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Research Article

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Lethal and sublethal effects of carlina oxide on *Tetranychus urticae* (Acari: Tetranychidae) and *Neoseiulus californicus* (Acari: Phytoseiidae)

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Abstract

BACKGROUND: *Tetranychus urticae* Koch, is a polyphagous and damaging pest, presenting several resistant populations worldwide. Among new and more environmentally friendly control tools, botanical pesticides represent a valuable alternative to synthetic ones within integrated pest management strategies. Accordingly, we investigated the lethal and sublethal effects of carlina oxide isolated from *Carlina acaulis* (Asteraceae) roots on *T. urticae* and its natural enemy, the predatory mite, *Neoseiulus californicus* (McGregor).

RESULTS: Carlina oxide (98.7% pure compound) was used for acaricidal tests on eggs, nymphs, and adult females of *T. urticae* (concentrations of 312.5, 625, 1250, 2500 and 5000 μL L^{-1}), and eggs and females of *N. californicus* (1250 and 5000 μL L^{-1} on eggs and females, respectively). Behavioral two-choice tests were also conducted on phytoseiid females. Carlina oxide toxicity was higher on *T. urticae* females than nymphs (median lethal dose 1145 and 1825 μL L^{-1}, respectively), whereas egg mortality and mean hatching time were significantly affected by all tested concentrations. A decreasing daily oviposition rate for *T. urticae* was recorded with concentrations ranging from 625 to 5000 μL L^{-1}, whereas negative effects on the population growth rate were recorded only with the three higher concentrations (1250, 2500 and 5000 μL L^{-1}). No toxic effect on *N. californicus* females was found, but a strong repellent activity lasting for 48 h from application was recorded.

CONCLUSION: Carlina oxide reduced longevity and fecundity of *T. urticae* adults, but not of *N. californicus*. This selective property allows us to propose it as a novel active ingredient of ecofriendly acaricides for *T. urticae* management.
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Keywords: Asteraceae; *Carlina acaulis*; green acaricides; side effects; two-spotted spider mite; phytoseiid mites

1 INTRODUCTION

The two-spotted spider mite (TSSM) *Tetranychus urticae* Koch (Acariformes, Tetranychidae) is a global and economically important pest on various crops, both in the field and in the greenhouse.[1,2] The phytoseiid mites *Phytoseiulus persimilis* Athias-Henriot and *Neoseiulus californicus* (McGregor) (Parasitiformes, Phytoseiidae) have been successfully used for decades in biological control programs against TSSM infestations.[3–5] However, TSSM biocontrol is unsatisfactory on some crops because of the intrinsic characteristics of the host plant species (e.g., tomato), or environmental conditions (e.g., low relative humidity),[6–8] forcing growers to still rely on chemical control. Synthetic acaricides have represented an appropriate control technique for phytophagous mites for decades.[9,10] However, rapid evolution of resistance to acaricides in spider mites has also been recorded.[11,12] In addition, acaricides also have detrimental effects on native phytoseiid populations inhabiting cultivated plants, causing major problems in integrated pest management (IPM) strategies,[13–15] and leading researchers to focus their interest on botanical-based products.[16–18]

Botanical pesticides were commonly used until World War II, but their role became marginal following the discovery of the synthetic pesticides.[19] The former have no or less-negative effects on human health and the environment, as well as on natural enemies, and show low persistence.[20,21] Among botanical products,

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essential oils (EOs) and their bioactive constituents have been re-evaluated in past years as promising pesticides.[22–24] EOs biocidal effects have been studied on many important arthropod pests such as tephritids,[25–31] ambrosia beetles,[32] mites, ticks[17,33–36], and on weeds.[37,38] Moreover, the high worldwide production of plant EOs for perfume and flavoring, makes them suitable from a commercial point of view.[39,40]

Most studies on botanical pesticides focus on plant species belonging to the Apiaceae, Myrtaceae and Lamiaceae families. However, Asteraceae have also received considerable attention.[41–44] The stemless carline thistle *Carlina acaulis* L., belongs to the latter family and is spread in xerothermic grasslands of Central and South Europe.[45] The plant was well known by ancient Greeks and Romans for its anthelmintic, antibiotic, dermatological and diuretic effects, and has been continuously included in the European Pharmacopoeia for more than two millennia.[46] Different attempts have recently been made to cultivate this plant on a large scale, with specific treatments to stimulate production of the active compounds in the plant.[47] The main constituent of the EO (>90%) is the polyacetylene carlina oxide [2-(3-phenylprop-1-ynyl) furan][48,49] and this has proven to be toxic on insect pests like *Lobesia botrana* (Denis & Schiffermüller),[50] *Ceratitis capitata* (Wiedemann)[29,30] and *Bactrocera oleae* (Rossi)[28] and on nematodes such as *Meloidogyne incognita* (Kof. & White).[51] Carlina oxide has potential as an ideal candidate ingredient in formulations for use in crop protection within the framework of sustainable agriculture aimed at reducing the risks from the use of conventional pesticides and to promote alternative strategies in IPM programs. *Carlina acaulis* and its EO are included in the list of botanicals to be used in food supplements shared with the health administrations of Belgium, France and Italy (BELFRIT) and this may facilitate biopesticide registration bypassing regulatory restrictions.[52]

The scientific community has recently begun to focus on the potentially hazardous effects of natural products on non-target organisms such as predatory mites, aquatic organisms and earthworms.[53–55] However, literature on the effects of botanical pesticides on non-target organism behavior is still poor.[53,56] To the best of our knowledge, little is known on the behavioral responses of predatory mites to carlina oxide.

Within this framework, this study aimed to evaluate the effects of carlina oxide toward *T. urticae* eggs, nymphs and adults, also adopting a population-level approach.[33,57] Moreover, because the study of side effects on natural enemies is essential when searching for biopesticides fitting with IPM principles, the toxicity of this polyacetylene was also evaluated on females and eggs of the phytoseiid predaceous mite *N. californicus*.

