CHAPTER 5- DISCUSSION

Candidiasis is the most common opportunistic fungal infection. In the past three decades with the use of potent antibacterial, immunosuppressive drugs and advancement in medical practices increased the frequency of invasive candidiasis. It is emerging as an important nosocomial agent with intrinsic and acquired drug resistance.

5.1 Isolation of Candida from clinical specimens

Candidiasis has come out as a disquieting opportunistic disease over the past two decades (6). At the same time many non-Candida albicans species have also emerged as a significant pathogen of clinical importance (1). The changing trends in Candida species from various clinical samples have made speciation of Candida compulsory in diagnostic microbiology. C. albicans was found to be the predominant species (31%) out of 336 Candida isolates in our study. The predominant non-C. albicans were C. tropicalis 87(26 %) followed by C.krusei 71(21 %) and C.glabrata (12%). Other researchers have also reported the evidence that C. tropicalis (29.4%) as the most common emerging pathogen from the group of non-candida albicans (173,174).

5.2 Vaginal candidiasis

The occurrence of vaginal candidiasis was 40%, which is significantly higher than other types of candidial infections in our study. Vaginal candidiasis is an extremely common infection in 60-70% women during their reproductive age at least once in their lives which are already reported in previous studies (175-178). The highest frequency of genitourinary candidiasis was observed in the age group of (26-35 years), followed by the age group (18-25years) in our study. The observation in this study is consistent with other studies which reported that genital candidiasis occurs most frequently in the age group of 20- 40 years (178,179).

We have studied the association of the risk factors associated with vaginitis. Pregnancy (48%) was the most common predisposing factor associated with vaginal candidiasis followed by the use of intrauterine devices (20%). This might be related to
the high level of reproductive hormones and increased glycogen content of vagina favors the growth of candida during pregnancy (180,174).

And the determination of the occurrence of vaginal candidiasis in pregnant women will be important in diagnosis and for giving better antenatal care.

The current findings of our study show *C.albicans* (31%) as the predominant strain from vaginal candidiasis. Previous studies too have revealed a higher rate of vaginal candidiasis caused by *C. albicans* (181-186). It is important to emphasize that in the past three decades there has been an increasing percentage of infections caused by *non albicans* species of *Candida* particularly, *C.tropicalis, C.glabrata*, and *C. krusei* which resists to conventional therapy. The emergence of *non albicans* species (69%) also supports this evidence. Most frequently isolated *non albicans* species in our study were *C.tropicalis* (27%), *C. krusei* (23%) *C.glabrata* (14%) and *C.dubliniensis* (2%). Studies have reported the emergence of *non candida albicans* from vaginal candidiasis by Saldanha *et al* and Mokkadas *et al* (187,188). We speculate this increasing detection of non albicans species is probably related to the widespread and inappropriate use of antimycotic treatments (self-medication, topical use, and long-term treatments) (189). Hence, the reliable and rapid identification method of *Candida* species is a fundamental goal of microbiology laboratories.

As *Candida* species differ in their antifungal susceptibility, it is essential to identify and differentiate them. Antifungal susceptibility of vaginal isolates in our study revealed that 100% susceptibility to Amphotericin B and Flucytosine in *C. albicans* which is in agreement with the earlier report (187). Fluconazole resistance was 53% which is more common in our study among the vaginal isolates. However, various studies observed the high resistance of fluconazole in vaginal candidiasis (190).

Studies from India reported that 30% fluconazole resistance in the susceptible dose dependent category with MICs between 16 to 32μg/ml (80,189). The treatment of fluconazole resistance is extremely difficult with limited options of antifungals. Prolonged therapy and increased use of antifungals for recurrent candidiasis are the most common risk factors for azoles resistance in vulvovaginitis candidiasis. Azoles have the advantage of being taken orally. Hence, the reliable and rapid identification and susceptibility testing of *Candida* species is a fundamental goal of microbiology laboratories.
5.3 Candidemia

Bloodstream infections due to *Candida* become the fourth-most frequent cause of septicemia (92,190). Significant geographical variation is observed among cases of candidemia in different parts of the world due to the emergence of various factors like immunosuppressive therapies, AIDS and the use of long-term antibiotic therapy. During the period of study, a total of 12% candidemia strains were isolated. The present study shows an increase in the rate of blood stream infection caused by *candida* with the present trends (67, 71, 191-193).

