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INTERACTION OF FLUCONAZOLE WITH *CANDIDA AURIS* AND *CANDIDA ALBICANS* LANOSTEROL 14 A- DEMETHYLASE (ERG11)

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ABSTRACT: *Candida auris* is an emerging pathogen which is being frequently isolated in Candidaemia. The organism poses serious threat due to its high resistance to fluconazole and amphotericin B. Not much studies are available on the resistance mechanisms of *C.auris*. Hence, this study aims in analysing the *erg*11 gene for mutations and the effect of those mutations on the structure and activity of Erg11 using homology modelling and protein-ligand docking studies. Antifungal susceptibility testing was done to determine the Minimal Inhibitory concentration of fluconazole, amphotericin B and other antifungal agents. The erg11 gene sequence of *C.auris* was acquired from the WGS data and the sequence was compared with fluconazole susceptible *C.albicans* and the presence of mutations and the impact of mutations were analysed. The 3D structure of Erg11 was determined by homology modelling. The differences in structure, interaction with fluconazole and binding energy between *C.auris* Erg11 and *C.albicans* Erg11 were analysed after structural alignment and protein-docking. *C.auris* Erg11 was 524 amino acid long. Two substitutions were found in the active site of the enzyme. Addition and deletion of various aminoacids were also observed. Alignment of 3D structures of *C.albicans* and *C.auris* Erg11 showed that the substitutions altered the structure of the protein. The change in structural conformation affected the binding of fluconazole to Erg11 which is shown by decreased binding energy in protein-ligand docking studies. The aminoacid substitutions, particularly Y132F, present in the active site of Erg11 affected the structure of the enzyme and decreased the binding affinity of fluconazole.

KEYWORDS: Lanosterol 14α demethylase, *erg*11, Erg11, *Candida auris*.

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1.INTRODUCTION

Candidaemia caused by new and less common *Candida* species, which exhibit resistance to one or more antifungal agents are being frequently reported recently [1-4]. *C.auris* is one such notable pathogen due to its high resistance to fluconazole [5]. Although various antifungal agents are available, fluconazole is considered the drug of choice for the treatment of candidaemia [6-8]. However, long-term exposure and underdosage during the empirical treatment leads to an increased resistance to fluconazole [9-16]. The molecular mechanisms involved in fluconazole resistance have been studied extensively in *C.albicans*. One major mechanism is that, the mutations in the *erg*11 gene can result in amino acid substitutions in the enzyme, which lead to the decreased affinity of fluconazole thereby leading to less susceptibility [17]. Among the many Erg11 substitutions described to date in *in-vitro* azole-resistant clinical isolates of *C. albicans* [18], only a few have been associated in resistance [12, 19-21]. However, studies have not been carried out in *C.auris* describing the mutations and aminoacid substitutions in Erg11. This study analyses the differences in Erg11 of *C.auris* and *C.albicans* with reference to the aminoacid substitution and its impact in structure and binding with fluconazole. This would aid in understanding the role of mutations in fluconazole resistance in *C.auris* which is necessary to discover newer antifungal agents that circumvent drug resistance [22, 23] and could guide the choice of the appropriate antifungal treatment at the onset of infection.

2. MATERIALS AND METHODS

Isolate and determination of Minimal Inhibitory concentration (MIC)

C.auris (MS 3054) isolated from blood specimen which came for routine culture to the Central Laboratory, Sri Ramachandra Medical Centre and Research Institute, Chennai, TamilNadu, India was used in this study. The MIC was determined by micro broth dilution method as per CLSI M27 A3 guidelines [24] for Fluconazole (Himedia, India), Voriconazole (Sigma-Aldrich, India), Posaconazole (Sigma-Aldrich, India), Itraconazole (Sigma-Aldrich, India), Amphotericin B (Himedia, India), Caspofungin (Sigma-Aldrich, India), Anidulafungin (Sigma-Aldrich, India) and Micafungin (Sigma-Aldrich, India). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains. The test was carried out in triplicates and the median was taken as the result.

2.2. Analysis of *C.auris erg*11 gene

*C. auris erg*11 gene sequence was obtained from the Whole genome sequence data done in our laboratory (GenBank accn No. KY410388) and *C.albicans erg*11 gene sequence was obtained from (GenBank accn no. GQ202082). The retrieved nucleotide sequences were translated to amino acid sequence using Mega7 software with standard genetic code as reference. The amino acid sequences were aligned using clustal omega program and analysed for amino acid substitutions in the protein, Erg11.

