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Isolation and Identification of Insecticidal Components from *Citrus aurantium* Fruit Peel Extract

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Three active components were identified by bioassay-guided fractionation of bitter orange (*Citrus aurantium* L.) fruit peel petroleum ether extract. Silica gel fractionation of the extract yielded a fraction that inflicted up to 96% mortality to adults of the olive fruit fly *Bactrocera oleae* (Gmelin) three days post-treatment. Subsequent HPLC purification of the active fraction resulted in the isolation of three components, eluted in fractions F_{222}, F_{224}, and F_{226}, that induced adult mortality. Considering the data obtained from UV, FTIR, MS, and ^{1}H NMR spectra, they were identified as 7-methoxy-8-(3′-methyl-2′-butenyl)-2*H*-1-benzopyran-2-one (osthol), 4-methoxy-7*H*-furo[3,2-*g*]benzopyran-7-one (bergapten), and 4-((*E*)-3′-methyl-5′-(3″,3″-dimethyloxiran-2″-yl)pent-2′-enyloxy)-7*H*-furo[3,2-*g*][1]benzopyran-7-one (6′,7′-epoxybergamottin). Our results are in concordance with those reported in the literature and were further verified by direct comparison to authentic components. 6′,7′-Epoxybergamottin was toxic when tested individually, while bergapten and osthol were found to act synergistically to 6′,7′-epoxybergamottin.

KEYWORDS: Bitter orange; *Citrus aurantium*; HPLC; coumarin; furanocoumarins; plant insecticides; fruit flies; *Bactrocera oleae*

INTRODUCTION

Peel and seed oils from several citrus plants contain chemicals that exhibit insecticidal and antifungal activity (*1–3*). Limonoids, extremely bitter chemicals present in citrus seeds, act as antifeedant or exhibit antagonistic to ecdysone action in many insect species (*4, 5*). Four major components have been isolated from lemon peel oil: 5,7-dimethoxy-2*H*-1-benzopyran-2-one, 9-[(3,7-dimethyl-2,6-octadienyl)oxy]-7*H*-furo[3,2-*g*][1]benzopyran-7-one, 4-[(3,7-dimethyl-2,6-octadienyl)oxy]-7*H*-furo[3,2-*g*][1]-benzopyran-7-one, and 5-[(3,7-dimethyl-2,6-octadienyl)oxy]-7-methoxy-2*H*-1-benzopyran-2-one. The blend of those compounds exhibited insecticidal activity against the adults of the beetle species *Sitophilus oryzae* L. and *Callosobruchus maculatus* (F.) (*6*). Recent studies reported that flavones and some phenolic compounds in certain citrus essential oils exhibit insecticidal activity (*7, 8*).

Bitter orange (*Citrus aurantium* L.) fruits have been reported to possess antibacterial, antifungal, and pharmacological properties (*9*). We have recently demonstrated that ripe *C. aurantium* peels homogenized in methanol and separated using solvents of increasing polarity exhibit strong insecticidal activity against the olive fruit fly *Bactrocera oleae* (Gmelin) (*10*).

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The objectives of the present study were (a) to isolate and identify the biologically active chemicals present in the bitter orange peels and (b) to test the effect of the isolated fraction(s) and the synthetic compound(s) on the viability of *B. oleae* adults.

MATERIALS AND METHODS

Chemicals. All chemicals used in the study, such as petroleum ether (PE), dichloromethane (DCM), *n*-hexane (Hex), ethyl acetate (EtOAc), and methanol (MeOH), were analytical grade. Sodium sulfate (Na_2SO_4) was purchased from Merck and 3-chloroperbenzoic acid (MCPBA) from Fluka (Buchs, Switzerland). The deuterated chloroform-*d* ($CDCl_3$) and the synthetic bergapten were purchased from Sigma-Aldrich Corp. (St. Louis, MO). The synthetic bergamottin was purchased from Carl-Roth, (Karlsruhe, Germany) and the synthetic osthol sample was kindly donated by Dr. M. R. Berenbaum.

Plant Material. Fruits were collected in September 2006 from *C. aurantium* trees located in the Zografos area of Athens, Greece. They were immediately transferred to the laboratory and washed in tap water containing 0.01% detergent using a soft brush, rinsed with tap water, and finally rinsed with distilled water. Peels were excised from 60 fruits using a razor blade, which left the white spongy portion (albedo) on the fruit.

