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Antifungal and antibiofilm potential of citral against *Candida albicans*

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This study demonstrates the potential of citral against the planktonic and sessile cells of *Candida albicans*. Various biochemical analysis and imaging techniques were applied in order to evaluate the antimicrobial potential of citral. Serial microdilution, disk diffusion and time kill assay were performed to estimate the antimicrobial effect of citral in planktonic cells. Concentration of 80 µg/ml and above showed a promising antimicrobial effect. Further effect of citral was tested against the *Candida* biofilm using XTT reduction, crystal violet, confocal analysis, SEM and AFM techniques. Various tests performed on planktonic and sessile biofilm cells gave a clear idea of antibiofilm potential of citral against *C.abicans*. Present investigation provides substantial evidences for the efficacy of citral; hence citral can be a molecule of choice for the prevention of *Candida* infection.

Key words: Antifungal; biofilm; citral; SEM; AFM; Confocal, XTT reduction

INTRODUCTION

Nosocomial infection or hospital infections are a major cause of mortality in developing countries. This has been attributed to the increased number of immunocompromised patients (Hagerty *et al*. 2003), extended hospital stay, indiscriminate use of antimicrobial agents and also to the intensified use of indwelling medical devices (Donlan 2001; Kojic and Darouiche 2004). Invasive candidiasis, caused mostly by *Candida albicans* is one of the most common causes of nosocomial infection with a substantial attributable mortality.

This disease is associated with formation of structured, multilayered microbial communities known as biofilms over indwelling devices. A biofilm is a structured microbial community of cells enclosed in a matrix of extracellular polymeric substances (EPS), that serves as a reservoir from which the cells disperse to the bloodstream and consequently cause infection in distant organs (Fanning and Mitchell 2012).

The increased resistance of pathogens to synthetic drugs (Kuhn and Ghannoum 2004; Ramage *et al*. 2012) and the need for more economic alternatives to manage fungal infections have driven the search for novel antifungal compounds. Medicinal plants have emerged as a valuable source of bioactive molecules with therapeutic application (Bansod and Rai 2008).

Citral (C10H16O), also called 3,7-dimethyl-2,6-octadienal, is a kind of monoterpene compound that was originally isolated from lemongrass oil and also found in many essential oils such as lemon myrtle, Litsea citrata etc. Because of its high bioreactivity, including antimicrobial (human pathogenic fungi and bacterium) and insecticidal properties, citral has been the focus of attention of numerous researchers. In the present study we have investigated the anti-biofilm potential of citral using various biochemical's and imaging techniques.

MATERIALS AND METHODS

For the present work *C. albicans* MTCC 227 was grown on Sabouraud dextrose agar (Himedia, India) plates and fungal suspension, was prepared in RPMI -1640 MOPS medium (Himedia) at concentration of 1×10^7 cell/ml. Citral was obtained from TCI Japan, and dissolved in 10% aqueous dimethylsulfoxide (DMSO). MICs of citral were determined for planktonic cells using the broth microdilution technique of Hadacek and Greger (2000). Briefly, serial 2-fold dilutions of the compounds were prepared in RPMI- 1640 MOPS medium (Himedia), in

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96-well microtitre trays to obtain concentration ranges from 10ug-1280 ug/ml. Minimum concentrations that inhibited 50% of the fungal yeast growth in relation to control was determined by visual analysis and confirmed by spectrophotometer at 492 nm in a microtitre plate reader. In order to determine the time required for citral action, the method described by (Klepser *et al.* 1998) was used. The starting inoculum was 105 cells/ml and drug concentrations ranged from 0.5 to 4 times the MIC. 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 48h.

The effect of citral on biofilm was estimated by using crystal violet assay and standard XTT reduction assay. Fungal biofilm were prepared as described (Ramage *et al*. 2001) on commercially available, pre-sterilized, flat-bottomed 96-well polystyrene microtitre plates (Himedia). Standard cell suspension of *Candida* (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 (40-640 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 citral 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 (80-1280 µg/ml) of citral 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 et al. 2001) and the colour change in the solution was measured with spectrophotometric readings at 490 nm. Total biofilm biomass was quantified by crystal violet staining methodology (Silva *et al*. 2009). The absorbance of suspensions was measured at 570 nm.

