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This study demonstrates the potential of juglone against the planktonic and sessile cells of *candida albicans*. Serial microdilution, disk diffusion and time kill assay were performed to estimate the antimicrobial effect of juglone in planktonic cells. Concentration of 160µg/ml and above showed a promising antimicrobial effect. Further effect of juglone was tested against the candida biofilm using XTT reduction, AFM, confocal analysis, and SEM techniques. Various tests performed on planktonic and sessile biofilm cells gave a clear idea of antibiofilm potential of juglone against the *C. albicans.* Present investigation provides substantial evidences for the efficacy of juglone; hence juglone can be a molecule of choice for the prevention of candida infection.

Key words: Anticandida, biofilm, confocal, juglone; SEM, XTT reduction assay

INTRODUCTION

Candida albicans, the leading cause of hospital acquired fungal infections is an opportunist pathogen that lives commensally within the human body. *Candida* bloodstream infection is the most common etiologic agent of fungal related biofilm infection. The use of broad-spectrum antibiotics, indwelling medical devices or the presence of immunosuppression or disruption of mucosal barriers due to surgery, chemotherapy and radiotherapy are among the most important predisposing factors for invasive candida infection.

This disease is associated with formation of biofilms over indwelling devices. A biofilm is a structured microbial community of cells enclosed in a matrix of extracellular polymeric substances (EPS), which provides protection against host defense mechanisms and also serves as reservoirs for persistent sources of infection in a patient (Fanning and Mitchell, 2012).The current antifungal drugs available in the market are associated with serious side effects like nephrotoxicity, photosensitivity, hepatitis, antibiotic resistance etc. (Ramage *et al,* 2012), hence the need for more safe and economic alternatives to manage fungal infections have driven the search for novel antifungal compounds. Juglone, also called 5-hydroxy-1, 4-naphthalenedione is an organic compound with the molecular formula $C_8H_6O(OH)_2$. Juglone occurs naturally in the leaves, roots, husks, fruit (the epicarp), and bark of plants in the Juglandaceae family, particularly the black walnut (*Juglans nigra*). Walnut tree has a long history of medicinal potential i.e. antimicrobial, sedative, herbiside, bioside and anti-tumor etc (Strugstad, 2012; Zakavi *et al,* 2013). Various reports with antimicrobial potential of juglone against wide range of microrganism are available, but all of them are against the planktonic form. In the present study we have investigated the anti-biofilm potential of juglone using various biochemical and imaging techniques and juglone has emerged as a poten-

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tial molecule with antibiofilm activity.

MATERIALS AND METHODS

Fungal strains and growth conditions

C. albicans MTCC 227 *was* grown on Sabouraud dextrose agar (Himedia, India) plates. To prepare a standard cell suspension, fungal cells were re-suspended at 1×10^7 cell/ml in RPMI-1640 MOPS medium (Himedia) supplemented with 2% glucose was used for various assay. Juglone was obtained from TCI Japan, and dissolved in dimethylsulfoxide (DMSO) and sterilized by filtration through a 0.45 ìm membrane filter.

Determination of antimicrobial activity by broth microdilution assay

MICs of juglone were determined for planktonic cells using the broth microdilution technique (Hadacek and Greger, 2000; CLSI, 2010). Briefly, serial 2-fold dilutions of the compounds were prepared in RPMI-1640 MOPS medium (Himedia), in 96-well microtitre plates to obtain concentration ranges from 10ug-1280 ug/ml. Yeasts were then added to each well at final concentrations of 1×10^3 cfu/ml. The microtitre plates were incubated at 35°C for 48 h in a dark, humid chamber. Minimum concentrations that inhibited 50% and 90% of the fungal yeast growth in relation to control (IC50 and IC90, respectively) were determined by visual analysis and confirmed by spectrophotometer at 492 nm in a microtitre plate reader.

The MFC is defined as the lowest concentration of compound that produces no fungal growth and was determined by transferring an aliquot (20µl) of each sample treated with concentrations higher than the MIC into an antifungal agent-free sabouraud dextrose agar plate and incubated at 35°C for 48 h.

Disk diffusion assay

Disk diffusion assay was performed as described previously (NCCL, 1999; 2004). A 200 µl suspension of each tested strain (1×10^6 cfu/ml) was spread uniformly onto muller hinton agar plates. Blank paper disks 6-mm in diameter was impregnated with 10 µl (50-400 ug) juglone. After drying, they were placed on the inoculated agar plates. The plates were then incubated at 35°C, and the diameters of the zones of inhibition were measured at 48 h.

