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Antifungal Effects of Different Organic Extracts from *Melia azedarach* L. on Phytopathogenic Fungi and Their Isolated Active Components

MARÍA C. CARPINELLA,*,† LAURA M. GIORDA,‡ CARLOS G. FERRAYOLI,† AND SARA M. PALACIOS†

CEPROCOR, Agencia Córdoba Ciencia S. E., Alvarez de Arenales 230, Barrio Juniors, X5004AAP Córdoba, Argentina, and Cátedra de Fitopatología, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, 5000 Córdoba, Argentina and EEA INTA Manfredi, 5988 Manfredi, Argentina

Extracts from different parts of *Melia azedarach* L. were studied as potential antifungal agents for selected phytopathogenic fungi. In a serial agar dilution method, hexanic and ethanolic extracts from fruit, seed kernels, and senescent leaves exhibited fungistatic activity against *Aspergillus flavus*, *Diaporthe phaseolorum* var. meridionales, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium verticillioides*, and *Sclerotinia sclerotiorum*. Both hexanic extract from senescent leaves and ethanolic extract from seed kernel were highly effective on all tested fungi, with minimum inhibitory concentration (MIC) values ranging from 0.5 to 25 mg/mL and 0.5 to 5 mg/mL, respectively. In addition, all of the above-mentioned extracts showed fungicidal activity on these fungi, with ethanolic seed kernel extract being the most active. Three compounds displaying activity against *F. verticillioides* were isolated from the ethanolic seed kernel extract and were characterized as vanillin (**1**), 4-hydroxy-3-methoxycinnamaldehyde (**2**), and (±)-pinoresinol (**3**), with MICs of 0.6, 0.4, and 1.0 mg/mL, respectively. These compounds also showed a synergistic effect when combined in different concentrations, needing four times less concentration to reach complete inhibition in the growth of *F. verticillioides*.

KEYWORDS: Melia azedarach; antifungal activity; vanillin; 4-hydroxy-3-methoxycinnamaldehyde; pinoresinol

INTRODUCTION

Plant pathogens, and particularly fungi, are responsible for yield reductions in food and crops throughout the world. Although these losses may be attenuated by the use of resistant cultivars, crop rotation, or sanitation practices, fungicides are often also needed to maximize crop yields (*1*). These antifungal chemicals also contribute substantially to the quality of food and human health by controlling many of the fungi that produce mycotoxins (*2*) or by interfering in their biosynthesis (*3*).

Despite these significant benefits, synthetic fungicides are also responsible for the generation of toxic residues (*4*) and the development of resistance in pathogens (*1*, *5*). There is therefore a continuing need to develop and release new fungitoxic chemicals that preserve the environment and enable a more efficient control of pathogenic fungi, improving crop yield and quality.

Despite the fact that many plants are affected by fungal diseases, some of them are able to synthesize their own antifungal compounds (*6*−*9*). These compounds can then become natural biodegradable pesticides in the future.

Much attention has been devoted to the Meliaceae family, described as one of the main sources of potentially active metabolites. Many of these active compounds are well-known for their excellent effects on insect pest control (*10*−*12*) and their low toxicity to nontarget organisms (*13*). However, not many studies have focused on their antifungal effects.

Melia azedarach L. (Meliaceae), commonly named Paraiso, is a tree that grows easily in Argentina where it is widespread. It is used for medicinal (*14*), ornamental, and timber purposes. The insect repellent property (*15*−*17*) attributed to its limonoids (*18*, *19*) is the most relevant of its biological activities. It has also been reported that the compound, 28-deacetylsendanin, purified from the fruit of *M. azedarach*, can act as a virus replication inhibitor (*20*). Compounds from some chemotypes of *M. azedarach* have been reported to be toxic to mammals (*21*), but studies on extracts from Argentinian trees have revealed no such toxicity (*22*).

In our search for natural agrochemicals of plant origin, we have previously demonstrated the antifungal activity of ethanolic FEs from *M. azedarach* (*23*) on plant and human pathogenic fungi such as *Aspergillus flavus*, *Fusarium moniliforme*, *Microsporum canis*, and *Candida albicans*.

* To whom correspondence should be addressed. Tel: 03541 489650/53. Fax: 03541 489650/53 Int. 129. E-mail: ccarpine@ceprocor.uncor.edu. or cecicarpi@yahoo.com.
† Agencia Córdoba Ciencia S. E..
‡ Universidad Nacional de Córdoba.

