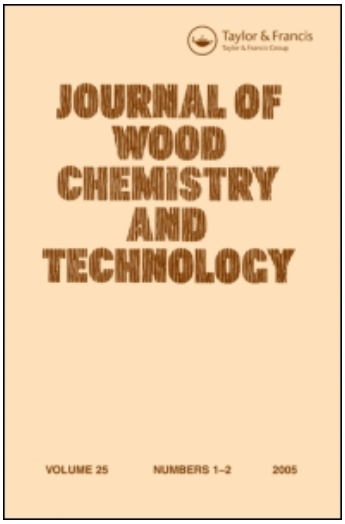

Journal of Wood Chemistry and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597282

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To cite this Article Hua, Yun , Bentley, Michael D. , Cole, Barbara J. W. , Murray, Kathleen D. and Alford, A. Randall(1991) 'Triterpenes from the Outer Bark of *Betula nigra*', Journal of Wood Chemistry and Technology, 11: 4, 503 — 516
To link to this Article: DOI: 10.1080/02773819108051090
URL: http://dx.doi.org/10.1080/02773819108051090

JOURNAL OF WOOD CHEMISTRY AND TECHNOLOGY, 11(4), 503-516 (1991)

TRITERPENES FROM THE OUTER BARK OF *BETULA NIGRA*

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ABSTRACT

Twelve pentacyclic triterpenes were isolated from the outer bark of river birch, *Betula nigra*. 3β-Acetoxyolean-11-oxo-12-ene-28-oic acid was isolated for the first time from a *Betula* species. 3β-Caffeatoxyolean-12-ene-28-oic acid has been spectrally characterized for the first time. 3β-Acetoxyolean-12-ene-28-oic acid and 3β-acetoxyolean-11-oxo-12-ene-28-oic acid have been demonstrated to be active as antifeedants for the Colorado potato beetle, *Leptinotarsa decemlineata*.

INTRODUCTION

Birch trees (*Betula spp.*) are widespread in North America and although they produce wood of commercial importance, the bark is currently a low value waste product. The availability and enormous supply of birch bark has thus generated considerable interest in exploring the chemistry of this underutilized natural resource.

In our laboratory, the outer bark of six birch species common in North America have been studied, including four white-barked birches

(*B. papyrifera, B. populifolia* Marsh, *B. cordifolia* Regel, *B.* x *caerulea*)[1] and two dark-barked birches (*B. lenta, B. alleghaniensis*)[2,3] from which we have isolated a total of twenty triterpenes. The compositions of the outer bark of these species are similar to those of European[4,5] and Japanese[6] birch species which have components belonging to the lupane class of triterpenes present in high concentrations. In the species we examined, betulin, lupeol and lupenone were the most common triterpenes, with small amounts of other triterpenes also present with some structural variation among the species.

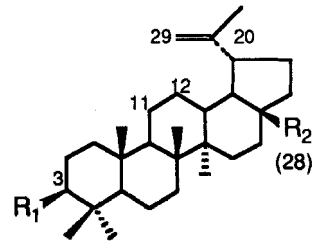
The predominant triterpene, betulin, found in all of the birches mentioned above, has been reported to exhibit some biological activities such as insect antifeedant and mammalian antitumor activities[7,8]. Certain betulin-derived semi-synthetic triterpenes also have high antifeedant activity against corn earworm (*Heliothis zea*)[9].

In further assessing the abundant triterpenes in birch bark for utilization as biologically active compounds, we have investigated the triterpene constituents in the outer bark of *Betula nigra* (river birch), a native to the southeastern United States. The bark of this species has a history of application in traditional folk medicine. The decoction of its bark was used by native Americans to treat their stomach pain[10] as well as the sore hooves of their horses[11]. While the flavonoids in buds of *B. nigra* have been studied[12], no research has been reported on the triterpenoid constituents in the bark of this birch species. In this paper, we report an analysis of the triterpenoids in the outer bark of *B. nigra* and the antifeedant properties against Colorado potato beetle (*Leptinotarsa decemlineata*) of the major components isolated from this species.

RESULTS AND DISCUSSION

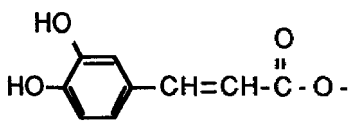
Our investigation resulted in the isolation and identification of twelve pentacyclic triterpenes from the outer bark of *B. nigra* (Tables 1 and 2). The structure of compound **XI**, which has not been found previously in *Betula* species, was confirmed by the oxidation with CrO_3 of the known compound, 3β-acetoxyolean-12-ene-28-oic acid (**IX**). The

TABLE 1. Lupane-Type Triterpenes from the Outer Bark of *Betula Nigra*.



