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ORIGINAL RESEARCH ARTICLE

Hibiscus sabdariffa Extract Inhibits Adhesion, Biofilm Initiation and Formation in *Candida albicans*

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Abstract Microbial biofilms act as reservoirs for pathogenic sessile microbes which reside inside the three dimensional matrix of the biofilm, and are thus protected against anti-microbial drugs. Most of the anti-microbial drugs fail to completely abolish the biofilm associated infections. In the present study, we provide evidence of *Hibiscus sabdariffa* (Hs) extract having possible anti-microbial activity, with emphasis on *Candida albicans* biofilm. The Hs extract was shown to be effective against *C. albicans* pre-formed biofilm at 3.125 mg/ml and was able to inhibit the hyphae initiation and adherence of cells. Furthermore, Hs extract was able to reduce the *C. albicans* load in *C. elegans* by effectively killing the *Candida* cells thereby reducing the viable colony count and effectively increasing the lifespan of worms. The percentage of viable hatched progeny of worms exposed to Hs extract (both at conc. 1.5 mg/ml and 6.25 mg/ml), was also comparable to that of the control untreated eggs. The Hs extract was also found to be significantly effective against fluconazole resistant *C. albicans* isolated from patients. Thus, we, for the first time, propose Hs extract as a prospective drug candidate and substitute for eradicating pre-formed biofilm and inhibiting the growth of *C. albicans*.

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Keywords *Candida albicans* · Bio-film · *Hibiscus sabdariffa* · Natural products · *Caenorhabditis elegans*

Abbreviations

CFU	Colony-forming units
DMSO	Dimethyl sulfoxide
Hs	*Hibiscus sabdariffa*
MDR	Multi drug resistant
MIC	Minimum inhibitory concentration
NP	Natural products
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide

Introduction

Candida albicans is both a member of the healthy human microbiome, and a major pathogen in immuno-compromised individuals. It is the fourth most prevalent pathogen implicated in nosocomial infections. The misuse of antibiotics and/or sub-optimal drug dosing, plays a pivotal role in drug resistance. In addition, environmental factors, horizontal gene transfer among microflora, and the evolution of several mutant strains with increased/altered virulence factors, has contributed towards the development of resistant strains. One of the most important virulence factors of microbes is their capacity for biofilm formation.

Biofilms are a structured population of microorganisms, encapsulated within a self-developed three-dimensional matrix adherent to a living or inanimate surface. A biofilm may compose of a single microbial species, but naturally biofilms generally consist of mixtures of microbial species, debris and corrosion products. *Candida* biofilms are multi-

layered, three dimensional components, essential for pathogenicity, and protected from anti-fungal agents in the extra-cellular matrix [1, 2]. They act as reservoirs to persistent fungal infections and it is estimated that approximately 80% of candidal infections are associated with biofilm formation [3]. Thus, biofilms play an important role in generating anti-fungal resistant pathogenic cells.

The screening of bio-active natural products (NP), obtained from marine organisms, microbial fermentation or extracts of plant tissues, has provided invaluable antibiotics, therapeutics and life-saving drugs to mankind. NP and their derivatives are used to treat various infectious diseases caused by micro-organisms. Till date, approximately 80% of all available antibiotics are derived from NP directly or indirectly [4]. However, antibiotic resistant infectious diseases and multi drug resistant (MDR) microorganisms have created a dire need for novel drugs. The discovery and screening of new beneficial NPs from bacteria, fungi and plants, that are capable of circumventing MDR with minimal adverse effects, is the need of the hour. Among all NPs, the plant-derived potential drugs have gained more attention, due to their versatile applications, easy availability and less toxicity.

One of the approaches to identify a novel drug is by screening herbal/botanical/phyto medicine derived from the seeds, roots, bark, leaves, flowers or berries of plants which are being used for medicinal purposes since ancient times. Herbs have been used as medicinal plants, from time immemorial, and have been associated with certain cultural traditions all over the world, especially in India. Antimicrobial properties of different plant extracts have been reported under laboratory trials, further demonstrating that plant extracts are effective antimicrobial agents [5–7]. Thus, selection of crude extracts in screening for antimicrobial properties has higher possibility of success than screening of pure compounds directly [8].

One such plant known for its various medicinal properties is *Hibiscus sabdariffa* (Hs). This plant has traditionally been used as a food, herbal drink (both as cold and hot beverages), flavoring agent, fiber source and as a herbal medicine for its anti-bacterial, anti-oxidant, diuretic, anti-cholesterol, anti-diabetic, anti-hypertensive and anti-cancer activities [9, 10]. The methanolic and aqueous extract of *Hibiscus* calyces have shown anti-bacterial activity against cariogenic *Streptomyces* mutants [11] and the aqueous-methanolic extract showed favourable effect against various pathogenic bacteria [12].

In the present study, the crude extracts of Hs calyces in water and in DMSO were screened against different pathogenic microbes such as *Bacillus atrophaeus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans,* to determine their anti-microbial activity. Further, the mechanism of Hs crude extract on growth of *C. albicans* and on biofilm formation was also elucidated. The study attempted to throw some light on the potential of Hs extract in removing the pre-formed *Candida* biofilm as well as its ability to inhibit biofilm initiation and adherence of *Candida* cells.

