**Scholars Journal of Applied Medical Sciences**
Abbreviated Key Title: Sch J App Med Sci
ISSN 2347-954X (Print) | ISSN 2320-6691 (Online)
Journal homepage: https://saspublishers.com/sjams/

OPEN ACCESS

**Botany and Zoology**

# Evaluation of Extracellular Proteinase and Phospholipase Activities of Indian Clinical Strains of *Candida Albicans* and Non-*Albicans* and Their Correlation with the Source of Isolation and Minimum Inhibitory Concentration Values of Antifungal Drugs

Shesh Rao Nawange[1,2,3*], Ruchika Yadu[1, 2], Shankar Mohan Singh[2,3], Ruchi Sethi Gutch[2,3], Richa Gumasta[2,3], Mahendra Nawange[4] and Arvind Kavishwar[5]

[1]Department of Botany and Zoology, NSCB Government Girls College, Seoni, Madhya Pradesh, India
[2]Centre for Medical Mycology, Fungal Disease Diagnostic and Research Center, Society for Research, Diagnosis, and Treatment of Human Fungal Diseases, Jabalpur–482002, Madhya Pradesh, India
[3]Medical Mycology Research Laboratory, Department of Biological Sciences, Rani Durgavati University, Jabalpur–482001, Madhya Pradesh, India
[4]Department of Microbiology, J.H. P.G. College, Betul, Madhya Pradesh, India
[5]National Institute for Research in Tribal Health (NIRTH), Jabalpur–482003, Madhya Pradesh, India

**DOI:** 10.36347/sjams.2020.v08i02.061 | **Received:** 06.02.2020 | **Accepted:** 14.02.2020 | **Published:** 28.02.2020

***Corresponding author:** Shesh Rao Nawange

**Abstract** **Original Research Article**

The present study was aimed to evaluate some of the virulence traits, *viz*., extracellular proteinase and phospholipase activities, of *Candida albicans* (n=130) and non-*albicans Candida* (n=60), such as *C. tropicalis, C. parapsilosis, C. glabrata, C. guilliermondii*, and *C. krusei*. The isolates of *Candida* species that were investigated in the current work were obtained from diverse clinical sources in Jabalpur, Madhya Pradesh, India. The correlation between the clinical sources of isolation and minimum inhibitory concentration of antifungal drugs was also determined. A screening for the production of extracellular proteinase and phospholipase enzymes was done using the Yeast Carbon Base–Bovine Serum Albumin medium and the Egg Yolk Plate method, respectively. The Minimal Inhibitory Concentration against the tested antifungal drugs was determined by the M–27A CLSI/NCCLS macrodilution method. Of the 190 *Candida* isolates, 150 (80%) were positive for extracellular proteinase and 141 (74.2%) for phospholipase secretion. A non-significant difference was observed for extracellular proteinase and phospholipase activities among *C. albicans* and non-*albicans* as determined by ANOVA ($p > 0.05$). The comparison of individual extracellular proteinase and phospholipase activities among the sources studied also demonstrated non-significant difference and almost comparable results using Dunnett's t-test and Tukey's HSD Post Hoc test for the secretion of both the enzymes. A significant positive correlation between enzyme secretion and MIC of antifungal was demonstrated ($p < 0.05$), which suggested some role of extracellular enzymes among the *Candida* spp. in increasing the resistance against commonly used antifungal drugs.

**Keywords**: *Candida albicans*, non-*albicans*, proteinase, phospholipase, virulence factors, minimum inhibitory concentration.



## INTRODUCTION

*Candida albicans* and non-albicans *Candida* species usually thrive as normal commensal flora in the human oral mucosa [1]. However, in immune compromised patients, the fungi can become opportunistic pathogens of the oral mucosa [2]. Nawange *et al*., previously reported a naturally acquired disseminated dual infection that was caused by *C. famata* and *C. catenulata* in a group of albino rats [3]. The transformation from a harmless to a virulent pathogen under the conditions of a dysfunctional host defense system can be attributed to an extensive repertoire of selectively expressed virulence determinants, including the ability of the yeast to produce extracellular hydrolytic enzymes, especially, extracellular proteinases and phospholipases that play important roles in adherence, penetration, and subsequent invasion of the infected tissues [3-6]. Hydrolytic enzymes, such as extracellular proteinases and phospholipases, have been regarded as the major determinants of pathogenicity of *C. albicans* [7-10].

However, only little is known about the pathogenicity of non-*albicans Candida* species.

The present study was conducted to determine the virulence traits, *viz*., extracellular proteinase and phospholipase enzyme activities, of *C. albicans* and non-*albicans Candida* species that were isolated from the patients of Jabalpur, Madhya Pradesh, India. In addition, minimum inhibitory concentration (MIC) values of antifungal drugs were also determined and correlated with the enzyme activities and the source of the yeast isolation.

## MATERIALS AND METHODS

One hundred and ninety *Candida* isolates that were obtained from cancer, vulvovaginal candidiasis, and maxillary sinusitis patients (all maintained in our laboratory stock collection) were included in this study. The sources of isolation are presented in the Table-1. Species distribution of the isolates was as follows: *C. albicans* (n=130), *C. tropicalis* (n=20), *C. parapsilosis* (n=21), *C. guilliermondii* (n=9), *C. glabrata* (n=5) and *C. krusei* (n=5). All the isolates were revived from the respective stock cultures by plating on fresh Sabouraud's Dextrose Agar plates followed by an incubation at 37 °C up to 48 h.

