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Effect of Paneer Booti During Kinetics on Candida

Tuba Batool[1], Sayyada Ghufrana Nadeem[1]*

[1]Department of Microbiology, Jinnah University for Women, Karachi-74600.

ABSTRACT

Candida albicans **is a dimorphic fungus and a commensal of skin in humans. It is opportunistic fungal pathogen that may cause localized as well as systemic infections. As the pathogenecity of fungus is increasing, the demand for effective antifungal agent is also increasing. In the present study the macrodilution, microdilution and time kill curve methods were used to evaluate the effect of paneer booti during growth kinetics of Candida. In macrodilution the most effective concentrations of paneer booti were found to be 10^{-1} and 10^{-2}. Similarly fluonazole 's concentration of 10^{-1} and 10^{-2} effect the growth after 4 hours and 10^{-3} and 10^{-4} effect the growth after 5 hours. In microdilution, magnesium and calcium enhanced the growth of** *C. albicans* **after 7 hours. In 10^{-3} and 10^{-4} concentration the inhibition of** *Candida albicans* **occured after 8 hours and fluconazole and paneer booti were found to be effective on its growth. The time kill curve of** *Candida albicans* **showed that paneer booti and fluconazole were effected against the** *Candida albicans* **and clearly showed the four phases of growth (lag, log, stationary and decline). As paneer booti was found to be effective against the pathogenic specie of Candida i.e.,** *Candida albicans,* **therefore, it can be use for the treatment of infections that are caused by this pathogenic agent.**

Keywords: Microdilution, Growth curve, Fluonazole, Paneer booti, *Candida albicans*.

INTRODUCTION

Candida albicans is a widespread opportunistic fungal pathogen that grows either as a yeast or as filamentous hyphae, depending on the environmental conditions (Pranjape *et al*.,1990). *Candida albicans,* the causative agent of mycotic infections collectively known as candidiasis, is a convenient organism for studying the regulation of differentiation. Antifungals are supplements that make use of their natural ingredient to inhibit harmful fungi. No treatment of Candida infection is complete without an effective antifungal. The rising incidence of serious fungal infections has created an increased demand for reliable methods of in vitro testing of antifungal agents that can assist in their clinical use. The National Committee for Clinical Laboratory Standards (NCCLS) has developed a standardized broth macrodilution method for the testing of *Candida spp.* and *Cryptococcus neoformans* which has greatly improved the reproducibility of antifungal susceptibility testing and serves as the "gold standard". Time-kill testing has become an indispensable tool for assessing the activity of antimicrobials against microorganisms. Standardized methods providing instruction on the implementation of time-kill methods have been proposed by the National Committee for Clinical Laboratory Standards to ensure the reproducibility and accuracy of test results.(Klepser *et al*. 1998).

Several studies have used spectrophotometric determination of endpoints to eliminate such subjective interpretation and revealed good agreement with the NCCLS recommended method (Blanco, *et al*. 1992, Pfaller, *et al*. 1995). Fungistatic agents as azoles (ketoconazole [KTC], fluconazole [FLC], and itraconazole [ITC]) and flucytosine (5FC) show less

*Corresponding author: tubabatool@live.com

defined endpoints and introduce significant subjectivity into the reading of results.

MATERIALS AND METHODS

Type of sample: Candida albicans was used as a sample.

Equipments: Glass wares (pyrex), syringes (mediocre), incubator (Mermmet), autoclave (Memmert), spectrophotometer (Germany), ELISA well reader (Germany), juster (Eppendroff), oven (Dawlance), filter paper (whattman no.1).

Chemicals and medias: RPMI 1640 medium, Sabouraud dextrose agar (SDA), Potato dextrose agar (PDA), magnesium sulphate ($MgSO_4$), calcium chloride ($CaCl_2$), herb (paneer booti).

Antifungal agent: Fluconazole was used as a antifungal. The stock solutions of fluconazole were prepared in sterile distilled water. Fluconazole we used that was 240 mg, it was dissolved in 20 ml of sterile distilled water and then made the working solution.

