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Research Article

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Prediction of novel drug targets in Ergosterol biosynthesis pathway: A proposed mechanism of anticandidal activity of green tea phytocompounds

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ABSTRACT

The emergence of antibiotic resistance in pathogenic Candida species has led to explore new alternative therapies to combat its associated life threatening infections. Synergistic anticandidal potentials of green tea (derived from Camellia sinensis) with antifungal agents is well known. Our study aims at predicting the novel inhibitory targets of green tea (GT) phytocompounds and pretending the inhibitory mechanism involved in synergistic inhibition of GT with antifungals. The interaction of GT phytocompounds and ergosterol synthesising proteins (ERG) of the Candida species has been assessed by in silico study using iGEMDOCK software which revealed ERG 26, ERG 6, ERG 25, and ERG 8 proteins as novel drug targets of kaempferitrin, EGCG, ECG, chlorogenic acid respectively present in GT.Supporting our investigation, in vitro studies have been done with GT leaves from different geographical locations. Catechins were purified and identified by HPLC and synergistic effect of solvent extracts and purified catechins with fluconazole (Flu), Amphotericin B (AmB) against Candida albicans and Candida glabrata depicted the convincing synergistic inhibitory effect of GT. The simultaneous interaction of GT and antifungals with ERG proteins could be the prohibitory mechanism that inhibits the growth of Candida species implying the candidacidal synergy between GT and antifungals.

Keywords: Green tea phytocompounds, ERG, antifungal drug, *Candida albicans*, *in silico*.

INTRODUCTION

Over the last decade, the fungal infections especially human infections associated with the yeast *Candida* infection have increased[1]. Candidiasis is a fungal infection caused by yeasts from the genus *Candida*. It is associated with diverse range of infections which includes mucosal, cutaneous, subcutaneous and systemic mycoses associated with high morbidity and mortality rates in immunosuppressed individuals, HIV patients, chemotherapy patients, and organ transplant patients [2].

With the advancement in modern medicine and patient management, use of prosthetic biomaterials has been increased which has led to the complication associated with biofilm formation on these materials. This biofilm production promotes persistence of Candida infection especially by *C. albicans* and non-*Candida albicans* species[3].

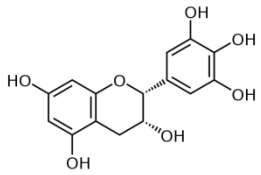
Numbers of drugs are available for treatment of these fungal infections; however, differences exist in terms of antifungal spectrum of activity, bioavailability, formulation, drug interactions, and side effects of these drugs[4]. Fungi are one of the most neglected pathogens since till today antibiotics like Amphotericin B, being the oldest antifungal drug, remains the drug of choice for many life-threatening invasive fungal infections[5] and its clinical use is impeded owing to its poor aqueous solubility and toxicity [2].

Recently, new antifungal agents and new therapy strategies such as antifungal prophylaxis, secondary prophylaxis, and pre-emptive therapy have come into use. However, these changes resulted in the alteration of *Candida* species

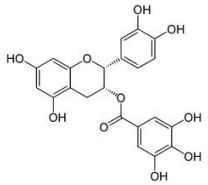
causing invasive infection [6]. With an increasing awareness of the hazards associated with the antibiotics and chemical agents, there has been an accelerated investigation on natural products, plants and their extracts as new sources of antimicrobial agents[7].

Herbal plants with their extracts or compounds provide unlimited opportunities for new drugs leads because normally they possess matchless chemical diversity [5]. Combinational drug therapy has emerged as a powerful strategy to enhance antifungal efficacy and to repudiate the drug resistance [8].

Synergistic effect of green tea (GT)and antibiotics combination could be efficient in solving drug resistance problem[9]. GT derived from leaves of *Camellia sinensis* is bestowed with natural phytocompounds such as flavanoids includes (-) - epigallocatechin gallate (EGCG), (-) - epigallocatechin (EGC), (-) - epicatechin gallate (ECG), and (-) - epicatechin (EC) which makes it a treasure for healthy lifestyle. Other polyphenols present in green tea are flavanols which include kaempferol, quercetine, isoquercetin, myricetin, myricitrin, rutin, kaempferitrin, and their glycosides, as chlorogenic acid, coumaroylquinic acid (Figure1)besides the water soluble anthocyanins [10].



(-)-Epigallocatechin (EGC)



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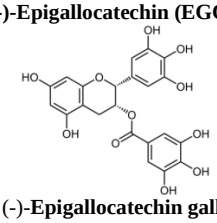
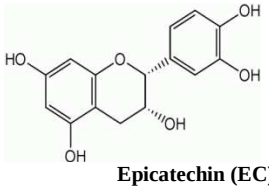
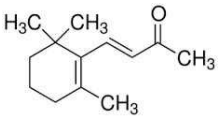


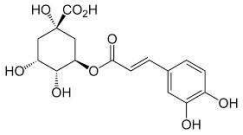
(-)-**Epigallocatechin gallate (EGCG)**



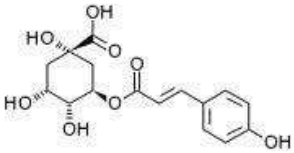
Epicatechin (EC)