Determination of Rate of Kill

In time kill experiments, the method described by Klepser (1998) was used. The starting inoculums were 10^5 cells/ml and drug concentrations ranged from 0.5 to 4 times the MIC. Test tubes were incubated with agitation in the dark at 35°C. At predetermined time points (0, 2, 4, 8, 12 and 24 h) samples (100 µl) were removed and serially diluted 10-fold in sterile saline; four 30 µl aliquots were subsequently plated onto muller hinton agar. Colony counts were determined after incubation of the plates at 35°C for 48 h. All assays were performed in triplicate and repeated at least twice.

Effects of juglone on biofilm formation and pre formed biofilm

The effect of juglone on biofilm was estimated by using standard XTT reduction assay.
Fungal biofilm were prepared as described previously (Ramage 2001) on 96-well polystyrene microtitre plates (Himedia). Standard cell suspension of C*andida* (100 µl) was transferred into the wells and incubated for 1.5 h at 37°C with agitation. After the adhesion phase, the liquid was aspirated and each well was washed twice with PBS to remove loosely attached cells. 200 ml of different antifungal agent concentrations (80-1280 µg/ml), diluted in RPMI 1640 buffered with MOPS was added to the wells and the plate was further incubated for 24 h at 37°C.

To investigate the effect of juglone on pre-formed biofilm, *C. albicans* biofilm were prepared for 24 h at 37°C as described above. The wells were washed twice with PBS and fresh RPMI-1640 medium buffered with MOPS (200 µl) containing different concentrations (80-2560 µg/ml) of juglone were added and the plate was further incubated for 24 h at 37°C. The effect of compound on biofilm was estimated by using a standard XTT reduction assay (Ramage, 2001) and the color change in the solution was measured with spectrophotometric readings at 490 nm.

Confocal laser scanning microscopy

Image acquisition was performed with a ZEISS LSM 780 confocal laser scanning microscope (ZEISS, Germany) equipped with an argon laser and GaAsP detectors. Before observation, the specimens were stained with propidium iodide (PI,

Himedia) and fluoresceine diacetate (FDA, Himedia) referred to previously literatures (Wu *et al*, 2011; Gabi *et al,* 2011; Li *et al,* 2012). The live cells will be stained with the FDA dye and visualized with a diffusely distributed green fluorescence, whereas those with damaged membranes (dead) will be stained with PI and with fluorescent red. Thus, the viability of cells could be assessed by this way. Each assay was performed in quadruplicate and repeated at three times.

Atomic Force Microscopy

Images of biofilms were taken with Atomic Force Microscopy (AFM) (NT-MDT Ntegra, Prima). Biofilms were established on glass coverslips. After washing the biofilms with PBS, different concentrations of juglone in PBS (4 MIC, 16 MIC, 32 MIC) were added. One sample without any juglone was used as a control. After 24 h of incubation, the liquid medium was withdrawn and the wells were washed twice with PBS. The biofilms were fixed with 2.5% glutaraldehyde in PBS, pH 7.4, at 37°C for 2 h. After washing with distilled water, the biofilms were dried in air. All images were collected in semi-contact mode using sharpened silicon nitride cantilevers NSG10S with spring constant about 10 N m^{-1}. Height and deflection images were simultaneously acquired at a scan rate of 250 kHz.

SEM

For scanning electron microscopy (SEM), biofilms were developed on glass coverslips. The coverslips were inoculated with *C. albicans* and incubated statically at 37°C for 90 min to allow adhesion. After removing non-adherent cells, the coverslips were incubated with fresh RPMI 1640 medium at 37°C for 24 h. For juglone treatment groups, mature biofilms were treated with juglone overnight. Biofilms were washed and placed in a fixative consisting of 2.5 % (vol/vol) glutaraldehyde in PBS (pH 7.2) for 2 h. The samples were rinsed twice in PBS, dehydrated in an ascending ethanol series, treated with hexamethyl-disilazane (Himedia, india), and dried overnight. The specimens were coated with gold and observed through a FE-SEM QUANTA 200FEG (FEI Neitherlands) in high-vacuum mode.