Extraction of Fruit Peels. Fresh peels (300 g) were homogenized with 600 mL of petroleum ether for 5 min in a blender. The homogenate was placed in a 2 L Erlenmeyer flask. Three hundred milliliters of petroleum ether were added in the flask and further extracted three times for 10 min each in an ultrasonic bath at room temperature. The

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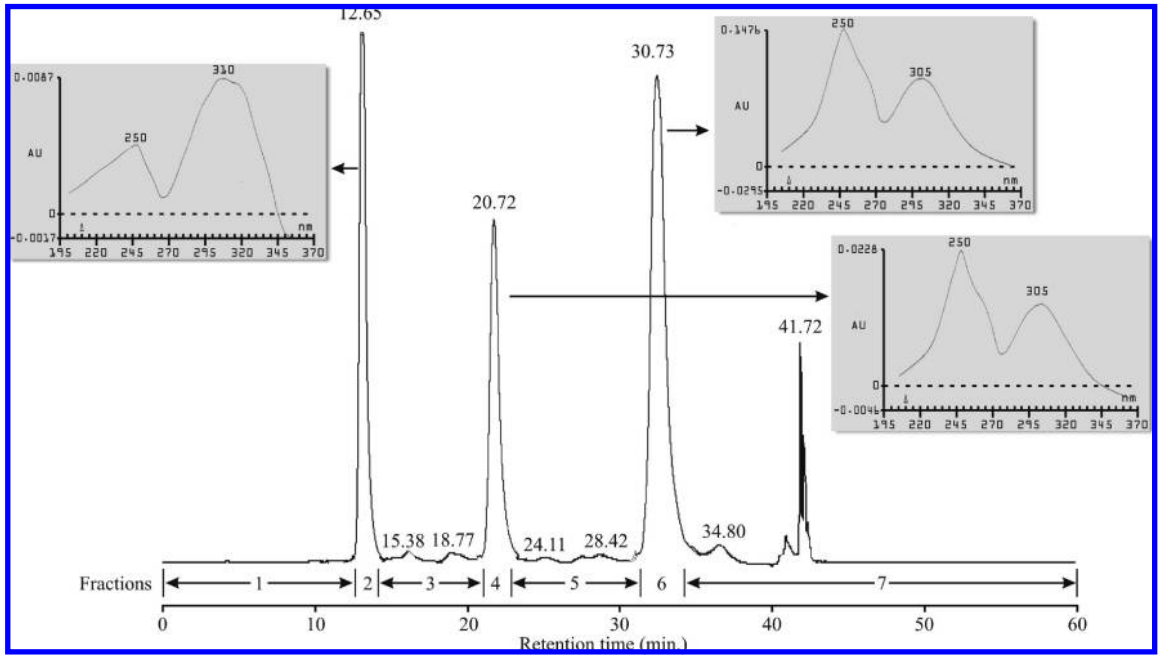

Figure 1. HPLC profile of the F_{22} fraction from silica gel column of the petroleum ether fruit peel extract of *C. aurantium* and UV absorbance wavelength spectra of (**A**) the coumarin osthol and the furanocoumarins (**B**) bergapten and (**C**) 6′,7′-epoxybergamottin, isolated from *C. aurantium* fruit peel extracts.

extract was filtered and evaporated under vacuum at 35 °C to dryness. The residue (2.22 g) was collected in 5 mL of DCM and stored at −20 °C until use.

Flash Chromatography. The petroleum peel extract was fractionated on a 500 × 45 mm glass column, packed with 200 g of silica gel (0.04−0.063 mm, 230−400 mesh; Macherey-Nagel, GmbH & Co. KG, Postfach, Germany) suspended in dichloromethane. A portion of the petroleum ether peel extract equivalent to 2 g in 20 mL of dichloromethane was introduced onto the column. Three fractions were collected by sequentially eluting the column with 1 L each of, dichloromethane (DCM-F_1 fraction, 542 mg), ethyl acetate (EtOAc-F_2 fraction, 1254 mg), and methanol (MeOH-F_3 fraction, 138 mg).

The biologically active (EtOAc-F_2) fraction was further fractionated. The same glass column packed with 100 g of silica gel (suspended in 20% EtOAc in Hex). One gram of the EtOAc-F_2 fraction dissolved in 50 mL of 20% EtOAc in Hex was introduced onto the column. Four fractions were collected by sequentially eluting the column with 500 mL each of, 20% EtOAc in Hex (F_{21} fraction, 41 mg), 40% EtOAc in Hex (F_{22} fraction, 243 mg), 60% EtOAc in Hex (F_{23} fraction, 633 mg), and 100% MeOH (F_{24} fraction, 80 mg).

