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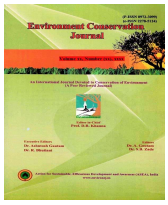

Antimycotic activity of green tea phytocompounds against *Candida glabrata*

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ABSTRACT

One of the medically important opportunistic fungal pathogen for humans is *Candida glabrata* that causes various types of candidiasis. Its environmental adaptations and antimicrobial resistance is now a great concern for public health. In the present study, the green tea phytocompounds; EGCg, Chlorogenic acid, Coumaroyl quinic acid and Rutin trihydrate along with a known antimycotic Fluconazole were studied for their antimycotic activity against *Candida glabrata.* The MIC_{90} for *C. glabrata* was observed at 125µg/ml for EGC g, 250 µg/mlf or Chlorogenic acid, 500µg/ml for Coumaroyl quinic acid and Rutin trihydrate while 12.5µg/ml for Fluconazole in macro dilution assay while the MFC values were 1000 µg/ml for EGC g, 500 µg/ml for Chlorogenic acid, Coumaroyl quinic acid, Rutin trihydrate and 50 µg/ml for Fluconazole. In microdilution assay, the MIC_{90} for *C. glabrata* was observed 125µg/ml for EGC g and chlorogenic acid, 500µg/ml for Coumaroyl quinic acid, Rutin trihydrate and 12.5µg/ml for Fluconazole while the MFC values were 31.25 µg/ml for Fluconazole, 250 µg/ml for chlorogenic acid and 500 µg/ml for EGC g, Coumaroyl quinic acid and Rutin trihydrate. EGCg and Chlorogenic acid was found to be more effective against *C. glabrata* and therefore these two were used for synergistic study along with Fluconazole. The viability of HeLa cells (in per cent) was observed ≥100% green tea phyto compounds. The viability of treated cells (in per cent) with a combination of Green tea, phytocompounds and fluconazole was observed between ≥98± 0.79 to ≥ 98± 0.87. Green tea phytocompounds mainly EGC g and chlorogenic acid can be used as synergistic molecules having antimycotic activity against *C. glabrata*.

Introduction

Candida albicans causes a high mortality rate of 10%-49% while Non-albicans Candida species including *C. glabrata, C. tropicalis*, *C. krusie* and *C. auris*is increasing in India. *C. albicans* is responsible for over 90% of infections, followed by *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei* (Pfaller *et al*., 2007). According to reports, India has one of the highest incidences of blood stream Candida infections worldwide (Gaffi - Global Action For Fungal Infections). About 35 to 40% of *C. tropicalis* species have been reported from clinical specimens like blood, urine, sputum, pus, lung aspirate, catheter tips, nail, throat swab, tested in patients in north India (Singh *et al*., 2011;

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Patel *et al*., 2012; Yapar, 2014). *Candida glabrata* (also now known as *Nakaseomyces glabrata*) has emerged as a very common bloodstream pathogen and recently it is reported as a widely prevalent pathogen in Asia (Gómez *et al*., 2023, Sarah *et al*., 2023). *C. glabrata* produces systemic fungal infection and its emergence has caused alarm because of its drug resistance and variations/mutations occurring in its genome. *C. glabrata is* continuously emerging as more serious pathogen for humans because of high-stress resistance and high adhesion characteristics. It has progressed a vigorous tolerance to cationic, oxidative, osmotic and nitrosative stresses. It has a large collection of adhesins, which enable its ability to colonize humans (Chew *et al*., 2019; Kumar *et al*., 2019; Pais *et al*., 2019., Lionakis *et al*., 2023). In addition, it has very high adaptability and can be found on catheters and inanimate hospital equipment. Particularly in ICU-recommended patients, such as elderly patients with underlying illnesses, candidemia has a significant fatality risk. (Ayhanım, 2020) and also detected in Hospitalized Patients with COVID-19 (Cattaneo *et al*., 2023). As a result, natural chemotherapeutic agents, such as plant extracts, represent a potential source for the creation of strong antifungal drugs and disease management strategies. To lessen side effects and toxicity, combining recognised beneficial medications with phytoconstituents is a great idea (Aboody and Mickymaray, 2020).

