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Research article

Comparative fitness trade-offs associated with azole resistance in *Candida auris* clinical isolates

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ABSTRACT

Multidrug-resistant yeast *Candida auris* is a serious threat to public health with documented survival in various hospital niches. The dynamics of this survival benefit and its trade off with drug resistance are still unknown for this pathogen. In this study we investigate the oxidative stress response (OSR) in fluconazole-resistant *C. auris* and compare its relative fitness with fluconazole-susceptible strains. A total of 351 *C. auris* clinical isolates (61 fluconazole-susceptible and 290 fluconazole-resistant) were screened for stress tolerance by spot assay and 95.08 % fluconazole-susceptible isolates were hyper-resistant to oxidative stress while majority (94.5 %) fluconazole-resistant isolates had lower oxidative tolerance. Expression of Hog1 and Cta1 gene transcript levels and cellular catalase levels were significantly higher in fluconazole-susceptible isolates and a corresponding higher intracellular reactive oxygen species level (iROS) was accumulated in the fluconazole-resistant isolates. Biofilm formation and cell viability under oxidative stress revealed higher biofilm formation and better viability in fluconazole-susceptible isolates. Fluconazole-resistant isolates had higher basal cell wall chitin. On comparison of virulence, the % cytotoxicity in A549 cell line was higher in fluconazole-susceptible isolates and the median survival of the infected larvae in *G. mellonella* infection model was higher in fluconazole-resistant (5; IQR:4.5–5 days) vs. fluconazole-susceptible *C. auris* (2; IQR:1.5–2.5 days). All organisms evolve with changes in their environmental conditions, to ensure an optimal balance between proliferation and survival. Development of tolerance to a certain kind of stress example antifungal exposure in yeast can leads to a compensatory decrease in tolerance for other stresses. This study provides useful insights into the comparative fitness and antifungal susceptibility trade off in *C. auris*. We report a negative association between H_2O_2 tolerance and fluconazole susceptibility. Using in-vitro cell cytotoxicity and in-vivo survival assays we also demonstrate the higher virulence potential of fluconazole-susceptible *C. auris* isolates corroborating the negative correlation between susceptibility and pathogen survival or virulence. These findings could also be translated to clinical practice by investigating the possibility of using molecules targeting stress response and fitness regulating pathways for management of this serious infection.

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1. Introduction

Over the last decade, *Candida auris* has emerged as a prominent nosocomial multidrug resistant pathogen worldwide [1]. After its first report in 2009 from a 70 year old female with otitis from Japan [2], this pathogen has been reported from 42 countries across six continents till date [3,4]. In India, infection due to *C. auris* was first reported at Delhi and subsequently spread across the entire country [5–7]. Chakrabarti et al. in 2015 reported a prevalence of 5.3 % in multicentre study across 27 intensive care units (ICUs) in India making *C. auris* the fifth major etiological agent among all candidaemia cases [6], where within few years *C. auris* candidemia topped the list (39.9 %) in another ICU-based study by Shastri et al. [6,8]. Despite attempts by national and international agencies to prevent the rapid spread of *C. auris* by generating awareness, emphasis on hospital surveillance during outbreaks and significant efforts from healthcare personnel, *C. auris* still continues to be a public threat.

Unlike other yeasts, *C. auris* exhibits characteristics resembling bacteria such as environmental persistence, multi drug resistance and rampant hospital transmission which threaten patient safety and global public health measures [9,10]. *C. auris* persists on medical equipment and survival on dry hospital surfaces for as long as four weeks [11]. *C. auris* immediately colonizes the body surfaces (the axillae, groin and nares) of hospitalized patients which further contributes to its horizontal inter and intra-hospital spread resulting in sporadic outbreaks [9,10,12–17].

This unassailable nature of *C. auris* transcends *in-vivo* as it is able to withstand various kinds of host immune stresses during infection. Like any other organism, it too encounters a plethora of stresses viz., oxidative, osmotic, thermal, cell wall and drug stresses along with exposure to a varied concentration of trace elements in body fluids. *C. auris* shows higher degree of resistance to various physiological stresses when compared to the other *Candida* species [18,19]. *C. auris* can tolerate high salt and fatty acid concentration and high temperatures up to 42 °C, allowing it to survive effortlessly on human skin surfaces even at high ambient temperatures [2,18]. *C. auris* also forms strong biofilms on the surface of inanimate objects contaminated with dried-up sweat and fatty acids with 30-fold higher cellular burden compared to *C. albicans* [20]. These biofilms are shown to be highly resilient to desiccation, osmotic stress and commonly used hospital disinfectants (chlorhexidine and hydrogen peroxide) [18,20–22]. The primary gene responsible for providing phenotypic plasticity for stress resistance and for maintaining cellular morphology in *C. auris* is Hog1 [18,19,23].

To understand the evolution of drug resistance, detailed molecular studies are warranted. Studying stress response may delineate new cellular pathways underlying the antifungal resistance in *C. auris* and this was the main rationale behind the present study. It should also be noted that the evolution of such multidrug-resistant pathogens often comes at the cost of their relative fitness when compared to the susceptible counterparts [24–31]. Since *C. auris* is reported to be highly drug resistant and there are also several reports of an adaptive response to stress by *C. auris*, this study was conceived to explore the comparative stress tolerance between azole resistant versus azole susceptible isolates and also to investigate the relative fitness trade-offs between these two phenotypes.

2. Materials and methods

2.1. Isolates and study design

A total of 351 clinical isolates of *C. auris* recovered from patients from 2009 through 2020 and persevered at our repository, National culture collection of pathogenic fungi (NCCPF), Chandigarh India were retrieved for this study. These comprised of two groups of *C. auris* isolates i.e., 61 fluconazole-susceptible and 290 fluconazole-resistant, which had been previously confirmed by antifungal susceptibility testing using micro broth dilution method as per CLSI guidelines (M27-A3). MIC for fluconazole was interpretated based on the tentative breakpoints given by Centers for Disease Control and Prevention (CDC), Atlanta [32].

Briefly, all the isolates were initially screened for oxidative stress tolerance by spot assay followed by expression analysis of the oxidative stress related genes, iROS measurement and catalase assay. 10 fluconazole-resistant (2 isolates each of MIC 64, 128, 256, 512 and 1024 μg/ml) and 10 fluconazole-susceptible isolates (MIC range 2–16 μg/ml) were compared using phenotypic assays such as analysis of growth parameters, morphology by scanning electron microscopy (SEM), biofilm production capacity and metabolic activity, in the presence and absence of oxidative stress. Finally, *in-vitro* cytotoxicity assay and *in-vivo* virulence using *Galleria mellonella* infection model were also performed to compare the two above mentioned groups. All procedural details are elaborated below. The work has been approved by the Institutional Ethics Committee (No. INT/IEC/2023/SPL-337, dated: 28th March 2023).

2.2. Stress tolerance spot assay

Spot assay was performed according to Song et al. [33] with modifications. Freshly grown *C. auris* (n = 351) (overnight at 35 °C at 200 rpm shaking) cells in Yeast extract peptone dextrose (YPD) broth (Hi Media, India) were washed with sterile phosphate buffered saline (PBS). Cell counts were adjusted to 10^6 cells/ml using spectrophotometer (OD600) and diluted from 10-fold to 1000-fold; 10 μl of each dilution were spotted on the YPD agar supplemented with hydrogen peroxide (H_2O_2, Merck Germany) from 5 to 50 mM (with 5 mM increments) and 8 %–20 % sodium chloride (NaCl extrapure, SRL Pvt Ltd, India). Plates were incubated at 37 °C for 2–4 days, colonies were counted and grading (+ to +++) was done in comparison to the growth control plate for each dilution of *C. auris* without H_2O_2. Oxidative tolerance was further confirmed with YPD broth assay using 40 randomly selected representative isolates (20 each from fluconazole-resistant and susceptible group) using similar protocol as above.

