"Glucosinolates As Antifungals: A Study On *Candida albicans*"

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IN

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UNDER THE FACULTY OF

INTERDISCIPLINARY STUDIES

SUBMITTED BY

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DECLARATION

I am Shivani Balasaheb Patil, hereby declare that the thesis entitled, **"Glucosinolates As Antifungals: A Study On *Candida albicans*"** submitted here for the award of **Doctor of Philosophy** in **Stem Cell and Regenerative Medicine,** faculty of **Interdisciplinary studies** under the guidance of **Prof. (Dr.) Sankunny Mohan Karuppayil** 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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This is to certify that the thesis entitled **"Glucosinolates As Antifungals: A Study On *Candida albicans*"**, which is being submitted here for the award of **Doctor of Philosophy** in **Stem Cell and Regenerative Medicine,** faculty of **Interdisciplinary studies,** under the guidance of **Prof. (Dr.) Sankunny Mohan Karuppayil,** 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. Shivani Balasaheb Patil** 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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LIST OF COPYRIGHTS, PATENT, PUBLICATIONS AND CONFERENCES ATTAINED

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1) **Anti- *Candida albicans* activity of Ethyl isothiocyanate**
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ABREVIATIONS

AbHog1 - *Alternaria brassicicola* high osmolarity glycerol 1 ortholog

AbAP1 - Armadillo BTB Arabidopsis Protein 1

AITC - Allyl isothiocyanate

AIC - Allyl isocyanate

ATCC - American Type Culture Collection

AME - Alternariol Monomethyl Ether

AOH - Alternariol

ALT - Altenuene

BCY1 - Bypass of cyclic AMP

BITC - Benzyl isothiocyanate

ButylITC - Butyl isothiocyanate

BZITC - Benzoyl isothiocyanate

DCFH-DA - 2′,7′ dichlorofluorescein diacetate

CDC35 - Cell-division-cycle 35

CEK1 - Extracellular signal regulated kinase 1

CFU - Colony Forming Unit

ºC - Degree Celsius

CLSI - Clinical Laboratory Standards Institute

CNS - Central Nervous System

cDNA - Complementary De-Oxy Ribose Nucleic Acid

CPH1 - Candida pseudo hyphal regulator 1

DMSO - Dimethyl sulphoxide

ECE1 - Extent of Cell Elongation protein 1

EFG1 - Enhanced filamentous growth protein 1

EITC - Ethyl isothiocyanate

EPC - Oesophageal Candidiasis

EPS - Extracellular Polymeric Substances

EGFRK - Epidermal Growth Factor Receptor Tyrosine Kinase

FBS - Fetal Bovine Serum

GSL - Glucosinolate

GSLs - Glucosinolates

h - Hour(s)

HCT - Human Colorectal Cancer

HST7 - Serine/Threonine- Protein Kinase homolog

HWP1 - Hyphal wall protein 1

H_2O_2 - Hydrogen peroxide

ITC - Isothiocyanate

ITCs - Isothiocyanates

3IA - 3 Indole acetonitrile

I3C - Indole -3- carbinol

IMTECH - Institute of Microbial Technology

IPITC - Isopropyl isothiocyanate

K_2HPO_4 - Dipotassium hydrogen phosphate

KH_2PO_4 - Dipotassium phosphate

MAPK - Mitogen-Activated Protein Kinase

MFC - Minimum Fungicidal Concentration

mM - Millimolar

MOPS - 3-(N-morpholino) propanesulfonic acid

mfsG - Major Facilitator Superfamily transporter

MIC - Minimum Inhibitory Concentration

MIG1- Multicopy Inhibitor of GAL gene

mg - Milligram

mg/ml - Milligram per millilitre

ml - Millilitre

mm - Millimetre

MTT - 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazoliumbromide

MTHFS - methyl tetrahydrofolate synthetase receptor

nm - Nanometre

NRG1 - Negative Regulator of Glucose controlled gene

OD - Optical Density

OPC - Oropharyngeal Candidiasis

PBS - Phosphate Buffered Saline

PDE2 - Cyclic nucleotide phosphodiesterase 2

PEITC - Phenyl ethyl isothiocyanate

PITC - Phenyl isothiocyanate

PI - Propidium Iodide

pH - Power of Hydrogen ion

PITC - Phenyl isothiocyanate

PEITC - Phenyl ethyl isothiocyanate

RAS - Ras like protein 1

RBC - Red Blood Cells

RMA - Relative Metabolic Activity

RNA - Ribose Nucleic Acid

RPMI 1640 - Roswell Park Memorial Institute 1640

RT-PCR - Real Time Polymerase Chain Reaction

ROS - Reactive Oxygen Species

SAP - Secreted Aspartyl Proteases

SEM - Scanning Electron Microscope

SDA - Sabouraud Dextrose Agar

TEN - Tentoxin

TEC1 - Transcription activator TEC1

TUP1 - Transcription repressor

VVC - Vulvovaginal Candidiasis

XTT - 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

YEPD - Yeast Extract Peptone Dextrose Broth

Y-H - Yeast to hyphal

% - Percentage

µg - Microgram

µg/ml - Microgram per millilitre

µl - Microlitre

µm - Micrometre

"Glucosinolates As Antifungals: A Study On *Candida albicans*"

ABSTRACT

Candidiasis, primarily caused by *Candida albicans*, presents a significant public health challenge, particularly due to the rising incidence of drug-resistant strains. This thesis explores the antifungal potential of glucosinolate derivatives, bioactive compounds derived from cruciferous vegetables, against *C. albicans*. The study systematically evaluates the antifungal properties of eleven glucosinolate derivatives, including allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), ethyl isothiocyanate (EITC), phenyl ethyl isothiocyanate (PEITC), butyl isothiocyanate (ButylITC), isopropyl isothiocyanate (IPITC), allyl isocyanate (AIC), benzoyl isothiocyanate (BZITC), phenyl isothiocyanate (PITC), 3 indole acetonitrile (3IA), and sulforaphane, focusing on their efficacy in inhibiting the growth and virulence of the *C. albicans* ATCC 90028 strain.

The introductory chapter emphasizes the opportunistic nature of *C. albicans*, particularly its ability to form resilient biofilms, complicating treatment strategies in immunocompromised patients and highlighting the urgent need for alternative therapies amid increasing antifungal resistance.

Subsequent chapters detail the antifungal potential evaluated through various assays, including Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC), Yeast-to-Hyphal Morphogenesis Assays, Adhesion Assays, and Biofilm Assays. Additional studies, including ergosterol assays, reactive oxygen species (ROS) production assays, cell cycle analysis, and Scanning Electron Microscopy (SEM), along with RT-PCR, elucidated the mechanisms of action of these derivatives. AITC, BITC, EITC, PEITC, ButylITC, IPITC, and AIC demonstrated potent antifungal activity, effectively inhibiting planktonic growth and biofilm formation, inhibiting ergosterol biosynthesis, and inducing ROS production while also arresting cell cycle progression. Furthermore, these compounds inhibit the developing biofilm by modulating genes involved in the signal transduction pathways related to virulence.

Hemolytic activity assessments revealed that AITC is non-hemolytic, while EITC and BITC exhibited mild hemolysis. Other derivatives showed hemolytic effects even at lower concentrations, raising concerns for therapeutic applications. *In vivo* study using a silkworm animal model confirmed the antifungal efficacy of AITC, which enhanced survival rates at a concentration of 0.125 mg/ml, while EITC exhibited toxicity at 0.5 mg/ml.

Overall, this research suggests that glucosinolate derivatives, particularly AITC, hold promise as alternative therapeutic agent against *C. albicans* infection. These findings suggest further investigation into their clinical applications, underscoring the need for innovative strategies to combat antifungal resistance and enhance public health outcomes.

Keywords

Candida albicans, Biofilm, Ergosterol biosynthesis, Glucosinolates, Scanning Electron Microscopy, Silkworm.

CHAPTER – 1

INTRODUCTION

1. Introduction

1.1. Fungal infections

Fungi, which are one of the largest groups of living organisms with complex cell structures, have various ways of living and changing to fit different environments (1). Even though there are millions of different types of fungi, only a small number of them can infect human beings. Among these, the Ascomycota group contains many harmful fungi. Some of these can infect healthy people, like *Histoplasma, Blastomyces*, *Coccidioides*, and *Paracoccidioides*. Others mainly affect people with weakened immune systems, like *Aspergillus*, *Fusarium, Scedosporium* and *Candida* (2). Fungal infections caused by *Candida* spp. are most common. These include *Candida albicans*, which is the most common cause of opportunistic infections, *Candida glabrata* which is resistant to some drugs, *Candida auris* which is a new global health threat, and others like *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* that are starting to become more common (3).

1.2. An opportunistic fungal pathogen *Candida albicans*

C. albicans is a ubiquitous fungus that is commonly found in the human body, particularly in the gastrointestinal tract, mouth, and vagina (4). Under normal circumstances, it doesn't cause any harm. However, when the balance of microorganisms in the body is disrupted, such as due to a weakened immune system, antibiotic use, or hormonal changes, *C. albicans* can pass through the epithelium and enter deep-seated anatomical niches to cause infection (5). Mucosal and systemic infections are the two main subgroups of *C. albicans*-related medically significant infections (6).

C. albicans can cause mucosal infections in various parts of the body, including the mouth, throat, oesophagus, genitals, and nails. Vulvovaginal candidiasis (VVC), oropharyngeal candidiasis (OPC), and oesophageal candidiasis (EPC) are among the most common types. While VVC primarily affects the vaginal area, OPC impacts the mouth and throat, and EPC involves the oesophagus. Additionally, nail infections (onychomycosis) are less common but still attributable to *C. albicans* (6). The development of mucosal candidiasis can be influenced by several factors. While

individuals with intact immune functions can experience VVC, those with compromised immunity are at a higher risk of experiencing more frequent, severe, or recurrent infections. Factors such as diabetes, HIV/AIDS, immunosuppressive therapy, antibiotic use, hormonal changes, and pregnancy can predispose individuals to mucosal candidiasis (7).

Systemic candidiasis occurs when *C. albicans* penetrates deep-seated anatomical niches, affecting areas of the body such as the bloodstream, central nervous system (CNS), liver, spleen, heart and kidneys (8). In some cases, the infection can extend into the intra-abdominal compartment, with or without bloodstream dissemination. Systemic candidiasis is associated with high mortality rates despite the administration of antifungal therapy, making it a significant clinical challenge (9). Recognising the distinction between mucosal and systemic candidiasis is crucial for accurate diagnosis and management. While mucosal infections may present with localised symptoms such as itching, burning, or white patches, systemic candidiasis can manifest with more severe symptoms such as fever, chills, hypotension, and organ dysfunction. Timely diagnosis, often through microbiological cultures or molecular testing, is essential for initiating appropriate antifungal therapy and preventing complications (6).

1.3. Virulence factors in *C. albicans*

In recent years, the incidence of candidiasis has significantly increased, posing a significant clinical challenge worldwide (10). However, under certain conditions, it can transition into a pathogenic form, leading to various infections known as candidiasis (11). Every year, approximately 1.6 million people experiences fungal infections, *C. albicans* a fungal pathogen, is responsible for over 150 million mucosal infections and approximately 200,000 infections occurs due to invasive and disseminated disease in susceptible populations (12). *C. albicans* is a dimorphic fungus, existing in two distinct forms: yeast and hyphal. The yeast form predominates in its commensal state, characterized by oval-shaped cells that reproduce by budding (13). Under specific environmental conditions such as elevated temperature, nutrient availability, and pH changes, *C. albicans* undergoes a morphological switch to the hyphal form, characterized by elongated, filamentous structures (14).

This morphological transition is crucial for its virulence and ability to invade host tissues. The pathogenesis of candidiasis involves a complex interplay between fungal virulence factors and host immune responses (15).

Adherence to host tissues, biofilm formation, tissue invasion, and immune evasion are among the key mechanisms employed by *C. albicans* to establish infection. Adhesins such as Als proteins mediate the attachment of *C. albicans* to host cells, facilitating colonization and subsequent invasion. After adherence to the surface *C. albicans* cells able to form biofilms on biotic and abiotic surfaces, rendering them resistant to antifungal therapy and host immune defences (16).

Biofilms are groups of micro-organisms that stick to surfaces and are covered by a protective layer of extracellular matrix (17). Biofilms formation is one of the primary virulence factors that contribute to the pathogenesis of *C. albicans* because it complicates therapy and raises morbidity and mortality rates (18). There are four basic phases in the *C. albicans* biofilm growth process: adhesion, proliferation, maturation, and dispersion (5).

During the adhesion stage, yeast cells adhere to a surface and form a base layer. In the cell proliferation stage, these cells stretch out and grow into long, filament-like structures called hyphae. This change in shape helps the cells invade the host's tissues or surfaces of medical devices. The yeast *C. albicans* releases various hydrolytic enzymes, like proteinases, phospholipases and hemolysins which help it invade tissues or other solid surfaces. Among these, secreted aspartyl proteases (SAPs), which come from a group of ten different genes (SAP 1-10), are the most studied (19).

In the maturation stage, as hyphae grow, *C. albicans* also produces extracellular polymeric substances (EPS). The EPS in its biofilm is complex and includes key polysaccharides like α-mannan, β-1,6-glucan, and β-1,3-glucan. Even though β-1,3-glucan is present in smaller amounts, it plays a major role in the biofilm's resistance to antifungal drugs because it can block the drugs from reaching their target (20). In the dispersion stage, yeast cells are released from the mature biofilm and can move to new areas to start forming new biofilms. This stage is very important in a clinical setting

because the released cells can not only begin new biofilm formations but also enter the bloodstream, leading to infections. This connection between biofilm formation and the spread of Candida infections is why biofilms are linked to conditions like candidemia and invasive candidiasis (17).

1.4. Biofilm and antimicrobial resistance: A big threat

To treat biofilm-related infection becomes a difficult challenge to clinicians. Since biofilms are drug-tolerant, increasing drug doses might be required, but this isn't ideal due to the potential side effects of the medications (21). Because biofilms are difficult to remove, the only option may be to physically remove the affected devices. Additionally, devices that become colonized, such as pacemakers, can malfunction. In diabetic or immunocompromised patients, *C. albicans* can clog central venous catheters or urinary catheters, leading to blockages that may necessitate replacement (22). Removal of prosthetics is expensive procedure for the patients and may require frequent visits to the doctor as well as hospitals contributing to the expenses. Many times mixed biofilms involving bacteria as well as fungi further complicate the situation (22). Prolonged anti- antibiotic therapy gives advantage to *C. albicans* and may lead to dominance of *C albicans* causing vaginal candidiasis or intestinal infections. Overall, *C. albicans* biofilm-related infections present a significant clinical challenge due to their resistance to antifungal treatment, ability to cause persistent infections, and impact on medical devices (23). Effective strategies for the prevention and treatment of these infections are areas of active research in the field of medical mycology.

1.5. Antifungals available for the treatment of candidiasis and their side effects

Antifungal drugs are used for the treatment of candidiasis, which is caused by *Candida* species such as *C. albicans*. There are several classes of antifungal agents, each with its own mechanism of action and spectrum of activity against *Candida*. Here's an overview of commonly used antifungals for the treatment of candidiasis, along with their mode of actions:

1.5.a. Azole

Examples: Fluconazole, Itraconazole, Voriconazole, Posaconazole

Mechanism of action: Azole antifungals work by blocking the production of ergosterol, which is crucial for the fungal cell membrane. This disruption causes the membrane to become unstable and leads to the death of the fungal cell.

1.5.b. Echinocandins

Examples: Caspofungin, Micafungin, Anidulafungin

Mechanism of action: Echinocandins inhibit the synthesis of β-(1,3)-D-glucan, a major component of the fungal cell wall, leading to cell wall disruption and cell death.

1.5.c. Polyenes

Example: Amphotericin B

Mechanism of action: Polyenes bind to ergosterol in the fungal cell membrane, causing membrane permeabilization and cell death.

1.5.d. Flucytosine (5-FC)

Mechanism of action: Flucytosine is converted into 5-fluorouracil within fungal cells, leading to inhibition of DNA and RNA synthesis and subsequent cell death.

Another promising antifungal medication currently undergoing Phase II clinical trials is fosmanogepix. It works by inhibiting a fungal enzyme called Gwt1, which plays a role in the production and placement of certain proteins on the fungal cell membrane and outer wall. Fosmanogepix has shown to be effective against a wide range of fungi in laboratory tests, including strains of *C. albicans* that are resistant to other drugs, as well as other types of yeast and mold. It has also demonstrated significant effectiveness in animal models of oral and disseminated *C. albicans* infections. Despite the challenges in developing antifungal drugs due to similarities between fungal and human biology, there are other promising candidates in various stages of development. For example, turbinmicin, discovered through screenings of marine animal microbiomes, shows potential as a new antifungal agent (24).

It is important for healthcare providers to consider the patients overall health status, comorbidities, drug interactions and potential adverse effects when selecting antifungal

therapy for candidiasis. Close monitoring and appropriate management of side effects are essential to optimize treatment outcomes and minimize complications.

1.6. Alternative approach in antifungal drug discovery: Use of bioactive molecules

The use of bioactive molecules of natural origin as antifungal agents is proposed by various workers as they inhibit biofilm formation. Essential oils and its components mainly terpenoids, phenolic acids, phenyl propenoids, sesquiterpene alcohols are promising. Terpenoids and phenyl propanoids has been shown to inhibit virulence factors of *C. albicans* (25).

Vegetables like cabbage, cauliflower, broccoli and mustard oil are rich in glucosinolate group of molecules. Glucosinolate is converted into a variety of bioactive molecules in presence of myrosinase enzyme when these vegetables are cut or chewed. Intestinal microflora also can convert the glucosinolates to various derivatives. Glucosinolates are rich in bioactive properties like anti-microbial, anti-cancer, anti-carcinogenic, anti-inflammatory, anti-oxidative, anti-platelet, anti-mutagenic and insecticidal properties (26). Epidemiological studies indicated that consumption of these vegetables may considerably reduce the incidences of cancer (27). In addition, they provide considerable protection for the plants from pathogenic fungi, bacteria and pests (28).

Isothiocyanate inhibits variety of bacteria including multiple drug resistant human pathogenic bacteria by targeting cell membranes and metabolic processes. Allyl isothiocyanate has been shown to inhibit at least one dozen human pathogenic bacteria (29). Sulfurophane, a derivative of glucosinolate has been shown to inhibit the ulcerogenic bacterium, *Helicobacter pylori* (30). The preventive as well as therapeutic properties of isothiocyanates, pharmacological properties of isothiocyanates are reviewed by Kala *et al.*,(2018) (31). The molecular targets of glucosinolate derivatives as anticancer agents and their potential targets are reviewed by Mitsiogianni *et al.,* (2019) (32). The potential targets of the isothiocyanates are detoxification of xenobiotics, carcinogens by activation metabolic enzymes, anti-inflammatory properties, inhibition of angiogenesis, inhibition of metastasis, inhibition of cell cycle, and epigenetic regulation. Isothiocyanates have been shown to inhibit cell cycle, affect

mitochondrial function, induce apoptosis, inhibit mitosis, and ultimately inhibit proliferation. Inhibition of signal transduction, metastasis and inhibition of metaloproteases is reported. Some of the isothiocyanates are known to regulate epigenetic processes like methylation of gene promoters of cancer associated genes. Histone deacetylase inhibition is exhibited by isothiocyanates (32). Available data on isothiocyanates suggest that isothiocyanates could be excellent candidates for anti-cancer drug development, and in clinical practice.

Human pathogenic dermatophytic fungi like, *Microsporum canis, Trichophyton rubruma* and *Epidermophyton* are reported to be inhibited by isothiocyanates (31). There are only few study report present which demonstrated anti-fungal activity of glucosinolate derivatives against human pathogenic yeast like *C. albicans.* Among that Allyl isothiocyanate alone and in combination with fluconazole inhibited growth and biofilm formation in *C. albicans*. The mode of action of allyl isothiocyanate against *C. albicans* is unknown (33). Specifically, bioactive compounds are valuable for creating new medicines to treat many human diseases. Current study focused on exploring natural substances that can combat planktonic growth and inhibit biofilms related infections caused by *C. albicans*. Further it was aimed to learn how these substances work by examining different mode of action studies and to assess their effectiveness using an animal model called silkworm (*Bombyx mori*).

1.7. Purpose of the present study

C. albicans is normally harmless members of human bodys natural flora. However, it become harmful, especially in people with weakened immune systems, causing infections. *C. albicans* is known for its ability to form biofilm, which became hard to kill with antibiotics or the bodys defences. This biofilm related infections becomes challenging task for antibiotics and antifungal medications to work effectively. Plants are the better options for obtaining variety of drugs and have been used in medicine for a long time. The use of plant molecules is the best option for treating biofilm associated infection. In current study it was expected that glucosinolate molecules may exert antifungal activity through multitargeting against *C. albicans*. With this regard objectives of the present work were formed and followed.

1.8. Objectives of the study

- To check the effect of glucosinolate derivatives and find out inhibitory concentrations against growth of *C. albicans*.
- To investigate the effect of selected three glucosinolate derivatives on the architecture of *C. albicans* biofilms.
- To study the effect of selected three glucosinolate derivatives on expression of genes involved in signal transduction.
- To study the effect of selected three glucosinolate derivatives in Silkworm animal model against *C. albicans* pathogenesis.

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CHAPTER – 2

REVIEW OF LITERATURE

2. Review of literature

2.1. Introduction

Plant-derived compounds, also called phytochemicals, have a unique property in preventing diseases such as diabetes, cardiovascular diseases and cancers that are a threat to global health (1). Phytochemicals are usually classified according to their functional group and chemical properties, like carotenoids, terpenoids, phenolic, nitrogen-containing, and organosulfur compounds. Organosulfur compounds grab special attention for their exclusive properties in cancer prevention and treatment (2). Volatile organosulfur compounds like isothiocyanates (ITCs) have been identified as good antimicrobial agents. ITCs have demonstrated significant inhibitory effects on pathogenic bacteria. ITCs are also tested for antifungal efficacy against oral infections and the results shows that these compounds have the strong antifungal action (3). The incidence of fungal infections in humans has significantly increased in the last several years. Worldwide, fungal diseases cause over 10 lakh deaths every year. Immunocompromised people are primarily affected by the fungal infections. Among these, *Candida albicans* is the most common pathogen which causes fungal infections. *C. albicans* is able to form biofilms on the human cell surfaces and implanted medical devices. Food spoiling, oral and clinical microbial infections, microbial drug resistance and pathogen resistance are the major problems increasing worldwide. Consequently, there has been a surge in the assessment of natural products as a source of chemicals that can combat antimicrobial agents. One of the most significant natural compounds whose antimicrobial mechanism of action has been identified is glucosinolates (GSLs) and their hydrolysis products (4). Allyl isothiocyanate (AITC) is one of the hydrolysis product of GSL. AITC inhibits growth and virulence factors of *C. albicans*. It inhibits ergosterol biosynthesis, causes cell cycle arrest at the G2/M pre-apoptic phase and induces ROS production in planktonic and biofilm cells (5). By upregulating *TUP1, MIG1*, and *NRG1* genes and downregulating *PDE2, CEK1*, and *TEC1* genes, AITC modifies the expression of genes involved in the signal transduction pathway that inhibits germ tube formation. Toxicity assay of AITC demonstrate no harmful effect to human red blood cells, therefore *in vivo* studies are carried out in silkworm model. Results depict that AITC boosts silkworm survival rates by preventing infection of *C. albicans.* From the above study it was concluded that AITC may have the potential to

eradicate biofilm related fungal infections (5). Vegetables like cabbage, broccoli, brussel sprouts, radish, and cauliflower, which belong to the family *Cruciferae*, *Brassicaceae* possess high content of GSL (**Fig. 2.1**) and plays essential role in many physiological processes like cardio-metabolic, neurological, and musculoskeletal processes (4, 6–8). The Sinalbin (4-hydroxybenzyl glucosinolate) and Sinigrin (prop-2-enyl glucosinolates) were the first GSL structures extracted from white and black mustard in 1956, also known as 4-hydroxybenzyl glucosinolate and prop-2-enyl glucosinolates respectively (9). The term GSL was first used in 1968 (by Ettlinger and Kjaer). GSLs, a mustard oil glycoside, have been a part of human life since ancient times. GSLs have been derived from angiosperm plants of the *Brassicaceae* family and *Capparales* order. Mustard oil has a characteristic spicy flavour, hence it is used as a condiment and food preservative (7). GSLs are organic compounds containing β-D-thioglucosidase linked to sulfonated aldoxime moieties (7, 10). The GSL compound consists of 3 parts in its structure (**Fig. 2.1**);

1. (Z)-N-hydroximinosulfate ester
2. β-D-glucopyranose residue linked to (Z)-N-hydroximinosulfate ester by a sulphur atom (β-D-thioglucose group)
3. R- group, which is variable

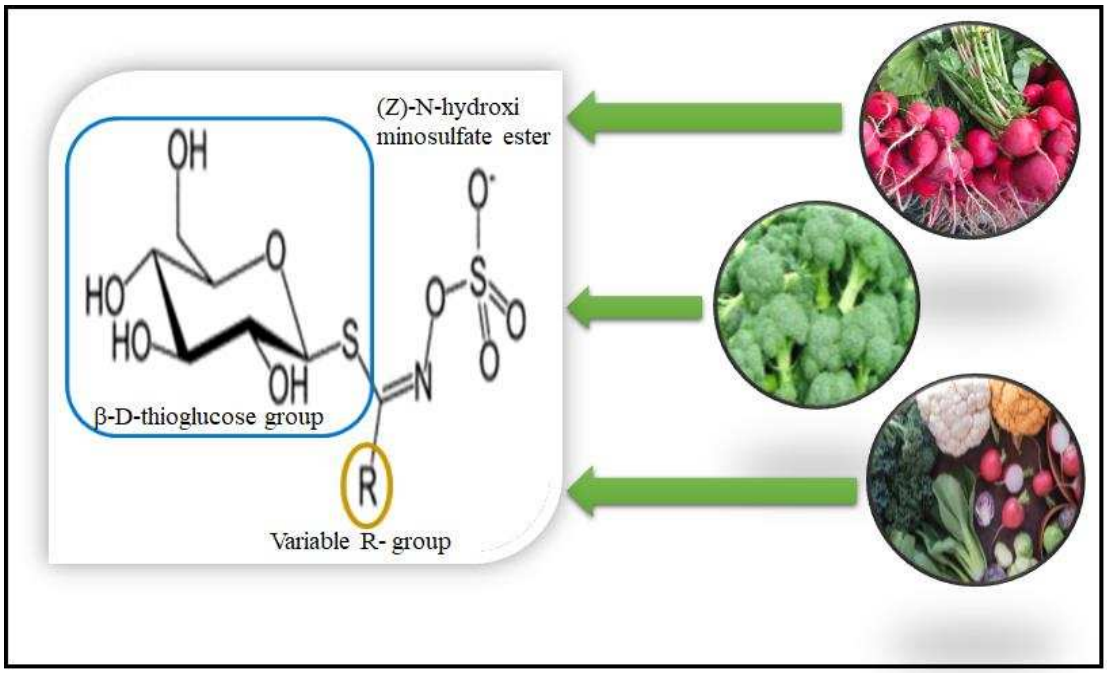


Fig. 2.1. Glucosinolates sources and structure.

and is synthesized in 3 steps;

1. Elongation of aliphatic and aromatic amino acids by addition of methylene group in their side chain
2. Formation of the core structure of glucosinolate by amino acid moiety, which reconfigured metabolically
3. And finally, initially formed glucosinolates are remodified by secondary metabolite transformation (**Fig. 2.2**) (11).

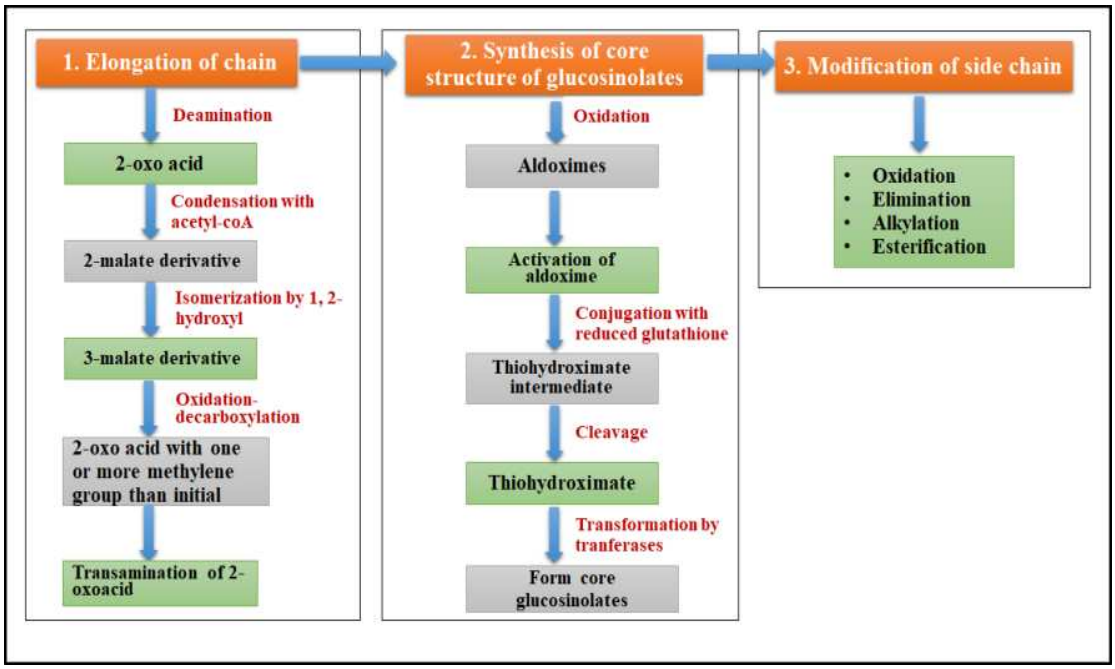


Fig. 2.2. Biosynthesis of glucosinolate derivatives.

The current review is based on articles that have been searched for in Scopus, SciFinder, PubMed, Research gate and Google Scholar. The aim of this report is to cover the literature up to December 2024 in detail. Therefore, on one of the platform searched for all known GSLs and their downstream products as compound names in Scopus abstracts or chemical entities in SciFinder. Search results were filtered to contain one of the text strings “Glucosinolates”, “Glucosinolate derivatives”, and “fungus” or “antifungal” or “fungistatic” in the titles or abstracts.

2.2. Hydrolysis of glucosinolates

GSLs derived from plants are observed as non-toxic compounds and have steady molecular structures. GSL are mechanically damaged by chewing, heating, or insect

attack and discharges a β-thioglucosidase which is also called myrosinase (12), and produces β-D-glucose and an unstable aglucone (thiohydroximate-O-sulfonate). The variety of natural GSLs is influenced by the side-chain of the O-sulfate thiohydroximate group, which is derived from different amino acids. Over 130 distinct GSL structures have been identified and confirmed. The breakdown products of GSLs, such as isothiocyanates, nitriles, epithionitriles and thiocyanates (**Fig. 2.3**), exhibit a broad spectrum of biological activities (13).

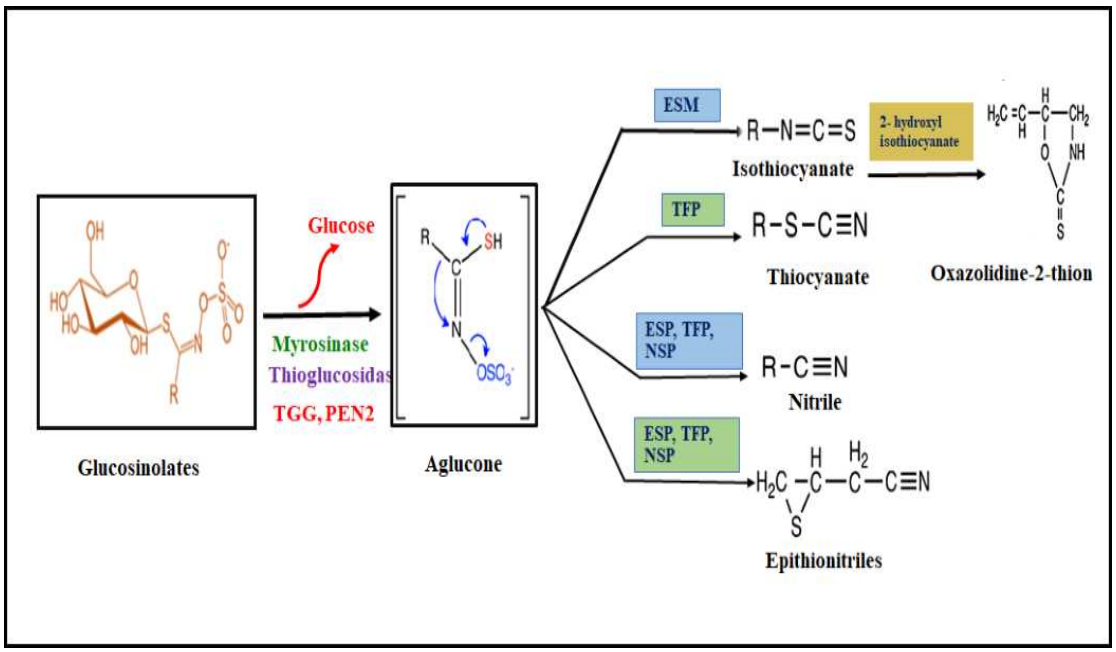


Fig.2.3. Glucosinolates and its hydrolysis products.

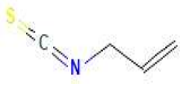
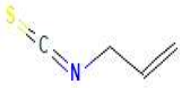
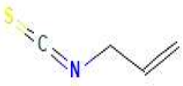
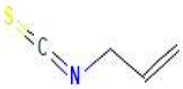
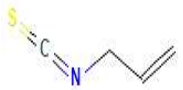
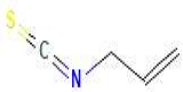
GSLs occur all over the tissues of plant organs, whereas myrosinases are restricted in scattered myrosin cells that appear to be GSL-free. Depending on the plant species, the enzyme is typically kept apart from GSLs in different cells or various intracellular compartments. The glucosinolate–myrosinase system provides plants with an effective defence system against herbivores and pathogens (14). GSLs are widely distributed in *Brassicaceae* plants. With its original culinary uses and medicinal advantages GSLs have received increased attention in recent years.

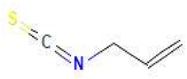
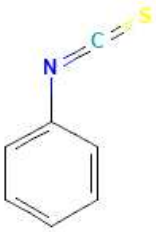
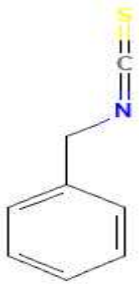
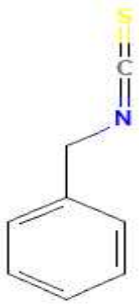
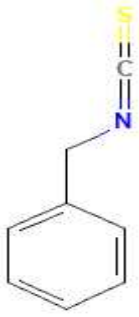
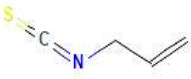
Natural GSLs are predominantly found in plants, with over 130 different types validated. The concentration of GSLs varies within different parts of the plant. For example, in *Brassica napus*, the seeds contain more GSLs than the leaves.

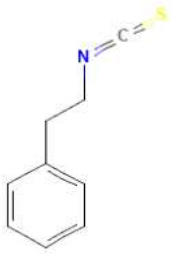
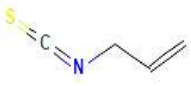
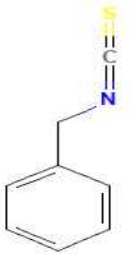
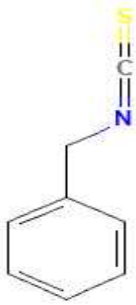
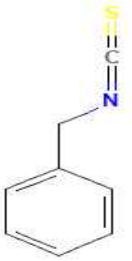
This variation is more pronounced in root vegetables (*Moringaceae* family) compared to oilseed crops (*Brassicaceae* family). Additionally, the GSL profile differs according to the kind of tissue. Although aliphatic GSLs prevail in both leaves and seeds, leaves have a higher concentration of indole GSLs than seeds (13). The study report by Troufflard *et al.*, (2010) (15) suggested that, due to abiotic stress, *Arabidopsis thaliana* accumulates higher levels of GSLs in its roots compared to its shoots. Reading the review of Martinez Ballesta *et al*., (2013) (16) for more information on plants' reactions to abiotic stress it was found that, GSLs and their hydrolysis products in the plant response to different abiotic stresses. Abiotic stresses change the plant GSL profiles, possibly through signaling and, affecting physiology of the plant. GSLs might help in stress response, but their exact role is unclear. Stress intensity, duration, and plant stage influence GSL levels, impacting interactions with pathogens and herbivores. Externally added GSLs can ease stress, but their molecular effects and precise function in environmental changes need more study (16). Breeding methods are commonly employed to develop crops with low GSL levels for food and feed purposes. Nevertheless, crops with elevated GSL levels remain valuable for non-food uses. The concentration of GSLs varies among different species within the same order. These variations can even be seen for the same crop. For instance, Ishida *et al.,* (17, 18) between 2005 and 2009 found that the quantity of GSLs in the same Japanese radish crops changed during this period (17, 18). It is assumed that environmental elements, such as the weather, which fluctuates slightly over time and affects the GSL contents of the crops, play a significant role in the accumulation of GSLs within plants. As a result, it is necessary to update the GSL content of the same crops once a year or more frequently if required. The downstream uses of raw resources should be carefully taken into account while choosing the species. It was also suggested that attention be paid to growth conditions to ensure that selected crops are well-suited to their environment.

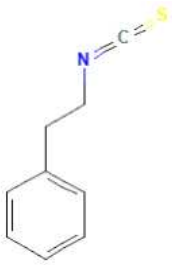
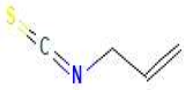
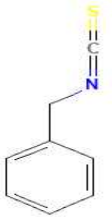
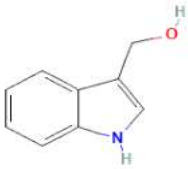
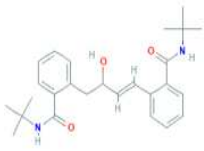
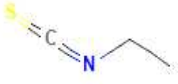
The GSLs and their derivatives have properties like antifungal, anti-bacterial, herbicidal, antioxidant, anti-mutagenic and anti-carcinogenic (10). The current review is focused on antifungal properties of GSLs and its derivatives (**Table 2.1**).

Table 2.1. Antifungal activity of glucosinolates

Sr. No.	Molecule	Structure	Organism	Antifungal activity	Ref.
1.	Allyl isothiocyanate		*Candida albicans*	Inhibit ergosterol biosynthesis, cell cycle arrest, ROS production, inhibit biofilm formation	(5)
2.	Allyl isothiocyanate		*Penicillium corylophilum*, *Penicillium roqueforti*, *Eurotium repens*, *Aspergillus flavus* and *Endomyces fibuliger*	Inhibit fungal growth	(7)
3.	Allyl isothiocyanate		*Aspergillus flavus*	Inhibit aflatoxin production	(22)
4.	Allyl isothiocyanate		*Gibberella zeae* , *Fusarium graminearum*, *Aspergillus flavus*, *Aspergillus parasiticus*, *Fusarium verticilloides*.	Inhibit aflatoxin production	(24, 25, 26, 27)
5.	Allyl isothiocyanate		*Penicillium roqueforti*, *Penicillium commune*, *Aspergillus flavus* and *Endomyces fibuliger*	Inhibit aflatoxin production	(28)
6.	Allyl isothiocyanate		*Penicillium roqueforti*, *Penicillium corylophilum*, *Eurotium repens*, *Aspergillus flavus*	Inhibit aflatoxin biosynthesis	(29)

			and *Endomyces fibuliger*		
7.	Allyl isothiocyanate,		*Aspergillus paraciticus*	Inhibit aflatoxin biosynthesis	(30)
8.	Phenyl isothiocyanate,		*Aspergillus paraciticus*	Inhibit aflatoxin biosynthesis	(30)
9.	Benzyl isothiocyanate		*Aspergillus paraciticus*	Inhibit aflatoxin biosynthesis	(30)
10.	Benzyl isothiocyanate		*Alternaria alternata*	Inhibit aflatoxin biosynthesis, Inhibit spore germination and mycelial growth, Cell membrane disruption	(32)
11.	Benzyl isothiocyanate		*Sclerotinia sclerotiorum*, *Rhizoctonia*, *Alternaria rot, Sclerotium rolfsi, Pythium irregulare* and *Rhizoctonia solani*	Inhibit aflatoxin biosynthesis, Inhibit mycelial growth and spore germination	(33, 34, 35)
12.	Allyl isothiocyanate		*Fusarium solani*	Hyphal deformity	(37)

13.	2-Phenyl Ethyl Isothiocyanate		*Alternaria alternate*	Inhibit spore germination and mycelial growth, Targets cell membrane	(38)
14.	Allyl isothiocyanate		*Monilia laxa, Botrytis cinerea, Aspergillus parasiticus*	Inhibit spore germination, mycelial growth and inhibit aflatoxin biosynthesis	(39, 40, 41)
15.	Benzyl isothiocyanate		*Aspergillus ochraceus*,	Inhibit mycotoxin production	(42)
16.	Benzyl isothiocyanate		*Phymatotrichopis omnivore*,	Inhibit hyphal growth	(43)
17.	Benzyl isothiocyanate		*Botrytis cinerea*	Efflux ability	(46)

18.	2-phenylethyl isothiocyanate		*Botrytis cinerea*	Efflux ability	(46)
19.	Allyl isothiocyanate + fluconazole		*Candida albicans*	Inhibits biofilm formation	(48)
20.	Benzyl isothiocyanate + Amphotericin B		*Saccharomyces cerevisiae*	Vacuolar membrane disruption	(49)
21.	Indole-3-carbinol		*Candida albicans*	Cell cycle arrest, DNA binding apoptosis	(50, 51, 52)
22.	2-hydroxy-but-3-enyl		*Pythium ssp. And Rhizoctonia solani*	Fungitoxic activity	(58)
23.	Thiocyanate		*Aspergillus fumigatus, Candida albicans*	Inhibits Mycelial growth	(59)
24.	Ethyl isothiocyanate		*Candida albicans*	Inhibit ergosterol biosynthesis, cell cycle arrest, ROS production, inhibit biofilm formation	(64)

Glucosinolates and their hydrolysis products have potent antifungal activity and wide application in many fields like in food preservations and pesticides. GSLs have different hydrolysis products like Allyl isothiocyanate (AITC), Benzyl isothiocyanate (BITC), Erucin, Phenyl hexyl isothiocyanate and Phenyl ethyl isothiocyanate (PEITC) have fungistatic and fungicidal properties against many plant pathogens. GSL and their derivatives are volatile compounds, and this characteristic is used in modern gas-based food packaging system to extend the shelf life of products. (7). Cereals often carry fungal spores on their surfaces and husks, meaning that whole milled cereal flours used in bread-making also contain these spores. Although baking deactivates these spores, fungal spoilage can still occur during storage or further processing of the baked goods. To address this issue, researchers have tested the use of rapeseed oil or mustard flour in bread production. Specifically, rapeseed brown mustard oil (from *Brassica juncea*) includes two main glucosinolates: butenyl isothiocyanate (10 %) and AITC (85 %), both of which are effective at killing fungi. In bakery settings, 2 ppm of AITC has been shown to inhibit the growth of various fungi, such as *Penicillium roqueforti, P. corylophilum, Eurotium repens, Aspergillus flavus*, and *Endomyces fibuliger* on rye bread stored in an airtight environment. Using modified atmospheric packaging with 85 % CO_2 and 1 % O_2, along with mustard oil vapors, has been effective in extending the shelf life of bread and bakery products while boosting their antifungal properties (7). Based on prior research indicating that glucosinolate derivatives might serve as effective antifungal agents, this review explores their antifungal properties and possible mechanisms of action (**Fig. 2.4**).