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In the present paper, we report the fungistatic and fungicidal activity exhibited by organic solvent extracts obtained from different plant structures of *M. azedarach* on several phytopathogenic fungi, among them, *A. flavus*, *Diaporthe phaseolorum* var. meridionales, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium verticillioides*, and *Sclerotinia sclerotiorum*. These fungi were selected because of their importance in causing major plant diseases affecting crop yields and quality, with a negative influence in the economy of our region.

The most active compounds against *F. verticillioides* were isolated from SKE using bioassay-guided fractionation. The growth inhibitory activity of each isolated compound and of the combinations between them are also described in this paper.

MATERIALS AND METHODS

Plant Material. Ripe fruits and senescent leaves from *M. azedarach* L. were collected in Cordoba, Argentina, in October 2000. A voucher specimen was deposited in the Botanical Museum of Córdoba (CORD 229, Córdoba, Argentina).

Chemicals. MCZ (ethylenebis(dithiocarbamic acid)manganese zinc complex) technical grade was purchased from Riedel-de Haën Company. Neem X (formulated Neem product, azadirachtin content 0.4%, Marketing Arm International, Port Charlotte, FL) was a gift from Agrimarketing, Argentina. A vanillin sample was obtained from Sigma Chemical Co., Inc. (St. Louis, MO).

General Experimental Procedures and Apparatus. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ (Aldrich Chemical Co., Inc.) with a Bruker AC 200 spectrometer operated at 200 MHz for ^{1}H and at 50 MHz for the ^{13}C nucleus. Chemical shifts (parts per million) are relative to internal tetramethylsilane used as a reference. Coupling constants are quoted in hertz. MS spectra were measured with a Finnigan 3300-f100 instrument, and optical rotation was measured using a JASCO DIP-370 spectropolarimeter (JASCO Co., Tokyo). High-performance liquid chromatography (HPLC) was performed on a Waters 2690, equipped with a Phenomenex Luna 5 μm ODS (4.60 mm i.d. $\times$ 250 mm) reversed-phase column and photodiode array detector. The mobile phase was 1% acetic acid/MeOH in a gradient of 75:25 to 50:50. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} Merck plates (Darmstadt, Germany). Silica gel grade 70−230 mesh, 60 Å, for column chromatography was purchased from Sigma Chemical Co., Inc. All solvents were purchased from Merck and Fischer Scientific (New Jersey, NJ).

Extraction and Isolation of Antifungal Principles. The crushed, air-dried, whole, ripe fruits and seed kernels were extracted in a Soxhlet apparatus with hexane. After the solvent was removed under reduced pressure, this yielded the FO (extract yield, 7.77 g %) and the SKO (extract yield, 9.26 g %). The remaining plant material was then extracted with ethanol, to obtain after removing the alcohol, the viscous FE (extract yield, 28.82 g %) and the SKE (extract yield, 3.17 g %). Senescent leaves were extracted either with hexane or with ethanol to yield, after evaporation of the solvent, the HSLE (extract yield, 5.69 g %) or the ESLE (extract yield, 8.74 g %).

For the isolation of the antifungal principles, a CH_2Cl_2 fraction (DCMF; 33.12 g), obtained by three time partitioning of the SKE (77.77 g) dissolved in 300 mL of MeOH:CH_2Cl_2:H_2O (12:3:15), was chromatographed using vacuum liquid chromatography and eluted with a petroleum ether−Et_2O−Me_2CO−MeOH mixture, with increasing gradient polarity. Ten fractions (F-1−F-10) were collected. To determine the antifungal activity of each fraction, direct bioautography was made on TLC (*24*) as follows. In each TLC, a line was draw in the middle of the plate dividing it into two zones. Each fraction to be tested was applied in both zones. The whole plate was then developed with petroleum ether/Et_2O 5:5 or $CHCl_3$/MeCN 2:1, and after the solvent was evaporated, the plate was cut into two pieces through the central line, after visualizing the spot position by UV detection. Spores of *F. verticillioides*, suspended in glucose−mineral salts medium to reach an absorbance of 0.022 at 530 nm (*25*), were sprayed directly onto one piece of the previously developed plate. On the other piece of the developed plate, no fungi suspension was applied, but vanillin−sulfuric acid reagent was sprayed followed by heating, to detect those compounds with no UV absorption. At the end of the incubation period, a clearly visible growth inhibition zone was observed in F-3 on the spot corresponding to an R_f 0.75 ($CHCl_3$/MeCN 2:1). The compound corresponding to this R_f was obtained by sublimation as a white crystal. According to ^{1}H and ^{13}C NMR spectra and by comparison with an authentic sample, the compound was identified as vanillin (**1**) (yield, 0.029 g/100 g of seed kernel, by HPLC) (**Figure 1**).

