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Antimicrobial Studies on Garlic Lectin

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Abstract
Allium sativum agglutinin (ASA) is an important lectin isolated from garlic bulbs and has shown promising therapeutic potential in earlier reports. It has a bulb-type lectin domain, and members of this protein family have been investigated for anti-cancer, antimicrobial and other effects. In our earlier study, we have reported ASA as an anti-cancer agent, and in the present study, we have evaluated it for its antifungal and antimicrobial effects. The effects of ASA on the opportunistic pathogens in humans *Candida auris* and *Candida glabrata* fungal strains have been evaluated, and efforts are made to evaluate the mechanistic basis of these antifungal effects. The antifungal activity of ASA on different strains of *C. glabrata* and *C. auris* was found with MIC_{50} concentration range of 30–70 µg/ml. Fungal growth was significantly suppressed upon treatment with ASA at MIC_{50} and $2MIC_{50}$. Hydrogen peroxide production was detected after ASA treatment in fungal cells and cell morphology, and integrity was affected when analysed through FE-SEM. Further, the anti-biofilm effect of ASA was investigated against *Candida* and three bacterial strains (*Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*), and promising results were obtained with maximal effect in case of *K. pneumoniae* among the bacterial strains. These results can form the basis for the development of ASA as antimicrobial agent.

Keywords *Allium sativum* agglutinin · Antifungal · Anti-biofilm · Cell wall · Hydrogen peroxide · Growth kinetics

Introduction

Allium sativum (garlic) is widely used in culinary and has been reported to have many potential therapeutic effects like activity against pathogenic microorganisms, modulation of cardiovascular risk and potential anti-cancer activity [1]. Lectins are glycan-interacting proteins found ubiquitously in nature, and many are reported to interact with carbohydrates like chitin, cellulose, glucans and mannoproteins which are present in the cell wall of the fungus leading to the inhibition of the cell growth, cell wall disruption and cell death [2–4]. ASA is reported to have many potent biological effects, and a variant of ASA, *Allium sativum* lectin (ASL50), showed antifungal activity against *Candida parapsilosis*, *Candida krusei*, *Candida tropicalis* and *Candida glabrata* in preliminary studies [5]. Another leaf lectin isolated from garlic leaves, i.e. mutant *Allium sativum* leaf agglutinin (mASAL), and its mutants have shown antifungal activity against *Rhizoctonia solani* [6].

Different opportunistic *Candida* species have been found to be associated with various fatal human infections. They may cause candidiasis and ultimately leads to fatal systemic mycosis [7]. In the condition of *candidemia* which is the fourth most common cause of blood nosocomial infection, the mortality rate is very high 30–60% [8, 9]. *Candida albicans* mainly causes *candidemia* infections, and other *Candida* species such as *C. glabrata*, *C. auris*, *C. tropicalis* and *C. krusei* are responsible for invasive infections. These species are also reported to be resistant to various echinocandin and azole-containing drugs, and hence, finding new antifungal agents becomes imperative [10]. *C. auris* has been identified and isolated a decade ago, but due to difficulties in detection, environmental resilience and multi-drug resistant, its treatment is problematic [11]. *C. auris* has the potential to develop biofilm on the medical devices and thus can easily cause infection in the hospitalised patients [12]. Various appropriate therapeutic agents are mandatory to terminate these fungal infections. As plant lectins are easy to extract and show anti-cancer, antibacterial, anti-inflammatory and antifungal activities,

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they can be potentially used as targets against these multi-drug resistant infectious agents.

In the present study, we have reported the antifungal and anti-biofilm activity of ASA on clinical isolates of *C. auris* and *C. glabrata*. A significant inhibition in the growth and biofilm formation in the fungal species was observed after ASA treatment. Three bacterial species *E. coli*, *K. pneumoniae* and *S. aureus* also showed a significant inhibition in biofilm formation after 24 h of lectin treatment. Further, the mechanistic effects of ASA effects on *Candida* species have been reported, and we hope these findings form the basis for the development of ASA as an anti-infective agent.

Materials and Methods

Candida Strains, Chemicals and Media

Clinical isolates of *C. auris* (NCCPF470153, NCCPF470197, NCCPF470200) and *C. glabrata* (NCCPF100037, NCCPF100033) were obtained from the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India (https://nccpf.in/catalogue/search). The environmental isolates of *C. glabrata* ATCC2001 and MTCC3019 (https://mtccindia.res.in/catalog) were also obtained from the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. They were preserved on Sabouraud dextrose agar plates (SDA) and in Sabouraud dextrose broth (SDB) for routine use in laboratory. For long-term preservation, 30% glycerol stocks were prepared and stored at −20 °C and −80 °C. *E. coli* (MTCC:585), *S. aureus* (MTCC:3160) and *K. pneumoniae* (MTCC:109) were kind gifts from Prof. Geeta Shukla, Department of Microbiology, Panjab University, Chandigarh. Sabouraud dextrose broth, nutrient broth, crystal violet, xylene, methanol, acetic acid, 3,3-diaminobenzidine etc. were procured from HiMedia (Mumbai, Maharashtra).

Purification of ASA

ASA was purified from fresh garlic bulbs using various chromatographic techniques such as size exclusion, ion exchange and hydrophobic interaction chromatography as reported in our previous work [13]. The protein was of molecular weight ~12 kDa and was analysed on SDS PAGE and further confirmed using MALDI intact mass analysis. Protein concentration was measured using Bradford reagent, and specific activity in haemagglutination assay was found to 975.24 HU/mg, and this further confirmed its lectin nature.

Antifungal Activity

Antifungal activity of ASA was determined by agar diffusion assay [14]. Strains of *C. glabrata* and *C. auris* were cultured in SDB medium for 24 h at 30 °C, and the log phase culture of cells containing 3×10^6 CFU/ml was spread on SDA plate, and wells were formed using sterile tips. Different concentrations of 100 µl purified ASA and PBS as negative control were loaded in the wells. The plates were allowed to incubate at 30 °C for 24 h, and antifungal activity was analysed by measuring the zone of inhibition in millimetres with a disc diffusion zone of 6 mm considered as no inhibition zone. Experiments were performed in triplicates.