2 MATERIALS AND METHODS

2.1 Carlina oxide isolation

Carlina oxide was isolated as a yellow liquid from 1 kg of dry *C. acaulis* roots by hydrodistillation using 10 L of distilled water and was recovered through a Clevenger-type apparatus for 8 h (yield 0.78% dry weight). The plant material was bought from Minardi & Figli S.r.l. (Bagnacavallo, Ravenna, Italy; https://www.minardierbe.it; batch no C-210920250920, collected in 2020). The purity of the compound (98.7%, Fig. 1) was assessed by gas chromatography–mass spectrometry (GC–MS) analysis using a previously validated method,[58] and the chemical structure was checked by MS and NMR data using a standard previously obtained in the authors' laboratory.[59] Once obtained, carlina oxide was stored at −20 °C until biological assays.

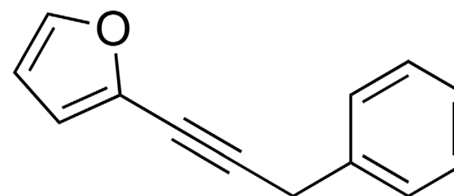

Figure 1. Chemical structure of carlina oxide.

2.2 *T. urticae* and *N. californicus* rearing

Tetranychus urticae was collected on weeds and *Solanum melongena* L., near Palermo, Italy (38°1′41.49″ N, 13°1′55.61″ E), in June 2020, and kept in laboratory cultures on potted bean plants (*Phaseolus vulgaris* L.). *Neoseiulus californicus* was collected on strawberries infested by *T. urticae* at Partinico (Palermo) (38° 4′22.21″ N, 13°6′12.27″ E) in December 2018 and reared on plexiglass arenas[60] supplying as food various stages of *T. urticae* and a mixture of pollens [*Carpobrotus edulis* (L.) N.E.Br., *Oxalis pes-caprae* L., *Typha latifolia* L.] as a food. Both rearings were maintained in a conditioned room [25 ± 1 °C, 70% ± 5% relative humidity (RH) and 16:8 h light/dark photoperiod]. The two rearings were regularly renewed or supplemented with field-collected specimens.

2.3 Toxicity of carlina oxide on *T. urticae*

Tests were carried out on eggs, nymphs and adults of *T. urticae*. The following concentrations of the carlina oxide were tested, i.e. 312.5, 625, 1250, 2500 and 5000 μL L^{-1}. Each experimental unit (EU) consisted of a bean leaf disk (3 cm in diameter) with the abaxial surface up, placed on wet cotton saturated daily with distilled water, in a Petri dish (diameter 100 mm, height 10 mm). For each EU, 8 mL of solution were sprayed using the Potter Precision Spray Tower,[61] at 6.89 kPa of pressure. Carlina oxide was dissolved in acetone. Negative control tests were treated with acetone only.

Toxicity tests on *T. urticae* were carried out following the methodology described by Tsolakis and Ragusa.[33] To obtain a cohort of TSSM eggs, three females were placed on each EU, allowed to lay eggs for 24 h and then removed. Ten eggs per EU were left for treatments. Each test was replicated 30 times and lasted 7 days (when more than 95% of eggs hatched in the control). The mean hatching time was calculated at the end of the test period. The above procedure was adopted for obtaining coetaneous (max 24 h old) protonymphs. Six replicates were adopted for each toxicity test on five nymphs per EU; the test lasted 4 days.

To obtain a cohort of coetaneous females, 50 females were transferred with a fine brush (4/0) to the abaxial surface of five bean leaves (ten females per leaf), allowed to lay eggs for 24 h and then removed. Mites obtained from these eggs were reared until reaching adulthood; afterwards 60 young females (max 24 h old) were transferred to the EUs (one female per EU + one male originating from the colony) to ensure mating. After 48 h males were removed, and fertilized females were used for tests. Each test was replicated 60 times and lasted 4 days. The mortality of nymphs and adult females was recorded daily and the mean survival time and survival rate were calculated at the end of tests. Moreover, the number of eggs/female/day was recorded over the test period. To calculate the total toxic effect of carlina oxide on female mortality and oviposition rate, the following formula

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proposed by Overmeer and van Zon[62] was applied to data obtained from each concentration:

$$E = 100\% - (100\% - M) \times R$$

Where M is corrected mortality of females at the end of the test[63] and R is the effect on the reproduction (no. of eggs per treated female/no. of eggs per untreated female).

Four toxicity categories, as proposed by Hardman *et al*.[64] were applied for *T. urticae* considering the Abbott[63] corrected mortality: 1 = not toxic (<25% mortality), 2 = slightly toxic (25%–50% mortality), 3 = moderately toxic (51%–75% mortality), and 4 = very toxic (>75% mortality).

2.4 Effect of carlina oxide on *T. urticae* population growth rate

To measure the effect of carlina oxide on the populations growth of *T. urticae*, we calculated the instantaneous rate of increase (r_i).[57,65] This index measures the population increase or decrease and is calculated adopting the following equation:

$$r_i = \frac{\ln\left(\frac{Nf}{No}\right)}{\Delta t}$$

where r_i is instantaneous rate of population increase, like that obtained with the intrinsic rate of increase (r_m),[57] *Nf* is final number of mites, *No* is initial number of mites and Δt is time that the experiment lasted. Positive values of r_i show a growing population, $r_i = 0$ indicates a stable population, and negative values of r_i indicate a declining population directed toward extinction.[57] The r_i was calculated after 4 days.

2.5 Side effects of carlina oxide on *N. californicus*

To obtain young females of *N. californicus*, 100 eggs from the colony were transferred to a new arena and provided with an abundant mixture of pollens until adulthood was reached. Newly emerged females and males were transferred with a fine brush (4/0) to a new arena for 48 h to ensure mating (sex ratio females/males 3:1). Afterwards, one female predator was transferred on each EU for subsequent tests.

Four different tests were carried out on phytoseiid females: A, spraying directly on the EUs bearing each one female (30 replications); B, spraying the leaf disk, the female was placed on the leaf disk 4 min later when the leaf disk was dry (15 replications); C, spraying the female which was then placed on untreated leaf disk (30 replications); D spraying the leaf disk, the female was placed on leaf disk 48 h later (15 replications). In all the above tests a concentration of 5000 μL L^{-1} was used, because only this caused a mortality rate of 95% on *T. urticae* females. Pollen grains were provided as food once the sprayed surface of leaf disk was dry. All tests lasted 3 days and were carried out at 25 ± 1 °C, 70% ± 5% RH and 16:8 h light/dark photoperiod.