2.3. Gene expression analysis

Representative isolates (5 flu-resistant and 5 flu-susceptible) were chosen randomly for the Hog1, Cta1, Mkc1, Sod1, Cek1, and Cnb1 gene expression study. In brief, overnight grown *C. auris* cells were washed with sterile PBS and an inoculum with 10^6 cells/ml was prepared using spectrophotometer (OD600). 10 mM H_2O_2 was added to the inoculum and incubated for 2 h at 35 °C under continuous shaking (200 rpm). Cells were washed with PBS and total RNA was extracted using TRIzol reagent (Invitrogen, USA). The concentration and quality of the extracted RNA was checked using NanoDrop (Thermo Scientific, USA) and 1 μg of extracted good quality RNA was used to prepare cDNA using High-capacity cDNA synthesis kit (Thermo Fisher Scientific, USA) in Eppendorf MasterCycler.

Gene expression was evaluated with the Roche Light Cycler 480 RT-qPCR system using the PowerUp SYBR Green Master Mix (Termo Fisher Scientific, United States). The RT-qPCR running protocol was as follows: One cycle initial denaturation at 95 °C for 1 min; 45 repeated cycles of denaturation, annealing and extension at 94 °C for 10 s, 56 °C for 10 s, 72 °C for 10 s respectively. Finally, melting curve was generated using the setup at 95 °C for 5 s, 56 °C for 1 min and 97 °C for 15 s. The gene transcript levels were analysed with $2^{-\Delta\Delta CT}$ method [34]. β-actin-1 gene was considered as the reference housekeeping gene. Supplementary Table 2 lists all the oligos used for gene expression analysis in this study.

2.4. Quantification of catalase

Overnight grown *C. auris* (5 flu-resistant and 5 flu-susceptible) cells (in YPD broth at 35 °C at 200 rpm shaking) were washed with sterile PBS and an inoculum (1X 10^8 cells/ml) was prepared using spectrophotometer. 10 mM H_2O_2 was added to the inoculum and incubated for 2 h at 35 °C with continuous shaking (200 rpm) in incubator-shaker (New Burnswick, Canada). Cells were washed with 1X PBS and again inoculum of 1X 10^6 cells/ml was adjusted using spectrophotometer. Cells were exposed to 10 mM H_2O_2 for 2 h, Catalase activity was measured using the EnzyChrom catalase assay kit (Universal Biologicals Ltd., UK), following manufacturer's instructions. All experiments were done in triplicate.

2.5. iROS measurement

iROS was measured using the DHR-123 fluorescent probe staining according to Pathirana et al. with modifications [35]. *C. auris* (10 flu-resistant and 10 flu-susceptible) cells were grown for 8 h to mid-log phase in YPD broth at 35 °C and washed twice with sterile 1X PBS. An inoculum of 1X 10^7 cells/ml were set using a spectrophotometer and cells were exposed to a final H_2O_2 concentration of 5mM–30mM (with an increment of 5 mM) for 2 h at 35 °C. After 2 h, cells were centrifuged, washed and a cell suspension was made with 1X PBS (5 X 10^6 cells/ml) and staining was done with 10 mM DHR-123 probe (Sigma Aldrich, St. Louis, MO, USA) in dark for 60 min. Stained cells were centrifuged, washed with PBS and the cellular pellet was re-suspended in 200 μl of 1X PBS. Total fluorescence of each sample (200 μl of final stained cell suspension) was measured at room temperature using excitation and emission spectra at 488 and 525 nm respectively in a multimode microplate reader (TECAN, Switzerland).

2.6. Analysis of growth parameters

Growth kinetics were measured in YPD broth for 10 flu-resistant and 10 flu-susceptible *C. auris* isolates according to Kasper et al. [36] with modifications. 200 μl of inoculum ($\sim 10^6$ cells/ml overnight grown cell prepared using a spectrophotometer at OD600) was added to the flat bottomed 96-well culture plates (Greiner, Sigma-Aldrich, Germany) and incubated for 32 h at both 37 °C and 42 °C in a kinetic microplate reader (BioTek Instruments, USA) under constant shaking condition. For, hyper-oxidative condition, H_2O_2 was added to the inoculum to achieve the final concentration of 5–20 mM (with 5 mM increments). Inoculum without H_2O_2 was kept as control in each experiment. The optical density (OD) was recorded at 600 nm by the kinetic reader after every 30 min interval. All experiments were performed in triplicate.

2.7. Scanning electron microscopy (SEM)

To examine cell morphology, representative isolates from both fluconazole susceptible (n = 2) and resistant (n = 2) groups were chosen. 50ul inoculum of cell suspension containing 1×10^8 cells/ml were spread on polystyrene coverslips (poly-L-lysine coated) that were placed in each well of a 6-well microplate plates and incubated with or without 10 mM H_2O_2 at 35 °C for 2 h. The coverslips were washed with 1X PBS and fixed in 2.5 % (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) overnight at 4 °C. The coverslips were subsequently washed with 1X PBS and treated with 1 % osmium tetroxide (OsO_4, Thermo Fisher Scientific, India) for 2.5 h at 4 °C, then washed and serially dehydrated in a series of gradually increasing concentration of ethanol (30 %, 50 %, 70 %, 90 % and 100 %). The coverslips were dried overnight in incubator. Finally, the samples were mounted on aluminium stubs and following sputter coating with platinum, samples were imaged in Joel scanning electron microscope (Tokyo, Japan) at 3 kV.

2.8. Biofilm formation assay

An inoculum of OD_{600} 0.2 was prepared from freshly grown *C. auris* (10 flu-resistant and 10 flu-susceptible) cells using RPMI-1640 (pH 7) buffered with 165 mmol/L MOPS (HiMedia, India) and 200 μL inoculum was inoculated per well in flat-bottomed 96-well

microtiter plates (Greiner, Sigma-Aldrich, Germany). H_2O_2 were added for all isolated at 5–30 mM final concentration (with 5 mM increments). The plates were incubated at 37 °C and 42 °C. Plates were incubated for 24 h. Biofilm-forming capacity was estimated according to Singh et al. [37]. Biofilms were washed twice with 1X PBS and after ethanol fixation were stained with 200 μL of safranin (0.5 %). After washing, elution was done using glacial acetic acid (30 %) and the absorbance was measured at 490 nm.

2.9. XTT reduction assay

Metabolic activity of *C. auris* (10 flu-resistant and 10 flu-susceptible) was measured by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay according to Uppuluri et al. [38] with modifications. Biofilms were formed as described above. H_2O_2 were added at 5–30 mM final concentration (with 5 mM increments) for all isolates during cell seeding for biofilm. XTT solution (0.5 g/L in PBS) was prepared and mixed with freshly prepared menadione solution (dissolved in acetone at 1 μM final concentration). 100 μl XTT-menadione aliquots were added to pre-washed formed biofilm and incubated in dark at 37 °C for 2h. 80 μL supernatant was pipetted out into another 96-well plate and absorbance was measured at 490 nm (Epoch Microplate Reader).

