Thidiazuron, a phenyl-urea cytokinin, inhibits ergosterol synthesis and attenuates biofilm formation of *Candida albicans*

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Abstract

Candida albicans is a common human fungal pathogen that colonizes mucosa and develops biofilm in the oral cavity which cause oral candidiosis. This study investigates the effects of a new molecule thidiazuron against the growth and biofilm formation properties of *C. albicans*. Preliminary molecular docking study revealed potential interaction between thidiazuron and amino acids residues of cytochrome P450 mono-oxygenase (CYP51). Further in vitro anti-fungal susceptibility test, scanning electron microscopy (SEM) and time kill analysis revealed potential anti-fungal activity of thidiazuron in both dose and time dependent manner. Crystal violet staining, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay and acridine orange staining visually confirmed biofilm inhibitory potential of thidiazuron. Gene expression study shows that thidiazuron treatment down regulated the expression of genes involved in ergosterol synthesis, cell adhesion and hyphae development in *C. albicans*. This study identified thidiazuron as a new CYP51 inhibitor and a novel antibiofilm agent against *C. albicans*.

Introduction

Fungal infections are increasing nowadays due to changes in lifestyle, pollution and increased systemic risk factors like diabetes, auto-immune diseases and immune-suppressant drugs. Many fungi form biofilm in human muco-dermal infections and on the biomaterials used inside the body. Varieties of anti-fungal agents are available and still drug resistance is often encountered. Conventional antibiotic therapy that leads to antimicrobial resistance is frequently responsible for biofilm-associated infections. So, there is a need for search of new biomolecules exhibiting good anti-fungal activity. One of the commonest opportunistic fungal infections in human and more so in the oral cavity is *Candida* species and prevention of its biofilm and recurrence is vital in the treatment of candidiasis. *Candida albicans* is a fungus that is a major source of device-associated infection due to its potential biofilm formation trait.

Biofilm is a community of microbes produced by the extracellular polymeric factors (Donlan 2001). It has two main components such as water and densely packed cells. Biofilm formation is a complex process to convert planktonic to sessile mode of cell growth (Okada et al. 2005). Biofilms are a potential cause for the establishment of infections and forms biological layering in medical devices such as catheter, suction tubes, prosthetic tubes etc., and it also forms reflux process during drug resistance (Sims et al. 2005; Ganguly and Mitchell 2011). *C. albicans* biofilm leads to superficial and systematic infections in the human host and it occur frequently in the mucosa or endothelial tissues (Donlan 2001). In *C. albicans*, the extracellular polymeric substances develop the pathogenic infections and multidrug resistance, by stages of adherence, proliferation, maturation and dispersion (Harikrishnan et al. 2013; Nett 2016; Lohse et al. 2018). Various antibiofilm studies reported that inhibition of biofilm formation in *C. albicans* by chemically synthesized azole molecules and natural plant molecules pretending the toxics and non-target effects. The search of new biomolecules against biofilm formation will improve the management of fungal diseases (Khan et al. 2017; Hu et al. 2018; Simonetti et al. 2019). Already few biomolecules were described against the *C. albicans* biofilms, such as *trans*-resveratrol, terpenes, catechins, stilbenes, quercetin, tannins, anthocyanins, and catechins, which inhibit the growth of extracellular matrix formation and reduce toxicity (Simonetti et al. 2019).

Ergosterol is a fungal cell membrane component, which regulates the membrane structure fluidity, permeability, mobility and stabilizes the membrane structure binding through phospholipids (Krumpe et al. 2012). It also functions as a fungal hormone which stimulates the growth and plays an essential role in oxidative stress during fungi maturation (Khan et al. 2017). An alteration in ergosterol biosynthesis revealed that modification of cell membrane fluidity and progression of drug efflux mechanisms leads to release of electrolytes from the endogenous cell membrane (Kumari et al. 2019). Taken together, these modifications depolarize the mitochondrial membrane potential activity and lead to reaction oxygen species (ROS) production (Hu et al. 2018). Lipid peroxidation activity is increased by mitochondrial stress that regulates the reciprocal formation of biofilm and cell membrane structural modifications in *C. albicans* (Khan et al. 2017). Ergosterol biosynthesis pathway is governed by expression pattern of different *ERG* genes (*ERG1*, *ERG3*, *ERG7*, *ERG9*, *ERG11* and *ERG25*), whose

products are involved in the synthesis of biofilms in *C. albicans* (Borecká-Melkusová et al. 2009). Among ERG genes, ERG11 gene encoding CYP51 enzyme plays a major role in ergosterol synthesis (Morschhäuser 2002). (Mukherjee et al. 2003). It was also reported that the levels of total ergosterol were significantly lower at later stages of biofilm formation than planktonic cells. But, it was noticed that fluconazole decreased the expression of ERG11 gene in fluconazole sensitive *C. albicans* biofilm, whereas it increased the expression of ERG11 gene in fluconazole-resistant *C. albicans* biofilm(Borecká-Melkusová et al. 2009).

Based on the earlier reports it is hypothesized that maturation of biofilm in fluconazole-resistant *C. albicans* was associated with increased ERG11 expression and subsequent ergosterol synthesis. Thus, screening of CYP51 inhibitor could be highly helpful for controlling Candida infections and its biofilm associated with artificially implanted medical devices. So, new molecules which can inhibit CYP51 will be of potential benefit in the treatment of candidiosis. Hence, in this present study, thidiazuron (TDZ) was docked against ergosterol biosynthesis enzyme (CYP51) and its invitro analysis was done by quantifying total sterol, biofilm fluorescent staining and mRNA quantification of biofilm responsive genes in *C. albicans*.

Materials And Methods

Preparation of protein, ligand and molecular docking study

The 3D X-ray crystallographic structures of sterol 14-alpha demethylase enzyme (CYP51, PDB code: 5TZ1) was retrieved from RCSB database (Hargrove et al. 2017). The structure of thidiazuron (PubChem CID: 40087) used in this study was retrieved from pubchem database (https://pubchem.ncbi.nlm.nih.gov/compound/) as .sdf format and latter converted to .pdb format using PyMOL software. Docking calculations were executed using AutoDock (version 1.5.2 revision 2) as described in the literature (Morris et al. 2009; Emeka et al. 2020). The docked conformations of each ligand were ranked into clusters based on the binding energy and the top ranked conformations were visually analyzed with PyMOL software.

