Insecticidal Fatty Acids and Triglycerides from *Dirca palustris*

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Five compounds, **1**−**5**, were isolated from the seed hexane extract of *Dirca palustris*. Compounds **1**−**3** were triglycerides, and **4** and **5** were linoleic and oleic acids, respectively. Compounds **1**−**3** were not biologically active; however, **4** (linoleic acid) and **5** (oleic acid) were insecticidal against fourth instar *Aedes aegyptii* larvae and exhibited potent feeding deterrent activity against neonate larvae of *Helicoverpa zea*, *Lymantria dispar*, *Orgyia leucostigma*, and *Malacosoma disstria*.

Keywords: *Dirca palustris; triglycerides; fatty acids; mosquitocidal compounds; antifeedant compounds*

INTRODUCTION

The genus *Dirca* (Thymelaeaceae) contains only three species: *Dirca mexicana*, *Dirca occidentalis*, and *Dirca palustris*, all of which are shade-tolerant shrubs indigenous to North America. *D. mexicana* and *D. occidentalis* are native to northeast Mexico and the Pacific states, respectively, where they are known by the colloquial name of western leatherwood (*1*). *D. palustris*, on the other hand, occurs in eastern North American mesic, deciduous forests, where it is known as leatherwood or moosewood. It has been reported that application of the fresh bark of *D. palustris* to skin causes redness, vessication, and sores that are very difficult to heal (*2*), and ingestion produces severe vomiting and purgation (*1*). Also, observations made by forest biologists in northern Michigan and Wisconsin indicate that deer, hares, and many other herbivores avoid eating all parts of this plant, strongly suggesting that *D. palustris* contains deterrent compounds. There are two reports on biologically active compounds from *D. occidentalis* (*1*, *3*), and we have recently reported biologically active phenolic glycosides from the winter dormant twigs of *D. palustris* (*4*).

In our continuous search for biologically active compounds from *D. palustris*, we now report three triglycerides (**1**−**3**) and two biologically active fatty acids (**4** and **5**) from the seeds of *D. palustris*. This is the first report on the constituents of the seed oil of *D. palustris* and the biological activities for **4** and **5**.

MATERIALS AND METHODS

General Experimental Procedures. NMR spectra (^{1}H, ^{13}C, DEPT) were recorded on a Varian INOVA 300 spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) or a Varian VXR 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shifts were recorded in $CDCl_3$ and coupling constants (J) in Hz, and the values are in δ (ppm) based on δ residual of $CDCl_3$: 7.24 and 77.0 for ^{1}H and ^{13}C NMR, respectively. Fast atom bombardment mass spectroscopy (FABMS) data were recorded on a JEOL JMS-HX 110 mass spectrometer where the ions were produced by bombardment with a beam of Xe atoms at 6 keV and an accelerating voltage of 10 kV. The silica gel used for medium pressure liquid chromatography (MPLC) was Merck Silica gel 60 (35−70 μm particle size). Analytical (5 × 20 cm) and preparative thin layer chromatography (TLC) (20 × 20 cm) were done on precoated silica gel 60 glass plates (Analtech, Inc.). All solvents were ACS reagent grade and were purchased from Aldrich Chemical Co., Inc.

Gas chromatographic analyses were performed using an HP 6890 gas chromatograph (Algilent Technology, Delaware). Samples (1 μL) were injected into a split injector at 200 °C (20:1 split ratio) and separated on an HP-5 capillary column (30 m × 0.25 mm id). Helium was used as the carrier gas at a flow rate of 22 mL min^{-1}. Compounds were detected using flame ionization at 250 °C. The temperature profile was 150 (2 min)−200 °C (1 min) at 10 °C min^{-1} and then to 250 °C (2 min) at 2 °C min^{-1}. Statistical analyses were performed on the larval antifeedant data using the F test and least significant differences.

Plant Material. Fresh, mature seeds of *D. palustris* (0.48 kg) were harvested in June from 1 to 2 m tall plants growing as understory shrubs in sugar maple, *Acer saccharum*, forests in eastern Gogebic County of the upper peninsula of Michigan. In this area, flowering typically occurs in late April and early to mid May, and the resulting fruits ripen in June to early to mid July. Seed dispersal occurs over 1−2 weeks in early to mid July. Fruits are borne in clusters (panicles) of 1−7, with 1−3/cluster most common. The fruit is a drupe that ranges in size from 9 to 15 mm long and 5−8 mm wide. Whole fruits ranged from 0.08 to 0.23 g fresh weight with a moisture content of 100−175% (dry weight basis). Seeds ranged from 6 to 9 mm in length, 3−5 mm in width, and vary from 0.04 to 0.08 mm at the time of dispersal with a moisture content of 40−55% of dry weight. Seeds were stored at −30 °C until extraction.