A recent trend noted in many hospitals is an increase in the prevalence of *non candida albicans* as a cause of candidemia. In India, *C.tropicalis* is the most common cause of nosocomial candidemia. Epidemiological data from the Indian subcontinent showed that 67–90% of nosocomial candidemia cases were due to *non- Candida albicans* species of which *C. tropicalis* was the most dominant isolate (194). Our study highlights the presence of 73% *non- Candida albicans* bloodstream infections. Among the *non-Candida albicans*, *C.tropicalis* (23%) is the predominant blood isolates. This finding is consistent with the reports from Asia (42%), UK (23%) and South America (21%) and also in the subcontinent of India (45%) (195). This is in line with previous data from India, which showed *C. tropicalis* as the most common blood stream isolates (191). The highest proportions of *C. tropicalis* were found in Eastern Asia (12.5%) and Argentina (28%) which supports our study (4). The proportion of infections due to *non- Candida albicans* species is persistently rising due to the increased use of fluconazole which is intrinsically resistant to certain *non -Candida albicans* species.

Host factors such as age and gender have also influenced the severity of infections. People at two extreme ends of life spectrum were more susceptible to frequent and severe infections. The predominant pre disposing factors associated with candidemia in our study were patients undergoing chemotherapy associated with malignancy (23%), followed by usage of central vascular catheters (18%) in long term stays in ICUs. This might be because of severely immunocompromised patients being cared for with most of them being on life support systems (4).
Amphotericin resistance was 5% in *C. albicans* with MIC value of 2µg/mL in our study followed by *C. tropicalis* (3%) with MIC value 0.38 µg/mL and *C. glabrata* 3% with MIC value 0.75 µg/mL. These observations were consistent with various studies comparing the MIC of candidemia isolates (71,196). We observed 35% fluconazole resistance with MIC values of >64 µg/mL. The similar pattern was found that 34% of bloodstream isolates were resistant to fluconazole (197). The resistance to fluconazole is of great concern because it is the most common azole used for the treatment of disseminated candidiasis including candidemia. The prophylactic use of fluconazole, especially, may be an important reason for the higher frequency of non-*Candida albicans* species with resistance. Similar data in the literature indicates that *C. krusei* is inherently resistant to fluconazole (198).

Voriconazole displayed the greatest spectrum of activity against candidemia isolates which is similar to the earlier study shows only 4.3% of resistance (199). An interesting finding of our study is caspofungin resistance was not encountered. Our finding is concurrent with the earlier study reported without any caspofungin resistance in 8197 *Candida* strains (4). Changes in the medical and surgical management of patients over the past 15 years are likely to influence the dissemination of *Candida* species that form part of commensal flora to a pathogen. With the increased incidence of candidemia and the growing number of resistant antifungal agents, our study also underlines the importance of *Candida* speciation and antifungal susceptibility for proper therapy in candidemia.

### 5.4 Candiduria

Candiduria is a common nosocomial infection, which involves the urinary tract system. The frequency of candiduria detected in this study is 21% which is in agreement with previous studies (200,201). This is in contrast with the study of Anchal *et al.*, that rate of occurrence of candiduria was 5.37% (202). Therefore the occurrence of candiduria varies considerably in the hospital settings. The present study demonstrated a significant rate of *C. albicans* (35%) strains from candidiasis. Similarly Kobayashi *et al* and Safdar *et al* reported incidence of *C.albicans* to be 35.6% (200,203). So it is reasonable to assume that *C. albicans* are the commonest species causing candiduria.
At the same time, the shift in Candida species to *non- Candida albicans* also noted in our study at the rate of 65% which is supported by the study of Mohammad *et al* at the rate of 55% (204). The increase in the frequency of *non- Candida albicans* was due to the prolonged or intermittent treatment with antifungal agents. Moreover drug resistance is a major cause of treatment failure in these patients.