Chemicals, microbial strains and culture conditions:

C. albicans strain MTCC 183 obtained from the Institute of Microbial Technology, Chandigarh, India were cultured in sabouraud dextrose broth (SDB) (1% yeast extract, 1% peptone, 4% glucose, 1% agar) and maintained at 4 °C. Standard cell suspensions were prepared by inoculating single colony of *C. albicans* in tryptone soya broth (TSB) medium containing 1% glucose and overnight incubation at 37 °C at 200 rpm in a shaker. Thidiazuron (TDZ) extrapure, 97% was purchased from Sisco research laboratories (SRL, Chennai, India). Unless indicated all the chemicals and media used in the present study were purchased from Himedia (Mumbai, India).

Antifungal Activity of thidiazuron:

Overnight grown fungal cells were collected by centrifugation and subsequently washed in phosphate- buffered saline (PBS) and resuspended to 1×10^6 CFU/mL using TSB medium with 1% glucose. Antifungal activity of thidiazuron (200 to 25µM) and fluconazole (25 µg) were screened using disk diffusion method in Mueller-Hinton agar. In brief, Mueller-Hinton agar was spread with *C. albicans* (1×10^6 CFU/mL) and the disk containing test antifungal agent was placed and subsequently incubated for 24 to 48 h at 37 °C. The results were interpreted based on measuring the diameter of the zone of inhibition.

Planktonic minimal inhibitory concentration (PMIC)

The minimal inhibitory concentration (MIC) values of planktonic suspending cells for *C. albicans* was determined in microtitre plates by broth micro-dilution according to the clinical and laboratory standards institute guidelines, document M27-S4 (Clinical and Laboratory Standards Institute [CLSI] 2008). Plates were prepared under aseptic conditions. To each well, 100 µL of thidiazuron (200µM) and fluconazole (100 µg/mL) in 10% (v/v) DMSO or sterile water was pipetted into the first row of the plate and serially diluted. Finally, 10 µL of fungal suspension (1×10^6 CFU/mL) was added to each well and incubated for 24 h at 37 °C. After incubation, 30 µL of resarzurin (0.015%) was added to each well and further incubated for

2-4 h for the observation of color change (Menon et al. 2012). Plate absorbance was then read at 570 nm and concentration of thidiazuron/fluconazole that inhibited 50% of cell growth was defined as PMIC.

Scanning electron microscopy (SEM) analysis

Planktonic cells of *C. albicans* (1 x 10^6 CFU/mL) were prepared in 2mL of SDB broth, to which thidiazuron and fluconazole at 1X $PMIC_{50}$ were treated and incubated for 24 h at 37 °C. Immediately after incubation, the cells were harvested by centrifugation and washed twice with sterile PBS. Immediately, the samples were dehydrated for 5 minutes with series of increasing concentration of ethanol (50, 70, 90, and twice at 100%). The dehydrated samples were placed overnight in a vacuum oven at 25 °C, then sputter-coated with gold, scanned and imaged using JEOL High Resolution Scanning Electron Microscope (HRSEM) (Thermoscientific Apreo S, Netherlands).

Time-Kill Assay for *C. albicans* towards thidiazuron

The time kill assay of *C. albicans* towards thidiazuron was performed as described earlier(Ali et al. 2010). A cell suspension of *C. albicans* (1 x 10^6 CFU/mL) was prepared in tubes containing 8 mL of SDB broth, to which different MIC folds of thidiazuron (0.5, 1, 2, 4 and 8X) were added and appropriate controls were maintained. All tubes were incubated in a shaking incubator at 37 °C for 24 h. Fungal cell suspensions (1 mL) were collected at time intervals of 0, 6, 12, 24, and 48 h, serially diluted in SDB, and then plated out on Sabouraud dextrose agar (SDA). After incubation for 24 h at 37 °C, colony-forming units (CFU) were counted for individual samples and analyzed.

Inhibitory potential of thidiazuron against *C. albicans* biofilm

The *C. albicans* biofilm was measured by crystal violet staining and XTT assay. In brief, the cell suspension of *C. albicans* was determined using a haemocytometer Neubauer improved chamber and adjusted to 1 x 10^6 CFU/mL in RPMI 1640 medium supplemented with 2% (w/v) glucose. To a 96 well flat bottom cell culture plate 200µl of cell suspension along with various concentration of thidiazuron (100 to 6.25 µM) in RPMI medium was co-incubated for 48 h at 37 °C. Appropriate media and culture controls were also maintained in parallel to the thidiazuron treatment. Later, the supernatant along with suspended planktonic cells were removed and the biofilm was washed twice with sterile PBS. Subsequently, the biofilm in each well was stained with freshly-prepared crystal violet solution (100 µL, 0.1%, w/v) and incubated for 10 min. Then, the unbound stains were removed and the wells were washed with sterile distilled water. Plates were then rocked in 95% ethanol at room temperature for 30 min and absorbance recorded at the wavelength of 595 nm (Emeka et al. 2020).

For XTT assay, the supernatant containing unbound cells and media components was removed from each well, washed twice using sterile PBS and 100 µL of fresh/sterile medium was added to each well. The phenazine methosulfate (PMS) solution was prepared by dissolving 3 mg PMS in 1 mL of 1X PBS. The XTT solution was prepared by dissolving 4 mg XTT in 4 mL of culture medium. Working detection solution was prepared by mixing 10 µL of the PMS solution to the 4 mL of XTT solution. Then immediately, 50 µL of detection solution made in the previous step was added to each well and incubated for 4 h at 37 °C. Plates were kept in a shaker for a short period of time (10 seconds) to mix the dye in the solution and absorbance recorded at 450 nm(Roehm et al. 1991). The percentage of biofilm inhibition was determined according to an earlier study (Subramenium et al. 2018) and 80% inhibition of biofilm formation was considered as minimum biofilm inhibitory concentration (MBIC).