Materials and Methods

Preparation of Plant Extract

The plant material used in this study was red calyces of *Hibiscus sabdariffa* flowers devoid of other floral parts. The material was purchased from a local vendor as dried calyx of *Hibiscus* flowers. The botanical identification of its species was performed by National Institute of Science Communication and Information Resource (NISCAIR), CSIR, Delhi (Reference No. NISCAIR/RHMD/Consult/2015/2884/77). The dried calyces were powdered using mortal-pestle and suspended at a concentration of 200 mg/ml in both DMSO and in water. The suspension was kept at 37 °C for at least 48 h with continuous shaking, filter sterilized and stored at room temperature for further use. Various concentrations of Hs extract ranging from 0.0976 to 50 mg/ml, were prepared in RPMI 1640 medium with L-glutamine w/o sodium bicarbonate, adjusted to pH 7.0 with 165 mM 3-[*N*-morpholine] propane sulphonic acid (MOPS) buffer, and used for all the assays described below, unless otherwise stated [13]. Wells without test compounds served as full-growth controls. Amphotericin B was used as the standard antifungal agent.

Test Microorganisms

Gram-positive bacteria used in the study were *Bacillus atrophaeus* and *Staphylococcus aureus*, gram-negative bacteria included *Escherichia coli* and *Pseudomonas aeruginosa*. All the tested strains were maintained on nutrient agar (BD Difco) at 37 °C and grown in nutrient broth (BD Difco) for experimental purposes. The fungal strain used was *Candida albicans* which was maintained routinely on Yeast Peptone Dextrose (YPD, BD Difco) agar plates at 30 °C and grown in YPD broth (BD Difco) for experimental procedures unless otherwise stated.

Antimicrobial Assay

Antimicrobial activity of Hs against various pathogenic organisms was determined according to Kirby–Bauer disc diffusion method recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. Different concentrations of Hs crude extracts, prepared either in water or DMSO, were tested for antimicrobial activity as described

previously [14]. DMSO or water was used as solvent control, along with Ampicillin (100 mg/ml) for bacteria and Amphotericin B (250 µg/ml) for *C. albicans,* as a standard drug control. The diameter of inhibition zone was measured and the total area of the inhibition was calculated according to the following equation:

$$\text{Area} = \pi r^2$$

where r is radius of the zone of inhibition, $\pi = 3.14$.

C. albicans Isolates from Patients

The *Candida* spp. was isolated from patients diagnosed with vulvo-vaginal candidiasis (VVC) by vaginal swab, examined microscopically by Gram stain, KOH preparation and further processed for fungal culture on Sabouraud's dextrose agar (without cycloheximide). A germ tube test was performed on all *Candida* isolates to separate the *Candida albicans* species from non albicans species. Further identification of colonies was done by growing cultures on CHROMagar Candida media (HiMedia, Mumbai, India) and studying the morphology on corn-meal agar media (CMA). A positive germ tube test, bright green colored colonies on CHROMagar Candida media and chlamydospore formation on corn meal agar were some of the parameters used in identification of the isolate as *C. albicans.* The identified *Candida albicans* isolates were further used for in vitro susceptibility testing.

In-Vitro Anti-fungal Susceptibility Test

The antifungal activity of Hs extract was tested by the broth micro-dilution method according to CLSI, M27-A3 [15]. The test was carried out in RPMI 1640 medium in 96-well round-bottomed plates. The overnight grown culture of *Candida* cells was used throughout the study, finally adjusted to a final inoculum of 0.5×10^3 cells/ml in RPMI 1640 [15]. The plates were incubated at 35 °C for 48 h after addition of Hs extract with appropriate controls. Minimum inhibitory concentrations (MICs) were determined by visual reading after agitating the cell suspension and measuring the optical density at 600 nm with the microplate reader (Tecan infinite M200). The background optical densities were subtracted and the MIC_{50} and MIC_{80} were determined.

In addition, the Minimum Inhibitory Concentration (MIC) strips were also used for performing the antifungal susceptibility test (E-test strips, Biomerieux) of the *C. albicans* isolated from patients against fluconazole, an antifungal agent used most commonly. The culture suspension of turbidity matching 0.5 Mc Farland, was made and spreaded on Petri dish with RPMI supplemented with 2% glucose agar for 48 h at 35 °C. The reading was observed by assessing the point of intersection between the halo formed and the E-test strip. The profile of the antifungal drug sensitivity was classified as sensitive (S), dose-dependent sensitivity (S-DD), and/or intermediate (I), and resistant (R) as defined by CLSI standard M27-A3 [15]. The MIC values of ≤ 8 µg/ml for fluconazole were considered susceptible (S), 16–32 µg/ml was considered as susceptible dose-dependent (SDD), and ≥ 64 µg/ml as resistant (R). For Amphotericin B (AMB), MICs ≤ 1 µg/ml were considered to be S and ≥ 1 µg/ml as R.

Filamentation Assay

For assessing effect of Hs extract on hyphae formation in *C. albicans*, spider media agar plates with or without Hs extract (MIC_{50}) was used. Overnight grown *Candida* in YPD broth was streaked onto the plates and were then incubated at 37 °C for 7–14 days. Images of colony edges were obtained using a stereo-zoom microscope (Nikon, SMZ1000).

For assessing the hyphae/germ-tube formation in liquid media, *Candida* was grown in RPMI 1640 medium with or without Hs extract. The 6-well flat bottom plates were incubated at 37 °C for 4 h, and the individual budding cells and the number of cells with hyphae were counted and results were expressed as percentage of germ tube formation using the following formula:

$$\text{Germ tube formation}\,(\%) = \frac{\text{No. of cells with germ tubes in cells grown in media with Hs extract}}{\text{No. of cells with germ tubes grown in control media}} \times 100$$

Adherence Assay on the Well Surface of Microtiter Plates

Cell suspension of *Candida* cells in RPMI 1640 was seeded in flat bottom 96-well plate with different concentrations of Hs extract and incubated at 37 °C for 4 h. Following the initial incubation, the medium was aspirated and non-adherent cells were removed by thoroughly washing the wells with sterile PBS thrice. A semiquantitative measurement of adhered cells was done using 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay as described previously [16, 17]. The colorimetric change at 490 nm was measured with a microplate reader (Tecan infinite M200) and MIC_{50} and MIC_{80} values were calculated.

