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Evaluation of Insecticidal Activity of Diterpenes and Lignans from *Aristolochia malmeana* against *Anticarsia gemmatalis*

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The insecticidal activity of hexane extracts from the roots and leaves of *Aristolochia malmeana* was evaluated against *Anticarsia gemmatalis* larvae by topical application. Extract from the roots was the most active and caused 50% mortality in larvae at 308.4 μg/μL. From this extract, a clerodane diterpene, (−)-kolavenic acid, and three lignans, (−)-kusunokinin, (−)-hinokinin, and (8*S*,8′*R*,9*S*)-cubebin, were isolated by chromatography and partition procedures and then evaluated for their insecticidal activities either individually or in pairs. (−)-Kusunokinin showed higher activity against *A. gemmatalis* (LD_{10} = 9.3, LD_{50} = 230.1 μg/μL) than the crude extract, and its activity was dose-dependent, whereas the other constituents did not exhibit any significant activity. Together with (−)-kusunokinin and (−)-hinokinin, (−)-copalic acid, (−)-2-oxokolavenic acid, (−)-*ent*-6-β-hydroxy-copalic acid, (8*R*,8′*R*,9*R*)- and (8*R*,8′*R*,9*S*)-cubebins, (−)-fargesin, and (−)-phillygenin were isolated from the hexane extract of the leaves. The compounds were identified on the basis of spectroscopic analysis.

KEYWORDS: Aristolochiaceae; *Aristolochia malmeana*; *Anticarsia gemmatalis*; insecticidal activity; lignan; diterpene; kusunokinin; cubebin; kolavenic acid; hinokinin

INTRODUCTION

Aristolochia species (Aristolochiaceae) have mainly been used as abortifacients, stomachics, antiophidians, antiasthmatics, and expectorants in Brazilian traditional medicine and, recently, in slimming therapies (*1–3*). They are known as "one thousand men" and are rich sources of diterpenes and lignans (*1, 4–8*). A previous study on the essential oil from the roots of *Aristolochia malmeana* Hoehne led to the identification of camphene, limonene, terpinolene, longicyclene, isosativene, and β-gurjunene by GC-MS (*9*). The monoterpenes camphene (46.3%) and β-gurjunene (17.1%) were the major compounds in the oil (*9*).

Various aristolochic acids, lignans, and diterpenes play a significant role in the defense of plants against insects (*10–14*). Although the precise mode of action of such compounds is mostly unknown (*11*), they can act largely as regulators of insect feeding, but in some cases they can also influence specific physiological functions of insects (*14–16*). The temporal variation in aristolochic acids defense exhibited by pipevine swallowtail [*Battus philenor* L. (Lepidoptera: Papilionidae)] has been shown to be both age- and sex-dependent (*17*).

Species belonging to Aristolochiaceae are active against and/or deterrents toward most insects. The fact that specific insects in the Troidini tribe and Parnassiinae subfamily, such as *B. philenor*, can tolerate toxic and/or deterrent aristolochic acids present in host plants of the Aristolochiaceae family (*17*) supports the hypothesis that such compounds represent barriers to colonization by other polyphagous *Lepidoptera* species (*18*).

Screening of natural insecticides from *Aristolochia* species has become a promising route for the discovery of new compounds and/or botanical preparations (part or extracts of plants) (*10, 19*), which could be used in crop protection against *Anticarsia gemmatalis* H. (Lepidoptera: Noctuidae). Larvae of

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this insect represent the major defoliator pest of soybean crops in Brazil (*20*).

In an attempt to identify management alternatives that mitigate environmental degradation, animal health, and farm-worker safety, while maintaining the agricultural productivity of soybean crops, we performed this study to evaluate the potential insecticidal activity of extracts and chemical constituents from *A. malmeana* against *A. gemmatalis* larvae.

