



Effect of β -Citronellol on Hydrolytic Enzyme Secretion, Ergosterol Biosynthesis and Biofilm Formation in *C. albicans*

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ABSTRACT

Antifungal potential of β -citronellol was studied on *C. albicans*, a commensal that causes both superficial and systemic infection in immunocompromised humans. This non-toxic natural compound displayed a minimum inhibitory concentration (MIC) of 200 μ g/ml and was fungicidal at 350 μ g/ml. On YPD agar, it formed inhibition zones (ZOI) of 16.2 mm, 18.15 mm and 19.25 mm, respectively at MIC, 2MIC and 3MIC while fluconazole (FLC) formed ZOI of 20.43 mm at its MIC (disc diffusion assay). No growth was observed above 2MIC on solid media (spot assay) and \geq MIC in liquid YPD media. Hydrolytic enzyme secretion decreased in the presence of β -citronellol and was concentration dependent. At MIC, the reduction in phospholipase activity (54.08 %) was greater than the reduction in proteinase activity (40.6 %). There was concentration dependent decrease in total ergosterol content by 19 %, 40 %, 91 % and 100 %, respectively at MIC/8, MIC/4, MIC/2 and MIC values of β -citronellol. FLC at MIC showed an inhibition of only 64%. Biofilm formation reduced by 71.13% at MIC. β -citronellol, hence has immense antifungal potential and significantly inhibits growth, ergosterol levels, hydrolytic enzyme secretion, adhesion and biofilm formation in *C. albicans* at MIC and sub-MIC values.

Keywords: *Candida albicans*; β -citronellol; antifungal susceptibility; cytotoxicity; biofilms; membrane integrity

I. INTRODUCTION

Candida albicans is an opportunistic commensal that can invade any part of the human body when conditions are favourable causing systemic infection with high mortality (Lim et al., 2012; Gow and Yadav, 2017). It becomes pathogenic in patients that are critically ill and immunocompromised like diabetics, HIV and cancer patients, people undergoing organ transplant, the elderly and people who get burnt (Enoch et al., 2017). The situation is alarming due to increasing resistance in hospital patients (Wiederhold, 2017). This pleomorphic fungus is the 4th most common source of nosocomial infections (Lim et al., 2012; Li et al., 2016). The different antifungal drugs available are toxic with unpleasant side effects. There are treatment issues due to multidrug resistance, high health care costs and low drug efficacy (Gow and Yadav, 2017). Therefore, a lot of work has to be done in the field of antifungal drug discovery and development.

Candida cells invade the host with the help of certain virulence factors. The first step of infection involves adhesion, hydrolytic enzyme secretion and biofilm formation. An increase in the secretion of hydrolytic enzymes (proteinases and phospholipases) has been linked to *Candida* virulence (Bramono et al., 2006). Subsequently the fungus invades the host cells with the help of hyphae to form resistant biofilms (Silva et al., 2011; Gow and Yadav, 2017) which constitute almost all morphological forms of the fungus enclosed in an extracellular matrix. Biofilms help the fungus to evade phagocytosis and make the fungus

less sensitive to conventional antifungals (Sardi et al., 2013; Lohse et al., 2018).

β -citronellol, a natural cyclic monoterpenoid, possesses, anti-inflammatory (Brito et al., 2012), insect repellent (Semmler et al., 2014), larvicidal (Hierro et al., 2004), and anti-bacterial (Lopez-Romero et al., 2015) properties. Like geraniol, it is a major component of rose oil and a number of other plant essential oils. Chemically, β -citronellol (dihydrogeraniol) is related to geraniol whose anti-*Candida* activity and mode of action was studied recently (Sharma et al., 2016). Although its antifungal potential has been reported (Pereira Fde et al., 2015) its mode of action is not clearly discussed. In the present study, we observed the effect of varying concentrations of β -citronellol on growth pattern, secretion of proteinases and phospholipases secretion, membrane ergosterol content and biofilm formation in *C. albicans* ATCC 10261.

