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Inhibition of planktonic and biofilm growth of *Candida albicans* reveals novel antifungal activity of caffeine

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Infections associated with drug resistant strains and biofilms of *Candida albicans* have necessitated search for novel molecules with antifungal properties. Caffeine, a major component of the most widely consumed beverages, coffee and tea, is known to possess various biological properties. To evaluate antifungal potential, its effect on growth and virulence attributes of *C. albicans* was studied using standard methodologies. Caffeine showed fungistatic effect on planktonic growth of two strains of *C. albicans* (including a fluconazole resistant strain), exhibiting minimum inhibitory concentration (MIC) at 12.5 mM concentration. Around 30% decrease in the adhesion of cells in the presence of caffeine indicated considerable anti-adhesion activity. Caffeine prevented formation of biofilms (which are drug resistant forms), in a concentration dependent manner. Analysis by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) metabolic assay and microscopic observations showed inhibition of biofilm development at 25 mM concentration. This study, for the first time demonstrates dietary chemical, caffeine, as a potential inhibitor of growth, adhesion and biofilm formation by *C. albicans*.

Key words: Adhesion, antifungal, biofilms, *Candida*, drug resistance.

INTRODUCTION

Emergence of drug resistant strains of pathogens and toxic side effects of the drugs are well known problems in antifungal chemotherapy (Kim and Sudbery, 2011). Infections caused by the fungal pathogen, *Candida albicans*, are difficult to treat because of the same reasons (Mishra et al., 2007). Additionally, this commensal easily colonizes host tissues as well as various prosthetic devices implanted in patient's body and develops into biofilm growth form. Device associated *Candida* biofilms do not respond to majority of the antifungal drugs and may act as a source of re-infections in immunocompromised patients, hence considered as a challenge to successful antifungal treatment (Ramage et al., 2005). Therefore, search for novel antifungal agents need to be continued. Plants are rich sources of bioactive molecules and are being explored for novel antimicrobial properties (Boroujeni et al., 2012; Cowan, 1999). Essential oils as well as their components have been shown to exert potential anti-*Candida* activities (Devkatte et al., 2005; Zore et al., 2011). Use of novel plant molecules against multiple drug resistant strains as well as biofilm growth of *C. albicans* may be an effective strategy (Kim and Lee, 2012; Raut et al., 2012). Caffeine or 1,3,7- trimethylxanthine, is a pseudoalkaloid found in leaves/fruits of at least thirteen plants, including coffee (*Coffea arabica* L.) and tea (*Camellia sinensis* (L.) Kuntze). It is one of the most widely consumed dietary chemicals in the form of coffee, tea, cola and chocolate (Baker et al., 2006; Esimone et al., 2008). Caffeine is used in a variety of pharmaceutical preparations due to

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various pharmacological properties (Hosseinzadeh et al., 2006; Kuranda et al., 2006).

Various *in vitro* and *in vivo* experiments have shown that extracts of tea and coffee possess potential inhibitory activities against bacterial pathogens, including drug resistant strains. Extracts of *C. arabica* L. and *Coffea canephora* P. were found to interfere with adsorption of *Streptococcus mutans* to saliva-coated hydroxy apatite beads. Water extracts prepared with Brazilian coffee showed significant reduction in adhesion of *S. mutans* to glass beads (Ferrazzano et al., 2009). Ethanol extracts of four species of *Cola* Schott and Endl. have shown *in vitro* antifungal properties against *C. albicans* and *Aspergillus niger* (Sonibare et al., 2009). The presence of different secondary metabolites, including caffeine in these plants/extracts is supposed to be the basis for their antimicrobial activities (Ferrazzano et al., 2009; Ibrahim et al., 2006). Few reports have described antibacterial and other antimicrobial activities of caffeine. It was found to act synergistically to reduce minimum inhibitory concentration (MIC) of antibiotics like, tetracycline, penicillin G, ceftizoxime, gentamicin and carbenicillin, against bacterial pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Esimone et al., 2008, Hosseinzadeh et al., 2006). Not many studies are available on the antifungal potential of caffeine. A study by Sundar-Raj and Dhala (1965) discussed the growth inhibitory effect of caffeine and caffeine citrate against different commensal bacteria as well as yeast. Caffeine was found to restrict growth and sporulation of the symbiotic fungus *Monacrosporium ambrosium*, while 6% concentration (w/v) of it was reported to inhibit aflatoxin production in *Aspergillus* species (Kumar et al., 1995; Maraqa et al., 2007). Structural alterations in *C. albicans*, at sub-inhibitory concentrations of caffeine are previously reported (Mittag, 1994). Interestingly, caffeinated soft drinks were found to reduce bacterial prevalence in voice prosthetic biofilms. However, it did not show any activity against biofilms of seven species of oral bacteria (Cogo et al., 2008). No systematic efforts are made to explore the effect of caffeine on fungal adhesion and biofilm formation. In this study, for the first time we report caffeine's potential to inhibit growth, adhesion and biofilm formation by the most common fungal pathogen, *C. albicans*.