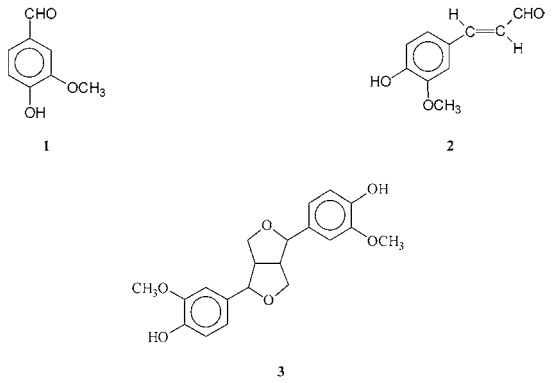

Figure 1. Chemical structures of vanillin (**1**), 4-hydroxy-3-methoxycinnamaldehyde (**2**), and pinoresinol (**3**).

Two compounds showing inhibition zones on direct bioautography TLC at R_f 0.77 and R_f 0.67 ($CHCl_3$/MeCN 2:1) were isolated, after successive vacuum liquid and radial preparative chromatography from F-4 to F-9. The first compound was identified by spectroscopic means as 4-hydroxy-3-methoxycinnamaldehyde (**2**) (**Figure 1**; yield, 0.054 g/100 g of seed kernel, by HPLC). The second one was obtained as a white crystalline solid (yield, 0.131 g/100 g of seed kernel, by HPLC) and identified as (±)-pinoresinol (**3**) (**Figure 1**) by NMR techniques and by comparison with previous literature (*26*, *27*).

4-Hydroxy-3-methoxycinnamaldehyde (2). $C_{10}O_3H_{10}$; t_R = 22.7 min (by HPLC). EI-MS *m*/*z* (Int. rel. %): 178 (M^+, 100), 161 (17), 147 (38), 135 (46), 124 (16). ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.65 (1 H, d, J = 7.7 Hz, H-1), 7.35 (1H, t, J = 17.9 Hz, H-3), 7.12 (1 H, dd, J = 8.1, 1.8 Hz, H-6′), 7.07 (1 H, d, J = 1.8 Hz, H-5′), 6.96 (1 H, d, J = 8.6 Hz, H-2′), 6.59 (1 H, q, J = 7.8 Hz, H-2), 3.94 (3 H, s, OMe). ^{13}C NMR (50.0 MHz, $CDCl_3$): δ 55.9 (ArOMe), 109.4 (C-2′), 114.9 (C-5′), 124.0 (C-6′), 126.4 (C-2), 126.6 (C-1′), 146.9 (C-3′), 148.9 (C-4′), 153.0 (C-3), 193.5 (C-1).

(±)-Pinoresinol (3). $C_{20}O_6H_{22}$; t_R = 38.3 min (by HPLC). EIMS *m*/*z* (Int. rel. %): 358 (M^+, 18), 327 (3), 205 (19), 196 (8), 180 (9), 163 (34), 151 (100), 150 (36), 137 (44), 133 (8), 131 (27). ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.78−6.90 (6 H, m, H-2′, 5′, 6′), 5.64 (2H, br s, ArOH), 4.73 (2 H, d, J = 4.2 Hz, H-2,6), 4.25 (2 H, q, J = 9.2, 6.9 Hz, H-4,8β), 3.89 (6 H, s, ArOMe), 3.86 (2 H, q, J = 9.2, 3.8 Hz, H-4,8α), 3.09 (2 H, m, H-1,5). ^{13}C NMR (50.0 MHz, $CDCl_3$): δ 54.2 (C-1,5), 55.9 (ArOMe), 71.7 (C-4,8), 85.8 (C-2,6), 108.6 (C-2′), 114.3 (C-5′), 118.9 (C-6′), 132.9 (C-1′), 145.2 (C-3′), 146.9 (C-4′).

