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**Original Research Article**

# Phospholipase, proteinase, esterase and haemolytic activity of *Candida* species isolated from oral cavity and its antifungal susceptibility pattern

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## ABSTRACT

**Background:** *Candida* species is a normal commensal flora of human body inhabiting the skin, mucous membrane and gastro intestinal tract but may be associated with superficial and deep-seated fungal infections. The switch of *Candida* species from commensal to a potent pathogen, is facilitated by various extracellular hydrolytic enzymes. The aim of this study was to estimate the phospholipase, proteinase, haemolysin and esterase activity of *Candida* species and to determine the antifungal susceptibility.
**Methods:** Total 100 isolates of *Candida spp.* were collected from diagnostic microbiology laboratories in central Kerala. Phospholipase, proteinase, esterase and haemolytic activity was determined by early defined methods of Price et al, Aoki et al, Walter Rudek and Manns et al, respectively.
**Results:** *C. tropicalis* exhibited highest phospholipase, proteinase and esterase activity followed by *C. albicans* and *C. krusei*. *C.albicans* shows highest haemolytic activity followed by *C. tropicalis* and *C. krusei*.
**Conclusions:** Extracellular enzymes, phospholipase, proteinase, esterase and haemolysin was detected among *Candida* species in the present study.

**Keywords:** Antifungal susceptibility, *Candida* species, Esterase, Haemolysin, Hydrolytic enzymes, Phospholipase, Proteinase

## INTRODUCTION

*Candida* is a ubiquitous human commensal yeast which resides mainly on mucosal surfaces of the oral cavity as well as in the urogenital, gastrointestinal, vaginal tracts and some cutaneous areas of healthy individuals without symptoms of disease. In immunocompromised patients, *Candida* species are frequently recognized as one of the main culprits causing infections.[1] *Candida* is a fungus that resembles yeast and comes in three different forms: pseudo-hyphae, yeast, and chlamydospores.[2] Currently, oral candidiasis is the most prevalent human fungal disease and is a common opportunistic infection. The primary human pathogen of the genus *Candida*, is *Candida albicans*, that colonizes human mucosal surfaces, especially those of the oral cavity and vagina, and may spread through the blood in immunocompromised individuals.[3,4] Although some non-albicans species like *Candida glabrata*, *Candida krusei*, *Candida dubliniensis*, *Candida parapsilosis*, and *Candida tropicalis* are recovered from infected individuals.[5]

*Candida* participates actively in the pathophysiology of the occurrence and advance of infection, thanks to its virulence factors. One group of virulence factors causes colonization to take place, or the initiation of an infection, whilst the other group helps to spread the

infection.[6] The ability of *C. albicans* to infect such diverse host niches is supported by a wide range of virulence factors and fitness attributes. A number of attributes, including the morphological transition between yeast and hyphal forms, the expression of adhesins and invasins on the cell surface, thigmotropism, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes are considered virulence factors. *Candida* species have the ability to produce a variety of hydrolytic enzymes, such as proteases, lipases, phospholipases, esterases, and haemolysins.[7-9] These enzymes have received much attention in the past, as they are known to mediate *Candida* pathogenesis, particularly by facilitating the hyphal invasion especially seen in disseminated candidiasis.[10] They appear to be crucial in the development of *Candida* overgrowth, since these enzymes promote host invasion through tissue penetration and adhesion. The present study investigated in vitro production of virulence factors namely phospholipase, proteinase, haemolysin and esterase activities in *Candida* species isolated from cases of oral candidiasis.

## METHODS

The present cross-sectional study was conducted at School of Medical Education (SME), Kottayam, Kerala between January 2022 and December 2022. 100 isolates of *Candida* species were collected from various diagnostic microbiology laboratories in central Kerala. Only *Candida* isolated from symptomatic candidiasis was included in the present study. Patients who had undergone any antimicrobial therapy in the past three months was exclude from the study.

