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Antifungal Clerodane Diterpenes from *Macaranga monandra* (L) Muell. et Arg. (Euphorbiaceae)

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Hexane and ethyl acetate phases of the methanol extract of *Macaranga monandra* showed fungal growth inhibition of *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides, Fusarium oxysporum, Botrytis cinerea*, *Phomopsis obscurans*, *and P. viticola*. Bioassay-guided fractionation led to the isolation of two active clerodane-type diterpenes that were elucidated by spectroscopic methods (1D-, 2D-NMR, and MS) as kolavenic acid and 2-oxo-kolavenic acid. A 96-well microbioassay revealed that kolavenic acid and 2-oxo-kolavenic acid produced moderate growth inhibition in *Phomopsis viticola* and *Botrytis cinerea*.

KEYWORDS: Fungicide; antifungal activity; natural products; phytopathogenic fungi; microtiter assay; bioautography

INTRODUCTION

Owing to the continuing development of microbial resistance in medicine and agriculture, discovery of new antimicrobial substances is an important, if not urgent, research objective. In addition, the desire for safer agrochemicals with less environmental and mammalian toxicity is a major concern. Particularly desirable is the discovery of novel prototype antimicrobial agents representing new chemical classes that operate by different modes of action than existing antifungal agents, and consequently, lack cross-resistance to chemicals currently used (*1*, *2*). Following natural product leads offers an efficient approach to discovering and optimizing new agrochemicals for disease control. After screening more than 75 different plants that were initially selected for their frequent application by indigenous people in Cameroon for medicinal needs, only two were active in the assays (*Macaranga mondra* and *Pterygota kamerunensis*). Therefore, we began a study to evaluate several natural products from *Macaranga monandra* for their potential use as disease control agents for filamentous pathogenic fungi of the genera *Colletotrichum*, *Botrytis*, *Phomopsis*, and *Fusarium*.

Macaranga monandra (L) Muell. et Arg. is a tree found in equatorial rain forest of Cameroon and other central African countries. The genus *Macaranga* is represented by 300 species (*3*), distributed throughout the tropical and subtropical regions. Previously reported phytochemicals from the genus include a prenylstilbene (*4*−*6*), flavonoids (*7*−*10*), a diterpene (*11*), tannins (*12*), and sterols (*13*, *14*).

The presence of diterpene clerodanes or antifungal activity has not been previously reported in *Macaranga monandra*. On the basis of the growth inhibition of *C. acucatum*, *C. fragariae*, and *C. gloeosporioides* in preliminary screens using direct bioautography, we selected to study in detail the hexane and EtOAc phases of the MeOH extract of *M. monandra*. We now describe the bioassay-guided isolation, antifungal activity using a novel 96-well microbioassay method, and the structure elucidation of kolavenic acid (**1**) (*15*) and 2-oxo-kolavenic acid (**2**) (*16*), which are *neo*-clerodane-type diterpenes previously isolated from *Aristolochia* species and *Eperua purpurea* (*15*, *16*).

MATERIALS AND METHODS

General Experimental. The 1D- and 2D Nuclear Magnetic Resonance (NMR) spectra were obtained on a Bruker Avance DRX 500 FT spectrometer operating at 500 and 125 MHz for ^{1}H and ^{13}C measurements, respectively. The chemical shift values are reported as parts per million (ppm) units relative to tetramethylsilane (TMS) for ^{1}H- and ^{13}C-; coupling constants are in Hz. For the ^{13}C NMR spectra, multiplicities were determined by a DEPT experiment. LC-ESIMS were obtained using a Bruker BioApex FT-MS in ESI mode.

Chromatographic Conditions. Thin layer chromatography (TLC), precoated Si 250F plates (Merck); developing system, hexanes/EtOAc/MeOH (10:10:2); developing temperature, 22 °C; visualization, vanillin/H_2SO_4 (1 g vanillin in 100 mL of 20% H_2SO_4 (in EtOH)). Column chromatography, silica gel 230−400 mesh (Baker).

Plant Material. *Macaranga monandra* (L) Muell. et Arg. (Euphorbiaceae) stem bark was collected in Cameroon in the dry season of 1998. The voucher specimen was identified and authenticated by Dr.