Biofilm Assay

Cell suspension (100 µl each) of *C. albicans* with inoculums of 1×10^6 cells/ml was inoculated into 96-well flat

bottom polystyrene plates at 37 °C for 4 h to score the effect of Hs extract on adhered cells and for 20–24 h for formation of mature biofilm. Non-adhered cells were removed by washing three times with sterile PBS and then 200 µl of desired concentration of Hs extract in RPMI 1640 medium was added to each well. The plates were incubated at 37 °C for 24 h to allow the effect of Hs extract on formation of biofilm and pre-formed biofilm respectively. Biofilm growth was analyzed using XTT as described above.

In-Vivo Toxicity, Antifungal Activity and Evaluation of Fungal Burden in *C. elegans*

In-vivo toxicity was assessed by measuring *Caenorhabditis elegans* lifespan as previously described in literature [18]. Briefly, L4-stage synchronized worms (75–100 per experimental group) were exposed to *C. albicans* culture mixed with *E. coli* OP50 (2:1) along with 12.5 mM of streptomycin. Plates lacking *C. albicans* were used as negative controls (uninfected). Kaplan–Meier survival analysis was used to compare the mean lifespan of different treatments and *P* values were calculated using the log rank (Mantel–Cox method).

Another assay that was performed to evaluate the toxicity of Hs extract is the percent hatching of worms in the presence of Hs extract and then scoring their viability. Gravid adult worms were collected from the plates using M9 buffer, and centrifuged at 1000 g for 3 min. The pellet was resuspended in 5 ml of bleaching solution (0.7 M NaOH with 2% Na-hypochlorite) to break the cuticle. The suspension was mixed thoroughly, centrifuged again and the pellet consisting of worm debris was then washed three times with M9 buffer. The pellet was resuspended to a final concentration of 8–10 eggs/µl; 10 µl of this solution containing eggs were added to M9 buffer in 6-well plates with or without Hs extract. The plates were incubated at 20 °C for 24 h to obtain hatched progeny; the hatched larvae were counted and compared with no-treatment control.

The colony-forming units (CFU) of *C. albicans* from *C. elegans* were quantified based on the protocol described previously [19] to determine the fungal burden on worms after exposure to Hs extract. Twenty worms per group were analyzed and colonies were counted to determine CFU per nematode.

All the microscopic examinations were performed in 6-well microtiter plates at MIC_{50} of Hs extract with similar procedure and a quantitative assessment of cellular morphology was performed with an inverted light microscope. All the experiments throughout the study were repeated at least thrice with each experiment having three replicates.

Results and Discussion

The use of ethano-medicinal plants is common worldwide, but the supporting scientific evidence proving their efficacy for the said use is lacking for many of these traditional medicinal plants. Experimental evaluation of their pharmacological use can provide evidence based alternative therapies for herbal drug and/or discovery of novel drug targets. *Hibiscus* is often taken as a beverage in many countries owing to its medicinal properties [9, 10, 20]. In this study, we provide evidence for the anti-microbial property of Hs extract with special emphasis on its effect on *C. albicans* growth and biofilm formation.

Anti-microbial Effect of Hs Crude Extracts

Previous studies by various groups have unanimously reported that Hs calyces collected from different varieties and different localities around the world have effective antimicrobial properties [14, 21, 22]. However, the bioactivity of the extract from same plant species may also vary depending on the extraction process which includes solvent, method of extraction, temperature, fresh or dried calyces, season of harvesting and age of plant [23, 24]. Keeping this in mind, we prepared the extract in two biologically compatible solvents; DMSO and water; and studied the antimicrobial effects of these extracts on different pathogenic microbes using disc diffusion assay. The results of antimicrobial assay showed that both the extracts are effective as anti-bacterial agents against both Gram positive and Gram negative bacteria showing significant area of inhibition ranging from 0.79 to 1.77 cm^2 (Table 1). The sensitivity against the tested bacteria differed, with *B. atrophaeus* being the most sensitive with inhibition zone of approx 1.5 cm in DMSO based extract and 1.2 cm in aqueous extract, while the least sensitive was *E. coli* with a zone of approximately 1.0 cm in both the extracts. Previous studies have shown the anti-bacterial effect of Hs calyces extract using different solvents such as methanol or ethanol [10, 25].

In addition, when the efficacy was checked against the eukaryotic microbe *C. albicans,* we observed that the extract prepared in DMSO was effective, with the area of inhibition as 0.5 cm^2, whereas the extract prepared in water failed to show any inhibition (Table 1). The difference in anti-*candida* activity, of DMSO based extract, may be attributed to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols which are preferentially extracted in DMSO rather than in aqueous phase.