Fluorescent microscopic analysis of biofilm inhibitory activity of thidiazuron

C. albicans biofilms were cultured in a 12 well culture plate with different concentrations of TDZ (0, 6.25, 12.5, 25, 50, 100 µM) in RPMI 1640 medium supplemented with 2% (w/v) glucose for 48 h at 37 °C. The wells were washed trice with sterile PBS to remove unbound cells and stained for 30 min in the dark with acridine orange. Fluorescent microscope (Optika, Germany) was used to record image stacks in five random locations at 40X magnification. In each experiment, the light intensity, background level, and contrast were maintained at the same level.

Ergosterol Biosynthesis assays:

The *C. albicans* cells (1 x 10^6 CFU/mL) were inoculated in 50 mL of SDB with TDZ (value µM) or DMSO as the control and incubated in a shaking incubator at 37°C for 18 h. Immediately after incubation, cell biomass centrifuged at 2700 rpm for 5 min, washed and weight of individual pellets were recorded. The pellet in each tube were treated with 3 mL of alcoholic potassium hydroxide solution (25%), vortexed for 1 min and incubated in a water bath at 85°C for 60 min. The tubes were then cooled and sterol extraction was conducted via vortexing the samples in water : n-heptane mixture (1:3) for 3 min. For analysis, 1 mL of sterol extracts with five-fold ethanol (100%) were subjected to scanning between wavelengths of 240 to 300 nm in UV/VIS Spectrophotometer (LABMAN Scientifics, India). The cells treated with fluconazole and DMSO were considered as positive and negative controls respectively. The levels of ergosterol was calculated and expressed as percentage in terms of weight of the pellet using the below equation:

$$\%\text{Ergosterol} + \%24(28)\text{DHE} = \left[\left(\frac{A281.5/290}{\text{Pellet weight}}\right) \times F\right]$$

$$\%24(28)\text{DHE} = \left[\left(\frac{A230/518}{\text{Pellet weight}}\right) \times F\right]$$

%Ergosterol = %ergosterol+%24(28)DHE−%24(28)DHE,

Where, F - factor for dilution in ethanol; 290 – E value (in percentages per centimetre) determined for crystalline ergosterol; 518 – E values (in percentages per centimetre) determined for 24 (28) DHE.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) based gene expression analysis of biofilm markers

C. albicans (1×10^6 CFU/mL) were cultured in a 12 well culture plate in RPMI 1640 medium supplemented with 2% (w/v) glucose and allowed for adhesion. The wells with 2 mL of RPMI 1640 medium containing TDZ (µM) or DMSO as the control were then incubated statically at 37°C for 24 h. On completion of incubation period, mycelial samples were collected and frozen in liquid N_2, and ground into fine powder. Total RNA extraction was performed according to the standard manufacturer's protocol using TRIzol reagent (Ambion, USA). For gene expression analysis, reverse transcription was performed using PrimeScript™ 1st strand cDNA Synthesis Kit (TAKARA BIO INC, Japan) following the manufacturer's instructions. Reverse transcription (RT) reactions contained 3 µg of total RNA samples, 1 µL of random Primer (50 µM), 1 µL of dNTP Mixture (10 mM each), 4 µL of 5X PrimeScript Buffer, 0.5 µL of RNase Inhibitor (40 U/µL), 1 µl of PrimeScript RTase (200 U/µL) and were topped off to 20 µL with Diethylpyrocarbonate (DEPC) treated water. The thermal profile for RT consisted of incubation at 30°C for 10 min, 42°C for 60 min and termination of the reaction at 95°C for 5 min. The quantitative PCR reactions, which were prepared to a final volume of 25 µL, included 12.5 µL of 2 × SYBR® Select Master Mix (Applied Biosystems, USA), 10 µM forward/reverse primers, and 1 µL of undiluted cDNA. Primers used in the present study were shown in Table 1 (Theberge et al. 2013). Quantitative RT PCR was performed using a Rotor-Gene Q 2PLEX HRM Real-Time PCR system (Qiagen, Netherlands). The amplification protocol involved enzyme activation at 50°C for 2 min, denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Three independent experiments were carried out and each cDNA sample was analyzed in triplicates. The average threshold cycle (CT) values were used to calculate relative expression levels normalized to b-tubulin using the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 1
List of primer sequences for biofilm marker genes used for qRT-PCR.

Gene	Forward primer	Reverse primer	Amplicon size (bp)	Reference
ALS3	AATGGTCCTTATGAATCACCATCTACTA	GAGTTTTCATCCATACTTGATTTCACAT	51	24
EAP1	CTGCTCACTCAACTTCAATTGTCG	GAACACATCCACCTTCGGGA	51	24
EFG1	TATGCCCCAGCAAACAACTG	TTGTTGTCCTGCTGTCTGTC	202	24
NRG1	CACCTCACTTGCAACCCC	GCCCTGGAGATGGTCTGA	198	24
HWP1	GCTCAACTTATTGCTATCGCTTATTACA	GACCGTCTACCTGTGGGACAGT	67	24
SAP5	CAGAATTTCCCGTCGATGAGA	CATTGTGCAAAGTAACTGCAACAG	78	24
ERG3	TCCAGTTGATGGGTTCTTCCA	GGACAGTGTGACAAGCGGTA	179	This study
ERG25	TGCTGCTCCATTTGGATTGG	GGAATGAGCATCAACGGCTT	175	This study
ACT1	GCTGGTAGAGACTTGACCAACCA	GACAATTTCTCTTTCAGCACTAGTAGTGA	87	24

ALS3 - Hyphal-specific Cell wall adhesin; EAP1 - hyphae-specific cell wall adhesin protein; EFG1 - hyphae-specific gene activator; NRG1 – transcriptional repressor of hyphae-specific genes; HWP1 - hyphae-specific cell wall protein; SAP5 - secreted aspartyl proteases; ERG3 - C-5 sterol desaturase; ERG25 - methylsterol monooxygenase and ACT1 - actin related gene 1

Statistical analysis

All experiments were performed in triplicates, and the results were expressed as the mean ± standard deviation. Statistical analyses of the differences between the means of two experimental groups were evaluated by an unpaired two-tailed Student's t-test using GraphPad Prism 5.0 and a p-value of less than 0.05 was considered significant.