Extraction and Isolation. The seeds (0.48 kg) were macerated using a Waring blender and then extracted exhaustively with hexane (2 × 1.5 L, 48 h) to yield a hexane extract (126 g), followed by ethyl acetate (2 × 1.5 L, 48 h) to yield an

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ethyl acetate extract (15.5 g), and finally, they were extracted with methanol (2 × 1.5 L, 48 h) to yield a methanol extract (23.6 g). The hexane extract (122 g) was extracted with methanol (3 × 300 mL) to yield a methanol soluble fraction (6.04 g) and a hexane soluble fraction (116 g). The hexane soluble fraction (400 mg) was purified by preparative TLC using hexane/ether (6:1) as the developing solvent and yielded one major band (320 mg, R_f 0.65). One of the components of this band was identified by ^{1}H and ^{13}C NMR spectral data as the triglyceride, 1,3-dilinoleoyl-2-olein, **1**. However, FABMS analyses revealed that the above band also contained two other triglycerides: 1,3-dioleoyl-2-linolein, **2** (*5*), and 1,2,3-trilinolein, **3**.

The biologically active, methanol soluble fraction (3.5 g) was further fractionated by MPLC using eluting solvents such as hexane with increasing amounts of acetone and finally methanol. This procedure yielded fractions I (2.05 g) and II (818.4 mg), both eluted with hexane−acetone (6:1, 800 mL); fractions III (39.2 mg) and IV (220 mg), eluted with hexane−acetone (1:1, 800 mL); and fraction V (266.1 mg), eluted with methanol (100 %, 1 L). Fraction IV, the biologically active fraction, was subjected to preparative TLC using hexane/acetone (2:1) as the developing solvent to afford three bands: band A (156 mg, R_f 0.40), band B (15.6 mg, R_f 0.55), and band C (3.4 mg, R_f 0.70). Band A was further purified by preparative TLC using hexane/acetone (4:1; ×4) as the developing solvent and yielded band 1 (6.8 mg, R_f 0.35), band 2 (91.2 mg, R_f 0.50), and band 3 (27.3 mg, R_f 0.65). Bands 2 and 3 were biologically active and characterized as linoleic acid, **4**, and oleic acid, **5**, respectively. The structures of **4** and **5** were confirmed by comparison of their ^{1}H NMR spectral data with those published in the Aldrich NMR library (*6*) and by gas chromatography (GC) analyses of their methyl esters with corresponding standards.

Compound 1: A pale yellow oil; FABMS *m/z* (relative intensity): $[M^+ + H]^+$ 881 (3), 866 (2), 601 (90), 339 (32), 265 (20), 263 (27), 109 (100). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.86 (bt, 6H, H-18′ × 2), 0.87 (bt, 3H, H-18″), 1.25 (m, 48H, H-(4′-7′ and 15′-17′) × 2 and H-(4″-7″ and 12″-17″)), 1.59 (m, 6H, H-3′ × 2 and H-3″), 2.02 (m, 12H, H-(8′, 14′) × 2 and H-(8″, 11″)), 2.28 (t, 4H, J = 7.6, H-2′ × 2), 2.29 (t, 2H, J = 7.6, H-2″), 2.75 (m, 4H, H-11″ × 2), 4.12 (dd, 2H, J = 14.0, 6.0, H-1a, 3a), 4.28 (dd, 2H, J = 14.0, 4.2, H-1b, 3b), 5.25 (m, 1H, H-2), 5.33 (m, 10H, H-(9′, 10′, 12′, 13′) × 2 and H-(9″, 10″)). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.0 (C-18″), 14.1 (C-18′ × 2), 22.4, 22.6, 24.8, 24.9, 29.0−29.7 (C-(4′-7′ and 15′-17′) × 2 and C-(4″-7″ and 12″-17″)), 25.6 (C-11′ × 2), 27.2 (C-(8′, 14′) × 2 and C-(8″, 11″)), 31.5 (C-3″), 31.9 (C-3′ × 2), 34.0 (C-2′ × 2), 34.2 (C-2″), 62.1 (C-1, 3), 68.9 (C-2), 127.8−130.2 (C-(9′, 10′, 12′, 13′) × 2 and C-(9″, 10″)), 172.8 (C-1″), 173.2 (C-1′ × 2). The spectral data confirmed that this compound is 1,3-dilinoleoyl-2-olein.

