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Antifungal Effect of Curcumin on Morphogenesis and Secretion of Hydrolytic Enzymes in *Candida* species

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Abstract:

In the present study antifungal activity of curcumin was systematically explored against several *Candida* isolates including its effect on morphogenesis and other pathogenicity factors. The clear zone of inhibition across the disc containing curcumin showed antifungal activity which further evaluated by microdilution method. Curcumin showed average sensitivity index 3.12 mm/mg and minimum inhibitory concentration (MIC) varied from 250 to 550 µg/mlagainst different *Candida* isolates. Curcumin also found to be effective for inhibition of proteinase and phospholipase secretion in *C. albicans*. Proteinase secretion was decreased by 17.4% and 34.0% at MIC (×0.25) and MIC (×0.50) concentration respectively. Similar concentrations also reduced phospholipase secretion by 16.1% and 27.2%.One of the most crucial pathogenicity attribute of *Candida* is to undergo yeast to hyphal transition. Dimorphic transition at 300 min in the *C. albicans* was 23% and 63% at MIC (×0.25) and MIC (×0.50) of curcumin as compared to control 86% transition. The results of present study suggest that curcumin can significantly inhibit growth, proteinase, phospholipase secretion and hyphal transition in *C. albicans.*

Keywords: Antifungal, curcumin, *Candida*, proteinase, phospholipase, hyphal transition

Introduction:

Fungal infections are one of the major concerns for human civilization became a serious threat in term of morbidity and mortality in developing and underdeveloped country [1]. Consumption or inhalation of fungal spore leads to cause life threatening diseases. Among pathogenic fungus, *C. albicans* is common for humans. *Candida* considered being an opportunistic fungus and natural habitat of human flora can causes superficial infections and life-threatening systemic Candidiasis in immunocompromised hosts mainly in women [2]. The Virulence factors mainly depend on ability to adhere to host tissues, tissue damaging secreted enzymes (phospholipase and proteinase) and changes in morphological form inside the host [3-5]. However till today real virulence factors for *C. albicans* are not fully understood. A number of genes are responsible for virulence and to spread systematic disease in human. The proteins encoded by genes on the pathogenicity islands contain a number of gens which lead to express several toxic products. Among the sectary protein, aspartyl proteinases can degrade epithelial and mucosal barrier proteins such as collagen, complement and cytokines [5-7]. Moreover,*C. albicans* have ability to undergo morphogenetic transition are reported more virulent [8, 9]. Limited treatments are available against *C. albicans.* Antifungal drug are most effective treatment till to date but have side effect and costly. These limitations pave the path to explore and develop less toxicity natural products with effective antifungal activity. Various studies have been carried out employing various natural products, but have limitation in term of toxicity, effectiveness, availability and cost [10-12]. So, finding of alternative easily available common natural product to prevent diseases caused by *Candida* species is an important for health safety.

On this concern, Curcumin is a common spices produced by the rhizome of Curcuma longa is a yellow orange polyphone compound widely used a spice in Asian country [13]. Curcumin is the principle curcuminoids of popular Indian spice commonly known as turmeric used as regular ingredients of food and natural medicine with an average uptake 0.5 to 1.5 g/day/person. Moreover, the polyphenole curcumin is the active ingredient in several herbal and traditional medicines of China and India can suppress carcinogenesis of the skin, liver, lung, colon, stomach and breast [14,15]. Studies have reported that curcumin can inhibit adhesion of *Candida* species to buccal epithelial cells [13, 16]. Curcumin inhibits several eukaryotic P-type ATPases: Na^+/K^+-ATPase and Ca^+-ATPase [17]. Exclusive study on antifungal activity of curcumin is very limited. Therefore, in the present study antifungal activity of curcumin was investigated against clinical isolates of *Candida*including its effect on secretion of proteinase, phospholipase and hyphal transition.

Material and Methods:

Strains and media

Candida isolates were obtained from regional Sexually Transmitted Disease (STD) Centre, Safdarjung Hospital New Delhi, India and grown in Sabouraud dextrose agar (SDA) medium in addition with 1% chloramphenicol. Among the isolates, *C. albicans* species was identified using different tests such as germinative tube development, growth characteristic on chromogenic medium, fermentation and sugar assimilation (Himedia, India). Further, all isolates were cultivated on yeast extract, peptone and dextrose (YPD) medium (Himedia, India). Curcumin powder was purchased from Sigma (USA) and stock concentration was prepared in 1% dimethyl sulphoxide (DMSO) solution.