The common predisposing condition is diabetes mellitus (48%) which correlates with the study of Mohammad *et al* and Deorukhkar *et al* showed 38% and 55% of candiduria in their studies (204,205). As in our study, the patients were elderly and female, and they had numerous co morbid medical conditions. The proliferation of *Candida* in diabetic patients may be attributed to high level of blood sugar, which acts as energy source to the yeasts (181). This clearly suggests that candiduria is common in elderly; debilitated, diabetics hospitalized populations with increased use of indwelling drainage devices (206,207). Hence, the studies from different parts of the world show that females have more predilections towards urinary tract infection, most probably due to short urethra in the females.

The antifungal spectrum of fluconazole resistance in candiduria showed 30% resistance in *C. glabrata* and *C. tropicalis* (23%). We observed no amphotericin resistance except *C. krusei* (4.2%). A study from India reported that 75% of all the isolates were resistant to fluconazole. They however not reported amphotericin B resistance (208). Paradoxically recent studies reported 6.3% of amphotericin resistance in *C. krusei* (196,202). A remarkable point of our study is that the most commonly isolated species was *non- Candida albicans* which can pose a serious threat due to resistance to the routine antifungal agents. Such agents are very effective, but in many countries especially in the developing nations, they are very expensive or not available to the respective patients.

### 5.5 Device related candidiasis

The occurrence of catheter-related infection varies to a great extent with the factors like the materials used, duration, adherence ability and virulence factors of pathogens and clinical situation of the patients (209,210). Catheterization is an essential feature of modern medical practice, particularly in the case of surgical treatment, transplant recipients and life threatening patients that require nutritional support, blood products and multiple drugs (211).
We obtained about 8% device related infections in our study. Contrary to our finding a study reported 15% of catheter related infections with a high mortality rate as 30% (212,213). In catheter related infections also *C.tropicalis* (35%) was the predominant isolate. The major source of catheter related infections are foley’s catheter (46%) followed by suction catheters (35%) (211). An interesting finding of our study is prolonged catheterization and mechanical ventilations predispose the colonization of *Candida* in these sites. So the duration of hospital stay was found to be directly proportional to the rate of candidiasis.

5.6 Oral candidiasis

In patients with oral candidiasis, a shift in species from *Candida albicans* to non-*Candida albicans* (14%) has been seen. The predisposing factors important for the development of oral candidiasis in our study is patients with an impaired immune system, such as those undergoing chemotherapy for cancer 43% which is similar to the earlier studies (214,215). Our data revealed only one amphotericin B resistant strain in *C. albicans* and fluconazole resistance was common in *C.krusei* (66%). Previous studies reported very high rate of fluconazole resistance in non-*Candida albicans* (216-218).

A significant finding of our study is fluconazole resistance is a major problem in the treatment of oral candidiasis. A newly raised concern about the wide spread use of fluconazole is the potential for development of azole-resistant *Candida albicans* and non-*Candida albicans* species which further complicate the management of infection.

5.7 Onychomycosis

Onychomycosis is a fungal infection of the fingernails or toenails that causes discoloration, thickening, and separation from the nail bed. Onychomycosis is a common infection due to candida species with an occurrence rate of 6% in our study. Our results are comparable with the study of Dyanne *et al* reported 10% rate of candida nail infections (219). Similar to the earlier reports nail infections are related to peripheral vascular disease and diabetes mellitus (220). Our study highlights that no antifungal resistance in *C.albicans* from nail isolates. Previous studies too have revealed that no antifungal resistance in *C.albicans* (221).
5.8 Central nervous system candidiasis

We reported two cases of neonatal meningitis caused by C. albicans and C. tropicalis which showed amphotericin and fluconazole resistance. The other two cases were originated from oncology patients undergoing chemotherapy and among that one case of C.tropicalis is reported from a patient with a prolonged external ventricular shunt. A similar finding is reported in the study of Jay et al (222). The isolation rate of Candida from cerebrospinal fluid in this study was 1% which correlates with the study of De et al (223). All the four patients were expired during the period of study. All the four isolates were resistant to fluconazole and flucytosine. Amphotericin resistance was seen in one isolate of each C.albicans and C.tropicalis.

5.9 Antifungal resistance

The resistance to fluconazole is distress, not only because it is a cost effective drug but it is also the most common azole used for the treatment of candidiasis. The high rate of nephrotoxicity of amphotericin B, and other adverse effects limited its use (224). It was determined that fluconazole-resistant Candida isolates were sensitive to caspofungin and the treatment ofazole-resistant candidiasis with caspofungin was successful (225).