### Determination of Phospholipase Activity

The phospholipase production by the *Candida* isolates was assessed according to the egg yolk agar plate method as previously described by Price *et al*., [11]. Sabouraud Dextrose Agar plates containing 1 M sodium chloride, 0.005 M calcium chloride and 8% sterile egg yolk emulsion were used. Each strain was spot inoculated (approx 6 mm) in triplicate. The Petri-dishes were incubated at 37 ºC and post 5 days of the inoculation, the diameters of the colonies and the colony-plus precipitation zones were measured. Three separate samples of each strain were used to determine the average phospholipase activity (Pz). Pz value represents the ratio of colony diameter to the sum of colony diameter and precipitation zone. The 190 *Candida* isolates, according to the values of their Pz coefficient, were grouped into five classes: (i) Pz ≥ 1 (−), negative; (ii) Pz 0.90–0.99 (+), very high Pz group; (iii) 0.80–0.89 (++), high Pz group; (iv) 0.70–0.79 (+++), low Pz group; and (v) Pz minor ≤ 0.69 (++++) group. According to this system, a low Pz ratio corresponds to a high phospholipase activity. The strains exhibiting (−) and (+) Pz values were considered as negative in our study. The reference strain, *C. albicans* MTCC/ATCC 10231, served as the positive control while the *C. glabrata* IHEM 22129 strain from our laboratory stock collection served as the negative control.

### Determination of Extracellular Proteinaseactivity

The production of extracellular proteinase enzyme was determined according to Aoki *et al.*, [12]. All the isolates were analyzed on a solid medium containing bovine serum albumin (BSA) for their ability to secrete extracellular proteinase. The medium (Hi Media Laboratories, Mumbai) containing 11.7% of yeast carbon base (YCB), 0.1% of BSA and 0.01% of polypeptone (pH adjusted to 5.0) was sterilized by filtration through a membrane filter of 0.2 µm pore size. For a preparation of 200 mL of the medium, 20 mL of YCB solution was added to the mixture. Of the final medium, 20 mL was poured into Petri-dishes. The medium was referred to as the YCB-BSA medium. Each isolate was a spot (6 mm) inoculated in triplicate and incubated at 37 ºC for 5 days. The plates were fixed with 10% trichloroacetic acid (TCA) for 2 h followed by staining with Coomassie Brilliant Blue G–250. The extracellular proteinase production was recorded as the appearance of clearance zones around the colonies. The diameters of the transparent zones around the colonies were considered as a measure of the proteinase production. The scoring of the enzyme activity was done based on the proteinase zone (Pz) value as for the phospholipase activity. The reference strain, *C. albicans* MTCC/ATCC10231, served as the positive control, while *C. parapsilosis* IHEM 22140 from our laboratory stock collection served as the negative control.

### *Invitro* Drug Sensitivity Testing

For *in vitro* testing of *Candida* isolates against antifungal drugs, the macrodilution method was performed, according to the recommendations of CLSI/NCCLS (formerly National Committee for Clinical Laboratory Standards) M27-A [13], using RPMI 1640 medium in MOPS buffered to pH 7.1. A two-fold drug dilution scheme was adopted with the concentration range of 0.03125–64 µg/mL. The yeast inoculum was adjusted to 0.5 McFarland standard at 530 nm. This procedure yielded a yeast stock suspension of $1 \times 10^6$ to $5 \times 10^6$ cells per milliliter. A working suspension was prepared by 1:100 dilution followed by 1:20 dilution of the stock suspension with RPMI 1640 broth medium that generally results in $0.5 \times 10^3$ to $2.5 \times 10^3$ cells per milliliter. The 10X drug dilutions were dispensed in 0.1 volumes into sterile glass bottles. Each bottle was then inoculated with 0.9 mL volumes of the diluted yeast inoculum suspensions. All the bottles were incubated at 35 °C and MICs were read after 24 and 48 h of incubation, as per the CLSI (NCCLS) M27-A protocol. The end point was determined at 80% inhibition in contrast to growth in the control bottles.

### Drugs

Five antifungal drugs were used in the assays: (a) amphotericin B (AMFOCAN, Dabur India Ltd.), (b) fluconazole (Flustan™, Dr. Reddy's Lab. Ltd.; trademark under registration), (c) ketoconazole (NIZRAL, Johnson and Johnson), (d) itraconazole (Candistat, E Merck India Ltd, licensed user of T.M.), and (e) terbinafine (DASKIL), Novartis India Ltd. As per the CLSI/NCCLS guidelines M27-A, thefollowing strains were tested in every batch to ensure the quality

control: *C. albicans* MTCC/ATCC 90028, *C. tropicalis* MTCC/ATCC 750, *C. glabrata* MTCC/ATCC 90030.

## STATISTICAL ANALYSIS

The experimental data were analyzed using Pearson's correlation coefficient, ANOVA, Dunnett's t-test, Tukey's HSD Post hoc test and the Kruskal-Wallis one-way analysis of variance by ranks. A p-value of $< 0.05$ was considered significant.

## RESULTS

A total of 130 *C. albicans* isolates and 60 non-*albicans Candida* species, isolated from various clinical sources (Table-1), were tested for the extracellular proteinase and phospholipase activities (Fig 1A and B). Table-2 exhibits the frequency of extracellular proteinase and phospholipase producers in *Candida albicans* and non-*albicans Candida* with respect to their source. The raw data for *C. albicans* and non-*albicans* isolates were compared followed by the analysis of sources of isolation of the two individual yeast categories. ANOVA test was applied to extracellular proteinase and phospholipase activities, while the individual comparisons were analyzed by Dunnett's t-test and Tukey's HSD Post Hoc tests. The analysis of Pz values for extracellular proteinase showed that in both the groups, no significant changes were seen for *C. albicans* ($F=0.780$; $p > 0.05$) and non-*albicans* ($F=0.939$; $p > 0.05$). A comparison among the yeast sources also demonstrated non-significant differences and exhibited almost comparable results in Dunnett's t-test and Tukey's HSD Post Hoc analysis. The comparison of the phospholipase activities by ANOVA showed no significant difference for *C. albicans* ($F=0.787$; $p > 0.05$) and non-*albicans* ($F=1.521$; $p > 0.05$). The comparison between two sources showed the non-significant difference ($p > 0.05$) by Dunnett's t-test and Tukey's HSD Post Hoc analysis.