To further establish the action of Hs extract on *C. albicans* we determined the minimum concentration

Table 1 In vitro antimicrobial activity of *Hibiscus sabdariffa* (Hs) calyces extract

S. no.	Microorganism	Diameter of inhibition (cm)		Area of inhibition (cm^2)	
		DMSO	Water	DMSO	Water
1	*Candida albicans*	0.8 ± 0.2	–	0.5 ± 0.031	–
2	*Pseudomonas aeruginosa*	1.2 ± 0.3	1.0 ± 0.2	1.13 ± 0.071	0.79 ± 0.031
3	*Escherichia coli*	1.0 ± 0.3	1.0 ± 0.1	0.79 ± 0.071	0.79 ± 0.007
4	*Bacillus atrophaeus*	1.5 ± 0.2	1.2 ± 0.5	1.77 ± 0.031	1.13 ± 0.2
5	*Staphylococcus aureus*	1.1 ± 0.3	1.2 ± 0.4	0.95 ± 0.071	1.13 ± 0.13

required to inhibit the growth of planktonic yeast cells to 50% (MIC_{50}) and 80% (MIC_{80}). We found MIC_{50} value to be 1.5 mg/ml and MIC_{80} value as 6.25 mg/ml against planktonic cells. However, when we assessed the effect of Hs extract on *C. albicans* and its ability of adhering to the substrate to form biofilm, biofilm maturation or effect on preformed biofilm, we found increased MIC_{50} and MIC_{80} values as shown in Table 2. The Hs crude extract prepared in DMSO (1.5 mg/ml) was effective against formation or initiation of biofilm as approximately 50% of *Candida* cells were not able to adhere to the plates for further biofilm formation and at 3.125 mg/ml the pre-formed biofilm was effectively abolished resulting in the cell death. We also observed that both for planktonic as well as preformed biofilm of *C. albicans,* the MIC_{80} value was four times higher than that of MIC_{50} value (Table 2). To the best of our knowledge for the first time, we are reporting the effect of Hs extract on pre-formed biofilms of *C. albicans.*

Hs Extract Affect the Yeast to Hyphae Transition in *C. albicans*

The important virulence-mediating attribute of *C. albicans* is transition from yeast cells to hyphae. This transition helps *C. albicans* to penetrate the host tissues and this invasive growth leads to the establishment of systemic infection. Since, multiple stimuli can induce hyphal morphogenesis [26], the study was initiated to observe the transition of yeast cells to hyphae formation in the presence of Hs extract on solid agar Spider's medium and liquid RPMI medium. The plate containing DMSO was taken as the solvent control and cells without any additive was the positive control for the experiment. The hyphae formation on the plates with Hs extract was significantly less as compared to the control plates after 7 days and 14 days of incubation (Fig. 1). Whatever limited hyphae were observed in the treated plates looked shrunken and curly in nature. This observation was further validated by microscopic observation of hyphae formation in liquid media, where the hyphae were completely inhibited (Fig. 1c). Thus, we propose that Hs extract not only inhibits the hyphae production, but also brings about significant morphological changes in the hyphae. There are various reports which concretely suggest that formation of hyphae is dependent on the pH, type of media (whether solid or liquid), temperature and other factors [27]. Thus, the retarded formation of hyphae on solid media and complete absence of hyphae formation in liquid media may be attributed to the fact that in solid media, hyphal growth recorded is from a colony [27] instead of individual cell. So, based on our results we suggest that Hs extract have the potential to inhibit yeast to hyphae transition of *C. albicans* cells. Equivalent concentration of DMSO used as solvent control had no effect on cell viability and hyphae formation of *C. albicans*.

Hs Extract Inhibits Adhesion of *C. albicans* Cells

Biofilm formation is a multicellular, complex process, involving various important steps such as cell adhesion, growth, morphogenic switching between yeast and filamentous states, and quorum sensing [1]. The pre-requisite for biofilm formation is the adhesion of *C. albicans* cells to materials or host cells that subsequently lead to cell–cell interactions in the hierarchical organization of cells within the formed biofilm. Since, this study results suggest that Hs extract can inhibit the biofilm formation (Table 2), the probable efficacy in circumventing the adherence of *C.*

Table 2 MIC_{50}, MIC_{80} and MIC_{80}/MIC_{50} ratios of DMSO extract of *H. sabdariffa* calyces against *C. albicans* ATCC 90028 strain

S. no.	Inhibition of	DMSO extract of *H. sabdariffa* (mg/ml)		
		MIC_{50}	MIC_{80}	MIC_{80}/MIC_{50}
1.	Planktonic cells	1.5 ± 0.2	6.25 ± 0.4	4.17
2.	Biofilm adherence	1.5 ± 0.3	6.25 ± 0.75	4.17
3.	Biofilm maturation	2.5 ± 0.5	10.0 ± 1.0	4.0
4.	Pre-formed biofilm	3.125 ± 0.75	12.5 ± 0.75	4

