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Attenuation of Pathogenicity in *Candida albicans* by Application of Polyphenols

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Abstract

Candida albicans is one of the most pathogenic and opportunistic class of fungi. Its emergence disturbs the human biome and can create serious damage in immunocompromised conditions. Hyphal growth and biofilm formation are mainly the core factors leading to progression of pathogenicity in *C. albicans*. The drug resistance towards azoles has created a demand of new novel pharmacologically active compounds. Although there are several pathways and factors known for causing pathogenicity there is none single connected pathway known for the whole mechanism such as SAP5, N-myrstyltransferase, Erg11 and Efg1 proteins. In the present study we have focussed on all the pathways and sorted major factors contributing in more than one pathways. The plant based polyphenols that have been studied here are non-toxic and more efficient as compared to the marketed azoles. These selected polyphenols were found to be capable of causing cell death of *C. albicans* up to 20% and inhibiting hyphal growth up to 90% as compared to Fluconazole. Biologically the polyphenols have been found to be more active on glyoxylate pathway.

Keywords: *Candida albicans*; Hyphal growth; Pathogenicity; Quercetin; Chalcone; Chromone; Curcumin

Introduction

Candida albicans is a major human pathogen and one of the most important causes of fungal infection in human beings. *C. albicans* is a harmless commensal of human biome in yeast form mainly found in skin and gastrointestinal cavity. It is a truly polymorphic organism and can undergo morphological changes between the yeast form (with rounded cells and daughter buds that physically separate from the mother cell), the pseudohyphal form (consisting of chains of cells with different degrees of elongation that still show constrictions between adjacent cells), and the true hyphal form (consisting of long tubes with parallel sides and no constrictions) [1]. Hyphal form proliferation makes it invasive and it can penetrate host tissues and these polymorphic changes aggravate in the immunocompromised conditions. The mutant's defective in hyphal growth are known to be less virulent [2].

C. albicans cannot be permanently removed from the human biome and nature. Furthermore, resistance to the currently available anti-fungal medications is rising [3,4]. This situation makes fungal infections a significant public health concern and thus novel anti-fungal drug targets are of great interest. Most anti-Candida drugs target cell membrane or cell wall synthesis, so identification of alternative physiological pathways that affects pathogenicity and genes involved in the yeast-to-hyphae transition is an important goal for the development of new antifungal agents. There are several factors and targets that have been known as the core factors responsible for virulence and pathogenicity in *C. albicans*. From several studies it has been found that the root cause of virulence in Candida is hyphal growth. The non-filamentous strains of *C. albicans* are non-virulent in nature [5]. So, the inhibition of the imbalance and secondary infections due to virulent strain can be prevented by inhibition or attenuation of pathways that leads to cause virulence. These causes are filamentation, white opaque phase transition and biofilm formation etc. This occurs through the contribution of many factors such as genetic, transcriptional, environmental, etc. Thus, coming down to the major facts we can take four principal pathway taking place in different cell organelles of *C. albicans* such as glyoxalate pathway, Ras1-pka pathway, Ergosterol pathway and signalling pathways. Further the key factors which can potentially affect maximum pathways have been listed below:

Efg1: Efg1 stands for enhanced filamentous growth. Efg1 is involved in many interactions such as stress resistance, Ras1 pka pathway, white opaque phase transition and biofilm formation. It's a transcriptional factor involved in linking many pathways. Role of Efg1 protein antagonises the action of WOR1, WOR2 and CZF1. WOR1 is required to maintain white cell identity. Efg1 is also required for TOR1 dependent cellular aggregation and causes hypoxial biofilm formation. The study of the *EFG1* gene showed that a reduced *EFG1* expression level suppresses formation of true hyphae [6]. Efg1 is required for expression of all hyphal-specific genes, and Tec1overexpression has been shown to restore filamentous growth in an efg1/efg1 mutant [7].

2QZX: Sap5 has been taken as the second target. SAP5 (Secreted aspartic proteinase5) is a class of hydrolytic enzymes. Various classes of enzymes participate in the process of virulence and invasion. The attenuation of hydrolytic enzymes in case of candida reduces virulence [8]. Proteolytic activity is an important virulence factor. Also, the hydrolytic enzymes mainly SAPs contribute for adoption and overcoming of residual host barriers and infection. SAP5 being an important hydrolytic enzyme non-likely to have similar homology in human and is a potential target of pathogenicity in *C. albicans*.

Erg11: Ergosterol biosynthesis pathway occurs in the cell membrane of *C. albicans*. Mostly the drugs available in the market against candida

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emergence is the class of azoles mainly fluconazole. The key factor of this pathway is Erg11. Erg11 mainly encodes for the enzyme cytochrome P450 lanosterol 14a-demethylase [9]. The inhibition of this protein can directly affect the pathways leading to cellulase membrane damage in candida. Most of the azole drug targets Erg11 though recently candida has developed drug resistance to azoles due to mutation in Erg11. Hence the target has been taken to find other inhibitors having better affinity than azoles.

Glyoxylate pathway: Glyoxylate pathway commonly known as TCA (Tri carboxylic acid) is an essential metabolic pathway taking place in *C. albicans*, since it is essential for it to survive in nutrient limited host niches and adverse host environment. This pathway occurs in three cell organelles such as cytoplasm, mitochondria and peroxisome [10]. There are cascade of reactions occurring but few enzymes like N-myristoyltransferase and Isocitrate lyase (ICL) are the core enzymes responsible for the pathways. Here, we have targeted N-myristoyltransferase for the inhibition of the pathway. Glyoxylate pathway has non-homologs with mammals which makes it a very safe and important target (Figure 1).

In the following study we have targeted *in silico* all these targets with a class of polyphenols such as flavonoids, coumarins, chalcones and curcumin analogs. Chalconesare precursor compounds for flavonoids biosynthesis in plants, and they can also be synthesized in laboratory. Chalcones possess a broad spectrum of biological activities including antioxidative, antibacterial, antihelmintic, amoebicidal, antiulcer, antiviral, insecticidal, antiprotozoal, anticancer, cytotoxic and immunosuppressive. Changes in their structure have offered a high degree of diversity that has proven useful for the development of new medicinal agents having improved potency and lesser toxicity and good pharmacological actions. Chalcones, Coumarins have close relationship with flavones, aurones, tetralones and aziridines. Flavonoids are present naturally as plant pigments, as secondary metabolites of plants and as conjugates with many other natural compounds [11-13]. These are anti-oxidant compounds and in the present scenario these have evolved as important lead molecules. Flavonoid possesses multiple anti-bacterial, anti-cancerous and anti-fungal properties.

Curcumin, the yellow pigment of spice turmeric is a polyphenol. *In vitro*, curcumin inhibits certain epigenetic enzymes (the histone deacetylases: HDAC1, HDAC3, HDAC8), transcriptional co-activator proteins [14]. Its analogues enjoy a range of biological activities such as antimicrobial, antioxidant, cytotoxic, anticancer, antiprotozoal, antihistaminic, antiulcer and anti-inflammatory activities which makes these compounds as special attraction for investigation. All these compounds are less toxic than synthetic drugs and contain biofilm degrading capacity. Hence, curcumin derivatives do have better potential over known commercial azole antifungal drugs. One of these analogues of curcumin, i.e., 1,7-di (1H-indol-3yl) hepta1,6-diene-3,5-dione has been used for therapy of varies cancers [15]. The difluoro boron complex has been used for cyanide ion sensing ability [16]. It can inhibit proliferation of various cancer cell lines (A549, K562, SW480) as compared to curcumin and it induced the activation of caspase-3,8,9 followed by down regulation of cyclin D1.

