Isothiocyanate Derivatives as Antifungals: A Study in *Candida albicans*

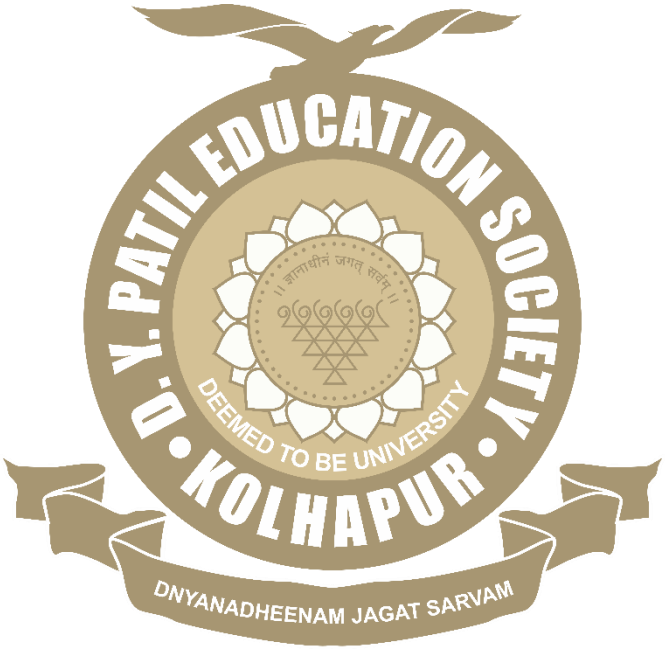
By

Dr. Rakesh Kumar Sharma

Under the Supervision of

Prof. S. Mohan Karuppayil

Thesis Submitted to



For the Degree of

Doctor of Philosophy

2024

Isothiocyanate Derivatives as Antifungals: A Study in *Candida albicans*

THESIS SUBMITTED TO

D. Y. PATIL EDUCATION SOCIETY (DEEMED TO BE UNIVERSITY), KOLHAPUR- 416 006 (MS) INDIA

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

OBSTETRICS AND GYNECOLOGY

BY

Dr. Rakesh Kumar Sharma

M.D., M.A.M.S.

UNDER THE SUPERVISION OF

Prof. S. Mohan Karuppayil

M.Sc., Ph.D.

(Former Head and Professor)

(CENTRE FOR INTERDISCIPLINARY RESEARCH)

2024

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

Faculty of Medicine

Certificate

This is to certify that the thesis entitled **"Isothiocyanate derivatives as antifungals: a study in *Candida albicans*"** which is being submitted herewith for the award of the degree of **Doctor of Philosophy (Ph.D.)** in **Obstetrics and Gynecology** of **D. Y. Patil Education Society (Deemed to be University), Kolhapur**, is the result of the original research work completed by **Dr. Rakesh Kumar Sharma** under my supervision and guidance. To the best of my knowledge and belief, the work embodied 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.

Place: **Kolhapur**

Date:

Research Guide

Prof. S. Mohan Karuppayil
(Former Head and Professor)

DECLARATION

I hereby declare that the thesis entitled **"Isothiocyanate derivatives as antifungals: a study in *Candida albicans*"** is original and carried out by me, in D. Y. Patil Education Society (Deemed to be University), Kolhapur.

It has not been submitted by me for any Degree or Diploma before this to any other University.

Place: Kolhapur **Dr. Rakesh Kumar Sharma**

Date: **(Research Student)**

LIST OF PUBLICATIONS

Articles in International Journals: (Published 03)

1. Sharma RK, Patil SB, Jadhav AK, Karuppayil SM. Isothiocyanates as potential antifungal agents: a mini review. Future Microbiology. 2023; 18:673-79.
2. Patil SB, Sharma RK, Gavandi TC, Basrani ST, Chougule SA, Yankanchi SR, Jadhav AK, Karuppayil SM. Ethyl Isothiocyanate as a Novel Antifungal Agent Against *Candida albicans*. Current Microbiology. 2024; 81:29-43.
3. Patil SB, Sharma RK, Basrani ST, Gavandi TC, Chougule SA, Yankanchi SR, Jadhav AK, Karuppayil SM. Antifungal Activity of Allyl Isothiocyanate by targeting signal transduction pathway, ergosterol biosynthesis and cell cycle in *Candida albicans*. Current Medical Mycology. 2023; 9:29-38.

Dedication

I dedicate my doctoral journey and the completion of my Ph.D. to my family and friends with sincere gratitude and deep affection. Their faith in me has driven me to overcome many challenges.

My parents, Dr. Ranbir Sharma, and Mrs. Krishna Rani, believed in my abilities and this has been the motivating force behind my pursuit of knowledge. Their guidance and encouragement have shaped me into the person I am today.

My parents-in-law, Er. R. K. Prasar and Mrs. Sneh Prabha constantly encouraged and inspired me to strive for excellence in every endeavor. Their firm support has been a source of strength and motivation throughout my academic journey.

My wife, Dr. Shimpa Sharma, has a strong work ethic, a positive attitude, and a constant urge to understand any subject thoroughly. Her presence has always inspired me and enabled me to pursue my dreams with determination and passion, especially during my moments of self-doubt and uncertainty. I am truly fortunate to have her as my partner and confidante.

