Cotton Boll Weevil Antifeedant Activity and Antifungal Activity (*Rhizoctonia solani* and *Pythium ultimum*) of Extracts of the Stems of *Wedelia biflora*

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Extracts of the stems of the Thai plant *Wedelia biflora* (Linn.) DC (Compositae) were shown to have antifeedant activity against the cotton boll weevil (*Anthonomus grandis* Boh.). This activity also prompted an investigation of antifungal properties. Five compounds with antifeedant and/or antifungal activity were isolated. They include 16-methylkaur-15-en-19-oic acid, 24-ethylcoprostanone, stigma-7-en-3-ol, stigmasterol, grandifloric acid, and *ent*-kauradienoic acid. 16-Methylkaur-15-en-19-oic acid and grandifloric acid showed antifungal activity against the fungi *Pythium ultimum* (240% and 70%) and *Rhizoctonia solani* (280% and 78%). 24-Ethylcoprostanone and *ent*-kauradienoic acid showed antifeedant activity against the cotton boll weevil (90% and 83%).

In a continuing search for compounds with antifungal and insect antifeedant activity, the Thai plant *Wedelia biflora* (Linn.) DC (Compositae) was investigated. *W. biflora* has shown both potent insect antifeedant activity against the boll weevil (*Anthonomus grandis* Boheman) and antifungal activity against the fungus *Rhizoctonia solani*. In Thailand, this plant is used to treat headaches and fevers. In the Philippines, a decoction of the fresh roots is administered as an emmenagogue and a diuretic (Quisumbing, 1951).

The genus *Wedelia* (Compositae, Heliantheae) belongs to the subtribe Ecliptinae. Only a few species of *Wedelia* have been chemically investigated. Six eudesmanolides and two *ent*-kaurenoic acid derivatives were isolated from *W. trilobata* (Bohlmann et al., 1981a). *W. grandiflora* was investigated and yielded the compounds steiractinolides, 6α-(angeloyloxy)-1α-hydroxysteiractinolide, and pseudoguaianolides (Bohlmann et al., 1980b). Kaur-16-en-19-oic acid and 15α-(tiglinoyloxy)-kaur-16-en-19-oic acid were isolated from *W. scaberrima* (Tomassini and Matos, 1979). Atractyloside was the proposed compound toxic to mice from *W. glauca* (Oberti et al., 1980). Lewis reported the isolation, toxicity, and potential antitumor activity of wedeloside, the major toxic constituent of the plant *W. asperrima* (Lewis et al., 1981). A series of known di- and triterpenoids were characterized in the whole plant of *W. buphthalmiflora* (Schteingart et al., 1981). From the aerial parts of *W. hookeriana*, eight new eudesmanokides, all derivatives of ivangustin, and a new *ent*-kaurenic acid derivative together with its methyl ester have been isolated (Bohlmann et al., 1982). *ent*-Kauranes and 10α-methyleudesman-8αH,12-olides have been found in *W. calycina* and *W. hispida* (Herz et al., 1984).

MATERIALS AND METHODS

Plant Material. Stems of *W. biflora* were collected in Samut Sakron, Thailand. The plant material was dried and milled to a coarse powder. A voucher specimen (no. 46042) has been deposited in the Herbarium of the Royal Forest Department, Flora of Thailand.

Cotton boll weevils and dehydrated cotton bud powder were supplied by the USDA Boll Weevil Research Laboratory at Mississippi State University. Fungi *R. solani* and *Pythium ultimum* were supplied by the Rohm and Hass Co.

Sequential Solvent Extraction Procedures. Chopped and air-dried plant material (2 kg) was defatted by continuous extraction with methylene chloride (10 L) for 7 days at room temperature. The extraction was repeated four times. The solvent from the combined extracts was removed in vacuo, yielding 17 g.

Chromatography. Column chromatography was performed with silica gel (Woelm 63–200 mesh). Thin-layer chromatography (TLC) was carried out on chromatoplates also prepared with 110 °C activated silica gel FG-254, 0.25-mm layers for analytical and 1.0-mm layers for preparative TLC. The methylene chloride extract was chromatographed on silica gel. The column was eluted with mixtures of methylene chloride in hexane (20, 30, 40, 50, 60, and 70%) affording compounds 1–6, respectively.

Instrumental. Ultraviolet spectra were recorded by using a Perkin-Elmer Model 555 UV–visible spectrophotometer. Infrared spectra were recorded by using KBr pellets on a Perkin-Elmer Model 283B infrared spectrophotometer. Nuclear magnetic resonance spectra were recorded on a General Electric Nicolet Model NT-200 high-resolution spectrometer, using $CDCl_3$ as the solvent with Me_4Si as the internal standard.