HPLC Fractionation. Analyses were performed on an HPLC system equipped with a binary LC pump 250, an LC-235 diode array detector (Perkin-Elmer, Norwalk, CT), and the Simple peak 202 Data System (SRI Instruments, Torrance, CA). The fractionation was carried out on a 250 mm × 10 mm i.d. 10 μm LiChrospher 100 CN column (Merck KGaA, Darmstadt, Germany), supplied with a 10 mm precolumn packed with the same stationary phase. The mobile phase consisted of *n*-hexane (solvent A) and ethyl acetate (solvent B). The gradient program commenced at 90:10 (A:B) for 35 min, followed by a linear gradient from A:B 90:10% to 100% B for 5 min and maintained at 100% B for 20 min. Prior to each sample injection, the column was re-equilibrated for 10 min at the initial conditions. The flow rate was adjusted to 4 mL/min, the column was maintained at ambient temperature, and the absorbance was monitored at 310 nm (optical bandwidth 15 nm). The active F_{22} fraction was introduced (1 mg/20 μL EtOAc per injection) onto the column via a Rheodyne 7125 injector (Rohnert Park, CA 94928) fitted with a 20 μL sample loop. Seven fractions were collected [F_{221} (18.9 mg), F_{222} (9.1 mg), F_{223} (18 mg), F_{224} (9.1 mg), F_{225} (9.4 mg), F_{226} (22.3 mg), and F_{227} 13 mg)]. One major peak was prominent in each of the fractions F_{222}, F_{224}, and F_{226}, while several minor ones were present in the other four fractions (**Figure 1**). One hundred milligrams of the F_{22} fraction was fractionated in total; the fractions were concentrated and stored at −20 °C until used.

Identification of the Major HPLC Active Fractions: UV Spectroscopy. UV spectra of fractions F_{222}, F_{224}, and F_{226} were obtained during the HPLC fractionation of the active fraction (F_{22}). They were recorded in the region of 245−365 nm and printed (GP-100 Graphics Printer, Perkin-Elmer) (**Figure 1**).

GC−FTIR. Analysis of the active fractions was carried out on a Perkin-Elmer Autosystem gas chromatograph interfaced to a Perkin-Elmer System 2000 (Perkin-Elmer Ltd., Buckinghamshire, England) Fourier transform infrared spectrometer equipped with a liquid-nitrogen cooled narrow-band (4000−750 cm^{-1}) infrared detector (mercury cadmium telluride). Helium was used as the carrier gas (2 mL/min) and the effluent from the GC mixed with the sweep gas helium (0.5 mL/min; transfer line temperature 280 °C) and passed through the IR light pipe with KBr windows. All spectra were obtained at 8 cm^{-1} resolution. All samples were chromatographed on a 30 m × 0.32 mm × 1 μm film thickness DB-5 column (J&W Scientific, Folsom, CA). The oven temperature program was 50 °C for 2 min and then 5 °C/min to 280 °C, where it was held for 60 min. Splitless injections were made (1 μL) at an injector temperature of 250 °C and a splitless period of 90 s.

GC−MS. Gas chromatography−mass spectrometry analysis of F_{222} and F_{224} fractions was carried out on a Hewlett-Packard 5890 Series II gas chromatograph interfaced to a Fisons VG Trio 1000 (Manchester M23 9BE, UK) quadrupole mass spectrometer. Electron impact ionization was used at 70 eV and a trap current of 200 μA. The interface was kept at 290 °C. All samples were chromatographed on a 60 m × 0.25 mm × 0.1 μm film thickness DB-5 column (J&W Scientific). The oven temperature was set at 50 °C for 2 min, raised to 250 at 5 °C/min, held for 1 min, raised 2 °C/min to 280 °C, and held there for 50 min. Helium was used as the carrier gas at a flow rate of 1 mL/min. Splitless injections were made (1 μL) at an injector temperature of 250 °C and a splitless period of 90 s. The mass spectrometric data for fraction F_{226} were obtained using the direct contact inlet (DCI) probe of the VG Trio 1000 mass spectrometer. The DCI was set at 0 mA for 2 min and then raised to 200 mA at 100 mA/min, held for 2 min, raised then 300 mA/min to 1000 mA, held for 4 min, and then ramped down at 200 mA/min to 0 mA, where it stayed for 5 min.

