Antifeedant Activity of Withanolides from *Salpichroa origanifolia* on *Musca domestica*

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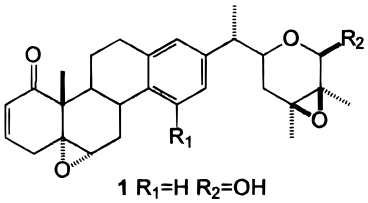
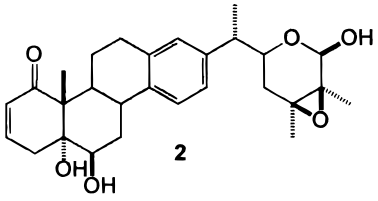
The antifeedant effect of several salpichrolides on larvae of *Musca domestica* was investigated. Three naturally occurring compounds, salpichrolide A (**1**), salpichrolide C (**2**), and salpichrolide G (**3**), previously isolated from *Salpichroa origanifolia*, and two known (**4**, **6**) and three new (**5**, **7**, **8**) synthetic analogues were tested. The maximal effect on development was observed for salpichrolide A (**1**), while salpichrolide G (**3**) was the most toxic. The content of the salpichrolides in *S. origanifolia* was monitored by HPLC during plant development, reaching a maximum during summer.

Environmental pressures and the rapidly developing resistance of insects to conventional insecticides have prompted the search for new, more ecologically acceptable methods of pest control. As a consequence of evolution and adaptation to the environment, plants have developed highly elaborate chemical defenses against insect attack, thus providing a rich source of biologically active compounds that may be used as novel insecticides. The withanolides are a group of C-28 steroidal lactones and lactols isolated from several genera of the Solanaceae family. Some of them exhibit activity as insecticides, feeding deterrents, and ecdysteroid antagonists, and they have been related to chemical defense mechanisms.[1−3] Withanolides with a six-membered aromatic ring D constitute a small group. A few examples (termed nicandrenoids) were first isolated from the peruvian "shoofly" plant *Nicandra physaloides* and were later related to the insecticidal activity of plant extracts.[4,5] A family of these compounds and related ergostane derivatives (termed salpichrolides) were recently isolated from *Salpichroa origanifolia* (Lam.) Thell (Solanaceae), the most widespread species of the genus *Salpichroa*. The major components were salpichrolide A (**1**), C (**2**), and G (**3**).[6−9] In view of the insect antifeedant and repellent properties of nicandrenone, the major withanolide isolated from *N. physaloides*, we have investigated the insect antifeedant properties of compounds **1**−**3**. In a study of structure−activity relationships, we also prepared and assayed several modified salpichrolide analogues. Compounds were tested against house fly larvae (*Musca domestica*), a well-known sanitary pest. To investigate the possible role of withanolides in a chemical defense mechanism, the content of salpichrolides **1**−**3** during plant development was monitored throughout the year.



1 R_1=H R_2=OH
3 R_1=OH R_2=OH
4 R_1=H R_2=OAc
5 R_1=R_2=OAc



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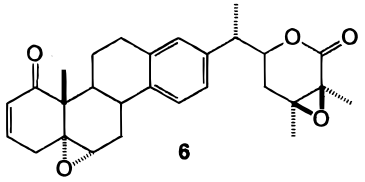

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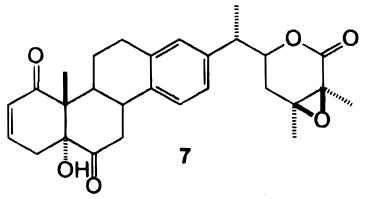

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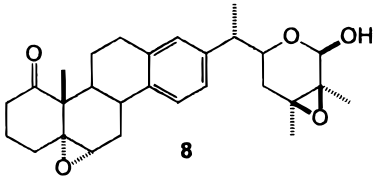

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Results and Discussion

Neonatae larvae of *Musca domestica* were grown on an artificial diet with varying concentrations of salpichrolide