2.3. Antifungal activity of isothiocyanate

ITC's antifungal properties have been documented since 1966 (19). From then, a rising body of scientific research has produced a large number of studies discussing the antifungal properties of different ITCs against diverse fungi. Usually, the chemicals used in the study have been included in two forms: pure compounds or plant extracts containing ITC (19). Antifungal properties of isothiocyanate with their possible mode of action is as follow.

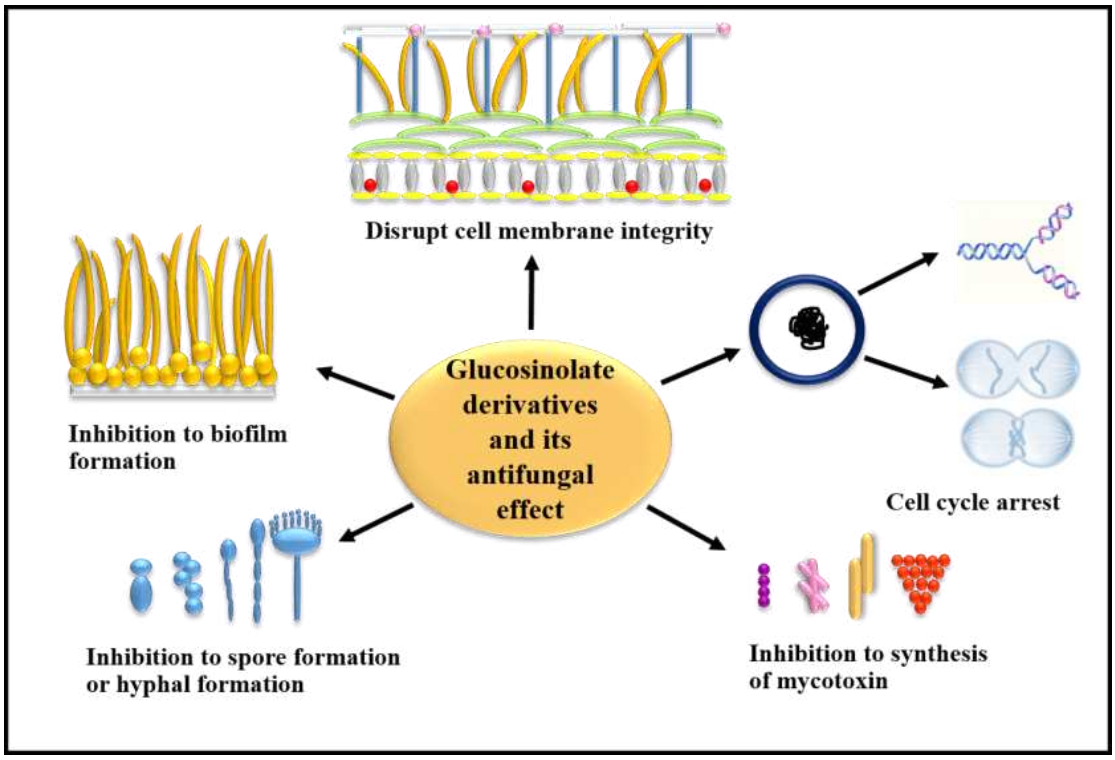


Fig. 2.4. Mechanism of antifungal action of glucosinolate derivatives.

2.3.a. Role of isothiocyanates in preservation by inhibiting Aflatoxin biosynthesis

A group of mycotoxins, i.e., Aflatoxins, are produced mainly by *Aspergillus parasiticus, Aspergillus flavus*, and *Aspergillus nomius*. These species are saprophytic in nature, present everywhere, grow on decaying animal and plant debris. Aflatoxin contamination mainly occurs in food, animal feed which is carcinogenic and is one of the main reasons of health and economic concern in many countries (20). There are many types of Aflatoxins among that Aflatoxin B1 is class I human carcinogen and the most toxic metabolite and has hepatotoxic, teratogenic and mutagenic properties (21). There are few studies that present the effect of isothiocyanate exposure on gene expression of mycotoxin biosynthesis. A comprehensive analysis of impact of allyl isothiocyanate on growth of *Aspergillus flavus* transcriptional profile and aflatoxin biosynthesis was done. Results suggests that allyl isothiocyanate remarkably inhibits the growth and Aflatoxin B1 production in *Aspergillus flavus* (22). Isothiocyanate inhibits fungal growth and toxin production and increases the shelf life of stored grain products. A study on stored soybean fumigated with mustard oil demonstrate that, there is reduction in fungal growth. Which was monitored by colony forming unit number

and free fatty acid content was less in the treated sample as compared to the non-treated sample during the storage period of 4.5 months (23). Along with this, vapors of AITC inhibits the fungal growth on grains that comprises *Gibberella zeae* (24), *Fusarium graminearum* (25), *Aspergillus flavus, Aspergillus parasiticus* (26) and *Fusarium verticilloides* (27). It was also reported that, isothiocyanate inhibits fungal growth on bread, when isothiocyanate was applied to the packages. Application of isothiocyanate in packaging helps in increasing the shelf life of a product in comparison with essential oils of plant-like monoterpenes or phenylpropanoids (28, 29). Nielsen and Rio (28) demonstrated that, mustard oil in in the form of gaseous AITC reduces the growth of fungi like *Penicillium roqueforti*, *Penicillium commune*, *Aspergillus flavus* and *Endomyces fibuliger* on bread at 1.8 to 3.5 µg/ml concentration range. Suhr *et al.,*(2003) (29) reported that, AITC and other volatile plant products successfully inhibit food-spoiling fungi *Penicillium roqueforti*, *Penicillium corylophilum*, *Eurotium repens*, *Aspergillus flavus* and *Endomyces fibuliger* on rye bread (29). Saladino *et al.,* (2017) (30) indicates that allyl isothiocyanate (AITC), phenyl isothiocyanate (PITC) and benzyl isothiocyanate (BITC) have antimicrobial activity against *A. paraciticus.* Among the AITC, PITC and BITC compounds, allyl isothiocyanate shows good activity. AITC soaked paper filter helps to improve the shelf life period of contaminated loaf bread by reducing the production of aflatoxin in packaging. Saladino *et al.,* (2016) (31) showed that AITC and para-hydroxy benzyl isothiocyanate reduce aflatoxin production in wheat. It reduces mycelial growth, which was produced by mycotoxigenic fungi (31). *Alternaria alternate* causes black spots on pear fruit. Which is one of the significant agents of post-harvest disease, causes fruit rotting. When the conidium of *A. alternata* adheres to the fruit wall, it germinates and forms hyphae.

Hyphal formation helps to absorb water, nutrition of fruit and produce toxic mycotoxins and most common toxins includes alternariol monomethyl ether (AME), alternariol (AOH), altenuene (ALT) and tentoxin (TEN). The use of non-organic fungicides may be fatal to human health and environment (32). Wang and his co-workers (32) tried to find organic fungicide for this, they studied isothiocyanates. As aromatic isothiocyanates show more antifungal activity than aliphatic, they selected benzyl isothiocyanate (BITC). BITC has shown biocidal activity against *Sclerotinia*

sclerotiorum, *Rhizoctonia* (33), *Alternaria* (34), *Sclerotium rolfsi, Pythium irregulare* and *Rhizoctonia solani* (35). BITC was found to suppress mycelial growth at 1.25 mM concentration. ≥ 50 % reduction in black spot development in wounded pear was seen which was inoculated with spores of *A. alternata*. BITC also significantly inhibited the production of alternaria toxins (AME, AOH, ALT, and TEN) at a concentration of 0.312 mM. The mechanisms underlying antifungal activity of BITC through membrane disruption and cell growth hindrance. BITC may thus offer an economical and environmental-friendly strategy to control postharvest diseases in fruits and vegetables (32). The study concludes that isothiocyanates prevent fungal growth and aflatoxin production.

2.3.b. Effect of isothiocyanate on Transcriptoms

The transcriptional response of fungi to various isothiocyanates has been investigated in numerous studies. Calmes *et al.,* (2015) (36) reported that, isothiocyanates elicit oxidative stress and mitochondrial dysfunction in fungal cells. Isothiocyanate is involved in fungal cell death as it acts as a protective coating against pathogen invasion. When *Saccharomyces cerevisiae* was exposed to isothiocyanate, the key regulators of oxidative stress MAPK (mitogen-activated protein kinase), AbHog1 (*Alternaria brassicicola* high osmolarity glycerol 1 ortholog) and AbAP1 (Armadillo BTB Arabidopsis Protein 1), the transcription factors of *S. cerevisiae* YAP1 (protein ortholog) gene expressions were significantly upregulated. Additionally, exposure to isothiocyanate decreased oxygen consumption, increases the accumulation of intracellular reactive oxygen species (ROS) and mitochondrial membrane depolarisation. Once ROS generated, AbAP1 also promotes the expression of other oxidative stress response genes, which helps to protect fungus against isothiocyanate. Moreover, if nutritional deficiency occurs in an organism, AbHog1 and AbAP1 become hypersensitive and leads to inhibition of organisms (36)**.** AITC caused inhibition in *Fusarium solani* by causing deformity of hyphae and leakage of electrolytes (37). A vacuolar transient receptor potential channel regulator (FsYvc1) was identified in this fungus. FsYvc1 was involved in the growth, development, and pathogenicity in *Fusarium solani*. FsYvc1 loss resulted in hypersensitivity to AITC and induced accumulation of reactive oxygen species (ROS). On treatment with 4.8 µg/ml AITC,

the expression FSYvc1 was 12-30 fold upregulated compared to the control. Hence FsYvc1 could be a new molecular target for drug development (37). ITCs can act as a good antifungal agent because it has multiple targets that include intracellular ROS accumulation, mitochondrial membrane disruption and activity on transcriptomes of fungal species.

2.3.c. Effect of isothiocyanates on cell wall and cell membrane

Zhang M. (2020) (38) reported the antifungal activity of 2-Phenyl Ethyl Isothiocyanate (2-PEITC) against *Alternaria alternate*. *A. alternate* is a filamentous fungus and one of the most important causal organisms of black spots on pear fruit at the time of harvest. The organism not only causes economic and industrial damage but also produces mycotoxins such as Alternariol (AOH), Alteuene (ALT), Alternariol Monomethyl Ether (AME) and Tentoxin (TEN). These mycotoxins can adversely affect both human and animal health. Therefore, inhibiting mycotoxin production is a challenge to control the situation. Some of the chemical fungicides like ortho-phenylphenate and imazalil were used, but continuous use of these fungicides produces resistant strains and pollution. A different approach for handling these circumstances is to use GSL or other substances with antimicrobial qualities and less adverse effects. AITC has antifungal activity on *Monilia laxa* of nectarine and peach (39), *Botrytis cinerea* of strawberry (40), *Aspergillus parasiticus* of pizza crust (41). BITC against *Aspergillus ochraceus* of peanuts, green coffee, grapes, soybeans (42), *Phymatotrichopis omnivore of cotton* (43), *Alternaria alternate* of tomato (34) 2-Propenyl isothiocyanate against *Penicillum expansum* of pears. 2-Phenyl ethyl isothiocyanate shown antimicrobial activity against many clinical isolates, soil microorganisms and plant pathogenic bacteria (44). Fumigation by 2-Phenyl ethyl isothiocyanate helps in inhibition of spore germination of *A. alternata* at 4.88 mM concentration. Mycelial growth of *A. alternate* is also inhibited, and MIC_{50} was achieved at a concentration of 1.22 mM. Treatment with 2-Phenyl ethyl isothiocyanate showed an effective reduction in the development of black spots at 1.22 mM concentration; the diameter of the lesion was only 39 % compared to the control in 7 days. To understand the precise mechanism of 2-PEITC in *A. alternata*, a plasma membrane integrity test was conducted using propidium iodide (PI). PI can penetrate compromised cell membranes and emit red fluorescence. The assessment of

plasma membrane permeability was carried out by measuring electrolyte leakage and nucleic acid content. After 30 minutes of treatment of 2-PEITC, the OD was measured at 260 nm. Results demonstrates that, the content of AOH, AME, ALT and TEN in *A. alternata* hypha notably decreased. In short, mechanism include steps like,

1. Disruption of cell membrane
2. Leakage of cytoplasmic content
3. Cell death

Finally, Zhang M. *et al.,* (2020) (38) concluded that 2-PEITC causes cell membrane disruption in *A. alternate* and it could be used as a fungicide.

Another study on black spot of pear fruit by *A. alternate* demonstrated by Tiaolan Wang *et al.,* 2020 (32). *A. alternate* causes black spots on pear fruit which is one of the significant agents of post-harvest disease and causes fruit rotting and leads to economic losses. When conidium of *A. alternate* adhere on the fruit wall, it germinates and forms hyphae. After hyphal formation, by continuous division, it will form colonies, on the fruit's cells and tissue, absorb water, nutrition and produce toxin like mycotoxins. The most common toxins are AME, AOH, ALT, and TEN. The use of non-organic fungicides is fatal to human health and the environment (45). *A. alternata* treated with BITC showed a loss of membrane integrity and cellular component leakage. This was identified in an effort to further clarify the processes behind the anti-fungal effect of BITC against *A. alternata*. BITC may damage membrane integrity as it is lipophilic in nature. When propidium iodide, a DNA stain, was introduced to cells, the cells that have lost their membrane integrity emits red fluorescence. PI-stained *A. alternata* spores were more numerous in response to BITC fumigation. At 0.625 mM of BITC, *A. alternata's* membrane integrity was nearly destroyed. Wang concluded that (32), BITC inhibit spore germination and mycelial growth of *A. alternate* by the mechanism of cell membrane disruption, reduction in growth of cell and exhibit antifungal activity via membrane targeting. Hence, isothiocyanates plays major role in inhibition of cell wall and cell membrane integrity in fungi.

2.3.d. Role of isothiocyanate in efflux

Botrytis cinerea is a necrotropic plant pathogen which also includes cruciferous plants. It causes gray molds during growth and harvesting and also responsible for economic

losses. *B. cinerea* is sensitive to GSL and most susceptible to BITC. BITC completely inhibits mycelial growth at 400 µM, whereas 2-phenethyl isothiocyanate, inhibits 70 % growth of *B. cinerea*. When *B. cinerea* is exposed to isothiocyanates, it triggers the production of Major Facilitator Superfamily transporter (mfsG) involved in the efflux of hazardous compounds from fungi. mfsG expression are induced when *B. cinerea* is injected into wild-type *Arabidopsis thaliana* plants, as compared to GSL-deficient *A. thaliana* mutants. An efflux-deficient strain of *B. cinerea* lacks in functioning mfsG transporter. It is more sensitive to isothiocyanates and accumulates more of them; also, it is less pathogenic towards plants that contain GSL. Additionally, *Saccharomyces cerevisiae* cells that express mfsG facilitate isothiocyanates efflux, which provides the yeast resistance to isothiocyanates. These results implies that the mfsG transporter is a virulence factor that enhances GSLs tolerance (46).

2.3.e. Role of isothiocyanates in synergistic activity

C. albicans can form biofilm on both biotic and abiotic surfaces. *C. albicans* biofilm is resistant to standard antifungals like fluconazole and Amphotericin-B (47). Biofilm-related infections are difficult to treat, so it is a threat to immunocompromised patients. To overcome this situation, there is an urgent need to find an alternative. A study report on the activity of allyl isothiocyanate (AITC) alone and in combination with fluconazole shows anti-candida activity. AITC is one of the good inhibitors of many pathogenic organisms, including fungi. Fluconazole (0.004 mg/ml) and AITC (0.125 mg/ml) in combination reduces the growth of *C. albicans* biofilm. The exact molecular mechanism is still unknown in this study; it is assumed that AITC-mediated membrane destabilization sensitises *C. albicans* cells and increases the fluconazole influx that inhibits biofilm growth (48)**.** In another study, Benzyl isothiocyanate helps to enhance the fungicidal activity of Amphotericin B in *Saccharomyces cerevisiae* by vacuole disruption. Amphotericin B binds to ergosterol and forms an ion channel through the plasma membrane. This helps in increasing intracellular leakage of K^+ ion and many other ionic substances and causes vacuolar membrane disruption, which results in lethal effects. Benzyl isothiocyanate shows no effect on plasma membrane permeability induced by Amphotericin B., but it boosts Amphotericin B-induced vacuolar disruption in *S. cervaceae*. In *C. albicans*, benzyl isothiocyanate helps to increase fungicidal

activity by decreasing cell viability due to disruption of vacuole (49). Synergism of isothiocyanates with standard antifungals may enhance isothiocyanates ability to inhibit fungal growth. Such combinations need to be explored for antifungal treatment.

2.4. Antifungal activity of Indole glucosinolates and its mechanism of action

In 2007, Sang and Lee (50) assessed the antifungal activity of indole-3-carbinol (I3C) against the human pathogenic fungus *C. albicans*. The outcomes demonstrated that I3C arrests the cell cycle in *C. albicans* at the G2/M phase. Fluorescent microscopy studies on *C. albicans* were used to track the changes in cell membrane dynamics by the of action of I3C. The findings imply that I3C may operate as an antifungal agent by altering the composition of the cell membrane. I3C demonstrated energy-independent candidacidal action without hemolysis on human erythrocytes. In order to understand the mode of action of I3C in *C. albicans*, experiments with fluorescence quenching and gel retardation were carried out on *C. albicans*. The findings imply that I3C may serve as an anti-candidal agent via binding to DNA in *C. albicans* (51). According to study by Hwang *et al*. (2011) (52), Reactive oxygen species (ROS) and hydroxyl radical production induces greatly when *C. albicans* cells were treated with I3C. I3C causes apoptosis by releasing cytochrome C, producing hydroxyl radicals, and activating the enzyme metacaspase (52). In conclusion indole glucosinolates exhibits fungicidal activity by altering cell membrane composition, intracellular ROS production which finally leads to apoptosis of fungal cell.

2.5. Antifungal activity of Nitriles and its mechanism of action

Numerous studies have demonstrated that substances derived from plant essential oils, such as nitrile, have antifungal qualities. However, because these mixtures often contain notable concentrations of the significantly stronger isothiocyanates, these cannot be interpreted as direct evidence of the antifungal action of nitriles. Interestingly, some nitrile compounds derived from GSL have been shown to inhibit fungal growth. Nitriles are substantially less effective as direct antifungal agents than isothiocyanates (53,54). A study by Zsolt Szucs *et al.,* 2023 (55) demonstrated that the potential synergistic antifungal interaction between various GSLs-derived nitriles and 2-phenylethyl isothiocyanate (PEITC). 45 fungal strains, including endophytes from horseradish roots

(*Brassicaceae*) and soil fungi. Study was carried out in an airtight system that allows for the precise study of extremely volatile antifungal agent. The results indicate that, the median minimal inhibitory concentrations (MICs) for 1H-indole-3-acetonitrile, 3-phenylpropanenitrile, 4-(methylsulfanyl)-butane nitrile, and 3-butenenitrile were 1.28, 6.10, 27.00, and 49.72 mM, respectively. The median MIC of 0.04 mM for nitriles indicated that they were significantly less effective as antifungal agents than PEITC. The median fractional inhibitory concentration indices (FICIs) for the combinations using the same nitriles were 0.562, 0.531, 0.562, and 0.625 mM. The examined fungal strains exhibited a synergistic antifungal activity (FICI $\leq$ 0.5) for the nitrile–isothiocyanate combinations. Regarding the GSL breakdown products and their combinations, strains of Hypocreales exhibited the least susceptibility. For Hypocreales, Eurotiales, Glomerellales, and Pleosporales, the mean MIC values for PEITC were 0.0679 ± 0.0358, 0.0400 ± 0.0214, 0.0319 ± 0.0087, and 0.0178 ± 0.0171 mM, respectively. Furthermore, notable variations were also seen in nitriles, particularly 1H-indole-3-acetonitrile. Depending on the nitrile, the median FICI values for the same fungus ranged from 0.61 to 0.67, 0.52 to 0.61, 0.40 to 0.50, and 0.48 to 0.67, respectively. Study findings depict that nitriles produced from glucosinolates may improve the antifungal action of isothiocyanates (55).

2.6. Antifungal activity of Epithionitriles

The epithionitriles are another GSL breakdown products. Epithionitriles' direct impact on fungi was investigated by many researchers. Epithionitrile was shown to be non-inhibitory at doses as high as 1 mg/ml, whereas isothiocyanates shows inhibition at MIC 1-4 µg/ml. Epithionitriles appear to play a less significant part in fungal defense against *Verticillium longisporum*. Witzel *et al*., (2015) (56) assessed the growth-inhibitory effect of volatile GLS breakdown products on *V. longisporum* in relation to *A. thaliana* accessions. Many *Verticillium* species of fungus cause plants to wilt when they penetrate the vascular system. They are capable of causing considerable losses in agricultural output and quality. Four *A. thaliana* accessions were evaluated for natural variation in their GLS composition. Bur-0, Hi-0 and Kn-0, Ler-0 these are the four accession of *A. thaliana*. Alkenyl GLS was accumulated in the accessions Bur-0 and Hi-0, while 3-hydroxypropyl GLS was more prevalent in the accessions Kn-0 and Ler0.

The majority of the isothiocyanates derived from GLS breakdown were formed by Hi-0 and Kn-0, while Bur-0 released epithionitriles and Ler-0 nitriles. After the plants were exposed to *V. longisporum* species, the composition of GLS and its breakdown products in the leaf and root showed several alterations specific to distinct organs and accessions. However, colonisation increased GLS accumulation in the roots but decreased it within the rosette leaves of the less sensitive accessions Bur-0 and Ler-0. Comparatively, in three of the four accessions, the amount of GLS breakdown products in the root reduced. This could mean that the chemicals were bound or conjugated to a fungal target molecule. The plant-pathogen interactions influenced the production of GLS degradation products specific to organs and accession (56). According to the above study, the colonisation of *A. thaliana* by *V. longisporum* had an impact on the accumulation of GLS and its breakdown products that were unique to each tissue and accession. It is possible that tolerant accessions possess a greater ability to accumulate GLS at the site of root infection. Although the study couldn't find a particular breakdown product or GLS associated with pathogen tolerance, additional work needs to be done on plants exposed to specific inhibitors so that they may produce a specific GLS prior to fungal inoculation.

2.7. Antifungal activity of oxazolidine -2- thion

A quick and spontaneous rearrangement occurs when an ITC side chain has a functional group that reacts with the isothiocyanate group's carbon, and the compound is flexible enough to support the reaction (**Fig. 2.3**). By this rearrangement process oxazolidine-2-thiones derived from 2-hydroxy-isothiocyanates (53). Many of the GSLs can produce isothiocyanates that are susceptible to this rearrangement, but only fraction of these have so far been reported as being of plant origin. Green, leafy rocket (*Eruca sativa L.*) is a member of the *Brassicaceae* family and is high in glucosinolates (GLSs) content. 1,3-thiazepane-2-thione is the major cyclized isothiocyanate breakdown product from rocket. Whereas other isothiocyanates from rockets disintegrate after thermal treatment, this molecule is relatively stable in comparison to other isothiocyanates. Sativin/1,3-thiaze-pane-2-thione might play a smaller function in GLS-related chemo-prevention compared to isothiocyanate. Because it exhibited no cytotoxicity on HepG2 cells at relevant doses for food. However, the results need to be confirmed with more cell lines.

Future research on these compounds needed to be explored for its bioactivities (57). In order to confirm the production of the corresponding degradation products, Lazzeri *et al.* (2004) (58) mentioned about plant hydrolases containing the isothiocyanates like 2-hydroxy-but-3-enyl glucosinolate isolated from *Crambe abyssinica* seeds with purity level higher than 85 %. *In vitro* analysis against *Pythium sp*. and *Rhizoctonia solani* found that nitrile and vinyl-oxazolidine-thione had good fungi toxic activity.

2.8. Antifungal activity of Thiocyanates

Data on antifungal activity of GSL-derived thiocyanates is very limited as compared to synthetic thiocyanates. Radulovic *et al.,* (2012) (59) reported the antifungal activity of synthetic thiocyanate against *A. fumigatus* and *C. albicans* which inhibits mycelial growth at 0.15 mg/ml and 1.25 mg/ml concentration respectively.

2.9. Cytotoxicity data

GLS hydrolysis products, especially isothiocyanates is reported to have cytotoxicity. The toxicity of GSLs hydrolysis products may be due to activity on CYP enzyme and glutathione levels (60). The isothiocyanates and indoles give chemo-preventive qualities to *Brassica* crops. Dominik Kołodziejski *et al.,* (2022) (61) demonstrated cytotoxicity and genotoxicity effect of GSLs. The genotoxic effects of glucotropaeolin, glucobrassicin, and sinigrin through comet assay in HT29 cells is investigated in HT29 cells. Perhaps because of their low bioavailability, intact GSLs did not show any cytotoxic activity. Nevertheless, GSLs were able to stop the development of HT29 cells in the presence of myrosinase. GSLs were hydrolysed by myrosinase and produce equivalent isothiocyanates or indole compounds with higher biological activity than parent GSLs. The most potent anti-proliferative effect was observed in pure isothiocyanate / indole solutions. Restrictions result analysis study revealed that in a condition without cells, GSLs modified DNA more than isothiocyanates. When it came to GSLs, metabolic activation by the S9 fraction enhanced this effect and caused a shift in the preferential binding site from the AT base pair region to the GC base pair region. Among all tested compounds, only benzyl isothiocyanate caused DNA damage detectable in the comet assay, but it required relatively high concentrations (61). A study by Cuellar-Nunez M. *et al.,* (2020) (62) demonstrated that, phytochemical profile

of four different extracts from the leaves of *Moringa oleifera*: aqueous (AE), methanolic (ME), glucosinolate-rich hydrolyzed (GEH), and glucosinolates (GE). Additionally, the extracts were compared for their anti-proliferative effects on human colorectal cancer (CRC) cells, HCT116 and HT-29. The constituents of moringa extracts included glucomoringin, quercetin glucoside, and quercetin-malonyl-glucoside. The results indicate that extracts from moringa leaves had anti-proliferative effects on colon cancer cells, with IC_{50} values ranging from 0.17 to 3.17 mg/ml. TNF-α and IL-1β, two pro-inflammatory cytokines, were reduced in production by GEH. In HCT116 (IC_{50}: 0.55 mg/mL) and HT-29 (IC_{50}: 0.59 mg/ml), GEH enhanced apoptosis to 58.1 % and 38 %, respectively. To produce the GEH inhibitory effect, cytotoxic activity was stimulated, which raised lactate dehydrogenase activity and started an inherent pro-apoptotic pathway that was dependent on ROS. Intracellular metabolites of GEH accumulated in the cells and caused damage to the mitochondria, which decreased Bcl-2 expression and increased Bax and cytochrome C release. The noteworthy outcomes found in HCT116 cells suggested that moringa extracts may be able to address colorectal cancer situations early on. This study advances our understanding of the vegetable moringa as a functional food with additional health advantages (62). Another study by Kadir *et al.,* (2023) (63) showed that one of the natural compounds that has been found to be a powerful anticancer agent is benzyl isothiocyanate (BITC). This study focused on the cytotoxicity effects of BITC exposure, the MTT assay, the measurement of reactive oxygen species (ROS), the amount of caspase 3/7, the Acridine orange/Ethidium bromide apoptosis assay, and Western blotting. Protein-ligand interactions were investigated by molecular docking of BITC with human 5.10 methyl tetrahydrofolate synthetase receptor, MTHFS (PDB:3HY3), and epidermal growth factor receptor tyrosine kinase (EGFRK) domain (PDB:1M17). The outcomes demonstrated that BITC had an EC_{50} value of 23.4 μM and demonstrated cytotoxicity against MCF-7 breast cancer cells. It's interesting to note that BITC had a minimally harmful effect on normal human fibroblast cells. The GST, BCL-2, p-ERK, p38, NRF-2, p-ERK, and ERK 1/2 protein expression, as well as the expression of the NF-κB and MAPK genes, were all regulated in the treated MCF-7 cell lines. The methanol hexane extraction of Miswak also yielded a 6.25 % BITC concentration. The importance of BITC as a strong cancer cell inhibitor may be clarified

by this new research. To further enhance comprehension of the mechanism behind BITC's lethal effect through MAPK protein activation, the *in silico* analysis offers a profile of protein and ligand interactions (63). The prevalence of candidiasis has sharply grown in recent years, primarily in people with weakened immune systems, which results in considerable mortality and morbidity. It has been observed that compounds of GLS exhibit antifungal properties. The anti-fungal activity of ethyl isothiocyanate (EITC) against *C. albicans* and its mode of action is still unclear. A study by Patil SB *et al.,* in (2024) (64) demonstrated *in vitro* and *in vivo* research to gain a molecular understanding of EITC's anti-*Candida* activity. The virulence factors of *C. albicans*, such as yeast to hyphal form morphogenesis, adherence to polystyrene surface and biofilm formation were significantly suppressed by EITC. EITC arrest *C. albicans* cells in their S-phase further it prevented the biosynthesis of ergosterol. Nuclear or DNA fragmentation and ROS-dependent cellular death were triggered by EITC. EITC upregulated *TUP1, MIG1*, and *NRG1* and downregulated *PDE2* and *CEK1* genes. These changes in gene expression controlled the signal transduction pathway and prevented yeast from hyphal form morphogenesis. At a dosage of 0.5 mg/ml, EITC exhibited hemolytic action to human red blood cells (RBCs). According to an *in vivo* investigation conducted in a silkworm model EITC causes toxicity to silk worm. From this investigation it became clear that EITC has antifungal action and to lower its minimum inhibitory concentration and toxicity further research combining it with other antifungal medications is necessary (64). In conclusion the toxicity of GSLs hydrolysis products on cells is confirmed, and it is primarily linked to ITCs.

2.10. Clinical trials study on glucosinolates

According to a recent study, there are various mechanisms by which GLS and isothiocyanates work, ultimately leading to the development of antioxidant, anti-inflammatory, and chemo-protective activities (4). Increasing vegetable intake improved glycemic control in a 12-week trial with 92 type 2 diabetes patients. However, vegetables with strong and bitter flavors (500 g/day), including kale and cabbage also significantly improved fasting glucose (by 4-fold), total cholesterol (by 2 -fold) and body fat mass (by 2- fold) (65). In a population-based study, it was found that allium vegetables affected the incidence of prostate cancer, with men who

consumed allium having a lower chance of developing prostate cancer (66). The majority of the clinical trials have been documented on broccoli when compared with brassica vegetables and glucosinolate extracts (65). However, only one study found that a 4-week supplementation with 300 g of Glucoraphanin-rich broccoli soup altered the expression of genes and related oncogenic pathways of prostate cancer (67). In another study it was found that consumption of brassica vegetables rich diet, increases the *lactobacilli* count in human gut along with reduction of sulfate – reducing bacteria count compared to the diet with low Brassica vegetable (68). 22 out of 29 clinical trials that used sulforaphane as an extract were registered during the most recent period, suggesting an increasing trend. 200 µmol of sulforaphane equivalents administration was found to inhibit the histone deacetylase activity *in vivo* (69,70). The latest randomized placebo-controlled trial provides supporting information of sulforaphane on psychiatric outcome. It showed that a 6-week sulforaphane intervention (30 mg/day) safely enhanced symptoms of depressive disorder in 66 participants who had previous experience with a cardiac procedure and mild-moderate depression, as measured by the Hamilton Rating Scale for depression (71). In another study performed in large women population with ≥70 years age group demonstrated that higher consumption of cruciferous (>44 g/day) and allium (>11 g/day) vegetable resulted in lowered relative risk of fracture-related hospitalization by 28 and 34 % over period of 14.5 years, respectively (72). The report suggests that, incorporation of Cruciferous vegetable in diet every week helps as to provide nutrient and supplement.

2.11. Conclusions

Many compounds, including ITCs, indole glucosinolates, nitriles, epithionitriles, oxazolidine-2-thione, and thiocyanate are produced as a result of the chemical diversity of the glucosinolate side chains. The medicinal, industrial and agricultural benefits of GSL and its breakdown products have recently received substantial research. However, due to their natural origin and biodegradability, make them a strong option for a wide range of potential uses. The majority of the published literature is focused on isothiocyanates, as demonstrated by an astonishing number of studies. Numerous applications of some excellent articles on transcriptomes demonstrate general stress responses and importantly the inhibition of the production of fungal toxins, is shown as

well. Its antibacterial activity, or potential uses have to be taken into consideration. This study sought to close this knowledge gap by compiling the most recent research on the interactions of fungi with isothiocyanates, indole glucosinolates, nitriles, epithionitriles, oxazolidine-2-thione, and thiocyanate chemicals that result from the glucosinolate hydrolysis route. These GLS compounds were found to inhibit a large number of human diseases, plant pathogens, and other fungi. Despite the significance of molecules, a lot of unanswered questions still exist. The exact composition of the glucosinolate-derived mixture of antifungal agents is not fully characterized, and it likely contains additional, currently unidentified natural products. These antifungal mechanisms of these chemicals are still poorly known. However, due to the benefits of isothiocyanates, indole glucosinolates, nitriles, epithionitriles, oxazolidine-2-thione, and thiocyanate, it is likely that there will be a significant increase in demand and interest for such applications. Cytotoxicity data depict that, the toxicity of GSLs hydrolysis products on cells is confirmed, and it is primarily linked to isothiocyanates. More extensive studies will be needed in this field to assess safety concerns and clinical efficacies of GSLs and its derivatives.

2.12. References

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CHAPTER – 3

MATERIALS AND METHODS

3. Materials and Methodology

3.1. Microorganism

C. albicans American Type Culture Collection (ATCC) 90028 reference strain was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India used in this study. Yeast extract peptone dextrose (YPD; 1 % yeast extract, 2 % peptone and 2 % dextrose) broth was used for culturing *C. albicans* strain. For long-term culture maintenance, fungal cultures were prepared as glycerol stocks and stored in ultra-deep freezer (-80 ºC). The organism was sub cultured on YPD agar plates and slants and stored at 4 ºC. *In vitro* assays like planktonic growth, developing biofilm and mature biofilm was performed using Roswell Park Memorial Institute (RPMI) 1640 medium. Yeast to hyphal (Y-H) morphogenesis assay performed with the help of Fetal Bovine Serum (FBS) media.

3.2. Media Preparation

3.2.a. Yeast Extract-Peptone-Dextrose (YPD) agar

50 ml distilled water was taken in 250 ml of flask. 2.5 gm of Yeast Extract-Peptone-Dextrose (YPD) broth and 0.5 gm of agar were added to 25 ml of distilled water and adjusted to 50 ml by adding distilled water. The flask was covered with cotton plug and media was sterilized at 120 ºC temperature and 15 lbs pressure by using autoclaved.

3.2.b. Sabouraud dextrose agar (SDA)

50 ml distilled water was taken in 250 ml of flask. 1.5 gm of sabouraud dextrose agar broth and 0.5 gm of agar were added to 50 ml of distilled water. The flask was covered with cotton plug and media was sterilized at 120 ºC temperature and 15 lbs pressure by using autoclave. SDA plate used for culture maintainance.

3.2.c. Roswell Park Memorial Institute (RPMI) 1640

1.72 gm 3-(N-morpholino) propanesulfonic acid (MOPS) powder was added to 50 ml of sterile distilled water. It was stirred by glass rod till the MOPS dissolves completely in the sterile distilled water. 0.52 gm of RPMI was added to 50 ml of MOPS buffer. The pH was adjusted up to 6.5. The medium was filter sterilized by using syringe filter

with pore size 0.22 µm. For filtration the medium was filled in syringe of 20 ml capacity and it was passed through sterile syringe filter in 50 ml of sterile falcon tube. 200 µl RPMI medium was added in 1.5 ml sterile eppendorf tube and kept in incubator for 24 h at 37 ºC to ensure its sterility. The clear medium confirms the sterility and used for the experiments. If medium found turbid the that batch of medium was desterilised. RPMI 1640 medium supports *C. albicans* growth by providing essential nutrients and maintaining a stable environment, making it suitable for various experimental applications.

3.2.d. XTT reagent

10 mg XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) weighed and added to the 10 ml of distilled water. It was filter sterilized by membrane syringe filter of pore size 0.22 µm. 10mM stock of menadione was used. 0.0172 gm menadione was added to 10 ml of acetone. To prepare working stock of XTT, 990 µl from the XTT stock and 10 µl of menadione from stock were mixed together to male 1 ml of working XTT solution. The XTT assay measures metabolic activity by detecting color changes in viable *C. albicans* cells, allowing for quantitative assessment of biofilm formation and adhesion. Its colorimetric output directly correlates with the level of cell activity within the biofilm.

3.2.e. Potassium phosphate buffer (PBS)

a) 0.68 gm of potassium hydrogen phosphate (K_2HPO_4) was added to 50 ml of distilled water.

b) 3.48 gm of potassium dihydrogen phosphate (KH_2PO_4) was added in 250 ml of distilled water.

40 ml of solution (a) and 160 ml of solution (b) were mixed together to make 200 ml of 10X Potassium phosphate buffer. pH was adjusted to 7. To make 1X Potassium phosphate buffer 10 ml of 10 X buffer was added to 90 ml of distilled water to make final volume 100 ml. Then it was sterilized by using autoclave at 120 ℃ temperature and 15 lbs. pressure. PBS is used in *C. albicans* experiments to provide a stable, isotonic environment that preserves cell integrity, facilitates washing and handling, and minimizes disruption of cellular processes.

3.2.f. 20 % Fetal Bovine Serum (FBS)

The fetal bovine serum used for yeast to hyphal morphogenesis was prepared by addition of 20 ml of fetal bovine serum in 80 ml of sterile distilled water. The serum was sterilized by using syringe filter 0.22 µm diameter before performing the experiments. FBS provides essential growth factor that support the transition of *C. albicans* from yeast to hyphal form. This facilitates accurate study of its developmental processes in experiments.

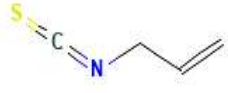
3.2.g. Alcoholic Potassium hydroxide (KOH)

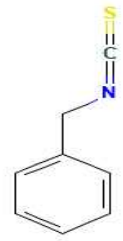
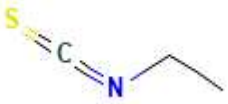
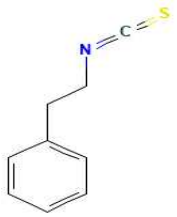
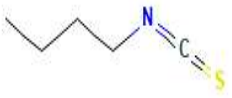
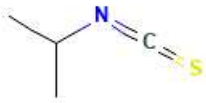
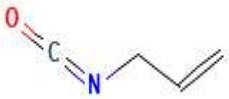
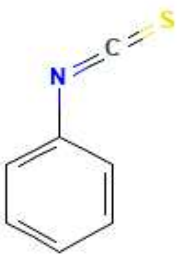
The alcoholic KOH was prepared by addition of 5 gm of KOH in 7 ml of sterile distilled water. Then 13 ml of 99.9 % absolute alcohol was added to the above solution to make final volume 20 ml. Alcoholic KOH solubilises fungal cell membranes, facilitating the extraction and quantification of ergosterol in *C. albicans*. This is essential for studying ergosterol levels and assessing drug effects.

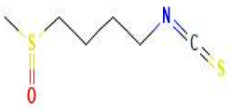
3.3. Glucosinolate derivatives used for screening

Glucosinolate derivatives for screening were chosen based on their availability. These compounds were purchased from Sigma Aldrich Chemical Ltd., Mumbai, India. Their list is provided in **Table 3.1** Stock solutions of each compound was prepared as 200 mg/ml concentration with appropriate solvent Dimethyl sulphoxide (DMSO) and then diluted in growth medium in the range of 0.0039 to 2 mg/ml range (total concentration of DMSO is not more than 1 %).

Table 3.1. Molecular formula, solubility, stock concentration and structures of glucosinolate derivatives used in the study.

Sr. No.	Compounds	Molecular formula	Solubility	Stock solution	Structure
1.	Allyl isothiocyanate (AITC)	C_4H_5NS	DMSO	200 mg/ml	

2.	Benzyl isothiocyanate (BITC)	C_8H_7NS	DMSO	200 mg/ml	
3.	Ethyl isothiocyanate (EITC)	C_3H_5NS	DMSO	200 mg/ml	
4.	Phenyl ethyl isothiocyanate (PEITC)	C_9H_9NS	DMSO	200 mg/ml	
5.	Butyl isothiocyanate (ButylITC)	C_5H_9NS	DMSO	200 mg/ml	
6.	Isopropyl isothiocyanate (IPITC)	C_4H_7NS	DMSO	200 mg/ml	
7.	Allyl isocyanate (AIC)	C_4H_5NO	DMSO	200 mg/ml	
8.	Benzoyl isothiocyanate (BZITC)	C_8H_5NOS	DMSO	200 mg/ml	
9.	Phenyl isothiocyanate (PITC)	C_7H_5NS	DMSO	200 mg/ml	

10.	Indole-3-acetonitrile (I3A)	$C_{10}H_9NO_2$	DMSO	100 mg/ml	
11.	sulforaphane	$C_6H_{11}NOS_2$	DMSO	40 mg/ml	

3.4. Minimum inhibitory concentration (MIC)

To analyse the effect of glucosinolate derivatives on planktonic cells of *C. albicans* micro broth dilution method was used as per the guidelines of clinical laboratory standard institute (CLSI) CLSI-M27-A3 (1). Inoculum of 1×10^3 cells and various concentrations of glucosinolate derivatives ranging from 0.0039 to 2 mg/ml prepared in RPMI-1640 medium were added in 96-well plate. Total volume of micro broth dilution assay was kept 200 µl, wells without test molecule served as control. The plates were incubated for 48 h. at 35 ºC. Absorbance was taken at 620 nm with the help of spectrophotometer (Multiskan Ex, Thermo Electron Corp., USA) to analyse the growth. The lowest concentration of glucosinolate derivatives which caused 50 % inhibition as compared to control was considered as MIC for growth of *C. albicans* (2).

3.5. Adhesion assay

Effect of glucosinolate derivatives on the adherence of *C. albicans* to the polystyrene surface was analysed using micro plate based assay. Inoculum of 1×10^7 cells and various concentrations of glucosinolate derivatives ranging from 0.0039 to 2 mg/ml prepared in PBS and were added in 96-well plate. Wells without glucosinolate derivatives considered as control. The plates were incubated for 90 min at 37 ºC and 100rpm in an orbital shaker incubator to allow attachment of cells on the polystyrene surface. After incubation wells were washed with PBS to remove non-adhere cells. Density of adherence in each well was examined by XTT metabolic assay and percentage of adherence of cells was calculated. The concentration which shows 50 % reduction in adherence as compared to control was considered as MIC concentration (3).

3.6. Determination of Minimum Fungicidal concentration (MFC)

Effect of glucosinolate derivatives on growth of *C. albicans* was visibly assessed using Minimum fungicidal concentration assay. 10 µl of cell suspension from the wells of MIC and sub MIC concentrations of glucosinolate derivatives were selected to assess MFC and spread on YPD agar plate. YPD agar plates were incubated at 30 ºC for 48 h. After 48 h., plates were observed. The ones showed fewer growth or no growth were selected and photograph (2).

3.7. Yeast to hyphal (Y-H) Morphogenesis

A microplate-based assay was used to investigate *C. albicans'* Y-H morphogenesis that was triggered by fetal bovine serum. In 20 % serum, glucosinolate derivatives was prepared at concentrations ranging from 0.0039 to 2 mg/ml. 1×10^6 cells/ml of cells were prepared and added to the control and test wells of the microtiter plate. Plates was incubated at 37 °C with 200 rpm for 2 h. Using an inverted microscope, the cells' production of germ tubes was observed. An Inverted microscope was used to count the quantity of yeast cells and hyphae (4).