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Extraction. Pulverized plant material (750 g) was extracted with MeOH at room temperature for 48 h with occasional stirring. The MeOH extract (10.3 g) was recovered by evaporation, dissolved in distilled water, and subsequently partitioned, with the following solvents recovering corresponding yields after separation and evaporation of the various phases: hexane (1.15 g), $CHCl_3$ (1.0 g), EtOAc (3.98 g), *n*-BuOH (2.2 g), and aqueous phase (0.5 g).

Isolation of the Active Principles. Two antifungal clerodane diterpenes were isolated by bioassay-guided fractionation. Bioautography of antifungal compounds on silica gel TLC plates was used to direct the fractionation and isolation of the active compounds. Hexane and EtOAc phases, determined by bioautography to contain antifungal compounds, were pooled together yielding 5.0 g of active crude extract. The extract was chromatographed on 40-μm silica gel, which was prestabilized with 100 mL of hexane before introducing the plant extract. The column was eluted with 200 mL of hexane and followed by 200 mL of hexane with increasing volume of EtOAc (from 10 to 50%), and final wash with 100% of EtOAc afforded five fractions (I−V). Fractions I (0.759 g) and V (0.0563 g) were the most active and were further chromatographed using hexanes/EtOAc/MeOH (7:3:0.5) as the mobile phase. This procedure afforded two active compounds (Mm-01 (39.9 mg) and Mm-05 (4.1 mg)).

Kolavenic Acid. ^{1}H NMR (500 MHz, in $CDCl_3$): β 5.70 (s, 1H, H-14), 5.21 (br.s, 1H, H-3), 2.19 (s, 3H, Me-16), 1.60 (s, 3H, Me-18), 1.02 (s, 3H, Me-19), 0.84 (d, J = 6.0 Hz, 3H, Me-17), 0.76 (s, 3H, Me-20). ^{13}C NMR (125 MHz): δ 171.9 (s, C-15), 164.9 (s, C-13), 144.8 (s, C-4), 120.8 (d, C-3), 115.1 (d, C-14), 46.9 (d, C-10), 39.2 (s, C-9), 38.6 (s, C-5), 37.2 (t, C-6), 36.7 (x2) (d, C-8; t, C-12), 35.3 (t, C-11), 27.8 (t, C-7), 27.3 (t, C-2), 20.3 (q, C-19), 19.8 (q, C-16), 18.7 (t, C-1), 18.6 (q, C-20), 18.3 (q, C-18) and 16.3 (q, C-17). LC-MS: m/z 305 $[M + H]^+$ (positive mode), 303 $[M - H]^-$ (negative mode) (*15*).

2-Oxo-Kolavenic Acid. ^{1}H NMR (500 MHz, in $CDCl_3$): δ 5.70 (s, 1H, H-3), 5.63 (s, 1H, H-14), 2.11 (s, 3H, Me-16), 1.85 (s, 3H, Me-18), 1.08 (s, 3H, Me-19), 0.80 (d, J = 6.3 Hz, 3H, Me-17), 0.78 (s, 3H, Me-20). ^{13}C NMR (125 MHz): δ 200.6 (s, C-2), 173.1 (s, C-15), 171.8 (s, C-4), 163.1 (s, C-13), 125.8 (d, C-3), 115.6 (d, C-14), 46.1 (d, C-10), 40.2 (s, C-5), 39.1 (s, C-9) 36.4 (d, C-8), 35.9(x2) (each t, C-1 and C-12), 35.2 (t, C-6), 34.6 (t, C-11), 27.2 (t, C-7), 19.8 (q, C-16), 19.3 (q, C-18), 18.7 (q, C-19), 18.1 (q, C-20), 16.1 (q, C-17). LC-MS: m/z 319 $[M + H]^+$ (positive mode), 317 $[M - H]^-$ (negative mode) (*16*).