MATERIALS AND METHODS

Chemicals and culture conditions

C. albicans, ATCC 90028 and 10231 strains were obtained from the Institute of Microbial Technology, Chandigarh, India. A single colony from the Yeast extract-Peptone-Dextrose (YPD) agar plates was inoculated in 50 ml of YPD broth in a 250 ml Erlenmeyer flask, and incubated at 30°C for 24 h, in a shaking incubator at 120 rpm. Cells from the activated culture were harvested by centrifugation for 5 min at 2000 *g* speed; washed three times with phosphate buffer saline (PBS, 10 mM; potassium chloride, 2.7 mM; and sodium chloride, 137 mM; pH 7.4) and resuspended in PBS. RPMI-1640 medium with L-glutamine and without sodium bicarbonate, buffered with 165 mM 3-[N-morpholine] propane sulphonic acid (MOPS) pH 7, was filter sterilized. Various concentrations of caffeine (AR, S.d. Fine-Chem. Ltd., Mumbai) from 1.56 to 50 mM were prepared in respective media in each assay. Fluconazole (Forcan, Cipla Pvt. Ltd., Mumbai, India) was purchased from local market. 2, 3-bis (2-methoxy-4-nitro-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and menadione were purchased from Sigma-Aldrich Chem. Ltd. Mumbai, India. All other media components and chemicals were from Hi-Media Laboratories Ltd., Mumbai, India.

Growth assay (MIC)

Effect of caffeine on the growth of planktonic cells of *C. albicans* was studied by using the standard broth micro dilution method as per Clinical and Laboratory Standards Institute Guidelines (CLSI, 2008). Inoculum of 1×10^3 cells/ml was added to each well with varying (1.56 to 50 mM) concentrations of caffeine and the microplates were incubated at 35°C for 48 h. Final volume of assay system in each well was kept at 200 µl. Wells without caffeine served as a control. Various concentrations (1 to 256 µg/ml) of fluconazole were used as a standard antifungal. To analyze the growth, absorbance was read spectrophotometrically at 620 nm using a microplate reader (Multiskan EX, Thermo Electron Corp., USA). The lowest concentration of caffeine which caused fifty percentage reduction in the absorbance compared to that of control, was considered as MIC for growth of *C. albicans*.

Minimum fungicidal concentrations (MFC)

To determine the minimum *Candida*-cidal concentrations of caffeine, cells from MIC concentrations and above were selected. An aliquot of 10 µl cell suspension from these wells were spread on YPD agar. These plates were incubated at 30°C temperature for 48 h and observed for the presence of colonies. Absence of visual growth (no appearance of colonies) was considered as fungicidal concentration (Raut et al., 2013).

Adhesion assay

Effect of caffeine on adherence of *C. albicans* to a solid surface (that is, polystyrene) was studied by using micro plate based assay (Raut et al., 2013). 1×10^7 cells/ml was allowed to adhere to polystyrene surface of 96 well plates, in the presence of 1.56 to 50 mM of caffeine in PBS. Wells without caffeine were kept as a control, while fluconazole was used as a standard antifungal. The plates were incubated at 37°C for 90 min at 100 rpm in an orbital shaking incubator to allow attachment of cells on the surface. After the incubation, wells were washed with PBS to remove non-attached cells. Density of the adherence in each well was analyzed by using XTT metabolic assay, and percentage of adhered cells was calculated as compared to that of control.

