Isolation and Characterization of Four Major Components from Insecticidally Active Lemon Peel Extract

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Four major components of insecticidally active lemon peel oil were isolated and identified. They were (A) 5,7-dimethoxy-2*H*-1-benzopyran-2-one, (B) 9-[(3,7-dimethyl-2,6-octadienyl)oxy]-7*H*-furo[3,2-*g*]-[1]benzopyran-7-one, (C) 4-[(3,7-dimethyl-2,6-octadienyl)oxy]-7*H*-furo[3,2-*g*][1]benzopyran-7-one, and (D) 5-[(3,7-dimethyl-2,6-octadienyl)oxy]-7-methoxy-2*H*-1-benzopyran-2-one. They were bioassayed against *Sitophilus oryzae* (L.) and *Callosobruchus maculatus* (F.) by a topical application method. Compounds B–D showed weak to moderate toxicity to the test insects but were less toxic when compared to that of the original extract, while compound A was nontoxic to *C. maculatus* or slightly toxic to *S. oryzae*.

The nonvolatile fraction of lemon oil extracted from fresh peels of Eureka lemon (*Citrus limon* L. Burm. f.) was found to show high toxicity against rice weevils, *Sitophilus oryzae* (L.), and cowpea weevils, *Callosobruchus maculatus* (F.) (Su et al., 1972b; Su, 1976). Lemon peel extract also gave long-lasting protection to black-eyed peas against infestation from cowpea weevils (Su et al., 1972a). Catar (1954) found that lemon peel extract killed *Ixodes* ticks. Abbassy and co-workers (1979) demonstrated the insecticidal and synergistic properties of lemon peel oil to confused flour beetles, *Tribolium confusum* Jacquelin du Val, and granary weevils, *Sitophilus granarius* (L.). The toxicity of lemon peel liquids (with the volatile components) to red imported fire ants, *Solenopsis invicta* Buren, was reported by Sheppard (1984).

In an effort to elucidate the insect biologically active nonvolatile chemical components of lemon peel hexane extract, we isolated four major components from the extract and identified their chemical structures. Their toxicity against rice weevils and cowpea weevils was then studied.

MATERIALS AND METHODS

Extract of Lemon Peels. The extract was prepared by hexane extraction of cleaned fresh Eureka lemon peels as described by Su et al. (1972a). Fresh Eureka lemons (36) were cleaned thoroughly in the following order: washed in alcohol to remove any surface insecticides and fungicides, rinsed in tap water, washed in detergent, rinsed with tap water, and then rinsed with distilled water. The peels were obtained by knife paring, which left the white spongy (albedo) portion on the fruit. These peels, about 1-cm wide, were cut into 1- to 3-cm lengths. The peels were extracted twice with hexane in a Waring blendor. The liquid extract was collected by suction filtration, and the combined filtrate was placed in a large separatory funnel. The lower aqueous layer was discarded. The upper layer was filtered, concentrated under reduced pressure, and then lyophilized.

HPLC Determination of Major Components. A Waters Associates Model ALC/GPC 244 high-pressure liquid chromatograph with a Model 6000A pump, a U6K injector, a Model R401 differential refractometer, and a Model 440 UV detector with a 300 × 7.8 mm (i.d.) μBondapak C_{18} column (octadecyltrichlorosilane covalently bonded to 10-μm μPorasil packing) was used. Methanol–water (80:20 v/v, degassed) was used as the eluting solvent at a flow rate of 1.2 mL/min. The effluent was monitored at 254 nm, and the response was recorded on a Waters Associates data module, Model 730.

Thin-Layer Chromatographic Fractionation. For TLC fractionation, Brinkmann EM reagent, precoated silica gel F_{254}, and analytical 0.25-mm chromatoplates, 20 × 20 cm, were used. About 15–20 mg of lemon extract dissolved in 0.2 mL of methanol–isopropyl alcohol (1:1 v/v) were applied on each plate in a straight line 2.5 cm above the lower edge. Each plate was developed twice with benzene–acetone–ethyl acetate (27:1.5:1.0 v/v) and then examined under UV light (254 nm). The zones corresponding to the major HPLC peaks were collected, extracted with acetone, filtered, and concentrated.

HPLC Purification of Components. Each TLC fraction of the lemon extract was purified by the HPLC method previously described. The purified fraction was collected. The solvent was removed under reduced pressure and then lyophilized to obtain the pure material.