Pathogen Production. Isolates of *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. were obtained from B. J. Smith, USDA, ARS, Small Fruit Research Station, Poplarville. MS. *Colletotrichum fragariae* (isolate CF63), *C. acutatum* (isolate CAGoff), and *C. gloeosporioides* (isolate CG162) were used for all pathogen and bioautography studies. Isolate CF63 is one of the most virulent isolates at infecting strawberry plants and inducing both crown and fruit rot (*17*). CF63, CAGoff, and CG162 were used as standard test isolates because of our extensive knowledge of these isolates and their known fungicide sensitivity profiles in both bioautography and microtiter formats (*18*). The three *Colletotrichum* species were isolated from strawberry (*Fragaria xananassa* Duchesne). *Botrytis cinerea* Pers.:Fr, was isolated from commercial grape (*Vitis vinifera* L.) and *Fusarium oxysporum* Schlechtend:Fr from orchid (*Cynoches* sp.). *Phomopsis viticola* (Sacc.) Sacc. and *P. obscurans* (Ellis & Everh.) Sutton were provided by Mike A. Ellis, Ohio State University, Wooster, OH. Fungi were grown on potato-dextrose agar (PDA, Difco, Detroit MI) in 9-cm Petri dishes and incubated in a growth chamber at 24 ± 2 °C, under cool-white fluorescent lights (55 ± 5 μmols/m^2/sec^1 light) with a 12-h photoperiod.

Inoculum Preparation. Conidia were harvested from 7−10-day old cultures by flooding plates with 5 mL of sterile distilled water and dislodging conidia by softly brushing the colonies with an L-shaped glass rod. Conidial suspensions were filtered through sterile miracloth (Calbiochem-Novabiochem Corp., La Jolla CA) to remove mycelia. Conidia concentrations were determined photometrically (*19*, *20*), from a standard curve based on the percent of transmittance (%T) at 625 nm and suspensions were then adjusted with sterile distilled water to a concentration of 1.0×10^6 conidia/mL.

Bioautography. Extracts containing antifungal compounds were indicated by clear zones of fungal growth inhibition directly on chromatographic plates using modifications of thin-layer chromatography (TLC) bioautographic assays (*21*−*23*). Extracts were dissolved as described above. Using a disposable glass micropipet for each sample, 4 μL of each test extract was placed on the TLC plate and chromatographed in one-dimension. Pure compounds were placed on the TLC plate in a dose−response format, where final concentrations were 3.12, 6.25, 12.5, 25.0, 50.0 and 100 μg/mL. To detect biological activity directly on the TLC plate, silica gel plates were sprayed with either of the three spore suspensions adjusted to a final concentration of 3.0×10^5 conidia/mL with liquid potato-dextrose broth (PDB, Difco, Detroit, MI) and 0.1% Tween-80. By use of a 50-mL chromatographic sprayer, each glass, 250-μm Silica Gel GF Uniplate TLC plate with a fluorescent indicator (Analtech, Inc. Newark DE) was sprayed lightly (to a damp appearance) three times with the conidial suspension. Inoculated plates were then placed in a 30 × 13 × 7.5-cm model 398-C moisture chamber (Pioneer Plastics, Inc. Dixon, KY) and incubated in a growth chamber at 24 ± 1 °C and 12-h photoperiod under 60 ± 5 μmols/m^2/sec^1 light. Inhibition of fungal growth was measured 4 d after treatment. Sensitivity of each fungal species to each test compound was determined by comparing size of inhibitory zones. Means and standard deviations of inhibitory zone size were used to evaluate antifungal activity of solvent fractions and pure compounds. Bioautography experiments were performed multiple times using both dose- and nondose−response formats. Fungicide technical standards benomyl, chlorothalonil, and captan (Chem Service, Inc. West Chester, PA) were used as controls. Benomyl and captan have been the commercial standards for control of strawberry anthracnose and many other small fruit diseases. Benomyl and chlorothalonil have been the commercial standards for control of many *Fusarium* and *Phomopsis* diseases of ornamental plants.

