Antigiardial Activity of Triterpenoids from Root Bark of *Hippocratea excelsa*[||]

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Received November 8, 2006

Two new triterpenoids, 21β-hydroxyolean-12-en-3-one (**1**) and a seco-dinor derivative of pristimerine named dzununcanone (**2**), were isolated from the root bark of *Hippocratea excelsa*. Their structures were assigned on the basis of spectroscopic evidence, mainly ^{1}H and ^{13}C 1D and 2D NMR including DEPT, COSY, ROESY, HSQC, and HMBC experiments, as well as EIMS and HREIMS. The known 21α-hydroxy-3-oxofriedelane (**3**), a compound new to the species, and the known methide quinones pristimerine (**4**) tingenone (**5**), and xuxuarine Eβ (**7**) were also isolated. The antiprotozoal activities were determined against *Giardia intestinalis*. Pristimerine and tingenone were the most active antigiardial compounds, with IC_{50} values of 0.11 and 0.74 μM, respectively, compared with metronidazole, the current drug of choice (IC_{50} 1.23 μM).

Giardia intestinalis (syn. *Giardia lamblia*, *Giardia duodenalis*) is a cosmopolitan protozoan that causes intestinal infections in humans, known as giardiasis. This infectious disease is considered a zoonosis that may be transmitted by dogs, cats, livestock, rodents, and other mammal reservoirs.[1] Symptomatic infection is characterized by diarrhea, epigastric pain, nausea, vomiting, cramps, and weight loss; chronic giardiasis causes malabsorption sydrome and, thus, malnutrition, especially in children.[2] Although, most infections are asymptomatic,[3,4] the transmission of *Giardia* cysts commonly occurs via the ingestion of contaminated water and food. Other types of contagion include close contact with infected people, fecal−oral contact, and sexual practices.[2,3]

WHO has estimated that 280 million people are infected each year.[5] The prevalence of infection varies from 2% to 7% in industrialized countries to more than 50% in developing countries in Asia, Africa, and Latin America.[6] In Mexico the prevalence of giardiasis varies from 3% to 60%. In Yucatan the prevalence varies depending on educational level and sanitary and climatic conditions.

The drugs used to treat this illness are 5-nitroimidazoles, such as metronidazole, the nitrofuran furazolidone, and more recently benzimidazoles and nitazoxanide. Although these drugs are effective, most of them have side effects such as headache, nausea, and vomiting during the period of treatment.[2,7] Previous studies have also demonstrated cytotoxic effects of metronidazole.[8] Furazolidone has a mutagenic potential in bacteria and could lead to mammary tumors in rats, while benzimidazoles could be teratogenic agents.[9,10] In addition, there are reports of drug resistance of *G. intestinalis* to these drugs.[5,11] Thus, there is an evident continuing need for effective and safe antigiardial agents, and medicinal plants are promising sources of such compounds.

As part of our search for bioactive compounds from Mayan medicinal plants, we have investigated *Hippocratea excelsa* H. B. K. (Celastraceae), a medicinal and insecticidal plant growing in the Yucatan Peninsula. The root bark is commonly known in the central part of Mexico as "cancerina", due to its popular use in the treatment of cancer, and "mata piojo" in Yucatan, Michoacan, and Guerrero, because of its use against lice and mites.[12] The plant is also used as a sedative, anti-inflammatory, and cicatrizing agent and for treatment of dysentery, gastritis, and gastric ulcers.[13] The species is restricted to Mexico and Central America. In Mexico the plant may be found in the states of Chiapas, Oaxaca, Guerrero, Michoacan, Jalisco, Mexico, Morelos, Durango, and Yucatan.[12] This paper describes the isolation and structure elucidation of active antiprotozoal triterpenes from the roots of this plant, including two new compounds, 21β-hydroxyolean-12-en-3-one (**1**) and a seco-dinor-triterpenoid named dzununcanone (**2**). The known compounds 21α-hydroxy-3-oxofriedelane (**3**), pristimerine (**4**), tingenone (**5**), and xuxarine Eβ (**7**)[14] were also isolated. The structures of the compounds were established by 1D and 2D NMR experiments and comparison of their physical and spectroscopic data with data in the literature.