3.8. Early biofilm

To assess the effect of glucosinolate derivatives on developing biofilm of *C. albicans*, biofilms were developed on tissue culture treated 96 well polystyrene plate by addition of 100 µL cell suspension of 1×10^7 cells/ml and plates were incubated at 37 ºC, 100rpm for 90 min. Non-adhere cells were removed by washing the wells with PBS. Various concentrations of glucosinolate derivatives ranging from 0.0039 to 2 mg/ml were prepared in RPMI 1640 medium and added in wells and incubated at 37 º C for 48 h. After 48 h. wells were washed with PBS and biofilm growth was analysed by using XTT metabolic assay (5).

3.9. Mature biofilm

C. albicans 24 h. old biofilms were used to analyse the effect of glucosinolate derivatives on mature biofilm. Various concentrations of glucosinolate derivatives ranging from 0.0039 to 2 mg/ml prepared and added along with RPMI 1640 medium.

Plate was incubated at 37 ºC for 48 h. after incubation wells were washed with PBS and mature biofilm was analysed by using XTT metabolic assay (6).

3.10. XTT analysis

The growth of the biofilm was quantified by performing an XTT metabolic analysis. The wells containing the biofilms were rinsed with PBS to get rid of any non-adherent cells before being incubated at 37 °C for 5 h. in the dark with 100 µl of the XTT-Menadione solution. A microplate reader was used to measure the coloration produced by the water-soluble formazan product at 450 nm (5) .

3.11. Scanning Electron Microscopy (SEM)

For visualization of effect of glucosinolate derivatives on biofilm of *C. albicans* scanning electron microscopy (SEM) was performed. 2 ml of cell suspension of *C. albicans* cells (1×10^7 cells/ml) were treated with and without glucosinolate derivatives at a biofilm inhibitory concentration and incubated a 37 ºC for 48 h. on Foley catheter. The cells were washed with sterile PBS and fixed in a mixture of 2 % glutaraldehyde in 0.1 M % phosphate buffer (P^H 7.2), and left at 4 ºC temperature for 24 h. Following this, cells were washed with 0.1 % of PBS, and post fixed in osmium tetroxide (OsO_4) for 4 h. Subsequently, the cells were dehydrated in grades of alcohol. After the samples were completely dry, samples were coated with gold coater and examined with SEM (TESCAN Model- VEGA 3) at Shivaji University Kolhapur (7).

3.12. Determination of ergosterol biosynthesis

To assess the effect of glucosinolate derivatives on ergosterol biosynthesis, a conventional technique was used to extract all of the intracellular sterols from *C. albicans*. For the control and planktonic MIC and sub MIC concentrations of glucosinolate derivatives. 50 ml of sabouraud dextrose broth was inoculated with a single colony of *C. albicans* from an overnight sabouraud dextrose agar plate culture. After 16 h. of incubation, the cultures were collected by centrifugation at 2700 rpm for 5 minutes. The cell pellet's net weight was noted. In each pellet 3 ml of a 25 % alcoholic potassium hydroxide solution was added before being vortexed for 3 min. Cell suspensions were put into sterile borosilicate glass screw-cap tubes and incubated at 85

°C in a water bath for an hour before being allowed to cool at room temperature. The next step was to extract the sterols by adding 1 ml of sterile distilled water and 3 mL of n-heptane, followed by 3 min. of vigorous vortex mixing. The n-heptane layer was transferred to a spotless borosilicate glass screw-cap tube and kept at 20 °C. Using a Microplate Spectrophotometer (Mutiskan SkyHigh), the concentration of sterol was scanned spectrophotometrically between 230 and 300 nm (8).

3.13. Intracellular reactive oxygen species (ROS) production measurement

To assess the effect of glucosinolate derivatives on intracellular ROS accumulation following experiment was performed. A fluorescent dye 2′,7′ dichlorofluorescein diacetate (DCFH-DA) was used to measure the ROS levels of *C. albicans*. The cells were cultured for 4 and 24 h. at 30 °C and 37 °C with and without the planktonic and biofilm MIC concentration of glucosinolate derivatives respectively, 1.5 mM/L of hydrogen peroxide. The cells were then collected, and washed twice with PBS buffer. Cells were put in 3 ml of PBS. In each cell suspension 10 µM of the fluorescent dye DCFH-DA added and incubated at 30 °C for an hour. A fluorescent spectrophotometer was used to measure the amount of fluorescence (Excitation-485 nm and Emission at 530 nm) (9).

3.14. Cell cycle analysis

Log-phase cells of *C. albicans* (1×10^6 cells) were collected and treated with planktonic MIC (2 mg/ml) concentration of glucosinolate derivatives without MIC_{50} concentration of glucosinolate derivatives considered as control, incubated at 30 °C for 4 h. The cells were washed with chilled PBS and fixed in 70 % chilled ethanol for overnight at 4 °C. Next day cells were washed with PBS and treated with RNase-A (10 µg). For DNA staining 50 µg/ml propidium iodide (PI) was added and incubated for 1 h. at 4 °C in the dark. The cells were analysed using FACS (FACS Diva Version 6.1.3) (10).

3.15. Gene expression study with quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

To assess the effect of glucosinolate derivatives on genes involved in signal transduction pathway for biofilm formation gene expression study performed with

qRT-PCR. For RNA extraction *C. albicans* biofilms were developed in tissue culture treated plate. The developed biofilm treated with and without biofilm inhibitory concentration of glucosinolate derivatives, incubated for 24 h. at 37 ^{o}C. After incubations cells were harvested by centrifugation and washed with PBS. Total RNA was extracted with RNeasy mini kit (QIAGEN, Valencia, CA, USA) and was reverse transcripted to cDNA using Super Script III (Invitrogen, Life technologies, Camarillo, CA, USA). With the help of UNI SYBR GREEN SUPERMIX, PCR were carried out (Biorad Real Time PCR Machine, 0.2 ml, 96 wells) in 96 well PCR plates. The qPCR reaction total volume was 10 µl. Primers purchased from geneombiome Technologies Pvt Ltd.; Pune (**Table 3.2**) was added in SYBR GREEN SUPER MIX in predetermined ratio. The genes used in this study were involved in signal transduction pathway and important for pathogenicity of *C. albicans*. The housekeeping gene ACTIN 1 was used as internal control. Expression of gene were analysed with the help of thermal cycler (Real Time System Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the relative gene expression of control and treated cells was determined using $2^{-\Delta\Delta CT}$ method (11).

Table 3.2. Gene specific primers used for real time polymerase chain reaction

Sr. No.	Primers	Sequences
1.	**ACTIN**	F- 5'ATGGACGGTGAAGAAGTTGC 3' R- 5'ACCTCTTTTGGATTGGGCTTCA 3'
2.	**RAS**	F- 5'GGCCATGAGAGAACAATATA 3' R- 5'GTCTTTCCATTTCTAAATCAC 3'
3.	**PDE2**	F- 5'ACCACCACCACTACTACTAC 3' R- 5'AAAATGAGTTGTTCCTGTCC 3'
4.	**BCY1**	F- 5′CCCAAGCTTATGTCTAATCCTCAACAGCA 3′ R- 5′GGGCTGCAGTTAATGACCAGCAGTTGGGT 3′
5.	**EFG1**	F- 5' TATGCCCCAGCAAACAACTG 3' R- 5' TTGTTGTCCTGCTGTCTGTC3'
6.	**TEC1**	F- 5' AGGTTCCCTGGTTTAAGTG 3' R- 5' ACTGGTATGTGTGGGTGAT 3'
7.	**ECE1**	F- 5'-CCCTCAACTTGCTCCTTCACC-3'

		R- 5'-GATCACTTGTGGGATGTTGGTAA-3'
8.	**CEK1**	F- 5' AGCTATACAACGACCAATTAA 3' R- 5' CATTAGCTGA ATGCATAGCT 3'
9.	**HST7**	F- 5' ACTCCAACATCCAATATAACA 3' R- 5' TTGATTGACGTTCAATGAAGA 3'
10.	**CPH1**	F- 5'ATGCAACACTATTTATACCTC 3' R- 5'CGGATATTGTTGATGATGATA 3'
11.	**CDC35**	F- 5'TTCATCAGGGGTTATTTCAC 3' R- 5'CTCTATCAACCCGCCATTTC 3'
12.	**HWP1**	F- 5'TGGTGCTATTACTATTCCGG 3' R- 5'CAATAATAGCAGCACCGAAG 3'
13.	**MIG1**	F- 5'CTTCAACTAGCCTATATTCCGATGG 3' R- 5'-CTTTCT GTAGGTACCAACAACTAC 3'
14.	**NRG1**	F- 5'CACCTCACTTGCAACCCC 3' R- 5'GCCCTGGAGATGGTCTGA 3'
15.	**TUP1**	F- 5' GAGGATCCCATGTATCCCCAACGCACCCAG 3' R- 5'GGCGACGCGTCGTTTTTTGGTCCATTTCCAAATTCTG 3'

3.16. Toxicity assay

Human Red Blood Cells (RBCs) were used to study the toxicity of glucosinolate derivatives. There were no ethical concerns involved in the toxicity assay process. The institutional ethical committee gave its approval to the toxicity assay protocol. Centrifugation was performed on the blood (5 ml) drawn from healthy volunteers in an ethylenediamine tetraacetic acid (EDTA)-containing tube at 2000 rpm for 10 min at 20 °C. RBC pellet was suspended in PBS at 10 % volume by volume. Before use, RBC Suspension was diluted in PBS 1: 10 proportion. 100 ml aliquots from the suspension were mixed with 100 ml of AITC at a different concentration in the same buffer in Eppendorf tubes. 1 % Triton X 100 was used for total hemolysis. After incubation of 1 h at 37 °C it was centrifuged for 10 min at 2000 rpm at 20 °C. Optical density (OD) was obtained at 450 nm after 150 µl of supernatant was transferred to a microtiter plate with a flat bottom (12). All the experiments were done in triplicates. The hemolysis percentage was calculated by following formula:

% of Hemolysis = [[A_{450} of test compound treated Sample - A_{450} of buffer treated sample] / [A_{450} of 1%TritonX 100 treated sample - A_{450} of buffer treated sample]] × 100.

3.17. *In vivo* study in silkworm animal model

Department of Zoology at Shivaji University in Kolhapur provided third-instar silkworm larvae (*Bombyx mori*), which were fed mulberry leaves and kept alive until they reached the fifth instar. For our study, we only selected 1.9–2.2 g of silkworm larvae. Cells of *C. albicans* were cultured in YPD broth for an overnight period before being washed and re-dissolved in phosphate-buffered saline. An insulin syringe was used to inject a 1×10^6 cells into the haemolymph through the dorsal surface of a silkworm larva. The planktonic MIC concentration of glucosinolate derivatives was injected into the haemolymph to assess its anti-*C. albicans* effectiveness. Silkworms injected with fluconazole and *C. albicans* were considered as standard. Silkworm injected with DMSO. The mortality of silkworms was measured every 8 h. up to 48 h. Throughout the studies, silkworm larvae were maintained at 25 °C, and survival was noted (13). Experiments were carried out in triplicates. Specification of groups used in *in vivo* silkworm animal model experiments were mentioned in **Table 3.3**.

Table 3.3. Specification of groups used in *in vivo* silkworm animal model experiment

Group I	Positive control	Silkworm injected with *C. albicans*
Group II	Negative control	Silkworm injected with PBS
Group III	Test	Silkworm injected with *C. albicans* + planktonic MIC concentration of glucosinolate derivatives.
Group IV	DMSO	Silkworm injected with DMSO (1%)
Group V	Standard	Silkworm injected with *C. albicans* and fluconazole MIC concentration (0.15µg/ml)

3.18. Statistical analysis

Values mentioned were the mean with standard deviations, obtained from three different observations. Values in the control and treatment groups for various molecules were compared using Student's *t* -test. A value of $P < 0.05$ was considered statistically significant.

3.19. Ethical statement

Ethical statement in the present study, Human Red Blood Cells (RBCs) were collected from the blood bank. The experimental protocol and the use of RBCs were assessed and approved by the Institutional Ethical Committee (IEC), D. Y. Patil Education Society (Deemed to be University), Kolhapur (IEC Ref No: DYPMCK/425/202/IEC). All methods were carried out in accordance with relevant guidelines and regulations.

3.20. References

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CHAPTER – 4

RESULTS

4. Results

4.1. Anti-*C. albicans* activity of Allyl isothiocyanate (AITC)

4.1.1. Antifungal activity of AITC on *C. albicans* planktonic growth

The inhibitory effect of AITC on planktonic growth of *C. albicans* was assessed. The minimum inhibitory concentration (MIC) of AITC for *C. albicans* was found to be at 0.125 mg/ml (**Fig. 4.1.1.a**).

4.1.2. Effect of AITC on adhesion of *C. albicans*

Adhesion plays vital role in biofilm formation and infection in *C. albicans*. The inhibitory effect of AITC on *C. albicans* to polystyrene surface were quantified by XTT metabolic assay. AITC inhibited adhesion to polystyrene surface to an extent of 58 % at 0.125 mg/ml and at concentrations of 0.25 mg/ml, 0.5 mg/ml, 1 and 2 mg/ml AITC significantly decreased adhesion to an extent of 28 %, 25 %, 21% and 21 %, respectively (**Fig. 4.1.1.a**).

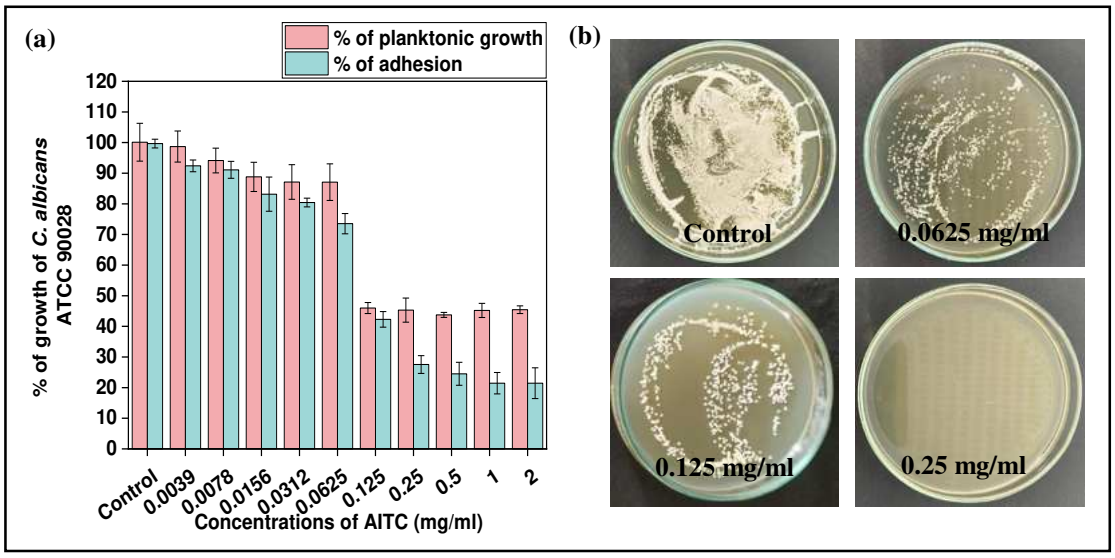

Fig.4.1.1. (a) Inhibition of *C. albicans* planktonic growth and adhesion to polystyrene surface by allyl isothiocyanate at 0.125 mg/ml concentration. **(b)** Bioassay to determine the minimum fungicidal concentration (MFC) for *C. albicans* 90028, allyl isothiocyanate at 0.25 mg/ml was found to entirely inhibit colony survival.

4.1.3. Minimum fungicidal concentration of AITC

The minimum fungicidal concentration of AITC was assessed by spread plate technique on YPD agar plate with the help minimum inhibitory concentration (MIC) concentration and results indicates that AITC was fungicidal in nature at its MIC and above concentrations (**Fig. 4.1.1.b**).

4.1.4. Inhibitory effect of AITC on germ tube formation of *C. albicans*

In this study, analysed the effect of AITC on germ tube formation of *C. albicans* using fetal bovine serum (FBS) growth media. AITC inhibited germ tube formation in a concentration dependent manner. The effect was studied at various concentrations ranging from 0.0039 to 2 mg/ml. At 0.125 mg/ml concentration AITC completely inhibited germ tube formation (**Fig. 4.1.2.a**).

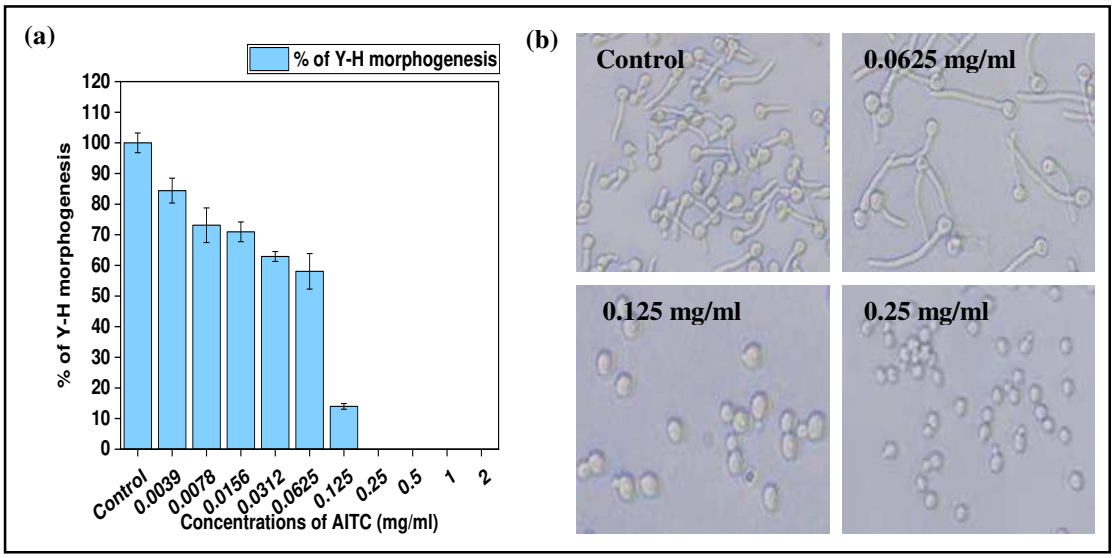

Fig.4.1.2. (a) Effect of allyl isothiocyanate against the yeast to hyphal (Y-H) morphogenesis of *C. albicans* ATCC 90028 strain treated by various concentrations of allyl isothiocyanate 0.0039 to 2 mg/ml. **(b)** Inhibition of fetal bovine serum induced yeast to hyphal growth was visualized by Inverted Microscopy at a concentration of 0.125 mg/ml.

4.1.5. Effect of AITC on biofilm formation (Developing and Mature biofilm)

AITCs anti-biofilm activity was evaluated against the *C. albicans* ATCC 90028 strain. At a dose of 0.5 mg/ml, AITC inhibits the developing biofilm and at same

concentration, AITC eradicate mature biofilm as shown by the XTT metabolic assay (**Fig. 4.1.3.a**).

4.1.6. Effect of AITC on developing biofilm with scanning electron microscopy

It is well known that *C. albicans* biofilm production plays a crucial role in human yeast infections, particularly those affecting implanted medical devices. When AITC treatment (0.5 mg/ml) was compared to the control sample, the scanning electron microscopy analysis showed a significant inhibition in the biofilm formation (**Fig. 4.1.3.b**). Additionally, while examining under a microscope, it was found that in control sample dense biofilm observed with hyphal structure, while AITC effectively inhibited biofilm formation at 0.5 mg/ml concentration.

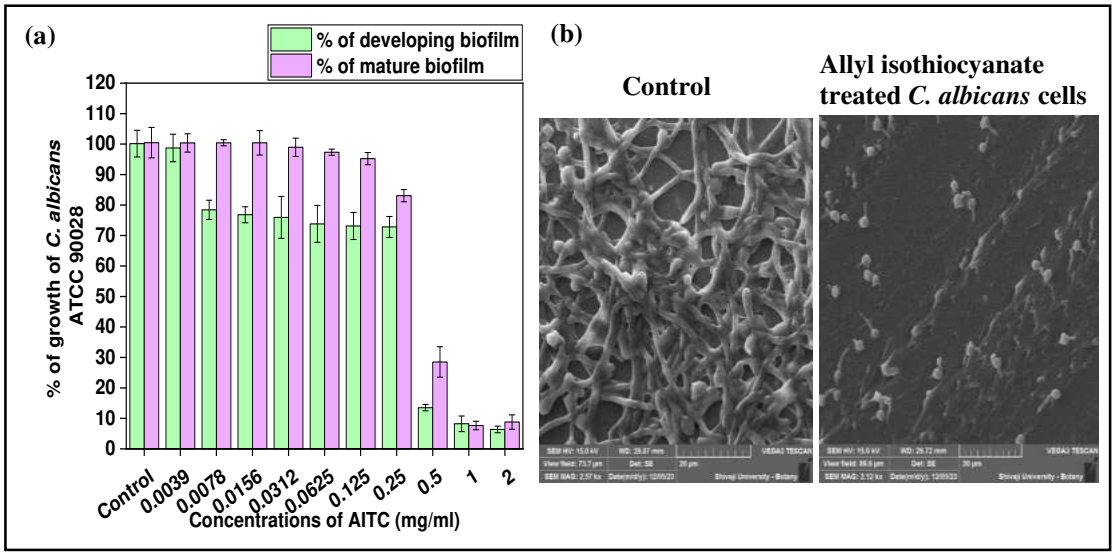

Fig. 4.1.3. **(a)** Effect on *C. albicans* developing and mature biofilm formation by the treatment of allyl isothiocyanate. **(b)** Scanning electron microscopy, showing the dense biofilm with hyphal structures of untreated cell (control) and inhibition of biofilm formation by the treatment of 0.5 mg/ml concentration of allyl isothiocyanate.

4.1.7. Effect of AITC on *C. albicans* cell membrane

Cell membrane is the primary target for many standard antifungal drugs like azoles and polyenes. Therefore, in present study observed ergosterol content in *C. albicans* cells upon treatment with AITC. Using ergosterol biosynthesis, the impact of AITC on the cell membrane of *C. albicans* was examined. We measured the ergosterol concentration

of AITC treated and untreated *C. albicans* cell membranes. Untreated control cells show characteristic peaks that signifies ergosterol production. However, ergosterol biosynthesis inhibition in *C. albicans* cells treated with AITC at planktonic MIC concentration and below MIC concentration in the range of 0.0078 to 0.125 mg/ml is represented by a flat curve (**Fig. 4.1.4.a**).

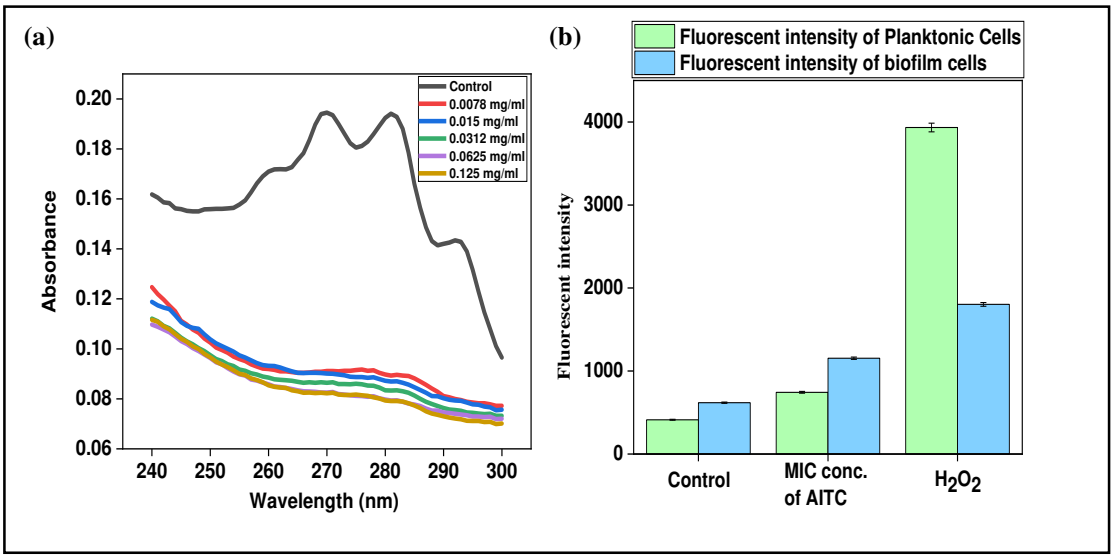

Fig. 4.1.4. (a) Inhibition to ergosterol biosynthesis by the treatment of planktonic inhibitory and sub inhibitory concentration of allyl isothiocyanate. **(b)** Detection of ROS level after treatment of allyl isothiocyanate using DCFH-DA staining involved in the planktonic and biofilm cells of *C. albicans*.

4.1.8. AITC induced intracellular reactive oxygen species (ROS) generation in *C.*

Under extremely stressful conditions, *C. albicans* cells are known to produce ROS. High concentrations of ROS, including superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, hypochlorous acid, and hydroperoxyl radicals, may interact with biological components such lipids, proteins, and nucleic acids, resulting in oxidative stress and ultimately cell death. The fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to assess AITCs ability to stimulate endogenous ROS generation in *C. albicans* (**Fig. 4.1.4.b**). In Figure 4.1.4.b it was demonstrated that AITC increased fluorescence intensity, which demonstrates AITCs capacity to boost endogenous ROS production. AITC promoted intracellular ROS generation at its 0.125 mg/ml planktonic inhibitory concentration. However, increase in the production of

ROS after the treatment of AITC as compared to non-treated *C. albicans* cells may be responsible for inhibition of *C. albicans* planktonic growth. Along with this AITC treatment also increased ROS production in *C. albicans* biofilm at 0.5 mg/ml concentration. Increase in ROS production in biofilm cells of *C. albicans* may be a reason for anti-biofilm activity of *C. albicans*.

4.1.9. Effect of AITC on cell cycle

In current study examined the impact of AITC on the *C. albicans* cell cycle. In order to understand how AITC affected DNA replication and cell division, two crucial and tightly controlled processes for a cells growth and multiplication, cell cycle study was conducted. Any irregularity in DNA replication and cell division triggers the DNA damage checkpoint pathway, which halts all processes related to growth and division until the damage is repaired. In a cell cycle analysis investigation, *C. albicans* cells exposed to a planktonic inhibitory dose of AITC (0.125 mg/ml) and compared them to untreated cells. DNA content present during various cell cycle stages was measured based on the fluorescence intensity produced by propidium iodide to confirm cell cycle arrest in *C. albicans*. In control, 61.1 % cells were arrested in G0/G1 phase, 30.7 % in S phase and 8.0 % in G2/M phase while in the test, 14.6 % cells were arrested in G0/G1 phase, 30.9 % in S phase and 54.1 % in G2/M phase (**Fig. 4.1.5.a**). The results suggest that AITC arrested cells in G2/M phase of *C. albicans*.

4.1.10. Effect of AITC on signal transduction gene expression involved in biofilm inhibition of *C. albicans*

By using qRT PCR analysis, the impact of AITC on *C. albicans* biofilm inhibition was evaluated at the transcriptional level. The impact of 0.125 mg/ml concentration of AITC on biofilm formation revealed a notable inhibition in *C. albicans* biofilm as compared to non-treated control. qRT-PCR analysis showed a relative fold change in the gene expressions. AITC downregulated the expression of *CPH1, PDE2, BCY1, HWP1, MIG1, RAS1, ECE1, NRG1, CEK1, TUP1* and *HST7* by 5.26, 2.44, 2.33, 12.50, 9.09, 3.03, 1.25, 1.79, 1.67, 625 and 1.25 respectively. AITC upregulated the expression of *CDC35, EFG1* and *TEC1* by 3.49, 2.46 and 1.20, respectively. AITC inhibited one of the important virulence factor i.e. biofilm formation may be due to downregulation of

CPH1, *PDE2, BCY1, HWP1, TEC1, RAS1, ECE1* and *HST7* genes (**Fig. 4.1.5.b**).

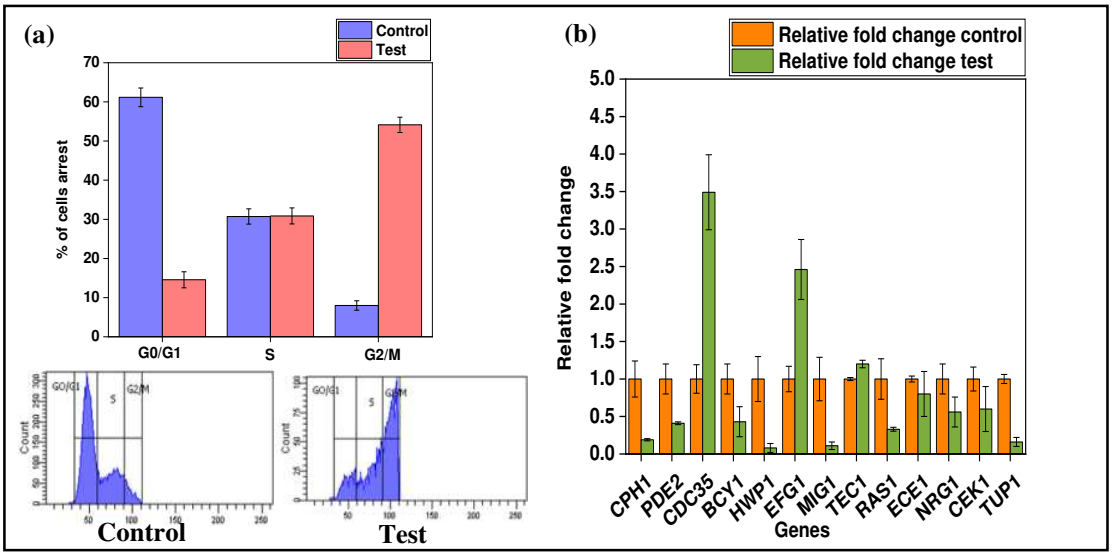

Fig.4.1.5. (a) Cell cycle arrest at G2/M phase by allyl isothiocyanate in *C. albicans* cells. **(b)** Effect of allyl isothiocyanate on signal transduction genes involved in biofilm formation.

4.1.11. Toxicity effect of AITC on human red blood cells (RBCs)

The toxicity of AITC was evaluated through an *in vitro* hemolysis assay on human red blood cells (RBCs). The test involved exposing the RBCs to AITC at concentrations ranging from 0.0039 mg/ml to 2 mg/ml. The results, as shown in Figure **4.1.6.a**, indicated that AITC did not cause hemolysis at any of these concentrations, meaning it did not significantly disrupt the RBC membranes or release hemoglobin into the surrounding solution. This finding demonstrates that AITC is non-hemolytic within the tested concentration range, suggesting it does not pose a significant risk to RBC integrity.

4.1.12. *In vivo* study using silkworm animal model

In vivo experiment was conducted in silkworm (*Bombyx mori*) to examine the antifungal efficacy of AITC against *C. albicans*. Silkworms injected with *C. albicans* cells were considered as positive control and silkworms injected only with PBS were considered as negative control. Silkworms injected with AITC (0.125 mg/ml) and *C. albicans* were considered as test. Positive control silkworms died within 1day while

negative control silkworms completed their life cycle and underwent cocoon phase. Whereas silkworm injected with AITC and *C. albicans* cells survived and completed their life cycle (**Fig. 4.1.6.b).**

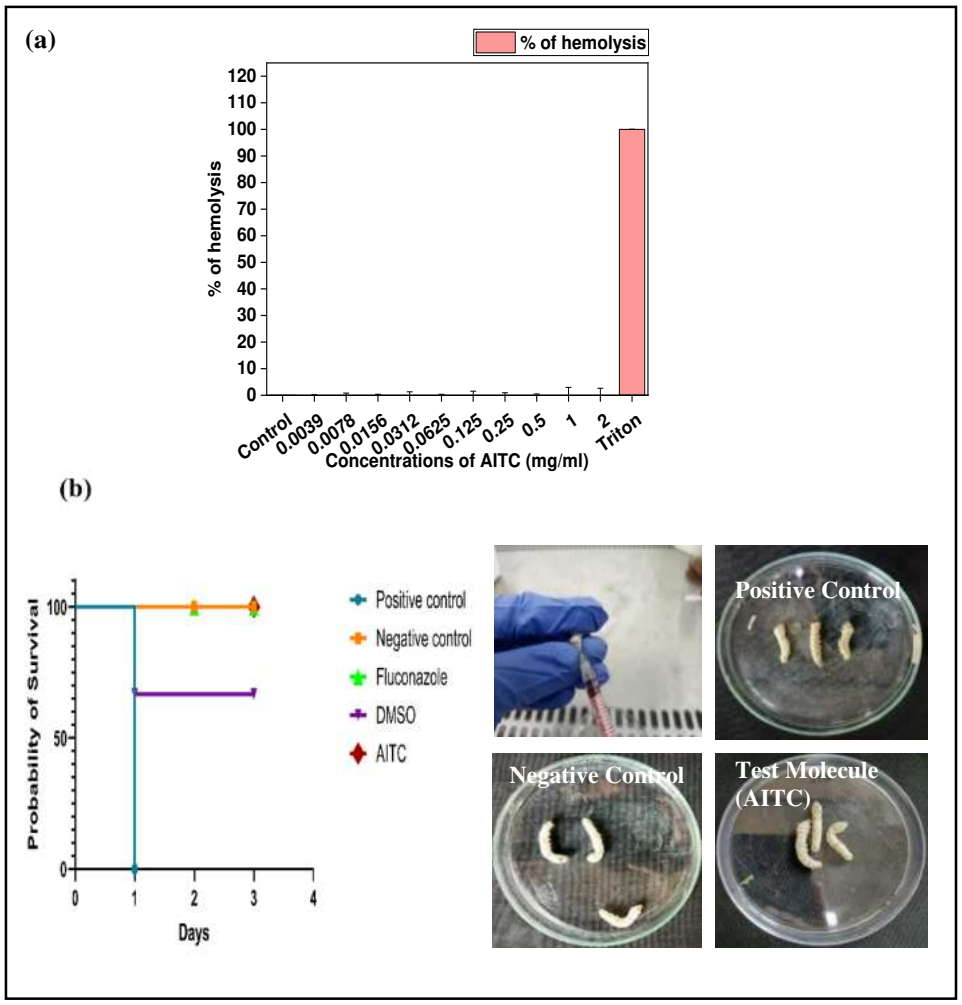

Fig.4.1.6. (a) Hemolysis assay on erythrocyte. A relative rate of hemolysis on human erythrocytes observed following 1 h incubation with different concentrations of allyl isothiocyanate at 37 ^{o}C. (**b)** Antifungal effect of allyl isothiocyanate on survival of silkworm infected with *C. albicans.*

4.1.13. Conclusions

AITC demonstrated potent antifungal activity against *C. albicans* against various factors. It inhibited planktonic growth at a minimum inhibitory concentration (MIC) of 0.125 mg/ml and hampers adhesion to polystyrene surfaces. AITC also showed

fungicidal properties at 0.25 mg/ml concentration and above. Moreover, it effectively suppressed germ tube formation and biofilm development, both in developing and mature stages. AITC's mode of action involved disrupting ergosterol biosynthesis, inducing intracellular reactive oxygen species (ROS) generation, arresting the cell cycle at the G2/M phase. Further gene expression study analysis revealed that AITC inhibits developing biofilm by downregulating the expression of signal transduction genes *CPH1*, *PDE2, BCY1, HWP1, TEC1, RAS1, ECE1* and *HST7*. Importantly, AITC exhibited no hemolytic activity on human red blood cells and demonstrated promising antifungal efficacy in an *in vivo* silkworm model. These findings suggest AITC as a potential candidate for combating *C. albicans* infections with multiple mechanisms of action and no toxicity profile.

Results

4.2. Anti-*C. albicans* activity of Ethyl isothiocyanate (EITC)

4.2.1. EITC inhibited planktonic growth of *C. albicans*

Initially we studied the effect of EITC on planktonic growth of *C. albicans* ATCC 90028 strain using micro broth dilution method. For this purpose, in current study exposed *C. albicans* cells to a series of concentrations of EITC in the range of 0.0039 to 2 mg/ml for 48 h. After 48 h of incubation 50 % reduction in planktonic growth of *C. albicans* was seen at 0.5 mg/ml concentration compared to non-treated control. At 1 mg/ml and 2 mg/ml concentrations 58 % and 73 % reduction was seen, respectively (**Fig. 4.2.1.a**).

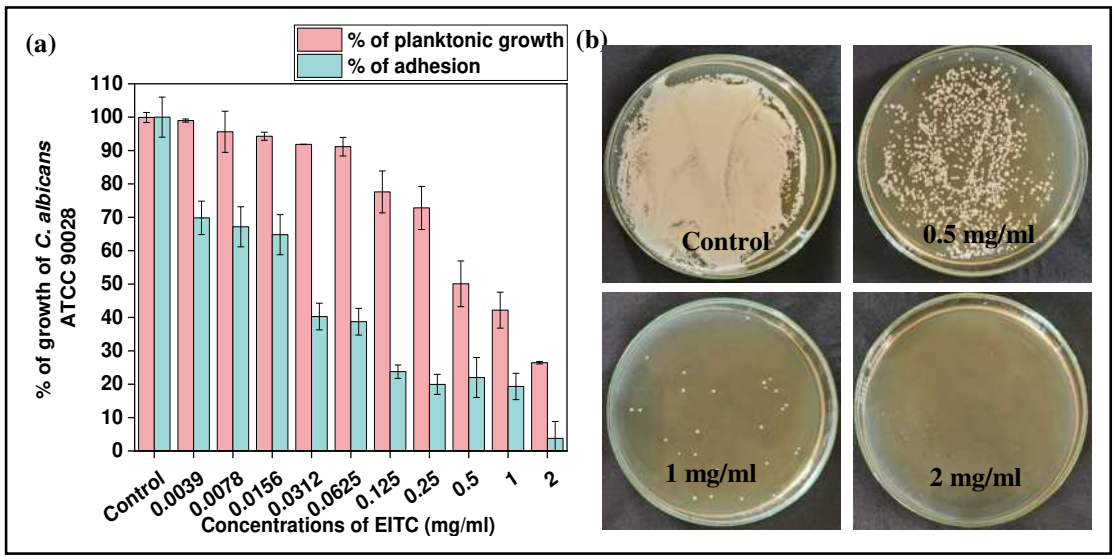

Fig. 4.2.1. (a) Determination of the antifungal activity of ethyl isothiocyanate against drug-resistant *C. albicans* planktonic growth and adhesion. **(b)** The candidacidal activity of ethyl isothiocyanate was seen at 2 mg/ml concentration by using minimum fungicidal concentration assay.

4.2.2. EITC inhibited of Adhesion *C. albicans* cells to polystyrene surface

We examined the effect of EITC on the adhesion of *C. albicans* to polystyrene surface because infections and biofilm formation of *C. albicans* start from adhesion. The result suggests that EITC at 0.0312 mg/ml concentration decreased 60 % viability of adhered cells to the polystyrene surface (**Fig.4.2.1.a**). Treatment above 0.0312 mg/ml

concentration inhibited approximately 80 % - 90 % of the adhesion compared to the non-treated control groups. From above observation it is concluded that EITC inhibits the adhesion of *C. albicans* cells to the polystyrene surface.

4.2.3. Minimum fungicidal concentration (MFC)

Exposure to EITC affected the viability of *C. albicans* cells. EITC was fungicidal in nature at 2 mg/ml concentration. 99 % of killing of *C. albicans* cells was observed on YPD agar plate (**Fig. 4.2.1b**).

4.2.4. EITC inhibited Yeast to Hyphal (Y-H) Morphogenesis in *C. albicans*

The yeast to hyphal form morphogenesis plays vital role in the pathogenesis of *C. albicans* infections and is considered as the important virulent trait of *C. albicans*. Therefore, in current study assessed the effects of EITC on the Y-H morphogenesis by using fetal bovine serum growth medium. Serum induced Y-H morphogenesis of *C. albicans* ATCC 90028 strain was inhibited by EITC at 0.0312 mg/ml concentration. It was observed that concentrations above MIC showed complete inhibition of Y-H morphogenesis of *C. albicans* compared to non-treated control (**Fig. 4.2.2. a, b**).

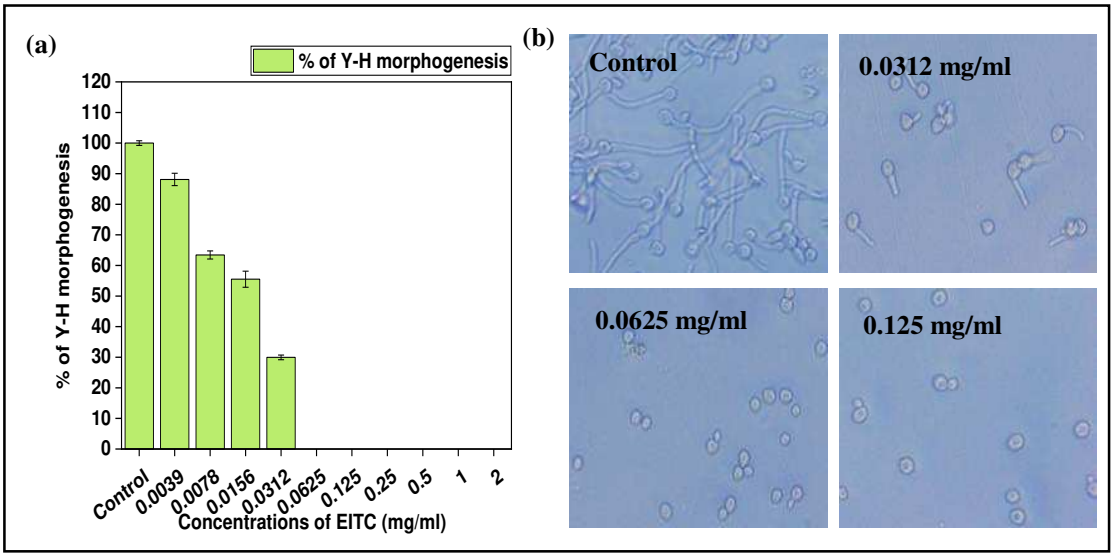

Fig. 4.2.2. (a) Effect of ethyl isothiocyanate against the yeast to hyphal (Y-H) morphogenesis of *C. albicans* 90028 strain treated by various concentrations of ethyl isothiocyanate 0.0039 to 2 mg/ml. **(b)** The inhibition of *C. albicans* observed with inverted microscopy.

4.2.5. Anti-biofilm activity of EITC

The anti-biofilm ability of EITC at different concentration ranges between 0.0039 to 2 mg/ml was analysed by XTT metabolic assay. As shown in **Fig. 4.2.3.a** formation of developing and mature biofilm was inhibited at 2 mg/ml by 74 % and 0.5 mg/ml concentrations, respectively compared to non-treated control.

4.2.6. Effect of EITC on developing biofilm with scanning electron microscopy

In current study, investigated the effect of EITC treatment (2 mg/ml) on the biofilm production of *C. albicans*, which is known to be a significant factor in yeast infections associated with implanted medical devices. Scanning electron microscopy analysis revealed that EITC treatment resulted in a notable inhibition of biofilm formation compared to the control sample (**Fig. 4.2.3.b**). Additionally, microscopic examination of cell morphology showed that the control sample exhibited dense biofilm with prominent hyphal structures. In contrast, EITC treatment effectively prevented biofilm formation at the 2 mg/ml concentration.