Compound 2: FABMS *m/z* (relative intensity): $[M^+ + H]^+$ 883 (1), 868 (1), 603 (60), 341 (10), 265 (20), 263 (27), 109 (100) (*5*).

Compound 3: FABMS *m/z* (relative intensity): $[M^+ + H]^+$ 879 (1), 864 (1), 599 (65), 337 (32), 263 (27), 109 (100).

Compound 4: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (t, 3H, J = 7.6), 1.35 (bs, 14H), 1.67 (m, 2H), 2.10 (m, 4H), 2.37 (t, 2H, J = 7.1), 2.80 (m, 2H), 5.40 (m, 4H). These spectral data were in agreement with the published data and confirmed that it was linoleic acid (*6*).

Compound 5: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, 3H, J = 7.4), 1.29 (bs, 12H), 1.34 (bs, 8H), 1.67 (m, 2H), 2.05 (m, 4H), 2.40 (t, 2H, J = 6.9), 2.80 (m, 2H), 5.30 (m, 2H). These spectral data were in agreement with the published data and confirmed that it was oleic acid (*6*).

Saponification and Methylation of Compound 1: Compound **1** (50 mg) was stirred with 5% methanolic KOH solution (5 mL) for 10 min. Methanolic 6 N HCl was then added to acidify this solution to pH 5.5. This acidic solution was then extracted with EtOAc, and the ethyl acetate extract was evaporated to yield a colorless oil (46.7 mg). Diazomethane was prepared by dissolving 0.5 *N*-nitroso-*N*-methylurea in 10% KOH solution (50 mL) with ether (50 mL) over an ice bath. The mixture was stirred gently for a few minutes and then

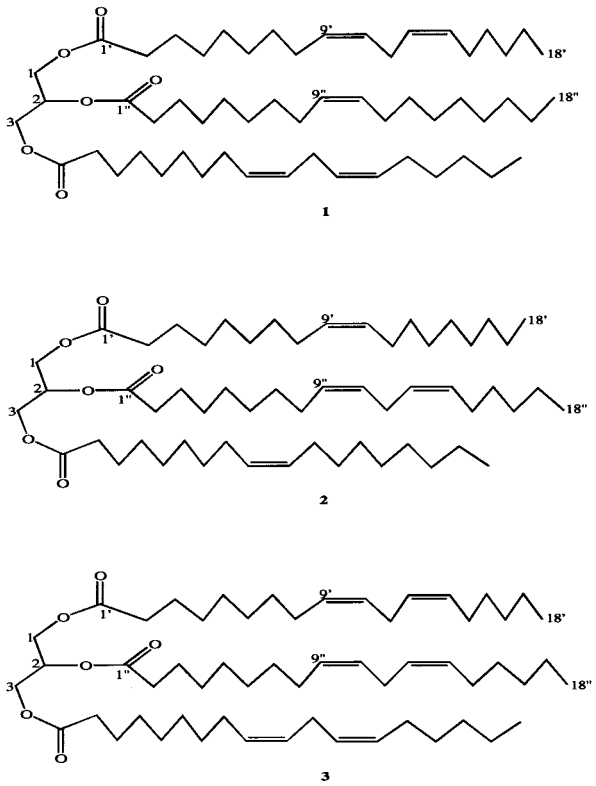

Figure 1. Structures of **1**−**3**.

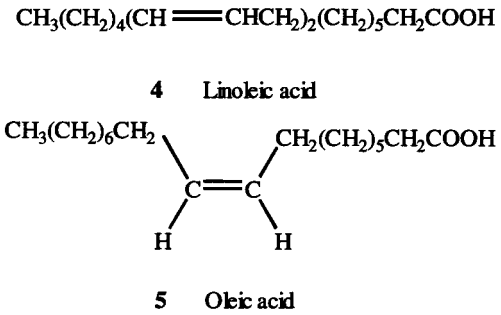

Figure 2. Structures of **4** and **5**.

poured into a 250 mL separatory funnel. The KOH solution layer was removed, and the diazomethane solution was then washed with 25 mL of ice-cold water. The yellow diazomethane solution was poured into a brown bottle containing KOH pellets and stored at −30 °C. The diazomethane solution was added to a sample of the ethyl acetate extract (20.2 mg) from the hydrolysis above until the solution maintained a permanent yellow color. The sample was capped and allowed to sit in the fumehood for 2 h. The ether was evaporated with N_2 gas, and the sample was dried in a vacuum desiccator to yield their methyl esters.