The present study has evaluated the anticandidal activity of green tea phytocomponds viz. EGCg, Chlorogenic acid, Coumaroylquinic acid and Rutin trihydrate along with a known antimycotic Fluconazole against *Candida glabrata.*

Material and Methods

Green tea phytocompounds

A purified form of (−)Epigallocatechin gallate (EGCg), Chlorogenic Acid, Coumaroylquinic Acid, Rutin trihydrate were procured from Sigma Aldrich and SRL.

Yeast strains

Candida glabrata (MTCC 3019) was procured from MTCC, IMTech, Chandigarh and employed for assessing the potential of Green Tea for anticandidal activity. It was grown and preserved in Yeast Peptone Dextrose (YPD) agar and broth.

Broth Macrodilution Assay

Two-fold dilution of EGCG, chlorogenic acid, Coumaroylquinic acid, and rutin trihydrate was prepared from 1000 µg/ml to 31.25 µg/ml and Fluconazole from 100 µg/ml to 3.125 µg/ml in YPD broth.*C. glabrata* culture was cultured in YPD broth at 37^0C for 18 h.Each diluted samples were inoculated with $1x10^3$ CFU of *C. glabrata*. The controls taken were YPD broth only, YPD broth with *C. glabrata*, 10% DMSO as a negative control. These were incubated at 37^0C for 18h after which two-fold dilutions of each sample were made and poured on YPD agar plates, incubated at 37^0C, colonies were counted and CFU/ml was calculated. Minimum inhibitory concentration (MIC) was calculated as the concentration which inhibited 90% growth of *C. glabrata*. Minimal fungicidal activity (MFC) was calculated as the lowest concentration which resulted in 99.9% death of cultures. For this 100µl of the test sample with culture was poured on YPD agar plates, incubated at 37^0C and CFU/ml was calculated (Kaya and Ozbilge, 2012).

Broth microdilution assay

The MIC of EGCg, Chlorogenic acid, Coumaroylquinic acid, Rutin trihydrate and Fluconazole were assessed following the micro dilution method (CLSI, 2002). The *C. glabrata* culture was pre-incubated in broth at 37°C for 18hr at 150rpm. Two-fold dilutions of EGCg, Chlorogenic acid, Coumaroylquinic acid, Rutin trihydrate was prepared as described above and 100µl of each sample was added into wells microtitre plate (Axygen, USA) followed by inoculation with 5µl of *C. glabrata* ($1x10^3$CFU) with gentle plate shaking.The 10% DMSO was taken as negative control and YPD broth in wells as blank. The microtitre plate was incubated at 37°C for 18hr and MIC was calculated by recording the absorbance at 600nm (Radji *et al*., 2013).

Assay of synergistic activity

The inhibitory effects of each GT phytocompounds with fluconazole combinations were evaluatedusing micro dilution checker board technique (CLSI, 2002). Fluconazole and GT phytocompounds were

produced and employed in several combinations at 2-fold serial dilutions that were equivalent to, below, and above their MICs for *C. glabrata*.

The effective MIC was considered for the combination that completely inhibited the growth of *C. glabrata* (Jin *et al*., 2010). The growth was quantified using spectrophotometer and then analysed for fractional inhibitory concentration index (FICI). The drugs combination that inhibited the growth completely was considered as an effective MIC for the combination. Fractional inhibitory concentration index (FICI) was evaluated using standard formula; FICI = FIC A + FIC B, where, FIC A = MIC of the combination A and B /MIC of drug A alone; FIC B = MIC of the combination A and B / MIC of drug B alone. The effects of the combinations were classified as synergistic if the FIC index was equal to 1, indifferent if it was between 1 and 4, and antagonistic if it was greater than 4.