Biofilm formation

C. albicans biofilms were developed on polystyrene surface of 96-well plates as per standard methodologies (Raut et al., 2013). 100 µl of 1×10^7 cells/ml cell suspension in PBS was inoculated and plates were incubated at 37°C for 90 min to allow attachment of cells on the surface. Non-adhered cells were removed by washing the wells with sterile PBS, two to three times. 200 µl of the RPMI-1640 medium was added to each well and the plates were incubated at 37°C for 48 h to allow biofilm formation. To observe effect on development of biofilms, RPMI-1640 medium with

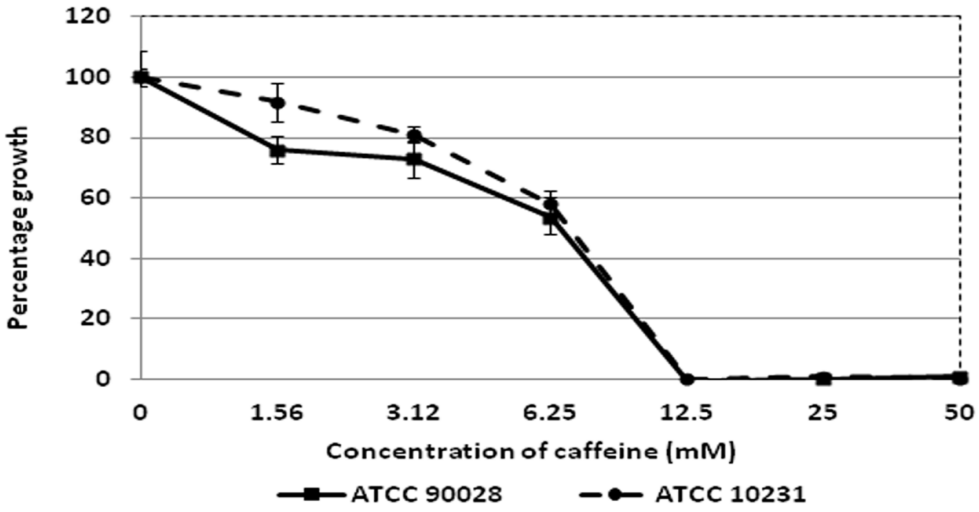

Figure 1. Growth of *C. albicans* (ATCC 90028 and ATCC 10231), in the presence of caffeine. MIC was determined as per the CLSI guidelines. Growth after 48 h was analyzed in terms of absorbance at 620 nm and percentage of growth as compared to that of the control was calculated.

concentrations of caffeine (from 1.56 to 50 mM), was added immediately after adhesion phase. To analyze activity against mature biofilms of *C. albicans*, caffeine was added to 24 h mature biofilms and the plates were incubated for 48 h, at 37°C. In both experiments, fluconazole was used as standard antifungal drug in the range 1 to 256 µg/ml. After incubation, wells were washed to remove any planktonic cells, and biofilms were observed using an inverted light microscope (Metzer, India). Photographs were taken by Labomed microphotography system at 200× magnification. Biofilm growth was analyzed with XTT metabolic assay.

Biofilm quantitation by XTT assay

Biofilm growth was quantitated using XTT. The XTT solution was prepared by mixing 1 mg/ml XTT salt in PBS and stored at -20°C. Prior to use, menadione solution prepared in acetone was added to XTT to a final concentration of 4 µM. The wells containing biofilms were washed with PBS to remove non-adhered cells and incubated for 5 h in 100 µl of XTT-menadione solution in dark. Absorbance of the color formation by water soluble formazan product was measured at 450 nm. Wells without caffeine were considered as control, while those without biofilms were the blank.

Statistical analysis

All the experiments were carried in three replicates and the values mentioned are mean with standard deviations.

RESULTS

Growth of *C. albicans* was inhibited in the presence of caffeine

Caffeine inhibited growth of *C. albicans* in a concentration dependent manner. Concentration of 6.25 mM showed 40 to 45% inhibition of growth in *C. albicans* ATCC 90028 and 10231, as compared to that of the control. Complete prevention of growth after 24 and 48 h of incubation was observed at 12.5 mM concentration of caffeine. Two strains, ATCC 90028 and 10231 were similar in their sensitivity to caffeine (Figure 1). Exposure to caffeine did not affect viability of *C. albicans*. Cells incubated in caffeine survived at concentrations as high as 50 mM, indicating that caffeine is not *Candida*-cidal. Strain ATCC 90028 was sensitive to fluconazole with MIC at 1 µg/ml while ATCC 10231 was resistant requiring 64 µg/ml concentration to inhibit planktonic growth.

