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Activity of *Solanum lycopersicum* against *Candida* Species Isolated from Retro-Positive Patients – An Invitro Study.

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Abstract

In the recent years, Human Immunodeficiency Virus (HIV) has infected millions of people around the globe, making it an important public health concern of the future. *Candida* species found as a commensal is the most common etiology of oropharyngeal candidiasis, an opportunistic fungal infection seen in HIV patients. The objective of the present study was to determine the anti-candidal activity of *Solanum lycopersicum* (tomato) extract on oral *Candida* species isolated from HIV positive patients. 30 oral rinse samples were collected from retro-positive patients attending the anti-retroviral therapy center. Standard culture techniques were followed for the identification of yeast. Of the total 30 samples, 13 samples grew *Candida* species belonging to *Candida albicans* (77%), *Candida guilliermondii* (15%) and *Candida lusitaniae* (8%). The mean CD4 count was 386. About 61% (n=8/13) of *Candida* species were susceptible to Fluconazole (MIC >0.50 µg/mL) and 39% (n=5/13) were resistant (MIC <0.50 µg/mL). Ethanolic extract of *Solanum lycopersicum* showed anti-candidal activity at MIC and MFC 6.25mg/mL for the drug-resistant *Candida* species. Further exploring the phytochemical components present in *Solanum lycopersicum* could help be an alternative treatment modality to fight the increase in drug resistance seen in *Candida* species.

Keywords: *Candida* species, Human Immunodeficiency Virus, minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), *Solanum lycopersicum*.

INTRODUCTION

Human Immunodeficiency Virus (HIV) has infected millions of people around the globe (36.7 million currently), making it an important public health concern of the future. Presently, only 60% of HIV infected individuals know their status and over 14 million people (40%) still need to access HIV testing services. Patients infected with HIV have a suppressed immune system, which makes them vulnerable to opportunistic infections [1]. An example of a common opportunistic pathogen in retro-positive patients (HIV) is the yeast - *Candida* species, causing oral thrush. *Candida* species are gram-positive microorganisms that are commonly present as normal flora of the body and cause opportunistic infections in immunocompromised hosts. The oral thrush treated with long-term, low-dose of anti-fungal drugs, and has led to the rise of drug resistance thereby impairing the therapeutic prognosis [1,2]. With the emergence of nonalbicans *Candida* in HIV patients in consort with their drug resistance, there is a critical need to find an efficient therapeutics.

Several plant products used in traditional medical practice have been studied for the management of several diseases like malignancy, diabetes, arthritis and infectious diseases. The indigenous medicinal plants as an alternative to antibiotics are said to play a significant role here. It is expected that plant extracts show target sites with the suitable therapeutic index for the advancement of novel drugs [3].

Solanum lycopersicum, known as tomato belongs to the Solanaceae family that comprises over 3,000 species. The genus *Solanum* entails of 13 species with *Solanum lycopersicum*, and the remaining 12 wild species. The world production of tomato is estimated to approximately 159 million tons. Tomato fruits are an essential source of nutrition including vitamins, minerals, and antioxidants, with known beneficial effects on health. Consumption of tomato fruit has been related to a reduced risk of the inflammatory process, carcinoma, coronary heart disease, hypertension, diabetes, and obesity [4]. Evidence-based studies have reported the role of tomato intake in plummeting the risk of oral and esophageal cancer [5].

With the emergence of drug-resistant *Candida* species in HIV-infected individuals, there is a need for the alternative therapeutic approach. Owing to the rich nutritional value of tomato and its non-seasonal availability, the present study intended to determine the anti-Candidal activity of *Solanum lycopersicum* (tomato) against the oral *Candida* species isolated from HIV patients.

MATERIALS AND METHODS

A cross-sectional study was carried out randomly on 30 retro-positive patients attending the anti-retroviral therapy

center. A standardized patient information form was used to retrieve demographic information and the latest CD4 counts from the patient records. A written informed consent was obtained from the participant. Ethical clearance was obtained by the Institutional Ethics Committee. The patients had no oral lesions at the time of specimen collection.