Microtiter Assay. A standardized 96-well microtiter plate assay developed for discovery of natural product fungicidal agents (*24*, *25*) was used to evaluate naturally occurring antifungal agents from *Macaranga monanara.* A 96-well microtiter assay was used to determine sensitivity of *B. cinerea, C. acutatum, C. fragariae, C. gloeosporioides, F. oxysporum, Phomopsis viticola, and P. obscurans* to the various antifungal agents in comparison with known fungicidal standards (*18*). Vinclozolin, captan, and thiabendazole, which represent three different modes of action, were used as standards in the microtiter experiments. Each fungus was challenged in a dose−response format using test compounds where the final treatment concentrations were 0.3, 3.0, and 30.0 μM. Nunc MicroWell untreated microtiter plates (Roskilde, Denmark) were covered with a plastic lid and incubated in a growth chamber as described previously for fungal growth. Growth was then evaluated by measuring absorbance of each well at 620 nm using a Packard Spectra Count microplate photometer (Packard Instrument Co., Downers Grove, IL). Each fungus was challenged in a dose−response format using test compounds where the final treatment concentrations were 0.3, 3.0, and 30.0 μM. Microtiter plates were covered with a plastic lid and incubated in a growth chamber at 24 ± 1 °C and 12 h photoperiod under 60 ± 5 μmol light. Growth was then evaluated by measuring absorbance of each well at 620 nm, using the microplate photometer.

Microbioassay Experimental Design. Chemical sensitivity of each fungus was evaluated using 96-well plate microbioassay format. Each chemical was evaluated in duplicate at each dose (0.3, 3.0, and 30.0 μM). Sixteen wells containing broth and inoculum served as positive controls, eight wells containing solvent at the appropriate concentration and broth without inoculum were used as negative controls. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 and 72 h, except for *P. obscurans* and *P. viticola,* for which the data were recorded at 120 h. Means for percent inhibition of each fungus at each dose of test compound (n = 4) relative the untreated positive growth controls (n = 32) were used to evaluate fungal growth inhibition. Treatments were arranged as a split-plot design replicated twice in time.

Table 1. Sensitivity of *Colletotrichum acutatum*, *C. fragariae*, and *C. gloeosporioides* to Sample Extracts from *Macaranga monandra* by Direct Bioautography

	solvent fractions[a]		
Colletotrichum spp.	MeOH	hexane	EtOAc
C. acutatum	20.5[b] ± 0.10	9.7 ± 0.05	12.8 ± 0.01
C. fragariae	11.0 ± 0.01	9.2 ± 0.11	12.6 ± 0.05
C. gloeosporioides	12.1 ± 0.17	9.0 ± 0.11	10.1 ± 0.12

[a] Mean dimensions of zones (mm) of fungal inhibition produced by sample extracts ± SD. [b] Slightly diffused zone.

Table 2. Dose-Response of Clerodane Diterpenes Shown Relative Range of Activity against *Colletotrichum fragariae* and *C. gloeosporioides*

	C. fragariae			*C. gloeosporioides*
	100 μg/mL	50 μg/mL	25 μg/mL	100 μg/mL
1	9.0	8.5	5.0[a]	9.5
2	8.0[a]	8.0[a]	6.0[a]	0.0[a]
negative control	0.0	0.0	0.0	0.0

[a] Indicates inhibition; however, slight or no definite dimensions of zones (mm) to measure.

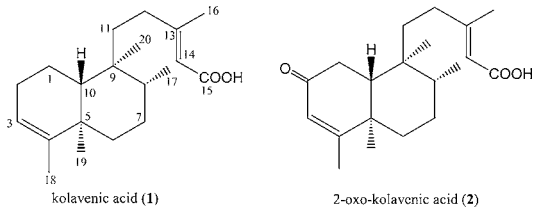

Figure 1. Structure of compounds kolavenic acid **(1)** and 2-oxo- kolavenic acid (**2**).

RESULTS AND DISCUSSION

Bioautography spot applications of MeOH, hexane, EtOAc, *n*-BuOH, and aqueous extracts tested in quadruplicate in a matrix format showed antifungal activity of MeOH, hexane, and EtOAc (**Table 1**). Butanol and the aqueous extracts were not active. Bioassay-guided isolation studies performed on active EtOAc fraction resulted in the isolation of two compounds. The isolated compounds were identified as kolavenic acid (**1**) and 2-oxo-kolavenic acid (**2**) by comparison of their ^{1}H- and ^{13}C NMR spectroscopic data with literature values (*15*, *16*). The assignments of ^{1}H and ^{13}C signals were also secured by G-DQF-COSY, G-HMQC, and G-HMBC spectra. The relative stereochemistry of the compounds was ascertained by the NOESY experiment.

