Supplementary Material

Coumarin (2H-1-benzopyran-2-one): a novel and eco-friendly aphicide

Abstract

Coumarin (2H-1-benzopyran-2-one) is a phenolic compound derived from the shikimate pathway and synthesized by various medicinal and aromatic plants as parent molecule of a large group of secondary metabolites, namely coumarins. Its main utilization is as fixative in perfumes and flavour enhancer. Given its role as phytoalexin and phagodepression activity, herein we evaluated for the first time its efficacy against several insect species: the green peach aphid, *Myzus persicae*, the moth *Spodoptera littoralis*, the housefly, *Musca domestica* and the filariasis vector *Culex quinquefasciatus*. Two non-target species were also included in our toxicity evaluation experiments: the ladybug *Harmonia axyridis* and the earthworm *Eisenia fetida*. Results highlighted remarkable selectivity of coumarin, being highly toxic to *M. persicae* aphids ($LC_{50(90)}$ values of 1.3(1.9) mg L^{-1}) and friendly to natural enemies of aphids as well as soil invertebrates.

Keywords: aphid; earthworm; insecticide; ladybug; mosquito; moth; non-target species

3. Experimental

3.1. Reagents

HPLC grade coumarin (99%) was purchased from Sigma-Aldrich (Milan, Italy).

3.2. Insect and earthworm rearing

The insect pest species tested in this study, i.e., *Culex quinquefasciatus* larvae, *Musca domestica* adults, *Myzus persicae* adults, and *Spodoptera littoralis* larvae, as well as non-target earthworms, *Eisenia fetida*, were reared following exactly the method recently reported by Benelli et al. (2019a). Non-target ladybugs (*Harmonia axyridis* larvae and adults) were reared as described by Benelli et al. (2019b). All invertebrates were obtained from an established laboratory colony (>20 generations; Crop Research Institute, Czech Republic) and maintained at 25±1 °C, 70±3% R.H. and 16:8 h (L:D).

3.3. Toxicity against mosquito larvae and adults

The acute toxicity of coumarin diluted in dimethyl sulfoxide (DMSO) on *C. quinquefasciatus* 3rd instar larvae was evaluated using the WHO method (WHO 1996) with slight adjustments as described by Pavela et al. (2017). Coumarin tested concentrations were 10, 25, 50, 100 and 150 mg L^{-1}; each concentration was replicated four times. The control was distilled water with the same amount of DMSO used for dissolving coumarin. Larval mortality was recorded after 24 h.

Moreover, the tarsal contact test was performed to evaluate the acute toxicity of coumarin to *C. quinquefasciatus* adult females. The test was done using the method by WHO (1996) with minor modifications by Benelli et al. (2019c). Coumarin was diluted with acetone (2 mL, Sigma-Aldrich, Germany) plus silicon oil (3.6 mg cm^{-2}). The blend was applied to filter paper (Whatman no. 1, size: 12×15 cm, Fisher Scientific, Czech Republic). Coumarin was tested at 500 µg cm^{-2} as the maximum dose. After drying (24 h at 25 °C), treated filter paper pieces were inserted into test tubes and 20 females (2-5 day-old) were exposed for 1 h; subsequently, the mosquitoes were moved to plastic cages (20×20×20 cm, 26±1 °C 16:9 (L:D)) for 24 h. Sucrose solution was supplied *ad libitum* as food. Mortality was determined 24 h after exposure.

3.4. Toxicity against housefly adults

Topical application tests were conducted to evaluate the acute toxicity of coumarin on *M. domestica* adult females (3–6 day-old). Following Benelli et al. (2019a), 1 µL of acetone (Sigma-Aldrich, Germany) plus coumarin at the following concentrations, 10, 25, 50, 100, 150 and 200 µg $adult^{-1}$ (each concentration was tested on four groups of 20

flies each), was applied using a microelectric applicator to the pronotum of houseflies anesthetized with CO_2. Acetone alone served as control. Houseflies were moved to a recovery box (10×10×12 cm, 26±1 °C 16:9 L:D) for 24 h, then mortality was recorded (Benelli et al. 2019c).

3.5. Toxicity against aphid adults

The toxicity of coumarin to *M. persicae* aphids was assessed treating *M. persicae* adults (2-5 days old) on potted cabbage plants with 3-4 true leaves, in agreement with Stepanycheva et al. (2014). Coumarin was mixed with Tween 85 (Sigma Aldrich, Czech Republic; ratio 1:9, w:w), and tested at 2, 5, 10, 20, 30, 40 and 50 mg L^{-1}, equivalent to 0.2, 0.5, 1, 2, 3, 4 and 5 mg L^{-1} of coumarin. The blend was applied to the plants using a manual sprayer, at 50 mL m^{-2} (about 500 L ha^{-1}). Tween 85 in water at 40 mg L^{-1} was the negative control (50 mL m^{-2}). Four groups of 50 aphids each were tested for each coumarin concentration, at 25±1 °C, 70±5% R.H., and 16:8 h (L:D) photoperiod. Aphid mortality was recorded 48 h after spraying (Benelli et al. 2019c).

3.6. Toxicity against moth larvae

The toxicity of coumarin to 3rd instar larvae of the moth pest *S. littoralis* was evaluated through topical application of the compound diluted in acetone. Following Pavela et al. (2017), moth larvae were treated on the dorsum with 1 µL of acetone containing 25, 50, 100, 150 and 200 µg $larvae^{-1}$ of coumarin. Four replicates (n=20 larvae per replicate) for each concentration were done. Acetone was the negative control. Larvae were moved to a recovery box (10×10×7 cm, with thin holes to avoid fumigation effects, 26±1 °C, 70±3% R.H., and 16:8 L:D) for 24 h, then mortality was noted (Pavela et al. 2017; Benelli et al. 2019c).

