Research Article

In-vitro antifungal activity of resorcinol against human fungal pathogen *Candida albicans*

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**Abstract:** The aim of this study was to evaluate the antifungal potential of Resorcinol (Res) against human fungal pathogen, *Candida albicans*. In this study, we explored that Res displayed its antifungal potential against two reference strains of *C. albicans* (ATCC 10261 and ATCC 24433) and five clinical isolates tested. We also showed that Res is equally effective against various non-*albicans* species of *Candida* as well thereby demonstrating its wide repertoire. Notably, the antifungal effect of Res seems to be independent of the activity of major drug efflux pumps transporter which is the major cause of drug resistance in *C. albicans*. Finally, we also demonstrated that Res inhibits the serum and nitrogen starvation dependent yeast to hyphal transition in *C. albicans* which is one of the major virulence attribute. Taken together, Res exhibited promising in vitro antifungal properties and potent inhibitor of yeast to hyphal transition that could be used for the treatment of *Candida* infections. Further cellular and in vivo studies are required to explore its precise mechanism of action.

**Keywords:** *Candida*, Resorcinol, MDR, Yeast to hyphal transition

**Introduction**

Fungal infections are one of the most common dilemmas that the world is facing and should be dealt with immediate attention. Many scientists have shown their efforts to cope up against the human fungal pathogen like *Candida albicans*, which is the fourth most leading cause of hospital acquired infections [1, 2, 3] Effective drugs such as azoles that target the sterol-14α-demethylase thereby inhibiting the ergosterol biosynthesis, echinocandins acting against β-1,3-glucan synthesis and inhibiting the cell wall synthesis and polyenes effectively disrupting membrane homeostasis are currently in use. However, present therapeutic drugs due to their excessive usage for the treatment against this fungal pathogen is facing the problem of multidrug resistance (MDR) rendering them ineffective [4, 5]. Therefore there is an urgent need for new antifungal chemical structures as remedy for the existing ones. For this many scientific studies have been conducted by workers, which showed that finding the novel drugs and their targets is in priority [6, 7, 8].

Benzene-1,3-diol commonly known as Resorcinol (Res) is a colorless synthetic compound that is commonly used in the dye and also in the treatment of acne and as seborrheic dermatitis [9]. In this study, we have scrutinized the antifungal properties of Res against *C. albicans* as well as other *Candida* species and also showed its effect on yeast to hyphal transition which is an important virulence trait.

**Materials and Methods**

All Media chemicals YEPD (Yeast Extract Peptone Dextrose), Agar, Horse Serum were purchased from Himedia (Mumbai, India). Sodium Chloride (NaCl), D-Glucose and Resorcinol (Res) were obtained from Fischer Scientific.

**Growth media and strains used**
The reference strains of *C. albicans* used in this study were ATCC 10261 and ATCC 24433. The clinical isolate strains of *C. albicans* were D1, D2, D4, D7, D18 and non-*C. albicans* species include ATCC 90030 (*Candida glabrata*), D9 (*Candida tropicalis*), D11 (*Candida parapsilosis*) and D46 (*Candida krusei*) [10]. All the strains of *Candida albicans* were cultured in YEPD broth with the composition of yeast extract 1% (w/v), peptone 2% (w/v) and dextrose 2% (w/v). For agar plates 2% (w/v) agar (Himedia, Mumbai, India) was added to the media. All *Candida* strains were stored in 30% (v/v) glycerol stock at -80°C. The cells were freshly revived on YEPD broth and transferred to agar plate. The cells were grown at 30°C on agar plate before each study to ensure the revival of the strains.

**Drug susceptibility testing**

Drug susceptibility was tested using spot assay and minimal inhibitory concentration (MIC) as described below:

**Spot assay**

Spot assays for the strains were determined using a method as described elsewhere [11, 12]. Briefly, for the spot assay, 5μL of fivefold serial dilutions of each yeast culture (each with cells suspended in normal saline to an OD₆₀₀ of 0.1) was spotted onto YEPD plates in the absence (control) and presence of the Res. Growth was not affected by the presence of solvent used in the examination (data not shown). Growth difference was measured after incubation at 30°C for 48 hours. The concentrations used in this study are specified in figure legends.

**Minimum Inhibitory Concentration (MIC)**

MIC was determined by broth dilution method as described in method M27-A3 from the Clinical and Laboratory Standards Institute (CLSI) formerly NCCLS (National Committee for Clinical Laboratory Standards) [13]. Briefly, 100μl of media was placed at each well of the 96 wells plate following with the addition of the drug with the remaining media and then was serially diluted. 100μl of cell suspension (in normal saline to an OD₆₀₀ 0.1) was added to each well of the plate and OD₆₀₀ was measured after 48 hours at 30°C. The MIC₈₀ was defined as the concentration at which the 80% of the growth was inhibited.

