

## In Vitro Susceptibilities of *Madurella mycetomatis* to Itraconazole and Amphotericin B Assessed by a Modified NCCLS Method and a Viability-Based 2,3-Bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenylamino)Carbonyl]-2H-Tetrazolium Hydroxide (XTT) Assay

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**Susceptibilities of *Madurella mycetomatis* against amphotericin B and itraconazole in vitro were determined by protocols based on NCCLS guidelines (visual reading) and a 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) assay for fungal viability. The XTT assay was reproducible and sensitive for both antifungals. Itraconazole (MIC at which 50% of the isolates tested are inhibited [MIC<sub>50</sub>]) of 0.06 to 0.13 mg/liter) was superior to amphotericin B (MIC<sub>50</sub> of 0.5 to 1.0 mg/liter).**

Little is known about the susceptibility of the fungus *Madurella mycetomatis*, the major cause of eumycetoma, to antifungal agents (6). In the past, ketoconazole was promoted as the drug of choice (4, 6, 8, 10, 15, 16), but clinical response to ketoconazole is often poor (5, 17, 18, 25, 26). Other studies show that early treatment with itraconazole (ITC) seems to be optimal (5, 10, 17). Here we evaluate the in vitro activities of ITC and amphotericin B (AMB) against 36 clinical isolates of *M. mycetomatis*. MICs were determined visually by a method based on the NCCLS (approved standard M38-A) (20). In addition, a quantitative colorimetric method using the dye 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) was used (12, 13, 24).

Independent clinical isolates ( $n = 34$ ) obtained from Sudanese mycetoma patients visiting the Mycetoma Research Clinic (University of Khartoum, Khartoum, Sudan) during the year 1999 were included. Two additional clinical isolates were derived from patients from Mali (2). Strains were cultivated on Sabouraud dextrose agar with or without 80 mg of gentamicin (Centrafarm, Etten-Leur, The Netherlands) per liter or on malt extract agar (Difco Laboratories, Paris, France). Species were identified as described previously (1, 9).

ITC was obtained from Janssen Pharmaceutica Products, Ghent, Belgium, and AMB was obtained from Bristol-Myers Squibb, Woerden, The Netherlands. The protocol for susceptibility testing (broth macrodilution) was based on the NCCLS procedure for filamentous fungi (approved standard M38-A [20]). To prepare inocula from cultures in RPMI 1640 with

L-glutamine (0.3 g/liter) and 20 mM morpholinepropanesulfonic acid, mycelia were harvested by 5 min of centrifugation at  $2,158 \times g$  and washed once with sterile saline. After sonification (20 s at 28- $\mu$ m maximum power; Soniprep, Beun de Ronde, The Netherlands) of the hyphal suspension, Tween 60 was added at 0.05% (vol/vol), and the transmissions were adjusted to 70% at 660 nm (Novaspec II; Pharmacia Biotech). The inoculated tubes were incubated at 37°C for 7 days. Inoculum reading controls (hyphal suspension in saline without antifungals) were included, as were growth controls without antifungals.

The viable fungal mass was determined colorimetrically with XTT as the substrate as described previously (19). Tubes containing final concentrations of 250  $\mu$ g of XTT/ml and menadione (58  $\mu$ M) were incubated for 2 h at 37°C and for another 3 h at room temperature. The tubes were then centrifuged, and the extinction coefficient of the supernatant was measured at 450 nm in a microplate reader.

Figure 1 shows the reproducibility of antifungal susceptibility testing of an *M. mycetomatis* strain (mm55) according to the XTT assay. For AMB, a sudden switch to full reduction in viable fungal mass was observed. Exposure to ITC resulted in a concentration-dependent gradual decrease. In Fig. 2, the results for the XTT assay and the modified NCCLS method were compared for *M. mycetomatis* strain mm55. A concentration-dependent pattern of antifungal activity, each being different for AMB and ITC, was observed. The MICs of AMB and ITC for *M. mycetomatis* mm-55 were 1 to 2 and 0.25 to 0.5 mg/liter, respectively.

