *C. acaulis* EO and carlina oxide were toxic to *D. magna* adults, the observed toxicity rates were
491 significantly lower if compared to those achieved by cypermethrin. It will therefore be important to
492 carry out further studies on other non-target organisms to clarify the impact of *C. acaulis* EO and
493 carlina oxide on non-target species as well as that of their encapsulated forms. However, it is crucial
494 to note that the application of larvicides may also be frequently carried out in local mosquito
495 hatcheries in close proximity of humans and pets (Marina et al., 2012). In such cases, the safety of
496 insecticide application is important, and botanical pesticides can guarantee such safety to a certain
497 degree (Pavela, 2016).

498 Besides, from a safety perspective, the use of *C. acaulis* EO should not be matter of concern
499 for human health since it has been included, together with the root from which it is obtained, in the
500 Italian list of botanicals to be used in food supplements
501 (http://www.gazzettaufficiale.it/eli/gu/2018/09/26/224/sg/pdf) as well as in the BELFRIT project
502 (Cousyn et al., 2013). Notably, it is indicated to enhance the diuretic, carminative, digestive and
503 diaphoretic functions. In line with the concepts outlined above, moderate cytotoxicity was found
504 testing the main oil component carlina oxide on human dermis cell lines. About this latter evidence,
505 in the literature there is only one paper (Hermann et al., 2011) where carlina oxide was tested on the
506 human HeLa cell line, showing an IC_{50} value of 446 $\mu g\ mL^{-1}$ (2.45 mM). The difference in activity
507 can be explained by the different incubation time in which the cells are exposed to the carlina oxide.
508 In HeLa cells the MTT test was performed after 24 h, in our experiment, the human cells are
509 exposed for 72 h in presence of the compound. However, it cannot be excluded that increasing the
510 exposure time of cells to the carlina oxide, cytotoxic or apoptotic processes leading to cell death

511 may be triggered. The cytotoxicity exhibited by carlina oxide on colon carcinoma and breast
512 adenocarcinoma cells may be worthy of further investigation to propose it as an anticancer agent as
513 well.
514
515 **5. Conclusions and prospects for future research and real-world applications**
516
517 *Carlina acaulis* is an overlooked traditional medicinal plant whose biological potential has been
518 little explored so far. Results of this work shed light on the possible utilization of *C. acaulis* EO and
519 its major compound carlina oxide as the active ingredients of highly effective and eco-friendly
520 botanical insecticides. Its toxicity seems to be partly related to the AChE inhibitory properties of
521 carlina oxide. However further studies on the precise mode of action of this polyacetylene are
522 needed. *In situ* cultivation of the plant together with *in vitro* micropropagation techniques and
523 utilization of the less rare *C. acanthifolia* containing the same active compound, make the
524 manufacturing of insecticides from this plant likely.
525 Although *C. acaulis* is less toxic respect to other species due to its old uses as a food, our
526 non-target assays showed a relevant toxicity on aquatic microcrustaceans, even if the observed
527 mortality was significantly lower when compared to cypermethrin. Therefore, further studies on the
528 possible toxicological effects of this EO and carlina oxide as well on non-target terrestrial and
529 aquatic organisms are still needed. Furthermore, the stabilization and efficacy improvement of EO
530 and carlina oxide using nanotechnologies, such as nanoemulsions, are particularly welcome, to
531 allow long-lasting efficacy in real-world conditions (Pascual-Villalobos et al., 2017; Mishra et al.,
532 2018; Pavela et al., 2019b,c). Furthermore, the evaluation of their safety for human health and the
533 assessment of potential synergistic effects of carlina oxide when co-formulated in binary blends
534 with other compounds (Pavela, 2015b) is urgently needed. It would be important to obtain
535 information about new technologies to increase the yields and content of EO in aromatic plants
536 (Pavela et al., 2018b) or allowing an optimized extraction of EO bioactivity constituents (Fiorini et

537 al., 2019). Finally, our work outlines highly promising perspectives related to the wide-scale
538 employ of carlina oxide as an active substance for future insecticide development.
539
540 **Author Contributions**
541 Conceptualization: G.B., F.M., A.C.; Methodology: Ro.P., Ri.P., F.K.N., L.C., G.L., L.Q., M.B.,
542 S.S., S.D., Formal Analysis: Ro.P., G.B., A.C., S.D., Ri.P.; Writing/Review/Editing: F.M., G.B.,
543 Ro.P.
544
545 **Acknowledgments**
546
547 The authors are grateful to University of Camerino (Fondo di Ateneo per la Ricerca, FAR
548 2014/2015, FPI 000044) for financial support. Dr. R. Pavela would like to thank the Ministry of
549 Agriculture of the Czech Republic for its financial support concerning botanical pesticide and basic
550 substances research (Project MZE-RO0418).
551
552 **Conflict of Interest**
553
554 The authors declare no conflict of interest.
555
556 **References**
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Table

Table 1. Chemical composition of the essential oil from *Carlina acaulis* roots.