Caffeine exhibits anti-adhesion properties

Adherence of *C. albicans* cells to polystyrene was not influenced by caffeine up to 6.25 mM concentration and XTT metabolic activity similar to that of the control was observed. Analyzing the density of adhered cells with XTT assay showed up to 25% decrease in adhesion at 12.5 to 25 mM caffeine. Addition of caffeine significantly inhibited adhesion of cells to the solid surface. Around 30% of *C. albicans* cells were found to adhere to polystyrene in the presence of 50 mM caffeine (Figure 2). Adhesion of two strains of *C. albicans*, ATCC 90028 and 10231 was found insensitive towards fluconazole treatment up to 64 µg/ml concentration, showing no significant reduction in relative metabolic activity (RMA) by XTT assay.

Biofilm development was inhibited by caffeine

Analysis of biofilm growth by using XTT-metabolic assay and microscopy showed that addition of caffeine at

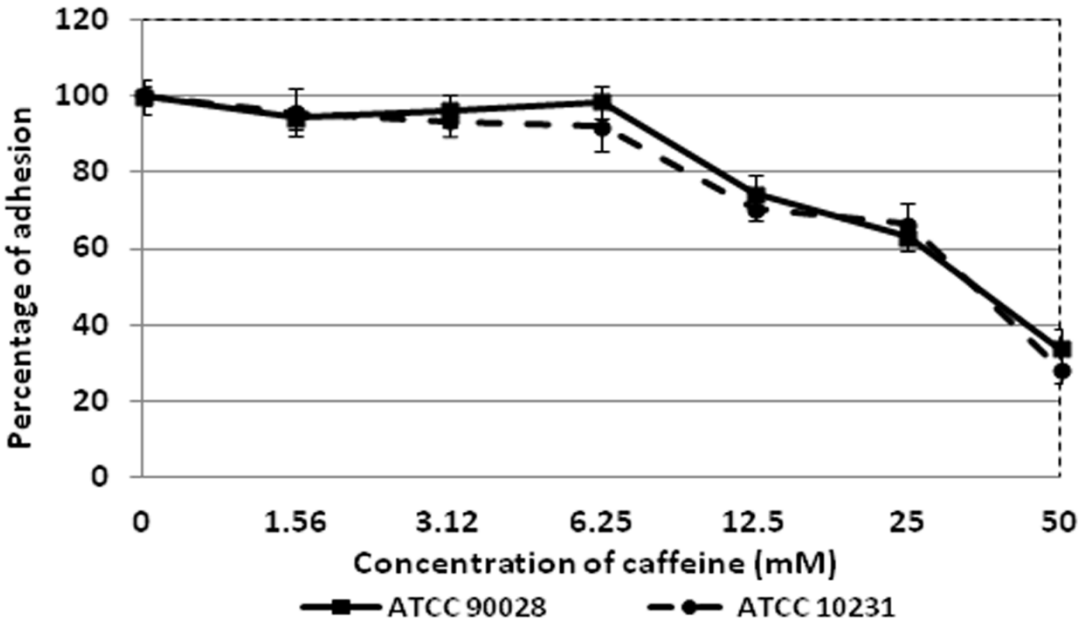

Figure 2. Effect of caffeine on adhesion of *C. albicans* cells to polystyrene surface was studied in a microplate based assay. Density of adhered cells was analyzed by using XTT metabolic assay, in terms of absorbance of colored end product at 450 nm. Relative metabolic activity (RMA) in treatment as compared to the control showed inhibition of adhesion in presence of caffeine.

adhesion phase prevented biofilm formation by *C. albicans* ATCC 90228 and 10231. Concentration of 6.25 mM of caffeine did not affect development of biofilms, while 12.5 mM caused 20 to 25% reduction in biofilm growth as compared to that of the control biofilms. Interestingly, at this concentration, most of the cells were found to be in yeast form and dense matrix of the filamentous forms was absent. At 25 mM of caffeine, biofilm formation was inhibited completely and only few adhered cells were observed. This was also confirmed by XTT assay which showed significant (50 to 60%) reduction in RMA as compared to that of the control (Figures 3 and 4A). Mature biofilms were comparatively less sensitive to caffeine's inhibitory activity. Caffeine at a concentration of 12.5 mM exhibited 20% reduction in metabolic activity for biofilms of *C. albicans* ATCC 90028 and 10231. Around 30% inhibition was observed in the presence of caffeine concentrations of ≥25 mM (Figure 4 B). Biofilm development and mature biofilms were well tolerant to fluconazole. No significant reduction in biofilm could be achieved even at 256 µg/ml concentration.