Currently, an increase in the number of yeasts that are resistant to antifungal drugs is recognized worldwide, therefore, the vitro antifungal-susceptibility may aid the clinician in choosing an appropriate therapy (226). Antifungal-susceptibility testing should be carried out routinely for all invasive infections caused by Candida species to observe the epidemiological trends and to detect the emergence of antifungal resistance. Candida isolates were more susceptible to amphotericin B and flucytosine to that of azoles with increase in resistance to fluconazole is a matter of great unease as it is the most commonly used azoles for the treatment. Voriconazole exhibited greatest activity against isolates of C. albicans, C. glabrata, C. tropicalis and C. krusei in our study. The data presented in this study indicated that caspofungin and voriconazole are more effective than either amphotericin B or fluconazole against all non-Candida albicans species. The prophylactic use of fluconazole in immunosupressed patients may be an important reason for the higher rate of resistance in non- Candida albicans species (224,225).
Although the CLSI M27-A broth micro dilution method remains the standard antifungal susceptibility testing method, it is not convenient to carry out on a routine basis. Alternative methods including the broth colorimetric micro dilution, flow cytometry and the disc agar diffusion methods, automations have been developed. Therefore this study emphasizes the need for rapid and precise speciation of Candida isolates for effective treatment and management strategies.

The present study also is in favor of the need for periodic surveillance of the antifungal susceptibility pattern of the prevalent Candida species. The emergence of non candida albicans resistant to azole confirms the importance of monitoring changes in the distribution of pathogenic Candida species. Captivating the relative importance of risk factors and the colonization by Candida species, an approach for the clinical diagnosis and practical management of critically ill patients suspected to have or at risk of severe candidiasis has to be made in the hospitals.

5.10 Virulence factors detection

Virulence factors particular for Candida species are directly involved in adhesion and invasion of host tissues, biofilm formation and hedging of the immune system. Our result also indicates that even though all the isolated strains were pathogenic, not all strains of Candida produced proteinase and phospholipase and biofilm as virulent factors. So the virulence of Candida species is attributed not to a single factor but to a combination of several factors, like proteinase, phospholipase and biofilm production (227,228,229).

5.10.1 Phospholipase

It was seen that 13% of C.albicans expressed strong phospholipase activity followed by 7% C tropicalis and 6% C.glabrata in our study. Maximum phospholipase production was seen in C.albicans isolated from high vaginal swabs (38%) and blood (24%). Studies reported that C. albicans species isolated from blood produced more phospholipase than non- Candida albicans (229,230). The virulence factors expressed by Candida to cause infections may vary, depending on the type of infection, stage of infection, the site of infection, and the nature of the host response. In Candida species phospholipase release the fatty acid by hydrolase and lysophospholipase transacylase seen mainly at the tip of hyphae (231). Phospholipase production was predominantly
seen in vaginal isolates in the present study possibly due to higher number of *C.albicans* in this specimen.

### 5.10.2 Proteinase

The proteinase appears to be an important virulence factor of *Candida* species by helping them in the colonization and invasion of host tissues (1). The rate of occurrence of proteinase producers of *Candida* in the present study was 43% out of 336 isolates. *Non- Candida albicans* (46%) were the major proteinase producers in our study. A previous study showed *C. albicans* were the predominant proteinase producers and only eight *non- Candida albicans* strains were proteinase positive (232). But in our study, that is entirely different with *C.tropicalis* (27%) were the major proteinase producers and followed by 25% isolates of *C. albicans*. Proteinase activity was seen in 38% of *non –Candida albicans* species in the study of Sachin Deorukhkar *et al* reported 40%, which is consistent with our study (233).

Proteinase production was more common among blood isolates (28%) in comparison with high-vaginal isolates (18%). This is more or less similar to the studies of Chaitanya *et al*, showed 35% blood isolates and 13% in high-vaginal isolates (234). It has been reported that the enzymatic activity of *Candida* species may vary depending on the species and source of isolates (235). Proteinase has specific functions during the infective process, includes digesting molecules for nutrient acquirement, alter the host cell membranes to facilitate adhesion and tissue invasion, and digesting cells and molecules of the host immune system to avoid or resist antimicrobial attack by the host (7,236).