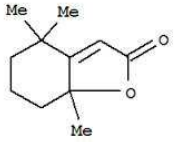
B-Ionone



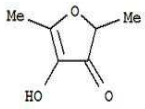
Chlorogenic acid



Coumaroylquinic acid

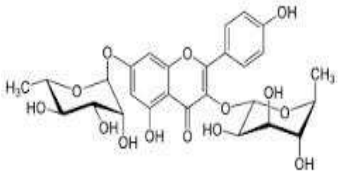


Dihydroactinidiolide

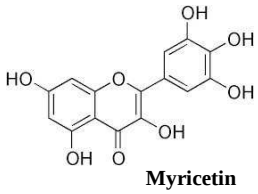


2, 5- Dimethyl-4- hydroxy-3- (2H) – furanone

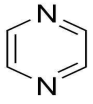
Gallic acid



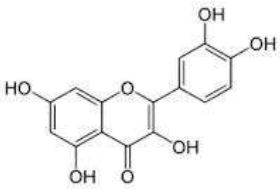
Kaempferitrin



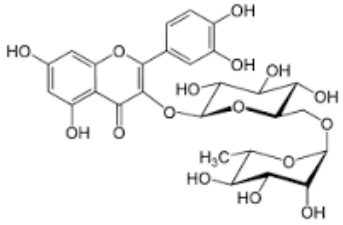
Myricetin



Pyrazine



Quercetin



Rutin

Vitexin

Figure 1: Structure of different phytocompounds present in Green tea

Among its many biological effects, is the ability of EGCG to inhibit ergosterol synthesis by disturbing the folate metabolism in *Candida albicans*. Earlier, it has been reported that by disturbing the folic acid metabolism, GT catechin EGCG interferes the ergosterol biosynthesis which suggests its inhibitory mechanism against *Candida albicans*[11]. However, in our study, attempt have been made to explore binding mechanism of GT phytocompounds as through molecular docking studies which were performed with the target proteins (key enzymes) involved in ergosterol biosynthesis The *in silico* analysis of the inhibitory mechanism of GT was also validated by performing *in vitro* assays to depict the inhibition of *Candida* species by GT alone and in combination with antifungal agents.

EXPERIMENTAL SECTION

In silico interaction of GT phytocompounds and ergosterol biosynthesis proteins of*Candida*

The mechanism of inhibition of *Candida* by GT was explored by assessing the binding affinity of GT phytocompounds with the proteins (key enzymes) involved in ergosterol biosynthesis of *Candida* (Figure 2). For *in silico* study, GT phytocompounds (as ligands) were docked with the ergosterol biosynthesis proteins and their interaction was assessed by determining their binding energies.

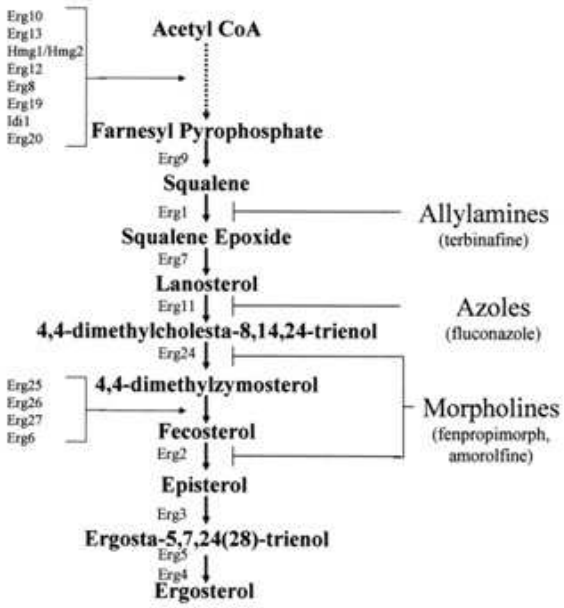


Figure 2: Linear Model of Ergosterol Biosynthesis Pathway [12]

Retrieval of 3D structures of the receptors

The three dimensional structure of the proteins of ergosterol biosynthesis pathway were obtained in PDB format (Table 1) with the help of PHYRE program (Protein Homology/analog Y Recognition Engine) which predicts the three-dimensional structure of a protein sequence using the principles and techniques of homology modelling [13]. It is an automatic fold recognition server that converts the given amino acid sequence of proteins into their respective PDB format. For the interaction analysis of Amphotericin B with ergosterol, the PDB structure of ergosterol was obtained from RCSB (PDB ID: 2AIB). The amino acid sequences were retrieved from the NCBI in FASTA format [14]. Expasy's Prot-param server was used for physico-chemical and functional characterization of these proteins [15] (Figure 3]. The proteins were validated with Ramachandran plot using Swiss Model workspace

which is a fully automated protein structure homology-modeling server and their active sites were predicted through automated active site prediction server AADS [16].

Table 1: List of enzymes involved in ergosterol biosynthesis in *Candida albicans* with their representing genes Enzymes commission (EC) number, total active sites and their molecular weight

S. No.	Gene	Protein	EC number	Amino acid Sequence Length	Total No. of Active site	Molar weight of protein	pI
1.	ERG 10	Acetyl coA C-acetyltransferase	2.3.1.9	402	50	41935.2	6.47
2.	ERG 13	3-Hydroxy-3-methylglutaryl-coA (HMG-coA) synthase	2.3.3.10	451	59	49755.2	5.65
3.	HMG1	3-Hydroxy-3 methylglutaryl-coenzyme reductase	1.1.1.34	-	NA	NA	-
4.	HMG2	3-Hydroxy-3 methylglutaryl-coenzyme reductase	1.1.1.34	-	NA	NA	-
5.	ERG12	Mevalonate kinase	2.7.1.36	431	52	46972.8	5.42
6.	ERG8	Phosphomevalonate kinase	2.7.4.2	432	60	48279.8	5.30
7.	MVD-1	Mevalonate diphosphate decarboxylase	4.1.1.33	-	NA	NA	-
8.	ERG20	Farnesyl pyrophosphate synthase	1.3.1.71	351	46	40711.6	4.82
9.	ERG9	Squalene synthase	2.5.1.21	448	61	51171.2	6.57
10.	ERG1	Squalene epoxidase	1.14.13.132	496	61	55298.2	8.89
11.	ERG7	Lansterol synthase	5.4.99.7	728	95	83724.6	5.67
12	ERG11	Lansterol 14-α- demethylase	1.14.13.70	528	69	60647.4	6.69
13.	ERG24	Sterol C-14 reducaase	1.3.1.70	448	65	51462.2	8.25
14	ERG25	C-4 methyl sterol oxidase	1.14.13.72	308	39	36560.9	6.83
15.	ERG26	C-3 Sterol dehydogenase	1.1.1.170	350	43	39183.7	6.52
16	ERG27	3- keto sterol reductase	1.1.1.270	346	47	39077.9	8.22
17.	ERG6	Sterol 24-C methyltransferase	2.1.1.41	376	48	43070.5	5.82
18.	ERG2	C-8 sterol isomerase	5.3.3.5	217	25	24543.4	5.21
19.	ERG3	C-5 sterol desaturase	1.14.21.6	386	44	45447.3	6.30
20.	ERG5	C-22 sterol desaturase	1.14.14.	517	67	59652.0	6.21
21	ERG4	C-24(28) sterol reducatse	1.3.1.71	469	63	54935.9	7.00