Table-3 shows the resistant strains of *Candida* spp. against amphotericin B, fluconazole, ketoconazole, itraconazole, and terbinafine. We observed that the *C. albicans* isolates were resistant to ketoconazole (n=128) and itraconazole (n=130) only at a wide Pz range of 0.51–1.4 and 0.54–1.3 against extracellular proteinase and phospholipase, respectively. *C. tropicalis* isolates were resistant to ketoconazole (n=16) only with a wide Pz range of 0.60–1.4 and 0.55–1.4 against extracellular proteinase and phospholipase, respectively. Similarly, *C. parapsilosis* isolates were resistant to ketoconazole (n=20) and itraconazole (n=10) only with a wide Pz range of 0.66–1.3 and 0.65–1.3 for extracellular proteinase and phospholipase, respectively.However, *C. guilliermondii* isolates were resistant to fluconazole (n=1), ketoconazole (n=9), and itraconazole with a wide Pz range of 0.71–0.97 and 0.76–1.4 against extracellular proteinase and phospholipase, respectively. Similarly, *C. glabrata* and *C. krusei*were also found to be resistant to fluconazole, ketoconazole, and itraconazole. *C. glabrata* isolates demonstrated resistance to fluconazole (n=1), ketoconazole (n=3), and itraconazole (n=3) with a broad Pz range of 0.66–1.3 and 0.72–0.98 against extracellular proteinase and phospholipase, respectively. *C. krusei*, on the other hand, showed resistance against fluconazole (n=5), ketoconazole (n=5) and itraconazole (n=2) with a wide Pz range of 0.64–1.2 and 0.76–1.1 against extracellular proteinase and phospholipase, respectively.

Table-4 summarizes the correlation between the enzyme secretion and antifungal drugs as calculated by Pearson's coefficient of correlation. The enzyme secretion as determined by the Pz values was compared to antifungal drug resistance using the MIC values obtained for different drugs studied herein. The values were considered significant at $p \leq 0.05$. A negative correlation was observed between the Pz values for *C. albicans*, *C.tropicalis*, *C. parapsilosis* and the MIC values for the drugs used in the present study. Further, among *C. guilliermondii* and *C. glabrata* isolates, a negative correlation was recorded between the Pz values and MIC values of amphotericin B. A negative correlation was also observed between the Pz values of phospholipase secretion and the MIC values of itraconazole in *C. guilliermondii* isolates. The Pz values of proteinase secretion and MIC values of itraconazole among the *C. krusei* isolates also demonstrated a negative correlation. However, *Candida* spp. showed a positive correlation between their extracellular proteinase activities and their sensitivities to the antifungal drugs, *viz*., fluconazole, ketoconazole, itraconazole, and terbinafine. An overall negative correlation between the Pz and the MIC values in our study indicate a positive correlation between the enzyme activity (as enzyme activity is inversely proportional to Pz values) and the MIC values. Such a correlation suggests that the secretion of these enzymes plays a major role in enhancing the resistance of *Candida* strains to the antifungal drugs studied in the current research work.

Table-5 enlists the frequencies of extracellular proteinase and phospholipase production by the various *Candida* isolates investigated in the present work. The Kruskal-Wallis one-way analysis of variance by ranks was used for statistical analysis of the data that displayed a statistically significant difference between the six different *Candida* spp. studied for extracellular proteinase and phospholipase activity ($p = 0.0008$ and $p < 0.0001$). The extracellular proteinase production was observed in 89.23% of *C. albicans* isolates and 60% of non-*albicans Candida*. isolates. Similarly, phospholipase production was found to be 86.92% in *C. albicans* isolates, whereas only 46.6% in non-*albicans Candida* species. Of the 190 *Candida* isolates from different clinical sources, 152 samples (80%) were positive for extracellular proteinase secretion, while 141 samples (74.2%) were positive for the production of phospholipase.

**Table-1: The source of isolation and the number of isolates obtained from the source**

| *Candida* spp. | Blood | Urine | Sputum | CSF | VVC | Maxillary sinus | Oral | Total |
|---|---|---|---|---|---|---|---|---|
| ***C. albicans*** | 40 | 30 | 5 | 30 | 10 | 10 | 5 | 130 |
| ***C. tropicalis*** | 4 | 2 | 2 | 5 | 4 | 2 | 1 | 20 |
| ***C. parapsilosis*** | 7 | 2 | 2 | 6 | 2 | 1 | 1 | 21 |
| ***C. guilliermondii*** | 2 | 2 | 1 | 0 | 2 | 0 | 2 | 9 |
| ***C. glabrata*** | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 5 |
| ***C. krusei*** | 2 | 1 | 1 | 0 | 0 | 1 | 0 | 5 |

**CSF=Cerebrospinal fluid; VVC=Vulvovaginal candidiasis; Oral=Buccal cavity swabs**

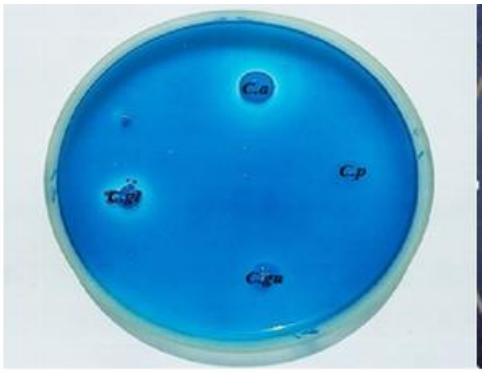

Figure A: Extracellular proteinase activity seen by formation of proteolytic zones around the *Candida* colonies on YCB BSA Agar plate. *C.a: C. albicans* (isolated from MS; Pz (++++):0.66; *C.gl: C. glabrata* (isolated from blood; Pz (+++):0.77; *C.gu: C. guilliermondi* (isolated from VVC; Pz(+):0.95); *C.p: C. parapsilosis* (isolated from sputum; Pz (-):1.1).

