Phytogrowth-Inhibitory Compounds from *Malmea depressa*[1]

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Fractionation of the $CHCl_3$ extract of the stem bark of *Malmea depressa*, guided by phytogrowth-inhibitory bioassays, led to the isolation of four known phenylpropanoids: 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene (**1**), 2,3,4,5-tetramethoxycinnamaldehyde (**3**), *trans*-isomyristicin (**4**), and 2,3,4,5-tetramethoxycinnamyl alcohol (**5**), and a new C_6-C_1 derivative that was characterized by spectral means as 2,3,4,5-tetramethoxybenzaldehyde (**2**). Compound **1** exhibited significant phytogrowth-inhibitory activity on seedlings of *Amaranthus hypochondriacus* (IC_{50} = 43 μg/mL) and *Echinochloa crusgalli* (IC_{50} = 810 μg/mL) and moderate antifungal activity against *Trichophyton mentagrophytes* (MIC 500 μg/mL) and *Fusarium oxysporum* (MIC = 250 μg/mL).

Malmea depressa (Baill.) R. E. Fries (Annonaceae) [syn. *Guatteria leiophylla* (Donn. Sm.) Saff. ex. Standl.], commonly known by the Maya people as "elemuy", "sufricaya", "elemuy-box", and "nazareno prieto", is a tree (up to 10 m high) found in Central America and Mexico, from Veracruz to the Peninsula of Yucatan. In the tropical forest it is a dominant species. The tree is used by local people as an analgesic agent and for the treatment of diseases such as pellagra and liver and kidney stones.[2] No previous studies on the phytochemistry and biological properties of this species have been reported. In a general screening of several plant extracts for antifungal, cytotoxic, and plant-growth-inhibiting agents, the $CHCl_3$ extract and the essential oil of the stem bark of *M. depressa* demonstrated a significant inhibition of the radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli*. In this investigation we describe the isolation and identification of the major phytotoxic constituents of *M. depressa*.

The initial phytogrowth-inhibitory activity of the $CHCl_3$ extract of the stem bark of *M. depressa* on seedlings of *A. hypochondriacus* and *E. crusgalli*[3−5] gave IC_{50} values of 134 and 457 μg/mL, respectively (Table 1). The essential oil of the stem bark also significantly inhibited the radicle growth of both species (Table 1). In addition, the $CHCl_3$ extract displayed moderate antifungal activity against *Fusarium oxysporum* and *Trichophyton mentagrophytes*, with MIC values of 400 and 300 μg/mL, respectively, brine shrimp lethality [(BST), LC_{50} = 62 μg/mL with a 95% confidence interval of 66−57 μg/mL] and equivocal cytotoxic activity against three human solid-tumor cell lines (lung carcinoma, A-549; breast carcinoma, MCF-7; and colon adenocarcinoma, HT-29; the ED_{50} values being 30, 21, and 21 μg/mL, respectively).[6,7]

Table 1. Phytogrowth Inhibitory Activity of the $CHCl_3$ Extract, the Essential Oil, and Isolates

	seedling growth IC_{50}, μg/mL (95% confidence intervals)	
sample	*E. crusgalli*	*A. hypochondriacus*
$CHCl_3$ extract	457 (500−414)	134 (164−114)
essential oil	475.2 (593−385)	116 (152−85)
1	810 (857−765)	43 (51−35)
2	>1000	822 (864−777)
3 + **4** + **5**	>1000	345 (425−269)
tricolorin A*[a]	12 (17−7)	37 (46−29)

[a] Tricolorin A served as the positive control.

The $CHCl_3$ extract was fractionated by column chromatography over Si gel to yield seven primary fractions (F-1 to F-7) with a direct bioautographic bioassay being used for activity-directed fractionation. Successive preparative TLC of the active fraction F-1 allowed the isolation of 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene (**1**) as the major active compound.

Preparative TLC of active fraction F-2 led to the isolation of the novel compound **2** and the known compounds 2,3,4,5-tetramethoxycinnamaldehyde (**3**), *trans*-isomyristicin (**4**), and 2,3,4,5-tetramethoxycinnamyl alcohol (**5**). Compounds **1** and **3**−**5** were identi-

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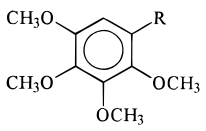
fied from their IR, NMR, and MS data, which were identical to those previously described.[8,9]



