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(-)-Olivil and (+)-1-Acetoxypinoresinol from the Olive Tree (Olea europaea LINNE; Oleaceae) as Feeding Stimulants of the Olive Weevil (Dyscerus perforatus)

Emiko KADOWAKI[a], Yasuhiro YOSHIDA[c], Teruhiko NITODA[a], Naomichi BABA[b] & Shuhei NAKAJIMA[b]

[a] Graduate School of Natural Science and Technology, Okayama UniversityTsushimanaka, Okayama 700-8530, Japan

[b] Department of Bioresources Chemistry, Faculty of Agriculture, Okayama UniversityTsushimanaka, Okayama 700-8530, Japan

[c] Nippon Olive Co., Ltd.3911-10 Ushimado, Ushimado-cho, Oku-gun, Okayama 701-4394, Japan

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Note

(−)-Olivil and (+)-1-Acetoxypinoresinol from the Olive Tree (*Olea europaea* LINNE; Oleaceae) as Feeding Stimulants of the Olive Weevil (*Dyscerus perforatus*)*

Emiko KADOWAKI,[1] Yasuhiro YOSHIDA,[3] Teruhiko NITODA,[1] Naomichi BABA,[2] and Shuhei NAKAJIMA[2,†]

[1]*Graduate School of Natural Science and Technology, Okayama University, Tsushimanaka, Okayama 700-8530, Japan*
[2]*Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Tsushimanaka, Okayama 700-8530, Japan*
[3]*Nippon Olive Co., Ltd., 3911-10 Ushimado, Ushimado-cho, Oku-gun, Okayama 701-4394, Japan*

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Guided by a feeding stimulant activity test on the olive weevil (*Dyscerus perforatus*), two compounds that showed potent feeding stimulant activity were isolated from the olive tree (*Olea europaea*). Based on their spectral data and a literature survey, they were identified as (−)-olivil (1) and (+)-1-acetoxypinoresinol (2). The activities of these minor lignans were significantly higher for the female than for the male weevil.

Key words: olive weevil; olive; feeding stimulant; (−)-olivil; (+)-1-acetoxypinoresinol

The most serious pest of the olive tree in Japan, an important plant as a source of fruit and oil production, is now the olive weevil [*Dyscerus perforatus* (ROELOFS); Coleopetera; Curculionidae] that is a native species in Japan. This weevil originally seems to have colonised *Ligustrum japonicum* Thunb. and *L. obtusifolium* Sieb. et Zucc, both of which belong to the oleacea family, like the olive. However, when olive trees were introduced to Japan in 1908, the weevils immediately attacked the plants and soon preferred them to the former hosts. Unlike in the former hosts, where the weevils lived in a low population density, it is extraordinary high in the case of the olive tree and the assault thereby becomes seriously damaging for the host plant. The adult weevils have a long life and can survive for over 900 days in a room.[1)] Moreover, the females have a long egg-laying period from spring to autumn, and the larvae attack the bark and trunk of olive trees, weakening and withering even adult trees as the assault progresses. During the course of our study on the relationship between the olive tree and olive weevil, we have been interested in the possible chemical constituents of this plant that are responsible for host selection and attraction of the insect.

We have previously found that a secoiridoid glucoside (oleuropein) isolated from the olive tree stimulated the feeding activity of the weevil.[2)] In this present report, we describe the isolation and characterization of two more feeding stimulants from the same plant.

The purification of the active compound(s) was guided by a feeding stimulant activity test on the olive weevil.[2)] After extracting a sample of olive tree with methanol, the methanol extract was evaporated and successively partitioned with hexane/water and EtOAc/water. Among these fractions, the EtOAc-soluble fraction exhibited feeding stimulant activity which seemed to be significantly higher for female than for male insects. Therefore, this fraction was further purified by column chromatography and preparative TLC on silica gel to give active compound **1** as colorless needles and active compound **2** as an amorphous solid. Active compound **1** was much more polar than **2** as indicated by its lower R_f value on silica gel TLC (solvent system of hexane:EOAc = 1:1).

