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Response of *Epilachna paenulata* to Two Flavonoids, Pinocembrin and Quercetin, in a Comparative Study

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Abstract We examined the effects of the flavonoids pinocembrin and quercetin on the feeding behavior, survival, and development of the Cucurbitaceae pest *Epilachna paenulata* (Coleoptera: Coccinellidae). In no-choice experiments, 48 hr-consumption of *Cucurbita maxima* Duch. leaves treated with pinocembrin at 1, 5, and 50 μg/cm^2 was less than one third of that for leaves treated with 0.1 μg/cm^2 of pinocembrin or untreated leaves. Larvae stopped feeding after 9 days of high doses of pinocembrin (5 and 50 μg/cm^2), and larval weight and survival were negatively affected by pinocembrin at 1–50 μg/cm^2. Delayed mortality in comparison to food-deprived larvae suggests that the mechanism of action for pinocembrin is chronic intoxication, rather than simple starvation from antifeedant effects. In contrast, leaf consumption and larval weight were not significantly affected by quercetin (at 0.1, 1, 5, and 50 μg/cm^2) while mortality rates were only slightly increased. The response of *E. paenulata* larvae in a choice-test to combinations of pinocembrin at antifeedant doses (5 and 50 μg/cm^2) and quercetin at phagostimulant doses (0.01 and 0.1 μg/cm^2) indicated that the feeding deterrent activity of the former completely overshadowed the stimulant activity of the latter. These results demonstrate the different responses of one insect species to two widely distributed plant flavonoids. Pinocembrin strongly affected survival of *E. paenulata* while quercetin had only a weak effect without major consequences on the insect life-cycle.

Key Words Flavonoids · Pinocembrin · Quercetin · *Epilachna paenulata* · Antifeedant · *Coleoptera coccinellidae*

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Introduction

Flavonoids are widely distributed in the plant kingdom and are involved in various aspects of plant interactions with other organisms. Increasing evidence suggests a role of flavonoids in plant defenses by contributing to disease resistance and protection against insect herbivory (Harborne and Williams, 2000; Treutter, 2006). The role of flavonoids in insect-plant interactions is complex, with some compounds apparently involved in host recognition, while others may deter or stimulate either feeding or oviposition (Simmonds, 2001).

Recently, we reported (Diaz Napal et al., 2009) on the differential effects of two flavonoids that are widely distributed in nature, pinocembrin (**1**), a flavanone which is found in conifers and Asteraceae species (Dillon and Mabry, 1977; Tewtrakul et al., 2003; Adelmann et al., 2007; Lin et al., 2007), and quercetin (**2**), a ubiquitous, abundant and intensively studied flavonol (Harnly et al., 2006; Harwood et al., 2007) (Fig. 1). When choice-tested at

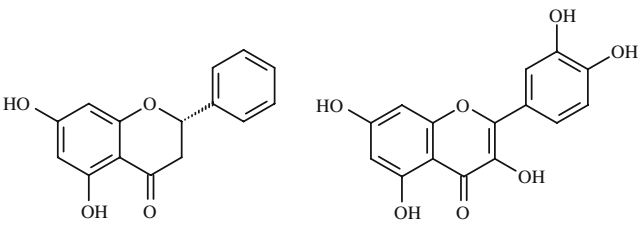
different doses on *Epilachna paenulata*, **1** demonstrated a clear antifeedant activity, whereas **2** elicited variable behavioral responses (phagostimulant or antifeedant) in the same insect species, depending on the dosage (Diaz Napal et al., 2009). *Epilachna paenulata* (Germar) (Coleoptera: Coccinellidae) is a folivorous South American pest of the Cucurbitaceae species (Camarano et al., 2009), which we have used extensively as a model insect for natural insecticide activity studies (Carpinella et al., 2002, 2003; Maggi et al., 2005; Palacios et al., 2007).

We asked whether the antifeedant (**1** and **2**) and phagostimulant (**2**) effects have consequences on the survival of insects. In order to answer these questions we carried out a comparative study on the effects of these compounds on feeding behavior and survival of *E. paenulata*.

Behavioral experiments have shown that insect responses to a particular compound can be modulated by the presence of other compounds (Simmonds, 2001, 2003). We examined how insects react to the simultaneous presence of both compounds. The system *E. paenulata*-Cucurbitaceae, in which the insect is sensitive to both compounds, and neither compound is found in the plant, provides a neutral study system.