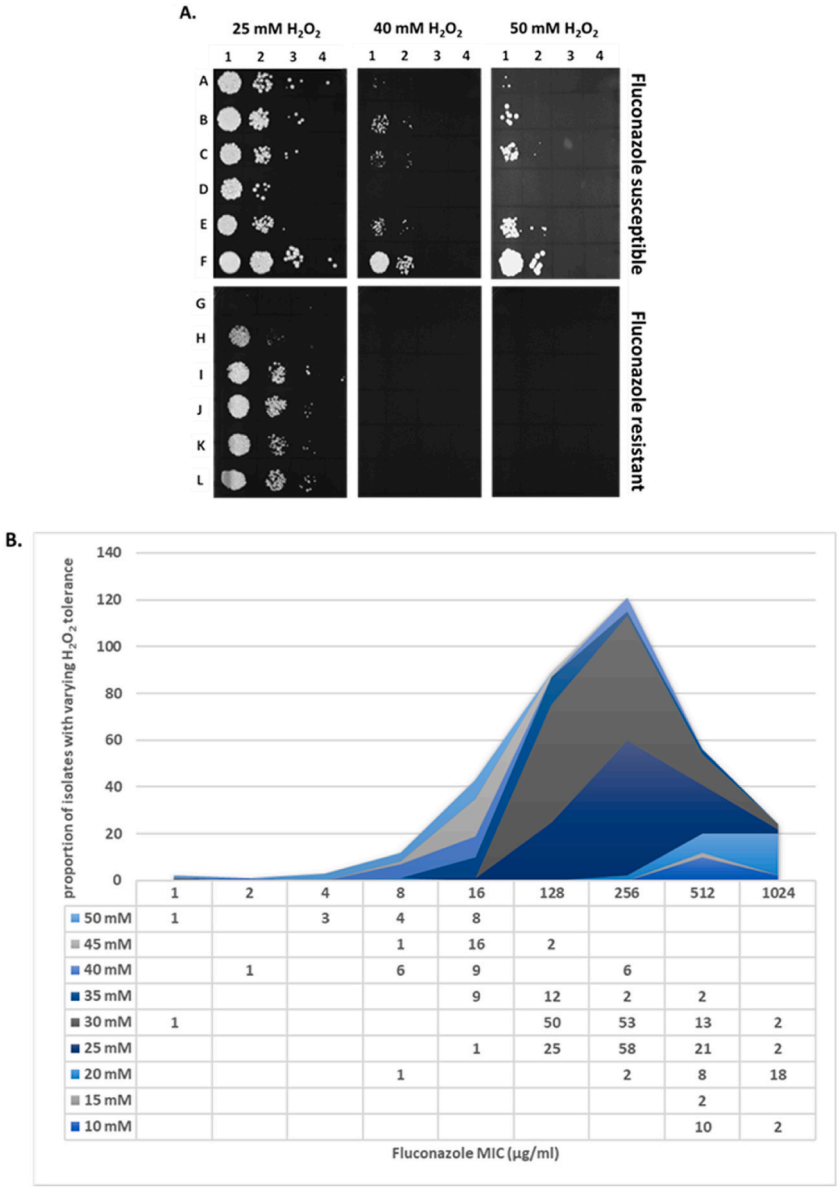

Fig. 1. A. YPD agar screening for tolerance to hyper-oxidative stress (hydrogen peroxide); B. Distribution of isolates of different fluconazole MIC vs. varying H_2O_2 tolerance among *C. auris* clinical isolates (n = 351). The table represents the number of isolates under individual fluconazole MIC from 1 to 1024 μg/ml *1, 2, 3 and 4 represents the inoculum cell count of 10^6, 10^5, 10^4 and 10^3 cells per ml.

2.10. Estimation of cell wall chitin

Baseline cell wall chitin contents of *C. auris* isolates (10 flu-susceptible and 9 flu-resistant) were measured following a flow cytometry-based method described by Oliveira et al. [39]. Overnight grown cells (in YPD broth) were washed with PBS, and ~1 X 10^7 cells were mixed with 2.5 mg/L calcofluor white (CFW) in dark at room temperature for 15 min. Washed cells were subjected to BD FACS CantoTM II flow cytometer (Becton, Dickinson, USA). Mean intensity (MI) was measured from stained and their respective unstained preparations and staining index was calculated using the equation: Staining index (SI) = (MI of stained cells - MI of unstained cells)/2 * standard deviation in MI of unstained cells.