Methylation of Standards and Compounds 4 and 5: Standard fatty acids (palmitic (4.1 mg), linolenic (5.3 mg), oleic (5.7 mg), stearic (4.9 mg), and linoleic (6.1 mg)) as well as **4** (9.2 mg) and **5** (7.3 mg) were methylated with diazomethane solution as described above. After the ether was removed, samples were further dried under vacuum for 24 h prior to GC analyses.

Mosquitocidal Assay (*7*). First instar mosquito, *Aedes aegyptii* (L) (Dipera:Culicidae), was provided by Drs. Alexander Raikel and Alan Hays, Department of Entomology, Michigan State University. The larvae were reared in reverse osmosis (RO) water in an incubator at 26 °C for 3 days. Ten to 12 fourth instar larvae were placed in 980 μL of RO water in test tubes. Twenty microliters of test material dissolved in dimethyl sulfoxide (DMSO), to give a concentration of 250 $\mu g \cdot 20\ \mu L^{-1}$, was added to each tube. A 20 μL aliquot of DMSO was used

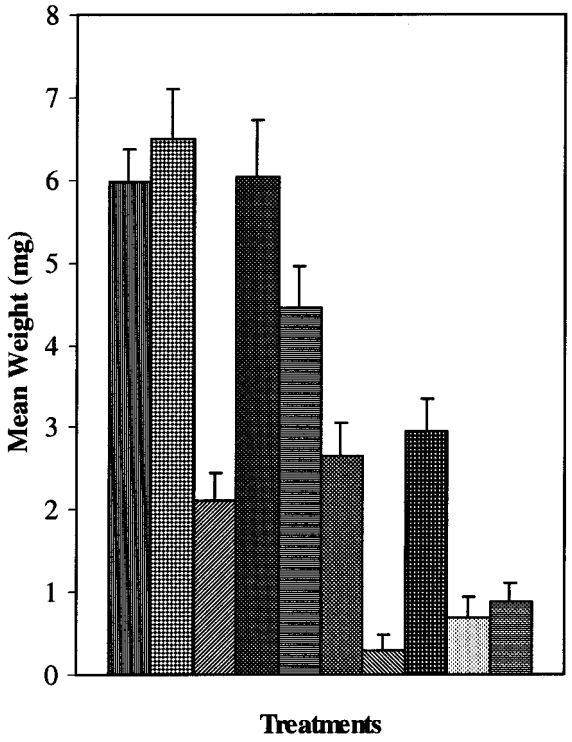

Figure 3. Antifeedant assay of purified fractions and pure compounds from the hexane extract of *D. palustris* seeds on *H. zea* at 250 μg mL^{-1} concentration after 6 days; $p \leq 0.01$. Bars represent DMSO control, the hexane soluble fraction, the methanol soluble fraction, fraction I, fraction II, fraction III, fraction IV, fraction V, **4**, and **5** from left to right.