### 5.10.3 Biofilm

In the current study *C. albicans* and other *non- Candida albicans* species were evaluated for their ability to produce biofilm. Out of 336 isolates 227(68%) were biofilm producers. *Candida albicans* (31%) were the predominant biofilm producers from vaginitis. *C. albicans* consistently produce more biofilm in vitro than *non* *C.albicans* isolates was well correlated with other studies reported 47% and 35% *C.albicans* biofilm producers from vaginitis (237,238).
Among the non Candida albicans species, biofilm production was more in C. tropicalis (27%) isolated from blood samples in the present study. Biofilm positivity occurred most frequently in C. tropicalis, in various studies of Vinitha et al (36%) and Agwan et al (42%) (190,228). Biofilm formation rate was higher in non Candida albicans species than in C. albicans strains by the micro plate method (239).

According to our study also, it was found that the biofilm formation rate was higher in non -Candida albicans because of the emergence of antifungal resistance. Biofilm producing Candida species are known to be more resistant to immune response and antimicrobial agents, which lead to treatment failures. The ability to form extensive biofilms on the surface of catheters, and other prosthetic devices, also contributes to the high prevalence of Candida as an etiologic agent of intravascular nosocomial infections (240). The presence of the matrix in the biofilm, which restricts the penetration of drugs by formation of a diffusion barrier; hence, only the most superficial layers are in contact with lethal doses of drugs. So biofilms are less susceptible to antimicrobial agents (241).

In this study, we used XTT staining method for biofilm detection that reduced by mitochondrial dehydrogenases of metabolically-active cells, and it represents the best method used for biofilms. XTT reduction is a very sensitive and quick reaction according to our results. It is supported by the studies which show XTT reduction is a very sensitive for Candida biofilm detection (242-245).

5.11 Characterization of Wickerhamomyces anomalous killer toxin

The advancement of antifungal resistance does not give off an impression of being an issue amid their fleeting use, yet is a chief concern for long term treatments.” Mutation of targets within the organisms or by transfer of resistance genes from other organisms by transformation or conjugation is the mechanism of drug resistance. And these resistances spread hastily among other microorganisms. So the increasing antifungal resistance of Candida species limited the choice of treatment options and stimulates the interest to search for an alternative anti-Candida agent from a natural source (141). Killer toxins are reported to have the potential for treatment of fungal infections, which are distributed in several environmental sources (246). Yeast killer strains are differentiated by a killing spectrum based on the sensitivity and resistance of other yeasts. In this study, we aimed to isolate and characterize the yeast killer
protein secreted by the yeast *Wickerhamomyces anomalous*. Several species of environmental yeasts *Wickerhamomyces* species have been reported to have the killer character (18,121,141,247,248). We used the yeast killing spectrum to identify the killer phenotype of an environmental strain of *Wickerhamomyces anomalous* against the pathogenic *Candida* species.

5.12 Growth conditions for killer toxin production.

In our study, we determined the optimum culture conditions for the production of *W. anomalus* killer toxin. The activity of the yeast killer proteins was depended on pH, temperature and composition of the medium. So the killer toxin assay was carried out in YEPD with methylene blue agar containing 5% glycerol buffered to pH 4.5 with 0.1 M citrate-phosphate buffer with glucose. *W. anomalus* killer toxin activity reached its peak after 72 hours of growth at pH of medium from 5.0 to 5.5 at 25 °C. This coincides with the findings of Young *et al* and Sawant *et al* which shows the optimum pH of 5.5 and temperature 25°C (11, 17). Our findings indicate that glucose is needed for toxin production which is supported by the study of Bar-Shimon *et al* (249). Killer toxins generally have a drawback related to their temperature and pH stability. Killer toxins generally stable at pH 4-5 and 20–25°C (3).

5.13 Purification of killer toxin.

In order to improve the reproducibility of procedures for killer toxin assay, a few authors suggested the use of more or less partially purified killer toxins (6, 17,250). Our study supported the evidence of purified form of killer toxin carries more antifungal ability. The primary method to obtain purified *W. anomalus* killer toxin from the YEPD supernatant is ultrafiltration at 4°C in our study. Similar results are documented in various studies with filtration at 4°C (251,252). Contrary to our findings were reported in previous studies that ultrafiltration at 25°C by using different environmental strains of killer toxin (115).