MATERIALS AND METHODS

Instrumentation. One-dimensional (^{1}H, ^{13}C, DEPT, and *g*NOESY) and two-dimensional (^{1}H−^{1}H *g*COSY, *g*HMQC, *g*HMBC, and *g*NOESY) NMR experiments were recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (^{1}H) and 126 MHz (^{13}C), with the residual solvent ($CHCl_3$) used as an internal standard for ^{1}H (δ 7.23) and $CDCl_3$ for ^{13}C (δ 77.0). Mass spectra [electrospray ionization−mass spectroscopy (ESI-MS)] were performed on a Fisons Platform II, and flow injection into the electrospray source was used for ESI-MS. Infrared spectra (IR) were obtained on a Nicolet Impact 400 spectrometer using KBr disks. Ultraviolet (UV) absorptions were measured on a Perkin-Elmer UV−vis Lambda 14P diode array spectrophotometer. HPLC analyses were performed using a Shimadzu liquid chromatograph 10Avp equipped with UV–vis (210 and 254 nm) and 341-LC polarimeter detectors and a Nova-Pak (Waters; silica 60 Å, 5 μm; 3.9 × 150 mm) column. Optical rotations were measured on a Perkin-Elmer 341-LC polarimeter. A 100 μL Hamilton syringe and a Burkard 900X microapplicator (Rickmansworth, U.K.) were used for topical application to the larvae.

Solvents. Nanopure water (>18.2 MΩcm) was obtained using a Millipore purifier (Bedford, MA) and filtered through Millipore. All of the HPLC-grade solvents were purchased from Mallinckrodt Baker Inc. (Paris, KY); all organic solvents were filtered through Millipore PTFE membranes (0.5 μm, 47.0 mm) prior to use, and samples were filtered through Millipore polyvinylidene fluoride (PVDF) membranes (0.45 μm, 13.0 mm). $CDCl_3$ 99.98% D for NMR analyses was purchased from Cambridge Isotope Laboratories, Inc. (CIL, Andover, MA).

Adsorbents. Silica gel 60 PF_{254} for thin-layer chromatography (PTLC) was purchased from Aldrich (Milwaukee, WI), and silica gel 60H for column chromatography (CC) was obtained from Merck (Darmstadt, Germany).

Plant Material. The plant material was collected in Ituiutaba, MG, Brazil, in February 2003 and identified as *A. malmeana* Hoehne (Aristolochiaceae) by Dr. Condorcet Aranha and by Dr. Lindolpho Capellari Júnior [Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Piracicaba, SP, Brazil]. A voucher specimen (ESA 88883) was deposited at the herbarium of the ESALQ, Piracicaba, SP, Brazil. The material was separated according to the plant parts, dried (∼45 °C), and ground.

Extraction and Isolation of the Chemical Constituents. The plant materials from the roots (496.0 g) and leaves (367.0 g) were extracted exhaustively at room temperature with hexane, acetone, and ethanol, successively, and the extracts were individually concentrated.

The root crude hexane extract (41.6 g) was washed with hexane. A precipitate (2.4 g) was separated, and a portion (50.0 mg) of it was subjected to PTLC (hexane/EtOAc, 7:3) to give **1** (15.0 mg), **2** (13.0 mg), and **3** (1.5 mg) (**Figure 1**). The hexane solution was concentrated (39.1 g) and crystallized in MeOH to give **4** (38.1 g).

The crude hexane extract (37.9 g) from leaves was also washed with hexane. The insoluble portion (1.6 g) was subjected to CC (60.0 by 4.8 cm; silica gel 60H; 151.0 g; hexane/EtOAc gradient, 95:5 to 100% EtOAc) to give 25 fractions (∼100 mL each). Fractions 5, 8, 13, 14, and 16 gave **5** (3.0 mg), **6** (4.0 mg), **8** (115.0 mg), **1** (23.0 mg), and **9** (7.0 mg), respectively. Fractions 11, 12, and 20 were individually subjected to PTLC (hexane/EtOAc, 7:3) and gave **2** (3.2 mg), **7** (2.0 mg), and **10** (10.0 mg), respectively (**Figure 1**).

A comparative analysis of isolated compounds (**1**−**6**, **8**–**10**), solutions of **1** + **2**, **1** + **4**, and **2** + **4**, and the crude hexane extracts was carried out by ^{1}H NMR and HPLC (hexane/EtOH at various proportions; detectors: UV–vis and 341-LC polarimeter) to establish the identity and purity of these compounds and to estimate their chemical composition in the extracts. When subjected to HPLC (hexane/EtOH, 97:3, 1.0 mL/min) compounds **1**−**4**, which were isolated from extract of the roots, showed retention times (t_R) at 7.8, 3.6, 5.0, and 2.8 min, respectively, whereas **5**, **6**, **9**, and **10** from the leaves showed t_R at 1.9, 9.5, 10.3, and 1.8, respectively, and lignans **8** showed t_R at 6.0 and 7.2 min.