Test isolate: Candida albicans was used in this experiment. We made the suspension of *Candida albicans* from 48 hours old culture and inoculated in 20 ml sterile distilled water. Compared the suspension with 0.5 McFarland tube (Moore *et al*., 2003).

Herb extraction: Paneer boti was used to check the effect on kinetics of Candida. The herb extraction was prepared in 15ml of sterile distilled water (3:15) dilution, then boiled it into the oven for atleast 5 to 10 minutes. After that passed the whole suspension from whattman filter paper (no.1) in a sterile flask and stored at 0-4 °C for further experiments and analysis (Huda-Faujan *et al*., 2007).

Metals dilution: Two dilutions of magnesium sulphate ($MgSO_4$) were made i.e., 0.5M and1M. For 0.5M, 0.6g of $MgSO_4$ were dissolved in 10 ml of distilled water, for 1M , 1.2g of $MgSO_4$ were dissolved in same quantity of distilled water (Pranjape *et al*.1990). Two dilutions of calcium chloride ($CaCl_2$)were made i.e., 0.5M and 1M. For 0.5M , 0.5g of $CaCl_2$ were dissloved in 10 ml of distilled water. For 1M, 1.1g dissolved in 10ml distilled water (Pranjape *et al*.1990).

Microtiter method: The method used was a microtitre modification of the NCCLS M27 (A method5 in flat-bottomed microtitre plates with either YNBG or RPMI-G broth). The yeast suspensions used for the macrodilution method were then adjusted further (Moore *et al*., 2003). Each well contain 40µl RPMI and same amount of yeast suspension(40µl). The first 2 well contain 40µl of 0.5M and 1M of $MgSO_4$ and other 2 wells contain same quantity of $CaCl_2$.

40µl of herb (paneer booti) was taken from other well and serially dilute it, same thing we done with fluconazole. Fluconazole was taken as a control. The microtitre plates were incubated in a moist chamber at 37°C for 48 hrs. After incubation, the microtitre plates were shaken for 5 min to obtain a uniform suspension before reading (Moore *et al*., 2003). Next day optical density was recorded at 492nm using ELISA plate reader at every 2 hours interval.

Macrobroth dilution method: The broth macrodilution method employed was that of the 'Report of a Working Group of the British Society for Mycopathology' (Moore *et al*., 2003). In this method 10 ml RPMI 1640 medium were taken in first test tube and 9 ml in other 3 tubes. In first tube put 1ml of fluconazole and serially diluted (10^{-1} to 10^{-4}), discarded from last one to get the equal quantity in all 4 tubes. After that add 1ml of yeast suspension in all tubes. The same procedure were performed with paneer booti (herb). Optical density(OD) was taken from spectrophotometer, first was taken at 0 hr. and then incubate test tubes into shaking water bath. Other OD readings were taken at every 1 hour of incubation and after taken OD put test tubes again into the shaking water bath till next OD reading.

Time- kill curve method: Time-kill curve studies were performed in standard RPMI by the method described by Klepser *et al*. (Klepser, *et al*.1998). Before the tests were performed, the isolates were subcultured at least twice and grown for 24 h at 35°C on SDA plates. The inoculum was adjusted spectrophotometrically to the density of a 0.5 McFarland turbidity standard at 530 nm. The adjusted inoculum suspension was diluted 1:20 in RPMI containing the appropriate concentrations of fluconazole and tubes with the test solution were incubated at 35°C without agitation; the final volume was 5 ml. Optical densities were taken at 1 hr. interval. Volumes of 0.1 ml, (depending on the dilution and the concentration of the drug) were spread onto SDA plates and incubated at 35°C for 24 to 48 hr. to determine the number of CFU per milliliter (Canto´n, *et al*., 2004.).