Disc diffusion assay

Candida isolates were inoculated into YEPD broth medium and incubated at 37ºC for 48 h. Then cells were pelleted by centrifugation at 3000 rpm for 5 min and washed three times with sterile Milli-Q water. Further, adjust the cells number (10^5cells/ml) and inoculated into molten agar medium at 40ºC and poured in sterile petriplates. The sterile filter disc (4 mm) were impregnated with test sample and placed on molten agar plates. 1% DMSO solution used as a control in each plate. The plates were incubated at 37ºC for 48 h and the zone of inhibition across the disc was measured at mm scale. Sensitivity Index (SI) was calculated for *Candida* isolates which is defined as

Zone of Inhibition (mm) /concentration of drug (mg/ml) = Clearing (mm/mg).

The minimum inhibitory concentration (MIC) of the test sample was determined against *Candida* isolates by microdilution method.

Proteinase assay

Candida strains were inoculated into 5 ml of YEPD broth medium containing different concentrations of curcumin and incubated at 37^0C for 18 h. The cells were pelleted by centrifugation at 3000 rpm for 5 min and washed two times with saline water. Proteinase assay was carried out as per previously described by [18]. Briefly, 1 µL of cells suspension placed in duplicate at equidistant points in proteinase agar medium containing BSA fraction V, 2g; yeast nitrogen base w/o amino acids; ammonium sulfate, 1.45g; glucose, 20g; distilled water added to 1000 ml respectively. The presence of proteinase was determined by the formation of a transparent halo around the colonies. The enzyme activity (precipitation zone, PZ) was measured by dividing the diameter of the colony by the diameter of the colony plus precipitation zone.

Phospholipase assay

The phospholipase activity of curcumin against *C. albicans* was determined by the method described by [19]Briefly, *C. albicans* were grown in YEPD medium and cells pelleted by centrifugation at 3000 rpm, for 5 min. The cells suspension (at index of 5 on the McFarland scale)was exposed to different concentrations of curcumin. Finally, 1 µl of cells suspension were placed on phospholipase agar media at equidistant points. Phospholipase agar media containing peptone, 10g; glucose, 30g; NaCl, 57.3g; $CaCl_2$ 0.55g; distilled water added to 1000 ml and 10% egg yolk enrichment 100 ml (Himedia). The plates were incubated at 37^0C for 2-4 days. The presence of phospholipase was determined by an opaque zone around the colonies and enzyme activity was measured by dividing the diameter of the colony by the diameter of the colony PZ.

Hyphal transition

C. albicans from agar slants were inoculated into amino acid rich Lee's simplified medium and incubated at 25^0C up to the late log phase of growth (5×10^8 cells/ml) [20]. Culture was further transferred into fresh medium and incubated up to 48 h (Stationary phase) for synchronous population cells at a final density of 2.5×10^8 cells/ml. Further, Stationary phase G_1 (1.5×10^9 cells) were transferred to fresh 300 ml media in a 500 ml Erlenmeyer flask and incubated 37^0C at pH 6.5 to induce hyphae formation. The desired concentrations of curcumin (MIC×0.25 and MIC×0.50) were introduced in to the culture flask, to see the effect on hyphal transition. The external pH of the culture flask was checked every half an hour and adjusted to the actual setting. The cells were taken from culture flask at different time intervals and their cell divergence was observed using microscopically (Motic AE31, Germany). The percentage of hyphae transition was obtained by ratio of the number of hyphae with total number of cells.

Results:

Disc diffusion Assay

Disc diffusion is one of the standard methods of measuring the antifungal activity of any compound on solid media. Curcumin diffuses from filter paper disc in agar and forms a ZOI of visible growth which is clearer near the disc and as the distance from disc increases zone of clearance is hazy. Discs impregnated with 1% DMSO, the solvent used for solubilizing curcumin powdershowed no ZOI. The concentration dependent ZOI observed up to 8 mg/ml against all tested isolates shown in Figure 1. Sensitivity index (S.I) of curcumin against all tested isolates represented in Table 1. Curcumin showed average clearing mm/mg of 4.43 mm/mg and 3.12 mm/mg for standard and clinical isolates respectively.