Table 1 shows the susceptibilities of 36 *M. mycetomatis* clinical isolates determined by both methods. Often, high susceptibility to ITC is accompanied by relatively low susceptibility to AMB. For AMB, the MIC values ranged from 0.13 to 4 mg/liter (MIC at which 50% of the isolates tested are inhibited [MIC<sub>50</sub>]) of 0.5 to 1 mg/liter). For ITC, the MIC values ranged

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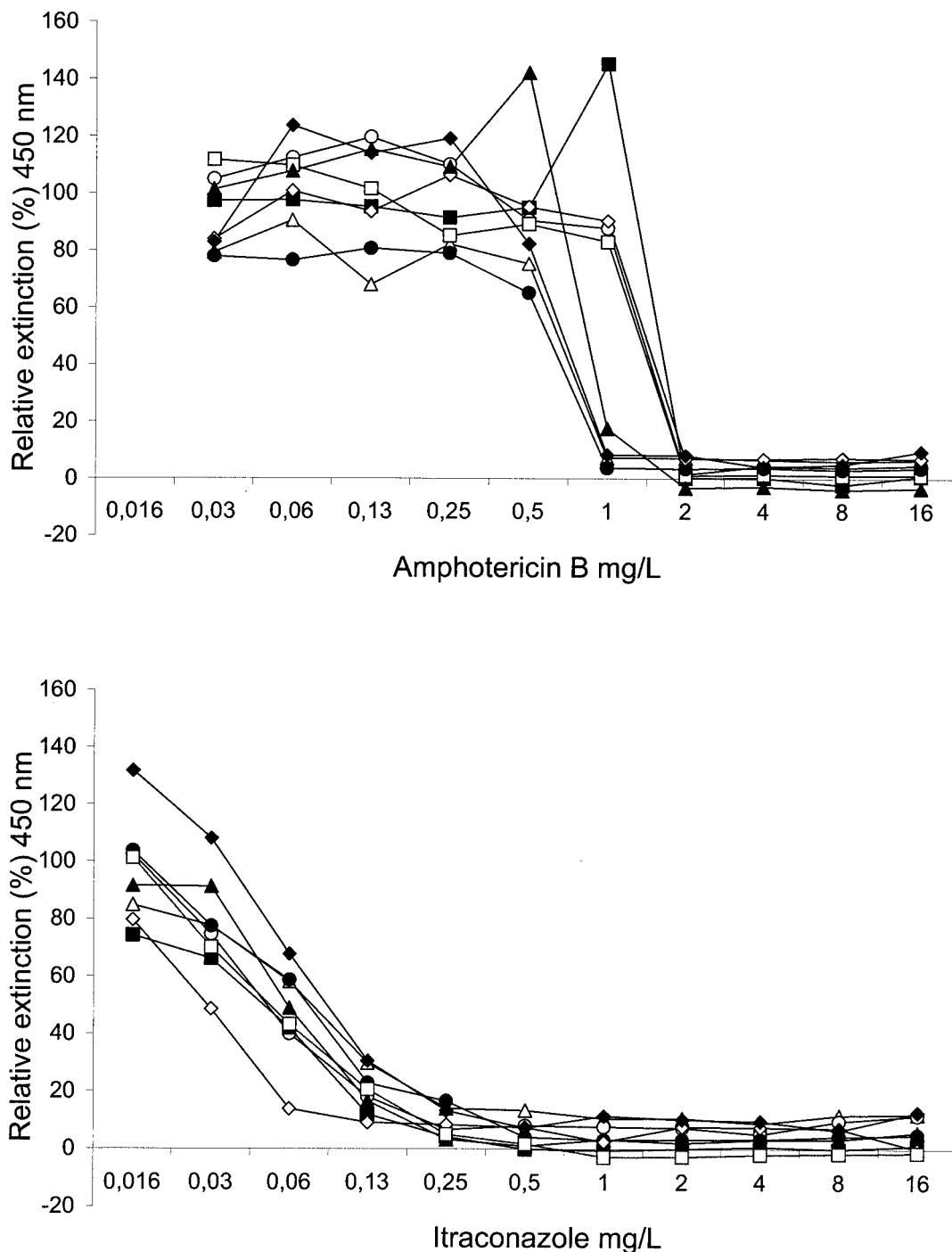


FIG. 1. Reproducibility of susceptibility testing of *M. mycetomatis* strain mm-55 against AMB and ITC by the XTT method. Curves represent the relative extinction at 450 nm for each drug concentration compared to the growth control (100%). Assays were repeated eight times.

from 0.016 to 1 mg/liter ( $MIC_{50}$  of 0.06 to 0.13 mg/liter). With the XTT assay, 100% reduction in viable fungal mass could not be determined as a number of strains produced pigments that influenced the color intensity. For all 36 *M. mycetomatis* isolates, 80% reduction was obtained with AMB ranging from 0.13 to 8 mg/liter and with ITC from 0.016 to 1 mg/liter. A wide range of MICs was obtained for AMB as well as ITC for our clinical isolates, irrespective of their clonal relatedness (3).

This finding implies that gene expression levels rather than differential gene presence are a driving factor in the development of resistance.