Materials

Sabouraud's Dextrose agar with chloramphenicol (Himedia, India), CHROM ID agar (Hi-Media), Fluconazole MIC-E strips (Himedia, India), Mueller Hinton Agar with glucose and methylene blue (Himedia), Sabouraud's Dextrose Broth (Himedia), Distilled Water, Ethanol (Merck), Di-Methyl Sulphoxide (DMSO) (Merck), ripe tomatoes from the local market.

Sample Collection and Processing:

Samples of saliva were obtained as oral rinses in buffered phosphate saline (PBS, 0.1 M, pH 7.2) vortexed, cultured onto Sabouraud's Dextrose agar with chloramphenicol and incubated overnight at 35°C. A gram stain of the sample was also performed to look for the presence of oral flora and yeast. Growth was observed for moist, cream-colored, pasty/ dry, wrinkled colonies showing gram-positive oval budding yeast cells. Yeast colonies were further identified to species by CHROM ID agar, carbohydrate assimilation test [6] and further confirmed by the VITEK 2 identification system. The isolated *Candida* species were preserved at 4^0C until further study.

Antifungal Susceptibility Test:

The antifungal susceptibility testing was done for Fluconazole by Minimum Inhibitory Concentration (MIC) E-test method. Mueller Hinton Agar with glucose and methylene blue was prepared and poured into sterile petri dishes. Yeast cell suspension was prepared by sub-culturing the isolated *Candida* strains onto SDA agar and the inoculum was standardized to Mac Farland standard 4. The strains were inoculated by lawn culture on the media and allowed to dry for 10 mins. Using sterile forceps, Fluconazole E-strips (0.016-256µg.mL) was placed onto the medium and the plates were incubated at 35^0C for 48h. The lowest concentration of fluconazole required to inhibit Candida growth was taken as the MIC [7].

CD4 Count:

CD4 counts were collected from each patient from their records. According to the World Health Organization, a normal CD4 count is 500-1,500 cells/cm3 of blood, a CD4 count between 200-500 cells/cm3 is symbolic of HIV, and a CD4 count of fewer than 200 cells/cm3is a diagnosis of AIDS syndrome.

Plant Material:

The ripe Tomatoes (*Solanum lycopersicum* family Solanaceae) was collected from the local market.

Preparation of the extract:

Ripe *Solanum lycopersicum* (tomato) were washed with sterile distilled water, sliced, sun-dried and crushed into a coarse powder. About 20g of the dried material was subjected to extraction using ethanol (300ml) as a solvent by Soxhlet apparatus, the solvent was distilled off and the dried crude extract (8.57g) was stored in a desiccator until use.

Culture:

Out of the 13 *Candida* species isolated, *C.albicans* (resistant to Fluconazole), *C. lusitaniae*, *C. guilliermondii* & ATCC *C.albicans* 90028 was studied. The inoculum was prepared in sterile distilled water containing10^6 to 10^7 viable *Candida* species per ml.

Anticandidal activity:

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the crude Ethanolic plant extract was determined [8]. The stock concentration of 50mg/ml was prepared by dissolving the plant extract in DMSO. The extract was then diluted 1:2 in Sabouraud's Dextrose Broth (SDB) and further diluted to seven doubling dilutions (1:256; 25mg/ml – 0.1mg/ml) in SDB. The final concentration of DMSO did not exceed 2.5%. The dilutions were from 25mg/mL – 0.1mg/mL. 198µl of each dilution was distributed into wells of a microtitre plate. 2µl of inoculum was added to all the wells and incubated at 37^0C for 48 h. The test was performed in triplicate. MIC was defined as the lowest concentration capable of visually inhibiting fungal growth seen in the wells.

Determination of Minimum Fungicidal Concentration (MFC)

Aliquots of 5 µL of supernatant from each well of the microtitre plate with no visible fungal growth were sub-cultured on SDA and incubated at 35°C for 24h. MFC was defined as the lowest concentration of the extract that inhibited the growth of Candida species. The test was performed in triplicate [8].