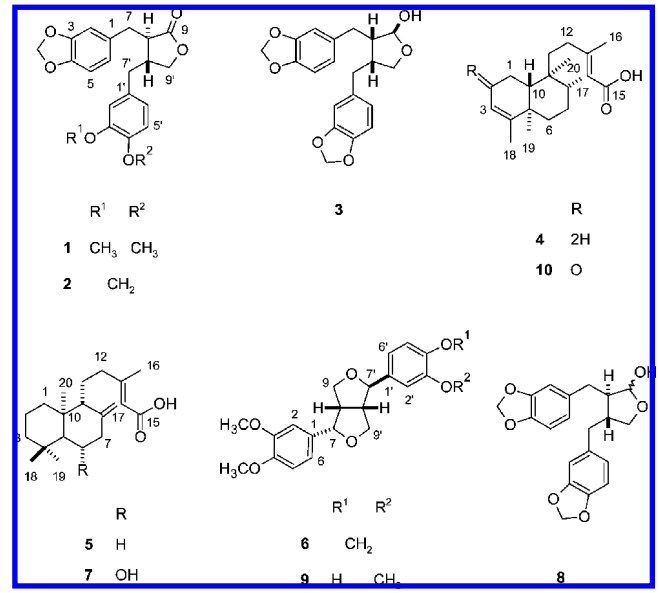

Figure 1. Chemical structures of compounds (**1**−**10**) isolated from the hexane extracts of *A. malmeana*.

(−)-Kusunokinin (1) was obtained as a yellow oil: $[\alpha]_D^{25}$ −40.0 (*c* 0.2, $CHCl_3$) [lit. −26.3 (*c* 0.1, $CHCl_3$) (*21*)]; IR, UV, and ^{13}C NMR data were consistent with those previously reported (*21*); ESI-MS, *m*/*z* 371 $[M + H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.54 (1H, d, J = 2.0 Hz, H-2), 6.65 (1H, d, J = 8.0 Hz, H-5), 6.53 (1H, dd, J = 8.0, 2.0 Hz, H-6), 5.87, 5.88 (2H, 2d, $w_{1/2}$ = 1.5 Hz, OCH_2O), 2.90 (1H, dd, J = 14.4, 5.5 Hz, H-7$_a$), 2.79 (1H, dd, J = 14.4, 7.0 Hz, H-7$_b$), 2.47 (1H, ddd, J = 8.7, 7.0, 5.5 Hz, H-8), 6.42 (1H, d, J = 2.0 Hz, H-2′), 6.70 (1H, d, J = 8.5 Hz, H-5′), 6.51 (1H, dd, J = 8.5, 2.0 Hz, H-6′), 3.77 (3H, s, 3′-OMe), 3.79 (3H, s, 4′-OMe), 2.55 (1H, dd, J = 12.5, 5.5 Hz, H-7$_a$′), 2.44 (1H, d, J = 12.5 Hz, H-7$_b$′), 2.44 (1H, ddt, J = 8.7, 5.5, 7.0 Hz, H-8′), 4.07 (1H, dd, J = 9.0, 7.0 Hz, H-9$_a$′), 3.81 (1H, dd, J = 9.0, 7.0 Hz, H-9$_b$′).