To obtain fresh eggs of *N. californicus* for toxicity tests, 50 females were placed in a new arena and allowed to lay eggs for 24 h. Afterwards, one egg was transferred with a fine brush (4/0) to each EU and then sprayed with carlina oxide at a concentration of 1250 μL L^{-1}, which was the dose causing 95% of mortality on *T. urticae* eggs. Each test was replicated 30 times and lasted 3 days.

The mortality of motile stages was daily recorded during the test period. We considered mortality to be natural or induced death (dead mites found on leaf disks), whereas mites found drowned in the wet cotton wool surrounding the leaf disk were ascribed to a repellent effect. Egg mortality was ascertained when more than 95% of eggs hatched in the control (after 3 days).

The four toxicity categories proposed by the International Organization for Biological Control for natural enemies, like those applied for *T. urticae*, were adopted for *N. californicus* considering Abbott[63] corrected mortality: 1 = harmless (<30% mortality), 2 = slightly harmful (30%–79% mortality), 3 = moderately harmful (80%–99% mortality) and 4 = harmful (>99% mortality).[66]

2.6 Y-tube olfactometer bioassays on *N. californicus* adults

2.6.1 *Y-tube apparatus*

To evaluate the potential attraction or repellency activity of carlina oxide on adult females of *N. californicus*, two-choice tests were performed in a Y-tube olfactometer as used by Canale *et al*.[67] and described in the Supplementary Information; the method of Fonseca *et al*.[68] with slight modifications was followed. Two different behavioral assays were carried out on *N. californicus* females: A, preliminary test on filter paper (50 replicates/dose); and B, spraying on a bean leaf disk and performing the test 15 min and 48 h after spraying (30 replicates/dose). The tested doses were 2500 and 5000 μL L^{-1}, the two highest doses tested on *T. urticae* females. All the bioassays were conducted under laboratory conditions (25 ± 1 °C, 70% ± 5% RH). Purified air was provided at 2 mL min^{-1} for both behavioral assays. Illumination was provided by a red LED light (12 W, 1050 lm) hanging vertically above the olfactometer unit (height 60 cm). In both behavioral assays, either the filter paper or the bean leaf were moistened with 10 μL of the product to be tested and the respective control. To avoid any bias, after testing five females the arena was flipped by 180° and cleaned as detailed in Carpita *et al*.,[69] the substances were then renewed.

2.6.2 *Preliminary bioassay*

Two doses (2500 and 5000 μL L^{-1}) of carlina oxide were formulated with Tween 80 (3%) (1:1) plus distilled water and applied to a filter paper (20 × 20 mm, Whatman 1). Negative controls (the solution without carlina oxide) were also prepared. Females of *N. californicus* were introduced individually in the main arm of the olfactometer and each was allowed to choose between the two arms. A choice was recorded when the female reached the end of one of the two arms. After making a choice, the female was removed and a new female was introduced. Each observation lasted 5 min; females that did not move at all within 3 min were removed and scored as no choice. A total of 50 adult females of *N. californicus* were tested at the two carlina oxide concentrations.

2.6.3 *Carlina oxide bioactivity over time*

The repellent or attractive effect of carlina oxide was also evaluated over time in Y-tube bioassays, following the method described above. Fresh bean leaf disks (20 mm in diameter) were used to evaluate how the attractive or repellent activity of carlina oxide toward *N. californicus* females changed over time. Leaf disks were sprayed with 10 μL of carlina oxide solution and the respective negative control using an airbrush, and were air-dried for 1 h. The spraying apparatus consisted of a compressor (FD-186 Piston Type 125 W Air Compressor, Fengda, Zhejiang, China) set at a pressure of 0.17 bar and an airbrush (Badger Air Brush 200-9-GFX, Chicago, IL, USA) positioned perpendicularly at a height of 30 cm from the sample using an adjustable metal stand

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(Frasconi *et al.*, unpublished data). The bioactivity of both doses was evaluated at 0 h (t0) and 48 h (t1) after the spraying treatment; at the end of the first bioassay, leaf disks were kept in the fridge for 48 h. A total of 30 adult females of *N. californicus* were tested for carlina oxide concentrations.

2.7 Statistical analyses

Binary logistic regression (BLR) was adopted to compare data on the mortality of *T. urticae* females and nymphs as well as on the mortality and repellence of *N. californicus* females. Data on egg mortality, mean oviposition rate and mean hatching time of *T. urticae* eggs and *N. californicus* females were transformed using an arcsine-square-root equation prior to generalized linear modeling (GLM) analysis. Abbott's formula[63] was used to correct mortality data when control mortality was recorded. When significant differences were found, the means were separated using Tukey's HSD test ($P = 0.05$). For instantaneous rate of increase and total toxic effect data, the jackknife method was used to create pseudo values,[70] considering that sample distributions on the various tests were unknown. Goodness-of-fit tests to ascertain the normality of distribution, and analyses of variance followed by Student's *t*-tests, were performed on these values. Differences were considered significant when 95% of fiducial limits did not overlap. Lethal concentrations necessary for 10% (LC_{10}), 30% (LC_{30}), 50% (LC_{50}) and 90% (LC_{90}) mortality were calculated using the probit model implemented in the Minitab program, adopting a 95% confidence level. All analyses were computed using the Minitab 17.0 software (Minitab Inc., State College, PA, USA).

Concerning behavioral assays on *N. californicus*, a contingency analysis was carried out to analyze the biological activity of carlina oxide toward *N. californicus* adults. If Pearson's χ^2 test was significant, a residual analysis was also carried out to determine which category (choices) majorly contributed to rejecting the null hypothesis. All statistical analyses on behavioral tests were done using RStudio software; $P = 0.05$ was set as the significance threshold.

3 RESULTS

3.1 Toxicity of carlina oxide on *T. urticae*

The BLR on survival rates of TSSM, showed significant differences among the various concentrations tested ($\chi^2 = 227.71$; $P < 0.001$) and the two stages ($\chi^2 = 14.63$; $P < 0.001$). No interactions between concentration and stage were noted ($\chi^2 = 0.72$; $P = 0.397$), indicating that each concentration had the same effect on females and nymphs (Table 1).