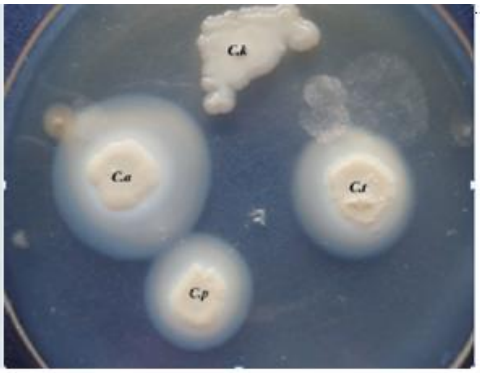

Figure B: Phospholipase activity of different *Candida* isolates on Egg yolk plate on 5th day of incubation. *C.a: C.albicans* (isolated from blood; Pz (++++): 0.25); *C.t: C.tropicalis* (isolated from sputum; Pz (+++): 0.76); *C.p: C. parapsilosis* (isolated from CSF; Pz (++):0.89; *C.k: C. krusei* (isolated from blood; Pz (-):1.1)

**Table-2: The frequency of extracellular proteinase and phospholipase activities *Candida albicans* isolates and non-*albicans Candida* isolates from different clinical sources**

| Source | Number of isolates | *C. albicans* | | *Candida*non *albicans* | | |
|---|---|---|---|---|---|---|
| | n | Proteinase | Phospholipase | n | Proteinase | Phospholipe |
| **Blood** | 40 | 36 (90%) | 35 (87.5%) | 16 | 11 (68.75%) | 10 (62.5%) |
| **Urine** | 30 | 22 (73.33%) | 21 (70%) | 8 | 5 (62.5%) | 4 (50%) |
| **Sputum** | 5 | 4 (80%) | 5 (100%) | 7 | 5 (71.43%) | 5 (71.43%) |
| **CSF** | 30 | 29 (96.67%) | 29 (96.67%) | 11 | 5 (45.45%) | 6 (54.54%) |
| **VVC** | 10 | 9 (90%) | 8 (80%) | 9 | 4 (44.44%) | 4 (44.44%) |
| **Maxillary sinus** | 10 | 9 (90%) | 9 (90%) | 5 | 3 (60%) | 2 (40%) |
| **Oral** | 5 | 4 (80%) | 4 (80%) | 4 | 4 (100%) | 2 (50%) |

**CSF= Cerebro spinal fluid; VVC= Vulvovaginal swabs; Oral= Buccal cavity swabs; n=number**

**Table-3: The distribution of resistant *Candida* strains against different antifungals used, Pz range, Average Pz value, and standard deviation for proteinase and phospholipase enzymes studied**

| *Candida* spp. | n | Proteinase | | Phospholipase | | Resistant strains | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Pz Range | AvgPz ±SD | Pz Range | AvgPz ±SD | Amp B | Flu | Keto | Itra | Terb |
| *C. albicans* | 130 | 0.51–1.4 | 0.74 ±0.161 | 0.54–1.3 | 0.75 ±0.162 | 0 | 0 | 128 | 130 | 0 |
| *C. tropicalis* | 20 | 0.60–1.4 | 0.87 ±0.255 | 0.55–1.4 | 0.91 ±0.245 | 0 | 0 | 16 | 0 | 0 |
| *C. parapsilosis* | 21 | 0.66–1.3 | 0.88 ±0.187 | 0.65–1.3 | 0.87 ±0.180 | 0 | 0 | 20 | 10 | 0 |
| *C. guilliermondii* | 9 | 0.71–0.97 | 0.80 ±0.1 | 0.76–1.4 | 0.96 ±0.207 | 0 | 1 | 9 | 7 | 0 |
| *C. glabrata* | 5 | 0.66–1.3 | 0.91 ±0.275 | 0.72–0.98 | 0.84–0.099 | 0 | 1 | 3 | 0 | 0 |
| *C. krusei* | 5 | 0.64–1.2 | 0.88 ±0.209 | 0.76–1.1 | 0.91 ±0.148 | 0 | 5 | 5 | 2 | 0 |

**Amp B= amphotericin B; Flu=fluconazole; Keto=ketoconazole; Itra=itraconazole; Terb=terbinafine**
**Pz=Zone of precipitation, SD= Standard deviation**

**Table-4: The correlation between the Pz values and MIC values of amphotericin B, fluconazole, ketoconazole, itraconazole, and terbinafine using bivariate correlation analysis**