My cherished daughters, Shweta and Rachna, and son-in-law Samin have brought me immeasurable joy and happiness. Their love, laughter, and support have given me purpose, prompting me to achieve my goals and aspirations.

As I stand on the verge of this remarkable achievement, I am filled with deep gratitude for each of you.

I dedicate this achievement to you all, with gratitude and love.

~Dr. Rakesh Kumar Sharma

Acknowledgment

I would like to express my deepest gratitude to those who have been pivotal in completing my Ph.D. journey.

First and foremost, I extend my heartfelt appreciation to my Guide**, Dr. Mohan Karuppayil,** Former Professor and Head of the Departments of Medical Biotechnology and Stem Cells and Regenerative Medicine, D. Y. Patil Education Society Deemed to be University, Kolhapur. Dr. Karuppayil's profound knowledge and unwavering belief in my abilities have been invaluable throughout my doctoral research. His guidance, encouragement, and insightful feedback have not only enriched my understanding of the subject but also instilled in me a passion for scholarly inquiry. I am deeply grateful for his mentorship and the invaluable lessons learnt under his tutelage.

I would also like to extend my sincere thanks to **Dr. Ashwini Jadhav,** Assistant Professor, Department of Medical Biotechnology and Stem Cells and Regenerative Medicine for her invaluable help and encouragement throughout my doctoral journey. Dr. Jadhav's expertise, constructive feedback, and scholarly insights have been instrumental in shaping the direction of my research.

I express my heartfelt appreciation to **Ms. Shivani Patil**, a fellow Ph.D. scholar in the Department of Medical Biotechnology and Stem Cells and Regenerative Medicine. Ms. Patil's collaboration, camaraderie, and willingness to share her knowledge and expertise have greatly enriched my research experience. Her support has been truly invaluable.

I would also like to extend my sincere thanks to **Dr. Meghnad Joshi**, Professor, and Head of the Department of Medical Biotechnology, and Stem Cell and Regenerative Medicine, for always being available for any advice and direction, throughout my Ph.D. journey.

I am thankful to **Mr. Sohel Shaikh**, a Ph.D. Scholar from the Department of Medical Physics, for his assistance. Mr. Shaikh's scientific and technical expertise and sincere collaboration have been invaluable in advancing my research endeavors, and I am deeply appreciative of his contributions.

I express my sincere gratitude to **Dr. Sanjay D. Patil**, Chancellor, D. Y. Patil Education Society Deemed to be University, Kolhapur for his visionary leadership, and unwavering promotion of research excellence. His encouragement and belief in my abilities have motivated me to strive for excellence in my research endeavors.

I would also like to extend my heartfelt appreciation to **Dr. Rakesh Mudgal**, Vice Chancellor of D. Y. Patil Education Society Deemed to be University, Kolhapur. His scholarly insights and encouragement have been invaluable in navigating the challenges of academic research.

I wish to express my deepest gratitude to **Dr. C. D. Lokhande**, Research Director, and Dean Centre for Interdisciplinary Research, for his invaluable guidance and research input. I am indebted to him for his generosity with his time, expertise, and resources.

I express my gratitude to **Dr. V. V. Bhosale**, Registrar of D. Y. Patil Education Society Deemed to be University, Kolhapur. His administrative acumen, expertise with regulations, and attention to detail have facilitated the progress of my doctoral studies.

I extend my appreciation to **Mr. Sanjay Jadhav**, Deputy Registrar, and **Mr. Yogesh More**, Personal Assistant to the Dean, for their efforts in streamlining many aspects of my workplace to facilitate my research work.

I would also like to extend my appreciation to **Mr. Suraj Vankundre**, System Analyst, **Mr. Rushi, Mr. Vikram**, and the entire IT Department for always providing timely solutions to any IT-related issues encountered during my academic journey.

I extend my thanks to **Mr. Ramdas, Mr. Arjun, Mr. Ashok Methe**, and **Mrs. Pooja Nimbalkar-Jadhav** for their cooperation and support.

I would like to express my deepest gratitude to my wife, **Dr. Shimpa Sharma**, for her unwavering encouragement, and invaluable contributions throughout my Ph.D. journey despite her busy professional responsibilities. Her critical and valuable input has enriched the quality of my research.

I extend my heartfelt appreciation to the many talented **Ph.D. Co-scholars** whose work and presentations I have witnessed. Each has offered practical tips, unique insights, perspectives, and methodologies that have broadened my research horizons.

I would like to express my sincere gratitude to D. Y. Patil Education Society Deemed to be University, Kolhapur for their generous support and for providing state-of-the-art resources to smoothen my journey. I am deeply appreciative of the opportunities afforded to me and remain committed to upholding the institutional values of academic excellence and integrity.

~Dr. Rakesh Kumar Sharma

CONTENTS

List of Tables

List of Images and Figures

Images

Figures

Fig. 4.3.3: Inhibition of *C. albicans* adhesion by the treatment of Allyl Isothiocyanate to an abiotic surface (polystyrene). *C. albicans* cells were incubated in phosphate buffered saline (PBS) in polystyrene wells for 90 min. and then washed with PBS to remove unattached cells. The attached cells were detected by measuring the reduction of XTT against *C. albicans.*

Fig. 4.3.4A: Inhibition of *C. albicans* early biofilm formation by the treatment of Allyl Isothiocyanate. The inhibitory effect of Allyl Isothiocyanate dilutions was evaluated on biofilms after a 48 h incubation with XTT metabolic.