II. MATERIAL AND METHODS

C. albicans ATCC 10261 was maintained on YPD media constituting yeast extract, peptone, dextrose, in a percentage ratio of 1:2:2 supplemented with 2.5% agar at 4°C. All chemicals were of analytical grade purchased from Merck (India). The media components, β -citronellol and fluconazole (FLC) were purchased from Sigma Aldrich (Germany).

2.1. Antifungal drug susceptibility assays

Broth dilution method (CLSI, 2008) was used to determine the minimum inhibitory concentrations (MIC) defined as the least possible concentration that causes 90% decrease in absorbance in comparison to that of the control (without test compound). After getting the MIC value, 15 μ L aliquots were removed from tubes that show absolutely no growth along with the last tube showing growth. These were sub-cultured on YPD agar plates and incubated at 35 °C until growth was visible in the control samples. The minimum fungicidal concentration or MFC value was determined as the minimum concentration of the test compound for which there was no visible growth (Samber et al., 2015). Results were calculated as mean of the two separate experiments with three different values.

To study antifungal susceptibility to β -citronellol (Ansari et al., 2014) by spot assay, *Candida* cells were grown overnight in YPD media at 37 °C, and suspended in 0.9% NaCl to achieve an optical density

of 0.1 at 600 nm. Aliquots (5 μ L) of five-fold serially diluted cultures were pipetted on YPD agar plates in the absence (control) and presence of MIC and sub-MIC values of β -citronellol. After 48 h of incubation at 30 °C, growth variations were observed.

Candida cells (1×10^5 cells/ml) were inoculated into YPD agar at 40 °C and poured into 90 mm petriplates to perform disc diffusion assay. Sterile filter discs (4mm) were laden with three different concentrations of test compound (MIC, 2MIC and 3MIC) and placed on agar plates. After 48 h, the average diameter of inhibition zones (ZOI) was measured. A disc impregnated with FLC at its MIC value was used as positive control.

Candida cells were sub-cultured at least two times and grown till stationary phase is reached at 35 °C on SDA plates. To study growth pattern, cell culture ($A_{595} = 0.1$) was inoculated into fresh media along with β -citronellol at MIC and sub-MIC concentrations in 50 ml total volume. Growth was monitored at 37 °C, 200 rpm and recorded after every 2 h till 48 h. Absorbance was recorded at 595 nm for each concentration using Labo-med Inc. spectrophotometer (USA) and plotted against time in hours.

2.2. WST-1 based cytotoxicity assay

The assay was performed as described previously (Khan et al., 2010). Cell culture (1×10^5 cells/ml) was taken along with β -citronellol (MIC and sub-MIC values) in 96-well plate (final volume $\sim 100 \mu$ l/well) and incubated for 24 h. WST-1/CEC dye (10 μ L) was added to each well and plates were again incubated at 37 °C for 2 h with shaking. The reaction was stopped by adding 10 μ L of 1% SDS. WST-1 salt was reduced to red coloured formazan by cellular dehydrogenases (Tsukatani et al., 2008), the absorbance of which was recorded at 450 nm using a micro plate Reader (BIORAD iMark, US) (reference was set at 655 nm). Experiment was repeated thrice and cytotoxicity was calculated using the following equation:

$$\% \text{ Cytotoxicity} = \left[\frac{\text{(cell control)} - \text{(cell with test compound)}}{\text{(cell control)}} \right] \times 100$$

2.3. Secretion of hydrolytic enzymes

For assessing the activity of proteinases and phospholipases, *Candida* cells were first inoculated into 5 ml YPD media and incubated for 18 h at 37 °C (Khan et al., 2014). Subsequently, cells were separated from culture media, washed twice and re-suspended in 0.9% NaCl. Cell suspensions (MacFarland 0.5 index) were exposed to desired

concentrations of β -citronellol ($1/8$ MIC, $1/4$ MIC, $1/2$ MIC and MIC). In case of proteinase assay, small aliquots (2 μ l) were placed at equidistant points on agar plates (2% agar, 0.2 g BSA, yeast nitrogen base, 20 g glucose, and distilled water to a final volume of 1000 ml) while in case of phospholipase assay, aliquots were pipetted on agar peptone media (2 % agar, 10 g peptone, 30 g glucose, 57.3 g NaCl, 0.55 g CaCl_2 , and distilled water to a final volume of 900 ml) enriched with 10 % (v/v) egg yolk emulsion (HiMedia). Plates were incubated at 37 °C till noticeable growth (2-4 days). Enzyme secretory activity was estimated by measuring degradation/precipitation zones formed and calculated in terms of Pz values which is the ratio of the colony diameter to the colony diameter plus diameter of the zone of degradation/precipitation (Price et al., 1982).