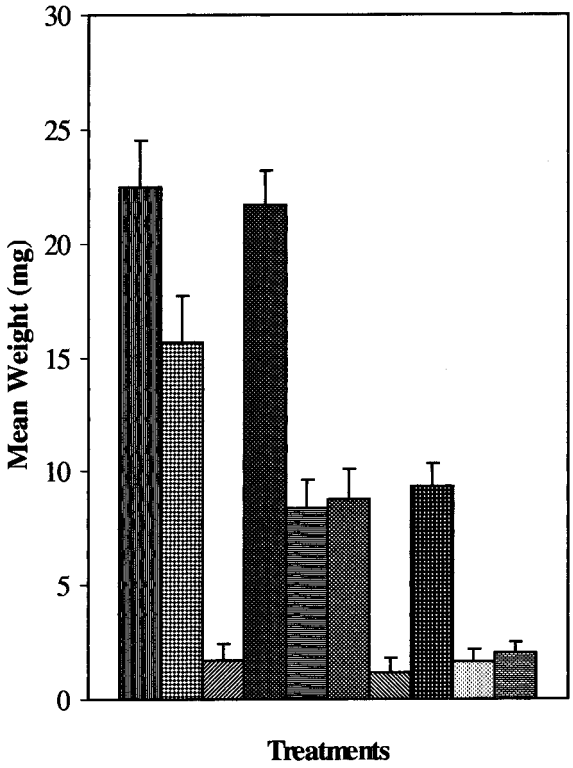

Figure 4. Antifeedant assay of fractions and pure compounds from the hexane extract of *D. palustris* seeds on *L. dispar* at 250 μg mL^{-1} concentration after 6 days; $p \leq 0.01$. Bars represent DMSO control, the hexane soluble fraction, the methanol soluble fraction, fraction I, fraction II, fraction III, fraction IV, fraction V, **4**, and **5** from left to right.

as a control. Tubes were covered and left at room temperature. Treatments and controls were run in triplicate. Larval mortality was recorded at 2 h intervals, up to and including 24 h.

Antifeedant Bioassay (*8*). Corn earworm eggs, *Helicoverpa zea* Boddie (Lepidoptera:Noctuidae), and dry corn earworm diet were purchased from North Carolina State Insectory, Department of Entomology, North Carolina State University, Raleigh, North Carolina. Eggs of gypsy moth, *Lymantria dispar* L. (Lepidoptera:Lymantriidae); whitemarked tussock moth, *Orgyia leucostigma* (J. E. Smith); and forest tent caterpillar, *Malacosoma disstria* Hübner were obtained from the Insect Production Unit of the Canadian Forest Service, Sault Ste. Marie, Canada. The eggs of all species were hatched in an incubator at 27 °C.

Dry diet was mixed in the Bioactive Natural Products Laboratory, Department of Horticulture, Michigan State University, and dispensed into scintillation vials (940 mg) for each treatment. The recipe was as follows: wheat germ (36 g), casein (7.5 g), Wesson's salt mix (2.4 g), sorbic acid (0.6 g), methylparaben (*p*-hydroxy-benzoic acid methyl ester) (0.3 g), and Hoffman-Larouche #26862 vitamin mixture (Hoffman-Larouche, Inc., Nutley, NJ; 3.0 g). Test material was dissolved in DMSO to give a concentration of 1250 μg · 25 μL^{-1}, unless otherwise stated. Twenty-five microliters of the test solutions was mixed thoroughly with 5 g portions of dry diet. An aliquot of 25 μL DMSO was used as a control. Agar solution (1.4%) was mixed and autoclaved for 5 min at 17 psi and 125 °C to melt the agar. This solution was held in a water bath at 50 °C and added to the dry diet until the total diet weighed 5 g. The final concentration of test extracts and compounds was 250 μg/mL. The wet diet was mixed thoroughly, and 3−4 drops of diet was dispensed into 3.5 mL polystyrene vials. The freshly poured aliquots of diet were allowed to cool and dry for at least 1 h. After the diets were dried, one neonate larva was placed in each vial, and the vials were capped. The treatment and control vials were held in a growth chamber at a photoperiod of 16 h day and 8 h night with day temperature at 28 °C and night temperature at 24 °C. Each treatment was replicated 15 times. The treatments were arranged in a completely randomized design. Larvae were weighed (mg) after 6 days.

RESULTS AND DISCUSSION

Five compounds, **1**−**5**, were isolated from the seed hexane extract of *D. palustris* by silica gel MPLC and repeated preparative TLC. Compounds **1**−**3** were triglycerides and were identified by ^{1}H and ^{13}C NMR spectroscopy and mass spectral data. The NMR data for all three compounds were identical since they were either linoleic or oleic acid esters. However, three molecular ions were observed from FABMS analyses at mass to charge ratios of 879, 881, and 883, respectively. Compound **1** had a mass to charge ratio of 881 $[M^+ + H]^+$, which indicated a molecular formula of $C_{57}H_{100}O_6$. NMR and mass spectral data indicated that this was the major triglyceride. Also, mass to charge ratio fragments at 263 and 265 in the mass spectrum gave further evidence to corroborate the presence of linoleic and oleic acid residues in this molecule. Saponification of **1** followed by methylation and GC analyses confirmed the presence of linoleic and oleic acid moieties. Six standards, methyl palmitate, linolenate, γ-linolenate, oleate, stearate, and linoleate, as well as the methylated hydrolysis product were analyzed by gas chromatography under the same conditions. The retention times (R_t) for the standards were 19.207, 22.280, 21.947, 21.220, 21.014, and 21.654 min, respectively. The methylated hydrolysis product gave two peaks at R_t = 21.198 and 21.643 min in the ratio of 2:3. These peaks were identified as methyl oleate and linoleate, respectively. The ratio of 2:3 for these peaks gave further evidence that there were other triglycerides present with the