RESULTS AND DISCUSSION

In this study the effect of herb i.e., paneer booti was observed on Candida. Herbs are found effective against different *Candida species* but here we used the only one specie of candida that was *Candida albicans*. In previous studies, it was reported that methanol was a better solvent for the consistent extraction of antimicrobial substances from medicinal plants compared to other solvents such as water, ethanol and hexane. The extract *P. endlicherianum* was observed against *B. megaterium. E. coli, K.pneumoniae, P. vulgaris, C. albicans, C. tropicalis.* Their results showed that the methanol extract of *V. georgicum* has inhibitory effect on the growth of all *Candida albicans* isolate (Sengul *et al*.2013). But in this study, only water extraction method was used and this showed the effective result on *Candida albicans* (Table I). In macrodilution method we serially diluted the herb (paneerbooti) from 10^{-1} to 10^{-4} and according to our observations all four concentrations were found to be effective on *Candida albicans* growth curve. 10^{-1} and 10^{-2} concentration of paneer booti was found to be more effective (Figure 1), because the first phase (lag) occurred and remained at 4 hours , the log phase started at 6 hours and after 6 hours the stationary phase started but it did not remained long and decline occurred after 8 hours (Figure 1). The concentration 10^{-3} and 10^{-4} of paneer booti showed that first phase (lag) started and remained at 2 hours, after 2 hours log phase started and remained at 4 hours and decline occured after 4 hours but slight differences were seen in concentration 10^{-4} , log phase was started after 2 hours and stayed for 4 hour and then stationary phase started and this phase remained longer and after 6 hours decline phase occurred (Figure 2). It means that the first two concentration rapidly affect the *Candida albicans* growth and *Candida albicans* was not be able to survive for longer time. Other concentrations of paneer booti effect slowly thats why Candida showed stationary phase for long time.

As control we used fluconazole (antifungal) to observed the difference between the fluconazole and paneer booti that how they both effect the growth of *Candida albicans*. Fluconazole exhibited fungistatic (99.9% decrease in the log10 of the number of CFU/milliliter compared with starting inoculum) activity against each of the test isolates; however, three distinct effects on growth were observed (Klepser, *et al*.1998). According to our result *Candida albicans* was found to be highly sensitive to fluconazole (Table I). Initially the growth of *Candida albicans* was increased but after some time it showed decrased growth (Figure 3). The same macrodilution method was done with fluconazole that was already done with paneer booti. In fluconazole we made serial dilution (10^{-1} to 10^{-4}) and according to (Figure 3 and 4) all concentrations showed the effectiveness on *Candida albicans* growth. At the starting Candida growth was high but gradually decrased with time. In the next method that was microdilution 96 wells plate was used. In this method magnesium and calcium was used to checked either they are effecting *Candida albicans* or not. If Mg values were to be expressed on a dry weight basis, cell Mg would remain relatively constant since the dry weight of *C. albicans* mass increases by 100% over this period. However, the gradual increase was interrupted by a

peak of Mg content per cell which coincided with the onset of germ-tube formation. This peak represents a three-fold increase indicating a transient, but net, increase in cell Mg at this time. In a further test, cells were added in $CaCl_2$, (10 mM) and incubated for 3 hrs. About 70% of the cells were able to form germ tubes (Pranjape *et al*.1990). According to our results magnesium and calcium both enhanced the growth of *Candida albicans*. We used two concentrations of magnesium and two concentrations of calcium that is 0.5M and 1M (Table II). This result showed that firstly Candida was in lag phase and growth was not increased and later on the magnesium and calcium effected on *Candida albicans* growth, both enhanced the growth of *Candida albicans*. There was little effect of concentration seen on growth i.e., 1M of magnesium and calcium effect was higher than 0.5 M, but there was not a big difference between both concentrations. In this method we used the magnesium and calcium as growth enhancing agent because the results clearly indicated that the *Candia albicans* growth increased after the incubation.