The main objective of the present work is to design and discover new plant based antifungal compounds specifically for inhibiting filamentation in Candida species and thus attenuate its pathogenicity since these would be non-toxic and better solve the problem of drug resistance as well.

Materials and Methods

In the present study all the proteins were either downloaded from

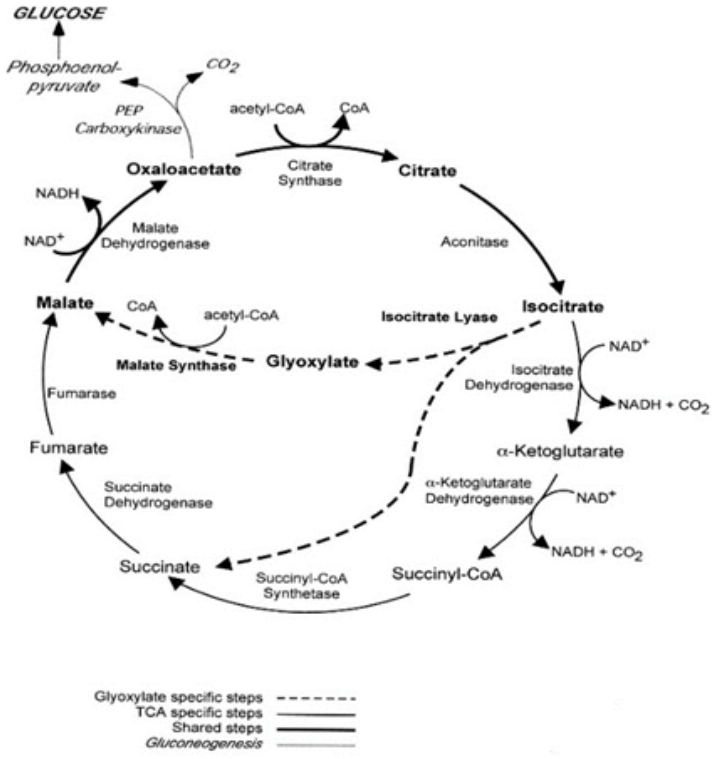


Figure 1: Glyoxylate pathway in *Candida albicans*.

RCSB or modelled by I-Tasser using the sequences of particular strains of the species that have to be considered for further *in vitro* studies. The strains were provided by the school of life sciences, JNU. The software's used for *in silico* analysis were Schrodinger, modules Glide, Virtual screening workflow, ADME prediction, etc. All the media and solvents used for *in vitro* studies were purchased from Sigma Aldrich.

All chemicals, reagents and Duterated solvents were purchased from Sigma Aldrich and Merck Monitored reactions of TLC plates (Aluminium silicagel coted) and it's purchased from Merck. 1H NMR, 13C NMR. Spectrum w recorded on a Bruker 400 MHz spectrometer, at 400 MHz and 100 MHz for 1H and 13C respectively. Chemical shifts (δ) values and tetramethylsilane is used as an internal standard in during of spectrum analysis. All the 1H and 13C spectra were reported, value of Coupling constant (J) was reported in Hz. The spots reaction mixture was visualized in UV chamber, and stain with solution of PMA, $KMnO_4$. Charring of it's with heat gun. Purification of products was carried out by silica gel column chromatography (100-200) mesh size.

The study began with the screening of targets for *C. albicans*. The targets were sorted based on the pathways that lead to virulence in *C. albicans*. The pathogenicity and drug resistance in *C. albicans* has become a serious problem in the present scenario. The whole methodology has been divided in three steps:

In silico

The protein modelling and preparation: The protein targets N-myristoyl transferase and SAP5 protein structures were downloaded from RCSB (Research Collaboratory for Structural Bioinformatics). N-myristoyl transferase protein pdb file has been downloaded from RCSB, pdb id-1IYK contains two chains A, B and 392 amino acids with two distinct inhibitor myristoyl-COA and peptidic inhibitor (p1). SAP5 (Secreted Aspartyl Proteinase5) protein pdb file has been downloaded from RCSB, pdb id-2QZX, containing macromolecule Candidapepsin-5, two chains A, B and 342 amino acids [17]. Sap5 clearly differs from Sap1-3 by its electrostatic overall charge as well as through structural conformation of its entrance to the active site cleft. Design of inhibitors specific for Sap5 should concentrate on the S4 and S3 pockets, which significantly differ from Sap1-3 in size and electrostatic charge. Saps show controlled expression and regulation for the individual stages of the infection process.

Efg1 and Erg11 protein has been modelled using I-Tasser online server. The protein sequence of *C. albicans* strain SC5314 was downloaded from NCBI [18]. I-TASSER server is an on-line platform that utilizes meta-threading approach to find similar folds and templates. The continuous fragments excised from the PDB templates are reassembled into full-length models by replica-exchange Monte Carlo simulations with the threading unaligned regions (mainly loops) built by ab-initio modelling. In cases where no appropriate template is identified by LOMETS, I-TASSER builds the whole structure by ab-initio modelling. The low free-energy states are identified by SPICKER through clustering the simulation decoys. The quality of the models was assessed using the DOPE (Discrete Optimized Protein Energy) method. The model with the lowest DOPE score was selected as the best model over the same query sequence. Then, the Ramachandran distribution plots were plotted for these two best initial models, and the one with better performance was selected for further validation by Verify 3D.

All the protein structures were prepared in ProteinPrep module of Schrodinger [19]. It modifies the non-bonded atoms and stabilizes the protein by removing extra water molecules. After the energy minimization further, the proteins were ready for virtual screening.

Ligand library preparation: The ligand library was prepared by collecting the different analogs of polyphenols. The molecules belonging to classes of coumarins, flavonoids and curcumin analogs were collected from data bases and the ligand molecules were stabilized and prepared in LigPrep module of Schrodinger. The module stabilizes the molecules based on Lipinski rule.

Virtual screening and docking: All the ligand libraries were docked in the specific pockets of the proteins. The binding sites of proteins were located using SiteMap module of Schrodinger [20]. SiteMap has proven algorithm for binding site identification and it generally proposes three to five active sites based on the volume and position. The best possible pocket was considered for further protocol.

Virtual screening was carried out in Glide [21]. Glide provides a rational workflow for virtual screening from HTVS to SP to XP, enriching the data at every level such that only an order of magnitude fewer compounds need to be studied at the next higher accuracy level. Glide assembles the programs highly configured to analyse large amount of data accurately.

Based on the activity of compounds such as docking scores and No of H-bonds in the protein ligand interaction the best compounds were sorted. The compounds Chalcone, Coumarin, tetraacetate quercetin, pentaacetate quercetin and (1E,6E)-1,7-di(1H-indol-3-yl) hepta-1,6-dience-3,5-dione) were screened based on their docking score, interaction with protein amino acids and their common affinity with all the protein targets. These compounds were synthesised in the laboratory using unambiguous methods and further tested *in vivo* for their sensitivity.

Chemical synthesis and spectroscopy

Synthesis of 7-hydroxy-2H-chromen-2-one4: A mixture of resorcinol (2.8 g, 25 mmol), malic acid (4 g, 29 mmol) and concentrated sulfuric acid (10mL) was stirred at room temperature for 1 h. The reaction mixture was heated to 100°C and stirred for another 3 h. After cooling to room temperature, ice water (50 g) was added to the reaction mixture and stirred for 0.5 h. The reaction mixture was filtered and dried to afford compound as white solid. Crude product was purified with column chromatography, make elution gradient was ethyl acetate: hexane (5:5) [22]. Yield 71%, mp 232–234°C. 1H NMR (400 MHz, DMSO) δ 10.57 (s, 1H), 7.96-7.92 (m, 1H), 7.53 (d, J=8.5 Hz, 1H), 6.82-6.74 (m, 1H), 6.72 (d, J=2.2 Hz, 1H), 6.21 (d, J=9.5 Hz, 1H). 13C NMR (101 MHz, DMSO) δ 161.77, 160.90, 155.97, 145.00, 130.19, 113.59, 111.88, 111.75, 102.63.

