Journal of Immunological Techniques and Infectious Diseases

Research Article A SCITECHNOL JOURNAL

Production of Biofilm In Vitro by Candida Species and its Inhibition from Natural Product

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Received date: May 5, 2021; **Accepted date:** May 20, 2021; **Published date:** May 27, 2021

Abstract

Candida species appear as a nosocomial fungal pathogen that causes infection predominantly in the individual having impaired immune system. It is the cause of invasive candidiasis with numerous cases reported among leukemia patients, after bone marrow transplantation. It naturally shows resistance from fluconazole, an antifungal agent which is prescribed frequently. The mortality rate caused by C. krusei is greater than the C. albican which is more common. Other Candida species that are similar to the profile of C. krusei are C. glabrata, C. parapsilosis, C. guillermondii, C. rugosa, and C. tropicalis.

The collection of the samples (n=50) was made by using Hi Chrome Candida differential agar medium. The presence of biofilm formation was identified by 96-well microplates by using Congo red agar and the test tube method. The inhibition of biofilm was performed by using garlic, ginger, and vinegar with different concentrations. Biofilm formation by 96-well micro titer plate showed positive results. The inhibition of biofilm of C. krusei by using different concentrations of ginger and vinegar showed higher activity against the biofilm while the garlic showed lower activity.

The purpose of the study is to check the capability of biofilm formation of C. krusei and other Candida species and analysis of the antifungal activity of natural products such as; garlic, ginger, and vinegar against the biofilm of Candida species.

Keywords: Candida species; Biofilm; Immuno compromised; Candidiasis; Hi chrome candida differential agar; Congo red agar

Introduction

The fungal infection is usually counted as opportunistic. It can reside in numerous parts of the body so it can exist as systemic or localized [1]. Fungi are the most common organism which can easily be found in the environment and it is the main cause of fungal infection [2]. The tropical region is the common place where fungal infections often occur. Few of them are severe and may cause the death of the individual. The risk factor of infection mainly depends on the immunological status of the patient and local factors such as trauma, moisture environment, or occlusive clothing that may increase the risk collectively within contact with the etiological fungi [3]. An individual having impaired immunity that has decreased the natural defense system or those who are heavily exposed to the fungus are more prone to infection [4]. The yeast is not usually causing any harm to humans unless the host's immunity, protective skin barrier, or normal flora of the body gets altered. Infection caused by Candida species mainly targets nails, skin, and also mucosal membrane. Clinically the superficial fungal infection can be diagnosed [5].

Candida is considered as normal flora of the body colonizing many anatomical parts such as the digestive tract, vagina, oral cavity, skin, and upper respiratory tract [6]. Globally the Candida species are the most common organism that is responsible for fungal infection. There are more than 17 various species of Candida that are identified as etiological agents of infection in humans. The genus is made of a heterogeneous group of organisms. More than 90% of invasive infections are occurred by different species of Candida like C. glabrata, C tropicalis, C. albican, C. krusei, C. parapsilopsis, C. lusitaniae, and C. dubliniensis. Yeast primarily invades and colonizes the body tissue by producing some pathogenic compound into the bloodstream that leads to symptoms like chronic diarrhea, lethargy, yeast vaginitis, muscles, and joint ache, infection in the bladder, cycle problems, the problem in passing stool, and anxiety or depression [7].

The principal-agent of candidiasis is C. Albicans, but other species are known generally as C. non-albicans (C. glabrata, C. tropicalis, C. krusei, C. parapsilosis, C. gullermondii) are also involved. C. Albicans and non-albicans species are closely related but differ from each other with respect to epidemiology, virulence characteristics, and fungal susceptibility, therefore Candida species identification is important for successful management of infection [8].

With time the candida spp. are associated with infection related to the biomaterial. The majority of manifestations of Candidiasis are linked with the development of biofilm either in one way or another on the biological surfaces, and both mucosal and systemic site infection is linked with this phenotype [9]. Biofilm formation is the surface attachment by microbial communities which is of different habitat. Biofilm defines as the community of microbe encased in a matrix of Extracellular Polymeric Substance (EPS), the phenotype of their free-floating counterparts is different from the displayed phenotypic feature [10].

The resistance to antimicrobial therapy is the main consequence of biofilm formation, for that reason, the infections associated with biofilm formation is often refractory to conventional antibiotic therapy [11].

Nowadays plants are the most focused object for the production of various beneficial drugs. There are various approaches to find out new innovative biologically active compounds in higher plants. The discovery of novel effective compounds is a result of the resources that are folk medicine and systematic screening [12].

