"IDENTIFICATION OF MOLECULAR TARGETS OF SELECTED BIOACTIVE MOLECULES IN *CANDIDA ALBICANS* BIOFILM FORMATION"

A THESIS SUBMITTED TO
D. Y. PATIL EDUCATION SOCIETY
(DEEMED TO BE UNIVERSITY), KOLHAPUR

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

UNDER THE FACULTY OF

INTERDISCIPLINARY STUDIES

SUBMITTED BY

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416 006, MAHARASHTRA, INDIA

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2025

DECLARATION

I am **Sayali Ashok Chougule**, hereby declares that the thesis entitled **"Identification of molecular targets of selected bioactive molecules in *Candida albicans* biofilm formation"** submitted here for the award of **Doctor of Philosophy** in **Microbiology**, faculty of Interdisciplinary Studies, under the guidance of **Prof. (Dr). Sankunny Mohan Karuppayil** and **Dr. Ashwini Khanderao Jadhav** in the **Department of Stem Cell and Regenerative Medicine and Medical Biotechnology, Centre for Interdisciplinary Research (CIR), D. Y. Patil Education Society (Deemed to be University), Kolhapur** is completed and written by me has not previously formed the basis for the degree or diploma or other similar title of this or any other university or examining body.

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CERTIFICATE

This is to certify that the thesis entitled **"Identification of molecular targets of selected bioactive molecules in *Candida albicans* biofilm formation"**, which is being submitted here for the award of **Doctor of Philosophy** in **Microbiology,** Faculty of **Interdisciplinary Studies** under the guidance of **Prof. (Dr). Sankunny Mohan Karuppayil, and Dr. Ashwini Khanderao Jadhav** in the Department of **Stem Cell and Regenerative Medicine** and **Medical Biotechnology**, **Centre for Interdisciplinary Research (CIR), D. Y. Patil Education Society (Deemed to be University), Kolhapur** (Estd. u/3 of the UGC Act 1956), is the result of original research work completed by **Miss. Sayali Ashok Chougule** under my supervision and guidance. To the best of my knowledge and belief the work bound in this thesis has not formed earlier the basis for the award of any degree or similar title of this or any other university or examining body.

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1. **Chougule, S.A**., Karuppayil, S.M., Jadhav, A.K. (2024). Molecules of Natural Origin as Inhibitors of Signal Transduction Pathway in *Candida albicans*. In: Manzoor, N. (eds) Advances in Antifungal Drug Development. Springer, Singapore.

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1. **Chougule S**, Gavandi T, Patil S, Basrani S, Sawant D, Yankanchi S, Jadhav A, Karuppayil SM. Nonanal inhibits growth and virulence factors in *C. albicans.* Pharmacological Research - Natural Products. 2025. **(I.F. 9.1)**
2. **Chougule S**, Basrani S, Gavandi T, Patil S, Yankanchi S, Jadhav A, Karuppayil SM. Zingerone effect against *Candida albicans* growth and biofilm production. Journal of Medical Mycology. 2025 Mar 1;35(1):101527. **(I.F. 2.2)**
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3. Participated in Two days virtual International Conference on **STEM CELLS AND REGENERATIVE MEDICINE–ACADEMIC AND INDUSTRIAL OUTLOOKS (SCRM-AIO-2021),** organized by Department of Stem Cell and Regenerative Medicine, Centre for Interdisciplinary Research, D. Y. Patil Education Society, Institution Deemed to be University, Kolhapur, 416006 on 19 to 20th March 2021

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6. Participated in the workshop & hands-on training on **BIO-ATOMIC FORCE MICROSCOPY (BIO-AFM)** organized by SAIF (CFC), Shivaji University, on 04-05th Jan, 2022.

7. Presented poster in international conference on **Emerging Trends in Applied Microbiology and Food Sciences (ETAMFS)** organized by Department of Microbiology and Food Processing and Packaging, Yashwantrao Chavan institute of Science, Satara, Maharashtra, India on 2-3rd December, 2022.

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LIST OF ABBREVIATIONS

ATCC	American type culture collection	**IMTECH**	Institute of Microbial Technology
BCY1	cAMP-dependent protein kinase regulatory subunit	**BD**	Biatriosporin D
BDSF	Cis-2-dodecenoic acid	**CAT1**	Catalase isozyme
CBD	Cyclase-associated protein (Cap1) binding domain	**CDC3**	Adenylate cyclase
CLEO	Cedar Leaf Essential Oil components	**CNMA**	Cinnamaldehyde
CPH	Transcription factor	**CST**	Serine/threonine-protein kinase
CYR1	Adenylate cyclase	**DMSO**	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid	**RNA**	Ribonucleic acid
ECE1	Endothelin converting	**EE**	Eucarobustol E
EFG1	Enhanced filamentous growth protein	**H_2DCF**	2',7'-dichlorofluorescin diacetate
H_2DCF	2',7'-dichlorofluorescin diacetate	**qRT-PCR**	Quantitative real time system
HGC1	Hypha-specific G1 cyclin-related protein 1	**HOG**	High-osmolarity glycerol
HST7	Serine/threonine-protein kinase STE7 homolog	**HWP1**	Hyphal wall protein
HYR1	Hyphal regulated cell wall protein	**IC**	Invasive candidiasis
K_2HPO_4	Dipotassium hydrogen phosphate	**KH_2PO_4**	Potassium dihydrogen phosphate
KOH	Potassium hydroxide	**MCA1**	Metacaspase 1
MAPK	mitogen-activated protein kinase	**HOG**	high-osmolarity glycerol
MFC	Minimum fungicidal concentration	**MIC**	Minimum inhibitory concentration
MIG1	Regulatory protein	**MOPS**	3-(N-morpholino) propane sulfonic acid
MOPS	3-(N-morpholino) propane sulfonic acid	**XTT**	2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5 carboxanilide)
NRG1	Transcriptional regulator	**PBS**	Phosphate Buffered saline
PDE	3',5'-cyclic-nucleotide phosphodiesterase	**PI**	Propidium iodide
RAB	Retigeric acid B	**RAS1**	Ras-like protein
RBCs	Red blood cells		
ROS	Reactive oxygen species	**RPMI1640**	Roswell Park Memorial Institute
S-8	Thiazolidinedione-8	**SAN**	Sanguinarine
SDA	Sabouraud dextrose agar	**SOD1**	Superoxide dismutase 1
SEM	Scanning electron	**FACS**	Flow cytometer

	microscopy		
SOD2	Superoxide dismutase 1	**TEC1**	Transcription activator
TET	Tetrandrine	**TUP1**	Transcriptional repressor
XTT	2, 3-bis (2-methoxy-4-nitro-sulfophenyl)-2H-tetrazolium-5-carboxanilide	**UME6**	Transcriptional regulatory protein
FLC	Fluconazole	**Amp B**	Amphotericin B
YPD	Yeast extract-Peptone-Dextrose		

ABSTRACT

This study focuses on the identification of anti-virulence potential of seven bioactive molecules such as zingerone, α-bisabolol, nonanal, undecanal, berberine, α-caryophyllene, and β-caryophyllene against *Candida albicans*. Among these, zingerone, α-bisabolol, nonanal, and undecanal have shown significant inhibitory activity on crucial virulence factors such as adhesion, yeast to hyphal morphogenesis and biofilm formation in *C. albicans*. However, zingerone, nonanal, and undecanal have candida-cidal activity. The mechanism of action study revealed that, these molecules affect Reactive Oxygen Species (ROS) level, cell cycle, and ergosterol synthesis in *C. albicans*. However, zingerone, α-bisabolol, nonanal, undecanal, and α-caryophyllene compounds have been found to interfere with the biofilm formation through Ras1-cAMP-Efg1 and Cek1-MAPK pathways in *C. albicans*. At effective concentrations, these molecules do not show any hemolytic activity, while α- and β-caryophyllene shows notable hemotoxicity. The *in vivo* antifungal efficacy of zingerone, nonanal, and undecanal have been confirmed by using silkworm as an animal model. Overall, this study offers significant value of natural molecules as alternative antifungal agents in effective treatments of biofilm-related infections. Further studies should be carried out to optimize their clinical applications to come up with innovative antifungal strategies against *C. albicans*.

Chapter I: Introduction

1.1. Pathogenicity of *Candida albicans*

C. albicans is a commensal present on human skin, oral, and gastrointestinal tract (Fig. 1.1). It may act as an opportunistic pathogen under immune-compromised conditions. Around 9 % of nosocomial bloodstream infections are caused by *C. albicans,* and it is known as 'candidemia' [1]. It is the most common fungal pathogen of humans worldwide and has become a major clinical problem because of the growing number of immunocompromised patients susceptible to infection. *C. albicans* can form biofilm on various abiotic and biotic surfaces. Biofilm has a highly heterogeneous structure made up of yeast, hyphae, pseudo-hyphae, elongated and cylindrical hyphal cells surrounded by an extracellular matrix [2]. Development of biofilm involves different stages, like adherence of yeast cells to a substrate, proliferation of yeast cells, formation of hyphal cells, accumulation of extracellular matrix, and dispersion of yeast cells from the biofilm complex [3]. *C. albicans* can turn into an opportunistic pathogen and cause crippling mucocutaneous disease and life-threatening systemic infections [4]. Invasive candidiasis (IC) is an important nosocomial infection with high morbidity and mortality rates. Clinical diagnosis of candidiasis could be difficult because of the lack of specific symptoms and clinical signs [4]. Invasive fungal infections cause severe problems due to a rise in the frequency of resistance towards standard antifungal drugs. Hence, no new drug has been approved from the year 2006 [5]. The intracellular signaling pathways activate morphological switching in *C. albicans*, which leads to the maintenance of hyphal growth in response to diverse environmental cues [6]. It can also modulate its metabolic pathways to adapt to the hostile environment [7].

1.2. Virulence factors in *C. albicans*

Many virulence factors expressed by *C. albicans* contribute to its pathogenesis. These include adhesin proteins, morphogenesis, release of phospholipases, and aspartyl proteases. Furthermore, 'phenotypic switching' affects antigen expression, colony shape, and tissue affinities in *C. albicans*. Through this switching mechanism, the organism may be able to adapt to the harsh environment created by the host and the medications used to treat the infection. Yeast to hyphal morphogenesis is regulated by a signal transduction pathway. Mainly, two pathways are involved in signal transduction: Ras1-cAMP-PKA and mitogen-activated protein kinase (MAPK) pathways.

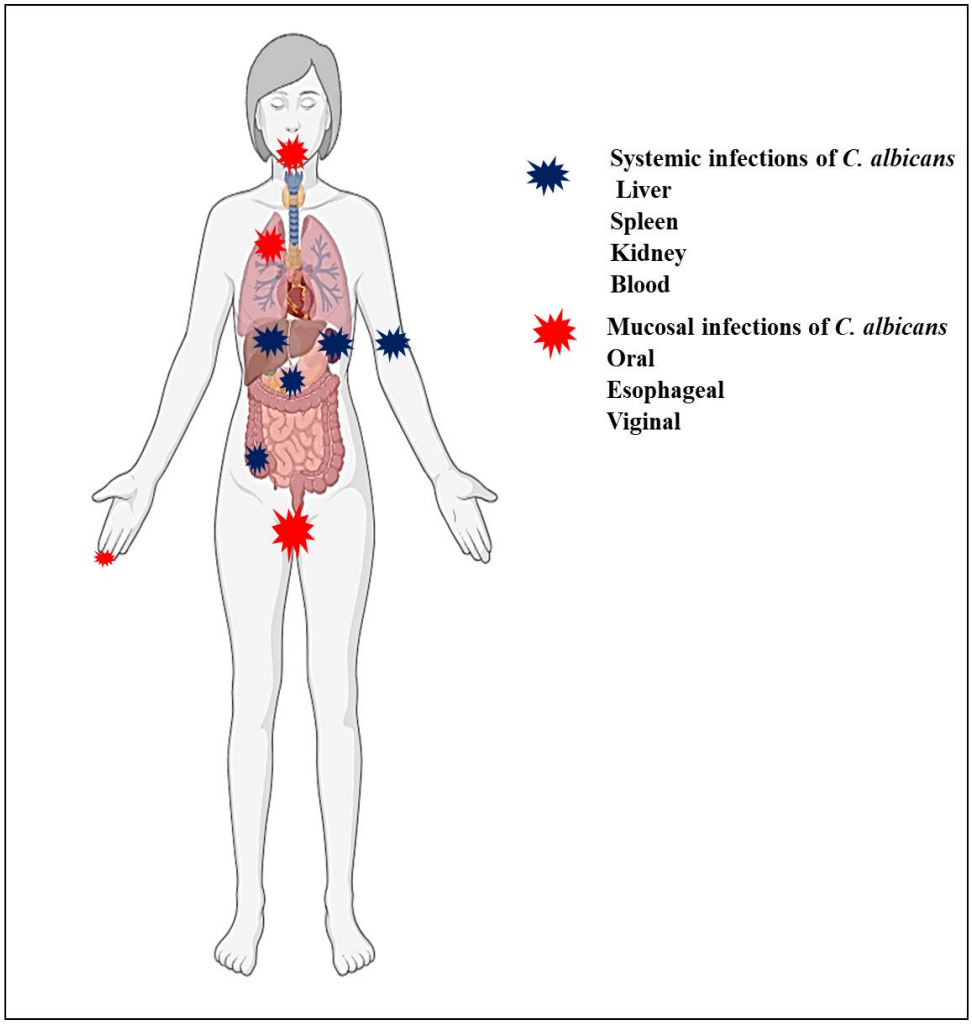

Fig. 1.1: Schematic representation of the *C. albicans* infections commonly occurring in the body, representing the dual role of the fungus as both a commensal organism and an opportunistic pathogen.

1.3. Pathways involved in signal transduction

Various signaling pathways are crucial in the regulation of pathogenesis and related characteristics, including environmental adaptations and morphological transitions. Among all these pathways, Ras1-cAMP-PKA and MAPK kinase pathways are the most important. The Ras1-cAMP-PKA pathway plays a vital role in regulating various biological processes in eukaryotic organisms. In fungi, these pathways are responsible for pathogenesis, morphological transitions, detection of nutrient availability and intake of available nutrients, sexual reproduction, and response to stress [8].

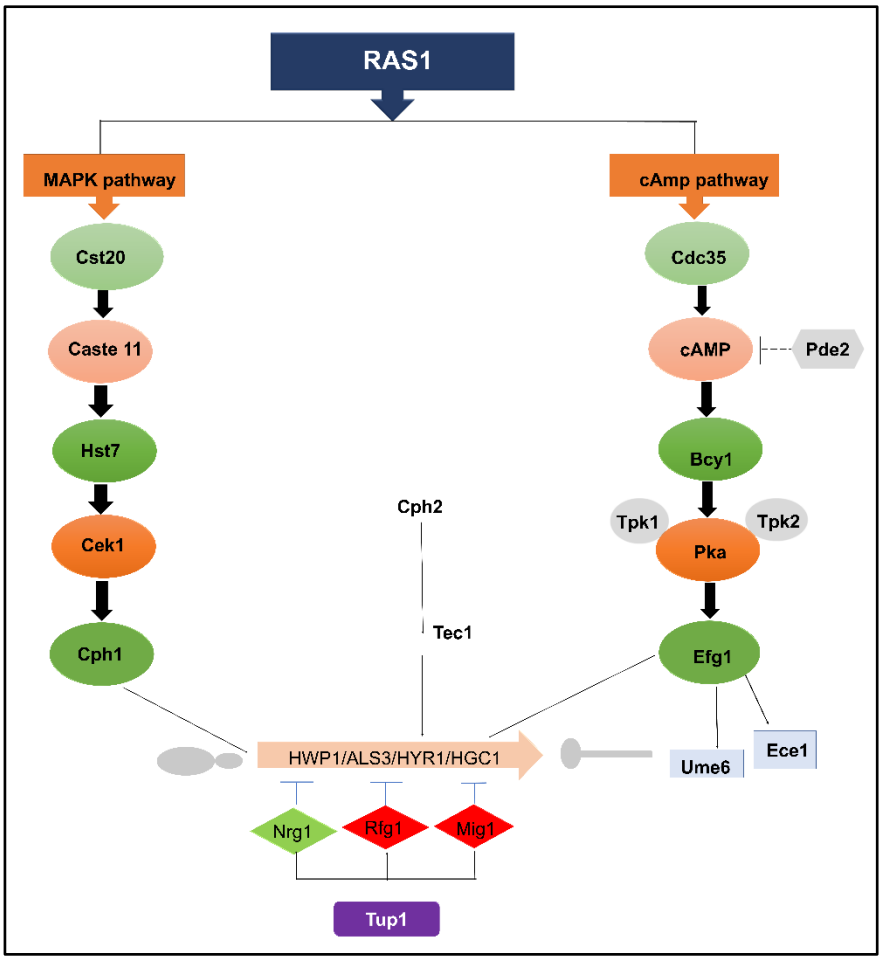

Fig. 1.2: RAS mediated signal transduction pathway involved in yeast to hyphal morphogenesis of *C. albicans* and showing potential molecular targets for antifungal drug development.

1.3.1. RAS mediated signal transduction pathway

The primary components of the Ras1-cAMP-PKA pathway (Fig. 1.2) in *C. albicans* include the RAS GTPases, *RAS1* and *RAS2* [9]. The RAS GTPases are considered molecular switches that become activated or inactivated in response to environmental signals. Inactive GDP gets converted into active GTP, which further binds to adenylate cyclase to produce cAMP. *RAS* signaling is important for the integration of environmental signals with morphogenesis, and *C. albicans* has two *RAS* genes, i.e., *RAS1* and *RAS2,* which encode for two proteins, *RAS1* and a highly different RAS-like protein, namely *RAS2*. *RAS* signaling is now known for the induction of hyphal growth in response to various environmental factors like 37 °C growth temperature, exposure to high levels of CO_2, N-acetyl glucosamine, and serum. These pathways are responsible for the control of genes which are involved in the control of yeast to hyphal transition genes like *ALS3* (adhesin), which is

responsible for the adhesion, *HWP1* involved in invasion, *HYR1,* which modulates host defense mechanisms, and *HGC1,* which is a hyphal specific gene [10].

1.3.2. MAPK Pathway

MAPK pathway (Fig. 1.2) is a major regulator of cellular physiology in *C. albicans*. Three different MAPK pathways have been characterized. The first one is the high-osmolarity glycerol (HOG) pathway, which is mainly activated due to osmotic and oxidative stress and also participates in regulating other pathways. The second Cek1 pathway has a role in the cell wall formation during vegetative and filamentous growth, while the third Mkc1-mediated pathway is involved in cell wall integrity. Fungi have to face hypoxia and high concentrations of reactive oxygen species. HOG pathway and Mkc1-mediated pathway are activated in response to oxidative stress, while the Cek1 pathway is deactivated. The kinetics and functional responses generated upon oxidative stress differ among them. However, they have essential functional consequences that severely influence pathogenesis. MAPK pathways are valuable targets to be explored in antifungal research [11].

1.4. Current antifungal therapy

Currently available classes of antifungal drugs that are used to treat *C. albicans* infections include polyenes, 5-flucytosine, azoles, and echinocandins. Azoles are fungistatic, and the largest class of antifungals used to date. Mainly, they inhibit ergosterol biosynthesis by targeting 14-α-lanosterol demethylase. Polyenes bind to ergosterol and form pores in the membrane, leading to osmotic lysis of the cell. The drug, like amphotericin B, causes oxidative damage to the cell. Next class echinocandins target 1,3-β-D-glucan synthase complex required for synthesis of the glucan polymer of the fungal cell wall. Lastly, flucytosine targets thymidylate synthase and interferes with DNA and RNA synthesis. Also, sordarins target protein synthesis, and grisofulvin targets microtubule assembly [12] (Fig. 1.3). Some *C. albicans* strains have developed drug resistance; hence, there has been a rise in the use of antifungals for treatment [13]. *C. albicans* has a variety of mechanisms to develop resistance to antifungal drugs [14]. The antifungal drug resistance is 1,000-fold higher in biofilm as compared to planktonic cells [15]. Most of the drugs that are available in the market target the cell wall and plasma membrane of *C. albicans*. Some drugs may cause severe side effects, nephrotoxicity by amphotericin-B, visual disturbances by

voriconazole, and congestive heart failure by itraconazole [16]. Hence, there is a need to find new and effective antifungal drugs against Candidiasis. Bioactive molecules from plants are considered the best source for the identification of antifungal molecules.

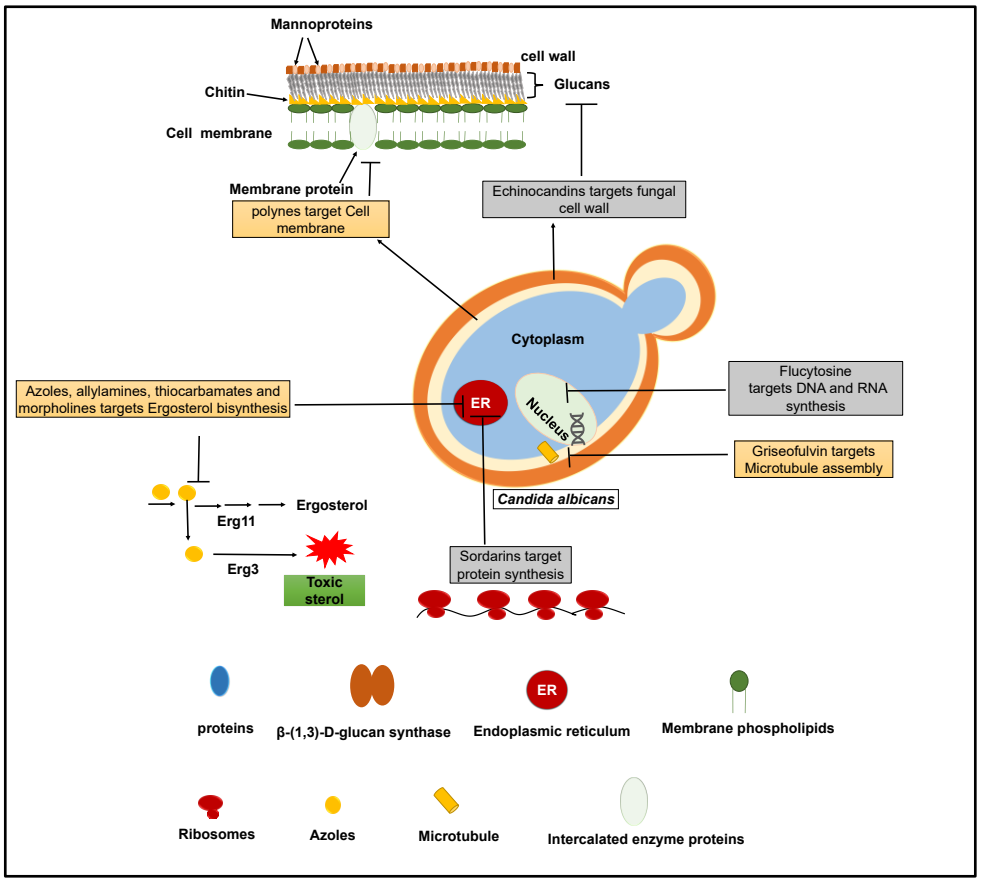

Fig. 1.3: Targets of antifungal drugs in *C. albicans,* highlighting key targets: ergosterol synthesis, DNA and RNA synthesis, cell membrane, cell wall, protein synthesis, and microtubule assembly, fungal survival, and pathogenicity.

Various plant molecules like carvacrol, geraniol, eugenol, linalool, and geranyl acetate are specific inhibitors of invasive growth of *C. albicans*. Similarly, indole, isatin, phenazine methosulphate, caprylic acid, and capric acid have already been reported for their antifungal activity against *C. albicans*. However, detailed studies need to be carried out to confirm their efficacy against Candidiasis.

1.5. Orientation and purpose of thesis

The plant kingdom is a rich source of bioactive molecules with immense therapeutic potential. Among these, essential oils extracted from plant materials through physico-chemical processes such as dry distillation or mechanical methods have been valued for their medicinal properties. These natural extracts contain diverse biologically active molecules that exhibit antimicrobial, anti-inflammatory, and antifungal properties.

Fungal infections, particularly those caused by *C. albicans*, pose a significant challenge in clinical settings. *C. albicans* is an opportunistic fungal pathogen responsible for infections ranging from superficial mucosal candidiasis to life-threatening systemic infections. The increasing prevalence of antifungal resistance necessitates the search for alternative antifungal agents, particularly from natural sources. Essential oils and their bioactive constituents have demonstrated significant antifungal activity, making them promising candidates for further investigation.

Objectives of the thesis

The primary objective of this study is to evaluate the antifungal properties of selected bioactive molecules from plant sources and identify their molecular targets within *C. albicans*. To achieve the aim following objectives were designed:

1. To identify antifungal activity of selected bioactive molecules against growth, adhesion, and biofilm formation of *C. albicans.*
2. To analyse biofilm architecture of *C. albicans* after treatment with selected bioactive molecules.
3. To identify molecular targets of effective bioactive molecules during biofilm formation in *C. albicans*.

Selected bioactive molecules

The study focuses on seven bioactive molecules known for their various properties:

1. Zingerone: A phenolic compound from *Zingiber officinale* (ginger), known for its antimicrobial and antioxidant effects.
2. α-Bisabolol: A sesquiterpene alcohol found in *Matricaria recutita* (German chamomile), known for its anti-inflammatory and antifungal effects.
3. Nonanal: An aliphatic aldehyde found in essential oils with reported antifungal potential.

4. Undecanal: A citrus-derived aldehyde (*Citrus reticulata*), recognized for its inhibitory effects on fungal growth.
5. Berberine: An isoquinoline alkaloid from the *Berberis* genus, extensively studied for its antifungal and antimicrobial activities.
6. α-caryophyllene: A sesquiterpene found in *Humulus lupulus* (hops), exhibiting antifungal activity.
7. β-caryophyllene: A bicyclic sesquiterpene present in *Syzygium aromaticum* (cloves), with antimicrobial and antifungal properties.

Significance of the Study

Understanding the antifungal mechanisms of these bioactive molecules can provide crucial insights into their potential as novel therapeutic agents. This study will contribute to:

- The development of alternative antifungal strategies, addressing drug resistance in *C. albicans*.
- Identifying natural compounds that disrupt fungal biofilm formation, a key factor in antifungal resistance.
- Establishing a foundation for future research in plant-derived antifungal drug discovery.

Finally, this research aims to bridge the gap between natural product-based antifungal therapy and modern drug discovery. By investigating the antifungal activity and molecular targets of these bioactive molecules, this study seeks to expand our understanding of plant-derived molecules in combating *C. albicans* infections, ultimately contributing to the development of novel antifungal agents.

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Chapter II: Review of literature

2.1. Introduction

Fungal infections kill about 1.6 million people per year [1]. *Candida albicans* accounts for 75 % of all infections caused by the *Candida* genus. It is a global health burden, and the severity continues to increase rapidly [2]. *C. albicans* is a human commensal fungus present on the skin, oral, genitourinary, and gastrointestinal tracts. It may act as an opportunistic pathogen under immune-compromised conditions [3]. *C. albicans* can grow in several morphological forms, like yeast, hyphal, and pseudo-hyphal forms. Under certain conditions, *C. albicans* switches to a filamentous morphology, characterized by the formation of germ tubes from the yeast cell, which is followed by growth of branching hyphae. This transition from yeast to hyphal growth has attracted the attention of researchers, as it has a role to play in the development of virulence of *C. albicans*. Yeast to hyphal form morphogenesis can be induced by internal cellular signals as well as external environments. The modulation of endogenous cellular signals can affect hyphal development, and some specific receptors respond to external stimuli [4,5].

When signaling pathways interact with one another, they form networks, which cause cellular coordinated responses [6]. Such responses include alterations in the transcription, translation, post-translational, and conformational changes in proteins. These events are essential for controlling growth, proliferation, metabolism, and many other processes. Many transcription factors are responsible for the transcriptional regulatory network that coordinates environmental cues and controls phenotypic characters [7]. The *RAS1* is a master regulator of growth, stress response and cell death in eukaryotic cells [8]. Recent investigations in the regulation of yeast to the hyphal form transition of *C. albicans*, by natural antifungal molecules, are reviewed herewith special reference to morphogenetic signals.

2.2. Role of pathways involved in signal transduction in the regulation of virulence factors

The Ras1-cAMP-PKA pathway is crucial for controlling virulence factors in *C. albicans*. *RAS* serves as a key regulator for both the cAMP-PKA signaling pathway and the Sre11-Hst7-Cek1/2-mediated pathway in *C. albicans* [9]. The deletion of the *RAS* gene in *C. albicans* has been shown to reduce virulence in a mouse model of systemic infection [10]. Deletion of *CYR1* completely terminated virulence in both mouse mucosal membrane model as well as the systemic infection model [11]. The

protein kinase A catalytic subunit Tpk2 and its target Efg1 are essential for virulence in mouse oropharyngeal candidiasis but *TPK1* is not essential for virulence [12]. The *TPK2/TPK2* mutants exhibit similar virulence to the wild-type strain in a mouse model of systemic infection [13].

Yeast to hyphal form transition is the most important virulence factor in *C. albicans* [14]. A variety of environmental factors like temperature, serum, CO_2, pH, and nutritional conditions regulate the hyphal development through the Ras1-cAMP-PKA pathway in *C. albicans* [9]. After deletion of *RAS1,* hyphal growth defects are observed in the presence of serum, but are unable to block pseudo-hyphal development in *C. albicans* [15]. Deletion of *RAS2* does not affect the induction of hyphal growth [16]. The deletion of *RAS1* and *RAS2* affects hyphal growth via cAMP-independent pathways and cAMP-dependent pathways. Deletion of *PDE2* or *BCY1* enhances filament formation in *C. albicans*. Deletion of *CYR1* or *TPK1* and *TPK2* inactivates filament formation in response to serum, high levels of CO2 and N-acetylglucosamine [14].

Adenylyl cyclase (Cyr1) is considered a center point of all the environmental signal sensing and integration in *C. albicans* [10]. Cyr1 is a large protein that contains multiple functional domains responsible for environmental stimuli. Moderate interaction of *RAS1* and Cyr1 is essential for an increase in cAMP level and hyphal growth in response to external stimuli [17]. Leucine-rich repeat domain is responsible for high temperature >37℃ and peptidoglycan sensing, regulating hyphal growth via physical interaction with HSP90 [18]. There is a protein phosphatase 2C domain, a cyclase catalytic domain, and a cyclase-associated protein (Cap1) binding domain at the C-terminal of *C. albicans* Cyr1. CO_2 or HCO_3^- directly binds to the cyclase catalytic domain and enhances the production of cAMP and hence promotes filamentation in *C. albicans*. Lys 1373 of Cyr1 act as a receptor for CO_2/bicarbonate, which is essential for CO_2-induced filamentation. Farnesol, a quorum-sensing molecule, may inhibit the activation of *CYR1* by targeting its cyclase catalytic domain, thereby suppressing hyphal growth [19]. The addition of cAMP, a non-hydrolyzable functional analog of cAMP, restores filamentation in a culture medium containing farnesol. This filamentation relies on the transcription factor *EFG1*, which is regulated by the Ras1-cAMP-PKA pathway. Farnesol enhances the expression of *CAT1* and *HSP12* in the wild-type strain, and the two genes *RAS* and *CYR1* are also

present at higher levels in the null mutants [20]. The deletion of *EED1* led to an increase in farnesol production and hypersensitivity to farnesol in *C. albicans* [21].

TPK1 and *TPK2* genes plays different roles in the regulation of hyphal growth in *C. albicans*. The *TPK1/TPK1* mutant shows defects in hyphal formation in solid media, whereas it forms normal hyphae in liquid media. The *TPK2/TPK2* mutant grows as yeast cells in liquid media but shows partial defects in growth on solid media [22]. Research on filamentation suggests that, *TPK2* is crucial for regulating filamentous growth in both liquid and solid media. Under several culture conditions, including spider media, Lee's glucose, and Lee's GlcNac, deletion of *TPK1* or *TPK2,* caused filamentous growth. However, no hyphal growth was observed in the *TPK1/TPK1,* and *TPK2/TPK2* double mutants under all inducing conditions. Hence, it reveals that the catalytic subunit is essential for hyphal growth [14].

The cAMP-PKA signaling and MAPK pathways regulate morphogenesis in *C. albicans*. In response to environmental cues, *RAS1* gives a signal to both pathways to promote filamentous growth [11]. The transcription factors *EFG1* and *FLO8* are potential targets of PKA and play a critical role in the control of filamentous growth [14]. The deletion of *EFG1* reduces the ability to form filamentous growth. The *EFG1/EFG1* mutant can produce elongated cells and filaments that exhibit morphological differences compared to the true hyphae formed by wild-type strains. The *EFG1/EFG1* mutants form pseudohyphae on solid medium with serum [23].

The 206 of Efg1 is a potent PKA phosphorylation site needed for hyphal growth in the presence of inducing conditions [24]. LisH motif containing the transcription factor *FLO8* is required for hyphal growth induced by serum, CO_2, and Lee's medium. It has been suggested that *FLO8* interacts with *EFG1* in yeast and hyphal cells and may function synergistically [14]. If the CO_2 level increases, the Ras1-cAMP-PKA interacts with the tricarboxylic acid cycle and transcription factor *SFL2* to regulate filament formation in *C. albicans*. cAMP and ATP may function as molecular linkers in this regulatory process [25]. The Ras1-cAMP-PKA pathway also has a negative role in the regulation under certain conditions. The *CYR1/CYR1* and *FLO8/FLO8* mutants show increased hyphal growth in microaerophilic conditions as compared to wild-type strains [14]. It is suggested that *FLO8* and *EFG1* also function as repressors of hyphal development under several cultural conditions.

2.3. Natural molecules as inhibitors of signal transduction in *C. albicans*

The inhibition of signal transduction pathways by natural molecules is an intriguing area of study, especially in fields like pharmacology and biochemistry. Many natural compounds, derived from plants, microorganisms, or marine sources, have been found to modulate signal transduction pathways in various ways. Each of these natural molecules has garnered attention due to its potential health benefits and its ability to modulate specific signaling pathways, which can have implications in treating various diseases and conditions. Some of these molecules are listed here (Fig. 2.1; Table 2.1). Also, the target of these molecules in signal transduction pathways is discussed (Fig. 2.2).

2.3.1. Tetrandrine

Tetrandrine (TET), a bis-benzyl isoquinoline alkaloid, is a calcium channel blocker. It originates from the plant *Stephania tetrandra.* At low concentrations, it inhibits hyphal growth of *C. albicans* in both liquid and solid spider media. TET also inhibits biofilm formation of *C. albicans* at 16 mg/L and mature biofilms at 32 mg/L, respectively. TET at 32 mg/L concentration down-regulates the expression of hyphal specific genes *ECE1, HWP1,* and *ALS3* by 0.050, 0.083, and 0.036-fold, respectively. TET is able to inhibit hyphal growth through the Ras1-cAMP-PKA pathway. It was confirmed when exogenous cAMP addition caused restoration of the normal phenotype under TET exposure [25].

2.3.2. Capric acid and caprylic acid

Capric acid and caprylic acids are present in mammalian milk, coconut oil, and palm kernel oils. Both compounds were effective against all the virulence factors of *C. albicans,* like morphogenesis and biofilm formation. Capric acid and caprylic acid inhibit planktonic growth at 0.25 and 0.5 mg/ml, respectively. Moreover, both compounds are fungicidal. At the minimum fungicidal concentration, RAS-cAMP-Efg1 and MAPK pathways are affected by both natural compounds. The expression of genes associated with serum-induced morphogenesis demonstrated a decrease in *Cdc35* expression, which encodes adenylate cyclase. It is required for cAMP production in Ras1-cAMP-PKA and Cek1-MAPK pathways. It is downregulated after the treatment of capric and caprylic acid by 1.57-fold and 6.84-fold, respectively. After exposure to capric and caprylic acids, Pde2 gene expression is reduced by 1.46 and 6.41-fold, respectively. It is observed that *HWP1* encodes for the hyphal wall

proteins and the genes involved in the Cek1-MAPK pathway (*HST7* and *CPH1*), and after treatment with capric acid and caprylic acid, were also downregulated. Capric acid treatment upregulates the expression of *CEK1* by 2.27-fold, whereas caprylic acid treatment downregulates it by 1.5-fold. Downregulation of *BCY1,* which has a role in cell proliferation and death, is observed. It is also found that cell elongation gene *ECE1* is downregulated by 52.08-fold and genes like *NRG1*, a negative regulator of hyphal induction overexpressed by both capric acid and caprylic acid up to 11.66 and 10.87-fold and *TUP1* which is a negative regulator was overexpressed with the treatment of two fatty acids by three to four times [26].

2.3.3. Cedar leaf essential oil components

Cedar leaf essential oil (CLEO) possesses remarkable antibiofilm activity against *C. albicans*. It inhibits biofilm formation by more than 85 % without any effect on planktonic cells. Components of CLEO namely, camphor, fenchone, ethyl alcohol, α-thujone, and borneol, significantly reduce *C. albicans* biofilm formation. After treatments with CLEO, camphor, or fenchyl alcohol at 0.01 %, hyphal formation is inhibited, and this inhibition is responsible for their antibiofilm effects. Transcriptomic analysis indicated that camphor and fenchyl alcohol downregulated hyphal-specific genes involved in signal transduction pathways like *ECE1, ECE2,* and *HWP1*. On the other hand, after camphor or fenchyl alcohol treatment, *HGC1, HYR1, RAS1, TEC1,* and *UME6* remained unaffected. Based on these results, CLEO, camphor, and fenchyl alcohol it was suggested for controlling *C. albicans* infections [27].

2.3.4. Sanguinarine

Sanguinarine (SAN) is a quaternary benzo-phenanthridine alkaloid present in plants of the *Papaveraceae* family. The MIC_{50} of SAN was 3.2 µg/ml. It was also observed that at 0.8 µg/ml, SAN is found to suppress *C. albicans* biofilm and hyphal formation significantly. Treatment of SAN caused suppression of *ALS3, HWP1, ECE1, HGC1,* and *CYR1* gene expression. The endogenous cAMP level of *C. albicans* is also downregulated after SAN treatment, and the addition of cAMP restored the SAN induced filamentation defect. SAN showed relatively low toxicity to the human umbilical vein endothelial cells [28].

2.3.5. Hinokitiol

Hinokitiol, also known as β-thujaplicin, is a natural monoterpenoid found in the wood of trees in the family *Cupressaceae* like *Chamacypari staiwanensis*. With an MIC value of 1.6 mg/ml, hinokitiol proved very effective in preventing the planktonic development of *C. albicans*. With an MFC of 100 mg/ml, hinokitiol has a high killing potential for *C. albicans*. It is capable of preventing biofilm formation in both fluconazole-resistant strains and fluconazole-susceptible *Candida* species. The expression levels of genes associated with adhesion, *HWP1,* and *ALS3* are downregulated by hinokitiol. Hinokitiol also led to a reduction in the expression of *HGC1* and *UME6*, which are the genes responsible for maintaining long-term hyphal growth. This monoterpenoid also inhibited the expression of *CYR1, UME6,* and *HGC1* genes, which are linked to long-term hyphal maintenance. Master regulator RAS1 was also suppressed by hinokitiol [29].

2.3.6. Cinnamaldehyde

Cinnamaldehyde (CNMA), found in *Cinnamomum zeylanicum.* It is very effective against the planktonic growth of *C. albicans.* It has shown synergistic activity with fluconazole. Time-dependent kill curve analysis showed that the MFC of CNMA was 0.62 mM. Further effects of the encapsulated preparation of CNMA have been determined in multilamellar liposomes against *C. albicans*. These encapsulated preparations showed more fungicidal activity than free CNMA. The antifungal activity is due to reactive oxygen species and cellular damage by sustained release of CNMA. RT-PCR study revealed that after treatment with ML-CNMA, the expression of the *HWP1* gene, encoding hyphal wall protein, was significantly reduced [30].

2.3.7. Thiazolidinedione-8

Thiazolidinedione-8 (S-8) is a bacterial quorum sensing quencher found in *Vibrio harveyi,* having antibiofilm and antiadhesion properties against *C. albicans* at four to eight-fold lower concentrations than MIC. The MIC of planktonic growth is observed at 64 mg/ml, and 50 % biofilm inhibition at 8 mg/ml concentration was observed. This compound concentration-dependently downregulates the transcriptional level of genes involved in the adhesion process, including the hyphal-specific genes *HWP1* and *ALS3*. It is interesting to note that S-8 treatment does not affect the expression of *EFG1*, a component of the cAMP-PKA signaling cascade. The *UME6* gene is also significantly downregulated. Signaling pathways involved in

hyphal formation, like cAMP-PKA and MAPK, are found to be interrupted by S-8. *RAS1* gene expression was also downregulated by the treatment of S-8. Along with this, the expression levels of MAPK signal components *CST20, HST7,* and *CPH1* are downregulated by this compound. Finally, transcriptional repressors of filament formation, *TUP1* and *NRG1*, were dramatically upregulated by S-8 [31].

2.3.8. Cis-2-dodecenoic acid

Cis-2-dodecenoic acid (BDSF) is a quorum-sensing molecule present in *Burkholderia cenocepacia*. It suppresses the formation of germ tubes and biofilms in clinical isolates of *C. albicans*. At concentrations up to 120 mM, BDSF does not significantly affect the viability of *C. albicans*. BDSF (90 mM) inhibited the adherence capacity of *C. albicans*. The *EFG1* gene expression was upregulated by roughly 1.8-fold, whereas its downstream gene *YWP1* was overexpressed by more than 4-fold [32].