2.4. Adhesion and biofilm formation

These experiments were performed according to Ramage et al., 2002 with modifications. An aliquot of 100 μ l cell suspension (1×10^7 cells/ml) in RPMI 1640 media was placed into each well of a sterile 96-well microtiter plate. β -citronellol was added to each well at desired concentrations except control. Plates were incubated at 37°C with gentle shaking to allow the cells to adhere to plate walls. After 90 min, non-adherent cells were washed out of each well with PBS (150 μ l). To see effect on biofilm formation, after the initial 1 h adhesion period, freshly prepared media (RPMI 1640) containing desired concentrations of β -citronellol were added to the wells containing adherent cells. The plates were then incubated for 24 h at 37°C. Estimation was done by semi-quantitative XTT reduction assay. A saturated solution of XTT (in PBS) was mixed with menadione-acetone solution (electron mediator). Pre-formed *Candida* bio films were washed with PBS first and taken in a 96-well plate with or without β -citronellol. A 100 μ l aliquot of XTT was then added to each well and mixed gently. The plates were incubated in dark for 5 h at 37 °C after which absorbance of each sample was recorded at 450 nm using a micro titre plate reader (BIO-RAD, iMark, US). The wells containing PBS only or PBS + XTT were used as blank. The results were expressed as percentage viability.

2.5. Sterol extraction and quantitation

Sterol content of treated and untreated *Candida* cells was evaluated as discussed earlier with slight changes

(Sharma et al., 2016). Briefly, 50 ml of SD broth (along with varying concentrations β -citronellol) was inoculated with a single colony from a culture plate grown overnight. After an incubation of 16 h at 35 °C (180 rpm), cells were harvested and washed with sterile distilled water. To each pre-weighed pellet, 3 ml of 25% alcoholic KOH was added and vortexed for 60 sec. Cell suspensions were then poured into sterile borosilicate glass tubes and incubated at 85 °C for 1 h. Tubes were cooled to room temperature and sterols were extracted by adding sterile distilled water and n-heptane in the ratio of 1:3. The contents were mixed thoroughly before transferring the heptane layer to a clean borosilicate glass tube and stored at 20 °C for 24 h. The extracted sterols were diluted five-fold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm using Labomed, Inc. spectrophotometer (USA). Both a positive control (FLC) and a negative control (without test compound) were also included. The ergosterol content (%) per wet weight of cells was calculated as done in previous studies (Sharma et al., 2016).

2.6. Statistical Analysis

All the assays were conducted in triplicate, and the results were exhibited as mean \pm standard deviation. The student's t-test was used to verify statistical significance ($p < 0.05$).

III. RESULTS AND DISCUSSION

3.1. Antifungal susceptibility of β -citronellol against *C. albicans*

The antifungal efficacy of β -citronellol was estimated by performing drug susceptibility tests on *C. albicans* ATCC 10261. MIC was evaluated using broth micro dilution assay. *Candida* cells showed susceptibility to β -citronellol at an MIC of 200 μ g/ml while the conventional drug FLC gave an MIC of 10 μ g/ml. This natural monoterpenoid was fungicidal above 350 μ g/ml (MFC). Disc diffusion assay was performed where sterile filter discs were impregnated with the test compound and placed on the YPD agar surface. After incubation the diameters of the zone of inhibition (ZOI) formed around the discs were measured and found to be concentration dependent (Fig. 1). The diameters were 16.2, 18.15 and 19.25 mm at MIC, 2MIC and 3MIC values of β -citronellol. FLC (positive control) gave a ZOI diameter of 20.43 mm at 10 μ g/disc.