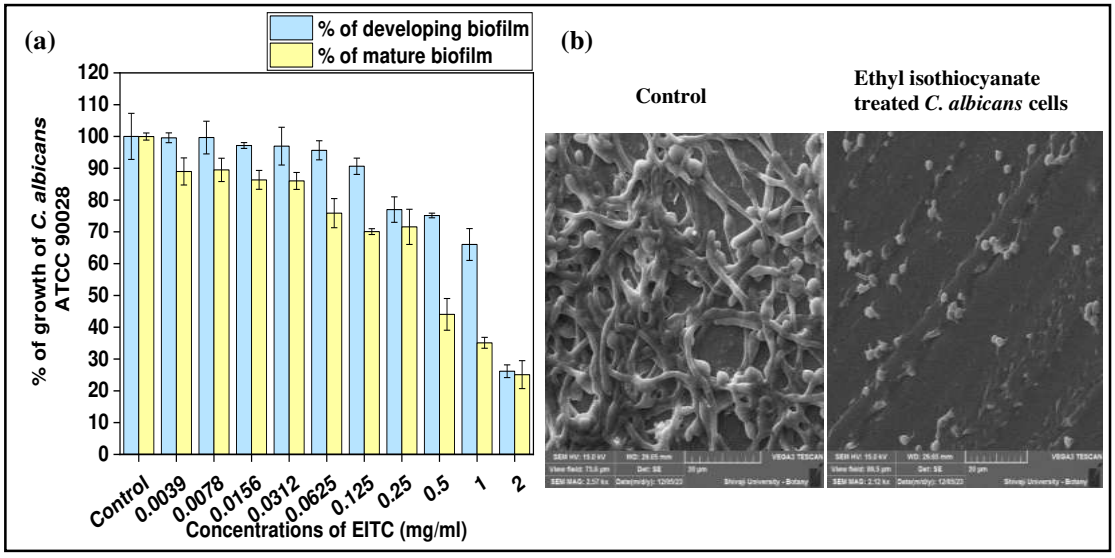

Fig. 4.2.3. **(a)** The inhibitory effect of ethyl isothiocyanate against developing and mature biofilm formation of *C. albicans*. **(b)** Scanning electron microscopy, showing the dense biofilm with hyphal structures of untreated cell (control) and eradication of biofilm by the treatment of 2 mg/ml concentration of ethyl isothiocyanate.

4.2.7. EITC inhibited ergosterol synthesis in *C. albicans*

Ergosterol is an important component of cell membrane and primary target of antifungal agents. EITC at its planktonic MIC concentration successfully inhibited ergosterol biosynthesis (**Fig. 4.2.4.a**). *C. albicans* cells which were not -treated with EITC (control) represent two sharp peaks which indicates ergosterol biosynthesis. However, *C. albicans* cells treated with planktonic MIC concentration and sub MIC concentration of EITC represent inhibition to ergosterol biosynthesis by flat curve. The result suggests that inhibition of ergosterol biosynthesis is a possible mechanism of action for *C. albicans* growth inhibition.

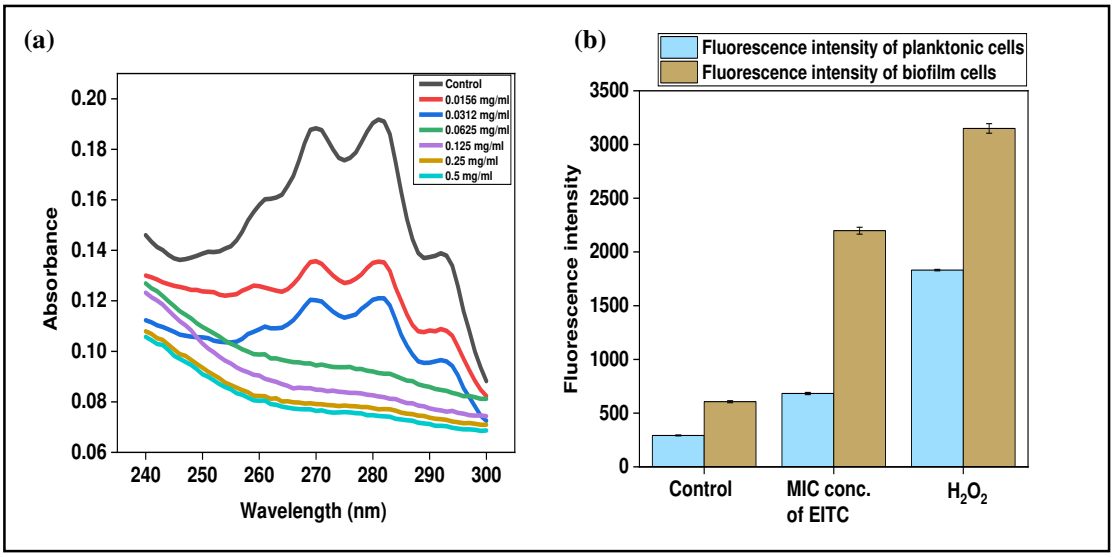

Fig. 4.2.4. (a) Inhibition to ergosterol biosynthesis by the treatment of planktonic inhibitory and sub inhibitory concentration of allyl isothiocyanate. **(b)** Detection of ROS level after treatment of ethyl isothiocyanate using DCFDA staining involved in the planktonic and biofilm cells of *C. albicans*.

4.2.8. EITC treatment induced intracellular reactive oxygen species (ROS) accumulation in *C. albicans*

The ability of EITC to induce endogenous ROS production in *C. albicans* was evaluated using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (**Fig. 4.2.4.b**). The enhanced fluorescence intensity in **Figure 4.2.4.b** reveals EITCs ability to stimulate endogenous ROS generation. *C. albicans* are known to produce ROS under severely stressed situations. When biological components like lipids, proteins, and

nucleic acids are exposed to high concentrations of ROS, such as superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, hypochlorous acid, and hydroperoxyl radicals, oxidative stress and eventual cell death can follow. EITC promoted intracellular ROS generation at its 0.5 mg/ml planktonic inhibitory concentration. However, increase in the production of ROS after the treatment of EITC as compared to non-treated *C. albicans* cells may be responsible for inhibition of *C. albicans* planktonic growth. Along with this EITC treatment also increased ROS production in *C. albicans* biofilm at 2 mg/ml concentration. Increase in endogenous ROS production in biofilm cells of *C. albicans* may be a reason for anti-biofilm activity of *C. albicans*.

4.2.9. EITC affected DNA replication and cell division

Cell cycle analysis was done to determine how EITC affected DNA replication and cell division, two highly important and regulated processes for a cell's growth and multiplication. Any abnormality in the order of these stages causes the DNA checkpoint pathway to activate, which stops all processes linked to growth and division until the damage is fixed. In cell cycle analysis study, *C. albicans* cells exposed to planktonic inhibitory concentration (0.5 mg/ml) of EITC and was compared with non-treated cells. In this study control shows 49.4 % cell in G1 phase, 36.0 % in synthetic S phase and 14.5 % in G2/M phase while cells treated with EITC showed 34.7 % in G1, 46.9 % in S and 17.2 % in G2/M phase (**Fig. 4.2.5.a**). From this study it was observed that EITC arrested the cells at synthetic S-phase.

4.2.10. Effect of EITC on signal transduction gene expression involved in biofilm inhibition of *C. albicans*

By using qRT-PCR analysis, the impact of EITC on *C. albicans* biofilm inhibition was biofilm formation revealed a notable inhibition in *C. albicans* biofilm as compared to non-treated control. qRT-PCR analysis showed a relative fold change in the gene expressions. Gene expression study demonstrated that developing biofilm inhibitory concentration of EITC significantly downregulated the expression of *CPH1*, *PDE2, BCY1, HWP1, TEC1, RAS1, ECE1, CEK1* and *HST7* by 2.00, 1.67, 1.35, 10.00, 1.67, 1.25, 1.39, 1.32, 1.35 and 2.50-fold change, respectively. EITC upregulated the expression of *CDC35, EFG1* and *MIG1* by 4.88, 1.08 and 1.20-fold change,

respectively. EITC inhibited one of the important virulence factor i.e. biofilm formation may be due to downregulating *CPH1*, *PDE2, BCY1, HWP1, TEC1, RAS1, ECE1, CEK1* and *HST7* genes and upregulating *MIG1* gene (**Fig. 4.2.5.b**).

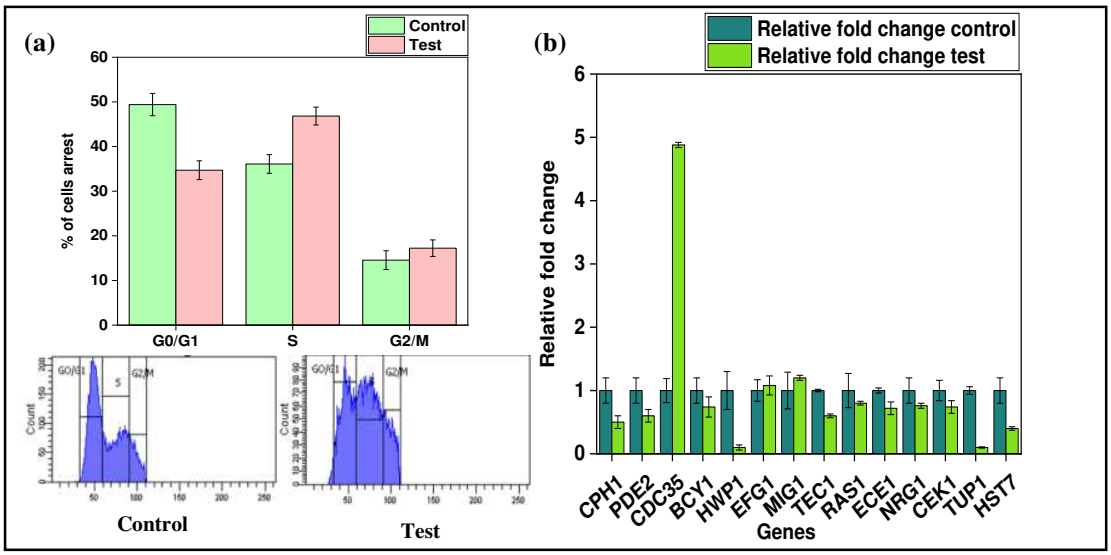

Fig.4.2.5. (a) S-phase arrest by ethyl isothiocyanate. **(b)** Effect of ethyl isothiocyanate on signal transduction genes involved in biofilm formation.

4.2.11. Hemolytic activity of EITC

Hemolytic analysis study was performed to assess the toxicity of EITC on human RBCs. EITC exhibited 19 % haemolysis at 0.5 mg/ml concentration and at 0.0156, 0.0078, 0.0039 mg/ml concentration it showed 8 %, 5 % and 5 % haemolysis respectively (**Fig. 4.2.6.a**). This indicates that EITC may be less toxic at lower concentrations and more toxic at higher concentration.

4.2.12. *In vivo* study in Silkworm animal model

In vivo study carried out to analyse the toxicity effect and survival potential on silkworm (*Bombyx mori*) after the treatment of EITC. Various control and test groups were kept to analyse the survival of silkworm. In positive control silkworm injected with *C. albicans* observed decreased survival within one day. whereas, in negative control silkworm injected with PBS complete their life cycle and undergoes cocoon phase. Silkworm injected with vehicle control i.e. DMSO (< 1 %) complete their

lifecycle. Silkworm injected with fluconazole completes their life cycle and undergoes cocoon phase. In test group silkworm injected with EITC survived up to 2 days. After 2 days silkworms were died this might be due to toxicity of EITC (**Fig. 4.2.6.b**). By reducing toxicity of EITC with various approaches like combining with standard antifungals or by preparing hydrogel EITC can be used as antifungal agent against *C. albicans*.

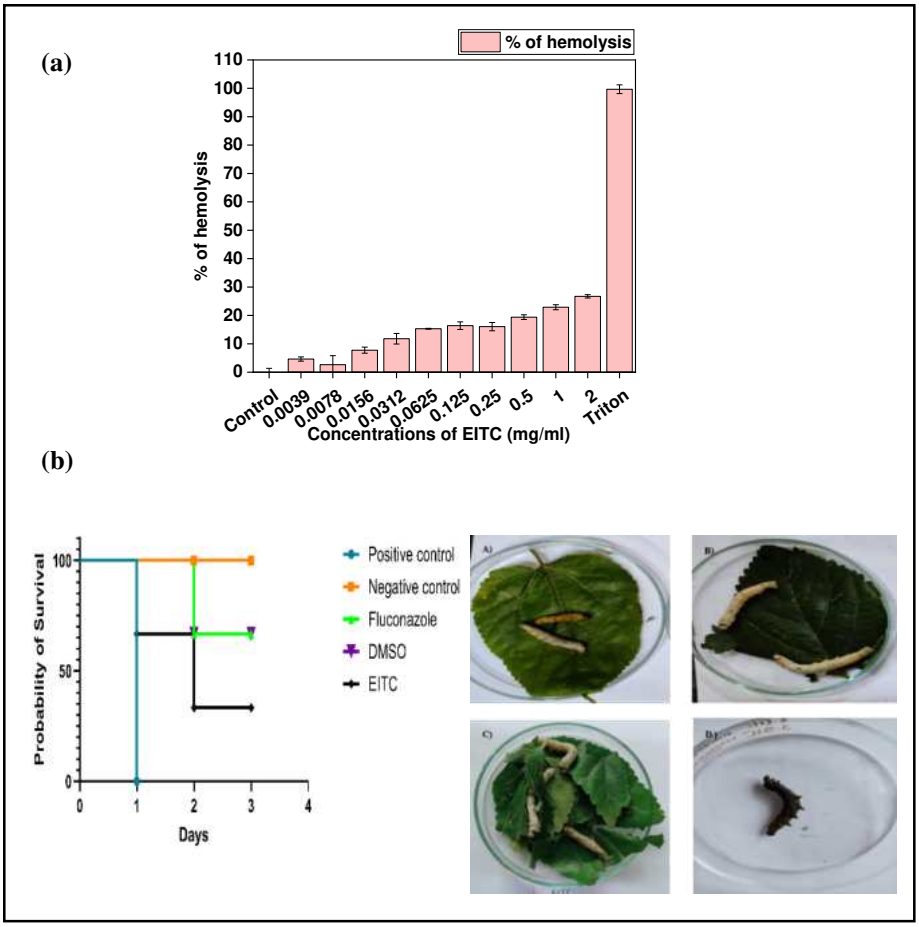

Fig. 4.2.6. **(a)** Effect of ethyl isothiocyanate on human Red Blood Cells. **(b)** Effects of ethyl isothiocyanate on silkworm infected *C. albicans*. The graph indicates percentage of worm survival after exposure of *C. albicans* for 3 days to ethyl isothiocyanate.

4.2.13. Conclusions

Current study reports for the first time, the anti *C. albicans* potential of EITC. This supports the possibility of using EITC as an anti-biofilm agent against *C. albicans*. In

conclusion, EITC inhibited planktonic growth and virulence factors like Y-H morphogenesis, adhesion to polystyrene surface, and biofilm formation. EITC is fungicidal in nature and its mode of action in *C. albicans* is through the effect on multiple targets including the disruption of cell membrane by inhibiting ergosterol biosynthesis. EITC arrested the *C. albicans* cells at S-phase. EITC caused ROS-dependent cellular death. Biofilm formation was inhibited by EITC by downregulating *CPH1*, *PDE2, BCY1, HWP1, TEC1, RAS1, ECE1, CEK1* and *HST7* genes and upregulating *MIG1* gene. By reducing the toxicity of EITC, it might be used as antifungal agent against *C. albicans* infection.

Results

4.3. Anti-*C. albicans* activity of Benzyl isothiocyanate (BITC)

4.3.1. Minimum inhibitory concentration (MIC) of BITC against planktonic growth

In the experiment, *C. albicans* cells were treated with a series of concentrations (0.0039 to 2 mg/ml) of BITC. BITC produced a strong and dose-dependent inhibitory effect on *C. albicans* and inhibit planktonic growth with MIC of 0.125 mg/ml concentration (**Fig.4.3.1.a**).

4.3.2. Inhibitory activity of BITC against adhesion to the polystyrene surface

Adherence of *C. albicans* cells to polystyrene was affected by BITC. *C. albicans* cells were exposed to series of concentration of BITC in the range of 0.0039 to 2 mg/ml. Analysing the density of adhered cells with XTT assay showed up to 65 % decrease in adhesion was seen at 0.5 mg/ml concentration. BITC significantly inhibited adhesion of cells to the solid surface (**Fig. 4.3.1.a**).

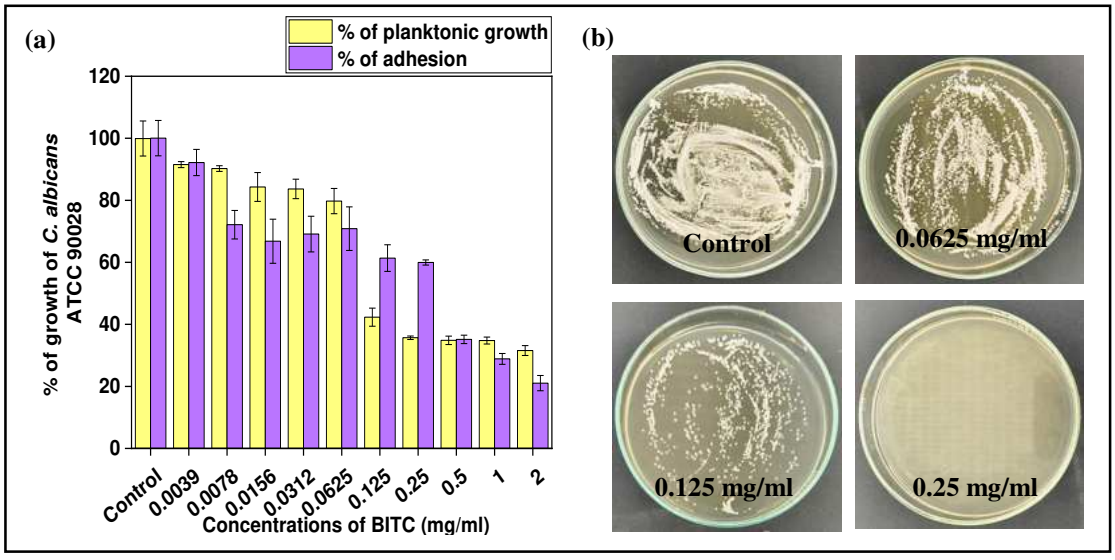

Fig.4.3.1. (a) Planktonic growth and adhesion of *C. albicans* inhibited at 0.125 mg/ml and 0.5 mg/ml by benzyl isothiocyanate respectively. **(b)** Minimum Fungicidal Concentration of benzyl isothiocyanate against *C. albicans* was obtained at 0.25 mg/ml by counting the colonies grown on YPD agar plate after performing planktonic growth assay.

4.3.3. Minimum fungicidal concentration of BITC

The minimum fungicidal concentration of BITC was assessed by spread plate technique on YPD agar plate with the help minimum inhibitory concentration (MIC) concentration and results indicates that BITC was fungicidal in nature at 0.25 mg/ml concentration (**Fig. 4.3.1.b**).

4.3.4. Inhibitory activity of BITC against the transition of *C. albicans* from yeast to hyphae

The transition from yeast to hyphae is an important virulence-mediating attribute of *C. albicans*. Conversion of yeast cells to hyphal forms is critical for biofilm biogenesis, which provides strength and support to the developing heterogeneous biofilm structure. In the present study, we observed that BITC at a low concentration of 0.0312 mg/ml effectively inhibited transition from yeast to hyphae (**Fig. 4.3.2. a, b**).

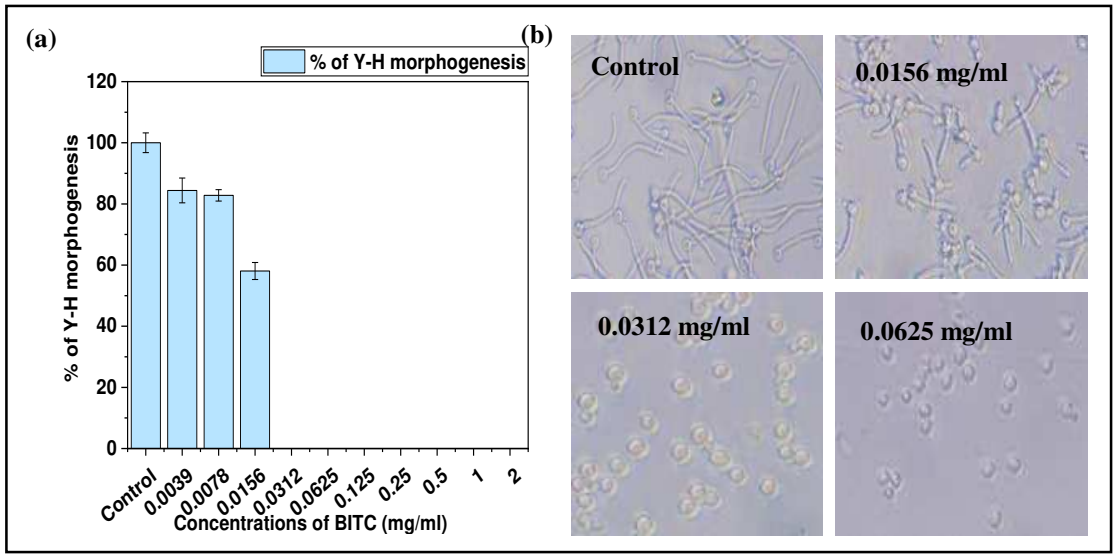

Fig.4.3.2. (a) Effect of benzyl isothiocyanate against *C. albicans* yeast to hyphal (Y-H) morphogenesis after performing morphogenesis assay. **(b)** Analysis of inhibition of yeast to hyphal morphogenesis at 0.0312 mg/ml observed with inverted microscopy.

4.3.5. BITC inhibits developing and mature biofilm

Analysis of biofilm growth by using XTT-metabolic assay showed that the addition of BITC at a series of concentration range from 0.0039 to 2 mg/ml prevented developing and mature biofilm formation of *C. albicans*. Treatment with a 2 mg/ml concentration

of BITC caused a 61 % decrease in the developing biofilm growth of strain ATCC 90028. And at 0.25 mg/ml concentration, BITC inhibited mature biofilm, which was evident from notable (> 50 %) lowering in relative metabolic activity (RMA) analysed by XTT assay, compared to that of control (**Fig.4.3.3.a**).

4.3.6. Effect of BITC on developing biofilm with scanning electron microscopy

It is well known that *C. albicans* biofilm production plays a crucial role in human yeast infections, particularly those affecting implanted medical devices. When BITC treatment (2 mg/ml) was compared to the control sample, the scanning electron microscopy analysis showed a significant change in the biofilm formation (**Fig. 4.3.3.b**). Additionally, while examining under a microscope, it was found that in control sample contains dense biofilm with hyphal structure, while BITC effectively inhibit biofilm formation at 2 mg/ml concentration.

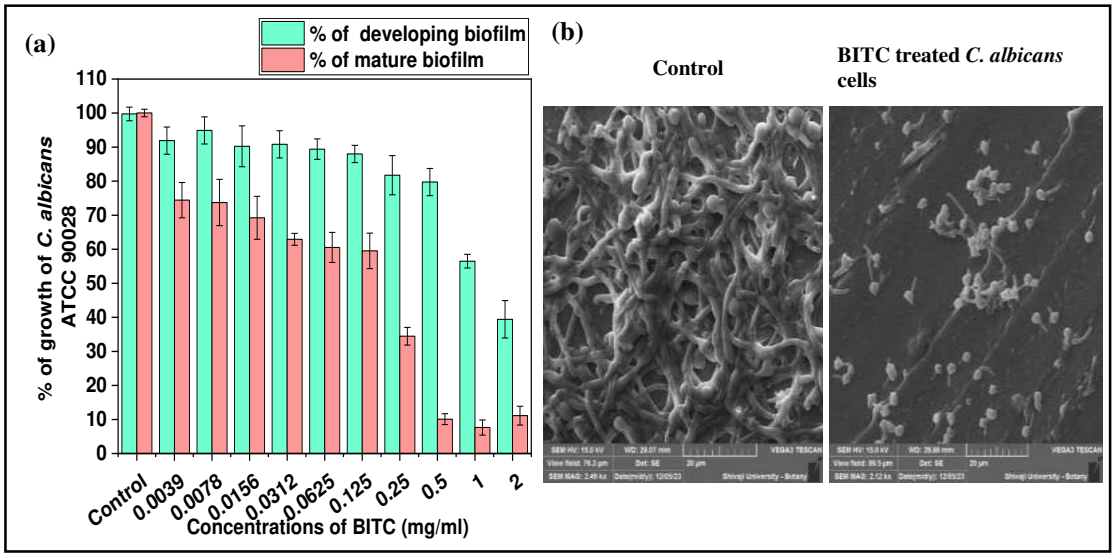

Fig. 4.3.3. (a) Effect of benzyl isothiocyanate on *C. albicans* biofilms. Treatment with BITC (0.0039–2 mg/ml) for 48 and 72 h reduced both developing and mature biofilms respectively in a 96-well plate. **(b)** Scanning electron microscopy, showing the dense biofilm with hyphal structures of untreated cell (control) and eradication of biofilm by the treatment of 2 mg/ml concentration of benzyl isothiocyanate.

4.3.7. Effect of BITC on *C. albicans* cell membrane

The cell membrane is a primary target for many standard antifungal drugs, such as azoles and polyenes. In the present study, examined the presence of ergosterol in *C. albicans* cells following treatment with BITC. By analysing ergosterol biosynthesis, we assessed the impact of BITC on the cell membrane of *C. albicans*. Untreated control cells displayed characteristic peaks of ergosterol production. However, cells treated with BITC at planktonic MIC concentrations, as well as at concentrations below the MIC (ranging from 0.0078 to 0.125 mg/ml), showed a significant inhibition of ergosterol biosynthesis, which is represented by a flat curve (**Fig. 4.3.4.a**).

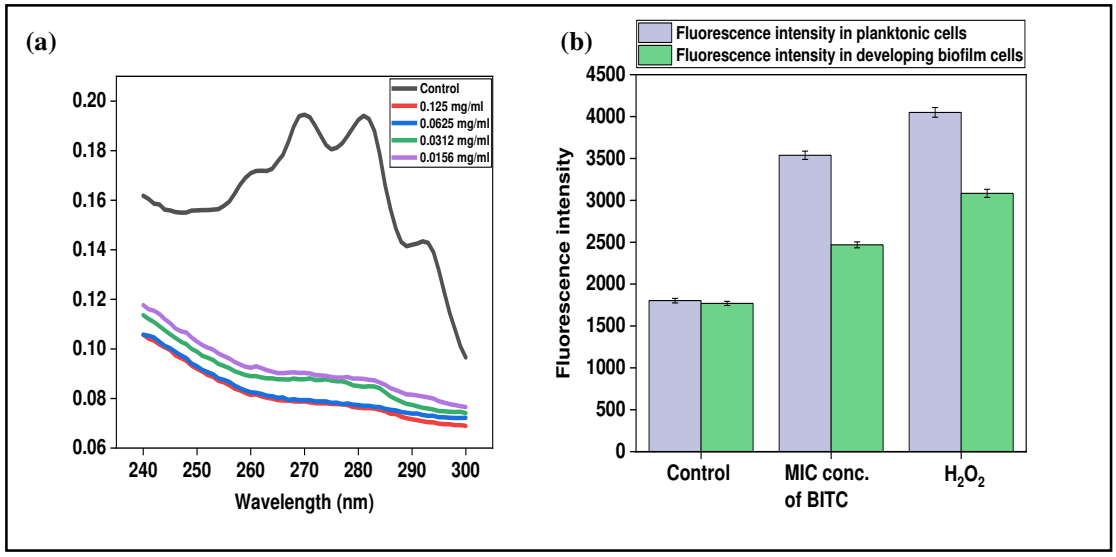

Fig 4.3.4. (a) Inhibition to ergosterol biosynthesis by the treatment of planktonic inhibitory and sub inhibitory concentration of benzyl isothiocyanate. **(b)** Detection of ROS level after treatment of benzyl isothiocyanate using DCFH-DA staining involved in the planktonic and biofilm cells of *C. albicans*.

4.3.8. BITC induces ROS production in *C. albicans* cell

The production of ROS in *C. albicans* planktonic and developing biofilm cells was examined in the current study in the presence of minimal inhibitory concentrations i.e. 0.125 mg/ml and 2 mg/ml BITC respectively. DCFH-DA dye used to measure the levels of intracellular ROS. After 4 h and 24 h of incubation, respectively. The ROS level in *C. albicans* planktonic and biofilm cells was considerably induced than the control group (**Fig.4.3.4b**).

4.3.9. Effect of AITC on cell cycle

In current study, examined the impact of BITC on the *C. albicans* cell cycle. In order to understand how BITC affected DNA replication and cell division, two crucial and tightly controlled processes for a cells growth and multiplication, cell cycle study was conducted. Any irregularity in DNA replication and cell division triggers the DNA damage checkpoint pathway, which halts all processes related to growth and division until the damage is repaired. In a cell cycle analysis, *C. albicans* cells exposed to a planktonic inhibitory dose of BITC (0.125 mg/ml) and compared them to untreated cells. DNA content present during various cell cycle stages was measured based on the fluorescence intensity produced by propidium iodide to confirm cell cycle arrest in *C. albicans*. In control, 61.1 % cells were arrested in G0/G1 phase, 30.7 % in S phase and 8.0 % in G2/M phase while in the test, 14.6 % cells were arrested in G0/G1 phase, 30.9 % in S phase and 54.1 % in G2/M phase (**Fig 4.3.4.a**). The results suggest that BITC arrested cells in G2/M phase of *C. albicans*.

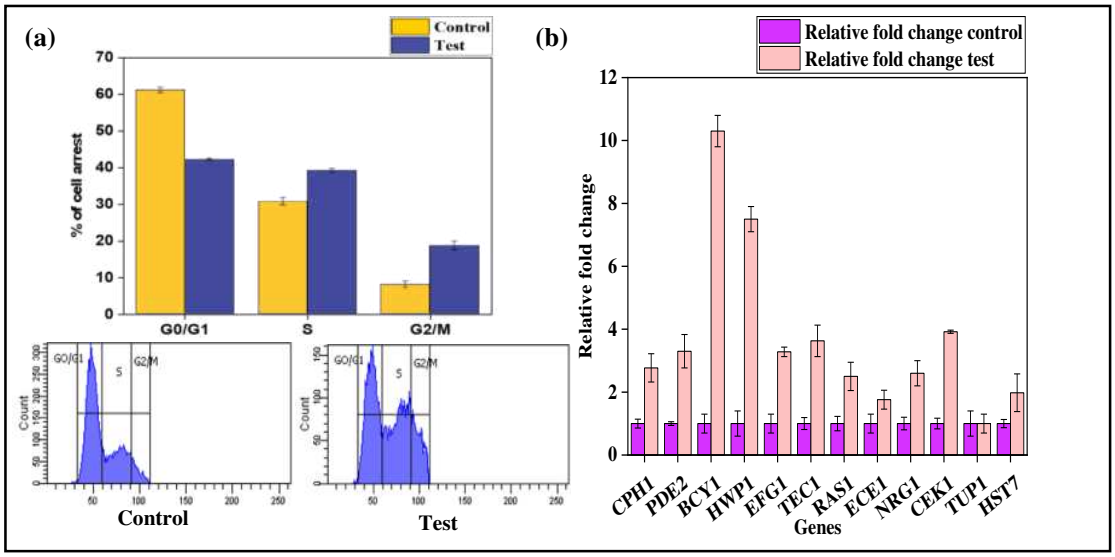

Fig.4.3.5. (a) Cell cycle arrest at G2/M phase by benzyl isothiocyanate in *C. albicans* cells. **(b)** Effect of benzyl isothiocyanate on signal transduction genes involved in biofilm formation.

4.3.10. Gene expression by BITC

Transcriptional level of hyphal-specific and biofilm related genes in *C. albicans* in the absence and presence of BITC at 2 mg/ml concentration was quantified by real time

PCR. BITC at biofilm inhibitory concertation significantly up regulated the expression of *CPH1, PDE2, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, NRG1, CEK1* and *HST7* by 2.77, 3.30, 10.30, 7.50, 3.28, 3.63, 2.50, 1.76, 2.60, 3.92 and 1.98, respectively. TUP1 remains unaffected. BITC inhibited one of the important virulence factor i.e. developing biofilm might be due to upregulation of negative regulator of yeast to hyphal transition i.e. *NRG1* gene (**Fig.4.3.5.b**).

4.3.11. Toxicity analysis of BITC

The toxicity of BITC was analysed by *in vitro* haemolytic activity on human RBCs. It was observed that BITC was haemolytic in a concentration range from 0.0039 to 2 mg/ml. BITC shows 20 % hemolysis at 2 mg/ml concentration (**Fig. 4.3.6**).

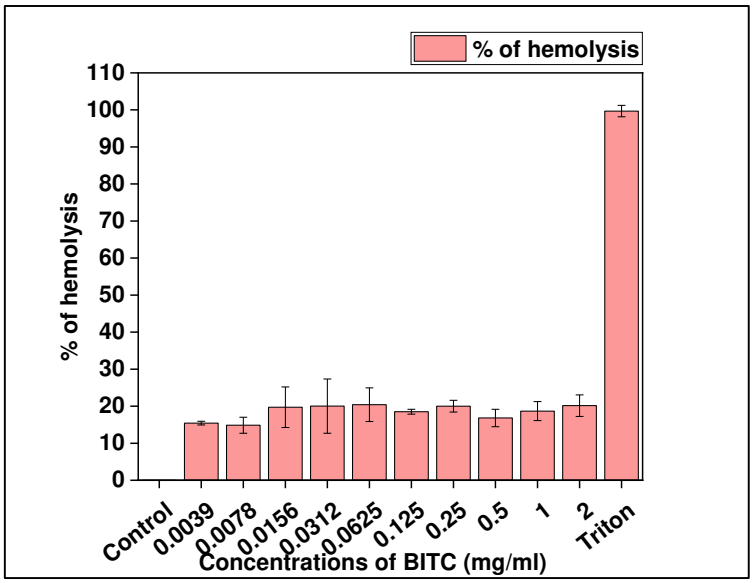

Fig.4.3.6. Effect of BITC on human Red Blood Cells.

4.3.12. Conclusions

BITC demonstrated potent antifungal activity against *C. albicans*. It exhibited a strong and dose-dependent inhibitory effect on planktonic growth, adhesion to surfaces, and transition from yeast to hyphal forms, with MIC concentrations 0.125mg/ml, 0.5 mg/ml and 0.0312 mg/ml respectively. BITC also showed fungicidal properties at 0.25 mg/ml concentration. BITC effectively inhibited biofilm formation, both in developing and mature stages, with notable reductions in metabolic activity. BITCs mode of action

involved inhibition to ergosterol biosynthesis, inducing ROS production, and arresting the cell cycle at the G2/M phase. Furthermore, BITC upregulated *NRG1* gene which might be associated in inhibition of biofilm. However, BITC exhibited hemolytic activity on human red blood cells. However, reducing the toxicity of BITC by using various approaches like combining with standard antifungals or by preparing gel formulations BITC might be used as an antifungal agent against *C. albicans* infections.

Results

4.4. Anti-*C. albicans* activity of Phenyl ethyl isothiocyanate (PEITC)

4.4.1. Minimum inhibitory concentration (MIC) of PEITC against planktonic growth of *C. albicans*

Impact of PEITC on the planktonic growth of *C. albicans* was assessed using micro broth dilution method according to the guidelines of Clinical and Laboratory Standards Institute. The MIC of PEITC for *C. albicans* was found to be at 1 mg/ml (**Fig. 4.4.1.a**).

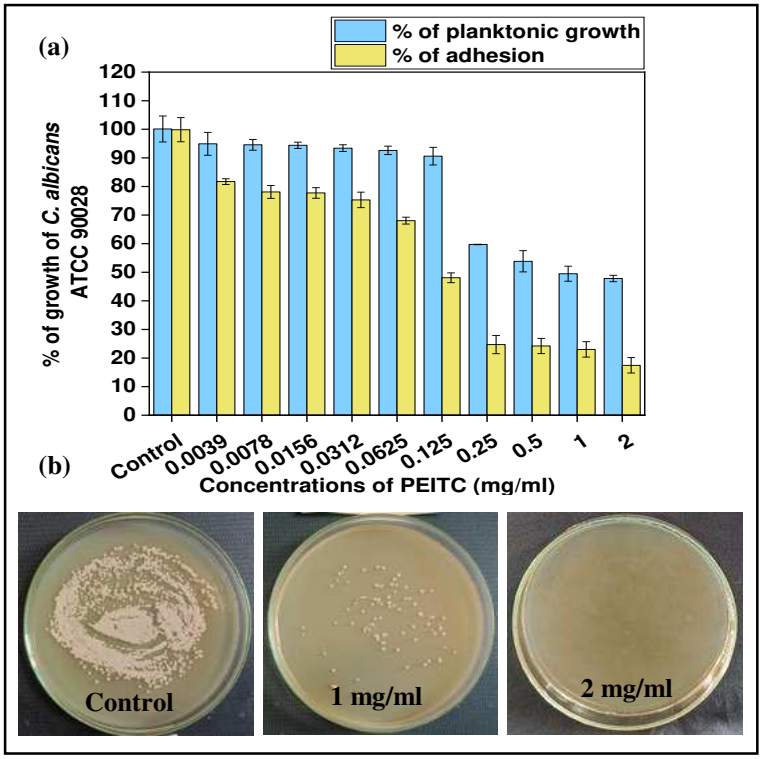

Fig. 4.4.1. (a) Inhibition of *C. albicans* planktonic and adhesion growth by phenyl ethyl isothiocyanate was observed at 1 mg/ml and 0.125 mg/ml concentration respectively. **(b)** Minimum fungicidal concentration against *C. albicans* growth after the treatment of phenyl ethyl isothiocyanate.

4.4.2. Inhibitory activity of PEITC against adhesion to the polystyrene surface

Adhesion ability is one of the primary independent factors contributing to the virulence of *C. albicans*, we detected the effect of PEITC on the adhesion of *C. albicans* at the bottom of the 96-well plates. Adherence of *C. albicans* cells to polystyrene was influenced by PEITC. *C. albicans* cells were exposed to series of concentration of

PEITC in the range of 0.0039 to 2 mg/ml. Analysing the density of adhered cells with XTT assay showed up to 52 % decrease in adhesion was seen at 0.125 mg/ml concentration (**Fig. 4.4.1.a**).

4.4.3. Minimum fungicidal concentration (MFC)

The minimum fungicidal concentration of PEITC was assessed by spread plate technique on YPD agar plate with the help MIC concentration and results indicate that PEITC was fungicidal at 2 mg/ml i.e. above MIC concentrations (**Fig. 4.4.1.b**).

4.4.4. Inhibitory activity of PEITC isothiocyanate against the transition of *C. albicans* from yeast to hyphae

Since hyphal development is the main pathogenic factor of *C. albicans*. In current study examined the effect of PEITC on hyphal formation *in vitro* with the help of inverted microscopy. PEITC effectively inhibited the morphological transformation from yeast to the hyphae. In present study, we observed that PEITC at a low concentration of 0.0312 mg/ml effectively inhibited transition from yeast to hyphae (**Fig.4.4.2. a, b**).

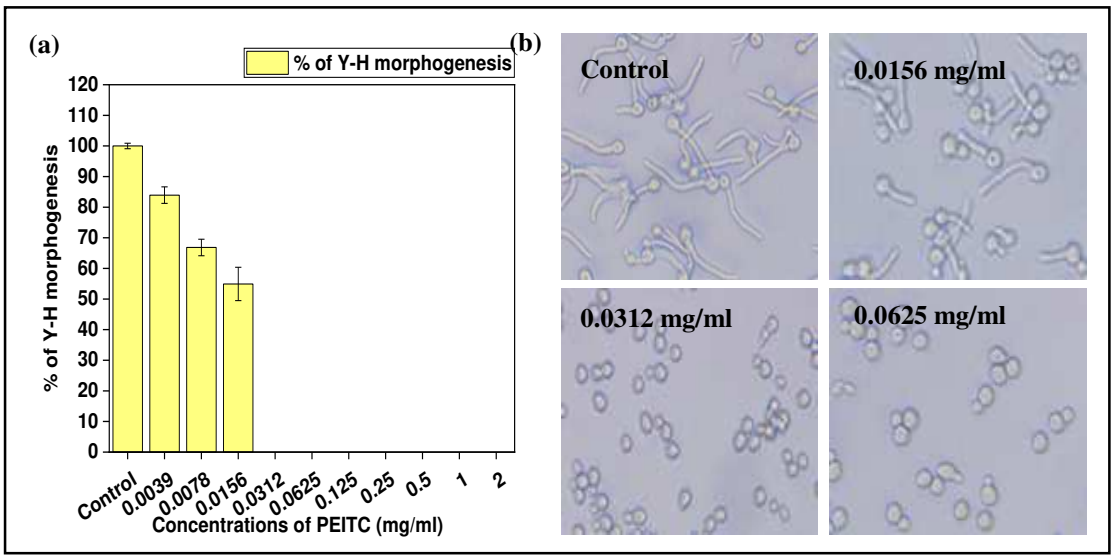

Fig.4.4.2. (a) Effect of phenyl ethyl isothiocyanate against the yeast to hyphal (Y-H) morphogenesis of *C. albicans* ATCC 90028 strains treated by various concentrations of phenyl ethyl isothiocyanate (0.0039 to 2 mg/ml). **(b)** Inhibition of *C. albicans* observed with inverted microscopy.

4.4.5. PEITC inhibit developing and mature biofilm

The capacity to form biofilms gives *C. albicans* strong resistance to drugs and immunological escape, suggesting that biofilm formation is a key component of the *C. albicans* virulence factor. As a result, we qualitatively examined how PEITC affects the production of biofilms growth by using XTT-metabolic assay. Addition of PEITC at a concentration ranges from 0.0039 to 2 mg/ml prevented developing and mature biofilm formation by *C. albicans*. Treatment with 0.125 mg/ml concentration of PEITC caused 84 % decrease in developing biofilm growth (**Fig.4.4.3.a**) of strain ATCC 90028. Eradication of mature biofilm is very difficult, but at 2 mg/ml concentration, PEITC eradicated mature biofilm compared to that of control.

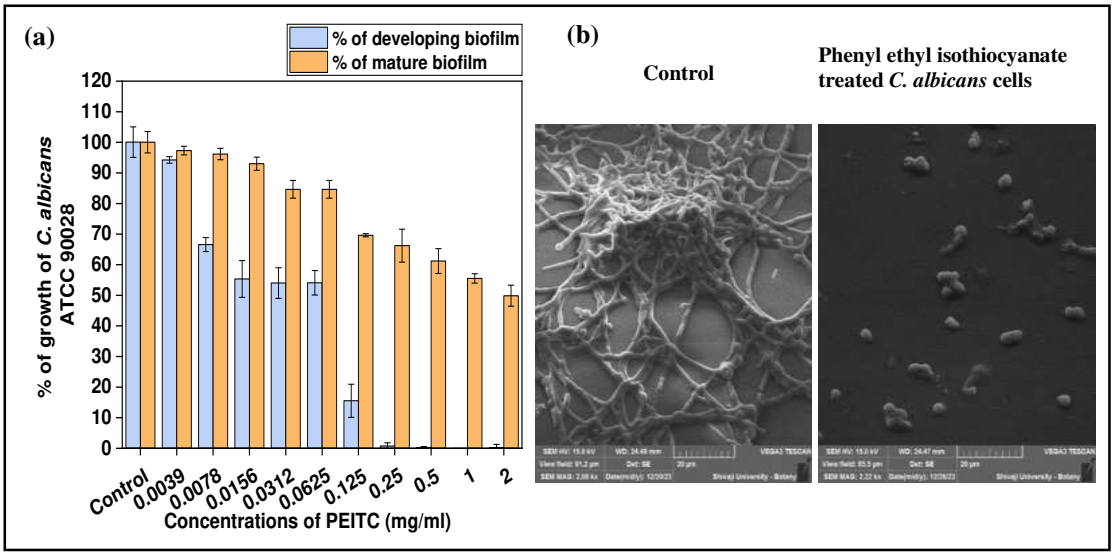

Fig. 4.4.3. (a) Effect of phenyl ethyl isothiocyanate on metabolic activity of developing and mature biofilm. The inhibitory effects of phenyl ethyl isothiocyanate dilutions were evaluated on biofilms after 48 and 72-h incubation with XTT metabolic respectively. **(b)** Scanning electron microscopy, showing the dense biofilm with hyphal structures of untreated cell (control) and eradication of biofilm by the treatment of 0.125 mg/ml concentration of phenyl ethyl isothiocyanate.