Synthesis of E-chalcone: To the stirred solution of NaOH (2.2 g, 0.05 mol) in aq.ethanol (EtOH:H_2O 50:2 ml) at 0°C a solution of benzaldehyde (7.2 g, 0.06 mol) and acetophenone (6.8 g, 0.05 mol) in ethanol (50 ml) was added during 10 min. The reaction mixture was stirred for 12 h and reaction followed with TLC. After completion of reaction, water (200 ml) was added to the reaction mixture. The obtained precipitate of chalcone was filtered and washed with cold water; product weight was 10 g, yield 71% [23]. ^{1}H NMR (800 MHz, $CDCl_3$) δ 8.04 (d, J=7.2 Hz, 1H), 7.83 (d, J=15.7 Hz, 1H), 7.65 (dd, J=6.4, 2.7 Hz, 2H), 7.59 (t, J=7.3 Hz, 1H), 7.56 (s, 1H), 7.54 (s, 1H), 7.52 (t, J=7.7 Hz, 2H), 7.45-7.41 (m, 3H). ^{13}C NMR (201 MHz, $CDCl_3$) δ 190.58, 144.88, 138.23, 134.90, 132.84, 130.60, 129.00, 128.67, 128.55, 128.50, 122.09, 77.25.

Synthesis of tetra acetate of quercetin

4-(5,7-diacetoxy-3-hydroxy-4-oxo-4H-chromen-2-yl)-1,2-phenylene diacetate: To the stirred solution of quercetin (1 g, 3.3

mmol) in dichloromethane (100 ml), 2-5 drops of pyridine were added followed with acetic anhydride (3.3 ml, 32 mmol). Reaction mixture was stirred at rt for 24 h and after completion of reaction the product was purified with column chromatography [24]. Elution was made with gradient of ethyl acetate: hexane (39:61). The yield of the pure product was 650 mg, (yield, 43%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.10 (s, 1H), 7.83-7.67 (m, 2H), 7.45-7.29 (m, 1H), 6.86 (d, *J*=2.0 Hz, 1H), 6.61 (d, *J*=2.0 Hz, 1H), 2.43-2.27 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.29, 168.21, 167.84, 167.76, 161.75, 156.37, 155.96, 144.66, 142.27, 132.23, 127.58, 126.57, 124.05, 108.83, 105.57, 101.19, 21.21, 20.70, 20.43 [24].

Synthesis of penta acetate of quercetin: 2-(3,4-diacetoxyphenyl)-4-oxo-4H-chromene-3,5,7-triyl triacetate: To the stirred solution of quercetin (1 g, 3.3 mmol) in acetic anhydride (10 ml, 98 mmol), pyridine (1-3 ml) was added and the mixture refluxed for 2 h and reaction monitored with TLC. After completion of reaction, 200 ml water was added in to reaction mixture. The product was extracted with ethyl acetate, concentrated under reduced pressure [25]. The product was purified with silica gel column chromatography. Elution was done with gradient of ethyl acetate: hexane (40:60). The pure product obtained was 1 g (yield 62%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (dd, *J*=8.5, 2.1 Hz, 1H), 7.69 (d, *J*=2.0 Hz, 1H), 7.36 (s, 1H), 7.34-7.33 (m, 1H), 6.88 (d, *J*=2.2 Hz, 1H), 2.43 (s, 3H), 2.35-2.33 (m, 12H).13C NMR (151 MHz, CDCl3) δ 170.07, 169.29, 167.90, 167.86, 167.84, 156.89, 154.30, 153.82, 150.45, 144.41, 142.24, 134.10, 127.81, 126.46, 123.96, 123.88, 114.81, 113.92, 109.01, 21.20, 21.06, 20.69, 20.53 [24].

Synthesis of 1,7-di(1H-indol-3-yl)hepta-1,6-diene-3,5-dione: To the stirred mixture of acetyl acetone (250 mg, 2.5 mmol) and boron trioxide (130 mg, 1.8 mmol) in Argon atmosphere a solution of indole-3-carboxaldehyde (725 mg, 5 mmol) in 35 ml of absolute ethyl acetate was added at rt during 15 min. Tributyl borate (5 ml, 18.5 mmol) was added in the reaction mixture and n-butyl amine (0.05 ml,0.47 mmol) dissolved in ethyl acetate (5 ml) was added dropwise and mixture stirred for 20 h at rt. Reaction was monitored with TLC, after completion of reaction, the mixture was concentrated under reduced pressure and crude product (5) diluted with 200 ml methanol, refluxed for 4 h. The reaction mixture was again concentrated under reduced pressure. Crude product was finally purified with column chromatography; elution was made with gradient of ethylacetate:hexane (45:55). The pure product was obtained in 79% yield (700 mg), 1H NMR (800 MHz, DMSO, 11.81 (s, 2H), 7.98 (dd, J=10.4, 5.3 Hz, 2H), 7.93 (d, J=2.7 Hz, 2H), 7.85 (dd, J=16.0, 4.7 Hz, 2H), 7.47 (t, J=7.3 Hz, 2H), 7.23-7.16 (m, 4H), 6.62 (d, J=15.9 Hz, 2H), 5.89 (s, 2H). 13C NMR (201 MHz, DMSO) δ 204.26, 194.65, 181.16, 137.98, 135.50, 133.39, 123.06, 121.12, 120.65, 117.07, 113.13, 112.45, 100.26, 55 (Schemes 1-4 and Figure 2).

In vivo

Sensitivity of compounds: The sensitivity of compounds was tested *in vivo*. Candidanian strain SC5314 has been taken for analysis. The wild strain was tested individually first to observe the behaviour for normal growth and filamentation. The compounds were dissolved in DMSO to create their molar solutions. Further the wild type strain

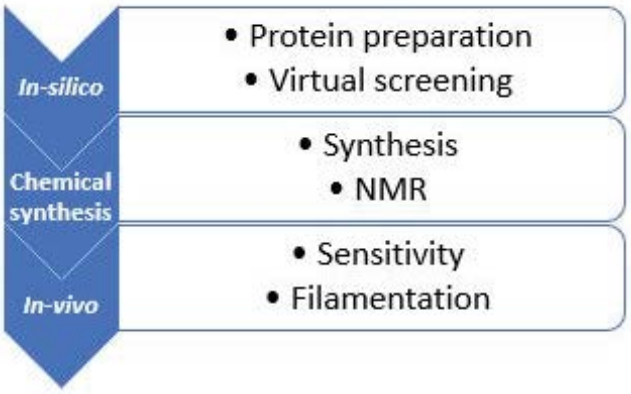
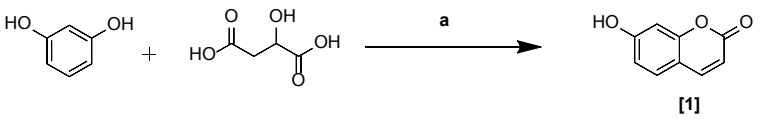


Figure 2: The workflow of methodology.