3.7. Non-target toxicity assays on ladybugs and earthworms

Following the method by Benelli et al. (2019b), 3rd instar larvae and adults (3-7 days old) of the non-target ladybug *H. axyridis* were tested to assess the acute toxicity of coumarin. For larvae and adults, the tested coumarin concentrations were 1, 3 and 5 g L^{-1} while the testing procedure was as described above for *M. persicae*, since the latter is a common prey of *H. axyridis* and they share the same habitats. Only a difference is noteworthy: at variance with aphid tests, coumarin was applied to *H. axyridis* individuals in open Petri dishes (diameter 9 cm, 10 insects per replicate, 4 replicates per concentration). 50 mL of the product was applied per m^2, corresponding to about 500 L ha^{-1}. The negative control was water plus equivalent of Tween 85. Ladybug individuals

were then moved to clean Petri dishes and fed with *M. persicae* aphids (25±1°C, 70±5% R.H., and 16:8 (L:D)). Mortality was noted after 48 h.

To evaluate the toxicity of coumarin on *E. fetida* adult earthworms, the standard OECD (1984) method was followed. The composition and pH of the artificial soil used in these experiments was detailed by Benelli et al. (2019a,c). The soil was prepared by adding coumarin at concentrations 10, 50 and 100 mg kg^{-1} mg a.i. per kg of dry weight basis soil. The negative control was distilled water alone. An aqueous formulation containing coumarin or pure water was mixed in the soil (650 g), then 10 *E. foetida* adults were added. The treated and control soil samples were stored in glass pots (1 L) covered with gauze to ensure aeration. Earthworm mortality was recorded after 7 and 14 days of exposure to the treatments at 20±1 °C, R.H. 80-85 %, 16:8 (L:D) and 600 lux (Benelli et al. 2019c).

3.8. Data analysis

If control mortality <20 %, the observed mortality rates were corrected using Abbott's formula (Abbott 1925) and probit analysis was used to estimate the $LD_{50(90)}$ and $LC_{50(90)}$ values, with associated 95 % confidence limits (CL) for each treatment (Finney 1971). The obtained data were analysed using the software BioStat v5.

References

Abbott WS. 1925. A method of computing the effectiveness of an insecticide. J Econ Entomol. 18: 265–267.

Benelli G, Pavela R, Maggi F, Wandjou JGN, N' Guessan BYF, Diénéba KB, Sagratini G, Vittori S, Caprioli G. 2019a. Insecticidal activity of the essential oil and polar extracts from *Ocimum gratissimum* grown in Ivory Coast: efficacy on insect pests and vectors and impact on non-target species. Ind Crops Prod. 132: 377-385.

Benelli G, Pavela R, Petrelli R, Cappellacci L, Bartolucci F, Canale A, Maggi F. 2019b. *Origanum syriacum* subsp. *syriacum*: from an ingredient of Lebanese 'manoushe' to a source of effective and eco-friendly botanical insecticides. Ind Crops Prod. 134: 26-32.

Benelli G, Pavela R, Zorzetto C, Sánchez-Mateo CC, Santini G, Canale A, Maggi F. 2019c. Insecticidal activity of the essential oil from *Schizogyne sericea* (Asteraceae) on four insect pests and two non-target species. Entomol Gen. doi: 10.1127/entomologia/2019/0662

Finney DJ. 1971. Probit Analysis. Cambridge University, London, pp. 68–78.

Pavela R, Maggi F, Lupidi G, Cianfaglione K, Dauvergne X, Bruno M, Benelli G. 2017. Efficacy of sea fennel (*Crithmum maritimum* L., Apiaceae) essential oils against *Culex quinquefasciatus* Say and *Spodoptera littoralis* (Boisd.). Ind Crops Prod 109: 603-610.

Stepanycheva EA, Petrova MO, Chermenskaya TD, Pavela R. 2014. Prospects for the use of *Pongamia pinnata* oil-based products against the green peach aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). Psyche 2014: 705397.

Table 1 Supplementary Material. Evaluation of the toxicity of coumarin on six invertebrate species of economic importance.

Insect species	**Unit**	**LC_{50}/LD_{50}**	**CI_{95}**	**LC_{90}/LD_{90}**	**CI_{95}**	**Chi-square**	**Mortality (%) at maximum tested dose or concentration**
Insect pests and/or vectors							
Culex quinquefasciatus 3rd instar larva	mg L^{-1}	>150	-	-	-	-	42.3±2.8 % at 150 mg L^{-1}
Culex quinquefasciatus adult (tarsal test)	µg cm^{-2}	>500	-	-	-	-	0.0±0.0 % at 500 µg cm^{-2}
Musca domestica adult female	µg $adult^{-1}$	>200	-	-	-	-	23.3±5.4 % at 200 µg $adult^{-1}$
Myzus persicae adult	mg L^{-1}	1.3	1.2-1.4	1.9	1.8-2.2	0.199 n.s.	100.0±0.0 % at 5 µg $adult^{-1}$
Spodoptera littoralis 3rd instar larva	µg $larva^{-1}$	>200	-	-	-	-	0.0±0.0 % at 200 µg $larva^{-1}$
Non-target invertebrates							
Harmonia axyridis 3rd instar larva	mg L^{-1}	>5000	-	-	-	-	0.0±0.0 % at 5000 mg L^{-1}
Harmonia axyridis adult	mg L^{-1}	>5000	-	-	-	-	0.0±0.0 % at 5000 mg L^{-1}
Eisenia fetida adult	mg kg^{-1}	>100	-	-	-	-	0.0±0.0 % at 100 mg kg^{-1}

n.s. = not significant (P>0.05)