*Candida albicans* MTCC 227, procured from Institute of Microbial Technology (IMTECH), Chandigarh, India, was used as standard control for enzymatic activities-phospholipase, proteinase, haemolysin, esterase and antifungal susceptibility testing. The isolates were reconfirmed by subculturing on to chromogenic media - HiCrome™ *Candida* Differential Agar, followed by Gram staining and colonies were confirmed to be Gram positive yeast like budding cells. Further identification was done by tests like germ tube and chlamydospore production. All reagents, culture media and antifungal disc were procured from HiMedia Laboratories, Mumbai, India.

### *Determination phospholipase activity*

The phospholipase production of the isolates was assayed according to Polak using the egg yolk agar plate method of Price et al.[11,12] *Candida* species were screened for extracellular phospholipase activity by measuring the size of the zone precipitation after growth on egg yolk agar. The egg yolk medium consisted of SDA -13.0g, NaCl -11.7g, $CaCl_2$ -0.11g and 10 % sterile egg yolk. First, the components without the egg yolk were mixed and sterilized, then the egg yolk was centrifuged at 500 g for 10 min at room temperature and 20 ml of the supernatant was added to the sterilized medium. After 24hr incubation period of *Candida* species grown on fresh SDA, yeasts were harvested and suspended in sterile phosphate buffer saline (PBS) to attain visible turbidity.10µl suspension of PBS is plated in triplicate on the surface of egg yolk medium and the plates are incubated at 37°C for 72 hours. The value of phospholipase production (Pz) is determined by:

$$Pz = \frac{Colony\ diameter}{Colony\ diameter + Zone\ of\ precipitation}$$

Pz coefficients were grouped into four classes: Very high (+); Pz of 0.90-0.99), High (++); Pz of 0.80-0.89), Low (+++); Pz of 0.70-0.79) and Very low (++++); Pz of 0.69 or lower.

### *Determination of proteinase activity*

Determination of proteinase production was performed according to Aoki et al.[13] Test medium consisted of agar plates containing bovine serum albumin (BSA). A solution containing 0.04g $MgSO_4.7H_2O$,0.5g $K_2HP04$,1g NaCl,0.2g yeast extract, 4g glucose is prepared in a volume of 60 ml distilled water. The medium is sterilized and allowed to cool down to room temperature.0.5g BSA is added onto the prepared media and pH is adjusted to 3.5. *Candida* strains are inoculated in triplicate and the plates are incubated at 37°C for 7 days. The presence of proteinase is determined by the formation of transparent halo around the yeast colonies. Proteinase activity is measured and calculated according to the method described by Price et al.[12]

### *Determination of haemolytic activity*

Determination of haemolysin activity was determined by Manns et al.[14] A 10µl aliquot is taken from each suspension and inoculated onto SDA supplemented with 3% glucose, containing 7% fresh blood and incubated at $37^0$C in the presence of 5% $CO_2$ for 48 hours. The presence of a distinct translucent halo and/or a greenish-black ring around the inoculum site was evaluated positive haemolytic activity when viewed with transmitted light. Haemolysis tests are repeated three times and the results represent mean values±SD. The diameters of the zone of haemolysis and colony were measured to evaluate the intensity of the haemolysin production exhibited by different *Candida* strains. The diameter of the translucent radial zone of haemolysis was divided by the diameters of the colony size, and this ratio (equal to or larger than 1) was used as haemolytic index:

$$\mathrm{HI} = \frac{Colony\ diameter}{Colony\ diameter + zone\ of\ precipitation}$$

Haemolytic Index were grouped into four classes: very high (++++); Haemolytic Index of 0.90-1), high (+++); Haemolytic Index of 0.80-1), low (++); Haemolytic Index

of 0.70 -1) and very low (+); Haemolytic Index of O.69 or lower).