1.2.1.Impact of amino acid substitutions

The amino acid substitutions detected were further analysed for their effect on the protein function. For this, the mutations were analysed using SIFT, PROVEAN and Polyphen2 online tools.

1.2.1.1. SIFT

Sorting **I**ntolerant **F**rom **T**olerant (SIFT) is a sequence homology-based tool that sorts intolerant from tolerant amino acid substitutions and predicts whether an amino acid substitution in a protein will have a phenotypic effect [25]. The mutations are categorised as 'Deleterious' or 'Tolerated' based on normalised probability scores.

1.2.1.2. PROVEAN

Protein **V**ariation **E**ffect **An**alyzer (PROVEAN) too computes the pairwise sequence alignment scores for amino acid substitutions and/or deletions to predict whether an amino acid substitution has an impact on the biological function of a protein. Here, the mutations are classified as 'Deleterious or Neutral' [26, 27].

1.2.1.3. Polyphen2

Polymorphism Phenotyping v2 (Polyphen2) uses straightforward physical and comparative considerations to predict the possible impact of an amino acid substitution on the structure and function of a protein [28]. Polyphen2 categorises the mutation as 'Possibly deleterious' or 'Benign' based on its effect on the protein.

1.2.2. 3D-Structure prediction of lanosterol 14 α demethylase (Erg11)

1.2.2.1. Homology modelling using Iterative Threading ASSEmbly Refinement (ITASSER)

Protein homology modelling generates 3D models for a target amino acid sequence based on previously available 3D structures of one or more related proteins. Here, homology modelling was used to predict the 3D structure of *C.auris* and *C.albicans* Erg11 using I-TASSER online server [29].

1.2.2.2. Assessment of homology model

The models are generated based on alignments between the target and the template amino acid sequences and have chances of error [30]. Therefore, model quality estimation methods were used to identify unreliable or erroneous regions in the predicted models, and to estimate its overall accuracy. The quality of the predicted models were assessed by Structural Analysis and Verification

Server (SAVES) for its internal consistency and reliability. PROCHECK [31], ERRAT [32] and Verify3D [33] tools of SAVES were used to check the quality of the predicted models. The models of *C. albicans* and *C. auris* Erg11 with the best score were used for further molecular modelling studies.

1.2.3. Structure alignment

The 3D structure of *C.albicans* and *C.auris* Erg 11 were aligned with each other using RaptorX structure alignment tool and the structural differences between them was analysed.

1.2.4. Molecular docking using Glide

In order to determine the interactions between Erg11 and fluconazole, the stereoisomer of fluconazole was retrieved from PubChem database (PubChem id. CID3365) and docked with the modelled Erg11 of *C.albicans* and *C.auris* using Glide distributed by Schrodinger.

1.2.4.1. Ligand preparation

The 2-Dimensional (2D) structure of fluconazole were drawn from the Pubchem database in sdf format. This structure was converted to 3-Dimension (3D) by optimizing the ligand using Ligprep software. Ligprep does the geometry optimization using optimized potentials for Liquid simulations (OPLS-2005) force field with the help of steepest descent and conjugate gradient protocol. Ligprep converts 1D or 2D structures to 3D by searching for tautomers, steric isomers and minimizing the ligand. This geometry minimized ligand was taken for docking studies.

1.2.4.2. Protein Preparation

The modelled protein Erg11 was geometry optimized using Protein preparation wizard of Schrodinger software. First, the protein was preprocessed to check the errors. Later hydrogen bonds, charges and protonation states were assigned and finally energy minimization was performed with root mean square deviation of 0.30 Angstroms using OPLS-2005 force field.

1.2.4.3. Receptor-grid generation and glide docking

The grid was generated for the active site residues of Erg11 protein using default options of receptor-grid generation program of Schrodinger software. Later both optimized ligand and protein was taken for docking studies. Docking was performed using Glide extra precision (XP) docking protocol by selecting the per-residue interaction. The docking results were analyzed using Glide XP viewer and per residue interactions were plotted to compare the energies of residues between two the proteins.

1.2.4.4. Interaction of fluconazole with *C.albicans* and *C.auris* Erg11

The drug was docked with the active site residues of Erg11 of *C.albicans* and *C.auris* and the per-residue interaction energy was compared.

3. RESULTS AND DISCUSSION

Minimal Inhibitory Concentration of *C.auris*

C.auris (MS 3054) was highly resistant to Fluconazole (>64µg/ml) and Amphotericin B (>16µg/ml). The isolate was susceptible to Posaconazole, Itraconazole Voriconazole (MIC 0.5, 2, 0.5µg/ml

respectively) and the echinocandins. However, it had a higher MIC for caspofungin and micafungin (1µg/ml) than Anidulafungin (0.5µg/ml).