Compound **1** was obtained as a gum with IR absorption bands at 3442 and 1701 cm^{-1}, suggesting the presence of hydroxyl and ketone groups in the molecule. The HREIMS gave an accurate ion peak at *m*/*z* 440.3639 corresponding to the molecular formula $C_{30}H_{48}O_2$ (calcd 440.3654), which was also in good agreement with the ^{13}C NMR and DEPT data. The ^{13}C NMR spectrum indicated the presence of 30 carbon atoms, which accounted for eight methyl groups, nine methylenes, five methines including one olefinic and one hydroxymethine, and eight quaternary carbons including one olefinic and one ketone. The ^{1}H NMR spectrum confirmed the presence of eight tertiary methyl groups. It also showed the presence of one methine proton (δ_H 5.24, t, J = 3.5 Hz, δ_C 122.4) attached to a trisubstituted double bond and one hydroxymethine proton (δ_H 3.52, dd, J = 12.0, 4.7 Hz, δ_C 74.0). On the basis of these data compound **1** was assumed to be a 12-oleanene type triterpene with a secondary hydroxyl group and a keto group. Two doublets of doublets of doublets at δ 2.55 (J = 15.9, 11.1, 7.3 Hz) and 2.37 (J = 15.9, 6.8, 3.7 Hz) suggested that the keto group was at C-3 (δ_C 217.8). The EIMS showed a characteristic fragment peak at *m*/*z* 234 due to retro-Diels−Alder cleavage, indicating that the secondary hydroxyl group must be at ring D or E.

Correlations observed in the HMBC experiment between the hydroxymethine signal and two methyl carbon signals at δ 29.1 (C-29) and 16.9 (C-30), a methylene signal at δ 45.3 (C-22), and a signal of a quaternary carbon at δ 36.3 (C-20) indicated that the secondary hydroxyl group must be at C-21. Accordingly, the proton methyl signals at δ 0.97 (H-29) and 0.86 (H-30) showed long-range correlations with the methine carbon signal at δ 74.0

[||] Taken in part from the Ph.D. dissertation of G.J.M.-R.

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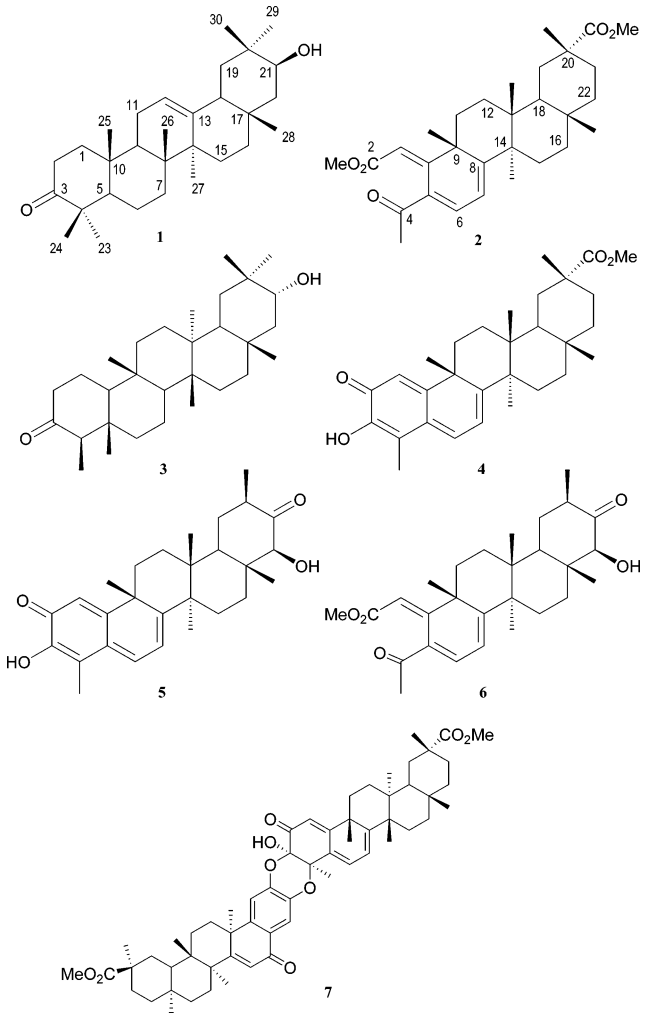
10.1021/np060559y CCC: $37.00
Published on Web 03/27/2007