MIC_{50} Estimation

The antifungal activity was evaluated through the broth micro-dilution method according to the guidelines from the Clinical and Laboratory Standards Institute (2021) [15] in 96-well round bottom plate (Greiner Bio-One, Germany) as described in earlier studies [16]. Fungal strains were cultured in SDB medium at 30 °C overnight, and bacteria were cultured in nutrient broth (NB) medium at 37 °C overnight. After growth, the density of the cells was adjusted to 3×10^6 colony-forming units/ml to each well of round bottom plate. These inoculums and protein solution in different concentrations (0–200 µg/ml concentration) with SDB and NB media medium were added to make 200 µl total volume in each well. A control without treatment was also maintained, and plates were allowed to incubate at respective temperatures for 24 h. The optical density (OD) was measured at 600 nm at the start of incubation and after 24 h to estimate the microbial growth. The MIC_{50} was determined as the lowest protein concentration at which 50% growth was inhibited at 600 nm as compared to control. Each treatment was given in triplicate, and readings were taken:

$$\text{Cell inhibition (\%)} = 100 - \left(OD_{600} \text{ of treated cells} / OD_{600} \text{ of control cells}\right) \times 100$$

Cell viability was measured by measuring colony-forming units (CFU) after treatment [17]. A 10^4 cells/ml fungal suspension was incubated with ASA (40 µg/ml) for 24 h at 30 °C, and 100 µl culture of 1000-fold dilution of this culture in SDB medium was spread on SDA plate and incubated at 30 °C for 24 h. Finally, CFU were counted, and cell viability percent was determined relative to the control (without treatment), which was considered 100%.

Growth Curve

Growth curves were analysed according to the established protocols [18]. The cell density of the culture was adjusted 3×10^6 CFU/ml in SDB medium, and 5 µl of these inoculums along with different dilutions of ASA (1/4MIC_{50}, 1/2MIC_{50}, MIC_{50} and 2MIC_{50}) was added to 96-well plate,

and the final volume was made 150 µl with growth medium. The plates were allowed to incubate at 30 °C. Appropriate controls were made without lectin treatment, and the optical density was measured at 600 nm from 0 to 24 h after each hour of incubation. The growth of fungal species at these four different concentrations was compared to controls, and the results were analysed.

Detection of Hydrogen Peroxide

Hydrogen peroxide released from fungal cells was estimated by staining procedure using DAB (3,3′-diaminobenzidine) [17]. Strains of *C. glabrata* and *C. auris* (1×10^5 cells/ml) were incubated at 30 °C in six wells plates with PBS (negative control) and ASA (MIC_{50}) in the presence of 0.5 mg/ml DAB. Appropriate time was given (about 4–5 h) for the incubation of fungal cells so that DAB interacted with the produced hydrogen peroxide. After incubation, the plates were microscopically observed for the detection of brown pellet which is the clear indication of hydrogen peroxide production.

Biofilm Assay

The biofilm assay was performed as already reported in earlier studies with some modifications [18, 19]. Bacterial cultures were grown in NB medium at 37 °C for 24 h and were diluted in the ratio of 1:100 with fresh medium so that the cell density was approximately 2×10^6 cells/ml. Similarly, fungal cultures were grown in SDB medium at 30 °C for 24 h to get a final cell density of about 1×10^6 CFU/ml. These inoculums were added in each well of 96-well flat bottom plates, different concentrations of ASA (0–100 µg/ml) were added, and the final volume was made 150 µl with respective media. The plates were allowed to incubate for 24 h at 37 °C for bacterial strains and 30 °C for fungal strains. The anti-biofilm effect of ASA on fungal strains was also evaluated by giving a treatment of protein (0–200 µg/ml) to a 24-h formed biofilm. After incubation, the medium was removed, and wells were washed twice with 200 µl sterilised water without disturbing the biofilm. After drying the wells, biofilms were fixed by adding 200 µl methanol in each well for 15 min. The methanol was removed, and the plates were air dried. The biofilm was stained with the addition of 200 µl of 0.1 % crystal violet for 5 min. The unbound dye was removed by washing the wells twice with water, and 200 µl of 33 % acetic acid was added in each well to dissolve the dye. Optical density was measured at 570 nm (OD_{570}) using SYNERGY|^{H1}microplate reader (Bio-Tek Instruments, USA), and each treatment was given in triplicate, and readings were taken:

$$\text{Biofilm formation inhibition or reduction in formed biofilm (\%)} = 100 - \left(OD_{570} \text{ of treated cells} \,/\, OD_{570} \text{ of control cells}\right) \times 100$$

Microscopic Analysis

Fungal cells (1×10^6 CFU/ml) were cultured in SDB medium and treated with ASA at MIC_{50} for 24 h at 30 °C in six well plate. The medium was then discarded, and cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min. After fixation, the morphology of the cells was analysed using a fluorescence microscope (Nikon ECLIPSE Ts2, Japan).

The effect on biofilm formation on ASA treatment was analysed using field emission scanning electron microscope (FE-SEM) as per established protocols [20]. The cells (1×10^6 CFU/ml) were incubated with medium and treated with ASA at MIC_{50} dose in six well plates having poly-lysine-coated cover slip at the bottom of each well. Cells without treatment were taken as control. The plates were allowed to incubate at 30 °C for 24 h under steady condition for biofilm analysis. To analyse the effect of ASA treatment on planktonic cells, the fungal cells were incubated at 30 °C for 24 h after ASA treatment in incubator shaker; cell pellet was taken, washed with PBS and fixed in 2.5% glutaraldehyde for 2 h at 30 °C and dehydrated using ethanol. The cell-coated cover slips (both biofilm and planktonic) were fixed on stub and air dried, and the cells were coated with gold using MC 1000 Ion Sputter coater (Hitachi, Japan) for 30 s. The effect of ASA on fungal cells in both the forms was analysed using SU 8010 (Hitachi, Japan) ultra-high-resolution FE-SEM along with controls.

Cellular Surface Hydrophobicity (CSH) Determination

The cellular surface hydrophobicity (CSH) of fungal cells was determined using a microbial adhesion to hydrocarbon method according to the reported method [21]. Fungal cell suspension having 0.4 OD at 600 nm was prepared using SDB medium. This suspension was incubated with ASA (40 µg/ml) and PBS as treated and control samples, respectively, for 3 h. After incubation, 300 µl of xylene was added in the treated and control tubes. The suspension was vortexed for 1 min and allowed to settle for 10 min, and again OD was measured at 600 nm. The experiments were performed in triplicate. The CSH index was calculated as

$$\text{CSH (\%)} = \left[\left(OD_{\text{Initial}} - OD_{\text{Final}}\right) / \, OD_{\text{Initial}}\right] \times 100$$

Statistical Analysis

All the graphs were made using GraphPad Prism 5.0 software. The data set for each experiment was taken in triplicate and repeated as explained in each experimental description, and average values were represented as mean ± standard deviation (SD). The statistical difference between two sets of data was analysed by unpaired two tailed *t* test with the assumption of unequal variance, and the data with *p* values less than 0.05 were considered significant.

Results

Antifungal Activity

The sensitivity test using well diffusion assay on ASA treatment showed significant effect on all fungal strains tested, and the effect was seen in the concentration range of 5–50 µg/ml of ASA treatment. A clear zone of inhibition of 8 mm and 11 mm at concentration of 20 and 50 µg/ml, respectively, in case of *C. auris* strain (NCCPF470200) (Fig. 1A) was observed, and similarly, a zone of clearance of 8 mm and 12 mm at concentration 10 µg and 40 µg/ml, respectively, was observed in *C. glabrata* (ATCC2001) (Fig. 1A). After treatment of ASA (40 µg/ml), a prominent decrease in the viable cells was observed (Fig. 1B, C), and a cell viability loss of 81–84% was observed in *C. glabrata* (ATCC2001) and *C. auris* (NCCPF470200) (Fig. 1D) as compared to control cells when treated cells were spread over SDA plates after making dilutions, as by spreading the cultures on SDA plates, only live cells were detected in the form of colonies.