Image acquisition was performed with an 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 (Gabi *et a*l. 2011; Li *et al*. 2012; Wu *et al*. 2009). For scanning electron microscopy (SEM), biofilms were developed on glass coverslips. The coverslips with mature biofilms were treated with citral 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 200 FEG (FEI Neitherlands) in high-vacuum mode. Images of biofilms were taken with Atomic Force Microscopy (AFM) (NT-MDT Ntegra, Prima). Biofilms were established on glass coverslips. After washing the biofilms were treated with different concentrations of citral in PBS (4 MIC, 16 MIC, 32 MIC). The biofilms were fixed with 2.5% glutaraldehyde and dried in air. All images were collected in semi-contact mode using sharpened silicon nitride cantilevers NSG10S with spring constant about 10 Nm^{-1}.

RESULTS AND DISCUSSION

Antifungal activity of citral was determined by the application of broth method. Citral exhibited a favorable anti-candida activity with a MIC50 value of 80 g ml-1 (Data not shown), whereas concentration of 200 µg ml-1 and above was fungicidal. These results are in agreement with the previous studies done by Leite *et al* (2014), where antimicrobial potential of citral was tested against the planktonic *Candida* cells. Time-kill assay was utilized in this study to verify MIC findings and to evaluate the ability of citral to eliminate *C. albicans* growth *in vitro*. The time required to achieve 3 log10 decrease in CFU is an acceptable index of bactericidal activity from time-kill analysis (NCCL 1999].In the case of two and four-times MIC concentrations, citral inhibited the yeast growth within 4 h and showed 3log reduction in fungal population (Fig. 1A). These results are in agreement with previous report (Leite *et al*. 2014) in which citral was proposed to be fungistatic and fungicidal at the concentrations of 64 µg/ml and 256 µg/ml respectively.

Apart from being effective against the planktonic candida cells; the potential of citral 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 citral proved to

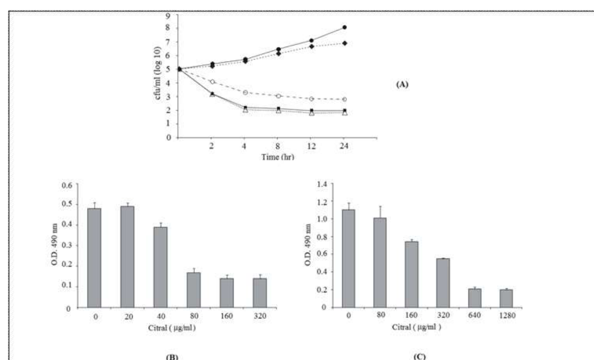

Fig. 1 : (A) Time kill curve of *Candida albicans* treated with citral. Symbols indicate inhibitory concentrations as follows: (—●—) control (no drug); (- -◆- -) 40 µg ml^{-1}; (–⊖ - -) 80 µg ml^{-1}; (—■—) 160 µg ml^{-1} and (···△···) 320 µg ml^{-1}. (B) Effect of citral on b**iofilm formation of *Candida*** *albicans*. Biofilm was quantified colorimetrically by XTT assay, which measures biofilm metabolic activity. Error bars represents the standard deviation. (C) Effect of citral on mature b**iofilm of *Candida*** *albicans*. Biofilm was quantified colorimetrically by XTT assay, which measures biofilm metabolic activity. Error bars represents the standard deviation.

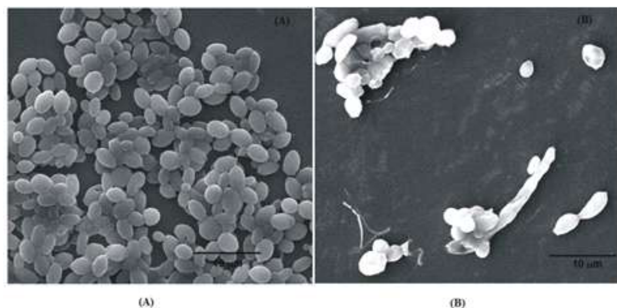

Fig. 2 : Scanning electron microscopy micrographs of the 48 h *C. albicans* biofilms on coverslip. (A) Biofilm in the absence of citral showing a dense network of healthy cells. (B) Established biofilm treated with citral (after 24 h) shows rough cell wall and shrinkage in cell membrane due to plasmolysis of cell.is illustrated. Bar indicates 10 µm.

be effective against both; biofilm formation and mature biofilms. Analysis of biofilm growth by using XTT- reduction assay showed that addition of citral after adhesion phase prevented formation of biofilm by *C.albicans*. Concentration less than 40 µg/ml citral did not affect development of biofilm, while 80 µg ml-1 and above caused a marked reduction (65-71%) in biofilm growth (Fig 1B) as compared to that of the control biofilm.

