Development and validation of *in vitro* microtiter-based viability assay incorporating resazurin for drug discovery and susceptibility testing against *Madurella mycetomatis*

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Objectives

Madurella mycetomatis is the major causative agent of mycetoma, which is considered as one of the most neglected tropical diseases. The lack of a therapeutically effective drug against *M. mycetomatis* coupled with the rise of resistance against azole antifungal agents in general prompted us to develop a rapid, reproducible, and inexpensive *in vitro* viability assay for drug susceptibility testing for *M. mycetomatis.* This assay will be an indispensable tool in the drug discovery of novel bioactive agents against this disfiguring infectious disease.[1] For this assay, we aimed to use resazurin (Alamar blue) as a read-out system, as it offers a simple and affordable method especially in resource-limited settings along the "*mycetoma endemic belt*". The mechanism of the resazurin method is based on targeting the mitochondrial enzymes in the live cells that convert resazurin to resorufin proportionally to the number of metabolically active viable cells (Figure 1).

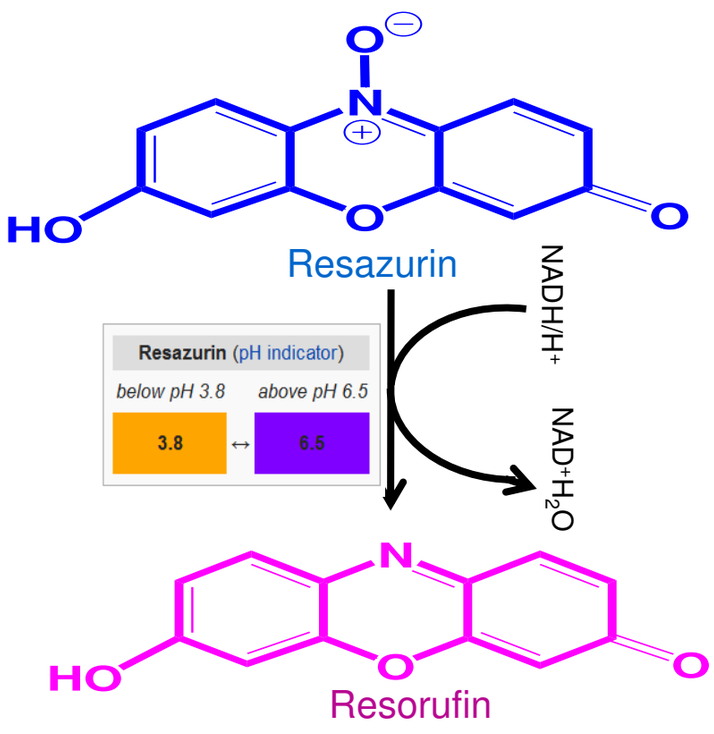

Figure 1. Conversion of resazurin into resorufin

Methods

The *in vitro* susceptibility of nine *M. mycetomatis* isolates (CBS132259, CBS132260, CBS132264, CBS132267, CBS132271, CBS132274, CBS132277, CBS131320, CBS132283) towards eight standard antifungal agents (Amphotericin-B, Caspofungin, Fluconazole, Itraconazole, Ketoconazole, Voriconazole, Posaconazole and Terbinafine) was determined with the resazurin assay and XTT assay.[2] In short, a fungal inoculum ranging between 0.4 x 10^4 to 5 x 10^4 CFU/ml with 68-72% transmission was exposed to different concentrations of the antifungal agents. When resazurin was used as a read-out system, it was added on the first day of incubation at a final concentration of 0.15 mg/ml with distinctive colour on the 7th day of its incubation at 37 ºC. When XTT was used as a read-out system, the dye was added at the end of incubation period.

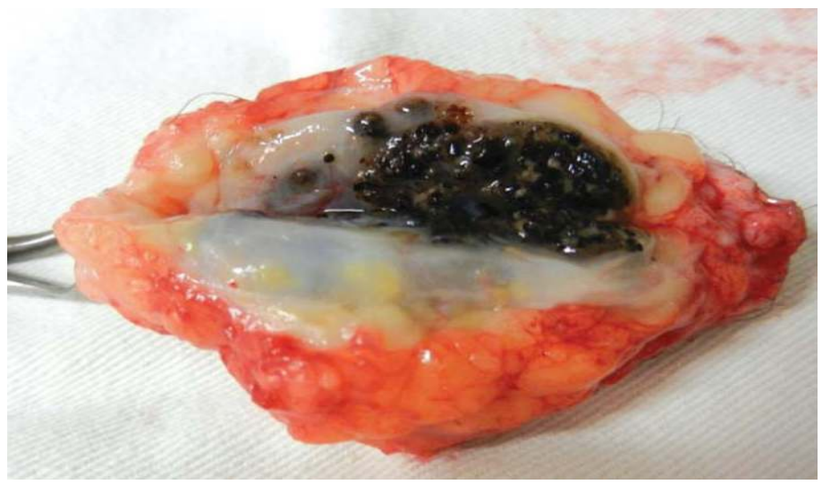

Figure 2. *M. Mycetomatis* lesion after excision with black grains inside[4]

Results

A sharp, reproducible end-point was detected resulting from the conversion of resazurin to resorufin (Figure 1 & Table 1). Visual detection of MIC provided quantitatively identical absorbance measurements in resazurin assay. Comparison of the overall MIC values of resazurin assay with its counterpart XTT assay generated comparable MIC values. The MIC of Amphotericin B ranges between >256-16 ug/ml while the azoles except for Fluconazole, exhibited varying inhibitory effect on different isolates. Itraconazole and Posaconazole exhibited the lowest MIC value (>8-0.031ug/ml). Caspofungin and Terbinafine, however, showed weak inhibition on most of the isolates examined (MIC >256ug/ml).

Table1. MIC (ug/ml) of *in vitro* antifungal susceptibility of nine clinical isolates of *M. mycetomatis*

Antifungal agent	MIC range (ug/ml)	MIC_{50}
Amphotericin B	>256-16	>256
Caspofungin	>256-256	>256
Fluconazole	>256-256	>256
Itraconazole	>8-0.031	0.125
Ketoconazole	>8-0.06	0.25
Posaconazole	>8-0.031	0.125
Terbinafine	>256-128	>256
Voriconazole	>8-0.125	0.25

Conclusion

A simple, reproducible resazurin-based *in vitro* assay was optimized for susceptibility testing of *M. mycetomatis*. The major advantages of the resazurin reduction assay are inherent in its more sensitivity in comparison with tetrazolium assays besides its limited variability across linear range, whiles much larger variations were observed for the XTT assays.

References

1. Khalid SA. 2nd ResNet NPND workshop on natural products against neglected diseases, Nov, 25-28th, 2014, Rio de Janeiro, Brazil.
2. Ahmed, A.O. et al. *Antimicrobial Agents and Chemotherapy*, 48 (7), 2742-46, 2004.
3. Sampaio et al., *An Bras Dermatol*. 2013 Nov-Dec; 88(6 Suppl 1; 88(6 Suppl 1): 82–84.