The highest concentration (5000 μL L^{-1}) killed all females within the first 2 days, whereas mortality with 2500 μL L^{-1} reached similar values after 4 days. Less toxic effects were shown by the other three concentrations, with scalar mortality over time. Analyzing data regarding the two stages together, three main groups were found: (i) control, (ii) 625 and 1250 μL L^{-1}, and (iii) 2500 and 5000 μL L^{-1}, being a concentration of 312.5 μL L^{-1} like both the first and second group ($P < 0.05$). All the dead motile stages were found on the leaf disk; no mites were found on the wet cotton wool surrounding the leaf disk, indicating the absence of a repellent effect of carlina oxide toward *T. urticae*. GLM analysis performed on mean survival time (with the carlina oxide concentration and survival rate at 24, 48, 72 and 96 h included as factors), showed significant differences in the survival trend among concentrations ($F_{15,1439} = 3.5$; $P < 0.001$); that is, TSSM death occurred at a different time regardless of the effect recorded for each concentration at the end of the test.

As regards the mean survival time of motile stages, we found significant differences among the tested carlina oxide concentrations ($F_{5,539} = 97.76$; $P < 0.001$) and the two stages ($F_{1,539} = 6.62$;

Table 1. Susceptibility of females and nymphs of *Tetranychus urticae* to different concentrations of carlina oxide

Ontogenetic stage	Carlina oxide concentration (μL L^{-1})	Survival/day (%, mean ± SE)				Survival time (days)	Overall mortality	Fitted probability of mortality	Toxicity classes*
		Day 1	Day 2	Day 3	Day 4	mean ± SE	(%)	(95% CI)	
								$Y' = -1.177 + 0.001232 \times \text{dose}$	
Female	Control	100.0 ± 0.0	98.3 ± 1.67	96.7 ± 2.34	85.0 ± 4.65	3.80 ± 0.070^{a}	15.0 ± 4.65	23.56 (18.2–29.9)	
	312.5	90.0 ± 3.91	80.0 ± 5.21	75.0 ± 5.64	68.3 ± 6.06	3.13 ± 0.185^{abc}	31.7 ± 6.06	31.16 (24.4–37.5)	1
	625	86.7 ± 4.43	81.7 ± 5.04	68.3 ± 6.06	48.3 ± 6.51	2.85 ± 0.184^{bc}	51.7 ± 6.51	39.96 (33.9–46.3)	2
	1250	83.3 ± 4.85	75.0 ± 5.64	56.7 ± 6.45	48.3 ± 6.51	2.63 ± 0.200^{c}	51.7 ± 6.51	58.97 (52.3–65.4)	2
	2500	61.7 ± 6.33	26.7 ± 5.76	11.7 ± 4.18	10.0 ± 3.90	1.10 ± 0.159^{d}	90.0 ± 3.91	87.02 (80.7–91.5)	3
	5000	38.3 ± 6.33	0.0	0.0	0.0	0.38 ± 0.063^{d}	100.0 ± 0.0	99.32 (98.1–99.8)	4
								$Y' = -2.073 + 0.001232 \times \text{dose}$	
Nymph	Control	100.0 ± 0.0	96.7 ± 3.33	90.0 ± 4.47	90.0 ± 4.47	3.78 ± 0.133^{ab}	10.0 ± 4.47	11.17 (7.2–16.9)	
	312.5	100.0 ± 0.0	100.0 ± 0.0	93.3 ± 4.22	86.7 ± 6.67	3.80 ± 0.101^{ab}	13.3 ± 6.67	15.59 (10.6–22.4)	1
	625	93.3 ± 4.22	90.0 ± 4.47	76.7 ± 8.03	73.3 ± 6.67	3.33 ± 0.216^{abc}	26.7 ± 6.67	21.36 (15.2–29.1)	1
	1250	80.0 ± 5.16	73.3 ± 9.89	66.7 ± 13.3	66.7 ± 13.3	2.90 ± 0.301^{abc}	33.3 ± 13.3	36.97 (28.4–46.4)	2
	2500	46.7 ± 11.2	36.7 ± 8.03	26.7 ± 6.67	16.7 ± 3.33	1.27 ± 0.291^{d}	83.3 ± 3.33	73.23 (62.4–81.9)	4
	5000	16.7 ± 6.15	16.7 ± 6.15	10.0 ± 4.47	10.0 ± 4.47	0.53 ± 0.234^{d}	90.0 ± 4.47	98.35 (95.6–99.4)	4

Different letters within the survival time column, show significant differences (analysis of variance followed by Tukey pairwise comparisons $P < 0.05$). Binary logistic regression (BLR) was performed on mortality data. $P_{(0)} = \exp(Y')/(1 + \exp(Y'))$ was used for probability of mortality after definition of Y' for females and nymphs.
*Calculated on corrected mortality.[63]

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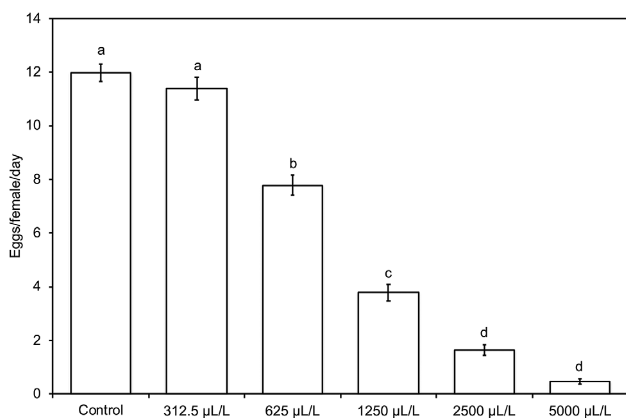

Figure 2. Oviposition rate (mean ± SE) of *Tetranychus urticae* exposed to different concentrations of carlina oxide. Above each column, different letters indicate significant differences (generalized linear model followed by Tukey's HSD test, $P < 0.05$).