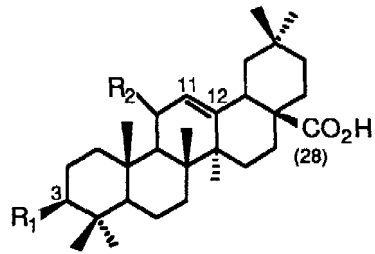
	R_1	R_2	NAME	%*
I	AcO	CO_2Me	methyl 3β-acetoxylup-20(29)-ene-28-oate	0.04
II	=O	CH_3	lupenone	0.08
III	=O	CHO	lup-20(29)-ene-3-one-28-al	0.27
IV	OH	CH_3	lupeol	0.44
V	AcO	CO_2H	3β-acetoxylup-20(29)-ene-28-oic acid	0.07
VI	OH	CH_2OH	betulin	0.96
VII	OH	CO_2H	betulinic acid	2.88
VIII	3β-caffeate	CH_2OH	3β-caffeatoxylup-20(29)-ene-28-ol	0.48

Caffeate =



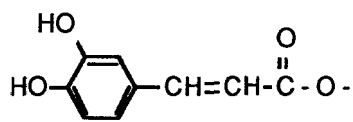
* Percentage of total extract

TABLE 2. Oleanane-Type Triterpenes from the Outer Bark of *Betula Nigra*.



	R_1	R_2	NAME	%
IX	AcO	H	3β-acetoxyolean-12-ene-28-oic acid	3.30
X	OH	H	oleanolic acid	0.92
XI	AcO	=O	3β-acetoxyolean-11-oxo-12-ene-28-oic acid	0.15
XII	3β-caffeate	H	3β-caffeatoxyolean-12-ene-28-oic acid	1.10

Caffeate =



• Percentage of total extract

other eleven triterpenes have been found either in European birch (*B. verrucosa, B. pubescens*)[4,5] bark or in Japanese birch (*B. platyphylla*)[6] bark. Compound **XII** has been isolated from the outer bark of *B. davurica*[13], but no spectral data of **XII,** other than infrared, have been reported, and it was characterized from the spectral data of its acetylated derivative. The IR spectrum of **XII** showed a carboxylic acid at 3400-2500 (OH) and 1695 cm^{-1} (C=O), an α,β-unsaturated carbonyl at 1686 cm^{-1} and an aromatic ring at 1603 and 1515 cm^{-1}. These data agree closely with the reported IR spectrum. The CIMS showed a $[M+1]^+$ ion at m/z 619 and a high resolution CIMS showed a peak at 439.3571 ($C_{30}H_{47}O_2$) corresponding to $[M+1\text{-caffeic acid}]^+$. The EIMS showed a fragment peak at m/z 438 corresponding to the loss of caffeic acid. The ^{1}H-nmr exhibited seven singlets at δ 0.83, 0.91, 0.92, 0.95, 0.96, 1.0 and 1.20 corresponding to seven tert-methyl groups, a multiplet at δ 4.58 due to a proton on carbon-3 and a triplet at δ 5.26 due to a vinyl proton on carbon-12 of the oleanolic acid moiety. Two trans olefinic protons at δ 6.29, 7.54 (d, 1H each, J=16Hz) and ABX-pattern proton signals at δ 6.86 (d,1H, J=8.2Hz), 7.03 (dd, 1H, J=2.3, 8.2Hz) and 7.16 (d, 1H, J=2.3Hz) are assignable to the 3',4'-dihydroxycinnamyloxy moiety. The ^{13}C-nmr data presented in Table 3 were assigned using the Attached Proton Test (APT)[14] and comparison with reported spectra of 3β-acetoxyolean-12-ene-28-oic acid and 3β-caffeatoxylup-20(29)-ene-28-ol[15,16].

This study showed that the composition of the triterpenes in *B. nigra* was quite different from those observed in the previously studied birch species. Instead of lupenone (**II**), lupeol (**IV**) and betulin (**VI**) being the major components, the oleanane-type triterpene, 3β-acetoxyolean-12-ene-28-oic acid (**IX**) was the predominant compound in *B. nigra* (3.3% of the total extract). The second most abundant compound was the lupane-type triterpene, betulinic acid (**VII**) (2.9% of the total extract), a minor component in other birch barks. Other oleanane-type triterpenes such as oleanolic acid (**X**) and 3β-caffeatoxyolean-12-ene-28-oic acid (**XII**) were also present in relatively high amounts (0.9% and 1.1% of the total extract, respectively).