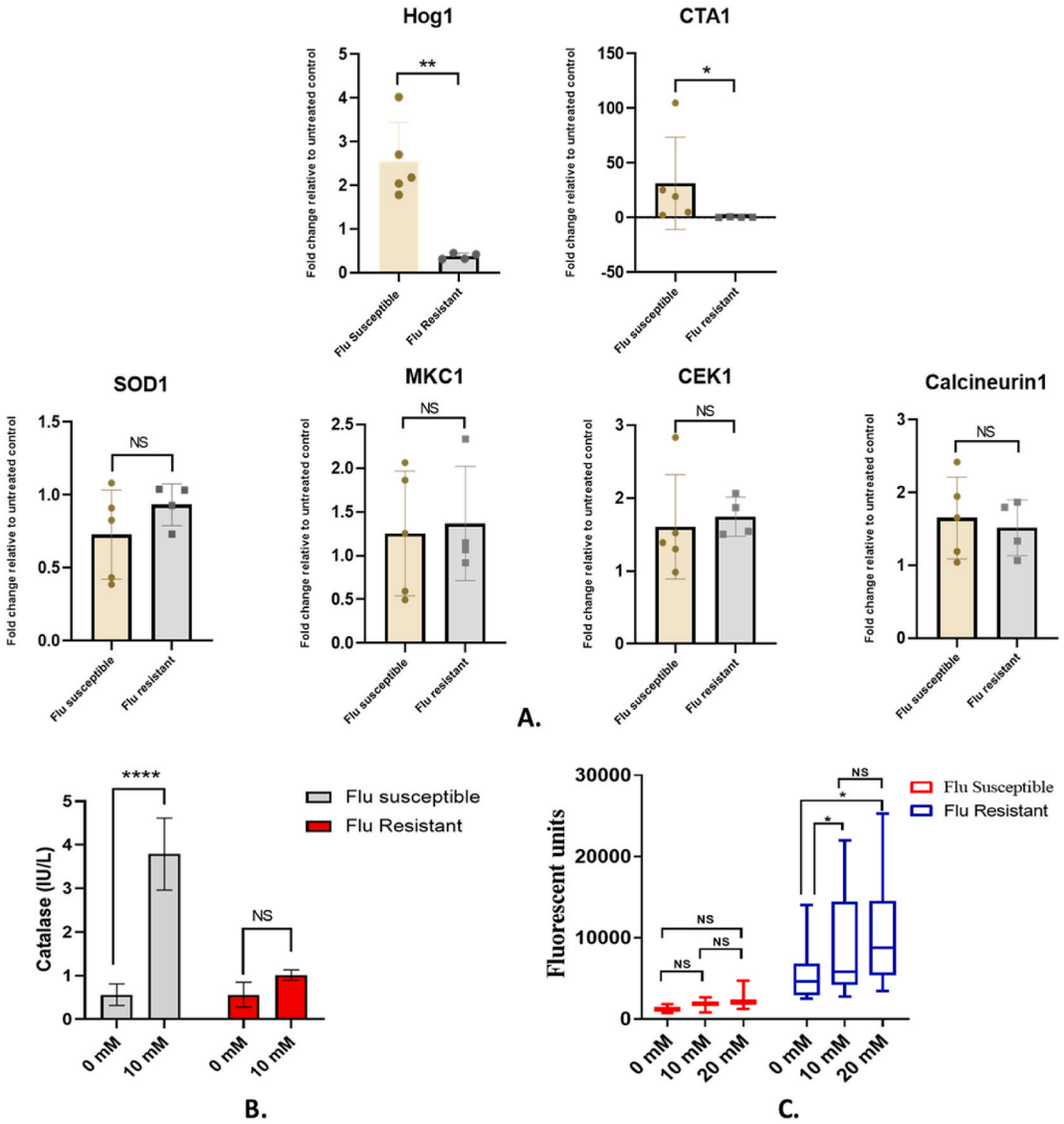

Fig. 2. Comparative gene expression, catalase estimation and iROS measurement among *C. auris* clinical isolates. A. represents relative fold-induction in the expression of oxidative stress related genes in *C. auris* (n = 10, 5 each from fluconazole susceptible and resistant groups) upon 10 mM H_2O_2 treatment compared to untreated controls. The data are represented as mean ± SD of three biological replicates for each isolate and analysed by Mann- Whitney *U* test; B. Estimation of intracellular catalase by *C. auris* isolates (n = 10, 5 each from fluconazole susceptible and resistant groups) under hyper-oxidative stress by commercial catalase kit and analysed by 2-way ANOVA corrected by Tukey's multiple comparison test.; C. iROS accumulation under hyper-oxidative condition among *C. auris* isolates (n = 19, 10 fluconazole susceptible and 9 fluconazole resistant) by DHR-123 assay and compared by 2-way ANOVA corrected by Tukey's multiple comparison test. NS- non-significant; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

2.11. Cytotoxicity by lactate dehydrogenase (LDH) measurement

Epithelial damage was determined by measuring lactate dehydrogenase (LDH) in the co-culture supernatant of *C. auris* (10 flu-susceptible and 9 flu-resistant isolates) according to Wächtler et al. [40] with necessary modifications. A549 cells [in Dulbecco's Modified eagle's media (DMEM) supplemented with 8 % Foetal bovine serum (FBS) and 1 % FBS for maintenance after monolayer formation] were seeded in the flat-bottomed 96 well microtiter culture plate and incubated for 24 h at 37 °C in 5 % CO_2. The monolayer was gently washed twice with Dulbecco's phosphate buffered saline (DPBS) and fresh media was added. An inoculum of 10 μl (1 X 10^6 cells/ml) was added and incubated for 24 h; 100 μl of the culture supernatant was removed and LDH activity was measured using the Cytotoxicity Detection Kit^{Plus} (Roche diagnostics) according to the manufacturer's protocol. Values were plotted as % LDH cytotoxicity. All experiments were performed in triplicates.

2.12. Galleria mellonella infection

Galleria larvae infection experiments was performed in the Department of Nematology, Indian Agricultural Research Institute (IARI), New Delhi. Survival assay was performed using sixth instar larvae (~300 mg weight), which did not have any melanisation and was maintained in dark at room temperature. Before inoculation, the body surfaces of the larvae were decontaminated using 70 % ethanol. Only disinfected larvae without inoculation were kept as control for absolute larval quality checking. 1X10^5 yeast cell (10 μL final volume) inoculated by insulin syringe in each larva through their left hind proleg. At least 10 larvae were inoculated for every isolate (10 flu-susceptible and 9 flu-resistant isolates) and 10 larvae were used as un-inoculated control in every batch. After inoculation with *C. auris* (10 flu-susceptible and 9 flu-resistant isolates), the larvae were kept at 37 °C incubator in 6 well cell culture plates in dark. larvae were scored daily for death for 5 days post infection. Kaplan–Meier survival plots were prepared and median survival were calculated [41].

2.13. Statistical analysis

GraphPad Prism 8.0.2 software and Microsoft excel software package were used for data analysis. Each experiment was performed in triplicates and results were presented as the mean ± SD. One-way and two-way analysis of variance (for multiple comparisons) and Mann-Whitney *U* test were utilized to determine statistically significant differences where appropriate. One-way and two-way ANOVA with appropriate recommended corrections was also used, wherever applicable. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Screening for stress tolerances

Among the total of 351 clinical isolates of *C. auris* (61 fluconazole susceptible and 290 fluconazole resistant) screened for oxidative stress tolerance by spot assay, 95.08 % (58/61) fluconazole susceptible isolates showed hyper-resistance to oxidative stress (30–50 mM) while 94.5 % (274/290) fluconazole resistant isolates showed lower tolerance to oxidative stress (10–25 mM) after 48–72 h of incubation at 37 °C. The result of agar screen for representative isolates are given in Fig. 1A and the distribution of the H_2O_2 tolerance vs. the fluconazole MIC of all isolates is given in Fig. 1B. To confirm the results of agar screen, 40 *C. auris* isolates (20 fluconazole susceptible and 20 fluconazole resistant) were randomly picked and the oxidative tolerance was reconfirmed with YPD broth and fluconazole susceptible isolates had grown up to 40–50 mM of H_2O_2 while the resistant isolates could withstand 10–25 mM of H_2O_2 (Supplementary Fig. 1) shows the results of the YPD broth screening. Pearson correlation index was calculated for the distribution of the H_2O_2 tolerance vs. the fluconazole MIC and the r value was −0.6315 [(−0.7146 to −0.5309), $p < 0.0001$] which suggests a strong negative correlation between H_2O_2 tolerance and fluconazole MIC i.e., with the increase in MIC, the tolerance to the oxidative stress gradually decreases. Regression analysis (Goodness of fit) calculated the R square value is 0.3988 (Supplementary Fig. 3).

Representative isolates (8 each from fluconazole susceptible and resistant group) were screened for cationic stress (sodium chloride incorporated with YPD agar, Supplementary Fig. 2). For, cationic stress screening, the results were similar as that of hyper-oxidative screening i.e., maximum NaCl tolerance for fluconazole-susceptible and resistant isolates were 18 % and 16 % respectively.

3.2. Gene expression analysis

Expression level of total six genes namely Hog1, Cta1, Sod1, Mkc1, Cek1 and Cnb1 were studied under hyper-oxidative condition (Fig. 2A). Upon induction with 10 mM H_2O_2, the transcript levels of Hog1 (2.548 ± 0.88 vs. 0.37 ± 0.06, $P = 0.0159$) and Cta1 (31.41 ± 42.2 vs. 0.47 ± 0.3, $P = 0.0159$) genes in flu-susceptible isolates were significantly higher compared to the resistant isolates. No significant difference among susceptible and resistant isolates were observed in the transcript level of the remaining genes Sod1, Mkc1, Cek1 and Cnb1 ($P = 0.412$, 0.904, 0.285 and 0.904 respectively).

3.3. Estimation of catalase and intracellular ROS

Since we observed a significant induction of catalase gene under hyper-oxidative stress in flu-susceptible isolates, we estimated the cytoplasmic catalase level at the baseline and after induction with 10 mM of H_2O_2 (Fig. 2B). Fluconazole-susceptible isolates had an

increased catalase content under hyper-oxidative stress (0.55–3.788 IU/L, P= <0.0001) while no significant or minimal increase in the catalase level was documented in fluconazole-resistant isolates (0.566–1.011 IU/L, P = 0.604). At 10 mM of H_2O_2 stress, fluconazole susceptible isolates produced higher amount of catalase compared to fluconazole resistant isolates (3.788 ± 0.82 vs. 1.011 ± 0.11 IU/L, P=<0.0001).