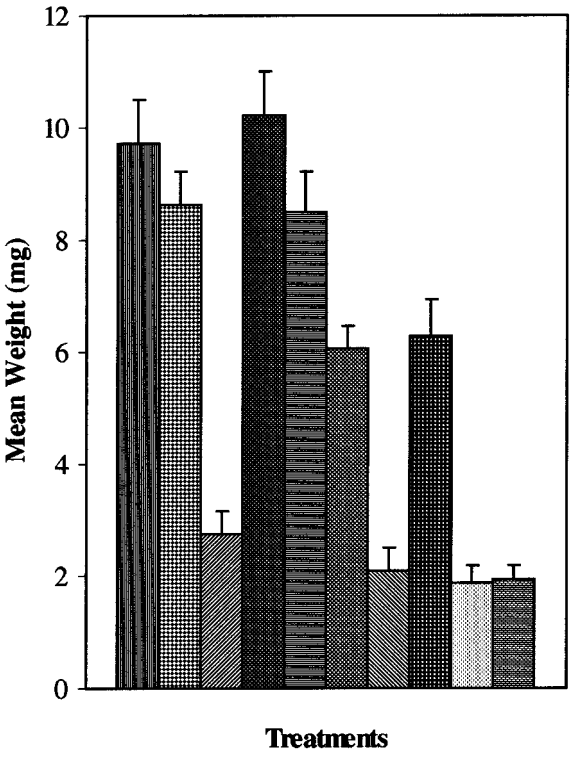

Figure 5. Antifeedant assay of fractions and pure compounds from the hexane extract of *D. palustris* seeds on *O. leucostigma* at 250 μg mL^{-1} concentration after 6 days; $p \leq 0.01$. Bars represent DMSO control, the hexane soluble fraction, the methanol soluble fraction, fraction I, fraction II, fraction III, fraction IV, fraction V, **4**, and **5** from left to right.

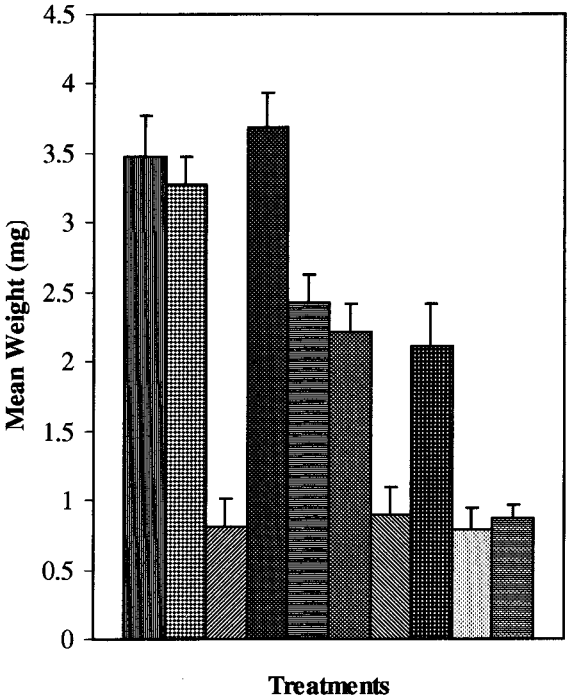

Figure 6. Antifeedant assay of fractions and pure compounds from the hexane extract of *D. palustris* seeds on *M. disstria* at 250 μg mL^{-1} concentration after 6 days; $p \leq 0.01$. Bars represent DMSO control, the hexane soluble fraction, the methanol soluble fraction, fraction I, fraction II, fraction III, fraction IV, fraction V, **4**, and **5** from left to right.

same side chains as **1**. Molecular ions at a mass to charge ratio of 883 and 879 $[M^+ + H]^+$ indicated a molecular formula of $C_{57}H_{102}O_6$ and $C_{57}H_{98}O_6$, which corresponded to **2** and **3**, respectively. Hence, **1** was determined as 1,3-dilinoleoyl-2-olein, **2** as 1,3-dioleoyl-2-linolein, and **3** as 1,2,3-trilinolein (Figure 1). These three triglycerides were not biologically active in our bioassays.