Many triterpenes with oleanane or lupane skeletons are biologically active. For example, compounds **V**, **VII** and **IX** have been found to display antitumor activity[17,18]; 3β-caffeatoxylup-20(29)-ene-28-

TABLE 3. ^{13}C NMR of Compound XII in d_4-Methanol.

atom	δ (ppm)	atom	δ (ppm)
1	39.3	20	31.6
2	24.6[a]	21	33.9
3	82.3	22	33.8
4	38.2[b]	23	28.7
5	56.8	24	17.7
6	19.4	25	15.9
7	35.0	26	17.4
8	39.0[b]	27	26.5
9	42.7	28	181.7
10	40.6[b]	29	33.6
11	24.7[a]	30	24.0
12	122.9[c]	CH=CHCO	169.2
13	145.2	CH=CHCO	116.5
14	42.7	CH=CHCO	146.6
15	28.8	1'	127.8
16	24.1[a]	2'	115.6
17	47.6	3'	145.2
18	42.7	4'	146.8
19	47.2	5'	115.1
		6'	123.5[c]

Chemical shifts having the same letter superscript may be interchanged.

oic acid and oleanolic acid (**X**) also showed anti-inflammatory activity[19,20]. In this study, the major compounds **VII** and **IX,** along with compound **V** and all other oleanane-type triterpenes isolated from *B. nigra,* were assayed for antifeedant activity against the Colorado potato beetle, an agriculturally important insect pest. Compounds **IX** and **XI** exhibited significant antifeedant activities (ED_{50} = 36.8 and 36.5 ug/cm^2). The activities of these two compounds are of the same order of

TABLE 4. Antifeedant Activity of Compounds IX, XI and X Against *Leptinotarsa decemlineata* (Colorado Potato Beetle).

Compound IX

Dosage ($\mu g/cm^2$)	N	Consumption (g) mean ± SE	%FR
0	22	0.0053(±0.0006)	
10	23	0.0039(±0.0005)	26
30	23	0.0025(±0.0004)	53
50	17	0.0021(±0.0003)	60

Compound XI

Dosage ($\mu g/cm^2$)	N	Comsumption (g) mean ± SE	%FR
0	10	0.0035(±0.0002)	
10	10	0.0034(±0.0003)	3
30	10	0.0026(±0.0004)	26
50	10	0.0010(±0.0003)	71

Compound X

Dosage ($\mu g/cm^2$)	N	Consumption (g) mean ± SE	%FR
0	22	0.0060(±0.0004)	
10	22	0.0054(±0.0004)	10
30	22	0.0042(±0.0004)	30
50	22	0.0036(±0.0003)	40

magnitude as the known antifeedant, limonin ($ED_{50} \simeq 8ug/cm^2$)[21]. Compound **X** showed somewhat lower activity ($ED_{50}=91.2ug/cm^2$). The dose response results of these three compounds are presented in Table 4. Compounds **V**, **VII** and **XII** exhibited no antifeedant activity at a dose of $50ug/cm^2$.

The biological activities of these triterpenes along with their natural abundance demonstrate that the bark of *B. nigra* is a potential source of

useful natural products. We are currently exploring structure-activity relationships in this system with the goal of designing model antifeedants, an approach which has proved successful in the case of the antifeedant, limonin[22].

EXPERIMENTAL

General Experimental Procedures: IR spectra were recorded on a Digilab FTS-60 spectrometer. ^{1}H-nmr and ^{13}C-nmr spectra were recorded on a Varian XL-200 spectrometer. All nmr spectra were obtained in $CDCl_3$ unless otherwise noted. Mass spectra were obtained on an HP-5985B mass spectrometer operating at 70 ev. Uncorrected melting points were determined on a Fisher-Johns melting point apparatus.

Plant Materials and Extraction: Outer bark of *Betula nigra* was collected near Atlanta, Georgia in April, 1990. The bark was then dried to constant weight at 100°C under air, pulverized, and 52g of this material was extracted with refluxing chloroform (700ml) three times. After evaporation of the chloroform, 7.28g of crude extract was obtained.

