Boll Weevil Antifeedants from *Eleocharis dulcis* Trin.

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Six compounds were isolated from the CH_2Cl_2 extract of *Eleocharis dulcis* Trin. They are hexacosanoic acid (**1**), 5α-stigmastane-3,6-dione (**2**), β-sitosterol (**3**), stigmasterol (**4**), betulin (**5**), and tricin (**6**). 5α-Stigmastane-3,6-dione, betulin, and tricin showed antifeedant activity against boll weevils.

As a part of a program to search for new agrochemicals from Thai plants, *Eleocharis dulcis* Trin. was shown to possess antifeedant activity against the boll weevil (*Anthonomous grandis* Boheman). *E. dulcis* Trin. is in the family Cyperaceae. It is called Haeo-song-krathiam and is commonly known as the Chinese water chesnut (Smitinand, 1980). *E. dulcis* is described as a rushlike plant with distinctly angled stems and is characteristically grown in marshy and boggy soils. It is tufted or solitary, often has long rhizomes, and is commonly used in Chinese cookery (Muenscer, 1967). There are about 150 other species of the genus grown in warm climates; most are perennials, although a few are annuals.

Examination of *Chemical Abstracts* showed no reference to natural products that have been isolated from this plant species. However, there are some reports of compounds isolated from this genus. *p*-Coumaric acid, dihydroacetinidiolide, ferulic acid, and luteolin were isolated from *Eleocharis coloradoensis* (Steven and Merril, 1981). 11-Hydroxy-14-(3,5-dihydroxy-2-methylcyclopentyl)tetradec-9-ene-12-ynoic acid was isolated from *Eleocharis microcarpa* and was active as an allelochemical for algae (Aller and Robert, 1983).

MATERIALS AND METHODS

Extraction of Plant Material. Plants (above ground) of *E. dulcis* Trin. were collected at Laxsi Bangkhen, Bangkok, Thailand, in March 1985 and were identified (Voucher 82224) by Pipat Patanaponbaiboon, Department of Botony, Chulalongkorn University, Bangkok, Thailand. The air-dried plant material (10 kg) was ground into coarse pieces and was extracted three times overnight (approximately 20 h) with 95% ethanol at room temperature. The solvent was removed in vacuo to give 130 g of crude material. This crude ethanolic extract was triturated in methylene chloride. The methylene chloride soluble material was separated, and then the solvent was removed in vacuo to yield 90 g of crude material.

Chromatography. Sixty grams of the crude methylene chloride extract was separated by open column chromatography. The column size was 7 × 150 cm with 600 g of silica gel (E. Merck, 60–200 mesh). One thousand milliliters of hexane was collected for each fraction, and the separation was performed by gradually increasing the solvent polarity with methylene chloride and then methanol, respectively. A total of 215 fractions were collected, and the combined fractions were monitored by thin-layer chromatography (TLC). TLC was then carried out on chromatoplates prepared with silica gel activated at 110 °C (Merck GF-254 type 60) with a thickness of 0.25 mm. After development with the appropriate solvent system, UV light (254 nm) and iodine vapor were used as detecting agents.

Instrumentation. Infrared (IR) spectra were recorded on a Perkin-Elmer Model 1420 ratio recording infrared spectrometer. Solid samples were prepared as potassium bromide (KBr) pellets. Ultraviolet (UV) spectra were obtained on a Perkin-Elmer Lambda 6, UV–vis spectrometer. Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) was performed on a Varian Gemini 200. The chemical shifts are reported as δ (parts per million downfield from TMS). Gas chromatographic (GC) analyses were obtained on a Perkin-Elmer 8500 gas chromatograph with a flame ionization detector. Mass spectra were recorded on a Finnigan 4510 quadropole mass spectrometer (direct probe). High-resolution mass spectra were obtained from the Midwest Center for Mass Spectrometry, University of Nebraska, Lincoln, NE. Elemental analyses were obtained from Atlantic Microlab Inc. Norcross, GA. Melting points were observed on a mel-temp melting point apparatus and are uncorrected.

Boll Weevil Antifeedant Bioassay. The agar plug bioassay feeding stimultant 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 then poured into glass tubes to allow the formation of a gel. Upon cooling, the plugs were extruded from the tubes and cut to the aforementioned lengths. Known quantities of the plant samples dissolved in ethanol were applied to 4-cm squares of Whatman No. 1 chromatography paper. Air-dry test papers were wrapped around the agar plugs and fastened with staples. Control papers were prepared in the same solvent and air-dried. 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 inhibition value. Three replications of the boll weevil antifeedant bioassay were performed on test and control samples, and the average percent inhibition was reported.