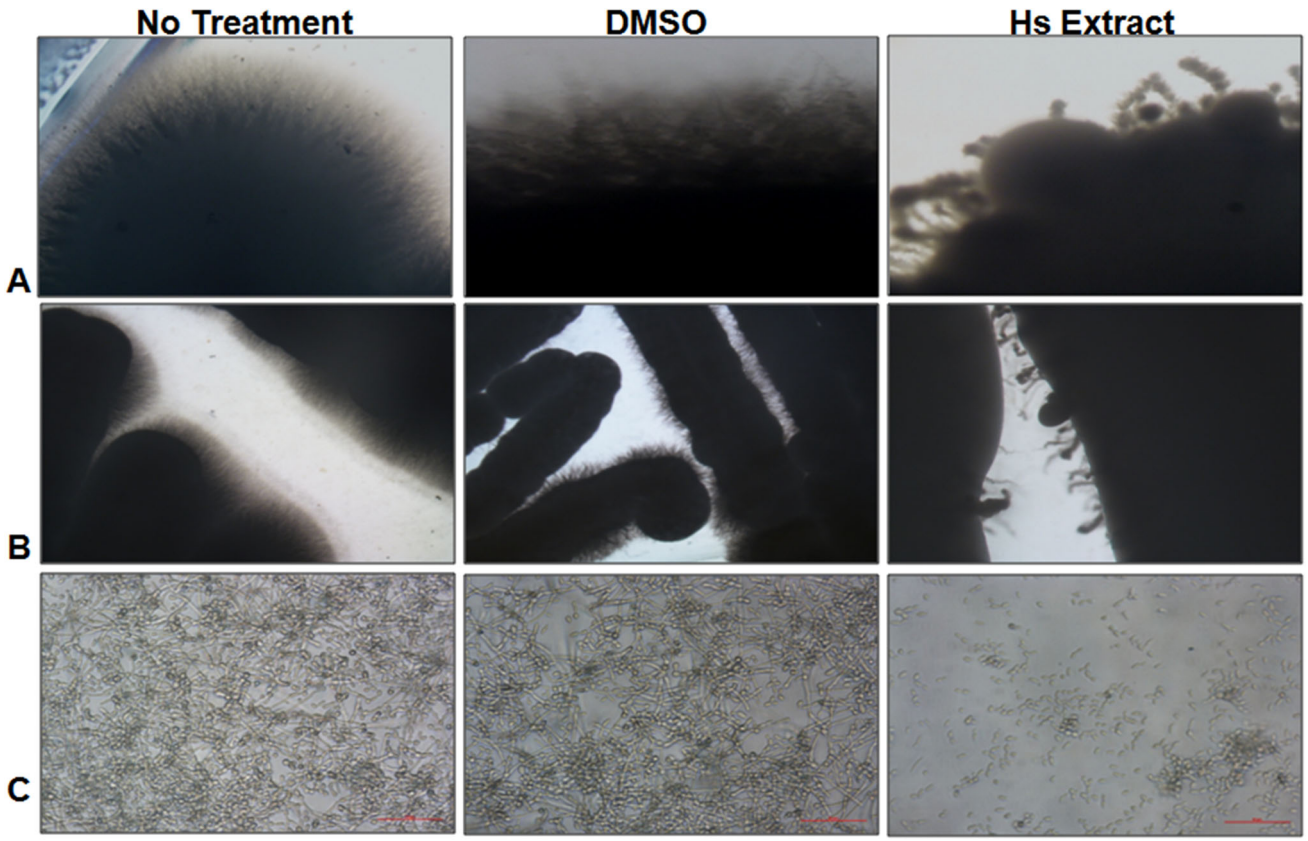

Fig. 1 Inhibition of *C. albicans* filamentation by Hs extract in different hyphal-inducing media. Colonies were grown on spider media agar plates containing either Hs extract or DMSO (solvent control) for 7 days (**a**) and 14 days (**b**). *C. albicans* with Hs extract was grown in (**c**) RPMI1640 medium at 37 °C for 6 h. Images of colony edges were obtained using a stereozoom microscope and *Candida* cells with hyphae using inverted microscope at 40 ×

albicans cells was then checked. The cells were grown in the presence and absence of Hs extract for 4 h, followed by washing, and then cells attached to the substrate were analyzed. It was found that cells grown in the presence of Hs extract lost their attachment and were easily washed away during the washing step (Fig. 2).

Hs Extract Inhibits the Biofilm Formation and Disrupts the Pre-formed Biofilm

The conversion of yeast cells to filamentous forms is a critical step in biofilm biogenesis, which provides strength and support to the developing heterogeneous biofilm structure, also defined as the three dimensional matrix of hyphae. In the present study, we checked for the effect of Hs extract on the formation of filamentous forms in *C. albicans* cells. The percentage of germ tube observed in Hs treated *C. albicans* cells was 2.85% at 2 h and 4.65% at 4 h. The microscopic observations of the untreated *Candida* cells start showing the formation of germ tube from 2 h onwards (Fig. 3a) which further elongated to characteristic hyphae within 4 h. Almost all the cells showed hyphae formation after 4 h (Fig. 3b). On the other hand, cells incubated with Hs extract (MIC_{50}) failed to initiate germ tube formation within two h and after 4 h the cells were arrested with a few budding or pseudo-hyphae formation (Fig. 3b). Based on these observations, it was concluded that presence of Hs extract effectively inhibited the initiation of hyphae, which subsequently lead to the formation of biofilm. Next, we wanted to check whether Hs extract could disrupt the pre-formed biofilm. The incubation of the pre-formed biofilm with Hs extract showed significant reduction with time. The pre-formed biofilm of control untreated cells kept on increasing with time (Fig. 4), while that of Hs extract treated cells started dislodging and decreasing from 4 h onwards (Fig. 4a–d, right panel) and was completely abolished by 24 h (Fig. 4d). These observations fall in line with the assumption that Hs extract is effective against pre-formed biofilm, and also has the ability to inhibit the formation of new biofilm. Rukayadi et al. [28] suggested the anti-fungal role of Hs using the disc diffusion assay in terms of inhibition zone but provided no observation on biofilm formation. Another study by Alshami and Alharbi [29], performed the MIC