Isolation and Identification: The above crude extract was chromatographed on a silica gel (230-400 mesh) column (2.5 x 28 cm) using hexane/toluene/ethyl acetate gradient. Fractions were further purified by preparative thin layer chromatography and each compound was finally purified by recrystallization. Twelve triterpenes were isolated and identified. The identifications were made either by comparison of the spectral data to those of authentic samples or by comparison with literature data.

Lupenone [**II**] (6mg), lupeol [**IV**] (32mg) and betulin [**VI**] (70mg) were purified by preparative TLC with toluene, toluene-ethyl acetate (10:1) and toluene-ethyl acetate (5:1), respectively. Each was recrystallized from 95% ethyl alcohol as white needles. Their melting points, ^{1}H-NMR, IR, as well as MS spectra, were identical to those of authentic samples.

Methyl 3β-acetoxylup-20(29)-ene-28-oate [**I**] (3mg) was purified by preparative TLC with toluene: ir υ max 1730 (C=O), 1641 (C=C), 1245, 1133 (C-O) cm^{-1}; eims m/z $[M]^+$ 512 (5.3), $[M-CO_2Me]^+$ 453 (10.1), $[M-AcOH]^+$ 452 (20.1), 437 (7.4), 293 (19.9), 262 (37.8), 189 (100), 149 (82.1), 105 (52.2); ^{1}H-nmr 0.83, 0.84 x 2, 0.91, 0.95 (3H each, s, CH_3 x 5), 1.69 (3H, bs, CH_3-30), 2.04 (3H, s, CH_3CO), 2.98 (1H, m, H-19), 3.67 (3H, s, OCH_3), 4.47 (1H, m, H-3α), 4.60 (1H, m, H-29), 4.73 (1H, m, H-29). The acetylation of methyl lup-20(29)-ene-28-oate gave a product exhibiting an ^{1}H-nmr spectra identical with that of compound **I**.

Lup-20(29)-ene-3-one-28-al [**III**] (20mg) was purified by preparative TLC followed by crystallization from 95% EtOH to give colorless needles: mp. 153-155°; ir υ max 1720, 1706 (C=O), 1640 (C=C) cm^{-1}; eims m/z $[M]^+$ 438 (11.5), $[M-CHO]^+$ 409 (20.0), 273 (3.2), 219 (20.2), 205 (38.4), 189 (39.6), 133 (35.6), 105 (55.8), 81 (65), 55 (100); ^{1}H-nmr 0.92, 0.93, 0.96, 1.02, 1.07 (3H each, s, CH_3 x 5), 1.70 (3H, bs, CH_3-30), 2.45 (2H, m, H-2), 2.87 (1H, m, H-19), 4.64 (1H, m, H-29), 4.76 (1H, m, H-29), 9.67 (1H, d, J=1.7Hz, H-28).

3β-Acetoxylup-20(29)-ene-28-oic acid [**V**] (5mg) was purified by preparative TLC (toluene/acetone10:1) to give a white solid: mp. 278-280°; ir υ max 3080-2500 (COOH), 1735 (CH_3CO), 1694 (COOH), 1640 (C=C), 1244, 1027 (C-O) cm^{-1}; eims m/z $[M]^+$ 498 (5.2), $[M-COOH]^+$ 453 (2.8), $[M-AcOH]^+$ 438 (19.2), 423 (11.4), 395 (12.3), 259 (10.9), 248 (39.8), 203 (35.9), 189 (100), 175 (37.5), 159 (22.5), 147 (30.6), 133 (40.6), 119 (50), 105 (60.0); ^{1}H-nmr 0.82, 0.83, 0.84, 0.92, 0.96 (3H each, s, CH_3 x 5), 1.68 (3H, bs, CH_3-30), 2.03 (3H, s, CH_3CO), 2.98 (1H, m, H-19), 4.46 (1H, m, H-3α), 4.59 (1H, m, H-29), 4.72 (1H, m, H-29). The ^{1}H-nmr of this compound was the same as that of the acetylated product of the known compound, betulinic acid.

Betulinic acid [**VII**] (210mg) was purified by preparative TLC with toluene and ethyl acetate (3:1) followed by recrystallization from 95% EtOH to give a white solid: mp. 280-282°; ir υ max 3470 (OH), 1688 (COOH), 1646 (C=C), 1237 (C-O) cm^{-1}; eims m/z $[M]^+$ 456 (3.9), $[M-H_2O]^+$ 438

(2.8), 423 (1.8), 410 (3.9), 257 (4.8), 248 (20.9), 234 (9.3), 207 (42.7), 189 (94.2), 175 (33.6), 147 (36.9), 135 (48.8), 119 (74.2), 105 (94.5), 91 (98.2), 81 (91.2), 67 (80.5), 55 (100); ^{1}H-nmr 0.75, 0.82, 0.93, 0.96, 0.97 (3H each, s, CH_3 x 5), 1.69 (3H, bs, CH_3-30), 3.0 (1H, m, H-19), 3.16 (1H, m, H-3α), 4.61 (1H, m, H-29), 4.74 (1H, m, H-29).