2.3.9. Biatriosporin D

Biatriosporin D (BD) is a small phenolic compound from an endolichenic fungus, *Biatriospora* sp. It was observed that BD prevents hyphal formation. At the low dosage of 2 mg/ml, BD was able to confine *C. albicans* in the yeast form after 24 h. Three genes that encode adhesins, *ALS3, HWP1*, and *ECE1*, had lower transcriptional levels at 1.5 and 6 h after the treatment with BD. Additionally, *RAS1, CDC35, EFG1,* and *TEC1* genes, which are linked to the Ras1-cAMP-Efg1 pathway, were dramatically downregulated by several orders of magnitude, while *PDE2* was upregulated in the presence of BD. Decreasing intracellular cAMP concentrations might be caused by up-regulating *PDE2* and down-regulating *CDC35* expression. Intracellular cAMP measurements revealed that BD regulated the Ras1-cAMP-Efg1 pathway by reducing cAMP levels to inhibit hyphal formation. It causes downregulation of *ECE1*. BD also stimulates the expression of Dpp3 to synthesize more farnesol that directly inhibits the *CDC35* activity, reducing intracellular cAMP and thereby disrupting the morphologic transition of *C. albicans* [33].

2.3.10. Eucarobustol E

Eucarobustol E (EE) is a formyl phloroglucinol meroterpenoid, an important class of secondary metabolite available in plants like *Eucalyptus* and *Psidium*. Its MIC_{50} against *C. albicans* ranged from 4 to 16 µg/ml for fluconazole-susceptible and

32 to 128 µg/ml for clinical drug resistant isolates. It is found that EE blocks yeast-to-hyphal transition in *C. albicans*. EE has a strong inhibitory effect against *C. albicans* biofilms at a concentration of 16 µg/ml. Exposure to the same concentration resulted in a marked reduction in the expression of genes like *EFG1, CPH1, TEC1, UME6, and HGC1* involved in hyphal growth, which are down-regulated by 0.49, 1.77, 1.54, 3.86, and 4.41-fold, respectively. The expression of genes encoding cell surface proteins like *ALS3* and *HWP*1 are also downregulated. Three key hyphal initiation regulators, *EFG1, CPH1,* and *TEC1,* are also downregulated by 1.58, 4.35, and 5-fold following EE treatment. The findings also revealed that EE upregulates negative regulator genes, *TUP1* and *NRG1,* in *C. albicans* [34].

2.3.11. Quinones and anthraquinone-related compounds

Quinones are found as biological pigments in sea urchins, aphids, lac insects, and certain scale insects, while anthraquinones are found in plants of families like *Rubiaceae* and *Leguminosae*. Alizarin and chrysazin suppressed biofilm formation in *C. albicans* at 2 µg/ml. The presence of a hydroxyl group at the C-1 position is essential for antibiofilm and anti-filamentation activities. The expression of the hypha-specific genes *ALS3* (2.4-fold), *ECE1* (3.7-fold), and *ECE2* (6.3-fold) was dramatically downregulated by the treatment of alizarin at 2 µg/ml. Furthermore, following 2 µg/ml treatment with chrysazin, the expression of *ECE1* and *ECE2* was dramatically downregulated by 2-fold and 2.3-fold, respectively. Alizarin treatment led to an increase in transcriptional expression of the hyphae-regulating gene *EFG1* in *C. albicans* [35].

2.3.12. Indole and Isatin

Indole is a compound synthesized by animals, plants, bacteria, and fungi. Isatin (1H-indole-2,3-dione), which is an important derivative of indole, has important physiological functions in humans. Both molecules are capable of inhibiting yeast to hyphal morphogenesis in *C. albicans* at a 0.25 mg/ml concentration. Indole downregulated eleven genes of the signal transduction pathway, while isatin upregulated the expression of twelve genes involved in the signal transduction pathway. Indole only caused an 11.6-fold downregulation of *ECE1*, but isatin caused a 25.5-fold downregulation. In contrast to the 35-fold downregulation caused by isatin, *HWP1* was downregulated 77-fold by Indole. Negative hyphal regulators, such as *NRG1* and *TUP1*, are upregulated by indole and isatin. However, indole reduced

*MIG*1 by 1.4 times. Isatin upregulates the expression of *NRG1, TUP1,* and *MIG1*. Out of the eleven genes downregulated after the treatment, four of them are from the Cek1-MAPK pathway. Isatin upregulates 4 genes from the Cek1-MAPK pathway. The expression of Ece1 and Hwp1 is downregulated by both molecules. Isatin generated a 78-fold overexpression of *NRG1,* but indole only produced a 1.5-fold increase. Isatin and indole upregulate *TUP1* expression by 12-fold and by 5.5-fold, respectively. Hyphal suppressor genes *NRG1* and *TUP1* are significantly upregulated and affect the expression of multiple genes in the signal transduction pathway of *C. albicans* in the presence of isatin and indole [36].

2.3.13. Phorbasin H

Phorbasin H is a diterpene acid of a bisabolene related skeletal class, isolated from the amarine sponge *Phorbas sp*. This compound acts as an inhibitor of yeast to hyphal transition in *C. albicans*. it inhibits the expression of genes related to the cAMP-Efg1 pathway. *CPH1, EFG1,* and *TUP1* mRNA expressions are not suppressed in phorbasin H-treated cells, according to a Northern blot study analysis. Interestingly, 125 mg/ml phorbasin H causes a total decrease in *HWP1* mRNA expression. The exogenous addition of cAMP to *C. albicans* cells did not affect hyphal formation. The expression of hypha-specific *HWP1* and *ALS3* genes, which are positively regulated by an important regulator of cell wall dynamics, *EFG1*, is significantly inhibited by Phorbasin H addition. This compound also has the ability of *C. albicans* cells to adhere in a dose-dependent manner. Phorbasin H affects the activity of the cAMP-Efg1 pathway, thus leading to inhibition of morphogenesis [37].

2.3.14. Farnesol

Farnesol is a quorum-sensing molecule produced by *C. albicans* extracellularly. It also acts as an autoregulatory component that prevents yeast cells from germination. It inhibits the Ras-cAMP pathway through direct inhibition of Cyr1. Though it acts as a regulator of yeast to hyphal morphogenesis, the exact mechanism is not clear [38]. Farnesol sensitivity was observed when growth media and conditions were altered. *C. albicans* gives a response to farnesol by altering gene expression. An increase in *TUP1* expression was observed after treatment with farnesol, while the expression of *HWP1* decreased. *NRG1* remains unaffected by the

farnesol. The expression of *EFG1* activates *HWP1*. The mRNA levels of *EFG1* are regulated during filamentation, but not affected by farnesol [38].

2.3.15. Purpurin

The food coloring agent purpurin is a natural red anthraquinone pigment commonly found in madder roots (*Rubia cordifolia*). The sub-lethal concentration of 3µg/ml was inhibitory for the yeast to hyphal transition in *C. albicans*. Purpurin inhibited biofilm formation and reduced the metabolic activity of mature biofilms. qRT-PCR analyses indicated that purpurin downregulated the expression of hypha-specific genes *ALS3*, *ECE1*and *HYR1* by 59 %, 61 %, and 40 %, respectively. The hyphal growth regulator *RAS1* was also reduced by 40%. *HWP1* expression dropped by more than 88 % [39].

2.3.16. Retigeric Acid B

Retigeric acid B (RAB) is a pentacyclic triterpene acid present in the lichen species *Lobaria kurokawae.* It has antifungal activity when applied alone or in combination with azoles, especially effective against azole-resistant strains. RAB demonstrated antifungal efficacy against these *C. albicans* strains *in vitro* at 8-16 mg/ml (MIC80). The gene that encodes phosphodiesterase, *PDE2*, is upregulated after the treatment of RAB. The results revealed that *RAS1* and RAB do not interact directly. RAB did not appear to have any impact on *EFG1*. The level of phosphodiesterase increased, which is encoded by the *PDE2* gene. In the MAPK cascade, *CST20* and *CPH1* are increased as a kind of feedback for the Ras1-cAMP-Efg1 pathway that is blocked. RAB also represses *CDC35* activity by stimulating farnesol production which leads to a decrease in cAMP synthesis, leading to retarded yeast to hyphal transition. Reduction in levels of intracellular cAMP resulted in the inhibition of downstream adhesions [40].

2.3.17. Piperine

Piperine is the main bioactive alkaloid in pepper seeds and is known for giving them their characteristic pungency. Even at the highest tested dose (2 - 1,024 µg/ml), piperine significantly affects the development of *C. albicans.* A maximum of 93 % of the biofilms were reported to be suppressed by piperine at a dosage of 32 µg/ml. It dramatically reduces the expression of signal transduction genes such as *HWP1, HST7, RAS1,* and *ECE1.* The filamentous growth transcriptional regulators, such as

CPH1, UME6 and *EFG1,* and *ALS3*, are also downregulated by piperine at biofilm inhibiting concentrations [41].

2.3.18. Ellagic acid

Ellagic acid is a phenolic compound found in various plants and fruits. It has antioxidant, antimicrobial, and anti-inflammatory activities. Ellagic acid inhibits *C. albicans* growth at 12.5 µg/ml after 48 h incubation. It inhibits biofilm formation at 25 µg/ml. Gene expression studies revealed that ellagic acid causes downregulation of two hyphal-specific genes, *HWP1* and *ALS3* [42].

2.3.19. Berberine

Berberine is a quaternary ammonium salt from the protoberberine group of benzyl isoquinoline alkaloids found in some plants like *Berberis*. Berberine is usually found in the roots, rhizomes, stems, and bark of these plants. Berberine hydrochloride is found to inhibit biofilms of *C. albicans*. It also inhibits the expression of three major genes in signal transduction pathways, including *EFG1*, *HWP1,* and *ECE1* [43,44].

2.3.20. Curcumin

Curcumin (CUR) is a natural plant polyphenol produced by the rhizome of *Curcuma longa*. Curcumin inhibits the growth of *C. albicans* and non-albicans *Candida* species through increased production of reactive oxygen species (ROS) and induction of early apoptosis. To study the antifungal mechanism of curcumin, various morphological, iron transporter, and oxidative stress mutants of *C. albicans* are used. The oxidative stress mutant is more susceptible than the other mutants. It is also observed that growth inhibitory effects and elevated ROS levels can be reversed if natural or synthetic antioxidants are present in the growth medium. ROS stimulates the pro-apoptotic regulatory machinery in *Candida* cells, and hence increases the number of pre-apoptotic cells. Even at low concentrations, CUR can block the hyphae development in both *albicans* and non-*albicans* species of *Candida*. CUR increases *TUP1* transcript levels in wild-type *C. albicans* cells [45].

2.3.21. Quinic acid

Quinic acid is a cyclic polyol, usually obtained from different medicinal plants including *Eucalyptus globules, Hymenocrater calycinus, Tara spinosa, Ageratina*

adenophora, Urtica dioica, coffee beans, and barks of *Cinchona* trees. Combinations of quinic acid and undecanoic acid significantly inhibit the virulence of *Candida* spp. such as extracellular polymeric substances production, biofilm formation, yeast to hyphae formation, secreted hydrolase production, and ergosterol biosynthesis. *In vivo* studies, carried out by using *Caenorhabditis elegans*, reveal the non-toxic nature of the quinic acid-undecanoic acid combination and its anti-virulence effect against *Candida* spp. The genes involved in biofilm formation are tested, and it is observed that *ALS1, HWP1, EFG1*, and *UME6* were majorly downregulated while *ALS3, CST20, RAS1, HST7,* and *CPH1* genes are moderately downregulated upon treatment with this combination. Slight upregulation is observed in the case of *NRG1* and *TUP1* genes [46].

Table 2.1: Regulation of genes in the signal transduction pathway of *C. albicans* by natural molecules having antifungal potential.

Natural compound	Upregulated genes	Downregulated genes	References
Tetrandrine	-	*HWP1* *ECE1* *EFG1*	[25]
Capric acid	*EFG1* *TEC1* *ECE1*	*RAS1* *CDC35* *PDE2* *HWP1* *BCY1*	[26]
Caprylic acid	*RAS1* *BCY1* *EFG1*	*CDC35* *PDE2* *TEC1* *ECE1* *HWP1*	[26]
Cedar leaf oil 1. Camphor 2. Fenchyl alcohol	-	*ECE1* *ECE2*	[27]
	-	*ECE1* *ECE2*	
Sanguinarine	-	*HWP1* *ECE1* *HGC1* *CYR1*	[28]
Hinokitiol	-	*HWP1* *UME6* *HGC1* *CYR1* *RAS1* *HWP1* *EFG1*	[29]
Cinnamaldehyde	-	*HWP1*	[44]

Thiazolidinedione-8	*TUP1* *NRG1*	*HWP1* *UME6* *CST20* *HST7* *CPH1* *RAS1*	[31]
Cis-2-dodecenoic acid	*EFG1*	-	[32]
Biatriosporin D	*PDE2*	*HWP1* *ECE1* *RAS1* *CDC35* *EFG1* *TEC1*	[33]
Eucarobustol E	*NRG1*	*ECE1* *UME6* *HGC1* *TEC1* *EFG1* *CPH1*	[34]
Quinones and anthraquinone related compounds	*EFG1*	*ECE1* *ECE2*	[27]
Indole	*NRG1* *TUP1*	*HWP1* *MIG1*	[36]
Isatin	*NRG1* *TUP1*	*HWP1*	
Phorbasin H	-	*HWP1* *EFG1*	[37]
Farnesol	*TUP1*		[38]
Purpurin	-	*HWP1* *HYR1* *ECE1* *RAS1*	[39]
Retigeric Acid B	*PDE2* *CST20* *CPH1*	*CDC35* *HWP1* *ECE1*	[40]
Piperine	-	*HWP1* *HST7* *RAS1* *ECE1* *CPH1* *UME6* *EFG1*	[41]
Ellagic acid	-	*HWP1*	[42]
Berberine	-	*EFG1* *HWP1* *ECE1*	[43]
Curcumin	*TUP1*	-	[45]
Quinic acid	*NRG1* *TUP1*	*HWP1* *EFG1* *UME6* *CST20* *RAS1*	[46]

		HST7 *CPH1*	

*HWP1:*Hyphal wall protein; *ECE1:* Endothelin converting; *EFG1:* Enhanced filamentous growth protein; *RAS1***:** RAS-like protein; *CDC35*: Adenylate cyclase; *PDE2*: 3',5'-cyclic-nucleotide phosphodiesterase; *TEC1*: Transcription activator; *BCY1*: cAMP-dependent protein kinase regulatory subunit; *HGC1*: Hypha-specific G1 cyclin-related protein1; *CYR1*: Adenylate cyclase; *UME6*: Transcriptional regulatory protein; *CST20*: Serine/threonine-protein kinase; *HST7*: Serine/threonine-protein kinase *STE7* homolog; *CPH1*: Transcription factor ; *NRG1*: Transcriptional regulator; *MIG1*: Regulatory protein; *TUP1***:** Transcriptional repressor; *HYR1*: Hyphal regulated cell wall protein.

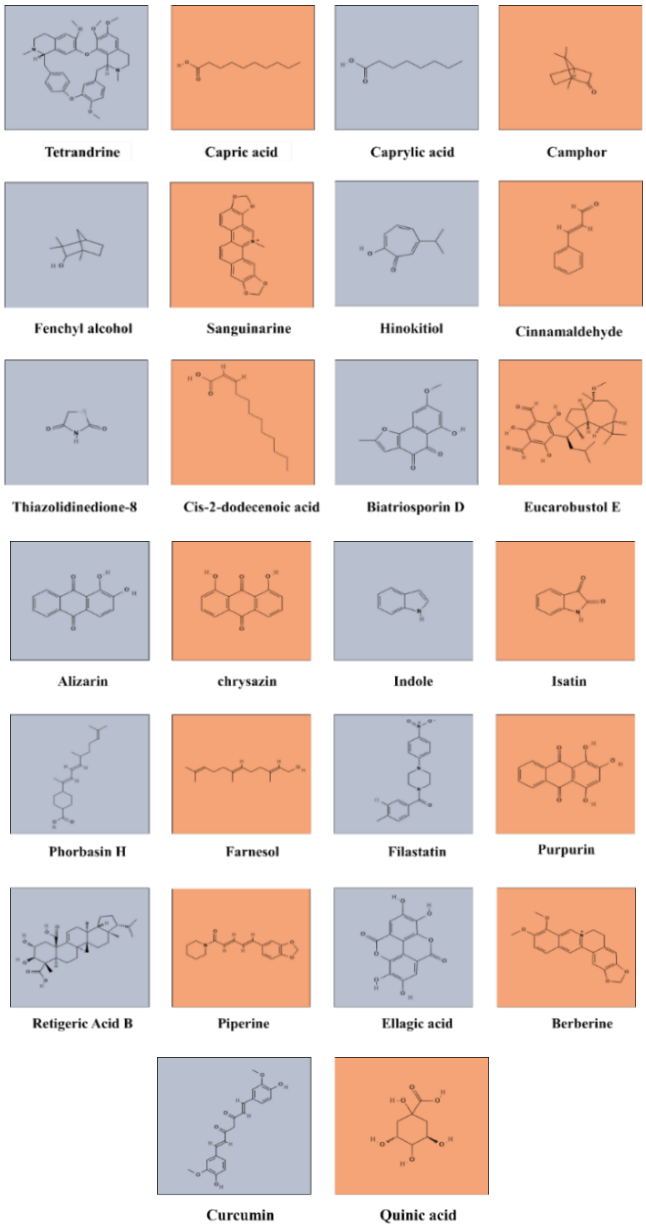

Fig. 2.1: Chemical structures of the bioactive molecules studied for their anti-virulence activity against *C. albicans*.

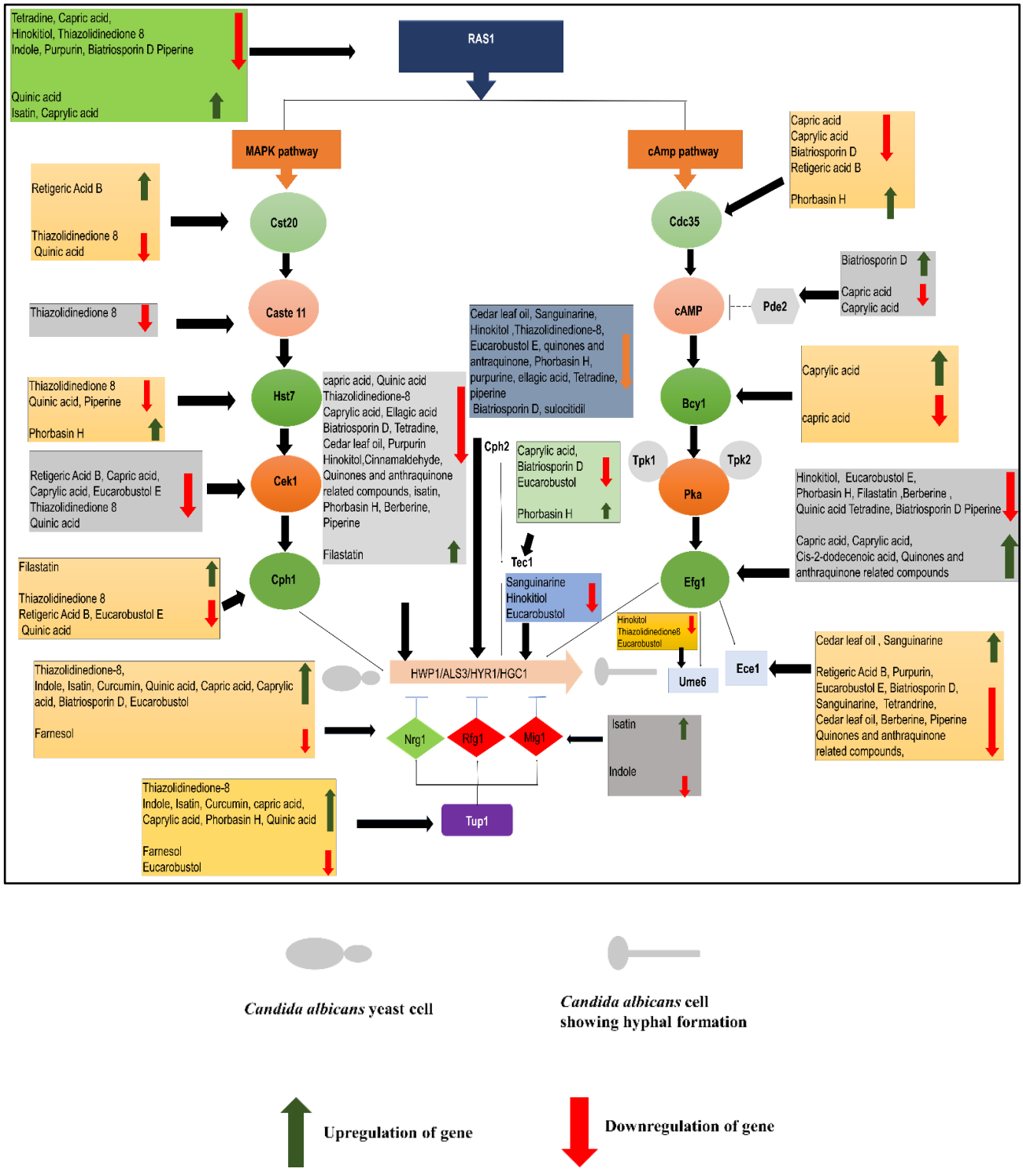

Fig. 2.2: Summary of the effect of different bioactive molecules on gene expression in *C. albicans* studied to date.

2.4. Conclusions

Colonization of prosthetic devices and the formation of biofilms by *Candida albicans* is a significant clinical problem faced by patients, physicians, and drug developers. The biofilms formed are resistant to most of the antifungal antibiotics. As such, there is a necessity to identify novel antibiofilm agents. Natural molecules derived from plant origin have various bioactive properties, including antifungal activity. Some of the molecules targeting signal transduction pathways involved in yeast to hyphal morphogenesis are reviewed here. These molecules can be good candidates for developing new antifungal drugs with no toxicity.

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Chapter III:
Materials and Methods

3.1. Culture and bioactive molecules

A strain of *C. albicans* American Type Culture Collection (ATCC) 90028 was purchased from the Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India. Yeast extract-Peptone-Dextrose (YPD) agar medium was used to cultivate the culture. The bioactive test molecules zingerone, undecanal, nonanal, α-bisabolol, α-caryophyllene, β-caryophyllene and berberine were purchased from Sigma Aldrich, USA.

Standard antifungal drugs: Fluconazole (FLC) and Amphotericin B (Amp B) were used as positive controls.

3.2. Media components and chemicals

All the media, media components, and chemicals were purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. 2, 3-bis (2-methoxy-4-nitrosulfophenyl)-2H-tetrazolium-5-carboxanilide was purchased from SRL Pvt. Ltd., India. All the chemicals are of high degree purity and used as received.

3.3. YPD

50 ml of distilled water was taken in a 250 ml conical flask. 2.5 g of YPD broth and 1.5 g of agar were added to 50 ml of distilled water. The flask was covered with a cotton plug, and the media was sterilized at 120 ºC temperature and 15 lbs pressure by using an autoclave.

3.4. Sabouraud dextrose agar (SDA)

50 ml of distilled water was taken in a 250 ml conical flask. 1.5 g of SDA broth and 0.5 g of agar were added. The flask was closed with a cotton plug, and the media was sterilized at 120 ºC temperature and 15 lbs pressure by using an autoclave.

3.5. 3-(N-morpholino) propane sulfonic acid (MOPS) buffer

1.72 g MOPS powder was added to 50 ml of sterile distilled water. It was stirred by a glass rod till the MOPS dissolved completely in the sterile distilled water.

3.6. Roswell Park Memorial Institute (RPMI 1640) medium

RPMI 1640 medium was used as the growth medium for *C. albicans*. 0.52 g of RPMI1640 (w/L- Glutamine, w/o sodium bicarbonate) was added to 50 ml of MOPS buffer (prepared by the above method). The pH was adjusted up to 6.5. The medium was filter sterilized by using a syringe filter with a pore size of 0.22 µm. For filtration,

the medium was filled in the syringe of 20 ml capacity syringe and it was passed through a sterile syringe filter in a 50 ml sterile Falcon tube. 200 µl RPMI medium was added to a 1.5 ml sterile Eppendorf tube and kept in an incubator for 24 h at 37 °C to ensure its sterility. The clear medium confirms the sterility, and the sterile medium was used for the experiments. If the medium shows turbidity, the respective batch of medium was re-sterilized.

3.7. 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide) (XTT) reagent

3.7.1. Stock solution

10 mg of XTT was weighed and added to 10 ml of distilled water. It was filter sterilized by a membrane syringe filter of pore size 0.22 µm.

3.7.2. Menadione stock

10 mM stock of menadione was used. 0.0172 g menadione was added to 10 ml of acetone.

3.7.3. Working XTT:

990 µl from the XTT stock and 10 µl of menadione stock were mixed to make 1 ml of working XTT solution.

3.8. Potassium phosphate buffer (PBS)

3.8.1 K_2HPO_4 solution (a)

0.68 g of K_2HPO_4 was added to 50 ml of distilled water.

3.8.2 KH_2PO_4 solution (b)

3.48 g of KH_2PO_4 was added to 250 ml of distilled water. 40 ml of solution (a) and 160 ml of solution (b) were mixed to make 200 ml of 10x Potassium phosphate buffer. To make 1x Potassium phosphate buffer, 10 ml of 10x buffer was added to 90 ml of distilled water to make a final volume of 100 ml. The pH of PBS was 7.2. Then it was filter sterilized by a membrane filter.

3.9. 20 % fetal bovine serum

The fetal bovine serum was utilized to induce yeast to hyphal morphogenesis. The 20 % serum was prepared by the addition of 20 ml of fetal bovine serum to 80 ml of sterile distilled water. The serum was sterilized by using a membrane syringe filter 0.22 µm pore size before performing the experiments.

3.10. Alcoholic KOH

Alcoholic KOH was used for the extraction of sterol content from *C. albicans*. The alcoholic KOH was prepared by the addition of 5 g of KOH to 7 ml of sterile distilled water. Then, 13 ml of 99.9 % pure ethanol was added to the above solution to make the final volume 20 ml.

3.11. Revival of the culture

The revival of *C. albicans* ATCC 90028 culture was necessary for growth and further experimentation. The care was taken in opening the ampoule as the contents were in a vacuum mark was made on the ampoule near the middle of the cotton wool with a sharp file. The surface around the mark is disinfected with alcohol. Thick cotton wool wrapped around the ampoule and broke at the marked area. The pointed top of the ampoule was removed. The cotton plugs were carefully removed, and 0.4 ml of sterile distilled water was added and allowed it stand for 20 min before being transferred onto solid medium. A few drops of the suspension were streaked on a sterile YPD agar plate and slants. The rest of the suspension was stored as water culture in a 1.5 ml sterile centrifuge tube. Incubated at 30 ºC for 24 h. After incubation slants and plates were stored as master cultures. By using master cultures, working cultures were made. All the remains in the original ampoule were sterilized before discarding.

3.12. Culture conditions

A single colony of *C. albicans* ATCC 90028 isolated from YPD agar was used to activate the culture by inoculating it into 50 ml of YPD broth in a 250 ml conical flask. The flask was incubated for 20 h on an orbital shaking incubator (100 rpm) at 30 ºC. *C. albicans* cells were collected by centrifugation at 5000 rpm for 5 min and washed three times using sterile PBS and suspended in 1 ml of PBS to use in experiments.

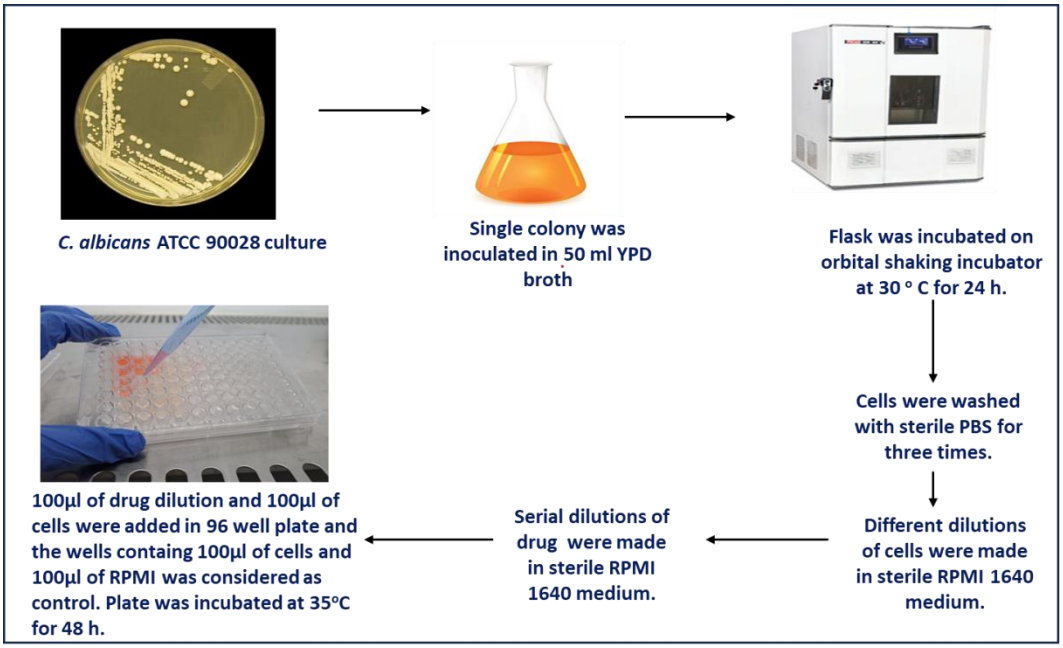

Fig. 3.1: Schematic representation of the experimental workflow for the broth microdilution method used to determine the MIC of test compounds against *C. albicans*.

3.13. Minimum inhibitory concentration (MIC) for planktonic growth

The National Council for Clinical Laboratory Standards CLSI M27-A2 technique for micro dilution broth-based (Fig. 3.1) antifungal susceptibility testing of yeasts was used to identify the minimum inhibitory concentrations (MICs) of all the molecules selected for this study [1 2]. 100 µl cell suspensions (1×10^3 cells/ml of RPMI1640) were also added to each well of polystyrene 96-well plates. Various concentrations of test molecules ranging from 0.125 to 4 mg /ml were prepared in RPMI-1640 medium in sterile Eppendorf tubes. Then, 100 µl of dilutions of the test molecule were added to the respective wells. The plates were incubated at 35 °C for 48 hours, and growth was assessed by measuring the absorbance at 620 nm with a microplate reader (Multiscan Sky, Thermo Scientific). Each experiment was performed in triplicate (Fig. 3.1).

3.14. Minimum fungicidal concentration (MFC)

MFC was determined to check the fungicidal concentration of the bioactive molecule [3]. Molecules for which the MIC was achieved (in the range 0.125-4 mg/ml) were selected for the MFC determination experiment. *C. albicans* cells from the MIC and wells containing concentrations above the MIC were used. Aliquots of 10 µl from these wells were spread on YPD agar plates. These plates were incubated

for 48 h at 30 °C and observed for the presence of colonies. The lowest concentration of the test molecule that exhibited no growth was identified as the MFC.

3.15. Kill curve assay

Time required to kill *C. albicans* cells after the treatment of test molecules was determined by the time-dependent kill curve assay method [4]. 2×10^3 cells/ml were added to 10 ml of sterile YPD broth. The MFC concentration of test molecules was added to the medium. The flask was incubated at 30 °C. 1 ml of broth from the flask was taken at different time intervals (0, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h). The cells were harvested by centrifugation and washed twice with PBS. The 20 µl of cell suspension was inoculated on a YPD agar plate. The plates were incubated at 30 °C for 24 h. The number of colonies grown for control and test was counted. The experiment was performed in triplicate.

3.16. Yeast to hyphal morphogenesis assay

The assay was used to determine the effect of test molecules on yeast to hyphal morphogenesis of *C. albicans* [5]. Yeast to hyphal morphogenesis was studied using 96-well polystyrene microtiter plate-based assay. Cell density of the stock suspension was determined by hemocytometer count. Cells were inoculated in 20 % serum prepared in deionized distilled water to get 1×10^6 cells/ml, and various concentrations of test molecules were added. Wells without any concentration of test molecules were kept as a control. The final volume of the assay system in each well was kept at 200 µl. The plates were incubated at 37 °C at 100 rpm on an orbital shaker for 90 min, and cells were observed microscopically. The numbers of yeast and hyphae were noted. The percentage of yeast to hyphal morphogenesis was calculated. The experiment was performed in triplicate.

3.17. Adhesion assay

The assay was used to determine the effect of test molecules on the adhesion of *C. albicans* to polystyrene surfaces [6]. The cells of *C. albicans* at a concentration of 10^7 cells/ml in RPMI-1640 were exposed to varying concentrations of bioactive molecules for 90 minutes at 37°C. Following this, the cells were washed three times with PBS. Subsequently, XTT assays were performed to assess the inhibitory effect of the test molecule on the adhesion of *C. albicans* cells. The experiment was performed in triplicate.

3.18. Biofilm development assay

Biofilm development assay was used to detect the inhibitory effect of test molecules on *C. albicans* biofilm development [7]. Biofilm was formed on a tissue culture treated 96-well microtiter plate. To allow the adherence of cells to the solid surface, 100 µl of cells (1 x 10^7 cells/ml) was poured into each well and incubated at 37 °C (100 rpm). The wells were washed with PBS, and RPMI1640 (100 µl) was added to the wells. Also, various concentrations of test molecules prepared in RPMI1640 medium were added to the wells and incubated (37 °C) for 48 h. The experiment was performed in triplicate.

3.19. Mature biofilm assay

The activity of test molecules on mature biofilm was studied by using 24 h old *C. albicans* biofilm [8]. It was prepared on tissue 96-well plates. The concentrations of test molecules made in RPMI 1640 were added in to the wells. The plates were incubated at 37 °C for 24 h. XTT metabolic assay was performed to analyse metabolic activity of *C. albicans* biofilm. The experiment was performed in triplicate.

3.20. XTT assay for biofilm quantification

XTT metabolic assay was used for the quantitative estimation of adhesion and biofilm growth of *C. albicans* [9]. The wells containing biofilms were washed with PBS to remove non-adherent cells and then incubated with 100 µl of XTT-Menadione solution in the dark at 37 °C for 5 h. Orange color development by the water-soluble formazan product was measured at 450 nm using a Multiskan sky Thermofisher (India) spectrophotometer. The experiment was performed in triplicate.

3.21. Scanning electron Microscopy (SEM)

Scanning electron microscopy was used to observe the effect of test molecules on the structure of biofilm during biofilm development [10]. The test molecules inhibiting biofilm development were selected for SEM. The small pieces of Foley's urinary catheter were made and sterilized by using absolute alcohol. After drying, the *C. albicans* biofilms were developed by seeding a Foley's catheter with 2 ml of a standardized cell suspension of 1×10^7 cells/ml in a 12-well plate. The plates were incubated at 37 °C at 50 rpm for 90 min. The catheter pieces were removed from the cell suspension and washed with sterile PBS solution to remove non-adhered cells. Then the pieces were kept in the wells containing RPMI1640 medium, as well as the

MIC concentration of test molecules. The plates were again incubated at 37 ℃ for 24 h. The pieces were removed from the wells and washed with sterile PBS solution and observed under a microscope for biofilm formation. Further, SEM samples were fixed in 2.5 % glutaraldehyde in PBS (pH 7.2) for 24 h at 4 ℃. Samples were fixed in 2 % aqueous solution of osmium tetroxide for 4 h, and then dehydrated in a series of graded alcohols. The samples were mounted on stubs, and gold sputtering was carried out using an automated gold coater. Images were obtained by scanning electron microscope JEOL 6360 (Tokyo, Japan).

3.22. Cell cycle analysis

Cell cycle analysis was used to study the effect of test molecules on the cell cycle propagation of *C. albicans*. The test molecules inhibiting planktonic growth were selected for the cell cycle analysis. The *C. albicans* cell cycle study was performed as described previously by Gupta et al. (2020). Log phase *C. albicans* cells were treated with the molecule for nearly 4 h 30 min at 35 °C in (10 ml RPMI/ 10^6 cells/ml, MIC_{50}) and then washed twice with PBS (pH 7.2). After washing, cells were fixed overnight in 70 % ethanol, and the next day were incubated with 50 µg/ml of PI and 10 µg RNase A, after washing with PBS. After 30 min of incubation at 4 °C, the cells were analyzed using a Flow cytometer (FACS). The experiment was performed in triplicate.

3.23. Ergosterol assay

Ergosterol assay was used to find the effect of test molecules on ergosterol synthesis in *C. albicans* [11]. The test molecules inhibiting planktonic growth were selected for the ergosterol assay. A colony from an overnight sabouraud dextrose agar (SDA) plate and inoculated in 50 ml sabrouds dextrose broth (SDB) for control and various concentrations. The culture was incubated for 16 h and harvested by centrifugation at 2700 rpm for 5 min. The net weight of the cell pellet was determined. 3 ml of 25 % alcoholic potassium hydroxide solution was added to each pellet and vortexed for one min. Cell suspensions were transferred to sterile borosilicate glass screw cap tubes and incubated in an 85 °C water bath for one hour. After incubation, the tubes were cooled. Sterols were extracted by the addition of a mixture of one ml of sterile distilled water and 3 ml of n-heptane, and mixed for 3 min. 0.6 ml aliquots of sterol were prepared and diluted fivefold in 100 % ethanol.

Scanned between 240-300 nm by using a spectrophotometer (Multiscan Sky Thermofisher).

3.24. Membrane integrity analysis

The membrane integrity analysis assay was used to evaluate plasma membrane damage by the treatment of test molecules [12]. The test molecules inhibiting planktonic growth were selected for the membrane integrity analysis. Post-treatment, cells in their log phase were collected via centrifugation and underwent washing with PBS to eliminate other cellular impurities. These cells were then suspended in PBS (50 µl) and exposed to propidium iodide (PI) in the dark for 20-30 min. Subsequently, the cells were observed under a fluorescence microscope at 40x magnification (Nikon, T1-SAM JAPAN). The experiment was performed in triplicate.

3.25. Assessing Reactive Oxygen Species (ROS) Generation in *C. albicans*

The ROS generation assessment was carried out to check the production of ROS after the treatment of bioactive molecules. The test molecules inhibiting planktonic growth were selected for the ROS generation assessment. The measurement of ROS was done according to the previously described method with slight modifications [13]. *C. albicans* cells underwent treatment with test molecules alongside 2',7'-dichlorofluorescin diacetate (H_2DCF) to assess the levels of endogenous ROS. Cells in logarithmic growth phase were exposed to test molecules at concentrations of MIC_{50} in YPD for durations of 4 h at 30 °C. Following this treatment, cells underwent three washes with PBS (pH 7.2) and then were exposed to H_2DCF at a concentration of 10 µM in PBS. After an incubation of 30 min (30 °C), the samples were quantitatively examined using a spectrofluorometer (486 nm excitation and 525 nm emission wavelengths). The experiment was performed in triplicate.

3.26. Gene expression studies

3.26.1. RNA extraction and cDNA synthesis

The gene expression studies were carried out to evaluate the effect of test molecules on gene expression of *C. albicans* during biofilm formation and planktonic growth for ROS generation [14]. The expression of hyphal genes during biofilm formation was measured using quantitative real-time polymerase chain reaction (qRT-PCR). *C. albicans* (1×10^7 cells/ml) cells were added to a 12-well plate and incubated

for 90 min. at 100 rpm. The plates were removed and the wells were washed with sterile PBS to remove non adhered cells. RPMI1640 medium and MIC_{50} concentrations of test molecules were added to the test. Wells without any test molecules served as controls. Total RNA was isolated by using RNeasy® Mini Kit (QIAGEN, USA) and converted to cDNA by using SuperScript® III First strand synthesis for qRT-PCR (Bio-Rad, USA). The experiment was performed in triplicate.

3.26.2. qRT-PCR

qRT-PCR reactions were carried out using the KAPA SYBR® Fast qPCR Kit Master Mix (2x) (BIOSYSTEMS, South Africa) in 96-well PCR plates [14]. The protocol included an initial denaturation at 95 °C for 3 min, followed by 32 amplification cycles consisting of denaturation at 95 °C for 30 sec, annealing at 60 °C for 20 sec, and primer extension at 72 °C for 30 sec, using the CFX 96 Quantitative Real Time System (Bio-Rad, USA).

3.26.3. Primers

Primers were purchased from Eurofins Genomics, Bangalore, India Pvt. Ltd. Actin, a housekeeping gene, was used as an internal control (Table 3.1) [14].

Table 3.1: Gene-specific primers used for qRT-PCR.