(C-21), a methylene signal at δ 47.0 (C-19), and the quaternary carbon at δ 36.3 (C-20). The methyl signal at δ_H 0.97 (H-29) showed correlation with the methyl signal at δ 16.9 (C-30), and the methyl signal at δ_H 0.86 (H-30) showed correlation with the methyl signal at δ 29.1 (C-29). Considering the *cis* junction and chair−chair conformation of the D and E rings in oleanane triterpenes,[15,16] the configuration at C-21 was assigned from the coupling constants of H-21, which showed axial−axial and axial−equatorial coupling values (J = 12.0, 4.8 Hz) with the C-22 protons, indicating that the hydroxyl group must be equatorial (β-oriented). Furthermore cross-peaks between signals at δ_H 3.53 (H-21α) and δ_H 0.97 (H-29α), 1.00 (H-16α), 1.50 (H-22α), and 1.75 (H-19α) in the ROESY experiment confirmed the α-orientation of H-21. Confirmation of the keto group at C-3 and assignment of the C-23 and C-24 *gem*-dimethyl group signals was substantiated by the long-range coupling between the carbonyl signal at δ 217.8 and both methyl signals at δ_H 1.10 and 1.06 (H-23 and H-24, respectively). Therefore, compound **1** must be 21β-hydroxyolean-12-en-3-one (21β-hydroxy-β-amyrinone).

Compound **2** was isolated as a colorless, amorphous solid. The high-resolution mass spectrum indicated the molecular formula $C_{30}H_{42}O_5$ (*m*/*z* 482.3487, calcd for $C_{30}H_{42}O_5$, 482.3548), which was in agreement with the ^{13}C NMR and DEPT data. Its IR spectrum showed absorption bands due to an aliphatic ester (1722 cm^{-1}), an α,β-unsaturated ester (1710 cm^{-1}), and an α,β-unsaturated ketone (1650 cm^{-1}). The ^1H NMR spectrum, which resembled that of pristimerine (**4**), showed five tertiary methyl singlets (δ 0.81, 1.07, 1.12, 1.19, 1.21), one deshielded methyl doublet at δ 2.08 (J = 1.2 Hz), two methoxy groups (δ 3.64 and 3.66), and three olefinic protons, one singlet at δ 5.93 and two coupled doublets at δ 5.87 and 6.51 (J = 9.2 Hz). The main differences between the two

Table 1. Antigiardial Activity of Triterpenoids **1**−**5** and **7**

compound	IC_{50} (μM)	CI_{95}[a]
1	27.4	27.2−27.6
2	22.4	22.3−22.4
3	19.8	19.8−19.9
4	0.11	0.09−0.13
5	0.74	0.71−0.76
7	11.3	11.2−11.3
metronidazole	1.23	0.88−1.59

[a] CI_{95}, 95% confidence interval.

compounds were in the chemical shifts and the presence of an extra methoxyl signal in compound **2**. The ^{13}C NMR spectrum and DEPT experiments of **2** indicated the presence of 30 carbon atoms, which accounted for eight methyl groups (including two methoxyl groups and a methyl ketone), seven methylenes, four methines (three of them olefinic), and 11 quaternary carbons including three carbonyl groups and three olefinic ones.

These data suggested that **2** was a bisnor-triterpenoid with 10 unsaturated carbon atoms closely related to pristimerine (**4**). The long-range $^1H-^{13}C$ correlations indicated that **2** had the same B−E ring system as **4**, but the chemical shifts of the olefinic protons indicated structural modification of the A ring. The HMBC experiment indicated that a methylketone was attached at C-5, since it showed correlations between the deshielded methyl signal at δ 2.08 (Me-23) and two quaternary carbon atoms, the carbonyl at δ 197.9 (C-4) and the sp^2 quaternary carbon at δ 135.5 (C-5). Furthermore, the olefinic proton singlet (H-1) exhibited correlations with the carbonyl ester at δ 166.1 (C-2) and two quaternary carbons, an olefinic carbon at δ 165.9 (C-10) and a sp^3 carbon at δ 39.4 (C-9). Further coupling between the carbonyl at δ 166.1 and the methoxyl protons at 3.64 confirmed the presence of an α,β-unsaturated methyl ester moiety at C-1, as in regelone (**6**), a trinor-seco-triterpenoid lacking C-3, isolated from *Tripterygium wilfordii*.[17] The configuration of the double bond was confirmed to be *E*, since a correlation between H-1 and H-11 in the NOESY experiment was observed. The chemical shifts of C-1, -2, -4, -5, -10, and -23 were very similar to those for the same carbon atoms in **6**. Therefore compound **2** was identified as 3,24-dinor-2,4-seco-4-oxo-friedelan-1(10),5,7-triene-2,29-dioic acid dimethyl ester, which we named dzununcanone (**2**).