4.4.6. Effect of PEITC on developing biofilm with scanning electron microscopy

C. albicans biofilm formation is a significant factor in human yeast infections, particularly those related to implanted medical devices. In current study, observed the effects of PEITC treatment (0.125 mg/ml) in comparison to a control sample. Scanning

electron microscopy analysis demonstrated a marked reduction in biofilm formation in the samples treated with PEITC (**Fig. 4.4.3.b**). Additionally, microscopic observation of cell morphology revealed that the control samples displayed a dense biofilm characterised by distinct hyphal structures, while PEITC treatment effectively inhibited biofilm formation at the 0.125 mg/ml concentration.

4.4.7. Effect of PEITC on *C. albicans* cell membrane

Cell membrane is the primary target for many standard antifungal drugs like azoles and polyenes. Therefore, in present study we examined ergosterol presence in *C. albicans* cells upon treatment with PEITC. Using ergosterol biosynthesis, the impact of PEITC on the cell membrane of *C. albicans* was examined. Untreated control cells show characteristic peaks that signifies ergosterol production. In contrast, *C. albicans* cells treated with PEITC at planktonic MIC concentrations and below (ranging from 0.125 to 1 mg/ml) exhibited inhibition of ergosterol biosynthesis, as evidenced by a flat curve (**Fig. 4.4.4.a**).

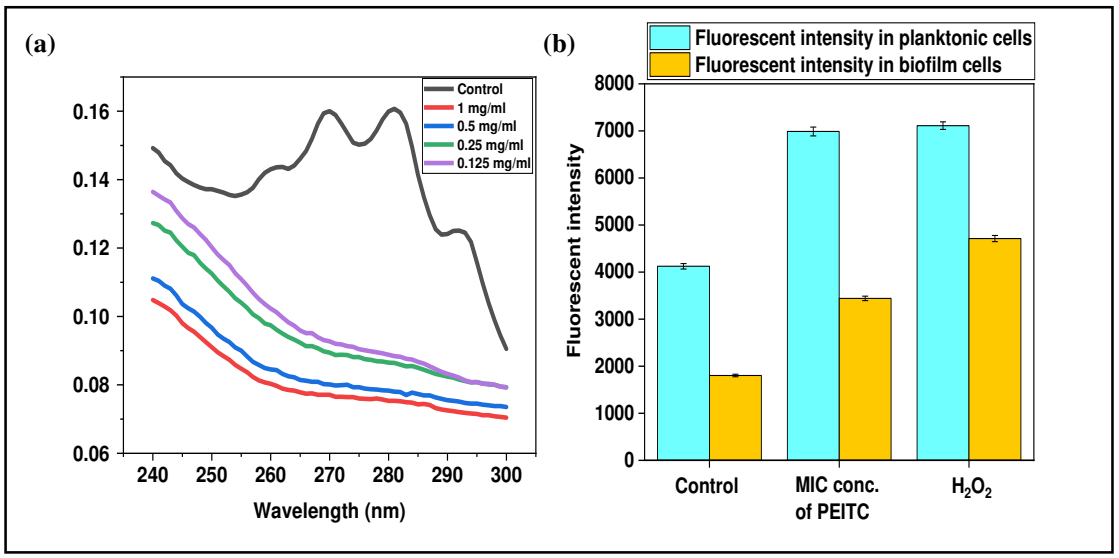

Fig 4.4.4. (a) Inhibition to ergosterol biosynthesis by the treatment of planktonic inhibitory and sub inhibitory concentration of phenyl ethyl isothiocyanate. **(b)** Effect of phenyl ethyl isothiocyanate on ROS production of *C. albicans* planktonic and developing biofilm growth.

4.4.8. Effect of PEITC on intracellular ROS accumulation in *C. albicans*

After treating *C. albicans* cells with planktonic (1 mg/ml) and developing biofilm (0.125 mg/ml) inhibitory doses for 4 h and 24 h respectively, the intracellular ROS generation was measured using a fluorescent dye, dichlorofluorescin diacetate (DCFH-DA). The oxidation of DCFH-DA to DCF is triggered by intracellular ROS. After 4 h and 24 h of incubation with PEITC, DCF fluorescence was found to significantly increase as compared to non-treated control, as Figure **4.4.4.b** illustrates. These findings suggest that PEITC induced intracellular reactive oxygen species (ROS), which resulted in intracellular oxidative damage and maybe membrane damage. This could be the likely cause of inhibition of planktonic growth and biofilm development.

4.4.9. Effect of PEITC on cell cycle

In current study examined the impact of PEITC on the *C. albicans* cell cycle. In order to understand how PEITC affected DNA replication and cell division, two crucial and tightly controlled processes for a cell's growth and multiplication, cell cycle study was conducted. Any irregularity in DNA replication and cell division triggers the DNA damage checkpoint pathway, which halts all processes related to growth and division until the damage is repaired. In a cell cycle analysis investigation, *C. albicans* cells exposed to a planktonic inhibitory dose of PEITC (1 mg/ml) and compared them to untreated cells. DNA content present during various cell cycle stages was measured based on the fluorescence intensity produced by propidium iodide to confirm cell cycle arrest in *C. albicans*. In control, 49.5 % cells were arrested in G0/G1 phase, 36.23 % in S phase and 14.4 % in G2/M phase while in the test, 65.6 % cells were arrested in G0/G1 phase, 24.8 % in S phase and 7.5 % in G2/M phase (**Fig.4.4.5.a**). The results suggest that PEITC arrested cells in G0/G1 phase of *C. albicans*.

4.4.10. Gene expression profiling though Real Time Polymerase Chain Reaction (RT-PCR)

Transcriptional level of hyphal-specific biofilm related genes in *C. albicans* in the absence and presence of PEITC was quantified by real time PCR. PEITC at its developing biofilm inhibitory concentration (0.125 mg/ml) significantly upregulated the expression of *CPH1, PDE2, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, NRG1,*

CEK1 and *HST7* genes by 12.09, 25.99, 3.37, 12.04, 17.05, 16.92, 9.56, 9.53, 9.19, 9.17 and 8.74-fold change, respectively. PEITC downregulated the expression of *CDC35* gene by 29.33-fold change. PEITC inhibited one of the important virulence factor i.e. developing biofilm might be due to upregulating the expression of negative regulator *NRG1* by 9.19-fold change and downregulating the expression of *CDC35* by 29.33-fold change (**Fig.4.4.5.b**).

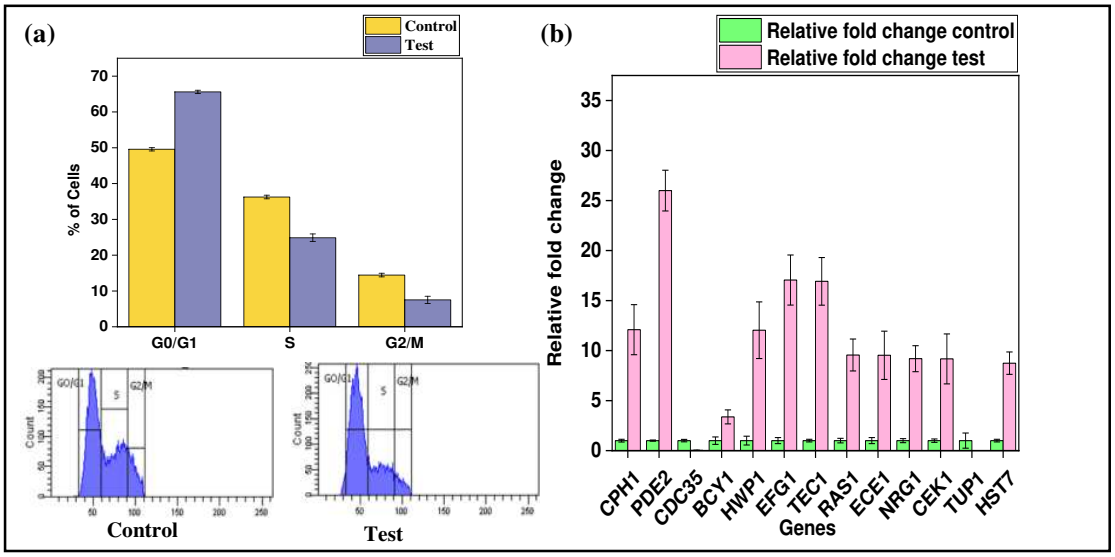

Fig. 4.4.5. (a) Cell cycle arrest at G0/G1 phase by phenyl ethyl isothiocyanate in *C. albicans* cells. **(b)** Effect of phenyl ethyl isothiocyanate on signal transduction genes involved in biofilm formation.

4.4.11. Toxicity analysis of phenyl ethyl isothiocyanate

The toxicity of phenyl ethyl isothiocyanate (PEITC) was evaluated by assessing its hemolytic activity on human red blood cells (RBCs). The results demonstrated that PEITC exhibited hemolytic properties across a concentration range of 0.0039 to 2 mg/ml (**Fig.4.4.6**). This finding suggests that while PEITC has potential anti-*C. albicans* properties, its hemolytic activity must be carefully considered when evaluating its safety for therapeutic use.

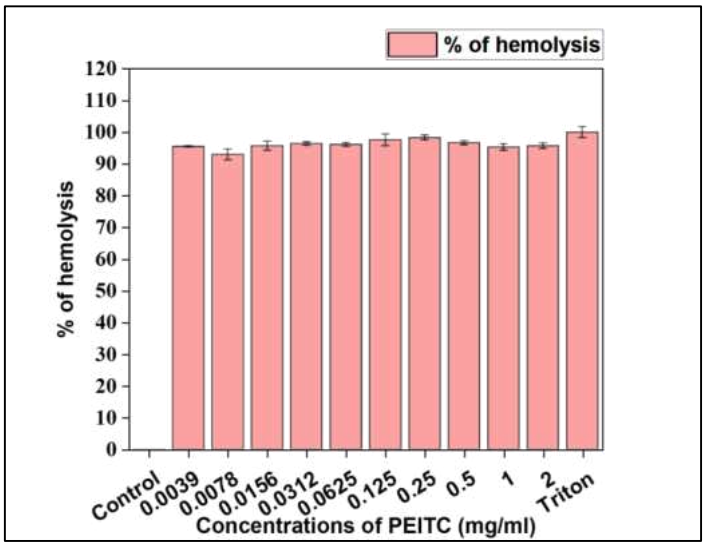

Fig.4.4.6. Effect of phenyl ethyl isothiocyanate on human red blood cells (RBCs). The diluted RBCs (100 µl) were incubated with series of concentrations (0.0039 to 2 mg/ml) of phenyl ethyl isothiocyanate at 37°C for 1 h.

4.4.12. Conclusions

PEITC exhibited significant antifungal activity against *C. albicans*, it inhibited planktonic growth at 1 mg/ml and fungicidal properties were observed at above 2 mg/ml. It effectively inhibited adhesion to surfaces and transition from yeast to hyphal forms, with notable effects seen even at low concentrations 0.125 mg/ml and 0.0312 mg/ml respectively. Additionally, PEITC showed promising inhibition to developing and mature biofilms, indicating its potential as a biofilm-disrupting agent. Mechanistically, PEITC suppressed ergosterol biosynthesis, induced intracellular ROS production, and arrested the cell cycle at the G0/G1 phase. Gene expression profiling revealed downregulation of *CDC35* gene and upregulation of *NRG1* gene, negative regulators responsible for biofilm inhibition. However, PEITC demonstrated hemolytic activity on human red blood cells. However, PEITC has a potential to act as antifungal agent against *C. albicans* but it is showing toxicity to human RBCs by reducing its toxicity PEITC might be used as promising therapeutic approach for the treatment of candidiasis.

Results

4.5. Anti-*C. albicans* activity of Butyl isothiocyanate (ButylITC)

4.5.1. Planktonic growth of *C. albicans* inhibited by ButylITC

This study investigated the antifungal activity of ButylITC against the *C. albicans* ATCC 90028 strain. ButylITC-treated *C. albicans* cells were compared with untreated control cells. The planktonic growth of *C. albicans* cells was shown to be reduced by an increasing concentration of ButylITC. After treatment with ButylITC, a 66 % reduction in planktonic growth of *C. albicans* was observed at a dosage of 2 mg/ml (**Fig. 4.5.1.a**).

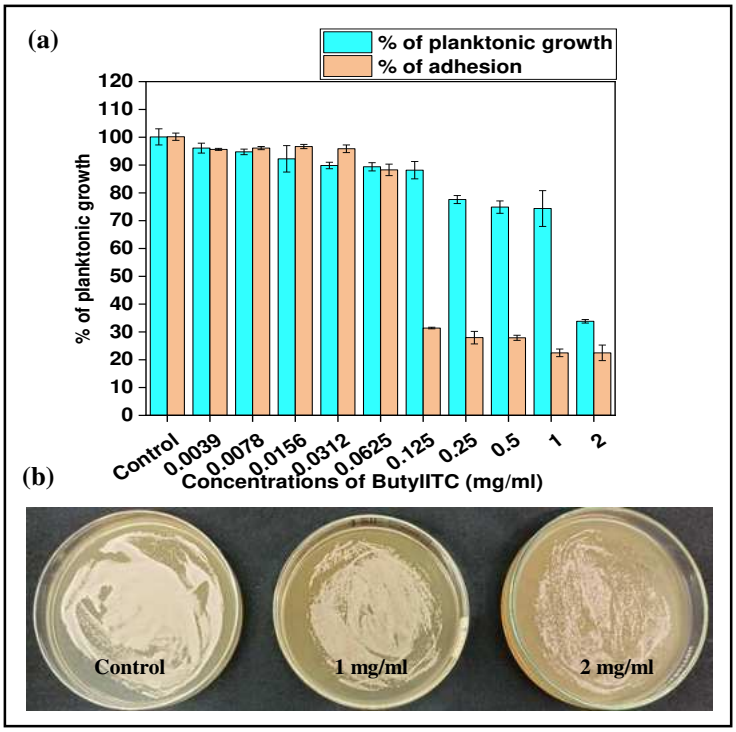

Fig. 4.5.1. (a) Effect of butyl isothiocyanate on the planktonic growth and adhesion of *C. albicans* cells. Butyl isothiocyanate inhibited planktonic growth significantly at 2 mg/ml and adhesion at 0.125 mg/ml concentration compared to control. **(b)** Minimum fungicidal concentration against *C. albicans* growth after the treatment of butyl isothiocyanate.

4.5.2. ButylITC inhibits adhesion of *C. albicans*

With the help of adhesion assay, activity of ButylITC on adherence of *C. albicans* cells to polystyrene surface was analysed. Various concentrations of ButylITC were

prepared and tested for anti-adhesion activity. ButylITC caused 69 % reduction to adhesion of *C. albicans* cells at 0.125 mg/ml concentration (**Fig. 4.5.1.a**).

4.5.3. Minimum fungicidal concentration of ButylITC

With the use of the MIC concentration, the spread plate technique was employed to determine the fungicidal concentration of ButylITC on YPD agar plates. But the results demonstrated that, ButylITC was fungistatic in nature at its MIC concentration 2 mg/ml (**Fig. 4.5.1.b**).

4.5.4. Assessment for effect of ButylITC on hyphal transition

The current study revealed the impact of ButylITC on yeast to hyphal (Y-H) transition of *C. albicans* using Fetal Bovine Serum (FBS) growth media. ButylITC was more sensitive to hyphal growth compared to planktonic growth. Anti-Y-H transition activity of ButylITC was observed in a concentration dependent manner. At 0.0039 mg/ml, 0.0078 mg/ml, 0.0156 mg/ml and 0.0312 mg/ml concentration 91 %, 81 %, 78 % and 66 % growth of yeast to hyphal (Y-H) was observed respectively. 92 % of yeast to hyphal (Y-H) transition was inhibited at 0.0625 mg/ml concentration. At 0.125 mg/ml concentration no hyphae were observed which indicates Y-H induction was inhibited by ButylITC. Inhibition was observed by using inverted light microscope at 40X (**Fig. 4.5.2. a, b**).

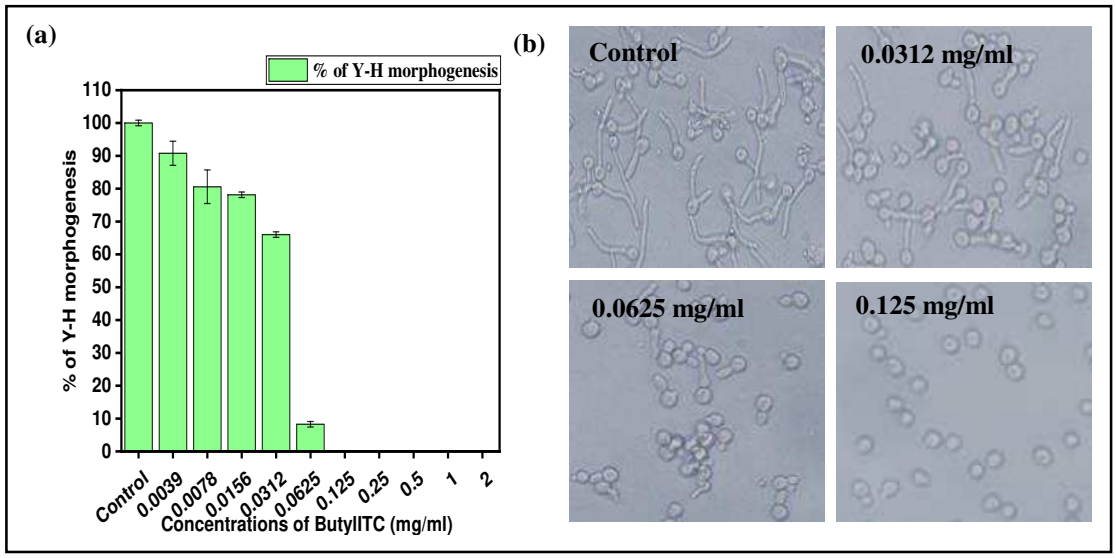

Fig. 4.5.2. (a) Inhibitory effects of butyl isothiocyanate on *C. albicans* yeast to hyphal morphogenesis. **(b)** Inhibition of yeast to hyphal form morphogenesis was observed at 0.0625 mg/ml concentration through inverted light microscopy.

4.5.5. Effect of ButylITC on developing and mature biofilm

The *C. albicans* ATCC 90028 strain was used to evaluate ButylITC anti-biofilm activity. At a dosage of 2 mg/ml, ButylITC suppressed the developing biofilm, as demonstrated by the XTT metabolic assay. However, ButylITC in the dosage range of 0.0039 to 2 mg/ml could not inhibit mature biofilm of *C. albicans* (**Fig. 4.5.3.a**).

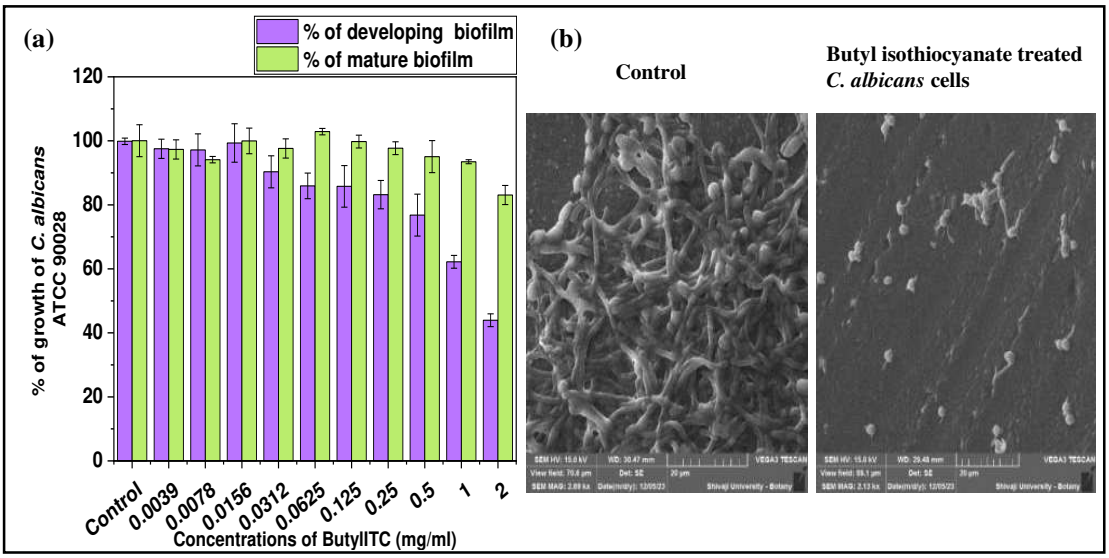

Fig. 4.5.3. (a) Anti-biofilm activity of butyl isothiocyanate against *C. albicans* developing and mature biofilm. Biofilm was quantified by XTT metabolic assay results are presented as mean percent of metabolic activities. **(b)** Scanning electron microscopy, showing the dense biofilm with hyphal structures of untreated cell (control) and eradication of biofilm by the treatment of 2 mg/ml concentration of butyl isothiocyanate.

4.5.6. Assessment of impact of ButylITC on developing biofilm with scanning electron microscopy (SEM)

It is widely recognised that, the biofilm formed by *C. albicans* grabs special attention in human yeast related infections, specifically those that impact medical implants. An examination using scanning electron microscopy revealed a notable defect in the biofilm growth after the treatment of ButylITC (2 mg/ml) when compared with the non-treated control sample (**Fig. 4.5.3.b**). Additionally, while examining the cells morphology under SEM, it was observed that, control sample consists dense biofilm with hyphal structure,

while ButylITC treatment effectively eradicate dense hyphal structure leads to inhibition of biofilm formation at 2 mg/ml concentration.

4.5.7. Inhibition activity of ergosterol biosynthesis by ButylITC

Effect of ButylITC on *C. albicans* cell membrane was analysed using ergosterol biosynthesis assay. In Figure 4.5.4a, untreated control cells showed characteristic peaks of ergosterol in the spectrum. However, *C. albicans* cells treated with ButylITC 2 mg/ml, 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml did not show any characteristic peaks of ergosterol in the spectrum as shown in **Fig. 4.5.4.a**.

4.5.8. ButylITC induced reactive oxygen species (ROS) generation

The ButylITC-induced ROS generation in the *C. albicans* ATCC 90028 strain was estimated using the fluorescent dye DCFH-DA. ButylITC increased intracellular ROS accumulation at its planktonic inhibitory concentration (2 mg/ml) and developing biofilm inhibitory concentration (2 mg/ml). These results demonstrated that oxidative stress after the treatment of ButylITC may be associated for the inhibition of *C. albicans* planktonic growth and developing biofilm (**Fig. 4.5.4.b**).

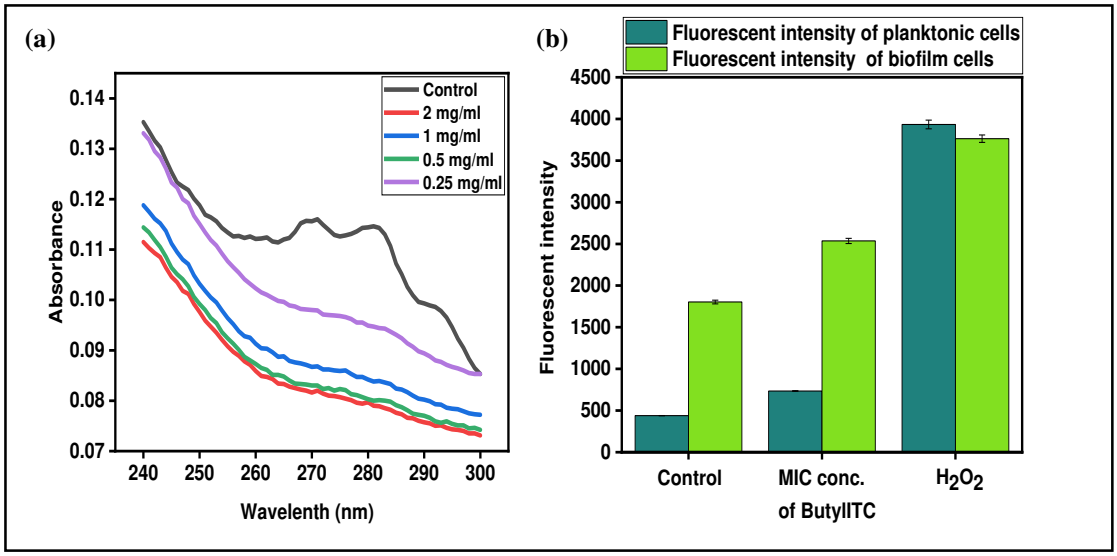

Fig. 4.5.4. (a) Ergosterol profile of *C. albicans* scanned between 240 to 300 nm from culture grown with and without the treatment of butyl isothiocyanate. **(b)** The treatment of butyl isothiocyanate significantly enhanced the production of ROS in *C. albicans* planktonic and biofilm cells observed using fluorescence spectrophotometer.

4.5.9. Cell cycle analysis

In cell cycle analysis study, *C. albicans* cells exposed to a planktonic inhibitory concentration (2 mg/ml) of ButylITC, and the results were compared with control (non-treated cells). ButylITC arrested (**Fig. 4.5.5.a**) 32.2 % cells at G0/G1 phase, 11.3 % at G2/M phase and 54.4 % at S phase. In non-treated cells, 49.4 % cells were present at G0/G1 phase, 14.5 % at G2/M phase and 36.0 % cells were arrested at S phase. Results showed that, ButylITC arrests *C. albicans* cell cycle at S phase.

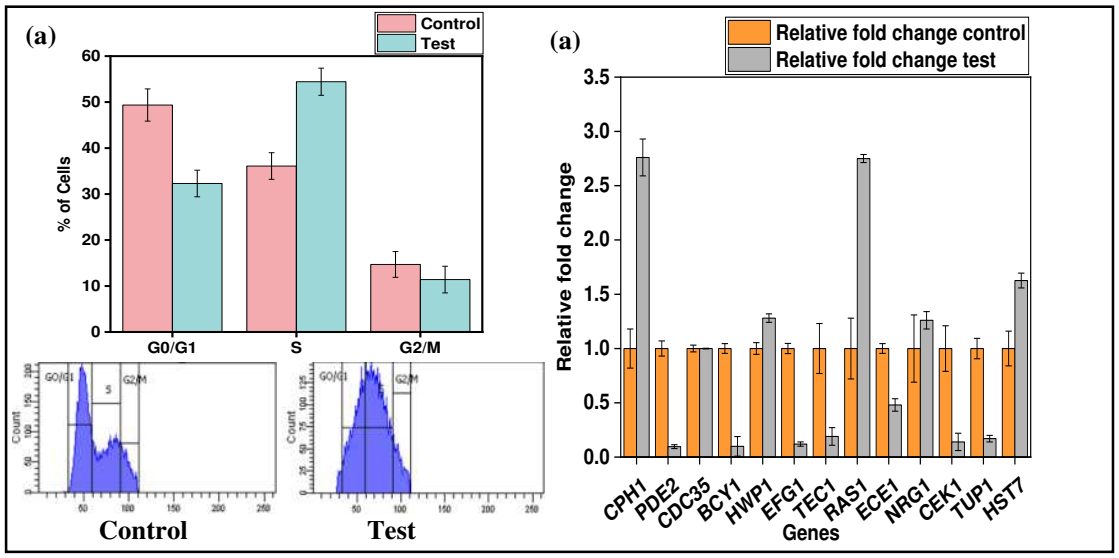

Fig. 4.5.5 (a) Cell cycle analysis and arrest with propidium iodide staining. The bars indicate the percent of cells in G1, S, and G2/M phases in control cells (pink bars) and in 2 mg/ml test i.e. butyl isothiocyanate treated cells (blue bars). Butyl isothiocyanate arrests the *C. albicans* cells at S- phase. **(b)** Effect of butyl isothiocyanate on signal transduction genes involved in biofilm formation.

4.5.10. Gene expression profiling through Real Time Polymerase Chain Reaction (RT-PCR)

Transcriptional level of hyphal-specific and biofilm related genes in *C. albicans* in the absence and presence of ButylITC was quantified by real time PCR. ButylITC at its developing biofilm inhibitory concentration (2 mg/ml) significantly down regulated the expression of hyphal specific and transcription factors gene expression like *PDE2, BCY1, EFG1, TEC1, ECE1* and *CEK1* by 10.42, 22.22, 8.33, 5.26, 2.08 and 7.14-fold change respectively. ButylITC upregulated the expression of *CPH1, HWP1, RAS1,*

NRG1 and *HST7* genes by 2.19, 1.28, 2.75, 1.26 and 1.62-fold change, respectively (**Fig.4.5.5.b**). ButylITC inhibits one of the important virulence factor i.e. ButylITC inhibits developing biofilm at 2 mg/ml concentration might be due to downregulation of *PDE2, BCY1, EFG1, TEC1, ECE1* and *CEK1* and upregulation of negative regulator of Y-H morphogenesis i.e. *NRG1*.

4.5.11. Hemolytic activity against human RBC

Hemolytic activity was evaluated using human RBC. It was observed that ButylITC is hemolytic in nature at concentration ranges from 0.0039 to 2 mg/ml (**Fig. 4.5.6.**). The result suggests that ButylITC has high toxicity towards human RBCs.

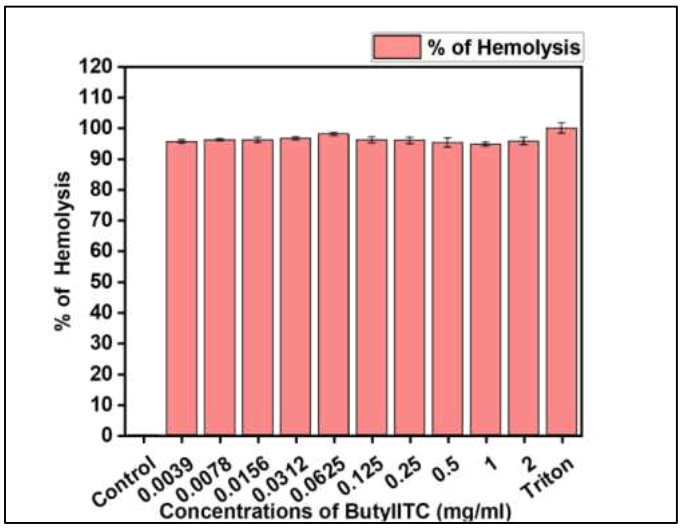

Fig. 4.5.6. Effect of butyl isothiocyanate on human red blood cells.

4.5.12. Conclusions

Current study demonstrated antifungal activity of ButylITC against *C. albicans* cells for the first time. It is unclear exactly how ButylITC works in *C. albicans*. The current investigation showed that, ButylITC suppresses *C. albicans* planktonic growth, morphological transition of yeast cells, adhesion, and biofilm formation. The mechanistic investigations demonstrated that ButylITC inhibits ergosterol biosynthesis, hence stopping the proliferation of *C. albicans* cells. The research work mentioned above showed that, ButylITC has antifungal activity which is linked to generation of ROS and FACS analysis which demonstrate S-phase arrest of *C. albicans* cells by the

treatment of ButylITC. ButylITC inhibits developing biofilm at 2 mg/ml concentration might be due to downregulation of *PDE2, BCY1, EFG1, TEC1, ECE1* and *CEK1* genes and upregulation of negative regulator of Y-H morphogenesis i.e. *NRG1* gene. However, by reducing toxicity, ButylITC based topical formulations may be employed in antifungal biomedical applications.

Results

4.6. Anti-*C. albicans* activity of isopropyl isothiocyanate (IPITC)

4.6.1. Determination of effect of IPITC using planktonic assay

The effect of treatment of isopropyl thiocyanate was determined by the serial micro-broth dilution method in 96 well microtiter plates with the help of spectrophotometer. IPITC exhibited inhibitory activity against *C. albicans* ATCC 90028 strain in the range of 0.0039 to 2 mg/ml. IPITC inhibited 50 % planktonic growth at a concentration of 2 mg/ml when compared to non-treated control (**Fig. 4.6.1.a**).

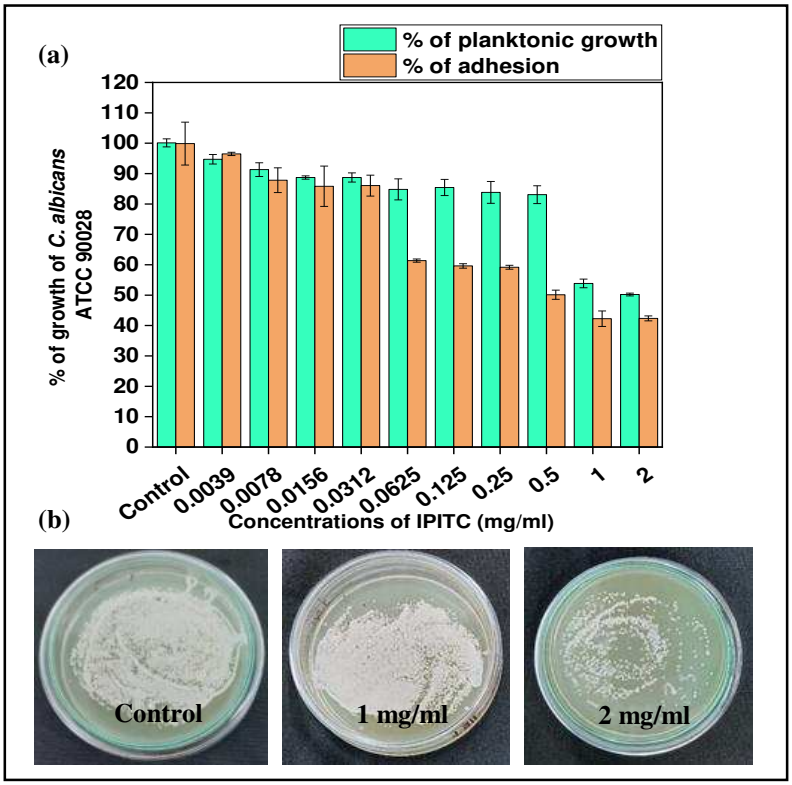

Fig. 4.6.1. (a) Effect of isopropyl isothiocyanate on the planktonic growth and adhesion of *C. albicans* cells. Isopropyl isothiocyanate inhibited planktonic growth and adhesion significantly at 2 mg/ml and 0.125 mg/ml concentration compared to control. **(b)** Minimum fungicidal concentration against *C. albicans* growth after the treatment of isopropyl isothiocyanate.

4.6.2. IPITC inhibits adhesion of *C. albicans*

The effect of IPITC on *C. albicans* adherence to polystyrene surface was analysed. Various concentrations of IPITC were prepared and tested. IPITC caused 50 % reduction to adhesion of *C. albicans* cells at 0. 5 mg/ml concentration (**Fig. 4.6.1.a**).

4.6.3. Minimum fungicidal concentration (MFC)

By performing MFC it was further assessed the effect of IPITC on growth of *C. albicans*. In this investigation it was observed that IPITC was fungistatic in nature up to 2 mg/ml concentration (**Fig.4.6.1.b**).

4.6.4. Determination of effect of IPITC on yeast to hyphal (Y-H) morphogenesis

The conversion of yeast to hyphal morphogenesis is an essential virulence factor in *C. albicans* infection. Hyphal formation helps *C. albicans* in penetrating host tissue which subsequently leads to systemic infection and biofilm formation. In current study it was observed that IPITC inhibited Y-H morphogenesis at 0.25 mg/ml concentration (**Fig.4.6.2. a, b**).

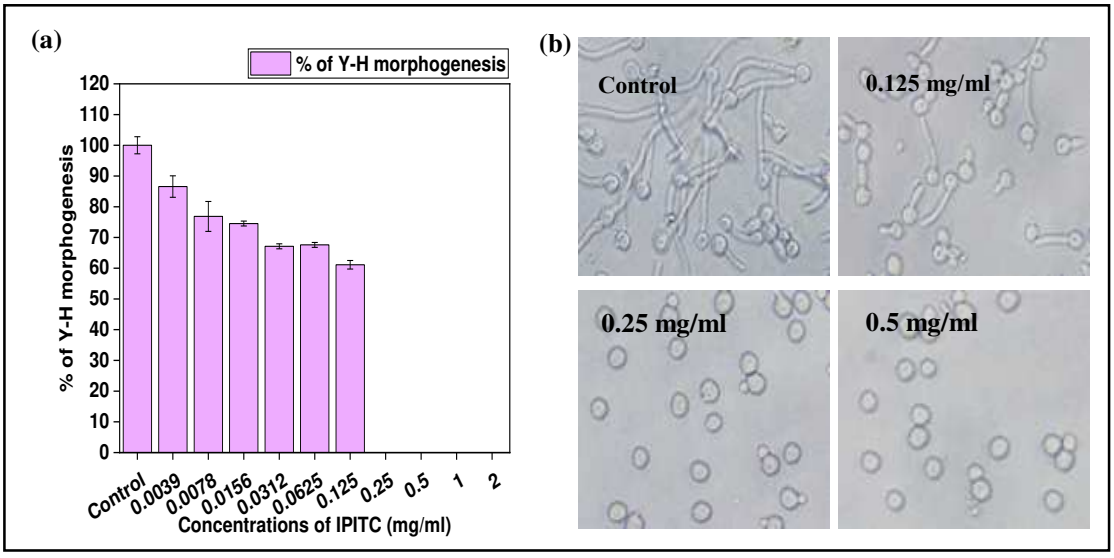

Fig. 4.6.2. (a) Inhibitory effects of isopropyl isothiocyanate on *C. albicans* yeast to hyphal morphogenesis in a concentration-dependent manner. **(b)** The inhibitory activity of isopropyl isothiocyanate against *C. albicans* yeast to hyphal form morphogenesis observed at 0. 25 mg/ml concentration through inverted light microscopy.

4.6.5. Determination of effect of IPITC on developing and mature biofilm formation

We further investigated for the first-time inhibitory effect of IPITC on *C. albicans* developing biofilm and mature biofilm formation with the help of XTT metabolic assay. Results of XTT assay suggest that metabolic activity of *C. albicans* developing biofilm was reduced by the activity of IPITC. At a concentration of 2 mg/ml IPITC showed 54 % reduction in developing biofilm growth of *C. albicans* as compared to non-treated control. Mature biofilm of *C. albicans* was not inhibited by IPITC concentration up to the range of 0.0039 to 2 mg/ml (**Fig. 4.6.3.a**).

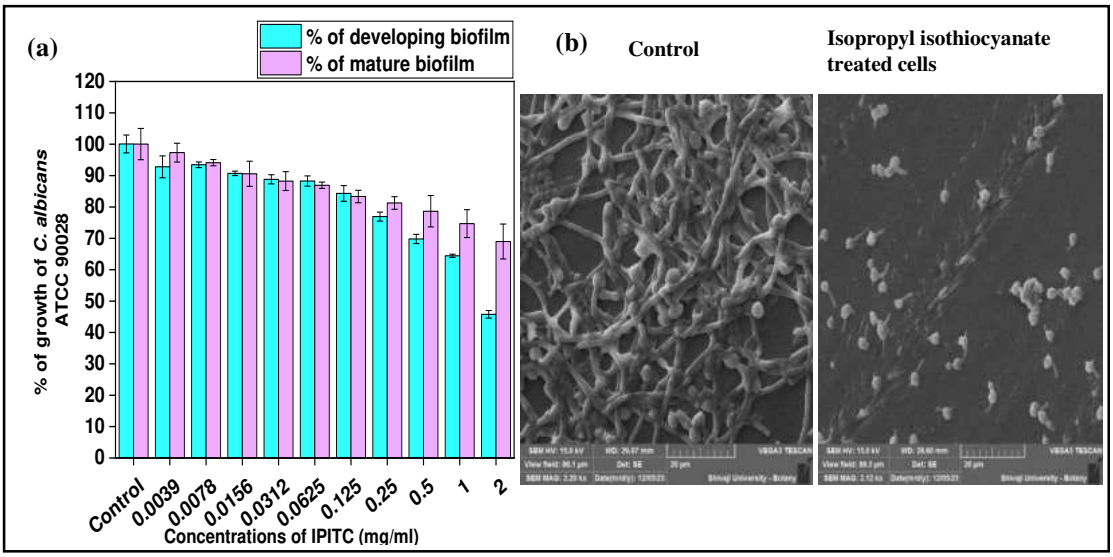

Fig. 4.6.3. (a) Anti-biofilm activity of isopropyl isothiocyanate against *C. albicans* developing and mature biofilm. **(b)** Scanning electron microscopy, showing the dense biofilm with hyphal structures of untreated cell (control) and eradication of biofilm by the treatment of 2 mg/ml concentration of isopropyl isothiocyanate.

4.6.6. Effect of IPITC on developing biofilm observed with Scanning Electron microscopy (SEM)

The SEM's images of the IPITC-treated cells displayed significant morphological alterations. Biofilm inhibitory dose (2 mg/ml) of IPITC has been added to the *C. albicans* cell. Figure **4.6.3.b** of the SEM monograph shows that untreated *C. albicans* cells possessed a thick hyphal structure. On the contrary, hyphal structure was eliminated and smooth-walled yeast cells were visible in the treated cells with IPITC at its MIC values for 24 hours.

4.6.7. IPITC inhibited ergosterol biosynthesis

In current study examined the presence of ergosterol in *C. albicans* cell membranes that were treated with various concentrations of IPITC (0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml and 2 mg/ml) compared to those that were not was treated. Figure **4.6.4.a** flat curve at 2 mg/ml concentration illustrates how the amount of ergosterol in *C. albicans* cell membrane has significantly decreased after the treatment of IPITC. On the other hand, the untreated control cells exhibited the ergosterols characteristic peaks. The dose-dependent reduction in ergosterol content suggested that IPITCs antifungal activity depended on its concentration and that its main mechanism of action was the inhibition of ergosterol biosynthesis.