Results

Computational analysis of CYP51 and thidiazuron interaction

The interactions between Thidiazuron and amino acid residues in CYP51 were discovered through molecular docking studies, as shown in Table 2 and Fig. 1. The interaction of CYP51 amino acid residues with thidiazuron was investigated in order to gain insight into the ligand-protein interactions of this enzyme. According to the results of the molecular docking analysis, thidiazuron has a high binding potential for CYP51 with a binding energy of -5.86G. (Table 2). An in-depth examination of the interactions revealed two hydrogen bonds, four hydrophobic bonds, and two miscellanous bonds, among other things (Fig. 1). It was found that the nitrogen and hydrogen groups of the eighth carbon from thidiazuron formed two hydrogen bonds with the amino acid residues His377 and Met508, which were separated by a distance of 1.97 microns in each case. Aside from that, we discovered two Pi-alkyl hydrophobic bond interactions between thidiazuron and the CYP51 enzyme's Leu121 and Leu376 residues (Fig. 1 and Table 2).

Table 2
Interactions of Thidiazuron and amino acid residues of CYP51

Sl.No	Ligand	Pubchem ID	Binding energy	Ligand efficiency	Intermole energy	Ligand atoms (ring)	Docked amino acid residue (bond length)
1.	Thidiazuron	CID_40087	-5.86	-0.39	-6.27	Conventional Hydrogen Bond: C8 S-N C8 N-H Pi-Alkyl Hydrophobic bond: O O Pi-Pi T shaped Hydrophobic bond: O O Miscellanous sulfur Bond: C8-S Miscellanous sulfur Bond: C8 S-N	Chain A: HIS`377 'HN'(1.97 Å) Chain A: MET`508 'O' (1.97 Å) Chain A: LEU`121 (5.32 Å) Chain A: LEU`376 (4.26 Å) Chain A: HIS`377 (4.04 Å) Chain A: TYR`118 (5.35 Å) Chain A: MET`508 'O' (2.89 Å) Chain A: HIS`377 'HN'(2.97 Å)

Antifungal activity of thidiazuron:

Through the use of the disc diffusion method, the antifungal activity of thidiazuron was investigated at three different concentrations (25, 50, and 100 µg) against *C. albicans* (Fig. 2A). The antifungal pontential of thidiazuron was determined by the zone in which fungal growth was inhibited. According to the findings of this study, a thidiazuron-mediated dose-dependent reduction in the zone of inhibition of *C. albicans* was observed (Fig. 2B). When compared to the standard drug (fluconazole), thidiazuron at a concentration of 100 µg has the most significant inhibitory effect against *C. albicans*.

Thidiazuron and fluconazole were tested against *C. albicans* and their planktonic minimal inhibitory concentrations (PMIC) were shown in Figs. 2C and D, respectively. When tested against *C. albicans*, the PMIC50 of thidiazuron fluconazole was discovered to be 41.9 M and 14.21 g/mL, respectively. We further confirmed the antifungal activity of thidiazuron by scanning electron microscopy (SEM) analysis of *C. albicans* treated with the drug. SEM analysis also revealed that the *C. albicans* cells in the control group were healthy, whereas cells with damaged membranes were found in both the thidiazuron and fluconazole treatment groups (Fig. 2E).

In order to evaluate the antimicrobial activity of the thidiazuron at concentrations of 0.5, 1 and 2 times the MIC from 0 to 48 hours, a time kill study was carried out, and a growth curve was plotted (Fig. 3). Furthermore, a time kill study revealed that thidiazuron inhibited the growth of Candida albicans cells in a concentration and time-dependent manner, indicating that it is a potent antifungal (Fig. 3). Growth of *C. albicans* cells in the presence of 21 M (0.5X PMIC) of thidiazuron was only marginally retarded compared to control cells in the absence of the compound (DMSO). The growth of C. albicans cells was drastically reduced in a time-dependent manner at higher concentrations (1X to 8X PMIC), in contrast to the lower concentrations. As shown in Fig. 3, at concentrations ranging from 1X to 4X PMIC, 100 percent growth inhibition of *C.*

albicans was achieved in 48 hours. In contrast, at 8X, 100 percent growth inhibition of *C. albicans* was completed within 24 hours of treatment.

In vitro C. albicans **biofilm inhibitory potential of thidiazuron**:

Crystal violet staining and the XTT reduction assay both revealed that thidiazuron inhibits the formation of C. albicans biofilms in a dose-dependent fashion. The crystal violet staining method showed that thidiazuron inhibited the formation of C. albicans biofilms starting at a concentration of 6 M. At a concentration of 50/100 M, 100 percent inhibition in biofilm formation was observed (Fig. 4A). The XTT reduction assay revealed a pattern of biofilm inhibition that was similar to what we observed (Fig. 4B). The fluorescent microscopic analysis of acridine orange-stained biofilms also revealed that thidiazuron inhibited the formation of C. albicans biofilms in a dose-dependent fashion, as previously reported (Fig. 4C).

Thidiazuron inhibited ergosterol synthesis in C. albicans

C. albicans ergosterol synthesis was investigated using spectrophotometric analysis of thidiazuron (0.5X PMIC, 1X PMIC, and 2X PMIC) on a spectral absorption pattern between 240 and 300 nm. When compared to untreated control cells, all concentrations of thidiazuron (0.5X PMIC, 1 X PMIC, and 2 X PMIC) caused a significant decrease in ergosterol biosynthesis in *C. albicans* in a dose-dependent manner. The findings of the current study demonstrated that thidiazuron has the potential to inhibit ergosterol synthesis in a cell culture model. Further, treatment with 0.5X PMIC thidiazuron reduced ergosterol content by 35%, whereas total ergosterol content was reduced to 93 and 100% in C. albicans treated with 1X and 2X PMIC thidiazuron, respectively, by 35% and 100%. (Fig. 5).