Accumulation of total iROS estimated using DHR-123 fluorescent probe revealed that catalase activity was inversely proportional to the accumulated iROS level inside the cell (Fig. 2C). With the exposure of increasing concentration of H_2O_2, there is a minimal increase in their iROS level [1200 ± 329.1 (0 mM) to 1815 ± 524.6 (10 mM), P = 0.9995; to 2291 ± 933 (20 mM), P = 0.9929] whereas there was a gradual increase in iROS level resistant isolates [5468 ± 3585 (0 mM) to 8914 ± 7485 (10 mM), P = 0.2581; 10510 ± 6982 (20 mM), P = 0.0719] but this gradual increase in iROS concentration was statistically insignificant. At basal level (without H_2O_2 exposure), fluconazole-susceptible isolates had lower intracellular ROS compared to the resistant isolates at basal level (1200 ± 329.1 vs. 5468 ± 3585, P=<0.0001) but no significant difference was observed in their catalase production (0.55 ± 0.22 vs. 0.56 ± 0.29, P = 0.925), the latter could be an underestimation because only 5 isolates from both the groups were tested for catalase production (half of the isolates tested for iROS) due to high cost of the catalase assay kit and in view of budget constraint.

3.4. Growth parameters under oxidative stress

A total of 19 (10 Flu-susceptible and 9 flu-resistant) *C. auris* clinical isolates were randomly selected and studied for growth kinetics at both 37 °C and 42 °C under hyper-oxidative condition (5, 10, 15, 20 mM of H_2O_2). Supplementary Figs. 4A and B shows the growth kinetics of all 19 *C. auris* isolates under 37 °C and 42 °C respectively. The doubling time of flu-susceptible vs. flu-resistant isolates at 37 °C are as follows: 1.5–3 h vs. 1.5–3.2 h in controls without H_2O_2, 4–5.2 h vs. 5–6.8 h with 10 mM H_2O_2, 3.8–5 h vs. 5.4–8.9 h with 20 mM H_2O_2. The duration of lag phase in flu-susceptible vs. flu-resistant isolates at 37 °C are as follows: 0.5 h vs. 1 h in controls, 2.5–7.5 h vs. 5.5–22.5 h in presence of 10 mM H_2O_2, 4–10 h vs. 9.5–27 h in presence of 20 mM H_2O_2. At 37 °C under no stress condition (control) and with 5 mM of H_2O_2, all isolates had similar overlapping growth curves. When the isolates were allowed to grow under higher H_2O_2 concentration (10–20 mM), the growth curve of the resistant isolates (except one flu-resistant isolate) had distinct delay in growth pattern (with increased doubling time and lag phase) in contrast to susceptible isolates (with shorter lag phase and steep log phase). The doubling time of flu-susceptible vs. flu-resistant isolates at 42 °C are as follows: 3.2–4.8 h vs. 2–6.9 h in control, 4.6–6 h vs. 3.6–8.6 h with10 mM H_2O_2, and 4.8–10.4 h vs. 5.4–10.5 h with 20 mM H_2O_2. The duration of lag phase of flu-susceptible vs. flu-resistant isolates at 42 °C are as follows: 1 vs. 1–2.5 h in control, 2–11 h vs. 5–20 h with 10 mM H_2O_2, and 4.5–23.5 h vs. 10.5 - >30 h with 20 mM H_2O_2. At 42 °C, no distinct difference in the different stages of the growth curve was observed between the two groups of isolates.

3.5. Scanning electron microscopy

In the absence of H_2O_2, the control cells of flu-susceptible isolate appeared as thin monolayer which was uniformly distributed coving the entire surface of the coverslip (Fig. 3 A1 & A2) whereas there were multiple degenerate focal gaps in the control biofilm of flu-resistant isolate (Fig. 3 B1 & B2). After treating the cells with 5 m H_2O_2, flu-susceptible cells formed three dimensional clusters coving almost 90 % of the surface (Fig. 3 C1 & C2) and thereby most of the cells remain inside the clusters being protected from the

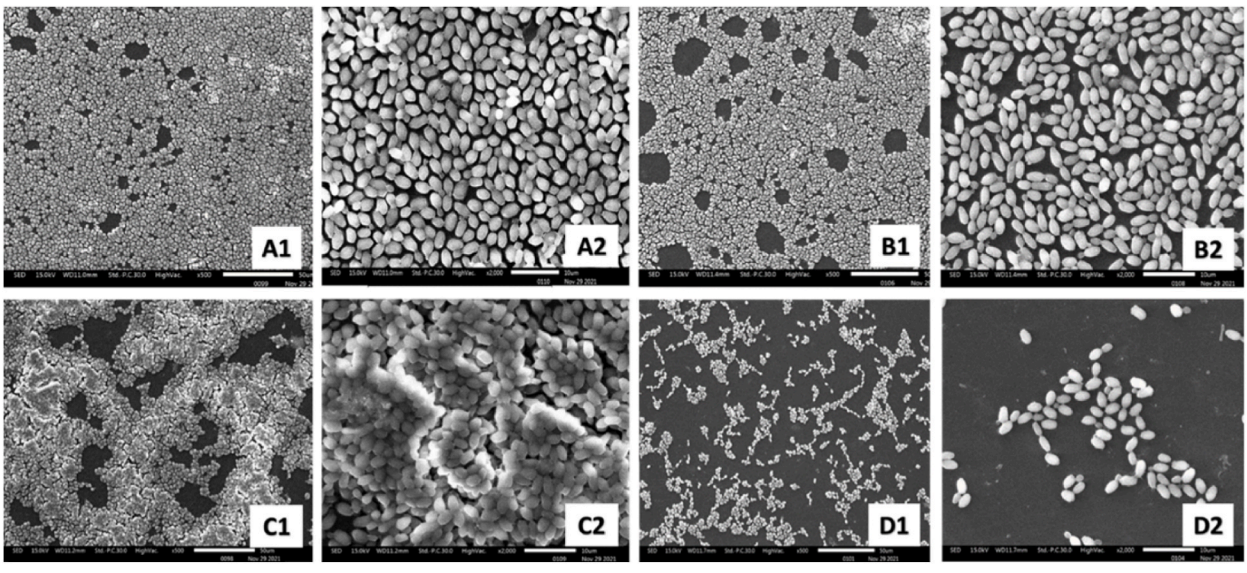

Fig. 3. Scanning electron microscopy images of *C. auris* Fluconazole susceptible and Fluconazole resistant isolates without (A & B) and with (C & D) hydrogen peroxide exposure (5 mM). 1 and 2 represents the lower (500X) and higher (2000X) magnification, respectively.

external oxidative stressors which is in advantage of their survival under hyper-oxidative stress. In contrast, after the H_2O_2 treatment, flu-resistant cells lost their adherence and detached from the coverslip surface and very less number of cells remain adhered to the surface (Fig. 3. D1 & D2).

3.6. Biofilm formation

Under no-stress (control) condition, flu-susceptible isolates formed significantly more biofilm compared to flu-resistant isolates at 37 °C (P= <0.0001, 0.917 ± 0.24 vs. 0.3 ± 0.19) whereas, this difference was not statistically significant (P = 0.4954, 0.54 ± 0.24 vs. 0.441 ± 0.25) at 42 °C (Fig. 4A). Under hyper-oxidative stress at 37 °C, fluconazole susceptible isolates formed significantly higher biofilms compared to flu-resistant isolates at 5 mM (P= <0.0001, 0.785 ± 0.22 vs. 0.245 ± 0.15), 10 mM (P= <0.0001, 0.694 ± 0.23 vs. 0.207 ± 0.10), 15 mM (P= <0.0001, 0.624 ± 0.20 vs. 0.165 ± 0.05), 20 mM (P= <0.0001, 0.547 ± 0.17 vs. 0.147 ± 0.04), 25 mM (P= <0.0001, 0.461 ± 0.14 vs. 0.134 ± 0.03) and 30 mM of H_2O_2 exposure (P = 0.0014, 0.373 ± 0.12 vs. 0.113 ± 0.02). At 42 °C, flu-susceptible isolates formed significantly more biofilms vs. fluconazole resistant isolates at 10 mM (P = 0.0025, 0.464 ± 0.19 vs 0.215 ± 0.07), 15 mM (P = 0.0099, 0.404 ± 0.13 vs. 0.184 ± 0.06) and 20 mM of H_2O_2 stress (P = 0.0277, 0.356 ± 0.13 vs 0.159 ± 0.05). At 42 °C, there was a significant reduction in biofilm among resistant isolates under 10 mM H_2O_2 compared to no-stress control (from mean OD 0.48 ± 0.26 to 0.23 ± 0.09) (P = 0.02).