3.2. Analysis of *C.auris erg*11 gene

On aligning and analysing *C.albicans* and *C.auris erg*11 gene, it was found that both were 70% identical to each other. *C.albicans* Erg11 was 528 amino acids long whereas, *C.auris* Erg11 was 524 amino acid long. A total of 162 amino acid substitutions were observed in *C.auris* Erg11 on comparison with *C.albicans* Erg11. Two substitutions, Y132F and I304V (*i.e.* the tyrosine in 132nd position was replaced by phenylalanine and Isoleucine in 304th position was replaced by Valine) were found in the active site. Also, deletion of 5 amino acid residues (Asparagine, Serine, Isoleucine, Serine and Phenylalanine) in the positions, 435 to 439 and addition of one amino acid (Aspartic Acid) was observed at the 493 position of *C. auris*. The alignment and substitution of amino acids are shown in Fig. 1.

Fig 1: Alignment of amino acid sequence of Erg11of *C. auris* and *C. albicans*

3.3. Aminoacid substitutions obtained and their impact

On analysing the effect of substitutions on the protein function, it was found that out of the 162 substitutions, 8 were found to affect the activity of protein in more than one tool. The substitutions which affected the protein function were Y132F, Y415W, N418R, S453E, V4K, Q21V, H242A and T352A. Out of these substitutions, Y132F was found in the active site of the protein. Another substitution, I304V was also found in the active site and substitutions, K119S, M372L, T384M and S506Q were found near the active site of the protein. But these substitutions did not affect the activity of the protein.

3.4. Homology modelling and assessment of homology model

I-TASSER predicted five structures for *C.auris* Erg11 and *C.albicans* Erg11. To choose the best structure, all the predicted structures were validated by Structural Analysis and Verification Server (SAVES). The best model was chosen for further analysis (Fig. 2.1 and 2.2). The minimum score

required, and the score obtained by the predicted 3D protein models are tabulated in table 1.

Table 1: Score obtained by the predicted 3D protein models in different assessment tools

Tool	Minimum score	*C.auris* Erg11	*C.albicans* Erg11
PROCHECK	80%	88.35%	92.4%
VERIFY 3D	80%	84.73%	87.31%
ERRAT	95	95.543	96.346

The PROCHECK, ERRAT Verify 3D results confirm the quality of predicted 3D structure as more reliable and within an acceptable range. These results suggest that the model developed is robust and can be used in subsequent stages of analysis.