MIC_{50} and Growth Kinetics

ASA showed antimicrobial activity against four strains of *C. glabrata*, three strains of *C. auris* and three bacterial strains. An MIC_{50} value in the range of 30–70 µg/ml was obtained for all the fungal strains tested and from 76 to 126 µg/ml was obtained for bacterial strains. The exact MIC_{50} values of ASA for all microorganisms are given in Table 1.

Individually growth kinetics studies on strain *C. glabrata* (ATCC2001) and *C. auris* (NCCPF470200) showed that upon ASA treatment with a concentration of ¼ MIC_{50}, ½ MIC_{50}, MIC_{50} and $2MIC_{50}$, the maximum effect was observed when cells were treated MIC_{50} and $2MIC_{50}$ as compared to normal cells (Fig. 2). In case of *C. glabrata*, growth inhibitory effect was observed after 10 h of incubation, and maximal effect was seen after 24 h of incubation. In case of *C. auris*, the effect was observed after 16 h, and the maximum effect was seen after 24 h of incubation.

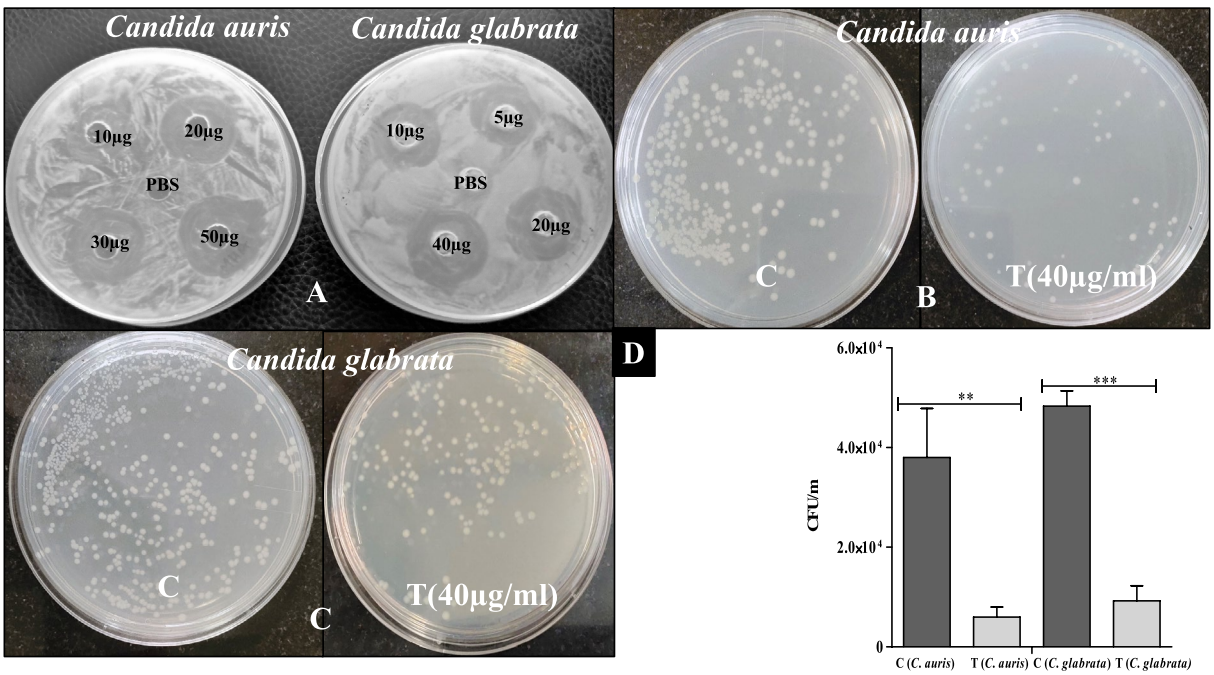

Fig. 1 Antifungal activity of ASA on fungal strains in the concentration range of 5–50 µg/ml. **A** Zone of clearance observed on treatment with ASA at a concentration of 20 µg/ml in *C. auris* (NCCPF470200) and at 5 µg/ml in *C. glabrata* (ATCC2001). **B**, **C** Colonies in control and treated plates after 40 µg/ml treatment of ASA on *C. auris* and *C. glabrata*. **D** Cell viability (%) obtained by counting colonies and obtaining CFU (**B**, **C**). The results were representative of three biological replicates. (*) significant difference $p < 0.05$

Table 1 MIC_{50} values of ASA protein against *Candida* and bacterial species

Sr. No	Microorganism name	MIC_{50}
1	*C. glabrata* (ATCC2001)	33 µg/ml
2	*C. glabrata* (MTCC3019)	49 µg/ml
3	*C. glabrata* (NCCPF100037)	43 µg/ml
4	*C. glabrata* (NCCPF100033)	56 µg/ml
5	*C. auris* (NCCPF470153)	42 µg/ml
6	*C. auris* (NCCPF470200)	30 µg/ml
7	*C. auris* (NCCPF470197)	69 µg/ml
8	*E. coli* (MTCC:585)	106 µg/ml
9	*S. aureus* (MTCC:3160)	126 µg/ml
10	*K. pneumoniae* (MTCC:109)	76 µg/ml

Detection of Hydrogen Peroxide

The mechanism behind the cytotoxic effect of ASA on fungal cells might be the reactive oxygen species (ROS) induction which was confirmed using DAB staining. When the yeast cells were treated with ASA at MIC_{50} concentration for 4 h, the oxidation of 3,3-diaminobenzidine (DAB) occurred, and a dark brown-coloured pellet was observed in the treated cells in the presence of hydrogen peroxide. In the images, it was clearly observed that negative control cells (treated with water) were unstained and bright, but in ASA-treated cells, arrows indicated the presence of dark brown pellet in *C. glabrata* and *C. auris* (Fig. 3) which was the indication of insoluble precipitate formation at the site of the reaction.

Morphological Changes

Under fluorescence microscopic analysis, even distribution of control fungal cells was observed, whereas the ASA-treated cells at MIC_{50} concentration showed cell clumping (Fig. 4A–D) which was a possible indication of agglutinating effect of ASA due to glycans present on fungal cell walls.

Under electron microscope, uniformity and regular size were observed in untreated planktonic cells. After the treatment with ASA (MIC_{50} concentration), there was shrinkage, loss of cellular integrity and bulge formation in the cells, and some cell fragments were also observed (Fig. 5A–D).