Microorganism and Growth Medium. Isolates of *A. flavus* obtained from peanut, *D. phaseolorum* var. meridionales from soybean, *F. oxysporum* isolated from bean, *F. solani* from potatoes, *F. verticillioides* from maize, and *S. sclerotiorum* from lettuce were used. All fungi cultures were kept in potato dextrose agar (Difco), except for *A. flavus* and *F. verticillioides* cultures, for which peanut agar and V8 medium, respectively, were used.

Antifungal Assays. *Minimum Inhibitory Concentration (MIC). M. azedarach* organic solvent extracts HSLE, ELSE, FE, FO, SKE, and SKO were evaluated for their antifungal activity, using an agar serial dilution method according to Shadomy et al. (*25*). Inocula were prepared by suspending conidia from cultures of each for 48−96 h old fungus (1×10^6 conidia per mL) in physiological saline solution. Similar size (1−2 mm) mature sclerotia of *S. sclerotiorum* weighing 5.80−14.3 $\times$ 10^{-4} g were also used for the assays.

Bioassays were conducted by adding the appropriate amounts of each *M. azedarach* extract or Neem X, a commercial product used for comparison purposes, to 5 mL of 10% glucose medium, to obtain a

Table 1. MIC of Different Extracts from *M. azedarach*

	MIC[a] (mg/mL)							
test organism	HSLE	ESLE	FE	FO	SKE	SKO	NEEM-X	MCZ
A. flavus	25	100	50	10	5	25	100	0.01
D. phaseolorum var. meridionales	2.5	0.25	25	10	0.5	2.5	50	0.01
F. oxysporum	5	10	25	5	5	25	100	0.01
F. solani	5	5	25	25	5	50	100	0.01
F. verticillioides	1	5	50	0.5	2.5	0.5	200	0.001
S. sclerotiorum	0.5	0.25	25	5	2.5	1	25	0.001

[a] MIC is defined as the lowest concentration providing complete inhibition of fungal growth. Results are the average of two replicates measured at 48 h after incubation.

final concentration of 0.25−200 mg/mL. The fungicide MCZ was used as a positive control. Dilutions of ELSE and SKE were prepared by previously dissolving the extracts in ethanol (final concentration of ethanol < 10%), while HSLE was dissolved in chloroform (final concentration of $CHCl_3$ < 4%) before mixing with the glucose media. Plates containing only the culture medium, with the addition or not of the above-mentioned solvents, were used as viability controls for each fungi studied.

The fungi inocula (10 μL) or the sclerotia of *S. sclerotiorum* were placed in a hole (diameter 0.4 mm^2) made in the center of each Petri dish after solidification of the medium. Plates were incubated at 28 °C and controlled every 24 h. MICs were determined as the lowest concentration that produces complete growth inhibition of the fungi studied.

A broth microdilution method (*5*) was used to determine the MIC of the purified compounds individually or in different combinations. Four day old conidia from a monosporic culture of *F. verticillioides* were added to glucose−mineral salts medium to reach 1×10^5 conidia/mL. Purified compounds dissolved in dimethyl sulfoxide (DMSO) were incorporated into each well, by duplicate, containing the spore suspension, resulting in concentrations ranging from 0.025 to 1500 mg/mL. The final concentration of DMSO did not exceed 2−4%. Wells containing spore suspension with the addition or not of DMSO were simultaneously made as controls. The percentage of fungi growth (recorded in percentage from 0 to 100) was visually determined with an inverted light microscope for each concentration of the compounds. The measurements were always done by the same operator. The inhibitory concentration (IC_{50}) values were calculated by Probit analysis on the basis of percentage of inhibition obtained at each concentration of the samples.

Synergism among the pure compounds was also measured, adding to each well different combinations of different concentrations of each compound to be tested. In all cases, the final concentration of DMSO was lower than 8%.

Minimum Fungicide Concentration (MFC). These tests were carried out to analyze the possible fungicidal activity exhibited by each organic solvent extract, while further studying their fungistatic activity. Fourteen days after the beginning of the MIC assay, a circle of agar around the central hole was obtained, as well as the sclerotia of *S. sclerotiorum*, from those plates that exhibited negative growth. Two replicates of the agar circle or the sclerotia were placed in the center of Petri dishes containing only the specific medium for each fungi and then controlled for 14 days. At the end of this period, the MFC values were recorded.