Primers	Sequence (5'⟶ 3')
ACTIN-F	5'ATGGACGGTGAAGAAGTTGC 3'
ACTIN-R	5'ACCTCTTTTGGATTGGGCTTCA 3'
RAS1-F	5'GGCCATGAGAGAACAATATA 3'
RAS1-R	5'GTCTTTCCATTTCTAAATCAC 3'
PDE 2-F	5' ACCACCACCACTACTACTAC 3'
PDE 2-R	5' AAAATGAGTTGTTCCTGTCC 3'
BCY 1-F	5′ CCC AAGCTTATGTCTAATCCTCAACAGCA 3′
BCY 1-R	5′ GGG CTGCAGTTAATGACCAGCAGTTGGGT 3′
EFG 1-F	5' TATGCCCCAGCAAACAACTG 3'
EFG 1-R	5' TTGTTGTCCTGCTGTCTGTC3'
TEC 1-F	5' AGGTTCCCTGGTTTAAGTG 3'
TEC 1-R	5' ACTGGTATGTGTGGGTGAT 3'
ECE 1-F	5'CCCTCAACTTGCTCCTTCACC3'
ECE 1-R	5'GATCACTTGTGGGATGTTGGTAA3'
CEK 1-F	5' AGCTATACAACGACCAATTAA 3'
CEK 1-R	5' CATTAGCTGA ATGCATAGCT 3'
HST 7-F	5' ACTCCAACATCCAATATAACA 3'
HST 7-R	5' TTGATTGACGTTCAATGAAGA 3'
CPH1-F	5'ATGCAACACTATTTATACCTC 3'

CPH2-R	5'CGGATATTGTTGATGATGATA 3'
HWP1-F	5'TGGTGCTATTACTATTCCGG 3'
HWP1-R	5'CAATAATAGCAGCACCGAAG 3'
MIG1-F	5'CTTCAACTAGCCTATATTCCGATGG 3'
MIG1-R	5'-CTTTCT GTAGGTACCAACAACTAC 3'
NRG1-F	5'CACCTCACTTGCAACCCC 3'
NRG1-R	5'GCCCTGGAGATGGTCTGA 3'
TUP1-F	5'GAGGATCCCATGTATCCCCAACGACCCAG3'
TUP1-R	5'GGCGACGCGTCGTTTTTTGGTCCATTTCCAAATTCTG3'
CDC35-F	5'TTCATCAGGGGTTATTTCAC3'
CDC35-R	5'CTCTATCAACCCGCCATTTC3'
SOD1 - F	5' TTGAACAAGAATCCGAATCC 3'
SOD1 - R	5' AGCCAATGACACCACAAGCAG 3'
SOD2-F	5' ATGTTTTCTATCAGATCATC 3'
SOD2-R	5' ACCACCACCTTGAGAGACAGGAGCC 3'
CAP1 -F	5'AGTCAATTCAATGTTCAAG 3'
CAP1 -R	5' AATGGTAATGTCCTCAAG 3'
CAT1-F	5' CCCAGAAAGAGTTGTCCACGC 5'
CAT1-R	5' CCATGATGGGTAGTTACCAGCAGC 5'
MCA1-F	5' TGGTACTGCCACTGGTGCTA 3'
MCA1-R	5' TGGGAAGCAGATAATTGTGG 3'

3.26.4. Quantification of gene expression

The gene expression levels of selected genes in *C. albicans* after the treatment of molecules were calculated using the formula of $2^{-\Delta\Delta CT}$. The relative fold change has been calculated by comparing with untreated *C. albicans* cells. This experiment was performed in triplicate [14].

3.27. Hemolytic Activity

Haemolytic activity is essential for the determination of a molecule's toxicity on human red blood cells (RBCs) [15]. The blood was collected in a tube containing EDTA from blood bank, and consent was taken from the same volunteer. The blood was centrifuged at 2000 rpm for 10 min (20 °C). The pellet of RBC was suspended in PBS (10 % v/v). Again, this suspension of RBC was diluted 1:10 in PBS. Aliquots of 100 µl from the suspension were added to 100 µl of a different concentration of test molecule in the same buffer in Eppendorf tubes. 1 % TritonX-100 used as positive control. After incubation of 1 h at 37 ºC it was then centrifuged for 10 min at 2000 rpm at 20 °C. 150 µl from the supernatant was transferred to a flat-bottomed

Microtiter plate, and O. D. was taken at 450 nm. The hemolysis percentage was calculated by the following formula: % of hemolysis = [(A_{450} of test compound treated Sample - A_{450} of buffer treated sample) / (A_{450} of 1 % Triton X 100 treated sample - A_{450} of buffer treated sample)] × 100. The experiment was performed in triplicate.

3.28. Silkworm survival assay

A silkworm survival assay was performed to identify the antifungal efficacy *in vivo* [16]. The test molecules inhibiting planktonic growth were selected for the silkworm survival assay. The silkworm, *Bombyx mori* larvae, were obtained from the Department of Zoology, Shivaji University, Kolhapur, and reared on mulberry leaves. Fifth instar day larvae weighing approximately 0.8 to 1 g were selected for the study. Four groups of larvae were made as shown in Table 3.2. Each group contained six larvae. In the treatment group, the larvae were subjected to a two-step injection process. In the negative control, they were injected with *C. albicans* only (Group 1). In the positive control group, larvae received injections of *C. albicans* cells plus fluconazole (Group 2). The vehicle control group was injected with 2 % DMSO (Group 3). In the test, they were injected with *C. albicans* (1×10^6 cells/ml) followed by the injection of the MIC_{50} of test molecules (Group 4). The administration of cells and drugs into silkworms was carried out with the help of a 27-gauge needle syringe through the 3rd abdominal leg, and then silkworms were maintained for survival period observation at laboratory conditions at 24ºC with 70 % relative humidity.

Table 3.2: Specification of silkworm larvae groups used in the experiment.

Group No.	Type of administration	Type of group
Group 1	Larvae injected with *C. albicans*	Negative control
Group 2	Larvae injected with 1×10^6 cells/ml of *C. albicans* + fluconazole	Positive control
Group 3	Larvae injected with DMSO	Vehicle control
Group 4	Larvae were injected with *C. albicans* and subsequently with the MIC_{50} concentration of the test molecule.	Test

3.29. Statistical analysis

Each experiment was carried out in triplicate. The values provided represent the means and standard deviations derived from three distinct observations. Statistical analysis was conducted using the Student's t-test to compare the values between the

control and treatment groups. A significance level of $P < 0.05$ was taken into account as significant from a statistical standpoint. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

3.30. References

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Chapter IV:
Antifungal activity of zingerone against growth and virulence factors of *Candida albicans.*

4.1. Introduction

Ginger (*Zingiber officinale*) is widely used in indigenous systems of medicine to treat various inflammatory conditions and other diseases. It is mainly used as a spice in food. It contains various bioactive molecules like gingerols, shogaols, and zingerone. Zingerone [4-(4-hydroxy-3-methoxyphenyl) butan-2-one] is found in dried ginger rhizome (Fig. 4.1). It is also called vanillyl acetone. In fresh ginger rhizome, it is present in small quantities, but after cooking or drying, gingerol gets converted into zingerone through a reaction called the retro-aldol reaction [1].

Zingerone has been reported to possess anti-cancer, anti-angiogenic, anti-oxidant, and anti-inflammatory properties [2,3] (Fig. 4.2). It also displays insect growth regulatory (IGR), anti-feedant activity against *Spilosomao bliqua,* and antifungal activity against *Rhizoctonia solani* [4]. Zingerone is the active constituent responsible for the anti-diarrheal efficacy. Heat-labile enterotoxin LT of *E. coli* induces diarrhea in infants, which is a leading cause of death in developing countries. Zingerone exerts a protective effect on *Escherichia coli* induced diarrhea. *In vivo* studies in mice revealed that ginger significantly blocked the binding of heat-labile enterotoxin LT to cell-surface receptor mono-sialotetrahexosyl ganglioside (GM1), resulting in the inhibition of fluid accumulation in the closed ileal loops of mice [5]. Studies also showed that zingerone, given to Pacific white shrimp (*Litopenaeus vannamei*) juveniles, showed good immunity and protection against *Vibrio alginolyticus* [6].

In the current study, zingerone was tested for its anti-*C. albicans* activity. *C. albicans* can cause life-threatening systemic fungal infections that occur mainly in immunocompetent and immunocompromised patients with AIDS, immunosuppressant-treated organ transplant patients, and recently patients with COVID-19 [7,8]. Invasive candidiasis is an important nosocomial infection with a high morbidity and mortality rate. Clinical diagnosis of candidiasis could be difficult because of the lack of specific symptoms and clinical signs [9]. *C. albicans* has various virulence factors, like yeast to hyphal (Y-H) form conversion, adhesion, and biofilm formation. Out of all this, Y-H form morphogenesis is one of the most important virulence factors in *C. albicans* [10]. This is essential for infiltrating epithelial tissues and evading immune responses [7]. Y-H morphogenesis involves two major pathways: Ras1-cAMP-PKA and C1-mitogen-activated protein pathway [10]. Many unique transcription factors play important roles in the transcriptional

regulatory network that integrates different environmental cues and determines which phenotypic state will be expressed [11]. Some hyphal-specific genes are upregulated during morphogenesis. Also, some negative regulators were downregulated during the hyphal formation. Hence, targeting these genes could be an effective strategy to develop new anti-*Candida* agents.

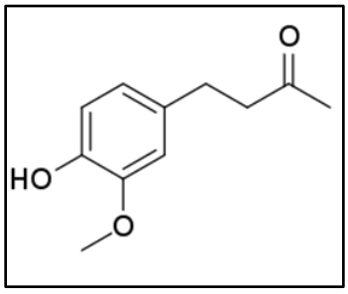

Fig. 4.1: Structure of zingerone.

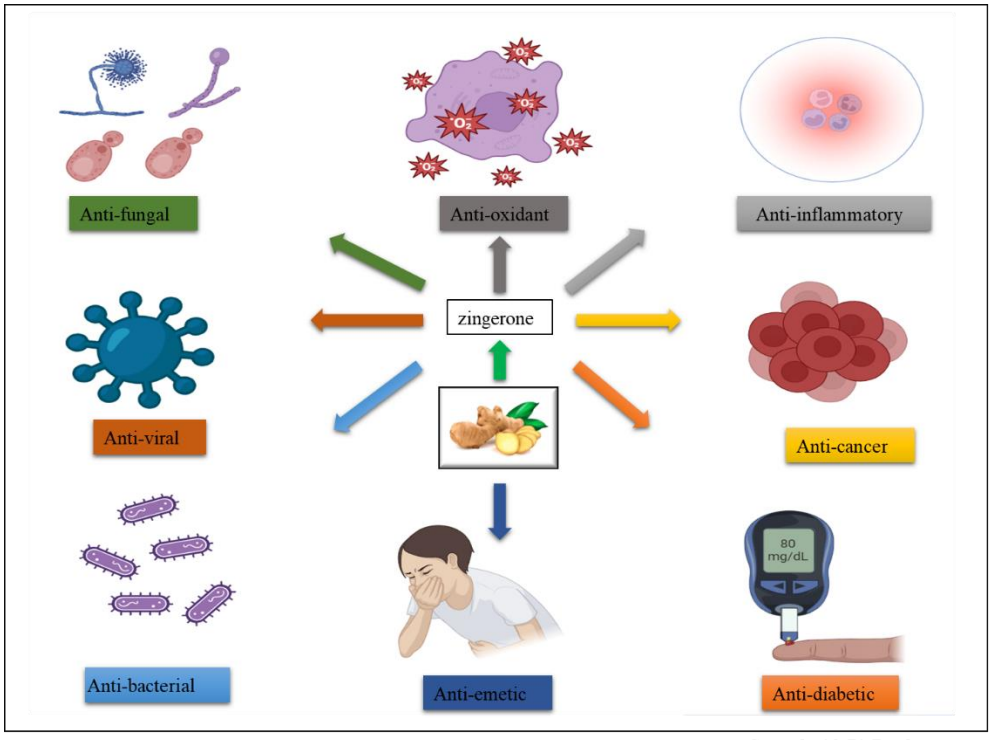

Fig. 4.2: Pictorial representation of anti-cancer, anti-angiogenic, anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral, anti-fungal properties of zingerone.

4.2. Materials and Methods

Methodology was followed as mentioned in chapter 3 from page no. 29 to 42.

4.3. Results

4.3.1. Minimum inhibitory concentration (MIC) of zingerone for planktonic growth

Zingerone exhibited inhibitory activity against the planktonic growth of *C. albicans* in a dose-dependent manner. The concentration at which 50 % inhibition of growth is observed, considered as MIC. Zingerone at 2 mg/ml concentration reduces 50 % *C. albicans* planktonic growth compared to control (Table 4.1, Fig. 4.3a), while the MFC is found at 4 mg/ml, as depicted in Fig. 4.3c. There is a 100 % decrease in *C. albicans* cell viability observed in the presence of zingerone at 4 mg/ml.

4.3.2 Kill curve for zingerone

The time-dependent killing of *C. albicans* cells by the treatment with zingerone is also observed. According to this experiment, it is noted that zingerone is able to kill 99.9 % of *C. albicans* cells due to the exposure of zingerone at a concentration of 4 mg/ml within 480 min (Fig. 4.3b).

4.3.3. Effect of zingerone on adhesion

The addition of zingerone had a noticeable impact on the adhesion of *C. albicans*, particularly up to 0.125 mg/ml. Analysis of the adhered cells using the XTT-reduction assay revealed that a 0.125 mg/ml concentration of zingerone inhibits 50 % of adhesion (Table 4.1). Suggesting a noteworthy decrease in the ability of *C. albicans* to adhere to the solid surface in a concentration-dependent manner, and it is considered as MIC of adhesion. At higher concentrations of zingerone, specifically 2 mg/ml and 4 mg/ml, it reduces adherence of *C. albicans* up to 90 % and 97 %, respectively (Fig. 4.3a).

Table 4.1: The antifungal efficiency of zingerone against *C. albicans* ATCC 90028 showing the MICs for planktonic growth, adhesion, yeast to hyphal morphogenesis, biofilm formation, mature biofilm, and MFC for planktonic growth.

Molecule	Planktonic growth	Adhesion	Yeast to hyphal morphogenesis	Developing biofilm	Mature biofilm	MFC
Zingerone MIC (mg/ml)	2	0.125	1	2	NA	4

NA not achieved up to 4 mg/ml; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

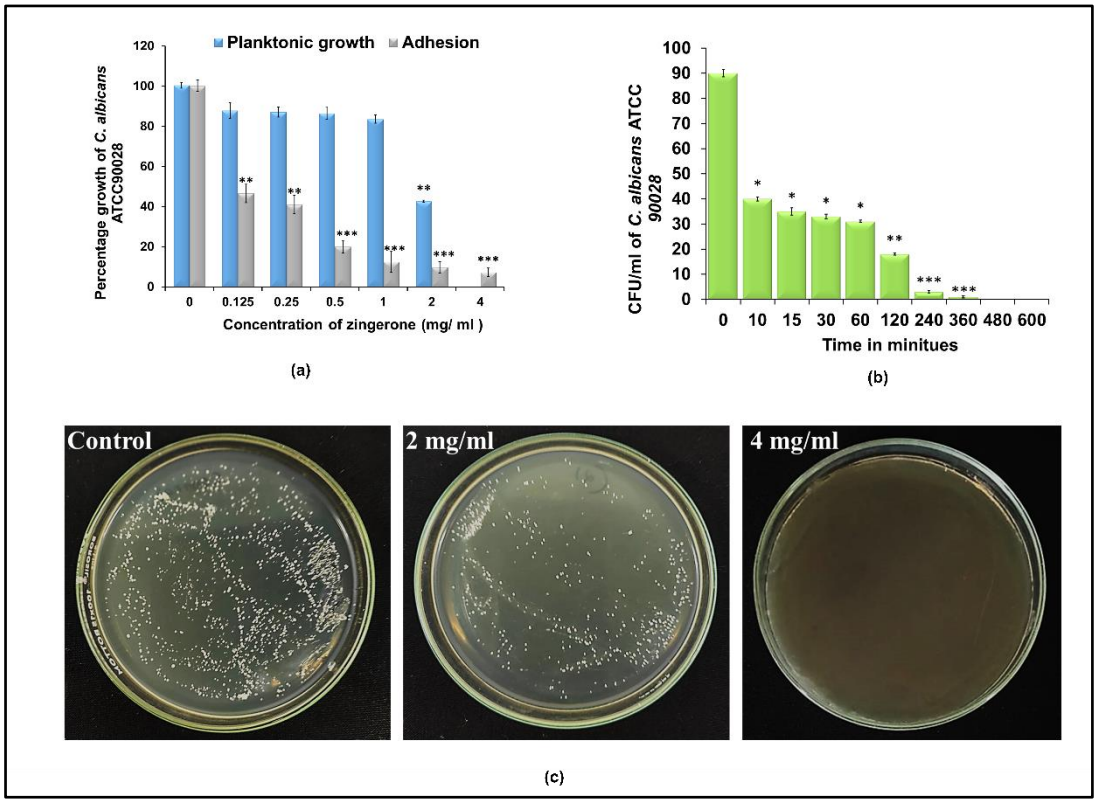

Fig. 4.3: (a) Concentration-dependent inhibition of planktonic growth and adhesion of *C. albicans* after the treatment of zingerone. (b) Time-dependent killing of *C. albicans* cells by the treatment of zingerone (4 mg/ml). $^{*}p<0.05$, $^{**}p<0.01$, *** $p<0.001$. (c) MFC of zingerone against *C. albicans* cells is observed at 4 mg/ml.

4.3.4. Effect of zingerone on Y-H morphogenesis

Zingerone displayed a concentration-dependent inhibition of the Y-H morphogenesis induced by fetal bovine serum (FBS) (Fig. 4.4a). The treatment with zingerone resulted in significant inhibition of the transformation from Y-H morphology. Specifically, at 1 mg/ml, 50 % inhibition of Y-H morphogenesis is observed (Table 4.1), while at higher concentrations of 2 mg/ml and 4 mg/ml, complete inhibition of this morphological transition is observed under a microscope (Fig. 4.4b).

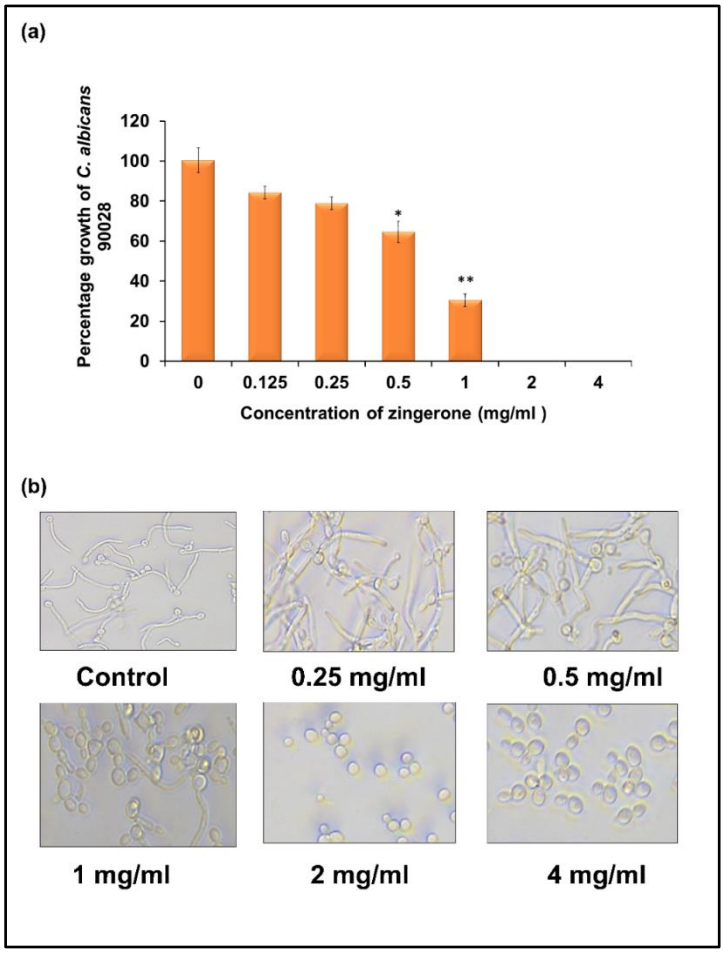

Fig. 4.4: (a) Concentration-dependent inhibition of serum-induced Y-H morphogenesis of *C. albicans* after the treatment of zingerone * p< 0.05, ** p<0.01. (b) Microscopic observation showing inhibition of Y-H morphogenesis at various concentrations of zingerone.

4.3.5. Effect of zingerone on biofilm development

The significant impact of zingerone on the biofilm development of *C. albicans* is found at 2 mg/ml and 4 mg/ml concentrations. However, the MIC of zingerone is observed at 2 mg/ml (Table 4.1). The addition of zingerone prevented the biofilm development of *C. albicans* in a concentration-dependent manner (Fig. 4.5a). This finding is further confirmed through scanning electron microscopy. After incubation, dense biofilm formed in a multilayered network, and a sophisticated three-dimensional structure is observed in the control. On the other hand, the treated test sample does not produce a significant hyphal network (Fig. 4.5b). There are no significant phenotypic changes between the *C. albicans* cells growing on the test and control materials; in both, ovoid, spherical blastoconidia type morphologies are observed. The use of silicon Foley's catheter discs allowed *C. albicans* to develop a biofilm characterized by a network of yeast and hyphal forms of cells. However, after

treatment with a MIC concentration of zingerone, only yeast cells are observed on the catheter, indicating the potent inhibitory effect of zingerone on biofilm formation.

In summary, zingerone, particularly at 2 mg/ml, effectively prevented the development of *C. albicans* biofilm on both polystyrene and urinary catheter surfaces.

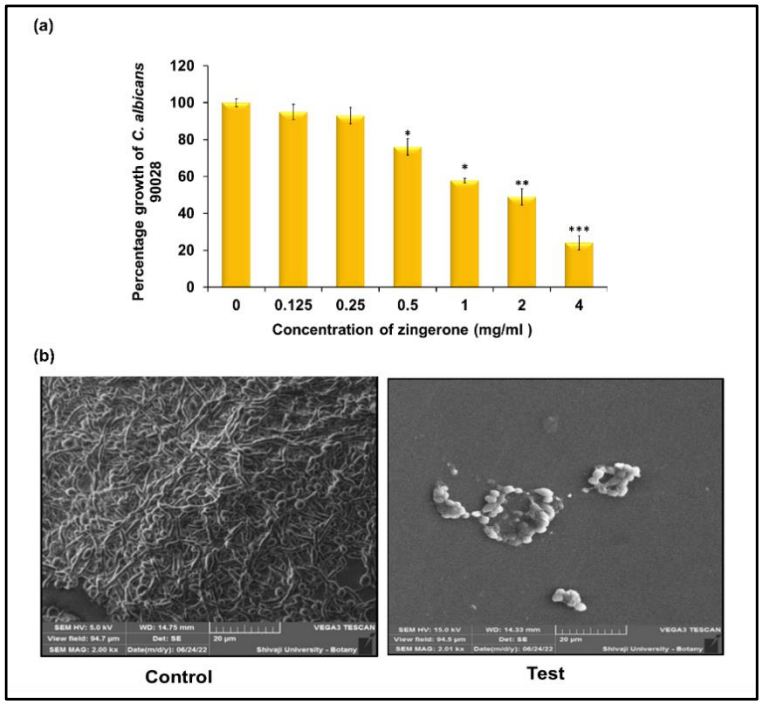

Fig. 4.5: (a) Effect of zingerone (0.125 mg/ml to 4 mg/ml) on developing biofilm of *C. albicans*. * p<0.05, ** p<0.01, *** p<0.001. (b) Scanning electron micrograph showing dense biofilm formation in control and complete inhibition of biofilm development by the treatment of zingerone in test (2 mg/ml) (Scale bar 20 µm).

4.3.6. Effect of zingerone on gene expression

To examine the influence of zingerone on gene expression in *C. albicans*, qRT-PCR studies were carried out. These experiments were performed following the treatment of zingerone throughout the formation of the biofilm, and the significance activity of zingerone is odserved in transcriptional profile of genes related to signal transduction and biofilm development (Fig. 4.6). The *RAS1,* which is a master regulator of the signal transduction pathway is downregulated by 7.84-fold by the treatment of zingerone. Along with *RAS1* expressions *PDE2*, *BCY1, EFG1, HST1, CPH1, CEK1, TEC1, ECE1* and *HWP1* gene expressions were also downregulated by 6.27-fold, 1.17-fold, 12.47-fold, 11.26-fold, 8.56-fold, 14.09-fold, 1.33-fold, 2.76-fold and 1.54-fold, respectively. Also, the negative regulators *MIG1* and *NRG1* were downregulated by 2.29-fold and 2.11-fold (Table 4.2). The zingerone treatment significantly altered the expression of key genes involved in signal transduction

pathways and biofilm development in *C. albicans*. These changes suggest the possible governing function for zingerone in modulating the fungal response and development processes.

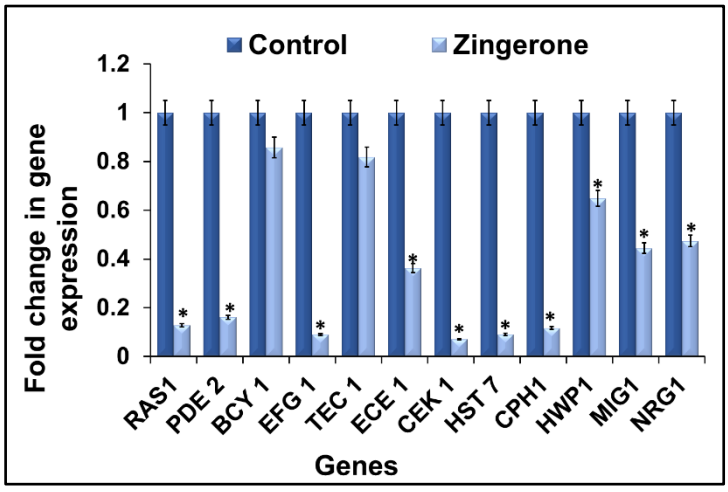

Fig. 4.6: Fold change of gene expressions during biofilm formation of *C. albicans* in the presence of zingerone (2 mg/ml) $^{*}p<0.05$.

Table 4.2: Downregulation of genes in biofilm after treatment with zingerone.

Gene	Zingerone induced relative fold Change	Regulation
RAS1	7.84	Downregulation
PDE2	6.27	Downregulation
BCY1	1.17	Downregulation
EFG1	12.47	Downregulation
TEC1	1.33	Downregulation
ECE1	2.76	Downregulation
CEK1	14.09	Downregulation
HST7	11.26	Downregulation
CPH1	8.56	Downregulation
HWP1	1.54	Downregulation
MIG1	2.29	Downregulation
NRG1	2.11	Downregulation

4.3.7. Effect of zingerone on cell division cycle

The flow cytometry analysis of the control sample and zingerone-treated test sample revealed that zingerone treatment affects the cell cycle of *C. albicans* (Fig. 4.7a). In the control group, it is observed that 54.04 % of the cells are found to be within the G0/G1 phase, 10 % are in the S phase, and 4 % are in the G2/M phase. Treatment with zingerone resulted in a notable change in the percentage of cells in particular cell cycle phases. Specifically, 89.84 % of the cells are present in the

G0/G1 phase, indicating a significant accumulation of cells at this stage. In contrast, only 2.4 % of cells are arrested in the S phase, and 4.9 % of cells are present in G2/M phase. This comparative cell cycle analysis data is depicted in the histogram (Fig. 4.7b). Zingerone also induced apoptosis in *C. albicans* cells, with approximately 20 % of cells undergoing apoptosis as a result of the treatment. In short, zingerone caused cell cycle arrest at the G0/G1 phase and also induced programmed cell death in *C. albicans*.

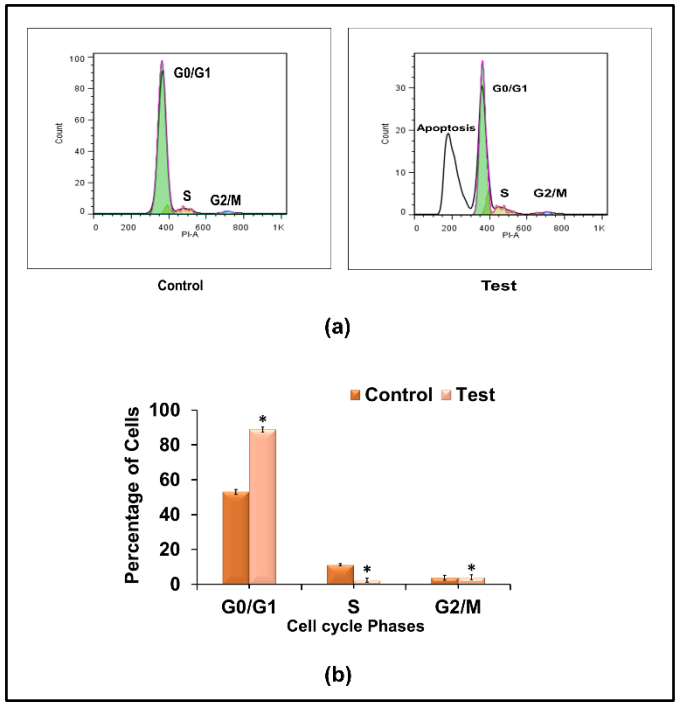

Fig. 4.7: (a) Cell cycle analysis of *C. albicans* control cells without treatment with zingerone and test with treatment of zingerone (2 mg/ml) for 4 h 30 min at 35ºC showing cell cycle arrest at G0/G1 phase. (b) Histogram for a percentage of cells present at different phases of the cell cycle. $^{*}p<0.05$.

4.3.8. Effect of zingerone on reactive oxygen species (ROS) generation

Intracellular ROS levels were assessed using H_2DCF, which is oxidized to produce green fluorescence in the presence of ROS. After treatment with zingerone, there was a significant increase in the population of green fluorescent cells. This increase was attributed to the intracellular accumulation of ROS induced by zingerone treatment when compared to untreated cells (control). In the positive control group (H_2O_2), the highest intensity of green fluorescence was observed. This enhanced fluorescence was a result of the accumulation of a large number of ROS, indicating a substantial oxidative stress response in these cells. These findings suggest that zingerone treatment leads to an accumulation of ROS in the treated cells, although to

a lesser extent than the positive control, indicating an oxidative stress response triggered by zingerone in *C. albicans* cells (Fig. 4.8a).

4.3.9. Effect of zingerone on genes involved in ROS production

The expression analysis of genes involved in protection against oxidative stress and apoptosis, *SOD1, SOD2, CAT1,* and *MCA1,* is carried out to support ROS generation and cell cycle results (Fig. 4.8b). Out of four genes, *SOD2* and *CAT1* are downregulated by 1.37-fold and 1.68-fold, respectively. *SOD1* is significantly downregulated following the treatment with zingerone. Whereas the expression of *MCA1* is significantly upregulated by 1.67-fold after the treatment of zingerone (Table 4.3). This indicates that zingerone treatment had differential effects on the gene expression associated with defending against oxidative stress and apoptosis. These gene expression changes could be linked to the observed ROS generation and alterations in the cell cycle, shedding light on the molecular mechanisms by which zingerone impacts *C. albicans*.

Table 4.3: Effect of zingerone on the gene expression involved in oxidative stress.

Gene	Zingerone induced relative fold Change	Regulation
SOD1	1.18	Downregulation
SOD2	1.37	Downregulation
CAT1	1.68	Downregulation
MCA1	1.67	Upregulation

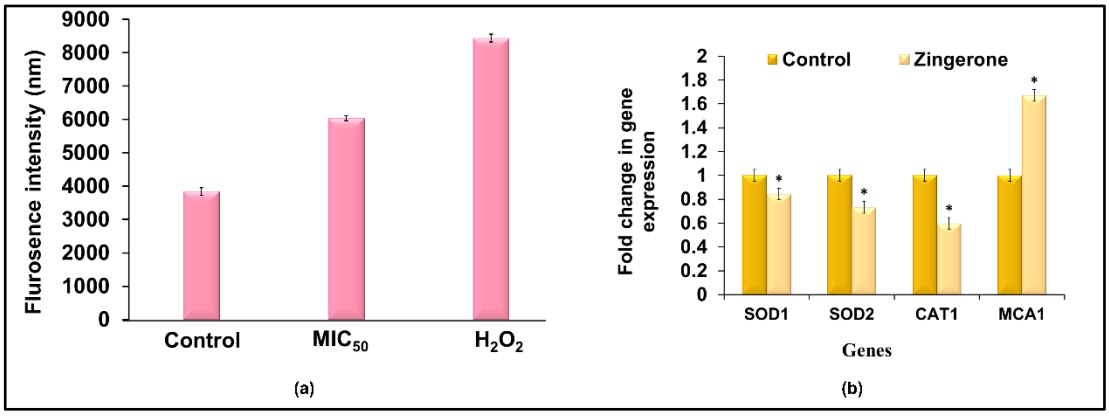

Fig. 4.8: (a) Enhancement of ROS level in *C. albicans* due to treatment with zingerone at MIC (2 mg/ml). (b) Fold change in gene expressions involved in preventing ROS damage after the treatment of zingerone * $p < 0.05$.

4.3.10. Effect of zingerone on the ergosterol biosynthesis

Ergosterol is indeed a critical lipid component found in the membrane of *C. albicans*, and it fulfills an essential function in regulating the fluidity, permeability, and overall integrity of the fungal membrane. The results indicate that the treatment of zingerone has a notable effect on the ergosterol profile in a dose-dependent manner (Fig. 4.9). Particularly, at 4 mg/ml and 2 mg/ml, there is a complete inhibition of ergosterol content. This suggests that zingerone treatment at these concentrations severely disrupted or inhibited the synthesis or maintenance of ergosterol in *C. albicans*. Additionally, at lower concentrations of zingerone, reductions in sterol content after zingerone treatment are observed. Even though complete inhibition is not achieved at these lower concentrations, there is still a significant decrease in sterol content.

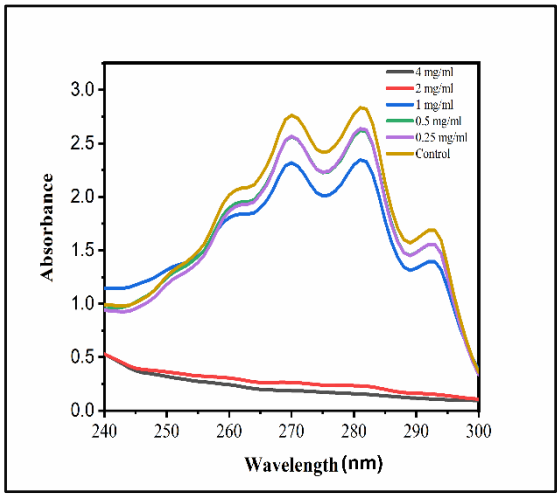

Fig. 4.9: Concentration-dependent inhibition of ergosterol biosynthesis in *C. albicans* after the treatment of zingerone (0.25 mg/ml to 4 mg/ml) measured by the spectrophotometric method.

4.3.11. Effect of zingerone on cell membrane integrity

A membrane-specific fluorescence dye, propidium iodide (PI), was utilized to examine the membrane integrity of *C. albicans* treated with zingerone. As concentration of zingerone increase flurocscence intensity of cells increase. Specifically, the control cells exhibited minimal PI accumulation in comparison to the treated cells, which were subjected to higher concentrations, MIC, and sub MIC levels of zingerone. The investigation on PI accumulation validated the compromised membrane integrity of *C. albicans* when treated with zingerone at 0.5 mg/ml, 1 mg/ml, and 2 mg/ml in increasing order (Fig. 4.10).

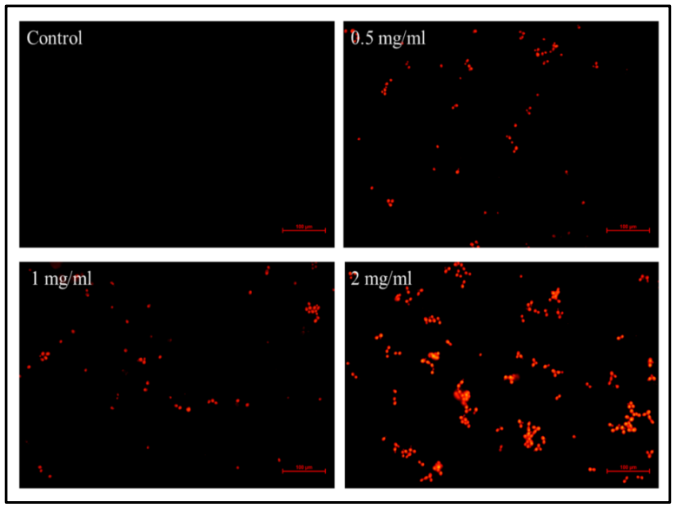

Fig. 4.10: Fluorescence microscopy images of *C. albicans* cells treated with various concentrations of zingerone stained with PI showing an increase in intensity due to membrane damage and PI uptake (Scale bar 100 µm).

4.3.12. Hemolytic activity of zingerone

In the context of assessing potential drug-induced immune hemolytic anemia, an *in vitro* hemolysis study was conducted as a precaution before considering any pharmaceutical applications [12]. Hemolysis is commonly employed as the initial assessment of toxicity in drug development because it can be indicative of cytotoxicity. Cell membrane disruption is often a primary cause of toxicity.

In the case of zingerone, the toxicity study revealed that zinerone induces 2 % hemolysis of human red blood cells (RBCs) when applied at a concentration of 4 mg/ml. However, at lower concentrations, zingerone does not cause hemolysis (Fig. 4.11). This information is essential for evaluating the safety profile of zingerone and its potential for pharmacological applications while considering the risk of immune hemolytic anemia as a rare side effect.

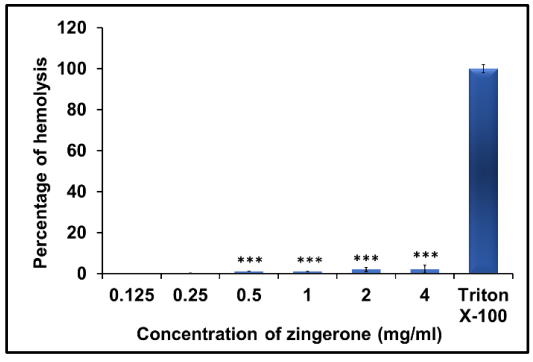

Fig. 4.11: Hemolytic activity of zingerone (0.125 mg/ml to 4 mg/ml) on human RBCs ***p<0.001.

4.3.13. Effect of zingerone on the survival of silkworms

The antifungal efficacy is evaluated using an *in vivo* silkworm model. The silkworm larvae of the negative control group injected with only *C. albicans* cells died within 24 h due to infection. The *C. albicans*-infected silkworm larvae of the positive control group treated with fluconazole survived up to 72 h. Additionally, silkworms of the vehicle control group injected with a 2 % DMSO also exhibited extended survival up to 72 h. Similarly zingerone-treated test group also survived up to 72 h (Fig. 4.12). This suggests that the use of zingerone alongside *C. albicans* had a protective effect on the survival of the silkworms. In this experiment, prolonging their lifespan compared to those treated with *C. albicans* alone. This indicates that the DMSO treatment does not result in substantial harm to the silkworms, and they can survive for the duration of the experiment, similar to the group treated with *C. albicans* and zingerone.

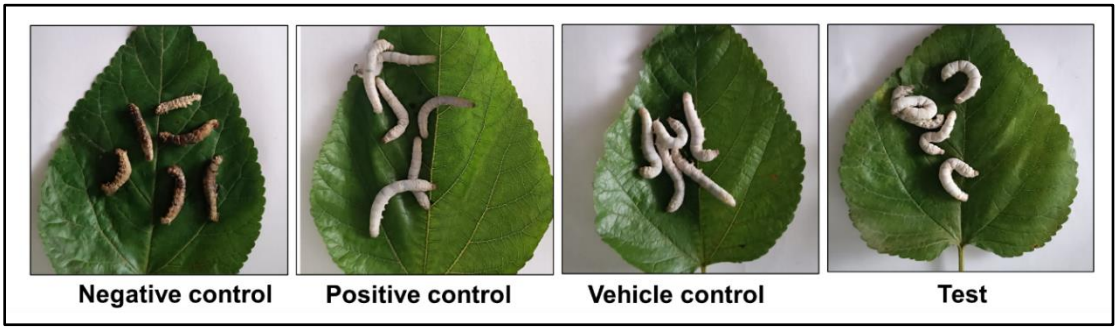

Fig. 4.12: *In vivo* antifungal efficacy of zingerone (2 mg/ml) in silkworm animal model. Silkworms in the negative control group died within 24 h due to *C. albicans* infection. Silkworms in the flucanozole-treated positive control group were alive up to 72 h, while silkworms from the vehicle control and zingerone-treated groups survived up to 72 h.

4.4. Discussion

Zingerone possesses an extensive array of bioactive properties, like antioxidant, anti-inflammatory, anticancer, and antimicrobial activity [13]. These bioactive properties make zingerone a compound of interest for various applications in health and medicine, including potential therapeutic uses in managing oxidative stress, inflammation, cancer, and microbial infections. Zingerone demonstrates the capacity to remove free radicals produced by food radiolysis. Zingerone is also known to prevent tumor formation and oxidative stress in animals. Its improved capacity to impede the growth of gram-positive as well as gram-negative bacteria is noteworthy [14]. This broad-spectrum antibacterial activity suggests its potential as an effective antimicrobial agent. The ability to target a diverse array of bacterial

species, including those with different cell wall structures, makes zingerone a valuable candidate for further

Exploration in the realm of antimicrobial investigations and development and its versatility in combating various bacterial strains could have important implications for the advancement of novel antibacterial agents. The metabolism of zingerone, as indicated by previous studies, involves the excretion of glucuronide and sulfate conjugates in the urine within 24 h after ingestion. This metabolic pathway suggests that zingerone is processed by the body and eliminated through these conjugation reactions, typically without causing significant side effects or harm to vital organs. The conjugation reactions are part of the body's natural detoxification processes, and they facilitate the safe elimination of substances like zingerone from the body. This information adds to the safety profile of zingerone when administered orally or intravenously, suggesting that it may be well-tolerated by the body. However, it is essential to consider individual variations and consult with healthcare professionals when using any substance for medicinal purposes [15]. In a recent study, the effect of dihydrozingerone was studied on growth, biofilm formation and ergosterol synthesis of *C. albicans* [16]. The transcriptome of *C. albicans* exhibited an enhanced response to ethylzingerone (hydroxyethoxyphenyl butanone) exposure, showing an upregulation of genes associated with amino acid biosynthesis and simultaneous activation of pathways related to chemical detoxification and oxidative stress response [17]. Also, it was found that the ethylated analog of zingerone exhibits the highest activity against *C. albicans* 10231 [18].