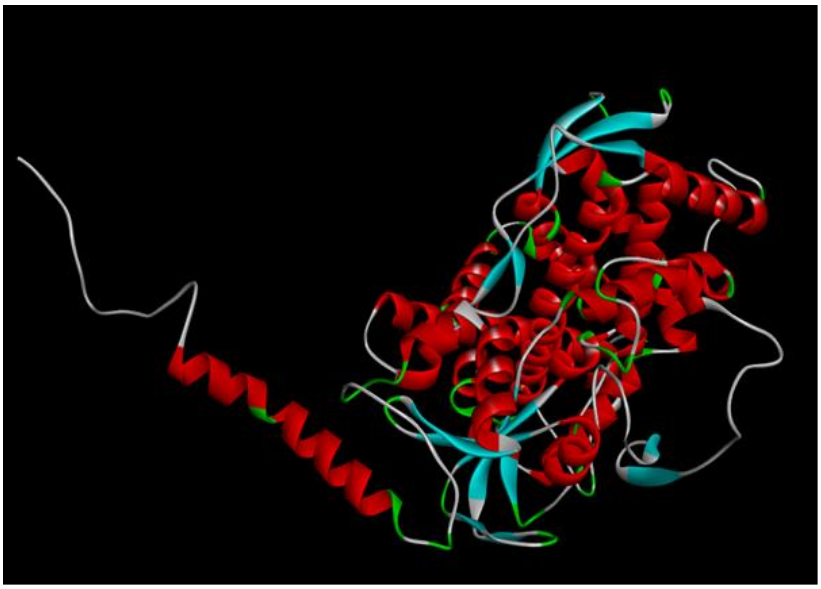

Fig 2.1: Predicted 3D structure of *C.albicans* Erg11

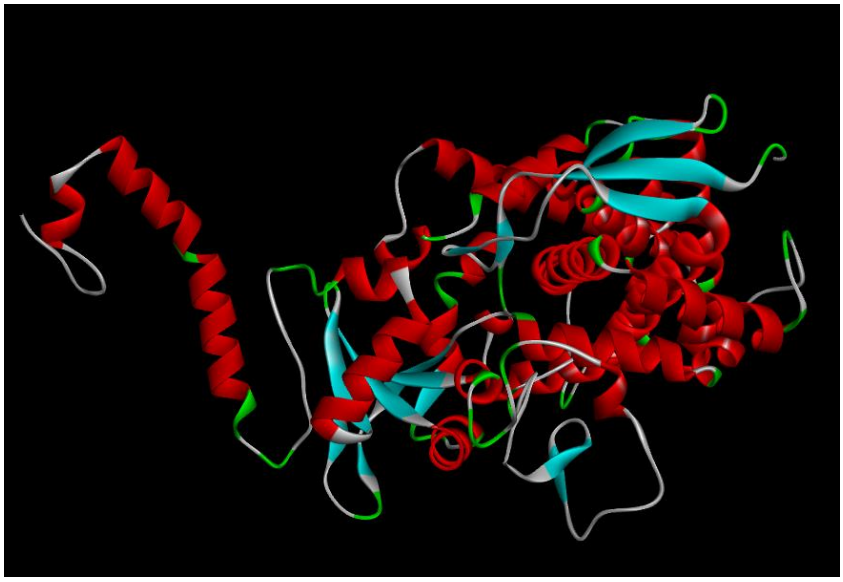

Fig 2.2: Predicted 3D structure of *C.auris* Erg11

3.5. Structure alignment

On aligning the 3D structures of *C.albicans* and *C.auris* Erg11 (Fig. 3) it was found that the α helices and the β sheets of both the proteins overlapped with each other completely. The regions where there was no overlapping observed was the loop region between the B,B' and C α helices. This is the hotspot 1 region where Y132F substitution was observed. The transmembrane tail region also was not overlapping with each other due to deletion of five aminoacid residues in *C.auris* Erg11.

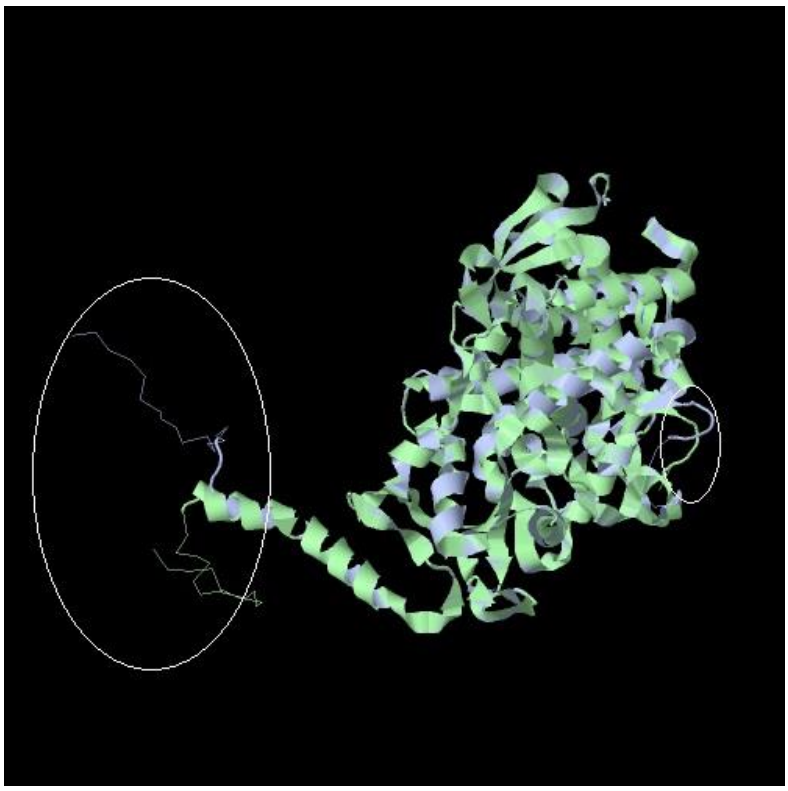

Fig 3: *C.auris* Erg11 (Green) aligned to *C.albicans* Erg11 (Blue)

3.6. Interaction of fluconazole with *C.albicans* and *C.auris* Erg11

The protein-ligand interaction was observed by docking fluconazole to the active site of Erg11 of *C.albicans* and *C.auris.* The per-residue interaction energy of all the active site residues and the substituted residues were compared (Fig 4.1 and 4.2). The mutation Y132F had a lower interaction energy in *C.auris* than *C.albicans*. Also, the overall docking score was less in *C.auris* than *C.albicans* (Fig. 5). Fluconazole formed two H-bonds with Erg11 of *C.albicans* whereas, only one bond was formed with Erg11 of *C.auris*.