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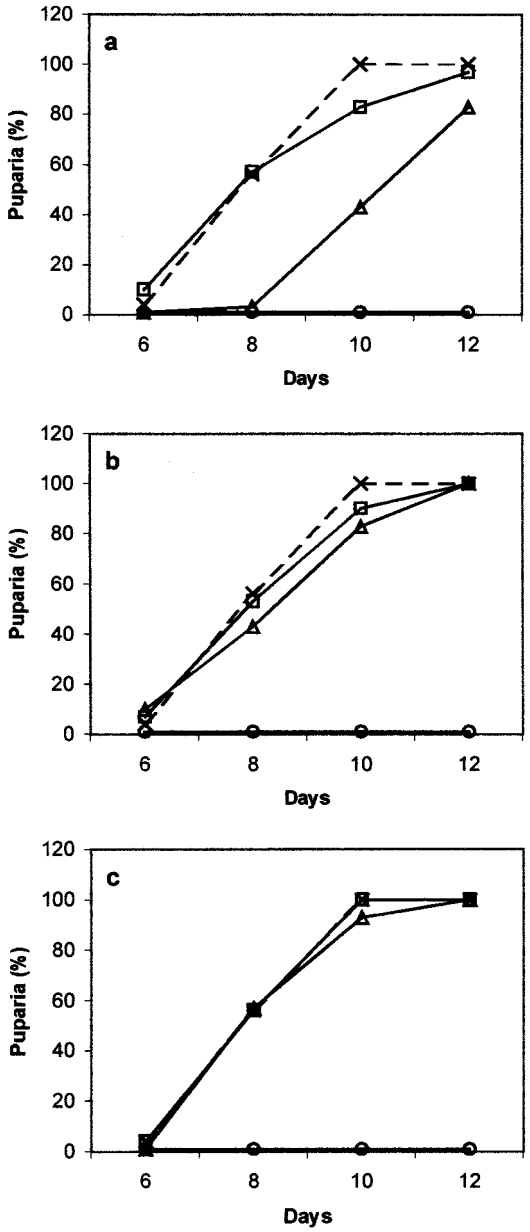

Figure 1. Percentage of puparia from *Musca domestica* larvae treated over a 12-day period with (a) Salpichrolide A (**1**), (b) Salpichrolide C (**2**), and (c) Salpichrolide G (**3**). Groups of 10 neonatae larvae were reared on an artificial diet[11] where the test compounds had been incorporated to give final concentrations of 100 ppm (□), 500 ppm (△), and 2000 ppm (■). Control experiments (×) were carried out under identical conditions but without addition of withanolides. All treatments were assayed in triplicate.

A (**1**), C (**2**), and G (**3**). Figure 1 shows the cumulative percentages of puparia for each withanolide over a 12-day period, at 100, 500, and 2000 ppm. The time needed for complete development of 50% of the surviving *M. domestica* larvae (PT_{50}) exposed to compounds **1**−**3** is summarized in Table 1. On the basis of the intermediate dose (500 ppm), compound **1** showed the greatest development delay, the effect being most dramatic after 8 days of exposure (Figure 1a). The 2000 ppm concentration produced in all cases 100% mortality before pupation occurred, not allowing the calculation of the PT_{50}. The necessary concentration to inhibit complete development in 50% of the larvae (EC_{50}) was calculated from the dose response curves in each experiment with the three natural withanolides, and salpichrolide G (**3**) was the most toxic compound (Table 2).

Table 1. Pupation Time in *Musca domestica* Larvae Exposed to Natural Withanolides **1**−**3**[a]

treatment[b]	conc (ppm)	n	PT_{50}[c] (days)
		30	7.7 (7.5−7.9)
1	100	30	7.9 (7.6−8.2)
1	500	30	10.3 (10.1−10.6)
1	2000	30	ID[d]
2	100	30	7.8 (7.6−8.1)
2	500	30	8.1 (7.8−8.4)
2	2000	30	ID
3	100	30	7.7 (7.5−7.9)
3	500	30	8.0 (7.8−8.2)
3	2000	30	ID

[a] The tests were performed on *Musca domestica* larvae, CIPEIN strain, from a laboratory colony reared in this laboratory since 1981. [b] Bioassays (three replicates) were conducted with groups of 10 freshly hatched larvae kept on an artificial diet (water, powdered milk, yeast, agar, and nipagin), containing the test compounds in the concentration indicated. [c]Every 2 days the number of puparia were counted to determine PT_{50} (pupating time or time needed to pupate 50% of exposed larvae). Probit analysis was used to assess development delays with 95% fiducial limit. [d]ID: Incomplete development.