Thidiazuron modulated gene expression in C. albicans

The inhibitory effect of thidiazuron on genes involved in erogosterol biosynthesis, regulating adhesion and hyphal growth in *C. albicans* biofilm formation was determined using quantitative real-time PCR (qRT-PCR) in Fig. 6. The presence of Thidiazuron at the PMIC50 concentration altered the expression pattern of genes involved in regulating adhesion, hyphal growth, and ergosterol synthesis compared to DMSO treatment of the cells. Thridiazuron, according to our findings, significantly decreased the expression of genes involved in adhesion and hyphae development, including hyphal-specific cell wall adhesion (ALS3), hyphae-specific cell wall adhesion protein (EAP1), hyphae-specific gene activator (EFG1), hyphae-specific cell wall protein (HWP), and secreted aspartyl proteases (SAP5) (Fig. 6). Thidiazuron also significantly affected the expression of the enzymes sterol desaturase (ERG3) and methylsterol monooxygenase (ERG25), both of which are involved in the synthesis of ergosterol. Thidiazuron treatment, on the other hand, resulted in a significant increase in the expression of the negative transcription regulator of hyphae (NRG1). Together, the thidiazuron-mediated inhibition of ERG11 resulted in the downregulation of other genes involved in ergosterol synthesis and hyphae growth, ultimately inhibiting the growth of the *C. albicans* biofilm.

Discussion

C. albicans is a commensal fungal species that colonies human mucosal surfaces. Increased colonization of *C. albicans* in root carious lesions was reported to promote tooth decay(Du et al. 2021). Emerging antifungal drugs resistance in *C. albicans* has been increasing due to biofilm phenotypes, which creates a need for identification of new antifungal agents. An earlier study demonstrated that synthetic plant cytokinin (forchlorfenuron) has capability to inhibit budding yeast cell division in *Saccharomyces cerevisiae*(Iwase et al. 2004). In our study, we applied computational and biological approaches to validate the antifungal and antibiofilm potential of thidiazuron against *C. albicans*.

Targeting ergosterol biosynthesis has been proven as a strategy to inhibit growth of different *Candida spp* including *C. albicans* and overcome antifungal drug resistance (Onyewu et al. 2003). Cytochrome P450 enzyme (CYP51) encoded by gene ERG11 is required for the biosynthesis of ergosterol and was also reported as a potential antifungal drug target in the treatment of *C. albicans* (Hargrove et al. 2017). In the present study, amino acid His-377 in K/β1−4 loop and Met-508 in β4

hairpin structure of CYP51 are involved in hydrogen bonded interaction with thidiazuron. Similar interactions have been reported between posaconazole or tetrazole-based drug candidate (VT-1161) and CYP51 (Hargrove et al. 2017). Hydrogen bonding plays a significant role in protein binding, thus hydrogen bonding between thidiazuron and 14-alpha demethylase enzyme affects the positioning of the natural ligands, which improves the drug binding capacity (Fig. 1). Hargrove et al. (Hargrove et al. 2017) noticed that CYP51 inhibitory potential of tetrazole-based modified drug candidate was enhanced by the H-bond between the imidazole ring of His-377 and the candidate drug. The Leucine residues in B-helix were reported to provide higher ability for interaction of C4-monomethyl sterols towards mammalian and fungal CYP51 that plays essential role in binding and metabolism of the sterol substrates (Lepesheva et al. 2006). Similarly, two Pi-Alkyl hydrophobic bond interactions were noticed between thidiazuron and Leucine residues of CYP51. Altogether our computational study revealed that thidiazuron has potency to bind with CYP51 via interaction to its functionally important amino acid residues.

Current pharmacological treatments against oral candidiasis fail in long-term efficacy against *C. albicans*. Also, the prevalence of resistance towards antifungal agents in usage has increased and emerged as a main limitation for antibiofilm activity (Madariaga-Venegas et al. 2017). Results from the present study confirmed the antifungal activity of thidiazuron in a dose dependent manner. In addition, SEM images clearly indicated that thidiazuron affected the cell membrane integrity and hypha morphogenesis associated with biofilm formation. Similar to our results, in few earlier studies also SEM observations of eugenol and cinnamaldehyde treatment revealed interference in cell membrane integrity in biofilm and planktonic cells of *C. albicans* (Bennis et al. 2004; Khan and Ahmad 2012).

In *C. albicans*, yeast-to-hyphae morphogenesis is associated with expression of genes encoding several virulence factors which are essential in biofilm formation on the oral mucosa and other surfaces that were used in prosthodontics (Bonilla Rodríguez et al. 2012). This study clearly showed that thidiazuron treatment significantly down regulated the expression of genes involved in adhesion and hyphae development that includes *ALS3, EAP1, EFG1, HWP* and *SAP5*. The *ALS3* and *HWP* are most highly expressed genes in *C. albicans* biofilm cells and responsible for the adhesion capacity (Nobile et al. 2006). Since adhesion trait of *C. albicans* is directly associated with the biofilm formation, it is mandatory for a new antibiofilm agent with an anti-adhesion property (Blankenship and Mitchell 2006). Thus, the pattern of thidiazuron in down regulating the expression of *ALS3* and *HWP* clearly demonstrated its potential as a new antibiofilm molecule against *C. albicans.* Attenuating from yeast-to-hyphae morphological transition or/and inhibition of hyphal development were suggested as a potential antifungal therapeutic strategy against *C. albicans* (Sionov et al. 2020). Down-regulation of genes *EAP1, EFG1 and SAP5* that are involved in regulation of hyphae development strongly supports the antifungal potential of thidiazuron. Expression of gene encoding EFG1 transcriptional activator is critical for yeast–hyphae transition (Ramage et al. 2002). Thidiazuron mediated down-regulation of *EFG1* expression is likely responsible for the significant decrease in expression and its downstream target genes including ALS3, HWP1 and SAP5 (Staib et al. 2002; James et al. 2016). Unlike other genes, thidiazuron treatment up-regulated the expression of NRG1, which is a hyphae-repressing transcription factor that inhibits filamentous growth and biofilm formation (James et al. 2016).