No.	Component[a]	RI[b]	RI Lit.[c]	%[d]	ID[e]
1	benzaldehyde	953	952	3.1±0.6	Std,RI,MS
2	acetophenone	1066	1059	tr[f]	RI,MS
3	benzyl methyl ketone	1129	1124	tr	RI,MS
4	camphor	1139	1141	tr	Std,RI,MS
5	carvone	1241	1239	tr	Std,RI,MS
6	*ar*-curcumene	1479	1479	0.4±0.1	RI,MS
7	carlina oxide	1597	1609[g]	94.6±1.4	NMR,RI,MS
	Total identified (%)	-	-	98.3±0.9	-

[a] Components are ordered according to their elution from a HP-5MS column. [b] Linear retention index calculated according to the Van den Dool and Kratz formula (1963). [c] Retention index taken from Adams. [d] Relative percentage values are mean of two independent distillations. [e] Identification methods: Std, comparison with available analytical standard; RI, coherence of the calculated RI with those stored in the ADAMS (2007) and NIST 17 (2017) libraries; MS, mass spectrum matching with respect to ADAMS, FFNSC2 (2012) and NIST 17 MS libraries. [f] Traces, % < 0.05. [g] RI taken from Zorica Stojanović-Radić et al. (2009).

Table 2. Lethal concentrations (LC) of root essential oil from *Carlina acaulis* and its main constituent carlina oxide against *Culex quinquefasciatus* 3rd instar larvae.

Treatment	LC_{50}±SE (CI_{95}) (µg mL^{-1})	LC_{90}±SE (CI_{95}) (µg mL^{-1})	χ^2	*d.f.*	*P*-value
Carlina acaulis root essential oil	1.31±0.03 (1.28-1.43)	1.83±0.07 (1.63-2.33)	0.927	3	0.831 n.s.
Carlina oxide	1.39±0.04 (1.34-1.43)	1.94±0.12 (1.84-2.07)	0.837	3	0.852 n.s.
Cypermethrin (positive control)	0.03±0.01 (0.02-0.04)	0.06±0.03 (0.05-0.09)	0.781	3	0.753 n.s.

SE= standard error

CI_{95}= 95% confidence interval

d.f.= degrees of freedom

n.s.=not significant

Table 3. Mortality over time of *Culex quinquefasciatus* larvae post-application of different concentrations of *Carlina acaulis* root essential oil. Larvae were left in contaminated water for 24 h. Then, they were transferred to clean water and provided with standard diet.

Concentration	**Mosquito larval mortality (%)[a]**					
(µg mL^{-1})	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**
2	97.5±4.3 [d]	100.0±0.0 [d]	100.0±0.0 [e]	100.0±0.0 [d]	100.0±0.0 [d]	100.0±0.0 [d]
1.75	87.5± 8.2 [d]	97.5±4.3 [d]	100.0±0.0 [e]	100.0±0.0 [d]	100.0±0.0 [d]	100.0±0.0 [d]
1.5	58.7±8.1 [c]	72.5±7.5 [c]	93.7±5.4 [e]	96.2±4.1 [d]	100.0±0.0 [d]	100.0±0.0 [d]
1.25	46.2±4.1 [c]	62.5±7.5 [c]	81.2±5.4 [d]	95.0±3.5 [d]	100.0±0.0 [d]	100.0±0.0 [d]
1	17.5± 2.5 [b]	35.0±3.5 [b]	57.5±4.3 [c]	86.2±4.1 [c]	88.7±2.1 [c]	91.2±2.1 [c]
0.75	0.0±0.0 [a]	5.0 ±3.5 [a]	26.2±2.1 [b]	45.0±3.5 [b]	70.0±5.0 [b]	73.7±5.4 [b]
0.5	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	3.75±4.1 [a]	5.0±3.5 [a]
Negative control	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]
ANOVA $F_{7,24}$; P	216.34; <0.0001	275.91; <0.0001	541.51; <0.0001	786.90; <0.0001	968.40; <0.0001	963.88; <0.0001

[a]Mosquito mortality was expressed as mean values (%) ±SE.