Biofilm Assay

In case of bacteria, the effect of ASA treatment from 0 to 200 µg/ml concentration for 24 h showed 70.3±1.4%, 55±.2.8% and 78±0.4% inhibitory effect in biofilm formation on *E. coli*, *S. aureus* and *K. pneumoniae*, respectively, at the maximum treatment concentration of 200 µg/ml (Fig. 6).

In fungal strains, the effect of ASA treatment on biofilm formation was observed under two conditions. When the treatment of 100 µg/ml ASA concentration was given at the start of incubation and the biofilm formation was measured after

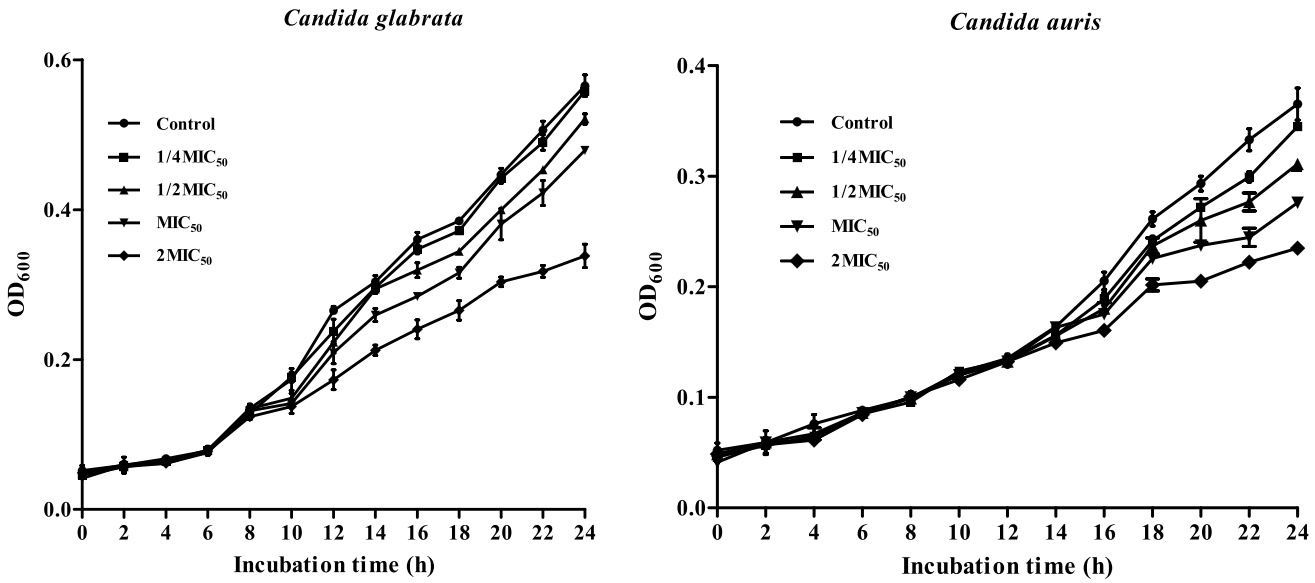

Fig. 2 Effect of ASA treatment on growth kinetics of *C. glabrata* (ATCC2001) and *C. auris* (NCCPF470200) strains. Results were expressed as mean ± standard deviation (SD) graph of optical density of cells in media at 600 nm vs incubation time in hours. OD was measured at 2-h interval after cells were treated at different concentrations of ASA, and the negative control cells were cultured in ASA-free medium, and details are provided in text

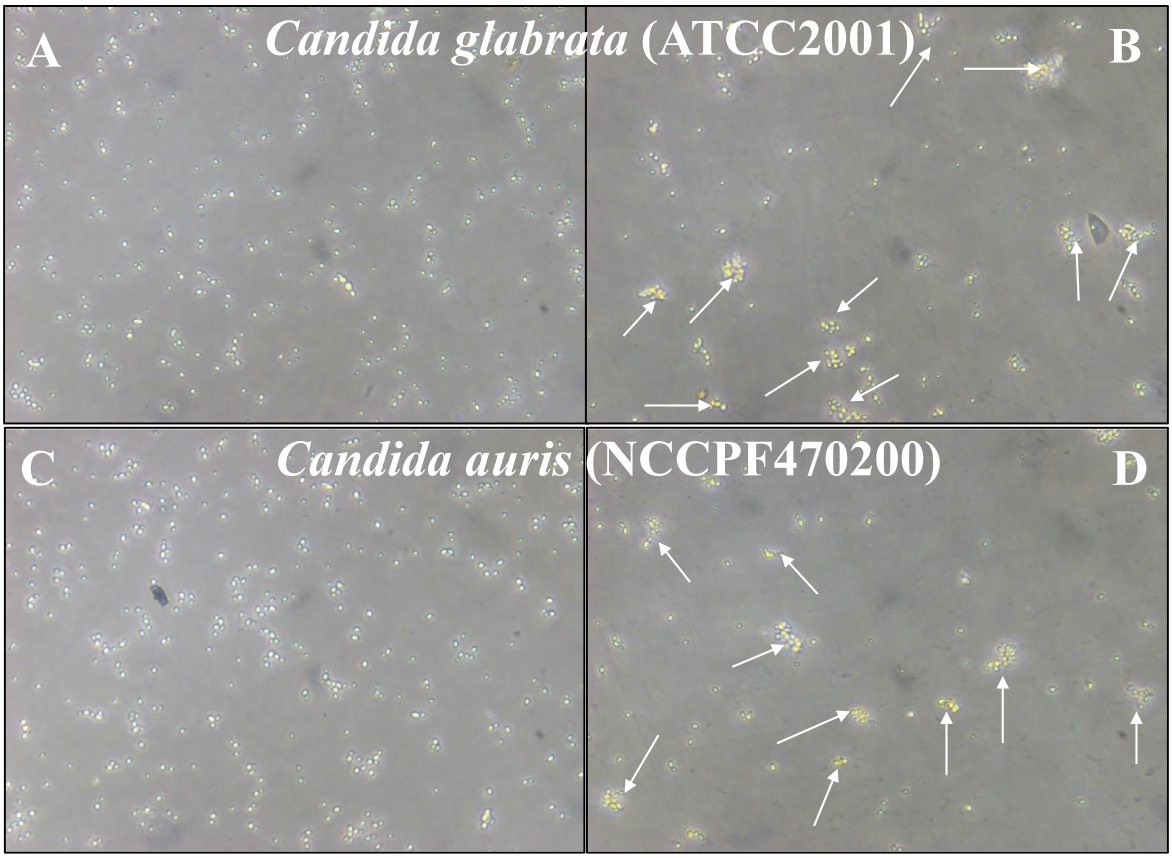

Fig. 3 Effect of ASA treatment on oxidative status of *C. glabrata* and *C. auris*. **A** Control cells of *C. glabrata* showing no significant pellet formation. **B** *C. glabrata* cells treated with ASA at MIC_{50}, showing significant brown pellet formation which is marked with arrow. **C** Control cells of *C auris* and **D** *C. auris* cells treated with ASA at MIC_{50}, showing significant brown pellet formation. Images were taken at 20 × magnification

24 h of treatment, then there was a reduction of 33.4 ± 2.3% to 83.3 ± 2.6% in biofilm formation. In *C. auris*, the minimum effect was observed in NCCPF470197, and a maximum inhibition was observed in NCCPF470153 strains. In *C. glabrata*, all the four strains (NCCPF100037, NCCPF100033, ATCC2001 and MTCC3019) formed ~60–70% reduced biofilm as compared to non-treated fungal cells (Fig. 7A).