Conclusion

The biomolecule Thidiazuron was evaluated for antifungal activity against *C. albicans*. Molecular docking analysis revealed that thidiazuron has the binding potential towards CYP51 enzyme. Thidiazuron exhibited a dose dependent reduction in the zone of inhibition. *C. albicans'* biofilm inhibition was also seen in a dose dependent manner by crystal violet staining and XTT reduction assay. Total ergosterol synthesis could be inhibited by 2X PMIC thidiazuron. qRT-PCR analysis of thidiazuron treated *C. albicans'* biofilm showed down-regulation of the genes involved in hyphae development and significantly up-regulated the negative transcription regulator of hyphae. Based on the findings of this study, we conclude that thidiazuron is a potential anti-fungal agent which could be evaluated further for clinical applications in the management of various systemic and local *C. albicans* infection.

Declarations

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Author contributions:

KT, PH, and HMI designed and conceptualized the experiment. KT, BA, VKJ, KE and EAA performed computational analysis and drafted the MS. PH, PS and HMI performed invitro biofilm inhibitory and enzymatic studies; KT, HMI, PH, AS and PS involved in sample preparation and SEM analysis; RB, H-GL, JK, AS and KK revised the first draft of the manuscript including tables and figures. KT and HMI involved in studies related to gene expression analysis. All authors drafted, contributed and approved the final manuscript.

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Conflict of Interest / Competing interests

The authors declare that they have no conflict of interest.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors

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Figures

Figure 1

Interaction of thidiazuron to the active site of *C.albicans* sterol 14α-demethylase (CYP51).

Figure 2

A. Disc-diffusion based antifungal susceptibility of *C.albicans* towards thidiazuron showing antifungal efficacy of thidiazuron (100, 50 and 25 µM) and fluconazole (25µg/ml) as zone of growth inhibition; B. Thidiazuron induced dose dependent inhibition of growth of *C.albicans*. C. Microbroth dilution based planktonic minimal inhibitory concentration of thidiazuron ($PMIC^{50}$ = 41.9 µM); D. Microbroth dilution based planktonic minimal inhibitory concentration of fluconazole ($PMIC^{50}$ = 14.21 µg/ml); E. Scanning electron microscope analyses of thidiazuron treated planktonic *C.albicans*. DMSO was used as the vehicle control and the values were expressed as the means ± standard error of three replicates and results were considered significant for *P≤0.05; **P≤0.01.

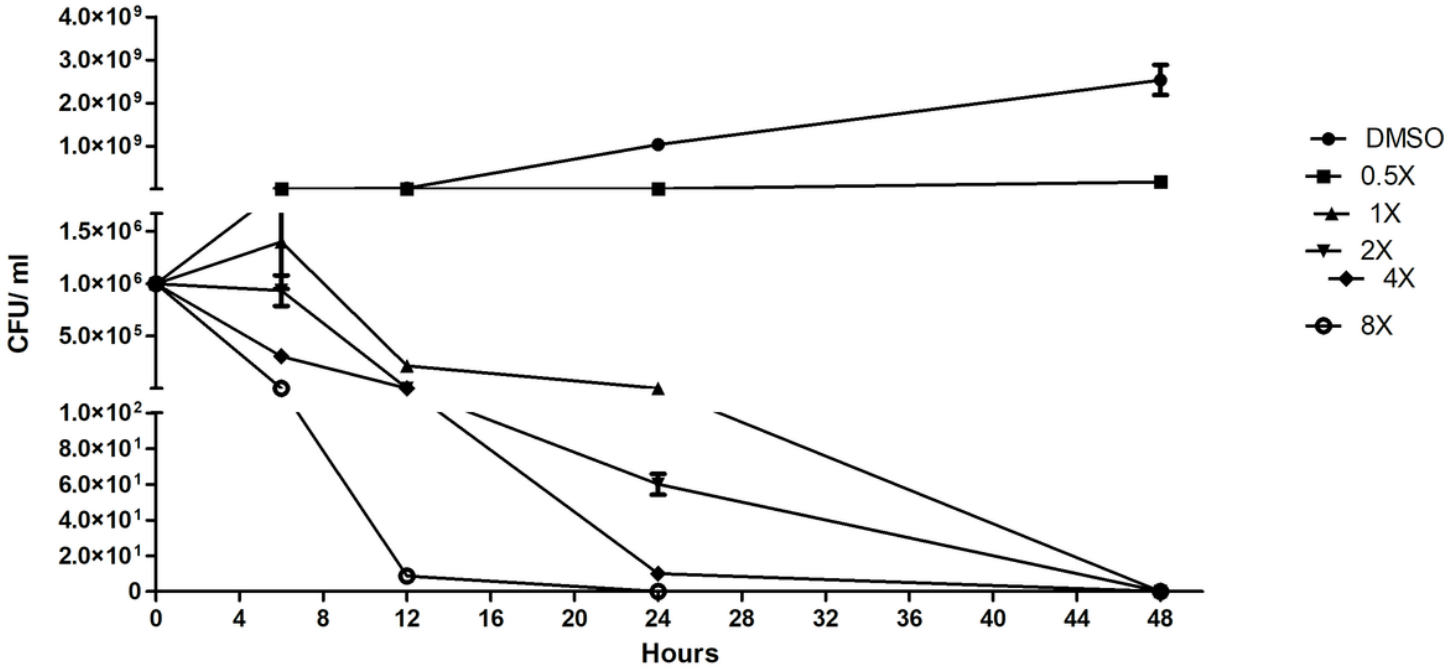

Figure 3

Time-killing curves of thidiazuron treated *C. albicans*. DMSO was used as the vehicle control and the values were expressed as the means ± standard error of three replicates.