RESULTS AND DISCUSSION

MIC of Extracts. Ethanolic and hexanic extracts of fruits, seed kernels, and leaves of *M. azedarach* were assayed on different phytopathogenic fungi in order to determine their potential antifungal activity. All extracts, depending on the concentration, exhibited a delay or a total inhibition in the growth of all of the test fungi *A. flavus*, *D. phaseolorum* var. meridionales, *F. oxysporum*, *F. solani*, *F. verticillioides*, and *S. sclerotiorum* in contrast to their controls.

As observed in **Table 1**, HSLE exhibited a significant inhibition in fungal growth with a MIC ranging from 0.5 to 25 mg/mL while the ESLE showed a similar activity (MIC = 0.25−10 mg/mL) except with *A. flavus* (MIC = 100 mg/mL). FE showed low antifungal activity with MIC values from 25 to 50 mg/mL, while SKE exhibited the most potent antimycotic effect with values of total inhibition ranging from 0.5 to 5 mg/mL.

The oils showed significant antifungal activity with MIC values from 0.5 to 50 mg/mL, with FO being more effective than SKO, which means that hydrophobic antifungal substances might be present in fruit pulp. The presence of hydrophobic antifungal compounds is in agreement with Locke and Walter (*28*) who have reported antifungal activity in hydrophobic solvent-extracted compounds from *Azadirachta indica* (Neem) seed. Neem -X exhibited very low activity against the studied pathogens, with MIC values (MIC = 25−200 mg/mL) lower than those obtained for *M. azedarach* extracts. The activities of the most active extracts were less than that of the control antifungal agent MCZ by at least 1 order of magnitude.

D. phaseolorum var. meridionales, *F. verticillioides*, and *S. sclerotiorum* were the fungi most affected (MIC = 0.25−25, 0.5−50, and 0.25−25 mg/mL, respectively), in contrast with *A. flavus*, which was the least inhibited (MIC = 5−100 g/mL), matching observations made by other authors (*29*). In concentrations lower than the MIC values, a delay in the growth of the fungi was observed with respect to the controls, showing that the extracts affected the optimum viability of the fungi (data not shown).

MFC of Extracts. All extracts studied showed fungicidal activity at 14 days from the beginning (**Table 2**) with SKE being the most effective, exhibiting an MFC from 10 to 25 mg/mL. Although the doses recorded in our experiments are high (the MFCs corresponded to 10−200 mg/mL for all studied extracts), these results are highly relevant, since a fungicidal effect is not often observed in products derived from plants.

In summary, these findings showed that the ethanolic and hexanic extracts obtained from different plant structures of *M. azedarach* L. have a significant fungistatic and fungicide effect on different phytopathogenic fungi.

Isolation of the Antifungal Active Principles. As mentioned before, HSLE and SKE exhibited the lowest MIC values on most tested fungi, but SKE showed more regular results in its fungistatic effect and the lowest fungicide activity. Therefore, we decided as a first step to isolate the active principles from the SKE, leaving for the next steps the isolation of the active compounds from the remaining extracts.

SKE was partitioned with CH_2Cl_2, and after the solvent was removed, the resultant residue (DCMF) was submitted to successive vacuum liquid and radial preparative chromatography directed by bioassay. Three compounds showing activity against the pathogenic fungi *F. verticillioides* were obtained and were

Table 2. MFC of Different Extracts from *M. azedarach*

	MFC[a] (mg/mL)							
test organism	HSLE	ESLE	FE	FO	SKE	SKO	NEEM-X	MCZ
A. flavus	100	>200	150	200	25	>200	>200	0.01
D. phaseolorum var. meridionales	50	50	50	50	10	25	100	0.01
F. oxysporum	50	200	100	50	25	100	>200	0.01
F. solani	50	200	100	50	25	>150	200	0.050
F. verticillioides	50	100	150	25	10	100	>200	0.025
S. sclerotiorum	100	50	50	200	10	50	200	600

[a] MFC is defined as the lowest concentration providing complete inhibition of fungal growth in a medium free of extract or compound. Results, the average of two replicates, were measured at 14 days after incubation.

Table 3. Antifungal Activity of **1**–**3**

compd	MIC[a] (mg/mL)	compd	MIC[a] (mg/mL)
1	0.60	**3**	1.00
2	0.40	MCZ	0.01

[a] Results, the average of two replicates, were measured at 48 h from the beginning.

identified as vanillin (**1**), 4-hydroxy-3-methoxycinnamaldehyde (**2**), and (±)-pinoresinol (**3**) (**Figure 1**).