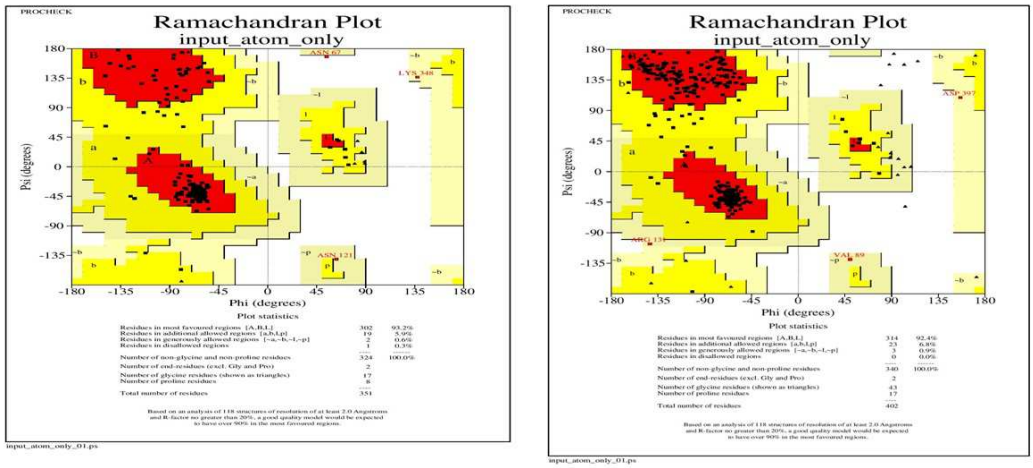


Figure3: Ramachandran plot of ERG10 and ERG 20

Preparation of ligands

The phytocompounds present in GT were used as ligands. The .mol2 structural formats of all the 15 selected herbal components were generated from the database ZINC AC [17] database which comprises the library of molecules in 3D structure and have been prepared in multiple protonation states and multiple tautomeric forms [18]. The ZINC I.D of the ligand compounds are given in Table 2. Also, the .mol2 formats of known inhibitors of ergosterol biosynthesis were generated and their binding energies were used as controls (Table 3). For amphotericin B, .mol2 format of 3-D structure was not accessible form ZINC AC database, therefore its 3D structure was obtained in .mol format from Chemspider [19] which then converted into .mol2 format through Open bable 2.3.2[20].

Table2: Phytocompounds in green tea (*Camellia sinensis*) used for the *in silico* study of *Candida albicans* inhibition

S.No.	Phytocompounds	Zinc ID
1.	Epigallocatechin gallate	3870412
2.	Epicatechin gallate	3978503
3.	Epigallate catechin	3870339
4.	Epicatechin	119978
5.	2, 5- Dimethyl-4- hydroxy-3- (2H) – furanone	15849045
6.	Quercetin	3869685
7	Myricetin	3874317
8.	Kaempferitrin	4216676
9.	Chlorogenic acid	2138728
10.	Coumaroylquinic acid	3804525
11.	Dihydroactinidiolide	1583510
12.	B-ionone	3881456
13.	Rutin	53683228
14.	Pyrazine	1692439
15.	Gallic acid	1504

Table 3: Positive controls as an inhibitor of Ergosterol biosynthesis and their binding energies with their respective target protein

S.NO	Inhibitors (antibiotics/drugs)		Zinc Id / Chemspider Id	Target receptor	Binding energy (kcal/mol)
1	Azoles	Fluconazole	4009	ERG11	**-77.6**
				ERG5	**-81.21**
		Ketoconazole	643138	ERG5	**-111.73**
		Itraconazole	4097343		**-111.69**
2	Morpholines	Amorolfine	13512582	**ERG9**	**-95.6**
		Fenpropimorph	12495255		**-87.41**
		Tridemorph	30727277		**-104.21**
3	Piperidine	1-aminopiperidine	19169685	**ERG24**	**-56.45**
		4-aminopiperidine	2381215		**-56.38**
		Piperidine	1529277		**-47.78**
4	Allylamines	Butenafine	1530975	**ERG1**	**-99.42**
		Naftifine	1530977		**-99.19**
		Terbinafine	1530981		**-91.97**
5	Thiocarbamate	Tolnaftate	57522	**ERG1**	**-98.37**
		Dithiocarbamate	3633221		**-54.14**
		Goitrin	5226611		
6	Amphotericin B		10237579 (Chemspider ID)	**ERG**	**-101.75**

Molecular Docking

Docking analysis was carried out for the ergosterol biosynthetic proteins with the selected phytocompounds and commercial drugs using iGEMDOCK software. The iGEMDOCK provides a graphical environment for recognizing pharmacological interactions and virtual screening[21] and based on the evaluated binding energies, it is presumed that how far the drug binds to the target macromolecule [22]. The molecular docking using iGEMDOCK generated the binding energies of all the 15 phytocompounds (in .mol2 format) of GT with the structural PDB proteins of ergosterol biosynthesis. The molecular interactions of the PDB proteins with the known commercial inhibitors served as a control. In order to get accurate docking, stable (slow) docking was used as a default setting.

Evaluation of anticandidal potential of green tea extracts and their purified catechins

Yeast strains and their culture condition

The yeast strains of *Candida* used for assessing the anticandidal potential of GT were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh. The yeast strains used were *Candida albicans* (CA)MTCC 3017 and *Candida glabrata* (CG)MTCC 3019. The yeast culture were routinely grown and maintained in Yeast Peptone Dextrose (YPD) agar and YPD broth. Antifungal agents viz. fluconazole (Flu) and Amphotericin B (AmB) from Hi-Media Pvt. Ltd., Mumbai were used as standard antimycotics. The standard compound (−)-epigallocatechin gallate (EGCG) was obtained from Sigma Aldrich.

Preparation of GT extracts and purified GT catechins

Green tea leaves of Himachal Pradesh (HP), Assam (AT) and Uttarakhand (IP) and (PN) were extracted in 70% methanol, 70% acetone and water in ratio plant: solvent (1: 10) with the help of rota vapour. The crude extract was stored at 4 °C until further use [23, 24, 10]. Purification of catechins from GT leaves was done using solvent partitioning method [25].