Methods and Materials

General Experimental Procedures and Apparatus ^{1}H (200 MHz) and ^{13}C (50 MHz) NMR spectra were recorded on a Bruker NMR with a Bruker AC 200 console (Bruker, Germany). Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^{1}H and ^{13}C NMR spectra measured in DMSO-d_6. Electron impact mass spectra (EI-MS) were obtained at 70 eV by GC–MS on a Hewlett–Packard 5970 Series mass spectrometer interfaced with a Hewlett–Packard 5890 gas chromatograph (Hewlett-Packard, Santa Clara, CA, USA) fitted with an HP-5MS, 15 m×0.25 mm i.d. column. The temperature was increased from 100 to 200°C at 10°C/min. The optical rotation angle was measured in a JASCO DIP-370 spectropolarimeter (JASCO Co., Tokyo, Japan).



1 **2**

Fig. 1 Chemical structures of pinocembrin (**1**) and quercetin. (**2**)

Chemicals and Chromatographic Media Pinocembrin, used as a reference sample, quercetin, used in the bioassays, and SiO_2 for column chromatography were purchased from Sigma Chemical Co. Inc. (St. Louis, MO, USA). Analytical TLC was performed on silica gel 60 F_{254} Merck plates (Darmstadt, Germany). All other solvents were purchased from Merck (Darmstadt, Germany) and Fisher Scientific (Jersey City, NJ, USA).

Isolation of Pinocembrin **1** was isolated from aerial parts of *Flourensia oolepis* S.F. Blake (Asteraceae) as reported previously (Diaz Napal et al., 2009). In brief, air-dried aerial parts of *F. oolepis* (97 g) were extracted with ethanol for 24 hr at room temperature. After solvent removal under reduced pressure, the extract (1.8 g) was fractionated by silica gel column chromatography until a crystalline solid was isolated (258 mg, 1.14% yield with respect to plant material) and identified as pinocembrin by ^{1}H NMR, ^{13}C NMR and MS.

Pinocembrin (**1**): white powder; mp 194–195°C; $[\alpha]_D^{20}$= −22.81 (*c* 0.86, EtOH); EI-MS: *m/z* (relative intensity, %) 256 (100 M^+) , 179 (82), 152 (67), 124(52), 96 (31), 69 (34); ^{1}H NMR (DMSO-d_6), 20.7°C, δ 2.77 (1H, dd, *J*=17.2, 3.2 Hz, H-3a), 3.06 (1H, dd, *J*=12.8, 17.2 Hz, H-3b), 5.44 (1H, dd, *J*=3.2, 12.8 Hz, H-2), 5.52 (1H, d, *J*=2.2 Hz, H-6), 6.01 (1H, d, *J*=2.2 Hz, H-8), 7.41 (5H, m, H-2′-6′); ^{13}C NMR 25°C, δ 40.45 (C-3), 80.17 (C-2), 95.94 (C-8), 96.84 (C-6), 102.69 (C-10), 127.47 (C-2′/6′), 129.39 (C-4′) 129.46 (C-3′/5′), 139.59 (C-1′), 163.59 (C-9), 164.41 (C-5), 167.62 (C-7), 196.75 (C-4). The spectral data were identical to previously published data of **1** (Bick et al., 1972; Neacsu et al., 2007; Adelmann et al., 2007).

Insects Epilachna paenulata larvae were obtained from a laboratory colony, reared on a natural diet of *Cucurbita maxima* Duch. leaves and maintained in a growth chamber at 24±1°C and 70–75% relative humidity, with a photoperiod of 16/8 hr light-dark cycle, and periodically renewed with field specimens (Maggi et al., 2005).

Leaf Treatments For all bioassays, cotyledon leaves of *C. maxima* seedlings were treated with acetone, which was allowed to evaporate before the insect trial began. The compounds were dissolved at sufficient concentration to achieve the desired doses, ranging from 0.01 to 50 μg/cm^2, in a single 10 μl aliquot. Control leaves were treated with pure acetone. The compounds were applied, and acetone was allowed to evaporate before starting the assay.