***Determination Esterase activity***

Lipolytic activities in *Candida* species was determined by Walter Rudek.[15] Tween 80 Opacity medium consisted of Peptone- 10 g, NaCl- 5 g, $CaCl_2$ - 0.1, Agar-15g in 1 litre distilled water. The medium is autoclaved at $121^0$C for 15 min, allowed to cool to about 50°C, mixed with 5 ml of pre-autoclaved and cooled Tween 80, and dispensed into sterile 90 mm-diameter petri dishes (25 ml of agar per plate). An overnight culture of each *Candida* isolate grown on SDA was transferred to the Tween 80 opacity medium and spread over a circular inoculation site of approximately 10 mm diameter. The inoculated agar plates were incubated aerobically at 35°C and were examined daily for up to 10 days. Detection of esterase activity on the test plates was performed by observing halos of precipitation around the inoculum under transmitted light.

***Antifungal susceptibility testing***

Antifungal disc diffusion susceptibility testing was done as prescribed by CLSI M44-A2.[16] Briefly, Mueller-Hinton agar supplemented with 2% glucose and 0.5µg/ml methylene blue was used for the sensitivity testing. Antifungals used were fluconazole, voriconazole and clotrimazole. Interpretive criteria for fluconazole and voriconazole was as prescribed by CLSI M44-A2 and for clotrimazole as per manufacturers' instructions.[16]

The data was analyzed using Microsoft excel 2019 and data were expressed as means ± standard deviation (SD).

## RESULTS

A total of 100 clinical isolates of *Candida* obtained from various diagnostic laboratories which belonged to four species viz *Candida albicans* (n=49), *Candida tropicalis* (n=32), *Candida krusei* (n=18) and *Candida auris* (n=1), Table 1. *Candida albicans* produced light green colour, *Candida tropicalis* blue colour, *Candida krusei* purple colour and *C. auris* cream in colour as shown in Figure 1.

***Phospholipase activity***

Precipitation zones around inoculated spots were measured for the positive samples (Figure 2). Mean were calculated and obtained results are exhibited in figure 3. The Pz value was calculated as 0.15, 0.13 and 0.12 for *C. albicans, C. tropicalis* and *C. krusei* respectively.

Of the 32 *Candida tropicalis* 9.37% shows high activity, 6.25% shows very high activity and 3.12% shows very low activity. While 49 *C. albicans* 6.12% shows high activity, 6.12% shows low activity and 4.08% shows very high activity. In case of C. *krusei* 5.55% shows high activity, 5.55% low activity and 5.55% very low activity among 18 isolates.

**Table 1: Demographic characteristics of the sample.**

| Variables | N (%) |
|---|---|
| **Sex** | |
| Male | 49 (49) |
| Female | 51 (51) |
| **Age (Years)** | 64 (59-72) |
| **Duration** | 12 months |
| **Total *Candida* isolates** | 100 |
| *Candida albicans* | 49 (49) |
| *Candida tropicalis* | 32 (32) |
| *Candida krusei* | 18 (18) |
| *Candida auris* | 1 (1) |

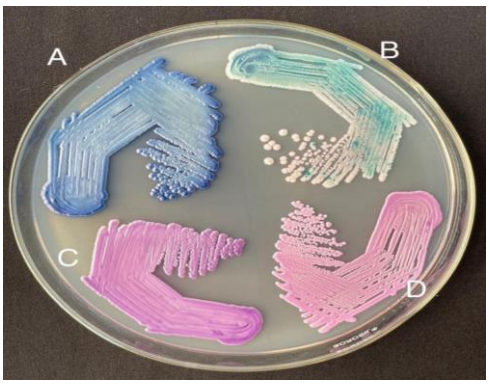


**Figure 1. Candida species on HiCrome™ *Candida* differential agar, A) *Candida tropicalis*, B) *Candida albicans*, C) *Candida krusei*, D) *Candida auris*.**