In the current investigation, it was found that zingerone inhibits *C. albicans* planktonic growth, adhesion, morphogenesis, and biofilm formation *in vitro* conditions (Fig. 4.3a, 4.4a, 4.5a). *C. albicans* biofilms play a crucial role in its pathogenicity. They serve as a protective environment where the yeast cells adhere to surfaces and form structured communities. This biofilm structure enhances the fungus's ability to persist and thrive in various environments, contributing significantly to its virulence and the development of persistent infections. Understanding and targeting biofilm formation is essential in combating *C. albicans* infections. Strategies aimed at disrupting biofilm integrity or preventing its formation hold promise in managing these infections, especially considering the increased resistance of biofilm-embedded microorganisms to conventional antifungal therapies. Therefore, the inhibition of biofilm formation by compounds like zingerone presents a

promising avenue for combating *C. albicans* infections and reducing their associated complications. The inhibitory effects of zingerone on yeast to hyphal morphogenesis and biofilm development, as observed under light microscopy and scanning electron microscopy, provide strong evidence of its anti-*C. albicans* properties (Fig. 4.5b). Microscopy techniques allow for a detailed examination of the fungal cells and the biofilm structure, providing visual confirmation of the inhibitory effects of zingerone. Inhibition of adhesion is crucial because it prevents *C. albicans* from adhering to surfaces, a process that involves an initial step in biofilm formation. Once attached, *Candida* cells can develop into complex biofilm communities that are often resistant to antimicrobial treatments. By preventing adhesion and disrupting biofilm development, zingerone may offer a promising approach to controlling *Candida* infections. *C. albicans* responds to many environmental variables by changing its morphology. This switching helps the organism for invasion and pathogenesis [13].

Yeast to hyphal form morphogenesis of *C. albicans* is regulated by two major signaling pathways, the Ras1-cAMP-PKA pathway and the MAPK pathway. These pathways play crucial roles in controlling the switch from yeast-like cells to filamentous hyphal forms, which is a key virulence factor for *C. albicans* and is associated with its pathogenicity [11]. Within the signal transduction pathways that regulate yeast to hyphal form morphogenesis in *C. albicans*, various transcription factors play critical roles as regulators. These transcription factors help to coordinate the expression of specific genes involved in the transition from yeast to hyphal form morphogenesis [8]. Unique transcription factors play crucial roles in the transcriptional regulatory network of *C. albicans*, integrating various environmental cues and determining the phenotypic states the organism will express. This regulatory network allows *C. albicans* to adapt and respond to changing conditions, enabling it to thrive in diverse host environments [19].

RAS1 is an upstream regulator that plays a crucial role in both the Ras1-cAMP-PKA (cyclic AMP-dependent protein kinase A pathway and the Cek1-MAPK pathway [13], it was downregulated 7.84-fold by the treatment with zingerone in developing biofilm. *RAS1* is a key element within the signaling pathway. When activated, *RAS1* promotes the activation of adenylate cyclase, which is encoded by the *CDC35* gene. Therefore, downregulation of *RAS1* can lead to reduced activation of adenylate cyclase, resulting in lower levels of cAMP. This, in turn, can affect the activation of PKA and the subsequent signaling events that drive filament formation.

Consequently, *C. albicans* may have difficulty transitioning to the hyphal form under such conditions [20]. Cell differentiation and cell death are significantly influenced by *BCY1*, a PKA regulatory component. It is also crucial for preserving the cell viability of *C. albicans.* 5.13-fold in biofilm formation, suggesting that zingerone may disrupt the normal signaling and regulatory pathways associated with the Ras1-cAMP-PKA pathway (Fig. 4.6). This disruption likely contributes to the suppressive impacts of zingerone on hyphal form morphogenesis and biofilm formation in *C. albicans*. *ECE1* is a significant downstream component in *C. albicans* that plays an essential role in cell elongation during biofilm formation. Biofilm development in *C. albicans* involves a complex process in which individual yeast cells transition to filamentous forms, including pseudohyphae and true hyphae, which are critical for the development of the biofilm structure. The expression of *ECE1* was downregulated in biofilm formation by 2.76-fold.

Invasion of epithelial cells during infection is indeed another important function of *ECE1* in *C. albicans*. *ECE1* contributes to the pathogenicity of *C. albicans* by facilitating the invasion of host epithelial cells. *ECE1* downregulation may have an impact on *C. albicans* cell elongation and invasion. *C. albicans* relies on the *HWP1* gene for adhesion to both biotic (living) and abiotic (non-living) surfaces. *HWP1* is an important virulence factor in *C. albicans*. It plays a key role in the adhesion and colonization of surfaces, which is crucial for the pathogenicity of this fungal species. Downregulation of *HWP1* in *C. albicans* can have a significant impact on adhesion and, consequently, biofilm formation. After treatment of zingerone, in biofilm formation, it was downregulated 1.54-fold, respectively. *PDE2* is a high-affinity cyclic nucleotide phosphodiesterase that plays a role in moderating signaling via cAMP. It is expressed shortly after hyphal induction, and it is required for normal hyphal growth [13]. In response to zingerone, *PDE2* was downregulated by 6.27-fold in biofilm formation, hence it may inhibit the hyphal formation in developing biofilm and morphogenesis.

The MAPK kinase pathway in *C. albicans* includes several key genes, including *HST7, CEK1,* and *CPH1*. *HST7* is a mitogen-activated protein kinase kinase (MAPKK) participating in both mating and hyphal growth in the signal transduction pathway of *C. albicans* [21]. Cek1-MAPK is responsible for cell wall biogenesis and vegetative growth in *C. albicans.* It helps fungi for successful establishment in the host [22]. *CPH1* is a transcription factor in *C. albicans* that plays a pivotal role in

regulating morphogenesis, particularly in the context of the MAPK kinase pathway. *CPH1* in *C. albicans* has a broad range of transcriptional targets that influence various aspects of fungal biology and virulence. The versatility of *CPH1* as a transcription factor in *C. albicans,* being able to function as both a positive and acts as a suppressor under certain morphological states and physiological circumstances, highlights its adaptability and the complexity of regulatory networks in the fungus. *HST7, CEK1* and *CPH1* are significantly downregulated in biofilm formation after the treatment in zingerone by 11.26-fold, 14.09-fold, and 8.56-fold (Fig. 4.6).

All multicellular organisms use the evolutionarily conserved process called apoptosis to control their development and differentiation [23]. Key features of apoptosis include cellular shrinkage, nuclear fragmentation, chromatin condensation, and generation of apoptotic bodies; these are ultimately engulfed by neighboring cells or macrophages, causing no inflammatory reaction. Apoptosis is tightly controlled by a network of genes and signaling pathways to ensure its proper execution. Zingerone-induced apoptosis in *C. albicans* cells. Cell cycle and apoptosis are interconnected processes and play essential roles in cell survival, growth, and reproduction. Zingerone-arrested *C. albicans* at the G0/G1 phase of the cell cycle may be connected to the failure of the cells to enter the S phase due to apoptosis in these cells. The inhibition of growth may be due to the triggering of apoptosis and inhibition of the cell cycle at the G0/G1 phase (Fig. 4.7a).

It is well known that most of the available antifungals act through the production of ROS, which helps them in their fungicidal actions. It was previously reported that by blocking the mitochondrial electron transport chain, farnesol, a quorum-sensing molecule in *C. albicans*, may cause the production of ROS [24]. Therefore, the effect of zingerone on ROS production was studied, and it is found that zingerone induces ROS accumulation in *C. albicans* (Fig. 4.8a). Since the mitochondria provide the constant flow of ATP necessary for cellular metabolism, any injury affecting the respiratory chain's performance may also affect the survival of the cell. As the main intracellular source of ROS, which is primarily produced in the mitochondrial respiratory chain, mitochondria and their role as the source of ROS are closely related. The disruption of electron transport and ATP synthesis caused by high ROS production is also a significant target for mitochondrial damage [25]. It suggests that inhibition of developing biofilm after the treatment of zingerone may

occur due to the downregulation of these genes, which are involved in cell growth and morphogenesis.

During planktonic growth, zingerone causes the downregulation of superoxide dismutase *SOD1* and *SOD2,* which are responsible for the reduction in ROS production by scavenging lethal superoxides and re-establishing the redox equilibrium necessary for cell viability [26]. Drugs used to treat fungal infections enhance oxidative stress, which prompts an adaptive response and increases *SOD1* expression. Zingerone causes downregulation of *SOD1* and *SOD2,* resulting in increased oxidative stress. The *CAT1* single catalase gene, which plays a role in resistance to oxidative stress, was also downregulated by the treatment of zingerone, ultimately leading to increased oxidative stress [27]. *MCA1* is upregulated by the zingerone treatment, which induces apoptosis in *C. albicans,* leading to cell death (Fig. 4.8b).

The plasma membrane in *C. albicans* is composed of a combination of proteins and lipids. This membrane serves as a physical barrier and plays key roles in cell wall synthesis, nutrient transport, influencing the virulence behavior, and structural transitions of the organism [28]. This study explored the impact of zingerone on the membrane integrity of *C. albicans*. Ergosterol serves a role in fungi that is somewhat analogous to the role of cholesterol in mammalian cells.

Ergosterol is a crucial element of fungal cellular membranes, and it plays several essential functions in maintaining membrane structure and function. It maintains the permeability, fluidity, and integrity of the membrane in fungi [29]. Zingerone inhibited ergosterol synthesis in a dose-dependent manner. Zingerone interferes with ergosterol synthesis; it can lead to changes in the membrane properties, compromising the fluidity, permeability, and structural integrity of the fungal cell membrane. This disruption can impede various cellular processes, including nutrient uptake, membrane transport, and cell division, ultimately inhibiting the growth and survival of *C. albicans*. Therefore, inhibition of ergosterol synthesis is a promising mechanism by which zingerone exerts its fungicidal effect on *C. albicans*. Disruption of membrane integrity is a common target for many antifungal agents as it is a vital component of fungal cells (Fig. 4.9). Our investigations, on varying doses of zingerone, showed that at the MIC, there was a significant accumulation of PI, indicating its maximum effect on membrane integrity. However,

as concentrations of zingerone increased in our current research, it was observed that more pronounced membrane damage and lesions following PI staining (Fig. 4.10)

Previously, it was reported that the silkworm model is commonly employed in scientific research to study the effectiveness of antifungal treatments in a living organism, providing valuable insights into the potential therapeutic benefits of the tested substances [30]. In current study, it is observed that *C. albicans* infection killed the silkworm within 24 h time interval but when the MIC concentration of zingerone is given to the silkworm the survival time increased up to 72 h even after the infection and went into the pupation stage and complete normal life cycle (Fig. 4.12). Hence, it is confirmed that zingerone has *in vivo* antifungal capacity against *C. albicans*. In addition to all these activities, zingerone does not show significant hemolysis up to 4 mg/ml concentration (Fig. 4.11).

4.5. Conclusions

In the present study, it is demonstrated that zingerone has a multifaceted effect on *C. albicans,* impacting various aspects of its biology and pathogenicity. Zingerone inhibited planktonic growth, adhesion, developing biofilm, and yeast to hyphal transition at concentrations of 2 mg/ml, 0.125 mg/ml, 2 mg/ml, and 1mg/ml, respectively. Zingerone-induced cell cycle arrest, primarily in the G0/G1 phase, impacts the proliferation of *C. albicans* cells. Zingerone treatment led to the creation of reactive oxygen species, which are signs of cellular stress and potential triggers for apoptosis. These combined effects make zingerone a potential anti-*Candida* agent. Its multifaceted action on the fungus could reduce the likelihood of treatment resistance. Zingerone was shown to modulate the gene expression involved in biofilm formation pathways. During biofilm zingerone treatment, caused a downregulation of *BCY1* and *EFG1* genes by 6.27-fold and 12.47-fold, respectively. This suggests that zingerone can influence the fungal transition between different growth forms and its ability to form biofilms. Zingerone effectively inhibited ergosterol biosynthesis, which is an essential lipid component of the fungal membrane. This disruption can affect the integrity and functions of the fungal cell membrane. Zingerone showed no hemolytic activity, indicating that it does not cause damage to human red blood cells. This is a positive indication of the safety of zingerone. Zingerone is a dietary food component and has demonstrated no toxicity issues in human studies. This non-toxic nature is promising for potential therapeutic applications. In conclusion, zingerone acts as a

potential antifungal agent with multiple targets in *C. albicans*. Its multifaceted actions on the fungus, coupled with its non-toxic nature, make it an intriguing candidate for further exploration as a therapeutic option for *Candida* infections.

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Chapter V:
Antifungal activity of α-bisabolol against virulence factors of *Candida albicans*

5.1. Introduction

C. albicans is a commensal fungus that naturally resides in the human microbiota, primarily in the gastrointestinal tract, skin, and mucosal surfaces. However, it can change into a pathogenic state when the host's immune system is compromised [1]. Opportunistic fungal infections caused by *C. albicans* are prevalent in immunocompromised individuals, particularly those undergoing chemotherapy, organ transplantation, or suffering from conditions like diabetes and HIV/AIDS. Current antifungal treatments are limited, often associated with adverse side effects, and challenged by emerging drug resistance [2]. The urgent need for novel antifungal agents is compounded by the cellular and biochemical similarities between fungal and human cells, making selective therapeutic targeting difficult [3].

The polymorphic nature is a key virulence factor of *C. albicans*, allowing it to tranform between yeast, pseudohyphal, and hyphal forms. This morphological plasticity facilitates tissue invasion, immune evasion, and biofilm formation factors that contribute significantly to its pathogenicity [4,5]. Biofilms, in particular, enhance resistance to antifungal treatments and host immune responses, making infections persistent and difficult to eradicate. Immunosuppression remains the primary risk factor for *C. albicans* infections, with contributing factors including prolonged use of broad-spectrum antibiotics, corticosteroids, neutropenia, and metabolic disorders such as diabetes [6]. These conditions disrupt microbial homeostasis, promoting fungal overgrowth and increasing susceptibility to systemic infections. Hence, there is a need to find effective therapeutic molecules to combat the situation.

α-Bisabolol (Fig. 5.1) molecule is a naturally occurring sesquiterpene alcohol, has gained attention for its diverse pharmacological properties. Originally isolated from *Matricaria chamomilla* (Asteraceae) in the twentieth century. It is also found in several aromatic plants, including *Eremanthus erythropappus*, *Smyrniopsis aucheri*, and *Vanillosmopsis* species. Notably, *Salvia runcinata*, a plant native to South Africa, contains substantial amounts of α-bisabolol in its essential oil (Fig. 5.2) [7,8]. This bioactive compound exhibits a broad spectrum of therapeutic properties, including anti-inflammatory, anti-bacterial, anti-oxidant, anti-diabetic, and anti-cancer effects. Additionally, its non-allergenic nature makes it a valuable ingredient in dermatological and cosmetic formulations such as lotions, deodorants, lip balms, and baby care products [9].

Given its promising bioactivities, the present study aims to investigate the antifungal mechanism of action of α-bisabolol against *C. albicans*. By elucidating its impact on fungal growth, morphology, and virulence factors, this research contributes to the development of alternative therapeutic strategies against *C. albicans* infections.

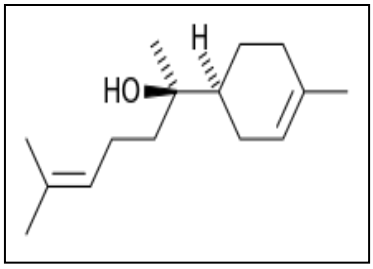

Fig. 5.1: Structure of α-bisabolol.

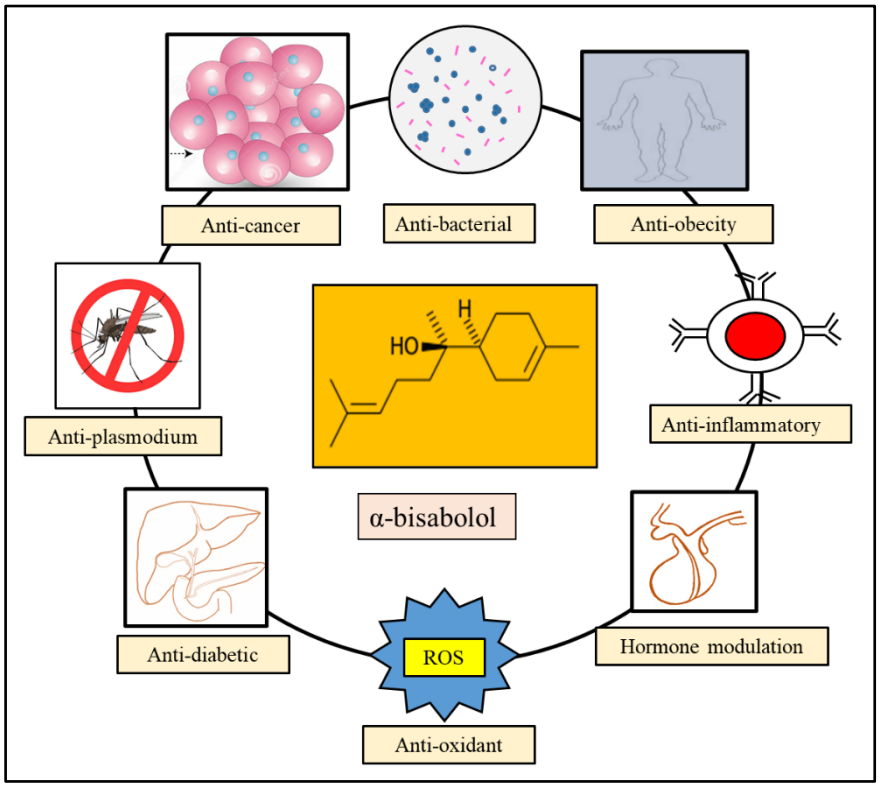

Fig. 5.2: Pictorial representation of anti-bacterial, anti-obesity, anti-inflammatory hormone modulation, anti-oxidant, anti-diabetic, anti-plasmodium anti-cancer activity of α-bisabolol.

5.2. Materials and methods

Methodology was followed as mentioned in chapter 3 from page no. 29 to 42.

5.3. Results

5.3.1. Minimum inhibitory concentration of α-bisabolol for planktonic growth

The impact of α-bisabolol on the planktonic growth of *C. albicans* was evaluated within a 0.125 mg/ml to 4 mg/ml concentration range (Fig. 5.3). The outcomes indicated that α-bisabolol did not exhibit inhibitory effects on the planktonic growth of *C. albicans*. Consequently, the MIC of α-bisabolol against planktonic growth is not reached even at the highest tested concentration of 4 mg/ml (Table 5.1).

Table 5.1: α-bisabolol influences growth, adhesion, yeast to hyphal morphogenesis, and biofilm formation in *C. albicans* ATCC 90028.

Molecule	Planktonic growth	Adhesion	Yeast to hyphal morphogenesis	Developing biofilm	Mature biofilm
α-Bisabolol MIC (mg/ml)	NA	1	0.25	0.125	NA

NA not achieved up to 4 mg/ml; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

5.3.2. Effect of α-bisabolol on adhesion of *C. albicans*

The presence of α-bisabolol, in 1 mg/ml to 4 mg/ml concentration range, has a noticeable impact on the attachment of *C. albicans* to polystyrene, and this effect is directly related to its concentration (Fig. 5.3). However, when α-bisabolol is at concentrations ranging from 0.5 to 0.125 mg/ml, it has no influence on the cell. Specifically, at 1 mg/ml, a MIC value is observed, indicating the concentration at which about 50 % inhibition of adhesion to polystyrene occurred (Table 5.1).

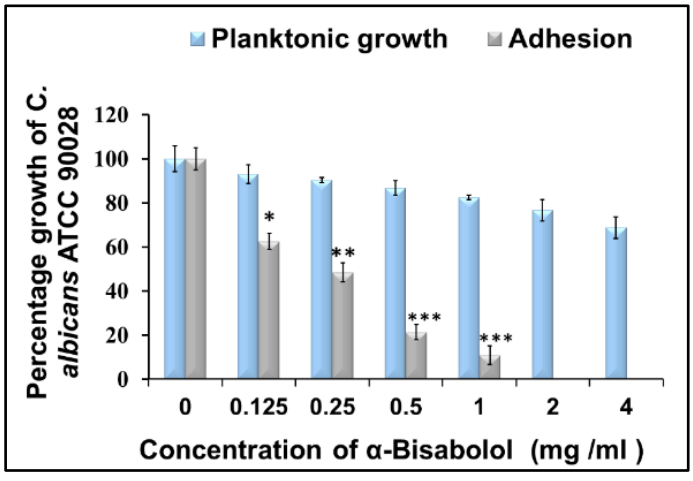

Fig. 5.3: Concentration-dependent inhibition of planktonic growth and adhesion of *C. albicans* after the treatment of α-bisabolol (0.125 mg/ml to 4 mg/ml). * p <0.05, ** p< 0.01, *** p <0.001.

5.3.3 Effect of α-bisabolol on yeast to hyphal morphogenesis in *C. albicans*

α-bisabolol effectively hindered the development of *C. albicans* hyphae in response to serum in a concentration-dependent manner (Fig. 5.4a). Substantial inhibition of the transformation from yeast to hyphal form, induced by serum, is evident after treatment with α-bisabolol. Notably, at 0.25 mg/ml, there is a 50 % inhibition of the yeast to hyphal morphology (Table 5.1). At higher concentrations, such as 2 mg/ml and 4 mg/ml, complete inhibition of this morphological change is observed (Fig. 5.4b).

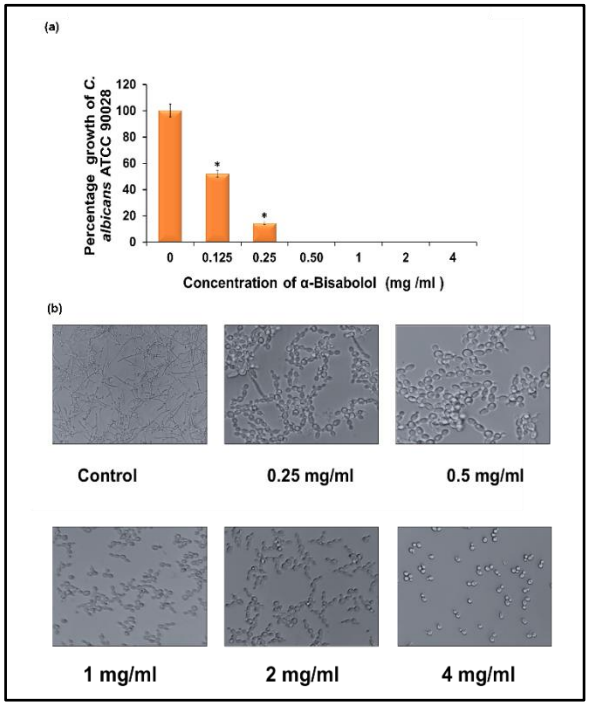

Fig. 5.4: (a) Effect of α-bisabolol yeast to hyphal morphogenesis of *C. albicans* (0.125 mg/ml to 4 mg/ml). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (b) Microscopic observation showing inhibition of yeast to hyphal morphogenesis.

5.3.4. Effect of α-bisabolol on biofilm development

The addition of α-bisabolol prevented the biofilm development of *C. albicans* in a concentration-dependent manner (Fig. 5.5a). The inclusion of α-bisabolol at 0.125 mg/ml caused a 50 % reduction in *C. albicans* biofilm formation as compared to the control group (Table 5.1). Scanning electron micrography revealed that at concentrations of 0.125 mg/ml of α-bisabolol, only adherent yeast cells are observed, indicating total suppression of biofilm growth (Fig. 5.5b).

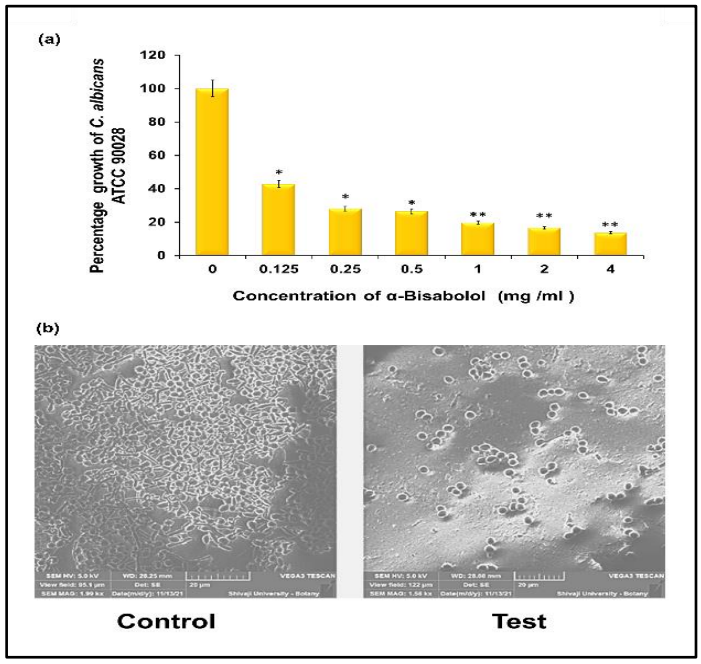

Fig. 5.5: (a) Effect of α-bisabolol on biofilm development of *C. albicans* (0.125 mg/ml to 4 mg/ml). * $p<0.05$, * $p<0.01$. (b) Scanning electron micrograph showing dense biofilm formation in control and complete inhibition of biofilm development by the treatment of α-bisabolol in test (0.125 mg/ml) (Scale bar 20 µm).

5.3.5. Effect of α-bisabolol on gene expression

The quantitative real time polymerase chain reaction (qRT-PCR) was used to scrutinize alterations in the expression of genes linked to the Ras1-cAMP-Efg1 and Cek1-MAPK pathways. The findings revealed that α-bisabolol treatment resulted in a notable 2.53-fold reduction in expression of *RAS1*, a key regulatory gene. *PDE2*, situated upstream in the cAMP-dependent PKA pathway, displayed a substantial 3.04-fold downregulation. Likewise, subsequent elements *BCY1, EFG1*, and *TEC1* were downregulated by 1.41-fold, 2.29-fold, and 1.41-fold, respectively (Fig. 5.6). *ECE1*, a downstream component of *EFG1,* exhibited a significant 4.88-fold reduction in expression. In contrast, *HWP1,* also situated downstream of *EFG1*, showed a 2.67-fold upregulation. In the context of the Cek1-MAPK pathway, genes *HST7, CEK1*, and *CPH1* displayed downregulation by 5.42-fold, 4.82-fold, and 5.39-fold, respectively, in response to α-bisabolol treatment. However, suppressors of hyphal initiation, *NRG1* and *MIG1,* were substantially downregulated with reductions of 1.55-fold and 1.34-fold, respectively (Table 5.2).

Table 5.2: Regulation of gene expression in *C. albicans* biofilm after treatment with α-bisabolol.

Gene	α-bisabolol induced fold Change	Regulation
RAS1	2.53	Downregulation
PDE 2	3.04	Downregulation
BCY 1	1.41	Downregulation
EFG 1	2.29	Downregulation
TEC 1	1.41	Downregulation
ECE 1	4.88	Downregulation
CEK 1	4.82	Downregulation
HST 7	5.42	Downregulation
CPH1	5.39	Downregulation
HWP1	2.67	Upregulation
MIG1	1.34	Downregulation
NRG1	1.55	Downregulation

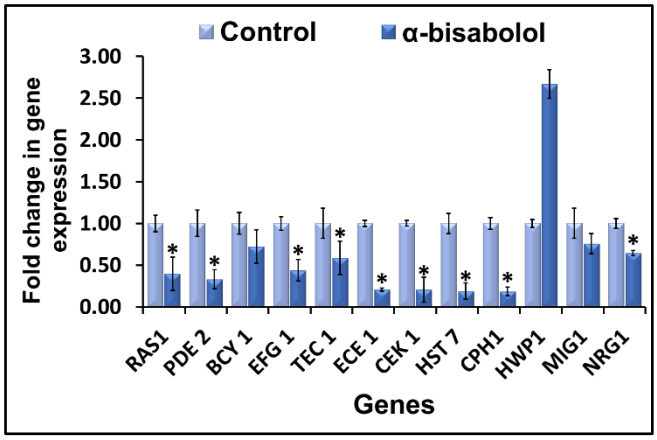

Fig. 5.6: Transcriptional profile of genes involved in biofilm formation of *C. albicans* after the treatment of α-bisabolol (0.125 mg/ml). * $p<0.05$.

5.3.6. Effect of α-bisabolol on cell division cycle

The flow cytometry analysis of the control sample and α-bisabolol treated test sample revealed that α-bisabolol treatment affects the cell cycle of *C. albicans*. Flow cytometry analysis of control *C. albicans* cells showed that 30 % of cells are present in the G0/G1 phase, 49.1 % are in the G2/M phase, and 20.4 % are in the S phase of the cell cycle. But, *C. albicans* cells treated with α-bisabolol exhibited an aggregation of cells in the G0/G1 phase, with a prominent peak showing 56.3 % of cells in this cell cycle phase and 10.9 % of cells are present in S phase, and 31.8 % of cells are present in G2/M phase (Fig. 5.7a). This comparative cell cycle analysis data is depicted in the histogram (Fig. 5.7b).

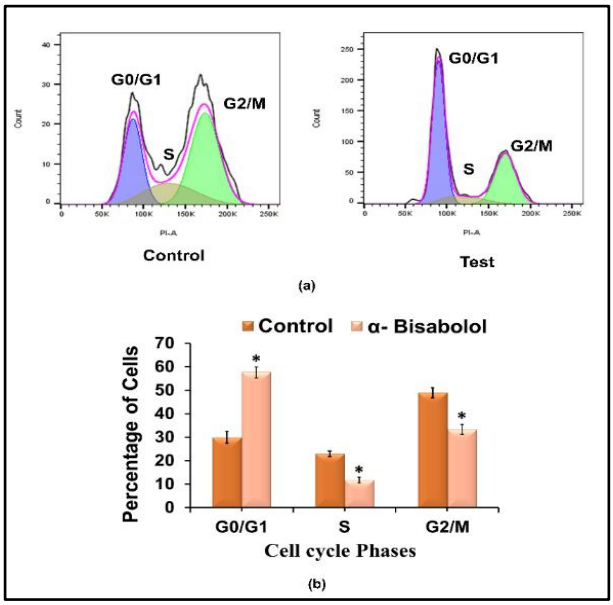

Fig. 5.7: (a) Cell cycle analysis of *C. albicans* showing control cells without treatment with α-bisabolol and test after treatment of α-bisabolol (2 mg/ml) for 4 h 30 min at 35ºC showing cell cycle arrest at G0/ G1 phase. (b) Histogram for percentage of *C. albicans* cells present at different phases of the cell cycle. * $p < 0.05$.

5.3.8. Hemolytic activity of α-bisabolol

As a precaution against potential pharmacologically induced toxicity, an *in vitro* hemolysis study was conducted before contemplating any pharmaceutical applications. The hemolysis test was conducted to evaluate the possible toxicity of the molecule, and it was observed that RBC hemolysis was not triggered by α-bisabolol at values of 0.125 mg/ml to 4 mg/ml. Hence, this molecule is considered safe for further investigation (Fig. 5.8).

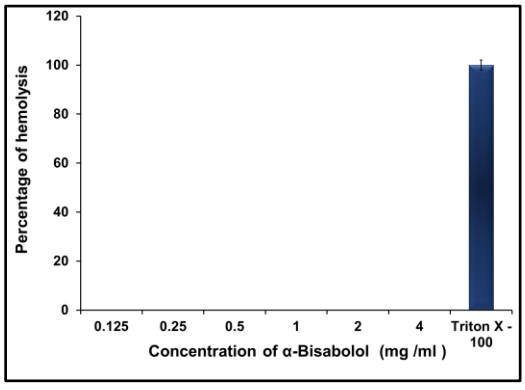

Fig. 5.8: Hemolytic activity of α-bisabolol (0.125 mg/ml to 4 mg/ml) on human RBCs.

5.4. Discussion

Attempts to explore the unknown bioactivities of existing natural molecules have provided insights into the field of drug development. Isaac and colleagues initially extracted α-bisabolol, a monocyclic sesquiterpene alcohol, from chamomile blossoms in 1951 (*Matricaria chamomilla*; *Asteraceae*). Since it has a lovely floral scent and has been demonstrated to have antibacterial and gastro-protective properties, it has attracted significant economic interest [10]. The molecule also has other bioactive properties like anti-inflammatory, anti-irritant, anti-oxidant, anti-plasmodial, and anti-diabetic [7,8].

The antifungal activity of α-bisabolol against *Aspergillus flavus, A. fumigatus, A. niger, A. terreus, Fusarium oxysporum, F. solani, and F. verticillioides* is alredy known [11]. Also, it has activity against dermatophytes, including: *Microsporum gypseum, M. canis, Trichophyton. violaceum, T. rubrum, and T. tonsurans T. mentagrophytes, N. ajetani, E. floccosum, A. magypseum* [12].

The antifungal activity of α-bisabolol on *C. albicans* against planktonic, adhesion, early biofilm, and mature biofilm is already known [13]. Marchi et al. (2017) reported the anti-*C. albicans* activity of chitosan-coated-nanocapsule containing α-bisabolol [12]. *Candida* infections, often referred to as candidiasis, can encompass a spectrum from mild, and surface-level conditions such as oral thrush or vaginal infections to more intense and invasive infections, particularly in individuals with weakened immune systems. In cases where the host's immune responses are compromised or when there are breaches in the body's natural barriers, fungal pathogens like *Candida* can exploit these vulnerabilities to move beyond the epithelial surfaces, such as the skin, mucous membranes, and linings of organs, and enter deeper anatomical niches within the body. This can lead to the spread of the fungal infection to internal organs and tissues, which can be more severe and life-threatening [14].

C. albicans undergoes a dimorphic transition in response to various environmental conditions. Morphogenetic changes are crucial for its ability to invade and cause disease. Morphogenesis additionally aids *C. albicans* in evading the human immune system. Ras1-cAMP-Efg1, MAPK, and other pathways influence the yeast to hyphal morphogenesis of *C. albicans* [15].

In current research, an effort has been undertaken to investigate the mechanism by which α-bisabolol acts against planktonic growth, adhesion, the formation of biofilms, and established biofilms (Table 5.1). The impact of α-bisabolol influence of treatment on gene expression associated with the development of biofilm formation was examined using qRT-PCR.

Current research demonstrates that α-bisabolol has an impact on gene expression related to the Yeast to hyphal morphogenesis during biofilm development in *C. albicans*. As a result of various environmental cues, *C. albicans* possesses the capability to transition from its yeast form to its hyphal form [16]. The yeast to hyphal morphogenesis plays a crucial role in enabling *C. albicans* to avoid detection by the host's immune system [17,18]. A promising avenue for the development of novel drugs is to focus on inhibiting the yeast to hyphal morphogenesis in *C. albicans*, aiming to decrease the disease-causing potential [19]. α-bisabolol inhibited adhesion and biofilm development at very low concentrations. It was observed under a light microscope and further confirmed by scanning electron microscopy. It is shown that α-bisabolol can block biofilm formation (Fig. 5.5b).

Ras1-cAMP-Efg1 and MAPK pathways favorably control the transformation of yeast to hyphal form [15]. *RAS1* is the key controller of the Ras1-cAMP-PKA and Cek1-MAPK pathways, and it strongly influences yeast to the hyphal transition [20]. α-bisabolol led to a downregulation of the *RAS1* gene expression by as much as 2.53-fold (Fig. 5.6). The signal transduction pathways influencing the suppression of biofilm formation in *C. albicans* may be impacted by the downregulation of *RAS1*. Under most hyphal inducing conditions, *C. albicans* is stopped in its yeast shape by inhibition of cAMP production [21], indicating that filamentation needs a specific concentration of cAMP. As cAMP plays a pivotal role in hyphal growth, targeting other components within the cAMP signaling system represents a promising approach for the development of antifungal medications [22]. The 3.04-fold downregulation of *PDE2* (Phosphodiesterase Activity) could lead to a reduction in cAMP synthesis, which might delay the yeast to hyphal transition [22].

In *C. albicans* PKA regulatory pathway subunit *BCY1* is essential for controlling cell differentiation and death [23]. Multiple cellular morphologies can develop when *BCY1* is deleted, and filament formation is encouraged. The *BCY1/BCY1* mutant typically has filamentous and smooth colonies, but it can also

spontaneously transition between the two forms. Following exposure to α-bisabolol, the *BCY1* gene exhibited a downregulation of 1.41-fold (Fig. 5.6). *EFG1,* which serves as a regulatory component of PKA, governs hyphal development. Its expression is observed to be downregulated by α-bisabolol (Fig. 5.6). *EFG1* is a regulator specific to fungi that is encoded by a basic helix-loop-helix transcription factor that participates in the morphogenesis process [23]. The significant but incomplete suppression of hyphal growth is seen in *EFG1* mutant strains. *EFG1* and *CPH2* control hyphal regulator *TEC1* transcription [15].

Treatment with α-bisabolol resulted in the downregulation of *TEC1* (Fig. 5.6). In *C. albicans*, the regulation of the *ECE1* gene is essential for promoting cell elongation in the process of filament formation. Infection and invasion are made easier by the damage to the epithelial cells by *ECE1* [24]. *ECE1* was downregulated on treatment with α-bisabolol by 4.88-fold (Fig. 5.6). Decreased *ECE1* expression could reduce the filamentation and invasive characteristics. α-bisabolol enhanced *HWP1* expression, which is essential for adherence to both biotic and abiotic surfaces. *CST20, CEK1*, and *HST7* control the activity of the MAP kinase pathway component *CPH1* of *C. albicans* [25,26]. α-bisabolol led to the reduction of *HST7, CEK1*, and *CPH1* gene expressions (Fig. 5.6) [27,28]. The suppression of yeast hyphal development may be caused by the downregulation of *HST7, CEK1,* and *CPH1*. *NRG1* and *MIG1* are examples of hyphal suppressor genes that prevent hyphal development. *NRG1* and *MIG1* are hyphal suppressor genes that prevent hyphal development. The downregulation of *NRG1* and *MIG1* after treatment with α-bisabolol was observed (Fig. 5.6).

According to cell cycle studies, α-bisabolol causes G0/G1 phase cell cycle arrest (Fig. 5.7). During the G0/G1 phase, the growth-dependent action of cyclin-dependent kinases promotes DNA replication and initiates the transition from G1 to the S phase [29] which is inhibited by the action of α-bisabolol. Remarkably, in an *in vitro* cytotoxicity study, it is observed that the concentration of α-bisabolol necessary to halt the cell cycle of *C. albicans* is not harmful. In addition to this, α-bisabolol is not showing any hemolysis at 0.125 to 4 mg/ml concentration (Fig. 5.8). Hence, it is considered safe for further exploration.

5.5. Conclusions

α-bisabolol effectively inhibited various pathogenic traits of *C. albicans*, adhesion, yeast to hyphal switching, and development of biofilm at 1 mg/ml, 0.25 mg/ml, and 0.125 mg/ml concentration, respectively. Also, α-bisabolol impedes adhesion and biofilm formation of *C. albicans* on polystyrene surfaces. The findings revealed that α-bisabolol treatment resulted in a notable 2.53-fold reduction in expression of *RAS1*, a key regulatory gene. *PDE2*, situated upstream in the cAMP-dependent PKA pathway, displayed a substantial 3.04-fold downregulation. Likewise, subsequent elements *BCY1, EFG1*, and *TEC1* are downregulated by 1.41-fold, 2.29-fold, and 1.41-fold, respectively. *ECE1*, a downstream component of *EFG1,* exhibited a significant 4.88-fold reduction in expression. It also interrupts the cell cycle at the G0/G1 phase in *C. albicans*. Moreover, gene expression studies reveal that α-bisabolol treatment results in modifications to the activity of genes participating in signaling pathways of *C. albicans*. Surprisingly, it operates against virulence factors without causing hemolysis of human RBCs. Given that it demonstrated anti-virulence activity and diverse targets in *C. albicans*, α-bisabolol holds promise as a prospective remedy against *C. albicans*. However, its ability to combat fungal infections needs to be validated through *in vivo* investigations.

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Chapter VI:
Antifungal activity of nonanal against *Candida albicans* and its targets

6.1. Introduction

C. albicans is the most prevalent opportunistic pathogenic fungus in humans [1]. *Candida* infections act as the primary culprit in bloodstream infections within clinical settings, constituting near about 18 % of nosocomial sepsis cases in hospitalized patients [2]. The occurrence of *C. albicans* leads to superficial mucosal infections in areas like the oral cavity, throat, and reproductive tract. It can also result in systemic invasive candidiasis affecting the circulatory system, bones, and brain [3,4]. More than 300 million individuals globally have serious fungal infections, which can result in 1.5 million fatalities annually and a mortality rate that is near about 50 % in severe systemic diseases. According to available data, there are more than 7,50,000 cases of invasive candidiasis reported annually, resulting in over 50,000 deaths worldwide per year [5]. Several antifungal drugs are used in clinical settings to manage invasive candidiasis. However, the emergence of drug resistance and the side effects significantly limit their application, leading to increased complications and the cost of treatment [6]. Hence, there is an increased need for new antifungal medications to be investigated.

Nonanal (Fig. 6.1) is found abundantly in various species like soybean, canola, *Senecio laetus, Minuarti ameyeri*, *Apium graveolens,* and *Haplophyllum tuberculatum.* The essential oils exhibited antifungal effects against various pathogenic fungi like *Alternaria alternate, Curvularia lunata*, *Bipolaris sp., Fusarium oxysporum,* and *C. albicans* [7]. It has also been reported for its antimicrobial activity against gram-positive and gram-negative bacteria [8]. The lipophilic nature of essential oils allows them to selectively travel from an aqueous environment to the membranes of fungi. This process causes expansion of membranes, increased fluidity and permeability, disruption of proteins embedded in the membrane, respiration inhibition, modification of ion transportation mechanisms in fungi, and the release of ions and other cellular constituents. Catalytic dehydrogenation reaction is also used for the synthesis of nonanal from nonanol. It is also prepared by 1-octene hydroformylation or formic acid and nonanoic acid reaction on a titanium dioxide catalyst [9]. The biological activities of nonanal have only been reported in a limited number of publications. Additional research is essential to study the antimicrobial and antifungal capabilities of this compound, particularly for its specific applications. It is

important to investigate the potential mode of action in resistant pathogenic fungi like *C. albicans*.

In literature, there is evidences of antimicrobial and antifungal activities of essential oils containing nonanal [10]. To the best of my knowledge, the present study is the first report to explore the *in vitro* and *in vivo* antifungal activity of nonanal against *C. albicans*. This study deals with the examination of the effect of nonanal on the growth and virulence factors of *C. albicans*. The investigation delves into the effect of varying concentrations of nonanal on morphology, cell cycle, cell membrane permeability, and gene expression. The goal is to understand its antifungal mechanisms against *C. albicans*.