Within each column, different letters indicate significant differences among values (P<0.05).

Table 4. Mortality over time of *Culex quinquefasciatus* larvae post-application of different concentrations of carlina oxide. Larvae were left in contaminated water for 24 h. Then, they were transferred to clean water and provided with standard diet.

Concentration ($\mu g\ mL^{-1}$)	**Mosquito larval mortality (%)[a]**					
	24 h	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**
2	96.2±4.1 [f]	100.0±0.0 [e]	100.0±0.0 [e]	100.0±0.0 [d]	100.0±0.0 [d]	100.0±0.0 [e]
1.75	76.2±2.1 [e]	86.2±4.1 [d]	98.7±2.1 [e]	100.0±0.0 [d]	100.0±0.0 [d]	100.0±0.0 [e]
1.5	58.7±2.1 [d]	78.7±2.1 [d]	95.0± 5.0 [e]	100.0±0.0 [d]	100.0±0.0 [d]	100.0±0.0 [e]
1.25	36.2±4.1 [c]	55.0±5.0 [c]	83.7±4.1 [d]	100.0±0.0 [d]	100.0±0.0 [d]	100.0±0.0 [e]
1	11.2±2.1 [b]	38.7±5.4 [b]	47.5±2.5 [c]	61.2±2.1 [c]	77.5±2.5 [c]	91.2±2.1 [d]
0.75	0.0±0.0 [a]	0.0±0.0 [a]	3.7±4.1 [b]	22.5± 2.5 [b]	46.25±4.1 [b]	68.7±6.4 [c]
0.5	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	12.5±2.5 [b]
Negative control	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]
ANOVA $F_{7,24}$; P	726.06; <0.0001	540.93; <0.0001	735.45; <0.0001	4.703.93; <0.0001	2.026.94; <0.0001	789.93; <0.0001

[a]Mosquito mortality was expressed as mean values (%) ±SE.

Within each column, different letters indicate significant differences among values (P<0.05).

Table 5. Evaluation of *Carlina acaulis* root essential oil and carlina oxide larvicidal efficacy against *Culex quinquefasciatus* in 10 L water tanks (10 μg of essential oil of carlina oxide per 8 L of water, 200 larvae per tank, 4 replicates).

Treatment	**Mosquito mortality (%)**[a]		
	24 h	**48 h**	**72 h**
Carlina acaulis root essential oil	64.7±3.4[b]	90.5±5.0[b]	98.2±5.4[b]
Carlina oxide	69.2±1.4[b]	95.3±0.6[b]	98.8±1.6[b]
Negative control	0.0±0.0[a]	0.3±0.4[a]	1.2±1.0[a]
ANOVA $F_{2,6}$; P	660.2; <0.0001	666.4; <0.0001	3248.3; <0.0001

[a]Mosquito mortality was expressed as mean values (%) ±SE.

Within each column, different letters indicate significant differences among values (P<0.05).

Table 6. Acetylcholinesterase inhibitory activity of carlina oxide, the major component of *Carlina acaulis* essential oil, over galantamine, tested as positive control.

Compound	**IC_{50} (mg mL^{-1})**	**mg GEIC/g[a]**
Carlina oxide	0.609±0.01	18.5±0.4
Galantamine	$11.3(\pm0.3)*10^{-3}$	

[a] GEIC=galantamine-equivalent inhibition capacity.

Table 7. *In vitro* radical-scavenging activity of carlina oxide and Trolox.

Compound	DPPH		ABTS	
	TEAC[a] (µmol TE/g)	**IC_{50}[b] (µg/mL)**	**TEAC[a] (µmol TE/g)**	**IC_{50}[b] (µg/mL)**
Carlina oxide	31.6±2	320.4±3.5	26.0±0.3	662.5±4.5
Trolox, positive control	-	2.6 ±0.2	-	4.2±0.2

[a] TEAC = Trolox equivalent (TE) antioxidant concentration.

[b] IC_{50} = The concentration of the compound affording a 50% reduction in the assay.

Table 8. Acute toxicity of *Carlina acaulis* essential oil and carlina oxide against *Daphnia magna* adults; *C. acaulis* root essential oil and carlina oxide were tested at the LC_{90} calculated on *Culex quinquefasciatus* 3rd instar larvae.