The killer toxin obtained with (NH₄)₂SO₄ precipitation showed high killer activity 70% in our study rather than acetone 11 %we used first in our procedure (7). This appears to be another rare finding of our study that the killer toxin is not stable in acetone. The purification procedures were not standardized because of the variations in the constituents of toxins of different genera (252). The pH and ionic strength are
essential to reduce the interaction between the column packing and sample in gel filtration. Our results are consistent with the reports that the column used G-100 is also very important factor for the separation of toxin in order to obtain the best resolution without losing the killing activity (13, 253,254).

5.14 Molecular weight determination of killer toxin.

Another interesting finding of our study is the purified killer toxin is a protein with molecular weight 42.6 kDa with a single band, estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protease treatment suggests that it is a protein with sulfide bonds like several other killer factors and papain has been revealed its toxin destruction activity. This appears to be another rare finding of our study that a new killer toxin is identified from W.anomalus species which is susceptible to pathogenic Candida species.

These findings are in agreement with other studies characterized different types of killer yeasts having molecular mass K1 (20kDa), K2 (21kDa), K3 (42kDa), PaKT (105kDa) type killer proteins (255-257). Young et al recognized distinct groups (K1-K11) of killer toxins with respect to cross-killing and immunity interactions among killer strains of the yeast genera (258). The present study demonstrated a different killer toxin from W.anomalus species rather than the previous discoveries. Killer toxins differ between species or strains, showing diverse characteristics in terms of genetic location, molecular size, structure of the protein and mode of action. The diversity of killer proteins of W.anomalus with molecular masses ranging from 8 kDa to more than 300 kDa is already established (259-261). However, this wide range in protein size may arise mainly from different levels of glycosylation (256).

5.15 pH and temperature stability of W. anomalus killer protein

However, the W. anomalus protein isolated from our study exhibited basic characteristics of mycocin with pH 5–5.5 and temperature 25°C. It is active only at moderate temperature (25°C for 10 h) and at acidic pH (5 to 5.5), whereas the killer protein of W. mrakii (designated HM-1 or HMK) is a monomer and is stable at higher temperatures (100°C for 10 min) and wider range of pH (2 to 11) (258,261). Thus, the killer protein of W. anomalus of present study is different from those of the K1 to
K10 classes (258). Like most toxins, \textit{W. anomalus} is unstable at both high temperatures and high pH values (262-265). These characteristics of killer toxin highlight its use as a novel antifungal agent. The protein isolated from \textit{W. anomalus} exhibited basic characteristics of mycocin with pH 5.5 and temperature ranging from 4 to 25°C.

\textbf{5.16 \textit{Wickerhamomyces anomalus} killer toxin action against pathogenic \textit{Candida} species}

Microorganisms gain resistance to conventional antimycotics especially in long term usages and these resistances spread very rapidly. Fluconazole has been widely used since 1988 for the treatment of \textit{Candida} infections. The increased fluconazole resistance has made difficult to treat candidiasis (266). We have purified and characterized the yeast killer toxin of \textit{W. anomalus} and determined the actual killing spectrum of this toxin on human pathogenic \textit{Candida} species. \textit{W. anomalus} producing anti-\textit{Candida} toxins have been reported in several studies (246,247,255).

One of the most important aspects of this study is the good activity (87%) of yeast killer toxin against the pathogenic strains of \textit{Candida} species including both clinical and standard isolates tested which correlates with the antagonistic action of \textit{W. anomalus} (WC65, NCYC 432, 434, YF07b) killer toxin against other fungus (253,267,268). \textit{Debaryomyces, Kluyveromyces, Pichia, and Saccharomyces} genera were characterized by their killer activity in different conditions were reported in different studies (269,270). \textit{W.anomalus} DBVPG 3003 secretes an ubiquitin like protein (Pikt) that has antimicrobial activity against different pathogenic yeast (270). Numerous reports are available about the antagonistic action of yeast killer toxins against same or closely related yeast or fungal species (272-274). So to the best of our knowledge, the present investigation reports a new \textit{W. anomalus} killer toxin against the human \textit{Candida} species.