Isolates **1**−**6** were evaluated for their antiprotozoal activity against *G. intestinalis* (Table 1). Pristimerine (**1**) was the most active compound (IC_{50} of 0.11 μM), even more active than metronidazole, the drug of choice for the treatment of giardiasis. Tingenone (**5**) was also very active, with an IC_{50} of 0.74 μM, similar to that of metronidazole. However, it is known that these compounds are cytotoxic, which could be a restriction for their use as antiparasitic drugs.[18−20] The other compounds had weak activity against *G. intestinalis*, with IC_{50} values ranging from 19.85 to 27.41 μM.

Experimental Section

General Experimental Procedures. NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 400 spectrometer. The chemical shifts are given in δ (ppm) with residual $CDCl_3$ as internal reference and coupling constants in Hz. EIMS were obtained on a Hewlett-Packard 5970 series II gas chromatograph as injection system. HREIMS were done on a Micromass VG Autoespect EBE at 70 eV. Optical rotations were measured in $CHCl_3$ solutions on a Rudolph Research Autopol IV polarimeter. IR spectra were recorded on KBr disks on a Nicolet Magna 750 Fourier transform IR spectrometer. UV−vis spectra were taken in methanol using a Spectronic Unicam α spectrophotometer. Precoated TLC silica gel 60 F_{254} aluminum sheets from Sigma-Aldrich were used for thin-layer chromatography (0.25 and 0.5 mm layer thickness for analytical and preparative TLC, respectively) and visualized under short (254 nm) and long (366 nm) wavelength UV light or oleum reagent (H_2SO_4−AcOH−H_2O, 1:20:4). Column chromatography was conducting using silica gel 60 (63−200 or 2−25 μm particle size) or Sephadex LH-20 from Sigma-Aldrich.

Plant Material. *Hippocratea excelsa* H.B.K. was collected in September 2003 at Dzununcán, 15 km south of the city of Mérida, in the state of Yucatán, México (N 20°51.8′, W 89°38.4′) and authenticated by Dr. José Salvador Flores. A voucher specimen (J. S. Flores 12350) has been deposited at the Herbarium "Alfredo Barrera Marin", Universidad Autónoma de Yucatán (UADY), Mérida, Yucatán, México.

Extraction and Isolation. The dried and powdered root bark of *H. excelsa* (900 g) was extracted with hexane−Et_2O (1:1) (4 L) in a Soxhlet apparatus. The extract (98 g) was dissolved in CH_2Cl_2, treated with MeOH (500 mL), and filtered to give 67 g of a gummy, white material, identified by NMR as *trans*-polyisoprene. The CH_2Cl_2−MeOH solution was concentrated at reduced pressure to give 31 g of crude residue.