The reduction in biofilm formation was also observed when ASA treatment at 0–200 µg/ml concentration was given a 24-h formed biofilm. A destruction of 29.8 ± 1.2% to 78.2 ± 1.8% in already formed biofilm was observed in all the fungal strains after 24 h treatment of ASA. In case of *C. auris*, the minimum effect was observed in strain NCCPF470197, and maximum effect was shown in NCCPF470200. In *C. glabrata*, 59.6–78.5% reduction in formed biofilm was observed in all the strains (ATCC2001, MTCC3019 NCCPF100033 and NCCPF10037) (Fig. 7B).

The morphological changes in the fungal cells during biofilm formation were also analysed by FE-SEM in which the untreated cells of *C. auris* showed regular morphology, well-defined size and a smooth morphological surface (Fig. 8A). After a treatment with ASA at concentration of MIC_{50}, the morphology of cells in the biofilm became irregular (Fig. 8B) as compared to normal cells.

Cellular Surface Hydrophobicity (CSH) Determination

To explore whether ASA induces changes in the CSH, this characteristic was determined by the method of microbial adhesion to hydrocarbons. In the experiments performed, there was a prominent difference in CSH of control and treated fungal cells (Fig. 9A). When fungal cells were analysed after treatment with purified lectin protein, then CSH

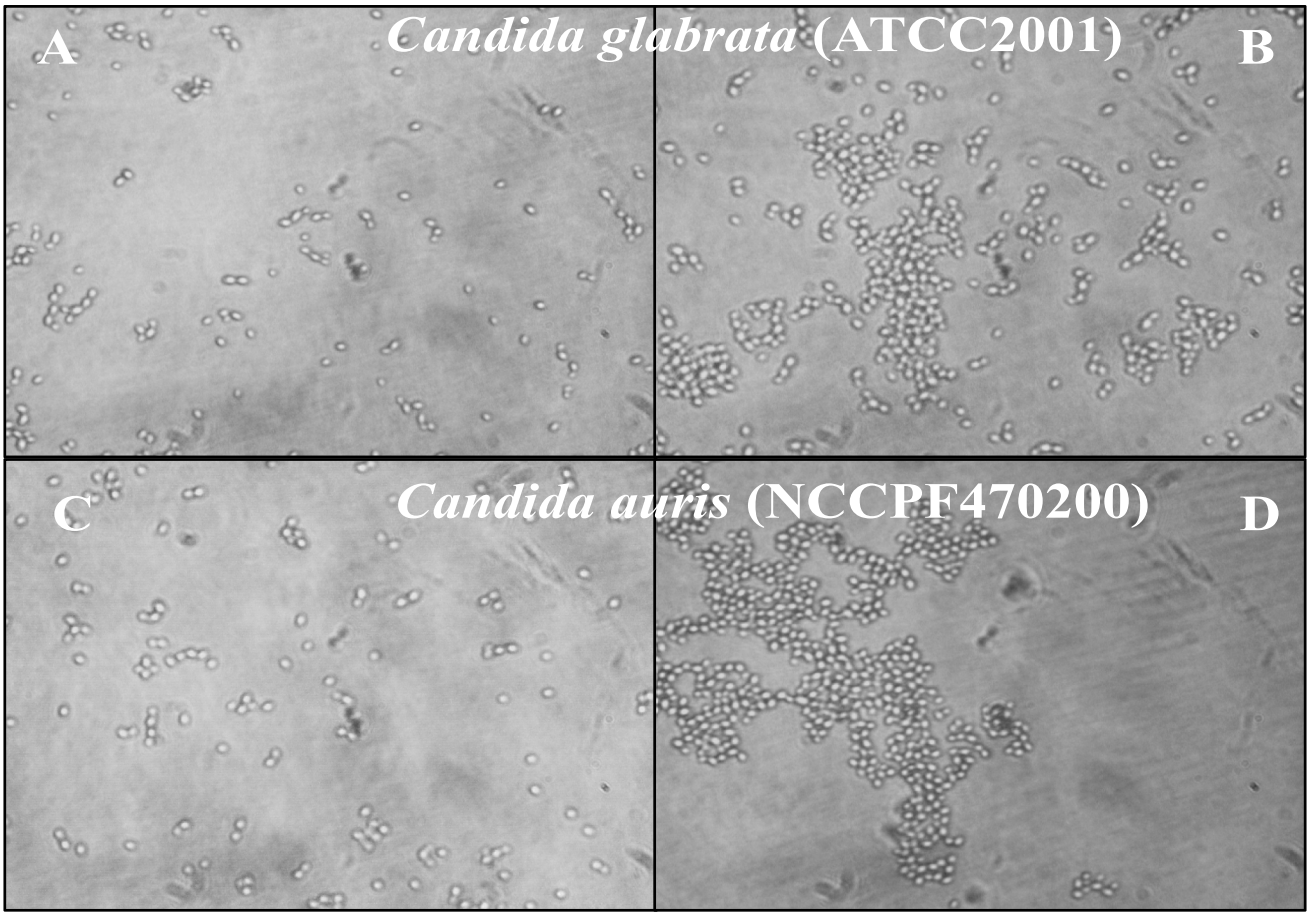

Fig. 4 The effect of ASA on the morphology of *C. glabrata* and *C. auris* observed microscopically. **A** Control cells of *C. glabrata* showing normal cell morphology. **B** ASA-treated *C. glabrata* cells showing cell clumping. **C** Control cells of *C. auris* with normal morphology. **D** ASA-treated *C. auris* cells showing cell clumping. Images were taken at 20× magnification

was found in the reduced form from 41.8 ± 1.3% in the control of *C. glabrata* to 4.8 ± 1.6% in ASA-treated fungal cells and from 54.3 ± 1.2% in the control of *C. auris* to 8 ± 1.1% in ASA-treated fungal cells (Fig. 9B).

Discussion

Due to increasing microbial infections and resistance of pathogenic microorganisms, the search for new therapeutic agents has increased in recent years. Lectins, isolated from various sources, have been evaluated for antifungal and antibacterial activities, and experiments have also been done to establish the mechanisms of action [22–24]. In the present work, we have shown the antifungal and antimicrobial effects of ASA from garlic in seven different strains of *C. auris* and *C. glabrata* and the bacterial species *E. coli*, *S. aureus* and *K. pneumoniae*. Significant effects of ASA treatment upon fungal growth and disruption of biofilm formation in both fungal and bacterial cells were observed. The data of cellular damage of fungal strains after ASA treatment was corroborated with microscopic and scanning electron microscopy techniques.