Gas chromatography was performed with a Varian Model 3300 gas chromatograph with a flow rate of 30.0 mL/min nitrogen carrier gas using a J & W DB 30 fused silica megabore column (30 m × 0.535 mm id). Gas chromatography mass spectra

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(GC–MS) were performed with a Finnigan 4510 quadropole mass spectrometer interfaced with a Finnigan gas chromatograph.

Boll Weevil Antifeedant Bioassay. The agar plug bioassay feeding stimulant procedure developed by Hedin et al. (1966) was used. Agar plugs (d = 1.3 cm, l = 3.6 cm) were formed by boiling 3 g each of agar and freeze-dried cotton bud powder in 100 mL of distilled water; the mixture was poured into glass tubes to gel. Upon cooling, the plugs were extruded from the tubes and cut to the aforementioned lengths. Known quantities of the plant samples were applied to 4-cm squares of Whatman No. 1 chromatography paper. The papers were wrapped around the agar plugs and fastened with staples. Twenty newly emerged boll weevils were placed in 14 × 2 cm Petri dishes containing test and control plugs. The bioassay was carried out in the dark at 80 °F for 4 h, after which time the papers were removed and the punctures counted.

Antifeedant activity was expressed as a percent T/C value:

$$\% \ T/C = \frac{\text{no. of punctures of test paper } (T)}{\text{no. of punctures of control paper } (C)} \times 100$$

Antifungal Bioassay. The bioassay was performed by using the preliminary "paper disc" method (AOAC, 1970). Papers that were soaked in test solution were placed on growth media which were inoculated with various fungi. As the fungal mycelium grew across the culture medium, any zone of growth inhibition surrounding the discs could be detected. The same method was used to prepare the control in the standard solution.

Two fungi were used in the bioassay—*P. ultimum* and *R. solani*.

The antifungal activity was shown as a % T/C value, where

$$\% \ T/C = \frac{\text{inhibition zone radius (mm) caused by sample}}{\text{inhibition zone radius (mm) caused by control}} \times 100$$

Color Test. The Liebermann–Burchard test was used to check for steroids and triterpenoids. One milligram of the sample was dissolved in chloroform and a few drops of acetic anhydride. Then one drop of concentrated sulfuric acid was added. Development of a color after a few minutes suggests the presence of steroids or triterpenoids.

Data for Isolated Compounds. *16-Methylkaur-15-en-19-oic acid* (1). The 20% methylene chloride–hexane eluant extract was evaporated and recrystallized by using hot MeOH followed by hexane to yield 120 mg of a white crystal: mp 171–172 °C; R_f 0.568, 5% MeOH:$CHCl_3$; IR (KBr) ν_{max} 3000–2500, 1700, 1450, 1410 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 4.86 (1 H, s), 2.09 (1 H, m), 1.99 (1 H, m), 1.68 (3 H, s), 1.33 (3 H, s), 1.22 (3 H, s), 0.98 (3 H, m), 0.90 (1 H, s); MS, m/e $[M]^+$ 302, 287, 257, 91, 79. Anal. Calcd for $C_{20}H_{30}O_2$: C, 78.29; H, 9.4; M_r 302.4658. Found: C, 79.42; H 9.998. The structure of compound 1 was assigned as 16-methylkaur-15-en-19-oic acid by comparison of the physical and chemical properties for 16-methylkaur-15-en-19-oic acid (Bohlman et al., 1981b; Hayman et al., 1986).

24-Ethylcoprostanone (2). Fraction number 27 was obtained by elution with 30% methylene chloride in hexane followed by recrystallization from MeOH to yield white crystals: mp 113–114 °C; IR (KBr) ν_{max} 2900, 2840, 1700, 1460 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.8–2.2, 1.75 (3 H, s), 1.39 (3 H, s), 0.97, (3 H, d); MS, m/e $[M]^+$ 414.55, 255, 213, 119, 81. Anal. Calcd for $C_{29}H_{50}O$: C, 83.99; H, 12.57; M_r 414.713. Found: C, 83.647; H, 11.97. The structure of compound 2 was assigned as 24-ethylcoprostanone by comparison of the physical and chemical properties published for this compound in the literature (Marker and Wittle, 1937).

Stigma-7-en-3-ol (3). The 40% methylene chloride–hexane eluate was recrystallized with hexane to yield 160 mg of white needles: mp 145–146 °C; IR (KBr) ν_{max} 3500–3200, 1460, 1380 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.25 (1 H, t), 3.69 (1 H, m), 3.45 (1 H, m), 2.44 (2 H, m), 2.00–0.7 (steroid type); MS, m/e $[M]^+$ 414, 396, 329, 255. Anal. Calcd for $C_{29}H_{50}O$: C, 83.99; H, 12.15; M_r 414.713. Found: C, 83.94; H, 12.02. The structure of compound 3 was assigned as stigmast-7-en-3-ol by comparison of published physical and chemical properties (Rubinstein et al., 1976). The acetyl derivative of compound 3 was recrystallized with hexane to yield a crystalline solid: mp 129 °C.