The crude residue was chromatographed over Sephadex LH-20 eluting with hexane−CH_2Cl_2−MeOH (2:1:1). Eluates (30) were collected, monitored by TLC, and combined into nine major fractions (A−I). Fraction D (eluates 7−14, 13.6 g) was rechromatographed on silica gel 60 (63−200 μm) using hexane, mixtures of hexane−AcOEt (9:1, 8:2, 7:3, 6:4, 4:6, 3:7, 2:8, 1:9), and AcOEt, providing 63 fractions. Fraction D4 (eluates 13−16, 1.4 g) was subjected to chromatography on a silica gel 60 (2−25 μm) column eluting with hexane, mixtures of hexane−Et_2O (85:15, 8:2, 7:3, 6:4, 1:1, 3:7), and Et_2O to afford 55 eluates. Eluates 30−34, 35−37, and 38−43 were combined, separately, to give fractions D4.7 (366 mg), D4.8 (181 mg), and D4.9 (138 mg). Fraction D4.7 was chromatographed over silica gel 60 (2−25 μm) using CH_2Cl_2, mixtures of CH_2Cl_2−Et_2O (9:1, 7:3, 1:1), and Et_2O; 51 eluates were collected and combined into five new fractions according to their TLC profiles. Fraction D4.7.2 (50 mg) was separated by preparative TLC (CH_2Cl_2−acetone 95:5) to afford the compound **1** (4.6 mg). Fraction (D4.7.3, 63.4 mg) was chromatographed on preparative TLC using hexane−dioxane (8:2), 3×, and hexane−diethyl ether (1:1), 2×, to give 2 mg of compound **2**. Fraction D4.7.5 (11 mg) eluted with CH_2Cl_2−AcOEt (95:5) gave **4** (3.8 mg) and **5** (3.3 mg). Fraction D4.8 was repeatedly chromatographed on silica gel 60 (2−25 μm) with hexane−AcOEt mixtures to afford **3** (5.3 mg). Fraction D4.9 was chromatographed on silica gel 60 (2−25 μm) eluting with hexane and mixtures of hexane−AcOEt, to give 88 fractions. Fractions 35−44 were combined (28.0 mg) and purified by preparative TLC (hexane−acetone 8:2, 3×) to afford 6.1 mg of xuxarine Eβ (**7**).

21β-Hydroxyolean-12-en-3-one (1): colorless gum; $[\alpha]^{25}_{D}$ +3.2 (*c* 0.1, EtOH); UV (EtOH) λ_{max} 202, 248, 285 nm; IR ν_{max} (KBr) 3442, 1035, 2949, 2860, 1383, 1701, 756 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.86 (3H, s, H-30), 0.88 (3H, s, H-28), 0.97 (3H, s, H-29), 1.00 (1H, m, H-15a, H-16a), 1.01 (3H, s, H-26), 1.06 (3H, s, H-24), 1.07 (3H, s, H-25), 1.10 (3H, s, H-23), 1.14 (3H, s, H-27), 1.15 (1H, m, H-19a) 1.33 (1H, m, H-5), 1.35 (1H, m, H-7a), 1.37 (1H, m, H-22a), 1.41(1H, m, H-1a), 1.48 (1H, m, H-6a), 1.50 (1H, m, H-22b), 1.52 (1H, m, H-7b), 1.55 (1H, m, H-6b), 1.65 (1H, dd, J = 11.5, 6.3 Hz, H-9), 1.75 (1H, m, H-19b), 1.76 (1H, m, H-15b), 1.88 (1H, m, H-11a), 1.90 (1H, m, H-1b), 1.96 (1H, m, H-16b), 1.97 (1H, m, H-11b), 2.00 (1H, dd, J = 14.8, 3.5 Hz, H-18), 2.36 (1H, ddd J = 16, 6.8, 3.7 Hz, H-2b), 2.55 (1H, ddd, J = 16, 11, 3.7 Hz, H-2a), 3.53 (1H, dd, J = 12, 4.7 Hz, H-21), 5.24 (1H, t, J = 3.5 Hz, H-12); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.2 (CH_3, C-25), 16.7 (CH_3, C-26), 16.9 (CH_3, C-30), 19.6 (CH_2, C-6), 21.5 (CH_3, C-24), 23.7 (CH_2, C-11), 25.8 (CH_3, C-27), 26.0 (CH_2, C-15), 26.5 (CH_3, C-23), 28.2 (CH_2, C-16), 28.3 (CH_3, C-28), 29.1 (CH_3, C-29), 32.1 (CH_2, C-7), 34.2 (CH_2, C-2), 35.0 (CH, C-17), 36.3 (C, C-20), 36.7 (C, C-10), 39.3 (CH_2, C-1), 39.7 (C, C-8), 41.8 (C, C-14), 45.3 (CH_2, C-22), 46.7 (CH, C-18), 46.8 (CH, C-9), 47.0 (CH_2, C-19), 47.5 (C-4), 55.3 (CH, C-5), 74.0 (CH, C-21), 122.4 (CH, C-12), 143.8 (C, C-13), 217.8 (C, C-3); HREIMS *m*/*z* 440.3638 [M]$^+$ (calcd for $C_{30}H_{48}O_2$, 440.3654).