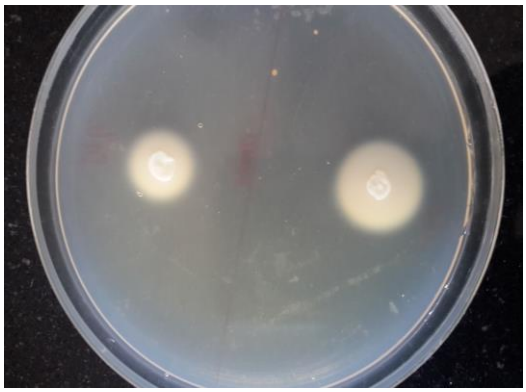

**Figure 2: Phospholipase activity of *Candida* species on egg yolk agar.**

***Proteinase activity***

Precipitation zones around inoculated spots were measured for the positive samples (Figure 4) Mean were calculated and obtained results are exhibited in Figure 3. The Pz values was calculated as 0.79, 0.86 and O.76 for *C. albicans, C. tropicalis* and *C. krusei* respectively.

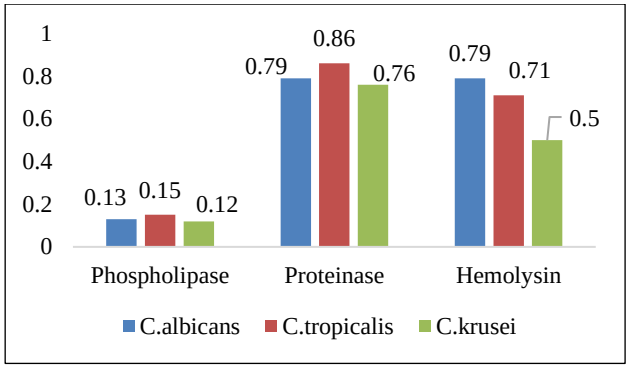


**Figure 3: Graph depicting Pz values for phospholipase and proteinase and haemolytic index of *Candida* species.**

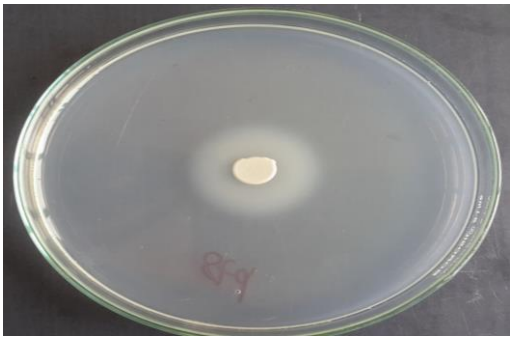

**Figure 4: Proteinase activity of *Candida* species on BSA agar.**

Of the 32 *C. tropicalis* 62.5% shows very high activity and 34.3% shows high activity. While 49 *C. albicans* 65.3% shows high activity and 24.48% shows very high activity. In case of *C. krusei* 55.5% shows high activity and 33.3% shows very high activity.

***Haemolytic activity***

Precipitation zones around inoculated spots were measured for the positive samples (Figure 5).

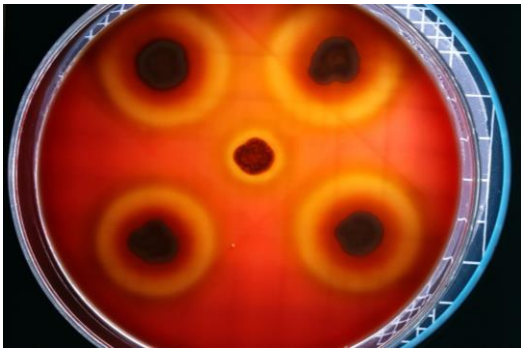

**Figure 5: Haemolytic activity of *Candida* species on SDA supplemented with blood and glucose.**

Mean were calculated and obtained results are exhibited in Figure 3. The haemolytic index was calculated as 0.79, 0.71 and 0.5 for *C. albicans*, *C. tropicalis* and *C. krusei* respectively.