(−)-Hinokinin (2) was obtained as a yellow oil: $[\alpha]_D^{25}$ −30.3 (*c* 0.6, $CHCl_3$) [lit. −26.3 (*c* 0.1, $CHCl_3$) (*21*)]; IR, UV, and ^{13}C NMR data were consistent with those previously reported (*21, 22*); ESI-MS, *m*/*z* 355 $[M + H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.39 (1H, d, J = 2.0 Hz, H-2), 6.66 (1H, d, J = 8.0 Hz, H-5), 6.53 (1H, dd, J = 8.0, 2.0 Hz, H-6), 2.91 (1H, dd, J = 14.0, 5.0 Hz, H-7$_a$), 2.77 (1H, dd, J = 14.0, 7.5 Hz, H-7$_b$), 2.46 (1H, ddd, J = 8.0, 7.5, 5.0 Hz, H-8), 6.56 (1H, d, J = 2.0 Hz, H-2′), 6.63 (1H, d, J = 8.0 Hz, H-5′), 6.39 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 2.52 (1H, m, H-7$_a$′), 2.39 (2H, m, H-7$_b$′, H-8′), 4.06 (1H, dd, J = 9.0, 7.0 Hz, H-9$_a$′), 3.86 (1H, dd, J = 9.0, 7.5 Hz, H-9$_b$′), 5.86 (4H, m, OCH_2O).

(−)-(8*S*,8′*R*,9*S*)-Cubebin (3) was obtained as a colorless oil: $[\alpha]_D^{25}$, IR, UV, ^{1}H NMR, and ^{13}C NMR data were consistent with those previously reported (*22*); ESI-MS, *m*/*z* 357 $[M + H]^+$.

(−)-Kolavenic acid (4) was obtained as colorless crystals: mp 97–98 °C; $[\alpha]_D^{25}$ −41.4 (*c* 1.0, $CHCl_3$) [lit. −44.0 (*c* 0.7, $CHCl_3$) (*23*)]; IR and ^{13}C NMR data were consistent with those previously reported (*24*); ESI-MS, *m*/*z* 305 $[M + H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.93 (1H, t, $w_{1/2}$ = 7.0 Hz, H-1$_a$), 1.36 (1H, m, H-1$_b$), 1.99 (1H, m, H-2$_a$), 1.94 (1H, m, H-2$_b$), 5.13 (1H, br s, H-3), 1.66 (1H, dt, J = 13.0, 3.0 Hz, H-6$_a$), 1.12 (1H, ddd, J = 13.0, 12.0, 4.2 Hz, H-6$_b$), 1.33 (1H, m, H-7$_a$), 1.39 (1H, m, H-7$_b$), 1.37 (1H, m, H-8), 1.27 (1H, dd, J = 12.0, 1.5 Hz, H-10), 1.34 (1H, ddd, J = 14.0, 13.0, 4.5 Hz, H-11$_a$), 1.48 (1H, ddd, J = 14.0, 12.5, 5.0 Hz, H-11$_b$), 1.97 (1H, td, J = 13.0, 4.5 Hz, H-12$_a$),

1.90 (1H, ddd, J = 13.0, 12.5, 5.0 Hz, H-12$_b$), 5.62 (1H, dq, J = 2.5, 1.0 Hz, H-14), 2.11 (3H, br d, J = 1.0 Hz, H-16), 0.76 (3H, d, J = 6.0 Hz, H-17), 1.53 (3H, br s, H-18), 0.94 (3H, s, H-19), 0.68 (3H, s, H-20).

(−)-Copalic acid (5) was obtained as a colorless oil: $[\alpha]_D^{25}$ −33.7 (c 0.4, $CHCl_3$) [lit. −14.7 (c 1.7, $CHCl_3$) (*25*)]; UV and ^{13}C NMR data were consistent with those previously reported (*25*); ESI-MS, m/z 305 $[M + H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.76 (1H, m, H-1$_a$), 1.01 (1H, ddt, J = 17.0, 13.0, 3.5 Hz, H-1$_b$), 1.58 (1H, m, H-2$_a$), 1.50 (1H, m, H-2$_b$), 1.40 (1H, dtd, J = 13.5, 4.5, 1.5 Hz, H-3$_a$), 1.19 (1H, ddd, J = 13.5, 13.4, 4.0 Hz, H-3$_b$), 1.09 (1H, dd, J = 12.5, 2.5 Hz, H-5), 1.74 (1H, m, H-6$_a$), 1.32 (1H, tdd, J = 12.5, 12.0, 4.0 Hz, H-6$_b$), 2.00 (1H, m, H-7$_a$), 2.40 (1H, ddd, J = 12.5, 4.0, 2.5 Hz, H-7$_b$), 1.58 (1H, m, H-9), 1.68 (1H, m, H-11$_a$), 1.50 (1H, m, H-11$_b$), 1.96 (1H, m, H-12$_a$), 2.33 (1H, ddd, J = 14.4, 9.7, 3.3 Hz, H-12$_b$), 5.68 (1H, q, J = 1.3 Hz, H-14), 2.17 (3H, d, J = 1.3 Hz, H-16), 4.85 (1H, d, J = 1.5 Hz, H-17$_a$), 4.50 (1H, d, J = 1.5 Hz, H-17$_b$), 0.88 (3H, s, H-18), 0.81 (3H, s, H-19), 0.69 (3H, s, H-20).