Mature C*andida* biofilms were also considerably sensitive to inhibitory effect of citral (Figure 1C). Citral at a concentration of 320µg/ml and above exhibited a significant antibiofilm potential (25-56% inhibition). Along with the reduction in metabolic

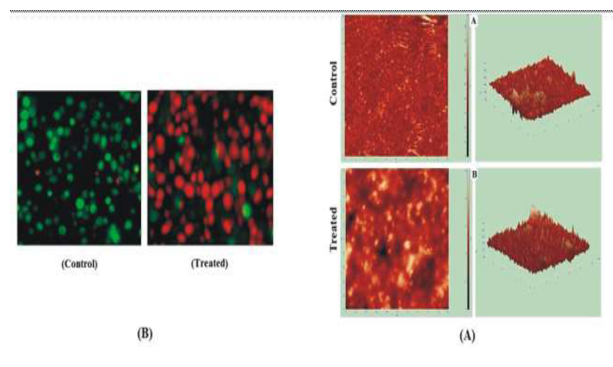

Fig. 3 : (A) Candida biofilm after the treatment of citral. The confocal laser scanning micrographs show the section in the xz plane. Live cells appear green because of fluorescein diacetate; dead cells in red, staining with propidium iodide. (B) Atomic force microscopy micrographs showing the variation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

activity total biofilm biomass was also reduced to a great extent. Around 18-480% decline was observed in biomass in the presence of citral at the concentrations of 640 µg/ml (Figure 1C).

Apart from *Candida*, antifungal effect of citral has also been tested against other fungi like *Geotrichum, Penicillium, Saccharomyces* etc. where it inhibited mycellium growth and work against the cell wall integrity and cell membrane permeability (Zhou *et al*. 2014; Droby *et al*. 2008; Belletti *et al* . 2010). These results hypothesized that the citral attacked the C. albicans cells and caused higher cell damage. To verify this hypothesis, *C. albicans* cells (control and citral treated) were subjected to SEM analysis. SEM showed that the cells treated with citral decreased in size, appeared irregular in shape with cell wall modifications and clear depressions on the cell surface with holes (Fig. 2) Such modifications may be related to the interference of the citral with enzymatic reactions of wall synthesis which affects yeast morphogenesis and growth as hypothyzed by several authors (Harris 2001, Tao *et al*. 2014, Zhou *et al*. 2014). Another evidence of citral's interference with cell morphogenesis comes from germ tube assay, where a sub lethal concentration of causes a marked reduction in size and percentage of germ tube formation in the presence of citral (Data not presented). Germ tube formation is considered as a potent virulent factor for candida infection as it is essential for invasion into host system.

Effectiveness of citral against the biofilm was further confirmed using confocal analysis; as used by various authers 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 citral treated mature biofilm. Not only had a marked reduction in metabolic activities, but citral also called a significant reduction in biofilm matrix (Fig.3A). Biofilm matrix which is mainly composed of exopolysaccharides forms an effective barrier against antimicrobial from penetrating the cells (Mishra *et al*. 2015). Any reduction in this matrix can facilitates the interaction of antimicrobial compound with *Candida* cells. Reduction in biofilm matrix was visible with crystal violet assay and it was further confirmed using AFM analysis. 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 (Fig. 3B). 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 citral is effective for the inhibition of biofilm formation and has fungistatic, fungicidal and antibiofilm potential. This potential is superior to that of most of the commonly used antifungal. Our results suggest that citral has potential as a promising therapeutic agent in the treatment and prevention of biofilm associated *C. albicans* infections. The significant antifungal activity of citral 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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