Table I. Boll Weevil Bioassay of the Compounds from *W. biflora* (Stems)

compd	dosage, mg	% T/C	% inhibition
1	13.0	0	100
2	2.0	10	90
3	1.0	70	30
4	5.6	100	0
5	1.0	150	0
6	5.0	16.6	83.4

[a] % T/C = [no. of punctures of test paper (T)/no. of punctures of control paper (C)] × 100.

Stigmasterol (4). The fractions eluted with 50% methylene chloride–hexane were recrystallized from hexane to yield 200 mg of a white crystalline solid: mp 167–168 °C; IR (KBr) ν_{max} 3500–3300, 2900, 1400, 1360, 1040 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.44 (1 H, t), 5.27 (1 H, d), 3.72 (1 H, m), 2.00–0.7 (steroid type); MS, m/e $[M]^+$ 412, 397, 369, 255, 213, 105. Anal. Calcd for $C_{29}H_{48}O$: C, 84.40; H, 11.93; M_r 412.697. Found: C, 84.68; H, 11.93. The structure of compound 4 was assigned as stigmasterol according to IR, NMR, MS, mix mp, and co-TLC comparison with those of an authentic sample (Sigma Chemical Co., St. Louis, MO).

Grandifloric Acid (5). The 60% methylene chloride–hexane eluant was recrystallized with hexane and methanol to yield a white crystalline solid: mp 232–233 °C; IR (KBr) ν_{max} 3400–2500, 1700, 1640, 1440 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.60 (1 H, s), 5.42 (1 H, s), 5.18 (2 H, s), 2.90 (1 H, s), 2.29 (4 H, s), 2.06 (6 H, s), 1.73 (9 H, m), 1.36 (3 H, m), 1.16 (2 H, s), 1.03 (3 H, m); high-resolution mass spectrum, m/e $[M]^+$ 318.2195 ($C_{20}H_{30}O_3$), 255, 91, 83. The structure of compound 5 was assigned as grandifloric acid by comparison of the physical and chemical properties published for grandifloric acid in the literature (Tomassini and Matos, 1979).

ent-Kauradienoic Acid (6). The 70% methylene chloride–hexane eluate was recrystallized with hexane to yield a white crystalline solid: mp 171–172 °C; R_f 0.40, solvent, 5% MeOH–$CHCl_3$; IR (KBr) ν_{max} 3500–2450, 1700, 1640, 890, 730 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.84 (1 H, s), 5.42 (1 H, s), 5.20 (2 H, s), 1.16, 1.04; high-resolution mass spectrum, m/e $[M]^+$ 300.2081 ($C_{20}H_{28}O_2$), 285, 255, 81. The structure of compound 6 was assigned as *ent*-kauradienoic acid by comparison of the physical and chemical properties published for *ent*-kauradienoic acid in the literature (Turnbull et al., 1986).

RESULTS AND DISCUSSION

W. biflora stems were extracted with methylene chloride. The initial extract showed boll weevil antifeedant activity (% T/C = 13 at 20 mg) (Table I). The extract was chromatographed on an open column with silica gel as the absorbent. The column was eluted with an increasing hexane in methylene chloride solvent system. The fractionation procedure yielded fractions containing six compounds, which were assigned compound numbers 1–6.

Compound 1 was identified as 16-methylkaur-15-en-19-oic acid. Compound 1 was isolated as white crystals. A molecular formula of $C_{20}H_3O_2$ was established by high-resolution mass spectrometry (M^+ m/e obsd 302.2248, calcd 302.4558). This compound was identified by MS, ^1H NMR, and IR. A computerized literature search utilizing this molecular formula and mp resulted in the assignment of the structure as 16-methylkaur-15-en-19-oic acid (1) since the physical properties were identical with those listed in the literature for this compound (Bohlman et al., 1981b; Hayman et al., 1986). This compound showed 100% inhibition of feeding of boll weevils at a dose of 2.9 mg (Table III), and compound 1 showed extremely high activity against the fungi *P. ultimum* (240%) and *R. solani* (280%) as shown in Table II.

Table II. Antifungal Activity of the Compounds from *W. biflora* (Stems)

compd	P, % *T/C*	R, % *T/C*
1	240	280
2	60	38
5	70	78
6	13	

[a] Dosage = 1 mg. P = *P. ultimum*. R = *R. solani*. % *T/C* = [inhibition zone radius (mm) caused by sample/inhibition zone radius (mm) caused by control] × 100.