Minimum inhibition concentration

Minimum inhibition concentration (MIC) values of curcumin against all tested isolates given in Table 2. MIC values in the range 300-400 µg/ml was recorded for standard strains, whereas250-550 µg/ml for clinical sensitive and resistant isolates.

Proteinase assay

Proteinase assay was carried out with various concentration of curcumin and average PZs were recorded. Secretion of proteinase by the exposure of curcumin at MIC (×0.25) and MIC (×0.50) concentrations is shown in (Figure 2). Curcumin at MIC (×0.25) and MIC (×0.50) concentration showed average PZ of 0.53 and 0.67 ascompared to control 0.43 (Figure3). The average percentage inhibition of proteinase secretion by MIC (×0.25) and MIC (×0.50) of curcumin was 17.4% and 34.0% (Table 3). Significant difference in PZs was observed with respect to control.

Phospholipase assay

Figure4 depicts the phospholipase secretion of three different *Candida* isolates by the presence and absence of MIC (×0.25) and MIC (×0.50) concentrations of curcumin.Significant difference in opaque zone around the colony was observed with respect to control. The average (PZ) opaque zone in different strains of *C. albicans* was observed at MIC (×0.25) and MIC (×0.50) concentration of curcumin (Figure 5). Inhibition of phospholipase secretion by MIC (×0.25) and MIC (×0.50) of curcumin was 16.1% and 27.2% with respect to control (Table 4).

Hyphal transition

Hyphal transition of *C. albicans* ATCC 10261 in the absence and presence of curcumin at MIC(×0.25) and MIC(×0.50)concentration for different time interval (0-300 min) of microscopic fields showed in Figure 6. In case of control condition, cells showed hyphal induction after 60 minutes post treatment of Lee' s media at 37^0C. In the presence of curcumin (MIC×0.25), induction was delayed and seen at 120 minutes. Induction was further delayed at curcumin (MIC×0.50) and seen at 180 minutes. The effect of curcumin at MIC (×0.25) and MIC (×0.50) concentrations in the length and hyphal induction of *C. albicans* ATCC 1026 is summarized in Table 5. At control condition, percentage population of hyphal transition continued to increase with respect to time periods up to 300 min. But induction was significantly affected in case of curcumin treated cells. When treated with MIC(×0.25) concentration of curcumin, host cells did not show any induction up to 60 min and suppressed transition dramatically to 63% at 300 min. Induction of hyphal transition was started at 180 min when treated with MIC(×0.50) concentration of curcumin. Population of cells showing conversion at 300 minutes was reduced to 26% as compared to control. In control condition,

hyphal length was increased with respect to time periods and maximum length seen at 300 minutes was arbitrarily taken as 10 units. The effect of curcumin on hyphal length was grade on this scale. The hyphal length was significantly reduced and found to be 6.8 and 3.3 in the presence of curcumin at MIC(×0.25) and MIC (×0.50) concentration respectively.

Discussion:

Fungal diseases in humans have increased significantly with increase in population of immunosuppressed and debilitating patients. Medical implants prolong anticancer and antibiotic therapies further dispose toward fungal infections. Hospital acquired fungal infections now accounts for as much as 10 to 15% of such infections, with candidosis as fourth most common cause of hospital-acquired infections [21, 22]. Among all *Candida* species four most frequently isolated in human infections are *C. albicans, C. tropicalis, C. glabrata* and *C. parapsilosis*. *C. albicans* with frequency of about 50% is the most abundant and significant species [23]. Current lines of antifungals are Polyenes and Azoles present severe side effects and resistance to them is frequently observed by [24]. These limitations emphasize the need to explore and develop new and more effective antifungal agents with less toxicity. Natural products are attractive prototype for this purpose. Such as curcumin is the principle curcuminoids of popular Indian spice turmeric have antifungal activity [13, 25]. But limited studies explored their efficacy on *Candia* species. Therefore, in the present study we investigated the antifungal activity of curcumin against clinical *Candida* isolates.