MIC of the controls:

A negative control consisting of DMSO (1:2 dilution in SDB) (198µl) and yeast inoculum (2µl) was included. Positive control of fluconazole was included whose MIC was determined [9]. Briefly, the stock concentration of the drug fluconazole was prepared in DMSO (128 µg/ml). 195µl of sterile distilled water was added to the wells in the first column and 100 µl to the remaining wells of the microtitre plate. To the first wells, 5µl of the drug was added and the serially diluted. 20µl of the inoculum and 80µl of the SDB was added to all the wells, mixed thoroughly and incubated 35^0C for 48 h (final volume - 200 µl). After incubation, aliquots from each well was cultured on an agar plate and examined for the presence of viable *Candida* species. The lowest concentration of the drug with no visible growth of organism was recorded as the MIC. Negative control (95µl water, 5µl DMSO, 100µl SDB) and yeast control (95µl water, 5µl DMSO, 80µl SDB, 20µl yeast) were included.

RESULTS AND DISCUSSION

Despite the challenges, there has been progression in various aspects regarding HIV. *Candida* species, a commensal in the oral and gastrointestinal flora of healthy individuals, are the most usual cause of opportunistic fungal infections causing Oropharyngeal Candidiasis (OPC) in HIV-infected patients and in those with acquired immunodeficiency syndrome (AIDS) [10]. Candida species as oral carriers in healthy individuals is about 30-50% and overgrowth of these *Candida* causes Oropharyngeal

Candidiasis which is strongly associated with immune suppression. OPC is a clinical marker and indicator of HIV disease progression [11]. With the availability of antiretroviral therapy (ART), the occurrence of oropharyngeal candidiasis has waned considerably for HIV-infected patients; but continues to be a problem for those with a deprived immunologic response or living in locations with limited resources [12, 13].

The present study evaluated 30 HIV-positive patients under anti retroviral therapy. One of the patients had oropharyngeal candidiasis and 29 did not have any *Candida* lesions. Among the 29 patients, 13 (44.8%) were positive for oral carriage of yeast of which, 77% (n=10) *Candida albicans*, 15% (n=2) *Candida guilliermondii* and 8% (n=1) *Candida lusitaniae* were isolated. Although the sample size is limited, oral colonization by yeast in HIV-positive patients is similar to the epidemiological studies across the globe. Similar reports from studies [12] support the fact that *C. albicans* was the most prevalent species of oral colonization along with the advent of non-albicans *Candida* species as a potential source of infection. All the patients except one were on antiretroviral therapy.

The mean CD4 counts in this study were 386. The absolute CD4+ T-lymphocyte count (< 200 cells/µL) is being used as a marker to assess the risk of development of oropharyngeal candidiasis from an asymptomatic yeast colonization. There was no correlation between CD4+ T-lymphocyte counts and yeast colonization as reported by similar studies [12].

Table 1: Minimum Inhibitory Concentration (MIC) of fluconazole against oral Candida isolates.

Isolate ID	*Candida* species	MIC fluconazole (µg/mL)	Interpretation
1	*Candida albicans*	1	Resistant
2	*Candida lusitaniae*	0.75	Sensitive
5	*Candida albicans*	0.5	Sensitive
6	*Candida albicans*	2	Resistant
7	*Candida albicans*	0.5	Sensitive
9	*Candida guilliermondii*	0.75	Sensitive
13	*Candida albicans*	256	Resistant
16	*Candida albicans*	0.75	Resistant
18	*Candida albicans*	0.5	Sensitive
22	*Candida albicans*	0.5	Sensitive
23	*Candida guilliermondii*	0.75	Sensitive
24	*Candida albicans*	256	Resistant

In this study, the MIC Fluconazole of the *Candida* strains [Table 1] ranged from 0.25µg/mL – 256µg/mL. Out of the 10 *C.albicans*, 5 were resistant to fluconazole (MIC 0.75 - 256µg/mL). The ATCC *C.albicans* showed a MIC of 0.25 µg/mL. However, the non albicans *Candida* was susceptible to fluconazole as per the epidemiological cutoff values [14]. The susceptibility testing aids in the treatment of oral candidiasis and are significant as variability in MIC values among strains of same species was observed.