Fig. 4.3.4B: Inhibition of *C. albicans* mature biofilm formation by the treatment of Allyl Isothiocyanate. The inhibitory effect of Allyl Isothiocyanate dilutions was evaluated on biofilms after a 72 h incubation with XTT metabolic.

Fig. 4.3.5: Hemolysis assay of Allyl Isothiocyanate, including triton 100 X as positive control and 0 as negative control. The relative rate of hemolysis in human erythrocytes was measured following a 1h incubation with concentrations ranging from 0.0039 to 2 mg/ml of Allyl Isothiocyanate at 37°C.

Chapter V: Study of Ethyl Isothiocyanate (EITC) On *Candida albicans*

Fig. 5.4.1A: Antifungal activity of Ethyl Isothiocyanate against planktonic growth of *C. albicans.* the planktonic growth of *C. albicans* 90028 strain treated by various concentrations of Ethyl Isothiocyanate (0.0039 to 2 mg/ml) and the growth of *C. albicans* measured by spectrophotometer.

Fig. 5.4.1B: Candidacidal activity of Ethyl Isothiocyanate using a minimum fungicidal concentration (MFC) assay. The *C. albicans* 90028 strain was treated with different concentrations of Ethyl Isothiocyanate. The significant Candidacidal activity of Ethyl Isothiocyanate was seen at 2 mg/ml concentration.

Fig. 5.4.2: The treatment of Ethyl Isothiocyanate significantly inhibits yeast to hyphal morphogenesis in *C. albicans* analysed with the help of scanning electron microscopy (SEM) **(A.)** *C. albicans* cells without treatment of Ethyl Isothiocyanate at 2.00 kx magnification, and **(B.)** *C. albicans* cells with the treatment of 0.0312 mg/ml of Ethyl Isothiocyanate at 1.00 kx magnification.

Fig. 5.4.3: XTT metabolic analysis-based polystyrene adhesion assay to evaluate the effect of Ethyl Isothiocyanate on *C. albicans* adherence. *C. albicans* cells were exposed to 0.0039

to 2 mg/ml concentrations of Ethyl Isothiocyanate for 90 min. at 37 °C. Control bar (0) indicates untreated cells, accepted as 100 % growth.

Fig. 5.4.4A: *C. albicans* cells were treated with 0.0039 to 2 mg/ml concentration of Ethyl Isothiocyanate during early biofilm, has shown concentration dependent inhibition of biofilm development quantified by XTT assay.

Fig. 5.4.4B: The inhibitory effect of Ethyl Isothiocyanate against mature biofilm formation of *C. albicans*. Cells were treated with concentrations ranging from 0.0039 to 2 mg/ml of EITC during biofilm development, revealing concentration-dependent inhibition of biofilm formation quantified by XTT assay.

Fig. 5.4.5: Hemolysis assay of Ethyl Isothiocyanate, including triton 100 X as positive control and 0 as negative control. The relative rate of hemolysis in human erythrocytes was measured following a 1h incubation with concentrations ranging from 0.0039 to 2 mg/ml of Ethyl Isothiocyanate at 37°C.

Chapter VI: Study of Benzyl Isothiocyanate (BITC) On *Candida albicans*

Fig 6.3.1A: Growth curve of *C. albicans* planktonic cells treated with Benzyl Isothiocyanate. Planktonic cells were co-incubated with various concentrations (0.0039 to 2 mg/ml) of Benzyl Isothiocyanate for 48 hrs. The graph shows that Benzyl Isothiocyanate (0.125 mg/ml) significantly inhibits the growth of *C. albicans*.

Fig. 6.3.1B: The treatment of Benzyl Isothiocyanate inhibits yeast to hyphal morphogenesis in *C. albicans* analysed with the help of Scanning Electron Microscopy, A) *C. albicans* cells without treatment of Benzyl Isothiocyanate; B) *C. albicans* cells with the treatment of 0.0312 mg/ml Benzyl Isothiocyanate.

Fig. 6.3.2: Minimum Fungicidal Concentration of Benzyl Isothiocyanate against *C. albicans* obtained by counting the colonies grown on YPD agar plate after performing planktonic growth assay. Minimum fungicidal concentration is achieved at 0.25 mg/ml concentration.

Fig. 6.3.3: The effect of Benzyl Isothiocyanate on the adhesion of *C. albicans* to polystyrene plates. *C. albicans* in PBS with 0.0039 to 2 mg/ml concentrations of Benzyl Isothiocyanate were added to 96-well plate and incubated at 37 °C for 90 min., followed by an XTT assay to assess the adhesion rate of the cells compared to the control group.

Fig. 6.3.4A: Anti-biofilm activity of Benzyl Isothiocyanate against early biofilm of *C. albicans*. Early biofilm of *C. albicans* were treated with Benzyl Isothiocyanate in 0.0039 to 2 mg/ml concentrations range and incubated at 48 h in 96 well polystyrene plate.

Fig. 6.3.4B: Anti-biofilm activity of Benzyl Isothiocyanate against mature biofilm of *C. albicans*. Mature biofilms of *C. albicans* were treated with Benzyl Isothiocyanate in 0.0039 to 2 mg/ml concentrations range for 48 h in 96 well polystyrene plate.