Pure kolavenic acid (**1**) and 2-oxo-kolavenic acid (**2**) were subsequently spotted onto the TLC plate in a dose−response format, where final concentrations were 100, 50.0, 25.0, 12.5, 6.25, and 3.12 μg/mL (**Table 2**). Clearly defined antifungal zones indicated that kolavenic acid (**1**) was more active than 2-oxo-kolavenic acid (**2**). Moreover, kolavenic acid was more active against *C. fragariae* than *C. gloeosporioides*, and it appeared to have an in vitro antifungal threshold between 25 and 50 μg/mL.

Microtiter assay of kolavenic acid (**1**) and 2-oxo-kolavenic acid (**2**) showed that **1** and **2** demonstrated minimal antifungal activity and were much less active than captan against the two *Phomopsis* species (**Figure 2**) and *Botrytis cinerea* (**Figure 3**). Compound **1** was slightly more active against *P. viticola* and showed 50% growth inhibition at 30 μM, whereas **2** showed

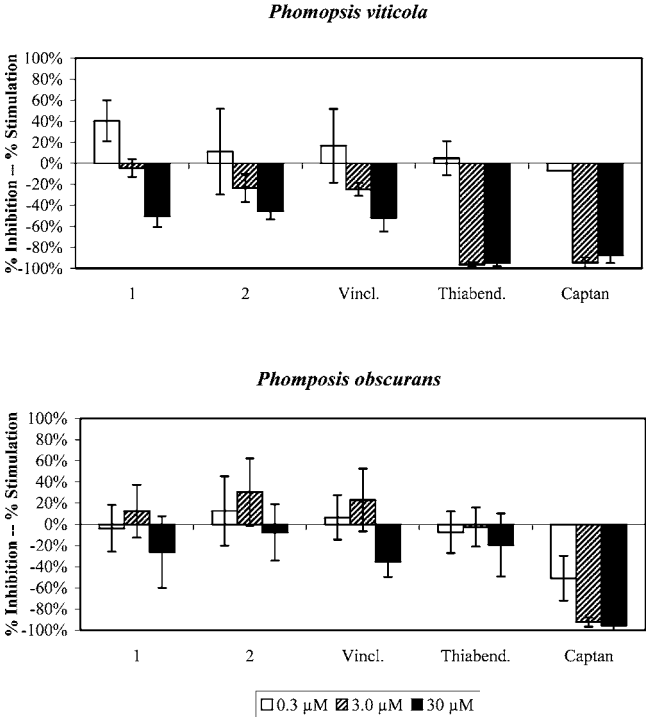

Figure 2. The antifungal activity of kolavenic acid (**1**) and 2-oxo-kolavenic acid (**2**) in comparison to commercial fungicide standards (vinclozolin, thiabendazole, and captan) at 120 h. Means for percent inhibition are listed for each concentration ($n = 4$) for *Phomopsis viticola* and *P. obscurans*. Positive values indicate growth stimulation, and negative values indicate growth inhibition relative to untreated control.

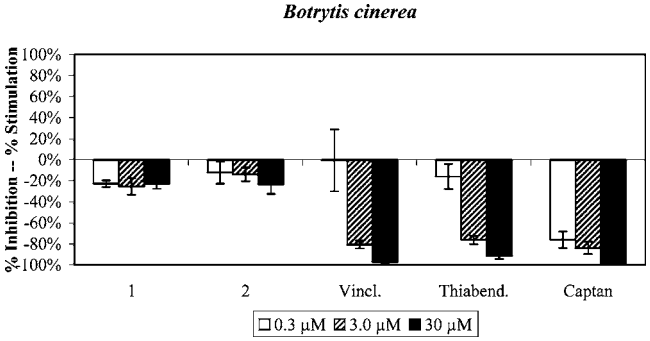

Figure 3. The antifungal activity of **1** and **2** in comparison to commercial fungicide standards (vinclozolin, thiabendazole, and captan). Means for percent inhibition are listed for each concentration ($n = 4$) for *Botrytis cinerea* at 48 h and *Colletotrichum fragariae* at 72 h. Positive values indicate growth stimulation, and negative values indicate growth inhibition relative to untreated control.