Table 4: Binding energies of the molecular docking between GT polyphenols and enzymes of ergosterol biosynthesis in *Candida* species

Ligands → / Genes ↓	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ERG1	**-135.98**	-123.92	-115.79	-109.82	-74.47	-97.01	-114.4	-147.12	-114.86	-119.52	-69.08	-69.87	-132.69	-55.44	-86.76
ERG2	**-128.89**	-119.88	-101.14	-99.55	-73.95	-136.5	-113.93	-144.49	-125.53	-115.51	-89.98	-91.46	-143.26	-54.81	-99.98
ERG3	-124.36	**-137.18**	-103.97	-103.92	-55.81	-121.57	-113.8	-140.95	-111.41	-95.55	-65.31	-82.65	-112.82	-53.19	-80.86
ERG4	**-128.85**	-124.58	-110.86	-103.47	-63.26	**-124.46**	-113.17	-129.9	-120.33	-104.12	7-71.92	-79.74	-118.01	-54.38	-89.29
ERG5	-116.24	-81.12	-98.2	-95.22	-60.7	-94.89	-105.61	-124.54	-121.06	-92.54	-61.56	-64.26	-136.4	-57.3	-88.9
ERG6	**-148.66**	-146.08	-127.95	-109.2	-75.66	-111.27	-127.38	-136.01	-120.14	-113.72	-87.45	-80.64	-133.78	-53.23	-97.66
ERG7	-115.82	-119.53	-101.72	-99.23	-69.11	-102.5	-100.78	**-123.23**	-94.99	-95.97	-69.5	-69.23	-115.91	-54.32	-72.37
ERG8	-126.79	-123.78	.119.28	-115.06	-69.3	-112.8	-127.41	-124.6	**-147.59**	-125.42	-81.03	-73.89	-141.18	-59.62	-93.46
ERG9	-118.7	-110.74	-91.85	-85.54	-62.05	-98.7	-104.47	**-129.48**	-98.68	-102.28	-67.65	-68.19	-128.96	-51.12	-72.65
ERG10	-108.17	-98.72	-87.78	-100.47	-63.51	-89.1	-109.35	**-120.59**	-94.65	-98.37	-65.93	-62.89	-117.71	-47.71	-76.54
ERG11	-125.12	-110.21	-106.0	-94.87	-65.99	-104.78	-107.9	**-132.67**	-124.41	-89.27	-63.14	-67.7	-120.59	-52.39	-76.45
ERG12	**-137.87**	-117.49	-95.82	-93.84	-62.65	-103.71	-105.7	-114.89	-105.86	-107.35	-80.21	-67.72	-130.23	-54.22	-76.4
ERG13	-116.11	-108.94	-101.1	-102.98	-64.92	-94.11	-110.19	-125.61	-112.38	-94.16	-58.78	-66.37	-52.84	**-116.85**	-92.5
ERG20	-130.67	**-136.73**	-109.5	-103.32	-64.47	-102.42	-99.6	-135.62	-110.97	-104.43	-65.66	-71.65	-136.18	-54.92	-96.49
ERG24	-123.71	-132.74	-127.35	-116.73	-67.89	-124.42	-121.83	**-125.5**	-101.56	-111.31	-80.59	-80.47	-123.14	-53.69	101.39
ERG25	-122.6	**-139.04**	-114.45	-99.25	-65.4	-113.88	-111.01	-128.2	-112.14	-105.41	-68.44	-71.52	-115.88	-48.91	-80.03
ERG26	-130.93	-132.42	-107.36	-102.53	-69.67	-105.91	-116.1	**-154.44**	-124.6	-119.83	-74.19	-76.48	-145.51	-56.12	-85.43
ERG27	-125.23	**-126.9**	-123.56	-112.03	-63.05	-117.74	-125.14	-119.47	-121.18	-119.32	-61.39	-67.63	-122.04	-50.76	-83.09
ERG	-104.97	-100.58	-89.72	-84.8	-50.92	-80.34	-93.13	**-116.65**	-89.77	-90.65	-58.02	-59.27	-107.28	-38.83	-60.91

Where, ligands are expressed as 1) EGCG, 2) ECG, 3) EGC, 4) EC, 5) 2, 5- Dimethyl-4- hydroxy-3- (2H) – furanone, 6) Quercetin, 7) Myricetin, 8) Kaempferitrin, 9) Chlorogenic acid, 10) Coumaroylquinic acid, 11) Dihydroactinidiolide, 12) β ionone, 13) Rutin, 14) Pyrazine and 15) Gallic acid

Agar well diffusion assay

The preliminary investigation of the anticandidal potential of different solvent extracts of GT leaves from Assam (AT), Himachal Pradesh (HP), Uttarakhand (IP and PN) was done against *Candida albicans* (CA) and *Candida glabrata* (CG) by agar well diffusion assay. Flu (2mg/ml), AmB (1mg/ml) were used as a positive control and DMSO was used as negative control. Wells were prepared using sterile cork borer. To each assigned wells, GT extract (100mg/ml), DMSO Flu and AmBwere added accordingly. The plates were duplicated in all the experiments. Anticandidal activity was determined by measuring zone of inhibition (ZOI) at cross-angles and the mean of two reading was taken [24]. The relative inhibition zone diameter (RIZD) for each GT extract was also measured where,

$$RIZD = \frac{\text{ZOI of GT extract and antibiotic}}{\text{ZOI of antibiotic}} \times 100$$

Broth Macro dilution assay

Two fold dilution of EGCG standard was prepared in YPD broth at varying concentration of 0.07mg/ml, 0.14mg/ml, 0.28mg/ml, 0.56mg/ml, 1.12mg/ml, 2.25mg/ml and 4.5mg/ml. Similarly, two fold dilution of purified catechin of GT in YPD broth was prepared at varying concentrations (0.07mg/ml to 4.5mg/ml). Each diluted sample of standard and purified GT was inoculated with diluted culture of CA (approx. $1x\ 10^3$cfu/ml) and left for incubation for 18 hrs at 150 rpm. The YPD tube without catechins served as a culture control. The incubated broths with their respective concentration were diluted to 10 folds and were poured on YPD agar plates in duplicates. The plates were kept for overnight incubation at 37°C for 18 hrs at 150rpm. EGCG free YPD medium plate was left for incubation as control. The assay was done similarly for CG culture [26]. The minimum concentration that inhibited the growth of CA and CG on YPD agar plates by 90%, as compared with the growth in EGCG free YPD medium, was defined as MIC_{90}. Whereas, the minimum fungicidal activity (MFC) was determined as the lowest concentration resulting in the death of 99.9% or more of the initial inoculum.