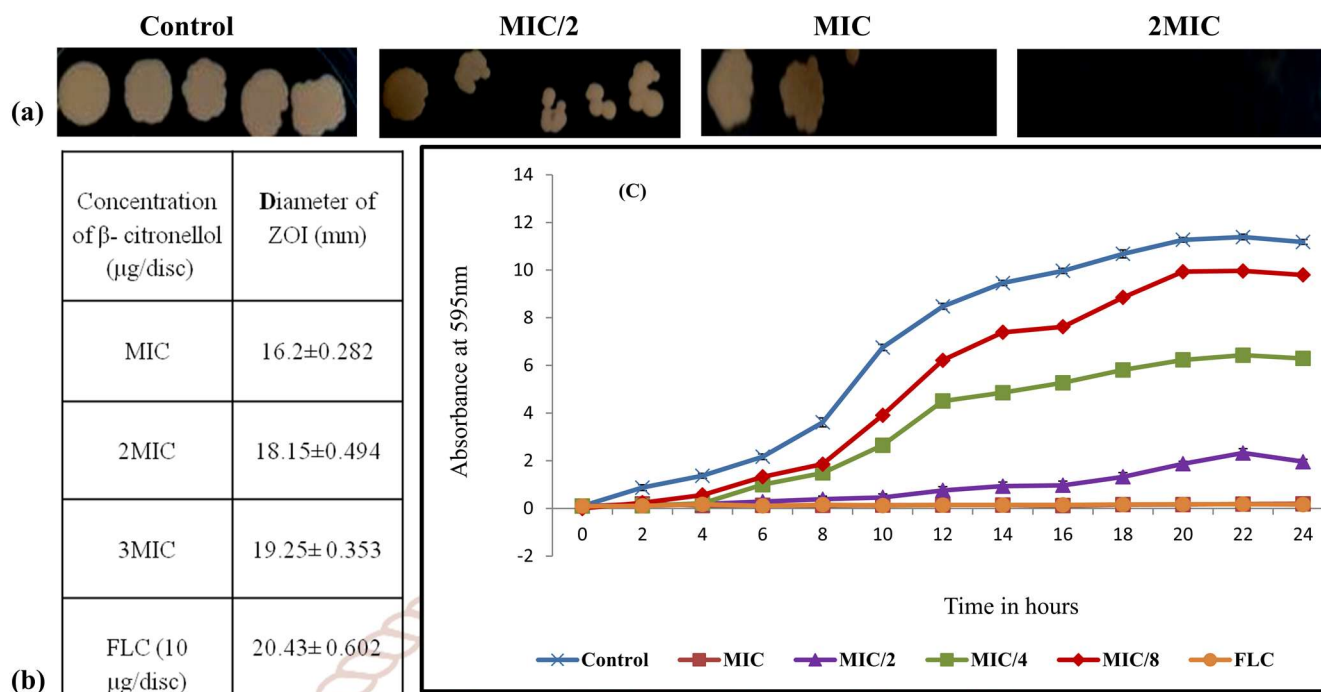
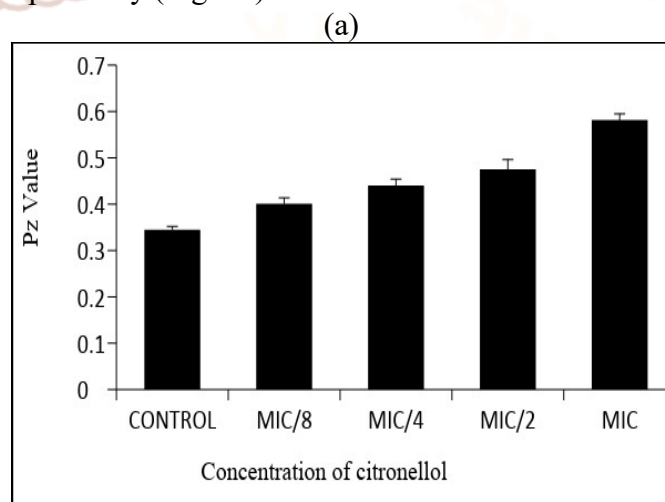


Figure 1: Antifungal susceptibility of β -citronellol to *C. albicans* ATCC10261. (a) Spot assay at MIC/2, MIC and 2MIC (b) Diameters of zone of inhibition formed on YPD agar in the presence of MIC, 2MIC and 3MIC concentrations of test compound and fluconazole (FLC) at MIC. (c) Effect of β -citronellol on *Candida* growth pattern.