Table 2. Development Inhibition of *Musca domestica* Larvae Exposed to Natural Withanolides[a]

	1[b]	**2**[b]	**3**[b]
EC_{50}[c] (ppm)	290.0 (249.0−335.0)	310.0 (264.0−363.0)	203.0 (168.0−244.0)

[a] The tests were performed on *Musca domestica* larvae (see footnote to Table 1 for details). [b]Concentrations assayed were 100, 500, and 2000 ppm. [c]Every 2 days the number of survivants was counted over an 18-day period. From the dose response curves in each experiment with natural withanolides, the parameter EC_{50} (concentration needed to inhibit complete development in 50% of the larvae) was calculated with Probit analysis to assess development inhibition (95% fiducial limit).

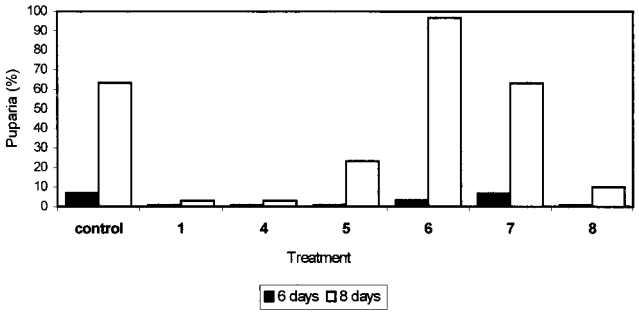

Figure 2. Percentage of puparia from *Musca domestica* larvae treated with 500 ppm of modified withanolides (see Figure 1 for details). Salpichrolide A (**1**), 500 ppm, was included as a positive control.

With both salpichrolide A (**1**) and G (**3**), adults failed to eclose from some puparia.

As a preliminary approach to assess structure−activity relationships, we assayed the acetylated derivatives **4** and **5**, the oxidized analogues **6** and **7**, and the reduced analogue **8**. On the basis of the results described above, a dose of 500 ppm was used and the effect evaluated after 6 and 8 days of exposure. Cumulative percentages of puparia for each treatment are shown in Figure 2 for compounds **4**−**8**. Results indicate that oxidation of the hemiacetal side chain to the lactone (compounds **6** and **7**) eliminated the biological activity. Acetylation of the hemiacetal (compound **4**) resulted in a nonsignificant decrease of the activity; however acetylation of salpichrolide G (compound **5**) caused an increase in this activity (compared with Figure 1c). The last result is probably due to a reduced toxicity, which increased the number of survivors. Reduction of the 2,3-

Table 3. Pupation Time in *Musca domestica* Larvae Exposed to Diets of Different Nutritional Value[a]

nutritional value	concentration[b] (ppm)	*n*	PT_{50}[c] (days)
high	80.000 yeast−80.000 milk	30	6.9 (6.8−7.1)
medium	8.000 yeast−8.000 milk	30	12.2 (11.4−13.7)
low	0 yeast−0 milk	30	ID[d]

[a] The tests were performed on *Musca domestica* larvae, CIPEIN strain, from a colony reared in this laboratory since 1981. Bioassays (three replicates) were conducted with groups of 10 freshly hatched larvae kept on artificial diets (water, powdered milk, yeast, agar, and nipagin) of different nutritional values without withanolides. [b]Nutritional values were obtained decreasing the amount of milk and yeast (100, 10, and 0%). [c]Every 2 days the number of puparia was counted to calculate the parameter PT_{50} (pupating time or necessary time to pupate 50% of exposed larvae). Probit analysis was used to assess development delays with 95% fiducial limit. [d] ID: Incomplete development.

double bond (compound **8**) had a small negative effect on the feeding deterrent activity.