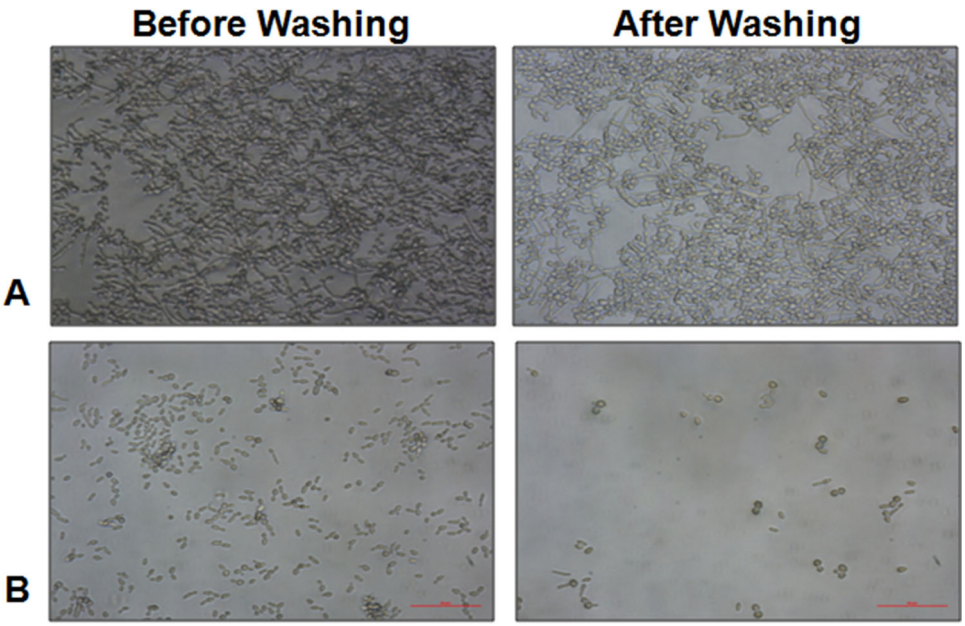

Fig. 2 Loss of adherence property of *C. albicans* in the presence of Hs extract. *C. albicans* ATCC 90028 cells were grown in the RPMI 1640 medium without any additive (**a**) and with Hs extract (**b**) at 37 °C for 4 h. The cells before and after washing with PBS were visualized by microscopy

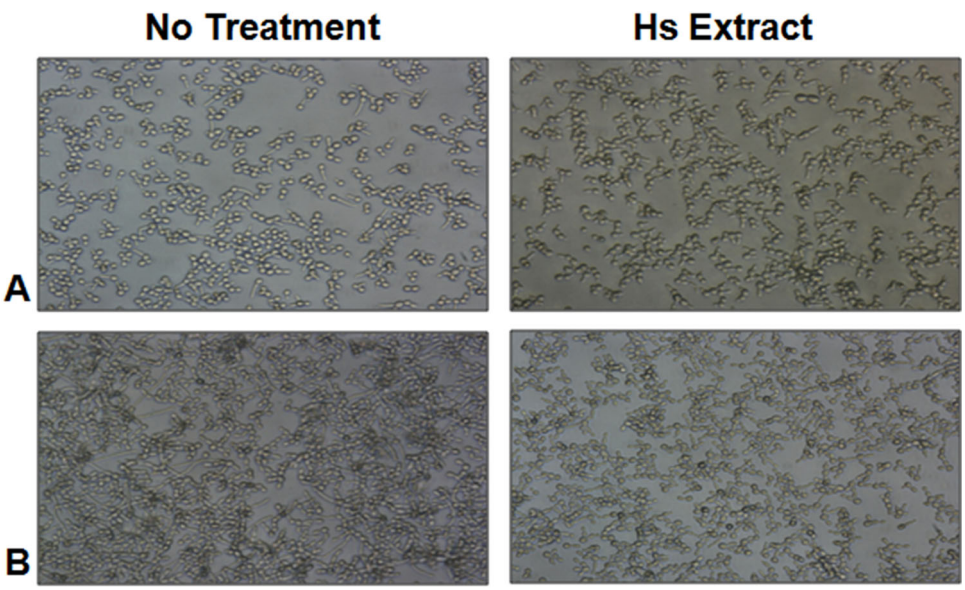

Fig. 3 Inhibition of *C. albicans* biofilm formation/hyphae initiation by Hs extract. *C. albicans* ATCC 90028 cells were grown continuously in the presence of extract at 37 °C for **a** 2 h and **b** 4 h

assay in broth dilution and also suggested the role of Hs in inhibiting the biofilm formation. Our study, for the first time, provides evidence not only for the inhibition of biofilm formation but also loss of adherence property and most importantly its effectiveness against pre-formed biofilm, which is the landmark of established *Candida* infection. Another major difference between previous two studies and the present study, is the solvent used to prepare Hs extract; DMSO was used in the present study, while alcohol/methanol were used to prepare the extracts in other studies.

Hs Extract Exhibited Anti-*Candida* Activity in the In Vivo *C. elegans* System

The next important evidence for any compound to be projected as a probable antimicrobial compound, is to determine its toxicity and anti-microbial nature under in vivo conditions. The toxicity test using cell lines is a cellular phenomenon, whereas use of *C. elegans* for the toxicity and anti-microbial assays provide information about the response from a whole animal. The data so obtained can be used indirectly for predicting mammalian host responses. The worms with their intact and metabolically active digestive, endocrine, reproductive, sensory