46% growth inhibition. Similar activity was observed in *P. obscurans*, where **1** showed 26% growth inhibition and **2** demonstrated only 8% growth inhibition. At the highest concentration (30 μM) kolavenic acid and 2-oxo-kolavenic acid showed approximately the same level of activity with 23% growth inhibition against *Botrytis cinerea*. There was no significant antifungal activity demonstrated against *C. acutatum*, *C. gloeospioides*, *C. fragariea* or *F. oxysporum* at concentrations ≤30 uM (data not shown).

Kolavenic acid (**1**) and 2-oxo-kolavenic acid (**2**) inhibited growth of *B. cinerea*, *C. fragariae*, and *Phomopsis viticola*, and stimulated growth in *C. acutatum*, *C. gloeosporioides*, and *P. obscurans*, depending on concentration and incubation time. At low concentrations, **1** and **2** caused some stimulation of *P. obscurans* growth, and this stimulatory effect was not observed

in *C. fragariae* or *B. cinerea.* Growth stimulation caused by low levels of potentially toxic compounds is termed "hormesis" (*26*) and occurs often in in vitro antifungal assays (*25*).

The structural similarity of these two compounds (kolavenic acid and 2-oxo-kolavenic acid) and the resemblance in their antifungal activities point to the fact that the activity could be due to the hydrogenation of the double bonds at positions C_3 and C_{13}. Because these plant acids have relatively low antifungal activity (<50% inhibition) at 3 μM concentrations, and we believe that they probably do not have a direct defensive role against fungal attack in *M. monandra*. While they are found on the stem bark, they are possibly there as nuisance compounds or indirect deterrents to potential plant pathogens. *M. monandra*, like other members of the Euphorbiaceae may be susceptible to certain diseases, such as gray mold on poinsettia, *Euphorbia pulcherrima* caused by *Botrytis cinerea*, and may not possess sufficient endogenous defense chemicals to prevent its successful pathogens. While these compounds may not have a demonstrated significant role in plant defense, plant acids have not been reported on for antifungal activity, and this study suggests that such plant acids may constitute a source for future evaluations of new antifungal agents.

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LITERATURE CITED

(1) McChesney, J. D. *Biological and chemical diversity and the search for new pharmaceuticals and other bioactive natural products*; ACS Symposium Series 534; American Chemical Society: Washington, DC, 1993; pp 38−47.

(2) Kirst, H. A.; Michel, K. H.; Mynderase, J. S.; Chio, E. H.; Yao, R. C.; Nakasukasa, W. M.; Boeck, L. D.; Occlowitz, J. L.; Paschal, J. W.; Deeter, J. B.; Thompson, G. D. In *Synthesis and chemistry of agrochemicals III*; Baker, D. R., Fenyes, J. G., Steffens, J. J., Eds.; ACS Symposium Series No. 504; American Chemical Society: Washington, DC, 1992; pp 214−225.

(3) Webster, G. The genus *Macaranga*. *Ann. Missouri Bot. Garden* **1994**, *81*, 33−144.

(4) Toison, O.; Hnawia, E.; GuŽritte-Voegelein, F.; Svenet, T. Plants of New Caledonia. Part 140. Vedelianin, a hexahydroxanthene derivative isolated from *Macaranga vedeliana*. *Phytochemistry* **1992**, *31*, 1439−1442.

(5) Beutler, J. A.; Shoemaker, R. H.; Johnson, T.; Boyd, M. R. Cytotoxic geranyl stilbenes from *Macaranga schweinfurthii*. *J. Nat. Prod*. **1998**, *61*, 1509−1512.

(6) Kaaden, J. E.; Hemscheidt, T. K.; Mooberry, S. L. Mappain, a new cytotoxic prenylated stilbene from *Macaranga mappa*. *J. Nat. Prod*. **2001**, *64*, 103−105.

(7) Hnawia, E.; Toison, O.; GuŽritte-Voegelein, F.; Bourret, D.; Svenet, T. T. Plants from New Caledonia. Part 133. A geranyl-substituted flavonol from *Macaranga vedeliana*. *Phytochemistry* **1990**, *29*, 2367−2368.