Alliums Sativum (also known as garlic), *Zingiber Officinale* (also known as ginger), and vinegar have also been reported to have antifungal activity [13]. Nowadays, at least a few hundred antibiotics are known, but this does not mean a sense of security. The discovery of the plant extract for the treatment of microorganism infections was one of the most significant medical achievements of the twentieth century [14].

Materials and Methods

Collection of isolates

The sample collection for Candida species was made from different laboratories and hospitals of Karachi, Pakistan. The isolation of these strains was done by using standard microbiological techniques. These Candida strains were isolated from different clinical specimens which include blood, urine, vaginal discharge, and sputum pus as shown in (Table 1).

All isolates were transported to the Jinnah University for women in a closed box with an appropriate transport medium. They were sub cultured immediately and stored in the refrigerator for further processing.

Identification of isolates

Primarily to confirm the isolate's type of species and for their identification, they were processed for purification. The experiment was initiated with streaking on Sabouraud Dextrose Agar (SDA) plate to get isolated colonies after incubation of 24 hours at 37°C. A distinguishing single colony was picked with the help of an inoculation loop and streaked on the SDA (Sabouraud Dextrose Agar) slant. Based on morphological and cultural characteristics all the samples were identified.

Morphological characteristics

The morphology of all Candida species was determined through gram staining. The morphological characteristics involve the shape, arrangement, presence, or absence of pseudohyphae (formed by budding yeast) and the gram reaction of the organism. On gram staining, gram-positive oval-shaped budding yeast is observed as shown in Figure 1.

Differentiation and purification of species

For differentiation and purification of the species, a differential medium is used which is Hi CHROM agar.

Biofilm Formation

Test tube method: The biofilm formation was qualitatively assessed by the TSB (10 ml) medium with loop full culture grown overnight after incubation of 24 hours at 37oC. On the next day, the tubes were decanted and washed with Phosphate Buffer Saline (PBS) (pH 7.2). The tubes were subjected to the process of drying. Crystal violet (0.1%) was used to stain the dried tubes. De-ionized water was used to wash off the excess stain. For the formation of biofilm, tubes were placed in an inverted position.

Congo red agar method: For the screening of isolates for biofilm formation, an alternative method was applied in which Congo red agar containing Brain Heart Infusion (BHI) broth with sucrose was used. The CRA medium is constructed by mixing 0.8 g of Congored and 36 g of sucrose to 37 g/L of Brain Heart Infusion (BHI) agar. After an incubation period that was 24 Hours at 37°C, the morphology of colonies that underwent different colors is differentiated as biofilm producers or not.

96-Well micro plate: Yeast culture was primarily transferred into a tube by using an inoculation loop. The tube was containing 2 ml of Brain Heart Infusion broth with 0.25% glucose; afterwards, it was incubated at 37°C for 24 Hours. Dilutions of all the tubes were made of 1:20 by using Brain Heart Infusion broth prepared freshly. On micro titer plate final solution, 200 µl was placed which was then incubated for 24 hours at 37°C. Right after incubation, the microtiter plate was washed three times with the Phosphate Buffer Saline (PBS) and placed in an inverted position to blot. 1% of crystal violet 200 µl was also added to each well and then incubated for 15 mins. The micro titer plate was again rinsed with PBS thrice after the incubation. 200 µl of ethanol solution was added to each well. Optical density was observed by using an Enzyme-Linked Immuno Sorbent Assay (ELISA) reader at 4500 nm.

Biofilm inhibition by using natural products: For the inhibition of Biofilm natural products such as pure garlic, ginger, and vinegar or with dilution used. The dilution of these natural products was made by the serial dilution method (Nine test tubes with 9 ml distilled water was taken. Three sets of the tubes were made and labeled, three with garlic, three with ginger, and three with vinegar respectively. All the sets of the tubes were labeled for serial dilutions as follows: 10-1, 10-2, and 10-3 respectively.

Anti-biofilm activity of natural products: The wells in the micro titer plate were also labeled with the respective dilution and products. The final solution of 200 µl was transferred into each labeled well of the micro titer plate and incubated at 37°C for 24 Hours. After incubation, the micro titer plate was washed thrice with Phosphate Buffer Solution (PBS) and then placed in an inverted position to blot. Optical density was observed by the usage of an Enzyme-Linked Immuno Sorbent Assay (ELISA) reader at 4500 nm and results were compared before and after the inhibition of Biofilm.