Fig. 6.3.5: Hemolysis assay of Benzyl Isothiocyanate, including triton 100 X as positive control and 0 as negative control. The relative rate of hemolysis in human erythrocytes was measured following a 1h incubation with concentrations ranging from 0.0039 to 2 mg/ml of Benzyl Isothiocyanate at 37°C.

Chapter VII: Study of Phenylethyl Isothiocyanate (PEITC) On *Candida albicans*

Fig. 7.3.1A: The inhibitory effects of series of concentrations ranges from 0.0039 to 2 mg/ml were evaluated on planktonic growth after 48 h incubation. Inhibition of *C. albicans* planktonic growth by Phenylethyl Isothiocyanate was observed at 1 mg/ml concentration.

Fig. 7.3.1B: Minimum fungicidal concentration against *C. albicans* growth after the treatment of Phenylethyl Isothiocyanate. Plate based bioassay to determine minimum fungicidal concentration (MFC) in *C. albicans* 90028 strains.

Fig. 7.3.2: The treatment of Phenylethyl Isothiocyanate inhibits yeast to hyphal morphogenesis in *C. albicans* analysed with the help of Scanning Electron Microscopy, A) *C. albicans* cells without treatment of Phenylethyl Isothiocyanate; B) *C. albicans* cells with the treatment of 0.0312 mg/ml Phenylethyl Isothiocyanate.

Fig. 7.3.3: Effect of Phenylethyl Isothiocyanate on adhesion of *C. albicans*. Cell adhesion to a polystyrene surface. *C. albicans* cells were incubated in phosphate buffered saline (PBS) in polystyrene wells for 90 min and then cells were detected by measuring the reduction of XTT against *C. albicans*.

Fig. 7.3.4: Effect of Phenylethyl Isothiocyanate on metabolic activity of early biofilm. The inhibitory effects of Phenylethyl Isothiocyanate dilutions were evaluated on biofilms after a 48 h incubation with XTT metabolic effect of Phenylethyl Isothiocyanate on metabolic activity of early biofilm. The inhibitory effects of Phenylethyl Isothiocyanate dilutions were evaluated on biofilms after a 48 h incubation with XTT metabolic.

Fig. 7.3.5: Effect of Phenylethyl Isothiocyanate on metabolic activity of mature biofilm. The inhibitory effects of Phenylethyl Isothiocyanate dilutions were evaluated on biofilms after a 48 h incubation with XTT metabolic.

Fig. 7.3.6: Hemolysis assay of Phenylethyl Isothiocyanate, including triton 100 X as positive control and 0 as negative control. The relative rate of hemolysis in human erythrocytes was measured following a 1h incubation with concentrations ranging from 0.0039 to 2 mg/ml of Phenylethyl Isothiocyanate at 37°C.

List of Abbreviations

Allyl Isothiocyanate	AITC
Adhesion Like Sequence	ALS
American Type Culture Collection	ATCC
Benzyl Isothiocyanate	BITC
Clinical and Laboratory Standards Institute	CLSI
Ethylene-diamine-tetra-acetic acid	EDTA
Ethyl Isothiocyanate	EITC
Extracellular Polymeric Substances	EPS
Fetal Bovine Serum	FBS
Fluconazole	FLC
Glucosinolates	GLS
Dimethyl Sulfoxide	DMSO
Highly Active Antiretroviral Therapy	HAART
Human Immunodeficiency Virus	HIV
Institute of Microbial Technology	IMTECH
Isothiocyanates	ITCs
Minimum Fungicidal Concentration.	MFC
Minimum Inhibitory Concentration	MIC
National Centre for Health Statistics	NCHS
3-[N-Morpholine] propane sulphonic acid	MOPS
Patchouli Alcohol	PA
Phenylethyl Isothiocyanate	PEITC
Phosphate Buffered Saline	PBS
Quantitative real time Polymerase chain reaction	qRT- PCR
Red Blood Cells	RBCs
Reactive Oxygen Species	ROS
Roswell Park Memorial Institute	RPMI
Scanning Electron microscopy	SEM
(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfonyl)-2*H*-Tetrazolium-5-Carboxanilide)	XTT
Yeast extract, Peptone, Dextrose	YPD
Yeast to hyphal form transition Morphogenesis	Y-H

Abstract

Globally, *C. albicans* is responsible for about 70% of overall fungal infections and is increasing in the vulnerable population. Increasing resistance to the existing antifungal drugs necessitates research for newer alternatives. Plant-based Isothiocyanates are an important group of glucosinolate derivatives exhibiting antioxidant, antibacterial, anticarcinogenic, antifungal, antiparasitic, herbicidal, and antimutagenic activity. The use of these bioactive compounds as antifungals could be a new therapeutic approach against human pathogenic fungi. The Analytical Research grade drugs *Allyl Isothiocyanate* (AITC), *Benzyl Isothiocyanate, Ethyl Isothiocyanate* (EITC), and *Phenylethyl Isothiocyanate* (PEITC) were studied for their effect on *C. albicans* strain ATCC 90028. Minimum Inhibitory Concentration (MIC_{50}) determination for Planktonic growth of *C. albicans*, Minimum Fungicidal Concentration (MFC) Assay, Yeast to Hyphal Morphogenesis (Y-H Morphogenesis) Assay, Scanning Electron Microscopy (SEM), Adhesion Assay, Biofilm Assays and Hemolytic assay were studied in vitro. The results suggest that all four molecules tested may potentially considered for therapeutic application against *C. albicans* due to their effects of anti-biofilm, anti-Y–H morphogenesis, anti-adherence to the polystyrene surface, and reduction of virulence factors of *C. albicans*. However, the hemolytic activity of PEITC at equivalent concentrations for antifungal activity raises concerns regarding its safety profile.