(−)-Fargesin (6) was obtained as colorless crystals: mp 135–136 °C [lit. 138–141 °C (*26*)]; $[\alpha]_D^{25}$ −54.7 (c 0.3, $CHCl_3$) [lit. +66.0 (c 0.1, $CHCl_3$) (*27*)]; IR data were consistent with those previously reported (*26*); ESI-MS, m/z 371 $[M + H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.87 (1H, br s, H-2), 6.79 (2H, br s, H-5, H-6), 3.82 (3H, s, 4-OMe), 3.83 (3H, s, 3-OMe), 4.81 (1H, d, J = 5.5 Hz, H-7), 3.27 (1H, dtd, J = 8.0, 7.0, 5.5 Hz, H-8), 3.25 (1H, t, J = 7.0 Hz, H-9$_a$), 3.77 (1H, t, J = 7.0 Hz, H-9$_b$), 6.81 (1H, d, J = 1.0 Hz, H-2′), 6.71 (1H, d, J = 8.0 Hz, H-5′), 6.78 (1H, dd, J = 8.0, 1.0 Hz, H-6′), 5.88, 5.89 (2H, 2 × d, $w_{1/2}$ = 1.5 Hz, OCH_2O), 4.37 (1H, d, J = 7.0 Hz, H-7′), 2.82 (1H, dddd, J = 8.0, 7.0, 6.5, 1.0 Hz, H-8′), 3.79 (1H, dd, J = 9.5, 6.5 Hz, H-9$_a$′), 4.06 (1H, dd, J = 9.5, 1.0 Hz, H-9$_b$′); ^{13}C NMR (126 MHz, $CDCl_3$) δ 130.9 (C-1), 108.9 (C-2), 148.8 (C-3), 148.0 (C-4), 111.0 (C-5), 117.6 (C-6), 55.8 (2 × OMe), 82.0 (C-7), 50.1 (C-8), 69.7 (C-9), 135.1 (C-1′), 106.5 (C-2′), 147.9 (C-3′), 147.1 (C-4′), 108.1 (C-5′), 119.5 (C-6′), 101.0 (OCH_2O), 87.6 (C-7′), 54.5 (C-8′), 70.9 (C-9′).

(−)-*ent*-6-*β*-Hydroxycopalic acid (7) was obtained as a colorless oil: $[\alpha]_D^{25}$ −25.3 (c 0.43, $CHCl_3$); ^{1}H NMR and ^{13}C NMR data were consistent with those previously reported and authentic sample (*28, 29*); ESI-MS, m/z 321 $[M + H]^+$.

(−)-(8*R*,8′*R*,9*R*)-Cubebin + (−)-(8*R*,8′*R*,9*S*)-cubebin (8) was obtained as colorless crystals: mp 127–128 °C [lit. 133–134 °C (*30*)]; $[\alpha]_D^{25}$ −41.5 (c 0.4, $CHCl_3$) [lit. −53.8 (c 2.6, $CHCl_3$) (*22*)]; ^{1}H NMR and ^{13}C NMR data were consistent with those previously reported (*22*); ESI-MS, m/z 357 $[M + H]^+$.