The filamentous nature of *M. mycetomatis* frustrates the straightforward use of the standardized NCCLS protocols since a conidial suspension is usually used as an inoculum (7, 11, 20). Preparing a standardized inoculum for the poorly differentiating and nonsporulating fungal species is problematic (9). To

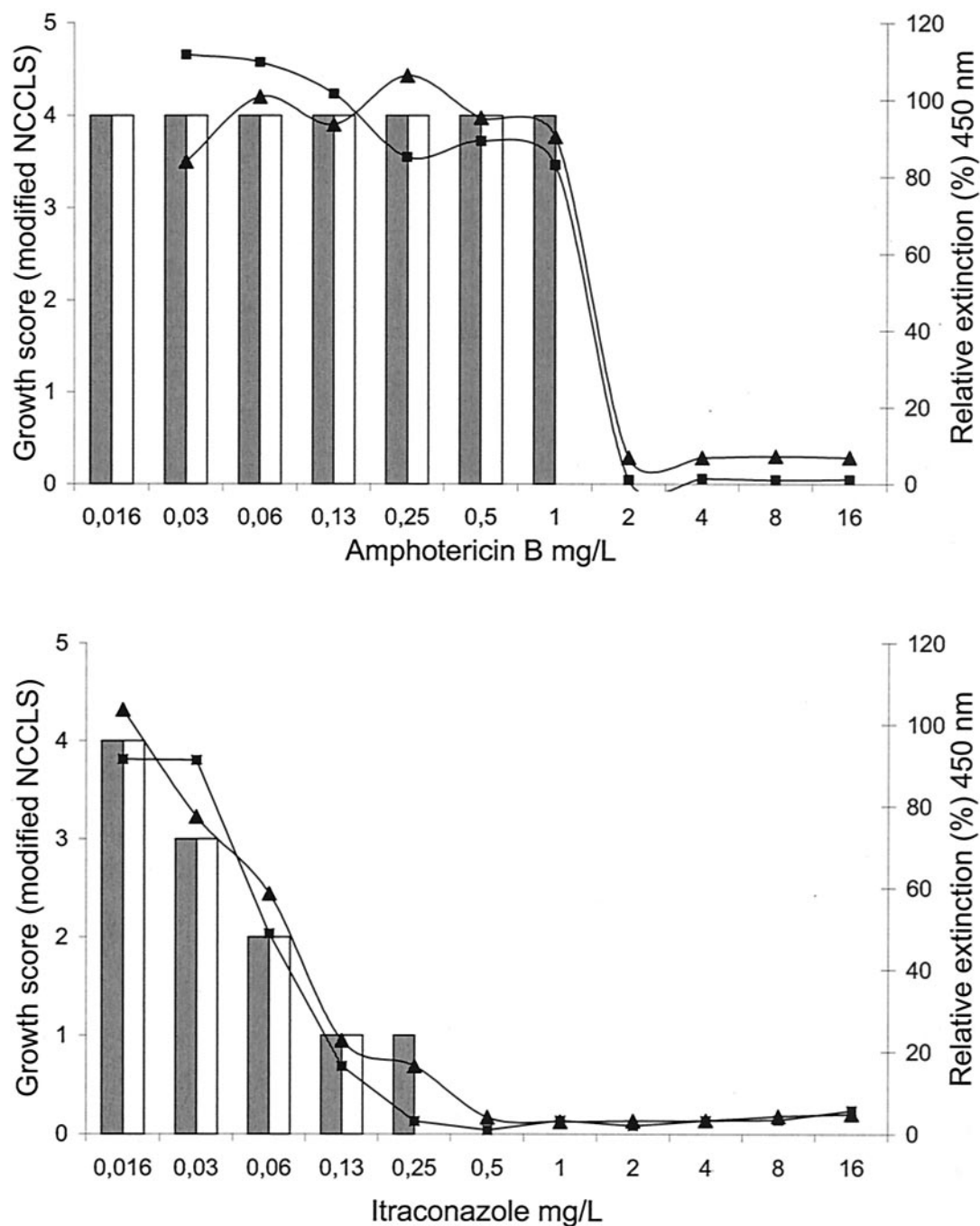


FIG. 2. Antifungal susceptibility testing of *M. mycetomatis* strain mm-55 against AMB and ITC in duplicate. Curves represent the relative extinction at 450 nm for each drug concentration compared to the growth control (100%) obtained by the XTT assay. Bars represent the growth levels obtained by the modified NCCLS method as determined in a separate experiment.

standardize the inoculum, the harvested hyphae were first homogenized. These inocula were found to be within the recommended range of  $0.4 \times 10^4$  to  $5 \times 10^4$  CFU per ml (20).