Development delays similar to those obtained with salpichrolide A (**1**) (Table 1) were observed when medium- and low-nutrition diets, without withanolides, were offered as food (Table 3), supporting the idea that these compounds act as feeding deterrents**.** Antifeedant effects have been previously observed with some withanolides from *Withania somnifera*, *Physalis peruviana*, *Nicandra physaloides*, and *N. physaloides* var. *albiflora* applied to the diet of larvae of the coleoptera *Epilachna varivestis*[10] and *Tribolium castaneum*[11] and the lepidoptera *Spodoptera frugiperda*.[12]

To study the possible role of salpichrolides in the chemical defense mechanisms of the plant *Salpichroa origanifolia*, the amounts of **1**, **2**, and **3** were determined throughout the year (Figure 3). A marked increase in the concentration of the active withanolides in the plant was observed in summer (Dec 21st to March 21st in the southern hemisphere) when insect populations are higher. When the content of withanolides was related to tissue water (μg/mL), we found levels of 350 ppm for **1** and 168 ppm for **2** in summer, which are comparable to the EC_{50} shown in Table 2. Salpichrolide A (**1**) and C (**2**) were also found in the fruit in concentrations of 300 and 150 ppm on a fresh weight basis. These results in conjuction with the observed toxic and feeding deterrent activities suggest that these compounds may provide protection against predation by certain phytophagous insects (not tested in this work) acting as a chemical defense. A similar role has been proposed for the withanolides in *Physalis peruviana* during fruit development.[2]

Experimental Section

General Experimental Procedures. Melting points are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions on a Bruker AC-200 NMR spectrometer at 200.13 and 50.32 MHz, respectively. Multiplicity determinations (DEPT) and 2D spectra (COSY) were obtained using standard Bruker software. Chemical shifts are given in parts per million (δ) downfield from TMS as internal standard. EIMS were collected on a VG TRIO-2 at 70 eV by direct inlet. HREIMS were measured on a VG ZAB-BEqQ mass spectrometer. HPLC separations were carried out on a Spherisorb ODS-2 reversed phase column (250 × 4.6 mm i.d., 5 μm particle size) with UV detection at 245 nm.

Plant Material. Aerial parts of *Salpichroa origanifolia* were collected in the surroundings of the University campus in Buenos Aires, Argentina. A voucher specimen is deposited at the Museo Botánico, Universidad Nacional de Córdoba, under No. CORD 89.

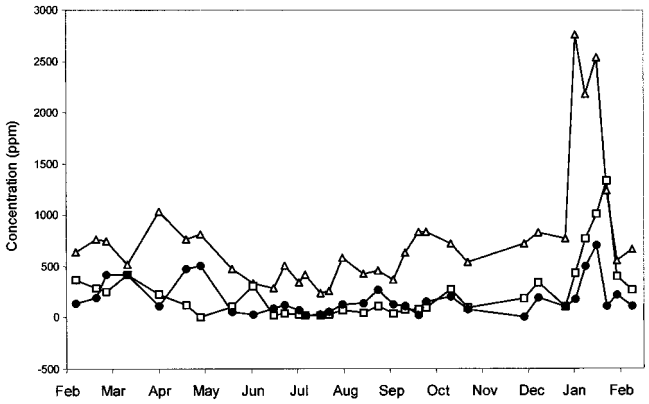

Figure 3. Concentrations (related to fresh weight) of **1** (△), **2** (●), and **3** (□) in leaves and stems of *S. origanifolia*, during plant development. Fresh leaves and stems were extracted with ether and the withanolide concentrations determined by reversed-phase HPLC (Dec 21st to March 21st is summer in the southern hemisphere).

Test Compounds. Salpichrolides A (**1**), C (**2**), and G (**3**) were isolated from fresh leaves, stems, and fruits as previously described.[9] Acetylation of compound **1** with Ac_2O−pyridine (1:1) at room temperature afforded acetate **4**.[6] Lactone **6** was obtained from salpichrolide A (**1**) by treatment with Jones' reagent as previously described.[6]