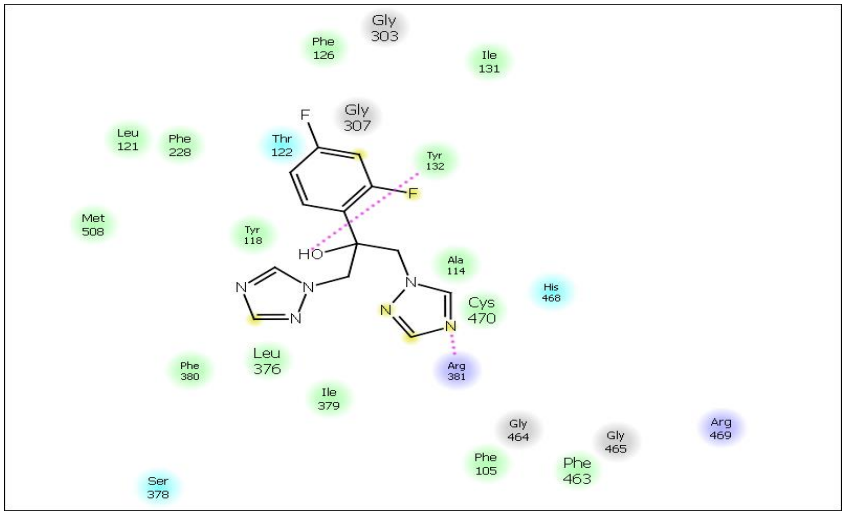

Fig 4.1: Interactions of active site aminoacid residues of *C.albicans* Erg11 with flucanozole

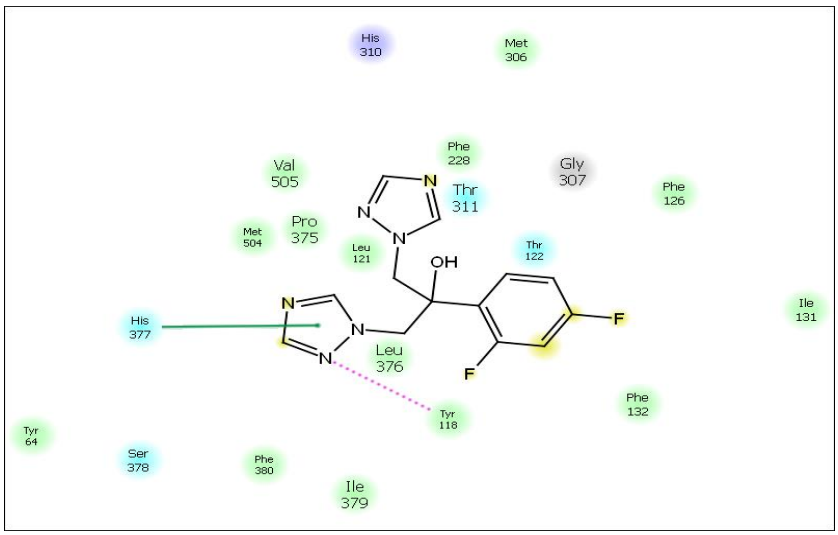

Fig 4.2: Interactions of active site amino acid residues of *C.auris* Erg11 with flucanozole