| nzyme | | | | Flu | Keto | Itra | Terb |
|---|---|---|---|---|---|---|---|
| ***C. albicans*** | **Proteinase** | Pearson's Correlation | -.047 | -.188* | -.449** | -.477** | -.565** |
| | | Sig. (2-tailed) | .599 | .032 | .000 | .000 | .000 |
| | | N | 130 | 130 | 130 | 130 | 130 |
| | **Phospholipase** | Pearson's Correlation | -.116 | -.213* | -.512** | -.327** | -.498** |
| | | Sig. (2-tailed) | .190 | .015 | .000 | .000 | .000 |
| | | N | 130 | 130 | 130 | 130 | 130 |
| ***C. tropicalis*** | **Proteinase** | Pearson's Correlation | -.561* | c | -.497* | -.045 | -.611** |
| | | Sig. (2-tailed) | .010 | | .026 | .851 | .004 |
| | | N | 20 | 20 | 20 | 20 | 20 |
| | **Phospholipase** | Pearson's Correlation | -.540* | .c | -.425 | -.058 | -.352 |
| | | Sig. (2-tailed) | .014 | | .062 | .807 | .128 |
| | | N | 20 | 20 | 20 | 20 | 20 |
| ***C. parapsilosis*** | **Proteinase** | Pearson Correlation | -.269 | -.561** | -.475* | -.595** | -.828** |
| | | Sig. (2-tailed) | .238 | .008 | .030 | .004 | .000 |
| | | N | 21 | 21 | 21 | 21 | 21 |
| | **Phospholipase** | Pearson's Correlation | -.359 | -.096 | -.296 | -.222 | -.625** |
| | | Sig. (2-tailed) | .111 | .678 | .192 | .332 | .002 |
| | | N | 21 | 21 | 21 | 21 | 21 |
| ***C. guilliermondii*** | **Proteinase** | Pearson's Correlation | -.008 | .207 | .069 | .065 | .093 |
| | | Sig. (2-tailed) | .983 | .593 | .860 | .869 | .812 |
| | | N | 9 | 9 | 9 | 9 | 9 |
| | **Phospholipase** | Pearson's Correlation | -.619 | .092 | .380 | -.195 | .153 |
| | | Sig. (2-tailed) | .075 | .813 | .313 | .616 | .695 |
| | | N | 9 | 9 | 9 | 9 | 9 |
| ***C. glabrata*** | **Proteinase** | Pearson's Correlation | -.076 | .571 | .955* | .954* | .524 |
| | | Sig. (2-tailed) | .903 | .314 | .011 | .012 | .365 |
| | | N | 5 | 5 | 5 | 5 | 5 |
| | **Phospholipase** | Pearson's Correlation | -.848 | .618 | .307 | .259 | .682 |
| | | Sig. (2-tailed) | .070 | .267 | .615 | .674 | .205 |
| | | N | 5 | 5 | 5 | 5 | 5 |
| ***C. krusei*** | **Proteinase** | Pearson's Correlation | .c | .c | .c | -.172 | .776 |
| | | Sig. (2-tailed) | | | | .783 | .123 |
| | | N | 5 | 5 | 5 | 5 | 5 |
| | **Phospholipase** | Pearson's Correlation | .c | .c | .c | .027 | .829 |
| | | Sig. (2-tailed) | | | | .966 | .083 |
| | | N | 5 | 5 | 5 | 5 | 5 |
| **. Correlation is significant at the 0.01 level (2-tailed). | | | | | | | |
| *. Correlation is significant at the 0.05 level (2-tailed). | | | | | | | |
| c: Not Calculated | | | | | | | |

**Amp B= amphotericin B; Flu=fluconazole; Keto=ketoconazole; Itra=itraconazole; Terb=terbinafine**
**A negative value indicates negative correlation**

**Table-5: The frequency of extracellular proteinase and phospholipase activity of various *Candida* spp. isolated from clinical samples**

| *Candida* spp. | Number of isolates | Proteinase positive | Phospholipase positive |
|---|---|---|---|
| ***C. albicans*** | 130 | 116 (89.23%) | 113 (86.92%) |
| ***C. tropicalis*** | 20 | 11 (55%) | 7 (35%) |
| ***C. parapsilosis*** | 21 | 13 (61.90%) | 12 (57.14%) |
| ***C. guilliermondii*** | 9 | 6 (66.67%) | 5 (55.56%) |
| ***C. glabrata*** | 5 | 3 (60%) | 2 (40%) |
| ***C. krusei*** | 5 | 3 (60%) | 2 (40%) |

## DISCUSSION

The extracellular hydrolases, like, proteinases and phospholipases, are major virulent factors of host tissue invasion. Mane *et al*., reported that out of 39 *C. albicans* isolates, which were obtained from HIV-positive patients with oral candidiasis in India, 89.7% were proteinase producers [14]. This finding concords with our results, wherein out of 130 *C. albicans* isolates recovered from cancer, vulvovaginal candidiasis, and maxillary sinusitis patients, 89.23% were found to be proteinase producers. Some earlier studies have also reported similar findings [5, 15-19]. Likewise, Mane *et al.*, reported that 59% of the isolates of *C. albicans* were phospholipase producers [14]. A study by Mattei *et al*., which reported 78% phospholipase activity and 97% proteinase activity in *C. albicans* isolates, further supports our results, which also exhibit a high secretion of phospholipase and proteinase enzymes [20]. The average value for proteinase activity reported by the authors was 0.71 (range, 0.49–1.00), which is similar to the values obtained in the present study (average Pz = 0.74; range, 0.51–1.4). Thomas *et al*., studied exoenzymes from *C. albicans* strains, which were isolated from oral cavities of HIV patients who were on highly active antiretroviral therapy (HAART) [21]. In that study, the Pz values for proteinase and phospholipase secretions ranged from 0.52 to 0.75 and 0.56 to 0.93, respectively.

Seifi *et al.*, studied extracellular enzymes in *Candida* strains isolated from vaginitis patients and healthy individuals [22]. The authors reported 36.4% and 42.4% strains obtained from the patients with vaginitis to secret extracellular proteinase and phospholipase enzymes, respectively. Mutlu *et al.*, demonstrated 73.7% and 94.7% cases of extracellular proteinase and phospholipase production, respectively in *C. albicans* isolated from blood cultures, thus strengthening our findings [23]. They also showed 48% and 38.4% extracellular proteinase and phospholipase producing strains, respectively, in non-albicans *Candida* strains.

However, our results are in contrast to those reported by Alenzi [24]. In his study, phospholipase activity was demonstrated in 12 out of 27 (44.5%) of the *Candida* isolates and the secretory aspartic proteinase activity was recorded in 13 out of 27 (48%) of the *Candida* isolates from the patients with urinary tract infection and obstructive uropathy. Our findings are in concordance with the previous reports that exhibited a higher percentage of phospholipase (86.92%) in *C. albicans* isolates [25-29]. Deepa *et al*., reported 100% phospholipase activity in *C. albicans* isolates. Interestingly, almost all of our *C. albicans* isolates produced a high percentage of extracellular proteinase and phospholipase enzymes [30]. Mane *et al*., reported extracellular proteinase and phospholipase production in 34.6% and 15.4% non-*Candida albicans* isolates [14]. Several studies have reported a wide variation in the production of these enzymes in non-*albicans Candida* isolates [4, 5, 15, 16, 29, 31, 32]. However, Jose *et al*., demonstrated phospholipase activity in 33% of *C. albicans* strains, whereas none of the non-*albicans Candida* showed the production of the enzyme [32]. The expression of virulence factors by *Candida* spp. may vary depending upon the type, the site and the stage of infection as well as the host response. This might explain the variability observed in the different studies [33].