Compound	***Daphnia magna* mortality (%)[a]**	
	After 24 h	**After 48 h**
Carlina acaulis essential oil (1.8 µg mL^{-1})	38.5±4.2[b]	44.4±4.1[b]
Carlina oxide (1.9 µg mL^{-1})	44.4±4.1[b]	51.9±2.4[c]
Positive control, α-cypermethrin (1.0 µg mL^{-1})	100.0±0.0[c]	100.0±0.0[d]
Negative control	0.0±0.0[a]	0.0±0.0[a]
ANOVA $F_{3,12}$; P	1,160.1; <0.0001	1,448.1; <0.0001

[a]*Daphnia magna* mortality was expressed as mean values (%) ±SE.

Within each column, different letters indicate significant differences among values (P<0.05).

Table 9. Cytotoxic activity of carlina oxide on normal human dermis (HuDe) and tumor (HCT116 and MDA-MB 231) cell lines.

	IC_{50} μM[a]		
	HCT116[b]	**MDA-MB 231[c]**	**HuDe[d]**
Carlina oxide	34.59	37.01	21.03
95% CI[e]	32.16-37.21	29.73-46.07	16.68-26.52
Cisplatin (positive control)	8.73	6.9	2.76
95% CI[e]	8.03-9.49	5.63-7.4	2.31-3.3

[a] IC_{50} = the concentration of compound affording a 50% reduction in cell growth (after 72 h of incubation).
[b] Human colon carcinoma cell line.
[c] Human breast adenocarcinoma cell line.
[d] Human dermis cell line.
[e] 95% confidence interval.

Figure

Fig. 1. GC-MS chromatogram of the essential oil extracted from roots of *Carlina acaulis*. Separation of peaks was achieved on a HP-5MS (5% phenylmethylpolysiloxane, 30 m l. x 0.25 mm i.d., 0.1 μm f.t.).