The susceptibility of *C. albicans* to β -citronellol was confirmed by the spot assay also (Fig. 1). In comparison to control, a significant decrease in growth was observed at MIC/2. At MIC, growth was observed only for the first two dilutions. No growth was seen at and above 2MIC. Growth pattern of *Candida* cells was studied in the absence and presence of the test compound at different concentrations. The inhibitory effect was concentration dependent leading to significant decline in growth of cells with late lag-phase, undifferentiated and delayed exponential-phase (Fig. 1). On the other hand, control cells showed a regular growth pattern with a 4 h lag-phase followed by an exponential-phase of 8-10 h and a stationary-phase. The growth was significantly suppressed in the presence of β -citronellol at sub-MIC concentrations of MIC/2 and MIC/4 while at MIC, the growth curve was seen as a flat line similar to that of FLC. At MIC/8 the growth pattern was similar to the control although the growth was inhibited to some extent. All the three growth studies corroborated well with each other.

3.2. Effect of β -citronellol on secretion of hydrolytic enzymes

Extracellular hydrolytic enzymes, especially proteinases and phospholipases play a vital role in fungal pathogenesis and tissue invasion (Silva et al., 2011). Proteinase and phospholipase activity was studied in the absence and presence of varying concentrations of β -citronellol. The Pz values were calculated and plotted for each concentration (Fig. 2). A concentration dependent inhibitory activity was observed for both the enzymes released by the fungus. Proteinase secretion decreased by 13.9 %, 21.3 %, 27.3 % and 40.6 % at MIC/8, MIC/4, MIC/2, and MIC values of β -citronellol, respectively (Fig. 2a). Similarly, secretion of phospholipases decreased by 9.4 %, 17.24 %, 24.75 % and 54.08 % in the presence of β -citronellol at the same concentrations respectively (Fig. 2b).



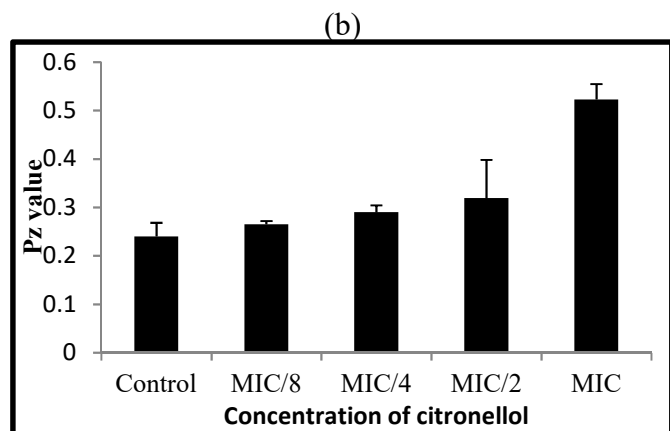
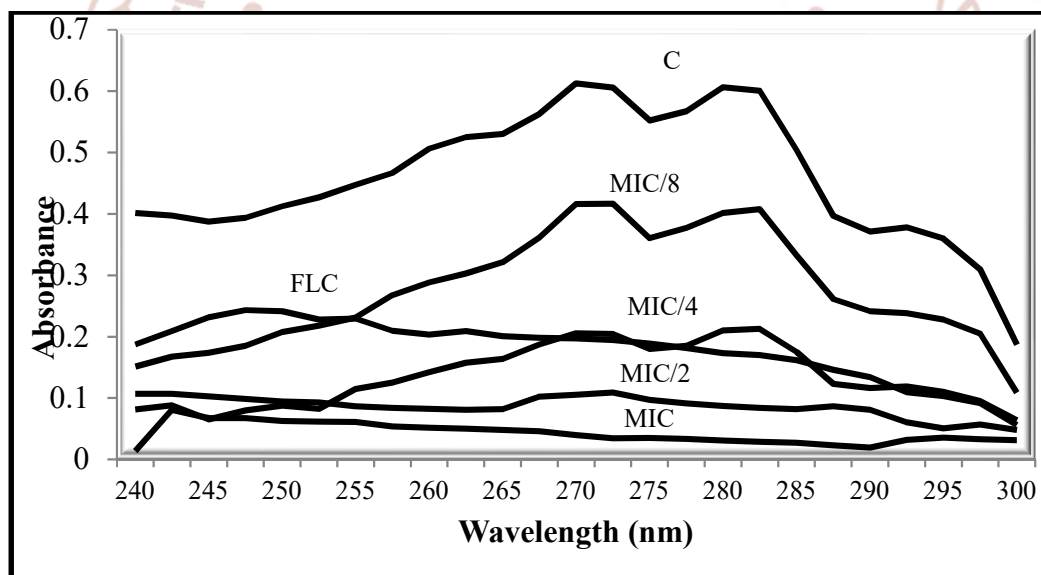