The high-resolution EI mass spectrum (HREIMS) of active compound **1** afforded a molecular ion at m/z 376.1525, indicating the molecular formula of $C_{20}H_{24}O_7$ which was consistent with the ^{1}H- and ^{13}C-NMR data. The IR spectrum of **1** exhibited strong absorption bands at 3400 cm^{-1} corresponding to a hydroxyl group(s) and at 1605, 1520 and 824 cm^{-1} suggesting the presence of a trisubstituted benzene ring. The UV spectrum (λ_{max} 230 and 280 nm) and positive $FeCl_3$ test implied the presence of a phenol chromophore. The presence of two phenolic

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† To whom correspondence should be addressed. Tel/Fax: +81-86-251-8302; E-mail: snaka24@cc.okayama-u.ac.jp

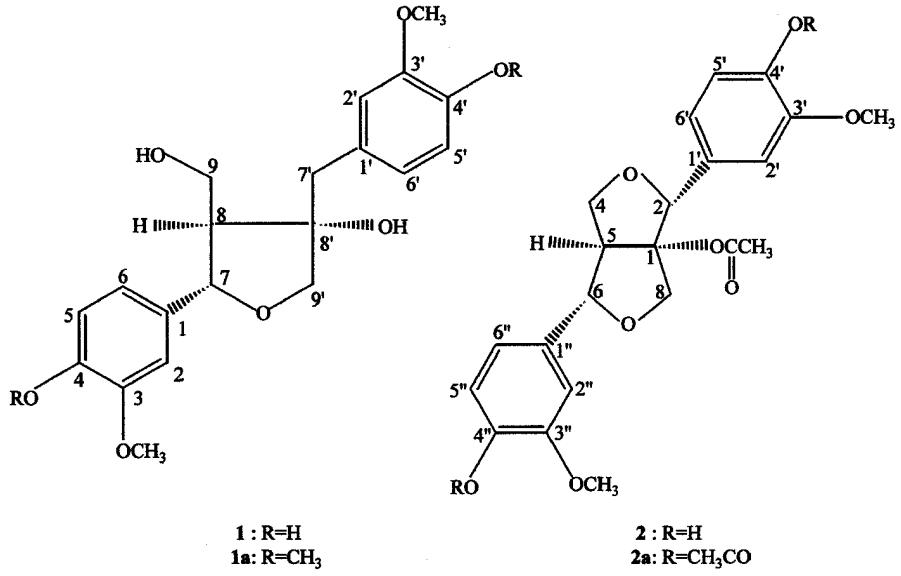

Fig. 1. Structures of Active Compounds **1** and **2**.

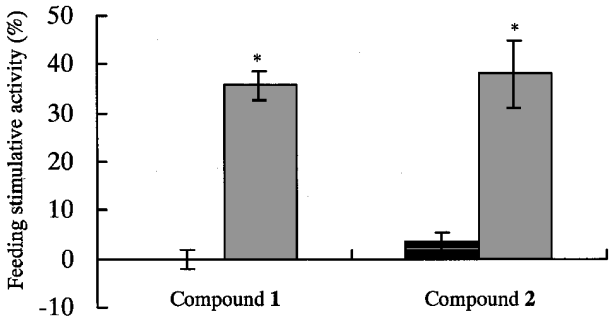

Fig. 2. Feeding Stimulative Activities of Compounds **1** and **2** toward the Male and Female Weevils.

The dose of compounds **1** and **2** on each disk was 10 μg. Each bar represents the mean ± SE ($n=20$). *Significantly different from male weevils by Student's t-test ($p<0.05$). ■ Male weevil; □ Female weevil.

hydroxyl groups in the molecule was suggested by the formation of a dimethyl ether (**1a**, Fig. 1) prepared by treating **1** with diazomethane.

The ^{1}H- and ^{13}C-NMR and HMQC spectra of **1** (see the experimental section) suggested the presence of two methoxy groups (δ_H 3.81, δ_C 56.1; δ_H 3.82, δ_C 56.2), two trisubstituted benzene rings (δ_H 6.68, 6.81, 7.05 for the A ring; δ_H 6.68–6.70, 6.86 for the C ring, respectively). The ^{1}H–^{1}H COSY spectrum showed that a methine proton (δ_H 2.35, H-8) was coupled with an oxygen-bearing methine proton (δ_H 4.65, H-7) and hydroxymethylene protons (δ_H 3.71 and δ_H 3.80, H-9a, b). Furthermore, in the HMBC spectrum, a cross peak between the proton signal (δ_H 4.65, H-7) and two aromatic methine carbons (δ_C 111.3 and 121.3; C-2 and C-6 respectively) on benzene ring A indicated the C_6-C_3 unit.