Synthesis of 7-Hydroxy-2H-chromen-2-one: [1]

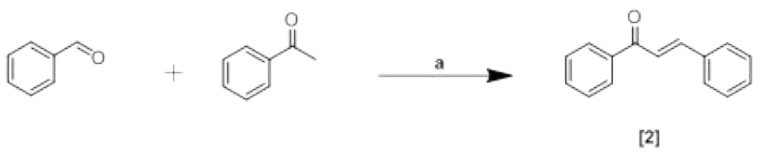


[1]

Reagent and Condition: (a) H_2SO_4, rt, 1h, 100 ^oC, 3h.

Scheme 1: Synthesis of 7-hydroxy-2H-chromen-2-one.

Synthesis of E-chalcone [2] :

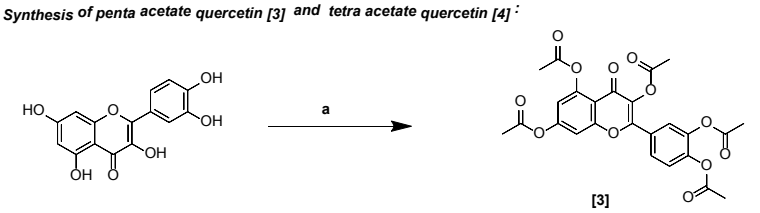


[2]

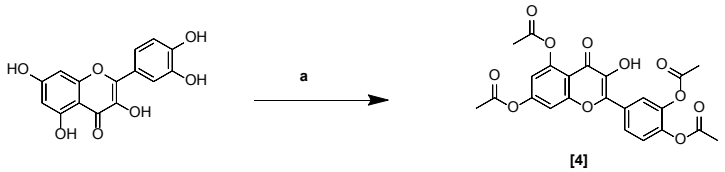
Reagent and condition: (a) Methenol, NaOH, rt, 12h.

Scheme 2: Synthesis of E-chalcone.

Synthesis of penta acetate quercetin [3] and tetra acetate quercetin [4]:



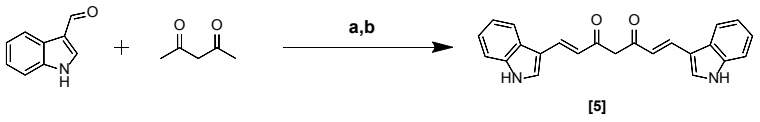
Reagent and condition: (a) Acetic anhydride, Pyridine, 100 ^{0}C, 2h



Reagent and condition: (a) DCM, Acetic anhydride, Pyridine, rt, 24h

Scheme 3: Synthesis of penta acetate quercetin and tetra acetate quercetin.

Synthesis of 1,7-di(1H-indol-3-yl)hepta-1,6-diene-3,5-dione [5]:



Reagent and condition: (a) acetyl acetone, boron trioxide, rt, 15 m (b) Ethyl acetate, n-butylamin, tributylborate, rt, 20h.

Scheme 4: Synthesis of 1,7-di(1H-indol-3-yl)hrpta-1,6-diene-3,5-dione.

capable of filamentation SC5314 was tested in 96 well plate methods with standard fluconazole. The solvent media used was RPMI; it is one the purest media used in sensitivity test. The plates were kept at 37°C incubation 24 h. The settled precipitate of candida cells was visible in the wells which showed less or no activity. To get the accuracy, the OD of the plate was taken to further calculate the IC80 values. The process was repeated five times to ensure the accurate sensitivity of the compounds. The molarity of the most active well (MIC) and 80% of control well (IC80) was calculated [26]. The process was carried out with around 8 compounds and the considerable activity was found in around 5 of them. These compounds were further tested for filamentation.

Filamentation test was performed further to validate whether the compounds are showing activity with the chosen targets biologically or not. The filamentation test was performed in spider media at pH 4.5 and 37 degree centigrade at 220 rpm. It provides the strain most compatible environment for filamentation. The filamentation was observed at several time intervals 1.5 h, 3 h, 5 h, 7 h and 12 h. Cells were imaged by DIC microscopy.

The compounds that show less or no growth of filaments should be targeting the pathways related to Ras1 pka pathway, glyoxylate pathway and hydrolytic enzymes. Otherwise if there is coagulates and still no filamentation that would refer to erg11 pathway as the compound will act on the cell membranes and the organelles will coagulate together without membrane.

Results and Discussion

In silico

The compounds that were found to have better affinity with all four targets were 7-hydroxy-2H-chromen-2-one (Coumarin), (E)-1,3-diphenylprop-2-en-1-one (Chalcone), 2-(3,4-diacetoxyphenyl)-4-oxo-4H-chromene-3,5,7-triyltriacetate (Pentaacetate quercetin), 4-(5,7-diacetoxy-3-hydroxy-4-oxo-4H-chromen-2-yl)-1,2-phenylene diacetate (Tetraacetate quercetin), (1E,6E)-1,7-di(1H-indol-3yl) hepta-1,6-diene-3,5-dione (Di indol-1,6-diene-3,5-dione),2-(2,4-Difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl) propan-2-ol (Fluconazole).

The most of H-bonds were occupied in the regions of phenyl ring. This shows the relevance of polyphenols and justifies their combination with most of the target proteins.The respective compound their 2D interaction, H-bonds and amino acids in their active site have been tabulated (Table 1). The detailed interaction and docking diagram have also been represented in (Figures 3-15).

Pentaacetate quercetin, tetraacetate quercetin and coumarins have been found active in all four targets illustrating their high affinity. Fluconazole which is an already known marketed drug for antifungal treatment has been reported with many side effects. Including fluconazole many azoles are very toxic for human biome. In last few years candida species has also shown drug resistance against azole compounds. In such scenario polyphenols seem to show promising results and also they are non-toxic.