Broth Micro dilution assay

Minimum inhibitory concentration (MIC) of purified GT catechins was determined by the microdilution method as described by National Committee for Clinical Laboratory Standards (NCCLS). The pre- incubated culture of CA in YPD broth (approx. $1x\ 10^3$ cfu/ml) was added into the wells of 96 well microtitre plate followed by adding serially diluted catechins (0.0625mg/ml to 4mg/ml). Well containing medium only served as blank. Serial dilution of Flu (0.0312 to 2mg/ml) and AmB (0.0156mg/ml to 1mg/ml) were also included in the experiment [27].

Anticandidal activity of combination of purified GT catechins and antimycotics

Agar well diffusion assay

The combinational anticandidal effect of GT extracts and antimycotics was assessed by agar well diffusion method with slight modifications. Combination of GT extracts (1000mg/ml) and Flu (2mg/ml) in 1: 1 ratio were prepared. Similarly, combination of GT and AmB (1mg/ml) were prepared in 1:1 ratio. Cultures of CA and CG were inoculated in YPD broth and were kept for overnight incubation at 37°C at 150 rpm. Flu and AmB were used as a positive control. For the negative control, DMSO was used. The plates were duplicated in all the experiments. Anticandidal activity was determined by measuring zone of inhibition (ZOI) at cross-angles and the mean of two reading was taken [28]. The activity index (AI) for each of the GT extract was also measured. The relative inhibition zone diameter (RIZD) for each of the GT extract was also measured.

Analytical determination of catechin in GT by HPLC

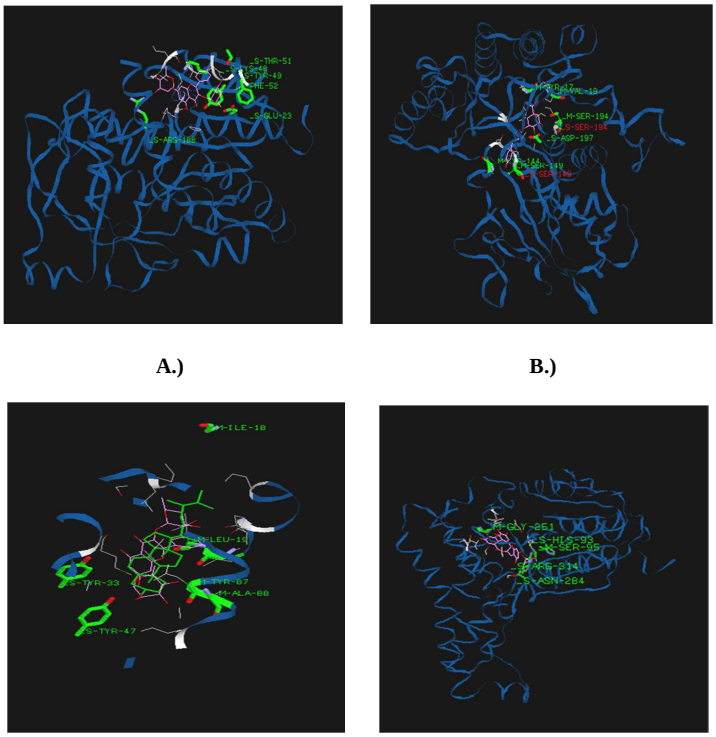
The presence of epigallocatechin gallate (ECGG) among the GT extracts was determined by HPLC analysis of the respective solvent extracts. The analytical determination of phytochemicals was performed using Dionex HPLC using varian, Microsorb- MV 100-5 C18 (250x 4.6 mm) reverse phase column fitted in thermostatic column Compartment TCC-100 oven. The detector used was UVD340U detector. Detection wavelength was set at 280 nm [29]. EGCG solution in acetonitrile was used as standard. 5µl each of standard and a purified sample were injected with a run time of 12 minutes. Specific polyphenol, EGCG was identified on the basis of their retention time.

RESULTS AND DISCUSSION

***In silico* analysis of the interaction of GT phytocompounds and ergosterol biosynthesis pathway proteins**

The *in silico* analysis of the selected GT phytocompounds revealed better interaction with the docked proteins (Figure4). The efficacy of the GT was assessed based on the binding energy of its interaction with proteins which was compared with the interaction analysis of the tested commercial drugs that act as inhibitors for ergosterol biosynthesis (Table 3, 4). Among all 15 phytocompounds tested, kaempferitrin (ZINC ID 4216676), epigallocatechin gallate (3870412), epicatechin gallate (ZINC ID 3978503), and chlorogenic acid (ZINC ID 2138728) were screened as the most active components against target proteins ERG26, ERG6, ERG 25 and ERG8

respectively. Based on the docking scores, Kaempferitrin interacted with ERG 26 at the amino acid residues Glu-23, Lys-28, Lys-48, Tyr-49, Thr-51, Phe-52 and Arg-188 with the highest binding energy of -154.44 kcal/mol. Most of the interacting amino acid residues were found within the active sites predicted by AADS (Table 5). This was followed by binding of epigallocatechin gallate and chlorogenic acid with ERG6 and ERG8 (binding energies of -148.66 kcal/mol and -147.59 kcal/mol respectively). The protein homology was validated by Swiss model workspace (Table 6).