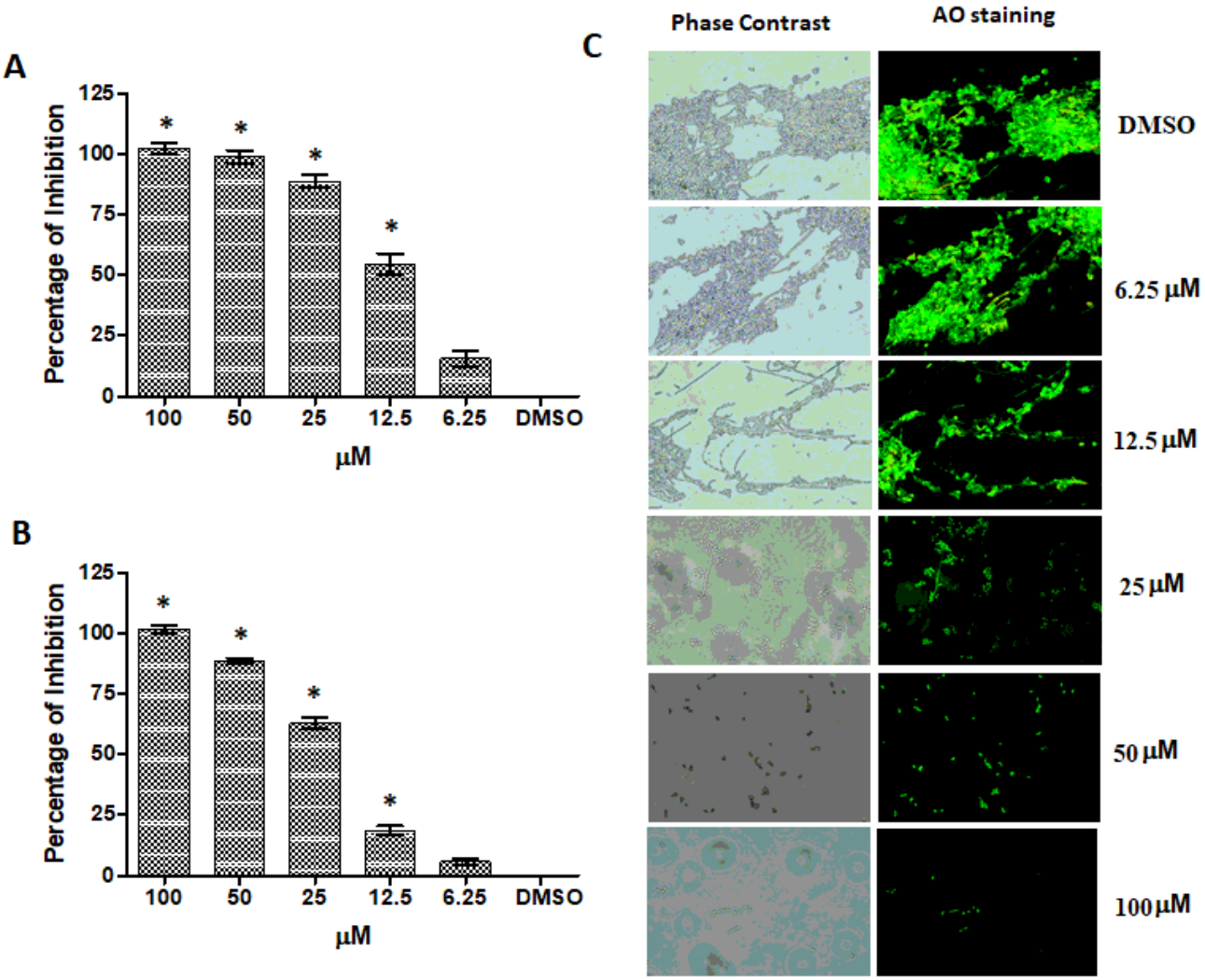

Figure 4

Effect of thidiazuron on *C. albicans* biofilm. A. crystal violet staining based quantitative measurement of the *C. albicans* biofilm; B. XTT reduction assay based quantitative measurement of the *C. albicans* biofilm; C. Acridine orange staining of assessment of *C. albicans* biofilm. DMSO was used as the vehicle control and the values were expressed as the means ± standard error of three replicates. Results were considered significant for *P≤0.05; **P≤0.01.