The AITC showed higher activity as an anti-biofilm agent by inhibiting early and mature biofilm at 0.5 mg/ml concentration while EITC, PEITC and BITC all three molecules showed similar effect on Y-H-morphogenesis in *C. albicans*. In conclusion, natural compounds like AITC and BITC offer a promising trajectory toward the development of transformative antifungal interventions. The potential of synergistic effects of combination therapy can further strengthen the possible role of isothiocyanates in antifungal treatment. Careful evaluation of the safety and efficacy in clinical settings is essential before translation into therapeutic applications.

Keywords:

C. albicans, Biofilm, *Allyl Isothiocyanate, Benzyl Isothiocyanate, Ethyl Isothiocyanate, Phenylethyl Isothiocyanate*.

CHAPTER – I

General Introduction

CHAPTER – I

General Introduction

1. Introduction

Candidiasis is a very serious problem in cancer patients as well Human Immunodeficiency Virus (HIV) patients. These patients may require the use of prolonged antifungal therapy often leading to acquired resistance to the drugs. *Candida albicans* can form drug-tolerant biofilms on prosthetic devices like central venous system catheters, urinary catheters, stents, Heart valves, Intra uterine devices, artificial heart valves, artificial kneecaps etc. Prolonged anti-bacterial antibiotic therapy gives advantage to *C. albicans and* may lead to dominance of *C. albicans* causing vaginal candidiasis or intestinal infections. Since 2006, no novel antifungal antibiotic has entered our clinics. *Isothiocyanate* derivatives are potential molecules worth exploring as anti-*Candida* agents. This will help us to mitigate one of the major challenges being faced in clinical practice today. The outcomes of this scientific work aim to explore the potential of *Isothiocyanate* derivatives as anti- biofilm agents in *C. albicans*.

The pattern of systemic fungal illnesses in people has changed in ways never anticipated a few decades ago. The relevance of these infections has expanded dramatically, mostly because major medical discoveries have made it possible to provide better long-term treatment for patients in critical care, transplant recipients, and other immunosuppressed individuals. Demographic trends are changing with an increase in the aging population. This population tends to have a higher incidence of chronic illnesses and debility. This places them at a higher risk for fungal infections. Apart from nosocomial and fomite infections, there is a rise in the occurrence of numerous endemic diseases **[1].** One of the main factors that has led to the increased prevalence of fungal illnesses is the AIDS epidemic. Up to 80% of HIV-positive individuals experienced mucosal candidiasis prior to the widespread use of highly active antiretroviral therapy (HAART) in developed nations. Other HIV-positive individuals experienced cryptococcosis, histoplasmosis, or coccidioidomycosis during their illness. These illnesses are often very contagious and potentially fatal in this population **[2].**

According to an analysis of U.S. National Center for Health Statistics (NCHS) death records, mycotic disease-related fatalities have increased more than threefold since 1980, and fungal infections were the seventh most common cause of infectious disease-related mortality in 1992. Further analysis showed that the majority of these deaths were caused by two specific diseases: aspergillosis and candidiasis **[3,4].** According to NCHS

statistics also revealed that fungi-related illnesses led to 30,000 hospital admissions in 1994, marking the fourth-highest yearly percentage increase (10 %) since 1980 **[5, 6].**

In the healthy gastrointestinal system, vagina, and mouth cavity, *Candida* sp. is a common resident. These species opportunistically infect humans and can spread in the blood and deeper tissues. Prolonged use of antibiotics, immunosuppressive drugs and HIV infection may lead to infection of *Candida* spp. Particularly in healthcare settings, medical gadgets and fomites can also transmit these yeast infections. Sufficient knowledge of the transmission path of these disorders influences decisions on preventative interventions. Such interventions may include the need for specific environmental conditions precautions or the use of antifungal medication prophylaxis. Additionally, a person's susceptibility to invasive fungal infections is significantly influenced by host variables **[7].** The genus *Candida* has over 200 species. *C. albicans, C. tropicalis, C. glabrata, C. parapsilosis, C. lusitaniae, C. krusei, C. stellatoidea, C. kefyr, C. guilliermondii* and *C. dubliniensis* are among the *Candida* species that are significant in medicine. The most prevalent opportunistic pathogen among all the species of *Candida* is *C. albicans*, which can cause a variety of illnesses, from minor superficial infections to potentially fatal systemic infections **[8].**

The likelihood of bloodstream infections caused by *Candida* has been linked to several factors. Hospitalization-related factors, such as central venous catheters, overuse of antibiotics, and surgical operations, can be separated from host factors, which include immunosuppression brought on by a variety of underlying diseases. Two important risk factors include the extensive use of antibiotics, which promote the growth of *Candida* organisms in the GI tract, and intravascular devices, such as catheters and pressure monitoring devices **[9].** Additional risk factors include immunosuppressive diseases, cytotoxic medication that causes neutropenia, GI tract integrity loss, and abdominal surgery **[10].** Numerous investigations have revealed an independent correlation between prior colonization with *Candida spp.* and an elevated risk of *Candida* bloodstream infection. In severely sick postoperative patients, the intensity of colonization measured as the total number of locations colonized has been useful in forecasting invasive *Candida* infections **[11-13].**