A.) **B.)**

C) **D)**

Figure 4: Molecular Docking images of interaction via *in silico* study

(A.) Kaempferitrin and ERG 26, (B) Chlorogenic acid and ERG8, C.) Ergosterol and Kaempferitrin, D.) EGCG and ERG 6. The pink colored molecular structures represent the bound GT phytocompounds on the blue colored ribbon structure representing the ERG proteins

Table 5: Most Favourable docking sites for the interaction between GT ligands and ergosterol biosynthesis pathway proteins

S. No.	Ligand	Proteins	Favorable Amino Acid Residues at Active Sites
1.	Kaempferitrin	ERG 26	Glu-23, Lys-28, Lys-48, Tyr-49, Thr-51, Phe-52, Arg-188
		ERG	Ile-18, Leu-19, Tyr-87, Ala-88, Tyr-47, Tyr-33
2.	EGCG	ERG1	Arg-259, Glu-47, Gly-46, Meth-345, Gly-344, Thr-346
		ERG2	Tyr-160, Val-143, Ser-166, Asp-148, His-150
		ERG4	Gly-275, Arg-354, Asn-346, Tyr-268, His-401, Ala-264
		ERG6	Gly-251, His-93, Ser-95, Arg-314, Asn-284
		ERG12	Asn-409, Arg-413, Arg-237, Lys-386, Thr-181, Asp-180, Pro-240, Gly-384
3.	ECG	ERG3	Lys-240, Tyr-382, Tyr-323, Tyr-383. Phe-321, Tyr-320, Leu-319
		ERG20	Lys-262, Gln-236, Asp-239, Lys-199, Thr-197, Ile-254, Asp-100, Thr-107, Arg-108, Tyr-200, Arg-54
		ERG25	Ser-15, Asp-50, Tyr-46, Ser-42, Lys-38, Asn-16
		ERG27	Ser-183, Met-185, Arg-200, Leu-184, Pro-236, Thr-136, ala-31, Leu-248, val-250
4.	Chlorogenic acid	ERG8	Tyr-17, Val-19, ser-194, Asp-197, Lys-144, Ser-149

The quantitative changes in ergosterol content that contribute to development of antifungal resistance includes decrease in amount of ergosterol because of inhibition of its synthesis or replacement of ergosterol with sterols having reduced affinity for polyenes as well as alteration in ratio of sterol and phospholipids [30]. The resistance to polyenes is also accompanied by changes in the cell membrane permeability and masking of ergosterol in the cell membrane which prevent the binding of polyenes [31]. In a clinical study of strains of *Candida* albicans, mutation in ERG2 genes (encoding C-8 sterol isomerase) and ERG 3 gene (encoding C-5 sterol desaturase) are found to be responsible for amphotericin B and fluconazole resistance in the tested strains [32].

Among all fungi species, ERG11 also known as ERG 16 or CYP 51A1 is the gene which encodes erg11 protein or lanosterol 14-α demethylase, an essential enzyme for ergosterol biosynthesis. Resistance to azoles is associated with

alteration in ergosterol biosynthesis pathway or over expression of ERG11 and /or point mutation which causes conformational changes that reduces effective binding of azoles and their targets in *Candida albicans*, *C. glabrata* and *C. dubliniensis* clinical isolates [33]. In a yet another study it has been reported that depletion of the ERG11 gene in *C.glabrata* results in accumulation of 4,14-demethylzimosterl which does not harm the growth of fungal cells *in vitro* and *in vivo* [34]. Also, up regulation of efflux pumps (ATP-binding cassette transporters) and the major facilitator's superfamilies (MFS) reduces the effective concentration of antifungal drugs which is correlated with the antibiotic resistance in *Candida* species [35].

Green tea from different geographical locations of India shows remarkable differences in their anticandidal activity. This regional variation due to difference with respect to the climate, physiological conditions prevailing under these regional sites might have contributed in the varying concentration of EGCG in GT leaves [10]. ERG 26 (C-3 sterol dehydrogenase), ERG 6 (sterol 24-C methyl transferase), ERG 25 (C-4 methyl sterol oxidase) and ERG 8 (Phosphomevalonate kinase) are the novel drug target for Green tea against *Candida* pathogens.

Table 6. Validation of protein Homology by Swiss model workspace

S. No.	Enzymes	Amino acid residue in most favored regions for interaction(%), based on Ramachandran Plot
1.	**ERG**	**96**
2.	ERG1	78.5
3.	ERG2	75.1
4.	ERG3	77.9
5.	ERG4	72.8
6.	ERG5	85.8
7.	ERG6	78.3
8.	ERG7	89.5
9.	ERG8	78.2
10.	ERG9	89.3
11.	**ERG10**	**92.4**
12.	ERG11	89.2
13.	ERG12	89.1
14.	ERG13	89.2
15.	**ERG20**	**93.2**
16.	ERG24	74.2
17.	ERG25	74.1
18.	ERG26	82.1
19.	ERG27	78.4

Anticandidal potentials of GT alone and in combination with antifungal drugs

The preliminary analysis of anticandidal effect of extracts (methanolic, acetone and aqueous) of Green tea (AT, HP, IP and PN) revealed that GT extracts alone does not have significant inhibitory effect against *Candida albicans* while it has significant inhibition against *Candida glabrata*. Extracts combined with antimycotics showed moderate to high inhibitory activity in comparison to antimycotics alone ($p \leq 0.05$). The Flu was found in-effective against both the *Candida* sp. with no significant zone of inhibition (ZOI) as observed by student T- test, ($p \leq 0.05$). When Flu (2mg/ml) and GT extracts (1000mg/ml) were used in combination (1:1), higher anticandidal effect with increased ZOI was observed [Table 7(A), 7(B), 7(C)]. AmB had a significant inhibitory effect against both the *Candida* sps. However, the combined application of GT (100 mg/ml) and AmB (1mg/ml) also revealed increased anticandidal effect. In *Candida albicans*, highest percentage of relative inhibition zone diameter (RIZD) was found at 150± 0.707 % when acetone extracts of AT was used in combination with AmB while for *Candida glabrata*, RIZD was 148.7± 0.353% with combination of methanolic extract of IP and AmB.

Antimicrobial activities of tea extracts are very selective. This difference in their activity depends upon the concentration and type of the extracts. These effects may also differ depending on the microorganism so that they may be either growth inhibitory or stimulatory [36]. The difference in antifungal potential of green tea leaves from *Camellia sinensis* plants can be attributed to the different biochemical activity, geographical region of plant collection, extraction method [37, 38, 39, 10]. The impairment in drug diffusion is a major limitation in the evaluation of the antimicrobial effect of plant extracts using agar well diffusion method [30]. Despite the fact that GT extracts showed weak inhibition against *Candida* species using agar well diffusion assay, the combined effect of antimycotics and plant extracts were mainly additive which could be attributed to the inability of higher concentration of plant extracts to diffuse through agar medium [40].