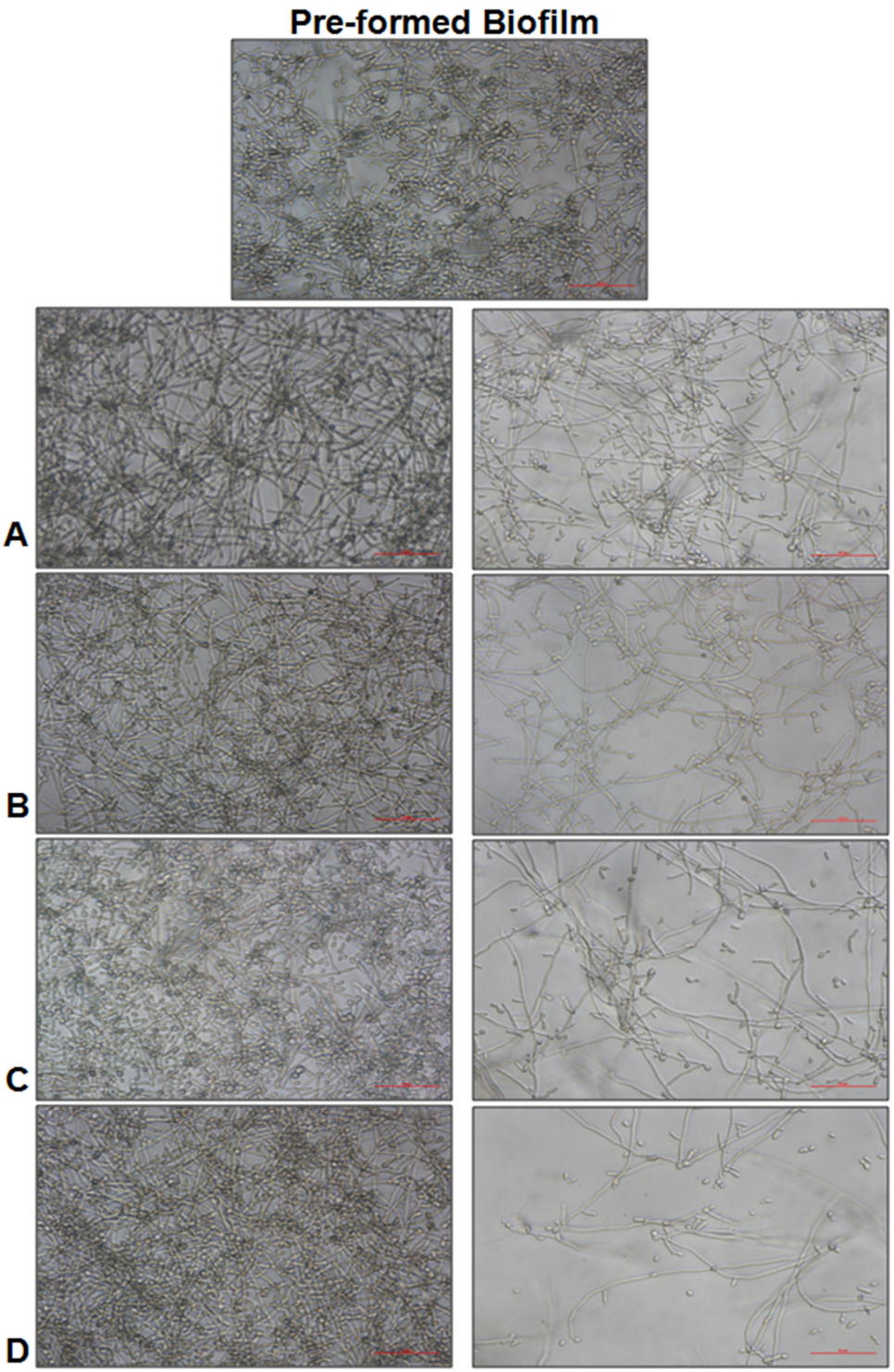

Fig. 4 Disruption of *C. albicans* pre-formed biofilm by Hs extract. *C. albicans* ATCC 90028 cells were grown in RPMI 1640 at 37 °C for 24 h to allow the formation of mature biofilm. These biofilms were then observed microscopically after addition of Hs extract at 4 h (**a**), 6 h (**b**), 12 h (**c**) and 24 h (**d**)

and neuromuscular systems have been shown to be as predictive of rat LD_{50} ranking as mouse LD_{50} ranking [30]. In the present study, the *C. elegans*, infected with *C. albicans* showed a significant decrease in lifespan with mean and maximum lifespan as 4 and 7 days, respectively, as compared to the uninfected nematodes (Fig. 5a, $P < 0.0001$). Exposing the infected worms to 1.5 mg/ml (MIC_{50}) of Hs extract increased both the mean and maximum lifespan of worms to 7 and 11 days, respectively. Moreover, it was found that treatment with 1.5 mg/ml (MIC_{50}) of Hs extract significantly reduced the CFU of *C. albicans* isolated from the infected worms (Fig. 5b). Thus, the Hs extract significantly reduced the colonization of *C. albicans* in the worms, and thereby increased the lifespan