On the other hand, in the HMBC spectrum of **1**, pairs of geminal proton signals (δ_H 2.90, 3.00, H-7′a, b; and δ_H 3.60, 3.80, H-9′a, b) of two methylene groups showed correlation with the quaternary carbon signal at δ_C 82.6 (C-8′) with which the methine proton (δ_H 2.35, H-8) also showed correlation. The HMBC spectral data also showed correlation of the geminal proton signals at δ_H 2.90 and 3.00 with two aromatic methine carbons at δ_C 115.1 and 124.0 (C-2′ and C-6′). These data indicated another C_6-C_3 unit. In addition, in the HMBC spectrum of **1**, the methylene proton (δ_H 3.60, H-9′a) showed correlation with the methine carbon signal at δ_C 86.1 (C-7) for forming the five-membered ring structure of this molecule. Based on these data and a comparison with data in the literature,[3-5] active compound **1** was identified as 4,4′,8′,9-tetrahydroxy-3,3′-dimethoxy-7,9′-cyclolignan, (−)-olivil (Fig. 1), which is a known as a minor lignan.

The HREIMS data of active compound **2** gave a molecular ion at m/z 416.1469, revealing its molecular formula as $C_{22}H_{24}O_8$, which is consistent with the ^{1}H- and ^{13}C-NMR data. The IR (3370, 1607, 1520 and 824 cm^{-1}) and the UV (λ_{max} 230 and 280 nm) spectra and positive $FeCl_3$ test also suggested the presence of phenolic groups and a trisubstituted benzene ring(s), as well as the ester carbonyl group (1738 cm^{-1}). The ^{1}H-NMR spectrum of the acetate (**2a**, Fig. 1) prepared by treating **2** with acetic anhydride/pyridine showed the presence of two hydroxyl groups.

The ^{1}H- and ^{13}C-NMR data of compound **2** correlated well with the data published by Owen *et al.*[6] Furthermore, the EIMS data of compound **2** were identical in all respects to those published.[7] Based on these results, the structure of **2** was identified as (+)-1-acetoxypinoresinol which is also a lignan like active compound **1**.

Figure 2 shows the feeding stimulant activities of compound **1** and **2** toward male and female olive

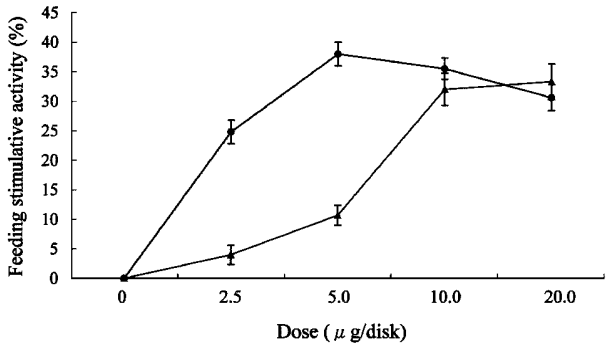

Fig. 3. Effect of the Dose of Active Compounds **1** and **2** on Female Weevils.

Each bar represents the mean ± SE ($n=25$). ▲ Active compound **1**; ● Active compound **2**.

weevils. Both compounds showed potent feeding stimulant activity toward the female at a dose of 10 μg/disk (35.6% for **1** and 38.0% for **2**, respectively). Figure 2 also shows that the activity was significantly higher for the female than for the male weevil ($p<0.05$). However, the activity for the male was not significant when compared with the control disk. Figure 3 shows the dose dependence of compounds **1** and **2** on the feeding stimulant activity of female weevils. The result shows that the activity was dependent on the dose of **1** on the paper disk in the range of zero-20 μg/disk, increasing with a gentle slope to a dose of 5 μg/disk, then by a steep slope between 5 and 10 μg, and finally becoming almost constant or saturated. In the case of active compound **2**, the maximum activity was attained at 5 μg/disk after a rapid increase, this being followed by a slow decrease to the same activity (~33%) as that of compound **1**. Although a significant difference in their activity profile was observed, the feeding stimulation was found to be totally dose-dependent.