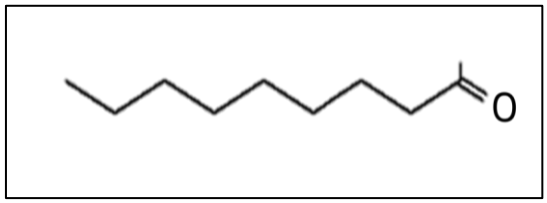

Fig. 6.1: Structure of nonanal.

6.2. Materials and methods

Methodology was followed as mentioned in chapter 3, from page no. 29 to 42.

6.3. Results

6.3.1. Minimum inhibitory concentration (MIC) of nonanal for planktonic growth

The activity of nonanal on the planktonic growth of *C. albicans* has been tested across a range of concentrations from 0.008 mg/ml to 4 mg/ml. It is observed that nonanal inhibited *C. albicans* growth in a concentration-dependent manner (Fig. 6.2a). At the concentration of 0.063 mg/ml nonanal is able to inhibit 50 % of the planktonic growth of *C. albicans* compared to the control (Table 6.1). This indicates that nonanal has an inhibitory effect on the growth of *C. albicans*. Despite inhibiting growth, the viability of *C. albicans* is affected by exposure to nonanal. This suggests that nonanal necessarily kills the cells. The observation that *C. albicans* cells are not able to survive even at the highest tested concentration of 4 mg/ml is considered as minimum fungicidal concentration (MFC) (Fig. 6.2b, Table 6.1).

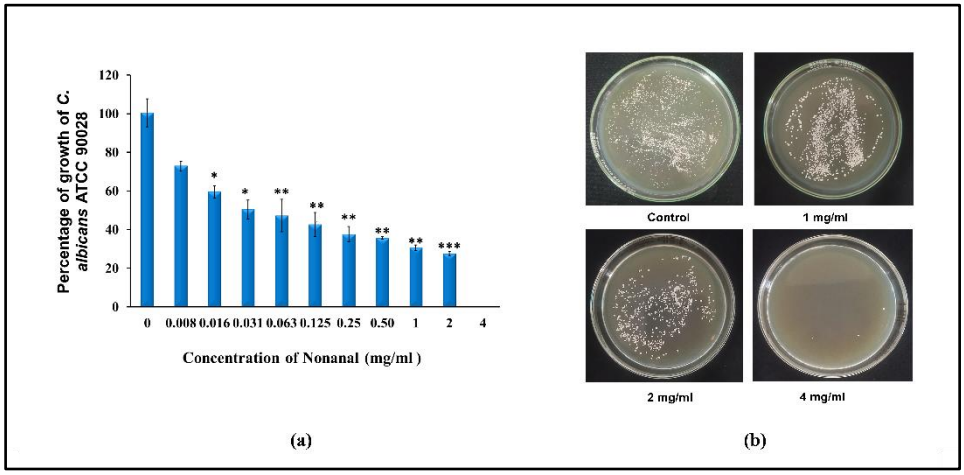

Fig. 6.2: (a) Concentration dependent inhibition of planktonic growth of *C. albicans* after the treatment of nonanal. * p <0.05, ** p <0.01, *** p <0.001. (b) MFC of nonanal against *C. albicans* is observed at 4 mg/ml.

Table 6.1: The antifungal efficiency of nonanal against *C. albicans* ATCC 90028 showing the MIC for planktonic growth, adhesion, yeast to hyphal morphogenesis, biofilm formation, mature biofilm, and MFC for planktonic growth.

Molecule	Planktonic growth	Adhesion	Yeast to hyphal morphogenesis	Developing biofilm	Mature Biofilm	MFC
Nonanal MIC (mg/ml)	0.063	0.25	0.125	0.016	NA	4

NA not achieved up to 4 mg/ml; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

6.3.2. Kill curve assay

The time-dependent killing of *C. albicans* cells by the treatment of nonanal is observed. According to the kill curve experiment, it is noted that nonanal is able to kill 99.9 % of *C. albicans* cells due to the exposure of zingerone at a concentration of 4 mg/ml within 240 min (Fig. 6.3).

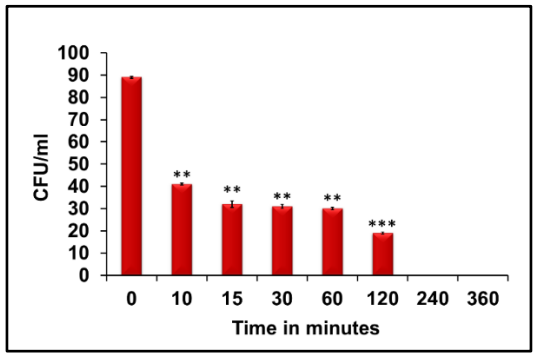

Fig. 6.3: Time dependent killing of *C. albicans* cells by the treatment of nonanal. * p <0.05, ** p <0.01, *** p <0.001.

6.3.3. Effect of nonanal on adhesion

The adhesion of *C. albicans* to polystyrene, a common surface in lab settings, is influenced by the presence of nonanal in a concentration-dependent manner (Fig. 6.4a). At concentrations ranging from 0.125 mg/ml to 0.5 mg/ml of nonanal, the adherence of *C. albicans* cells to the polystyrene surface is not influenced. Additionally, the metabolic activity measured by the XTT assay remained similar to that of the control. This suggests that at the lower concentrations, nonanal does not impact the adhesion or metabolic activity of the *C. albicans* cells. However, as the concentration of nonanal increases (1 mg/ml to 4 mg/ml), there is a noticeable influence on *C. albicans* adhesion to the polystyrene surface. This effect is concentration-dependent, indicating that higher concentrations of nonanal led to a decrease in the adherence of *C. albicans* to the surface. The MIC of nonanal for inhibiting the adhesion of *C. albicans* to polystyrene is observed at 0.25 mg/ml (Table 6.1). This investigation suggests that nonanal, particularly at higher concentrations and at the observed MIC level, has an inhibitory effect on the adhesion of *C. albicans*. This could have implications in preventing or controlling *C. albicans* colonization on surfaces, which is significant in various contexts, especially in preventing biofilm formation or infections.

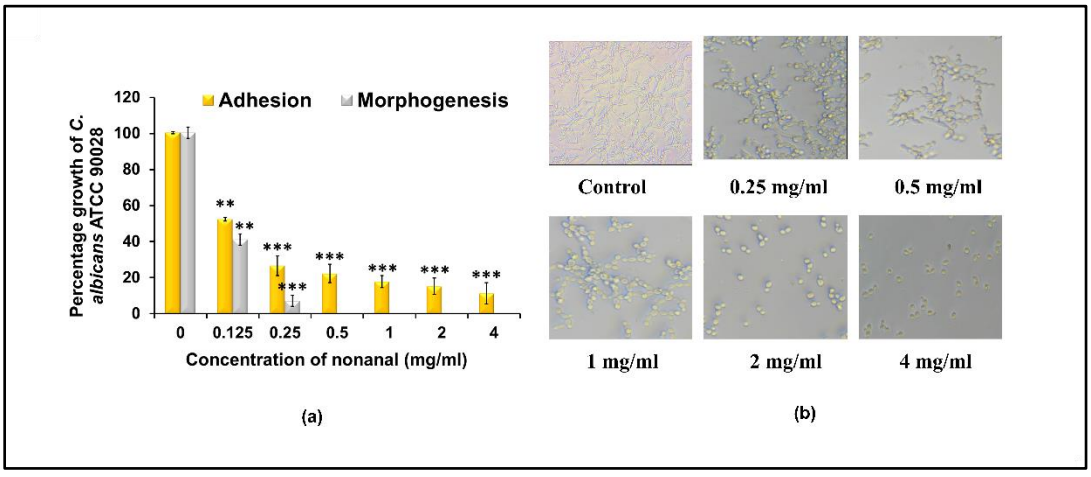

Fig. 6.4: (a) Concentration-dependent inhibition of adhesion and yeast to hyphal morphogenesis of *C. albicans* after the treatment of nonanal. ** p <0.01, *** p <0.001. (b) Microscopic images showing effect of nonanal on yeast to hyphal morphogenesis of *C. albicans.*

6.3.4. Effect of nonanal on yeast to hyphal morphogenesis

Nonanal inhibits serum influenced yeast to hyphal morphogenesis of *C. albicans*. The inhibitory effect of nonanal on the yeast to hyphal form transition is

observed to be dose-dependent (Fig. 6.4a). At a lower concentration of 0.25 mg/ml, nonanal inhibited 50 % of the transition from yeast to hyphal morphogenesis, indicating a partial inhibition of this process. At higher concentrations, such as 2 mg/ml and 4 mg/ml, there is complete inhibition of the yeast to hyphal morphogenesis (Fig. 6.4b). This means that at these concentrations, nonanal effectively prevented the transition of *C. albicans* from yeast form to the hyphal form induced by serum. This observation is significant as the transition from yeast to hyphal forms is a critical aspect of *C. albicans* virulence and pathogenesis. The ability of nonanal to impede this transition suggests its potential as a candidate for controlling or modulating the virulence of *C. albicans* by inhibiting its morphological transition, thereby potentially mitigating its pathogenicity.

6.3.5. Effect of nonanal on biofilm development by *C. albicans*

At 0.016 mg/ml, the inclusion of nonanal led to a 50 % decrease in normal biofilm formation by *C. albicans* compared to the control biofilms (Fig. 6.5). Interestingly, when nonanal is present at concentrations of 4 mg/ml and 2 mg/ml, only a few adhered yeast cells are detected, signifying complete inhibition of biofilm development. Examination of biofilm microstructure via SEM images indicated that the biofilm is comparatively less dense in nonanal treated sample than the control after 24 h. The control displayed a complex three-dimensional structure with long hyphae forming a multi-layered network, while the treated sample exhibited a lack of significant hyphal formation (Fig. 6.6).

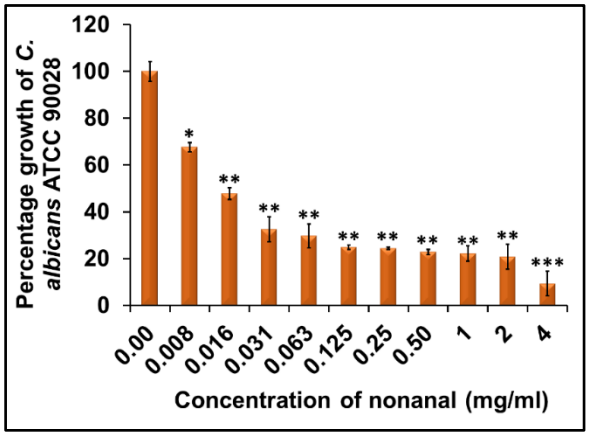

Fig. 6.5: Concentration dependent inhibition of biofilm formation in *C. albicans* during the treatment of nonanal (0.008 mg/ml to 4 mg/ml). * p <0.05, ** p <0.01, *** p <0.001.

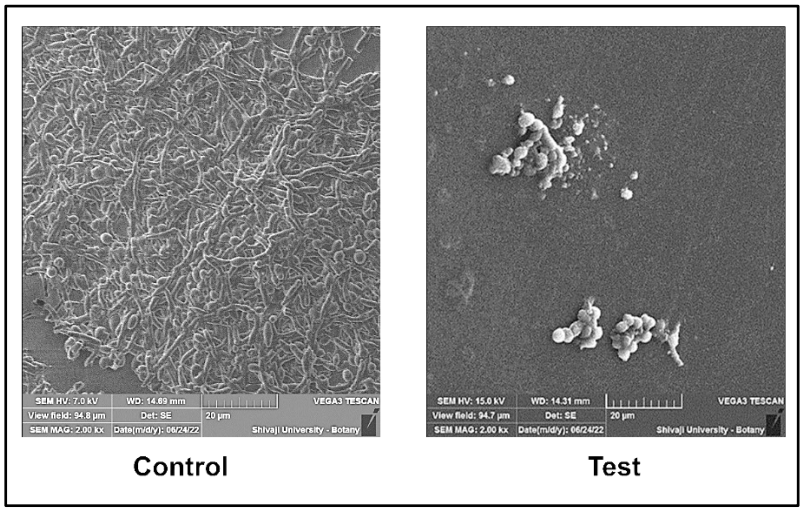

Fig. 6.6: Scanning electron micrograph showing dense biofilm formation in control sample and complete inhibition of biofilm development by the treatment of nonanal in test sample (Scale bar 20 µm).

6.3.6. Effect of nonanal on gene expression

Since nonanal prevents formation of developing biofilm in *C. albicans* quantitative real-time PCR technique was used to analyse expression of the genes involved in Ras1-cAMP-Efg1 and Cek1-MAPK pathways. *RAS1* gene and its signalling pathways in *C. albicans* are essential for unravelling the mechanisms behind its pathogenicity and developing potential antifungal strategies targeting this crucial regulator. It has been observed that treatment with nonanal suppressed the expression of *RAS1* by 11.29-folds (Fig. 6.7). In *C. albicans, PDE2* is necessary for hyphal growth, and cell wall integrity is upregulated by 2.40-fold. The expression of *EFG1*, *BCY1,* and *TEC1* was downregulated by 3.62-fold, 7.18-fold, and 5.91-fold. *ECE1* downstream of *Efg1* is significantly downregulated by 35.26-fold. On the other hand, *HWP1,* which is also downstream of *EFG1,* is downregulated by 39-fold, respectively. Expression of *CPH1* and *HST7,* involved in Cek1-MAPK pathways are downregulated by 1.21 and 3.25-fold, respectively. The expression of *CEK1* was drastically downregulated by 21.42-fold in presence of nonanal. Suppressor genes of hyphal induction, *NRG1, MIG1,* and *TUP1* are also downregulated by 11.79-fold, 11.47-fold, and 7.75-fold, respectively (Table 6.2) (Fig. 6.7).

Table 6.2: Regulation of genes in biofilm after treatment with nonanal.

Gene	Relative fold change induced by nonanal	Regulation
RAS1	11.29	Downregulation
PDE2	2.40	Upregulation
BCY1	7.18	Downregulation
EFG1	3.62	Downregulation
TEC1	5.91	Downregulation
ECE1	35.26	Downregulation
CEK1	21.42	Downregulation
HST7	3.25	Downregulation
CPH1	1.21	Downregulation
HWP1	39.00	Downregulation
MIG1	11.47	Downregulation
NRG1	11.79	Downregulation
TUP1	7.57	Downregulation
CDC35	7.60	Upregulation

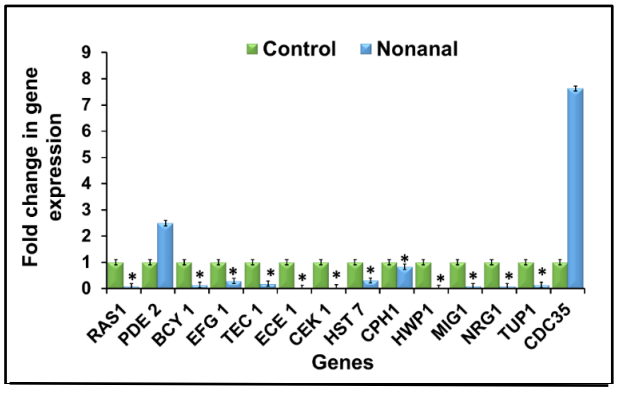

Fig. 6.7: Transcriptional profile of genes involved in biofilm formation of *C. albicans* after the treatment of nonanal (0.016 mg/ml). * $p < 0.05$.

6.3.7. Effect of nonanal on cell division cycle

The results of a Flow Cytometry analysis (FACS) comparing cell cycle phases in untreated (control) cells versus cells treated with nonanal. In the control group, 32.7 % of cells are in G0/G1, 59.5 % in G2/M, and 7.32 % in the S phase. This distribution suggests a normal cell cycle distribution. In the nonanal treated cells, 23.2 % cells are present in G0/G1 phase 17.1 % cells in the S phase, and 58.1 % cells in

G2/M phase (Fig. 6.8a). This comparative cell cycle analysis data is depicted in the histogram (Fig. 6.8b). This indicates that nonanal treatment might be causing cell cycle arrest at the S phase, causing damage to the DNA due to challenges during DNA replication, such as encountering obstacles on the DNA strands or inadequate nucleotide availability. This information is valuable as it suggests that nonanal might have an impact on cell cycle progression, potentially causing arrest at S phase, which could have implications in various biological processes or potential therapeutic uses. Understanding alterations in cell cycle distribution can provide insights into how different compounds or treatments affect cellular processes, aiding in further research or potential therapeutic developments (Fig. 6.8).

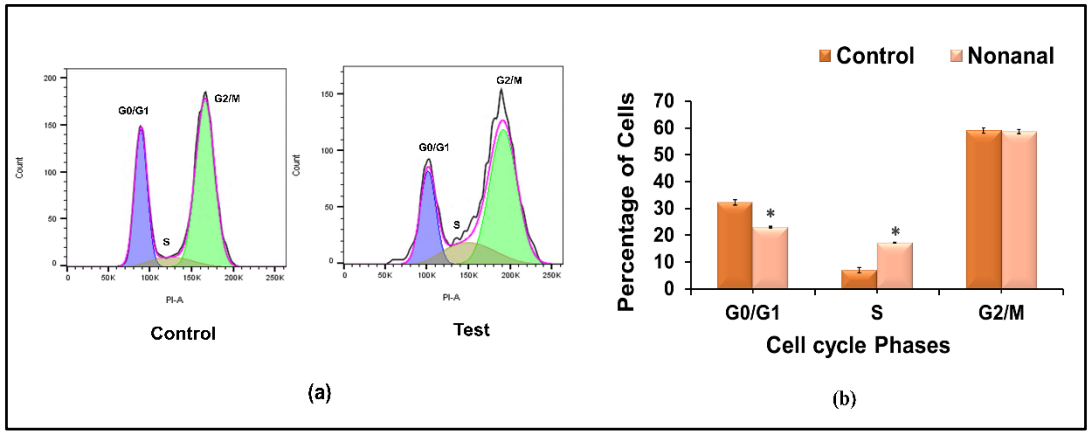

Fig. 6.8: (a) Cell cycle analysis of *C. albicans* control cells without treatment with nonanal and test after treatment of nonanal. (b) Histogram for percentage of cells present at different phases of the cell cycle showing S phase arrest after the treatment of nonanal. * $p < 0.05$.

6.3.8. Effect of nonanal reactive oxygen species (ROS) generation

H_2DCF was employed to evaluate intracellular reactive oxygen species levels, generating green fluorescent DCF upon oxidation in the presence of ROS. Following nonanal treatment, a notable rise in the population of green fluorescent is observed. This increase in fluroscence is linked to the intracellular buildup of ROS induced by nonanal, contrasting with untreated cells (negative control). The positive control group (H_2O2) exhibited the highest intensity of green fluorescence, signifying a significant oxidative stress response due to the accumulation of a substantial number of ROS in these cells (Fig. 6.9a). These results implyes that nonanal treatment leads to ROS accumulation in the treated cells, although to a lesser degree than the positive control, suggesting an oxidative stress response triggered by nonanal in *C. albicans* cells.

6.3.9. Effect of nonanal on genes involved in ROS production

The study involved analyzing the gene expression associated with preventing oxidative stress, *SOD1, SOD2, CAP1, CAT1*, and *MCA1* to correlate findings related to ROS generation and cell cycle outcomes (Fig. 6.9a). Among these genes, *SOD2* and *CAT1* exhibited upregulation by 1.89-fold and 1.35-fold, respectively, following nonanal treatment (Table 6.3). Interestingly, *MCA1* demonstrated an upregulation of 1.38-fold after exposure to nonanal. These varying gene expression patterns highlight the differential impact of nonanal treatment on genes associated with protecting against oxidative stress and apoptosis. These alterations in gene expression likely contribute to the observed changes in ROS generation and cell cycle dynamics, offering insights into the molecular mechanisms through which nonanal influences *C. albicans*.

Table 6.3: Upregulation of genes involved in ROS generation after treatment with nonanal.

Gene	Relative fold change induced by nonanal	Regulation
SOD1	1.90	Upregulation
SOD2	1.89	Upregulation
CAP1	1.27	Upregulation
CAT1	1.35	Upregulation
MCA1	1.38	Upregulation

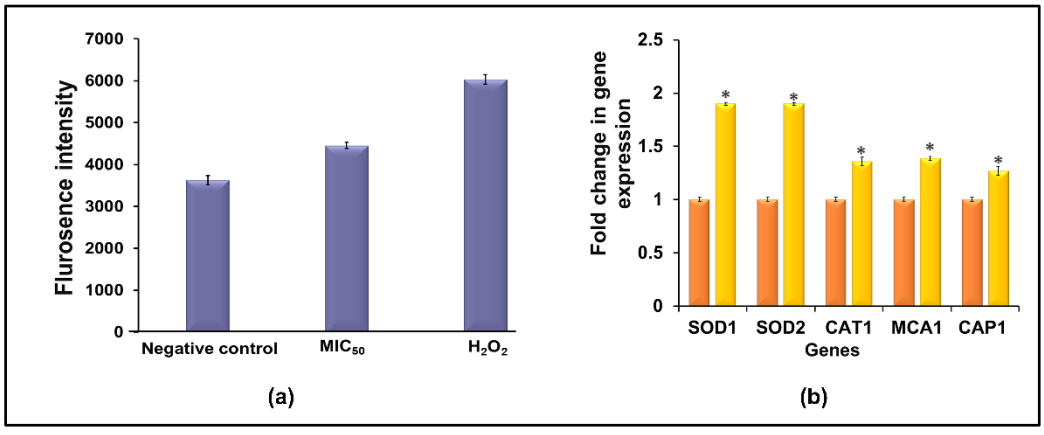

Fig. 6.9: (a) MIC concentration of nonanal enhances endogenous ROS accumulation in *C. albicans*. (b) Upregulation of genes involved in preventing ROS damage after the treatment of nonanal in *C. albicans*. * $p < 0.05$.

6.3.10. Effect of nonanal on the ergosterol biosynthesis

Ergosterol is a vital lipid within the membrane of *C. albicans*, plays a pivotal role in regulating its fluidity, permeability, and overall integrity. The introduction of nonanal led to a notable alteration in the ergosterol profile, showing a clear dose-dependent relationship. As the concentration of nonanal increases (0.5 mg/ml to 4 mg/ml), there was a complete inhibition of ergosterol content (Fig. 6.10). Even at lower concentrations, there is a discernible reduction in the sterol content. This observation highlights the sensitivity of ergosterol to varying doses of nonanal, indicating a potential mechanism through which nonanal affects the membrane integrity and functionality of *C. albicans*. This dose-dependent impact suggests a significant correlation between the concentration of nonanal and its influence on ergosterol levels within the fungal membrane.

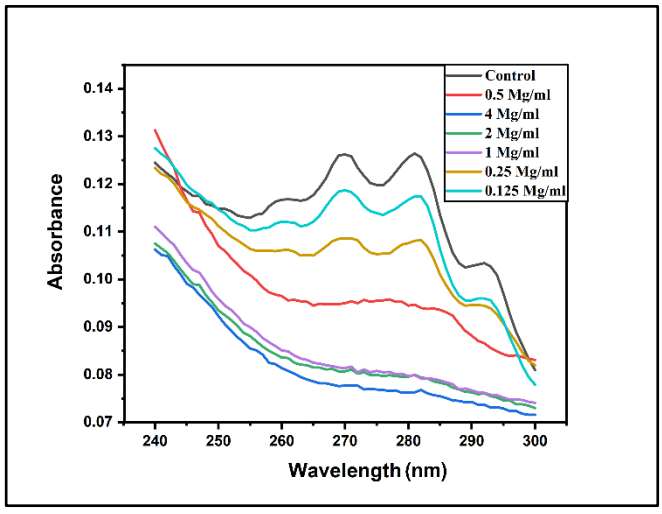

Fig. 6.10: Concentration-dependent inhibition of ergosterol biosynthesis after the treatment of nonanal measured by the spectrophotometric method.

6.3.11. Effect of nonanal on cell membrane integrity

Propidium iodide (PI) is an intercalating fluorescent dye that binds to DNA. It is unable to enter the healthy cells. Once the cell membrane is damaged, propidium iodide enters the cytoplasm, facilitating its binding to DNA, resulting in a vivid red fluorescence when observed under a fluorescence microscope. The fluorescence microscope analysis documented an elevation in number of damaged *C. albicans* as the concentration of nonanal increased from 0.5 mg/ml to 2 mg/ml (Fig. 6.11). the PI uptake assay revealed a significant rise in the PI-positive cells following nonanal treatment in comparison to the cells in control. This indicates that nonanal induced cell membrane damage in *C. albicans*.

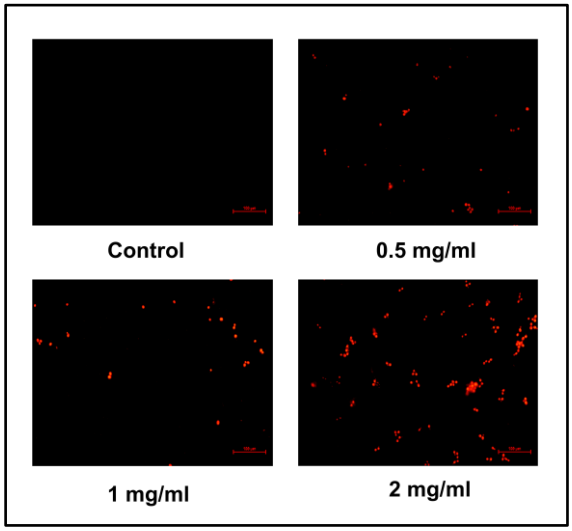

Fig. 6.11: Fluorescence microscopy images of propidium iodide-stained *C. albicans* cells treated with various concentrations of nonanal proves the membrane damage effect of nonanal on cell membrane integrity of *C. albicans* (scale bar 100 µm).

6.3.12. Hemolytic activity of nonanal

The hemolysis assay is an important test to assess the potential toxicity of a molecule, especially its effect on human red blood cells (RBCs). The hemolysis assay results indicate that nonanal demonstrated a lack of significant hemolytic activity on human RBCs up to a concentration of 0.25 mg/ml. At concentrations ranging from 0.062 mg/ml to 2 mg/ml, no significant hemolysis was observed, while at 4 mg/ml, 20 % hemolysis is observed (Fig. 6.12). Based on these findings, the molecule nonanal is considered safe for further investigation. This suggests that at the tested concentrations, nonanal does not induce substantial rupture or damage to red blood cells, indicating a favorable safety profile for potential exploration in further studies or applications. While these results are promising, it's important to conduct additional studies to comprehensively assess the molecule's safety across various concentrations and biological systems before concluding its safety for broader use or application.

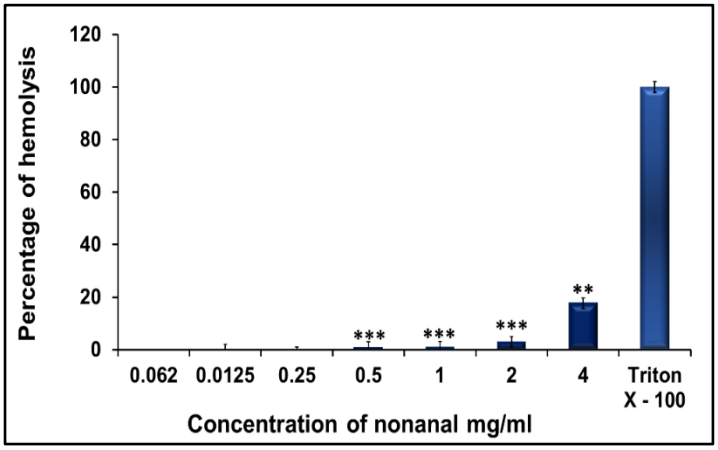

Fig. 6.12: Hemolytic activity of nonanal on human RBCs. ** p <0.01, *** p <0.001.

6.3.13. Effect of nonanal on the survival of silkworms

The *in vivo* assessment of antifungal efficacy utilized a silkworm model, a common tool in scientific research for gauging the effectiveness of antifungal treatments in a living organism. This model yields crucial insights into the potential therapeutic advantages of tested substances. The silkworm larvae of the negative control group injected with only *C. albicans* cells died within 24 h due to infection. The *C. albicans*-infected silkworm larvae of the positive control group treated with fluconazole survived up to 72 h. Additionally, silkworms of the vehicle control group injected with a 2 % DMSO also exhibited extended survival up to 72 h. Also, results indicated that silkworms treated with a combination of *C. albicans* and nonanal-treated test group survived for up to 72 h, indicating a protective effect of nonanal against *C. albicans*, extending their lifespan compared to those treated with *C. albicans* alone (Fig. 6.13).

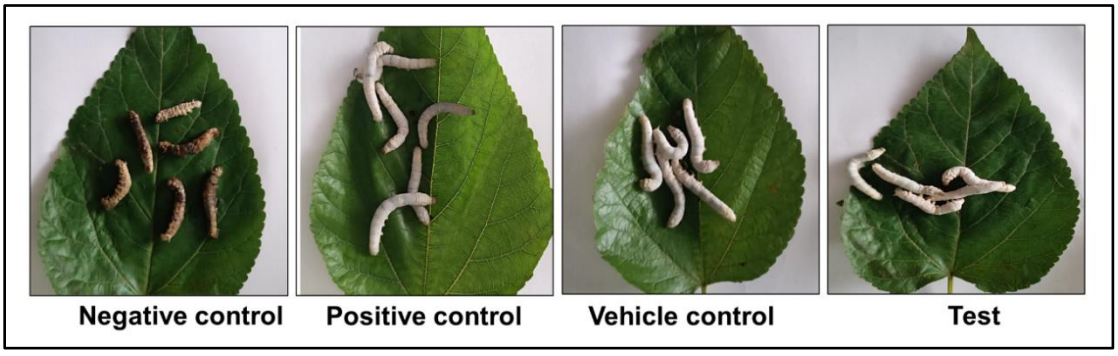

Fig. 6.13: *In vivo* antifungal efficacy of nonanal (0.063 mg/ml) in silkworm animal model. Silkworms in the negative control group died within 24 h due to *C. albicans* infection. Silkworms in the flucanozole-treated positive control group were alive up to 72 h, while silkworms from the vehicle control and nonanal-treated groups survived up to 72 h.

6.4. Discussion

Each year, fungal infections from pathogens such *as Aspergillus*, *Cryptococcus,* and *Candida* result in over a million deaths, despite the limited availability of medications, in the face of increasing drug resistance [11]. While *C. albicans* can form biofilms on living and non-living surfaces, these biofilms also contribute to increased resistance against antifungal medications. Thus, it is obvious that the discovery of novel antifungal drugs is urgently needed. Natural compounds, especially those found in herbs or medicinal plants, are becoming more and more recognized as potential building blocks for the development of innovative antifungal drugs [12]. Medicinal plants and phytochemicals are frequently mentioned as innovative antifungal treatments for effectively managing *Candida* infections, often with minimal or no adverse effects, according to widespread reports [13].

Previously the antifungal activity of nonanal was tested against *Penicillium cyclopium* and to ascertain anti-fungal effectiveness for a particular application, more research is required [8]. In our present investigation we thoroughly examined the antifungal effects of nonanal on *C. albicans* with *in vitro* approach. The preliminary study showed that planktonic growth was found to be inhibited at 4 mg/ml (Fig. 6.2a). A key pathogenic feature of *C. albicans* is its ability to form biofilms, which serve as a foundation for enhanced adhesion to body surfaces and act as a protective barrier against environmental stressors and the host immune system, and frequently lead to heightened tolerance to antifungal agents [14]. Our research findings suggest that a low dose of nonanal effectively prevents Y-H transition and biofilm formation. Nonanal had significant biofilm inhibitory activity against *C. albicans* with 0.016 mg/ml concentration. It is further confirmed by SEM. In SEM, it is observed that in control, an intricate three-dimensional configuration characterized by lengthy hyphae forming a multi-layered network was present. In contrast, the treated sample displayed a notable absence of significant hyphal formation (Fig. 6.6).

Further going in to detailed study to find out molecular mechanism of action of nonanal in biofilm formation qRT-PCR studies are conducted to identify molecular targets in signal transduction pathway includes Ras1-cAMP-PKA pathway and the MAPK pathway. These pathways are involved in regulating the transition from yeast-like cells to filamentous hyphal forms, which ultimately leads to biofilm development, a critical virulence factor for *C. albicans* that is linked to its

pathogenicity [15]. Various transcription factors serve as crucial regulators within the signal transduction pathways governing the morphogenesis of yeast to hyphal form in *C. albicans* [16]. *RAS1* serves as a pivotal upstream regulator, playing a significant role in both the Ras1-cAMP-PKA pathway and the Cek1-MAPK pathway [17]. *RAS1* is tremendously downregulated after the treatment nonanal by 11.29- fold. *RAS1* facilitates the activation of adenylate cyclase, an enzymatic process governed by the *CDC35* gene. Consequently, the downregulation of *RAS1* may result in decreased activation of adenylate cyclase, leading to reduced levels of cAMP. This may affect filament formation during biofilm formation. During infection, *ECE1* plays an important role in the invasion of epithelial cells. Ultimately leading to pathogenicity. Downregulation of *ECE1* has an impact on cell elongation and invasion. Nonanal treatment leads to downregulation by 35.26-fold. *HWP1* plays a crucial role in the adhesion and colonization of surfaces, which is essential for the pathogenicity of *C. albicans*. After the treatment of nonanal it is downregulated by 39-fold leading to significant impact on adhesion and, consequently, biofilm formation. Hence, inhibition of biofilm may occur due to downregulation of all these genes involved in the Ras1-cAMP-PKA pathway (Fig. 6.7, Table. 6.2).

The genes from MAPK pathway like *CEK1, CPH1,* and *HST7* are also downregulated after the treatment of nonanal. The Cek1-MAP kinase is accountable for cell wall biogenesis and vegetative growth in *C. albicans*. *CPH1* is a transcription factor in *C. albicans* that plays a pivotal role in regulating morphogenesis. Also, the adaptability and complexity of regulatory networks in *C. albicans* are underscored by the versatile nature of *CPH1* as a transcription factor. It exhibits the ability to function both positively and as a suppressor under specific morphological states and physiological circumstances. In case of nonanal treatment it may acts as suppressor of morphological states (Fig. 6.7, Table. 6.2).

Nonanal effectively inhibited cell cycle propagation at S phase (Fig. 6.8a). It is also called as synthesis phase, in this stage of the cell cycle, DNA replication takes place. During this phase, the cell duplicates its DNA in preparation for cell division. The cell cycle arrest at this stage may lead to programmed cell death or apoptosis [18].

It is known that several current antifungals work by producing ROS, which increases their efficacy against *C. albicans*. Multiple research studies propose that, at

high concentrations, phytochemicals have the potential to stimulate the production of ROS [19]. Hence, we have studied effect of nonanal on ROS generation. The presence of nonanal led to the accumulation of reactive oxygen species in *C. albicans* (Fig. 6.9a). After nonanal treatment, the upregulation of *SOD1* and *SOD2* indicated heightened oxidative stress within the cell (Fig. 6.9b, Table. 6.3). Molecular and cellular analyses have highlighted the importance of *CAP1* gene expression in resisting oxidative stress and its role in activating genes associated with this mechanism, including *CAT1*. *CAT1* is crucial for catalase production, serving as an essential enzyme in detoxifying *C. albicans* cells by eliminating hydrogen peroxide (H_2O_2) from the intracellular environment. The elevated expression of both genes affirmed the increased oxidative stress induced by nonanal treatment (Fig. 6.9b, Table. 6.3).

The examination of the *S. cerevisiae* genome has unveiled the existence of a caspase-like protein falling in the type I classification of metacaspases, which has been designated as *YCA1* (*MCA1* in *C. albicans*). Initially, Endonuclease (EndoG) was suggested to play a role in mitochondrial DNA replication, and is implicated in caspase-independent apoptosis [20]. Upon initiation of apoptosis, EndoG is liberated from the mitochondria into the cytosol, subsequently translocating to the nucleus and causing oligonucleosomal DNA fragmentation. Caspase activation is not involved in the entire process, as it occurs independently of caspase activation. The gene *MCA1* for caspase-dependent pathway was upregulated after the treatment of nonanal (Fig. 6.9b, Table. 6.3).

Ergosterol plays a vital role as a key component of the fungal cell membrane, serving to maintain membrane fluidity, permeability, and overall integrity in fungi [21]. Nonanal has be en found to inhibit ergosterol biosynthesis (Fig. 6.10). Consequently, this may affect the membrane integrity and permeability of *C. albicans.* To further validate the loss of membrane integrity, a propidium iodide (PI) staining was conducted by subjecting *C. albicans* to different concentrations of nonanal and subsequently stained with PI. PI is a fluorescent dye known for its high cell membrane impermeability, thus making it incapable of staining healthy cells. However, it can rapidly penetrate damaged cell membranes and stain cells with a vivid red fluorescence by binding to DNA [22]. Absorption of PI by the *C. albicans* cells treated with nonanal rises with an increase in the dosage (Fig. 6.11). These

results additionally suggest an increased permeabilization of the membrane and a loss of membrane integrity as a result of nonanal treatment.

Nonanal exhibited no hemolytic activity at concentrations inhibiting virulence factors, suggesting it does not harm human RBCs (Fig. 6.12). This non hemolytic activity is favorable and indicating safety of nonanal offering promise for its potential therapeutic applications. It was previously documented that pathogenic fungi were responsible for the mortality of silkworms [23].

This study revealed that *C. albicans* infection resulted in the death of the silk worm within a 24 h timeframe. However, when administered with the MIC concentration of nonanal the survival time extended 72 h after the infection (Fig. 6.13). Furthermore, the silkworm progressed into the pupation stage and completed its normal life cycle. This confirmed the ability of nonanal to enhance the survival of the silkworm.

6.5. Conclusions

This study demonstrated that, nonanal has a multifaceted effect on *C. albicans,* impacting on various aspects of its biology and pathogenicity. The results indicated that nonanal effectively impeded growth, yeast to hyphal morphogenesis and biofilm formation at concentrations of 0.063 mg/ml, 0.125 mg/ml and 0.016 mg/ml, respectively in a dose dependent manner.

Nonanal was shown to modulate the gene expression involved in biofilm formation pathways in *C. albicans*. The results revealed that nonanal at 0.016 mg/ml led to a suppression of *RAS1* expression. In contrast, the expression levels of *EFG1*, *BCY1,* and *TEC1* are significantly downregulated. The downstream effector *ECE1* of *EFG1* exhibited a remarkable reduction of 35.26-fold in expression, while *HWP1*, another downstream target of *EFG1*, is downregulated by 39.0-fold. Furthermore, the expression of *CPH1* and *HST7*, key components of the Cek1-MAPK pathway, is decreased. Notably, the expression of *CEK1* is drastically reduced in the presence of nonanal. Additionally, suppressor genes involved in hyphal induction, such as *NRG1*, *MIG1*, and *TUP1*, are downregulated. This suggests that nonanal can inhibit the biofilm formation.

Nonanal effectively inhibited ergosterol biosynthesis, which is an essential lipid content of the fungal membrane. This disruption can affect the integrity and functions of the fungal cell membrane. At 0.008 mg/ml, nonanal-induced cell cycle

arrest, primarily in the S phase, impacts the DNA proliferation of *C. albicans* and leads to the generation of reactive oxygen species at 0.008 mg/ml, which are signs of cellular stress.

Nonanal showed no hemolytic activity, indicating that it does not cause damage to human red blood cells. This is a positive indication of its safety of nonanal. This non-toxic nature is promising for potential therapeutic applications.

In conclusion, nonanal acts as a potential antifungal agent with multiple targets in *C. albicans*. Its multifaceted actions on the fungus, coupled with its non-toxic nature, make it an intriguing candidate for further exploration as a therapeutic option for *C. albicans* infections.

6.6. References

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Chapter VII:
Antifungal activity of undecanal against growth and virulence factors of *Candida albicans.*

7.1. Introduction

Invasive candidiasis is a prevalent fungal infection in immunocompromised patients, significantly contributing to mortality in severely ill patients [1]. The disease manifests across a broad spectrum, spanning from fungal bloodstream infections to candidiasis deeply rooted within tissues. and progressing to septic shock, characterized by failure of multiple organs. accompanied by a mortality rate exceeding 70 % [2]. In the clinical management of systemic fungal infections, substantial doses and extended periods of administering antifungal medications are frequently necessary. Despite these efforts, the mortality rate for severe patients persists at a high range of 40–50 % [3]. The treatment of invasive fungal diseases has become a significant clinical challenge.

Several strains of *C. albicans* exhibit resistance to existing antifungal medications, including azoles and echinocandins [4]. The virulence of *C. albicans* is defined by its capacity to create biofilms, densely populated cell communities that adhere to surfaces [5]. Extended exposure to antifungals, especially the development of *C. albicans* resistance, is largely caused by excessive use of azoles and echinocandins for prophylaxis or as an empirical treatment for invasive candidiasis in high-risk individuals [4].

Essential oils are known to contain a wide range of therapeutic compounds. Procuring natural compounds in adequate quantities for research and clinical trials proves challenging due to their limited availability, stemming from their low abundance in plants. It is essential to thoroughly investigate the use of these compounds for the bioavailability, toxicity, and any potential side effects. Therefore, our goal is to find pure natural compounds that will serve as building blocks for the development of antifungal drugs of the future.

Undecanal (Fig. 7.1) is naturally present in citrus oils, emitting a floral scent with waxy undertones. It also holds some commercial value. In their pure form, it is a colorless liquid with a green, fatty essence, harmonizing effectively with woody and mossy fragrances. Undecanal is synthesized through the hydroformylation process of decene, or it can be obtained through the catalytic dehydrogenation of undecanol [6]. Previously, it was stated that the volatile organic compounds (VOCs) from antagonistic microbes containing undecanal exhibit significant antifungal properties against soil-borne fungal pathogens [7]. Also, the essential oil derived from

Coriandrum sativum leaves, comprising 3.23% undecanal, demonstrated notable antibacterial effects against *E. coli*, *S. typhi*, *K. pneumoniae*, *P. mirabilis*, and *P. aeruginosa*, along with antifungal activity against *C. albicans* [8].