Compound **1**, which is already known to exhibit antifungal activity (*30*, *31*), has previously been obtained from *M. azedarach* seeds (*32*). Compound **2** has already been described as an antifungal compound (*33*), but its isolation from *M. azedarach* extracts has not previously been reported. Compound **3** has already been isolated from seeds of *M. azedarach* (*34*) and showed a variety of bioactivities (*35*−*37*), but its fungitoxic activity has not been described in the literature, to the best of our knowledge.

The presence of vanillin, cinnamic acid, or ferulic acid has already been described in *M. azedarach* fruit and leaves (*32*), and it is well-established that they have antifungal properties (*31*, *38*). However, among the above-mentioned compounds, only compound **1** showed fungal inhibitory effect in our guided bioassay procedure, in addition, compounds **2** and **3** emerged as the compounds responsible for the antimycotic activity of *M. azedarach* SKE.

Vanillin (GRAS) is present in food or acts as a naturally derived food flavoring (*39*, *40*). This suggests a low toxicity to mammals, giving its capacity in protecting crops from fungal attack greater importance. The activity reported here is of interest for the potential use of this compound in organic and ecological crop production.

Antifungal Activity of Compounds 1−**3.** MIC values of isolated compounds **1**−**3** measured by the broth microdilution method are detailed in **Table 3**. Compounds **1** and **2** showed a total growth inhibition of the pathogenic fungi *F. verticillioides* at 0.60 (MIC 1) and 0.40 (MIC 2) mg/mL, respectively, while the MIC of compound **3** was observed at 1.00 mg/mL (MIC 3).

At 0.40 mg/mL, compound **1** developed 65% of growth inhibition (see **Figure 2**) while at 0.20 mg/mL a 20% inhibition was still observed. The same percentage of inhibition was detected for compound **2** at 0.20 mg/mL. Fungi growth was inhibited by 20 and 40% by compound **3** at concentrations of 0.60 and 0.80 mg/mL, respectively. As observed, the inhibitory activity of compounds **1**−**3** increased in a dose-dependent manner. The IC_{50} indicated a similar performance for **1** (0.30 mg/mL; 95% confidence interval = 0.18−0.51) and **2** (0.24 mg/mL; 95% confidence interval = 0.07−0.85), and this effect was better than that for **3** (0.76 mg/mL; 95% confidence interval = 0.37−1.56).

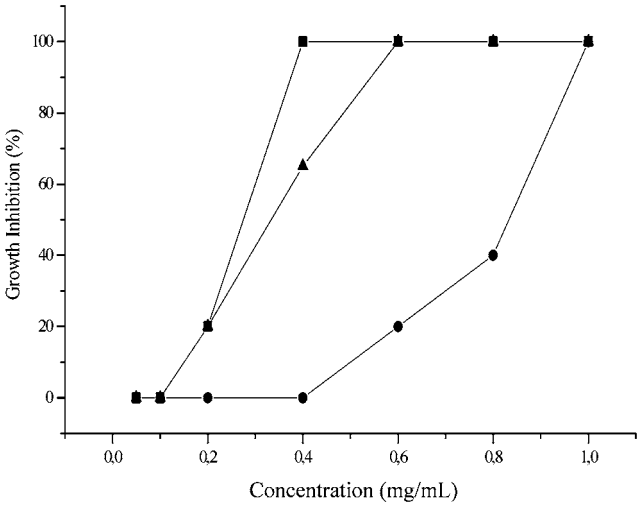

Figure 2. Effect on fungi growth inhibition of different concentrations of **1** (▲), **2** (■), and **3** (●). See text for additional experimental details.

Table 4. Synergism among Different Concentrations of **1**–**3** Combined in Pairs

compd	growth inhibition[a] (%)				
(mg/mL)	**2** (0.200)	**2** (0.100)	**2** (0.050)	**3** (0.400)	**3** (0.200)
1 (0.300)	100	100	100	100	100
1 (0.200)	97	95	84		
1 (0.150)	100	90	85	95	80
3 (0.700)	100	85	80		
3 (0.500)	90	85			
3 (0.400)	50				
3 (0.200)		2			

[a] Results, the average of two replicates, were obtained at 48 h from the beginning.