The bark of the olive tree has been traditionally used in the Orient as an antipyretic, antirheumatic, tonic and remedy for scrofula,[8] while olive oil and the fruits are very important food products nowadays. Our problem for the cultivation of this plant in Japan is that the olive trees have been attacked by the olive weevils, a typical insect pest of this plant.

It is well known that plants have a chemical defense systems.[9,10] One method seems to be the synthesis of lignans when being attacked by pests. Lignans and neolignans, which are produced through a biosynthetic pathway starting from *E*-coniferyl alcohol,[11] are a widely distributed and structually diverse phytochemical class. Most of them and their intermediate products exhibit various biological activities.[12] For example, 7′-hydroxymatairesinol inhibits fungal growth,[13] (+)-5′-demethoxyepixcelsin, anti-HIV activity,[14] and (−)-trachelogemin, an antiviral property.[15] Some acyclic and furofuran lignans with methylenedioxy phenyl groups such as sesamin have a synergistic effect on a range of insecticides.[16] Furthermore, kobusin and sesamin have been shown to inhibit the growth of silkworm (*Bombyx mori*) larvae.[17] As an another example, (−)-parabenzlactone, one of the piperolignanolides, from *Parabenzoin trilobum* Nakai is known to suppress the feeding activity of insects.[18] Thus, lignan biosynthesis plays important roles in the host plant defense system.

In our present study, however, the two active compounds, **1** and **2**, which also had a lignan structure, did not show any defensive role against the insect pest, but strongly stimulated their feeding behavior (Fig. 2).

In fact, as shown in Fig. 2, isolated compounds **1** and **2** showed 30–40% feeding stimulant activity toward female weevils in particular, although in the case of males, the activity was not significant. Figure 3 show that, in the case of the female, the feeding activity respectively increased with increasing dose of active compounds **1** and **2**.

Although we do not have a reasonable explanation for this clear difference, these two compounds appear to play an important role in the preferential stimulation by olive trees of the female weevil. This result seems to be the first example in which feeding stimulant activity was observed in compounds having a lignan structure. No report describing the same phenomenon has so far been found.

The activity of the two compounds in the lignan family discovered in the present study might be applied to attract the olive weevils to a limited area where the compounds are provided as a dummy.

Experimental

General procedure. All the NMR experiments were conducted with a Varian VXR500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C). High-resolution electron ionization mass spectra (HREIMS) were recorded by a JEOL SX-102A mass spectrometer, IR (KBr) spectra were measured by a JASCO FT/IR-5000 spectrometer, optical rotation values were determined with a JASCO Dip-360 digital polarimeter, and UV spectra were measured by a Shimadzu UV-3000 spectrophotometer.

Insect material. The weevils were field collected as newly emerged adults from infested olive trees in August 2000 and July 2001. Male and female weevils were separately reared in plastic containers (27 cm × 20 cm × 13 cm) with a piece of young branch from an olive tree (*ca.* 20 cm length, 5 mmϕ) and wet cotton under gregarious conditions at 25°C with a 12L:12D photoperiod.

Plant material. The olive tree (*Nevadillo Blanco*)

was obtained from Nippon Olive Co. Ltd. in April 2001. It was cut into pieces for extraction.

Extraction and purification of the plant material. Olive trunk sections (9.1 kg) were extracted with MeOH (26.4 l) at room temperature (r.t.) for 7 days. The extract was filtered, concentrated under reduced pressure and partitioned first with hexane (500 ml × 3) and then with EtOAc (500 ml × 3) to obtain hexane-soluble (2.6 g), EtOAc-soluble (160 g) and aqueous (547 g) fractions. The EtOAc fraction was separated by column chromatography on silica gel 60 (Nacalai Tesque, 230–400 mesh), successively eluting with hexane (100%)→hexane:EtOAc = 7:3→1:1→EtOAc (100%)→MeOH (100%). The feeding stimulatory activity was found in the MeOH fraction. This fraction was further separated in the silica gel column, eluting with $CHCl_3$:MeOH = 4:1, and then in another silica gel column, eluting with $CHCl_3$:MeOH = 10:0.5→10:1, to afford two active fractions (Fr. a and Fr. b). Fr. a (2.1 g) was further purified by preparative TLC on silica gel with EtOAc:MeOH = 10:1, and then by preparative TLC on silica gel with $CHCl_3$:MeOH = 10:1 to give active compound **1** (colorless needles, 40.5 mg, 4.5 μg/g of olive). Fr. b (10.9 g) was separated by column chromatography (silica gel, 136 g), eluting with $CHCl_3$:MeOH = 10:0.5, and then by eluting with hexane:EtOAc = 1:1→3:7→0:100. Active compound **2** (66.8 mg, 7.3 μg/g of olive) was obtained as an amorphous solid.