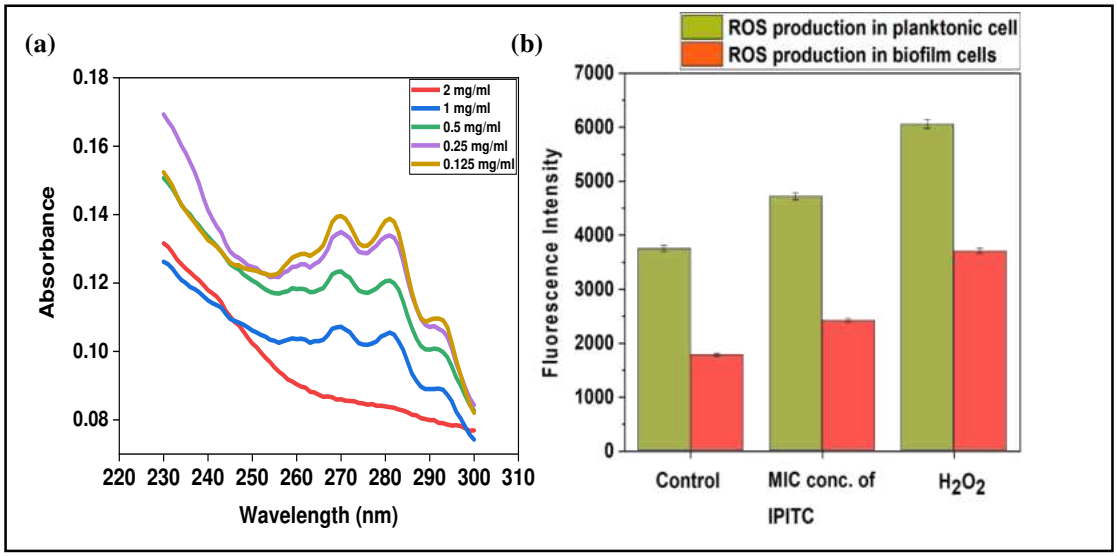

Fig. 4.6.4. (a) Ergosterol profile of *C. albicans* scanned between 230 to 300 nm from culture grown with and without the treatment of isopropyl isothiocyanate. **(b)** The treatment of isopropyl isothiocyanate significantly enhanced the production of ROS in *C. albicans* planktonic and biofilm cells observed using fluorescence spectrophotometer.

4.6.8. ROS production induced by IPITC

The amount of ROS in *C. albicans* cells has been determined using DCFH-DA fluorescent dye. For analysis, a fluorescence spectrophotometer was used. The generation of ROS within the cell is a sign of cellular stress brought on by external factors. Therefore, in current study we have exposed *C. albicans* cells to planktonic (2

mg/ml) and biofilm (2 mg/ml) inhibitory concentrations of IPITC for respective time duration mentioned in methodology (**Fig. 4.6.4.b**). By the treatment of IPITC cumulative increase of ROS in the cells were noted as compared to non-treated control.

4.6.9. Cell cycle analysis after the treatment of IPITC

To find out how IPITC affects DNA replication and cell division a cell cycle analysis was conducted. If there is a deviation in the sequence of these phases, the DNA checkpoint pathway gets triggered, halting all growth and division-related activities until the damage is repaired. In cell cycle analysis study, we have exposed *C. albicans* cells to planktonic inhibitory concentration of IPITC and was compared with non-treated cells. IPITC arrested 26.6 % cells at G0/G1 phase, 31.3 % at G2/M phase and 39.2 % at S phase where as in non-treated cells 61.1 % cells were arrested at G0/G1 phase, 8.0 % at G2/M phase and 30.7 % cells were arrested at S phase. Which shows that IPITC arrest *C. albicans* cells at G2/M phase (**Fig.4.6.5.a**).

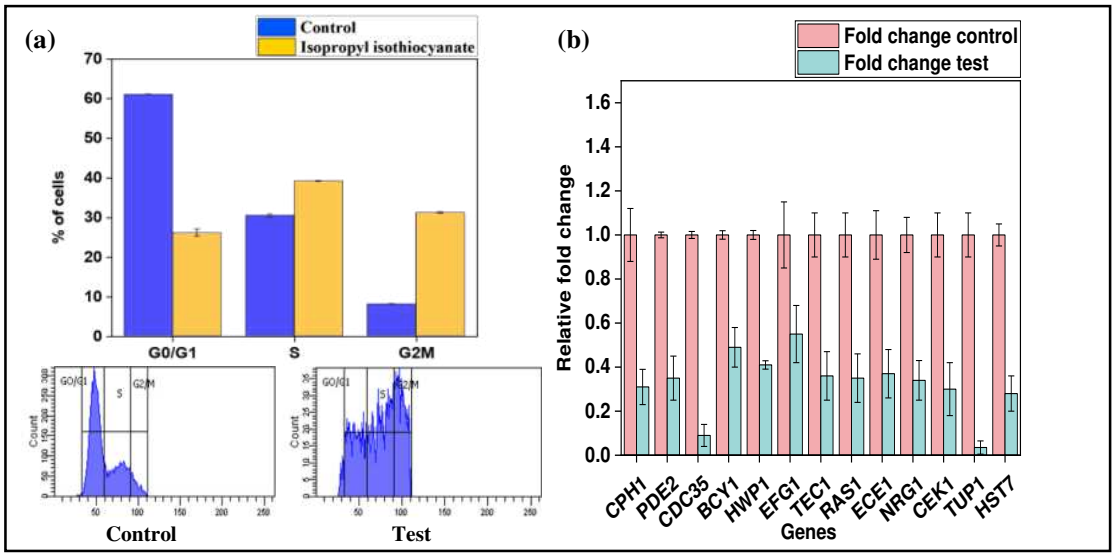

Fig. 4.6.5. (a) Cell cycle analysis and arrest with propidium iodide staining. The bars indicate the percent of cells in G1, S, and G2/M phases in control cells (blue bars) and in 2 mg/ml test i.e. isopropyl isothiocyanate treated cells (yellow bars). Isopropyl isothiocyanate arrests the *C. albicans* cells at S- phase. **(b)** Effect of isopropyl isothiocyanate on signal transduction genes involved in biofilm formation.

4.6.10. Gene expression profiling through Real Time Polymerase Chain Reaction (RT-PCR)

Transcriptional level of hyphal-specific and biofilm related genes in *C. albicans* in the absence and presence of IPITC was quantified by real time PCR. IPITC at its developing biofilm inhibitory concentration (2 mg/ml) significantly down regulated the expression of gene. Further IPITC downregulated hyphal specific and transcription factors gene expression like *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, NRG1, CEK1, TUP1* and *HST7* genes by 3.23, 2.86, 11.11, 2.04, 2.44, 1.82, 2.78, 2.86, 2.70, 2.94, 3.33, 28.57 and 3.57-fold-change, respectively (**Fig. 4.6.5.b**). IPITC inhibits one of the important virulence factor i.e. biofilm formation might be due to downregulation of *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, CEK1* and *HST7* genes.

4.6.11. Hemolytic activity against human RBC

Hemolytic activity was evaluated using human RBC. It was observed that IPITC is hemolytic in nature at concentration ranges from 0.0039 mg/ml to 2 mg/ml (**Fig. 4.6.6**.). The result suggests that IPITC has high toxicity towards human RBCs.

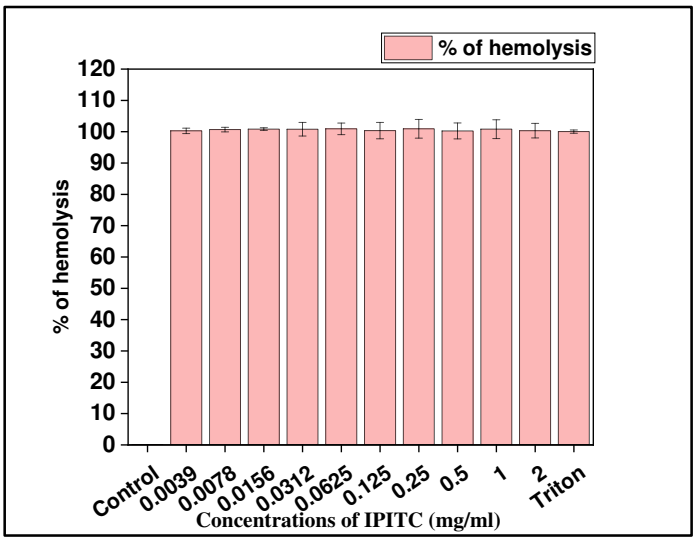

Fig. 4.6.6. Effect of isopropyl isothiocyanate on human red blood cells.

4.6.12. Conclusions

IPITC demonstrates potent antifungal activity against *C. albicans* by inhibiting planktonic growth, adhesion to surfaces, and transition from yeast to hyphal forms. IPITC effectively inhibited biofilm formation, particularly in developing biofilms, and induces significant morphological alterations in biofilm structures. Mechanistically, IPITC inhibited ergosterol biosynthesis, induces ROS production, and arrested the cell cycle at the G2/M phase. Gene expression profiling revealed downregulation of genes *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, CEK1* and *HST7* in the presence of IPITC. However, IPITC demonstrates hemolytic activity against human red blood cells. However, by reducing toxicity, IPITC based topical formulations may be employed in antifungal biomedical applications.

Results

4.7. Anti-*C. albicans* activity of Allyl isocyanate (AIC)

4.7.1. Planktonic growth of *C. albicans* was inhibited by AIC

AIC inhibited planktonic growth (**Fig. 4.7.1.a**). The absorbance of growth indicates that AIC inhibited *C. albicans* planktonic growth in a concentration dependent manner ranging from 0.0039 to 2 mg/ml. At 2 mg/ml concentration 50 % reduction was observed in growth of *C. albicans* ATCC 90028 strain.

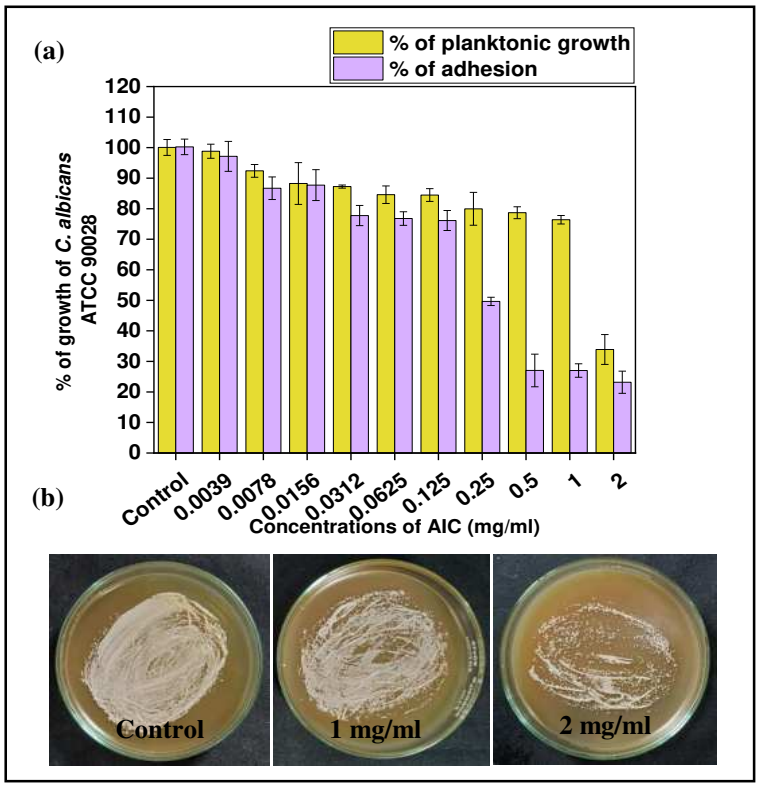

Fig. 4.7.1. (a) Growth curve of *C. albicans* planktonic and adhesion cells with allyl isocyanate. *C. albicans* cells were co-incubated with various concentrations (0.0039 to 2 mg/ml) of allyl isocyanate for 48 h and 90 min. **(b)** Fungistatic nature of allyl isocyanate against *C. albicans* was obtained by observing colonies grown on YPD agar plate after performing planktonic growth assay.

4.7.2. Inhibition of Adhesion by AIC

The process by which *C. albicans* colonises host tissues and forms a biofilm on devices that are embedded begins with cell adhesion. With the help of XTT metabolic assay

adhesion of *C. albicans* cells on the polystyrene surface was observed. AIC inhibited adhesion of *C. albicans* at 0.25 mg/ml concentration (**Fig. 4.7.1.a**).

4.7.3. Minimum fungicidal concentration

After performing MFC experiment (**Fig. 4.7.1.b**) it was observed that there was no complete killing of *C. albicans* cells observed on YPD agar plate up to 2 mg/ml concentration so it was confirmed that AIC was fungistatic in nature.

4.7.4. Yeast to Hyphal Morphogenesis

Serum induced Y-H morphogenesis of *C. albicans* ATCC 90028 strain was observed with the help of inverted microscopy. *C. albicans* cells were treated with series of concentrations of AIC in the range of 0.0039 to 2 mg/ml concentration. AIC inhibited Y-H morphogenesis at 0.5 mg/ml concentration (**Fig. 4.7.2. a, b**). It was observed that concentration above MIC shows complete inhibition to Y-H morphogenesis of *C. albicans.*

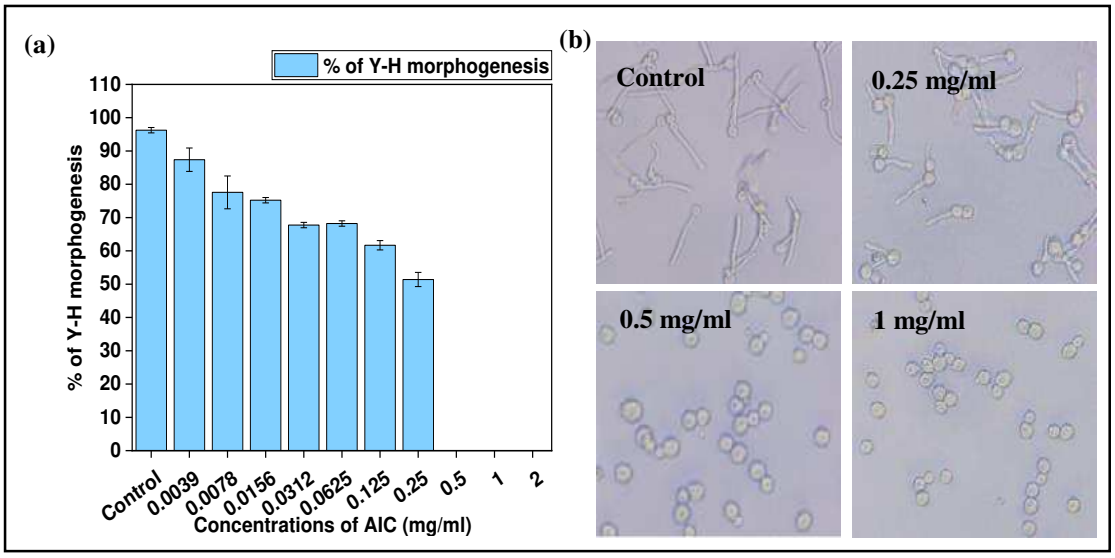

Fig. 4.7.2. (a) Effect of allyl isocyanate against the yeast to hyphal (Y-H) morphogenesis of *C. albicans* 90028 strains treated by various concentrations of allyl isocyanate (0.0039 to 2 mg/ml) and the inhibition of *C. albicans* observed with inverted light microscopy. **(b)** Inhibition of yeast to hyphal form morphogenesis was observed at 0. 5 mg/ml concentration through inverted light microscopy.

4.7.5. Effect of AIC on developing and mature biofilm formation

The XTT metabolic assay was used to evaluate inhibition of developing and mature biofilm of *C. albicans* in the presence and absence of AIC in order to support the anti-biofilm activity. AIC inhibits developing biofilm of *C. albicans*. The concentration ranges between 0.0039 to 2 mg/ml was screened for anti- biofilm activity. Reduction in developing biofilm formation was seen at 2 mg/ml concentration. The MIC for developing biofilm was found at 2 mg/ml concentration. Eradication of mature biofilm is very difficult; in current study it was observed that AIC fails to inhibit mature biofilm up to 2 mg/ml concentration (**Fig. 4.7.3.a**).

4.7.6. Scanning Electron Microscopy analysis

Scanning Electron Microscopy (SEM) analysis performed to investigate the effect of AIC on developing biofilm of *C. albicans* sample. SEM analysis showed that (**Fig. 4.7.3.b**) in control image dense biofilm observed with hyphal structure, while 2mg/ml concentration of AIC eradicate dense biofilm only smooth walled spherical bodies were seen.

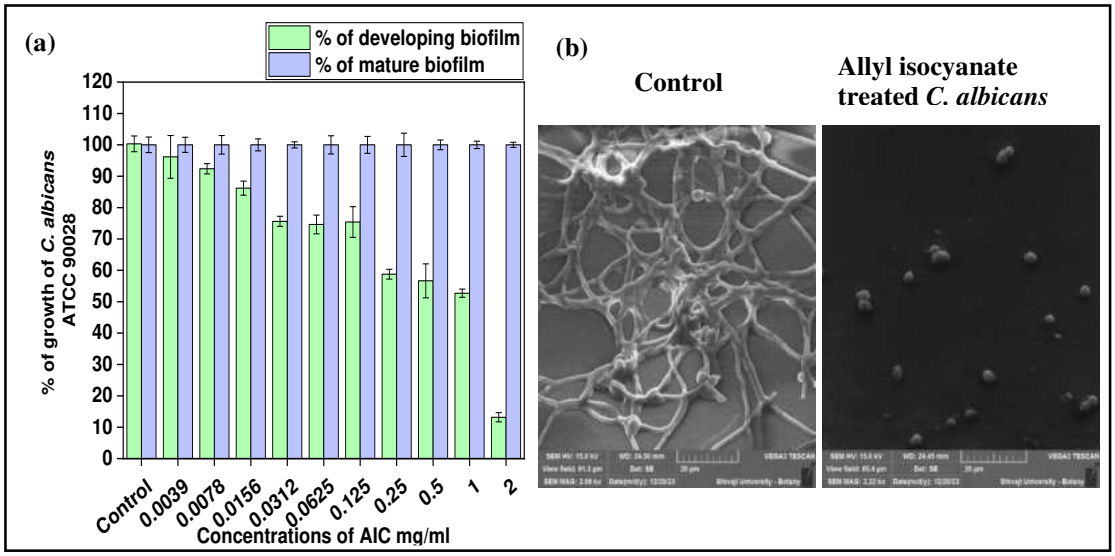

Fig. 4.7.3. (a) Effect of allyl isocyanate against developing and mature biofilm of *C. albicans* 90028 strains treated by various concentrations of allyl isocyanate (0.0039 to 2 mg/ml) and the inhibition of *C. albicans* developing biofilm observed with XTT-metabolic assay. **(b)** Scanning electron microscopy, showing the dense biofilm with hyphal structures of untreated cell (control) and eradication of biofilm by the treatment of 2 mg/ml concentration of allyl isocyanate.

4.7.7. AIC inhibits ergosterol synthesis

Ergosterol is an important component of fungal cell membrane and primary target of antifungal agents. The presence of ergosterol in fungal cell membrane was observed with characteristic peaks, which was inhibited by the action of drug and observed with flat curve. AIC inhibits ergosterol biosynthesis in a concentration dependent manner and AIC at its MIC concentration (2 mg/ml) successfully inhibit ergosterol biosynthesis (**Fig.4.7.4.a**).

4.7.8. ROS production induced by AIC

The amount of ROS in *C. albicans* cells has been determined using DCFH-DA fluorescent dye. For analysis, a fluorescence spectrophotometer was used. The generation of ROS within the cell is a sign of cellular stress brought on by external factors. Therefore, in current study we have exposed *C. albicans* cells to planktonic (2 mg/ml) and biofilm (2 mg/ml) inhibitory concentrations of AIC for respective time duration mentioned in methodology (**Fig. 4.7.4.b**). By the treatment of AIC cumulative increase of ROS in the cells were noted as compared to non-treated control.

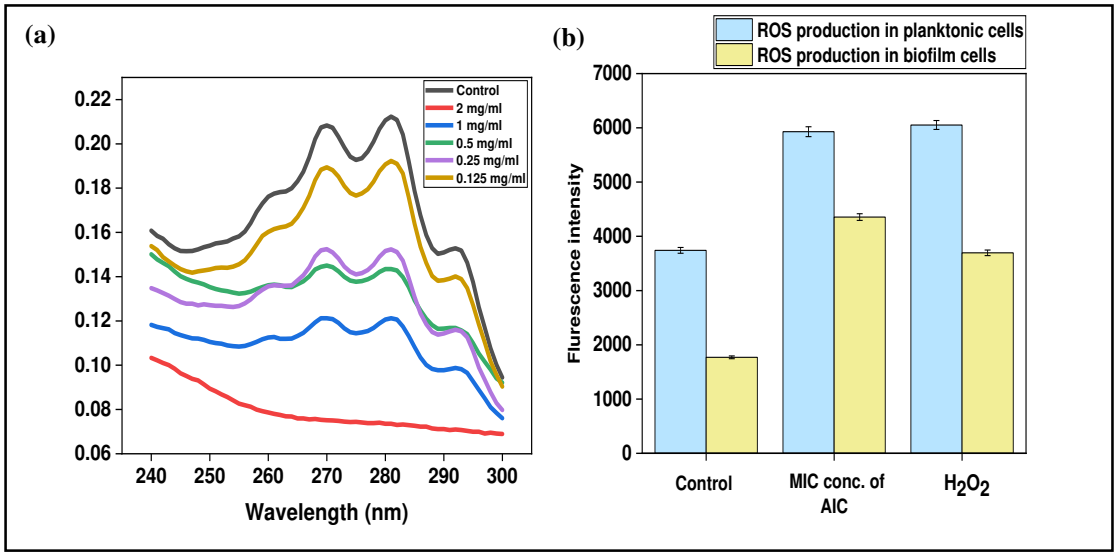

Fig. 4.7.4. (a) Ergosterol profile of *C. albicans* scanned between 240 to 300 nm from culture grown with and without the treatment of allyl isocyanate. **(b)** The treatment of allyl isocyanate significantly enhanced the production of ROS in *C. albicans* planktonic and biofilm cells observed using fluorescence spectrophotometer.

4.7.9. Cell cycle analysis results

Cell cycle analysis study shows that (**Fig.4.7.5.a**) *C. albicans* cells were arrested in different phases of cell cycle. In this study control shows 49.4 % cell in G1 phase, 36.0 % in synthetic S phase and 14.5 % in G2/M phase while cells treated with 2 mg/ml concentration of AIC shows 34.7 % in G1, 46.9 % in S and 17.2 % in G2/M phase. From this study it was observe that AIC arrest the cells at G0/G1- phase.

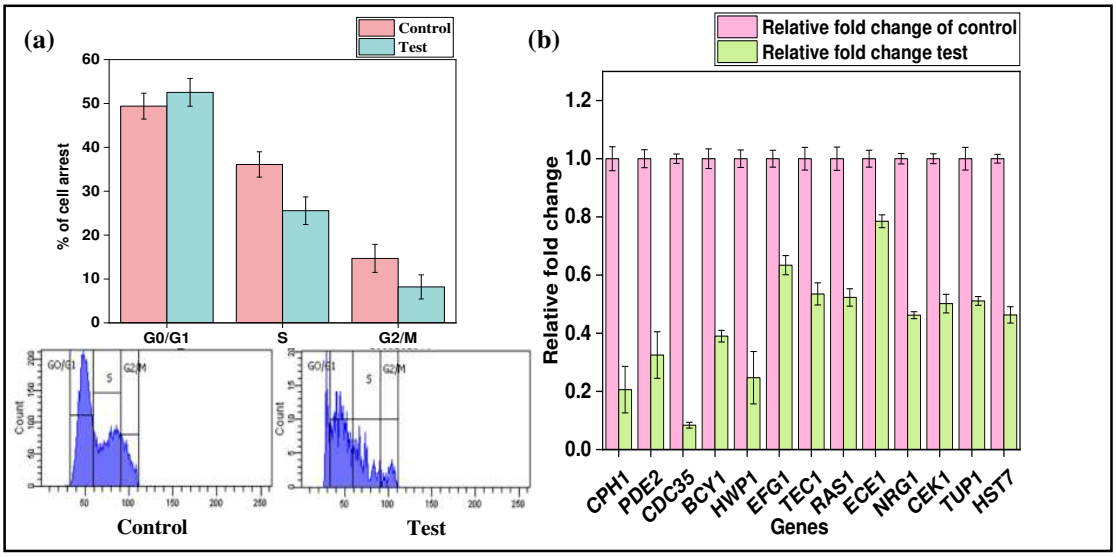

Fig. 4.7. 5. (a) Cell cycle analysis and arrest with propidium iodide staining. The bars indicate the percent of cells in G1, S, and G2/M phases, in control cells and in allyl isocyanate treated cells. Allyl isocyanate arrest *C. albicans* cells at G0/G1- phase. **(b)** Effect of allyl isocyanate on signal transduction genes involved in biofilm formation.

4.7.10. Gene expression profiling through Real Time Polymerase Chain Reaction (RT-PCR)

Transcriptional level of hyphal-specific and biofilm related genes in *C. albicans* in the absence and presence of AIC was quantified by real time PCR. AIC at its developing biofilm inhibitory concentration (2 mg/ml) significantly down regulated the expression of hyphal specific and transcription factors gene expression like *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, NRG1, CEK1, TUP1* and *HST7* by 4.85, 3.08, 11.93, 2.56, 4.05, 1.58, 1.87, 1.91, 1.27, 2.16, 1.99, 1.96 and 2.16-fold change, respectively (**Fig.4.7.5.b**). AIC inhibits one of the important virulence factor i.e. biofilm formation by downregulating *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, CEK1* and *HST7* genes.

4.7.11. Toxicity Analysis of AIC

Hemolytic assay helps to analyse the effect of AIC on host cell i.e. on human Red Blood Cells (RBCs). It was observed that AIC was hemolytic in nature in a concentration ranges between 0.0039 to 2 mg/ml (**Fig. 4.7.6**).

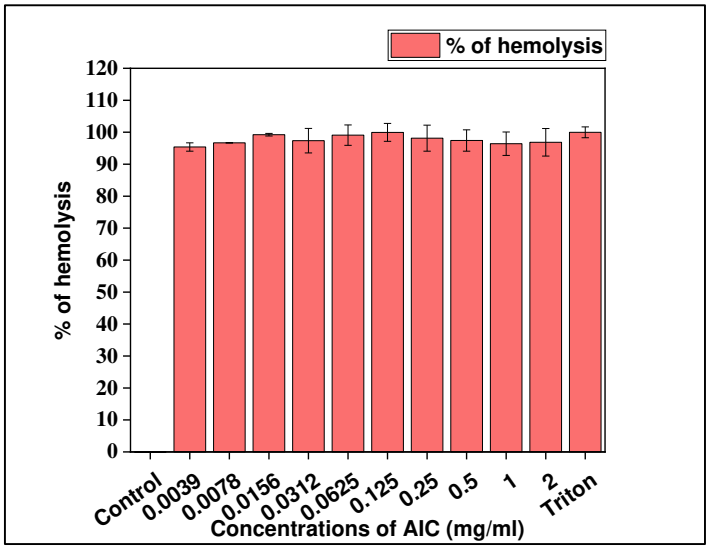

Fig. 4.7.6. Effect of allyl isocyanate on human Red Blood Cells (RBCs).

4.7.12. Conclusions

AIC demonstrated significant antifungal activity against *C. albicans*, inhibiting planktonic growth and adhesion in a concentration-dependent manner. It also effectively inhibits the transition of *C. albicans* from yeast to hyphal forms and disrupts developing biofilms. However, it didn't inhibit mature biofilm. AIC acts by inhibiting ergosterol synthesis, inducing ROS production, and arresting the cell cycle at the G0/G1 phase. Gene expression analysis reveals downregulation of biofilm-related genes in the presence of AIC by downregulating *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, CEK1* and *HST7* genes. AIC exhibited hemolytic activity against human red blood cells. Further research is needed to explore its clinical applicability while addressing its cytotoxic effects.

Results

4.8. Anti-*C. albicans* activity of benzoyl isothiocyanate (BZITC)

4.8.1. Minimum inhibitory concentration (MIC) of BZITC

C. albicans cells were treated with varying concentrations of BZITC ranging from 0.0039 to 2 mg/ml to evaluate its impact on planktonic growth. As illustrated in **Fig. 4.8.1.**, BZITC did not significantly reduce the planktonic growth of *C. albicans*, even at the maximum concentration of 2 mg/ml. Suggesting that BZITC lacks effective antifungal properties against the planktonic growth of *C. albicans*.

4.8.2. Inhibitory activity of BZITC against adhesion to the polystyrene surface

Adherence of *C. albicans* cells to polystyrene was influenced by BZITC. *C. albicans* cells were exposed to a series of concentrations of BZITC in the range of 0.0039 to 2 mg/ml. Analysis of adhered cell density using the XTT assay revealed up to a 52% reduction in adhesion at a concentration of 1 mg/ml. BZITC effectively inhibited the adhesion of cells to the solid surface (**Fig. 4.8.2**).

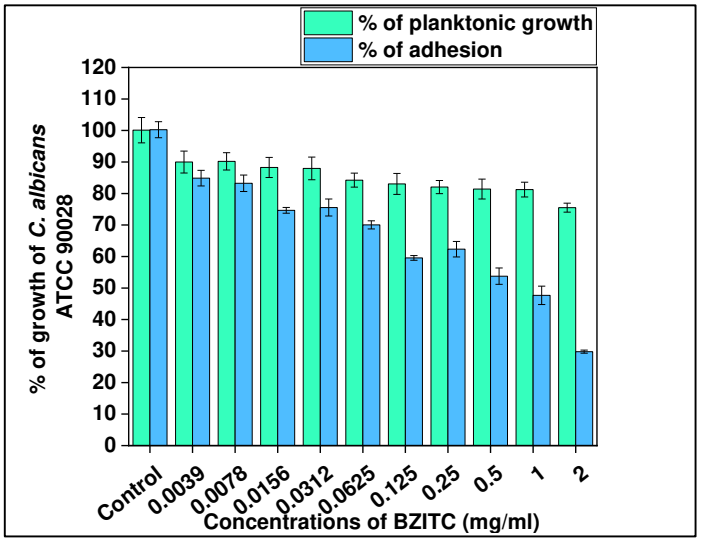

Fig. 4.8.1. Growth curve of *C. albicans* planktonic and adhesion cells with benzoyl isothiocyanate. For growth yeast cells were co-incubated with various concentrations (0.0039 to 2 mg/ml) of benzoyl isothiocyanate for 48 h and 90 min, respectively.

4.8.3. Inhibitory activity of BZITC against the transition of *C. albicans* from yeast to hyphae

The transition from yeast to hyphae is an important virulence-mediating attribute of *C. albicans*. Conversion of yeast cells to hyphal forms is critical for biofilm biogenesis, which provides strength and support to the developing heterogeneous biofilm structure. In the present study, we observed that BZITC at a concentration of 1 mg/ml effectively inhibited transition from yeast to hyphae (**Fig.4.8.2 a, b**).

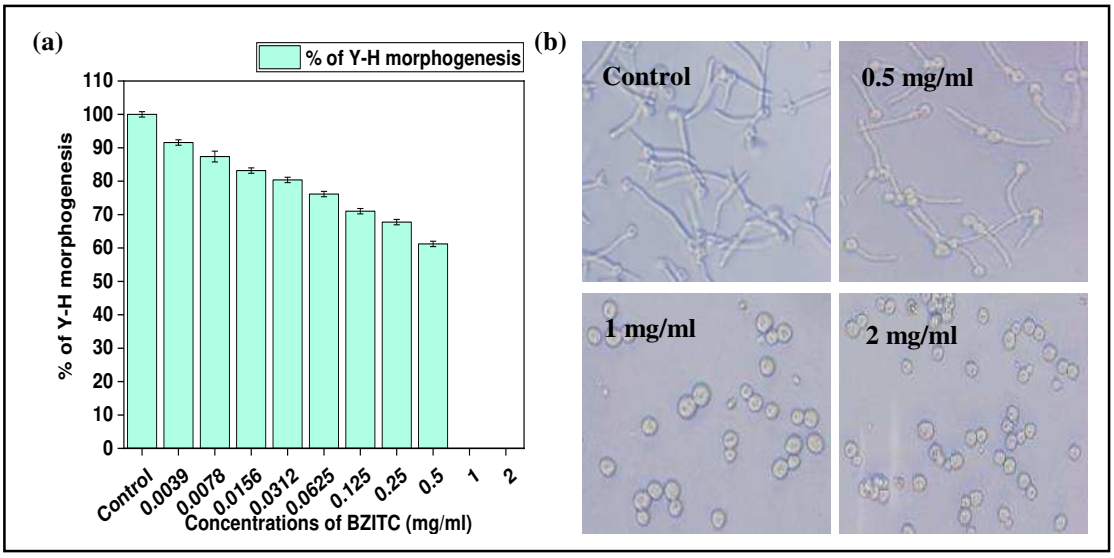

Fig. 4.8.2. (a) Effect of benzoyl isothiocyanate against *C. albicans* yeast to hyphal (Y-H) morphogenesis after performing morphogenesis assay. **(b)** Inhibition of yeast to hyphal form morphogenesis was observed at 1 mg/ml concentration by benzoyl isothiocyanate through inverted light microscopy.

4.8.4. Effect of BZITC on developing and mature biofilm

The XTT metabolic assay revealed that BZITC at concentrations from 0.0039 to 2 mg/ml failed to inhibit biofilm formation by *C. albicans*. Figure **4.8.3.** illustrates that both developing and mature biofilms showed similar metabolic activity to the untreated controls, suggesting that BZITC is ineffective in preventing or disrupting developing or mature biofilm growth. These results demonstrate the necessity for alternative approaches to address *C. albicans* biofilms in clinical settings.

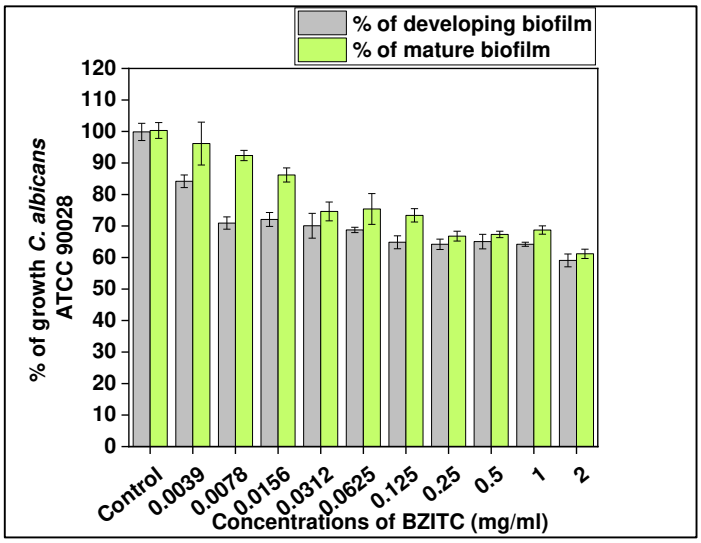

Fig. 4.8.3. Effect of benzoyl isothiocyanate against developing and mature biofilm of *C. albicans* 90028 strains with XTT-metabolic assay.

4.8.5. Toxicity analysis of BZITC

The toxicity of BZITC was analysed by *in vitro* hemolytic activity on human RBCs. It was observed that BZITC was hemolytic in a concentration range from 0.0039 to 2 mg/ml (**Fig.4.8.4**).

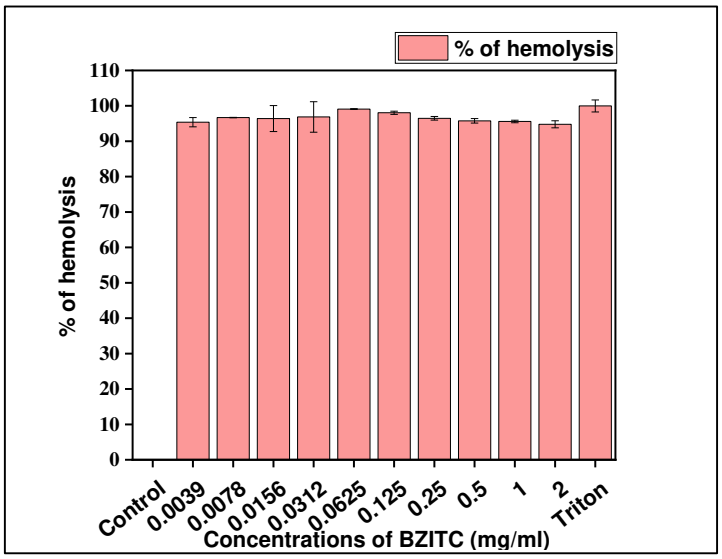

Fig. 4.8.4. Effect of benzoyl isothiocyanate on human Red Blood Cells (RBC).

4.8.6. Conclusions

BZITC showed promising anti-*C. albicans* activity *in vitro*. While it failed to inhibit *C. albicans* growth at concentrations up to 2 mg/ml, it significantly reduced adhesion to

polystyrene surfaces by up to 50 % at 1 mg/ml. Additionally, BZITC effectively inhibited the transition of *C. albicans* from yeast to hyphal forms at 1 mg/ml concentration. However, it did not inhibit developing and mature biofilm formation by *C. albicans* at concentrations ranging from 0.0039 to 2 mg/ml. Toxicity analysis revealed that BZITC exhibited hemolytic activity on human red blood cells across the same concentration range. These findings suggest that BZITC has potential as an anti-*C. albicans* agent, but its use may be limited by its toxicity and fails to prevent biofilm formation.

Results

4.9. Anti-*C. albicans* activity of Phenyl isothiocyanate (PITC)

4.9.1. Minimum inhibitory concentration (MIC) of PITC

C. albicans cells were subjected to various concentrations of PITC ranging from 0.0039 to 2 mg/ml to evaluate its effects on planktonic growth. The results indicated that PITC did not effectively inhibit the growth of *C. albicans*, even at the highest concentration tested i.e. 2 mg/ml (**Fig. 4.9.1.**).

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4.9.2. Inhibitory activity of PITC against adhesion to the polystyrene surface

Adherence of *C. albicans* cells to polystyrene was influenced by phenyl isothiocyanate. *C. albicans* cells were exposed to a series of concentrations of PITC in the range of 0.0039 to 2 mg/ml. Analysis of adhered cell density using the XTT assay demonstrated a reduction in adhesion of up to 54 % at a concentration of 0.5 mg/ml. PITC effectively inhibited the adhesion of cells to the solid surface (**Fig. 4.9.1.**).

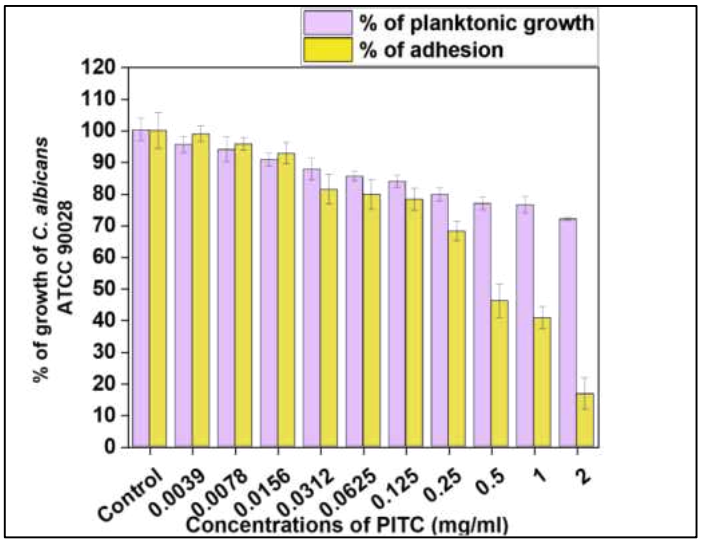

Fig. 4.9.1. Antifungal activity of PITC against planktonic growth and adhesion of *C. albicans* cells.

4.9.3. Inhibitory activity of PITC against the transition of *C. albicans* from yeast to hyphae

The transition from yeast to hyphae is an important virulence-mediating attribute of *C. albicans*. Conversion of yeast cells to hyphal forms is critical for biofilm biogenesis, which provides strength and support to the developing heterogeneous biofilm structure. In the present study, we observed that PITC at a concentration of 0.125 mg/ml effectively inhibited transition from yeast to hyphae (**Fig. 4.9.2. a, b**).

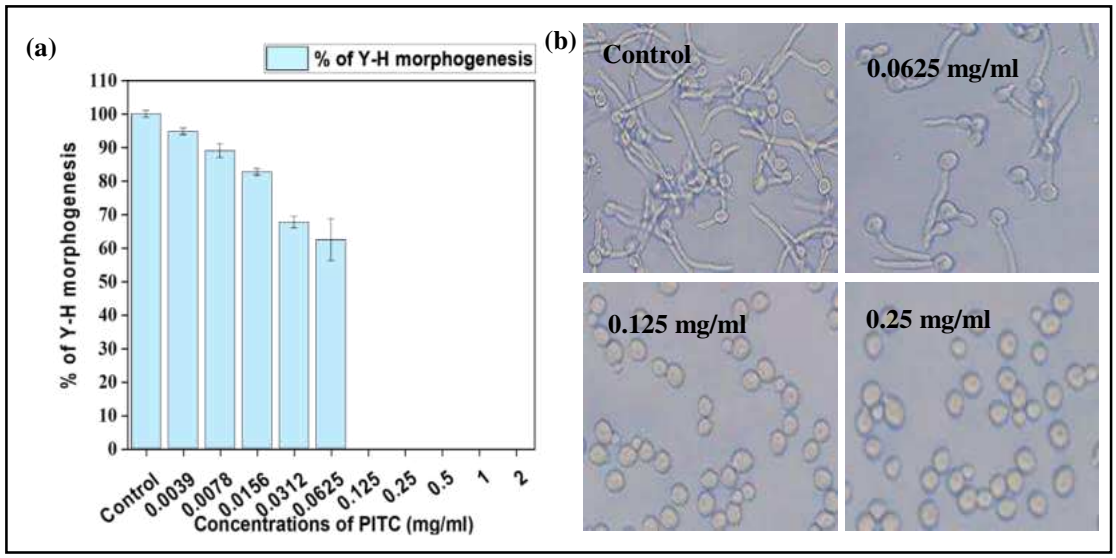

Fig. 4.9.2. (a) The inhibitory activity of isopropyl isothiocyanate against *C. albicans* yeast to hyphal form transition in a concentration dependent manner. **(b)** Inhibition of yeast to hyphal form morphogenesis was observed at 0.125 mg/ml concentration through inverted light microscopy.

4.9.5. PITC fails to inhibits developing and mature biofilm

The analysis of biofilm growth using the XTT metabolic assay demonstrated that the addition of PITC at concentrations ranging from 0.0039 to 2 mg/ml did not inhibit the formation of either developing or mature biofilms of *C. albicans* **(Fig. 4.9.3.)**. The metabolic activity of the biofilms treated with PITC was not considerably decreased, showing that PITC does not effectively disrupt the structure of the biofilm or reduce the activity of *C. albicans* cells. These findings suggest that despite the anti morphogenic and anti-adhesive properties of PITC, it fails to combat developing and mature biofilm formation, highlighting the need for alternative strategies in addressing biofilm-related infections.

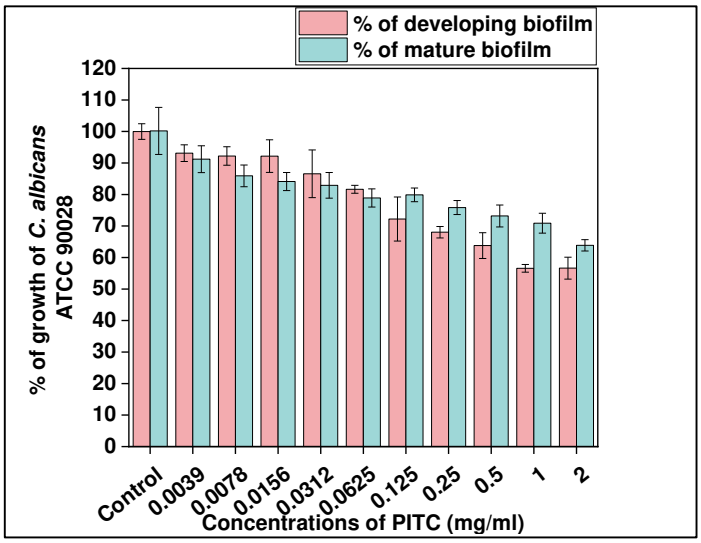

Fig. 4.9.3. The effect of PITC on the developing and mature biofilm of *C. albicans* to polystyrene plates with the help of XTT metabolic assay.