Effectiveness of curcumin was evaluated on both solid and liquid media to check their antifungal property. This was evaluated by disc diffusion assay in which filter paper disc in impregnated with various concentrations of curcumin and put on agar plates. Mean sensitivity index of curcumin towards standard and clinical isolates was 4.43 mm/mg and 3.12 mm/mg respectively. The MIC values for curcumin are generally higher against all tested isolates, as previously reported by Martins et al., 2009 [13]. Curcumin was found to be slightly less effective against clinical isolates than standard strains. This can be attributed to robust nature of clinical isolates as they have been receiving exposure of curcumin in food of patients.*C. albicans* have remarkable ability to adhere, penetrate and invade the host cells which is aided by hydrolytic enzymes. Secretion of hydrolytic enzymes was tested on solid media and result assessed by quantitating PZ value. More the PZ value more is the percentage decrease in proteinase and phospholipase secretion. Our results have good agreements with previous studies carried out with curcumin [18].

Secretion of proteinase and phospholipase reported to be affected by functioning of membrane ATPase and membrane integrity [26, 27]. In the present study inhibition of proteinase and phospholipase secretion was almost equal magnitude indicates that it does not inhibit

synthesis of these pathogenicity markers. But preventing their secretion may be due to suppression of PM-ATPase activity or alteration of membrane integrity or bringing about changes in ROS [28-30]. Mode of antifungal activity of curcumin and its effect on pathogenicity marker of *Candida* has not been fully understood. Curcumin also induces reactive Oxygen species and has been reported to suppress dimorphism in *C. albicans* [29].

One of the crucial pathogenicity attribute of *Candida* is to undergo yeast to hyphal transition which considered being more pathogenic [31]. Morphogenesis in *Candida* is associated with functioning of PM-ATPase and pHi [32]. Under control conditions, induction of hyphae was seen at 60 minutes. Dimorphic transition kept on increasing till 300 minutes where 86% transition was observed. At MIC (×0.25) and MIC (×0.50) concentration curcumin suppressed transition by 23% and 63%, respectively. These results are very encouraging as yeast to hyphal transition is very important pathogenicity attribute of *C. albicans*. Inhibition of hyphal transition by curcumin has been also reported by [29].

Conclusion:

In conclusion, antifungal activities of curcumin have been investigated against several standard and clinical *Candida* isolates. It is found to be effective both in solid and liquid media with MIC_{90} in the range of 250-550 µg/ml against all tested strains. Important pathogenicity attributes of proteinases, phospholipase secretion and yeast to hyphal transitions were significantly suppressed by even sub-MIC of curcumin. Above finding taken together with its low toxicity make curcumin a potential antifungal of clinical interest.