Table 7(A). ZOI of *Candida albicans* MTCC 3017 (CA) and *Candida glabrata* MTCC 3019 (CG) by Green tea (GT) methanolic extract alone and in combination with antimycotics by Agar well diffusion assay

Methanolic extract	ZOI of extract (alone) in mm		ZOI of combination (GT and Flu) in mm		ZOI of combination (GT and AmB) in mm	
	CA	CG	CA	CG	CA	CG
AT	-	14	11.75± 0.353	11.25±0.353	18.5±0.707	12.25±0.353
HP	-	14.5±0.070	17.9±0.141	16.5±0.707	21.50±1.414	15.5±0.707
IP	-	15.5 ±0.070	14.25±0.353	15.75±0.353	20.5±0.707	15.25±0.353
PN	-	10.5±0.070	14.25±0.353	16	19.5±0.707	15.25±0.353
Methanolic extract	**ZOI of Flu (alone) in mm**		**ZOI of AmB (alone) in mm**		**Activity Index (AI)**	
	CA	CG	CA	CG	CA	CG
AT	-	-	14.5±0.707	10.7±0.353	1.23±0.042	1.13±0.007
HP	-	-	15±0.707	11.5±0.353	1.44±0.155	1.37±0.103
IP	-	-	14.5±0.707	10.25±0.353	1.36±0.049	1.48±0.09
PN		-	15±0.707	10.75±0.353	1.40±0.077	1.41±0.077

Table7 (B). ZOI of *Candida albicans* MTCC 3017 (CA) and *Candida glabrata* MTCC 3019 (CG) by Green tea (GT) acetone extract alone and in combination with antimycotics by Agar well diffusion assay

Acetone extract	ZOI of extract (alone) in mm		ZOI of combination (GT and Flu) in mm		ZOI of combination (GT and AmB) in mm	
	CA	CG	CA	CG	CA	CG
AT	-	13.5±0.070	11.75± 0.707	11. 5±0.707	22.5±0.707	14.25±0.707
HP	-	14.5±0.070	14.25±0.353	14.25±0.353	18±1.414	14.25±0.353
IP	-	15	15.5± 0.707	15.5±0.707	15	15
PN	-	13.25±0.282	14.75±0.353	14.75±0.353	17±1.414	14.25±0.707
Acetone extract	**ZOI of Flu (alone)in mm**		**ZOI of AmB (alone)in mm**		**Activity Index (AI)**	
	CA	CG	CA	CG	CA	CG
AT	-	-	15	10.25±0.353	1.44±0.077	1.21±0.021
HP	-	-	14.5±0.707	10.5±0.707	1.23±0.155	1.31±0.056
IP	-	-	14±0.707	10.5±0.707	1.07±0.049	1.43±0.09
PN		-	15.5±0.707	10.75±0.353	1.13±0.042	1.34±0.106

Table 7(C): ZOI of *Candida albicans* MTCC 3017 (CA) and *Candida glabrata* MTCC 3019 (CG) by Green tea (GT) aqueous extract alone and in combination with antimycotics by Agar well diffusion assay.

Aqueous extract	ZOI of extract (alone)in mm		ZOI of combination (GT and Flu) in mm		ZOI of combination (GT and AmB) in mm	
	CA	CG	CA	CG	CA	CG
AT	-	-	-	-	16±0.141	11.25±0.353
HP	-	-	-	-	15	11
IP	-	-	-	-	14.9± 0.141	10.25±0.353
PN	-	-	-	-	14.75 ±0.353	10.75±0.707
Aqueous Extract	**ZOI of Flu (alone) in mm**		**ZOI of AmB (alone) in mm**		**Activity Index (AI)**	
	CA	CG	CA	CG	CA	CG
AT	-	-	14.5±0.707	11.25±0.353	1.01±0.021	1
HP	-	-	15.5±0.707	10.25±0.353	0.96±0.049	1.07±0.042
IP	-	-	14.5±0.707	10	1.02±0.353	1.025±0.353
PN		-	16.5±0.707	10.25±0.353	0.96±0.044	1.05±0.707

Broth Macrodilution assay

The concentration of purified catechins ranging from 1.12mg/ml to 4.5 mg/ml was found inhibitory against *Candida albicans* and *Candida glabrata*. The purified catechins from AT were depicted as the most effective at lowest concentration. MIC_{90} at 1.12 mg/ml while minimum fungicidal activity (MFC) at 2.25 mg/ml was recorded against *Candida albicans*, while MIC90 for *Candida glabrata* was recorded at 2.25mg/ml while concentration of 4.5mg/ml was found to be MFC (Table 8).

Table 8: Broth Macrodilution assay of GT purified catechins

	EGCG standard (mg/ml)		AT (mg/ml)		HP (mg/ml)		PN (mg/ml)		IP (mg/ml)	
	MIC_{90}	MFC	MIC_{90}	MFC	MIC_{90}	MFC	MIC_{90}	MFC	MIC_{90}	MFC
CA	0.562	2.25	**1.12**	2.25	2.25	4.5	1.12	4.5	1.12	2.25
CG	1.12	2.25	2.25	4.5	**2.25**	4.5	4.5	4.5	2.25	4.5

Where, the minimum concentration that inhibited the growth of CA and CG on YPD agar plates by 90%, as compared with the growth in EGCG free YPD medium, was defined as MIC_{90} and the minimum fungicidal activity (MFC) was determined as the lowest concentration resulting in the death of 99.9% or more of the initial inoculum.

Broth Microdilution Assay

Table 9: Broth micro dilution assay of GT purified catechins

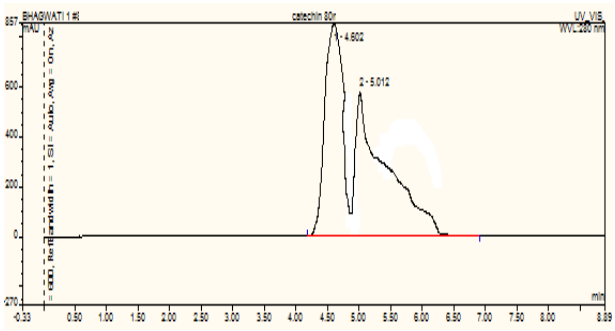
	EGCG standard (mg/ml)		AT (mg/ml)		HP (mg/ml)		PN (mg/ml)		IP (mg/ml)	
	MIC_{90}	MFC	MIC_{90}	MFC	MIC_{90}	MFC	MIC_{90}	MFC	MIC_{90}	MFC
CA	0.125	0.5	**0.12**	0.25	0.25	0.5	0.5	1	0.25	0.5
CG	0.25	0.5	0.5	0.25	**0.25**	0.5	0.5	1	0.5	1

The minimum concentration of purified catechins (MIC_{90}) from AT, 0.12mg/ml to 1mg/ml was found inhibitory against *Candida albicans* while minimum fungicidal activity (MFC) of all the purified catechins from different GT was found between 0.25mg/ml to 1mg/ml. The MIC_{90} for *Candida glabrata* of purified catechins from HP was recorded at lowest concentration of 0.25 mg/ml, while concentration ranging between 0.25mg/ml to 1mg/ml was found to be MFC (Table 9).