**Rhodamine 6G Efflux**

The efflux of Rhodamine 6G (R6G) was determined by using protocol described elsewhere [1, 14]. Briefly, approximately 1x10⁸ yeast cells from an overnight-grown culture were transferred to YEPD medium and allowed to grow for 5 h. Cells were harvested, washed twice with phosphate-buffered saline (PBS) (without glucose), and resuspended as a 2% cell suspension, which corresponds to 10⁸ cells (w/v) in PBS without glucose. The cells were then de-energized for 1h in 2-DOG (5 mM) and 2,4 DNP (5 mM) in PBS (without glucose). The de-energized cells were pelleted, washed, and again resuspended as a 2% cell suspension (w/v) in PBS without glucose, to which R6G was added at a final concentration of 10μM and incubated for 40 min at 30°C. The equilibrated cells with R6G were then washed and resuspended as a 2% cell suspension (w/v) in PBS without glucose. Samples with a volume of 1 ml were withdrawn at the indicated time and centrifuged at 10,000 x g for 1 min. The supernatant was collected, and absorption was measured at 527nm. Energy dependent efflux (at the indicated time) was measured after the addition of glucose (2%) to the cells resuspended in PBS (without glucose). Glucose-free controls were included in all the experiments.

**Yeast to hyphal transition**

Studies of hyphal induction on *C. albicans* were carried out on hyphal induction media like Spider (1% nutrient broth, 1% mannitol, 0.2% K₂HPO₄), 10% (v/v) horse serum and SLAD (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, 50 μM ammonium sulfate, 2% Bacto Agar) media. The dimorphic switching was performed using the protocol described elsewhere [15]. Briefly, the culture was grown overnight at 30°C in YEPD broth before each study. The revived cell were harvested by centrifugation at 5000g rpm for 3 minutes and washed twice and incubated at 37°C for 6h with PBS to induce starvation. After incubation the cells were transferred to the required media for hyphal growth and hyphae were observed under microscope at magnification 40X.

**Results**

**Res acts as effective antifungal against *C. albicans***
Fig. 1

(a) Spot assay of C. albicans reference strains (ATCC 10261, ATCC 24433) in the absence (control) and presence of Res. (b) Broth microdilution assay to determine the MIC\textsubscript{80} of C. albicans reference strains (ATCC 10261, ATCC 24433) in presence of Res.

To study the antifungal effect of Res against C. albicans, we first of all performed the drug susceptibility testing by two independent methods viz. spot assay and minimum inhibitory concentration (MIC). Spot assay results (Fig 1a) revealed that Res was antifungal against C. albicans at 2.75mg/ml concentration. Broth microdilution assay also correlates with the above result and it was observed that the growth of C. albicans (ATCC10261 and ATCC24433) was inhibited in presence of Res at 2.75mg/ml (Fig 1b). The antifungal effect of Res was also assessed against five clinically isolated species of C. albicans by performing the above mentioned drug susceptibility testing methods. Both spot assays and broth microdilution estimating MIC\textsubscript{80} confirmed that similar to the laboratory reference strains, Res was also affecting the growth of all the clinically isolated species of C. albicans. The effective concentration at which Res was showing its antifungal property was variable and ranges from 2.75mg/ml to 11mg/ml (Fig 2).

Fig. 2

(a) Spot assay of C. albicans clinically isolated strains (D1, D2, D4, D7, D18) in the absence (control) and presence of Res. (b) Broth microdilution assay to determine the MIC\textsubscript{80} of C. albicans clinically isolated strains (D1, D2, D4, D7, D18) in presence of Res.
Res is antifungal against the non-\textit{albicans} species of \textit{Candida}

To further intricate our studies, we tested the antifungal effect of Res against four non-\textit{albicans} species of \textit{Candida} viz. \textit{Candida glabrata}, \textit{C. krusei}, \textit{C. parapsilosis} and \textit{C. tropicalis} by performing the similar drug susceptibility tests. We showed that Res not only inhibited the growth \textit{C. albicans} but also other \textit{Candida} species as well (Fig 3a). It was observed that MIC\textsubscript{80} of Res for all the non-\textit{albicans} species of \textit{Candida} lies in the range of 2.75mg/ml to 11mg/ml (Fig. 3).

Inhibitory effect of Res is not linked with multidrug efflux transporter activity

Over expression of the drug efflux pumps are one of the most predominant mechanisms of drug resistance attained by \textit{Candida} against the drugs administered to it [16, 17]. To study whether the antifungal mechanism of Res involves disruption of major drug efflux pumps activity, we performed R6G efflux assay. Our results in Fig. 4 showed that there was no significant difference (\textit{P} value > 0.05) in the extracellular concentration of R6G between the cells grown in presence or absence of Res. This suggests that the efflux of R6G remained unchanged irrespective of the presence of Res, hence the antifungal activity of Res is independent of the functioning of these efflux pumps.