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Figure 1

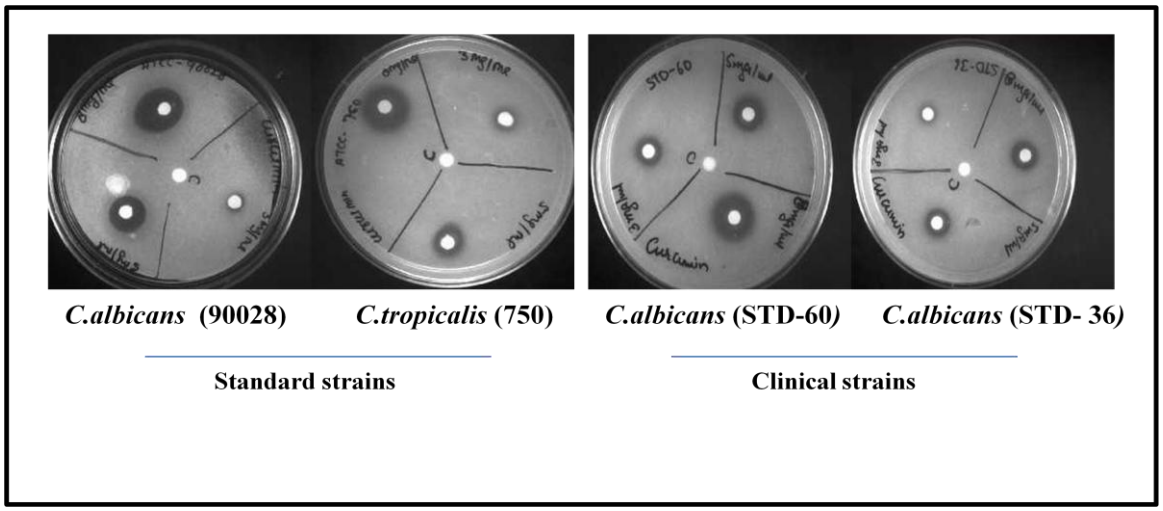

Figure1. Plates showing clear zone of inhibition on agar plates against different *Candida* isolates.

Figure 2

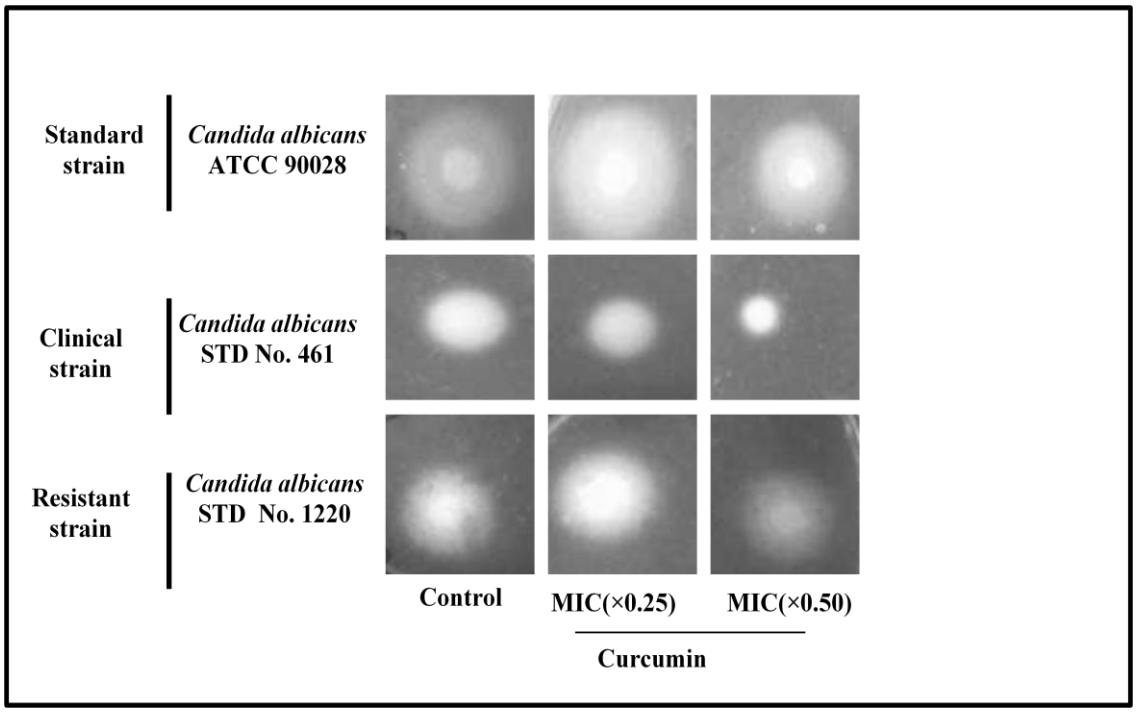

Figure 2. Photographs showing proteinase secretion by different isolates of C. *albicans* at presence of MIC(× 0.25) and MIC(× 0.50) concentration of curcumin.