From the methanol soluble fraction of the seed hexane extract, biologically active compounds **4** and **5** were isolated. ^{1}H NMR spectral data for these two compounds as well as GC analyses of their methyl esters confirmed that they were linoleic (**4**) and oleic acids (**5**), respectively (Figure 2). Also, the ^{1}H NMR data were compared with the Aldrich Library of ^{13}C and ^{1}H FT NMR spectra for these compounds and they were identical.

The mosquitocidal assay showed that both **4** and **5** had an LD50 value of 100 μg mL^{-1} when tested against fourth instar *A. aegyptii* larvae at 24 h. Control larvae experienced no mortality. In the caterpillar bioassays, **4** and **5** reduced the growth of *H. zea* by 88 and 85%, *L. dispar* by 93 and 91%, *O. leucostigma* by 81 and 80%, and *M. disstria* by 77 and 75%, respectively (Figures 3−6). The assay was terminated after 1 week, and survival data were not recorded.

Although the triglycerides were not biologically active, this is the first investigation of these compounds in the seeds of *D. palustris*. Similar fatty acids obtained from other plant sources have been shown to have pesticidal or insect growth regulatory activities (*9*−*11*), but to our knowledge, this is the first report of biological activity of these acids against insect pests.

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LITERATURE CITED

(1) Badawi, M. M.; Handa, S. S.; Kinghorn, A. D.; Cordell, G. A.; Farnsworth, N. R. Plant Anticancer Agents XXVII: Antileukemic and Cytotoxic Constituents of *Dirca occidentalis* (Thymelaeceae). *J. Pharm. Sci.* **1983**, *72*, 1285−1287.

(2) White, J. C. *Dermatitis Venenata*: *An Account of the Action of External Irritants upon the Skin*; Cupples and Hurd: Boston, 1887.

(3) Suh, N.; Luyengi, L.; Fong, H. H. S.; Kinghorn, A. D.; Pezzuto, J. M. Discovery of Natural Product Chemopreventive Agents Utilizing HL-60 Cell Differentiation as a Model; *Anticancer Res.* **1995**, *15*, 233−240.

(4) Ramsewak, R. S.; Nair, M. G.; DeWitt, D. L.; Mattson, W. J.; Zasada, J. Phenolic Glycosides from *Dirca palustris*. *J. Nat. Prod.* **1999**, *62*, 1558−1561.

(5) Chandra, A.; Nair, M. G. Chemical Characterization of Pit Oil from Montmorency (*Prunus cerasus* L.) Cherry. *J. Agric. Food Chem.* **1993**, *41*, 879−881.

(6) Pouchert, C. J.; Behnke, J. The Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra, 1st ed.; Aldrich chemical Co.: Milwaukee, WI, 1993; Vol. 1, 782 and 785.

(7) Ramsewak, R. S.; Erickson, A. J.; Nair, M. G. Bioactive *N*-Isobutylamides from the Flower Buds of *Spilanthes acmella*. *Phytochemistry* **1999**, *51*, 729−732.

(8) Zhang, D.; Nair, M. G.; Murry, M.; Zhang, Z. Insecticidal Activity of Indanomycin. *J. Antibiot.* **1997**, *50*, 617−620.

(9) Fatope, M. O.; Adoum, Q. A.; Takeda, Y. C18 Acetylenic Fatty Acids of Ximenia Americana with Potential Pesticidal Activity. *J. Agric. Food Chem.* **2000**, *48*, 1872−1874.

(10) Pari, K.; Subrahmanyam, B.; Rastogi, J. N.; Devakumar, C.; Rao, P. J. Insecticidal (*Z*)-6-Methyl-12-heptadecenoic Acid from the Essential Oil of *Ageratum conyzoides*. *Ind. J. Chem., Sect. B* **2000**, *39*, 451−454.

(11) Harada, H.; Suomalainen, M.; Uchida, H.; Masui, H.; Ohmura, K.; Kiviranta, J.; Niku-Paavola, M.-L.; Ikemoto, T. Insecticidal Compounds against Mosquito Larvae from *Oscillatorai agardhii* Strain 27. *Environ. Toxicol*. **2000**, *15*, 114−119.

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