In vitro cytotoxicity

The HeLa cell line procured from NCCS, Pune was cultured in 25cm^2 culture flasks having DMEM media with 10% FBS, Gentamycin (5µg/ml) in an incubator with 5% CO_2tension at 37˚C until confluent and was used in cytotoxicity study by MTT assay.The HeLa monolayer was trypsinized with TVS before cells were planted into each well of the microtitre plate at a density of 1x 10^5 cells/ml. Two-fold dilutions of GT phytocompounds and fluconazole as described above were prepared in DMEM. 100µl of various test concentrations were added into the partial monolayer in 96-well plates and incubated at 37^{0}C (5% CO_2) for 24 hr. The cells are examined under microscope at 0 and 24hrs. Wells with no media added are used as controls, along with untreated cells. As a positive control, H_2O_2 was employed, and as a negative control, 10% DMSO. After 24 hr, the solutionswere discarded. 20µl of MTT (5mg/ml) was incorporated to wells and kept for 4hr at 37^{o}C in a 5% CO_2 atmosphere. 100µl of 0.04N HCl was added to solubilise the formazan. The absorbance was recorded at 540 nm**.** The percentage viability was calculated as: Percentage viability (%) = [(At-Ab) / (Ac-Ab)] X 100; where, At is Absorbance of treated wells, Ab is Absorbance of blank, Ac is Absorbance of control wells.Lethal Concentration 50 percent was defined as the lowest concentration of the GT phytocompounds that caused a 50% reduction of cell development (LC50) (Fadeyi *et al*., 2013).

Results and Discussion

Antimycotic effect of green tea using broth macrodilution assay

The MIC_{90} for *C. glabrata* was observed at 125µg/ml for EGCg, 250 µg/mlfor Chlorogenic acid, 500µg/ml for Coumaroylquinic acid and Rutin trihydrate while 12.5µg/ml for Fluconazole (Table 1). The MFC values were 1000 µg/ml for EGCg, 500 µg/ml for Chlorogenic acid, Coumaroylquinic acid, Rutin trihydrate and 50 µg/ml for Fluconazole.

Table 1: MIC_{90} and MFC of GT Phytocompounds against *C. glabrata* using broth macrodilution

GT Phytocompounds	MIC_{90} (µg/ml)	MFC (µg/ml)
EGCg	125	1000
Chlorogenic acid	250	500
Coumaroylquinic acid	500	500
Rutin trihydrate	500	500
Fluconazole	12.5	50

Antimycotic effect of green tea using broth microdilution assay

The MIC_{90} for *C. glabrata* was observed 125µg/ml for EGCg and chlorogenic acid, 500µg/ml for Coumaroylquinic acid, Rutin trihydrate and 12.5µg/ml for Fluconazole. The MFC values were 31.25 µg/ml for Fluconazole, 250 µg/ml for chlorogenic acid and 500 µg/ml for EGCg, Coumaroylquinic acid and Rutin trihydrate (Table 2).

Table 2: MIC_{90} and MFC ofGT phytocompounds against *C. glabrata*using broth microdilution assay

GT Phytocompounds	MIC_{90} (µg/ml)	MFC (µg/ml)
EGCg	125	500
Chlorogenic acid	125	250
Coumaroylquinic acid	500	500
Rutin trihydrate	500	500
Fluconazole	12.5	31.25

Synergistic activity by Checkerboard susceptibility assay

In macrodilution and microdilution assays, it was found that EGCg and Chlorogenic acid gave better results and found more effective and therefore these two were used for synergistic study along with Fluconazole. The results obtained are shown in

Table 3. On HeLa cell lines, the cytotoxicity experiment for various mixtures of GT phytocompounds and fluconazole was carried out. When HeLa cells were exposed to GT Phytocompounds alone at concentrations ranging from 15.625 g/ml to 500 g/ml, they displayed growth viability in a dose-dependent manner. HeLa cells' vitality was observed to be 100 percent.
(table 4). At 0 hours and 24 hours of incubation, the cells were seen to be stable and in good condition. When cells were treated with a mixture of GT phytocompounds and antimycotics, the % viability was assessed, and results ranged from 98 to 98.87.
(Table 4). Additionally, the cytotoxicity of pure catechins on HeLa cells was assessed; the results revealed an increase in cell viability after 24 hours of incubation (Fig.1 to 4).The percentage vitality of HeLa cells treated with GT Phytocompounds and fuconazole was evaluated at 0 hours and 24 hours following treatment.HeLa cell viability was measured as a percentage and ranged from 98.7 to 99.8.