3.7. XTT metabolic reduction assay

Flu-susceptible *C. auris* isolates had significantly higher metabolic rate compared to flu-resistant isolates at both 37 °C (P = 0.0187,

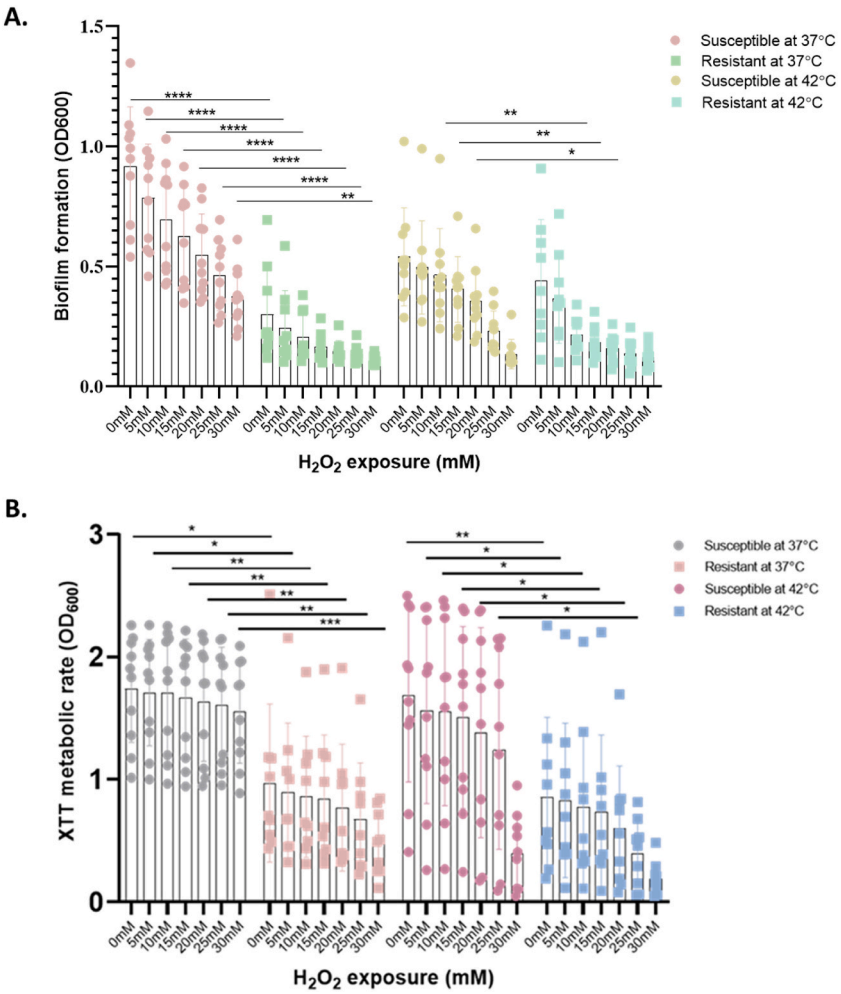

Fig. 4. A. Biofilm formation capacity and B. XTT metabolic reduction assay of *C. auris* clinical isolates (n = 19, 10 fluconazole susceptible and 9 fluconazole resistant) under hyper-oxidative condition at 37 °C and 42 °C compared by 2way ANOVA corrected by Tukey's multiple comparison test. NS- non-significant, *, $P < 0.05$, **, $P < 0.01$, ****, $P < 0.0001$.

1.737 ± 0.43 vs. 0.969 ± 0.64) and at 42 °C (P = 0.0086, 1.686 ± 0.70 vs. 0.852 ± 0.65) under no-stress condition (Fig. 4B). Under hyper-oxidative condition at 37 °C, flu-resistant isolates had significantly reduced metabolic activity at 5 mM H_2O_2 (P = 0.0108, 1.708 ± 0.43 vs. 0.892 ± 0.56), 10 mM H_2O_2 (P = 0.0069, 1.708 ± 0.49 vs. 0.856 ± 0.49), 15 mM H_2O_2 (P = 0.0096, 1.664 ± 0.48 vs. 0.838 ± 0.52), 20 mM H_2O_2 (P = 0.0061, 1.631 ± 0.48 vs. 0.768 ± 0.51), 25 mM H_2O_2 (P = 0.0024, 1.607 ± 0.47 vs. 0.673 ± 0.45) and 30 mM H_2O_2 (P = 0.0003, 1.549 ± 0.42 vs. 0.478 ± 0.26). Under hyper-oxidative condition at 42 °C, flu-resistant isolates had significantly reduced metabolic activity at 5 mM H_2O_2 (P = 0.0284, 1.557 ± 0.75 vs. 0.827 ± 0.62), 10 mM H_2O_2 (P = 0.0165, 1.550 ± 0.76 vs. 0.770 ± 0.64), 15 mM H_2O_2 (P = 0.0179, 1.505 ± 0.74 vs. 0.732 ± 0.62), 20 mM (P = 0.0165, 1.381 ± 0.85 vs 0.601 ± 0.50) and 25 mM H_2O_2 (P = 0.0078, 1.237 ± 0.80 vs. 0.395 ± 0.27). At both 37 °C and 42 °C, significant metabolic reductions were observed in flu-resistant isolates under hyper-oxidative condition.

3.8. Cell wall chitin estimation

Baseline chitin content was measured using calcofluor staining and flow cytometry and Flu resistant isolates had overall higher chitin content in their cell wall compared to the susceptible isolates (16525 ± 7508 FI vs. 6215 ± 2642 FI, P = 0.0076) (Fig. 5A).