(−)-Phillygenin (9) was obtained as colorless crystals: mp 130–131 °C [lit. 128–130 °C (*31*)]; $[\alpha]_D^{25}$ −120.0 (c 0.2, $CHCl_3$) [lit. −114 (c 0.1, $CHCl_3$) (*31*)]; IR data were consistent with those previously reported (*31*); ESI-MS, m/z 373 $[M + H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.96 (1H, d, J = 1.5 Hz, H-2), 6.87 (2H, br s, H-5, H-6), 3.93 (3H, s, 3-OMe), 3.90 (3H, s, 4-OMe), 4.89 (1H, d, J = 5.5 Hz, H-7), 3.34 (2H, m, H-8, H-9$_a$), 3.85 (1H, m, H-9$_b$), 6.93 (1H, d, J = 2.0 Hz, H-2′), 6.91 (1H, d, J = 8.0 Hz, H-5′), 6.86 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 3.92 (3H, s, 3′-OMe), 4.46 (1H, d, J = 7.0 Hz, H-7′), 2.93 (1H, m, H-8′), 4.15 (1H, dd, J = 10.0, 1.0 Hz, H-9′$_a$), 3.87 (1H, m, H-9′$_b$); ^{13}C NMR (126 MHz, $CDCl_3$) δ 131.0 (C-1), 109.1 (C-2), 148.9 (C-3), 148.1 (C-4), 111.2 (C-5), 117.8 (C-6), 82.1 (C-7), 50.3 (C-8), 69.7 (C-9), 133.1 (C-1′), 108.6 (C-2′), 146.7 (C-3′), 145.4 (C-4′), 114.6 (C-5′), 119.2 (C-6′), 87.7 (C-7′), 54.5 (C-8′), 71.0 (C-9′), 2 × 56.0, 55.9 (3 × OMe).

(−)-2-Oxokolavenic acid (10): ^{1}H NMR and ^{13}C NMR data were consistent with those previously reported (*24, 32*); ESI-MS, m/z 319 $[M + H]^+$.

Insects. The insecticidal experiments performed with *A. gemmatalis* (velvetbean caterpillar) larvae complied the appropriate laws and institutional guidelines and were approved by the Ethical Committee for Animal Welfare (CEBEA-023071-07), UNESP.

Larvae of *A. gemmatalis* (body weight = 23 ± 2 mg) were obtained from a laboratory colony that had been reared on an artificial diet under controlled conditions (25 ± 2 °C, 75 ± 10% relative humidity, 14:10 h light/dark photoperiod) at the Insect Biology Laboratory of the Faculdade de Ciências Agrárias e Veterinárias, UNESP, Jaboticabal, SP, Brazil.

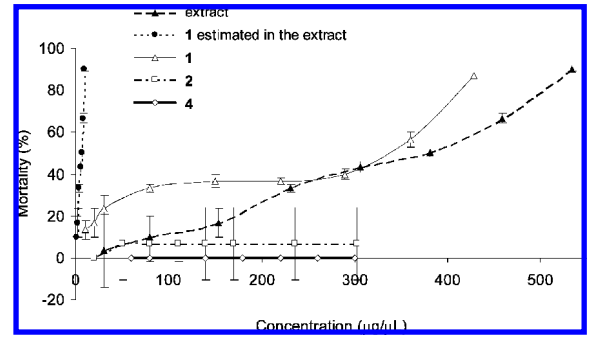

Figure 2. Effects of the hexane extract of the roots, of **1** estimated in the extract, and of **1**, **2**, and **4** on mortality of *A. gemmatalis*. Bars indicate standard deviation (SD) of observations.

Bioassay. The crude hexane, $(CH_3)_2CO$, and EtOH extracts from the roots and leaves of *A. malmeana* were dissolved in acetone (382 ± 1 and 154 ± 1 mg/mL, respectively) and then individually applied topically to the mesothorax of third-instar larvae at doses of 1.0 μL per larva. Control larvae were treated with 1.0 μL of water or acetone. For each treatment, three glass Petri dishes, each with 10 larvae, were used. Larval mortality was scored after exposure for 24, 48, and 72 h. The responses were interpreted as surviving or death, based on the larval response to mechanical stimulation. The hexane extract from the roots was also evaluated using eight different doses (from 30.5 to 534.0 μg/μL) (**Figure 2**).

The susceptibility of *A. gemmatalis* larvae to the three compounds (**1**, **2**, and **4**) dissolved in acetone was evaluated using different doses of 1.0 μL, which contained from 10.0 to 427.5 μg of each compound per insect (**Figure 2**). Solutions of 381.7 ± 0.3 mg/mL of **2** + **4** (A), **1** + **4** (B), and **1** + **2** (C), at different proportions, were also applied topically to the mesothorax of third-instar larvae (1.0 μL/larva), as well as the solvent (acetone) to the larvae of control groups. The treatments were replicated three times, and the responses were analyzed as described for the extracts.