In this microtiter method we also used the fluconazole as control. With NCCLS breakpoints, seven isolates were resistant, one intermediate and the remainder susceptible. With BSMM breakpoints (Moore *et al*., 2003), seven isolates were resistant, five intermediate and the remainder susceptible. The seven isolates were classed as resistant regardless of breakpoint use (Moore *et al*., 2003). According to our results *Candida albicans* showed sensitivity against fluconazole and both the dilutions were found to be effective. In this way, we also checked the effect of paneer booti on *Candida albicans*. The survival of resting cells decreased immediately and rapidly with EGC, GC, EGCg and GCg, and the survival rate was <1% after 4 h. A few colonies still survived after 24 h of culturing (Hirasawa *et al*., 2003). *Candida albicans* is sensitive to paneer booti in method as well. In (Figure 4) show effect of paneer booti on Candida. Here we diluted the paneer booti in RPMI 1640 broth, the both concentrations clearly indicate that *Candida albicans* were not able to grow further. Colonial changes of *Candida albicans* was also futher checked. It was cultured on SDA or PDA to see the changes appeared on growth. In general, worse reproducibility was observed with visual endpoint determinations than with spectrophotometric readings (Cuenca-Estrella *et al*., 2001). When we observed the plates after incubation it shows the colonial changes (Figure 1). The colonies were large and white in colour but after several period of time there was gradual changes occurred in colonies. In Figure 2 changes were clearly shown and the colonies become small and mucoid. These are herb concentrations that effected the *Candida albicans*. Figure 3 and 4 showed the effect of fluconazole on *Candida albicans*. Initially the colonies were large in number but later on the number of colonies were decreased and morphologically changed. A time-kill plot of the activity of voriconazole against a representative isolate from each of the fungal species tested is presented in a previous paper in which fungistatic activity was observed with voriconazole against all seven isolates. For the isolates of *C. neoformans* and *C. glabrata* and the isolate of *C. tropicalis*, voriconazole concentrations greater than the MIC did not appreciably increase the rate or extent of fungistatic activity. Against both isolates of *C. albicans*, however, the maximal fungistatic activity was observed with voriconazole concentrations greater than or equal to four times the MIC (Klepser *et al*., 1998) According to our results all four phase appeared that is lag , log stationary, and decline. The growth curve of *Candida albicans* is obtained by using both methods. The time period given to paneer booti and herb that were from 1 to 9 hours and in between these hours all four phase have observed.

At the end we concluded that the herb (paneer booti) we used against the *Candida albicans*, was effective, not only one concentration but all were found to be effective. It means that paneer booti can be use for the treatment of infections caused by *Candida albicans.*

Table I. Compare the effect of fluconazole and paneer booti (herb) on *Candida albicans* at different concentrations by spectrophotometer.

Time Period	Fluconazole				Paneer Booti			
	Different Concentrations				Different Concentrations			
Hours	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	0.400	0.271	0.226	0.366	0.112	0.147	0.103	0.120
2	0.336	0.233	0.196	0.209	0.110	0.155	0.112	0.126
3	0.225	0.189	0.179	0.154	0.102	0.150	0.199	0.122
4	0.153	0.154	0.154	0.132	0.111	0.146	0.254	0.172
5	0.119	0.132	0.144	0.120	0.138	0.164	0.266	0.180
6	0.114	0.118	0.132	0.101	0.273	0.89	0.252	0.179
7	0.102	0.101	0.125	0.099	0.280	0.325	0.211	0.172
8	0.100	0.100	0.111	0.100	0.179	0.305	0.152	0.119
9	0.103	0.102	0.110	0.101	0.098	0.101	0.099	0.089
10	0.102	0.101	0.109	0.100	0.064	0.099	0.045	0.024

Table II. Comparison of fluconazole and paneer booti (herb) on *Candida albicans* by microtiter plates method.

Time Period	Fluconazole		Paneer Booti	
	Different Concentrations		Different Concentrations	
Hours	**1**	**2**	**1**	**2**
2	0.912	0.933	2.186	1.007
4	0.668	0.849	2.097	0.944
6	0.975	0.868	2.097	0.953
8	0.981	0.846	2.072	0.918
10	1.588	0.780	2.071	0.807
12	1.033	0.812	2.040	0.842
14	8.590	7.386	11.311	7.491
16	8.687	7.775	11.214	7.321
18	5.980	4.867	7.428	6.428
20	4.360	3.287	5.237	5.652
22	2.887	2.790	2.891	3.211