Because DCMF presented a higher antifungal activity (MIC and MFC = 0.50 mg/mL) than those exhibited by pure compounds, the presence of a synergistic effect between **1**, **2**, and **3** was suspected. The compounds were added in different combinations of two or three that in most cases were equivalent to half or less of their MIC values.

As seen in **Table 4**, when compound **1** at 0.300 mg/mL (0.5 MIC 1) was combined with **2** at 0.100 mg/mL (0.25 MIC 2) or with **3** at 0.400 mg/mL (0.4 MIC 3), a 100% inhibition in *F. verticillioides* growth was observed. The same results were obtained when, at the same concentration of **1**, concentrations of **2** or **3** were decreased to 0.050 mg/mL (0.125 MIC 2) and 0.200 mg/mL (0.2 MIC 3), respectively. When compound **1**

Table 5. Synergism among Different Concentrations of **1**–**3** Combining All Three

compd (mg/mL)	growth inhibition[a] (%)		
	2 (0.200)	**2** (0.100)	**2** (0.050)
1 (0.300) **3** (0.400)	100		
1 (0.150) **3** (0.200)		100	88
1 (0.075) **3** (0.100)	100	82	0

[a] Value at 48 h from the beginning; average of two replicates.

was added at 0.200 mg/mL, equivalent to 0.33 of its MIC value and **2** at 0.100 mg/mL, a 95% inhibition was detected. When **1** was added at a concentration four times lower than the value of MIC 1 (0.150 mg/mL) and **2** at 0.200 mg/mL (0.5 MIC 2), the inhibition was complete. When the concentration of **2** decreased to 0.050 mg/mL, significant inhibition (85%) was still observed. When the same concentration of **1** was combined with **3** at 0.400 mg/mL, a 95% growth inhibition was obtained, decreasing to 80% when **3** was added at 0.200 mg/mL. These results might indicate that the combination between **1** and **2** has a slightly more synergistic effect than **1** and **3**. Adding **2** and **3** at 0.200 (0.5 MIC 2) and 0.500 mg/mL (0.5 MIC 3), respectively, 90% inhibition was observed, reaching 100% when the concentration of **3** was 0.700 mg/mL. Lower concentrations of both compounds exhibited inhibition values ranging from 2 to 85%, meaning that the synergism between **2** and **3** is lower than that observed between **1** and **2** or between **1** and **3**.

As is seen in **Table 5**, when all three compounds were combined, each at a concentration of about 0.25 of its MIC, 100% control of the growth of *F. verticillioides* was obtained, demonstrating a better control than that observed when pairs of compounds were combined at approximately 0.25 of their MIC values. There was no inhibition on fungal growth when **1** and **2** were assayed at 0.075 (0.125 MIC 1) and 0.050 mg/mL (0.125 MIC 2), respectively, and **3** at a concentration of 0.100 mg/mL (0.1 MIC). If the concentration of at least one of the compounds was increased, the inhibition also increased, obtaining 100% inhibition when compounds **1** and **3** remained at the same concentration (0.125 MIC 1 and 3) and **2** was increased to 0.200 mg/mL (0.5 MIC 2).

In summary, when the antifungal active principles **1**−**3** were combined in pairs or all three together in concentrations below their MIC values, an unexpected fungal growth inhibition almost as high as that observed with the synthetic compound MCZ was observed. This result showed a clear synergistic effect between them, which was not observed till now. This effect enabled the quantities of each compound needed for a total inhibition to be decreased at least 4-fold. The synergistic effect is one of the most important characteristics exhibited by natural extracts, increasing their efficacy in contrast to that which could be obtained with the equivalent amount of the active constituents alone (*41*).

In conclusion, this study demonstrates that extracts from *M. azedarach* and their respective bioactive compounds showed an important fungitoxic effect against the microorganisms studied, increasing the antifungal potency of the active principles when they are combined. Current research offers the possibility of developing strategies for controlling plant pathogen fungi with natural extracts or bioactive metabolites, in response to the ongoing search for novel environmentally safe agrochemicals of plant origin.

ABBREVIATIONS USED

HSLE, hexanic senescent leaves extract; ESLE, ethanolic senescent leaves extract; FE, fruit extract; FO, fruit oil; SKE, seed kernel extract; SKO, seed kernel oil; MCZ, Mancozeb.

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LITERATURE CITED

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