Insect Bioassay Method. Each purified fraction was tested with rice weevils and cowpea weevils by the method described in Su (1976). The test material was dissolved in acetone–methanol (2:1 v/v) to obtain concentrations of 35 and 15 mg/mL. Newly emerged rice weevil adults (<24 h) and cowpea weevil adults (<5 h) from the laboratory colonies were anesthetized briefly with CO_2, picked up individually with a vacuum pencil, and treated by topical application of 1 μL of solution to the dorsum of the thorax of each insect. Control insects were anesthesized and treated with 1 μL of the solvent. Each of the solutions prepared from the various fractions and the original extract were used to treat an individual group of 20 insects of each species (unsexed rice weevils; 10 males and 10 females of cowpea weevils). After treatment, insects were held in 100-mm-diameter petri dishes (5 males and 5 females/dish with cowpea weevils and 10 kernels of wheat were placed in each dish with 10 rice weevils) in a room maintained at 27 ± 1 °C and 60 ± 5% RH with fluorescent daylight under alternating 12-h light and dark cycles. The insects were examined for mortality every 24 h for 5 days. Insects that did not move when gently disturbed were considered dead.

Instrumental Analyses. A Du Pont Model 21-490B mass spectrometer was used with a direct-insertion probe at a temperature of 150–250 °C (below 70 °C for the thermally unstable compound). For compounds B and C, a probe temperature of at least 150 °C was required. Other conditions used were as follows: ion source temperature, 150 °C; ionizing voltage, 70 eV; ion source pressure 4×10^{-6} Torr; scan rate 100 s/decade from 15 to 500 amu. Infrared

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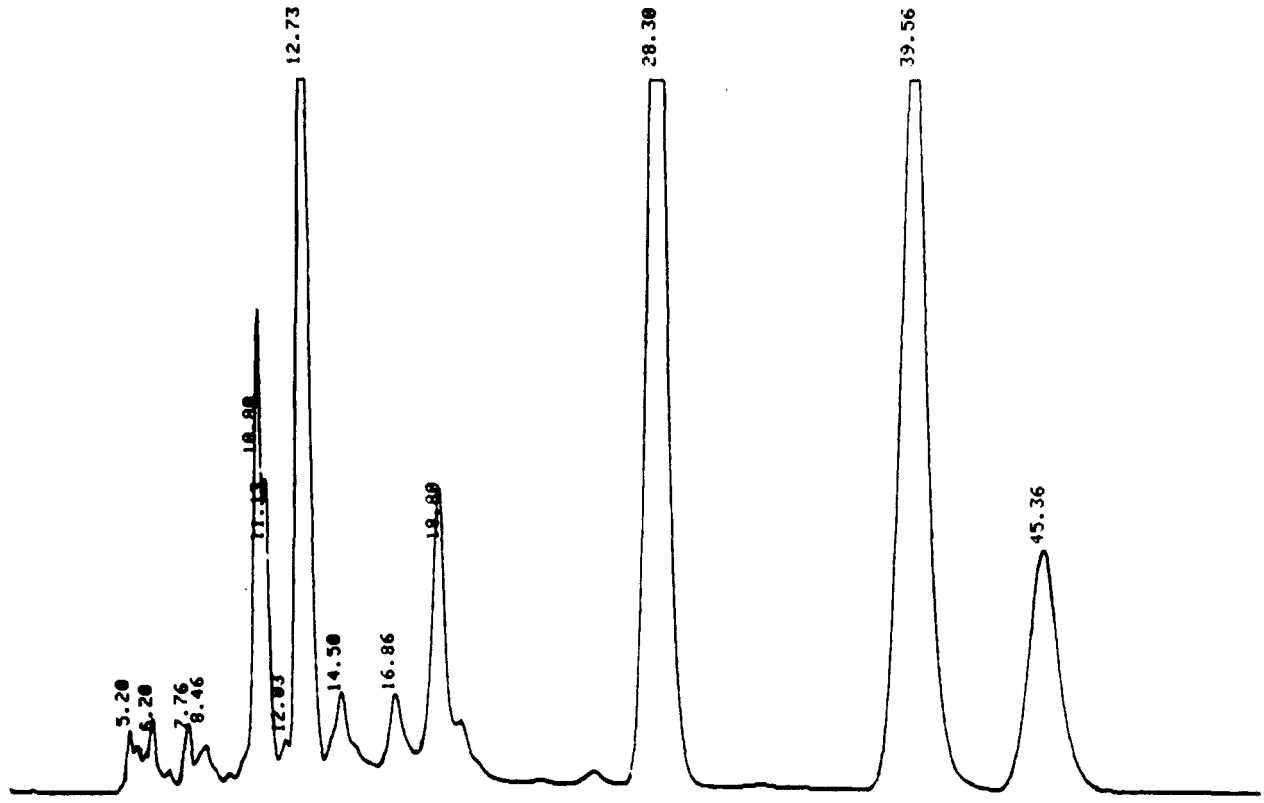