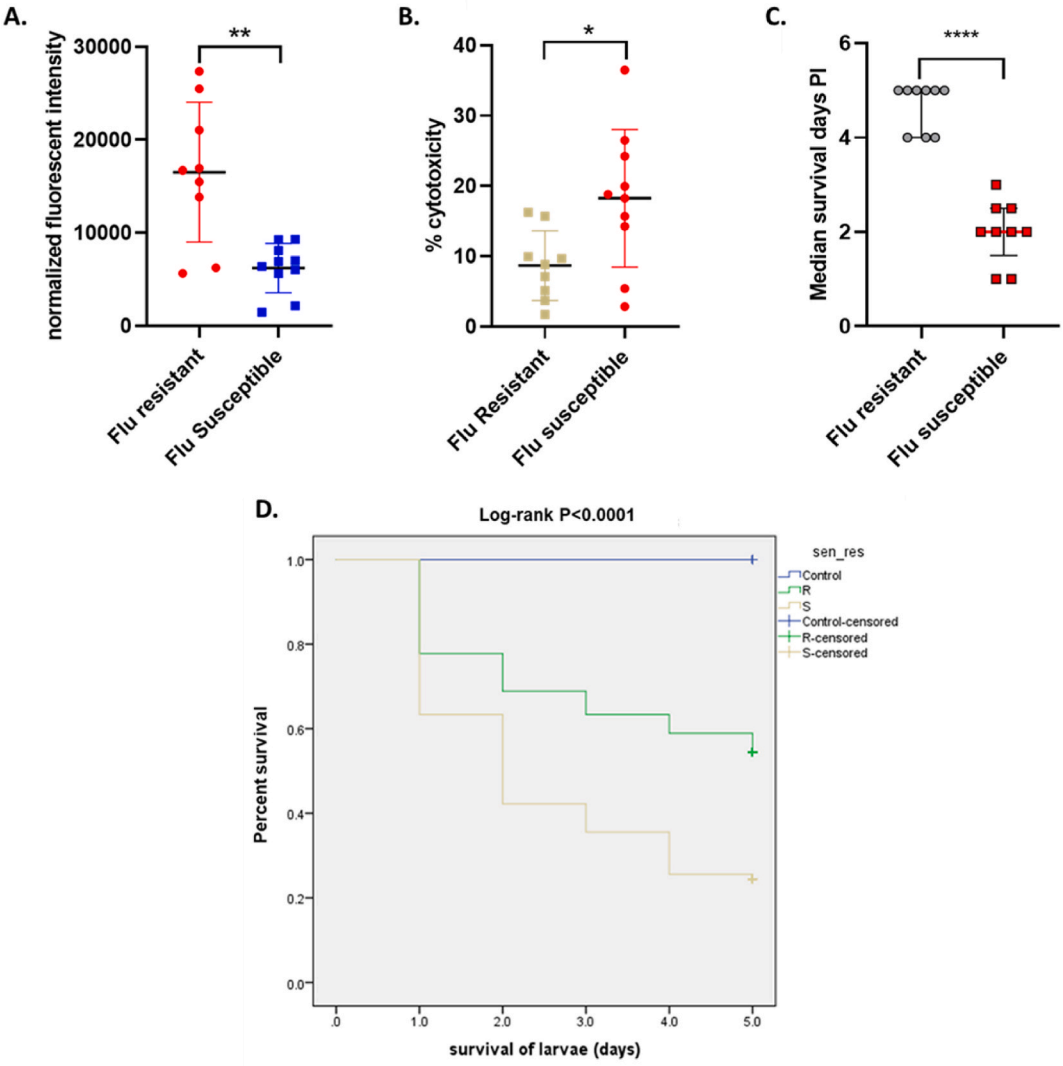

Fig. 5. A. Basal cell wall chitin content and B. %cytotoxicity among *C. auris* clinical isolates (n = 19, 10 fluconazole susceptible and 9 fluconazole resistant) analysed by Mann-Whitney *U* test.; C. comparison of median survival days of *G. mellonella* larvae infected with *C. auris*, compared by Unpaired *t*-test; D. Kaplan-meier survival curve illustrating the differential survival of the larvae infected with *C. auris* in systemic infection model. Kaplan-Meier survival curves were compared by Log-rank test. Green: *G. mellonella* larvae infected with fluconazole resistant isolates; Ochre: *G. mellonella* larvae infected with fluconazole sensitive isolates; Blue: *G. mellonella* larvae infected with sterile PBS (control). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Among 9 resistant isolates, only 2 had chitin content comparable to Flu-sensitive isolates.

3.9. LDH cytotoxicity assay

LDH released in the supernatant of *C. auris* (10 flu-susceptible and 9 flu-resistant isolates) infected A549 cell line culture which is considered to be directly proportional to the intensity of epithelial cell damage, was measured using commercial Roche LDH assay kit. The overall calculated mean % cytotoxicity was higher in flu-susceptible vs. flu-resistant isolates (18.23 ± 9.79 vs 8.66 ± 4.96, P = 0.0234) (Fig. 5B). Six out of 10 susceptible isolates had comparatively higher cytotoxicity while 4 showed mean % cytotoxicity score (9.54 ± 6.36) similar to that of those flu-resistant isolates.

3.10. Galleria mellonella infection model

The two control groups larvae (untouched larvae: control group 1 or larvae health control and larvae inoculated with PBS: control group 2 or trauma control) did not show any mortality by the +5 days post infection. The median survival of the larvae infected with fluconazole resistant and susceptible *C. auris* isolates were 5 days (IQR 4.5–5 days) and 2 days (IQR 1.5–2.5 days) respectively (P < 0.0001, Fig. 5C).

We also classified the virulence of the isolates according to the median survival days (Median Survival classification or MS classification) (Supplementary Table 1). Isolates with a median survival < P_{25} were classified as highly virulent and isolates with a median survival ≥ P_{75} were considered as of low virulent; isolates showing survival in between were classified as of moderately virulent. Among fluconazole resistant isolates, 66.6 % (n = 6), 22.2 % (n = 2) and 11.1 % (n = 1) showed low, moderate, and high virulence respectively whereas, 88.9 % (n = 8) of fluconazole susceptible isolates had moderate to high virulence. Despite some inter-strain variability, a significantly better survival was observed ($p = 0.0012$) in larvae infected with flu-resistant vs. susceptible isolates on Kaplan Meier survival curve analysis (Fig. 5D). Percent mortality of the larvae infected with fluconazole susceptible isolates were higher than those infected with fluconazole resistant isolates (75.5 % vs. 45.5 %).

4. Discussion

In this study, we have explored the comparative phenotypic profile of fluconazole-sensitive and fluconazole-resistant isolates of *C. auris* to study the fitness trade-offs between these two groups. Overall, we report a strong negative correlation between H_2O_2 tolerance and fluconazole MIC which was corroborated with higher transcript levels of stress response genes: Hog1 and Cta1 in fluconazole-susceptible isolates compared to the resistant isolates. Thus, fluconazole-susceptible isolates of *C. auris* are more tolerant to stress-tolerance compared to flu-resistant isolates. The SEM revealed formation of protective three-dimensional clusters of fluconazole-susceptible cells on exposure to external oxidative stressors which could be advantageous for their survival. Additionally, more biofilm formation was noted among susceptible isolates at baseline and in the presence of hyper-oxidative stress at 37 °C or 42 °C. Fluconazole susceptible isolates also showed higher metabolic activity and faster growth rate under hyper-oxidative condition. These findings suggest that although isolates may be phenotypically susceptible to fluconazole, they have other phenotypic stress responses which could improve survival under such conditions. Interestingly, virulence assessment using *in-vitro* cytotoxicity assay revealed higher % cytotoxicity in fluconazole-susceptible isolates with a significantly worse survival, higher melanisation and lower cocoon formation score in the *in-vivo* infection model.

Although azole is the most widely used antifungal drug for the treatment of invasive candidiasis, majority of the *C. auris* isolates are resistant to it [30]. We compared the oxidative and cationic stress tolerance of fluconazole susceptible and resistant *C. auris* clinical isolates due to its potential relevance for both, the escape from human host immune response and environmental survival. A unique pattern of higher resistant to oxidative and cationic stress with over-expression of Hog1(stress-activated protein kinase) and Cta1 (catalase) - genes was noted in in flu-susceptible isolates. Day et al. demonstrated that higher Hog1 activation in aggregative strains of *C. auris* strains provides oxidative and cationic stress resistance and also observed decreased virulence traits and altered morphology in *C. auris* Hog1Δ cells [18]. Horton et al. demonstrated that Hog1 offers resistance against desiccation and also maintains the morphology of the cell when *C. auris* is introduced in the environment leading to rapid colonization on the dry abiotic surfaces [19]. Highly resistant *C. auris* cells became susceptible to amphotericin B and caspofungin when Hog1 along with regulatory Ssk1 genes were removed [21], suggesting that Hog1 has serious impact in the pathogenesis of *C. auris*. Pradhan et al. described the role of catalase gene as an essential factor to combat the oxidative stress [42]. Besides Hog1, the overexpression of Cta1/catalase gene found in our study was directly correlated by the reduced iROS accumulation in cytoplasm in flu-susceptible isolates with gradual increase in extracellular ROS (increasing H_2O_2 exposure). Both Hog1 and Cta1 have been implicated to the oxidative stress fighting machinery in *Candida* in multiple studies [37–40,43–46].