3,24-Dinor-2,4-seco-4-oxo-friedelan-1(10),5,7-triene-2,29-dioic Dimethyl Ester (dzununcanone) (2): amorphous solid; $[\alpha]^{25}_{D}$ −3.6 (*c* 0.03, MeOH); UV (EtOH) λ_{max} 227, 263, 329 nm; IR ν_{max} (KBr) 2905, 2840, 1722, 1710, 1650, 1460, 1447, 1365, 1250, 1205, 1170, 1110, 1075, 990, 870, 835, 660 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.81 (3H, s, H-27), 0.98 (1H, m, H-22a), 1.07 (3H, s, H-28), 1.12 (3H, s, H-26), 1.19 (3H, s, H-30), 1.21 (3H, s, H-25), 1.30 (1H, m, H-12a), 1.40 (1H, m, H-21a), 1.48 (1H, m, H-16a), 1.52 (1H, m, H-11a), 1.53 (1H, m, H-18), 1.55 (2H, m, H-15), 1.72 (1H, m, H-19a), 1.75 (1H, m, H-12b), 1.80 (1H, m, H-16b), 2.06 (1H, m, H-22b), 2.08 (3H, d, J = 1.2 Hz, H-23), 2.21 (1H, m, H-21b), 2.22 (1H, m, H-11b), 2.43 (1H, d, J = 15.6 Hz, H-19b), 3.64 (3H, s, C-2OOMe), 3.66 (3H, s, C-29OOMe), 5.87 (1H, d, J = 9.2 Hz, H-7), 5.93 (1H, s, H-1), 6.51 (1H, dd, J = 9.2, 1.2 Hz, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.1 (CH_3, C-23), 20.3 (CH_3, C-27), 24.0 (CH_3, C-26), 25.6 (CH_3, C-25), 28.5 (CH_2, C-15), 29.2 (CH_2, C-12), 29.7 (CH_2, C-21), 30.4 (C, C-17), 31.1 (CH_2, C-19), 31.2 (CH_2, C-11), 31.5 (CH_3, C-28), 32.8 (CH_3, C-30), 34.4 (CH_2, C-22), 36.6 (CH_2, C-16), 38.3 (C, C-13), 39.2 (CH, C-9), 40.4 (C, C-20), 44.6 (CH, C-18), 47.6 (C, C-14), 51.8 (6H, C-2OOMe, C-29OOMe), 116.0 (CH, C-1), 118.6 (CH, C-7), 135.5 (C, C-5), 136.0 (CH, C-6), 163.3 (C, C-8), 165.9 (C, C-10), 166.1 (C, C-2), 179.1 (C, C-29), 197.9 (C, C-4); HREIMS *m*/*z* 482.3487 [M]$^+$ (calcd $C_{30}H_{42}O_5$, 482.3548).

Antiprotozoal Assay. *G. intestinalis* IMSS:0696:1, isolated from an individual with symptomatic giardiasis, was used.[21] Trophozoites were cultured in TYI-S-33 modified medium, supplemented with 10% calf serum, and subcultured twice a week; for the assay, trophozoites were tested in their log phase of growth.[22]

In vitro susceptibility assays were developed using a method described previously.[23] The compounds (1 mg) dissolved in 1 mL of dimethylsulfoxide (DMSO) were added to microtubes containing 1.5 mL of medium in order to reach concentrations of 1.6, 3.3, 6.6, and 13.3 μg/mL. The solutions were inoculated with *G. intestinalis* to achieve an inoculum of 5×10^4 trophozoites/mL. Metronidazole was used as the reference drug, culture medium with trophozoites and DMSO was the negative control, and culture medium was the blank. Inoculated solutions were incubated for 48 h at 37 °C. After, parasites were detached by chilling and trophozoites were counted with a hemocytometer. Experiments were carried out using duplicate tubes and were repeated three times.

The 50% inhibitory concentrations (IC_{50}) and confidence limits (95%) were calculated by Probit analysis.

Acknowledgment. The authors thank Consejo Nacional de Ciencia y Tecnología (CONACyT, México) for the financial support (Grant 32595-N).

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NP060559Y