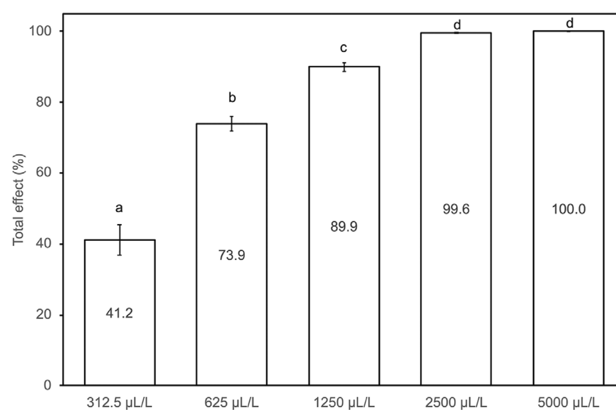

Figure 3. Total toxic effect (E) (mortality and reduction of fertility) of carlina oxide on *Tetranychus urticae* females. Different letters above columns indicate significant differences (one-way analysis of variance followed by Tukey's HSD test, $P < 0.05$).

$P < 0.001$), although no interaction of concentrations × stage was found ($F_{5,539} = 0.88$; $P = 0.497$). Tukey's HSD test showed no significant effect of the lowest concentration (312.5 µL L^{-1}) on females and of the lower three concentrations (312.5, 625 and 1250 µL L^{-1}) on nymphs, compared with control. However, the two higher concentrations (2500 and 5000 µL L^{-1}) significantly reduced the mean survival time of both motile stages (Table 1). The highest concentration of carlina oxide (5000 µL L^{-1}) showed very toxic effects (class 4), after 4 days, toward females, but was moderately toxic (class 3) toward *T. urticae* juveniles. Concentrations of 2500 and 1250 µL L^{-1} showed the same trend for females and nymphs (classes 3 and 2, respectively). By contrast, the two lowest concentrations (625 and 312.5 µL L^{-1}) showed no toxic effect for juveniles but were slightly toxic for *T. urticae* females.

The different concentrations of carlina oxide also showed a secondary effect on the oviposition performance of treated TSSM females (Fig. 2). In fact, the tested concentrations significantly reduced the mite daily oviposition rate ($F_{5,872} = 105.63$; $P < 0.001$) and this was obviously reflected on the total toxic effect (*E*) of the various concentrations (Fig. 3). All concentrations showed significant negative effects toward TSSM eggs ($F_{5,179} = 455.0$; $P < 0.001$) (Table 2). Slightly toxic effects were found with 312.5 µL L^{-1} (22% mortality), whereas the remaining concentrations induced mortality rates of 83.7% to 100% (Table 2). Also, the mean hatching time was significantly affected by carlina oxide in a concentration-dependent manner ($F_{4,609} = 57.83$; $P < 0.001$) (Table 2).

Carlina oxide LC_{50} was calculated by probit analysis for all *T. urticae* stages. Female and nymph mortalities fitted well with both the normal and Weibull distributions, whereas egg mortality did not fit a linear model. We adopted the Weibull distribution to define percentiles for the two motile stages and the normal distribution for eggs. The LC_{50} values obtained for *T. urticae* females and nymphs were 1145.1 and 1825.0 µL L^{-1}, respectively (Table 3), showing that females were more susceptible than nymphs ($z = -2.53$, $P = 0.012$). As regards eggs, the LC detailed in Table 3 should be considered indicative because the goodness-of-fit test (Pearson) showed a lack of fit to the linear model of the probit analysis ($\chi^2 = 84.0$, $P < 0.001$).

3.2 Effect of carlina oxide on *T. urticae* population growth rate

The different carlina oxide concentrations significantly influenced the instantaneous rate of population growth ($F_{5,359} = 752.69$; $P < 0.001$). In particular, the growth rate calculated for the lowest concentration (312 µL L^{-1}) did not differ from the control ($r_i = 0.847$ and 0.955, respectively). A positive growth index was also obtained with the 625 µL L^{-1} concentration ($r_i = 0.343$), even if it was statistically different from the lowest concentration.

Table 2. Susceptibility of *Tetranychus urticae* eggs to different concentrations of carlina oxide

Carlina oxide concentration (µL L^{-1})	Cumulative percentage of hatched eggs (mean ± SE) — Elapsed days after egg laying: 4	5	6	7	Hatching time (days) (mean ± SE)	Mortality (%)
Control	93.7 ± 1.55	97.7 ± 0.92	98.3 ± 0.84	100.0 ± 0.0	4.10 ± 0.026^{a}	0.0^{a}
312.5	55.7 ± 4.69	71.3 ± 3.77	76.7 ± 3.44	78.0 ± 2.89	4.39 ± 0.044^{b}	22.0 ± 2.89^{b}
625	2.7 ± 1.43	13.3 ± 2.81	16.3 ± 3.13	16.3 ± 3.13	5.02 ± 0.085^{c}	83.7 ± 3.13^{c}
1250	0.0	2.0 ± 0.88	4.3 ± 1.14	4.3 ± 1.14	5.60 ± 0.131^{d}	95.7 ± 1.14^{d}
2500	0.0	1.0 ± 0.56	2.3 ± 0.92	2.3 ± 0.92	5.57 ± 0.202^{cd}	97.7 ± 0.92^{de}
5000	0.0	0.0	0.0	0.0	0.0^{e}	100.0 ± 0.0^{e}

Different letters within a column show significant differences (one-way analysis of variance followed by Tukey pairwise comparisons, $P < 0.05$).

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Table 3. Lethal concentrations (LC) of carlina oxide against different ontogenetic stages of *Tetranychus urticae*

Ontogenetic stage	LC_{10} μL L^{-1} (95% CI)	LC_{30} μL L^{-1} (95% CI)	LC_{50} μL L^{-1} (95% CI)	LC_{90} μL L^{-1} (95% CI)	LC_{95} μL L^{-1} (95% CI)	Intercept ± SE	Slope ± SE	χ^2 (d.f.)
Female	181.5 (116.4–254.0)	560.7 (415.5–708.4)	1145.1 (918.1–1395.1)	5137.4 (3880.2–7516.7)	7434.5 (5330.9–11 893.5)	3.2447 ± 0.045	6.21436 ± 0.601	7.85 (7) $P = 0.346$ ns*
Nymph	256.1 (162.2–366.8)	852.3 (604.5–1152.1)	1825.0 (1333.6–2520.9)	9042.3 (5848.0–16 332.0)	13 409.4 (8195.0–26 771.3)	3.4594 ± 0.075		
Egg	196.2 (170.8–220.1)	313.8 (286.1–339.6)	434.3 (405.5–462.6)	961.3 (887.2–1055.6)	1204.2 (1093.5–1351.0)	−9.79632 ± 0.560	3.7138 ± 0.203	84.03 (3) $P < 0.001$

*Not significant.