Table 3: Synergistic activity of green tea phytocompounds and fluconazole by checkerboard antimycotic susceptibility assay for *C. glabrata*

GT Phytocompounds (µg/ml)	MIC_{90}	FICI
EGCg +Chlorogenic acid (62.5 each)	62.5	1.5(I)
EGCg + Fluconazole (15.625/12.5)	15.625	0.5 (S)
Chlorogenic acid + Fluconazole (15.625/12.5)	15.625	0.5 (S)

Table 4: Assay of the viability of HeLa cells treated with the combination of GT Phytocompounds with antimycotics after 24 hr of incubation

GT phytocompounds (µg/ml)	% Viability
EGCg +Chlorogenic acid (62.5/62.5)	≥98±0.79
EGCg + Fluconazole (15.625/12.5)	≥98±0.87
Chlorogenic acid + Fluconazole	≥99.8±0.19
Healthy HeLa cells	≥ 100

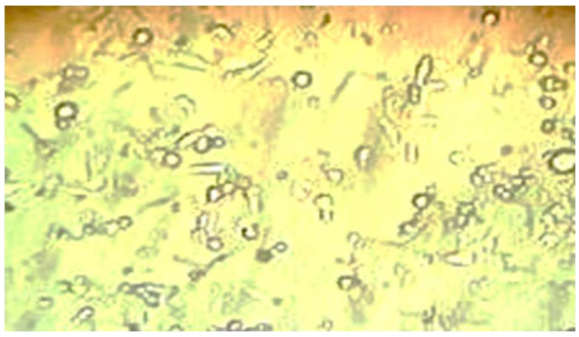

Figure 1: Untreated healthy HeLa cells at 24 hr

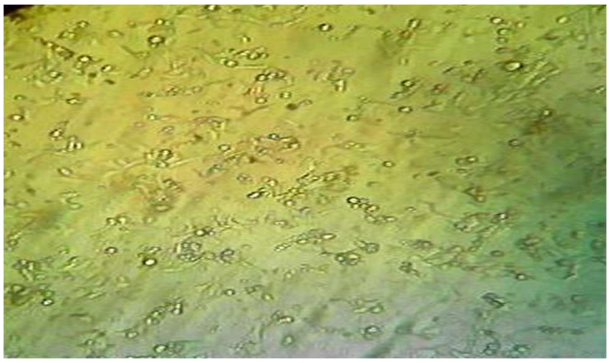

Figure 2: HeLa cells treated with EGCg and Chlorogenic acid at 24hr

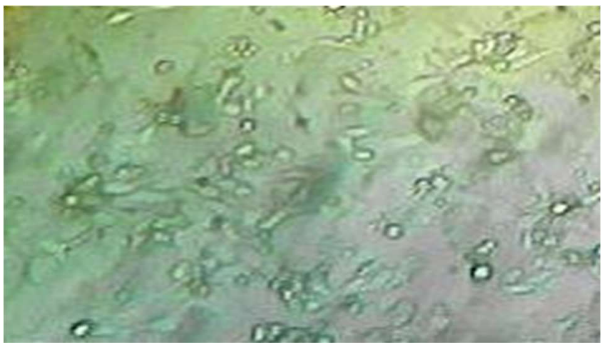

Figure 3: HeLa cells treated with EGCg and Fuconazole at 24 hr

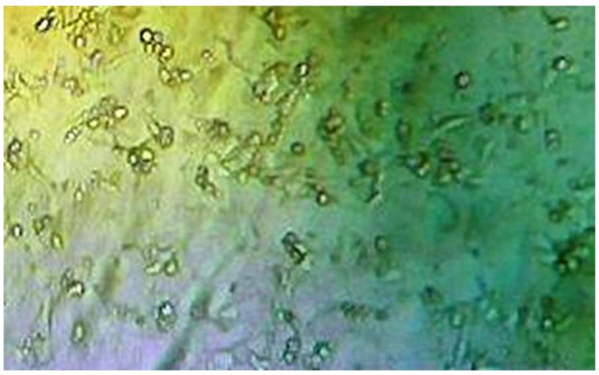

Figure 4: HeLa cells treated with Chlorogenic and Fluconazole at 24 hr

The green tea is known for its active phytocompounds with multiple benefits including antimicrobial properties (Anand, 2012, Sirari *et al.*, 2021). In the present investigation effective anticandidal synergistic activity of green tea compounds in combinations is evaluated (Hirasawa et al., 2004, Behbehani *et al.*, 2019, Kane *et al.*, 2022). In macrodilution assay, the MIC_{90} for *C. glabrata* was observed at 125µg/ml for EGCg, 250

µg/mlfor Chlorogenic acid, 500µg/ml for Coumaroylquinic acid and Rutin trihydrate while 12.5µg/ml for Fluconazole. The MFC values were 1000 µg/ml for EGCg, 500 µg/ml for Chlorogenic acid, Coumaroylquinic acid, Rutin trihydrate and 50 µg/ml for Fluconazole. Using microdilution,the MIC_{90} for *C. glabrata* was observed 125µg/ml for EGCg and chlorogenic acid, 500µg/ml for Coumaroylquinic acid, Rutin trihydrate and 12.5µg/ml for Fluconazole. The MFC values were 31.25µg/ml for Fluconazole, 250 µg/ml for chlorogenic acid and 500 µg/ml for EGCg, Coumaroylquinic acid and Rutin trihydrate. In macrodilution and microdilution assays, it was