The antifungal drugs have not been updated for many years, in the face of rising drug resistance and emerging fungal pathogens **[14].** These days, *C. albicans* is developing resistance to antifungal medications. *C. albicans* can cause prosthetic device biofilm. Additionally, the *C. albicans* biofilm is resistant to several antifungal drugs. Therefore,

treatment of drug-resistant candidiasis is difficult due to the limited options of antifungals. There is a need to find alternatives for treating infections caused by drug-resistant *C. albicans.*

Isothiocynates 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 vegetables containing *Isothiocynates* considerably reduces 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. Hence in the present study, the antifungal activity of *Isothiocyanate* derivatives has been explored against *C. albicans* pathogenesis.

1.1 References:

1. Dismukes WE, Pappas PG, Sobel JD, editors. Laboratory aspects of medical mycology. Clinical mycology.2003;03-22.
2. Rajasingham R, Smith RM, Park BJ, Jarvis JN, Govender NP, Chiller TM, Denning DW, Loyse A, Boulware DR. Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. The Lancet infectious diseases. 2017; 17:873-81.
3. McNeil MM, Nash SL, Hajjeh RA, Phelan MA, Conn LA, Plikaytis BD, Warnock DW. Trends in mortality due to invasive mycotic diseases in the United States, 1980–1997. Clinical infectious diseases. 2001; 33:641-47.
4. Pinner RW, Teutsch SM, Simonsen L, Klug LA, Graber JM, Clarke MJ, Berkelman RL. Trends in infectious diseases mortality in the United States. Journal of the American Medical Association. 1996; 275:189-93.
5. https://www.cdc.gov/nchs/nchs_for_you/researchers.htm
6. Pfaller MA, Diekema D. Epidemiology of invasive candidiasis: a persistent public health problem. Clinical microbiology reviews. 2007; 20:133-63.
7. https://microbewiki.kenyon.edu/index.php/*Candida_albicans*_%28Pathogenesis%29
8. https://drfungus.org/knowledge-base/*Candida*-species/
9. Zhong L, Dong Z, Liu F, Li H, Tang K, Zheng C, Wang L, Zhang K, Cai J, Zhou H, Cui W. Incidence, clinical characteristics, risk factors and outcomes of patients with mixed *Candida*/bacterial bloodstream infections: a retrospective study. Annals of Clinical Microbiology and Antimicrobials. 2022; 21:45-58.
10. Patolia S, Kennedy E, Zahir M, Patolia S, Gulati N, Narendra D, Vadde R, Pokharel S, Schmidt FM, Enriquez D, Quist J. Risk factors for *Candida* blood stream infection in medical ICU and role of colonization-A retrospective study. British Journal of Medical Practitioners. 2013:618-24.
11. Wey SB, Mori M, Pfaller MA, Woolson RF, Wenzel RP. Hospital-acquired candidemia: the attributable mortality and excess length of stay. Archives of internal medicine. 1988; 148:2642-45.
12. Pittet D, Tarara D, Wenzel RP. Nosocomial bloodstream infection in critically iii patients: Excess length of stay, extra costs, and attributable mortality. Journal of the American Medical Association. 1994; 271:1598-601.

13. Marr CA, French L, Thompson D, Drum L, Greening G, Mormon J, Henderson I, Hughes CW. Animal-assisted therapy in psychiatric rehabilitation. Anthrozoös. 2000; 13:43-47

14. Gow NA, Johnson C, Berman J, Coste AT, Cuomo CA, Perlin DS, Bicanic T, Harrison TS, Wiederhold N, Bromley M, Chiller T. The importance of antimicrobial resistance in medical mycology. Nature communications. 2022; 13:5352-364

CHAPTER – II

Review of Literature

CHAPTER – II

Review of Literature

2. Review of Literature

Within a few decades, species of *Candida* that were once uncommon and mostly thought of as nuisance pollutants have evolved into substantial and prevalent human infections that cause a wide spectrum of surface and profound diseases. Often acquired in the community, superficial infections cause significant morbidity. On the other hand, systemic, invasive, and deep-seated *C. albicans* infections are typically nosocomial. Although superficial and deep candidiasis share many similarities, systemic disease seldom results from superficial infection because of their distinct pathogenesis and risk variables.

All of the species, *C. albicans* is the most common causative agent of mucosal infections and systemic infection, and it is responsible for about 70 % of fungal infections around the world **[1].** Over the past decades, the prevalence of *Candida* infections has increased. This necessitated the availability of newer generations of antifungal medications. The predominance of the stiff cell wall of the unicellular yeast *C. albicans* is made up of complex polysaccharides known as chitin and glucans. The steroid molecule in animal cell membranes that takes the place of cholesterol is called ergosterol. In addition to chitin the cell wall also contains mannans, beta-glucans and other polysaccharides. Membrane sterol is one of the best targets for antifungal drugs. It can reproduce by sexual and asexual methods. On agar plates and in blood culture bottles, *Candida* organisms proliferate easily. For the most part, they don't need particular growth conditions. *Candida* species grow into glossy, smooth, creamy white colonies on culture media **[2].**