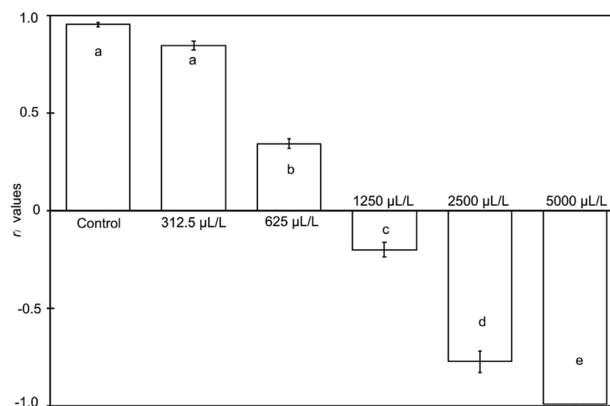

Figure 4. Instantaneous rate of increase values estimated for *Tetranychus urticae* mites exposed for different concentrations carlina oxide. Different letters within columns denote significant differences among tests (generalized linear model followed by Tukey's HSD test, $P < 0.05$).

By contrast, negative values were obtained with the other three concentrations ($r_i = -0.202, -0.775$ and -0.991 for the 1250, 2500 and 5000 μL L^{-1}, respectively), showing a decreasing trend in population toward extinction (Fig. 4).

3.3 Side effects of carlina oxide on *N. californicus*

Side effects of carlina oxide on *N. californicus* females are reported in Table 4. Mortality in the four tests was low and not statistically different from the control ($\chi^2 = 0.00$; $P = 0.993$), whereas a different repellent effect was recorded among tests ($\chi^2 = 12.63$; $P < 0.001$). The oviposition rate of *N. californicus* in test D (females placed 48 h after the treatment) was the same as that recorded in the control, whereas it was significantly lower in the other tests ($F_{4,355} = 53.57$; $P < 0.001$).

The toxic effect (mortality) on *N. californicus* females occurred entirely in the first day, and the four tests were not significantly different from control showing a harmless effect (class 1). However, a strong repellent effect was recorded both when females were directly sprayed and remained on the sprayed leaf disk, and when unsprayed females were placed on freshly sprayed leaf disk (tests A and B, Table 4). In these tests, females trying to escape the treated leaf disk, drowned on the wet cotton wool surrounding the leaf disk. A significant repellent effect was also recorded when treated females were put on untreated leaf disks (test C, Table 4). It should be mentioned that the repellent effect of carlina oxide lasted for about 2 days. In fact, when unsprayed females were put on treated leaf disks 2 days after spraying (test D, Table 4), only a slight repellent effect was recorded within the first day ($P < 0.05$) and the oviposition rate did not differ from the control.

Carlina oxide caused no side effects on phytoseiid eggs (100% hatching) ($F_{1,166} = 0.57$; $P = 0.453$) (Table 5). However, the highest hatching percentages were recorded on the second day in the control (93.4%), and the third day in treated eggs (70.0%), indicating a significant slowdown in egg hatching ($F_{2,166} = 57.4$; $P < 0.001$) (Table 5).

3.4 Y-tube olfactometer bioassays on *N. californicus* adults

3.4.1 *Preliminary bioassays*

The contingency analysis highlighted a significant difference among the mite choices made at different carlina oxide concentrations ($\chi^2 = 46.192$, d.f. $= 2$, $P < 0.001$) (Fig. 5).

The residual analysis indicates a correlation between the escape of *N. californicus* females and the highest dose, which indicates a

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Table 4. Side effects of carlina oxide (5000 μL L^{-1}) on *Neoseiulus californicus* females in the four different tests and in the control

Test	No.	Daily mortality (%) (mean ± SE) 1	2	3	Toxicity classes*	Daily repellence (%) (mean ± SE) 1	2	3	Eggs/ female/ day	Total negative effect (%)
A. Spraying on a female placed on the leaf disk	30	10.0 ± 5.57	—	—	1	90.0 ± 5.57^{a}	—	—	0.00^{a}	100.0^{a}
B. Spraying on the leaf disk; the female was placed on leaf disk 4 min later	15	6.7 ± 6.67^{a}	0.0	—	1	80.0 ± 10.7^{a}	13.3 ± 9.09^{a}	—	0.02^{a}	96.7^{a}
C. Spraying on a female which was then moved on an untreated leaf disk	30	6.7 ± 4.63^{a}	0.0	0.0.	1	23.3 ± 7.85^{b}	30.0 ± 8.51^{a}	0.0	0.09^{a}	86.8^{b}
D. Spraying on the leaf disk; the female was placed on leaf disk after 48 h	15	0.0^{a}	0.0	0.0	1	13.3 ± 9.09^{b}	0.0^{b}	0.0	0.62^{b}	1.6^{c}
Control	30	6.7 ± 4.63^{a}	0.0	0.0	1	0.0^{c}	0.0^{b}	0.0	0.63^{b}	

Different letters denote significant differences among tests for oviposition rate and total negative effect (one-way analysis of variance followed by Tukey pairwise comparisons for $P < 0.05$).
*Calculated on corrected mortality.[63]

repellent activity of the compound at 5000 μL L^{-1}. Indeed, 58% of predatory mites responded by escaping immediately after the release.

By contrast, there was a positive correlation between the choice of the control arm and the lowest dose (2500 μL L^{-1}).

3.4.2 *Carlina oxide bioactivity over time*

Investigating how the biological activity of carlina oxide changed in relation to a different substrate (the fresh bean leaf) and over time, the previously obtained results were partially corroborated. As in preliminary bioassays, a significant difference was found between the highest tested dose and the choice made by *N. californicus*, immediately after the leaf was sprayed (t0) ($\chi^2 = 24.649$, d.f. = 2, $P < 0.0001$). Residual analysis highlighted only an association between mite response and treatment (5000 μL L^{-1}), which translates to a greater preference for escape (non-random) than for the control or the treated arm.