Results

The results of the followings methods are depicted in the figures below (Figures 1-9).

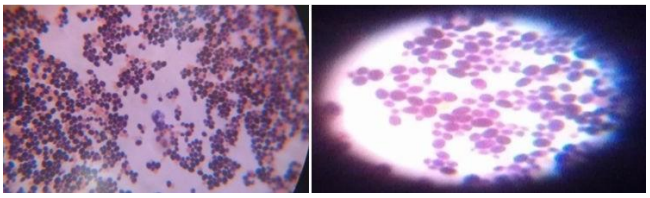

Figure 1: Gram reaction, microscopy of Candida species.

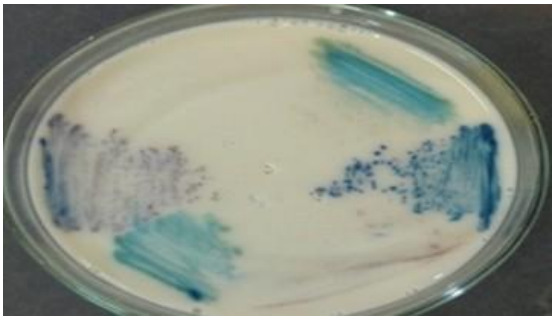

Figure 2: Candida species colonies on Hi CHROM agar.

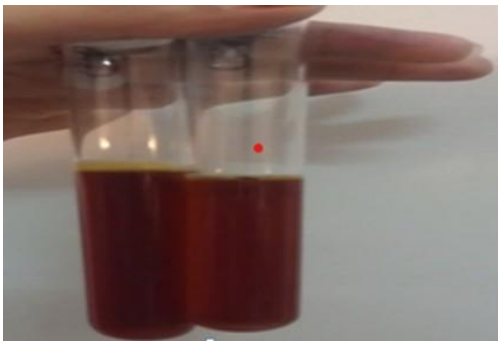

Figure 3: Biofilm formations by C. krusei and C. tropicalis.

Figure 4: Biofilm formations by C. albicans and C. glabrata.

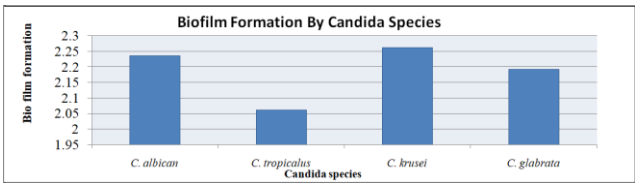

Figure 5: Graphical representation of biofilm formation by Candida species.

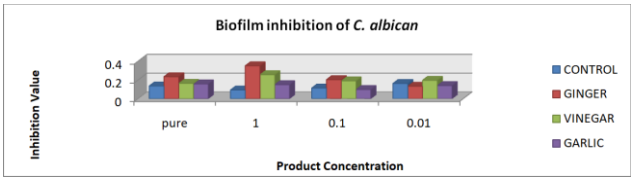

Figure 6: Graphical representation of biofilm inhibition by C. albicans.

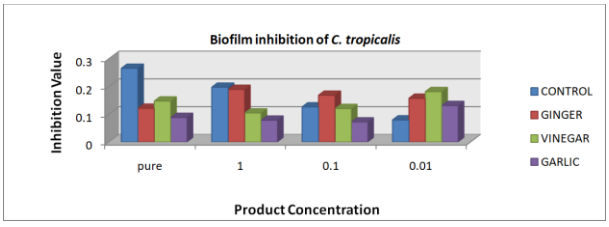

Figure 7: Graphical representation of biofilm inhibition by C. tropicalis.

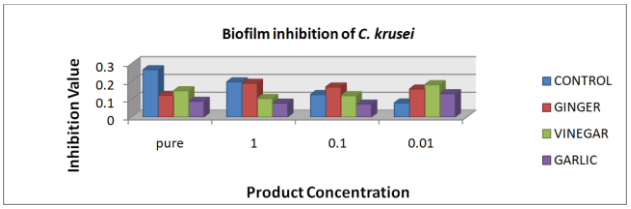

Figure 8: Graphical representation of biofilm inhibition by C. krusei.

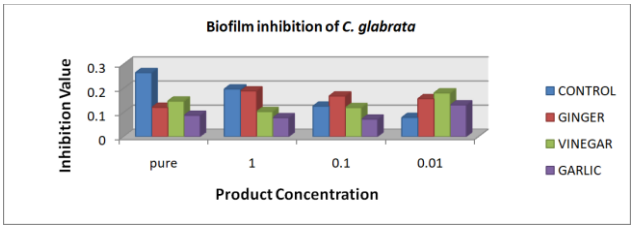

Figure 9: Graphical representation of biofilm inhibition by C. glabrata.