With the evidence of flu-susceptible isolates being more tolerant to stress and the involvement or overexpression of Hog1 in it, our second question was whether there is difference in relative fitness associated with this. Fitness is affected by phenotypic plasticity which facilitates endurance towards various adverse environments such as high temperatures, limiting nutrients, reactive oxygen species (ROS) and others. In a drug-naïve condition, isolates with acquired drug resistance pays the price with reduction in fitness while the susceptible counterparts are reported to thrive [47]. While studying the effect of oxidative stress on growth parameters, we observed that most flu-resistant isolates had significantly longer lag phase and doubling time compared to susceptible isolates at 37 °C. This may be extrapolated *in-vivo* to explain survival of fluconazole susceptible *C. auris* isolates by faster multiplication in hyper-oxidative conditions such as those encountered inside macrophages and neutrophils. No such pattern was observed when the

same experiment is done at 42 °C (dual stress H_2O_2 + temp) and can be explained as elevation in temperature could act as an additional selective pressure on growth [23]. Further, it may be hypothesised that in conditions associated with higher temperature, both in environment (due to higher ambient temperature in tropical regions) and human body (febrile-range hyperthermia), oxidative survival responses of *C. auris* may not be replicated [48]. However, gradient oxidative stress response with rising temperatures needs to be tested to confirm this.

Under hyper-oxidative condition, higher biofilm formation was observed in flu-susceptible isolates at both 37 °C and 42 °C. Pemmaraju et al. demonstrated that polysaccharide content and extracellular DNA secretion into the biofilm matrix is enhanced under oxidative stress and *C. albicans* adapts to the stress by producing increased biofilms with such rich exopolymeric matrix [49]. Seneviratne et al. showed the increased activity of ROS neutralizing enzymes like hydroperoxide reductase, thioredoxin peroxidase and thioredoxin in *C. albicans* biofilms under oxidative stress [50]. Deschaine et al. found a robust fitness advantage of biofilm strains of *S. cerevisiae* that outcompete non-biofilm strains in direct competition [51]. As mentioned in these studies, increased biofilm observed in our study indeed provide survival advantages to the flu-susceptible isolates over resistant isolates under oxidative stress. Higher adhesion index is related to strong biofilm formation. Adhesion of *C. glabrata* was shown to be regulated by Hog1 during iron homeostasis and Hog1 deletion also resulted in defective adherence of *C. albicans* in a mouse gut colonization model [52,53]. A significant Hog1 overexpression in flu-susceptible isolates in our study thereby justifies the enhanced biofilm formation. Our XTT reduction assay revealed that metabolic activity of flu-susceptible isolates remains higher compared to the resistant isolates under hyper-oxidative condition. Metabolic activity attributes to fitness during colonization or disease progression by providing precursors and energy required for many essential cellular activities for example, the generation of antioxidants, cell division, hyperthermal stress adaptation, cellular repair and biosynthesis of fungal cell wall [54]. Tendency of higher biofilm formation, adhesion and higher metabolic activity of flu-susceptible under stress condition altogether contributes to enhanced fitness compared to resistant counterparts.

Estimation of cell wall chitin and cell cytotoxicity assay were done to establish a more convincing direct link to the enhanced pathogenicity of the susceptible isolates. Shahi et al. found that flu-resistant *C. auris* clinical isolates had comparatively higher cell wall chitin content than flu-susceptible isolates which is exactly the similar finding we observed in our study [55]. In *C. albicans* and *C. glabrata*, it is already proven that enhanced cell wall chitin level is associated with attenuated growth attributing to lower virulence in systemic animal infection models [56].

It was imperative to replicate these findings in *in-vitro* and *in-vivo* experiments. LDH cytotoxicity assay was used to quantify the damage to the A549 lung epithelial cell line caused by *C. auris* revealed that flu-susceptible isolates were more cytotoxic compared to resistant isolates. Finally, in *G. mellonella* infection model, majority of the flu-resistant isolates showed significantly better survival of the larvae. The collective data from our *in-vitro* and *in-vivo* experiments indicate towards the fitness cost of the flu-resistant isolates which could be the consequences of evolution and development of azole resistance in these isolates. Additionally, flu-resistant *C. auris* isolates have been reported to be extremely susceptible to salivary cationic peptide Histatin 5 (Hst 5) where two out of three flu-susceptible isolates had 100 % resistant to Hst5 killing [57].

Fluconazole-susceptible strains were seen to have better physical adaptation (stress tolerance are virulence attributes) compared to resistant strains in this study. This would allow better survival in harsh environments when exposed to external stressors such as disinfectants in hospital settings or even on exposure to in-vivo oxidative stress. This provides a competitive advantage to the susceptible isolates allowing them to persist in environments that would otherwise be inhospitable. Such isolates would thereby have better opportunity to gain entry into critical drug naïve patients and cause infection. We also observed a higher virulence among susceptible isolates as per *Galleria mellonella* and LDH cytotoxicity study which could translate to poor clinical outcomes in infected drug naïve patients.

The emergence of *C. auris* as a human pathogen, its evolutionary diversification and development of drug resistance are still poorly understood. Stress responses are pivotal for fungal growth and virulence in different ecological niches and are also potentially affected by the development of antifungal resistance or tolerance [58]. Majority of the studies have focused on the drug resistance mechanisms whereas, only few reports are available on *C. auris* stress tolerance towards various abiotic stresses. Recently, by structure-guided approaches it is possible to identify specific inhibitory molecules *in silico*, that can interrupt the stress response in *C. auris* thereby crippling its virulence [59]. Selectively targeting these fungal stress responses in combination with current antifungals has the potential to disarm fungal virulence, reduce antifungal drug resistance, and sensitize drug-resistant pathogens [60].

Although an elaborate attempt to uncover various aspects of fitness and its relation with azole-resistance using a large number of isolates was made in this study, it still has a few limitations. While oxidative stress response was screened in all isolates, only representative isolates were screened for other stress such as cationic stress. Screening for metal stress may further elaborate on the iron homeostasis in *C. auris.* Only clinical *C. auris* isolates were included in this study where the addition of environmental isolates may strengthen our findings. This study involved only clade I isolates where screening of *C. auris* isolates from all five clades is warranted for better understanding of associated relative fitness.

5. Conclusion

In summary, this study provides previously unexplored and useful insights into the comparative fitness dynamics of *C. auris* isolates with respect to their antifungal susceptibility profile. Further studies using environmental isolates and other clades could strengthen our findings. These findings could also be translated to clinical practice by investigating the possibility of using molecules targeting stress response and fitness regulating pathways for management of this serious infection.

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Institutional review Board Statement

The work has been approved by the Institutional Ethics Committee (No. INT/IEC/2023/SPL-337, dated: 28th March 2023).

Data availability Statement

All data generated in this study has not been deposited into any publicly available repository. Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Sourav Das: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shreya Singh:** Writing – review & editing, Visualization, Supervision, Investigation. **Yamini Tawde:** Validation, Methodology, Investigation, Data curation. **Tushar K. Dutta:** Resources, Methodology, Investigation. **Shivaprakash M. Rudramurthy:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition. **Harsimran Kaur:** Writing – review & editing, Supervision. **Tushar Shaw:** Writing – review & editing, Formal analysis. **Anup Ghosh:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32386.

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