RESULTS AND DISCUSSION

MIC and Disk diffusion assay

Antifungal activity of juglone was determined by the application of broth dilution and disc diffusion method. In this study, the antimicrobial activities of juglone at different concentrations of 10 µg/ml to 1280 µg/ml were tested and juglone showed a clear inhibition in visible growth at concentration of 160 ug/ml and above, also sample taken from 320 ug/ml and above did not show any growth on plate culture. These finding were further supported by disk diffusion data; where juglone posed a clear zone of inhibition with concentration of 100 ug/ml and above (Fig. 1A).

Time kill assay

The time-kill kinetics profile of the *C. albicans* by juglone is shown in Figure 1B. The time required to achieve 3 log10 decreases in CFU is an acceptable index of bactericidal activity from time-kill analysis (NCCL 1999). The results illustrated that juglone did not showed significant bactericidal activity in first hour. Further 3log10 reduction in viability of Candida was clearly observed after 4-8 hours of exposure. The log10 CFU of Candida was reduced to zero after 24 hours of exposure with juglone.

Effect on Candida Biofilm

The effect of juglone on candida biofilm metabolic activity was estimated by using standard XTT reduction assay and crystal violet assay was used for the determination of biofilm biomass. Analysis of biofilm growth by using XTT- reduction assay showed that addition of juglone after adhesion phase prevented formation of biofilm by *C. albicans*. Concentration less than 80 µg/ml juglone did not affect development of biofilm, while 320 µg/ml and above caused a marked reduction (28-54%) in biofilm growth (Figure 2A) as compared to that of the control biofilm.

Mature candida biofilms were also considerably sensitive to inhibitory effect of juglone (Figure 2B). Juglone at a concentration of 640 µg/ml and above exhibited a significant antibiofilm potential (33-60% inhibition). Along with the reduction in metabolic activity total biofilm biomass was also reduced to a great extent.

Confocal analysis

Analysis of control and treated candida biofilm revealed a clear cell death and treated group. In con-

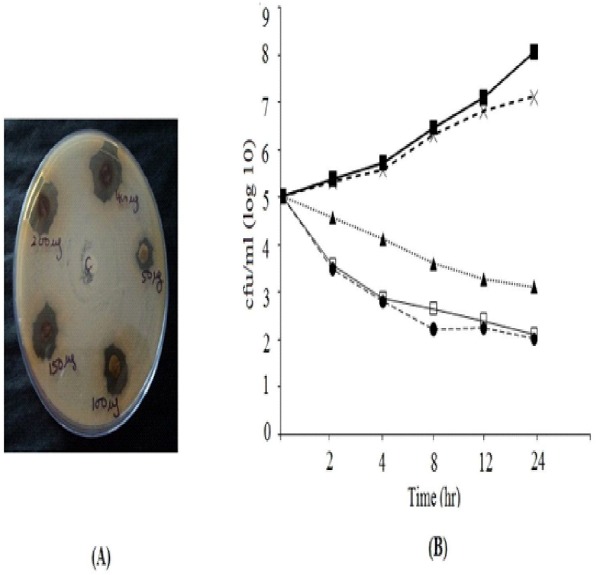

Fig. 1 : (A) Disk diffusion assay for Juglone against *C. albicans.* Juglone concentration as follow : C. control; 1:50 mg; 2:100 mg; 3: 150 mg; 200 mg and 4:200 mg. (B) Time kill curve of *Canadia albicans* treated with Juglone. Symbols indicate inhibitory concentrations as follows : (-■-) control (no drug); -)(- 80 mg ml^{-1}; (... ♦ ...) 160 mg ml^{-1}; (-□-) 320 mg ml^{-1} and (-●-) 640 μg ml^{-1}.

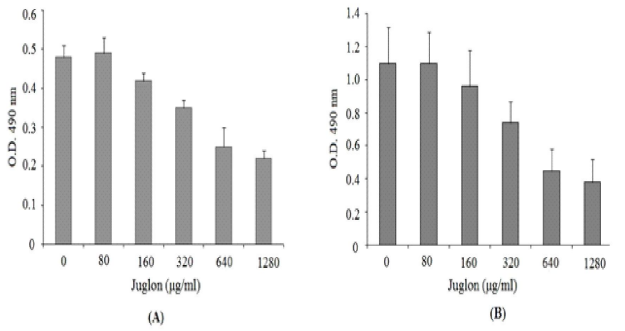

Fig. 2 : (A) Effect of juglone on biofilm formation of *Candiada albicans.* Biofilm was quantified calorimetrically by XTT assay, which measures biofilm metabolic activity. Error bars represents the standard deviation. (B) Effect of juglone on mature biofilm of *Candida albicans.* Biofilm was quantified calorimetrically by XTT assay, which measures biofilm metabolic activity. Error bars represents the standard deviation.

trol group viable cells with diffused FDA dye were clearly visible with higher cell number, at the same time few cells dyed in red color represents dead or dying cells (Fig.3A). In treated biofilm there was a clear reduction in number of green living cells with a higher number of PI dyed red cells.