The aim of the present study was to explore the impact of pure undecanal on the growth and virulence factors of *C. albicans*. This was accomplished through *in vitro* assays, including the determination of minimum inhibitory concentrations, cell cycle studies, qRT-PCR analyses, ergosterol assays, and membrane permeability assays.

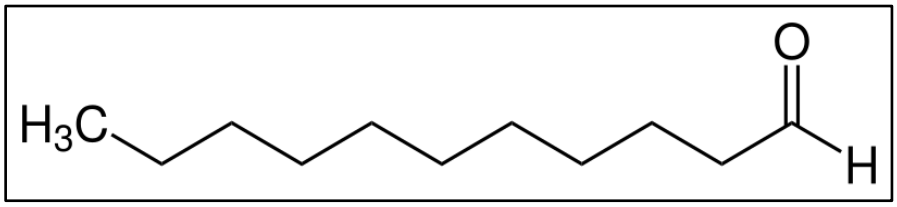

Fig. 7.1: Structure of undecanal.

7.2. Materials and Methods

The methodology was followed as mentioned in Chapter 3 on page no. 29 to 42.

7.3. Results

7.3.1. Minimum inhibitory concentration (MIC) of undecanal for planktonic growth

In this study, the effect of undecanal (concentrations ranging from 4 to 0.125 mg/ml) on the growth of *C. albicans* was assessed using a broth dilution assay. It is observed that undecanal exhibited a concentration-dependent inhibition in growth, and MIC is observed at 0.125 mg/ml (Fig. 7.2a, Table 7.1). Moreover, undecanal at 2 and 4 mg/ ml significantly prevented visible growth of *C. albicans*. It is observed that at the highest tested concentration of 4 mg/ml, *C. albicans* cells are not able to survive, indicating that undecanal is *Candida*-cidal in nature (Fig. 7.2c). Further, this is verified by a time-kill assay, which demonstrated that undecanal effectively eliminated *C. albicans* after 60 min (Fig. 7.2b).

Table 7.1: The antifungal efficiency of undecanal against *C. albicans* ATCC 90028 showing the MICs for planktonic growth, adhesion, yeast to hyphal morphogenesis, biofilm formation, mature biofilm, and MFC for planktonic growth.

	MIC					
Molecule	**Planktonic growth**	**Adhesion**	**Yeast to hyphal morphogenesis**	**Developing biofilm**	**Mature biofilm**	**MFC**
Undecanal (mg/ml)	0.125	1	0.125	0.002	NA	4

NA not achieved up to 4 mg/ml; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

7.3.2. Effect of undecanal on adhesion

The presence of undecanal, within the concentration range of 1 mg/ml to 4 mg/ml, significantly affected the attachment of *C. albicans* to polystyrene surfaces, with the impact being directly proportional to its concentration (Fig. 7.2a). However, when undecanal is present in 0.5 to 0.125 mg/ml, it does not affect cell adhesion and exhibits XTT metabolic function similar to the control group. Notably, at a concentration of 1 mg/ml, a minimum inhibitory concentration value is observed, indicating the concentration at which inhibition of adhesion to polystyrene occurred (Table 7.1).

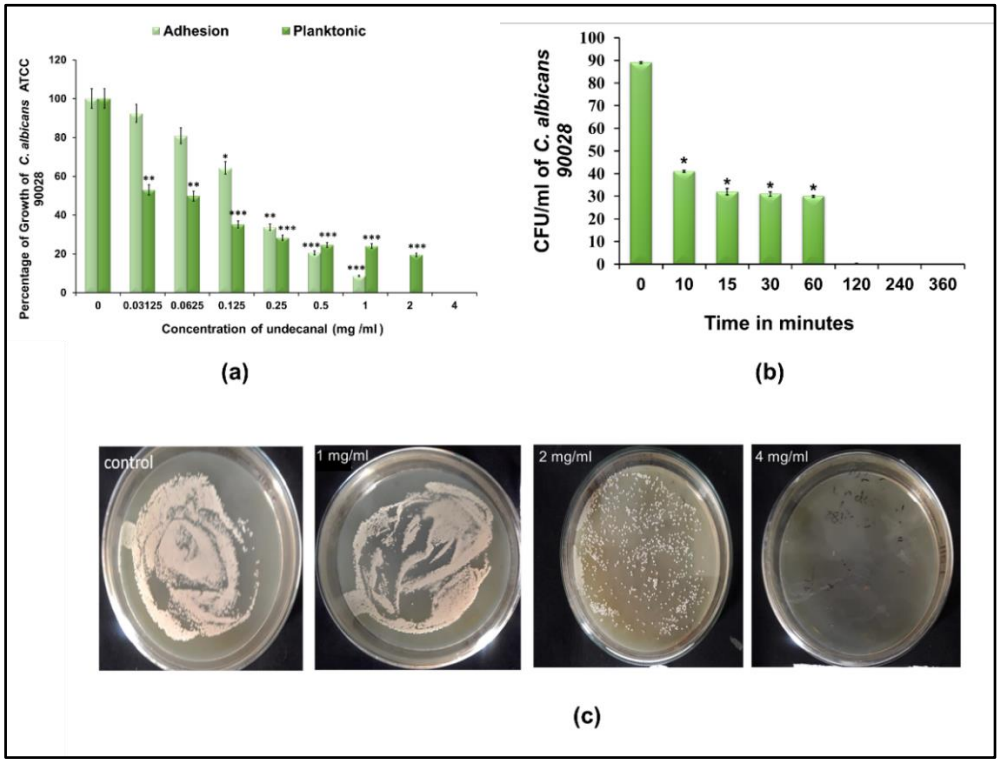

Fig. 7.2: (a) Concentration-dependent inhibition of planktonic growth and adhesion of *C. albicans* after the treatment of undecanal (b) Time-dependent killing of *C. albicans* cells by the treatment of undecanal. (c) Minimum Fungicidal Concentration (MFC) of undecanal against *C. albicans* 4 mg/ml. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

7.3.3. Effect of undecanal on yeast to hyphal morphogenesis

Undecanal displayed a concentration-dependent inhibition of the transition from yeast to hyphal morphogenesis induced by fetal bovine serum (FBS) (Fig. 7.3a). Undecanal inhibited 50 % of the transition from yeast to hyphal morphogenesis at a lower concentration of 0.125 mg/ml in *C. albicans* (Table 7.1). At higher concentrations, such as 2 mg/ml and 4 mg/ml, there is complete inhibition of the yeast to hyphal morphogenesis, suggesting a strong inhibitory effect (Fig. 7.3b). These results highlight its potential as an inhibitor of hyphal morphogenesis in *C. albicans,* which could be significant in combating its virulence.

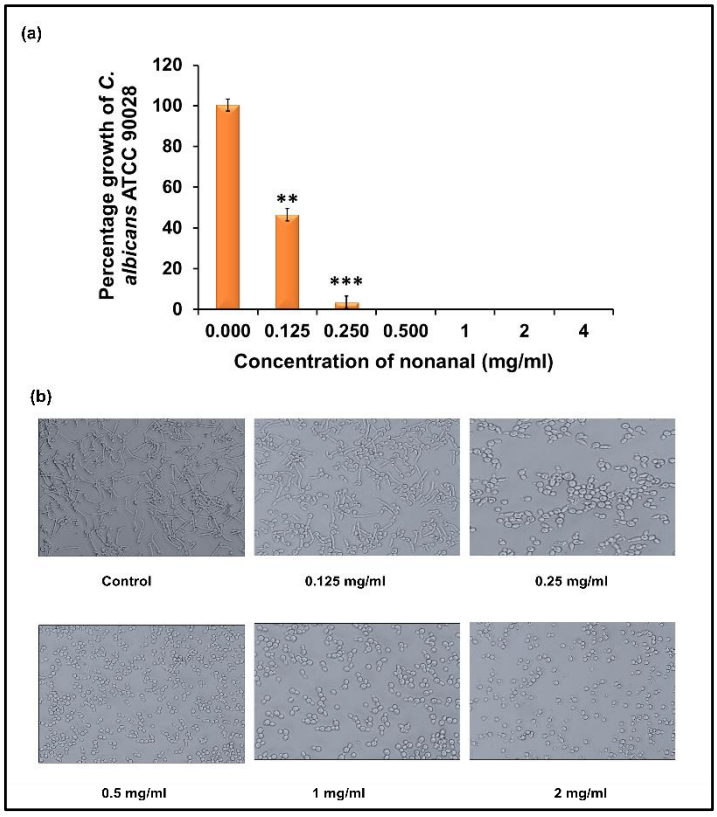

Fig. 7.3: (a) Effect of undecanal on yeast to hyphal morphogenesis of *C. albicans.* ** $p < 0.01$, *** $p < 0.001$. (b) Microscopic images showing the effect of undecanal on yeast to hyphal morphogenesis of *C. albicans.*

7.3.4. Effect of undecanal on *C. albicans* biofilm formation

The activity of undecanal against biofilm is tested against *C. albicans* at different concentration ranges from 0.002 to 4 mg/ml (Fig. 7.4). At 0.002 mg/ml, the addition of undecanal resulted in a 50 % reduction in normal biofilm development by *C. albicans* in comparison to the control biofilms (Table 7.1). Remarkably, at concentrations of 4 mg/ml and 2 mg/ml, the presence of undecanal resulted in the detection of only a few adhered yeast cells, indicating complete inhibition of biofilm

development. Analysis of biofilm microstructure through SEM images revealed that the biofilm treated with undecanal appeared less dense compared to the control after 24 h. The control biofilm exhibited a complex three-dimensional structure characterized by long hyphae forming a multi-layered network. In contrast, the treated sample showed a lack of significant hyphal formation. On the other hand, undecanal does not show inhibition against the mature biofilm of *C. albicans* (Fig. 7.5).

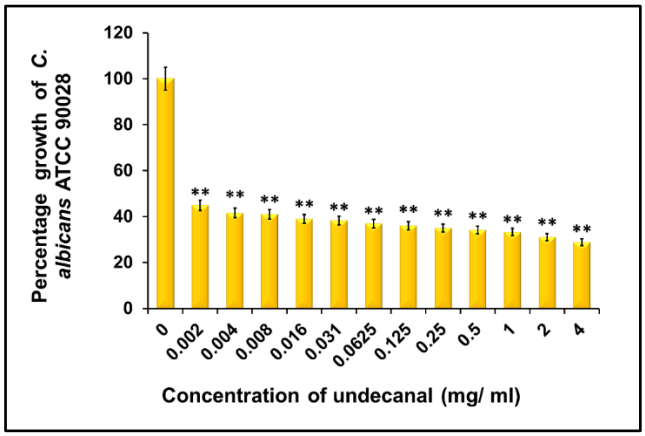

Fig. 7.4: Effect of undecanal on developing biofilm of *C. albicans*. $^{**}p < 0.01$.

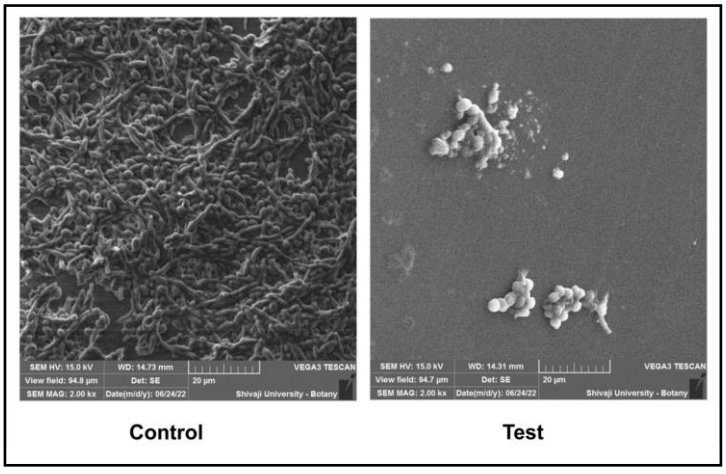

Fig. 7.5: Scanning electron micrographs showing dense biofilm formation in control and complete inhibition of biofilm development by the treatment of undecanal in test at 0.002 mg/ml (Scale bar 20 µm).

7.3.5. Effect of undecanal on gene expression

Given that undecanal impedes the formation of developing biofilms in *C. albicans*, quantitative real-time PCR was employed to analyze gene expression in the Ras1-cAMP-Efg1 and Cek1-MAPK pathways (Fig. 7.6). The *RAS1* gene and its associated signaling pathways in *C. albicans* are crucial for elucidating the mechanisms underlying its pathogenicity and for devising potential antifungal

strategies targeting this pivotal regulator. It is found that treatment with undecanal suppressed the expression of *RAS1* by 13.96-fold. Additionally, the expression of *PDE2*, essential for hyphal growth and cell wall integrity in *C. albicans*, is upregulated by 1.40-fold. Furthermore, the expression of *EFG1*, *BCY1*, and *TEC1* was downregulated by 2.30-fold, 3.52-fold, and 8.55-fold, respectively. *ECE1*, downstream of *EFG1*, exhibited a significant downregulation of 35.26-fold, while *HWP1*, also downstream of *EFG1*, showed a downregulation of 17.30-fold, respectively. Moreover, the expression of *CPH1* and *HST7*, participating in the Cek1-MAPK pathways, *CPH1* is upregulated by 1.40-fold and *HST7* is downregulated by 1.69-fold, respectively (Table 7.2). Notably, the expression of *CEK1* was downregulated by 6.19-fold after the treatment with undecanal.

Table 7.2: Fold change in gene expression of *C. albicans* in biofilm after treatment with undecanal.

Gene	undecanal induced relative fold change	Regulation
RAS1	13.96	Downregulation
PDE 2	1.40	Upregulation
BCY 1	3.52	Downregulation
EFG 1	2.30	Downregulation
TEC 1	8.55	Downregulation
ECE 1	35.26	Downregulation
CEK 1	6.19	Downregulation
HST 7	1.69	Downregulation
CPH1	1.40	Upregulation
HWP1	17.30	Downregulation
NRG1	7.46	Downregulation

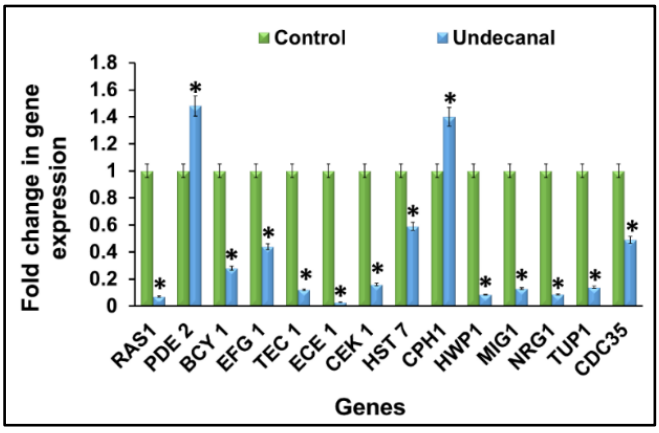

Fig. 7.6: Transcriptional profile of genes involved in biofilm formation of *C. albicans* after the treatment of undecanal (0.016 mg/ml). * $p < 0.05$.

7.3.6. Effect of undecanal on the cell division cycle of *C. albicans*

To find an antifungal activity with a particular mode of action, the effect of undecanal on the cell cycle of *C. albicans* is investigated. The results of a Flow Cytometry analysis (FACS) comparing cell cycle phases in untreated (control) cells versus cells treated with undecanal (test) (Fig. 7.7a, b). In the control group, 26 % of cells are in G0/G1, 26.8 % are in G2/M, and 46.9 % are in the S phase. This distribution suggests a normal cell cycle distribution. In the undecanal-treated 26.2 % of cells are in G0/G1, 10.1 % of cells are in S phase, and 66.1 % of cells are in G2/M. This comparative cell cycle analysis data is depicted in the histogram (Fig. 7.7c). This indicates that undecanal treatment might be causing cell cycle arrest at the G2/M phase.

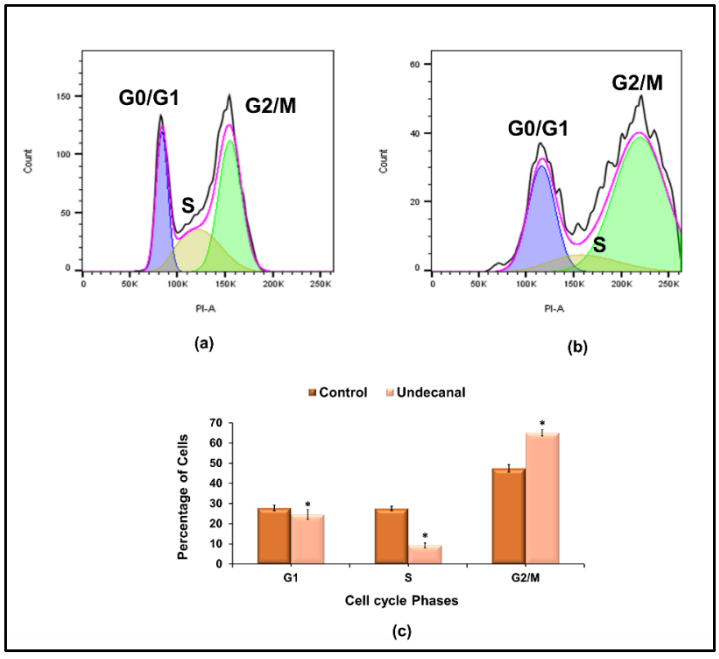

Fig. 7.7: Cell cycle analysis of *C. albicans* (a) control without treatment, (b) test after treatment of undecanal (0.125 mg/ml) for 4 h 30 min at 35ºC showing cell cycle arrest at G2/M phase. (c) Histogram for percentage of cells at different phases of the cell cycle. * $p \leq 0.05$.

7.3.7. Effect of undecanal on the production of reactive oxygen species in *C. albicans*

Reactive oxygen species are measured intracellularly using H_2DCF, which oxidizes to produce green fluorescence. Green fluorescence is seen to significantly rise after undecanal treatment, suggesting that the cells had higher quantities of ROS. This increase in fluorescence, as opposed to the untreated cells (negative control), was ascribed to the intracellular build-up of ROS caused by undecanal. Because of the

large build-up of ROS in these cells, the positive control group showed the strongest intensity of green fluorescence, suggesting a considerable oxidative stress response. These data indicate that undecanal treatment causes ROS accumulation in the treated cells, but not as much as in the positive control. This suggests that undecanal causes an oxidative stress response in *C. albicans* cells (Fig. 7.8a).

7.3.8. Undecanal modifies the expression of genes related to the production of reactive oxygen species

One of the objectives of the study was to evaluate the expression levels of genes, such as *SOD1*, *SOD2*, *CAP1*, *CAT1*, and *MCA1*, that are linked to oxidative stress protection (Fig. 7.8b). Significant increases in *SOD2, CAP1,* and *CAT1* expression are seen after undecanal treatment by 95.30-fold, 44.31-fold, and 30.87-fold, respectively, indicating overexpression. Furthermore, after undecanal treatment, *SOD1* upregulation is seen by 2.94-fold. Interestingly, after being exposed to undecanal, the expression of *MCA1* increased by 26.82-fold (Table 7.3). These unique patterns of gene expression highlight the diverse impacts of undecanal therapy on genes involved in oxidative stress resistance and apoptosis. These variations in gene expression are probably the cause of the observed changes in ROS production and cell cycle dynamics, providing information about the molecular processes by which undecanal influences *C. albicans*.

Table 7.3: Upregulation of genes involved in ROS generation after treatment with undecanal.

Gene	Relative fold change induced by undecanal	Regulation
SOD1	2.94	Upregulation
SOD2	95.30	Upregulation
CAP1	44.31	Upregulation
CAT1	30.87	Upregulation
MCA1	26.82	Upregulation

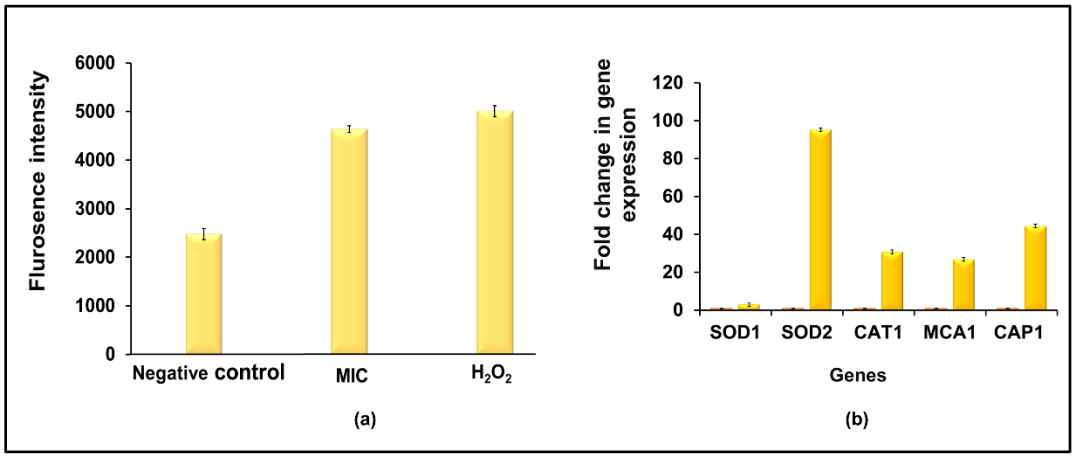

Fig. 7.8: (a) MIC concentration of undecanal enhances endogenous ROS accumulation. (b) Transcriptional profile genes involved in preventing ROS damage after the treatment of undecanal. * p <0.05.

7.3.9. Effect of undecanal on ergosterol biosynthesis

Ergosterol, a crucial lipid component in the membrane of *C. albicans*, plays a critical role in regulating membrane fluidity, permeability, and overall integrity. The introduction of undecanal resulted in a significant alteration in the ergosterol profile, demonstrating a clear dose-dependent relationship (Fig. 7.9). With increasing concentrations of undecanal (0.125 mg/ml to 2 mg/ml), ergosterol content decreased. Even at lower concentrations, a noticeable reduction in sterol content is observed. This finding underscores the sensitivity of ergosterol to varying doses of undecanal, suggesting a potential mechanism by which undecanal impacts the membrane integrity and functionality of *C. albicans*. The observed dose-dependent effect implies a substantial correlation between the concentration of undecanal and its influence on ergosterol levels within the fungal membrane.

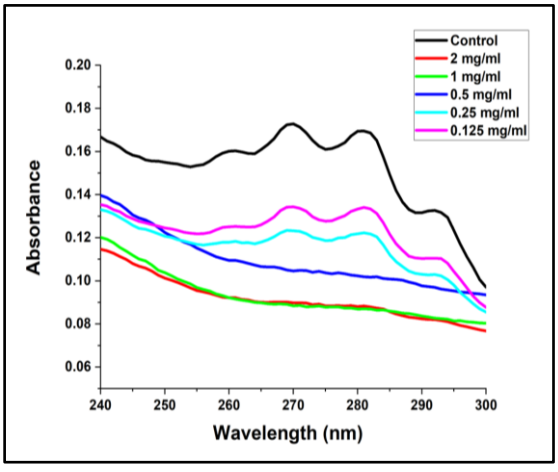

Fig. 7.9: Concentration-dependent inhibition of ergosterol biosynthesis after the treatment of undecanal measured by the spectrophotometric method.

7.3.10. Effect of undecanal on membrane permeability

A fluorescent dye, propidium iodide (PI), intercalates with DNA and is unable to penetrate healthy cells. However, when the cell membrane is compromised, propidium iodide gains access to the cytoplasm, where it binds to DNA, resulting in a red fluorescence observed by a fluorescence microscope. Analysis with a fluorescence microscope revealed an increase in the number of damaged *C. albicans* cells as the concentration of undecanal increased from 0.125 mg/ml to 0.5 mg/ml (Fig. 7.10). The propidium iodide uptake assay demonstrated a significant increase in the number of propidium iodide-positive cells following undecanal treatment as compared to the control cells. This suggests that undecanal induces cell membrane damage in *C. albicans*.

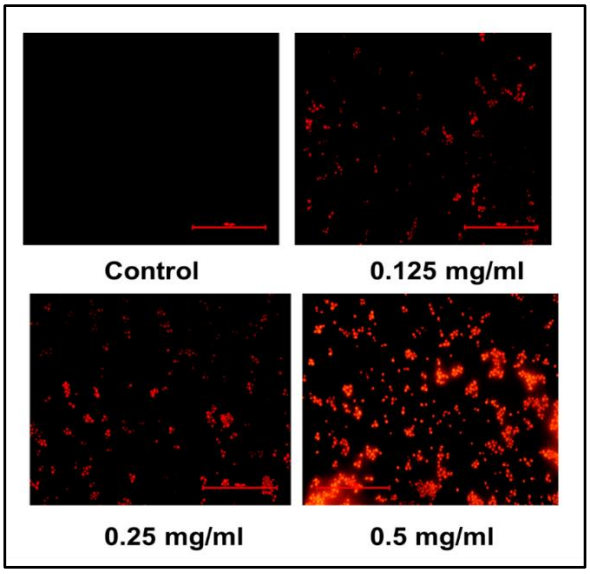

Fig. 7.10: Fluorescence microscopy images of propidium iodide-stained *C. albicans* cells treated with various concentrations of undecanal prove the effect of undecanal on the cell membrane damage of *C. albicans* (Scale bar 100 µm).

7.3.11. Hemolytic activity of undecanal

An important test for evaluating a compound's possible toxicity is the hemolysis assay, which examines how a substance affects human red blood cells (RBCs). The assay's results demonstrated hemolysis at higher dosages of 2 mg/ml and 4 mg/ml, however, undecanal does not exhibit hemolytic action against RBCs at concentrations that inhibited virulence factors, specifically up to 0.125 mg/ml (Fig. 7.11). According to these results, undecanal has promise for future study and applications and hence merits further consideration. However, before any judgments

can be made about the molecule's ability for wider use or application, more research must be done to fully assess its safety across a range of doses and biological systems. This is especially true in light of these encouraging results.

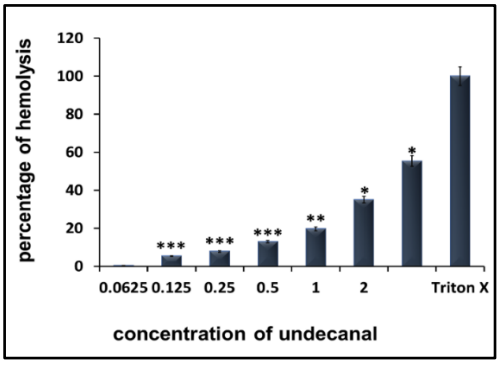

Fig. 7.11: Hemolytic activity of undecanal (0.0625 mg/ml to 4 mg/ml) on human RBCs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

7.3.12. Effect of undecanal on the survival of silkworms

The *in vivo* antifungal efficacy is evaluated using a silkworm model. This model is commonly employed in scientific research to assess the effectiveness of antifungal treatments in a living organism, providing valuable insights into the potential therapeutic benefits of the tested substances. The silkworm larvae of the negative control group injected with only *C. albicans* cells died within 24 h due to infection. The *C. albicans*-infected silkworm larvae of the positive control group treated with fluconazole survived up to 72 h. Additionally, silkworms of the vehicle control group injected with a 2 % DMSO also exhibited extended survival up to 72 h. Similarly, silkworms treated with a combination of *C. albicans* and undecanal test group at its MIC concentration were able to survive for up to 72 h. This suggests that the use of undecanal alongside *C. albicans* had a protective effect on the survival of the silkworms (Fig. 7.12).

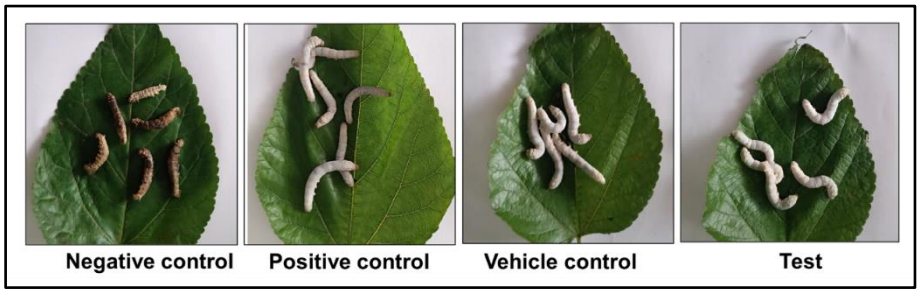

Fig. 7.12: *In vivo* antifungal efficacy of undecanal (0.125 mg/ml) in silkworm animal model. Silkworms in the negative control group died within 24 h due to *C. albicans* infection. Silkworms in the flucanozole-treated positive control group were alive up to 72 h, while silkworms from the vehicle control and undecanal-treated groups survived up to 72 h.

7.4. Discussion

Historically, natural resources have provided a wide array of antimicrobial agents. The effectiveness of natural compounds against microorganisms has been confirmed by recent research. Plant products have demonstrated inhibitory effects against filamentous fungi, yeast, and bacteria, including extracts, essential oils, and isolated chemicals [10–12]. The fact that many microbes can develop biofilms, which increases their resistance to medications, emphasizes the need for more research to examine the role that these natural compounds play in inhibiting the pathways that lead to biofilm formation. In a previous study, it was found that undecanal inhibits the growth of *S. cerevisiae* [13].

The effectiveness of undecanal, which has already been identified as having antifungal qualities, was evaluated in this investigation against planktonic cells and virulence factors of *C. albicans,* and it is found that undecanal inhibits the planktonic growth at a concentration of 0.125 mg/ml (Fig. 7.2a). At MFC concentration, undecanal is able to eradicate *C. albicans* after 60 min (Fig. 7.2b). In this study, the effect of undecanal on cell adhesion was determined, and it is observed that undecanal inhibited adhesion at 1 mg/ml concentration (Fig. 7.2a). The results of the study indicated that undecanal at low doses can effectively prevent Y-H transition (Fig. 7.3a).

The first stage of *C. albicans* biofilm growth, which is essential to the overall process of *C. albicans* biofilm formation, is the attachment of individual cells to the basal layer [14]. Undecanal is found to inhibit biofilm formation in *C. albicans*. SEM study provided additional proof for the antibiofilm effectiveness of undecanal. There is a noticeable variation from usual *C. albicans* biofilms having genuine hyphae, at 0.004 mg/ml resulted in a significant reduction in filamentation and cell density (Fig. 7.5).

The levels of expression of genes linked to the transition between yeast and filamentous states are examined in order to get more insight into the molecular mechanisms underlying the suppression of *C. albicans* biofilms after the undecanal therapy (Fig. 7.6). Based on the findings, several genes like *ECE1, HYR1, HWP1*, which are all controlled by the Ras1-cAMP-Efg1 pathway and MAPK signaling pathway are significantly downregulated. An essential function of the Ras1-cAMP-Efg1 pathway is to control *C. albicans* biofilms. It is activated by External

environmental signals, causing the *RAS1* protein to be activated. This then starts *Cyr1* to generate a second messenger, cAMP. As a result, transcription factors *TEC1* and *EFG1* are phosphorylated and activated as a result of the activation of the protein kinase A complex. Adhesion, mycelial growth, biofilm formation, and numerous other biological processes are all regulated by this process [15]. Through inhibition of the Ras1-cAMP and MAPK pathway, several natural compounds have shown the ability to prevent *C. albicans* adhesion, yeast-hyphae transition, and biofilm development [15]. Additionally, the study showed that undecanal dramatically lowered the expression of *RAS1*, *EFG1*, and *TEC1*, genes connected to the Ras1-cAMP signalling pathway (Fig. 7.6).

Following undecanal treatment, the MAPK pathway genes *CEK1*, *CPH1*, and *HST7* are also downregulated. In *C. albicans*, the Cek1-MAP kinase is responsible for both vegetative development and cell wall synthesis. *HST7* is a mitogen-activated protein kinase kinase (MAPKK) participating in both mating and hyphal growth in the signal transduction pathway of *C. albicans* [16]. After undecanal treatment, *HST7* and *CEK1* expression levels are significantly reduced during the biofilm-forming process. This suggests that the decreased expression of these genes, which are involved in cell development and morphogenesis, may be the cause of the inhibition of biofilm formation after undecanal treatment (Fig. 7.6).

Undecanal arrested the cell cycle at G2/M phase. The correlation between controlling the cell cycle and triggering apoptosis in higher eukaryotes remains unexplored. Earlier, it was reported that proapoptotic treatment in *C. albicans* leads to G2/M cell cycle arrest [17]. DNA damage repair checkpoint coincides with G2/M phase. ROS generation results in DNA fragmentation, which leads to cellular apoptosis thus initiating G2/M cell cycle arrest [18]. Hence, accumulation of *C. albicans* cells in G2/M of cell cycle after the treatment of undecanal may leads to DNA damage which ultimately resulted in to apoptosis (Fig. 7.7a).

According to a number of studies, phytochemicals may be able to increase ROS generation at high doses [19]. The addition of undecanal caused *C. albicans* to accumulate ROS (Fig. 7.8a). The enhanced expression of *SOD1* and *SOD2* after undecanal treatment indicated increased oxidative stress in the cells. Molecular and cellular investigations have underscored the significance of *CAP1* gene expression in conferring resistance against oxidative stress and its involvement in the activation of

genes linked to this process, such as *CAT1* (Fig. 7.8b). *CAT1* plays a pivotal role in catalase production, serving as a critical enzyme in detoxifying *C. albicans* cells by removing hydrogen peroxide (H_2O_2) from the intracellular environment [20]. Increased level of both the genes ensures the increased oxidative stress after the treatment of undecanal.

Undecanal is an inhibitor of ergosterol production. As a result, this might affect membrane permeability and the integrity of *C. albicans*. The loss of membrane integrity was further confirmed using a PI staining technique. After being exposed to different undecanal concentrations, *C. albicans* was stained with PI. PI is a fluorescent dye that is recognized for its unceasing ability to pierce intact cell membranes, thus keeping healthy cells impervious. It does, however, easily pass through damaged cell membranes and attach to DNA to stain cells with a vibrant red fluorescence [21]. As the dosage is increased, the amount of PI absorbed by the undecanal-treated *C. albicans* cells increased (Fig. 7.10). These findings also point to a loss of membrane integrity and enhanced permeabilization of the membrane as a consequence of undecanal treatment. Undecanal exhibited no hemolytic activity at concentrations inhibiting virulence factors, suggesting it does not harm human RBCs (Fig. 7.11). At higher concentrations, undecanal exhibited significant hemolytic activity. However, in future research, combining undecanal with other antifungal agents may mitigate its toxicity at elevated concentrations. Within this study it is demonstrated that *C. albicans* infection killed the silkworm within 24 h time interval but when the MIC concentration of undecanal is given to the silkworm the survival time increased up to 72 h, even after the infection and went into the pupation stage and complete normal life cycle (Fig. 7.12). Hence, it is confirmed that undecanal increased the survival of silkworm.

7.5. Conclusions

The current study demonstrates that undecanal exerts a multifaceted influence on *C. albicans*, affecting various aspects of its biology and pathogenicity. At concentrations of 0.125 mg/ml, 1 mg/ml, 0.125 mg/ml and 0.02 mg/ml, respectively, undecanal efficiently inhibited planktonic growth, adhesion, yeast to hyphal morphogenesis and biofilm formation. Undecanal is found to modulate gene expression involved in pathways related to biofilm formation in *C. albicans*, indicating its potential to inhibit biofilm formation.

These findings are confirmed by qRT-PCR investigations, which showed that undecanal downregulated the genes like *RAS1*, *PDE2, CEK1 and TEC1* by during biofilms formation. Moreover, undecanal effectively inhibited ergosterol biosynthesis, a crucial lipid component of the fungal membrane, which can disrupt membrane integrity and function. Undecanal induced cell cycle arrest, particularly in the G2/M phase of *C. albicans* cells. Additionally, treatment with undecanal resulted in the generation of reactive oxygen species, indicative of cellular stress. It also affects gene expression during biofilm formation. These combined effects position undecanal as a promising anti-*Candida* agent with the potential to reduce the likelihood of treatment resistance.

In conclusion, undecanal emerges as a potential antifungal agent with multiple targets within *C. albicans*. Its diverse effects on the fungus, coupled with its non-toxic nature, make it a promising candidate for further research as a treatment for *Candida* infections.

7.6. References

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Chapter VIII:
Antifungal activity of berberine on *Candida albicans* planktonic growth.

8.1. Introduction

C. albicans is typically a symbiotic organism found on the mucosal surfaces of most healthy individuals [1]. However, in those with compromised immune systems, it can lead to severe and life-threatening systemic infections. *C. albicans* infection ranks as the fourth most common type of hospital-acquired bloodstream infection. The formation of biofilm by *C. albicans* enhances its drug resistance and virulence [2]. Recent studies have shown an increasing trend of invasive fungal infections in hospitalized patients caused by *C. albicans*, which is closely associated with the ongoing HIV pandemic. Although antifungal drugs are readily available for clinical use, the incidence of *C. albicans* infections, coupled with high mortality rates, continues to pose a significant health threat worldwide. This outcome is primarily attributed to the emergence of *Candida* clinical isolates resistant to the currently available therapeutic drugs [3]. Treatment options for *Candida* infections are largely limited to a few antifungal classes, including azoles, polyenes, and echinocandins. The development of new natural antifungal agents could effectively control *C. albicans* infections by providing efficient and safe derivatives.

Berberine (Fig. 8.1), an isoquinoline alkaloid present in several plants such as *Berberis vulgaris* (barberry), *Hydrastis canadensis* (golden seal), and *Coptis chinensis* (golden thread), has been studied for its antimicrobial properties, particularly its antifungal activity against *Candida* species [4]. In the current study, the effect of berberine on the planktonic growth of *C. albicans* was investigated through various methods, including determining the minimum inhibitory concentration (MIC), conducting a membrane integrity assay, detecting ROS generation, and performing qRT-PCR analysis.

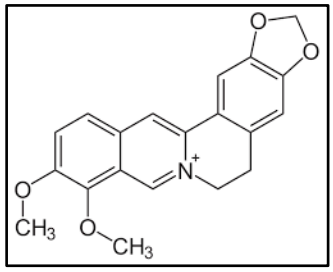

Fig. 8.1: Structure of berberine.

8.2. Materials and methods

The methodology was followed as mentioned in chapter 3 from page no. 33 to 46.

8.3. Results

8.3.1. MIC of berberine for planktonic growth

Berberine exhibited inhibitory activity against the planktonic growth of *C. albicans* in dose dependent manner at 0.004 mg/ml to 0.125 mg/ml (Fig. 8.2). Berberine at 0.004 mg/ml concentration reduced 50 % growth of *C. albicans* compared to control (Table 8.1), while the Minimum Fungicidal Concentration (MFC) was not achieved up to 4 mg/ml concentration.

8.3.2. Effect of berberine on adhesion

The addition of berberine has no noticeable impact on the adhesion of *C. albicans*, particularly up to 0.125 mg/ml (Table 8.1). Analysis of the adhered cells using the XTT-reduction assay revealed that at 0.125 mg/ml of berberine, approximately 70 % inhibition of adhesion is observed, suggesting a noteworthy decrease in the ability of *C. albicans* to adhere to the surface (Fig. 8.2).

Table 8.1: MIC values of berberine for planktonic growth, adhesion, yeast to hyphal morphogenesis, developing biofilm, mature biofilm, and MFC of berberine against *C. albicans* ATCC 90028.

Molecule	Planktonic growth	Adhesion	Yeast to hyphal morphogenesis	Developing biofilm	Mature biofilm	MFC
Berberine (MIC mg/ml)	0.004	NA	NA	NA	NA	NA

NA not achieved up to 4 mg/ml; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

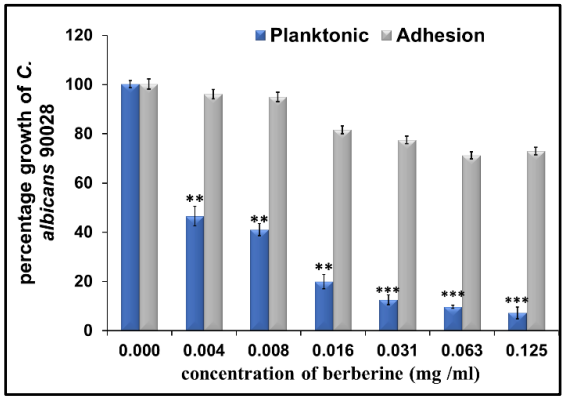

Fig. 8.2: Concentration-dependent inhibition of planktonic growth and adhesion of *C. albicans* after the treatment of berberine (0.004 mg/ml to 0.125 mg/ml). ** $p < 0.01$, *** $p < 0.001$.

8.3.3. Effect of berberine on yeast to hyphal morphogenesis

Berberine does not show significant effect on the transition from yeast to hyphal morphogenesis induced by FBS serum in *C. albicans* (Table 8.1). The treatment with berberine resulted in no significant inhibition of the transformation from yeast to hyphal morphology. Specifically, at a higher concentration of 0.125 mg/ml, only 40 % inhibition of yeast to hyphal morphogenesis is observed (Fig. 8.3).

8.3.4. Effect of berberine on biofilm development

Addition of berberine after the adhesion phase (90 minutes) did not prevent the normal biofilm formation, indicating that berberine did not act as an inhibitor at this stage up to 0.125 mg/ml (Table 8.1). Berberine does not significantly inhibit the development of biofilm. This suggests that higher concentrations of berberine have no significant impact on biofilm formation. It is also observed that berberine could not eradicate the mature biofilm of *C. albicans*completely (Fig. 8.3).