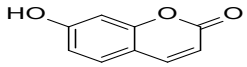
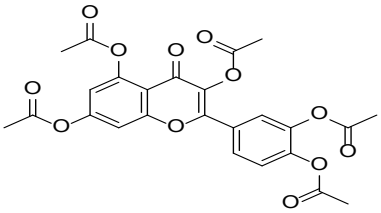
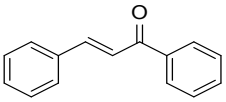
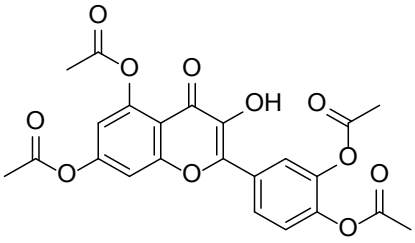
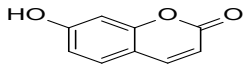
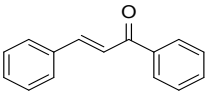
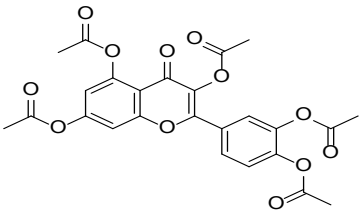
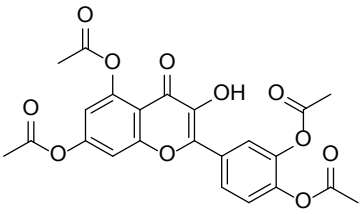
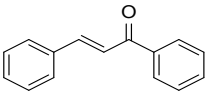
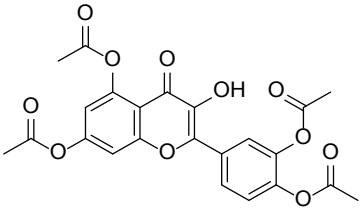
Protein Targets	Compounds	2D Structures	Docking Score	H-Bonds	Amino acids in active site
N-myristoyl transferase	Coumarin (1)		-5.541	1, GLY429	Charged(-ve) ASP428; Charged(+ve) LYS433; Hydrophobic MET427, LEU220, PRO426, PRO214, ILE431; Polar THR221, THR222
	Pentaacetate Quercetin (3)		-5.368	4, LYS436, SER214, PHE414	Charged(-ve) ASP432, ASP412; Charged(+ve) LYS433, LYS436; Hydrophobic ILE431, PHE414, ILE111; Polar THR221, THR222, SER214, GLY429
ERG11	Chalcone (2)		-7.825	3, TYR118, HIE377, SER378	Hydrophobic PHE228, MET508, LEU376, VAL509, PHE380, TYR64, TYR132, LEU121, TYR118; Polar HIE377, SER378, THR122
	Tetraacetate Quercetin (4)		-6.676	1, PHEA:233	Charged(+ve) LYS:A90, HEM1; Hydrophobic LEU:A87, TYR:A64, PRO:A230, ILE:A231, PHE:A380, PHE:A233, ILE:A379, TYR:A32,LEU:A376, VAL:A510, TYR:A505; Polar SER:A506, SER:A378,SER:A233, THR:A311
	Coumarin (1)		-6.349	1, GLYA:303	Charged(-ve) HEM1; Hydrophobic ILE:A304, ILE:A31, TYR:A132, LEU:A376; Polar THR:A311
SAP5	Chalcone (2)		-5.484	2, SERA:180, LYSB:271	Charged(-ve) ARG:B204, LYS:B271; Charged(+ve) ASP:B263; Hydrophobic PRO:A275, TYR:A180, VAL:A274, VAL:A330, PRO:A329; Polar SER:B259, SER:A313, THR:B261, SER:A180, SER:B273
	Pentaacetate Quercetin (3)		-6.069	3, THRB:261, LYSB:271, LYSA:271	Charged(-ve) ARG:B20, LYS:A331, LYS:B271, LYS:A271; Charged(+ve) GLU:A278, ASP:A263, ASP:B263; Hydrophobic PRO:A275, VAL:A274, PRO:B329,PRO:A329, TYR:A332, VAL:A274; Polar SER:B259, THR:B261, SER:A273, SER:A313
	Tetraacetate Quercetin (4)		-6.233	3, THRB:261, LYSB:271, THRA:261	Charged(-ve) LYS:A331, ARG:B204, LYS:B271; Charged(+ve) GLU:A278, ASP:B263; Hydrophobic PRO:A275,VAL:A261, PRO:B275,PRO:B329, ILE:B272, VAL:A274, TYR:A332, PRO:A329; Polar SER:B259, SER:A313, SER:B273, THR:A261, SER:A273, SER:A180
Efg1	Chalcone (2)		-4.651	1, MET272	Charged(-ve) ARG254, LYS242, LYS275, LYS268; Hydrophobic VAL227, LEU258, MET272; Polar ASN245,HIE273, THR241
	Tetraacetate Quercetin (4)		-3.314		Charged(-ve) ARG251, , ARG254, LYS242, LYS275, LYS268; Charged(+ve) ASP255; Hydrophobic VAL227, LEU258, MET272; Polar SER228, ASN245, HIE273, ASN239,THR241

Table 1: Docking score, H-bonds of compounds docked with protein targets showing amino acids in active sites.

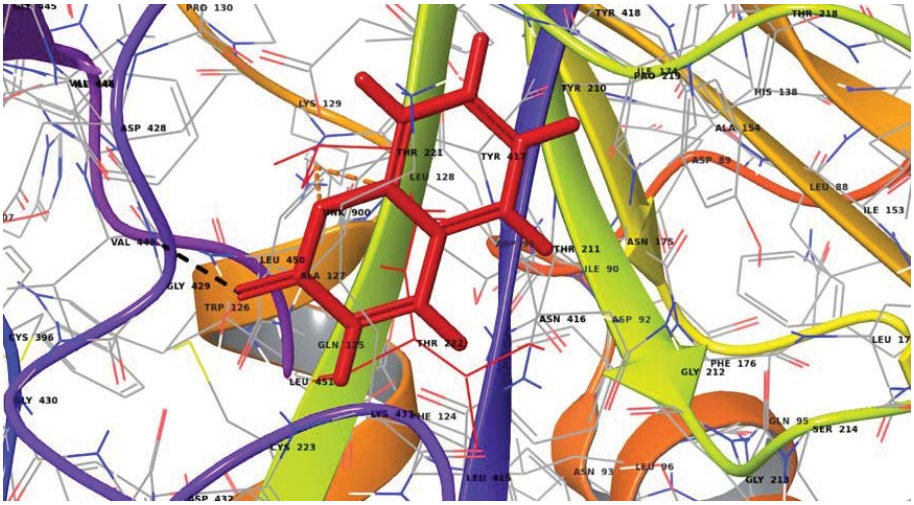


Figure 3: Showing the 3D docking interaction of 1IYK with coumarin.

Figure 4: Showing the 3D docking interaction between 1IYK and pentaacetate quercetin.

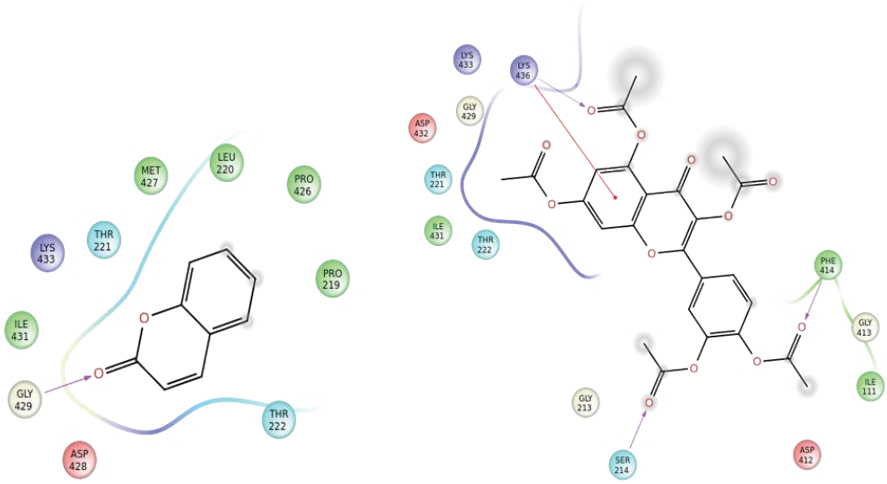


Figure 5: 2D interaction diagrams of coumarin and pentaacetate quercetin with N-myristoyl transferase.

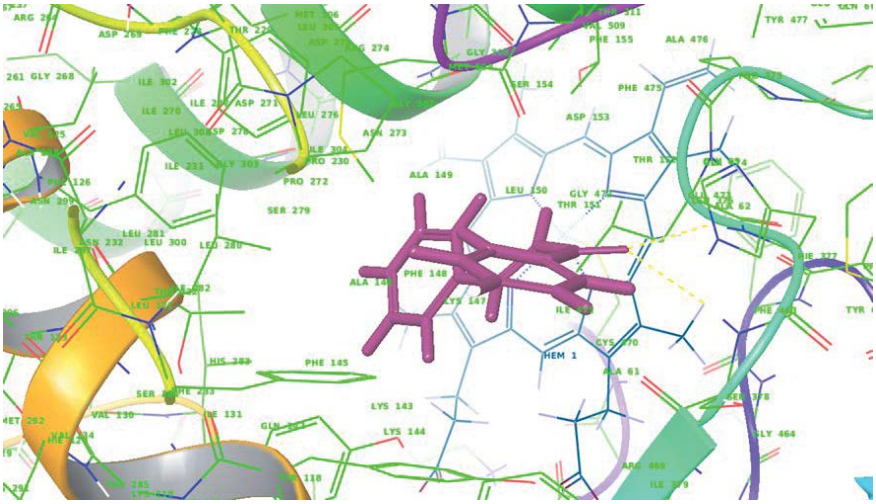


Figure 6: Showing the 3D docking interaction erg11 with chalcone.