Mane *et al*., reported that among the non-*albicans Candida* spp., maximum production of extracellular proteinase (50%) was seen in *C. krusei* followed by *C. tropicalis* (44.4%), *C. parapsilosis* (33.3%), with least activity recorded for *C. glabrata* (12.5%) isolates; whereas, phospholipase activity was detected in 33.3% and 12.5% of the isolates of *C. tropicalis* and *C. glabrata*, respectively [14]. None of the isolates of *C. krusei* and *C. parapsilosis* exhibited phospholipase activity. On the contrary, in the present study, all the five non-*albicans Candida* isolates exhibited both extracellular proteinase and phospholipase activities. However, inter-species variation in the amount of extracellular proteinase and phospholipase produced varied significantly. Similar findings were reported by Mohandas and Ballal [34]. Deepa *et al*., reported 44.4% and 33.3% strains of *C. tropicalis* and *C. krusei*, respectively, to produce phospholipase activity, which is quite similar to our results. However, the researchers reported phospholipase activity in all *C. glabrata* isolates, which is contrary to our findings for the enzyme (40% *Candida* isolates) [30]. On the contrary, Aher *et al*., reported 60% and 56% phospholipase producing isolates of *C. glabrata* and *C. tropicalis*, respectively [29].

A study conducted by Brilhante *et al*., aimed at assessing *in vitro* antifungal susceptibility profile and *in vitro* production of virulence factors, *viz*., biofilm production, phospholipase, and proteases in 28 isolates of *C. parapsilosis* [35]. Twenty-three (82.1%) of the fungal strains showed protease activity; however, none of those produced phospholipase; this is contrary to the present findings.

Ying and Chunyang [36] reported 80% of fluconazole-resistant *C. albicans* strains (n=15) to exhibit phospholipase activity. Brilhante *et al*., observed that more than 80% of *C. albicans* strains were resistant to fluconazole and 50% were positive for the production of phospholipase enzyme [37]. Nevertheless, in the present study, no resistance for fluconazole was seen in *C. albicans* strains; however, of the strains, 89.23% showed proteinase and 86.92% showed phospholipase activity.

In contrast to the findings of Aher and colleagues (2014), where in 14.9% *Candida* isolates displayed resistance to amphotericin B, no such resistance was recorded for the *Candida* isolates studied in the present work [29]. The authors also revealed that *C. tropicalis* was the most resistant species among all *Candida* isolates to itraconazole; however, no such resistance was observed by *C. tropicalis* isolates against itraconazole in the present study. Among the *Candida* isolates, maximum resistance was exhibited by *C. krusei* isolates against ketoconazole that is similar to our findings as well.

Ozkan *et al*., reported that *C. parapsilosis* and *C. albicans* displayed the negative correlation between the proteinase activity and the MIC values of amphotericin B and fluconazole [39]. However, a significant difference was found between the proteinase activity and the MIC values of ketoconazole ($p = 0.007$). Ollert *et al*., reported a correlation between proteinase activity and MIC values of azoles [19]. Nayak *et al*., observed that the majority of the mycotic keratitis causing filamentous fungi exhibiting higher MIC values were proteinase producers [39]. The researchers concluded that proteinase seems to be an important virulent factor of filamentous fungi and are correlated significantly with amphotericin B resistance. In the present analysis, except for *C. glabrata*, *C. guilliermondii*, and *C. krusei*, all other *Candida* species tested showed a negative correlation between the Pz and the MIC values. This is in concordance with our hypothesis that with a decrease in the Pz values (i.e., with an increase in enzyme production), an increase in MIC values (or resistance) among the isolates was observed. Thus, a positive correlation between the enzyme secretion and the MIC values was exhibited, as hypothesized. However, in the case of *C. glabrata*, a positive correlation was observed between the Pz value of extracellular proteinase activity and the MIC of ketoconazole and itraconazole, while no such correlation was observed between phospholipase production and the MIC values. Such a negative correlation between extracellular proteinase secretion and MIC values for ketoconazole might be due to a small number of *C. glabrata* isolates that were studied in the present work (n=5). Therefore, further analysis with a large number of isolates is necessary to make a true conclusion. A similar study was conducted by Gutch *et al*., who examined the antifungal susceptibility of clinical and environmental *Cryptococcus neoformans* and *Cryptococcus gattii* isolates [40].

Ibrahim *et al.*, reported that *C. albicans*, isolated from blood, showed high phospholipase activity [25]. Price *et al*., reported that 55% of blood, 50% wound and 30% urine isolates of *Candida* demonstrated phospholipase activity [11]. Kumar *et al*., also reported 100% phospholipase activity in *C. albicans* isolates that were obtained from the respiratory tract [5]. Our results showed that 100% sputum isolates of *C. albicans* were phospholipase producers. On the contrary, among the non-*albicans Candida*, it was not possible to delineate the site of phospholipase production by five *Candida* species due to their low numbers included in the study. Overall, *C. glabrata* isolates were the highest (80%) phospholipase producers followed by *C. parapsilosis* (55%), *C. tropicalis* (40%), and *C. krusei* (40%).

Bernardis *et al*., found that the cutaneous isolates of *C. parapsilosis* exhibited elevated extracellular proteinase activity, which was more than four times higher than the enzyme activity of the blood isolates [41]. This result suggests that the extracellular proteinase activity is correlated with the source of the isolate. In our study, the maximum number of *C. albicans* isolates (96%) from CSF was reported as the producers of extracellular proteinase enzyme followed by blood (92%), VVC (90%), MS (90%), sputum (80%) and oral (80%). Our analysis revealed a non-significant difference between the extracellular proteinase and phospholipase activity between each clinical source studied ( $p > 0.05$).

