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

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Received: 22 April 2022 / Accepted: 4 September 2022 / Published online: 17 September 2022
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Abstract

*Candida albicans* is a common human fungal pathogen that colonizes mucosa and develops biofilm in the oral cavity that causes oral candidiasis. It has been reported that cytochrome P450 enzyme (CYP51), a vital part of the ergosterol synthesis cascade, is associated with *Candida* infections and its biofilm formation. Thidiazuron, a phenyl-urea cytokinin, exhibits anti-senescence and elicitor activity against fungal infection in plants. However, how Thidiazuron impacts *C. albicans* biofilm formation is still uncertain. Here, we aimed to investigate the effects of a Thidiazuron against the growth and biofilm formation properties of *C. albicans* using in silico and in vitro experimental approaches. A preliminary molecular docking study revealed potential interaction between Thidiazuron and amino acid residues of CYP51. Further in vitro antifungal susceptibility test, scanning electron microscopy (SEM) and time kill analysis revealed the 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 revealed 50% inhibition in *C. albicans* biofilm by Thidiazuron at concentrations 11 and 19 µM respectively. Acridine orange staining assay visually confirmed the biofilm inhibitory potential of Thidiazuron. The gene expression study showed that Thidiazuron treatment down regulated the expression of genes involved in ergosterol synthesis (ERG3, ERG11, ERG25), cell adhesion (ASL3, EAP1), and hyphae development (EFG1, HWP1, SAP5) in *C. albicans*. Whereas, the expression of negative transcription regulator of hyphae (NRG1) was upregulated (5.7-fold) by Thidiazuron treatment. Collectively, our data suggest that Thidiazuron is a robust antifungal compound and an outstanding biofilm inhibitor, which may promise further therapeutic development due to CYP51 binding and inhibition of ergosterol formation against *C. albicans*.

**Keywords** Anti-fungal · Biofilm · *Candida albicans* · CYP51 · Ergosterol · Thidiazuron
Introduction

Fungal infections are more common due to changing lifestyles, pollution, and increasing systemic risk factors like diabetes, auto-immune diseases, and immune-suppressant drugs. Many fungi form biofilm in human mucosal-dermal infections and on the biomaterials used inside the body. *C. albicans* biofilm formation impacts antifungal sensitivity that leads to resistance to the currently available antifungal medicines and highlights the necessity of research focused on preventing and controlling these clinical microbial communities. Although varieties of anti-fungal agents are available, drug resistance is often encountered (Cowen et al. 2014). Conventional antibiotic therapy that leads to antimicrobial resistance is frequently responsible for biofilm-associated infections (Jabra-Rizk et al. 2004). So, there is a need to search for new biomolecules exhibiting good anti-fungal activity.

Candida yeasts are single-celled. The form and size of the cells are mostly determined by the species, the growth phase, the physiological condition, and the culture’s ambient parameters. Candida yeast biomass contains anti-genotoxic, anti-cancer, and anti-oxidant compounds such as b-glucans, glucomannan, and mannoproteins (Kieliszek et al. 2017). Candida species is also one of the most prevalent opportunistic fungal infections in humans, especially in the oral cavity, and preventing its biofilm and recurrence is critical in the treatment of candidiasis (Akpan 2002). *Candida albicans* is a major source of device-associated infection due to its potential biofilm formation trait. Adhesion to the cell surface and biofilm formation are reported as the mode of transmission of *C. albicans* in a neonatal intensive care unit (Zhang et al. 2021).