3β-Caffeatoxylup-20(29)-ene-28-ol **[VIII]** (35mg) was purified by preparative TLC with toluene and ethyl acetate (2.5:1) to give a light yellow solid: ir υ max 3380 (OH), 1701, 1684 (C=O), 1630, 1604, 1515 (aromatic CH), 1272, 1178, 1013 (C-O) cm^{-1}; eims m/z [M-caffeic acid]$^+$ 424 (1.6), 409 (1.2), 393 (1.6), 381 (1.6), 355 (3.4), 248 (100), 233 (11.0), 207 (31.9), 189 (55.2), 175 (29.6), 163 (43.9), 147 (31.0), 135 (35.0), 121 (45.7), 107 (50.9); ^{1}H-nmr 0.86, 0.87, 0.90, 0.98, 1.02 (3H each, s, CH_3 x 5), 1.68 (3H, bs, CH_3-30), 3.35 (1H, d, J=11.5Hz, H-28), 3.82 (1H, d, J=11.5Hz, H-28), 4.59 (2H, m, H-3, 29), 4.68 (1H, m, H-29), 6.25 (1H, d, J=15.9Hz, CH=CH-CO), 6.86 (1H, d, J=7.8Hz, H-5'), 7.0 (1H, dd, J=7.8, 2.0, H-6'), 7.09 (1H, d, J=2.0, H-2'), 7.54 (1H, d, J=15.9, CH=CH-CO).

3β-Acetoxyolean-12-ene-28-oic acid **[IX]** (240mg) was purified by preparative TLC with toluene and ethyl acetate (5:1) followed by recrystallization from 95% EtOH to give white needles: mp. 255-257°; ir υ max 3400-2500 (COOH), 1732 (CH_3CO), 1694 (COOH), 1215, 1027 (C-O) cm^{-1}; eims m/z [M]$^+$ 498 (0.8), [M-COOH]$^+$ (0.4), [M-AcOH]$^+$ 438 (2.5), 423 (1.5), 300 (2.5), 250 (3.5), 248 (100), 233 (11.2), 203 (90.2), 189 (25.8), 175 (15.9), 147 (12.5), 133 (59.9), 119 (24.9); ^{1}H-nmr 0.74, 0.84, 0.86, 0.89, 0.92, 0.93, 1.12 (3H each, s, CH_3 x 7), 2.04 (3H, s, CH_3CO), 2.82 (1H, dd, J=3.7, 13.0Hz, H-18), 4.48 (1H, m, H-3α), 5.28 (1H, t, H-12).

Oleanolic acid **[X]** (67mg) was purified by preparative TLC with toluene and ethyl acetate (3:1) followed by recrystallization from acetone to give a white solid: ir υ max 3600-2500 (COOH), 1694 (COOH), 1232 (C-O) cm^{-1}; eims m/z [M]$^+$ 456 (1.7), 300 (2.6), 257 (2.0), 248 (83.8), 233 (11.1), 207 (16.9), 203 (66.6), 189 (20.1), 175 (14.5), 149 (29.3), 133 (52.0), 119 (39.0), 105 (52.9); ^{1}H-nmr 0.76, 0.77, 0.90, 0.91, 0.92, 0.98, 1.13 (3H each, s, CH_3 x 7), 2.82 (1H, dd, J= 3.6, 12.5Hz, H-18), 3.20 (1H, m, H-3α), 5.28 (1H, t, H-12).

3β-Acetoxyolean-11-oxo-12-ene-28-oic acid [**XI**] (11mg) was obtained by preparative TLC with toluene/ethyl acetate (3:1) followed by recrystallization from 95% EtOH to give white needles: mp. 278-280°; ir υ max 3500-2500 (COOH), 1729 (CH_3CO), 1693 (COOH), 1657 (C=CHCO), 1211, 1029 (C-O) cm^{-1}; eims m/z $[M]^+$ 512 (14.5), $[M-AcOH-CH_3]^+$ 437 (10.0), 303 (47.3), 262 (100), 257 (40.0), 217 (41.8), 189 (31.8), 175 (47.3), 161 (25.5), 159 (24.5), 149 (20.0), 135 (30.0), 121 (29.1), 107 (37.3); ^{1}H-nmr 0.86 x 2, 0.91, 0.93 x 2, 1.13, 1.35 (3H each, s, CH_3 x 7), 2.04 (3H, s, CH_3CO), 2.33 (1H, s, H-9), 2.76-3.03 (1H, m, H-18), 4.50 (1H, m, H-3α), 5.63 (1H, s, H-12).