AFM

Atomic force microscopic analysis revealed that the

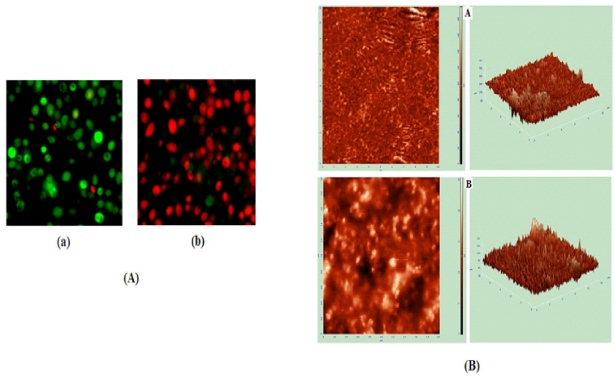

Fig. 3 : (A) Candida biofilm after the treatment of juglone. The confocal laser scanning micrographs show the section in the xz plane. Live cells appear green because of fluorescein diacetate; dead cells in red, staning with propidium iodide. (B) Atomic force microscopy micrographs showing the variation in the roughness and height of *C. albicans* biofilms on cover slip (a) untreated biofilm after 48 h 9height 200 nm). (b) citral treated established biofilm (48h) (height 90 nm).

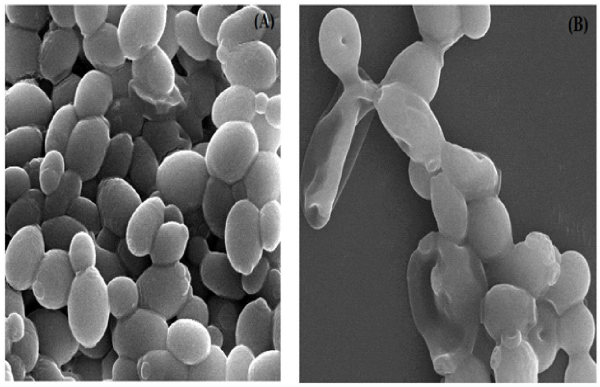

Fig. 4 : Scanning electron micrographs of the 48 h *C. albicans* biofilms on coverslips. (A) Biofilm in the absence of juglone showing a dense network of healthy cells. (B) established biofilm treated with juglone (after 24 h) shows cell wall and shrinkage in cell membrane due to plasmolysis of cell is illustrated.

Candida cells were embedded within a sticky layer of exopolysaccharides distributed around the cell surface, whereas this layer was absent in treated *Candida* biofilms. The 3D image of control group revealed that this layer surrounded the cells residing in the biofilm (Figure 3B Control) and provides better image resolution than SEM, providing both the height and roughness of the biofilm. A significant variation in the height of the biofilm was observed. The heights of the untreated and treated biofilms were 200 nm and 90 nm, respectively (Figure 3B Treated).

SEM

In order to evaluate the effect of juglone on *C.*

albicans biofilm growth, SEM was performed. SEM images of a control *C. albicans* biofilms and of a biofilm treated with juglone are shown in Figure 4**.** Untreated sessile cells of biofilm showed a smooth cell wall and large size, whereas cells from treated group had deformed and lysed cells with reduced density in biofilm.

C. albicans is one of the important fungal pathogen responsible for the most of serious nosocomial infections. *Candida* has the tendency to adhere to indwelling medical devices and forms biofilm on them; which is resistant to antifungal agents and protects the fungus from host defenses, which have important clinical repercussions.

Rising incidences of *Candida* infection, increasing resistance to commercial antifungal agents and associated side effects are an issue of serious concern. In recent years, the plant medicines are getting a new thrust for the treatment of different diseases since the herbal drugs are mostly free of side effects and cost effective. Juglone being an active constituents from various species of walnut tree, including the black walnut (*Juglans nigra*), English or Percian walnut (*J. regia*), Japanese walnut (*J. sieboldiana*), butternut (*J.cinerea*) and also by hickory tree (*Carya ovata*) has been tested by various authors (Soderquist, 1973) for its antimicrobial potential. In the present work, apart from the planktonic form, juglone was tested for its antibiofilm potential against *C. albicans*.