(8) Shuetz, B. A.; Wright, A. D.; Rali, T.; Sticher, O. Prenylated flavones from the leaves of *Macaranga pleiostemona*. *Phytochemistry* **1995**, *40*, 1273−1277.

(9) Sultana, S.; Ilyas, M. Chromenoflavones from *Macaranga indica*. *Phytochemistry* **1986**, *25*, 953−954.

(10) Beutler, J. A.; McCall, K. L.; Boyd, M. R. A novel geranylflavone from *Macaranga schweinfurthii*. *Nat. Prod. Lett.* **1999**, *13*, 29−32.

(11) Hui, W. H.; Ng, K. K.; Fukamiya, N.; Koreeda, M.; Nakanishi, K. Isolation and structure of macarangonol, a diterpene ketol from *Macaranga tanarius*. *Phytochemistry* **1971**, *10*, 1617−1620.

(12) Lin, J. H.; Nonaka, G. I.; Nishioka, I. Tannins and related compounds. XCIV. Isolation and characterization of seven new hydrolyzable tannins from the leaves of *Macaranga tanarius* (L.) Muell. et Arg. *Chem. Pharm. Bull*. **1990**, *38*, 1218.

(13) Han, G. Q.; Che, C. T.; Fong, H. H. S.; Farnsworth, N. R.; Phoebe, C. H. Chemical constituents of *Macaranga carolinensis*. *Fitoterapia* **1988**, *LIX*, 242−244

(14) Asharani, T.; Seetharaman, T. R. Polyphenols from the leaves of *Macaranga peltata*. *Fitoterapia* **1994**: LXV, *2*, 184.

(15) Lucia, M.; Lopes, X.; Bolzani, V. S.; Trevian, L. M. V. Clerodane diterpenes from *Aristolochia* species. *Phytochemistry* **1987**, *26*, 2781−2784.

(16) Avila, D.; Medina, J. D. A *cis*-clerodane diterpenic acid from *Eperua purpurea*. *Phytochemistry* **1991**, *30*, 3474−3475.

(17) Smith, B. J.; Black, L. L. Morphological, cultural, and pathogenic variation among *Colletotrichum* species isolated from strawberry. *Plant Dis*. **1990**, *74*, 69−76.

(18) Meazza, G.; Dayan, F. E.; Wedge, D. E. Activity of quinones on *Colletotrichum* species. *J. Agric. Food Chem*. **2003**, *51*, 3824−3828.

(19) Espinel-Ingrof, A.; Kerkering, T. M. Spectrophotometric method of inoculum preparation for the in vitro susceptibility testing of filamentous fungi. *J. Clin. Microbiol.* **1991**, *29*, 393−394.

(20) Wedge, D. E.; Kuhajek, J. M. A microbioassay for fungicide discovery. *SAAS Bull. Biochem. Biotechnol.* **1998**, *11*, 1−7.

(21) Homans, A. L.; Fuchs, A. Direct bioautography on thin layer chromatography as a method for detecting fungitoxic substances. *J. Chromatogr*. **1970**, *51*, 327−329.

(22) Osborne, A. E.; Chase, B. R.; Lunness, P.; Scott, P. R.; Daniels, M. J. An oat species lacking avenacin is susceptible to infection by *Gaeumannomyces graminis* var. *tritici*. *Physiol. Mol. Plant Pathol*. **1994**, *45*, 457−467.

(23) Wedge, D. E.; Nagle, D. G. A new 2D-TLC bioautography method for the discovery of novel antifungal agents to control plant pathogens. *J. Nat. Prod.* **2000**, *63*, 1050−1054.

(24) Wedge, D. E.; Galindo, J. C. G.; Marcias, F. A. Fungicidal activity of natural and synthetic sesquiterpene lactone analogues. *Phytochemistry* **2000**, *53*, 747−757.

(25) Oliva, A.; Meepagala, K. M.; Wedge, D. E.; Hale, A. L.; Harries, M. D.; Aliotta, G.; Duke, S. O. Natural fungicides from *Ruta graveolens* L. leaves, including a new quinolone alkaloid. *J. Agric. Food Chem*. **2003**, *51*, 890−896.

(26) Stebbing, A. R. Hormesis-the stimulation of growth by low levels of inhibitors. *Sci. Total Environ.* **1982**, *22*, 213−34.

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