Active compound 1. Mp 111–115°C from EtOAc-MeOH; $[\alpha]_D^{20}$ −41.3° (*c* 0.91, MeOH); EIMS *m/z* (rel. int.): 376 $[M]^+$ (2), 196 (16), 180 (9), 151 (6), 137 (100), 122 (13). HREIMS *m/z* $[M]^+$: calcd. for $C_{20}H_{24}O_7$, 376.1522; found, 376.1525; UV λ_{max} (EtOH) nm (ε): 230 (1.2×10^3), 280 (4.5×10^2); IR ν_{max} (KBr) cm^{-1}: 3400 (–OH), 1605, 1520, 1456, 824 (aromatic); NMR δ_H (500 MHz, CD_3OD): 2.35 (1H, q, *J* = 7.6, 5.5 Hz, H-8), 2.90 (1H, d, *J* = 14.0 Hz, H-7′), 3.00 (1H, d, *J* = 14.0 Hz, H-7′), 3.60 (1H, d, *J* = 9.5 Hz, H-9′), 3.71 (1H, dd, *J* = 11.3, 5.5 Hz, H-9), 3.80 (2H, m, H-9,9′), 3.81 (3H, s, $-OCH_3$), 3.82 (3H, s, $-OCH_3$), 4.65 (1H, d, *J* = 7.6 Hz, H-7), 6.68 (1H, d, *J* = 7.9 Hz, H-5), 6.68–6.70 (2H, m, H-5′,6′), 6.81 (1H, dd, *J* = 7.9, 1.8 Hz, H-6), 6.86 (1H, d, H-2′), 7.05 (1H, d, *J* = 1.8 Hz, H-2); NMR δ_C (125 MHz, CD_3OD): 40.6 (C-7′), 56.1 ($-OCH_3$), 56.2 ($-OCH_3$), 60.7 (C-9), 61.5 (C-8), 77.7 (C-9′), 82.6 (C-8′), 86.1 (C-7), 111.3 (C-2), 115.1 (C-2′), 116.9 (C-5′), 117.2 (C-5), 121.3 (C-6), 124.0 (C-6′), 127.9 (C-1′), 131.1 (C-1), 149.5 (C-4′), 149.7 (C-4), 150.7 (C-3′), 152.7 (C-3).

Active compound 2. $[\alpha]_D^{20}$ +29.2° (*c* 1.01, EtOH); EIMS *m/z* (rel. int.): 416 $[M]^+$ (13), 358 (1), 221 (9), 207 (16), 205 (15), 204 (100), 189 (7), 173 (6), 163 (9), 152 (16), 151 (33), 137 (19), 131 (34), 103 (17); HREIMS *m/z* $[M]^+$: calcd. for $C_{22}H_{24}O_8$, 416.1471; found, 416.1469; UV λ_{max} (EtOH) nm (ε): 230 (1.2×10^4), 280 (4.4×10^3); IR ν_{max} (KBr) cm^{-1}: 3370 (–OH), 1738 (C=O), 1607, 1520, 824 (aromatic); NMR δ_H (500 MHz, $CDCl_3$): 1.70 (3H, s, CH_3CO_2-), 3.32 (1H, dt, *J* = 4.9, 7.6, H-5), 3.77 (1H, dd, *J* = 4.9, 9.4, H-4b), 3.88 (3H, s, $-OCH_3$), 3.91 (3H, s, $-OCH_3$), 4.23 (1H, d, *J* = 10.7, H-8b), 4.42 (1H, dd, *J* = 7.6, 9.4, H-4a), 4.43 (1H, d, *J* = 10.7, H-8a), 4.74 (1H, d, *J* = 4.9, H-6), 5.06 (1H, s, H-2), 5.74 (2H, –OH × 2), 6.85–6.86 (3H, m, H-2′, 5′, 6′), 6.88 (1H, dd, *J* = 1.5, 7.9, H-6″), 6.91 (1H, d, *J* = 7.9, H-5″), 6.96 (1H, d, *J* = 1.5, H-2″); NMR δ_C (125 MHz, $CDCl_3$): 20.9 ($\underline{C}H_3CO_2-$), 55.9 (3′, 3″–OCH_3), 58.8 (C-5), 69.8 (C-4), 75.2 (C-8), 85.8 (C-6), 87.0 (C-2), 97.2 (C-1), 108.9 (C-2″), 111.6 (C-2′), 113.9 (C-5′), 114.3 (C-5″), 119.3 (C-6″), 121.4 (C-6′), 128.3 (C-1′), 131.9 (C-1″), 145.5 (C-4″), 145.7 (C-4′), 146.2 (C-3′), 146.7 (C-3″), 169.5 ($CH_3\underline{C}O_2-$).