Juglone shows a favorable anti-candida activity with a MIC50 value of 160{C}{C}{C}{C}g/ml, whereas concentration of 320 µg/ml and above was fungicidal (MFC). These results are in agreement with the previous studies done by various authors *Pseudomonas aeruginosa*, *Burkholderia capacia*, *Staphylococcus aureus*, *Basillus subtilis* etc. (Krajci and Lynch, 1978; Clark and Jurgens, 1990; Preira *et al,* 2001; Sharma *et al,* 2009). Further the dose dependent effect of juglone was showed using disk diffusion assay, where increasing concentration of juglone resulted In a wider zone of inhibition. This observation was comparable with the result obtained by Zakavi and coworker, who obtained successively wider zone of inhibition while using a increasing concentration of juglone regia bark extract (Zakavi *et al,* 2013).

Time kill assay was utilized in this study to verify MIC findings and to evaluate the ability of juglone to eliminate *C. albicans* growth *in vitro*. In the case of two and four-times MIC concentrations, juglone inhibited the yeast growth within 8 h and showed 3log reduction in fungal population. Although the mechanism for the toxic effect of juglone is still not fully understood but the high toxicity of juglone has been attributed to it high redox potential (-93mV) and redox recycling (Inbaraj and Chignell 2004; O'Brien, 1991). This produces hydrogen peroxide, which is a strong oxidant and can cause oxidative damage to cell resulting in cell death.

To verify the effect of juglone on cell morphology, *C. albicans* cells (control and juglone treated) were subjected to SEM analysis. SEM showed that the cells treated with juglone decreased in size, appeared irregular in shape with cell wall modifications and clear depressions on the cell surface with holes. Such modifications may be related to the interference of the juglone with enzymatic reactions of wall synthesis which affects yeast morphogenesis and growth.

Apart from being effective against the planktonic candida cells; the potential of juglone was also tested against the biofilm form of *Candida albicans*. As the microbial biofilms are more resistance against the general antimicrobial compounds, new effective prevention measures are of a great use. When tested for the antibiofilm potential juglone proved to be moderately effective against both; biofilm formation and mature biofilms. XTT reduction assay which measures the metabolic status of tested cells showed a clear and profound reduction in metabolic activities of treated cells when compared to control biofilm.

Effectiveness of juglone against the biofilm was further confirmed using confocal analysis; as used by various authors previously (Ansari *et al,* 2013), where fluorescence dyes FDA/PI was used for a clear visual of live/dead cells. Confocal images showed a clear enhanced cell death in juglone treated mature biofilm. Not only had a marked reduction in metabolic activities, but juglone also caused a significant reduction in biofilm matrix. Biofilm matrix that is mainly composed of exopolysaccharides, forms an effective barrier against antimicrobial from penetrating the cell (Bridier *et al,* 2011). Any reduction in this matrix can facilitates the interaction of antimicrobial compound with candida cells. Reduction in biofilm matrix was visible with atomic force microscopy im-

ages in treated group. AFM due to easy and fast sample preparation, emerging as a test of choice for the study of biofilm architecture (Chandra *et al,* 2001). AFM gives detailed information regarding the thickness and surface architecture of biofilm. In control group cells are embedded in polysaccharide surface matrix hence the surface appears to be smooth and leveled and at the same time with high thickness, whereas in treated biofilm the loss of matrix caused a rough and uneven texture with reduced biofilm thickness.

Candida biofilms are quite resistant to antifungal molecules and difficult to treat. The matrix secreted by cells creates a barrier for antifungal compounds. Due to the development of resistance towards the commercially available antifungal compounds scenario has changed towards the natural antimicrobial compounds. Our findings indicate that the juglone is effective for the inhibition of biofilm formation and has fungi static, fungicidal and antibiofilm potential. This potential is superior to that of most of the commonly used antifungal. Our results suggest that juglone has potential as a promising therapeutic agent in the treatment and prevention of biofilm associated *C. albicans* infections. The significant antifungal activity of juglone suggests that this could serve as a source of compounds which have a therapeutic potential for the treatment of *Candida*-related infections. Further evaluation *in vivo* is required to determine whether these findings can be exploited in treating biofilm-associated candidiasis.

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