Biofilm is a community of microbes produced by 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 the sessile mode of cell growth (Okada et al. 2005). Biofilms are a potential cause for the establishment of infections and form 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). *Candida sp.* biofilms consist of a structured mixture of yeast-form, pseudohyphal, and hyphal cells encased in an extracellular matrix made up of proteins, carbohydrates, lipids, and nucleic acids (Zarnowski et al. 2014). The biofilm matrix serves as a physical barrier between the biofilm and the environment, as well as providing structural integrity, and it is essential for mature biofilm resistance to mechanical disruption. During and after the maturation stage of biofilm growth, the matrix is most noticeable. The biofilm seems thick and structured at this stage, with discrete layers of solid surface, hyphal and pseudohyphal cells spreading away from the surface (Cavalheiro and Teixeira 2018). The hyphae that grow in the later stages of *C. albicans* biofilm development serve as a scaffold that supports the biofilm’s many components, adding to the biofilm’s overall architectural integrity. Once fully developed, the biofilm progressively disperses, primarily yeast-form cells that bud off from hyphae, contributing to the infection’s spread (Gulati and Nobile 2016). *C. albicans* biofilm leads to superficial and systemic infections in the human host and it occurs frequently in the mucosa or endothelial tissues (Donlan 2001). In *C. albicans*, the extracellular polymeric substances develop pathogenic infections and multidrug resistance, through stages of adherence, proliferation, maturation, and dispersion (Harikrishnan et al. 2013; Nett 2016; Lohse et al. 2018).

Several antibiofilm studies reported inhibition of biofilm formation in *C. albicans* by chemically synthesizedazole molecules and natural plant molecules pretending the toxicities and non-target effects. The search for 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). Exogenous Tyrosol, alone or in combination with antifungals, reduces the formation of planktonic cells and *Candida* biofilms. Tyrosol alone or in combination with amphotericin reduced mature biofilms, however, Tyrosol plus azoles boosted biofilm activity through a dose-dependent mechanism (Cordeiro et al. 2015). Vanillin was noticed to decrease cellular ergosterol in *C. albicans* and was also reported as a potential inhibitor of its biofilm formation (Raut et al. 2013).

The expression of genes responsible for ergosterol biosynthesis were reported to be involved in fluconazole resistance and biofilms in *Candida albicans* (Borecká-Melkusová et al. 2009). Ergosterol is a fungal cell membrane component, which regulates the membrane structure fluidity, permeability, and mobility and stabilizes the membrane structure binding through phospholipids (Krumpe et al. 2012). It also functions as a fungal hormone that stimulates growth and plays an essential role in oxidative stress during fungus 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 the 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 reactive 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). The ergosterol biosynthesis pathway is governed by the 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). It was also reported that the levels of total ergosterol were significantly lower at later stages of biofilm formation than in planktonic cells. But, it was noticed that fluconazole decreased the expression of the *ERG11* gene in fluconazole-sensitive *C. albicans* biofilm, whereas it increased the expression of the *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 inhibitors 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 candidiasis. Thidiazuron, a substituted phenylurea used predominantly as a cotton defoliant, has been demonstrated to have cytokinin-like action in several cytokinin bioassays (Thirukkumaran et al. 2009). Thidiazuron, a cytokinin, has been shown to reduce the growth, development, and virulence of fungal phytopathogens (Schmidt et al. 2017; Gupta et al. 2021). In this present study, Thidiazuron was docked against ergosterol biosynthesis enzyme (CYP51) and its in vitro 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 structure of sterol 14α-demethylase enzyme (CYP51, PDB code: 5TZ1) was retrieved from the RCSB database (Hargrove et al. 2017). The structure of thidiazuron (PubChem CID: 40087) used in this study was retrieved from the pubchem database (https://pubchem.ncbi.nlm.nih.gov/compound/) as .sdf format and later 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 a 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 extra pure (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 $1 \times 10^6$ CFU/mL using TSB medium with 1% glucose. Antifungal activity of thidiazuron (100–25 μM) and fluconazole (326 μM) were screened using the disk diffusion method in Mueller–Hinton agar. In brief, Mueller–Hinton agar was spread with *C. albicans* ($1 \times 10^6$ CFU/mL) and the disk containing test antifungal agent was placed and subsequently incubated for 24–48 h at 37 °C. The results were interpreted based on measuring the diameter of the zone of inhibition. The cells treated with fluconazole and DMSO were considered positive and negative controls respectively.