^{1}H NMR. ^{1}H NMR spectra of F_{222}, F_{224}, and F_{226} fractions were recorded on Bruker Advance DPX-400 spectrometer. $CDCl_3$ was used

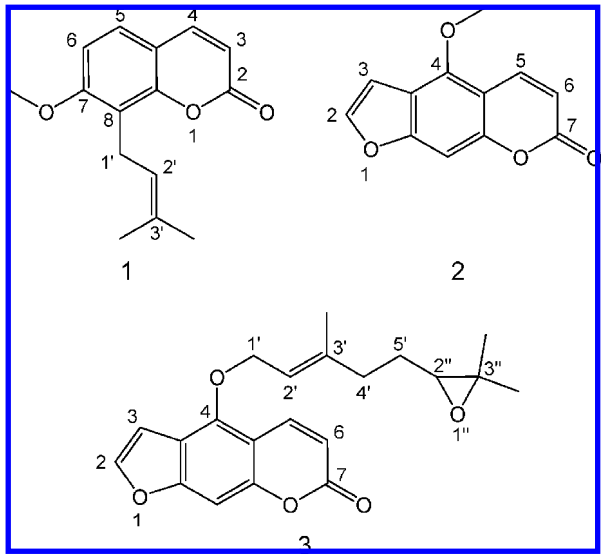

Figure 2. Chemical structures of osthol (**1**), bergapten (**2**), and 6′,7′-epoxybergamottin (**3**).

Table 1. Toxicity of *Citrus aurantium* Flash Chromatography Fractions F_2 and F_{22}; the Three Major HPLC Fractions F_{222}, F_{224}, F_{226}; and the Corresponding Synthetic Components against Olive Fruit Fly *Bactrocera oleae* Adults in a Petri Dish Exposure Bioassay 72 h Post-Treatment[a,b]

	I. concentration set to 5 μg/cm^2	II. concentration equivalent to natural presence	
fractions	% mortality	concentration (μg/cm^2)	% mortality
F_2	96 ± 3.1a	20.0	94 ± 2.4a
F_{22}	98 ± 2.0a	4.9	90 ± 3.2a
F_{222}	0 ± 0b	0.45	2 ± 2c
F_{224}	0 ± 0b	0.45	2 ± 2c
F_{226}	100 ± 0a	1.12	46 ± 6.8b
F_{222} + F_{224} + F_{226}		0.45 + 0.45 + 1.12	80 ± 3.2a
6′,7′-epoxybergamottin		1.12	48 ± 7.3b
osthol + bergapten + 6′,7′-epoxybergamottin		0.45 + 0.45 + 1.12	74 ± 8.1a
control	0 ± 0b		2 ± 2c

[a] Data were transformed into arcsine$\sqrt{p}$ value before ANOVA and Tukey's studentized range honestly significant difference (HSD) test. Values followed by the same letter within a column are not significantly different at the 0.05 level by HSD test. SE = standard error of mean. [b] Concentrations of all fractions were selected according to their participation in the preceding purification step, respectively. Concentrations of synthetic compounds were equal to those of their corresponding HPLC fractions.

as solvent. Tetramethylsilane or residual solvent peaks (e.g., chloroform) were used as frequency standards. Coupling constants were determined using the computer program Multiplet and are quoted in hertz (Hz). Values were reported to 0.1 Hz, but had an uncertainty of ca. ±0.3 Hz (at 400 MHz), due to the digital resolution of the FID accumulation and Fourier transformation. ^{1}H NMR spectra were simulated using RACCOON.

Synthesis of 6′,7′-Epoxybergamottin. We synthesized 6′,7′-epoxybergamottin using synthetic bergamottin and 3-chloroperbenzoic acid (MCPBA) (*11, 12*). The synthetic 6′,7′-epoxybergamottin was HPLC purified using the same chromatographic conditions as the natural isolate from the peel extract.