Figure 1

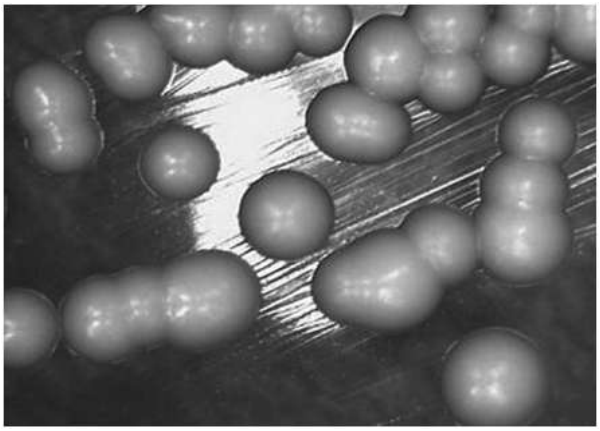

Figure 2

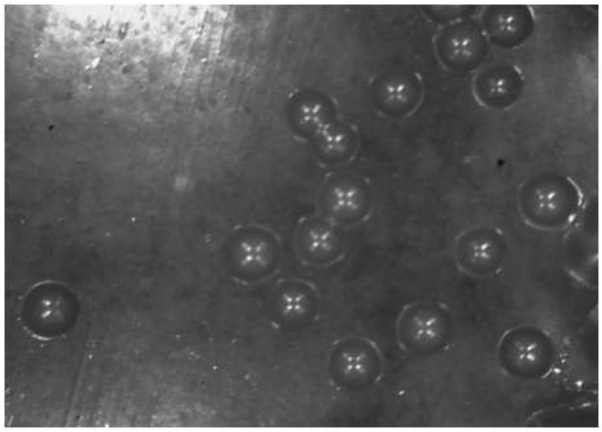

Figure 1. It is the early stage picture that clearly shows that the herb (paneer booti) affect very slightly on *Candida albicans* growth and due to which the large white colonies appeared.

Figure 2. It is the late phase picture in which the *Candida albicans* was affected more than in the early phase. It means that paneer booti (herb) effect gradually on *Candida albicans.*

Figure 3

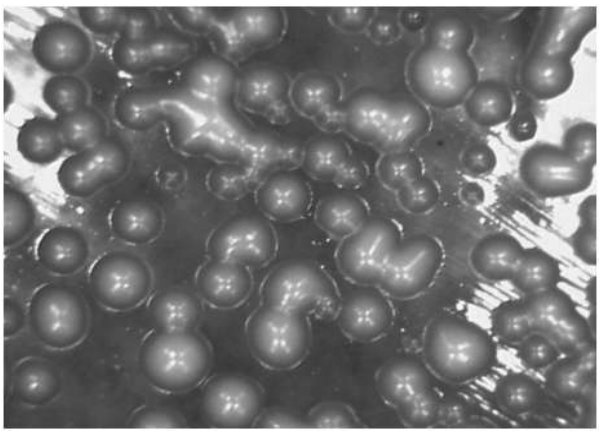

Figure 4

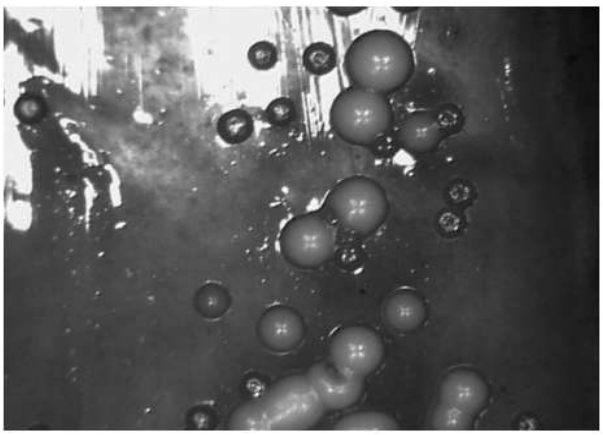

Figure 3. This is the early phase picture which shows the large number of colonies but slightly effected not white in colour.

Figure 4. This is the late phase picture in which the number of colonies are less in number and the colonies were morphologically changed.

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