Figure 1. HPLC separation of nonvolatile hexane extract of lemon peels with μBondapak C_{18} semipreparative column, eluted by methanol–water (80:20, v/v) [RT/min, area/cm^2,%]: 5.20, 1014.89, 0.35; 6.20, 845.02, 0.29; 7.76, 1712.42, 0.60; 8.46, 1528.46, 0.54; 10.80, 13608.00, 4.80; 11.13, 7972.44, 2.81; 12.03, 918.32, 0.32; 12.73, 43746.40, 15.45; 14.50, 4470.48, 1.57; 16.86, 3081.11, 1.08; 18.80, 11485.00, 4.05; 28.30, 81285.60, 28.72; 39.56, 82659.90, 29.20; 45.36, 28658.20, 10.12.

spectra were determined as KBr pellets with an Analet FTIR Model fx6160 spectrophotometer. The UV spectra were obtained with a Varian Cary Model 210 spectrophotometer. The samples were prepared in anhydrous methanol and spectra obtained by using cells with 1-cm paths. The melting points were determined with a Fisher-Johns melting point apparatus.

Chemicals and Reagents. HPLC-grade methanol (Fisher Scientific Co.) was filtered through a Waters Associates solvent clarification kit with a 0.5-μm Millipore organic filtration system. All other solvents were reagent grade.

RESULTS AND DISCUSSION

Hexane extraction of the blended peels from 3 dozen fresh Eureka lemons yielded 1.58 g of light brown viscous liquid. HPLC of the crude extract indicated four major components (Figure 1) as follows (RT/min, percent): A, 12.73, 15.45; B, 28.30, 28.72; C, 39.56, 29.20; D, 45.36, 10.12. All other peaks were below 5% with two dominant peaks at RT 10.80 (4.8%) and 18.80 (4.05%). TLC fractionation of the crude extract obtained four fractions in zones corresponding to HPLC major compounds as follows: A, R_f 3.4–4.0; B, R_f 4.2–4.6; C, R_f 5.2–5.5; D, R_f 4.7–5.1.

Compound A was obtained as colorless needles, mp 146–148 °C. Its UV absorption in methanol showed 246 nm (ε 6060), 255 (ε 5961), and 326 (ε 14300). Strong and moderate infrared absorptions were observed (cm^{-1}) at 3086, 3014, 1612 (aryl CH), 2988, 2952, 2844 (CH_3 and CH_2), 1723 (lactone C=O), 1458, 1363, 1226, 1152 (aryl and aryl alkyl ether), 1113, 1098, and 828 (aryl multisubstitution). The mass spectrum showed the following peaks (relative peak intensity in parentheses): 206 (M^+, 100), 178 (93), 163 (70), 149 (16), 135 (49), 120 (20), 92 (22), 89 (21), 79 (15), 69 (43), 63 (15), 53 (11), and 51 (18). Compound A was identified to be 5,7-dimethoxy-2*H*-1-benzopyran-2-one (5,7-dimethoxycoumarin). It was first isolated in 1890 and named limettin and again in 1901 and named citropten from *Citrus limetta* (Dean, 1952).

Compound B was obtained as white crystals, mp 57–58.5 °C. Its UV absorption in methanol showed 218 nm (ε 27642), 243 (sh, ε 21279), 249 (ε 22368), 263 (sh, ε 13137), and 300 (ε 11395). Strong and moderate infrared absorptions were observed (cm^{-1}) at 3130, 3107 (aryl CH), 2962, 2911, 2854 (CH_3 and CH_2), 1718 (lactone C=O), 1586, 1329, 990 (–C=C–), 1437 and 1401 (*gem*-dimethyl), 1149, 1085 (aryl and aryl alkyl ether), 1026, and 874 (aryl multisubstitution). The mass spectrum showed the following peaks (relative peak intensity in parentheses): 338 (M^+, 1), 203 (23), 202 (100), 174 (22), 145 (9), 137 (36), 136 (36), 121 (22), 93 (66), 92 (35), 81 (57), 79 (37), 77 (23), 69 (53). Compound B was identified to be 9-[(3,7-dimethyl-2,6-octadienyl)oxy]-7*H*-furo[3,2-*g*][1]benzopyran-7-one (8-geranoxypsoralen).

Compound C was obtained as colorless needles, mp 58–60 °C. Its UV absorption in methanol showed 221 nm (ε 23397), 243 (sh, ε 15577), 250 (ε 17314), 259 (ε 15417), 268 (ε 14878), and 309 (ε 13045). Strong and moderate infrared absorptions were observed (cm^{-1}) at 3150, 3122 (aryl CH), 2985, 2911, 2853 (CH_3 and CH_2), 1723 (lactone C=O), 1622, 1602, 1316 (–C=C–), 1466 and 1350 (*gem*-dimethyl), 1152, 1129, 1075 (aryl and aryl alkyl ether), 1579, 805, and 748 (aryl multisubstitution). The mass spectrum showed the following peaks (relative peak intensity in parentheses): 338 (M^+, <1), 203 (17), 202 (100), 174 (35), 145 (7), 137 (18), 136 (14), 121 (10), 93 (53), 81 (46), 79 (27), 77 (21), 69 (75). Compound C was identified to be 4-[(3,7-dimethyl-2,6-octadienyl)oxy]-7*H*-furo[3,2-*g*][1]benzopyran-7-one (5-geranoxypsoralen). It was first found in bergamot oil and named bergamottin or bergaptin. Späth and Kainrath (1937) noted that bergamottin decomposed at 180–190 °C to give bergaptol, 4-hydroxy-