Res inhibits serum and nitrogen starvation dependent dimorphism in \textit{C. albicans}

Yeast to hyphal switching in \textit{C. albicans} remains one of the major factors of virulence [18]. We scrutinized the effect of Res on this morphological switching by providing various hyphae inducing conditions at 37°C. We found that despite Res treated cells were able to express filaments in spider media, \textit{Candida} cells in presence of serum and SLAD media completely lacks filaments and appears only in yeast form in contrast to the untreated cells (Fig 5). The data suggests that Res targets specific signaling cascade governing morphogenetic switching for this dimorphic fungus.

Discussion

With ever increasing burden of MDR and lack of the effectiveness of current antifungal drug regimes, the crisis for discovery of new antifungal becomes immediate priority. The present study explored the possible antifungal activities of Res against human fungal pathogen, \textit{C. albicans}. Benzene-1,3-diol commonly known as resorcinol is used as a common component in dyes and also used for the treatment of acne and seborrheic dermatitis as already mentioned above. However, its antifungal activities have not yet been experimentally demonstrated. Hence, the present study focuses on elucidating the antifungal activity of Res against \textit{C. albicans} which is the fourth most leading cause of hospital acquired infections, as well as non-\textit{albicans} species of
Candida. We also explored that Res was able to inhibit the morphogenetic switching when grown in the presence of serum and SLAD but no effect of Res on this transition was observed when incubated and grown with the spider medium.

To assess the antifungal activity of Res, firstly we performed the two independent methods of drug susceptibility testings and found that Res was acting as an efficient antifungal against both the laboratory strains (ATCC10261 and ATCC24433) of C. albicans. To further confirm whether Res is efficient enough to show its antifungal behavior against the clinical isolates of C. albicans we again performed drug susceptibility against five strains isolated from different patients and found that Res was also effective against all five clinical isolates of C. albicans. Despite the fact that C. albicans is the predominant species among all the Candida infections, other species of Candida do contribute towards disease incidence up to considerable extent [19]. Therefore, to validate whether the antifungal action of Res is restricted to C. albicans only or effective on other non-albicans species of Candida, we performed drug susceptibility tests against four different species of Candida viz. C. krusei, C. parapsilosis, C. tropicalis and C. glabrata. Our results (Fig 3) clearly indicate that Res was an effective antifungal against non-albicans species of Candida also and has broad antifungal spectrum.

Next, we assessed whether growth inhibition due to Res could be the result of abrogated drug efflux pump activities. One of the most common mechanisms responsible for drug tolerance of Candida and several other fungi is the over expression of drug efflux pumps like ATP binding cassettes (ABC) and Major facilitator superfamily (MFS) transporters [20, 21]. Therefore we investigated the efflux of R6G which is a substrate for the above transporters and found that anticandidal activity of Res is not linked with the

Fig. 5

**Fig. 5. Effect of Res on Dimorphic Switching of C. albicans.** Hyphal morphogenesis in the liquid hyphal inducing media (YEPD with 10% Serum and Spider media) in the absence (control) and presence of Res (15mM) in the C. albicans (ATCC 10261) at 4 hours and hyphal morphogenesis in the solid hyphal inducing medium (Spider agar medium, 10% Serum and SLAD medium) in the absence (control) and presence of Res (15mM) in the C. albicans (ATCC 10261) at 4 hours.
disruption of efflux pumps activity as demonstrated in Fig 3. Thus, any contribution of efflux pump protein activity in enhancing the drug susceptibilities of Candida cells upon Res exposure was excluded by efflux assay which suggest that antifungal action of Res involves some other pathway which needs to be explored.

Finally, we assessed the effect of Res on yeast to hyphal transition which is another crucial attribute of C. albicans governing virulence. Our data revealed that Res is inhibiting the conversion of yeast to hyphal form in Serum and SLAD medium but not with spider medium (Fig 5). As a known fact, C. albicans morphology is dependent upon various environmental cues which stimulate independent signal transduction pathways, required to initiate hyphal growth [22]. For instance serum dependent transition is mediated through Ras1 which is an important regulator for hyphal development pathway while the filamentation phenotype through spider (carbon source as mannitol) and SLAD (nitrogen starved) media is mediated through separate pathways [23]. Hyphal morphogenesis being an integral part of the overall virulence strategy of C. albicans, inhibition of yeast to hyphal conversion under nitrogen starved condition and serum in presence of Res represents yet another unknown phenomenon that still requires further validation.

Despite the fact that Res displays efficient antifungal potential its safety concerns still remains an issue and needs to be tested for clinical trials. However, the in-vitro activity of Res in present study has provided sufficient evidence to establish its antifungal nature and therefore elucidating its mechanism of action could be an interesting area of research. Moreover, from pharmacological point of view, Res could also provide a lead structure based on which molecules having similar properties and even better activities could be synthesized that can be used for antifungal therapy.

Conclusion

Given the limited number of effective antifungal drugs and simultaneous emergence of MDR in C. albicans, the findings generated through this study clearly ascertain the antifungal nature of Res. Furthermore, Res could also hold promise to act as chemosensitizing agents that can be beneficial in reducing the dosages of current antifungal regimes.

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Conflict of interest

The authors declare that there is no conflict of interest.

References