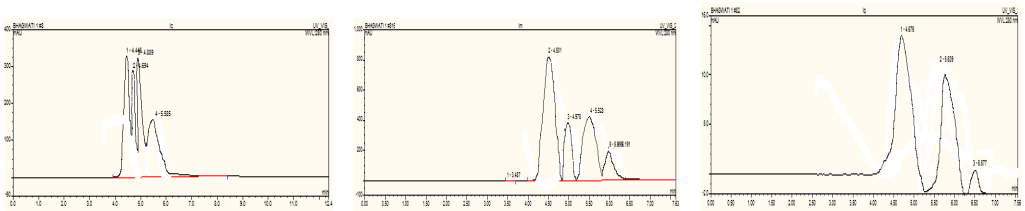
Where, the minimum concentration that inhibited the growth of CA and CG on YPD agar plates by 90%, as compared with the growth in EGCG free YPD medium, was defined as MIC_{90} and the minimum fungicidal activity (MFC) was determined as the lowest concentration resulting in the death of 99.9% or more of the initial inoculum.

HPLC chromatograph

Calibration curves were obtained at detection wavelength of 280nm. The HPLC chromatogram of methanolic, acetone and aqueous extract of GT depicted the separated peaks which were identified based on the retention time correlated with the standard EGCG peak. The EGCG was found to be present in all the extracts with the retention time ranging between 4.501 min to 4.676 min. (Figure 5).



Standard EGCG



IC **IM** **IQ**

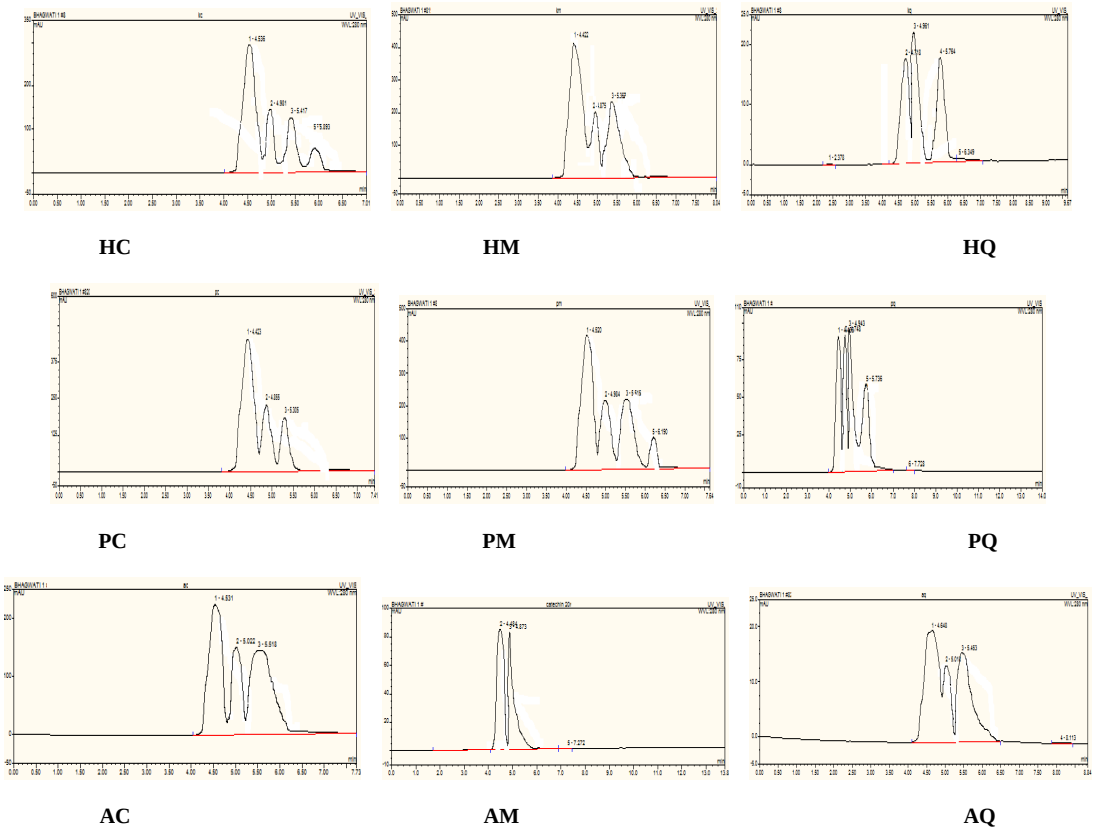


Figure5: HPLC chromatograph of standard and the different solvent extracts of GT

Where, IC, IM and IQ are acetone, methanolic and aqueous extracts of IP green tea, HC, HM and HQ are acetone, methanolic and aqueous extracts of HP green tea, PC, PM and PQ are acetone, methanolic and aqueous extracts of PN green tea while, AC, AM and AQ are acetone, methanolic and aqueous extracts of AT green tea respectively

CONCLUSION

The *in silico* study represents green tea as a potent herbal candidate with marked inhibitory activities against the *Candida* species. The identified novel protein targets of GT will be useful for anticandidal drug therapy. Also, it can be pretended that simultaneous interaction of GT and antifungals with ERG proteins could be the prohibitory mechanism that inhibits the growth of *Candida* species implying the candidacidal synergy between GT and antifungals. There is an urgent need to check the cytotoxic effect of the GT catechins alone and in combinations with antimycotics against *Candida* species to further confirm the safety of the desired herbal combination.

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