At 2500 μL L^{-1} of carlina oxide, the repellent action found in the preliminary results may be mitigated by the volatile compounds released by the leaf (Fig. 6). However, analyzing data after 48 h (t1), no significant differences were found between the dose and the choice made by the mite ($\chi^2 = 0.7213$, d.f. = 2, $P = 0.6972$), meaning that carlina oxide no longer exerted a repellent effect on the predatory mite (Fig. 7).

Y-Tube results showed a significant repellent activity of the highest dose of carlina oxide at the time of application on both fresh bean leaf and filter paper. However, the repellent activity was reduced or even disappeared after 48 h.

Table 5. Side effects of carlina oxide on eggs of *Neoseiulus californicus*

Treatment	Daily percentage of hatched eggs (mean ± SE) 1	2	3	Hatching (%) (mean ± SE)
1250 μL L^{-1}	13.3 ± 6.31^{a}	16.67 ± 6.92^{a}	70.0 ± 4.66^{a}	100.0
Control	3.3 ± 3.33^{b}	93.4 ± 4.63^{b}	3.3 ± 3.33^{b}	100.0

Within a column, different letters indicate significant differences among values (generalized linear model followed by Tukey pairwise comparisons, $P < 0.05$).

4 DISCUSSION

Research for eco-compatible methods to control insect or mite infestations on crops are desirable in modern agriculture to achieve four main goals: (i) provide viable alternatives to synthetic

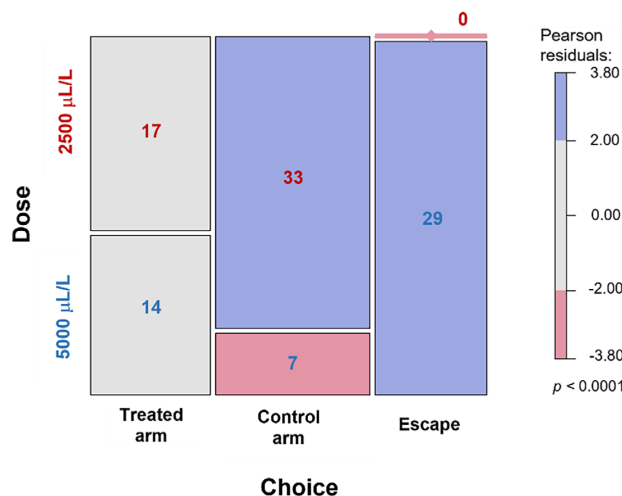

Figure 5. Contingency analysis on the dose of carlina oxide sprayed on filter paper and *Neoseiulus californicus* female choices in Y-tube olfactometer assays (based on a Pearson chi-squared test of independence, *P*-value reported in the plot). The area of each tile is proportional to the count of choices made by the predatory mite. The shading of the cells refers to the sign and magnitude of the respective Pearson residuals. The number of observations per cell is presented and each value is color-coded according to the dose ($P < 0.05$).

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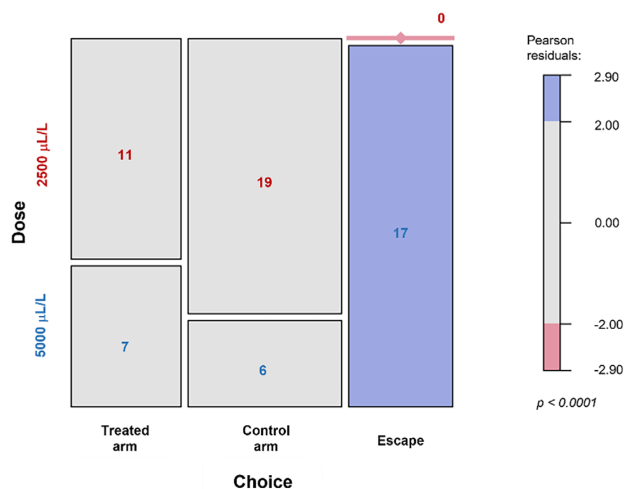

Figure 6. Contingency analysis on the dose of carlina oxide just sprayed (t0) on fresh bean leaves and *Neoseiulus californicus* female choices in Y-tube olfactometer assays (based on a Pearson chi-squared test of independence, *P*-value reported in the plot). The area of each tile is proportional to the count of choices made by the predatory mite. The shading of the cells refers to the sign and magnitude of the respective Pearson residuals. The number of observations per cell is presented and each value is color-coded according to the dose ($P < 0.05$).

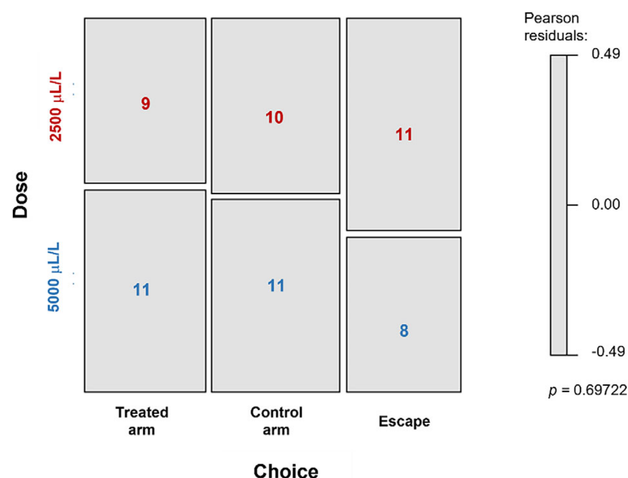

Figure 7. Contingency analysis on the dose of carlina oxide sprayed 48 h before (t1) on fresh bean leaves and *Neoseiulus californicus* female choices in Y-tube olfactometer assays (based on a Pearson chi-squared test of independence, *P*-value reported in the plot). The area of each tile is proportional to the count of choices made by the predatory mite. The shading of the cells refers to the sign and magnitude of the respective Pearson residuals. The number of observations per cell is presented and each value is color-coded according to the dose ($P < 0.05$).

chemicals, (ii) reduce environmental pollution, (iii) reduce chemical residues in the agri-food chain, and (iv) tackle the thorny problem of resistant strains induced by the continuous use of synthetic pesticides. Moreover, the use of acaricides that are selective for natural enemies is particularly useful in IPM strategies.[6,71]

Several studies have investigated the acaricidal activity, repellence and life-time parameters of different EOs and their bioactive constituents against *T. urticae*[23,72–76] and, to a lesser extent, the side effects on predatory mites.[33,73,75,77] To the best of our knowledge, this work is the first to study the toxic effects of carlina oxide on *T. urticae*, as well its side effects on the important biocontrol phytoseiid *N. californicus*.