As the initial hyphal suspension already shows significant turbidity, which complicates visual reading of the antifungal activity, the XTT assay was also used. It generated reproducible data and showed good agreement with the MIC data for AMB. This overall agreement was also documented for various other fungal species (19).

The antifungal effect of ITC is superior to that of AMB. Approximately 45% of the 36 *M. mycetomatis* isolates showed susceptibility to ITC concentrations of less than 0.13 mg/liter, whereas AMB was not effective at those concentrations. Prevention of growth of all isolates was obtained with ITC at 1 mg/liter or less and with AMB at 8 mg/liter or less; these results are in agreement with earlier findings for other filamentous ascomycetes (14, 18, 20). Activities of ITC against dermatophytes (11) and agents of hyalohyphomycosis, phaeohyphomy-

TABLE 1. Antifungal susceptibilities of 36 *M. mycetomatis* clinical isolates

Isolate	MIC (mg/liter) <sup>a</sup> :					
	AMB			ITC		
	XTT assay at reduction indicated		NCCLS modified	XTT assay at reduction indicated		NCCLS modified
	>50%	>80%		>50%	>80%	
mm71	0.13	0.13	0.13	0.03	0.06	0.13
p2	0.13	0.13	0.13	0.06	0.13	0.25
mm46	0.13	0.13	0.25	0.01	0.01	0.01
mm54	0.13	0.25	0.5	0.01	0.01	0.03
mm30	0.13	0.5	0.5	0.13	1	0.5
mm49	0.13	0.5	0.5	0.01	0.01	0.06
mm58	0.13	0.5	1	0.06	0.13	0.06
mm63	0.25	0.25	0.25	0.03	0.03	0.06
mm39	0.25	0.5	0.25	0.06	0.06	0.06
mm36	0.25	0.5	0.5	0.06	0.06	0.06
mm41	0.25	0.5	0.5	0.06	0.13	0.13
mm83	0.25	0.5	0.5	0.03	0.06	0.06
mm22	0.25	0.5	1	0.06	0.13	0.13
mm14	0.5	0.5	0.5	0.06	0.13	0.13
mm28	0.5	0.5	0.5	0.06	0.13	0.06
mm10	0.5	0.5	1	0.25	1	1
mm29	0.5	0.5	1	0.03	0.06	0.06
mm25	0.5	1	0.5	0.06	0.13	0.06
mm18	1	1	1	0.01	0.03	0.01
mm55	1	1	1	0.06	0.25	0.25
mm50	1	1	2	0.01	0.03	0.03
mm52	1	1	4	0.06	0.5	0.5
mm26	1	2	1	0.06	0.25	0.25
mm72	1	2	1	0.06	0.06	0.03
mm78	1	2	1	0.06	0.06	0.06
mm43	1	2	2	0.13	0.25	0.25
mm64	1	2	2	0.03	0.03	0.06
mm68	1	2	2	0.06	0.5	0.5
p1	1	2	2	0.25	0.5	1
mm45	1	4	2	0.25	0.5	0.5
mm73	2	2	1	0.06	0.06	0.25
mm56	2	2	2	0.03	0.06	0.06
mm13	2	4	4	0.5	1	0.5
mm35	4	4	4	0.5	1	0.5
mm16	4	8	4	0.25	0.5	0.5
mm31	4	4	4	0.13	0.5	0.25

<sup>a</sup> For the XTT assay, the concentrations resulting in more than 50% or 80% reduction in viable fungal mass are shown. For the NCCLS modified, the concentrations resulting in complete inhibition of fungal growth (MIC) are shown. Data are median values for three determinations.

cosis, chromoblastomycosis, and mycetoma were also demonstrated (18). Recently, ITC has been effectively used for the treatment of a case of fungal mycetoma due to *Fusarium solani* (27). Compared to ITC, voriconazole showed comparable or increased in vitro activity against a number of emerging and less common mold pathogens (21). The high in vitro susceptibility of the *M. mycetomatis* isolates may nominate ITC as the drug of choice for treatment. About 33% of the *M. mycetomatis* isolates included in this study had an AMB MIC that is higher than the average peak level in plasma of 1.5 mg/liter (22). In addition to the relatively low antifungal activity, the requirement for long-term treatment of mycetoma patients together with the potential toxic side effects of AMB further limits its use as a first-line therapeutic agent.

In conclusion, the XTT assay is optimal for antifungal susceptibility testing of *M. mycetomatis* since it avoids visual read-

ing. It also provides additional insight into the dynamics of killing. The assessment of antifungal susceptibility may be particularly useful for patients not responding to initial medical treatment.

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