1: R = - CH_2 - CH = CH_2

2: R = - CHO

3: R = - CH = CH - CHO

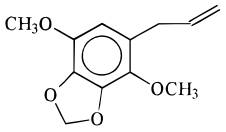
4: R = - CH = CH - CH_3

5: R = - CH = CH - CH_2OH

Compound **2** was obtained as a yellow oil. Its molecular formula, $C_{11}H_{14}O_5$, was deduced from elemental analysis and EIMS measurements. The IR spectrum showed absorption bands attributable to an aromatic aldehyde (2825, 1724 cm^{-1}) and an aromatic ether (1265 cm^{-1}). The NMR spectra supported the presence of the aldehyde functionality (δ_H 10.36; δ_C 188.80) and clearly indicated that the natural product contained a tetramethoxyphenyl moiety. The signals attributable to the aromatic methoxyl groups appeared at δ_H 3.95 (OMe-2), 3.87 (OMe-5), 3.99 (OMe-3), and 3.97 (OMe-4) in the ^{1}H-NMR spectrum and δ_C 61.25 (OMe-2), 56.10 (OMe-3 and 5), and 62.85 (OMe-4) in the ^{13}C-NMR spectrum. The only aromatic proton resonance was observed at δ_H 7.10, which showed a cross peak with the methine signal at δ_C 103.80 in the HETCOR spectrum. The other aromatic carbons observed in the ^{13}C NMR were quaternary and appeared at δ_C 124.00 (C-1), 149.37 (C-2), 149.83 (C-3), 146.63 (C-4), and 152.30 (C-5). The analysis of the ^{1}H−^{1}H NOESY correlations confirmed the substitution pattern of the aromatic ring. In fact, the NOESY spectrum of **2** showed that the aromatic proton (δ_H 7.10) had cross peaks both with the aldehyde hydrogen (δ_H 10.36) and with the methoxyl protons at δ_H 3.87. In addition, the aldehyde proton (δ_H 10.36) correlated not only with H-6 but also with the signal at δ 3.95; thus, it was assigned to the methoxyl group at C-2. These observations were in agreement with the disposition of the methoxyl groups at C-2, C-3, C-4, and C-5 of the aromatic ring.

The essential oil obtained by H_2O and steam distillation of the stem bark was also investigated by GC−MS analysis, which showed the presence of **1**−**4** and an unidentified compound. Identification was made by comparison of the GC mobilities, by study of mass fragmentation, and by coinjection with compounds **1**−**4** isolated during the course of this study from the $CHCl_3$ extract. It should be noted that tetraoxygenation of simple aromatic compounds is rare in nature,[8] and their presence in *M. depressa* could be of chemotaxonomic relevance.

Compound **1** produced significant inhibition of radicle growth in *A. hypochondriacus* (IC_{50} = 43 μg/mL) but was less sensitive against *E. crusgalli* (IC_{50} = 810 μg/mL). These results were in agreement with those of Horada and Nakayama,[10] who demonstrated that this compound reduced radicle growth of rice seedlings by 55% at a concentration of 100 μg/mL. Also, the growth-regulatory activity of **1** was comparable to that previously demonstrated for several phenylpropanoids, including apiol (**6**), which also possess a tetraoxygenated aromatic ring.[10−14] The new natural product 2,3,4,5-tetramethoxybenzaldehyde (**2**) and the mixture of phenylpropanoids **3** + **4** + **5** were markedly less active in the phytogrowth-inhibitory bioassays than **1**. The level of activity against *A. hypochondriacus* is significant, inasmuch as the concentration threshold required for most of the natural phytogrowth-inhibitors tested in similar experiments has often been in the 100−1000 μg/mL range.[15,16] Thus, **1** may be considered for development into an environmentally safe herbicide. Its mode of action is under investigation and will be communicated in due course.



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The cytotoxic, brine-shrimp-lethality, and antifungal properties of the major phenylpropanoid **1** were also evaluated. It was inactive in the BST (LC_{50} 144 μg/mL) and did not exhibit cytotoxic activity against the A-549 (ED_{50} = 29 μg/mL), MCF-7 (ED_{50} = 46 μg/mL), and HT-29 (ED_{50} = 71 μg/mL) cell lines but showed moderate antifungal activity against *T. metagrophytes* and *F. oxysporum* with MIC values of 500 and 250 μg/mL, respectively.

Experimental Section

General Experimental Procedures. IR spectra were obtained on a Perkin-Elmer 599 B spectrophotometer. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra were registered on a Varian VXR-300S spectrometer in $CDCl_3$ with TMS as internal standard. GC−MS analyses were accomplished on a Hewlett-Packard Model 5890 gas chromatograph interfaced with a JEOL JMS AX50HA mass spectrometer.

The GC column was a PAS-1701-tested 1701 silicone column (25 m × 0.32 mm i.d.) programmed from 1−150 °C at the rate of 7 °C × min; the carrier gas was He (7 psi, 1 mL/min). Analytical and preparative TLC were performed on Si gel 60 F_{254} E. Merck plates, and the spots were visualized by spraying with a 10% solution of H_2SO_4, followed by heating at 110 °C. Column chromatography was carried out on Si gel 60 (70−230 mesh, E. Merck).

Plant Material. The plant material (stem bark) of *M. depressa* was collected in Municipio de Carrillo Puerto, Quintana Roo, Mexico, in March 1994. A voucher specimen (*Anaya 93-4*) has been deposited in the National Herbarium (MEXU), Instituto de Biología, Universidad Nacional Autónoma de México, México D.F.