Carlina oxide showed greater toxicity against females than nymphs of *T. urticae* (LC_{50} = 1145.1 and 1825 µL L^{-1} for females and nymphs, respectively), but the effects on mean survival time were the same for both stages, indicating a similar action against all mobile stages.

Carlina oxide can be considered the chemical main responsible for the plant insecticidal and acaricidal activity previously reported for *C. acaulis*.[29,58,59] The triple bond of the propynyl chain in the molecule produces radicals generating oxidative damage.[78] This can be boosted under ultraviolet light.[30] Carlina oxide may also interact with the insect γ-aminobutyric acid (GABA) receptor.[78] Further studies on its mechanism of action are needed.

The toxic effects of other Asteraceae species have also been reported on *T. urticae* females. Chiasson *et al.*[17] reported the lethal effects of *Artemisia absinthium* L. and *Tanacetum vulgare* L. EOs on tetranychid females, adopting 16-fold higher concentrations (80 000 µL L^{-1}) in comparison with the highest (5000 µL L^{-1}) used in our experiments. In addition, carlina oxide showed 26-fold more toxicity compared with *Lippia gracilis* Schauer (Verbenaceae) EO, 34-fold more toxicity than azadirachtin, and 5-fold more toxicity than the synthetic acaricide fenpyroximate.[79]

In addition to toxicity, a negative effect on the daily oviposition rate by all but the lowest concentration was also noted. However, toxic effects recorded on treated eggs from doses as low as 625 µL L^{-1} indicate ovicidal activity of carlina oxide, which was confirmed by probit analysis estimating lethal concentrations. The lack of fit to a linear model was due to the high mortality (>80%) observed by four of the five concentrations of carlina oxide (625–5000 µL L^{-1}). These data indicate that small concentration increments between 312.5 and 625 µL L^{-1} result in a large increment in eggs mortality. Three days after treatment, *T. urticae* eggs were empty, with a dry residue inside the chorion, indicating arrest of the embryo development. Over time, the negative impact of carlina oxide on *T. urticae* females was reflected in the population growth rate. The negative values recorded with the three highest doses showed a combined effect of toxicity and decreased female fecundity, as demonstrated by the total effect of carlina oxide on females (Fig. 3). In contrast to various EOs and their major bioactives,[33,80,81] no repellent effects on *T. urticae* have been observed for carlina oxide, indicating the absence of disturbing olfactory cues for all mobile stages. Otherwise, carlina oxide elicited a strong repellent effect on *N. californicus*, but no toxic effect was observed against phytoseiid females. The different behavioral responses of the two mite species exposed to the same compound dose could be linked to their chemoreceptor types. Recently, Su *et al.*[82] reported that predatory mites, like *N. cucumeris*, rely mostly on gustatory receptors and ionotropic receptors for chemosensation. Ionotropic receptors represent the largest class of chemoreceptors in Acari and are particularly abundant in phytoseiid mites, with approximately 60 homologs, ten times those in spider mites.[82] Thus, the abundance of chemoreceptor genes in predatory mites may imply a different chemosensory ability.[82] The repellent effect, as also confirmed by our behavioral tests, vanishes after 48 h, allowing the predatory mite to again colonize the treated area.

Carlina oxide was shown to be harmless to *N. californicus* eggs, but all concentrations of the compound caused a significant delay in predator egg hatching. This has also been observed on tetranychid eggs. The harmless effects on predator eggs in comparison with the high toxicity toward the *T. urticae* eggs could be due to

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the different exposure time as also seen for *P. persimilis* eggs treated with caraway EO.[33] In fact, *N. californicus* eggs hatch within 2 days, whereas tetranychid eggs hatch after 4–5 days; this longer period allows the active ingredient to penetrate the chorion and block embryonic development. The significative delay in eggs hatching recorded for *N. californicus* eggs, strengthens this hypothesis.

5 CONCLUSION

Carlina oxide was shown to be a new candidate ingredient for the development of sustainable products for controlling *T. urticae* infestations. Because cultivation of *C. acaulis* has been scarcely developed so far, the supply chain of this compound relies only on spontaneous plant populations. Thus, to boost the scalability of carlina oxide-based insecticidal formulations, several cropping systems should be carried out, especially in marginal areas, in addition to possible sustainable synthetic routes for the production of this polyacetylene[83] by agrochemical companies. At the same time, new advanced extraction techniques should be pursued to increase the yield of the active ingredient, as well as novel formulations to boost its bioactivity over time.[52,84] Our findings show that carlina oxide reduces the longevity and fecundity of *T. urticae* adults. However, to predict the fate of a treated population over several generations, it is necessary to consider other important aspects, especially the development of resistance to these botanical products. Obviously, a reiterated use of the same compound increases the possibility of resistance development in *T. urticae*. Also, semi-field and field studies aiming to evaluate the efficacy of carlina oxide are needed for practical implementation under field and greenhouse conditions. Furthermore, the low negative impact on *N. californicus* makes this polyacetylene a good candidate in IPM programs, despite its repellent effects that, however, last for a very short period.

AUTHOR CONTRIBUTIONS

R Rizzo: *conceptualization, methodology, investigation, validation, visualization, writing – original draft preparation, reviewing and editing*. E Ragusa: *investigation, data curation, reviewing and editing*. G Benelli: *conceptualization, methodology, resources, validation, formal analysis, supervision, writing – original draft preparation, reviewing and editing*. G Lo Verde: *conceptualization, methodology, validation, formal analysis, visualization, writing – original draft preparation, reviewing and editing*. V Zeni: *investigation, formal analysis, data curation, writing – original draft preparation, reviewing and editing*. F Maggi: *resources, writing – original draft preparation, reviewing and editing*. R Petrelli: investigation, reviewing and editing. E Spinozzi: investigation, reviewing and editing. M Ferrati: investigation, reviewing and editing. M Sinacori: investigation, reviewing and editing. H Tsolakis: supervision, conceptualization, methodology, validation, formal analysis, visualization, writing – original draft preparation, reviewing and editing.

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CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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