Statistical Analysis. Concentrations that gave cumulative mortality rates of 10 and 50% compared to the control were defined as LD_{10} and LD_{50}, respectively. Differences between LD_{10} and LD_{50} values were evaluated by a Probit analysis using POLO-PC (LeOra software) (*33*). $P \leq 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

The insecticidal activity of the crude hexane, $(CH_3)_2CO$, and EtOH extracts from the roots and leaves of *A. malmeana* was evaluated against *A. gemmatalis* larvae (velvetbean caterpillar) by topical application to their mesothorax. Only the hexane extracts showed significant activities; that from the roots was the most active and caused 50% mortality in larvae at 381.7 μg/μL, whereas that from leaves caused only 8.6% mortality at the same concentration. From the former extract, two butyrolactone lignans, (−)-kusunokinin (**1**) and (−)-hinokinin (**2**), a butyrolactol lignan, (8*S*,8′*R*,9*S*)-cubebin (**3**), and a clerodane diterpene, (−)-kolavenic acid (**4**), were isolated by thin-layer chromatography and recrystallization. Their structures were identified by comparison of their spectroscopic data (ESI-MS, IR, UV, ^{1}H and ^{13}C NMR) and optical activities (α_D) with those reported in the literature (*21–24*), as well as with the assistance of *g*NOESY, *g*HMQC, and *g*HMBC experiments, which helped to establish the positions of methoxy and methylenedioxy groups on the aromatic rings for **1** and the relative configurations for **1**–**4**. Furthermore, the ^{1}H NMR spectra simulated using the program FOMSC3 (*34*) for first-order coupling between hydrogens of **1** and **4** were in total agreement with the experimental

spectra and the relative configurations determined. Moreover, X-ray crystallography analysis of **4** corroborated the structural identification of this diterpene (data to be published elsewhere).

Together with lignans **1** and **2**, (−)-copalic acid (**5**), (−)-fargesin (**6**), (−)-*ent*-6-β-hydroxy-copalic acid (**7**), (8*R*,8′*R*,9*R*)- and (8*R*,8′*R*,9*S*)-cubebins (**8**), (−)-phillygenin (**9**), and 2-oxokolavenic acid (**10**) were isolated from the hexane extract of the leaves by partition and chromatographic procedures. The structures of these compounds were identified by comparison of their spectroscopic data (ESI-MS, IR, ^{1}H and ^{13}C NMR) and optical activities (α_D) with those reported in the literature (*21, 22, 24–32*). Although furofuran lignans have been known for some time, the determination of their configurations is still a subject of controversy (*26, 27*). Analysis of ^{1}H−^{1}H COSY, *g*HMQC, and *g*HMBC data enabled us to determine of the relative configurations for **6** and **9**.

(−)-Kolavenic acid (**4**) was the major constituent of the hexane extract of the roots (89% w/w), followed by **1** (<2%) and **2** (<2%). Traces of **3** (<0.5%) accounted for the remaining constituents in the extract, as shown by the yield of the compounds isolated from the extract and by ^{1}H NMR experiments when we analyzed the integration shown in the ^{1}H NMR spectra. In particular, the characteristic signals at δ 5.62, 5.13, and 2.11 corresponded, respectively, to H-14, H-3, and 3H-16 of **4**, that at δ 5.04 corresponded to H-9 of **3**, and those at δ 3.77 and 3.79 were characteristic of methoxyl hydrogens of **1**. The HPLC analyses of extracts and the isolated compounds obtained at diverse elution and detection conditions are in agreement with this yield determination.

Clerodane diterpenes have already been isolated from *Aristolochia* species (*1, 5, 24, 28*). Clerodanes and related model compounds have been reported as sources of natural insect antifeedants (*12*). Although a detailed analysis of the structure–activity relationships has not yet been presented, there are some interesting trends based on the structures of the strongest antifeedants. A mixture of (sensory) antifeedant activity and toxic effects has also been attributed to clerodanes (*12*). (+)-Kolavenic and (+)-2-oxokolavenic acids, for example, were evaluated against *Reticulitermes speratus* K. (Isoptera: Rhinotermitidae, subterranean termite) and showed antifeedant activity (*35*).