4.9.5. Toxicity analysis of PITC

The toxicity of PITC was analysed by *in vitro* hemolytic activity on human RBCs. It was observed that PITC was hemolytic in a concentration ranges from 0.0039 to 2 mg/ml (**Fig. 4.9.4.**).

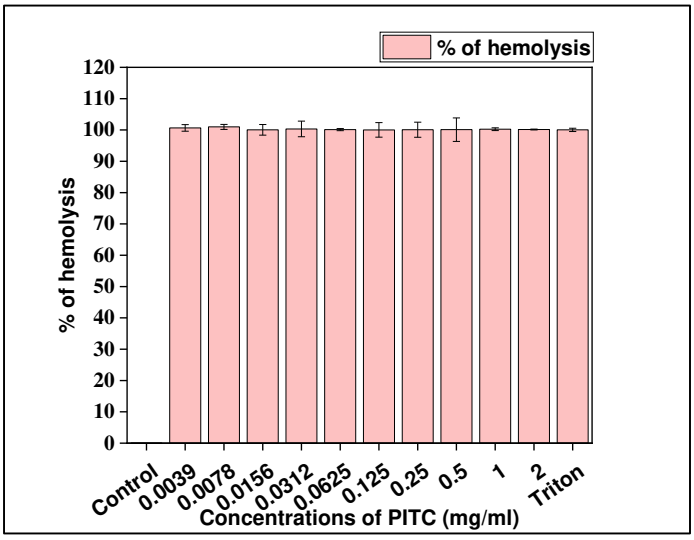

Fig. 4.9.4. Effect of PITC on human Red Blood Cells.

4.9.6. Conclusions

PITC demonstrates limited antifungal activity against *C. albicans*. While it fails to inhibit planktonic growth, it significantly reduced the adhesion of *C. albicans* cells to solid surfaces at 0.5 mg/ml concentration. However, it is ineffective against the transition of yeast to hyphal form at 0.125 mg/ml concentration. PITC does not prevent the formation of developing or mature biofilms at 0.0039 to 2 mg/ml concentrations. Additionally, PITC exhibits hemolytic activity against human red blood cells, suggesting potential toxicity. Overall, its efficacy against *C. albicans* is modest, and further research is needed to explore its therapeutic potential while considering its cytotoxic effects.

Results

4.10. Anti-*C. albicans* activity of 3 Indole acetonitrile (3IA)

4.10.1. Minimum inhibitory concentration (MIC) of 3IA

In this experiment, *C. albicans* cells were treated with various concentrations of 3IA, ranging from 0.0039 to 2 mg/ml, to assess its effects on fungal growth. The results demonstrated that 3IA did not effectively inhibit the planktonic growth of *C. albicans*, even at its highest concentration i. e. 2 mg/ml. This ability to fail inhibition suggests that 3IA may not possess sufficient antifungal properties to affect *C. albicans* planktonic growth, indicating the need for further exploration of more effective agents against this pathogen (**Fig. 4.10.1**).

4.10.2. Inhibitory activity of 3IA against adhesion to the polystyrene surface

Adherence of *C. albicans* cells to polystyrene was influenced by 3IA. *C. albicans* cells were exposed to series of concentration of 3IA in the range of 0.0039 to 2 mg/ml. Analysing the density of adhered cells with XTT assay showed up to 51 % decrease in adhesion was seen at 0.25 mg/ml concentration. 3IA significantly inhibited adhesion of cells to the solid surface (**Fig. 4.10.1**).

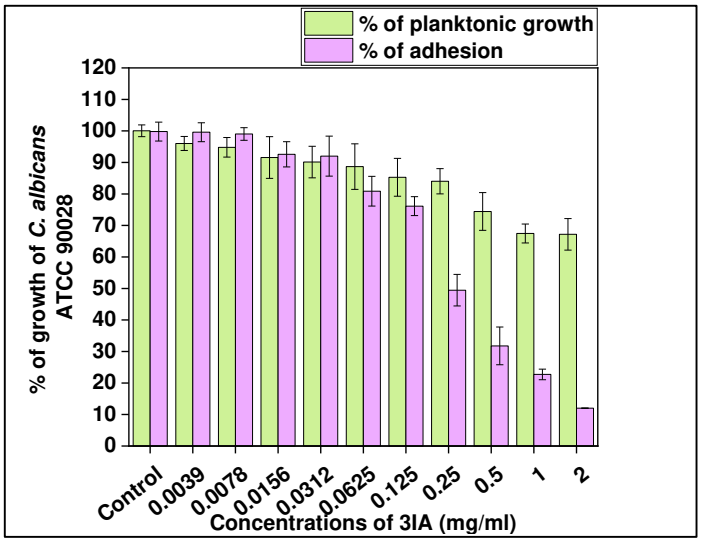

Fig. 4.10.1. Antifungal activity of 3 indole acetonitrile against planktonic growth and adhesion of *C. albicans* cells.

4.10.3. Inhibitory activity of 3IA against the transition of *C. albicans* from yeast to hyphae

The transition from yeast to hyphae is an important virulence-mediating attribute of *C. albicans*. Conversion of yeast cells to hyphal forms is critical for biofilm biogenesis, which provides strength and support to the developing heterogeneous biofilm structure. In the present study, we observed that 3IA at a concentration of 0.25 mg/ml effectively inhibited transition from yeast to hyphae (**Fig. 4.10.2 a, b**).

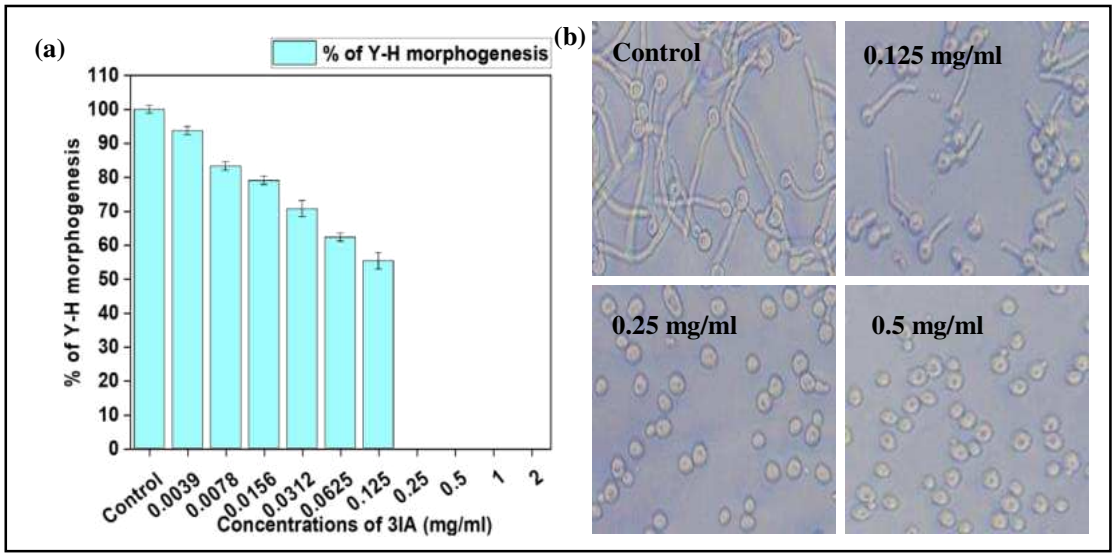

Fig. 4.10.2. (a) The inhibitory activity of 3 indole acetonitrile against *C. albicans* yeast to hyphal form transition in a concentration dependent manner. **(b)** Inhibition of yeast to hyphal form morphogenesis was observed at 0.25 mg/ml concentration through inverted light microscopy.

4.10.4. 3IA fails to inhibit developing and mature biofilm

The XTT-metabolic assay showed that adding 3IA at concentrations from 0.0039 to 2 mg/ml was not effective in inhibiting the formation of developing and mature biofilms by *C. albicans*, as depicted in Figure **4.10.3**. The results indicated that the metabolic activity of *C. albicans* biofilms treated with 3IA was similar to that of the untreated controls, suggesting that 3IA does not disrupt biofilm formation or decrease biofilm viability. This finding highlights the limitations of 3IA for *C. albicans* biofilms inhibition. Additional research is needed to explore more effective compounds for addressing *C. albicans* biofilms in clinical contexts.

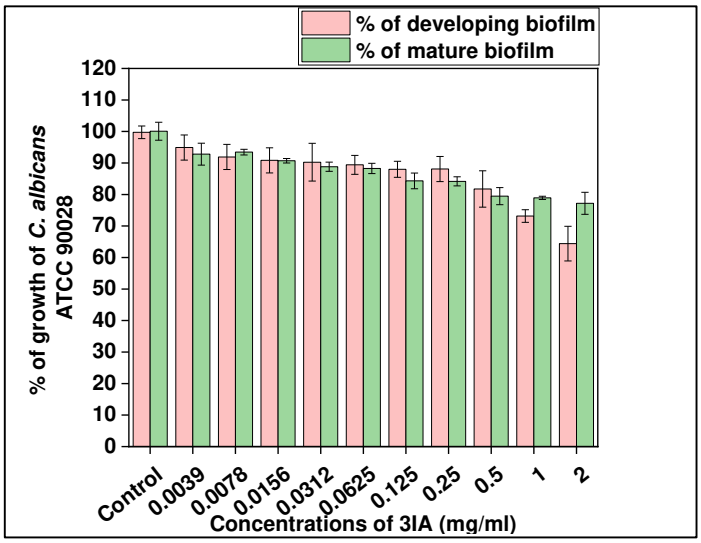

Fig. 4.10.3. The effect of 3 indole acetonitrile on the developing and mature biofilm of *C. albicans* to polystyrene plates with the help of XTT metabolic assay.

4.10.5. Toxicity analysis of 3IA

The toxicity of 3IA was analysed by *in vitro* hemolytic activity on human RBCs. It was observed that 3IA was hemolytic at a concentration range from 0.0039 to 2 mg/ml (**Fig.4.10.4**).

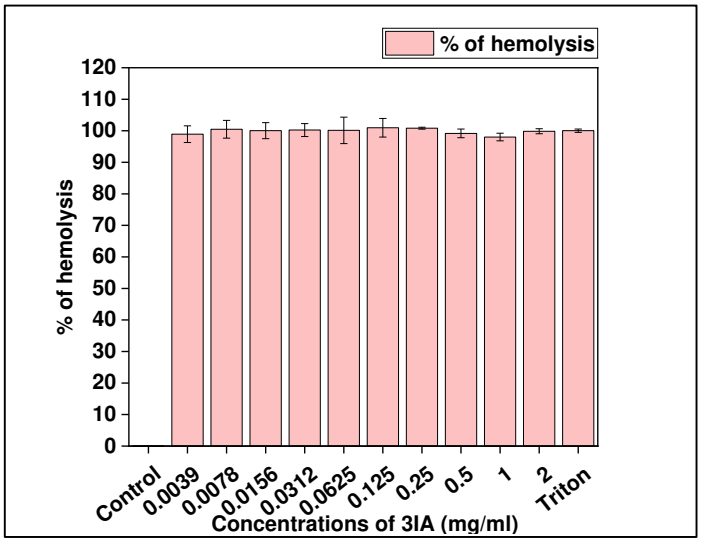

Fig. 4.10.4. Effect of 3 indole acetonitrile on human Red Blood Cells.

4.10.6. Conclusions

3IA showed limited antifungal activity against *C. albicans*. While it failed to inhibit fungal growth, it significantly reduced the adhesion of *C. albicans* cells to solid surfaces and inhibited the transition from yeast to hyphal forms at a concentration of 0.25 mg/ml. However, it does not prevent the formation of developing or mature biofilms. Additionally, 3IA exhibited hemolytic activity against human red blood cells, indicating potential toxicity. Overall, its efficacy against *C. albicans* is modest, and further research is needed to explore its therapeutic potential while considering its cytotoxic effects.

Results

4.11. Anti-*C. albicans* activity of Sulforaphane

4.11.1. Minimum inhibitory concentration (MIC) of sulforaphane

In this experiment, *C. albicans* cells were treated with varying concentrations of sulforaphane, ranging from 0.3 µg/ml to 200 µg/ml, to evaluate its effects on planktonic growth. The results showed that sulforaphane did not inhibit the growth of *C. albicans* up to 200 µg/ml. This suggests that sulforaphane lacks significant antifungal activity against *C. albicans* planktonic growth (**Fig. 4.11.1**).

4.11.2. Inhibitory activity of sulforaphane against adhesion to the polystyrene surface

Adherence of *C. albicans* cells to polystyrene was influenced by sulforaphane. *C. albicans* cells were exposed to series of concentrations of sulforaphane in the range of 0.3 to 200 µg/ml µg/ml. Sulforaphane failed to inhibit adhesion of cells to the solid surface up to 200 µg/ml. (**Fig.4.11.1**).

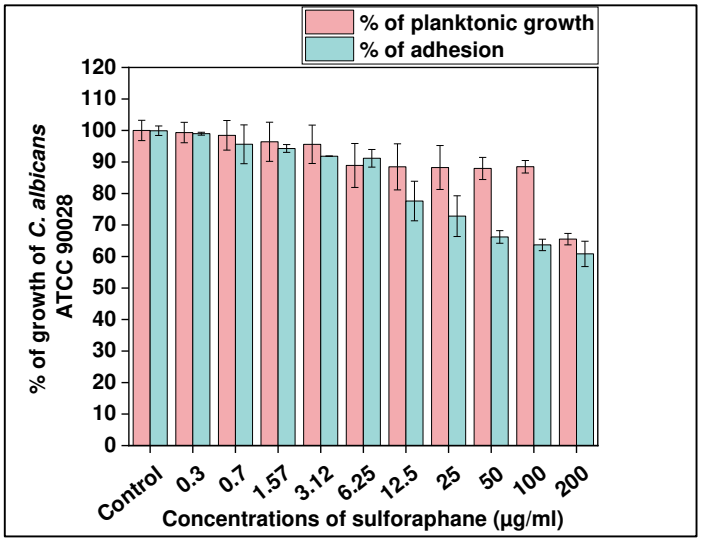

Fig. 4.11.1. Screening antifungal activity of sulforaphane against planktonic growth and adhesion of *C. albicans* cells.

4.11.3. Inhibitory activity of sulforaphane against the transition of *C. albicans* from yeast to hyphae

The transition from yeast to hyphae is an important virulence-mediating attribute of *C. albicans*. Conversion of yeast cells to hyphal forms is critical for biofilm biogenesis, which provides strength and support to the developing biofilm structure. In the present study, we observed that sulforaphane at a concentration of 200 µg/ml effectively inhibited transition from yeast to hyphae (**Fig. 4.11.2 a, b**).

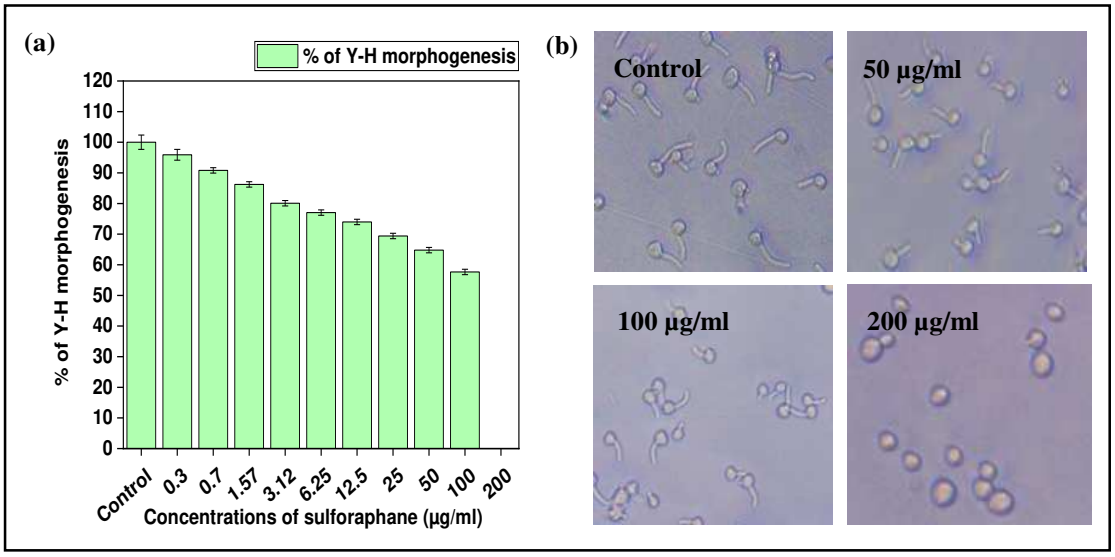

Fig. 4.11.2. (a) The inhibitory activity of sulforaphane against *C. albicans* yeast to hyphal form transition in a concentration dependent manner. **(b)** Inhibition of yeast to hyphal form morphogenesis was observed at 200 µg/ml concentration through inverted light microscopy.

4.11.4. Sulforaphane fails to inhibits developing and mature biofilm

The analysis of biofilm growth in *C. albicans* using the XTT metabolic assay revealed that the addition of sulforaphane, across a concentration range of 0.39 µg/ml to 200 µg/ml, did not inhibit either the developing or mature biofilm formations. This outcome indicates that sulforaphane, despite its recognized antimicrobial properties, is ineffective at disrupting the biofilm. Specifically, the results showed no significant decrease in biofilm viability at any of the tested concentrations, highlighting the resilience of *C. albicans* in its biofilm state. These findings suggest that sulforaphane may inhibit biofilm formation by increasing the concentration and used as an alternative

therapeutic strategy to address biofilm-related infections (**Fig. 4.11.3**).

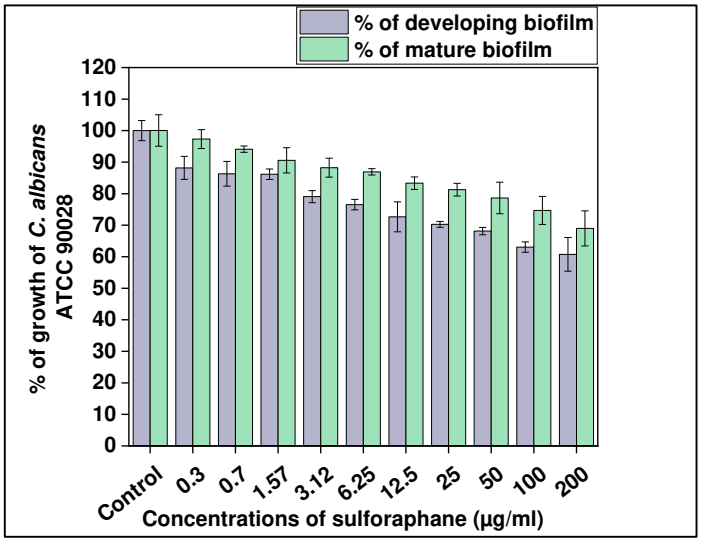

Fig. 4.11.3. The effect of sulforaphane on the developing and mature biofilm of *C. albicans* to polystyrene plates with the help of XTT metabolic assay.

4.11.5. Toxicity analysis of sulforaphane

The toxicity of sulforaphane was analysed by *in vitro* haemolytic activity on human red blood cells (RBCs). It was observed that sulforaphane was non hemolytic in nature at a concentration range from 0.3 to 200 µg/ml (**Fig. 4.9.4**).

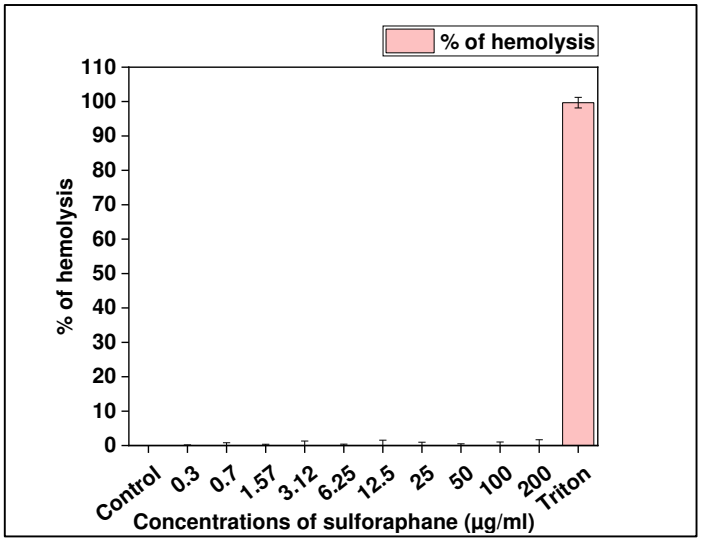

Fig. 4.11.4. Effect of sulforaphane on human Red Blood Cells (RBCs).

4.11.6. Conclusions

Sulforaphane, ineffective in inhibiting *C. albicans* planktonic growth and adhesion to surfaces, demonstrates promising inhibitory effects on the transition of yeast to hyphal forms at a concentration of 200 µg/ml. However, it does not prevent the formation of developing or mature biofilms. Notably, sulforaphane showed no hemolytic activity against human red blood cells at concentrations ranging from 0.3 to 200 µg/ml, suggesting a favourable safety profile. Further research is warranted to explore its potential as an antifungal agent and understand its mechanism of action against *C. albicans*.

CHAPTER – 5

DISCUSSION

5. Discussion

A human fungal pathogen called *Candida* causes candidiasis; recurrent infections, mainly in patients who have impaired immune systems. Every year, approximately one million people experiences candidiasis, which is mainly brought on by *C. albicans*. Over time, the prevalence of candidiasis has significantly grown due to the inappropriate use of antifungals and the emergence of drug-resistant forms. To improve available antifungal medications and stop the growth of increasing strains that are drug-resistant, plant-derived active components represent most secure and suitable option for new antifungal drugs (1). Glucosinolates, is prevalent secondary metabolites. Present in botanical order Brassicales, are hydrolysed enzymatically to form isothiocyanates (ITCs), which are beneficial compounds. Very few research has been published regarding the antimicrobial potential of ITCs against human pathogens, despite their extensive reporting of their antimicrobial activity against plant and foodborne pathogens (2). In current study we reported the anti-candidal activity of glucosinolate derivatives against growth and virulence factors of the human pathogenic fungus *C. albicans.* Glucosinolates inhibit *C. albicans* planktonic growth, adhesion to polystyrene surface and biofilm.

5.1. Effect of glucosinolate derivatives on planktonic Growth of *C. albicans*

C. albicans is a part of natural flora, especially in the mucous membranes. But in other cases, it can overgrow and lead to infections, especially in people with weakened immune systems or underlying medical issues (3). Planktonic growth, when referring to the growth of *C. albicans*, usually means that it is growing as a single-celled, free-floating form rather than producing multicellular structures like biofilms. In a liquid media, individual yeast cells multiply during planktonic development. The presence of other microbes, temperature, pH, and nutrition availability all affect *C. albicans* ability to develop plankton (4). For instance, *C. albicans* prefers warm, humid conditions, which increases the risk of infection in the mouth, vagina, and skin folds. For the purpose of preventing and treating Candida infections, it is critical to understand the factors that support the planktonic growth of *C. albicans* (5). These strategies might involve taking antifungal drugs, keeping oneself clean, and taking care of any

underlying medical issues that might make a person more susceptible to Candida overgrowth (6).

Table 5.1. Effect of glucosinolate derivatives on planktonic growth of *C. albicans*

Glucosinolate derivatives	**AITC**	**EITC**	**BITC**	**PEITC**	**ButylITC**	**IPITC**
MICs for planktonic growth	0.125 mg/ml	0.5 mg/ml	0.125 mg/ml	1 mg/ml	2 mg/ml	2 mg/ml
Glucosinolate derivatives	**AIC**	**BZITC**	**PITC**	**3IA**	**Sulforaphane**	-
MICs for planktonic growth	2 mg/ml	NA	NA	NA	NA	-

(Note: NA- Not Achieved)

The findings imply that, allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), ethyl isothiocyanate (EITC), phenyl ethyl isothiocyanate (PEITC), butyl isothiocyanate (ButylITC), isopropyl isothiocyanate (IPITC) and allyl isocyanate (AIC) possess antifungal activity against the *C. albicans* ATCC 90028 strain's planktonic growth at their respective inhibitory concentrations (**Table 5.1.**). Benzoyl isothiocyanate (BZITC), phenyl isothiocyanate (PITC), 3 indole acetonitrile (3IA) and sulforaphane fails to inhibit planktonic growth of *C. albicans*.

5.2. Effect of glucosinolate derivatives on adhesion to polystyrene surface by *C. albicans*

The initial step in infection and biofilm formation is adhesion to a substrate through certain cell surface adhesins. Thus, preventing *C. albicans* cells from adhering to surface could be an effective way for inhibit the early phases of Candida biofilm formation (7). Glucosinolate derivatives demonstrated anti-adhesion capacity, which is consistent with numerous antifungal agents demonstrating the ability to prevent the adherence of *C. albicans*.

Invasive growth and biofilm formation of *C. albicans* requires adhesion to tissue surfaces. Adhesion of yeast cell to host tissue or medical devices plays important role in colonization and systematic infection. Adhesion plays important role in colonization, biofilm formation and multidrug resistance inhibiting adhesion ultimately leads to prevention of biofilm formation (8).

Table 5.2. Effect of glucosinolate derivatives on adhesion to polystyrene surface by *C. albicans*

Glucosinolate derivatives	**AITC**	**EITC**	**BITC**	**PEITC**	**ButylITC**	**IPITC**
MICs for Adhesion	0.125 mg/ml	0.0312 mg/ml	0.5 mg/ml	0.125 mg/ml	0.125 mg/ml	0.5 mg/ml
Glucosinolate derivatives	**AIC**	**BZITC**	**PITC**	**3IA**	**Sulforaphane**	-
MICs for adhesion	0.25 mg/ml	1 mg/ml	0.5 mg/ml	0.25 mg/ml	NA	-

(Note: NA- Not Achieved)

In our study allyl isothiocyanate (AITC), ethyl isothiocyanate (EITC), benzyl isothiocyanate (BITC), phenyl ethyl isothiocyanate (PEITC), butyl isothiocyanate (ButylITC), isopropyl isothiocyanate (IPITC), allyl isocyanate (AIC), Benzoyl isothiocyanate (BZITC), phenyl isothiocyanate (PITC), 3 indole acetonitrile (3IA) inhibit adhesion to polystyrene surface at their respective minimum inhibitory concentrations (**Table 5.2.**) and sulforaphane up to 200 µg/ml didn't inhibit adhesion.

5.3. Yeast to hyphal (Y-H) morphogenesis of *C. albicans* inhibited by Glucosinolate derivatives

One of the key component of *C. albicans'* pathogenicity and a critical element in the formation of biofilms is its capacity to go through a morphogenic transition from yeast

to hypha (9). In our study It was observed that, in a concentration dependent manner glucosinolate derivative could restraint the shift of yeast to hyphal phase and can revert the hyphal cells to yeast morphogenesis. Allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), ethyl isothiocyanate (EITC), phenyl ethyl isothiocyanate (PEITC), butyl isothiocyanate (ButylITC), isopropyl isothiocyanate (IPITC), allyl isocyanate (AIC), Benzoyl isothiocyanate (BZITC), phenyl isothiocyanate (PITC), 3 indole acetonitrile (3IA) and sulforaphane inhibit Y-H morphogenesis (**Table 5.3.**).

Table 5.3. Effect of glucosinolate derivatives on Yeast to hyphal (Y-H) morphogenesis of *C. albicans*

Glucosinolate derivatives	**AITC**	**EITC**	**BITC**	**PEITC**	**ButylITC**	**IPITC**
MICs for Y-H Morphogenesis	0.125 mg/ml	0.0312 mg/ml	0.0312 mg/ml	0.0312 mg/ml	0.125 mg/ml	0.25 mg/ml
Glucosinolate derivatives	**AIC**	**BZITC**	**PITC**	**3IA**	**Sulforaphane**	-
MICs for Y-H Morphogenesis	0.5 mg/ml	1 mg/ml	0.125 mg/ml	0.25 mg/ml	200 µg/ml	-

(Note: NA- Not Achieved)

5.4. Impact of Glucosinolate derivatives on developing and mature biofilm of *C. albicans*

The complex structure known as the *C. albicans* biofilm is composed of many cell types, including yeast, pseudo-hyphal and hyphal forms, which are covered in extracellular exopolysaccharide (EPS) produced by the cells inside the biofilms. The biofilm formation of *C. albicans* is responsible for most infections, especially those associated with medical devices like prosthetic joints, dentures, pacemakers and catheters. Compared to planktonic cells, *C. albicans* biofilm cells have a markedly higher resistance to antifungal therapies (4). Therefore, we have studied anti-biofilm

ability of glucosinolate derivatives by XTT metabolic assay and scanning electron microscopy study. In this study it is seen that allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), ethyl isothiocyanate (EITC), phenyl ethyl isothiocyanate (PEITC), butyl isothiocyanate (ButylITC), isopropyl isothiocyanate (IPITC) and allyl isocyanate (AIC), inhibit developing biofilm formation at respective concentrations (Table 5.4). Eradication of mature biofilm is one of the challenging task for clinician because mature biofilm shows resistance to standard antifungals. In current study, allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), ethyl isothiocyanate (EITC) and phenyl ethyl isothiocyanate (PEITC) (**Table 5.4.**) concentrations shows inhibition to mature biofilm. The results suggest that above molecules may act as good anti-candidal agent for the treatment of candidiasis.

Table 5.4. Effect of glucosinolate derivatives on developing and mature biofilm of *C. albicans*

Glucosinolate derivatives	**AITC**	**EITC**	**BITC**	**PEITC**	**ButylITC**	**IPITC**
MICs for developing biofilm	0.5 mg/ml	2 mg/ml	1 mg/ml	0.125 mg/ml	2 mg/ml	2 mg/ml
MICs for mature biofilm	0.5 mg/ml	0.5 mg/ml	0.25 mg/ml	2 mg/ml	NA	NA
Glucosinolate derivatives	**AIC**	**BZITC**	**PITC**	**3IA**	**Sulforaphane**	-
MICs for developing biofilm	2 mg/ml	NA	NA	NA	NA	-
MICs for mature biofilm	NA	NA	NA	NA	NA	-

(Note: NA- Not Achieved)

5.5. Effect of glucosinolate derivatives on ergosterol biosynthesis

Glucosinolate derivatives that inhibits planktonic growth were tested for their effect on ergosterol biosynthesis. Allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), ethyl isothiocyanate (EITC), phenyl ethyl isothiocyanate (PEITC) are fungicidal in nature and inhibits ergosterol biosynthesis. Earlier studies suggest that most of the molecules that showed inhibition to ergosterol synthesis were fungicidal in nature and the fungicidal nature might be due to cell membrane damage. Ergosterol is a component of lipid membrane and supports membrane fluidity. A decrease in its concentration after drug treatment could cause reduction of membrane permeability, which would make cells more susceptible or potentially cause cell death (10). The azole class possesses fungistatic properties and restricts growth by preventing lanosterol from being demethylated to ergosterol. On the other hand, a few members of azole class, like miconazole and clotrimazole, are fungicidal at higher quantities. A well-known fungistatic medication that stops the synthesis of ergosterol is itraconazole. The lipids that make up the yeast cell membrane must contain ergosterol (11). Targeting ergosterol is an attractive and successful therapeutic option because it is absent in animals. Ergosterol is a key component of cell membranes, and the absence in fungal cell membrane affects a variety of intracellular biological processes as well as the fluidity, permeability, and thickness of membranes. In *C. albicans*, ergosterol is also crucial for the function of mitochondria, vacuoles, and lipid rafts (12). Butyl isothiocyanate (ButylITC), isopropyl isothiocyanate (IPITC) and allyl isocyanate (AIC) are fungistatic in nature inhibits ergosterol biosynthesis at its planktonic-MIC and sub-MIC concentration.

5.6. Effect of glucosinolate derivatives on reactive oxygen species (ROS) production

Glucosinolate derivatives that inhibits planktonic growth were tested for their effect on intracellular ROS accumulation. Glucosinolate derivatives also tested for the development of oxidative stress in *C. albicans* planktonic cells and biofilm cells by measuring ROS level. ROS is one of the by-product of cellular metabolism, mostly manufactured by mitochondria. In response to external stimuli like heat shock, metal ions, and oxidants, *C. albicans* cells are reported to increase ROS generation. Further

leads to cell death via apoptosis or necrosis, occur when a cell produces more ROS than it can metabolise. (11,13). In current study we found that planktonic inhibitory concentration and biofilm inhibitory concentration of Allyl isothiocyanate, Ethyl isothiocyanate, Benzyl isothiocyanate, Phenyl ethyl isothiocyanate, Butyl isothiocyanate, Isopropyl isothiocyanate and allyl isocyanate shows antifungal activity by enhancing oxidative stress in *C. albicans* planktonic and biofilm cells. Additionally, significant cell membrane damage is correlated with increased formation of ROS, which can lead to oxidation of various macromolecules and compromise the integrity of the cell membrane which may be a probable reason for inhibition of planktonic growth and eradication of developing biofilm.

5.7. Effect of glucosinolate derivatives on cell cycle by FACS analysis

Glucosinolate derivatives that inhibits planktonic growth were tested for their effect on cell cycle progression. An essential mechanism for eukaryotic cells is cell cycle arrest or delay. By maintaining the integrity of the cell throughout growth and replication, it reduces the possibility of abnormal mutations and unfavourable cellular proliferation. Cell cycle arrest or delay may result from a variety of situations that *C. albicans* cells may experience in the human host. During the cell cycle events switching to filamentous growth may be beneficial since it frees cells from such unfavourable conditions (14). The cell cycle progression in *C. albicans* encompasses four main phases: G1, S, G2, and M, followed by cytokinesis. The transition between yeast and hyphal forms involves distinct patterns of cell cycle progression, reflecting the morphological changes associated with these different growth states (14). In yeast cells, bud emergence occurs during the transition from G1 to S phase, marked by the formation of a septin ring. Nuclear division and cytokinesis lead to the formation of fully separated rounded cells. Pseudohyphae exhibit a similar cell cycle progression to yeast cells but remain attached and elongate due to a prolonged G2 phase. During hyphal growth, a germ tube forms before the G1-to-S transition, and nuclear division occurs within the growing tube. However, cytokinesis does not occur, resulting in the elongation of the hyphal tip and the formation of tubular cells. Regulation of cell cycle progression in *C. albicans* involves various proteins such as cyclins, cyclin-dependent kinases (CDKs), and checkpoint proteins. Proper cell cycle progression is crucial for

regulating morphogenesis. Depletion of genes involved in the cell cycle machinery or pharmacological treatments causing cell cycle arrest can induce filamentation, underscoring the tight connection between cell cycle regulation and morphogenesis in this fungal pathogen (15,16).

To find out how glucosinolate derivatives influenced DNA replication and cell division processes for a cell's growth and multiplication, a cell cycle analysis was conducted. If there is a deviation in the sequence of these phases, the DNA checkpoint pathway becomes activated, halting all activities (like growth, division) until the damage is restored (17,18).

In current study AITC (0.125 mg/ml), BITC (0.125 mg/ml), IPITC arrests at G2/M phase. In higher eukaryotes, the relationship between cell cycle regulation and the induction of apoptosis is still unknown. According to reports, pro-apoptotic therapies cause the cell cycle of *C. albicans* to arrest in the G2/M phase. The DNA damage repair checkpoint and the G2/M phase coincide. Induction of cellular death in yeast cells may cause DNA breakage by the production of ROS which, in turn, causes G2/M phase cell cycle arrest.

EITC and ButylITC enters in *C. albicans* cell interacts with intracellular DNA stops DNA replication process and shows antifungal activity by arresting cells at S phase.

PEITC and AIC arrest at G0/G1 phase which responsible for inhibition of filamentous growth.

5.8. Effect of glucosinolate derivatives on gene expression involved in signal transduction pathway

Further investigation was conducted on glucosinolate derivatives that exhibit inhibitory effects on developing biofilms to identify the specific genes responsible for this inhibition. In current study investigated the expression of genes that are involved in the signal transduction pathway of germ tube formation. RAS1-cAMP-PKA and CEK1-MAPK are two components of the germ tube formation signal transduction pathway.

CPH1- *CPH1* is a MAPK cascade gene, transcription factor involved in pseudo hyphal and hyphal formation (19) .

PDE2 -A high-affinity phosphodiesterase, is necessary for *C. albicans* hyphal growth and cell wall integrity. The downregulation of *PDE2* elevates cAMP levels, prevents

normal hyphal development in hypha-inducing liquid medium, and inhibits the formation of biofilms (20).

CDC35- Cdc35p, an adenylyl cyclase in *C. albicans*, is a key component in regulating cellular functions through cAMP. It plays a crucial role in vegetative growth and is essential for the transition from yeast to hypha morphogenesis, influencing the virulence of this fungus (21).

BCY1- *BCY1* involved in regulation of filamentation, cell growth, and carbon source utilisation and found that *BCY1* regulates white-opaque switching in *C. albicans* (22)

HWP1- HWP1 is a hyphal specific adhesion protein involved in hyphal development and biofilm formation. *Hwp1* allows binding of *C. albicans* to host epithelial cells by serving as a transglutaminase substrate, thus permitting covalent cross-linking of *C. albicans* germ tubes to host cell surfaces (23).

EFG1- EFG1 is a transcription factor in *C. albicans* that regulates morphological transitions, biofilm formation, and virulence factor expression. It promotes the yeast-to-hyphae transition, contributes to biofilm formation. Overall, EFG1 is essential for *C. albicans'* pathogenicity (24).

TEC1- TEC1 is a transcription factor and regulates the virulence of *C. albicans* like regulates genes involved in hyphal development, cell adhesion and biofilm development (25).

RAS1- *RAS1* necessary for the control of both a MAP kinase signalling pathway and a cAMP signalling pathway, which play vital roles in the pathogenicity of the cells by inducing the morphological shift from yeast to polarized filamentous form (26).

ECE1- ECE1 is a protein specifically expressed during the formation of hyphae in *C. albicans*. Recent research has revealed that the secretion of a cytotoxic peptide called candidalysin from hyphal structures significantly enhance the pathogenicity of this fungus. Candidalysin is derived from a precursor protein encoded by the ECE1 gene, whose transcription is closely linked to the process of hyphal formation (27).

CEK1- In the *C. albicans*, the Cek1 MAP kinase (MAPK) is involved in both vegetative growth and cell wall synthesis. CEK1 involved in invasive growth and filamentation. down regulation of *CEK1* inhibit filamentation (28).

HST7- *HST7* acts as a mitogen-activated protein kinase (MAPK), essential for transmitting signals involved in mating and hyphal growth, crucial for opaque mating and white biofilm formation (29).

MIG1, TUP1 and NRG1- MIG1, TUP1, and NRG1 plays crucial role in the suppression of the Y-H form transition as they are negative regulators (30).

Table 5.5. Relative fold changes in the gene expressions in *C. albicans* with or without treatment of allyl isothiocyanate

Sr. No.	Genes	Relative fold change
1.	CPH1	5.26 (Downregulated)
2.	PDE2	2.44 (Downregulated)
3.	CDC35	3.49 (Upregulated)
4.	BCY1	2.33 (Downregulated)
5.	HWP1	12.50 (Downregulated)
6.	EFG1	2.46 (Upregulated)
7.	MIG1	9.09 (Downregulated)
8.	TEC1	1.20 (Upregulated)
9.	RAS1	3.03 (Downregulated)
10.	ECE1	1.25 (Downregulated)
11.	NRG1	1.79 (Downregulated)
12.	CEK1	1.67 (Downregulated)
13.	TUP1	6.25 (Downregulated)
14.	HST7	1.25 (Downregulated)

Gene expression analysis on developing biofilm by the treatment of AITC (0.5 mg/ml) revealed that, downregulate the expression of *CPH1*, *PDE2, BCY1, HWP1, RAS1, ECE1, CEK1* and *HST7* by 5.26, 2.44, 2.33, 12.50, 3.03, 1.25, 1.67 and 1.25-fold change, respectively (**Table 5.5**). Eradication of developing biofilm in *C. albicans* may be due to downregulation of These Genes.

Table 5.6. Relative fold changes in the gene expressions in *C. albicans* with or without treatment of Ethyl isothiocyanate

Sr. No.	Genes	Relative fold change
1.	CPH1	2.00 (Downregulated)

2.	PDE2	1.67 (Downregulated)
3.	CDC35	4.88 (Upregulated)
4.	BCY1	1.35 (Downregulated)
5.	HWP1	10.00 (Downregulated)
6.	EFG1	1.08 (Upregulated)
7.	MIG1	1.20 (Upregulated)
8.	TEC1	1.67 (Downregulated)
9.	RAS1	1.25 (Downregulated)
10.	ECE1	1.39 (Downregulated)
11.	NRG1	1.32 (Downregulated)
12.	CEK1	1.35 (Downregulated)
13.	TUP1	10.00 (Downregulated)
14.	HST7	2.50 (Downregulated)

Gene expression analysis on developing biofilm by the treatment of EITC (2 mg/ml) revealed that, downregulate the expression of *CPH1*, *PDE2, BCY1, HWP1, TEC1, RAS1, ECE1, CEK1* and *HST7* by 2.00, 1.67, 1.35, 10.00, 1.67, 1.25, 1.39, 1.35, 10.00 and 2.50-fold change, respectively and upregulating *MIG1* by 1.20-fold change (**Table 5.6**). Eradication of developing biofilm in *C. albicans* may be due to downregulation of *CPH1*, *PDE2, BCY1, HWP1, TEC1, RAS1, ECE1, CEK1* and *HST7* these genes and upregulating *MIG1* gene.

Table 5.7. Relative fold changes in the gene expressions in *C. albicans* with or without treatment of Benzyl isothiocyanate

Sr. No.	**Genes**	**Relative fold change**
1.	CPH1	2.77 (Upregulated)
2.	PDE2	3.30 (Upregulated)
3.	BCY1	10.30 (Upregulated)
4.	HWP1	7.50 (Upregulated)
5.	EFG1	3.28 (Upregulated)
6.	TEC1	3.63 (Upregulated)

7.	RAS1	2.50 (Upregulated)
8.	ECE1	1.76 (Upregulated)
9.	NRG1	2.60 (Upregulated)
10.	CEK1	3.92 (Upregulated)
11.	TUP1	0.00 (Unaffected)
12.	HST7	1.98 (Upregulated)

Gene expression analysis on developing biofilm by the treatment of BITC (1 mg/ml) revealed that, the expression of upregulating *NRG1* by 2.60-fold change (**Table 5.7**). Eradication of developing biofilm in *C. albicans* may be due to upregulating *NRG1* gene.

Table 5.8. Relative fold changes in the gene expressions in *C. albicans* with or without treatment of Phenyl ethyl isothiocyanate

Sr. No.	**Genes**	**Relative fold change**
1.	CPH1	12.09 (Upregulated)
2.	PDE2	25.99 (Upregulated)
3.	CDC35	29.33 (Downregulated)
4.	BCY1	3.37 (Upregulated)
5.	HWP1	12.04 (Upregulated)
6.	EFG1	17.05 (Upregulated)
8.	TEC1	16.92 (Upregulated)
9.	RAS1	9.56 (Upregulated)
10.	ECE1	9.53 (Upregulated)
11.	NRG1	9.19 (Upregulated)
12.	CEK1	9.17 (Upregulated)
13.	TUP1	0.00 (Unaffected)
14.	HST7	8.74 (Upregulated)

Gene expression analysis on developing biofilm by the treatment of PEITC (0.125 mg/ml) revealed that, the expression of downregulating *CDC35* by 29.33-fold change

upregulating *NRG1* by 9.19-fold change (**Table 5.8).** Eradication of developing biofilm in *C. albicans* may be due to downregulation of *CDC35* and upregulating *NRG1* gene.