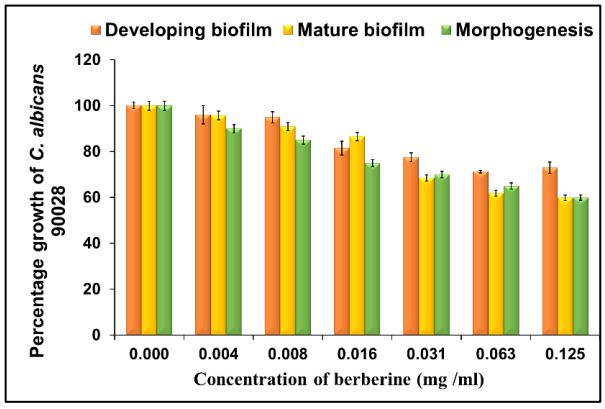

Fig. 8.3: Effect of berberine (0.004 mg/ml to 0.125 mg/ml) against yeast to hyphal morphogenesis, developing biofilm, and mature biofilm of *C. albicans.*

8.3.5. Effect of berberine on cell division cycle

The flow cytometry analysis indicated that berberine has an impact on the cell cycle of *C. albicans,* leading to cell cycle arrest at various stages. In the control group, it is observed that 31.7 % of the cells are found to be within the G0/G1 phase, 6.32 % are in the S phase, and 58.5 % are in the G2/M phase (Fig. 8.4a). Treatment with berberine resulted in a notable effect on the cell cycle, particularly arresting cells in the S phase. Specifically, 16.7 % of the cells are arrested in the S phase, indicating accumulation of cells at this stage. In contrast, a number of cells are arrested in the G2/M phase, suggesting that berberine does not have a substantial impact on the

G2/M phase (Fig. 8.4a). This comparative cell cycle analysis data is depicted in the histogram (Fig. 8.4b).

In short, berberine cell cycle disruption primarily caused of cells in the G0/G and G2/M phase, and an increase in the population of *C. albicans* cells in the S phase.

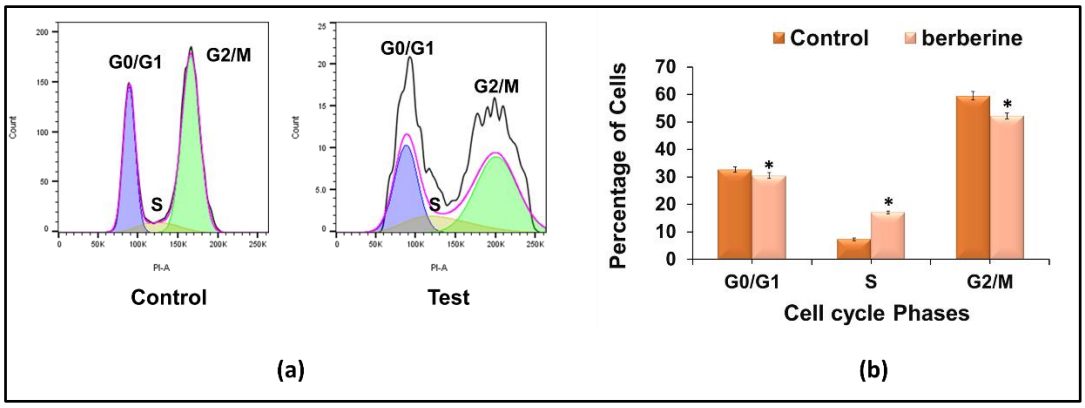

Fig. 8.4: (a) Cell cycle studies of *C. albicans* control cells without treatment with berberine and test after treatment of berberine (0.004 mg/ml) for 4 h 30 min at 35ºC showing cell cycle arrest at S phase. (b) Histogram of cells present at different phases of the cell cycle. * $p < 0.05$.

8.3.6. Effect of berberine on reactive oxygen species (ROS) generation

Intracellular reactive oxygen species (ROS) levels were assessed using H_2DCF, which is oxidized to produce green fluorescence in the presence of ROS. After treatment with berberine, there was a significant increase in the population of green fluorescent cells. This increase was attributed to the intracellular accumulation of ROS induced by berberine treatment when compared to untreated cells (negative control). In the positive control group (H_2O_2), the highest intensity of green fluorescence was observed. This enhanced fluorescence was a result of the accumulation of a large number of ROS, indicating a substantial oxidative stress response in these cells. These findings suggest that berberine treatment leads to an accumulation of ROS in the treated cells, although to a lesser extent than the positive control, indicating an oxidative stress response triggered by berberine in *C. albicans* cells (Fig. 8.5a).

8.3.7. Effect of berberine on genes involved in ROS production

The expression analysis of genes involved in protection against oxidative stress and apoptosis, *SOD1, SOD2, CAT1,* and *MCA1,* was carried out to support ROS generation and cell cycle results (Fig. 8.5b). Out of four genes *CAT1* gene expression is upregulated by 25.52-fold. *SOD1* is upregulated by 1.18-fold following the

treatment with berberine. Whereas the expression of *MCA1* is upregulated by 3.27-fold after the treatment of berberine (Table 8.2). It is also observed that the expression of *CAP1* is upregulated by 5.29-fold. This indicates that berberine treatment had differential effects on the expression of genes associated with defending against oxidative stress and apoptosis. These gene expression changes could be linked to the observed ROS generation and alterations in the cell cycle, shedding light on the molecular mechanisms by which berberine impacts *C. albicans*.

Table 8.2: Effect of berberine on the gene expression involved in oxidative stress.

Gene	Berberine induced relative fold change	Regulation
SOD1	1.18	Upregulation
SOD2	1.37	Upregulation
CAT1	25.52	Upregulation
MCA1	3.27	Upregulation
CAP1	5.29	Upregulation

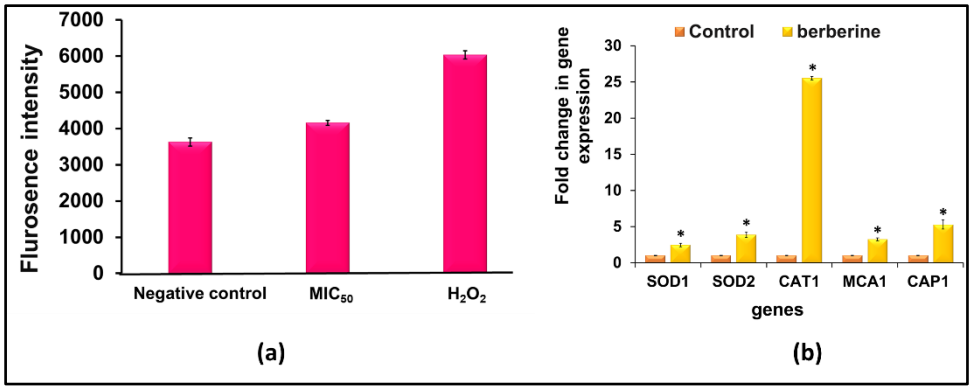

Fig. 8.5: (a) Berberine (0.004 mg/ml) enhances endogenous ROS accumulation in *C. albicans* cells. (b) Change in transcriptional profile genes involved in preventing ROS damage after the treatment of berberine. * $p < 0.05$.

8.3.8. Effect of berberine on the ergosterol biosynthesis

Ergosterol is indeed a critical lipid component found in the membrane of *C. albicans*, and it fulfills an essential function in regulating the fluidity, permeability, and overall integrity of the fungal membrane. This lipid is essential for maintaining the structural and functional properties of the membrane, making it an important target for understanding and controlling fungal biology. The results indicate that the treatment of berberine had a notable effect on the ergosterol profile in a dose-dependent manner. Particularly, at 0.063 mg/ml and 0.125 mg/ml concentration, there

is a complete inhibition of ergosterol content (Fig. 8.6). This suggests that berberine treatment at these concentrations severely disrupted or inhibited the synthesis or maintenance of ergosterol in *C. albicans*. Additionally, at lower concentrations of berberine, the study observed reductions in sterol content after berberine treatment. Even though complete inhibition is not achieved at these lower concentrations, there were still significant decreases in sterol content. This demonstrates that berberine has an impact on ergosterol is not limited to higher concentrations, but also occurs at lower levels, to a lesser extent. This wide range of reductions highlights the dose-dependent nature of the effect, with higher berberine concentrations leading to more pronounced reductions in sterol content in *C. albicans*.

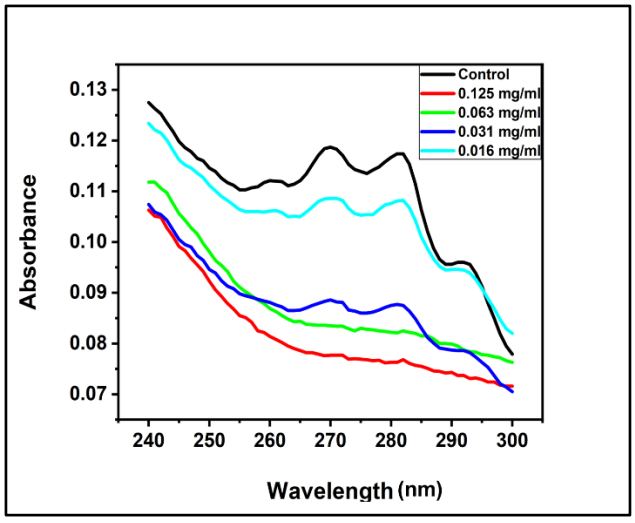

Fig. 8.6: Concentration-dependent inhibition of ergosterol biosynthesis after the treatment of berberine measured by the spectrophotometric method.

8.3.9. Effect of berberine on cell membrane integrity

A membrane-specific fluorescence probe, propidium iodide (PI), was utilized to examine the membrane integrity of *C. albicans* treated with berberine. As depicted in fig. 8.7, the intensity of red fluorescence progressively rises with the increase in berberine concentration. Specifically, the control cells exhibited minimal PI accumulation in comparison to the treated cells, which were subjected to higher concentrations, MIC, and sub MIC levels of berberine. The investigation on PI accumulation validated the compromised membrane integrity of *C. albicans* when subjected to berberine.

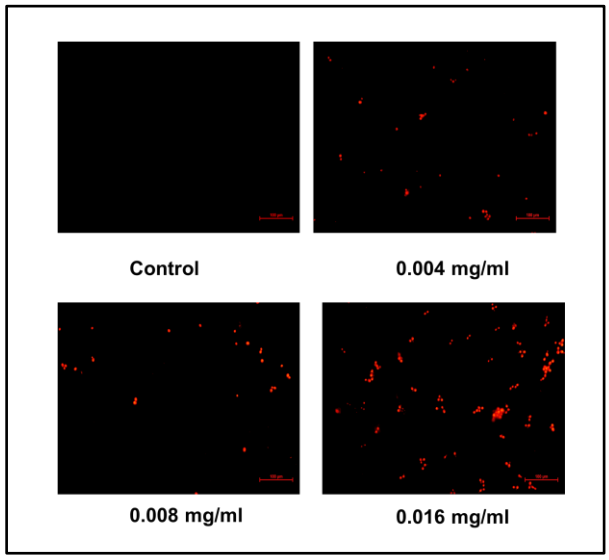

Fig. 8.7: Fluorescence microscopy images of PI-stained *C. albicans* cells treated with various concentrations of berberine prove the membrane damage effect of berberine on *C. albicans* (Scale bar 100 µm).

8.3.10. Hemolytic activity of berberine

In the context of assessing potential drug-induced immune hemolytic anemia, an *in vitro* hemolysis study was conducted as a precaution before considering any pharmaceutical applications. Hemolysis is commonly employed as the initial assessment of toxicity in drug development because it can be indicative of cytotoxicity. Cell membrane disruption is often a primary cause of toxicity. In the case of berberine, the study revealed that it induces 13 % hemolysis of human red blood cells (RBCs) when applied at a concentration of 0.125 mg/ml (Fig. 8.8). However, at the MIC concentration, berberine does not cause hemolysis. This information is essential for evaluating the safety profile of berberine and its potential for pharmacological applications while considering the risk of immune hemolytic anemia as a rare side effect.

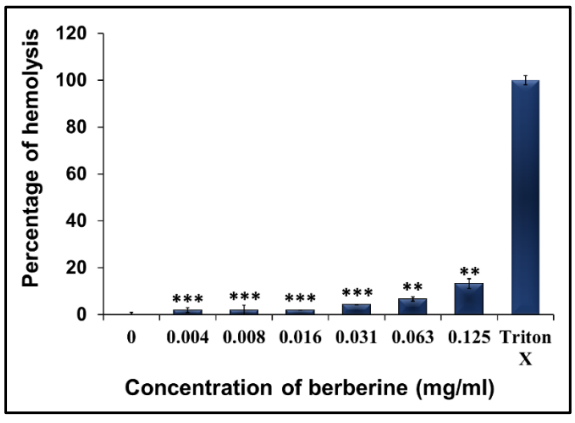

Fig. 8.8: Hemolytic activity of berberine on human RBCs. ** $p < 0.01$, *** $p < 0.001$.

8.3.11. Effect of berberine on the survival of silkworms

The *in vivo* antifungal efficacy is evaluated using a silkworm model. This model is commonly employed in scientific research to study the effectiveness of antifungal treatments in a living organism, providing valuable insights into the potential therapeutic benefits of the tested substances. The silkworm larvae of the negative control group injected with only *C. albicans* cells died within 24 h due to infection. The *C. albicans*-infected silkworm larvae of the positive control group treated with fluconazole survived up to 72 h. Additionally, silkworms of the vehicle control group injected with a 2 % DMSO also exhibited survival up to 72 h. Similarly, *C. albicans*-infected silkworms treated with berberine test group were able to survive for up to 72 h (Fig. 8.9). This suggests that the use of berberine alongside *C. albicans* has a protective effect on the survival of the silkworms. In this experiment, prolonging their lifespan compared to those treated with *C. albicans* alone.

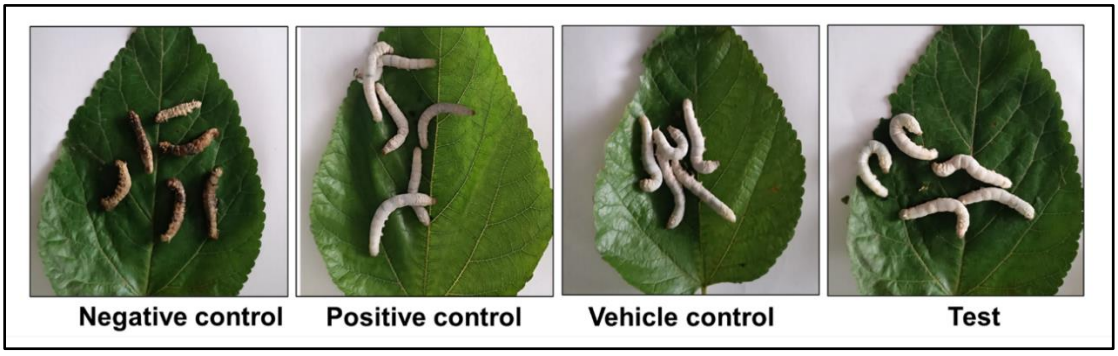

Fig. 8.9: *In vivo* antifungal efficacy of berberine (0.004 mg/ml) in silkworm animal model. Silkworms in the negative control group died within 24 h due to *C. albicans* infection. Silkworms in the flucanozole-treated positive control group were alive up to 72 h, while silkworms from the vehicle control and berberine-treated groups survived up to 72 h.

8.4. Discussion

Few studies have examined the inhibitory effects of berberine on *C. albicans* biofilms. Most research focuses on the morphological changes in *C. albicans* cells treated with berberine hydrochloride (BH), the impact of BH on cell viability and biofilm architecture, and the susceptibility of sessile cells within *C. albicans* biofilms [5].

In the current investigation, it is found that berberine inhibits *C. albicans* planktonic growth (Fig. 8.2). Berberine effectively inhibited cell cycle propagation at S phase (Fig. 8.4). It is also called as synthesis phase; in this stage of the cell cycle,

DNA replication takes place. During this phase, the cell duplicates its DNA in preparation for cell division. The cell cycle arrest at this stage may lead to programmed cell death or apoptosis [6]. It is known that a number of current antifungals work by producing reactive oxygen species, which increases their efficacy against *C. albicans*.

Multiple research studies propose that, at high concentrations, phytochemicals have the potential to stimulate the production of ROS [7]. Hence, we have studied the effect of berberine on ROS generation. The presence of berberine led to the accumulation of reactive oxygen species in *C. albicans* (Fig. 8.5a). After berberine treatment, the upregulation of *SOD1* and *SOD2* indicated heightened oxidative stress within the cell (Fig. 8.5b, Table. 8.2). Molecular and cellular analyses have highlighted the importance of *CAP1* gene expression in resisting oxidative stress and its role in activating genes associated with this mechanism, including *CAT1*. *CAT1*, crucial for catalase production, serves as an essential enzyme in detoxifying *C. albicans* cells by eliminating hydrogen peroxide (H_2O_2) from the intracellular environment. The elevated expression of both genes affirmed the increased oxidative stress induced by berberine treatment (Fig. 8.5b).

The examination of the *S. cerevisiae* genome has unveiled the existence of a caspase-like protein falling in the type I classification of metacaspases, which has been designated as *YCA1* (*MCA1* in *C. albicans*). Initially, Endonuclease (EndoG) was suggested to play a role in mitochondrial DNA replication, and is implicated in caspase-independent apoptosis [8]. Upon initiation of apoptosis, EndoG is liberated from the mitochondrion into cytosol, subsequently translocating to the nucleus and causing oligonucleosomal DNA fragmentation. Caspase activation is not involved in the entire process, as it occurs independently of caspase activation. The gene *MCA1* for caspase-dependent pathway was upregulated after the treatment of berberine (Fig. 8.5b, Table 8.2).

Berberine has been found to inhibit ergosterol biosynthesis in *C. albicans* (Fig. 8.6). Consequently, this may affect the membrane integrity and permeability of *C. albicans.* To further validate the loss of membrane integrity, a PI staining was conducted by subjecting *C. albicans* to different concentrations of berberine, followed by staining with PI. PI is a fluorescent dye known for its high cell membrane impermeability, thus making it incapable of staining healthy cells. However, it can

rapidly penetrate damaged cell membranes and stain cells by a vivid red fluorescence by binding with DNA [9]. Absorption of PI by the *C. albicans* cells treated with berberine rises with an increase in the dosage (Fig. 8.7). These results additionally suggest an increased permeabilization of the membrane and a loss of membrane integrity as a result of berberine treatment.

Berberine exhibited no hemolytic activity at concentrations inhibiting planktonic, suggesting it does not harm human red blood cells (Fig. 8.8). This lack of damage is a favorable indicating safety of berberine offering promise for its potential therapeutic applications. Within this study it was demonstrated that *C. albicans* infection killed the silkworm within 24 h time interval but when the MIC concentration of berberine was given to the silkworm the survival time increased up to 48 to 72 h even after the infection and went into the pupation stage and complete normal life cycle (Fig. 8.9). Hence it was confirmed that berberine increased the survival of silkworm.

8.5. Conclusion

This study confirms that berberine exhibits antifungal activity against the planktonic growth of *C. albicans*. *In vitro* tests demonstrated that the MIC of berberine chloride against *C. albicans* is 0.004 mg/ml. At 0.125 mg/ml, berberine chloride strongly inhibited the planktonic growth of *C. albicans*. Furthermore, it induces oxidative stress in *C. albicans* cells. The findings also illustrate that berberine influences the gene expression of major antioxidant enzymes like *SOD1*, *SOD2*, *CAP1*, *CAT1,* and *MCA1* in *C. albicans*. Additionally, berberine effectively inhibits ergosterol biosynthesis, crucial for fungal membrane integrity. This disruption affects the membrane's functions in fungal cells. Berberine-induced cell cycle arrest, particularly in the S phase, hampers the proliferation of *C. albicans* cells. Notably, berberine demonstrates no hemolytic activity, suggesting its safety for human RBCs. The survival time of the silkworm increased up to 72 h even after the infection. These collective findings highlight berberine as a promising antifungal agent with multiple targets in combating *C. albicans*.

8.6. References

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Chapter IX:
Antifungal activity of α-caryophyllene and β-caryophyllene against growth and virulence factors of *Candida albicans.*

9.1. Introduction

α-caryophyllene or β-caryophyllene (Fig. 9.1) is a naturally occurring monocyclic sesquiterpene found in the essential oils of various aromatic plants, such as hops (*Humulus lupulus*), sage (*Salvia officinalis*), and ginseng (*Panax ginseng*) [1]. It is known for its anti-inflammatory, analgesic, and antibacterial properties. Recent studies have suggested that α-caryophyllene may possess antifungal activity, making it a candidate for further investigation against fungal pathogens such as *C. albicans* [2]. *C. albicans* is an opportunistic fungal pathogen responsible for a range of infections, particularly in immunocompromised individuals. The ability of α-caryophyllene to inhibit fungal growth and biofilm formation could offer a new avenue for antifungal therapy [3]. β-caryophyllene is another prominent sesquiterpene, commonly found in the essential oils of plants like black pepper (*Piper nigrum*), cloves (*Syzygium aromaticum*), and cannabis (*Cannabis sativa*) [4]. It is known for its anti-inflammatory, analgesic, and antioxidant properties. In addition to its broad-spectrum antimicrobial effects, β-caryophyllene has shown potential as an antifungal agent [3]. Its mechanism of action is believed to involve the disruption of microbial cell membranes and interference with microbial metabolic processes [2]. Given the increasing incidence of antifungal resistance, β-caryophyllene's potential to inhibit fungal growth and morphogenesis, particularly in *C. albicans*, warrants thorough investigation.

This study aims to study the antifungal activity of α-caryophyllene and β-caryophyllene against *C. albicans*, focusing on their effects on planktonic growth, yeast to hyphal morphogenesis, and biofilm development. By elucidating the potential of these natural compounds as antifungal agents, this research seeks to contribute to the development of novel and effective treatments for fungal infections.

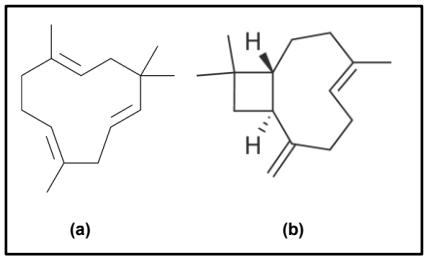

Fig. 9.1: Structure of (a) α-caryophyllene (b) β-caryophyllene.

9.2. Materials and methods

Methodology was followed as mentioned in chapter 3 from page no. 33 to 46.

9.3. Results

9.3.1. Minimum inhibitory concentration (MIC) of α-caryophyllene and β-caryophyllene for planktonic growth and adhesion

This study investigated the antifungal properties of α-caryophyllene and β-caryophyllene against the planktonic growth of *C. albicans*. Despite the known antimicrobial properties of these compounds, the results demonstrate that neither α-caryophyllene (Fig. 9.2a) nor β-caryophyllene (Fig. 9.2b) exhibits significant inhibitory activity against the planktonic growth of *C. albicans* (Table 9.1). However, α-caryophyllene significantly inhibits the adhesion of *C. albicans* cells to the polystyrene surface in a concentration-dependent manner, and the MIC is observed at 0.25 mg/ml concentration (Fig. 9.2a).

Table. 9.1: Effect of α-caryophyllene and β-caryophyllene influences growth, adhesion, yeast to hyphal morphogenesis, and biofilm formation in *Candida albicans* ATCC 90028.

Molecule	Planktonic growth	Adhesion	Yeast to hyphal morphogenesis	Developing biofilm	Mature biofilm
α- caryophyllene	NA	0.25 mg/ml	NA	0.25 mg/ml	NA
β- caryophyllene	NA	NA	NA	NA	NA

NA not achieved up to 4 mg/ml; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

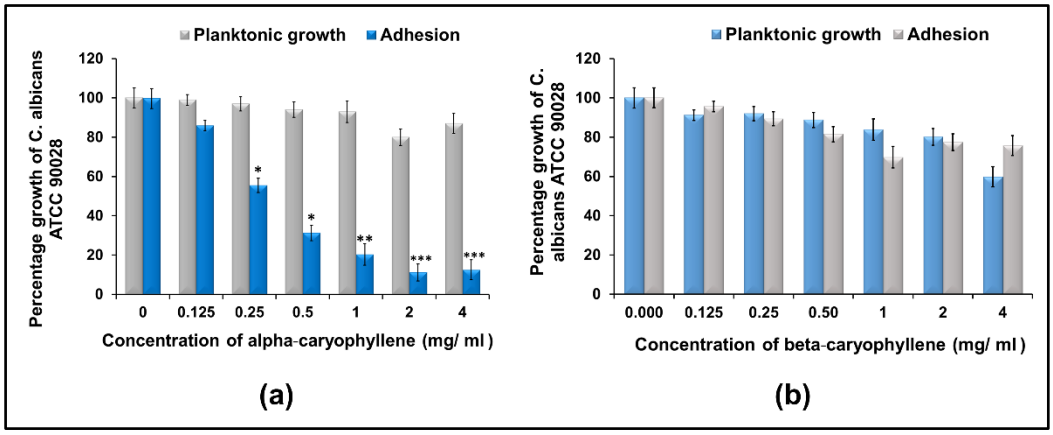

Fig. 9.2: (a) Effect of α-caryophyllene on planktonic growth and adhesion of *C. albicans* (0.125 mg/ml to 4 mg/ml). (b) Effect of β-caryophyllene on planktonic growth and adhesion of *C. albicans* (0.125 mg/ml to 4 mg/ml). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

9.3.2. Effect of α-caryophyllene and β-caryophyllene on yeast to hyphal morphogenesis

In this study, the effects of α-caryophyllene and β-caryophyllene on the yeast to hyphal transition in *C. albicans* are investigated (Fig. 9.2a, b). The yeast to hyphal morphogenesis is a critical virulence factor present in *C. albicans*, enabling it to form biofilms and invade host tissues. Despite their known antimicrobial properties, the findings reveal that neither α-caryophyllene nor β-caryophyllene significantly inhibited this morphological transition in *C. albicans* (Fig. 9.2c, d). When *C. albicans* cells are treated with α-caryophyllene and β-caryophyllene under conditions promoting hyphal growth, there is no significant reduction in the formation of hyphae observed under the microscope. This suggests that these compounds do not affect the yeast to hyphal transition (Table. 9.1).

9.3.3. Effect of α-caryophyllene and β-caryophyllene on early biofilm

This study investigated the effects of α-caryophyllene and β-caryophyllene (0.125 mg/ml to 4 mg/ml) on the early biofilm of *C. albicans* (Fig. 9.3a, b). Biofilm formation is a crucial factor in the pathogenicity of *C. albicans*, contributing to its resistance against antifungal treatments. The findings reveal that α-caryophyllene exhibits significant inhibitory activity against the developing biofilm at 0.25 mg/ml concentration (Fig. 9.3a, Table 9.1), whereas β-caryophyllene does not show inhibition. Viability assays, such as the XTT reduction assay, indicated reduced metabolic activity in biofilms treated with α-caryophyllene, correlating with its inhibitory effect on biofilm formation. Whereas β-caryophyllene treated biofilms do not show a significant reduction in metabolic activity compared to controls, aligning with the observation that β-caryophyllene does not inhibit biofilm development (Fig. 9.3b).

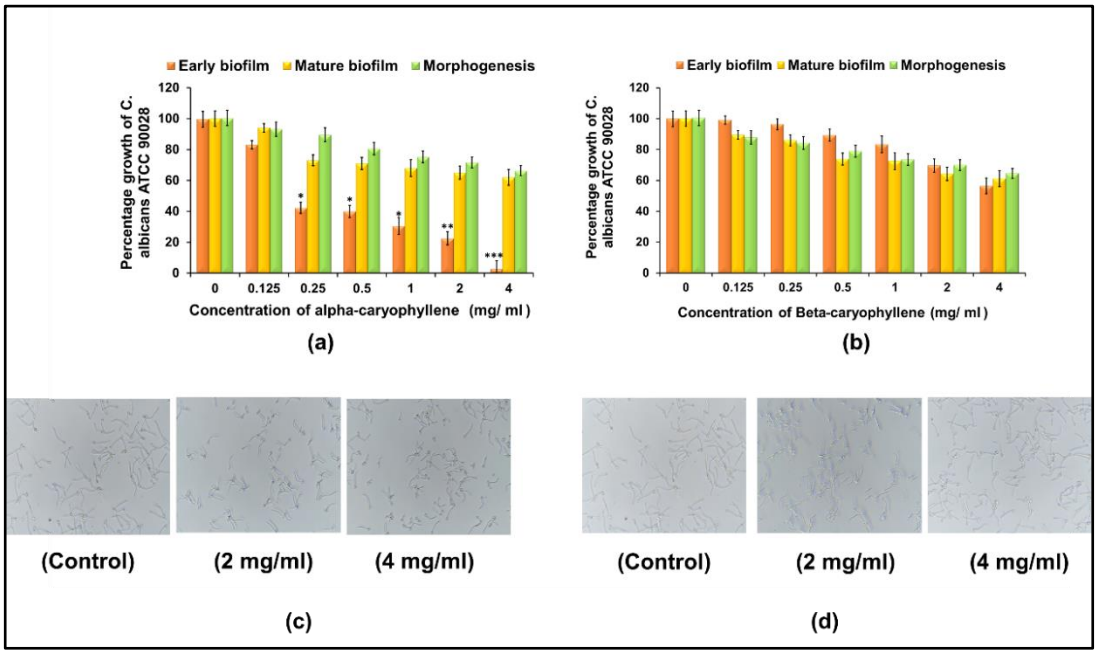

Fig. 9.3: (a) Effect of α-caryophyllene (0.125 mg/ml to 4 mg/ml) against early biofilm, mature biofilm, and yeast to hyphal morphogenesis of *C. albicans*. * p <0.05, ** p <0.01, *** p <0.001 (b) Effect of β-caryophyllene on early biofilm, mature biofilm, and yeast to hyphal morphogenesis of *C. albicans.* (c) Microscopic observation of the effect of α-caryophyllene on yeast to hyphal morphogenesis. (d) Microscopic observation of the effect of β-caryophyllene on yeast to hyphal morphogenesis.

9.3.4. Effect of α-caryophyllene on gene expression

To examine the influence on gene expression within *C. albicans*, qRT-PCR studies were carried out. These experiments were performed following treatment of α-caryophyllene throughout the formation of the biofilm, and the results revealed significant in the activity of genes related to signal transduction and biofilm development (Fig. 9.4). The manifestation of *RAS1,* which is a master regulator the signal transduction pathway, is downregulated by 17.10-fold by the treatment of α-caryophyllene. Along with *RAS1* expressions *BCY1, EFG1, HST1, CPH1, CEK1* and *HWP1* gene expressions are also downregulated 10.97-fold, 5.92-fold, 8.46-fold, 30.65-fold, 6.33-fold and 10-fold, respectively (Table. 9.2). The α-caryophyllene treatment significantly altered the expression of key genes involved in signal transduction pathway and biofilm development in *C. albicans*. These changes suggest a possible governing function for α-caryophyllene in modulating the fungal response and development processes.

Table 9.2: Downregulation of genes in biofilm after treatment with α-caryophyllene.

Gene	α-caryophyllene induced relative fold change	Regulation
RAS1	17.10	Downregulation
PDE2	6.91	Downregulation
BCY1	10.97	Downregulation
EFG1	5.92	Downregulation
TEC1	6.47	Downregulation
ECE1	68.71	Downregulation
CEK1	6.33	Downregulation
HST7	8.46	Downregulation
CPH1	30.65	Downregulation
HWP1	10.00	Downregulation
NRG1	3.03	Downregulation

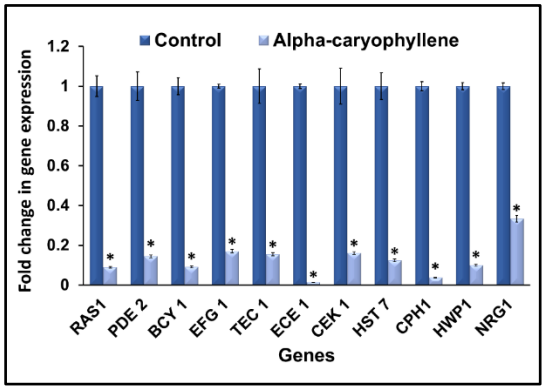

Fig. 9.4: Transcriptional profile of genes involved in biofilm formation of *C. albicans* after the treatment of α-caryophyllene (0.25 mg/ml). * $p < 0.05$.

9.3.5. Hemolytic activity of α-caryophyllene and β-caryophyllene

In the context of assessing potential drug-induced immune hemolytic anemia, an *in vitro* hemolysis study was conducted as a precaution before considering any pharmaceutical applications. Both α-caryophyllene and β-caryophyllene exhibited significant hemolysis at higher concentrations (Fig. 9.5a, b). This indicates potential cytotoxic effects on human red blood cells (RBCs), raising concerns about the safety of these compounds when used at elevated doses.

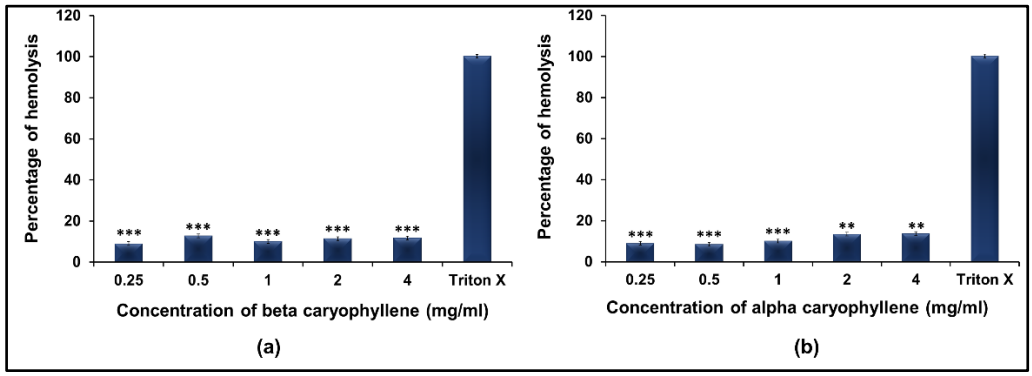

Fig. 9.5: Hemolytic activity of (a) α-caryophyllene and (b) β-caryophyllene on human RBCs. ** p <0.01, *** p <0.001.

9.4. Discussion

The findings of this study reveal critical insights into the antifungal properties of α-caryophyllene and β-caryophyllene against *C. albicans*, focusing on their effects on biofilm formation, planktonic growth, and yeast to hyphal morphogenesis. The study showed that neither α-caryophyllene nor β-caryophyllene inhibited the planktonic growth of *C. albicans* (Fig. 9.2a b). This suggests that these compounds do not affect the proliferation of free-floating fungal cells at the concentrations tested. Similarly, both compounds failed to inhibit the yeast to hyphal transition, a process essential for *C. albicans* virulence (Fig. 9.3). The yeast to hyphal transition allows the fungus to invade host tissues and establish infections [5]. The lack of impact on this morphogenetic switch implies that the mechanisms of action for α-caryophyllene and β-caryophyllene do not interfere with the regulatory pathways involved in hyphal formation.

A significant finding of this study is the inhibitory effect of α-caryophyllene on the developing biofilm of *C. albicans* (Fig. 9.3a). Biofilms are complex communities of cells embedded in a protective extracellular matrix, which contribute to the pathogenicity and antifungal resistance of *C. albicans* [6]. The ability of α-caryophyllene to disrupt biofilm formation suggests that it interferes with the initial adhesion of cells, biofilm maturation, or matrix production. This effect is crucial as biofilms are notoriously difficult to eradicate and are a major cause of persistent infections. The significant reduction in biofilm biomass and metabolic activity indicates that α-caryophyllene could be an effective agent in targeting biofilm-associated infections. Biofilm formation in *C. albicans* is a complex, multi-step process regulated by various signal transduction pathways. These pathways include

the cyclic AMP (cAMP)-protein kinase A (PKA) pathway, the mitogen-activated protein kinase (MAPK) pathway, and. These pathways regulate gene expression and cellular behaviors essential for biofilm development, such as cell adhesion, matrix production, and morphological transitions [7]. α-caryophyllene's ability to inhibit biofilm formation may involve the downregulation of genes associated with these crucial pathways. Studies have shown that biofilm formation is significantly influenced by the expression of specific regulatory genes. For instance, the cAMP-PKA pathway, which includes the genes *RAS1, BCY1, EFG1, HST1, CPH1, CEK1,* and *HWP1*, plays a vital role in the regulation of hyphal growth and biofilm development. Similarly, the MAPK pathway is crucial for stress responses and biofilm integrity [8]. By downregulating these genes, α-caryophyllene may disrupt the signaling processes required for biofilm formation (Fig. 9.4). This disruption could result in decreased cell adhesion, impaired extracellular matrix production, and inhibited morphological changes necessary for biofilm maturation. Consequently, α-caryophyllene ’s interference with these pathways hampers the ability of *C. albicans* to form robust and resilient biofilms, making the fungal cells more susceptible to antifungal treatments and immune responses. In contrast, β-caryophyllene did not show significant inhibition of biofilm development. This suggests that while β-caryophyllene may possess antimicrobial properties, its efficacy against *C. albicans* biofilms is limited. The lack of impact on biofilm biomass and metabolic activity implies that β-caryophyllene does not affect the critical steps involved in biofilm formation. The study also found that at higher concentrations, both α-caryophyllene and β-caryophyllene exhibited significant hemolytic activity (Fig. 9.5). Hemolysis indicates the disruption of red blood cell membranes, which raises concerns about the cytotoxicity of these compounds at elevated doses. This finding is critical as it highlights the potential side effects and risks associated with their use in therapeutic applications [9]. Ensuring the safe and effective use of these compounds necessitates a careful balance between antifungal efficacy and cytotoxicity.

9.5. Conclusions

The study demonstrates that α-caryophyllene exhibits significant inhibitory activity at 0.25 mg/ml developing biofilm of *C. albicans*, disrupting biofilm formation and reducing biomass and metabolic activity. In contrast, β-caryophyllene does not inhibit biofilm development. Neither compound affects planktonic growth or yeast-to-

hyphal morphogenesis. To examine the influence of α-caryophyllene on gene expression within *C. albicans*, quantitative real-time PCR (qRT-PCR) studies were conducted. Notably, the expression of *RAS1*, a master regulator of the signal transduction pathway, was downregulated by 7-fold following α-caryophyllene treatment. In addition to *RAS1*, the expressions of *BCY1, EFG1, HST1, CPH1, CEK1* and *HWP1* gene expressions were also downregulated 10.97-fold, 5.92-fold, 8.46-fold, 30.65-fold, 6.33-fold and 10-fold, respectively. These findings indicate that α-caryophyllene treatment significantly alters the expression of key genes involved in the signal transduction pathway and biofilm development in *C. albicans* at 0.25 mg/ml.

Collectively, these changes suggest a potential regulatory role for α-caryophyllene in modulating the fungal response and developmental processes. However, both α-caryophyllene and β-caryophyllene show significant hemolytic activity at higher concentrations, indicating potential cytotoxicity.

These findings highlight α-caryophyllene's potential as an antifungal agent for biofilm related infections, though its therapeutic use, along with β-caryophyllene, may be limited by toxicity concerns. Further research is necessary to optimize their safe and effective application.

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Chapter X:

Summary and Conclusions

The current study explored the antivirulent capacities of zingerone, α-bisabolol, nonanal, undecanal, berberine, α-caryophyllene, and β-caryophyllene which are naturally occurring bioactive molecules against *Candida albicans*. These molecules exhibited strong inhibitory effects on virulent factors of *C. albicans*, such as adhesion, yeast to hyphal formation, and biofilm formation.

Chapter 1. The context and significance of developing novel therapeutic agents against *C. albicans* have been intrduced in chapter 1. *C. albicans* poses a serious clinical challenge due to its ability to form biofilms, transition between morphologies, and develop resistance to conventional antifungal treatments. Understanding the mechanisms that govern its pathogenicity, including biofilm formation, morphogenetic switching, and gene regulation via key pathways like Ras1-cAMP-Efg1 and Cek1-MAPK, is essential for identifying new treatment strategies.

Chapter 2. The reviewed literature highlights the growing challenge posed by *C. albicans*, particularly its ability to form biofilms, which contributes significantly to antifungal resistance and persistent infections. Current treatments are challenged by the ability of *C. albicans* to adapt and resist conventional antifungal therapies, particularly in biofilm-associated infections. This underscores the need for novel therapeutic strategies. Natural compounds, particularly those derived from plant sources, show promise due to their bioactive properties and ability to interfere with key signal transduction pathways involved in morphogenesis and biofilm formation. Continued research into these molecules may provide a pathway to developing safer, more effective antifungal agents capable of overcoming the limitations of existing treatments.

Chapter 3. This chapter outlines the methodologies employed to investigate the antifungal activity of bioactive molecules against *C. albicans*. By using a standard strain of *C. albicans*, MIC concentrations, and molecular targets identified, the study ensures the reliability and reproducibility of the findings with statistical significance. The application of gene expression analysis and ROS measurement techniques provides insights into the molecular mechanisms of the test compounds, particularly their effects on signaling pathways related to biofilm formation and oxidative stress responses. These methods lay a strong foundation for the comprehensive evaluation

of potential antifungal agents, advancing the field toward the discovery of new therapeutic strategies *against C. albicans*.

Chapter 4. In this study effect of zingerone on *C. albicans* growth has been investigated. Zingerone inhibits planktonic growth, adhesion, biofilm development, and yeast to hyphal transition at concentrations of 2 mg/ml, 0.125 mg/ml, 2 mg/ml, and 1 mg/ml, respectively. Zingerone has been shown to modulate the gene expression involved in biofilm formation pathways. Zingerone treatment significantly caused downregulation of *BCY1*, *PDE2,* and *EFG1* genes. It also inhibits ergosterol biosynthesis, induces cell cycle arrest, and triggers the generation of reactive oxygen species in *C. albicans*. The non-toxic nature of zingerone and its efficacy against *C. albicans* make it a promising therapeutic agent for *C. albicans* pathogenesis.