Table I. Toxicity of Lemon Peel Hexane Extract and Its Four Major Components Applied Topically to Rice Weevil (RW) and Cowpea Weevil (CW) at 5 Days

material	dose, μg/insect	% mortality ± SD[a,b] RW	CW
lemon peel extr	15	20.0 ± 7.07	10 ± 0
	35	52.5 ± 3.54	40 ± 0
A	15	0	0
	35	10.0 ± 0	0
B	15	20.0 ± 7.07	20 ± 0
	35	22.5 ± 3.54	15 ± 7.07
C	15	0	0
	35	12.5 ± 3.53	20 ± 14.14
D	15	20.0 ± 14.1	0
	35	20.0 ± 0	35 ± 4.07
control[c]		0	0

[a] Mean of two replicates, 20 insects/replicate. [b] Standard deviation. [c] Treated with solvent (acetone–methanol, 2:1 v/v).

7*H*-furo[3,2-*g*][1]benzopyran-7-one. We also observed this phenomenon in the studies of compounds B and C. Whether this was caused by electron impact or thermal breakdown of the original compounds in the mass spectrometer probe is still unknown.

Compound D was obtained as white shiny crystals, mp 86–87 °C. Its UV absorption in methanol showed 245 nm (ε 5909), 256 (ε 5594), and 327 (ε 10175). Strong and moderate infrared absorptions were observed (cm^{-1}) at 3091 (aryl CH), 2968, 2914, 2852 (CH_3 and CH_2), 1736 (lactone C=O), 1612, 1306 (-C=C-), 1386 and 1363 (*gem*-dimethyl), 1440, 1229, 1203, 1159, 1116 (aryl and aryl alkyl ether), 1087, and 838 (aryl multisubstitution). The mass spectrum showed the following peaks (relative peak intensity in parentheses): 328 (M^+, 2), 193 (59), 192 (100), 164 (13), 149 (19), 137 (29), 95 (13), 93 (12), 81 (63), and 69 (79). Compound D was identified to be 5-[(3,7-dimethyl-2,6-octadienyl)oxy]-7-methoxy-2*H*-1-benzopyran-2-one (5-geranoxy-7-methoxycoumarin).

Caldwell and Jones (1945) isolated A and D from West Indian lime oil (*Citrus aurantifolia*, Swingle) and determined their melting points and UV_{max}. Stanley and Vannier (1957, 1967) were able to isolate these four compounds from the cold pressed lemon oil and gave their UV_{max} and IR spectra. Fisher and Nordby (1965) isolated A and C from Florida grapefruit peel oil and studied their NMR data and partial mass data.

These four compounds were bioassayed against rice weevils and cowpea weevils by the topical application method for their contact toxicity (Table I). At the dosage of 35 μg/insect, compounds B–D were toxic to the insects, while A was toxic to cowpea weevils and slightly toxic to rice weevils. The toxicities of B–D were lower when compared to the toxicity of the original extract. The result indicated that the compounds that contain geranoxy substitution were biologically active to these insects; and synergism could be involved between several of the four compounds (A–D) or between these compounds and other minor compounds of the peel oil.

The biological activities of the coumarin and furocoumarin (psoralen) derivatives were reported. Levin (1976) and Yajima and Munakata (1979) reported previously that some coumarins and furocoumarins deterred the phytophagous insects. Späth and Kuffner (1936) showed that simple coumarins had little effect upon freshwater fish but that the more complex heterocyclic derivatives, particularly the furocoumarins, were toxic in small doses. Furocoumarin was found to be toxic to larvae of the Southern armyworm, *Spodoptere eridania* (Cramer), in the presence of UV light (Berenbaum, 1978). We also found that the furocoumarin derivatives B and C are more toxic than A to rice weevils and cowpea weevils. Ito and Kitagawa (1953) indicated that couramin compounds with low melting points between 70 and 100 °C possess good anthelmintic activity. In this study we found that compounds B–D, which contain an unsaturated geranoxy group with low melting point, are toxic to rice weevils and cowpea weevils. Compound A, which does not contain a geranoxy group and has a high melting point, is nontoxic or very slightly toxic to these insects. These data are consistent with those of Ito and Kitagawa (1953).

Registry No. A, 487-06-9; B, 71612-25-4; C, 7380-40-7; D, 7380-39-4.

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