Some preliminary reports on antifungal effects of *Allium sativum* lectin 50 (ASL50), lectin isolated from garlic on *C. parapsilosis*, *C. krusei*, *C. tropicalis* and *C. glabrata*, have been reported [5]. A second report on a mutant of *Allium sativum* leaf agglutinin (mASAL) isolated from garlic leaves reported the effects upon *Rhizoctonia solani* [6]. In the present study, we have used our previously isolated and well-studied protein ASA (~12 kDa), which is highly stable and showed optimum biological activity up to 50 °C temperature and between a range of pH 4–10 [13] for a detailed investigation of its antimicrobial potential.

Candida species are widely distributed in nature which are present in saprophytic form in human microbiota and in favourable conditions can be opportunistic pathogens in humans [25]. Candidiasis is the infectious stage caused by any type of *Candida* which leads to vaginal infection, oral thrush, skin infection, joints infections, blood stream

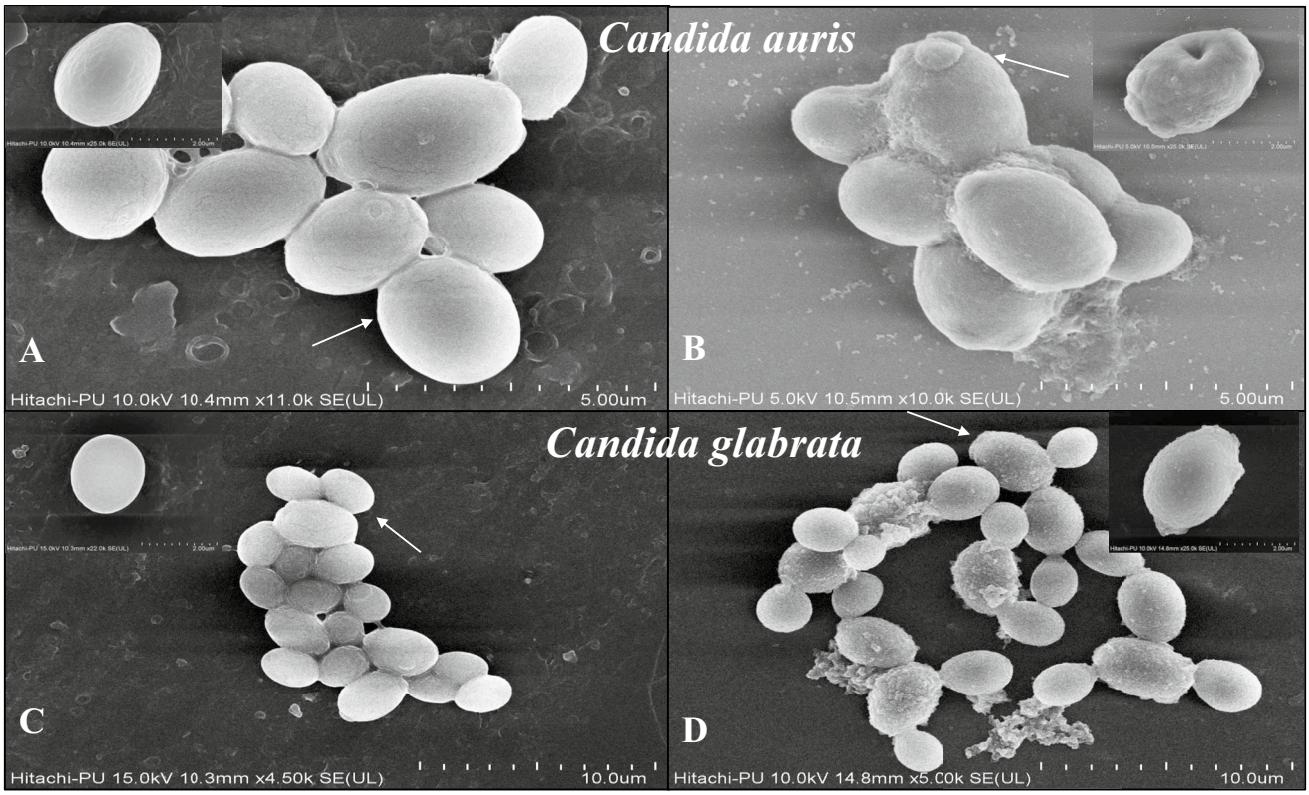

Fig. 5 Scanning electron microscopy of planktonic cells of *Candida glabrata* (ATCC2001) and *C auris* (NCCPF470200). **A**–**C** Control cells of *C. auris* and *C. glabrata*, respectively, with smooth and uniform cell morphology. **B**–**D** *C. auris* and *C. glabrata* cells treated with ASA at MIC_{50} concentration showing loss of cell integrity, and arrows highlight these cells

infections etc. The knowledge of the action mechanism of antimicrobial agents can assist in the sustainable management of fungal disease [6].

E. coli is a Gram-negative bacterium that may cause foodborne disease; *S. aureus* is a Gram-positive bacterium which may cause various types of infections such as bone, endovascular tissue, respiratory and endocarditis [26]. *K. pneumoniae*, a Gram-negative bacterium can colonise the gastrointestinal tract and skin. Other infections which are caused by *K. pneumoniae* may include pneumonia, bacteraemia,

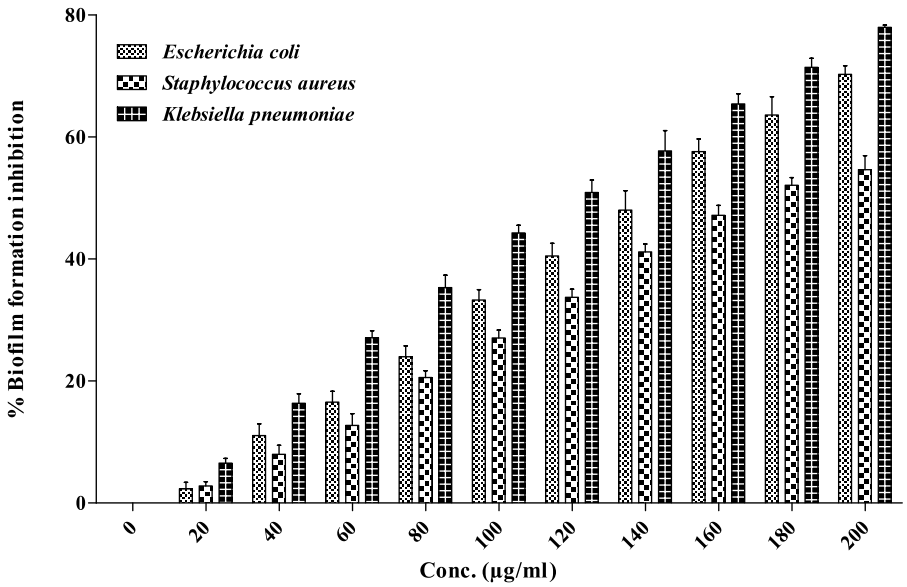

Fig. 6 Dose-dependent effect of ASA treatment on biofilm formation of *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. The anti-biofilm effect was observed in the order of *K. pneumoniae* > *E. coli* > *S. aureus.* ASA reduced 78 ± 0.4% (*K. pneumoniae*), 70.3 ± 1.4% (*E. coli*) and 55 ± 2.8% (*S. aureus*) biofilm formation in the three treated bacterial strains as compared to control. Data is expressed as the mean ± standard deviation (SD), and the results are representative of three biological replicates