**Planktonic minimal inhibitory concentration (PMIC)**

The minimal inhibitory concentration (MIC) values of planktonic suspending cells for *C. albicans* were 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 (200 μM) and fluconazole (652 μM) 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 ($1 \times 10^6$ 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 the 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 × 10⁶ CFU/mL) were prepared in 2 mL of SDB broth, to which thidiazuron and fluconazole at 1X MIC₃₀ 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 min with a series of increasing concentrations 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 Apreo two SEM High-Resolution Scanning Electron Microscope (HRSEM) (Thermo Scientific, Netherlands) at SRM Institute of Science and Technology, Tamil Nadu, India.

Time-kill assay for *C. albicans* towards thidiazuron

The time kill assay for *C. albicans* towards thidiazuron was performed as described earlier (Ali et al. 2010). A cell suspension of *C. albicans* (1 × 10⁶ 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 hemocytometer Neubauer improved chamber and adjusted to 1 × 10⁶ CFU/mL in RPMI 1640 medium supplemented with 2% (w/v) glucose. Cell suspension (200 µl; 1 × 10⁶ CFU/mL) and various concentrations of thidiazuron (100–6.25 M) in RPMI medium were co-incubated for 48 h at 37 °C in a 96 well flat bottom cell culture plate. Appropriate media and culture controls were also maintained in parallel to the thidiazuron treatment. Later, the supernatant along with suspended planktonic cells was 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 was recorded at the wavelength of 595 nm (Emeka et al. 2020).

For the 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. The working detection solution was prepared by mixing 10 µL of the PMS solution with 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 (10 s) to mix the dye in the solution and absorbance was 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 Thidiazuron (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 × 10⁶ CFU/mL) were inoculated in 50 mL of SDB with Thidiazuron or DMSO as the control and incubated in a shaking incubator at 37 °C for 18 h. Immediately after incubation, cell biomass was centrifuged at 2700 rpm for 5 min, washed and the weight of individual pellets was recorded. The pellet in each tube was 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 fivefold ethanol (100%) were subjected to scanning between wavelengths of 240–300 nm in a UV/VIS Spectrophotometer (LABMAN Scientifics, India). The cells treated with fluconazole and DMSO were considered positive and negative controls respectively. The levels of ergosterol were calculated and expressed as a percentage in terms of the weight of the pellet using the below equation:
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 × 10⁶ 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 Thidiazuron or DMSO as the control were then incubated statically at 37 °C for 24 h. On completion of the incubation period, mycelial samples were collected and frozen in liquid N₂ 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⁻ΔΔCT method (Livak and Schmittgen 2001).

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.

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

| Gene     | Forward primer      | Reverse primer               | Amplicon size (bp) |
|----------|---------------------|------------------------------|--------------------|
| ALS3     | AATGGTCCCTTAGAATCCACCATCTACTA | GAGTTTTTCATCCACTATGTTTCACAT | 51                 |
| EAP1     | CGTCCACTCACTCAATTTGCG | GAACATCCATTTCCGGAAGGA | 51                 |
| EFG1     | TTGGCCGCGAAGAAACTG  | TTGGTCTGCTGACGTAACGT | 202                |
| NRG1     | ACCTCACTTGGACACCC  | GCCCTGGAGATGGCTGTA | 198                 |
| HWP1     | GCTCAACATATGCGTACCCCTTACAT | GACGCTCCTATCGTGACGTA | 67                 |
| SAP5     | CAGGAATTTGCGTATGAGA | CATTTGCAAGTACAATCAG | 78                 |
| ERG3     | TCACGTTGATTGGTCTTCCA | GGAACATGTTGACAGCTGTA | 179                |
| ERG11    | TTTTGATTGGTACGATCATGAT | TAATCCGGTACGATCTT | 128                |
| ERG25    | TTTGCAGGCTAGCTTTGGAAGT | GGAATGAGCATACAGCG | 175                |
| ACT1     | GCTGTTAGAGACAGTACCAACCA | GAAATTTTCATTCTGAGATG | 87                 |