found that EGCg and Chlorogenic acid gave encouraging results and therefore these two were used for synergistic study along with Fluconazole. The interaction between an antibiotic and GT depends on a number of variables, including the type of bacteria involved (Haghjoo *et al*., 2013). In the present study, percentage viability of HeLa cells was observed between ≥98± 0.79 to ≥ 99.8± 0.19 alone at concentrations ranging from 31.25µg/ml to 500µg/ml. The percentage viability of HeLa cells was reported to be >100%.

HeLa cells were used to test the cytotoxicity of GT phytocompounds; the results revealed an increase in cell viability after 24 hours of treatment. The synergistic inhibition of a combination of fluconazole and cylcosporine was studied against *Candida albicans* based on checkerboard assay and no significant change in MIC of fluconazole was seen (Marchetti *et al*., 2000). However, in the present study the MIC of fluconazole decreased to half when combined with GT Phytocompounds. However, it is clear from the current results that EGCg induced higher percentage inhibition of *Candida glabrata* (99.8 0.19) when fluconazole was present. The present findings support that effective synergistic GT based combinations are potential candidate against many human pathogens (Anand and Rai, 2017, Mengjiao et al., 2017, Haji et al., 2022, Kováč et al., 2023).

Conclusion

The green tea phytocomponds EGCg, Chlorogenic acid, Coumaroylquinic acid and Rutin trihydrate along with a known antimycotic Fluconazole were studied for their antimycotic activity against *C.glabrata.* It was found that these showed antimycotic activity against *C. glabrata*. EGCg and chlorogenic acid showed enhanced activity against *C. glabrata*. Their combination with fluconazole also showed enhanced activity. Therefore, it is evident that green tea phytocompounds mainly EGCg and chlorogenic acid can be used as synergistic molecules having antimycotic activity against *C. glabrata*.

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Conflict of interest

The authors declare that they have no conflict of interest.

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