Feeding Choice Assay The antifeedant experiments were carried out by the leaf-disk choice test (Carpinella et al., 2002). The dosages applied for **1** and **2** were 1, 5 and 50 and 0.1 and 0.01 μg/cm^2, respectively. Two cotyledon leaves

from a *C. maxima* seedling were placed in a Petri dish; a glass disk with two 1 cm^2 diam holes was placed on top. A third-instar *E. paenulata* was placed equidistant from both a treated (with 10 μl of test solution) and an untreated (10 μl of acetone, solvent control) leaf disk and was allowed to feed for 24 hr. Ten replicates were run for each binary combination tested. Relative amounts (recorded in percentages from 0 to 100) of the treated and untreated substrate area eaten in each test were estimated visually by dividing the food area into imaginary quarters. A feeding index (FI%) was calculated as $[(C-T)/(C+T)] \times 100$ (e.g., Mazoir et al., 2008), where T and C represent consumption on treated and untreated foods, respectively. Effective dose 50 (ED_{50}) values for **1** were determined from the FI% data, using Probit analysis.

No-choice Feeding Assay One *E. paenulata* larva (first instar) was placed in a Petri dish containing *C. maxima* leaves that had been treated with acetone solutions of either pure **1** or pure **2,** at dosages of 0.1, 1, 5, or 50 μg/$cm^{2.}$ Leaves were replaced every 24 hr. Ten replicates were used for each treatment. Leaf consumption and body weight were recorded every 24 hr and 48 hr, respectively (Carpinella et al., 2003).

Mortality Assay A group of 10 larvae of *E. paenulata* (first instar) was fed continuously with leaves treated either with 0.1, 1, 5, and 50 μg/cm^2 of pure **1**, pure **2** or acetone (control). A similar set of larvae was not fed at all and acted as food-deprived controls. Three replicates were performed for each treatment. Mortality was recorded every 24 hr. From mortality data, lethal dose 50 (LD_{50}) and lethal time 50 (LT_{50}) values for **1** and **2** were determined by Probit analysis. LD_{50} and LT_{50} were calculated at doses or times at which **1** provoked mortality values below as well as above 50%, thus enabling the Probit calculation.

Statistical Analysis Data did not conform to requirements of normal distribution and homocedasticity, therefore non-parametric tests were used. Results from choice tests were analyzed by the Wilcoxon Signed Rank Test. For each compound, results from no-choice tests on average larval body weight, accumulated consumption values, and average mortality rates at each date, were compared among concentrations by the Kruskal Wallis non-parametric analysis of variance, followed by the Dunn test. Statistical analyses were performed using the SPSS statistical package. Differences were considered significant at $P \leq 0.05$.

Results and Discussion

After 48 hr of exposure to leaves treated with **1** at 1, 5, or 50 μg/cm^2, larvae of *E. paenulata* had consumed less than one third the amount of those offered leaves with lower doses or untreated leaves (Fig. 2a) ($H=26.07$; $P<0.001$). Differences between treatments increased with time, so that after 9 days of exposure to treated food, larvae receiving **1** at higher doses were either not feeding at all (5 or 50 μg/cm^2) or were consuming at rates an order of magnitude below those of control larvae (1 μg/cm^2). Lower doses (0.1 μg/cm^2) of **1** did not affect insect feeding. The present results confirm previous observations in feeding choice experiments regarding the inactivity of **1** at 0.1 μg/cm^2 and its marked antifeedant effects at doses above 1 μg/cm^2 (Diaz Napal et al., 2009). Moreover, such effects may lead to starvation if *E. paenulata* larvae do not have access to untreated food.

In contrast, the food consumption by larvae fed with **2** did not differ significantly from that of the controls at any dosage or at any time during the experiment (Fig. 3a). In the 12-day experiment, larvae faced with leaves treated with 0.1, 1, 5, and 50 μg/cm^2 of **2** ate as much food as the controls ($H=6.50$; $P=0.066$). Thus, neither the antifeedant nor the dose-dependent phagostimulant activity previously