Figure 3

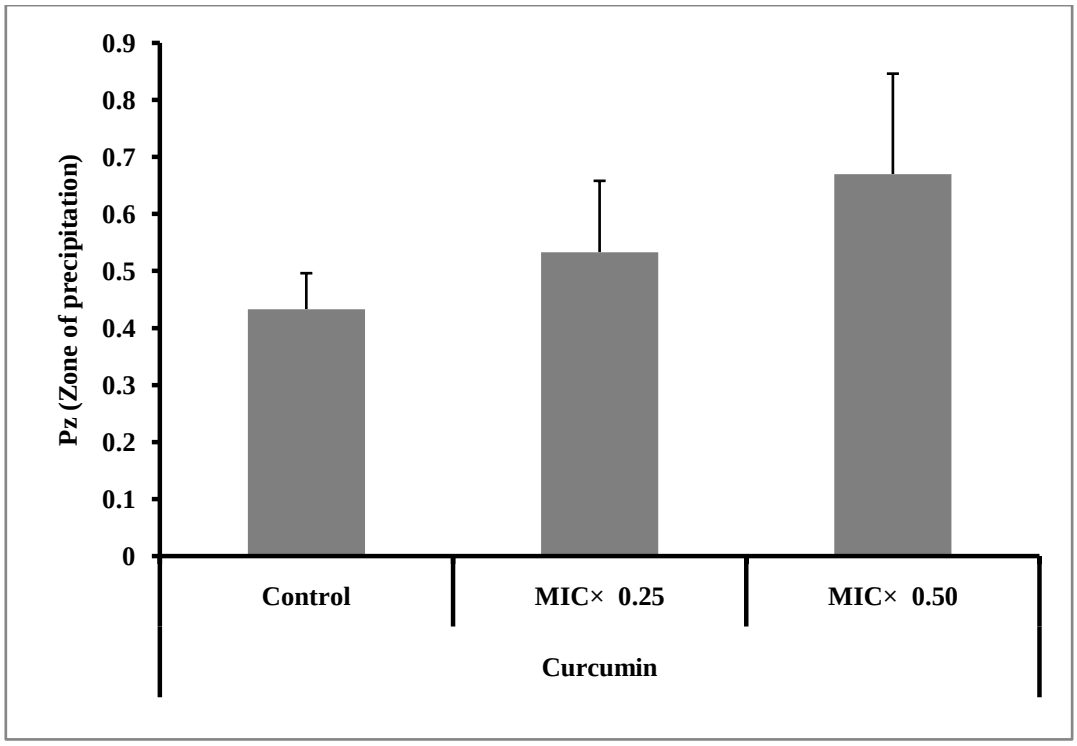

Figure 3. Average precipitation zone by different *Candida* strains in the presence at MIC (× 0.25) and MIC (× 0.50) of curcumin.