Component 1: Osthol ($C_{15}H_{16}O_3$). Component 1 was obtained as a white powder, mp 83−84 °C. UV (λ_{max}): (250, 310). GC−MS *m/z* (% intensity): 244 (100), 229 (77), 213 (48), 201 (72), 189 (81), 159 (40), 131 (62). IR (cm^{-1}): 3000, 2800, 1770, 1600, 1450, 1300, 1050. ^{1}H NMR δ_H ($CDCl_3$, 400 MHz): 7.55 (1H, d, J = 9.4 Hz, 4-H), 7.20 (1H, d, J = 8.6 Hz, 5-H), 6.77 (1H, d, J = 8.6 Hz, 6-H), 6.17 (1H, d, J = 9.4 Hz, 3-H), 5.16 (1H, t, J = 7.2 Hz, 2′-H), 3.85 (3H, s, 6′-H), 3.45 (2H, d, J = 7.2 Hz, 1′-H), 1.78 (3H, s, 4′-H), 1.59 (3H, s, 5′-H). The UV, IR, and GC−MS spectra were similar to that reported for osthol isolated from the wild celery *Apium graveolens* and the nonvolatile fraction of the grapefruit essential oil (*13, 14*).

Component 2: Bergapten ($C_{12}H_8O_4$). Component 2 was obtained as white needles, mp 188−191 °C. UV (λ_{max}): (250, 305). GC-MS *m/z* (% intensity): 216 (100), 201 (35), 188 (15), 173 (64), 145 (33). IR (cm^{-1}): 3000, 2800, 1770, 1600, 1450, 1300, 1050. ^{1}H NMR δ_H ($CDCl_3$, 400 MHz) 8.14 (1H, d, J = 9.9 Hz, 5-H), 7.57 (1H, d, J = 2.4 Hz, 2-H), 7.11 (1H, s, br, 9-H), 7.01 (1H, dd, J = 2.4 and 0.8 Hz, 3-H), 6.27 (1H, d, J = 9.9 Hz, 6-H), 4.27 (3H, s, 1′-H). The ^{1}H NMR and the GC−MS spectra was consistent with that reported for bergapten isolated from *Glehnia littoralis* (Umbelliferae) and from *Euodia borbonica* (Rutaceae) (*15, 16*).

Component 3: 6′,7′-Epoxybergamottin ($C_{21}H_{22}O_5$). Component 3 was obtained as white needles, mp 55−56 °C. UV (λ_{max}): (250, 305). GC−MS *m/z* (% intensity): 202 (69), 174 (42), 153 (30), 145 (27), 71 (100). IR (cm^{-1}): 3000, 2800, 1770, 1600, 1450, 1300, 1050. ^{1}H NMR δ_H ($CDCl_3$, 400 MHz) 8.09 (1H, d, J = 9.9 Hz, 5-H), 7.53 (1H, d, J = 2.5 Hz, 2-H), 7.09 (1H, s, 9-H), 6.88 (1H, dd, J = 2.5 and 0.9 Hz, 3-H), 6.21 (1H, d, J = 9.9 Hz, 6-H), 5.52 (1H, m, 2′-H), 4.89 (2H, d, J = 6.9 Hz, 1′-H_2), 2.64 (1H, dd, J = 7.0 and 5.4 Hz, 6′-H), 2.15 (2H, m, 4′-H), 1.67 (3H, s, 10′-H), 1.55 (2H, m, 5′-H), 1.25 (3H, s, 9′-H), 1.23 (3H, s, 8′-H). The ^{1}H NMR spectrum of 6′,7′-epoxybergamottin was consistent to that reported for 6′,7′-epoxybergamottin isolated from *Citrus macroptera* and that from grapefruit juice (*11, 17*).

Insects. Adult olive fruit flies *B. oleae*, were obtained from an artificially reared colony maintained in our laboratory at NCSR "Demokritos" for many generations. All the insects' stages were kept under the same rearing conditions: 25 ± 2 °C, 65 ± 5% relative humidity, and 12:12 light:dark regime.

Toxicity Studies. The Petri dish exposure bioassay described elsewhere (*10*) with slight modifications was used to evaluate the insecticidal activity of the fractions eluted from the silica gel and the HPLC columns and their blends, plus the three synthetic active components. The tested concentrated samples diluted in 1 mL of dichloromethane plus olive oil (0.65% v/v) were prepared. Groups of 5 pairs of 2−3-day-old flies of *B. oleae* were chill-immobilized for 5 min and subsequently introduced into each of the treated Petri dish and exposed for 12 h. Control Petri dishes were treated in a similar manner with dichloromethane plus olive oil only. Five replicates of 10 flies were employed for each treatment and the control.