\textit{C.albicans} was more sensitive (83%) to \textit{W. anomalus} killer toxin than \textit{C.tropicalis} (48%) at high temperature while \textit{C. tropicalis} (79%) were more sensitive than \textit{C. albicans} (32%) at low temperature in our study. This was similar to the earlier reports that the crude killer toxin produced by \textit{D. hansenii} P41 was tested at 37°C against
different *Candida* species and showed 100% killer activity against *C. glabrata, C. haemulonii, C. incospicua,* and *C. parapsilosis* (246).

More recently, killer toxin produced by *W. anomalus* has shown killer activity against *C. albicans* at pH 3.5 and 16°C in the study of Guo *et al* (265). The results confirmed that *W. s anomalus* isolated from cashew leaves demonstrated killer activity against *Candida species,* and the same killer toxin from *W. anomalus* can act differently in different species, temperature and pH conditions.

This coincides with the findings of various studies that proved the anti *Candida* activity in yeast killer toxins (130,255 257,262, 265,274). Therefore our study emphasizes the need for the detection of yeast killer toxins from environmental strains, which can be used as antimicrobial agents.

We evaluated the killer toxin assay of biofilm producers and non-biofilm producers of pathogenic *Candida* species including standard strains as control and it was found to be effective on 87% of biofilm producers and 90% of non-biofilm producers. Antifungal resistant and sensitive strains were also compared with killer toxin action and it showed maximum killing capacity on both resistant (91%) and sensitive strains (90%). From this we can assume that drug resistance or biofilm formation is not interfering with the killer toxin action and this feature is highlighting the future use of these natural killer toxins in therapeutic area (265,274)

This paper validates the observation that different killer toxins are produced by different isolates of the same species (267,268). They can produce multiple toxins with different spectrum of action and stability. Yeast killer toxins are proteins that are too large to be used in systemic antifungal therapy without eliciting an immune response (24). If they could be delivered without encountering the immune system with adverse effects on human cells they might prove useful. The susceptibility of pathogenic yeasts to killer toxin produced by yeasts, belonging to the genera *Wickerhamomyces* and *Williopsis,* could lead to the design of synthetic derivatives to be used as antifungal agents (125,275).

A recent study by Serena *et al* showed that the extracellular exoglucanase-encoding genes WaEXG1 and WaEXG2 from the three strains of *W. anomalus* were sequenced and were found to display noticeable similarities to those from known potent *W.*
anomalus killer strains (275). A study reported from Kashmir, India, that P. kudriavzevii RY55 toxin exhibited excellent antibacterial activity against several pathogens of human health significance such as Escherichia coli, Enterococcus faecalis, Klebsiella species, Staphylococcus aureus, Pseudomonas aeruginosa and Pseudomonas alcaligenes (276).

The environmental yeast killer protein of W. anomalus can be used for alternative treatment of candidiasis with further assessments has to be evaluated in future studies. Yeast killer protein was proved to have a wide antifungal spectrum on pathogenic Candida species and can be proposed as a novel antifungal agent for alternative treatment of candidiasis, especially for the mucosal fungal infections such as vulvovaginal candidiasis (278).

The extensive use of antifungal agents to treat fungal infections lead to the development of acquired resistance in C. albicans and non-Candida albicans species (113). The development of newer antifungal formulations especially with high selectivity on pathogenic yeast cells picked up significance. This study reveals the potential of yeast killer protein as a potent antifungal for alternative treatment of candidiasis in future. The broad killing spectrum of W.anomala killer toxin with high stability at pH 3- 5.5 and at temperatures up to 25°C highlights the potential use of this protein in environmental, industrial and medical field as an antifungal agent (279). Izgu et al have shown that the K5 killer toxin of W. anomalus displays activity against dermatophytes like Microsporum species and Trichophyton species (165).

Yeast killer toxin production and preservation methods also depends on several factors like culture medium, temperature and pH along with diversity of toxins between species or strains, its genetic location, molecular size, structure, mode of action and cost of production has to be evaluated (280,281). These uncertain inquiries in regards to the killer toxins can determine and resolved in future by this study.