It can be concluded that the extracellular proteinase and phospholipase production in *Candida* isolates may be a useful prognostic marker, especially, among the immunocompromised individuals due to the severity of the yeast infections in such patients. The high amount of enzyme secretion, as exhibited in the present study, suggests that it definitely has a role in the pathogenesis of the disease and is one of the important virulence factors to be studied extensively. A non-significant difference, exhibited for extracellular proteinase and phospholipase secretion among *C. albicans* and non-*albicans* isolates using individual comparisons for different sources using Dunnett's t and Tukey's HSD Post Hoc tests, revealed that a comparable number of samples belonging to each source needs to be studied extensively. It can be

inferred that antifungal susceptibility testing is necessary to study the pattern of susceptibility and resistance against antifungal drugs by *Candida* strains. It is also essential to choose the right drug for effective treatment of patients. Our findings also highlight the role of extracellular enzymes in enhancing the resistance to *Candida* spp. against most commonly used antifungal drugs. However, further investigation in this direction is required to establish the role of such enzymes on drug efficacy and their involvement in the treatment of invasive *Candida* infections in immunocompromised patients.

**Conflict of interest:** The authors report no potential conflict of interest relevant to this study.

## ACKNOWLEDGEMENTS

The authors are thankful to the Vice-Chancellor, R. D. University, for providing the necessary laboratory facilities to conduct the research work. The authors are also grateful to the Director-General, Indian Council of Medical Research (ICMR), New Delhi for awarding the Post Doctoral Research Associate ship SRN and to the Department of Science and Technology, Ministry of Science and Technology, Government of India, for awarding the Young Scientist SERC FAST Track Scheme to SRN.

## REFERENCES

1. Luque AG, Biasoli MS, Tosello ME, Binolfi A, Lupo S, Mangaró HM. Oral yeast carriage in HIV-infected and non-infected populations in Rosario, Argentina. Mycoses. 2009;52 (1):53–9.
2. Hung CC, Yang YL, Lauderdale TL, McDonald LC, Hsiao CF, Cheng HH, Ho YA, Lo HJ. Colonization of human immunodeficiency virus-infected outpatients in Taiwan with Candida species. Journal of clinical microbiology. 2005 Apr 1;43(4):1600-3.
3. Nawange SR, Singh K, Naidu J, Singh SM. Naturally acquired systemic dual infection caused by Candida famata (Debaryomyceshansenii) and Candida catenulata in albino rats bred for sale in the market at Jabalpur (Madhya Pradesh), India.Mycoses. 2010; 53(2):173–5.
4. Kalkancı A, Kuştimur S, Bozdayı G, Biri A. Virulence factors and susceptibility pattern of Candida strains isolated from patients with vulvovaginal candidiasis. T Mikrobiol Cem Derg. 2003;33:323-8.
5. Kumar CG, Kumar SS, Menon T. Phospholipase and proteinase activities of clinical isolates of Candida from immunocompromised patients. Mycopathologia. 2006 Apr 1;161(4):213-8.
6. Haynes K. Virulence in Candida species. Trends in microbiology. 2001 Dec 1;9(12):591-6.
7. LaFleur MD, Kumamoto CA, Lewis K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. Antimicrobial agents and chemotherapy. 2006 Nov 1;50(11):3839-46.
8. Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clinical microbiology reviews. 2000 Jan 1;13(1):122-43.
9. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol. Mol. Biol. Rev.. 2003 Sep 1;67(3):400-28.
10. Schaller M, Borelli C, Korting HC, Hube B. Hydrolytic enzymes as virulence factors of *Candida albicans*. Mycoses. 2005; 48(6):365–77.
11. Price MF, Wilkinson ID, Gentry LO. Plate Method for detection of Phospholipase activity in *Candida albicans*. Sabouraudia. 1982; 20:7–14.
12. Aoki S, Ito-Kuwa S, Nakamura K, Kato J, Ninomiya K, Vidotto V. Extracellular proteolytic activity ofCryptococcus neoformans. Mycopathologia. 1994 Dec 1;128(3):143-50.
13. CLSI (NCCLS) National committee for clinical laboratory standards. Reference method for broth dilution antifungal susceptibility testing of yeast (M 27) A, Approved Standard NCCLS document, Wayne Pa. 1997.
14. Mane A, Pawale C, Gaikwad S, Bembalkar S, Risbud A. Adherence to buccal epithelial cells, enzymatic and hemolytic activities of Candida isolates from HIV-infected individuals. Med Mycol. 2011; 49(5):548-51.
15. Costa CR, Passos XS, e Souza LK, LucenaPde A, Fernandes Ode F, Silva Mdo R. Differences in exoenzyme production and adherence ability of Candida spp. isolates from catheter, blood and oral cavity. Rev Inst Med Trop Sao Paulo. 2010; 52(3**)**:139-43.
16. Kumar VG, Latha R, Vedhagiri K, Sathiamoorthi T, Jayarani G, Sasikala R, Selvin J, Natarajaseenivasan K. Phospholipase C, proteinase and hemolytic activities of Candida spp. isolated from pulmonary tuberculosis patients. Journal de Mycologie Médicale. 2009 Mar 1;19(1):3-10.
17. Koga-Ito CY, Lyon JP, Vidotto V, de Resende MA. Virulence factors and antifungal susceptibility of *Candida albicans* isolates from oral candidosis patients and control individuals. Mycopathologia. 2006 Apr 1;161(4):219-23.
18. Menezes EA, Monteiro MN, Parente TM, Cunha FA, Augusto KL, Freire CC. Frequency and enzymatic activity of *Candida albicans* isolated from the oral cavity of HIV-positive patients at Fortaleza, Ceará. Jornal Brasileiro de Patologia e Medicina Laboratorial. 2006 Aug;42(4):253-6.
19. Ollert MW, Wende C, Görlich M, McMullan-Vogel CG, Borg-von Zepelin M, Vogel CW, Korting HC. Increased expression of *Candida albicans* secretory proteinase, a putative virulence factor, in isolates from human immunodeficiency virus-positive patients. Journal of clinical microbiology. 1995 Oct 1;33(10):2543-9.