DISCUSSION

Considerable amount of caffeine ingested through beverages (Baker et al., 2006) may exert interesting effects on oro-pharyngeal and gastrointestinal micro flora including *C. albicans*. The aim of this study was to analyze the effects of this common dietary chemical on planktonic and biofilm growth as well as adhesion in *C. albicans*. Results indicated that caffeine exhibits fungistatic activity against planktonic growth of *C. albicans* at low concentrations. Strain ATCC 10231 which showed fluconazole resistance was also susceptible to caffeine. Exposure to caffeine did not affect viability of *C. albicans*. Cells incubated in caffeine survived at concentrations as high as 50 mM, indicating that caffeine is not *Candida*-cidal. Adhesion to tissue surfaces and prostheses inside the body is an important step in *Candida* colonization and infection. Interestingly, cells of *C. albicans* were found resistant to the antifungal drug fluconazole immediately after adhesion (Shinde et al., 2012). Analysis with XTT metabolic assay showed that adhesion is significantly (70%) prevented by caffeine, while it was insensitive to high (64 µg/ml) concentrations of fluconazole. Anti-adhesion property of caffeine on oral bacteria has already been reported. Our observation that caffeine significantly lowers adhesion of cells to solid surface may be used as a strategy to prevent colonization and oral infections by *C. albicans*.

A characteristic feature of biofilms is their resistance to antifungal antibiotics, including the most commonly prescribed drug fluconazole (Ramage et al., 2005; Shinde et al., 2012). Our results showed that fluconazole do not have significant effect on biofilm development and mature biofilms. While, addition of 12.5 to 25 mM of caffeine immediately after adhesion phase prevented biofilm formation by *C. albicans.* Mature biofilms were comparatively less sensitive to the inhibitory activity;

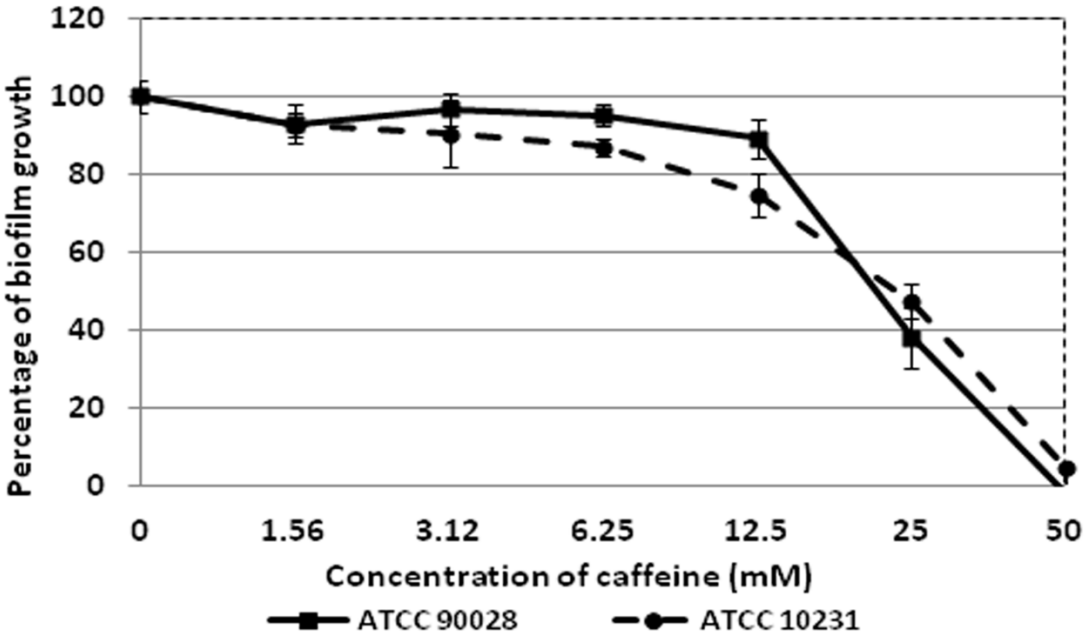

Figure 3. Biofilm formation of *C. albicans* (ATCC 90028 and ATCC 10231) in the presence of caffeine. Effect on biofilm development was analyzed in terms of relative metabolic activity (RMA) by XTT-assay. Concentration dependent decrease in biofilm growth was achieved in presence of caffeine.