Oxidation of **IX**: 20mg **IX** was refluxed in 12ml glacial acetic acid, to which CrO_3 (18mg) in 0.8 ml glacial acetic acid was added dropwise and the resulting mixture was refluxed for 45 minutes. Upon workup, the major product (8mg) was separated by TLC using toluene/ethyl acetate (5:1). The ^{1}H-nmr data of this product was identical to that of compound **XI**.

3β-Caffeatoxyolean-12-ene-28-oic acid [**XII**] (80mg) was purified by preparative TLC with toluene/ethyl acetate (2:1) followed by recrystallization from $CHCl_3$ to give a white solid: mp. 293-294°; ir υ max 3400-2500 (COOH), 1695 (COOH), 1686 (CH=CHCO), 1603, 1515 (aromatic CH), 1270, 1180 (C-O) cm^{-1}; cims m/z $[M+1]^+$ 619 (1.0), [M+1-caffeic acid]$^+$ 439 (2.1); eims m/z [M-caffeic acid]$^+$ 438 (3.2), 394 (6.5), 379 (2.0), 250 (2.5), 248 (74.2), 235 (2.8), 203 (77.3), 190 (40.8), 175 29.6), 163 (100), 135 (27.6), 133 (44.3), 121 (31.1), 105 (48.6); ^{1}H-nmr (d_6-acetone) 0.83, 0.91, 0.92, 0.95, 0.96, 1.0, 1.20 (3H each, s, CH_3 x 7), 2.82 (1H. m, H-18), 4.58 (1H, m, H-3α), 5.26 (1H, t, H-12), 6.29 (1H, d, J=16Hz, CH=CHCO), 6.86 (1H, d, J=8.2, H-5'), 7.03 (1H, dd, J=2.3, 8.2Hz, H-6'), 7.16 (1H, d, J=2.3Hz, H-2'), 7.54 (1H, d, J=16Hz, CH=CHCO). ^{13}C-nmr see Table 3.

Bioassay: Colorado potato beetle larvae used in the assays were reared at 24°C, ca 50% RH, 16:8 (L:D) photoperiod on potato foliage (*Solanum tuberosum* L. cv Katahdin) from greenhouse-grown plants.

No-choice feeding assays to assess antifeedant activity[23] were conducted in feeding arenas made of plastic petri dishes (15x90mm) lined with moistened filter paper. Disks (11cm diameter, ca 1cm^2) cut from potato leaves with a no. 8 cork hole borer were coated on the upper surface with 30ul of a solution of the test compound in acetone (treated), or with 30ul of acetone only (control). Three treated or three control disks were placed equidistant from each other on the top of the filter paper in each dish. At the beginning of each test, two fourth instars (which had molted within 24 hrs prior to the test) were placed into each arena for 6-8 hr. Assays began 4-5 hr after the start of photophase. Arenas were placed into clear plastic ventilated boxes containing moist paper toweling, and were kept at 24^{o}C in an environmental chamber.

Each compound was tested at four dosage levels (0 [control], 10, 30 and 50 ug/cm^2) in ten to twenty-three arenas for each compound and dosage. The amount of leaf material consumed in each arena was determined by weighing to the nearest 0.1 mg the oven-dried (24hr at 100^{o}C) leaf material remaining uneaten at the end of the test. Initial leaf disk weight was estimated from the mean dry weight of an additional 30-60 leaf disks. Consumption was calculated as the mean initial leaf disk weight minus the uneaten leaf disk weight. A treatment / control consumption ratio was calculated for each compound at each dosage level. Percentage feeding reduction (%FR) was calculated as:

%FR =[1-(mean treatment consumption/mean control consumption)] x100

The effective dose required to produce 50% FR (ED_{50}) was calculated by linear regression of the logarithm of the dosage against consumption using a computer statistical program[24].

ACKNOWLEDGEMENTS

We thank the USDA for support of this project through grants 87-CSRS-2-3053 and 88-34158-3276. We also thank the Maine Agricultural Experiment Station for partial support.

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