20. Mattei AS, Alves SH, Severo CB, Guazzelli LD, Oliveira FD, Severo LC. Determination of germ tube, phospholipase, and proteinase production by bloodstream isolates of *Candida albicans*. Revista da Sociedade Brasileira de Medicina Tropical. 2013 Jun;46(3):340-2.
21. Thomas S, Tsang CSP, Mathew A, Bhaskar S, Priya SP, Varughese N. A study on exoenzyme activities of *Candida albicans* isolated from oral cavities of HIV-infected patients on HAART. J Hum Virol Retrovirol. 2015; 2(2): 37.
22. Seifi Z, Mahmoudabadi AZ, Zarrin M. Extracellular enzymes and susceptibility to fluconazole in Candida strains isolated from patients with vaginitis and healthy individuals. Jundishapur journal of microbiology. 2015 Mar;8(3):e20162.
23. Sariguzel FM, Berk E, Koc AN, Sav H, Demir G. Investigation of the relationship between virulence factors and genotype of Candida spp. isolated from blood cultures. The Journal of Infection in Developing Countries. 2015 Aug 29;9(08):857-64.
24. Alenzi FQ. Virulence factors of Candida species isolated from patients with urinary tract infection and obstructive uropathy. Pakistan journal of medical sciences. 2016 Jan;32(1):143-146.
25. Ibrahim AS, Mirbod F, Filler SG, Banno Y, Cole GT, Kitajima Y, Edwards JE Jr, Nozawa Y, Ghannoum MA. Evidence implicating phospholipase as a virulence factor of *Candida albicans*. Infect Immun. 1995;63 (5):1993–8.
26. Kantarcioglu SA, Yucel A. Phospholipase and Protease activities in clinical Candida isolates with reference to the sources of strains. Mycoses. 2002; 45:160–165.
27. Oksuz S, Sahin I, Yildirim M, Gulcan A, Yavuz T, Kaya D, Koc AN. Phospholipase and proteinase activities in different Candida species isolated from anatomically distinct sites of healthy adults. Japanese journal of infectious diseases. 2007 Sep 1;60(5):280-283.
28. Samarnayke LP, Raeside JM, Mac Farlane TW. Factors affecting the phospholipase activity of Candida species in vitro. Sabouraudia. 1984; 22:210–207.
29. Aher CS. Species distribution, virulence factors and antifungal susceptibility profile of Candida isolated from oropharyngeal lesions of HIV infected patients. Int. J. Curr. Microbiol. App. Sci. 2014;3(1):453-60.
30. Deepa K, Jeevitha T, Michael A. In vitro evaluation of virulence factors of Candida species isolated from oral cavity. J Microbiol and Antimicro. 2015; 7(3):28–32.
31. Gokce G, Cerikcioglu N, Yagci A. Acid proteinase, phospholipase, and biofilm production of Candida species isolated from blood cultures. Mycopathol. 2007; 164: 265–269.
32. Jose NV, Mudhigeti N, Asir J, Chandrakesan SD. Detection of virulence factors and phenotypic characterization of Candida isolates from clinical specimens.J Curr Res Scient Med. 2016; 1(1):27–31.
33. Marija B. Oral candidiasis-adhesion of non albicans Candida species. Proc Nat Sci. 2008; 114:69–78.
34. Mohandas V, Ballal M. Distribution of Candida species in different clinical samples and their virulence: biofilm formation, proteinase and phospholipase production: a study on hospitalized patients in southern India. J Glob Infect Dis. 2011; 3(1):4–8.
35. Brilhante RS, Rodrigues TD, Castelo DD, Teixeira CE, de Brito Macedo R, Bandeira SP, de Alencar LP, Monteiro AJ, de Aguiar Cordeiro R, Bandeira TD, Moreira JL. Antifungal susceptibility and virulence attributes of animal-derived isolates of Candida parapsilosis complex. Journal of medical microbiology. 2014 Nov 1;63(11):1568-72.
36. Ying S, Chunyang L. Correlation between phospholipase of *Candida albicans* and resistance to fluconazole. Mycoses, 2011; 55:50–55.
37. Brilhante RS, de Alencar LP, de Aguiar Cordeiro R, Castelo DD, Teixeira CE, de Brito Macedo R, Lima DT, Paiva MD, Monteiro AJ, Alves ND, de Oliveira MF. Detection of Candida species resistant to azoles in the microbiota of rheas (Rhea americana): possible implications for human and animal health. Journal of medical microbiology. 2013 Jun 1;62(6):889-95.
38. Ozkan S, Kaynak F, Kalkanci A, Abbasoglu U, Kustimur S. Slime production and proteinase activity of Candida species isolated from blood samples and the comparison of these activities with minimum inhibitory concentration values of antifungal agents. MemInst Oswaldo Cruz. 2005; 100 (3): 319–324.
39. Nayak N, Satpathy G, Prasad S, Vajpayee RB, Pandey RM. Correlation of proteinase production with amphotericin B resistance in fungi from mycotic keratitis. Ophthal Res. 2010; 44(2):113–8.
40. Gutch RS, Nawange SR, Singh SM, et al. Antifungal susceptibility of clinical and environmental Cryptococcus neoformans andCryptococcusgattii isolates in Jabalpur, a city of Madhya Pradesh in Central India. Braz J Microbiol. 2015;46(4):1125–33.
41. Bernardis F, Mondello F, San Millan R, Ponton J, Cassone A. Biotyping and virulence properties of skin isolates of C. parapsilosis. J Clin Microbiol. 1999; 37: 3481–3486.