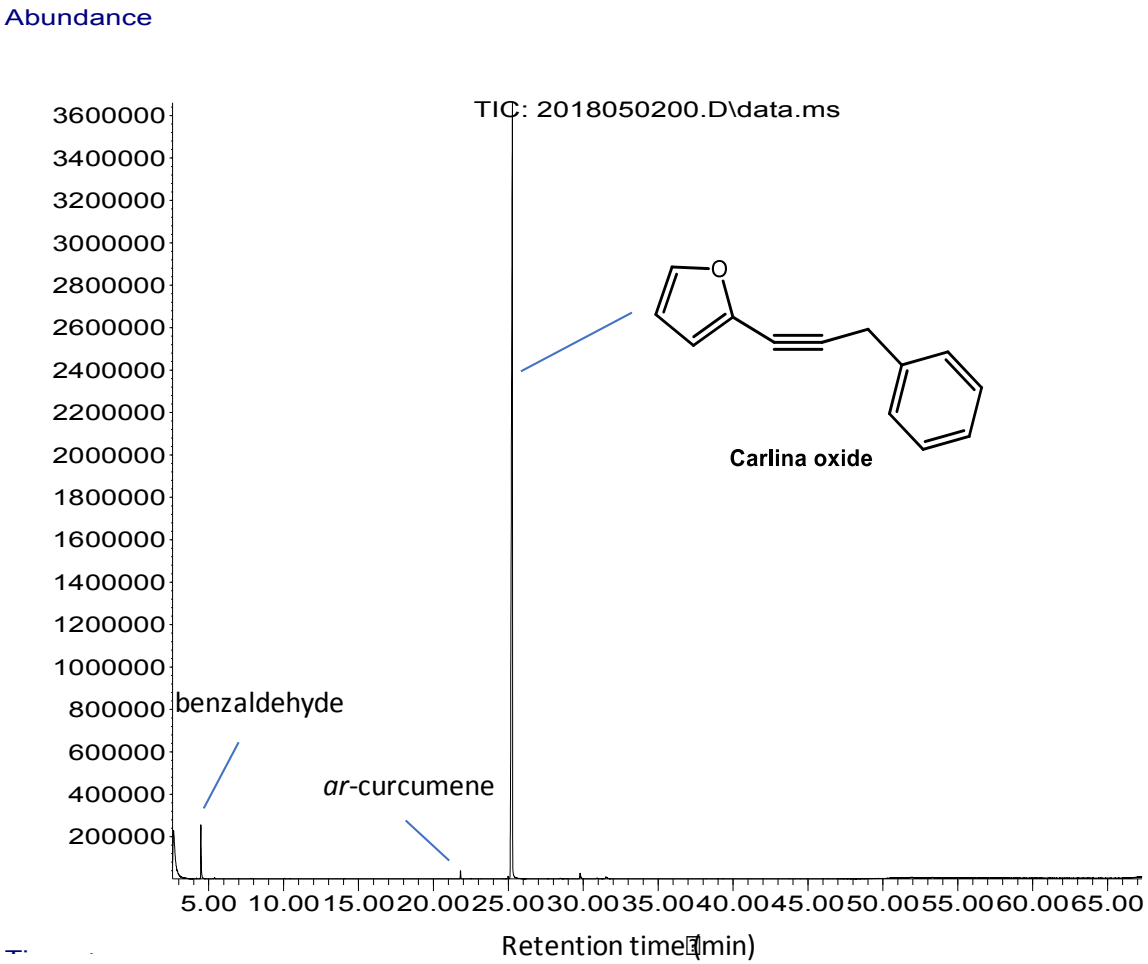

Fig. 2. ^{1}H-NMR spectrum of the *Carlina acaulis* root essential oil.

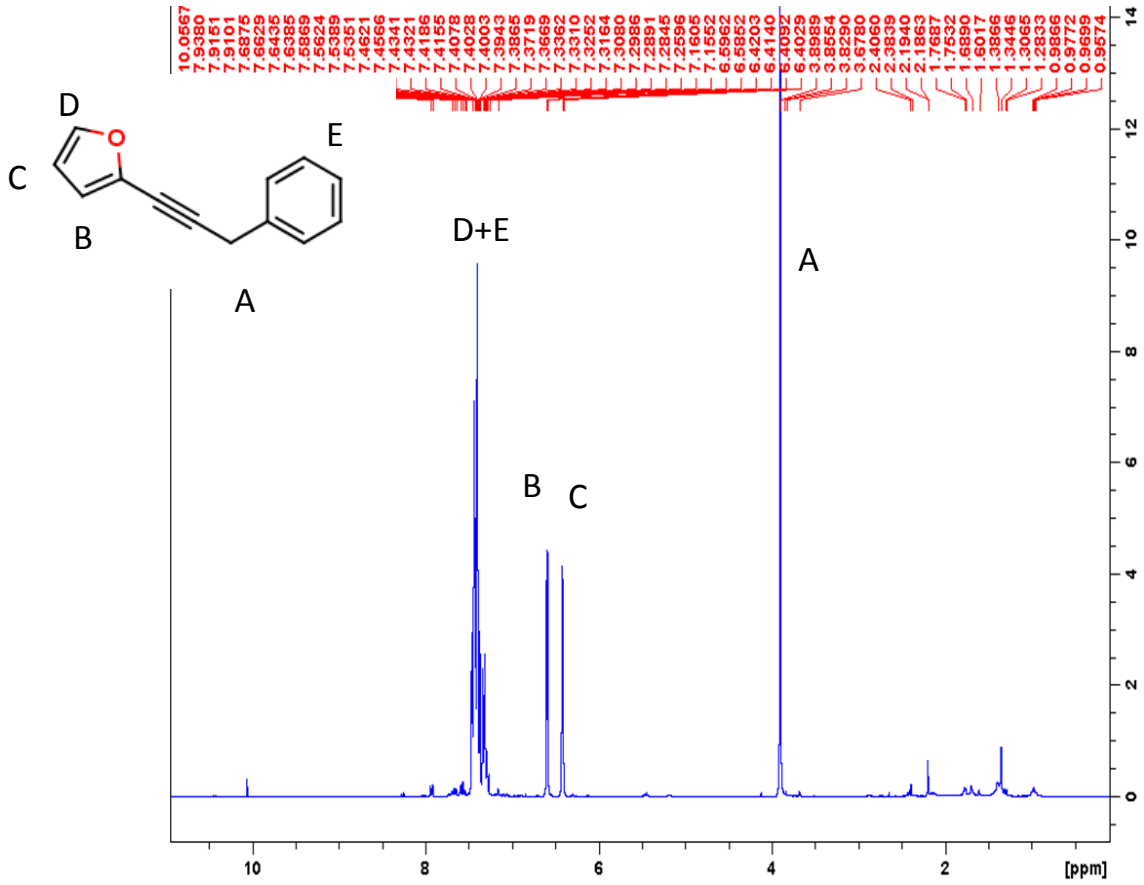

Fig. 3. Concentration-dependent inhibition of acetylcholinesterase (AChE) enzyme triggered by carlina oxide. Galantamine was the positive control. Within each data series, different letters above columns indicate significant differences (ANOVA, Tukey's HSD test, $P<0.05$).