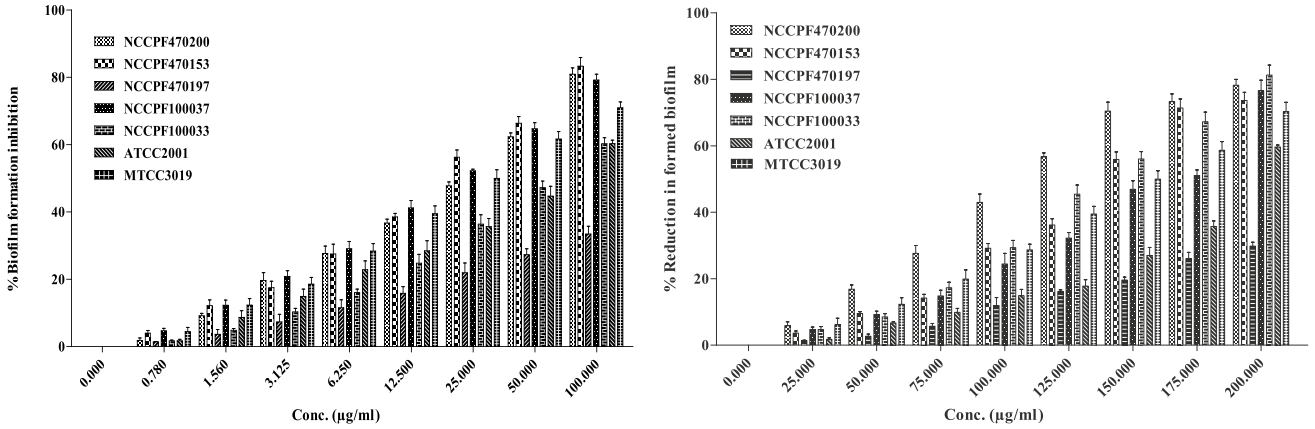

Fig. 7 Effect of ASA treatment on biofilm formation in fungal strains. **A** Graph of % reduction in biofilm formation on ASA treatment from 0–24 h of incubation showed the inhibitory effect of ASA against biofilm formation. Maximum effect was observed on strains NCCPF470153, and minimum effect was observed on NCCPF470197 at 100 µg/ml concentration. **B** Graph of % reduction in biofilm formation on ASA treatment after 24 h of incubation showed the effect of ASA on a 24-h already formed biofilm. Maximum effect was observed in the strain NCCPF470200 and minimum in NCCPF470197. Control cells were cultured in a lectin-free medium. Data expressed as the mean ± standard deviation (SD), and the results are representative of three biological replicates

infection of the urinary tract and suppurative infections, and in the immunocompromised persons or diabetic patients, it may cause osteomyelitis or meningitis [27].

There are various virulence factors upon which the pathogenicity of any microorganism depends. The mechanisms through which infection occurs include structural disruption strategies, production of virulence factors such as certain enzymes or substances through which microorganisms can invade indifferent tissues and also strategies used by microbes which help them to evade from immune system [28, 29]. Biofilms are the communities of microorganisms in which they adhere to the biotic or abiotic surface with higher resistance against various drugs and other adverse conditions than planktonic form [30, 31]. In many studies, it has already been reported that lectins inhibit the biofilm formation in bacteria and fungi by interfering with the attachment of cells to the surface and affecting the cell–cell interaction [32, 33]. Various types of human infections such as dental plaque, urinary, cystic fibrosis and endocarditis are caused by biofilm formation [34]. There are various stages of biofilm formation

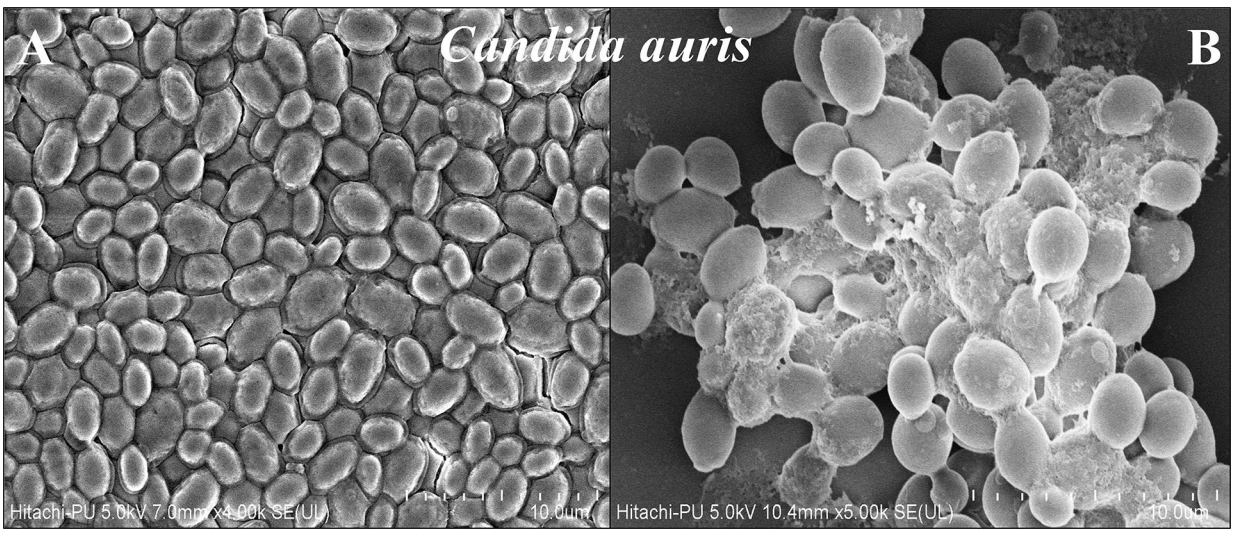

Fig. 8 Scanning electron microscopy of biofilm formation inhibition in *Candida auris* (NCCPF470200). **A** The control cells (without treatment of ASA) showed smooth and intact morphology. **B** Treatment with ASA at MIC_{50} showed morphological changes in the biofilm cells. There was bulging in some cells, and other cells showed rupturing