With regard to lignans, the effects of several of them have been reviewed in a variety of insect species with special focus on the recent advances on the determination of toxicity, feeding deterrence, growth disruption, excretion, and interactions between insects (*11, 16*). Several lignans of diverse structural types have been described in the literature as insecticide synergists, even though they were individually inactive. Dibenzylbutyrolactone-type lignans have been described as feeding inhibitors, feeding deterrents, or larval growth inhibitors (*11, 14, 36*). Hinokinin (**2**), a dibenzylbutyrolactone type that contains two methylenedioxyl groups, has been shown to be a pyrethroid−synergistic insecticide (*37, 38*). The importance of the methylenedioxyl substituent in the structures for a synergistic effect has been a subject of some debate in the literature (*38*). Hinokinin, together with (8*R*,8′*R*,9*R*)- and (8*R*,8′*R*,9*S*)-cubebins (**8**), butyrolactol lignans, has also shown a feeding deterrent effect toward selected stored-product pests: *Sitophilus granaries* L. (Coleoptera: Curculionidae), *Tribolium confusum* V. (Coleoptera: Tenebrionideae), and *Trogoderma granarium* E. (Coleoptera: Dermestridae) (*14, 38, 39*).

In our previous work, the insecticidal activity of (8*R*,8′*R*,9*R*)- and (8*R*,8′*R*,9*S*)-cubebins (**8**) against *A. gemmatalis* was evaluated topically, and these compounds did not exhibit significant activity (*10*). Therefore, except for (8*S*,8′*R*,9*S*)-cubebin (**3**), the minor root extract constituent, the activities of the other isolated compounds were evaluated against *A. gemmatalis*. (−)-Kusunokinin (**1**) showed higher activity against *A. gemmatalis* (LD_{10} = 9.3, LD_{50} = 230.1 μg/μL) than the crude extract (LD_{10} = 92.0, LD_{50} = 308.4 μg/μL), and its activity was shown to be dose-dependent. No significant mortality was observed for **2** or **4**, and the activity determined for **1** alone was lower than that estimated for it in the extract at diverse concentrations (**Figure 2**). To verify if there was a synergistic or antagonist effect, solutions containing 381.7 ± 0.3 μg/μL of **1**, **2**, and **4** (solutions A−C) at several proportions were bioassayed (**Figure 3**). These experiments showed that the effect of **1** on mortality was still dose-dependent, but that its activity (expressed as a function of the concentration of **1** in **Figure 3**) diminished when **2** and **4** were present. Therefore, compounds **2** and **4** have an antagonistic effect. These results suggest that the crude extract contains compounds, such as (8*S*,8′*R*,9*S*)-cubebin (**3**), that at specific concentrations may increase the bioavailability of **1** and render it more effective. In general, lignans that contain a methylenedioxyl group show higher antifeedant or deterrent activity against insects than those with polar substituents on the aromatic rings, especially hydroxyl or glycosyl groups, and even higher than those with methoxyl groups. The results, seen here with topical application, support the notion that the methoxyl groups are important for activity. Thus, structural changes in an insecticidal molecule may affect the activity in different manners for different insect species and for different methods of application.

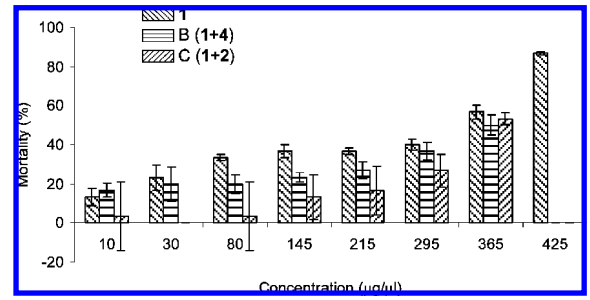

Figure 3. Effect of kusunokinin (**1**) and solutions B (**1** + **4**) and C (**1** + **2**), as a function of **1** concentration, at doses of 381.7 ± 0.3 μg/larva, on mortality of *A. gemmatalis*. Bars indicate standard deviation (SD) of observations.

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