Figure 2: Hydrolytic enzyme secretion by *C. albicans* ATCC 10261 in the absence and presence of different concentrations of β -citronellol. (a) Proteinase secretion (b) Phospholipase secretion. Pz value is the ratio of the diameter of the colony to the diameter of the colony plus zone of degradation/precipitation. The values are mean of 3 different recordings.

3.3. Effect β -citronellol on ergosterol biosynthesis

Ergosterol is unique to fungi and hence has been

crucial as an antifungal target. The mechanism of action of some antifungal drugs involve either their binding to this sterol as in polyenes or inhibition of its biosynthesis as in azoles (Ghannoum & Rice, 1999). Although ergosterol is not found in the human host, these drugs besides being toxic with side effects also induce resistance in the long run. Hence total ergosterol levels were estimated in the presence of varying concentration of β -citronellol, a non toxic natural compound that has promising antifungal properties. Fig. 3 shows the sterol profiles of *C. albicans* in the presence of MIC and sub-MIC concentrations of β -citronellol. 10 μ g/ml FLC was also taken as positive control. A significant percentage decrease was observed with increasing concentrations of the test compound. The decrease in total ergosterol content was 19%, 40%, 91% and 100%, respectively at MIC/8, MIC/4, MIC/2 and MIC concentration values. Interestingly, although FLC is a conventional antifungal drug whose mechanism of action involves inhibition of ergosterol biosynthesis, showed an inhibition of only 64% at its MIC value against *C. albicans* ATCC 10261 (Fig. 3).



Test Compound	Ergosterol Content (% wet weight of cells \pm SD)
Control	0.004723 \pm 0.16 (0)
MIC/8	0.00383 \pm 0.09 (19)
MIC/4	0.0028419 \pm 0.13 (40)
MIC/2	0.000441 \pm 0.003 (91)
MIC	0 (100)
FLC	0.0017366 \pm 0.041 (64)

Figure3: Total ergosterol content and spectrophotometric profile of *C. albicans* ATCC 10261 when grown in the presence of MIC and sub-MIC values of β -citronellol. Student's t- test was used for statistical analysis ($p < 0.05$).

3.4. Effect of β -citronellol on biofilm formation

C. albicans associated biofilms are well protected in an extracellular matrix. They are hence difficult to treat with acquired resistance towards available antifungal drugs (Mukherjee et al., 2005; Hirota et al., 2017). Majority of the biofilms are commonly formed by *C. albicans* rather than the non-*albicans Candida* species (Kuhn et al., 2002). XTT reduction by the metabolic activity of cells was used to study the inhibitory activity of β -citronellol on *Candida* biofilms. Biofilm formation was inhibited by 71.13 % when treated with MIC concentration of β -citronellol. At sub-MIC concentrations of MIC/2 and MIC/4, the inhibition in biofilm formation was reduced to 42.94 % and 20.5 %, respectively (Fig. 4). Inhibitory effect of test compound on biofilm formation was clearly dose dependent. The effect of DMSO (solvent control) against biofilms was only 5 % inhibition.