Salpichrolide G Diacetate (**5**). Acetylation of **3** with Ac_2O−pyridine (1:1) at room temperature afforded acetate **5**: crystals from EtOAc−hexane, mp 150−151 °C; ^{1}H NMR ($CDCl_3$) δ 6.80 (1H, s, H-18), 6.64 (1H, s, H-16), 6.74 (1H, ddd, J = 10.5, 5.0, 2.5 Hz, H-3), 6.06 (1H, s, H-26), 5.98 (1H, dd, J = 10.5, 2.5 Hz, H-2), 3.95 (1H, ddd, J = 11.2, 6.3, 2.3 Hz, H-22), 3.20 (1H, d, J = 5.0 Hz, H-6), 3.13 (1H, dt, J = 19.6, 2.5 Hz, H-4β), 2.32 (3H, s, CH_3CO-15), 2.03 (3H, s, CH_3CO-26), 1.35 (3H, s, CH_3-28 and CH_3-19), 1.31 (3H, s, CH_3-27), 1.18 (3H, d, J = 7 Hz, CH_3-21); ^{13}C NMR ($CDCl_3$) δ 212.9 (C-1), 166.6 (CH_3*C*O-26 and CH_3*C*O-15), 149.6 (C-15), 142.3 (C-3), 141.7 (C-13), 140.6 (C-17), 128.8 (C-2), 126.5 (C-14), 120.3 (C-18), 113.6 (C-16), 91.6 (C-26), 70.6 (C-22), 64.4 (C-24 and C-5), 63.8 (C-25), 59.7 (C-6), 48.9 (C-10), 42.5 (C-20), 38.1 (C-9), 34.1 (C-23), 33.3 (C-4), 32.4 (C-8), 31.7 (C-12), 29.7 (C-7), 24.2 (C-11), 21.2 (*C*H_3CO-15 or *C*H_3CO-26), 21.1 (*C*H_3CO-26 or *C*H_3-CO-15), 18.1 (C-28), 16.5 (C-27), 14.8 (c, CH_3-21); EIMS *m*/*z* 365 (3) $[M - 185]^+$, 305 (4), 288 (3), 185 (17), 141 (7), 43 (100); HREIMS *m*/*z* found $[M]^+$ 550.2559 ($C_{32}H_{38}O_8$ requires 550.2567).

Preparation of Compound 7 from 1. Salpichrolide A (**1**) (30 mg) was dissolved in Me_2CO (3 mL) and treated with 8 N CrO_3−H_2SO_4 (0.5 mL) at 0 °C for 3 h. Extractive workup followed by purification by flash chromatography afforded pure lactone **7** (25 mg): white crystals from EtOAc−hexane, mp 154−155 °C; ^{1}H NMR ($CDCl_3$) δ 7.11 (1H, d, J = 7.0 Hz, H-15), 7.02 (1H, d, J = 7.0 Hz, H-16), 7.00 (1H, s, H-18), 6.62 (1H, ddd, J = 10.2, 5.2, 2.5 Hz, H-3), 5.95 (1H, dd, J = 10.2, 2.5 Hz, H-2), 4.63 (1H, dt, J = 12.0, 4.0 Hz, H-22), 3.19 (1H, dt, J = 19.0, 2.5 Hz, H-4β), 2.92 (1H, m, H-20), 2.30 (1H, d, J = 19.0, 5.2 Hz, H-4α), 2.06 (1H, dd, J = 12.0, 4.0 Hz, H-23α), 1.87 (1H, t, J = 12.0 Hz, H-23β), 1.52 (3H, s, CH_3-28 or CH_3-27), 1.42 (3H, s, CH_3-27 or CH_3-28), 1.13 (3H, s, CH_3-19); ^{13}C NMR ($CDCl_3$) δ 209.2 (C-6), 201.5 (C-1), 169.7 (C-26), 141.0 (C-3), 139.3 (C-17), 137.5 (C-13 or C-14), 137.4 (C-14 or C-13), 129.1 (C-2), 128.2 (C-18), 125.9 (C-15), 125.4 (C-16), 82.2 (C-5), 77.8 (C-22), 62.7 (C-25), 59.3 (C-24), 55.4 (C-10), 42.6 (C-20), 41.0 (C-7), 39.5 (C-8), 37.6 (C-9), 32.6 (C-23), 31.2 (C-4), 30.6 (C-12), 26.3 (C-11), 17.7 (C-28), 16.4 (C-21), 13.6 (C-27), 13.2 (C-19); EIMS *m*/*z* 464 (2) $[M]^+$, 323 (60), 169 (4), 141 (6), 43 (100); HREIMS *m*/*z* $[M]^+$ 464.2193 ($C_{28}H_{32}O_6$ requires 464.2199).