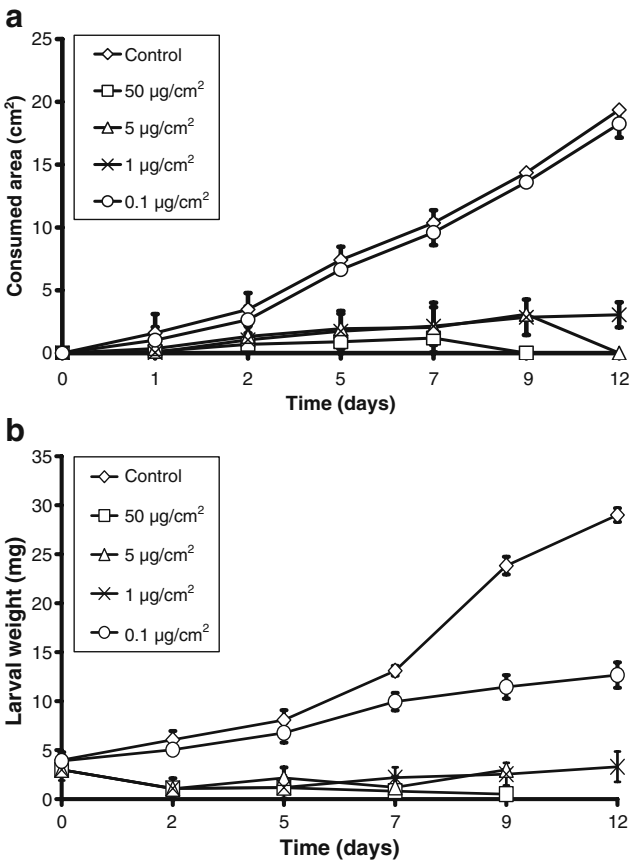


Fig. 2 Average leaf area consumed (**a**) and average body weight (**b**) of *Epilachna paenulata* larvae fed on leaves treated with **1** in no-choice assay. Error bars show standard deviation, where error is not shown it is smaller than the symbol

observed in choice assays with **2** (Diaz Napal et al., 2009), were detected in this system where insects could not switch to untreated food. These results confirm the necessity of comparing different methodologies when studying insect response to plant allelochemicals (Stuart and Polavarapu, 1998).

Larval weight data (Fig. 2b) demonstrated that whereas control larvae body weight steadily increased, larvae treated with **1** at 1 μg/cm^2 or higher doses either did not gain weight or lost weight. From 48 hr onward, significant differences in body weight (H=38.62; P<0.001) were observed between control larvae and those receiving food treated with **1** at doses of 1 μg/cm^2 or higher. After 7 days of receiving food treated with 5 or 50 μg/cm^2 of **1**, larvae had lost about half their original weight (Fig. 2b). Larvae fed on leaves treated with 0.1 μg/cm^2 of **1** gained some weight, but less weight (H=4.20; P<0.001) than control larvae starting at day 9. Compound **2** did not affect the body weight of *E. paenulata* larvae (Fig. 3b) (H=5.51; P=0.2379).

Treatment of leaves with **1** increased larval mortality (Fig. 4a), particularly at the highest dose (50 μg/cm^2), which induced 100% mortality after 12 days. At lower doses of **1**, mortality reached between 20 and 60%. After 5 days of receiving treated food, mortality rates were significantly higher than those observed in the controls (H=30.20; P<0.001) for all concentrations higher than 0.1 μg/cm^2. Food-deprived larvae did not survive longer than 5 days. These results indicate that the presence of **1** on the food of *E. paenulata* larvae strongly affected their survival through feeding inhibition, consistent with the well-established role of flavonoids in the defense of plants against herbivory (Harborne and Williams, 2000).

Larvae offered food treated with **2** at any of the doses tested died at the same rate as the controls (P>0.05) (Fig. 4b). All treatments were significantly different (P< 0.001) from the food-deprived group. According to these results, **1** strongly affected survival of *E. paenulata* larvae, whereas **2** had no major effects on the insect life-cycle.

The LD_{50} of **1** at 124 hr was 18.4 (6.0–56.6) μg/cm^2. A comparison of this value with those reported for the same insect for meliartenin (LD_{50}=0.76 μg/cm^2) and azadirachtin (LD_{50}=1.24 μg/cm^2) (Carpinella et al., 2003), indicates that pinocembrin is 24 and 15 times less active than those compounds, respectively. In molar units, LD_{50} for pinocembrin (70 nmol/cm^2) was 50 and 41 times higher than the