Of the 32 *C. tropicalis* 64% shows high activity, 18% shows very high activity, 14% shows low activity and 4% shows very low activity. While 49 *C. albicans* 58% shows very high activity, 23% shows high activity, 15% shows low activity and 4% shows very low activity. In case of *C. krusei* 46% shows high activity, 36% shows low activity and 18% shows very low activity.

***Esterase activity***

*C. tropicalis* shows highest esterase activity (81%) followed by C. albicans (76%) and C. krusei (22%) were shown in Figure 6.

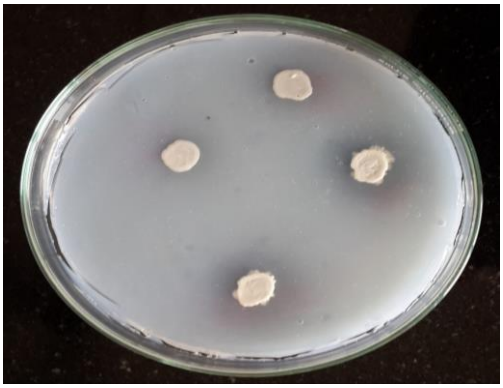

**Figure 6: Esterase activity of *Candida* species on Tween 80 opacity medium.**

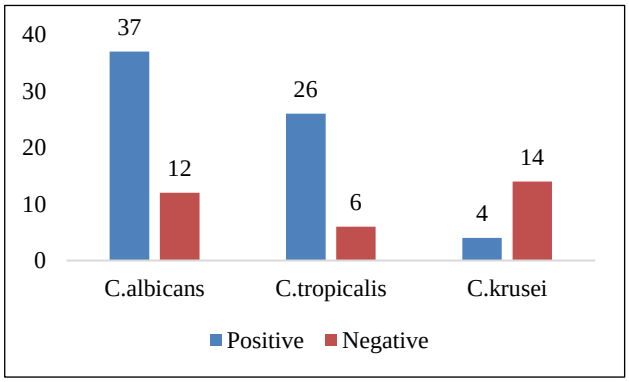


**Figure 7: Esterase activity of *Candida* species.**

***Antifungal susceptibility testing***

Antifungal susceptibility of *Candida spp.* were summarized in Figure 8. The rate of susceptibility to fluconazole were 100% for *C. albicans*. *C. tropicalis* show 96.8% susceptibility to fluconazole and 3.12% Susceptible Dose Dependent (SDD). *C. krusei* are intrinsically resistant to fluconazole. The rate of susceptibility to voriconazole were 100% for *C. albicans* and *C. krusei*. In case of *C. tropicalis*, voriconazole was sensitive to 96.8% and 3.12% resistant. The antifungal

susceptibility pattern of *C. albicans* against clotrimazole was 93.8% sensitive and 6.12% intermediate. In case of *C. tropicalis*, clotrimazole was sensitive to 81.25%, intermediate to 9.37% and resistant to 9.37%. *C. krusei* shows 83.3% sensitivity, 11.11% intermediate and 5.55% resistance to clotrimazole. *C. auris* was sensitive to voriconazole and clotrimazole while resistant to fluconazole.