Figure 4

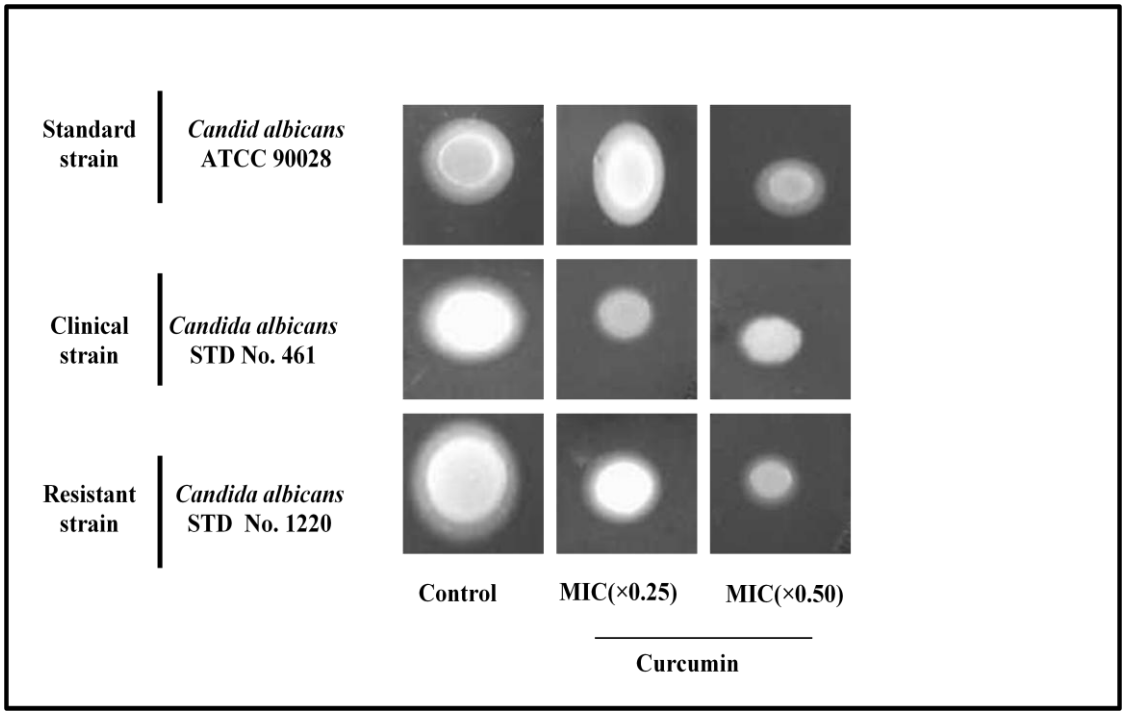

Figure 4. Photographs showing phospholipase secretion by different isolates of *C. albicans* at presence of MIC(× 0.25) and MIC(× 0.50) concentration of curcumin.

Figure 5

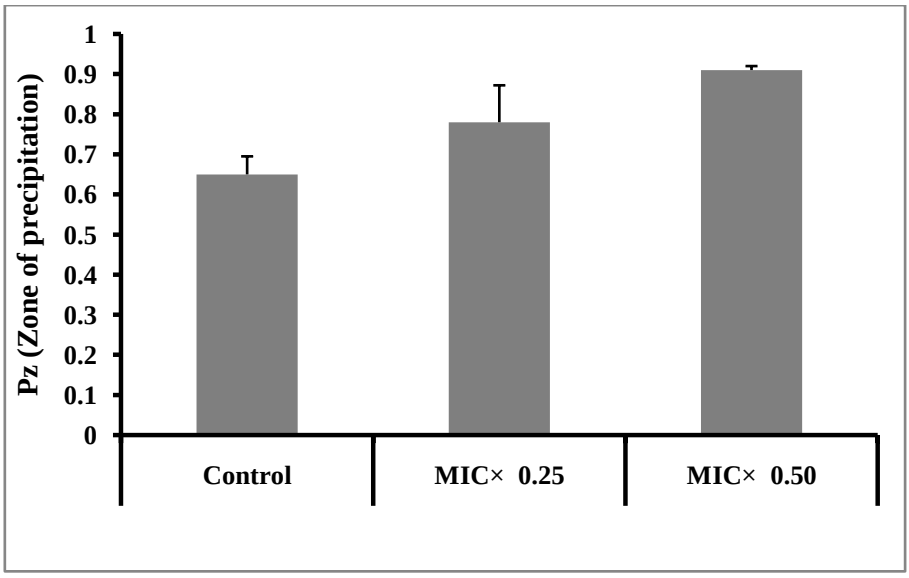

Figure 5. Average phospholipase secretion by three *Candida* strains in the presence at MIC (×0.25) and MIC (×0.50) of curcumin.