Figure 7: Showing the 3D docking interaction erg11 with tetraacetate quercetin.

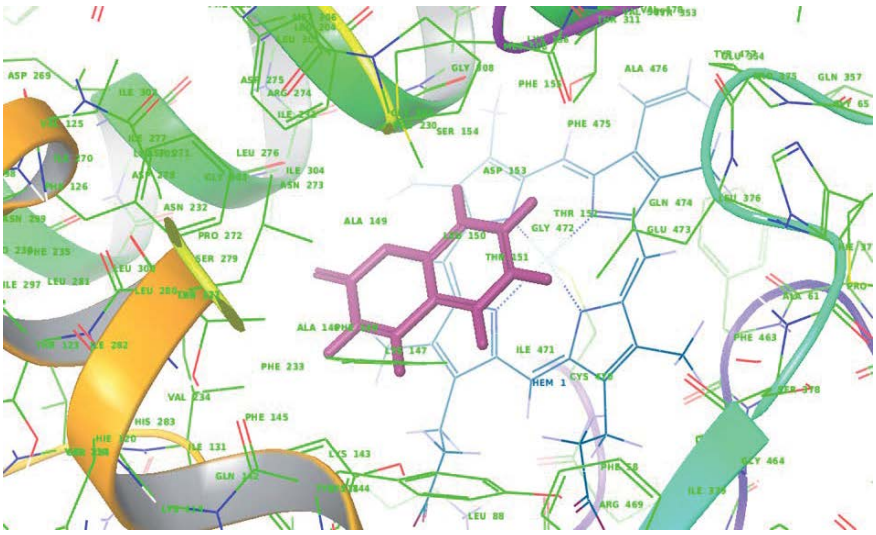


Figure 8: Showing the 3D docking interaction erg11 with coumarin.

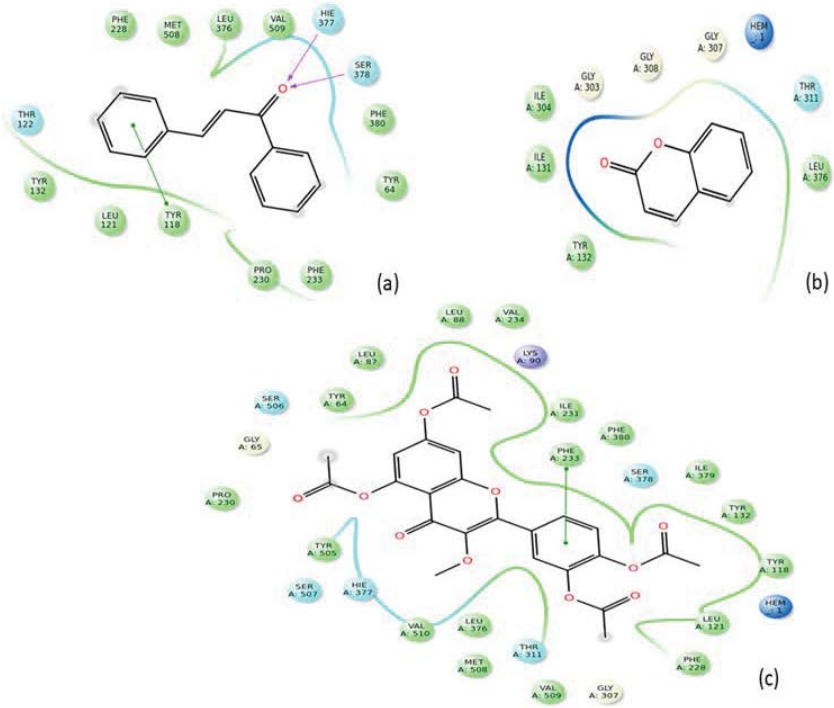


Figure 9: 2D interaction diagrams of (a) chalcone (b) coumarin and (c) tetraacetate quercetin with erg11.

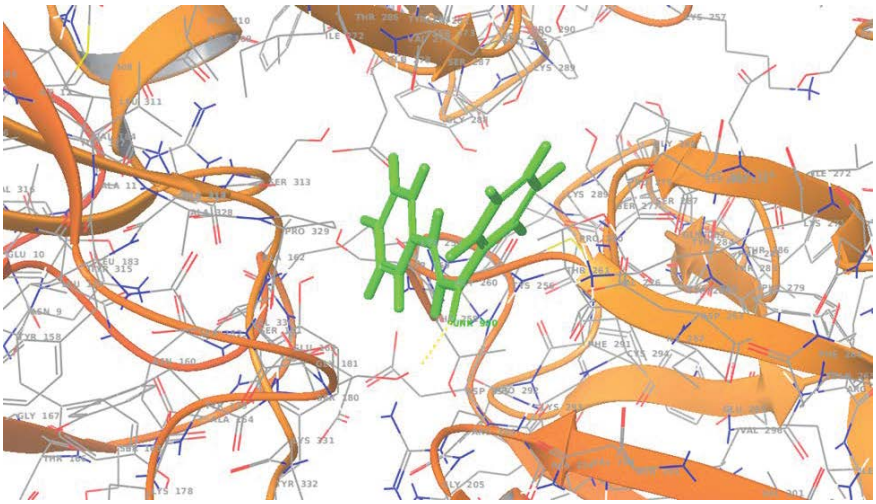


Figure 10: Showing the 3D docking interaction chalcone with sap5.

These naturally occurring compounds also proved their activity *in vitro*. The results of their sensitivity and effect on filamentation have been shown in Table 2.

Only the compounds that have made combination with more than one target were selected for *in vivo* analysis. The first target molecule N-myristoyl transferase had made combination with Coumarin and Penta acetate quercetin with the respective docking score -5.541 and -5.368. Mainly amino acids forming H-bonds were GLY429 and (LYS436, SER214, PHE414) respectively. Secondly target ERG11 i.e. one of the lead target of ergosterol pathway. It mainly makes combination with all types of azoles. However, in the experiments conducted for the present research, few polyphenols were also found making combination such as Chalcone, Tetraacetate quercetin and Coumarin with the respective docking scores -7.825, -6.676 and -6.349. The amino acid making H-bonds in case of each respective compound were (TYR118, HIE377, SER378) Chalcone, (PHE:A233) Tetraacetate quercetin and (GLY:A303) Coumarin.

The third target SAP5 had potential combination with Chalcone, Pentaacetate Quercetin and Tetraacetate quercetin with the respective docking scores -5.484, -6.069 and -6.349. The respective binding site H-bonding amino acids were (SERA:180, LYS:B271) Chalcone, (THR:B261, LYS:B271 and LYS:A271) Pentaacetate quercetin and

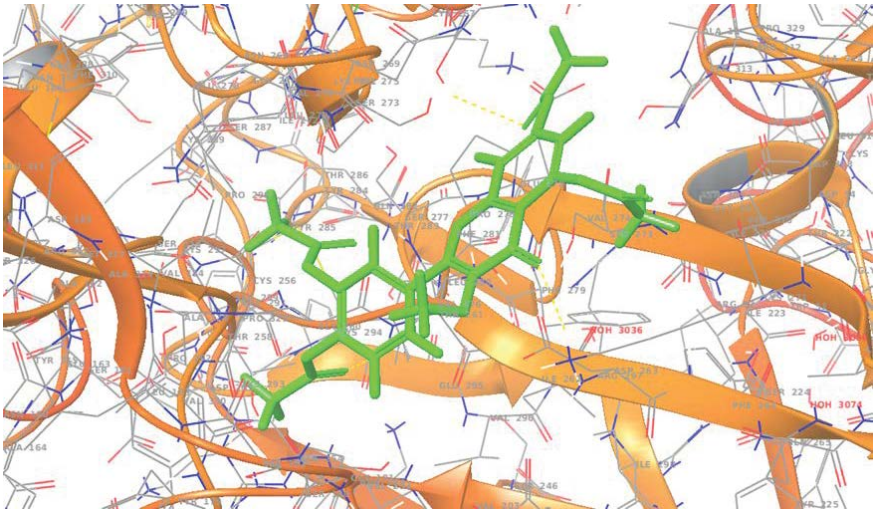


Figure 11: Showing the 3D docking interaction tetraacetate quercetin with SAP5.