C. albicans (Robin) is still the most frequent fungal pathogen in human beings and is the primary cause of mucosal and systemic fungal infection. Synonyms for this species include Monilia albicans, Zopf, 1890; and *Endomyces albicans, Vuillemin,* 1898 **[3].** Amongst the *Candida* species, *C. albicans* has the finest characterization (Odds, 1994). *C. albicans* is commonly found in the oral cavity, genital area, and gastrointestinal tract. Infection can be caused by endogenous invasion or acquired from external sources **[4].**

2.1 Pathogenicity of *Candida*:

The pathophysiology of *Candida* unfolds with adhesion to the surfaces of the host cells. Contact with the host cells triggers the yeast-to-hyphae transition and directs growth via thigmotropism, which means the ability to sense and respond to changes in surface contours. Fungus enters the host cells through induced endocytosis. The attachment of yeast cells to abiotic (e.g., catheters) or biotic (host cells) surfaces can lead to biofilm formation. In these biofilms, yeast cells are in the lower part and hyphal cells are in the upper part of the biofilm. The following stages include the overall advancements in *C. albicans* tissue infiltration.

a. Attachment to the cell membrane.

b. Settlement.

c. Hyphal invasion/epithelial penetration.

d. The spread of blood vessels.

e. Colonization and penetration of endothelium.

Invasive candidiasis refers to bloodstream infections caused by *Candida spp*. Most often, it occurs after passing through the intestinal barrier. The invasive disease is usually the result of increased or abnormal colonization, along with local or generalized lack of host defense **[5].**

Some of the significant contributors to pathogenesis includes:

- Receptor molecules such as, TLR recognition, TLR signaling, mannose receptor, mincle, DC sign, C type lectin receptors, Dectin 1, 2, and 3, Nod-Like Receptors (NLRs), and inflammasomes.
- Other factors included in the host-pathogen interaction are soluble molecules in the recognition of *Candida*, and cellular responses to *Candida* incited by neutrophils and macrophages **[6].**

Because different safe cells recognize distinct infectious Pathogen Associated Molecular Patterns (PAMP) and antigens at different disease locations, host responses to *C. albicans* are significantly varied. The correct structures of *C. albicans* ' PAMPs and Pattern Recognition Receptors (PRR) are now much clearer to us. Further investigation is necessary to fully comprehend the complex interaction that occurs between PAMPs and their associated receptors. Certainly, co-stimulation, which is a secondary signal that immune cells rely on to activate an immune response in the

presence of antigen-presenting cells, through several PAMP–PRR interactions may improve the immune recognition process's sensitivity and specificity simultaneously **[7].**

2.2 Virulence factors of *C. albicans*:

Previously phenotypic definitions of the biological characteristics that support the capacity of *C. albicans* to cause disease were established. The entire genome sequencing of *C. albicans* has now been done and this has enabled the assessment of the consequences of gene knockouts on the pathogenicity of *C. albicans* in animal models. Mekalanos claims that certain environmental cues that are unique to the host environment regulate the genes required for virulence of the *C. albicans.* These signals include those related to temperature, pH, osmotic pressure, and the amounts of calcium and iron ions **[8].** *C. albicans* capacity to adjust to shifting environmental conditions helps it to flourish in its host niche **[9].**

According to Brown and Gow, *C. albicans* can change their morphology reversibly, evolving from budding pseudo-hyphal to hyphal growth forms. Clinical disease tissue specimens contain all kinds **[10].** Though hyphae are considered to help invade both epithelial tissues and endothelial tissues and prevent macrophage engulfment, yeast cells might be more widely dispersed **[11].** Double mutants of *Candida* (chp1/chp1, efg1/efg1) locked in the yeast state are found to be non-virulent in a mouse model **[12].** The potential of the organism to spread disease appears to be directly impacted by its ability to change forms.

2.3 Adhesion:

Epithelial colonization is the initial stage of a *C. albicans* infection. This process is dependent on microbe adhesion to proteins and epithelial cells, which enables them to endure fluid forces that help to remove particles **[13].** The pathophysiology of infection has been linked to *C. albicans* ' sticky ability. There is a hierarchy among the species of *Candida*, with more often isolated pathogenic species having higher adhesive capacity. The genes and products that are involved in cell adhesion are the adhesion-like sequence (ALS) family of proteins, which encodes cell-surface adhesion glycoproteins (x-agglutinins), and HWP-1, which produces a protein (Hwp1), an adhesin that attaches to buccal epithelial cells **[14].** *C. albicans* can stick to abiotic surfaces such as medical catheters as well as the host's gastrointestinal tissues, cutaneous epithelial cells, vaginal

endothelium cells, and buccal tissues. A variety of substances, including proteins, mannans, and chitin, may be in charge of adhesion **[15].**

C. albicans invades host cells by penetrating and damaging the outer cell membrane. Most likely, enzymatic or physical pathways mediate trans-migration. The membrane of the host cell has main chemical components which are proteins and phospholipids. By cleaving phospholipids, phospholipases induce tissue invasion resulting in cell lysis **[16].** The activity of extracellular phospholipase is concentrated close to the hyphal growth tip, and it is believed that this enzyme is necessary for tissue invasion. Three cloned genes encode candid phospholipases. According to a 1969 study by Staib, the pathogenicity of the strain was linked with the proteolytic activity in *C. albicans* [28]. The secreted aspartyl proteinase isoenzymes (SAP) are a family of at least nine genes **[17].** Regulated during phenotypic transition include SAP1 and SAP3. *C. albicans* tissue invasion is facilitated by the secreted aspartyl proteinases. Its role in the pathophysiology of *C. albicans* tissue invasion has been confirmed by numerous studies. In mouse and guinea pig models of disseminated candidiasis, experiments incorporating SAP-gene deletion showed decreased pathogenicity. One characteristic that makes *Candida* species virulent is their capacity to withstand the suppressive effects of antifungal chemotherapy **[18].**