ALS3—Hyphal-specific Cell wall adhesion, 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, ERG11—lanosterol 14-alpha-demethylase and ACT1—actin related gene 1
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 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 miscellaneous 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 Miscellaneous sulfur Bond: C8 S Miscellaneous 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) |

Fig. 1 Interaction of thidiazuron to the active site of *C.albicans* sterol 14α-demethylase (CYP51)

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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 µM) against C. albicans (Fig. 2A). The antifungal potential 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). Similar to the standard drug (fluconazole), thidiazuron at a concentration of 100 µM 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 Fig. 2C, D, respectively.

Fig. 2  A Disc-diffusion based antifungal susceptibility of C. albicans towards thidiazuron showing antifungal efficacy of Thidiazuron (100, 50 and 25 µM) and fluconazole (326 µM) 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 (PMIC50 = 41.9 µM); D Microbroth dilution based planktonic minimal inhibitory concentration of fluconazole (PMIC50 = 85.12 µM); 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
When tested against *C. albicans*, the PMIC50 of thidiazuron and fluconazole was discovered to be 41.9 and 85.12 µM, 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).

To evaluate the antimicrobial activity of the thidiazuron at concentrations of 0.5–8 times the MIC from 0 to 48 h, 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 agent (Fig. 3). The growth of *C. albicans* cells in the presence of 6.25 µM (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–8X PMIC), in contrast to the lower concentrations. As shown in Fig. 3, at concentrations ranging from 1 to 4X PMIC, 100 percent growth inhibition of *C. albicans* was achieved in 48 h. In contrast, at 8X PMIC, 100 percent growth inhibition of *C. albicans* was completed within 24 h 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 µM, 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, 1X PMIC, and 2X 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.

![Fig. 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](image-url)
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 (Fig. 5).