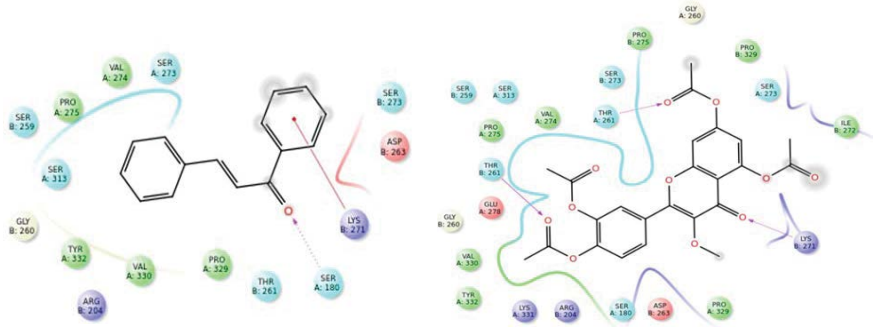


Figure 12: 2D interaction diagrams of chalcone and tetraacetate quercetin with SAP5, respectively.

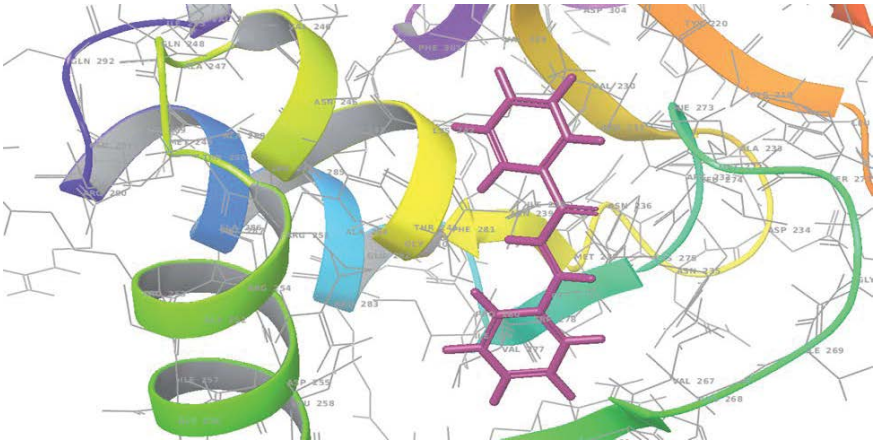


Figure 13: Showing the 3D docking interaction chalcone with efg1.

(THR:B261, LYS:B271, THR:A261) Tetraacetate quercetin. The last target protein Efg1 was found making combination with Chalcone and Tetraacetate quercetin with respective docking scores -4.651 and -3.314.

In vitro

As azoles mainly target Ergosterol pathway they do not show effect on the filamentation instead they cause cell death. While here the compounds that have been taken in our study have shown significant results with filamentation and have shown sensitivity in Candida growth. As we can see that chalcones might have shown not higher sensitivity as compared to fluconazole but is giving better effect with filamentation as shown in Figures 16a and 16b. Further the effect of fluconazole as compared with the polyphenols has been represented in Figure 16c. The yeast form of *C. albicans* is non-pathogenic and thus

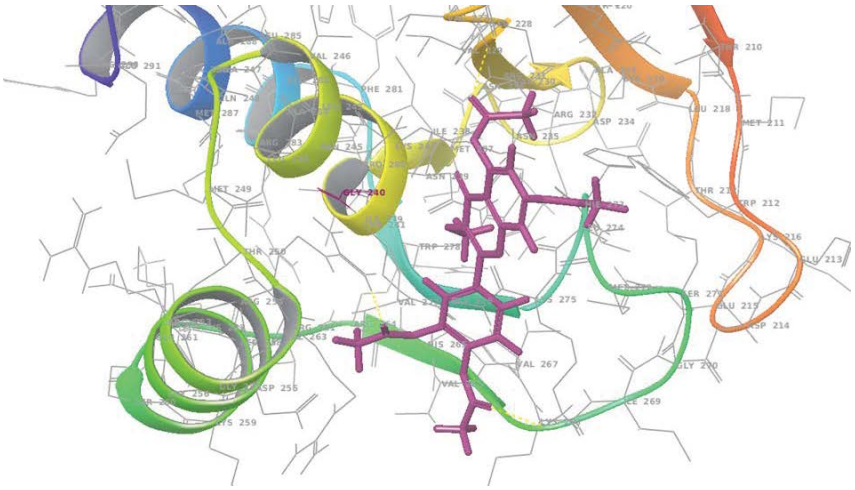


Figure 14: Showing the 3D docking interaction tetraacetate quercetin with efg1.

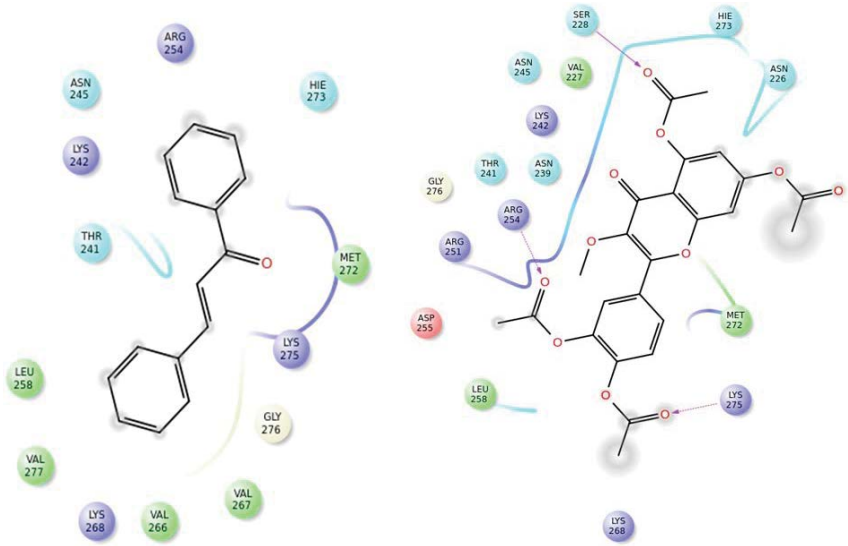


Figure 15: Showing the 3D docking interaction chalcone and tetraacetate quercetin with efg1, respectively.