Statistics. Mortality data were analyzed by one-way analysis of variance (ANOVA). Raw mortality data were transformed to arcsine$\sqrt{p}$ before ANOVA analysis, where p is the proportion of dead insects, in order to equalize the variance among treatments. Comparisons and separations of means were performed by Tukey's honestly significant difference (Tukey's HSD) test (*18*) at P = 0.05. SPSS 8.0 software (SPSS Inc., Chicago, IL) was used for our statistical analyses.

RESULTS AND DISCUSSION

Isolation and Identification of the Active Compounds. The fractions EtOAc-F_2 and 40% EtOAc in Hex (F_{22}) from the two flash chromatographic steps induced a substantial mortality level to the olive fruit flies. Further HPLC fractionation of the F_{22} fraction yielded three active components (fractions F_{222}, F_{224}, and F_{226}) (**Figure 1**). When these three fractions were tested as a blend on the flies (in the same proportions as they were in fraction F_{22}: 0.45 + 0.45 + 1.12 μg/cm^2) high mortality rates were recorded. The chemical structures of the compounds in F_{222}, F_{224}, and F_{226} were assigned as 7-methoxy-8-(3′-methyl-2′-butenyl)-2*H*-1-benzopyran-2-one (osthol) in F_{222}, 4-methoxy-7*H*-furo[3,2-*g*]benzopyran-7-one (**bergapten**) in F_{224}, and 4-((*E*)-3′-methyl-5′-(3″,3″-dimethyloxiran-2″-yl)pent-2′-enyloxy)-7*H*-furo[3,2-*g*][1]benzopyran-7-one (**6′,7′-epoxybergamottin**) in F_{226} (**Figure 2**).

Toxicity Studies. The active fractions F_2 and F_{22} eluted from the silica gel columns were tested and attained a 96% (EtOAc-F_2) and 98% (F_{22}) mortality 72 h after treatment at

a concentration of 5 μg/cm^2 (**Table 1.I**). When 5 μg/cm^2 of F_{226} was tested, the same insect mortality was attained. However, when F_{226} was tested at concentrations equivalent to that of its natural presence in fraction F_{22}, a lesser degree in insect mortality was achieved (**Table 1.II**). 6′,7′-Epoxybergamottin (fraction F_{226}) exhibited substantial toxicity when tested individually, while the other major components, osthol in fraction F_{222} and bergapten in F_{224}, were inactive. This is the first report of the insecticidal activity of 6′,7′-epoxybergamottin. However, 6′,7′-epoxybergamottin has been reported to inhibit the activity of the enzyme cytochrome P450 3A4 (CYP3A4) (*19, 20*). Comparison of the insecticidal activity of 6′,7′-epoxybergamottin and its precursor, bergamottin, revealed that the epoxide group is essential for the insecticidal properties of 6′,7′-epoxybergamottin, since bergamottin was inactive even at high concentrations (data not shown). Although bergamottin did not show any significant effect on the olive fruit flies when tested individually in the Petri dish exposure bioassay, it has been reported to exert antifeedant activity to several species of *Lepidoptera* (*21, 22*). The coumarin osthol when tested alone did not exhibit any activity on *B. oleae* flies. However, osthol is known as an antibacterial, antimalarial, antimutagenic, cytostatic, and cytotoxic agent (*23, 24*). Also the antifeedant activity of bergapten is well-documented (*21, 22, 25*)

Mixing of the three components (osthol, bergapten and 6′,7′-epoxybergamottin) increases the adults' mortality to the level of the initial fraction (F_{22}), indicating the occurrence of a synergistic effect (**Table 1.II**). Furanocoumarins have been reported to elicit behavioral responses and physiological effects in herbivorous insects. They are repellents and feeding deterrents (*5*) as well as toxins (*26*). In addition to their inherent toxicity, they may act synergistically (*21*) or antagonistically (*27*) when mixed with other coumarins or furanocoumarins. Toxicity in most instances is greatly enhanced in the presence of UV light, suggesting that photoactivation, possibly involving DNA, is a major mechanism of toxicity (*28*). However, there are also toxic effects of furanocoumarins, presently known, that are independent of UV light (*26*).

Considering the advantages of using botanical insecticides for pest management, it can be concluded that *C. aurantium* peel extracts could have great potential for the control of insect pests, particularly taking into account the effect of synergism present in this case. It is well-established that the evolution of insect resistance to plant extracts is extremely slow, in contrast to resistance to pure compounds, which develops rapidly (*29*).

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