Table III. Boll Weevil Bioassay of 16-Methylkaur-15-en-19-oic Acid

dosage, mg	% *T/C*[a]	% inhibition
1	54	46
1.5	20	80
2.9	0	100
3.3	0	100
6.6	0	100

[a] % T/C = [no. of punctures of test paper (*T*)/no. of punctures of control paper (*C*)] × 100.

Compound **2** was identified as 24-ethylcoprostanone, mp 113–114 °C. The high-resolution mass spectrometry showed a molecular formula of $C_{29}H_{50}O$ (M^+ *m/e* 411.550). The data for this compound were compared to those published of 24-ethylcoprostanone (Marker and Wittle, 1937). Compound **2** (at 2.0 mg) showed excellent boll weevil antifeedant activity (90%) (Table II), but was only moderately active against fungi.

Compound **3** was assigned as stigmast-7-en-3-ol. A molecular formula of $C_{29}H_{50}O$ was established by high-resolution mass spectrometry (M^+ *m/e* obsd 414.00, calcd 414.713). The acetyl derivative of this compound had a melting point of 129 °C. Examination of the literature revealed that this compound possessed properties which were identical with those of stigmast-7-en-3-ol (Rubinstein, 1976). This compound at 1.0 mg demonstrated low boll weevil antifeedant activity (Table I).

Compound **4** was identified as stigmasterol. The ^1H NMR and IR suggested the presence of a steroid. The color reaction developed with the Liebermann–Burchard reagent was also in accord with a steroid structure. The mass spectrum gave a parent ion at M^+ 412 ($C_{29}H_{48}O$). Compound **4** was identified as stigmasterol (Figure 1) by comparison of the mp, ^1H NMR, IR, and MS spectra data with those of an authentic sample.

Compound **5** was identified as grandifloric acid. Compound **5** was isolated as white crystals. The presence of a carboxylic acid was indicated by the IR spectrum. The high-resolution mass spectrum showed a molecular formula of $C_{20}H_{30}O_3$ (*m/e* [M^+] 318.2195). This compound was shown to be grandifloric acid by comparison with data contained in the literature (Tomassini and Matos, 1979). Grandifloric acid (1.0 mg) showed activity against the fungi *P. ultimum* (70%) and *R. solani* (78%) as shown in Table II.

Compound **6** was identified as *ent*-kauradienoic acid. The presence of a carboxylic acid was indicated by IR bands at 3500–2450 and 1700 cm^{-1}. The molecular formula was shown to be $C_{20}H_{28}O_2$ (M_r 300.2081) by the high-resolution mass spectrum. This compound was identified as *ent*-kauradienoic acid by comparison with literature data (Turnbull et al., 1986). This compound shows antifeedant activity against boll weevils (83%) (Table II).

In summary, six compounds were isolated, of which four had boll weevil antifeedant activity. While these compounds have been isolated previously, this is the first report of the investigation of their bioactivity against boll weevils and fungi. Particularly noteworthy is the high antifungal activity of 16-methylkaur-15-en-19-oic acid at 240% (1.0 mg) and 280% (1.0 mg) against *P. ultimum* and *R. solani*, respectively.

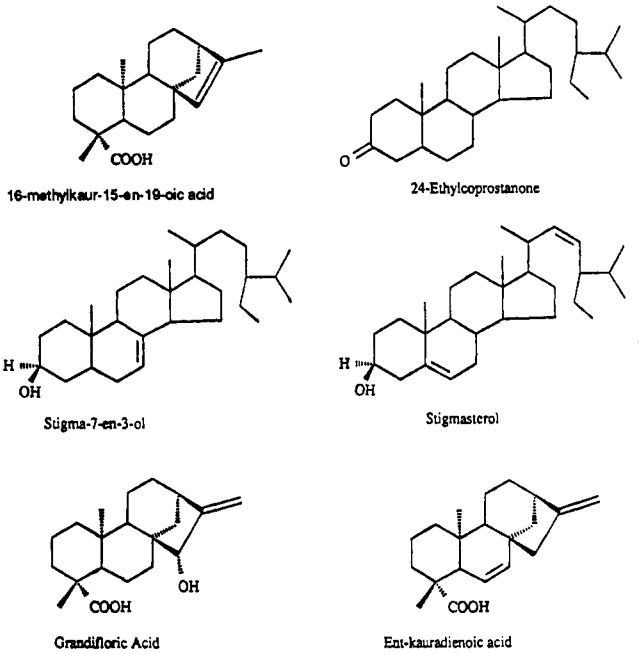

Figure 1. Compounds isolated from *W. biflora*.

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