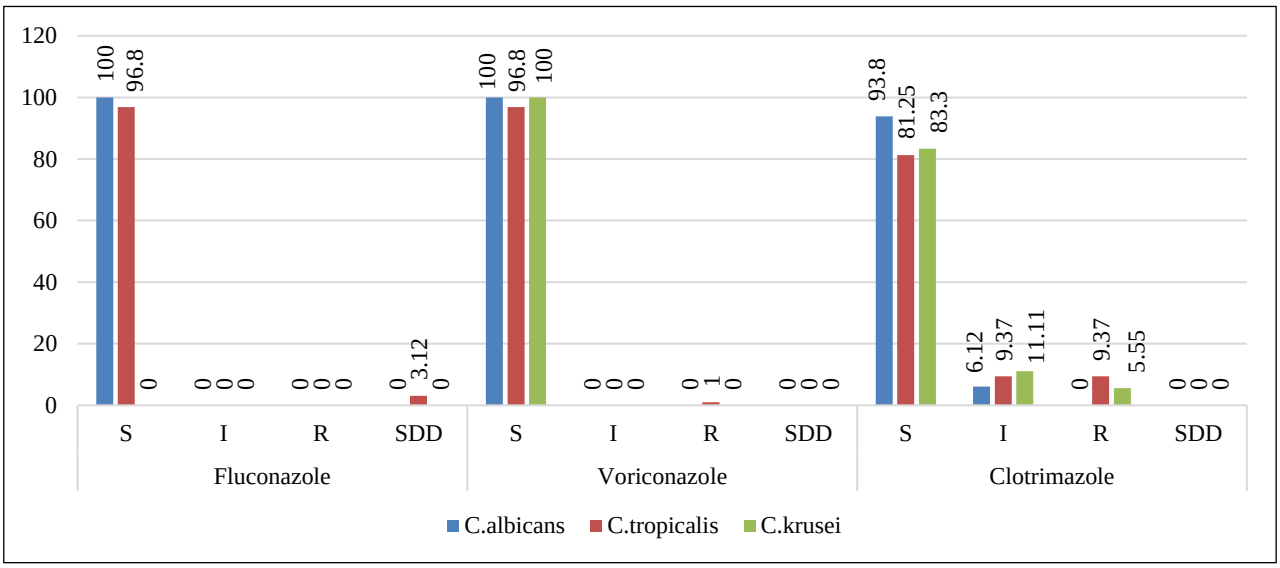


**Figure 8: Antifungal susceptibility pattern of candida species against fluconazole, voriconazole and clotrimazole (S-sensitive, I-intermediate, R-resistant and SDD- susceptible dose dependent).**

## DISCUSSION

Numerous hydrolytic enzymes, including proteinases, lipases, phospholipases, esterases, and haemolysins, can be produced by *Candida* species. Extracellular hydrolytic enzymes appear to be crucial in the development of *Candida* overgrowth, since these enzymes promote host invasion through tissue penetration and adhesion. Phospholipases, secreted aspartyl proteinases, haemolysin and esterase are the most significant hydrolytic enzymes. The phospholipases could play an active role in the invasion of host tissue in lesions of candidiosis by disrupting the epithelial cell membranes and allowing the hyphal tip to enter the cytoplasm.[17] Proteinase production is considered to enhance the organism's ability to colonise and penetrate host tissues, and to evade the host's immune system by degrading a number of proteins important in host defence such as immunoglobulins, complement and cytokines.[18-20] The ability of *Candida* to acquire elemental iron through haemolysin production is pivotal to its survival and ability to establish infections in human in particular in disseminated candidiasis and it is reported that *Candida albicans* excretes a haemolytic factor that causes haemoglobin to be released and is then used by the organism as an iron source. Invasive infections with *Candida* occur when host defence mechanisms are compromised, allowing a colonizing strain to access the blood and deeper structures. Virulence attributes of the colonizing organism also likely contribute to the risk of disseminated infection. Due to the increasing incidence of invasive *Candida* infections there is a great interest on *Candida* virulence factors including phospholipase, proteinase, haemolysin and esterase production to establish strategies for control and prevention of candidosis and also as a possible target for developing novel therapeutic interventions.[21,22]