With the emergence of drug resistance, the need for alternative therapy is on the upsurge. Herbal medicines have been known since ages and their therapeutic efficacy has been described by traditional specialists. As per WHO estimate, about 80% of the population use herbal extracts or their active constituents as folklore medicine. Plants have a rich source of antimicrobial properties [15]. There are several studies on the antimicrobial activity of these medicinal plants against *Candida* species [16, 17, 18, 19, 20, 21].

Solanum lycopersicum (tomato) belonging to the family Solanaceae, is one of the universal, non-seasonal vegetable, rich in antioxidants and nutrients. The fruits are consumed regularly in the Asian diet. Tomato and its products are the rich substrata of nutrients and phytochemicals that are beneficial for health. Evidence-based studies on tomato report the reduction of risk related to oral and esophageal carcinoma [5].

In the current study, the MIC and MFC [Fig. 1] of *Solanum lycopersicum* (tomato) against the oral *Candida* species were 6.25mg/ml. The MIC of the positive control was in accordance with the MIC of fluconazole. A literature search revealed no much studies on the antimicrobial activity of *Solanum lycopersicum* extract against drug-resistant pathogens, especially opportunistic pathogens. AL-Oqaili *et al* investigated the antibacterial activity of aqueous extracts of *Solanum lycopersicum* against Gram-negative pathogenic bacteria - *Escherichia coli*, *Klebsiella* species, *Pseudomonas* species and *Acinetobacter* species [22].

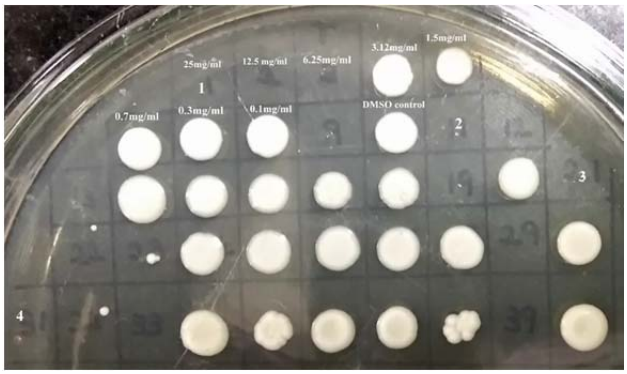

Figure 1: Minimum Fungicidal Concentration (MFC) of *Solanum lycopersicum* extract against oral *Candida* isolates.

1 = ATCC *C.albicans* 90028,
2 = *Candida lusitaniae*,
3 = Candida *guilliermondii*,
4 = *Candida albicans*.

Presently, there are many methods for antifungal testing of natural products such as - disk-diffusion test, broth macro-dilution and microdilution methods which are labor-intensive, time-consuming and thus not suitable for routine screening of natural products. The MIC broth dilution method described here was adapted to study the anti-candidal activity of ethanolic tomato extract since the extracts were screened at microgram scale. There were several technical glitches faced during the screening of the extract. Firstly, Candida species was sensitive to DMSO and its growth was considerably inhibited at a concentration higher than 2.5% of the final well volume (0.2 ml). Secondly, the extract was insoluble in 2.5% DMSO: water mixture. This was resolved by serially diluting the extract in appropriate media (SDB) and then transferring the dilutions onto the microtitre plate, thus reducing the chances of precipitation of the extract. In addition, a suitable micro-dilution method for screening of natural products against *Candida* species was optimized and method validated.

CONCLUSION

Since plants yield a range of compounds with antimicrobial properties, screening for antifungal activity may yield candidate amalgams for developing novel antifungal drugs. In the current study, the findings suggest the promising anti-candidal activity of crude *Solanum lycopersicum* (tomato) extract against drug-resistant *Candida* species. Further exploration is required in analyzing the phytochemical components present in *Solanum lycopersicum* (tomato) along with its toxicity and pharmacokinetic properties that could provide a potential therapeutic agent for HIV patients and help to combat the surge of drug resistance seen in *Candida* species.

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