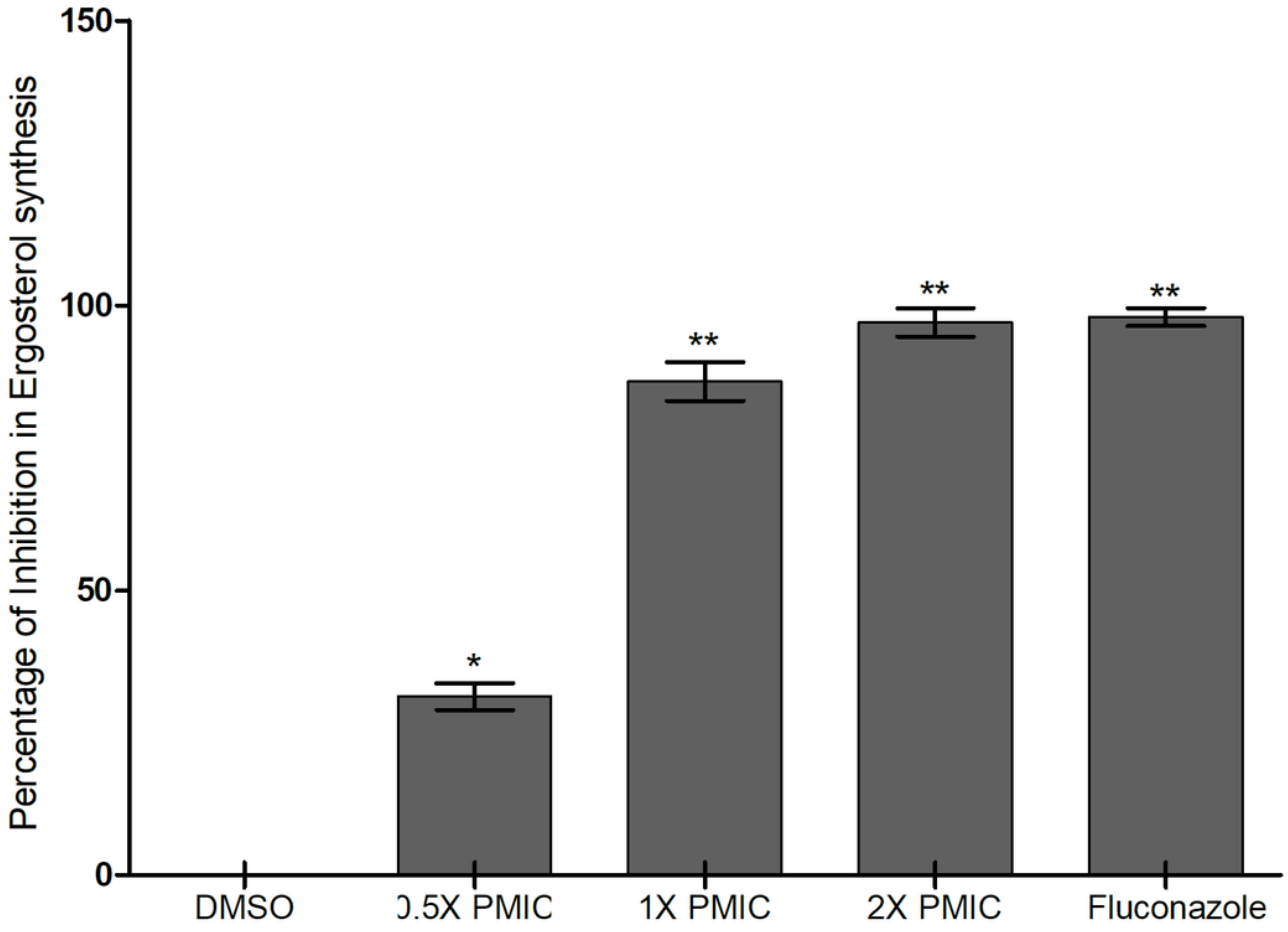

Figure 5

Effect of thidiazuron on ergosterol synthesis in *C. albicans*. DMSO and fluconazole (14.21 µg/ml) treated cells were represented as negative and positive control respectively. Values were expressed as the means ± standard error of three replicates and results were considered significant for *P≤0.05; **P≤0.01.

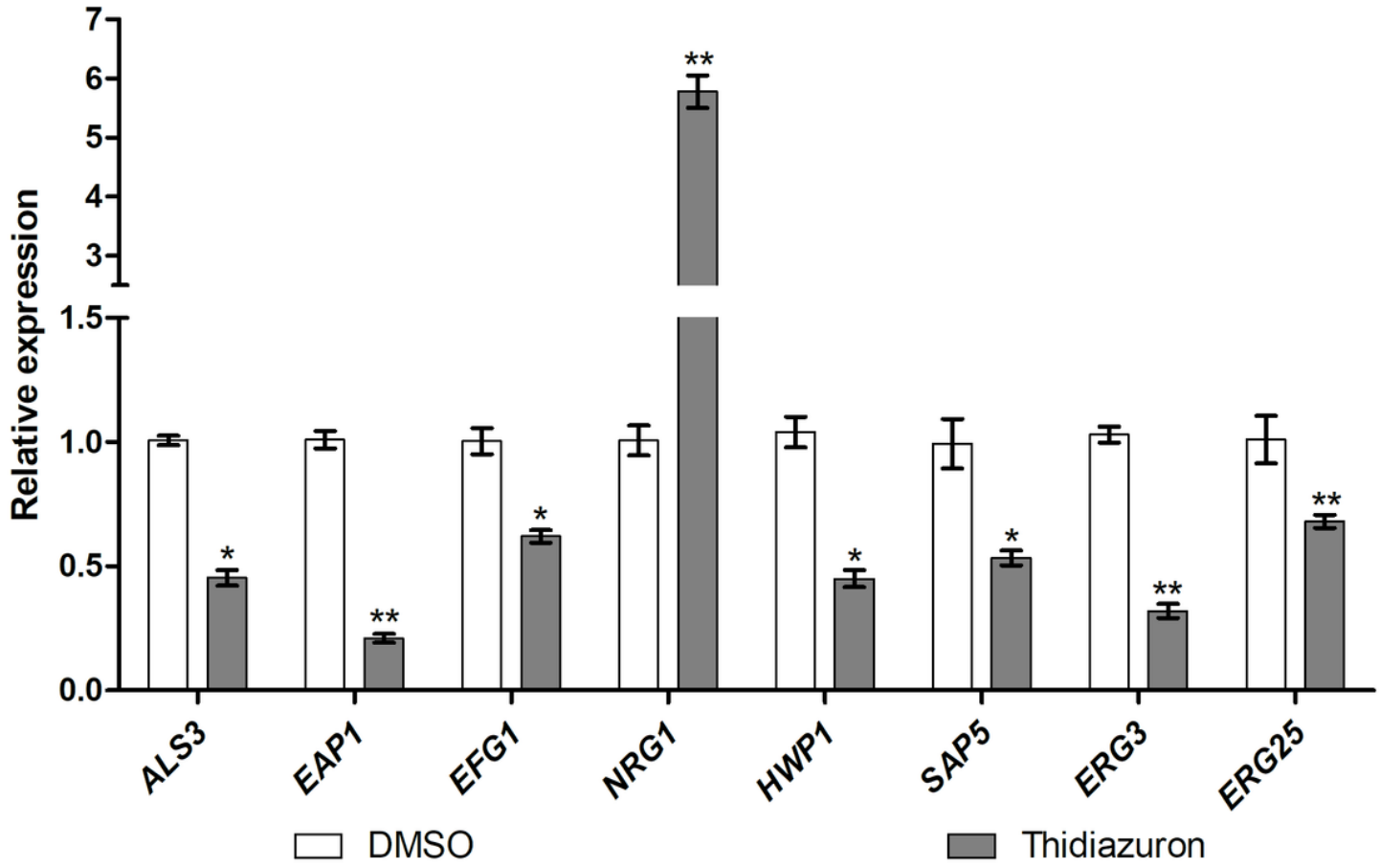

Figure 6

Quantitative real time PCR analysis of genes involved in *C. albicans* biofilm formation (*ALS3* - Hyphal-specific cell wall adhesion; *EAP1* - hyphae-specific cell wall adhesion protein; *EFG1* - hyphae-specific gene activator; *NRG1* – transcriptional repressor of hyphae-specific genes; *HWP1* - hyphae-specific cell wall protein; *SAP5* - secreted aspartyl proteases; *ERG3* - C-5 sterol desaturase; *ERG25* - methylsterol monooxygenase and *ACT1* - actin related gene 1). Results were expressed as mean relative expression ± standard error of three replicates. Results were considered significant for *P≤0.05; **P≤0.01.