The present study was aimed at determining *in vitro* phospholipase, proteinase, haemolysin and esterase activities in 100 clinical isolates of various *Candida* species [(*C. albicans* (49%), *C. tropicalis* (32%), *C. krusei* (18%) and *C. auris* (1%)]. It is in accordance with the study of Kalkanci et al, which also has *C. albicans* as the most common isolate.[23] In our present study, *C. tropicalis* shows highest phospholipase activity (18.7%) followed by *C. albicans* (16.3%) and *C. krusei* (16.6%). The $P_z$ value of *C. albicans* ranges from 0.71 -0.91. It is comparable to the activity of *C. albicans* isolated from oral cavity obtained from Tsang et al, while comparing the activity of phospholipase production of *C. tropicalis* to the study of Deorukhkar et al, almost comparable results of 13.8% and 18.7% respectively.[24,25] Researchers have reported contradictory findings regarding phospholipase activity in *C. tropicalis*. Investigators like Thangam et al reported high phospholipase activity in *C. tropicalis* isolates among NAC species.[26] While others like Samaranayake et al reported no activity.[27] These inconsistencies in observations may be due to biological differences among the isolates tested.

In our study 44 of 49 *C. albicans* (89.7%) strains yielded positive proteinase activity. For *C. tropicalis* 31 of 32 strains (96.8%) shows transparent halo around yeast colonies. In case of *C. krusei* 16 out of 18 strains (88.8%) shows positive proteinase activity. In the present study the $P_z$ value of *C. albicans* ranges from 0.85 -0.91. It is

comparable to the activity of *C. albicans* isolated from oral cavity obtained from Tsang et al, proteinase production in present study for *C. tropicalis* was 96.8% which is higher comparing to the study Deorukhkar et al, 30.7% which may be due to higher number of samples in the present study.[24,27] Study of Deorukhkar et al, had only 20 oropharyngeal isolates. But the overall positivity in the above study was 65%.

Current study reveals that *C. albicans* shows highest haemolytic activity (96%) followed by *C. tropicalis* (87.5%) and *C. krusei* (55.5%). The Haemolytic Index (HI) values ranges from 0.6 to 0.91 which is in accordance with the study of Tsang et al whose values ranged from 0.60 to 0.79.[28] Studies on the activity of haemolysin in *Candida spp* are limited. Manns et al defined the conditions under which *C. albicans* can display haemolytic activity, but found out that haemolysis is non-existent when no glucose is available in the culture medium.[14] On the other hand, Luo et al have tested 80 *Candida* isolates from clinical sources in different geographical locales and detected only alpha, and not any beta, haemolysis in experiments with glucose-free sheep blood agar.[29] An increased blood glucose concentration may contribute, directly or indirectly, to increased haemolysin activity among *C. albicans* isolates.

In our study of esterase activity, 37 of 49 *C. albicans* (76%) strains yielded positive results in 2-10 days; for *C. tropicalis* 26 of 32 strains (81%) shows halo production in 2 or 3 days and 4 of 18 *C. krusei* strains (22%) produces halo within 10 days. While comparing with the study of Aktas et al, who obtained 58 of 59 *C. albicans* strains (98%) and 38 *C. tropicalis* strains (100%) yielded positive results due to larger sample size.[30] 2 *C. krusei* strains (100%) yielded negative results due to smaller sample size. The patterns of precipitation resulting from reactions of fatty acids hydrolysed from Tween 80 with calcium ions in the medium were useful in distinguishing some *Candida* species.

The limitations of present study include relative smaller sample size, the test performed are phenotypic which may require genotypic confirmation. Only four species of *Candida* was used in the present study, a study with a greater number of *Candida spp.* may provide further a better picture of these enzymes as virulence factors.

## CONCLUSION

In conclusion, we report phospholipase, proteinase and esterase activity higher in *C. tropicalis* contrary to most of the studies which indicates *C. albicans*. But haemolytic activity was higher in *C. albicans*. Extracellular enzyme secretion can obviously be considered a potential virulence factor, but the level of their synthesis and their impact in host depend largely on host constitution. To answer more definitively whether these extracellular enzymes contribute to virulence of *Candida* species these strains should be compared in suitable animal models. So we could understand the natural history and host pathogen relationship associated with mucosal candidial infections.

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