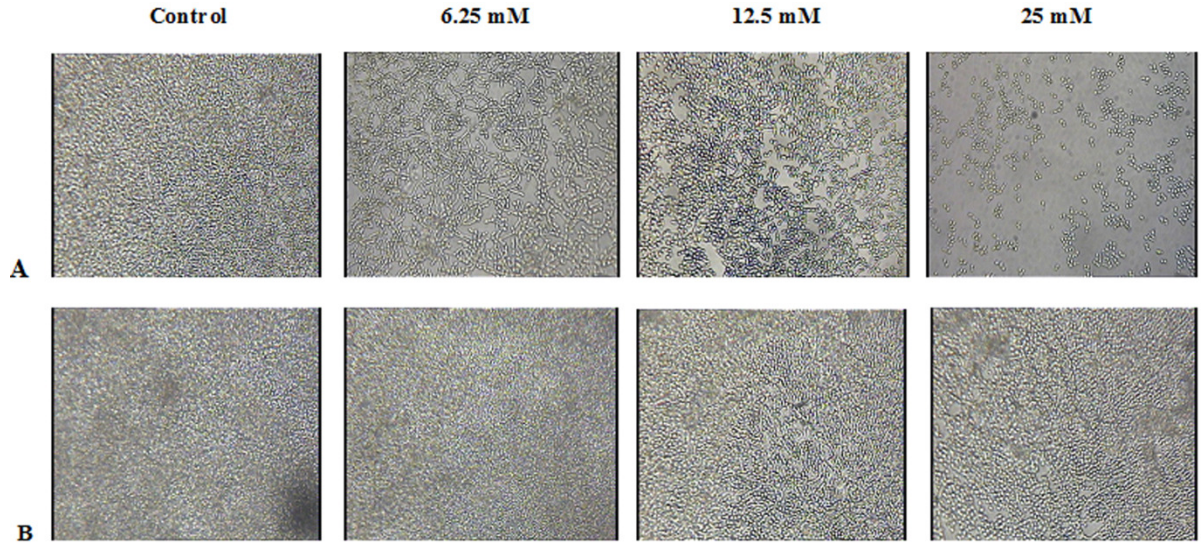

Figure 4. Panels A and B describe biofilm development and mature biofilms of *C. albicans* ATCC 90028, in the presence of various concentrations of caffeine ranging from 6.25 to 25 mM (left to right). First microphotograph in each panel shows control without caffeine (Magnification 200×). (A) Development of biofilm is inhibited in presence of caffeine. No dense biofilm matrix was observed at 12.5 mM concentration due to prevention of filamentous forms in biofilm structure. XTT assay showed 20 to 25% reduction in RMA. Complete inhibition of biofilm was achieved at 25 mM concentration and only isolated yeast form cells were seen adhered on the surface. (B) Growth of mature biofilms was less sensitive to caffeine. At 25 mM of concentration, up to 30% reduction in biofilms was observed as compared to that of control.

however, up to 30% inhibition was obtained in presence of ≥25 mM caffeine. About 400 to 600 mg/day is considered as a moderate intake of caffeine and may not have toxic effects (Healy et al., 1989). The effective concentrations in this study are in the range of caffeine content of commonly used caffeinated drinks, that is,

around 10 mM (Winter et al., 2008). Hence, even though high (in mM range), these concentrations are significant. Exact mechanism behind the antimicrobial activities of caffeine is not yet clear. A good account of work in eukaryotic model organism, *Saccharomyces cerevisiae*, speculated that caffeine's mode of action resembles that of rapamycin, a well known immuno-modulator. Target of rapamycin is TOR kinase, interfering in its function impairs TOR signaling activity in eukaryotes (Homann et al., 2009; Winter et al., 2008). TOR pathway regulates cellular activities including growth, nutrient acquisition, filamentation, cell-cell adhesion and aggregation of yeasts, in response to environmental stimuli. There is a possibility that caffeine exert anti-*Candida* effects through modulation of TOR pathway (Bastidas et al., 2009; Homann et al., 2009).

Conclusively, our study analyzed the activity of caffeine against fluconazole sensitive as well as resistant strains of *C. albicans*. Its anti-adhesion property and potential to inhibit *Candida* biofilms is revealed for the first time. Further work in this direction may give insight into novel antifungal targets and molecules for prophylaxis and treatment of *C. albicans,* especially against biofilms.

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