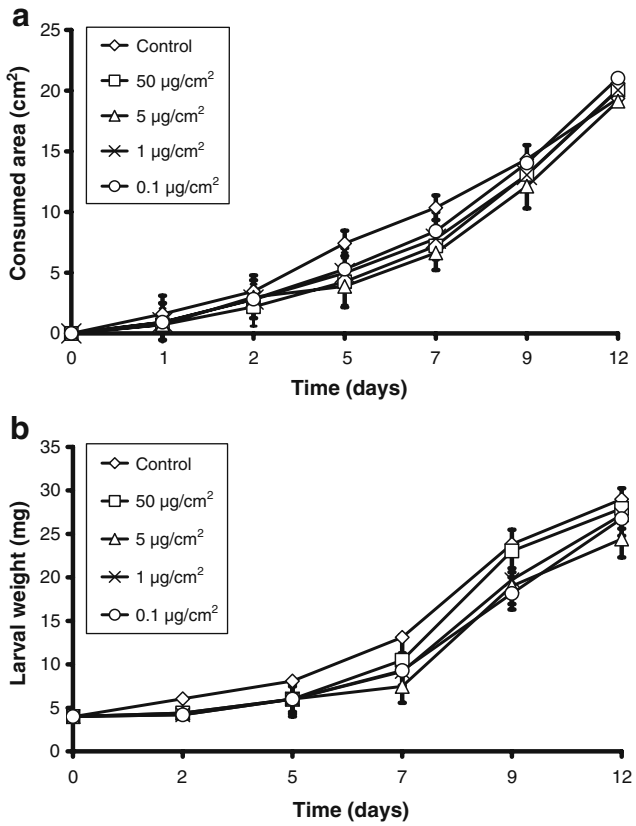


Fig. 3 Average leaf area consumed (**a**) and average body weight (**b**) of *Epilachna paenulata* larvae fed on leaves treated with **2** in no-choice assay. Error bars show standard deviation, where error is not shown it is smaller than the symbol

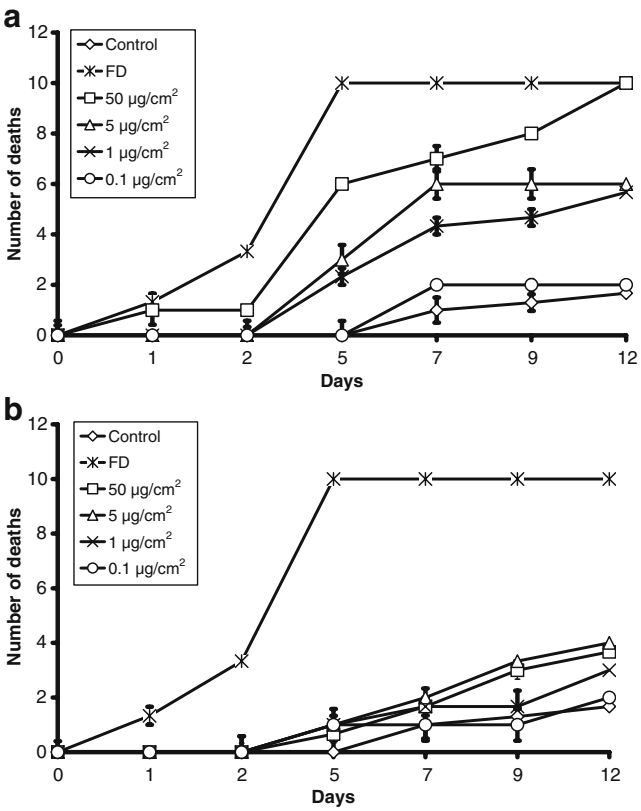


Fig. 4 Cumulative mortality rates (%) of *E. paenulata* larvae fed with leaves treated with **1** (**a**) and **2** (**b**). FD represents food-deprived treatment. Error bars show standard deviation, where error is not shown it is smaller than the symbol

Table 1 Lethal time (LT_{50}) for compound **1** and for food-deprived control in larvae of *Epilachna paenulata* in no-choice feeding assay

Dosage (μg/cm^2)	LT_{50}[a] in days (values and 95% confidence interval)	
	1	Food-deprived larvae
50	5 (4.73–5.87)	
5	8 (6.47–9.47)	
1	10 (8.10–11.85)	
		4 (3.07–4.23)

[a] LT_{50} is the time required to obtain 50% mortality

value for meliartenin (1.4 nmol/cm^2) and azadirachtin (1.7 nmol/cm^2), respectively.