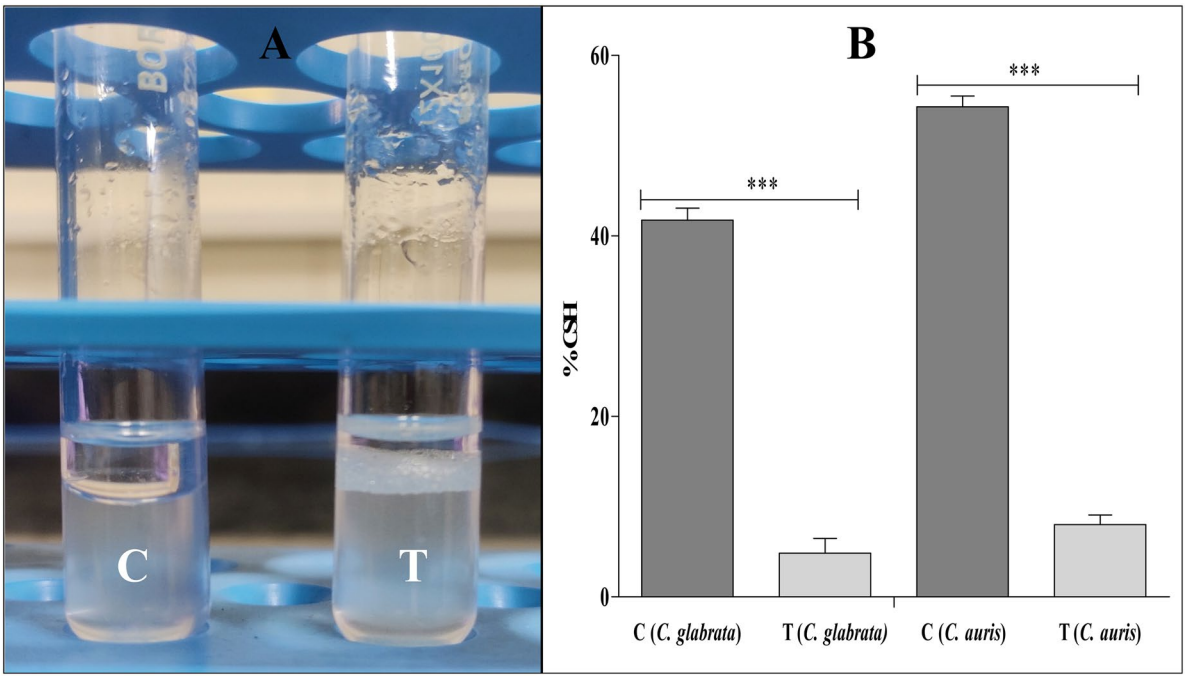

Fig. 9 ASA effect on the cellular surface hydrophobicity of *C. glabrata* (ATCC2001) and *C. auris* (NCCPF470200). **A** Fungal cells (*C. glabrata*) were incubated in the absence and presence of 40 µg/ml of ASA. Treated cells showed less attachment to the tube surface as compared to control cells. **B** CSH was reduced from 41.8 ± 1.3% in the control of *C. glabrata* to 4.8 ± 1.6% in ASA-treated fungal cells and 54.3 ± 1.2% in the control of *C. auris* to 8 ± 1.1% in ASA-treated fungal cells. The experiments were performed in triplicate. (*) significant difference $p < 0.05$

by microorganisms, and the conventional use of antibiotics for the extermination of biofilm is unfeasible due to the high concentration required for the effect, toxicity and other side effect related to human health [30, 35]. Due to these reasons, the initial stages of biofilm formation in which the microbes adhere to the surface and form colonies are the stages at which anti-biofilm agents are more effective and biofilm-associated infections can be controlled [36].

Although lectinology is a very important field of modern biology and many lectins have been investigated for various biological activities, the data on antifungal activities of lectins is still scanty [4]. Some examples of evaluation of antifungal studies of lectins include the mannose binding lectin *Moringa oleifera Lam.* (Moringaceae) Mo-CBP2 which exhibited antifungal activity by increasing cell membrane permeability and reactive oxygen species production in *Candida* [37]. A chitin-binding lectin from *Punica granatum* (PeGT) showed antifungal activity against *C. albicans* and *C. krusei* by promoting oxidative stress and rupturing cell wall in another study [18]. ASA is bulb-type lectins (BTLs), and members of this family have not been investigated in detail for antifungal activity. The few examples include mASAL (mutant *Allium sativum* leaf agglutinin) against *Rhizoctonia solani* where the effect is attributed to the loss of mitochondrial membrane potential loss and the accumulation of reactive oxygen species in the microbial cells [6] and on ASL50 (*Allium sativum* lectin 50) [5] is important leads.

The main motive of the study was to investigate the anti-infective properties of the ASA against highly pathogenic bacteria and yeast strains. *C. glabrata* was first identified in 1917, and its different strains are well-known for biofilm formation (ATCC2001 and NCCPF100037) [38]. *C. auris* was isolated and identified in 2009, and a clearly formed biofilm has already been reported in some strains of *C. auris* (e.g. from the vaginal discharge sample of a woman) [39], but till now, the effect and mechanism of action of lectins on them have not been thoroughly investigated. There is a possibility that the manno-saccharide specificity of ASA could play an important role in the interaction with the cell wall of the *Candida*. Our results showed that upon ASA treatment, *Candida* cells show agglutination, shrinkage and production of reactive oxygen species (ROS). There is also a reduction in CSH which might affect the attachment of fungal cells to the target tissue as per earlier reports [17]. These are preliminary studies which need to be developed further. ASA has already been tested on cancerous cells, and at the mentioned MIC_{50} concentration in the present work, it was not showing toxic effect in control cell line [13]. Garlic has also been used in culinary for many years, so it can be presumed that it has no

toxic effect on human health, and it can be investigated as an antimicrobial therapeutic agent in the future.

Conclusion

In the present study, ASA was investigated as antimicrobial agent against *C. glabrata*, *C. auris* and three bacterial strains, i.e. *E. coli*, *S. aureus* and *K. pneumoniae*. The antifungal and anti-biofilm effect was observed for both *Candida* species, and there was a significant reduction in biofilm formation in bacterial strains. Mechanistic studies were performed to analyse the underlying basis of these effects, and an increase in hydrogen peroxide production and cellular surface hydrophobicity alteration after ASA treatment was observed. The morphological changes and disruption of cell wall of *Candida* species with ASA treatment were shown using FE-SEM. We hope the significant effect on fungal growth and on the biofilm formation in *Candida* species and three pathogenic bacteria found in the present study can form the basis for development of ASA as an important antimicrobial agent.

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Availability of Data and Materials All relevant data and materials that support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics Approval No animal was used in the work.

Conflict of Interest The authors declare no competing interests.

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