2,3-Dihidrosalpichrolide A (**8**). Salpichrolide A (**1**) (25 mg) was dissolved in absolute EtOH (8 mL) and hydrogenated over 10% Pd/C (2.5 mg) at room temperature and 1 atm for 3 h. The catalyst was filtered off and the solvent evaporated to yield **8** (20 mg): crystals from EtOAc−hexane, mp 175−176 °C; ^{1}H NMR ($CDCl_3$) δ 7.10 (1H, d, J = 8.0 Hz, H-15), 6.99 (1H, d, J

= 8.0 Hz, H-16), 6.90 (1H, s, H-18), 5.00 (1H, bs, H-26), 3.84 (1H, ddd, J = 11.3, 5.6, 2.6 Hz, H-22), 3.23 (1H, d, 5.0 Hz, H-6), 1.46 (3H, s, CH_3-19), 1.37 (3H, s, CH_3-28), 1.35 (3H, s, CH_3-27), 1.24, (d, J = 7.0 Hz, CH_3-21); ^{13}C NMR ($CDCl_3$) δ 212.0 (C-1), 140.3 (C-17), 138.1 (C-13), 136.8 (C-14), 128.7 (C-18), 126.4 (C-16), 125.5 (C-15), 91.7 (C-26), 67.4 (C-22), 64.8 (C-24), 64.8 (C-5), 63.6 (C-25), 59.9 (C-6), 52.2 (C-10), 42.9 (C-20), 38.0 (C-4), 35.6 (C-9), 33.6 (C-23), 32.4 (C-8), 30.5 (C-7), 30.3 (C-12), 28.9 (C-2), 25.0 (C-11), 22.8 (C-3), 18.3 (C-28), 17.1 (C-21), 16.5 (C-27), 15.7 (C-19); EIMS m/z 452 (1) $[M]^+$, 310 (21), 171 (3), 144 (4), 43 (100); HREIMS m/z found $[M]^+$ 452. 2567 ($C_{28}H_{36}O_5$ requires 452.2563).

HPLC Determinations. HPLC experiments were performed using a Spherisorb ODS-2 reversed phase column (250 × 4.6 mm, 5 μm particle size) and a mixture of MeOH−H_2O (70:30) as eluant at a flow-rate of 1.5 mL/min. The salpichrolides A (**1**), C (**2**), and G (**3**) were detected at 245 nm; retention times were 10.4, 5.2, and 5.8 min respectively. Fresh leaves and stems (15 g) of *S. origanifolia* were triturated and extracted with ether at room temperature. The residue obtained after evaporation of the solvent was diluted with MeOH (10 mL), and an aliquot (1 mL) of the solution was filtered using a solid-phase extraction column, RP-18 (LiChrolut), to eliminate the less polar compounds. The eluate (5 μL) was chromatographed as indicated above. The withanolide concentrations were determined using authentic samples as external standards.

Insects. The tests were performed on *Musca domestica*, CIPEIN strain, maintained in the laboratory since 1981. Groups of eggs deposited during an 8 h period were collected from adult house flies 3−6 days old, and freshly hatched larvae (16−20 h old) were used in the bioassays.

Bioassays. Groups of 10 neonatae larvae were reared on an artificial diet (12.3 g) where the test compounds had been incorporated. The artificial diet was prepared according to Keiding,[11] with powdered milk, beer yeast, agar, nipagin, and water (1:1:0.2:0.1:10). Test compounds were dissolved in ethanol and mixed in the rearing medium to obtain final concentrations of 100, 500, and 2000 ppm for compounds **1**−**3** and 500 ppm for compounds **4**−**8**. Control larvae were exposed to 12.3 g of artificial diet without test compounds. All treatments were assayed in triplicate. Larvae were kept at 25−27 °C, 50−60% RH, and a 12:12 h photoperiod. The number of puparia was recorded every 48 h. Mortality was assessed over a period of 18 days of exposure to compounds **1**−**3**.

Another bioassay was conducted with three different levels of nutrients in the diet to compare larval development in a low-nutrient medium with that in one containing withanolides. Low-nutrient medium was obtained by decreasing the percentage of powdered milk and beer yeast (100, 10, and 0%). The number of puparia was registered every 48 h.

Statistical Analysis. The average percentage of puparia for each concentration was used to calculate the parameter PT_{50} (pupating time or necessary time needed to pupate 50% of exposed larvae) by Probit analysis.[14] The average larvae mortality for each concentration of the natural compounds was used to calculate EC_{50} (necessary concentration to inhibit complete development in 50% of the larvae).

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