Discussion

Candida species are the normal flora of the human but sometimes they become opportunistic pathogen and cause severe infection when they get favorable conditions. Antifungal drugs are used for the treatment of Candidal infections. The most potent threat is Candida species are becoming resistant to antifungal drugs which are used for treating fungal infections. Many of the Candida species form a biofilm, which is considered a significant virulence factor of the Candida species. It has also been observed that very few antifungal showing activity against Candida biofilm. Moreover, these antifungals also have some toxic effects on the host body. The present study has been designed to observe in vitro antifungal activity of the garlic, ginger, and vinegar against biofilm of Candida species through a 96-well micro plate.

From the isolates, four different species of Candida were identified; these species are C. albican, C. krusei, C. tropicalis, and C. glabrata. These species were identified based on the growth color on the Hi CHROM agar medium. Green, blue, pink, and purple color colonies have been produced by C. albican, C. tropicalis, C. krusei, and C. glabrata respectively.

When we compared the biofilm formation, it has been observed that after 24 Hours C. Tropicalis produced a minimum amount of biofilm as compared to C. albicans whereas C. Glabrata produced a little less amount of biofilm as compared to C. albican. However, C. krusei has produced the maximum amount of biofilm as compared to the other species.

According to reported researches regarding biofilm biomass production after 24 Hours C. albicans and C. glabrata produced a low quantity of biomass than C. tropicalis and C.krusei and C. krusei produced a higher quantity of biomass than C. tropicalis which shows a contradictory result in our study.

However, according to the results of biofilm production by tube method, C. krusei formed a greater quantity of biofilm as compared to the C. tropicalis which is second higher while the C. albican and the C.

glabrata shows the minimum amount of biofilm production, which is similar to the standard result.

The method of Congo red agar usage was reliable and was easy to perform to determine whether the isolates have the ability for the production of biofilm or not. It correlated with the study of Naveen Saxena reported in 2014.

In our study, the detection of the biofilm by the Congo red agar method showed a negative result. Black colonies with a dry crystalline consistency indicated a positive result.

Different concentrations of ginger, garlic, and vinegar were used against biofilm. All these natural products have antifungal activity and have also shown activity against the Candida biofilm.

In the result of the biofilm production of C. albican in the case of the pure extracts used against the biofilm; the ginger showed the highest inhibition rate of biofilm, followed by the vinegar that showed these cond-highest activity while the garlic showed the lowest activity of inhibition of biofilm. When all the products were taken in the form of the pure extract, the concentration of 10-1 and 10-2 gave the same result, and the ginger showed the highest activity against bio-film. However, when these products were diluted with the concentration of 10-3, the vinegar showed the highest activity followed by garlic and ginger respectively.

In the result of bio-film production of C. tropicalis, when the pure extracts used against the bio-film; the vinegar showed the highest inhibition of bio-film, followed by ginger and garlic respectively. As the extracts were taken with the concentrations of 10-1 and 10-2, ginger showed the highest activity, followed by vinegar and garlic respectively. But when the product is diluted with a concentration of 10-3, give the same result as a pure extract.

In the result of bio-film production of C. krusei when the pure extracts were used against the biofilm; the vinegar showed the highest inhibition of bio-film, followed by ginger and garlic respectively. As the extracts were with the concentrations of 10-1 and 10-2 ginger showed the highest activity, followed by vinegar and garlic respectively. However, when the extracts were is diluted with concentrations of 10-3 give the same result as a pure extract.

In the result of biofilm production of C. glabrata when the pure extracts were used against the biofilm; the vinegar showed the highest inhibition of biofilm, followed by ginger and garlic respectively. When the extracts were taken in the concentrations of 10-1 and 10-2, ginger showed the highest activity, followed by vinegar and garlic respectively. However, when the extract was diluted with a concentration of 10-3, gave the same result as a pure extract.

Conclusion

After performing this study it is concluded that the ginger, garlic, and vinegar showed positive results against the biofilm of Candida species isolates from the clinical sample. These natural products can be replaced by antifungal drugs as these antifungal drugs have some side effects on the host body. Further studies are necessary to explain conflicting results and to understand the anti-biofilm the activity of garlic, ginger, and vinegar.

Moreover, the number of natural products was not so high, but still many spices and other products may show antifungal or anti biofilm activity.

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