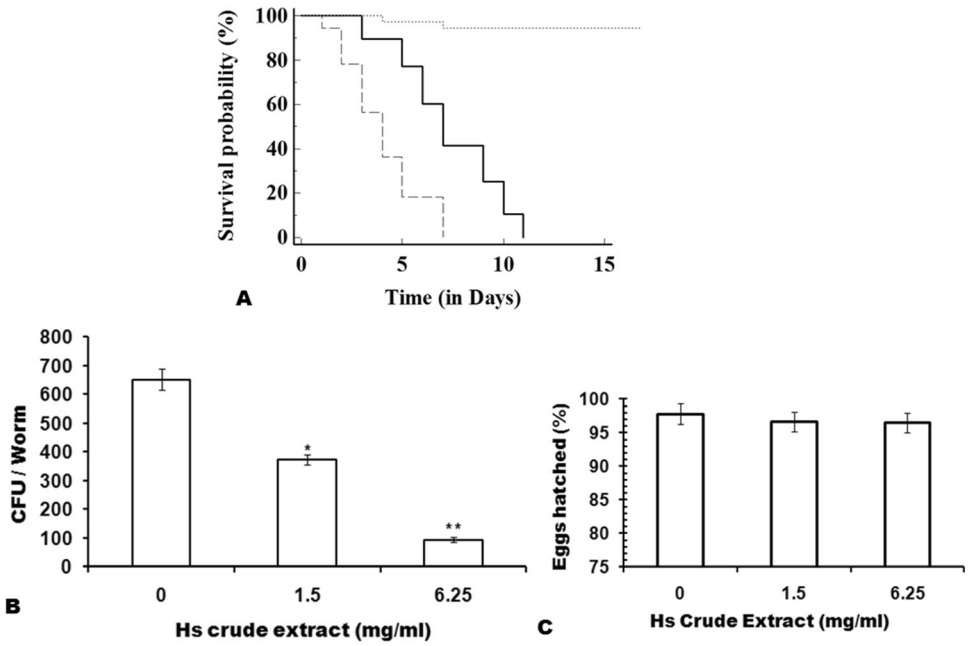

Fig. 5 Anti-*Candida* activity of Hs extract in *C. elegans*. **a** Kaplan–Meier survival curve showing the lifespan of uninfected worms (dotted line), infected with *C. albicans* (dashed lines) and infected worms exposed to Hs extract (solid lines). **b** Fungal Burden of *C. albicans* in nematodes. The infected nematodes were grown with Hs extract at concentration of 0, 1.5 and 6.25 mg/ml and CFU of *C. albicans* was quantified. Bars represent mean ± S.E.M.; $P < 0.001$

Table 3 Antifungal susceptibility testing of 30 *C. albicans* isolates from patients visiting OBG Department, VMMC and Safdarjung Hospital, New-Delhi

	E-test (μg/ml)	Planktonic cells (MIC_{50})			Pre-formed biofilm (MIC_{50})		
		Fluconazole (μg/ml)	Amp B (μg/ml)	Hs extract (mg/ml)	Fluconazole (μg/ml)	Amp B (μg/ml)	Hs extract (mg/ml)
C. albicans sensitive strain (10)	1.5–8	2.0–8.0	0.5	1.5	> 64	2	3.125
C. albicans intermediate sensitivity (16)	12.0–32.0	14.0–32.0	0.5	1.72–1.82	> 64	2	3.25–3.9
C. albicans resistant strain (4)	48–> 256	> 64	0.5	1.9–2.15	> 64	2	3.8–4.25

of infected nematodes. To further ascertain the feasibility to use Hs extract in *Candida* infections, it was necessary to evaluate the toxicity of Hs extract in the in vivo system. For this, the percentage of worm's eggs that hatched, and were alive in the media containing Hs extract were checked. It was observed that at both the MIC_{50} (1.5 mg/ml) and MIC_{80} (6.25 mg/ml), the difference in egg hatching was insignificant and the larvae were alive till 12 h of observation. This again supports the hypothesis that Hs extract, being a natural plant extract, is safe and effective against *C. albicans* biofilm. The significant anti-*candida* biofilm activity and less toxicity of Hs extract further motivated us to test the efficacy of Hs extract on *C. albicans* isolated from patients.

In-Vitro Susceptibility of Clinical Isolates of *Candida*

In the present study, 30 *C. albicans*, isolates obtained from patients visiting gynae-department at VMMC and Safdarjung Hospital, New-Delhi were checked for in vitro anti-fungal susceptibility by different methods. They were then tested for antifungal susceptibility and effect of Hs extract on mature biofilm. In the Fluconazole susceptibility test performed using the E-test method, *C. albicans* isolates were grouped as 13% resistant, 53% with intermediate sensitivity and 33% sensitive. For the isolates that were grouped as sensitive, the sensitivity to Amphotericin B and Hs extract was similar to that of control *C. albicans* strain, when assessed for antifungal sensitivity at planktonic and biofilm stage. However, the isolates showing intermediate sensitivity and resistance towards fluconazole were still sensitive to Hs extract at slightly increased dose from 1.5 to 2.15 mg/ml in planktonic cells (Table 3). Similarly, we observed that Hs extract was effective in inhibiting preformed biofilms in these isolates at higher concentration (from 3.125 to 4.25 mg/ml). Thus, based on the study results and clinical samples, it was deduced that DMSO extract of *Hibiscus sabdariffa* is also effective on the fluconazole resistant *C. albicans* strains.

Conclusion

One of the most important virulence traits of *C. albicans* is its ability to switch reversibly between its filamentous forms (hyphae) to budding yeast forms. This transition is the key factor for *C. albicans* infections. These hyphae ultimately form biofilms which protect sessile yeast cells from anti-fungal agents and are the source of new infections. Therefore, new anti-fungal agents should have the ability to impair hyphal development and, in addition, should have the ability to abolish the pre-formed biofilm, thereby targeting the sessile yeast cells.

Our results for the first time indicate that DMSO extract of *Hibiscus sabdariffa* has the potential to alleviate *C. albicans* pathogenesis, which is in line with the current anti-fungal paradigm to target virulence trait of microbial cells [31]. The Hs extract not only prevents adhesion, biofilm initiation and formation, but also destroys the preformed biofilm. Our results also point towards differences in antimicrobial activity of different extracts of Hs, which highlights the importance of choosing the correct combination of solvent and chemical compound, for testing antimicrobial activity. A detailed pharmacological analysis is needed to identify the active compounds that can be used as drugs against *Candida*.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical Approval The study protocol was as per the Guidelines and Standards for Research. The study was approved by the Ethics Committee of ACBR (ACBR/IHEC/DS-02/09-18), University of Delhi and VMMC and Safdarjung Hospital (IEC/VMMC/SJH/Project/November/2018-1104).

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