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

$$\%\ \text{inhibition} = 100 - \%\ \mathrm{T/C}$$

Cotton boll weevils and dehydrated bud powder were supplied by the USDA Boll Weevil Research Laboratory at Mississippi State University.

Data for Isolated Compounds. *Hexacosanoic acid* (**1**) was eluted by 10% methylene chloride in hexane (fractions 21–29). Recrystallization of this compound from ethanol yielded a white amorphous solid (100 mg): mp 87–88 °C; IR (KBr) ϑ_{max} 2500–3050, 1698, 1465, 721 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.88

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(3H, t, CH_3), 1.25 (44H, br, CH_2), 1.6 (2H, m), 2.3 (2H, t), 10.5 (1H, br, COOH); ^{13}C HNMR (50 MHz, $CDCl_3$) δ 14.2, 22.8, 24.9, 29.3, 29.5, 29.8, 32.1, 34.1, 180.1; LRMS El, *m/e* (rel intensity) $[M]^+$ 396 (3), 368 (10), 129 (30), 73 (72), 60 (5 4), 57 (100). Anal. Calcd for $C_{26}H_{52}O_2$: C, 78.78; H, 13.13. Found: C, 78.84; H, 13.13 The structure of compound 1 was assigned as hexacosanic acid by comparison of the physical and chemical properties published for this compound in the literature (Heiborn et al., 1965).

5α-Stigmastane-3,6-dione (2). The 10% methylene chloride–hexane eluate (fractions 30–36) was recrystallized with 5% ethyl acetate–hexane to yield 120 mg of white crystals: mp 199–200 °C; R_f 0.69, 25% ethyl acetate–hexane; IR (KBR) V_{max} 2945, 1700, 1454, 1374 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.77 (3H, s, CH_3), 0.8–0.9 (12H, m, $4CH_3$), 1.15–2.15 (26H, m, protons of steroid skeleton); ^{13}C NMR (50 MHz, $CDCl_3$) δ 38.65 (C-1), 37.90 (C-2), 209.55 (C-3), 4 6.52 (C-4), 57.43 (C-5), 211.71 (C-6), 53.37 (C-7), 37.96 (C-8), 55.91 (C-9), 42.87 (C-10), 21.48 (C-11), 41.13 (C-12), 45.66 (C-13), 56.52 (C-14), 23.83 (C-15), 39.24 (C-16), 55.97 (C-17), 12.35 (C-18), 11.8 (C-19), 33.66 (C-20), 18.81 (C-21), 35.91 (C-22), 22.86 (C-23), 42.87 (C-24), 27.87 (C-25), 21.49 (C-26), 19.63 (C-27), 25.83 (C-28), 11.8 (C-29); LRMS El, *m/e* (rel intensity) $[M]^+$ 428 (34), 287 (32), 245 (73), 231 (22), 98 (71), 55 (100). Anal. Calcd for $C_{29}H_{48}O_2$: C, 81.31; H, 11.21. Found: C, 81.17; H, 11.26. Compound 2 and synthetic 5α-stigmastane-3,6-dione, which was prepared by the method of Marker (1942), showed indentical R_f values by TLC and showed no depression of the melting point on mix mp.

Mixture of β-Sitosterol (3) *and Stigmasterol* (4). The 20% methylene chloride–hexane eluate (fractions 37–41) yielded 350 mg of colorless needles. The GC (DB-1701 megabore column, initial column temperature 250 °C, carrier gas He) gave two peaks with retention times of 18.40 and 20.43 min, which corresponded to the retention times of authentic stigmasterol and β-sitosterol (obtained from Sigma Chemical Co.).