Insects consuming leaves treated with **1** died later than food-deprived larvae, suggesting that the mechanism of activity for **1** is chronic intoxication rather than simple starvation (Defagó et al., 2009). Lethal times 50 (LT_{50}) for **1** (Table 1) were 5, 8, and 10 days for 50, 5 and 1 μg/cm^2, respectively. The LT_{50} for food-deprived larvae was 4 days. Comparing the LT_{50} at 1 μg/cm^2 with the values obtained for meliartenin and azadirachtin on the same insect (Carpinella et al., 2003), **1** acted approximately 4 and 2 times more slowly than meliartenin and azadirachtin, respectively.

It was not possible to calculate LD_{50} and LT_{50} for **2,** since mortality rates did not reach 50% at any point of the experiment (Fig. 4b).

Responses to plant secondary metabolites often play a crucial role in the feeding behavior of herbivore insects. Such responses result from the integration of information provided by taste and olfactory cells (van Loon, 1990) as well as from other extrinsic and intrinsic factors operating on and in the insect (Bernays and Chapman, 2001). Responses of chemical receptors to a specific stimulus may be modified by the presence of other plant constituents, so it may be impossible to predict the response to complex stimuli on the basis of single compound responses (Schoonhoven and van Loon, 2002). Combinations of **1** and **2** were assayed in a choice test, at doses at which **2** was expected to act as phagostimulant and **1** as an antifeedant. Choice assays of these mixtures with *E. paenulata* revealed that the ED_{50} of **1** was approximately the same when offered with or without **2** (Table 2). This insect recognized **1** as a feeding deterrent, and this effect was not modified by the presence of **2** at phagostimulant levels. Increased concentration of a deterrent compound can not only increase activity of the deterrent-sensitive cells, but may also suppress the activity of cells responding to phagostimulants (Bernays and Chapman, 2000), and therefore, negative inputs may dominate insect response to feeding stimulation (Chapman, 2003).

It is not possible to predict the effect of different flavonoids on insects from structural features of the compounds, although empirical evidence suggests that the structure can be important (Simmonds, 2001). According to Morimoto et al. (2000), hydroxyl groups at any position favor antifeedant activity against *Spodoptera litura*, but this

Table 2 Feeding choice assay for mixtures of **1** and **2** against *Epilachna paenulata*

Dosage (μg/cm^2)		FI%[a]	ED_{50} of **1** in μg/cm^2 (95% confidence interval)
1	**2**		
1		6	
5		12	10.08 (2.42–41.9)
50		98**	
	0.01	−48.72**	
	0.1	−16.22*	
1	0.01	5	
5	0.01	15.4	13.55 (2.71–67.74)
50	0.01	87**	
1	0.1	2	
5	0.1	20	13.18 (2.29–75.62)
50	0.1	82**	

[a] Feeding index for pinocembrin, calculated as $(FI\%) = [(C - T)/(C + T)] \times 100$, values are means of 10 replicates. Significant differences between consumption on treated and control leaves (Wilcoxon signed rank test *P<0.05, **P<0.01)

idea was not supported by our results since **2** has three more hydroxyl groups than **1**. A further complication arises from the variability in the responses of different insects to the same flavonoid (Simmonds, 2001, 2003). Our results support this observation and suggest that such effects might also depend on compound dosage. The growth of the corn borer *Ostrinia nubilalis* Hubner was reduced by 10 mg/g of **1** added to an artificial diet (Abou-Zaid et al., 1993). In contrast, **1** was reported as attractant and phagostimulant to *Scolytus mediterraneus* (Levy et al., 1974). Insect response to **2** varies greatly. This flavonoid may act as a phagostimulant for some insects, may be either a phagostimulant or a deterrent depending on the concentration tested for other insects (Lattanzio et al., 2000; Green et al., 2003; Simmonds, 2003), and may be inactive in other cases (Morimoto et al., 2000).

In summary, we have shown that compound **1** inhibited feeding and negatively affected survival of the cucurbit insect pest *E. paenulata*. We also found that **1** masked the phagostimulant effects of **2** in a choice test. According to the present results, **1** is less active than other well-known antifeedants such as azadirachtin or meliartenin, but its availability in a variety of plant species and its molecular simplicity, which could allow inexpensive chemical synthesis, suggest that **1** could be applied as an feeding deterrent or could be a target for plant improvement programs to increase natural crop resistance to herbivory.

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