Figure 6

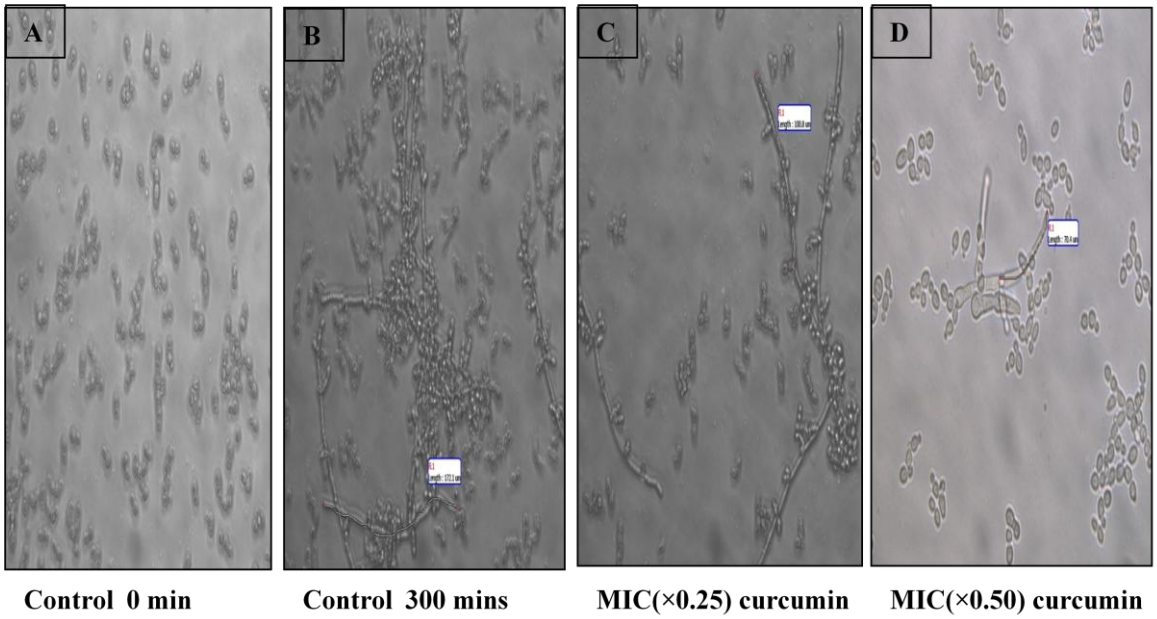

Figure 6. Representative microscopic fields showing hyphal transition of *C.albicans* ATCC 10261 in absence (A and B) and presence of curcumin (C and D) at different time intervals. L1 represents the length of hypha.

Table 1

Table 1. Sensitivity index (SI) for curcumin against different *Candida* isolates.

Sensitivity Index (clearing mm/mg)		
Type of strains	**Standard sensitive (n=2)**	**Clinical-sensitive (n=2)**
Curcumin	4.43	3.12

Table 2

Table 2. Minimum inhibitory concentration (MIC) of curcumin against different *Candida* isolates.

Strains	**Species**	**Curcumin MIC range (µg/ml)**
Standard (n=3)	*C. albicans* ATCC 90028, *C. tropicals* ATCC 750, *C. kruesi* ATCC 6258,	300-400
Clinical Sensitive (n=11)	*C. albicans* STD No 010, *C albicans* STD No 1220 , *C. albicans*STD No 06, *C.albicans* STD No 05, *C. albicans* STD No 9, *C. albicans*STD No 02, *C. glabrata* STD No 90, *C. glabrata* STD No 96, *C. tropicals* STD 36, *C. krusei* STD No 116, *C. krusei* STD No 132,	250- 550
Clinical Resistant (n=6)	*C. albicans*STD No 1128, *C.glabrata* STD No 64, *C. tropicalis*STD No 1118, *C. guilliermondii* STD No 1685, *C. krusei* STD No 1413,	250-500

Table 3

Table 3. Average percentage decrease in proteinase secretion of three different *Candida* strains at sub-MIC concentrations of curcumin.

	Concentration	**Average %age decrease**
Curcumin	Control	0
	MIC(×0.25)	17.4
	MIC(×0.50)	34.0

Table 4

Table 4. Average percentage decrease in phospholipase production in different *Candida* strains at sub-MIC concentrations of curcumin.

	Concentration	**Average %age decrease**
Curcumin	Control	0
	MIC(×0.25)	16.1
	MIC(×0.50)	27.2

Table 5

Table 5. Hyphal transition in *C. albicans* ATCC 10261 when treated with curcumin.

Time interval →			**0**	**60**	**120**	**180**	**240**	**300**
(minutes)								
Control		I*	-	2.2	3.9	5.9	8.6	10
		I%	-	1.6 ± 0.1	12.6 ± 0.2	22.6 ± 1.1	61.1 ± 3.0	86 ± 4.0
Curcumin	MIC (× 0.25)	I	-	-	3.1	5.1	6.2	6.8
		I%	-	-	0.5 ± 0.6	30 ± 1.6	5.1 ± 1.8	63 ± 1.2
	MIC (× 0.50)	I	-	-	-	1.9	2.5	3.3
		I%	-	-	-	12 ± 0.8	19 ± 0.6	26 ± 1.7

I*: Mean length of hyphae on the arbitraryscale 10 and I%: refers to the percentage of cells showing hyphal growth.