Betulin (5). The 35% methylene chloride–hexane eluate (fractions 54–60) was recrystallized with 20% hexane–chloroform to yield 130 mg of white amorphous solid: mp 248–250 °C; R_f 0.51, 10% methanol–chloroform; IR (KBr) V_{max} 3092–3600, 2931, 1634, 1444, 1371, 1015, 877 cm^{-1}; ^1H. NMR (200 MHz, DMSO-d_6) δ 0.75 (3H, s, CH_3), 0.84 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.98 (3H, s, CH_3), 1.03 (3H, s, CH_3), 1.70 (3H, s, $C{=}CCH_3$), 1.11–2.10 (25H, m, protons of lupene skeleton), 4.59 (2H, d, olefenic protons); ^{13}C NMR (50 MHz, DMSO-d_6) δ 38.52 (C-1), 27.51 (C-2), 77.61 (C-3), 38.90 (C-4), 54.99 (C-5), 17.95 (C-6), 33.97 (C-7), 40.82 (C-8), 49.98 (C-9), 36.97 (C-10), 20.55 (C-11), 24.92 (C-12), 37.50 (C-13) , 42.57 (C-14), 27.01 (C-15), 29.03 (C-16), 47.57 (C-17), 48.55 (C-18), 47.67 (C-19), 150.50 (C-20), 29.54 (C-21), 33.97 (C-22), 27.90 (C-23), 15.63 (C-24), 16.02 (C-25), 16.05 (C-26), 14.64 (C-27), 59.50 (C-28), 109.60 (C-29), 19.01 (C-30); HRMS El, *m/e* (rel intensity) $[M]^+$ 442.3818 ($C_{30}H_{50}O_2$), 411 (8), 234 (16), 207 (56), 203 (50), 189 (85). The structure of compound 5 was assigned as betulin according to mixed mp, co-TLC with an authentic sample obtained from Aldrich Chemical Co.

Tricin (6). The 40–50% methylene chloride–hexane eluate (fractions 76–110) was rechromatographed using column chromatography. The column was eluted with hexane, methylene chloride, and methanol, respectively. Two-hundred fifty milliliters of the eluting solvent was collected for each fraction, and a total of 175 fractions were collected. The 90% methylene chloride–hexane eluate (fractions 86–98) was recrystallized with 50% methanol–chloroform to yield 70 mg of fine yellow needles: 289–291 °C; IR (KBr) V_{max} 3413, 2906, 1639, 1612, 1555, 1495 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 3.98 (6H, s, $2OCH_3$), 6.15 (1H, s of d, H-8), 6.32 (1H, s of d, H-6), 7.05 (1H, s, H-3), 7.4 (2H, s, H-2′, H-6′), 9.54 (1H, s, OH); ^{13}C NMR (50 MHz, DMSO-d_6) δ 164.09 (C-2), 103.96 (C-3), 182.7 (C-4), 161.80 (C-5), 99.07 (C-6), 164.58 (C-7), 94.43 (C-8), 157.75 (C-9), 103.96 (C-10), 120.68 (C-1′), 104.58 (C-2′), 148.56 (C-3′), 140.18 (C-4′), 148.56 (C-5′), 104.58 (C-6′), 56.45 (OCH_3); UV λ_{max} MeOH 244, 268, 299, 330 nm; NaOMe 263, 275, 330 nm; $AlCl_3$ 258, 278, 303, 366, 395 nm; NaOAc/H_3BO_3 270, 305, 350 nm; $AlCl_3$/HCl 259, 277, 302, 360, 385 nm; HRMS El, *m/e* (rel intensity) $[M]^+$ 330.0787 ($C_{17}H_{14}O_7$), 178 (18), 153 (34). Compound 6 was identified as tricin according to mixed mp, IR, UV, ^1H NMR, and ^{13}C NMR with an authentic sample which was isolated from *Spartina cynosuroides* by Bhattacharyya et al. (1978).

RESULTS AND DISCUSSION

Dried plant material, *E. dulcis* Trin., was extracted with 95% ethanol to give a crude ethanol extract. The ethanol extract was further separated by methylene chloride to obtain a crude methylene chloride extract. Column chromatography of this crude extract was performed on a silica gel column, and 215 fractions were collected. Six compounds were isolated and were identified as hexacosanoic acid (1), 5α-stigmastane-3,6-dione (2), β-sitosterol (3), stigmasterol (4), betulin (5), and tricin (6). Compounds 1, 2, 5, and 6 inhibited the feeding activity of boll weevils at levels of 73% (at 1.5 mg), 77/82/90% (at 1/3/10 mg), 30/90/98% (at 1/3/8 mg), and 53/78/96% (at 1/3/10 mg), respectively. Betulin was reported to be present in *Alnus oregona*, which showed antitumor activity for the Walker 256 (5WA16) tumor system (Sheth et al., 1973). Betulin was also reported as a constituent in *Sarracenia flava*, which showed antitumor activity against human epidermoid carcinoma of the nasopharynx (KB) Miles et al., 1974). Tricin was reported to have an effect on guinea pig intestine (Bickoff et al., 1964) and was isolated from a fraction enriched in antileukemia activity of *Spartina cynosuroides* (Bhattacharyya et al., 1978).

In summary, four of the six compounds isolated 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.

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