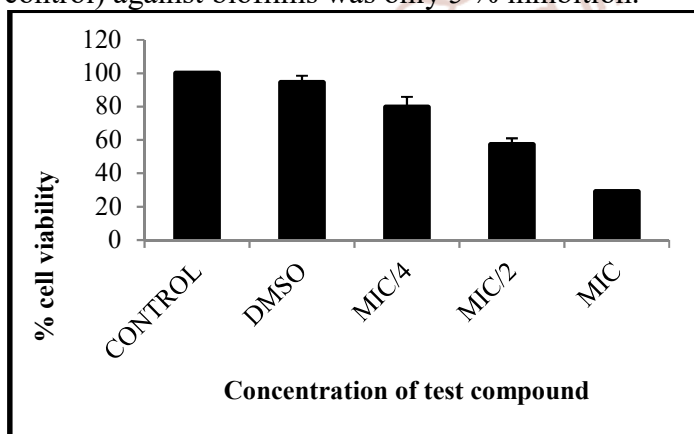


Figure 4: Effects of varying concentration of β -citronellol (MIC/4, MIC/2, and MIC) on biofilm formation in *C. albicans* ATCC 10261. Cell viability was estimated using the XTT reduction assay. DMSO was used as control.

3.5. Effect of β -citronellol on cell viability

The viability of *Candida* cells was studied in the presence of β -citronellol by a colorimetric method (WST1 cytotoxic assay) based on the metabolizing activity of mitochondria of living fungal cells (Kuhn et al., 2003). XTT is converted to formazan, a water soluble dark yellow coloured product that was measured in supernatants at 420-480nm (optimal at 440nm). This value directly correlates with the cell number. Cells cultured in micro plates were incubated with WST-1 and the assay was monitored spectrophotometrically. β -citronellol subjected $\geq 90\%$ cytotoxicity at its MIC value (Fig. 5). It recorded a cytotoxicity of 70-82 % at MIC/2 and only 29-51 % at MIC/4. β -citronellol hence possesses high cytotoxic potential towards *C. albicans* and affects the metabolic activity of yeast cells leading to decreased

growth and viability. The fact that β -citronellol causes only 1-2 % haemolysis in contrast to FLC and AmB, which causes $\sim 6.48\%$ and 10.35% respectively at 5MIC (unpublished data), shows that this compound has negligible toxicity at its MIC and MFC values.

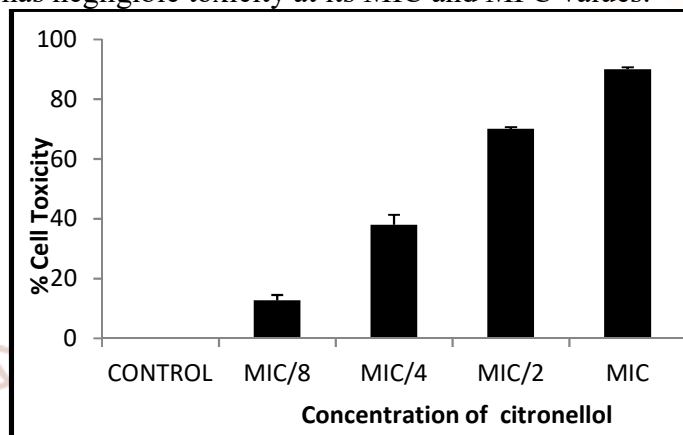


Figure 5: WST-1 cytotoxicity activity of β -citronellol against *C. albicans* ATCC 10261. Error bars represent SD to the mean value from 3 different recordings

III. CONCLUSION:

Infections caused by the pathogenic fungus *C. albicans* can be controlled by using β -citronellol, a plant essential oil constituents having significant antifungal potential. It has inhibitory effects on the growth pattern, adhesion and biofilm formation properties of this commensal. It also lowers the total ergosterol content of the cell and decreases its viability. Additional studies should to be performed to understand mechanism of antifungal action of β -citronellol both *in vitro* and *in vivo*.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest from the authors regarding publication of this article.

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