Treatment of active compound 1 with diazomethane. Active compound **1** (12.5 mg) was treated with diazomethane in ether at r.t. for 24 hr. The dimethylether (**1a**, 2.1 mg) of **1** was obtained by silica gel column chromatography, eluting with $CHCl_3$ and then with $CHCl_3$:MeOH = 10:1. EIMS *m/z*: 404 $[M]^+$, 151; NMR δ_H (500 MHz, $CDCl_3$): 2.52 (1H, q), 2.98 (1H, d), 3.08 (1H, d), 3.70 (1H, d), 3.87 (3H, s, $-OCH_3$), 3.88 (3H, s, $-OCH_3$), 3.88 (3H, s, $-OCH_3$), 3.89 (3H, s, $-OCH_3$), 3.86–3.90 (2H, m), 3.92 (1H, dd), 4.74 (1H, d), 6.83 (1H, d), 6.83–6.85 (3H, m), 6.93 (1H, dd), 7.26 (1H, d).

Acetylation of active compound 2. Active compound **2** (3.3 mg) was acetylated with acetic anhydride-pyridine at r.t. for 12 hr, the reaction mixture being worked up in the usual mannar, and the acetate (**2a**) was isolated by column chromatography. NMR δ_H (500 MHz, $CDCl_3$): 1.69 (3H, s, CH_3CO_2-), 2.30 (3H, s, CH_3CO_2-), 2.32 (3H, s, CH_3CO_2-), 3.35 (1H, dt), 3.67 (1H, dd), 3.83 (3H, s, $-OCH_3$), 3.86 (3H, s, $-OCH_3$), 4.31 (1H, d), 4.47 (1H, dd), 4.50 (1H, d), 4.82 (1H, d), 5.11 (1H, s), 6.90–6.93 (3H, m), 6.95 (1H, dd), 6.99 (1H, d), 7.04 (1H, d).

Bioassay. Male and female insects were preconditioned in separate Petri dishes (40 mmϕ) containing moistened paper disks (Advantec Toyo No. 2, 5 mmϕ) and were given distilled water every 12 hr for 24 hr. After the 24 hours of starvation, the moistened paper disks were replaced with paper disks containing sucrose and the active compounds. The paper disks were applied uniformly with a methanolic solution of each sample and an aqueous solution of sucrose (5 mg/10 μl) and then air dried. A paper disk with MeOH and sucrose was used as a control. The Petri dishes were kept at 25°C with a 12L:12D pho-

toperiod for 48 hr. Distilled water at 20 μl/disk was added every 12 hr. Each test was repeated 3–5 times. The extent of feeding was evaluated by measuring the area of the disk bitten, and is expressed by a score from zero to +3, in which zero is for no biting, +1 is for a bitten track being found, +2 is for less than 50% bitten and +3 is for more than 50%. The feeding stimulative activity is defined as $[(A-B)/(+3\times C)]\times 100(\%)$, where A = the total score of the sample disks, B = the total score of the control disks, and C = number of insects used. The results of the test were analyzed by Students' *t*-test.

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