Extraction and Bioassay-Directed Fractionation. The air-dried stem bark (200 g) was extracted with $CHCl_3$ at room temperature. After filtration, the solution was evaporated under reduced pressure to yield 5 g of residue, which was subjected to column chromatography over Si gel (100 g). Elution was accomplished with a mixture of solvents of increasing polarity (C_6H_6 gradually enriched with EtOAc); fractions of 100 mL each were collected and pooled based on TLC profiles to yield seven major fractions (F-1 to F-7). Bioautographic bioassay of the primary fractions showed that F-1 (R_f 0.75) and F-2 (R_f 0.45) were phytotoxic. Successive preparative TLC of the active fraction (F-1) (2.0 g) allowed the isolation of compound **1** (1.42 g) as a yellow oil. Preparative TLC of F-2 (380 mg), using the same solvent system, led to the isolation of compounds **2**−**5**: **2** (23 mg), **3** (19 mg), **4** (20 mg), and **5** (17 mg).

Compounds **1** and **3**−**5** were identified by comparison of their IR, NMR, and MS data with those previously described.[8,9]

2,3,4,5-Tetramethoxybenzaldehyde (2): pale yellow oil; IR ν_{max} ($CHCl_3$) 2825, 2720, 1724, 1265 cm^{-1}; EIMS *m/z* (rel int) M^+ 226 (100), 211 (54.4), 196 (6.4), 195 (5.3), 193 (14.4), 183 (12); ^{1}H-NMR δ 10.36 (s, CHO), 7.10 (s, H-6), 3.95 (OMe-2), 3.87 (OMe-5), 3.99 (OMe-3), 3.97 (OMe-4); ^{13}C-NMR δ 188.80 (CHO), 61.25 (OMe-2), 56.10 (OMe-3 and 5), 62.85 (OMe-4), 124 (C-1), 149.37 (C-2), 149.83 (C-3), 146.63 (C-4), 152.30 (C-5), 103.80 (C-6).

Essential Oil. The essential oil was prepared by H_2O and steam distillation from 200 g of plant material, yielding 1.45 g.

Phytogrowth-Inhibitory Activity. The phytogrowth-inhibitory activity of the $CHCl_3$ extract, fractions, and pure compounds was evaluated on seedlings of *A. hypochondriacus* and *E. crusgalli* by using a Petridish bioassay (PDPIB) according to the procedure previously described.[3−5,15] The seeds of *E. crusgalli* and *A. hypochondriacus* were purchased from Valley Seed Service, Fresno, CA, and Mercado de Tulyehualco, D.F., México, respectively. The data were analyzed by ANOVA ($p < 0.05$), and IC_{50} values were calculated by Probit analysis on the basis of the percentage of inhibition obtained. The organic extract and isolates were evaluated at 50, 100, 250, and 500 μg/mL. Tricolorin A was used as a positive control.[15] In addition, a direct bioautographic phytogrowth-inhibitory bioassay (BPIB),[17,18] in which the seeds of the target species grow directly on the TLC plates (Si gel G60 F254 plates 5 × 20 cm, E. Merck) was employed for activity-guided fractionation. Samples (*ca.* 10 mg each) were applied as a band, and then the developed plates (benzene-EtOAc 9:1) were dried, coated with agar (20 mL), and left to solidify. The seeds (*ca.* 150/plate) of the target species were distributed over the coated TLC plates and incubated at 27 °C in the dark for 24 h for *A. hypochondriacus* and for 48 h for *E. crusgalli*. In each experiment, a plate with the agar served as the control.

Bioassays with Phytopathogen Fungi. Bioassay was carried out using a previously described procedure[3] employing *Pythium* sp., *Helminthosporium* sp., and *F. oxysporum*. Test samples were evaluated at 50, 100, 200, 300, 400, and 500 μg/mL. Activity criteria: MIC values <500 μg/mL.[3]

Bioassays with Human Fungal Pathogens. *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16888), and *Trychophyton mentagrophytes* (ATCC 9129) were obtained from ATCC. Positive controls: amphotericin B (Sigma, 15 μg/mL) for *C. albicans* and *A. niger* and griseofulvin (Sigma, 8 μg/mL) for *T. mentagrophytes*. The test material was dissolved in MeOH and evaluated by the agar-dilution method as described.[19] MIC values represent the lowest concentration of test sample at which complete inhibition of growth occurs. The MIC value for griseofulvin was 2.60 μg/mL and for amphotericin B was 3 μg/mL.

Brine Shrimp Lethality and Cytotoxic Activity. The extracts, fractions, and isolates were evaluated for lethality to brine shrimp larvae, and *in vitro* cytotoxic activity was determined according to previously described protocols.[6,7] The cell lines used were lung carcinoma (A-549), breast carcinoma (MCF-7), and colon adenocarcinoma (HT-29), with Adryamicin as the positive control ($DE_{50} = 3 \times 10^{-2}$, 3×10^{-1}, and 7×10^{-3} μg/mL, respectively). Activity criteria: ED_{50} values of <20 μg/mL for extracts and <4 μg/mL for pure compounds.

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