Table 5.9. Relative fold changes in the gene expressions in *C. albicans* with or without treatment of butyl isothiocyanate

Sr. No.	Genes	Relative fold change
1.	CPH1	2.19 (Upregulated)
2.	PDE2	10.42 (Downregulated)
3.	CDC35	0.00 (Unaffected)
4.	BCY1	22.22 (Downregulated)
5.	HWP1	1.28 (Upregulated)
6.	EFG1	8.33 (Downregulated)
7.	TEC1	5.26 (Downregulated)
8.	RAS1	2.75 (Upregulated)
9.	ECE1	2.08 (Downregulated)
10.	NRG1	1.26 (Upregulated)
11.	CEK1	7.14 (Downregulated)
12.	TUP1	5.88 (Downregulated)
13.	HST7	1.62 (Upregulated)

Gene expression analysis on developing biofilm by the treatment of ButylITC (2 mg/ml) revealed that, downregulate the expression of *PDE2, BCY1, EFG1, TEC1, ECE1* and *CEK1* by 10.42, 22.22, 8.33, 5.26, 2.08 and 7.14-fold change respectively. ButylITC upregulated the expression of *NRG1* 1.26-fold change (**Table 5.9**). Eradication of developing biofilm in *C. albicans* may be due to downregulation of *PDE2, BCY1, HWP1, EFG1, TEC1, ECE1,* and *CEK1* genes and upregulating *NRG1* gene.

Table 5.10. Relative fold changes in the gene expressions in *C. albicans* with or without treatment of isopropyl isothiocyanate

Sr. No.	Genes	Relative fold change
1.	CPH1	3.23 (Downregulated)
2.	PDE2	2.86 (Downregulated)
3.	CDC35	11.11 (Downregulated)
4.	BCY1	2.04 (Downregulated)
5.	HWP1	2.44 (Downregulated)
6.	EFG1	1.82 (Downregulated)
7.	TEC1	2.78 (Downregulated)
8.	RAS1	2.86 (Downregulated)
9.	ECE1	2.70 (Downregulated)
10.	NRG1	2.94 (Downregulated)
11.	CEK1	3.33 (Downregulated)
12.	TUP1	28.57 (Downregulated)
13.	HST7	3.57 (Downregulated)

Gene expression analysis on developing biofilm by the treatment of IPITC (2 mg/ml) revealed that, downregulate the expression of *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, NRG1, CEK1, TUP1* and *HST7* by 3.23, 2.86, 11.11, 2.04, 2.44, 1.82, 2.78, 2.86, 2.70, 2.94, 3.33, 28.57 and 3.57-fold-change respectively (**Table 5.10**). Eradication of developing biofilm in *C. albicans* may be due to downregulation of *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, CEK1* and *HST7* gene.

Table 5.11. Relative fold changes in the gene expressions in *C. albicans* with or without treatment of allyl isocyanate

Sr. No.	Genes	Relative fold change
1.	CPH1	4.85 (Downregulated)

2.	PDE2	3.08 (Downregulated)
3.	CDC35	11.93 (Downregulated)
4.	BCY1	2.56 (Downregulated)
5.	HWP1	4.05 (Downregulated)
6.	EFG1	1.58 (Downregulated)
7.	TEC1	1.87 (Downregulated)
8.	RAS1	1.91 (Downregulated)
9.	ECE1	1.27 (Downregulated)
10.	NRG1	2.16 (Downregulated)
11.	CEK1	1.99 (Downregulated)
12.	TUP1	1.96 (Downregulated)
13.	HST7	2.16 (Downregulated)

Gene expression analysis on developing biofilm by the treatment of AIC (2 mg/ml) revealed that, downregulate the expression of *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, NRG1, CEK1, TUP1* and *HST7* by 4.85, 3.08, 11.93, 2.56, 4.05, 1.58, 1.87, 1.91, 1.27, 2.16, 1.99, 1.96 and 2.16-fold change respectively (**Table 5.11**). Eradication of developing biofilm in *C. albicans* may be due to downregulation of *CPH1, PDE2, DCD35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, CEK1* and *HST7* genes.

5.9. Effect of glucosinolate derivatives on human Red Blood Cells (RBCs)

Hemolytic activity of glucosinolate derivatives were analysed for further therapeutic application. AITC is non- haemolytic in nature, EITC shows 19 % hemolysis at 0.5 mg/ml concentration, BITC shows 13 % hemolysis at 2 mg/ml concentration. Remaining all molecules i. e. phenyl ethyl isothiocyanate (PEITC), butyl isothiocyanate (ButylITC), isopropyl isothiocyanate (IPITC), allyl isocyanate (AIC), Benzoyl isothiocyanate, phenyl isothiocyanate, indole 3 acetonitrile are hemolytic in nature even at its lower concentrations.

To confirm antifungal efficacy of AITC and EITC, the silkworm infection model was used. Silkworms are used in many studies on pathogenic microorganisms around the world.

5.10. *In vivo* study in silkworm model

The silkworm infection model and therapeutic efficacy of antiviral, antifungal, and antimicrobial agents (ED_{50}) are similar with the mammalian model. Silkworms have cytochrome P450s and conjugating enzymes like glutathione S-transferases, UDP-glucosyltransferase, ubiquitin-conjugating enzyme E. Silkworms can be used in detecting tissue damage caused by drugs and chemicals, in antidiabetic drugs, and herbal studies (31). In present study, silkworm survived after the treatment of 0.125 mg/ml concentration of AITC. Whereas, silkworms died after 24 hr with the treatment of EITC (0.5 mg/ml) may be due to its toxicity. The infection model was failed to prove antifungal efficacy in systemic infection of *C. albicans.* Combinatorial approach can be used to reduce the MIC subsequently toxicity of EITC. *In vivo* experiments for systemic or tropical candidiasis need to be done mice to confirm the antifungal efficacy. Based on the *in vivo* study, it was concluded that Planktonic MIC concentration (0.125 mg/ml) of AITC inhibited *C. albicans* infection and did not cause toxicity to the silkworm. The findings of this study suggested that AITC may be a promising molecule for the development of a future antifungal drug.

5.11. Conclusion

In summary, the study highlights the promising antifungal properties of glucosinolate derivatives against *C. albicans*, targeting various stages of fungal growth and virulence. These derivatives exhibit differential effects on *C. albicans*, suggesting multiple mechanisms of action and potential therapeutic targets. The assessment of their safety profile is crucial for therapeutic application. Further research to clarify their mechanisms of action and effectiveness *in vivo* is suggested for their development as antifungal agents.

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CHAPTER – 6

SUMMARY & CONCLUSIONS

6. Summary and Conclusions

C. albicans commonly resides in the human body, typically without causing harm. However, under certain conditions such as a weakened immune system or imbalance in the microbiome, it can cause infections ranging from superficial skin infections to life-threatening systemic infections. Over the years, there has been a rise in drug-resistant strains of *C. albicans*, posing challenges in the treatment of candidiasis, the infections caused by this yeast.

Drug resistance in *C. albicans* primarily arises due to prolonged and inappropriate use of antifungal medications, allowing the yeast to develop mechanisms to evade the effects of these drugs. This resistance can manifest through various mechanisms such as alterations in drug targets, efflux pumps that pump out the drugs from the cell, or changes in the cell wall composition to prevent drug entry. As a result, conventional antifungal drugs like fluconazole, amphotericin B, and echinocandins may become less effective against resistant strains. To combat this challenge, researchers are exploring alternative treatment strategies, one of which involves the use of bioactive molecules like glucosinolates. Glucosinolates are sulfur-containing compounds found in cruciferous vegetables such as broccoli, cabbage and kale. These compounds have been shown to possess antimicrobial properties, including activity against *C. albicans*. Studies have demonstrated that glucosinolates can inhibit the growth of *C. albicans* by disrupting its cell membrane integrity, interfering with essential cellular processes, and inducing oxidative stress. Moreover, glucosinolates have shown synergistic effects when used in combination with conventional antifungal drugs, potentially overcoming drug resistance and enhancing the efficacy of treatment.

Chapter 1 provides the introduction of *C. albicans*, an opportunistic fungal pathogen known for its ability to form resilient biofilms, complicating treatment options for candidiasis, especially in immunocompromised individuals. The research focuses on the potential of bioactive molecules derived from glucosinolates to inhibit *C. albicans* growth and biofilm formation through multitargeting mechanisms. By exploring the effects of these natural compounds on gene expression and pathogenicity in model organisms, the study aims to identify effective strategies for combating candidiasis.

Chapter 2 deals with the rising threat of fungal infections, driven by antibiotic overuse and increased immunocompromised populations, underscores the urgent need for novel antifungal therapies, especially in light of emerging drug resistance. Plant secondary metabolites, particularly glucosinolate (GSL) derivatives, present a promising avenue for the development of new antifungal agents due to their diverse chemical structures, low cost, and potent antimicrobial properties. This review highlights the multifaceted mechanisms of action of isothiocyanates and other GSL derivatives against fungi, including their effects on aflatoxin biosynthesis, transcriptomic changes, and interactions with fungal cell membranes. While initial studies show promise, further research is essential to fully assess the safety and clinical efficacy of these compounds, ultimately contributing to more effective strategies for combating fungal infections and enhancing public health outcomes.

Chapter 3 deals with different methodology to determine the antifungal activity of glucosinolate derivatives against *C. albicans*. For screening we performed planktonic growth, yeast to hyphal formation, adhesion and developing biofilm to confirm the activity of antifungal drugs. The effective glucosinolate derivatives were selected to find out its mode of action against *C. albicans* by using cell cycle studies, ergosterol assay, ROS assay, SEM and RT-PCR studies were performed. Toxicity was analysed with the help of haemolytic assay and nontoxic glucosinolate derivatives were further explored in *in vivo* assessments using a silkworm model confirmed the potential of these compounds to enhance antifungal activity, with findings indicating a promising avenue for the development of novel antifungal agents derived from glucosinolates.

Chapter 4 deals with results of glucosinolate derivatives.

Chapter 4.1 contains potent antifungal activity of AITC against *C. albicans*, a notorious pathogen known for causing various fungal infections in humans. AITC exhibited multifaceted inhibitory effects on different stages of *C. albicans* growth and virulence, suggesting its potential as a therapeutic agent against candidiasis. Firstly, AITC displayed remarkable inhibition of *C. albicans* planktonic growth with a minimum inhibitory concentration (MIC) as low as 0.125 mg/ml. This inhibitory effect extended to the adhesion of *C. albicans* to surfaces, a crucial step in biofilm formation,

further highlighting the efficacy of AITC in preventing biofilm establishment. Moreover, AITC demonstrated fungicidal activity at concentrations above its MIC i.e. at 0.25 mg/ml, ensuring effective eradication of *C. albicans* cells. It effectively suppressed germ tube formation, a key virulence factor of *C. albicans* at 0.125 mg/ml concentration, and inhibited both developing and mature biofilms at 0.5 mg/ml concentration. Scanning electron microscopy revealed significant disruption of biofilm structure upon AITC treatment. Further mechanistic insights revealed that AITC interferes with *C. albicans* cell membrane integrity by suppressing ergosterol biosynthesis, a target shared by conventional antifungal drugs. Additionally, AITC induced intracellular ROS production, contributing to its antifungal efficacy both in planktonic and biofilm forms of *C. albicans*. Cell cycle analysis indicated that AITC arrested *C. albicans* cells predominantly in the G2/M phase, disrupting DNA replication and cell division. At the transcriptional level, AITC downregulated key signal transduction genes i.e. *CPH1*, *PDE2, BCY1, HWP1, RAS1, ECE1, CEK1* and *HST7* by 5.26, 2.44, 2.33, 12.50, 3.03, 1.25, 1.67 and 1.25-fold change, respectively involved in biofilm formation. Importantly, AITC demonstrated no hemolytic activity on human red blood cells, indicating its safety profile. *In vivo* studies using a silkworm model confirmed the antifungal efficacy of AITC, as evidenced by the survival and completion of the life cycle of AITC-treated silkworms infected with *C. albicans*. Overall, the findings from this study highlight the promising therapeutic potential of AITC as a novel antifungal agent against *C. albicans* infections, further exploration and development for clinical applications is needed.

Chapter 4.2 contains the detailed study on the antifungal properties of EITC against *C. albicans* provides significant insights into its potential as a therapeutic agent. Here are the key conclusions drawn from the experimental findings EITC demonstrated notable inhibition of planktonic growth of *C. albicans* at 0.5 mg/ml, with a concentration-dependent effect. It displayed fungicidal activity at 2 mg/ml concentration, indicating its potency in combating *C. albicans* infections. EITC effectively hindered the adhesion of *C. albicans* cells to surfaces, a crucial step in biofilm formation at 0.0312 mg/ml concentration. Additionally, it exhibited significant anti-biofilm activity, disrupting both developing and mature biofilms, which are

notorious for their resistance to conventional antifungal agents at 2 mg and 0.5 mg/ml concentrations respectively. EITC inhibited the transition of *C. albicans* from yeast to hyphal morphology, essential for its virulence and pathogenesis at 0.0312 mg/ml concentration. This inhibition suggests EITC's potential in inhibiting the invasiveness of *C. albicans* infections. EITC exerted its antifungal effects through multiple mechanisms. It inhibited ergosterol biosynthesis, crucial for fungal membrane integrity, and induced intracellular ROS accumulation, leading to oxidative stress and eventual cell death in planktonic and biofilm cells. Furthermore, it disrupted DNA replication and cell division, causing cell cycle arrest at the synthetic S-phase. EITC downregulated the expression of key genes involved in *C. albicans* virulence and biofilm formation i.e. *CPH1*, *PDE2, BCY1, HWP1, TEC1, RAS1, ECE1, CEK1* and *HST7* by 2.00, 1.67, 1.35, 10.00, 1.67, 1.25, 1.39, 1.35, 10.00 and 2.50-fold change, respectively and upregulating *MIG1* by 1.20-fold change, further confirming its efficacy in inhibiting fungal growth and pathogenesis. While EITC showed some hemolytic activity at higher concentrations, it exhibited promising *in vivo* efficacy in a silkworm model of *C. albicans* infection. Although the survival rate of infected silkworms was limited compared to fluconazole treatment, optimization of EITC concentration could potentially enhance its protective effects. In conclusion, EITC emerges as a promising candidate for further exploration as an antifungal agent against *C. albicans* infections. Its multifaceted mode of action, along with its ability to inhibit biofilm formation and modulate gene expression, enhances its potential utility in combating fungal infections, further investigation into its safety and efficacy in clinical applications is needed.

Chapter 4.3 contains the findings of this study enhances the potent antifungal properties of BITC against *C. albicans*, an opportunistic pathogen responsible for various infections in humans, particularly in immunocompromised individuals and those with implanted medical devices. By performing various experiments, several key insights into the mechanism of action and efficacy of BITC have been elucidated. Firstly, BITC demonstrated significant inhibitory effects on *C. albicans* planktonic growth at 0.125 mg/ml concentration and BITC is fungicidal in nature at 0.25 mg/ml concentration. Further BITC inhibits adhesion to surfaces at 0.5 mg/ml concentration, transition from yeast to hyphae at 0.0312 mg/ml concentration. Notably, even at

concentrations of 2 mg/ml and 0.25 mg/ml BITC effectively disrupted developing and mature biofilm formation, a critical factor in *C. albicans* virulence and resistance. Further investigations revealed the impact of BITC on *C. albicans* cell membrane integrity, where it disrupted ergosterol biosynthesis, a vital component of fungal cell membranes. Additionally, BITC induced the production of ROS in *C. albicans* cells, leading to oxidative stress and subsequent cell damage. Moreover, BITC treatment altered the cell cycle dynamics of *C. albicans*, causing arrest in the G2/M phase, which could contribute to its antifungal effects. At the molecular level, BITC modulated the expression of gene *NRG1* by upregulating 2.60-fold change respectively involved in biofilm formation, suggesting its regulatory role in fungal virulence factors. However, it is crucial to consider the toxicity profile of BITC, as evidenced by its hemolytic activity on human red blood cells at higher concentrations. This highlights the importance of further research to optimize BITC concentrations for therapeutic use while minimizing the toxicity. In summary, the multifaceted antifungal mechanisms of BITC make it a promising candidate for the development of novel antifungal agents against *C. albicans* infections. Future studies should focus on elucidating its efficacy *in vivo* and exploring potential synergistic interactions with existing antifungal drugs to combat fungal infections effectively.

Chapter 4.4 based on the detailed study conducted on the effect of Phenyl Ethyl isothiocyanate (PEITC) on *C. albicans*, several significant conclusions can be drawn. PEITC demonstrates potent antifungal activity against *C. albicans* by inhibiting planktonic growth, as evidenced by its MIC (1 mg/ml), and is fungicidal in nature at 2 mg/ml concentration. Further PEITC inhibits adhesion to surfaces at 0.125 mg/ml concentration. PEITC effectively inhibits the transition of *C. albicans* from yeast to hyphae, which is crucial for its pathogenicity. PEITC not only prevents the developing biofilms but also eradicates mature biofilms at 0.125 mg/ml and 2 mg/ml concentrations respectively, which are notoriously resistant to conventional antifungal treatments. PEITC disrupts ergosterol biosynthesis, a key component of the fungal cell membrane, leading to membrane damage and ultimately cell death. PEITC induces intracellular ROS, causing oxidative damage and membrane disruption. It also arrests *C. albicans* cells in the G0/G1 phase of the cell cycle, hindering their growth and proliferation.

PEITC modulates the expression of genes by upregulating the expression of negative regulator *NRG1* by 9.19-fold change and downregulating the expression of *CDC35* by 29.33-fold change associated with biofilm formation, further inhibiting the virulence of *C. albicans*. While PEITC exhibits potent antifungal properties, it also demonstrates hemolytic activity at concentrations tested on human red blood cells, indicating a potential for toxicity. In conclusion, PEITC shows promising potential as an antifungal agent against *C. albicans* by targeting multiple facets of its pathogenicity, including growth inhibition, biofilm disruption, membrane damage, and modulation of gene expression. However, further studies are warranted to assess its efficacy and safety for clinical applications.

Chapter 4.5 contains the results of antifungal potential of Butyl isothiocyanate (ButylITC) against *C. albicans*, a common fungal pathogen. Here are the key findings and conclusions drawn from the results. ButylITC demonstrated significant inhibition of planktonic growth of *C. albicans* in a concentration-dependent manner. A 66 % reduction in planktonic growth was observed at a dosage of 2 mg/ml. ButylITC also effectively inhibited the adhesion of *C. albicans* cells to surfaces, with a 69 % reduction observed at a concentration as low as 0.125 mg/ml. While ButylITC showed fungistatic activity at its MIC concentration of 2 mg/ml, it was not fungicidal. ButylITC demonstrated sensitivity towards inhibiting the transition of *C. albicans* from yeast to hyphal form in a concentration-dependent manner. Complete inhibition of hyphal induction was observed at a concentration of 0.125 mg/ml. ButylITC effectively suppressed the development of biofilm (2 mg/ml), although it did not show significant activity against mature biofilm. Scanning electron microscopy revealed a notable reduction in biofilm formation after treatment with ButylITC. ButylITC disrupted the ergosterol biosynthesis of *C. albicans* cells, indicating its impact on cell membrane integrity. ButylITC induced intracellular ROS accumulation in *C. albicans* cells, which may contribute to its antifungal mechanism. ButylITC arrested the cell cycle of *C. albicans* at the S phase, which could affect its growth and proliferation. ButylITC modulate the expression of signal transduction genes associated with hyphal formation and biofilm development. ButylITC downregulated the expression of genes *PDE2, BCY1, EFG1, TEC1, ECE1* and *CEK1* by 10.42, 22.22, 8.33, 5.26, 2.08 and 7.14-fold

Change, respectively and upregulating negative regulator *NRG1* by 1.26-fold change which may be a probable reason for inhibition of biofilm formation. ButylITC exhibited hemolytic activity against human red blood cells, suggesting potential toxicity at certain concentrations. In conclusion, Butyl isothiocyanate demonstrates promising antifungal properties against *C. albicans* by inhibiting planktonic growth, adhesion, hyphal transition, biofilm formation, ergosterol biosynthesis, inducing ROS generation, altering cell cycle progression, and modulating gene expression. However, its hemolytic activity against human red blood cells suggests further investigation to evaluate its safety and potential clinical applications.

Chapter 4.6 deals with investigation of antifungal activity of isopropyl isothiocyanate (IPITC) against *C. albicans*, a common fungal pathogen. Here's a breakdown of the findings IPITC showed inhibitory activity against *C. albicans* planktonic growth in the range of 0.0039 to 2 mg/ml. It inhibited of planktonic growth at a concentration of 2 mg/ml. IPITC reduced the adhesion of *C. albicans* cells to polystyrene surfaces at a concentration of 0.5 mg/ml. IPITC exhibited fungistatic activity up to a concentration of 2 mg/ml. IPITC inhibited the transition of *C. albicans* from yeast to hyphal form at a concentration of 0.25 mg/ml. IPITC reduced the metabolic activity of developing *C. albicans* biofilms at a concentration of 2 mg/ml. However, it did not eradicate mature biofilms. SEM images showed significant morphological alterations in *C. albicans* biofilms treated with IPITC, leading to the elimination of hyphal structures. IPITC suppressed ergosterol biosynthesis in *C. albicans* cells in a dose-dependent manner, indicating its antifungal mechanism of action. IPITC induced the production of ROS in both planktonic and biofilm forms of *C. albicans*, indicating cellular stress. IPITC arrested *C. albicans* cells at the G2/M phase of the cell cycle. IPITC downregulated the expression of biofilm-related genes *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, CEK1* and *HST7* by 3.23, 2.86, 11.11, 2.04, 2.44, 1.82, 2.78, 2.86, 2.70, 3.33 and 3.57-fold-change, respectively in *C. albicans*. Downregulation of these genes by the action of IPITC may be a probable reason for inhibition of biofilm formation. IPITC exhibited hemolytic activity against human red blood cells in the concentration range of 0.0039 to 2 mg/ml, indicating high toxicity. These findings collectively suggest that IPITC possesses significant antifungal activity against *C.*

albicans, targeting various stages of its growth and virulence factors. However, its hemolytic activity against human red blood cells raises concerns regarding its high toxicity.

Chapter 4.7 study has shown the potential anti-*C. albicans* properties of Allyl isocyanate (AIC) across various parameters including planktonic growth inhibition, adhesion inhibition, yeast to hyphal morphogenesis inhibition, biofilm formation inhibition, ergosterol synthesis inhibition, ROS production induction, cell cycle arrest at G0/G1 phase, gene expression profiling, and toxicity analysis. The findings imply that, Allyl isocyanate (AIC) demonstrates promising anti-*C. albicans* properties by inhibiting various crucial aspects of Candida planktonic growth and pathogenicity including adhesion, morphogenesis, biofilm formation. AIC inhibit developing biofilm by downregulating the expression of genes involved in signal transduction pathway *CPH1, PDE2, CDC35, BCY1, HWP1, EFG1, TEC1, RAS1, ECE1, CEK1* and *HST7* by 4.85, 3.08, 11.93, 2.56, 4.05, 1.58, 1.87, 1.91, 1.27, 1.99 and 2.16 fold-change, respectively. However, it also inhibits ergosterol biosynthesis and induces ROS production in both planktonic and biofilm cells which may be responsible for inhibition of planktonic growth and biofilm formation. AIC shows hemolytic activity on human RBCs, suggesting a need for further investigation to balance its efficacy with high toxicity. Nonetheless, these findings highlight AIC as a potential candidate for the development of novel antifungal agents targeting Candida infections after reducing the toxicity.

Chapter 4.8, 4.9, 4.10 and 4.11 explored the anti-*C. albicans* activity of benzoyl isothiocyanate (BZITC), Phenyl Isothiocyanate (PITC) 3 indole acetonitrile (3IA) and sulforaphane revealed less significant findings across various parameters. In conclusion, BZITC, PITC and 3IA showed promising inhibitory effects on the adhesion at 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml concentration of *C. albicans* cells. Yeast to hyphal transition, was inhibited by BZITC, PITC, 3IA and sulforaphane at 1 mg/ml, 0.125 mg/ml, 0.25 mg/ml and 200 µg/ml concentrations. BZITC, PITC, 3IA and sulforaphane did not display significant antifungal activity in terms of growth inhibition or biofilm eradication. Additionally, its observed hemolytic activity suggests potential

toxicity concerns. Further research is suggested to elucidate its mechanism of action and explore strategies to enhance its efficacy while mitigating adverse effects before considering its therapeutic applications against *C. albicans* infections.

Chapter 5 contains the discussion of glucosinolate derivatives which possess promising antifungal properties against *C. albicans*, targeting various stages of fungal growth and virulence through multiple mechanisms. Further, explained about their safety profile and *in vivo* effectiveness is essential for their potential development as antifungal agents.

Conclusion and Summary

From current study report it is concluded that, AITC is a potential inhibitor of growth and virulence factors in *C. albicans*. AITC alters sterol profile and blocks ergosterol biosynthesis. AITC produces ROS in both planktonic and biofilm cell and arrest cells at G2/M pre-apoptic phase. AITC alters the expression of genes involved in signal transduction pathway which inhibits biofilm formation. Toxicity assay revealed that AITC can be used as an alternate therapeutic option to treat candidiasis as it in nontoxic to human RBCs. *In vivo* study demonstrated that AITC also increases survival rate of silkworm by inhibiting *C. albicans* infection. Therefore, AITC can be further explored for biomedical application by *in vivo* studies in mice.

RECOMMENDATIONS

Recommendations

The present investigation established the anti-infective potential of bioactive molecules Allyl isothiocyanate (AITC) against *C. albicans*. The future perspective for AITC in antifungal research can be enhanced by focusing on several key areas:

1. Formulation Development: Investigate various formulation strategies such as nanoparticles, liposomes, or hydrogels to improve AITC's stability, solubility, and targeted delivery. These formulations could enable controlled release and minimize off-target effects, enhancing its antifungal efficacy.

2. Exploration of Other Fungal Pathogens: Expand research to evaluate the antifungal activity of AITC against a broader range of clinically significant fungal pathogens, including non-albicans Candida species, *Cryptococcus neoformans*, *Aspergillus spp*., and emerging multidrug-resistant fungi. This will help ascertain its potential as a versatile antifungal agent.

3. Bioavailability and Pharmacokinetics: Conduct studies to investigate the bioavailability and pharmacokinetic profile of AITC *in vivo*, examining its absorption, distribution, metabolism, and excretion. This information will be critical for establishing optimal dosing regimens and understanding potential drug interactions.

4. *In Vivo* Efficacy and Safety Studies: Implement preclinical studies using animal models of Candida infections to assess the *in vivo* efficacy and safety of AITC. Key outcomes could include its ability to reduce fungal burden, inhibit biofilm formation, and improve survival rates, alongside monitoring for potential adverse effects and toxicity.

By addressing these areas, AITC can be further developed as a viable antifungal agent, paving the way for its eventual translation into clinical applications for managing fungal infections.

ANNEXURE

D. Y. PATIL MEDICAL COLLEGE KOLHAPUR

Constiuent Unit of D. Y. Patil Education Society (Deemed to be University),Kolhapur.
Re-accredited by NAAC with 'A' Grade

Dr. Rakesh Kumar Sharma
Dean & Professor (Obst. & Gyn)

Padmashree Dr. D. Y. Patil
Founder president

Dr. Sanjay D. Patil
President

No. DYPMCK/425/2021/IEC

Date: 24 MAR 2021

INSTITUTIONAL ETHICS COMMITTEE, D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR.

This is to certify that the research project titled,

"Glucosinolates as Antifungals: A Study on *Candida Albicans*."

Submitted by : **Ms. Shivani Balasaheb Patil**

Under the supervision of appointed Guide (if any): **Prof. S. Mohan Karuppayil**

Has been studied by the Institutional Ethics Committee (IEC) at its meeting held on **24/03/2021** and granted approval for the study with due effect with the following caveats:

1. If you desire any change in the protocol or standard recording document at any time, please submit the same to the IEC for information and approval before the change is implemented.
2. As per recommendations of ICMR, you must register your study with the Central Trials Registry- India (CTRI), hosted at the ICMR's National Institute of Medical Statistics (http://icmr-nims.nic.in). The registration details as provided by the website are to be submitted to the Institutional Ethics Committee within a period of 3 months from issue of this letter.
3. All serious and/or unexpected adverse events due to the drug/procedures tested in the study must be informed to the IEC within 24 hours and steps for appropriate treatment must be immediately instituted.
4. In case of injury/disability/death of any participant attributable to the drug/procedure under study, all compensation is to be made by the sponsor of the study.
5. The Chief investigator/Researcher must inform the IEC immediately if the study is terminated earlier than planned with the reasons for the same.
6. The final results of the study must be communicated to the IEC within 3 months of the completion of data collection.
7. The researcher must take all precautions to safeguard the rights, safety, dignity and wellbeing of the participants in the study.
8. The researcher must be up to date about all information regarding the risk/benefit ratio of any drug/procedure being used and any new information must be conveyed to the IEC immediately. The IEC reserves the right to change a decision on the project in the light of any new knowledge.
9. Before publishing the results of the study, the researcher must take permission from the Dean of the Institution.
10. Annual progress report should be submitted for all sponsored projects to the committee.
11. Unethical conduct of research in non-sponsored projects will result in withdrawal of the ethics approval and negation of all data collected till that date.

Prof. C. D. Lokhande
(Act. Member Secretary, IEC)

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Fax : (0231) 2601238, I **Email.** dypatilmedicalcollege@gmail.com I **Website** : www.dypatilmedicalkop.org

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. Shivani Balasaheb Patil Ph.D. student working under guidance of Prof. (Dr.) Sankunny Mohan Karuppayil (Former Head and Professor), department of Stem Cell & Regenerative Medicine and Medical Biotechnology, 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 "Glucosinolates As Antifungals: A Study On *Candida albicans*".

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 Stem cell & Regenerative Medicine and Medical Biotechnology, 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;

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

(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:

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REVIEW

WILEY

Glucosinolate derivatives as antifungals: A review

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Abstract

Fungal infections are becoming a severe threat to the security of global public health due to the extensive use of antibiotic medications and the rise in immune-deficient patients globally. Additionally, there is an increase in the development of fungus resistance to available antifungal medications. It is necessary to focus on the development of new antifungal medications in order to address these problems. The wide range of chemical structures, low cost, high availability, high antimicrobial action, and lack of adverse effects are the characteristics of plant secondary metabolites. In order to find and develop new antifungal medications, plant secondary metabolites like glucosinolate (GSL) derivatives are crucial sources of information. These natural compounds are enzymatically transformed into isothiocyanates (ITCs), nitriles, epithionitriles, oxazolidin-2-thion, and thiocyanate when they get mechanically damaged. The current review offers a thorough understanding of how isothiocyanates affect fungi with detailed mechanism. Along with this antifungal activity of nitriles, epithionitriles, oxazolidin-2-thion, and thiocyanate are mentioned. The review summarizes our present understanding of the following subjects: role of isothiocyanate by inhibiting aflatoxin biosynthesis, effect of isothiocyanate on transcriptomes, isothiocyanate targets cell membrane, role of isothiocyanate in efflux, and the role of isothiocyanate in synergistic activity. Antifungal activity of nitrile, epithionitrile, oxazolidine-2-thion, and thiocyanate is mentioned. Cytotoxicity study and clinical trials data were also added. More extensive studies will be needed in this field to assess safety concerns and clinical efficacies of GSL derivatives.

KEYWORDS
aflatoxin, *Arabidopsis thaliana*, aspergillus, fungi, glucosinolates, mycotoxins

1 | INTRODUCTION

Plant-derived compounds, also called phytochemicals, have a unique property in preventing diseases such as diabetes, cardiovascular diseases, and cancers that are the threat to global health (Zhang et al., 2015). Phytochemicals are usually classified according to their functional groups and chemical properties, like carotenoids, terpenoids, phenolic, nitrogen-containing and organosulfur compounds. Organosulfur compounds grab special attention for their exclusive properties in cancer prevention and treatment (Mitsiogianni et al., 2019). Volatile organosulfur compounds like isothiocyanates (ITCs) have been identified as good antimicrobial agents. Because, interestingly ITCs have demonstrated significant inhibitory effects on pathogenic bacteria. ITCs, also tested for antifungal efficacy against oral infections, show that these compounds have the strongest antifungal action (Khameneh et al., 2019). The incidence of fungal infections in humans has significantly increased in the last several years. Worldwide, fungal diseases cause over 10,00,000 deaths every year.

Shivani Patil was first author.

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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]

Received: 15 February 2024 / Revised: 25 April 2024 / Accepted: 26 April 2024

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*

Shivani Balasaheb Patil[1] · Rakesh Kumar Sharma[2] · Tanjila Chandsaheb Gavandi[1] · Sargun Tushar Basrani[1] · Sayali Ashok Chougule[1] · Shivanand Ramappa Yankanchi[3] · Ashwini Khanderao Jadhav[1] · Sankunny Mohan Karuppayil[1]

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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

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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

Special Report

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Isothiocyanates as potential antifungal agents: a mini-review

Rakesh K Sharma[1], Shivani B Patil[2], Ashwini K Jadhav[2] & Sankunny M Karuppayil*,[2]

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Cruciferous vegetables and mustard oil are rich in the glucosinolate group of molecules. Isothiocyanates are an important group of glucosinolate derivatives. These derivatives have various bioactive properties, including antioxidant, antibacterial, anticarcinogenic, antifungal, antiparasitic, herbicidal and antimutagenic activity. Previous studies indicate that regular intake of such vegetables may considerably reduce the incidence of various types of cancer. These studies have inspired studies where the bioactive agents of these plants have been isolated and explored for their therapeutic applications. The use of these bioactive compounds as antifungals could be a new therapeutic approach against human pathogenic fungi. Isothiocyanates have been studied for their antifungal activity and have the potential to be used for antifungal therapy.

Plain language summary: Vegetables like cabbage, cauliflower and broccoli have a distinct flavor because of chemicals called glucosinolates. Whenever we cut and eat these vegetables, glucosinolates are broken down into isothiocyanates. Glucosinolates and isothiocyanates have health benefits because they stop the growth of bacteria, parasites and fungi that cause disease, such as *Candida albicans*. They may also prevent cancer, as regularly eating these vegetables has been shown to reduce the development of some types of cancer in humans. Investigation is needed to explore how glucosinolates and isothiocyanates could be used to treat fungal infections.

Tweetable abstract: Vegetables like cabbage, cauliflower and broccoli are rich in glucosinolates and their derivatives, such as isothiocyanates, which have bioactive properties, including antifungal, antibacterial, antiparasitic, antioxidant, herbicidal, antimutagenic and anticarcinogenic activity.

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Keywords: anticarcinogenic • antifungal • *C. albicans* • glucosinolate • isothiocyanates

Vegetables like cabbage, cauliflower and broccoli as well as mustard oil are rich in glucosinolates (GSLs). GSLs have been reported in 16 families of flowering plants, mainly from the Brassicaceae family. More than 100 GSLs have been reported thus far. When GSL-rich vegetables are cut or chewed, an enzyme known as myrosinase (thioglucoside glucohydrolase) converts the GSLs into nitriles, thiocyanates, epithionitriles and isothiocyanates (ITCs) [1,2]. GSLs have a wide range of biological properties, including antifungal, antibacterial, antiparasitic, antioxidant, herbicidal, antimutagenic and anticarcinogenic activity (Figure 1). Cabbage, cauliflower and broccoli are thought to defend against fungal infections, repel pests and insects and reduce postharvest damage because of the presence of GSLs. Epidemiological studies indicate that intake of vegetables that contain GSLs may significantly reduce the incidence of various types of cancer.

ITCs are the most extensively studied GSL derivatives. These substances react with nucleophiles, causing a variety of pharmacological effects. ITCs are utilized in a wide range of agricultural and food-related industrial applications, have substantial antibacterial action and have a considerable impact on the soil microbiota [1]. The antifungal properties of ITCs were identified as early as 1966. Since that time, a rising body of scientific

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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]*

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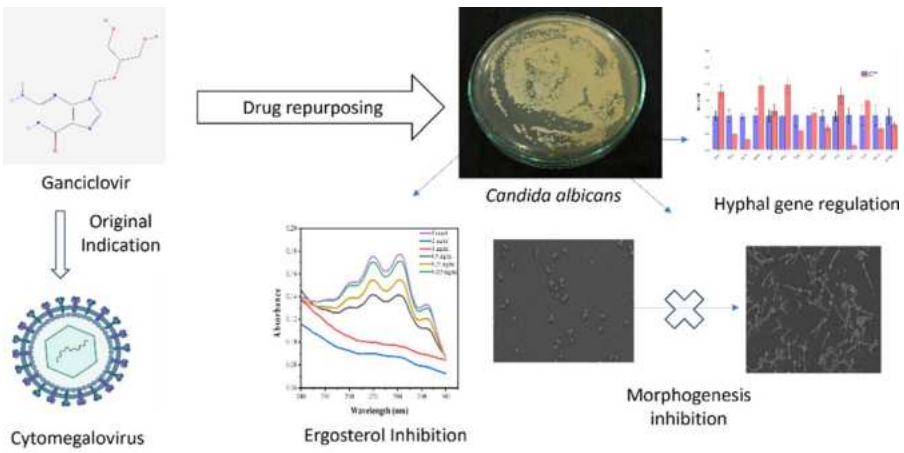
Abstract

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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Antifungal Activity of Mefloquine Against *Candida albicans* Growth and Virulence Factors: Insights Into Mode of Action

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Abstract

The antimalarial drug Mefloquine has demonstrated antifungal activity against growth and virulence factors of *Candida albicans*. The current study focused on the identification of Mefloquine's mode of action in *C. albicans* by performing *cell* susceptibility assay, biofilm assay, live and dead assay, propidium iodide uptake assay, ergosterol quantification assay, cell cycle study, and gene expression studies by RT-PCR. Mefloquine inhibited the virulence factors in *C. albicans*, such as germ tube formation and biofilm formation at 0.125 and 1 mg/ml, respectively. Mefloquine-treated cells showed a decrease in the quantity of ergosterol content of cell membrane in a concentration-dependent manner. Mefloquine (0.25 mg/ml) arrested *C. albicans* cells at the G2/M phase and S phase of the cell cycle thereby preventing the progression of the normal yeast cell cycle. ROS level was measured to find out oxidative stress in *C. albicans* in the presence of mefloquine. The study revealed that, mefloquine was found to enhance the ROS level and subsequently oxidative stress. Gene expression studies revealed that mefloquine treatment upregulates the expressions of *SOD1*, *SOD2*, and *CAT1* genes in *C. albicans.* In vivo, the antifungal efficacy of mefloquine was confirmed in mice for systemic candidiasis and it was found that there was a decrease in the pathogenesis of *C. albicans* after the treatment of mefloquine in mice. In conclusion, mefloquine can be used as a repurposed drug as an alternative drug against Candidiasis.

Introduction

Fungal pathogens cause adverse effect on human health worldwide, particularly in compromised immune patients, leading to significant morbidity and mortality [1]. The increasing drug resistance and undesirable side effects caused by existing antifungal agents have greatly enhanced the interest in pursuing alternative antifungal compounds [2]. Development of new antifungal agents is a time-consuming and costly process. Reuse of an already FDA-approved drug called as repurposing can be explored to find out an alternative option to treat fungal infections. Repurposing initiatives have typically concentrated on finding medicine that can be directly converted to a new clinical use without alteration to the structure or formulation [3]. In previous reports, it was found that the antimalarial drug chloroquine had capacity to inhibit the growth, morphogenesis, and ergosterol biosynthesis of *C. albicans* [4]. The combination of antimicrobial light (108 J/cm^2) with quinine (1 mg/ml) inactivates the planktonic growth and mature biofilm of *C. albicans* (strain CEC 749). Mefloquine is highly lipid soluble hence its distribution capacity is high [5]. Mefloquine is reported to have antifungal activity for *C. albicans* by targeting the vacuole, morphogenesis and disrupting mitochondrial proton motive force [6]. Mefloquine has been reported to exhibit activity against *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, and *Mycobacterium avium* [7]. The current work explores the antifungal efficacy and probable mode of action of mefloquine in *C. albicans* including inhibition of germ tube formation, adhesion, biofilm, ergosterol synthesis, DNA condensation, ROS production, cell cycle arrest, and

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RESEARCH

Vidarabine as a novel antifungal agent against *Candida albicans*: insights on mechanism of action

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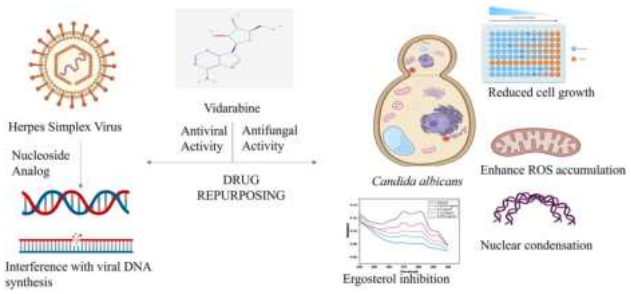
Abstract

Around 1.5 million mortality cases due to fungal infection are reported annually, posing a massive threat to global health. However, the effectiveness of current antifungal therapies in the treatment of invasive fungal infections is limited. Repurposing existing antifungal drugs is an advisable alternative approach for enhancing their effectiveness. This study evaluated the antifungal efficacy of the antiviral drug vidarabine against *Candida albicans* ATCC 90028. Antifungal susceptibility testing was performed by microbroth dilution assay and further processed to find the minimum fungicidal concentration. Investigation on probable mode of vidarabine action against *C. albicans* was assessed by using the ergosterol reduction assay, reactive oxygen species (ROS) accumulation, nuclear condensation, and apoptosis assay. Results revealed that *C. albicans* was susceptible to vidarabine action and exhibited minimum inhibitory concentration at 150 µg/ml. At a concentration of 300 µg/ml, vidarabine had fungicidal activity against *C. albicans*. 300 µg/ml vidarabine-treated *C. albicans* cells demonstrated 91% reduced ergosterol content. Annexin/FITC/PI assay showed that vidarabine (150 µg/ml) had increased late apoptotic cells up to 31%. As per the fractional inhibitory concentration index, vidarabine had synergistic activity with fluconazole and caspofungin against this fungus. The mechanism underlying fungicidal action of vidarabine was evaluated at the intracellular level, and probably because of increased nuclear condensation, enhanced ROS generation, and cell cycle arrest. In conclusion, this data is the first to report that vidarabine has potential to be used as a repurposed antifungal agent alone or in combination with standard antifungal drugs, and could be a quick and safe addition to existing therapies for treating fungal infections.

Graphical Abstract



Keywords *Candida albicans* ATCC 90028 · Drug repurposing · Ergosterol · ROS · Synergism · Vidarabine

Introduction

Candida albicans is one of the most common invasive fungal pathogens and the fourth most common cause of bloodstream infections. It has been further identified as the most prevalent cause of life-threatening disseminated candidiasis,

Extended author information available on the last page of the article

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This is to certify that **Ms. Shivani Patil** of Centre for Interdisciplinary Research, D. Y. Patil Education Society (Deemed to be University), Kolhapur has delivered ~~invited talk/ chaired the session/~~ presented oral~~/ presented poster/ participated~~ in the **International Conference on Nanotechnology Addressing the Convergence of Materials Science, Biotechnology and Medical Science (IC-NACMBM-2024)** held at the Centre for Interdisciplinary Research, D. Y. Patil Education Society (Deemed to be University), Kolhapur, Maharashtra, India during 12th to 14th February 2024. His/Her contribution to the conference is highly appreciated.

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We appreciate your hard work, dedication, creativity and achievement.

BEST WISHES

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