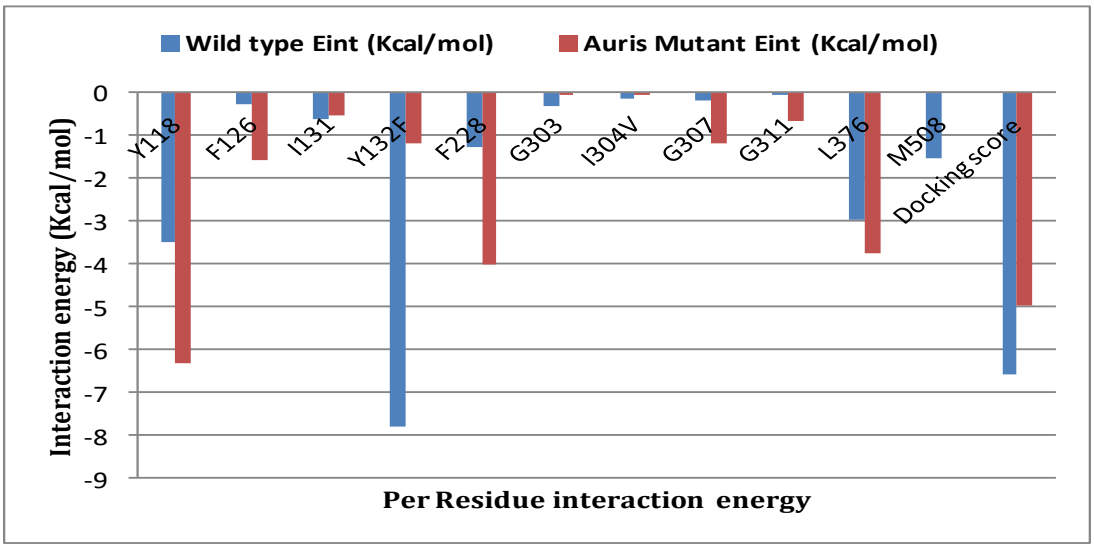

Fig 5: Per- residue interaction energies of fluconazole with Erg11 of *C. auris* and *C.albicans*

DISCUSSION

In the study, we found that *C.auris* was highly resistant to fluconazole and amphotericin B as previously reported [34]. The *erg*11 gene sequence was acquired from the WGS data and analysed. *C.auris erg*11 was 70% identical to *C.albicans erg*11 which was similar to another study where it was 78% - 85% identical with *C.albicans erg*11 and *C.glabrata erg*11 [35]. Multiple sequence alignment showed that *C.albicans* Erg11 is 528 amino acids in length whereas *C.auris* Erg11 is 524 amino acid in length. Of the observed 8 deleterious substitutions, Y132F was found to be in the active site of the enzyme. This mutation was previously reported in fluconazole resistant *C.albicans*, *C.tropicalis* and other species [12]. This region is also referred as Hot spot 1, which is prone to mutation in *C.albicans*. Other substitutions which were previously reported to be associated with resistance in *C.albicans* (N136Y, K143R, G307S, S405F, Y447H, G448E, G450E and V456I) [12] were not observed in *C.auris.* Furthermore, *C.auris* Erg11 lacked 5 amino acid residues (NSISF) in the positions, 435 to 439 of *C.albicans*. Also, an additional amino acid (D) was present in the 493 position in *C.auris*. These addition and deletion of amino acids are present in the Hotspot 3 (405 to 488) region [13]. This deletion of amino acids may result in conformational change in the enzyme, thereby preventing the proper binding of fluconazole to its active site, rendering the organism to resist the drug. *C.albicans* and *C.auris* Erg11 protein structures were aligned to check the structural difference between the two. This showed that both the proteins overlapped with each other throughout except the region where the Y132F substitution was observed. The transmembrane tail region, which had the addition of five aminoacids and deletion of one aminoacid was also not overlapping with each other. From the study, it is evident that amino acid substitutions, particularly, Y132F and deletion of aminoacids, N435, S436, I437, S438, F439 and an additional aminoacid D493, causes structural changes in Erg11 of *C.auris* which might alter the interaction with the protein. The protein-ligand docking study showed that the overall binding energy of fluconazole is less in *C.auris* than *C.albicans.* The substitution, Y132F, in *C.auris* has 6 times lower binding energy than *C.albicans*. This substitution lies in the active site of the Erg11 enzyme. Also, the overall docking score was less in *C.auris* than *C.albicans*. These findings suggest that the interaction and binding of fluconazole with *C.auris* is lesser than *C.albicans* and this might possibly be the reason for fluconazole resistance in *C.auris.* This shows that fluconazole binds with less affinity to *C.auris* Erg11, which could be the reason for resistance. Also, the predicted structure has more cavities where fluconazole can bind. This binding of fluconazole to cavities other than the active site doesn't affect the enzyme activity, which leads to continuous production of ergosterol even in the presence of fluconazole thereby making the organism resistant to fluconazole.

4. CONCLUSION

From the study, it is evident that *C.auris* is highly resistant to fluconazole and amphotericin B. The *erg*11 gene of *C.auris* had more mutations than *C.albicans*, this leads to substitution of many

aminoacids among which the substitution, Y132F lies in the active site of Erg11 and interferes with the binding of fluconazole with Erg11. This makes *C.auris* resistant to fluconazole.

CONFLICT OF INTEREST

Authors have no any conflict of interest.

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