**Thidiazuron modulated gene expression in C. albicans**

The inhibitory effect of thidiazuron on genes involved in ergosterol 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. Thidiazuron, according to our findings, significantly decreased the expression of genes involved in adhesion and hyphae development, including hyphal-specific cell wall adhesion (ALS3; 0.45-fold), hyphae-specific cell wall adhesion protein (EAP1; 0.21-fold), hyphae-specific gene activator (EFG1; 0.62-fold), hyphae-specific cell wall protein (HWP; 0.45-fold), and secreted aspartyl proteases (SAP5; 0.53-fold) (Fig. 6). Thidiazuron also significantly affected the expression of the mRNA encoding enzymes sterol desaturase (ERG3; 0.32-fold), lanosterol 14-alpha-demethylase (ERG11; 0.59-fold) and methyl sterol monooxygenase (ERG25; 0.68-fold), 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 on human mucosal surfaces. Increased colonization of *C. albicans* in root carious lesions was reported to promote tooth decay (Du et al. 2021). Emerging antifungal drug resistance in *C. albicans* has been increasing due to biofilm phenotypes, which creates a need for the identification of new antifungal agents. An earlier study demonstrated that synthetic plant cytokinin (forchlorfenuron) can 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 the 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). A recent computational study revealed that 1,2,4-triazine and its derivatives, either alone or in combination with the different compounds high binding affinity to CYP51 protein and suggested for future antifungal drug development (Verma et al. 2022). 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 the 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 a provide higher ability for the interaction of C4-monomethyl sterols towards mammalian and fungal CYP51 which plays an essential role in the binding and metabolism of the sterol substrates (Lepecheva et al. 2006). Similarly, two Pi-Alkyl hydrophobic bond interactions were noticed between thidiazuron and Leucine residues of CYP51. Furthermore, in mutagenesis experiments, the amino acid residue (LEU376) was cited as a critical residue for enzyme activity, and their mutants were allegedly associated with lower azole susceptibility (Morio et al. 2010). Altogether our computational study revealed that thidiazuron has the potency to bind with CYP51 via interaction with 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 to antifungal agents in usage has increased and emerged as the main limitation of biofilm activity (Madariaga-Venegas et al. 2017). The rise in antifungal resistance has sparked research into the molecular mechanisms that led to this situation, as well as potential treatment targets. When there is an established biofilm, treating *C. albicans* infections is more challenging, and biomaterial infections continue to be an increasingly serious problem of conventional therapy (Morio et al. 2010). In biofilms, only the cells of the surface are destroyed because the extracellular matrix acts as a mechanical barrier, preventing the antibiotic or other compounds from diffusing into the biofilm. Thus, weakening the biofilm matrix is an efficient method for biofilm eradication because it enhances antifungal susceptibility. Results from the present study confirmed the antifungal activity of thidiazuron in a dose-dependent manner. In our study, Thidiazuron was applied to Candida cells before biofilm formation and pre-biofilm activities were investigated by crystal violet staining, XTT reduction assay, fluorescence microscopy, and SEM analyses. The results from these analyses revealed the biofilm inhibitory potential of Thidiazuron. Our earlier study using the above-mentioned techniques revealed a similar pattern of biofilm reduction in other microbial pathogens under treatment with biomolecules (Emeka et al. 2020; Hairul Islam et al. 2020). In addition, SEM images indicated that thidiazuron affected the cell membrane integrity and hypha morphogenesis associated with biofilm formation. Similar to our results, in a 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). Because Thidiazuron possesses anti-biofilm and anti-fungal properties against *C. albicans*, it reduces crosslinking density and *C. albicans* survival by disrupting the extracellular matrix. Furthermore, *C. albicans* biofilms have been shown to impede neutrophil response via an extracellular matrix-induced inhibitory mechanism (Morio et al. 2010). As a result of Thidiazuron-mediated biofilm suppression, *C. albicans* is vulnerable to neutrophil response and other antifungal drugs. Similarly, 2-chloro-N-phenyacetamide was reported to have antifungal action against fluconazole-resistant *Candida* sp. biofilms and planktonic cells (Diniz-Neto et al. 2022). The present study also revealed that Thidiazuron treatment significantly reduces the ergosterol biosynthesis in *C. albicans*. The above result was supported by the earlier study, where the results revealed a decrease in ergosterol synthesis as a molecular mechanism in the anti-fungal activity of essential oils (Chelappandian et al. 2018).

In *C. albicans*, yeast-to-hyphae morphogenesis is associated with the 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 Rodriguez et al. 2012). This study clearly showed that thidiazuron treatment significantly reduced the expression of genes involved in adhesion and hyphae development including *ALS3, EAP1, EFG1, HWP*, and *SAP5*. The *ALS3* and *HWP* are the most highly expressed genes in *C. albicans* biofilm cells and are responsible for the adhesion capacity (Nobile et al. 2006). Since the 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* demonstrated its potential as a new antibiofilm molecule against *C. albicans*. Attenuating from yeast-to-hyphae morphological transition or 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 the 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 toward the 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. Thidiazuron, which has the best antimicrobial and anti-biofilm properties against C. albicans, could be a potential candidate for new antifungal formulations, particularly for topical therapies or in combination therapeutic techniques. In addition, our study also revealed that TDZ is biologically compatible on mouse fibroblast cell line (L929) (Supplementary data). 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 infections.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s11274-022-03410-5.

Acknowledgements

The authors are thankful to the Pondicherry Center for Biological Science and Educational Trust (PCBS), Pondicherry, India for providing the necessary facility to carry out the work. The authors also acknowledge the SRM Institute of Science and Technology, Tamil Nadu, India for providing access to utilize scanning electron microscopy. Experimental help by Mr. Premraj, Research Officer, PCBS is also gratefully acknowledged.

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.

Funding

This work was not funded by any external funding agencies.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

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

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