Compound Names	Compound IUPAC	Physical properties	IC80 (μM)	Filamentation
Coumarin [1]	7-hydroxy-2H-chromen-2-one	White crystalline solid Soluble in alcohol, acetone, DMSO No precipitate in RPMI	20	++
Chalcone [2]	(E)-1,3-diphenylprop-2-en-1-one	Light green solid Soluble in alcohol, acetone No precipitate in RPMI	10	++++
Pentaacetate Quercetin [3]	2-(3,4-diacetoxyphenyl)-4-oxo-4H-chromene-3,5,7-triyl triacetate	White crystalline solid Soluble in alcohol, acetone, DMSO Slight precipitate in RPMI	5	+++
Tetraacetate Quercetin [4]	4-(5,7-diacetoxy-3-hydroxy-4-oxo-4H-chromen-2-yl)-1,2-phenylene diacetate	White crystalline solid Soluble in alcohol, acetone, DMSO Slight precipitate in RPMI	8	++
Di indol-1,6-diene-3,5-dione [5]	(1E,6E)-1,7-di(1H-indol-3yl) hepta-1,6-diene-3,5-dione	Orange crystalline solid Soluble in alcohol, acetone, DMSO Slight precipitate in RPMI	5	+++
Fluconazole	2-(2,4-Difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl) propan-2-ol	White solid Soluble in DMSO, acetone No precipitate in RPMI	2	–

Table 2: Compound sensitivity and inhibition of *Candida albicans* filamentation with SC5314.

polyphenols show a better way to solve the problem of pathogenicity caused by *C. albicans* (Figure 16a).

In the present scenario immergence of fungal infections has become a global problem. The gene modification in *C. albicans* has led to imbalance in human biome. In addition, the drug resistance and biofilm formation has made it a big concern to human health. In the present study a naturally occurring compound based non-toxic solution has been worked out. Since the experiments have been conducted on targets in major pathways leading to emergence of pathogenicity in *C. albicans*, it can be considered as a more prominent solution. Further all results have been validated *in vitro* and polyphenols viz. quercetin acetates, E-Chalcone, 7-hydroxy coumarin and (1E, 6E)-1,7-di(1H-

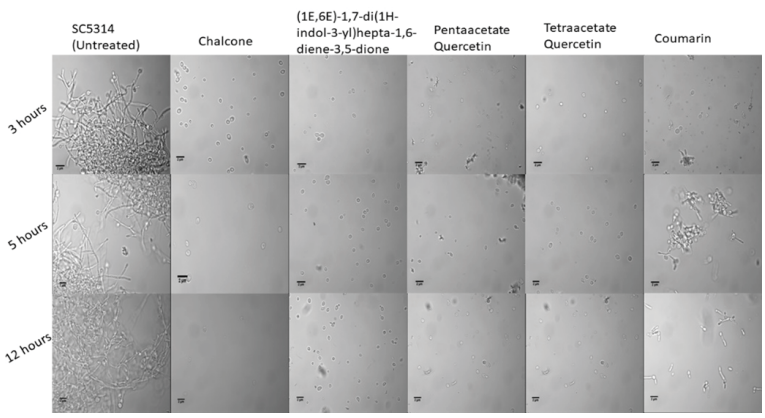


Figure 16a: Showing the multiple filamentation growth of SC5315 *Candida albicans* untreated strain against the same with our proposed lead molecule.

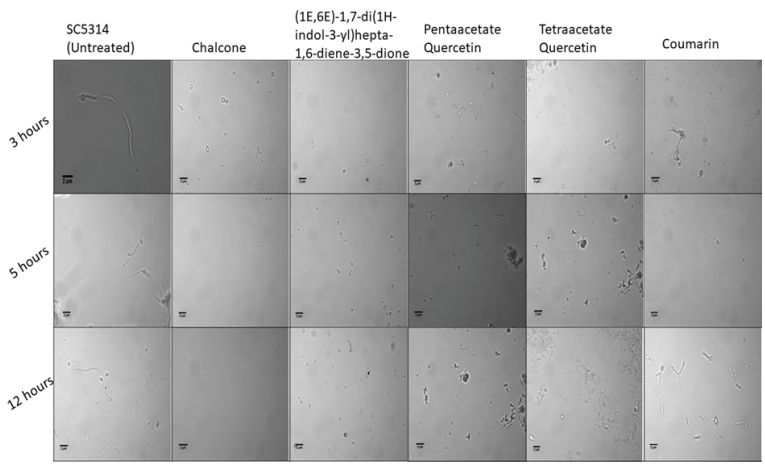


Figure 16b: Showing the filament length growth of SC5315 *Candida albicans* untreated strain against the same with our proposed lead molecules.

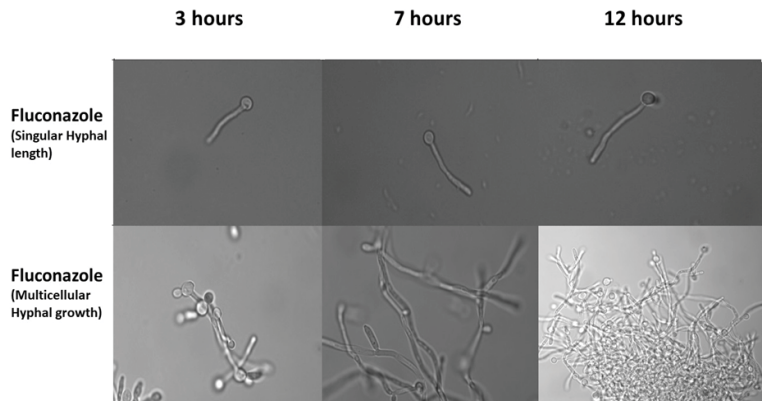


Figure 16c: Representing the filament length and complex growth in *Candida albicans* treated with fluconazole.

indol-3yl) hepta-1,6-diene-3,5-dione (curcumin analogue) have been proved to be the safe alternative of toxic azoles. Through the *in silico* study it has been found that most of H-bonds were occupied in the regions of phenyl ring as shown in Figures 3-15. It represents the relevance of polyphenols and justifies their combination with most of the target proteins (Figure 16b).

Pentaacetate of quercetin, tetraacetate of quercetin and coumarins have been found active in all four targets illustrating their high affinity. Fluconazole which is an already known marketed drug for antifungal treatment has been reported with many side effects. Including fluconazole many azoles are very toxic for human biome. In such scenario polyphenols seem to show promising results and also, they are non-toxic. These naturally occurring compounds also proved their activity *in vitro*. The polyphenols are naturally occurring antioxidants and can also be synthesised in laboratory. It has relevance in both ways as a drug or a supplement to help body in making a balance of biome. These will prove as better and safe alternative medicines in case of drug resistance of commercial azoles. As azoles mainly target Ergosterol pathway they do not show effect on the filamentation instead they cause cell death. While here the compounds that have been taken in our study have shown significant results with filamentation and have shown sensitivity in Candida growth. Representing the filament length and complex growth in *Candida albicans* treated with fluconazole (Figure 16c).

Conclusion

In the present scenario immergence of fungal infections has become a global problem. The gene modification in *C. albicans* has led to imbalance in human biome. In addition, the drug resistance and biofilm formation has made it a big concern to human health. In the present study a naturally occurring compound based non-toxic solution has been worked out. Since the experiments have been conducted on targets in major pathways leading to emergence of pathogenicity in *C. albicans*, it can be considered as a more prominent solution. Further all results have been validated *in vitro* and polyphenols viz. quercetin acetates, E-Chalcone, 7-hydroxy coumarin and (1E, 6E)-1,7-di(1H-indol-3yl) hepta-1,6-diene-3,5-dione (a curcumin analogue) have been proved to be the safe alternative of toxic azoles. The polyphenols are naturally occurring antioxidants and can also be synthesised in laboratory. It has relevance in both ways as a drug or a supplement to help body in making a balance of biome. These will prove as better and safe alternative medicines in case of drug resistance of commercial azoles.

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