Chapter 5. This chapter explored the anti-virulence activity of α-bisabolol against *C. albicans.* α-bisabolol also demonstrated significant anti-virulence activity, inhibiting the yeast to hyphal transition, adhesion, and biofilm formation of *C. albicans*. α-bisabolol effectively inhibits various pathogenic traits of *C. albicans*, adhesion, yeast to hyphal switching, and development of biofilm at 1 mg/ml, 0.25 mg/ml, and 0.125 mg/ml concentration, respectively. Gene expression study revealed that α-bisabolol treatment resulted in a notable 2.53-fold reduction in expression of *RAS1*, a key regulatory gene. *PDE2*, situated upstream in the cAMP-dependent PKA pathway, displayed a substantial 3.04-fold downregulation. Likewise, subsequent elements *BCY1, EFG1*, and *TEC1* are downregulated by 1.41-fold, 2.29-fold, and 1.41-fold, respectively. *ECE1*, a downstream component of *EFG1,* exhibits a 4.88-fold reduction in expression by the treatment of α-bisabolol. Its diverse targets within the fungus, coupled with its safety profile, suggest its potential as a remedy against *C. albicans* infections after further validation through *in vivo* studies.

Chapter 6. This chapter presents compelling evidence of nonanal which serves as a multifaceted antifungal agent against *C. albicans*, demonstrating significant impacts on various aspects of the fungus's biology and pathogenicity. The results indicated that nonanal effectively impeded growth, yeast to hyphal morphogenesis and biofilm formation at concentrations of 0.063 mg/ml, 0.125 mg/ml and 0.016 mg/ml respectively. The results reveales that nonanal treatment led to a suppression of *RAS1* expression.

In contrast, the expression levels of *EFG1*, *BCY1,* and *TEC1* are significantly downregulated. The downstream effector *ECE1* of *EFG1* exhibits a remarkable reduction in expression, while *HWP1*, another downstream target of *EFG1*, is downregulated. Furthermore, the expression of *CPH1* and *HST7*, key components of the Cek1-MAPK pathway, was decreased. Notably, the expression of *CEK1* is drastically reduced in the presence of nonanal. Additionally, suppressor genes involved in hyphal induction, such as *NRG1*, *MIG1*, and *TUP1*, are downregulated in *C. albicans* biofilm.

Chapter 7. Another bioactive molecule, undecanal, exerts a multifaceted influence on *C. albicans*, impacting various aspects of its biology and pathogenicity. At concentrations of 0.125 mg/ml, 1 mg/ml, 0.125 mg/ml, and 0.020 mg/ml, undecanal efficiently inhibits planktonic growth, adhesion, yeast to hyphal morphogenesis, and biofilm formation, respectively, highlighting its effectiveness as a potential antifungal agent. Furthermore, undecanal is shown to modulate gene expression involved in pathways related to biofilm formation, evidenced by qRT-PCR investigations that revealed downregulation of expression of key genes such as *RAS1*, *PDE2, CEK1,* and *TEC1* during biofilm development. This modulation underscores undecanal's capacity to inhibit biofilm formation in *C. albicans*. Additionally, undecanal effectively inhibited ergosterol biosynthesis, a crucial component of the fungal cell membrane, disrupting membrane integrity and function in *C. albicans*. The study also identified that undecanal induces cell cycle arrest, particularly in the G2/M phase of *C. albicans* cells, and generates reactive oxygen species (ROS), indicating cellular stress. These combined effects, alongside the modulation of gene expression during biofilm formation, position undecanal as a promising anti-*Candida* agent capable of reducing the likelihood of treatment resistance.

Chapter 8. The bioactive molecule berberine chloride demonstrated strong antifungal activity against *C. albicans* planktonic growth, oxidative stress induction, and inhibition of ergosterol biosynthesis. *In vitro* assays revealed a minimum inhibitory concentration (MIC) of berberine chloride at 0.004 mg/ml, with a strong growth inhibition observed at 0.125 mg/ml.

Additionally, berberine induces oxidative stress in *C. albicans* cells at 0.004 mg/ml, further contributing to its antifungal efficacy. The findings demonstrate that

berberine affects the gene expression of key antioxidant enzymes, *including SOD1, SOD2, CAP1, CAT1,* and *MCA1*, in *C. albicans*.

Moreover, berberine effectively inhibits ergosterol biosynthesis, which is essential for maintaining fungal membrane integrity, thereby compromising the membrane's functionality. Berberine also induces cell cycle arrest at 0.004 mg/ml, particularly during the S phase, which hinders the proliferation of *C. albicans* cells. Importantly, berberine exhibits no hemolytic activity, indicating its safety for human red blood cells. Collectively, these findings underscore berberine as a promising antifungal agent with multiple targets for combating *C. albicans*.

Chapter 9. The study demonstrates that alpha-caryophyllene exhibits significant inhibitory activity against the developing biofilm of *Candida albicans*, effectively disrupting biofilm formation and reducing both biomass and metabolic activity. In contrast, beta-caryophyllene does not inhibit biofilm development. Additionally, neither compound affects planktonic growth or yeast-to-hyphal morphogenesis.

However, both α-caryophyllene and β-caryophyllene exhibit significant hemolytic activity at higher concentrations, indicating potential cytotoxicity. To further examine the influence of α-caryophyllene on gene expression within *C. albicans*, quantitative real-time PCR (qRT-PCR) studies were conducted. These experiments revealed significant alterations in the activity of genes related to signal transduction and biofilm development.

Notably, the expression of *RAS1*, a master regulator of the signal transduction pathway, is downregulated following α-caryophyllene treatment. In addition, the expressions of *BCY1*, *EFG1*, *HST1*, *CPH1*, *CEK1*, and *HWP1* are also significantly reduced. These findings highlight α-caryophyllene's potential as an antifungal agent for biofilm-related infections, although its therapeutic use, along with that of β-caryophyllene, may be limited by toxicity concerns. Further research is necessary to optimize their safe and effective application in clinical settings, particularly to evaluate the regulatory role of α-caryophyllene in modulating the fungal response and developmental processes.

Table 10.1: The efficacies of the bioactive molecules against *C. albicans* ATCC 90028, showing the minimum inhibitory concentrations for planktonic growth, adhesion, yeast to hyphal morphogenesis, and biofilm formation.

Sr. No.	Name of molecules	MIC of Planktonic	MFC	MIC of Adhesion	MIC of Early Biofilm	Yeast to hyphal morphogenesis	Percentage hemolysis
1.	FLC	1.56 µg/ml	NA	12.5 µg/ml	NA	6.25 µg/ml	-
2.	Am B	0.39 µg/ml	0.7 µg/ml	3.12 µg/ml	12.5 µg/ml	0.39 µg/ml	-
3.	Zingerone	2 mg/ml	4 mg/ml	0.125 mg/ml	2 mg/ml	1 mg/ml	2 %
4.	α-bisabolol	NA	NA	1 mg/ml	0.125 mg/ml	0.25 mg/ml	-
5.	**Nonanal**	**0.0063 mg/ml**	**4 mg/ml**	**0.25 mg/ml**	**0.0016 mg/ml**	**0.125 mg/ml**	-
6.	**Undecanal**	**0.125 mg/ml**	**4 mg/ml**	**1 mg/ml**	**0.02 mg/ml**	**0.125 mg/ml**	-
7.	Berberine	0.004 mg/ml	NA	NA	NA	NA	2 %
8.	α-caryophyllene	NA	NA	NA	0.25 mg/ml	NA	9 %

(NA: Not achieved up to 4 mg/ml)

In conclusion, the molecular targets of bioactive molecules were identified in this study through ROS, cell cycle, and gene expression studies. The current study explored the antivirulent capacities of naturally occurring seven bioactive molecules zingerone, nonanal, undecanal, berberine, α-caryophyllene, β-caryophyllene, α-bisabolol against *C. albicans*. All these molecules, except β-caryophyllene, exhibited strong inhibitory effects on various virulent factors of *C. albicans* (Table 10.1). Among these, zingerone, α-bisabolol, nonanal, and undecanal have shown significant inhibitory activity on crucial virulence factors such as adhesion, yeast to hyphal morphogenesis and biofilm formation in *C. albicans.* However, zingerone, nonanal, and undecanal have candida-cidal activity. Zingerone, α-bisabolol, nonanal, undecanal, and α-caryophyllene compounds have been found to interfere with the biofilm formation through Ras1-cAMP-Efg1 and Cek-MAPK pathways in *C. albicans* (Table 10.2). The mechanism of action study revealed that these molecules affect the Reactive Oxygen Species level (ROS), cell cycle, and ergosterol synthesis in *C. albicans* (Table 10.3). At effective concentrations, these molecules do not show any hemolytic activity, while α- and β-caryophyllene show notable hemotoxicity. The

in vivo antifungal efficacy of zingerone, nonanal, and undecanal confirmed by using silkworm as an animal model. However, nonanal and undecanal have emerged as promising candidates for further exploration in antifungal therapy against *C. albicans*.

Overall, this study offers significant value of natural molecules as alternative nonanal and undecanal in effective treatments of biofilm-related infections. Further studies should be carried out to optimize their clinical applications to come up with innovative antifungal strategies against *C. albicans*.

Table 10.2: The relative fold change in gene expression of signaling intermediates after the treatment of bioactive molecules against *C. albicans* ATCC 90028.

Sr. No.	Gene	Zingerone	α-bisabolol	nonanal	undecanal	α-caryophyllene
1.	*RAS1*	7.84 -fold ↓	2.53-fold ↓	11.29-fold ↓	13.96- fold ↓	17.10- fold ↓
2.	*PDE 2*	6.27-fold ↓	3.04-fold ↓	2.40-fold ↑	1.40- fold ↑	6.91- fold ↓
3.	*BCY 1*	1.17 -fold ↓	1.41-fold ↓	7.18 -fold ↓	3.52- fold ↓	10.97- fold ↓
4.	*EFG 1*	12.47- fold ↓	2.29-fold ↓	3.62-fold ↓	2.30-fold ↓	5.92- fold ↓
5.	*TEC 1*	1.33- fold ↓	1.41-fold ↓	5.91-fold ↓	8.55-fold ↓	6.47-fold ↓
6.	*ECE 1*	2.76- fold ↓	4.88-fold ↓	35.26-fold ↓	35.26-fold ↓	68.71-fold ↓
7.	*CEK 1*	14.09- fold ↓	4.82-fold ↓	21.42-fold ↓	6.19-fold ↓	6.33-fold ↓
8.	*HST 7*	11.26- fold ↓	5.42-fold ↓	3.25-fold ↓	1.69-fold ↓	8.46-fold ↓
9	*CPH1*	8.56- fold ↓	5.39-fold ↓	1.21-fold ↓	1.40-fold ↑	30.65-fold ↓
10.	*HWP1*	1.54- fold ↓	2.67- fold ↑	39.00-fold ↓	17.30- fold ↓	10.00- fold ↓
11.	*MIG1*	2.29- fold ↓	1.34-fold ↓	11.47-fold ↓	7.46-fold ↓	3.01-fold ↓
12.	*NRG1*	2.11- fold ↓	1.55-fold ↓	11.79-fold ↓	11.63- fold ↓	3.03- fold ↓

Note: -↑-fold Upregulation ↓- fold Downregulation

Table 10.3: The relative fold intensity showing the effect of bioactive molecules on endogenous ROS accumulation in *C. albicans* ATCC 90028.

Name of molecules	Negative Control (nm)	Test (nm)	Positive control H_2O_2 (nm)
Zingerone	4404	6035	8438
Nonanal	1628	4457	6035
Undecanal	2476.5	4638	5007
Berberine	2128	4156	6035

Chapter XI:

Recommendations

In light of the promising findings presented in this study regarding the antivirulent properties of natural bioactive molecules against *C. albicans*, several recommendations emerge for further exploration and development. Nonanal and undecanal have emerged as promising candidates for further exploration in antifungal therapy against *C. albicans*. Their multifaceted actions, including the inhibition of planktonic growth, disruption of biofilm formation, modulation of critical gene expression, and induction of oxidative stress at very low concentrations, highlight their potential as effective therapeutic agents. These recommendations aim to advance our understanding of the therapeutic potential of these molecules.

1. ***In vivo* Studies:** Further *in vivo* studies need to be conducted to validate the efficacy and safety of the nonanal and undecanal as antifungal agents against *C. albicans* infections.
2. **Safety assessment:** Comprehensive safety assessments, including cytotoxicity studies and evaluation of potential adverse effects, to ensure the safety profile of the nonanal and undecanal for clinical use in animal model.
3. **Dose optimization:** Determination of the optimal dosage of the nonanal and undecanal to achieve maximal efficacy while minimizing potential side effects in animal model.
4. **Combination therapy:** Exploration of the potential synergistic effects of combining nonanal and undecanal to improve efficacy. Also, a combination with standard antifungal drugs to reduce their toxicity and reduce the likelihood of treatment resistance.
5. **Translational study:** Development of different formulations (e.g., nanoparticles, liposomes) to enhance the stability, bioavailability, and targeted delivery of nonanal and undecanal. The study will be helpful to prove translation potential of these molecules.
6. **Use of identified targets:** This comprehensive approach may lead to the discovery of novel molecules that target different aspects of *C. albicans* pathogenicity, such as adhesion, biofilm formation, and cell cycle regulation. By integrating advanced screening techniques, including high-throughput

assays and genomic analysis, researchers can efficiently identify and characterize promising bioactive compounds. Such efforts are essential for developing innovative and effective therapeutic options to combat *C.* infections and address the growing issue of antifungal resistance.

These recommendations can guide for further research and development efforts aimed at utilizing the therapeutic potential of natural bioactive molecules for the treatment of *C.* infections.

Limitations of the study

The study has several limitations.

1. More clinical isolates *C. albicans* required to claim the anti-*C. albicans* activity.
2. Toxicity study on animal cell line and *in vivo* system.
3. Drug resistance study.
4. The use of the silkworm model has some disadvantages like lack of organ system.

Despite these limitations, the study provides valuable insights into the anti-virulence effects of bioactive molecules on *C. albicans*, contributing to the understanding of potential therapeutic agents against *C. albicans* infections.

Annexures

PATIENT CONSENT FORM

D Y Patil Education society (Deemed to be University) Department of Medical Biotechnology and Stem cell & Regenerative Medicine, Center for Interdisciplinary Research, Kolhapur

I Miss. Sayali Ashok Chougule Ph.D. student working under guidance of Prof. Sankunny Mohan Karuppayil (Former Head and Professor), and Dr. Ashwini Khanderao Jadhav (Assistant Professor)department of Medical Biotechnology and Stem Cell & Regenerative Medicine, Center for Interdisciplinary Research, has prepared the following consent form to be filled by patient, at the time of sample collection needed for my research work entitled "Identification of molecular targets of selected bioactive molecules in *C. albicans* biofilm formation".

I, Mr/Mrs/Ms ……..... Gender …… Age: ……

Residing at.....

Do hereby confirm that:

(i) I have been asked by the Ph.D. student whether I wish to participate in a study under the aegis of D Y Patil Education society (Deemed to be University) Department of Medical Biotechnology and Stem cell & Regenerative Medicine, Center for Interdisciplinary Research.

(ii) The nature of the study being undertaken by the student as well as the extent of my participation in it, have been duly explained to me in a language that I understand.

(iii) The potential risks and consequences associated with this study have also been duly explained to me in a language that I understand.

(iv) I also understand that my participation in this study is only for the benefit of advancement in the field of research and that at no point in time is my participation being solicited for any pecuniary gain by the student.

(v) I have also been explained that I am in no way obliged to participate in the study and that, once I have agreed to participate in the study, I am still free to withdraw from participation in the study at any point in time upon notifying the Ph.D. student in writing in the prescribed form without assigning any reason.

(vi)There will be no financial transaction between myself and Ph.D. student for my participation in that study;

(vii) I have been explained that any data collected out of my participation in the study will only be used for research work.

(viii) I have also been reassured that any publication of the data collected during the course of the study or any publication of its conclusions, shall be done on a 'no names' basis and shall under no circumstances reveal my personal identity. Any personal details likely to reveal my personal

identity shall at all times remain confidential;

(ix) The contents and effect of this consent form have also been duly explained to me in a language that I understand.

By affixing my signature/thumb print hereto, I am therefore freely and voluntarily signifying my consent, intent and willingness to participate in the study of the student researcher for the purposes of the research work. I also certify that my right to privacy has not been infringed in any manner.

[SIGNATURE/THUMB PRINT OF PARTICIPANT]

DATE:

WITNESSED BY:

(1) NAME:

TITLE/CAPACITY:

SIGNATURE:

(2) NAME:

TITLE/CAPACITY:

SIGNATURE:

PATIENT CONSENT FORM

D Y Patil Education society (Deemed to be University) Department of Medical Biotechnology and Stem cell & Regenerative Medicine, Center for Interdisciplinary Research, Kolhapur

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Nonanal inhibits growth and virulence factors in *Candida albicans*

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ABSTRACT

The recent surge in fungal infections, particularly caused by *Candida albicans*, is an important public health concern, especially among immunocompromised individuals. The current study aims to elucidate antifungal activity of nonanal against *C. albicans* (ATCC 90028) and clinical isolates. A range of quantitative methods was employed, including minimum inhibitory concentration (MIC) determination, cell cycle analysis, biofilm assays, microscopic observation of yeast to hyphal transitions, scanning electron microscopy of biofilms, and assessments of ergosterol inhibition and cell membrane damage. The results demonstrated that nonanal effectively inhibits the growth, morphogenesis, and biofilm formation at concentrations of 63 μg/ml, 125 μg/ml, and 16 μg/ml, respectively for *C. albicans* ATCC 90028, and at 125 μg/ml, 500 μg/ml, and 63 μg/ml, respectively for C1 and C2. Nonanal was able to induce cell cycle arrest and increase the reactive oxygen species (ROS) level in all strains of *C. albicans*. qRT-PCR analysis showed the downregulation of expression of key genes *RAS1, BCY1, ECE1, CEK1*, and *HWP1* in ATCC 90028 and C1 isolte, while the negative regulator *TUP1* was significantly upregulated ATCC 90028. Nonanal also affected the ergosterol synthesis and cell membrane in all the tested strains. Moreover, nonanal exhibited *in vivo* antifungal efficacy in silkworm animal model. These findings advance our understanding of nonanal as a novel potential antifungal agent, laying the groundwork for future antifungal strategies.

Introduction

Candida albicans remains the most prevalent opportunistic pathogenic fungus in humans, causing infections that range from mucosal to systemic candidiasis, in immunocompromised patients [1]. *C. albicans* causes a range of infections, from superficial mucosal infections such as oral thrush, esophagitis, and vaginitis, to more severe systemic infections like invasive candidiasis. It can affect multiple organs, including the bloodstream, bones, liver, and central nervous system, potentially leading to conditions like candidemia and meningitis [2]. Globally, invasive infections caused by less common *Candida* species contribute to a significant burden, particularly among high-risk populations such as those in intensive care, receiving broad-spectrum antibiotics, or undergoing immunosuppressive therapy [3]. Several antifungal drugs are used to manage invasive candidiasis, but the emergence of drug resistance to conventional antifungal agents, such as azoles and polyenes, has necessitated the search for alternative therapeutic strategies and their side effects significantly limit their effectiveness, leading to increased treatment complications and costs [4]. Hence, there is an increased need for new antifungal medications to be investigated.

In recent years, essential oils plant-derived (EOs) have gained increasing attention as natural antimicrobial agents due to their broad-spectrum activity, low toxicity, and reduced potential for resistance development [5]. Essential oils are mixtures of volatile compounds extracted from various plant parts, including leaves, flowers, seeds, and bark. These essential oils have been widely used in traditional medicine and food preservation due to their antimicrobial and antioxidant properties. The antifungal activity of essential oils is primarily attributed to

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Research Paper

Zingerone effect against *Candida albicans* growth and biofilm production

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ABSTRACT

Background: The increasing resistance of *Candida albicans* biofilms underscores the urgent need for effective antifungals. This study evaluated the efficacy of zingerone and elucidated its mode of action against *C. albicans* ATCC 90028 and clinical isolate C1.
Experimental Procedure: Minimum inhibitory concentrations (MICs) of zingerone were determined using CLSI methods against planktonic cells, biofilm formation, and yeast-to-hyphal transition. The mode of action was investigated through fluorescent microscopy, ergosterol assays, cell cycle analysis, and RT-PCR for gene expression.
Key Results: Zingerone inhibited planktonic growth and biofilm formation at in *C. albicans* ATCC 90028 and clinical isolate C1 at 2 mg/mL 4 mg/mL and 1 mg/mL and 2 mg/mL respectively. Treatment with the MIC concentration caused significant cell cycle arrest at the G0/G1 phase, halting proliferation in both the strains. Propidium iodide Staining revealed compromised membrane integrity in both the strains. Also, acridine orange and ethidium bromide dual staining showed increased dead cell proportions in *C. albicans* ATCC 90028. RT-PCR studies showed downregulation of *BCY1, PDE2, EFG1*, and upregulation of negative regulators *NRG1, TUP1* disrupting growth and virulence pathways. Zingerone induced elevated reactive oxygen species (ROS) levels, triggering apoptosis, evidenced by DNA fragmentation and upregulation of apoptotic markers. It also inhibited ergosterol synthesis in a concentration-dependent manner, crucial for membrane integrity. Importantly, zingerone exhibited minimal hemolytic activity. In an *in vivo* silkworm model, zingerone demonstrated significant antifungal efficacy, protecting silkworms from infection. It also modulated stress response genes, highlighting its multifaceted action.
Conclusions: In vitro and *in vivo* findings confirm the potent antifungal efficacy of zingerone against *C. albicans* ATCC 90028 and clinical isolate C1, suggesting its promising potential as a therapeutic agent that warrants further exploration.

Introduction

C. albicans is indeed a commensal organism commonly established on human skin, oral cavity, and within the alimentary canal [1]. It is a major opportunistic fungal pathogen responsible for a wide range of infections, from superficial mucosal infections to life-threatening systemic diseases [2]. Candidiasis, caused by *Candida albicans*, is a prevalent opportunistic fungal infection that poses a significant threat to immunocompromised individuals, such as those undergoing chemotherapy, organ transplants, or living with acquired immunodeficiency syndrome (AIDS). The global burden of candidiasis is considerable, with systemic infections exhibiting mortality rates exceeding 40 %, even with the availability of antifungal treatments [2]. The increasing resistance of *C. albicans* to antifungal drugs, particularly in the case of invasive candidiasis, has led to prolonged hospital stays and elevated healthcare costs [3].

A key challenge in managing *C. albicans* infections is its ability to form biofilms, structured microbial communities that are highly resistant to antifungal therapies [4]. Biofilm formation on medical devices such as catheters and implants complicate treatment, as biofilm-associated infections are resistant to azoles and polyenes due to factors such as reduced drug penetration and altered metabolic states [5]. This resistance, combined with the emergence of multi-drug-resistant strains, underscores the need for new therapeutic strategies [6].

Despite the availability of drugs like fluconazole and Amphotericin B, these treatments have limitations. Fluconazole's efficacy

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ORIGINAL RESEARCH

Alpha-bisabolol inhibits yeast to hyphal form transition and biofilm development in *Candida albicans*: in vitro and in silico studies

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Abstract
In recent years, there has been growing concern about infections caused by *Candida albicans*, which pose a significant threat to human health. This intensifies the concern that can be largely attributed to the increasing number of people with compromised immune systems and the emergence of drug-resistant strains. Natural molecules are considered to be good alternatives to synthetic antifungal agents. The present study explored the effectiveness of alpha-bisabolol as an antifungal agent and its mechanism of action against *C. albicans* ATCC90028. α-bisabolol effectively inhibited various pathogenic traits of *C. albicans* like, adhesion, yeast to hyphal switching, and development of biofilm at 1 mg/ml, 0.25 mg/ml, and 0.125 mg/ml concentration, respectively. In addition, α-bisabolol demonstrated inhibition of cell cycle propagation at the G1 phase. Ergosterol production in the *C. albicans* was suppressed by α-bisabolol treatment in a dose-dependent manner. The molecular docking study revealed α-bisabolol has a good binding energy of − 7.11 kcal/mol with 14-α-demethylase enzyme, which is crucial for ergosterol synthesis. Therefore, the cell membrane integrity may be affected by treatment with α-bisabolol. qRT-PCR studies proved that α-bisabolol treatment affects gene expression in *C. albicans*. In silico binding affinity was also analyzed for *RAS1*, *TUP1* and *CST20* in the signal transduction pathway and exhibited binding affinities for at − 7.7 kcal/mol, − 8.21 kcal/mol, and for − 5.79 kcal/mol respectively. In conclusion, α-bisabolol caused reduced biofilm, ergosterol synthesis along with altered gene expressions in *C. albicans* with no hemolysis. This study proposed α-bisabolol as an alternative antifungal agent.

Keywords Biofilm inhibition · Cell cycle arrest · Signal transduction pathway · cAMP-PKA signaling pathway · Ergosterol synthesis

Introduction

Candida albicans is a commensal organism that naturally resides in the human body but can become pathogenic when the host's immune system is compromised (Alastruey-Izquierdo et al. 2015). It is responsible for a variety of infections, ranging from superficial mucosal infections to life-threatening systemic infections, particularly in immunocompromised individuals. Despite the availability of antifungal drugs, their use is often limited by issues such as toxicity, the development of resistance, and poor efficacy against biofilms (Shinde et al. 2012).

One of the most significant challenges in managing *C. albicans* infections is its ability to switch between different morphological forms like yeast, pseudohyphal, and hyphal form allowing it to adhere to surfaces and form biofilms. These biofilms, complex structures encased in an extracellular matrix, confer increased resistance to most antifungal agents, making treatment of biofilm-associated infections difficult (Camacho et al. 2007; Bachmann et al. 2003). Furthermore, the development of new antifungal therapies is hampered by the similarity between fungal and human cells at the molecular level, which limits the range of safe and effective drug targets (Ramage et al. 2007).

In recent years, natural products have gained attention as potential sources of novel antifungal agents. Among

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Molecules of Natural Origin as Inhibitors of Signal Transduction Pathway in *Candida albicans*

7

Sayali A. Chougule, S. Mohan Karuppayil, and Ashwini K. Jadhav

7.1 Introduction

About one and a half million people die every year as a result of fungal infections (Bar-Yosef et al. 2017). It is a universal health problem that has been increasing worldwide (Bradshaw and Dennis 2009). Of all the several *Candida* species, *Candida albicans* accounts for 75% of all cases of Candidiasis. Being a commensal, this opportunistic fungal pathogen is found at several places in the human body particularly the skin, oral, genitourinary and gastrointestinal tracts. Its proliferation increases manifold under immunocompromised conditions (Papin et al. 2005). *C. albicans* is polymorphic and can be seen in different forms like yeast, hyphae and pseudohyphae. Under certain conditions, germ tubes form ultimately branching into a hyphal network. Morphological transition can be induced by both intracellular and extracellular signals and plays a significant role in *Candida* pathogenicity. The inhibition of hyphae-specific gene expression can suppress hyphal extension showing that this phenomenon can be modulated by endogenous cellular signals with the help of proteins that function as receptors or sensors (Brown et al. 2014; Villa et al. 2020).

Signaling pathways form coordinated networks as they interact with each other leading to synchronized combinatorial signaling events (Richardson 2022). Such responses include alterations in the transcription, translation, post-translational and conformational changes in the proteins involved in controlling fungal growth, proliferation, metabolism and various other processes. Many transcription factors are responsible for the regulation and coordination of

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ORIGINAL PAPER

Butyl isothiocyanate exhibits antifungal and anti-biofilm activity against *Candida albicans* by targeting cell membrane integrity, cell cycle progression and oxidative stress

Shivani Balasaheb Patil[1] · Sargun Tushar Basrani[1] · Sayali Ashok Chougule[1] · Tanjila Chandsaheb Gavandi[1] · Sankunny Mohan Karuppayil[1] · Ashwini Khanderao Jadhav[1]

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Abstract

The prevalence of *Candida albicans* infection has increased during the past few years, which contributes to the need for new, effective treatments due to the increasing concerns regarding antifungal drug toxicity and multidrug resistance. Butyl isothiocyanate (butylITC) is a glucosinolate derivative, and has shown a significant antifungal effect contrary to *Candida albicans*. Additionally, how butylITC affects the virulence traits of *C. albicans* and molecular mode of actions are not well known. Present study shows that at 17.36 mM concentration butylITC inhibit planktonic growth. butylITC initially slowed the hyphal transition at 0.542 mM concentration. butylITC hampered biofilm development, and inhibits biofilm formation at 17.36 mM concentration which was analysed using metabolic assay (XTT assay) and Scanning Electron Microscopy (SEM). In addition, it was noted that butylITC inhibits ergosterol biosynthesis. The permeability of cell membranes was enhanced by butylITC treatment. Moreover, butylITC arrests cells at S-phase and induces intracellular Reactive Oxygen Species (ROS) accumulation in *C. albicans*. The results suggest that butylITC may have a dual mode of action, inhibit virulence factors and modulate cellular processes like inhibit ergosterol biosynthesis, cell cycle arrest, induces ROS production which leads to cell death in *C. albicans*.

Keywords *Candida albicans* · Butyl isothiocyanate · Ergosterol biosynthesis · Membrane integrity · ROS production

Introduction

Candida albicans is the most prevalent opportunistic fungal pathogen in humans. It has ability to cause life-threatening invasive fungal infections as well as superficial fungal infections, especially in those with impaired immune systems (Mayer et al. 2013). *C. albicans* infection has four phases. On epithelial surface *C. albicans* colonises, which causes superficial infections. After that, in second phase or intermediate phase germ tube or hyphal formation takes place which helps to invade the tissue, in third phase *C. albicans* invades the epithelial tissue to cause profound infections if the host has a compromised immune system. Finally, *C. albicans* can induce disseminated infections, which can be life threatening and allow the fungus to colonise and infect more host tissues (McCall et al. 2019; Talapko et al. 2021). *C. albicans* is polymorphic fungus that may appear in the form of a yeast-like budded form, a pseudo hyphal form or a filamentous true hyphal form (Mukaremera et al.

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Ethyl Isothiocyanate as a Novel Antifungal Agent Against *Candida albicans*

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Abstract

In the recent years, occurrence of candidiasis has increased drastically which leads to significant mortality and morbidity mainly in immune compromised patients. Glucosinolate (GLS) derivatives are reported to have antifungal activities. Ethyl isothiocyanate (EITC) and its antifungal activity and mechanism of action is still unclear against *Candida albicans*. The present work was designed to get a mechanistic insight in to the anti-*Candida* efficacy of EITC through in vitro and in vivo studies. EITC inhibited *C. albicans* planktonic growth at 0.5 mg/ml and virulence factors like yeast to hyphal form morphogenesis (0.0312 mg/ml), adhesion to polystyrene surface (0.0312 mg/ml) and biofilm formation (developing biofilm at 2 mg/ml and mature biofilm at 0.5 mg/ml) effectively. EITC blocked ergosterol biosynthesis and arrested *C. albicans* cells at S-phase. EITC caused ROS-dependent cellular death and nuclear or DNA fragmentation. EITC at 0.0312 mg/ml concentration regulated the expression of genes involved in the signal transduction pathway and inhibited yeast to hyphal form morphogenesis by upregulating *TUP1, MIG1,* and *NRG1* by 3.10, 5.84 and 2.64-fold, respectively and downregulating *PDE2* and *CEK1* genes by 15.38 and 2.10-fold, respectively. EITC has showed haemolytic activity at 0.5 mg/ml concentration. In vivo study in silk worm model showed that EITC has toxicity to *C. albicans* at 0.5 mg/ml concentration. Thus, from present study we conclude that EITC has antifungal activity and to reduce its MIC and toxicity, combination study with other antifungal drugs need to be done. EITC and its combinations might be used as alternative therapeutics for the prevention and treatment of *C. albicans* infections.

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Antifungal activity of Allyl isothiocyanate by targeting signal transduction pathway, ergosterol biosynthesis, and cell cycle in *Candida albicans*

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A B S T R A C T

Background and Purpose: In recent years, the inclusion of *Candida albicans* on the list of infections that pose a threat due to drug resistance has urged researchers to look into cutting-edge and effective antifungal medications. In this regard, the current study investigated the probable mode of action of allyl isothiocyanate (AITC) against *Candida albicans*.

Materials and Methods: In this study, planktonic assay, germ tube inhibition assay, adhesion, and biofilm formation assay were performed to check the growth and virulence factors. Furthermore, ergosterol assay, reactive oxygen production analysis, cell cycle analysis, and quantitative real-time polymerase chain reaction analysis were performed with the aim of finding the mode of action. A biomedical model organism, like a silkworm, was used in an *in vivo* study to demonstrate AITC anti-infective ability against *C. albicans* infection.

Results: Allyl isothiocyanate completely inhibited ergosterol biosynthesis in *C. albicans* at 0.125 mg/ml. Allyl isothiocyanate produces reactive oxygen species in both planktonic and biofilm cells of *C. albicans*. At 0.125 mg/ml concentration, AITC arrested cells at the G2/M phase of the cell cycle, which may induce apoptosis in *C. albicans*. In quantitative real-time polymerase chain reaction analysis, it was found that AITC inhibited virulence factors, like germ tube formation, at 0.125 mg/ml concentration by downregulation of *PDE2*, *CEK1*, *TEC1* by 2.54-, 1.91-, and 1.04-fold change, respectively, and upregulation of *MIG1*, *NRG1*, and *TUP1* by 9.22-, 3.35-, and 7.80-fold change, respectively. The *in vivo* study showed that AITC treatment successfully protected silkworms against *C. albicans* infections and increased their survival rate by preventing internal colonization by *C. albicans*.

Conclusion: *In vitro* and *in vivo* studies revealed that AITC can be an alternative therapeutic option for the treatment of *C. albicans* infection.

Keywords: Allyl isothiocyanate; *Candida albicans*; Ergosterol biosynthesis; RT-PCR; Silkworm

➢ **How to cite this paper**
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Introduction

In the past few years, incidence rates of fungal infections have increased tremendously. Annually, around 10 lakh deaths occur due to fungal infections across the world. The fungal infection mainly occurs in immunocompromised individuals [1,2]. Among these, fungal infections caused by *Candida albicans* are the most common threat to human beings [3,4]. *Candida albicans* is a pleiomorphic fungal pathogen that has the capacity to produce biofilms on the cell surfaces of mammals as well as implanted medical devices [5–7].

It has the ability to form biofilm on both biotic and abiotic surfaces, like central venous system catheters, urinary catheters, stents, porcine heart valves, artificial heart valves, intrauterine devices, and artificial knee caps. The colonized prosthetics may act as a permanent source of bloodstream infections. The majority of the studies suggest that biofilm-associated infections in patients are difficult to eradicate as biofilms are resistant to standard antifungals [8]. Therefore, the treatment of biofilm-related infections has become a major challenge to clinicians [9]. Due to drug tolerance, it may be necessary to increase the dosages of the drugs beyond the therapeutic range. This is not always advisable due to the

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Hydroxychloroquine an Antimalarial Drug, Exhibits Potent Antifungal Efficacy Against *Candida albicans* Through Multitargeting

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Abstract

Candida albicans is the primary etiological agent associated with candidiasis in humans. Unrestricted growth of *C. albicans* can progress to systemic infections in the worst situation. This study investigates the antifungal activity of Hydroxychloroquine (HCQ) and mode of action against *C. albicans*. HCQ inhibited the planktonic growth and yeast to hyphal form morphogenesis of *C. albicans* significantly at 0.5 mg/ml concentration. The minimum inhibitory concentrations (MIC_{50}) of HCQ for *C. albicans* adhesion and biofilm formation on the polystyrene surface was at 2 mg/ml and 4 mg/ml respectively. Various methods, such as scanning electron microscopy, exploration of the ergosterol biosynthesis pathway, cell cycle analysis, and assessment of S oxygen species (ROS) generation, were employed to investigate HCQ exerting its antifungal effects. HCQ was observed to reduce ergosterol levels in the cell membranes of *C. albicans* in a dose-dependent manner. Furthermore, HCQ treatment caused a substantial arrest of the *C. albicans* cell cycle at the G0/G1 phase, which impeded normal cell growth. Gene expression analysis revealed upregulation of *SOD2*, *SOD1*, and *CAT1* genes after HCQ treatment, while genes like *HWP1*, *RAS1*, *TEC1*, and *CDC 35* were downregulated. The study also assessed the in vivo efficacy of HCQ in a mice model, revealing a reduction in the pathogenicity of *C. albicans* after HCQ treatment. These results indicate that HCQ holds for the development of novel antifungal therapies.

Keywords HCQ · Gene expression · Virulence factors · Cell cycle · Ergosterol · ROS production · In vivo

Introduction

Fungi causes 1.5 million infections annually and affects human health, especially in immunosuppressed individuals or patients in intensive care units. The resistance against antifungal drugs necessitates the development of new therapies. Among the pathogenic fungi, *Candida* species causes mortality reaching up to 50 % in systemic fungal infections. The fungal pathogen *Candida. albicans* causes ≥ 150 million mucosal infections and 200,000 deaths annually because of the invasive and disseminated disease in susceptible populations (Garvey & Rowan, 2023). The virulence traits of opportunistic fungus *C. albicans* enhances its capacity to survive under drastic environmental conditions and its pathogenicity. The morphological changes between yeast and filamentous forms, the production of proteolytic and lipolytic enzymes, formation of biofilms, and the expression of host-recognizing proteins are the virulent traits present in *C. albicans*. Biofilm formation is an important virulence characteristics of *C. albicans* (Robbins & Cowen, 2023). *C. albicans* biofilms are resistant to various antifungal drugs (Fan et al., 2022). To overcome this problem, there is a need for alternative drugs. Developing new drugs costs around $100–800 million and it is a time-consuming process. This strategy has been thoroughly investigated in antifungal drug research (Mogire et al., 2017). In previous study, it is reported that, the antimalarial drug Chloroquine has capacity to inhibit the growth, morphogenesis, and ergosterol biosynthesis

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BACTERIAL AND FUNGAL PATHOGENESIS - RESEARCH PAPER

MIG1, *TUP1* and *NRG1* mediated yeast to hyphal morphogenesis inhibition in *Candida albicans* by ganciclovir

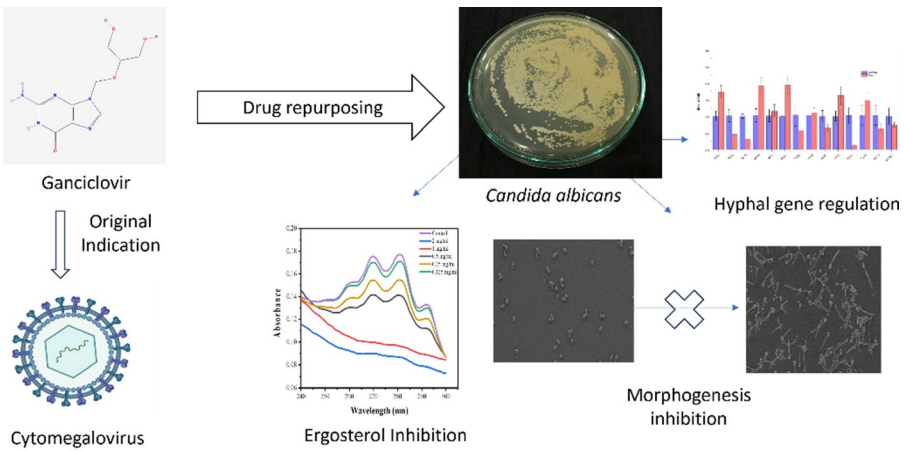
Tanjila Gavandi[1] · Shivani Patil[1] · Sargun Basrani[1] · Shivanand Yankanchi[2] · Sayali Chougule[1] · S. Mohan Karuppayil[1] et al. *[full author details at the end of the article]*

Received: 6 November 2023 / Accepted: 9 April 2024

Abstract

Candida albicans is a polymorphic human fungal pathogen and the prime etiological agent responsible for candidiasis. The main two aspects of *C. albicans* virulence that have been suggested are yeast-to-hyphal (Y-H) morphological transitions and biofilm development. Anti-fungal agents targeting these virulence attributes enhances the antifungal drug development process. Repositioning with other non-fungal drugs offered a one of the new strategies and a potential alternative option to counter the urgent need for antifungal drug development. In the current study, an antiviral drug ganciclovir was screened as an antifungal agent against ATCC 90028, 10231 and clinical isolate (C1). Ganciclovir at 0.5 mg/ml concentration reduced 50% hyphal development on a silicon-based urinary catheter and was visualized using scanning electron microscopy. Ganciclovir reduced ergosterol biosynthesis in both strains and C1 isolate of *C. albicans* in a concentration-dependent manner. Additionally, a gene expression profile study showed that ganciclovir treatment resulted in upregulation of hyphal-specific repressors *MIG1*, *TUP1*, and *NRG1* in *C. albicans*. Additionally, an in vivo study on the *Bombyx mori* silkworm model further evidenced the virulence inhibitory ability of ganciclovir (0.5 mg/ml) against *C. albicans*. This is the first report that explore the novel anti-morphogenic activities of ganciclovir against the pathogenic *C. albicans* strains, along with clinical isolates. Further, ganciclovir may be considered for therapeutic purpose after combinations with standard antifungal agents.

Graphical abstract



Keywords *Candida albicans* · Drug repurposing · Ganciclovir · in vivo · Polymerase chain reaction · Yeast to hyphal morphogenesis

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SHIVAJI UNIVERSITY, KOLHAPUR

SOPHISTICATED ANALYTICAL INSTRUMENT FACILITY (SAIF)– COMMON FACILITY CENTRE (CFC)
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SHIVAJI UNIVERSITY, KOLHAPUR

SOPHISTICATED ANALYTICAL INSTRUMENT FACILITY (SAIF)– COMMON FACILITY CENTRE (CFC)
WORKSHOP & HANDS-ON TRAINING ON BIO-ATOMIC FORCE MICROSCOPY (BIO-AFM) ORGANISED BY SAIF-CFC UNDER MAHARASHTRA ACADEMY OF SCIENCE AND STRIDE PROGRAMME

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