The hydrolytic enzymes that have been found in *C. albicans* that are connected to the pathogenesis of the organism are aspartyl protease, hemolysin, and phospholipase **[19].** The phospholipase enzyme improves an organism's capacity to destroy host cell immunological factors, creating a greater opening for invasion and (obtaining nutrients). Another important component of *Candida* spp. pathogenicity is the hemolysin enzyme, which aids the pathogen in absorbing iron from molecules containing hemin or hemoglobin **[20].** A study was conducted to isolate *C. albicans* yeast from oral infections, aiming to diagnose them accurately using modern techniques and explore factors contributing to its virulence **[21].**

2.4 Biofilm formation:

An accumulation of microorganisms adhered to a surface (or existing at the interface between liquid and air) is called a biofilm, encased in an extracellular matrix, and possesses unique characteristics compared to their free-floating counterparts. Phenotypic plasticity (switching) has been proposed to influence antigenicity and biofilm formation of *C. albicans.* Although biofilms are almost universal, treating them

in clinical settings is difficult due to their increased resistance to physical and chemical damage **[22].**

Disseminated bloodstream infections (candidemia), can result from biofilms. This invasion causes an invasive systemic infection of tissues and various organs. Biofilms can also serve as a nidus for pathogenic cells and are extremely resistant to medications and the host immune system **[23].**

After adhesion to the surfaces *C. albicans* can grow, colonize and have the ability to form biofilm. Biofilm is complex matrix consist of proteins and carbohydrates, extracellular DNA and resistant to antifungal drugs like Azole. The ALS1 gene is thought to be a key player in biofilm formation and organism adhesion. It contributes to the synthesis of glycoproteins on the cell surface, which increases the organism's adherence to host cells. A complex network of hyphal cells attached to both abiotic and animal tissues is known as the biofilm. It leads in fungal infection that spreads swiftly and is a key cause of virulence. Biofilm-associated yeast cells have enormous therapeutic value since they can either start new biofilms or travel throughout the host cell and tissues, which can lead to the spread of invasive diseases like candidemia. Influencing the development and maturation of biofilm in light of the difficulties connected with diseases linked to biofilms. To remove infections linked to biofilms, several techniques have been developed **[24-25].**

Several approaches have been undertaken in the fight against antifungal medication resistance. But despite all of our efforts, we are still at a stalemate as far as biofilms are concerned. Biofilms hinder the cure as they limit the ability of the medicines to penetrate. There is also a lack of specific antifungal drugs. In order to solve this problem, scientists are currently investigating methods to improve the absorption of medications into the extracellular matrix of biofilms. A variety of metal nanoparticles have surfaced as viable treatments for microbial diseases in recent years due to their high antibacterial capabilities. In this context, numerous research groups have been examining the antibiofilm properties of diverse nanomaterials in relation to *C. albicans* [**26].** Using the polymethylmethacrylate biofilm model, Recently, the *C. albicans* biofilm associated with catheters is exceedingly resistant to antifungal drugs such as fluconazole, amphotericin B, nystatin, and chlorhexidine **[27].**

The ability of *Candida* species to build biofilms, which shields them from outside effects like host immune system defenses and antifungal drugs, is one unique property of their pathogenicity. The ability of each species to produce extracellular

polymeric substances (EPS), display dimorphic development, biofilm substratum and the availability of carbon sources determine the biofilm formation by various *Candida* species. Additionally, transcriptional control over activities like adhesion, filamentation, biofilm formation, and EPS production shows a remarkable difference within the pathogenic yeasts of the *Candida* genus **[28].**

These differences impact the length of colonization and infections as well as the common antifungal resistance seen in *Candida* biofilm cells. The upregulation of transporters implicated in drug resistance and EPS functions as a barrier to drug diffusion. Both of these factors contribute to the resistance. *Candida*'s ability to interact with different species in in vivo biofilms is of a critical consideration when we are handling these issues **[29].**

2.5 Antifungal Resistance through Biofilm Formation:

Biofilm production and the emergence of antifungal medication resistance have been closely associated. In a study, 48-hour biofilms of *C. albicans* were found to have five-to eight-fold higher resistance to all antifungals compared to planktonic cells. This indicates that resistance acquisition occurs during biofilm development. There are reports that mixed biofilms can be produced by different species combinations of *Candida* or by mixtures of *Candida* and bacteria. *C. glabrata, C. krusei, C. tropicalis,* and *C. albicans* have all been shown to produce biofilms together. Biofilm production is maximum for most combinations of *Candida* species. A significant rise is noted between *C. tropicalis* and *C. albicans* [**28-30].**

Furthermore, it has been shown that *C. albicans* is favored in biofilm formation when four bacterial species are combined: *Actinomyces viscosus, Streptococcus sanguinis, Streptococcus mutans and Actinomyces odontolyticus* biofilms **[31].** The digestive enzymes like *aspartyl proteinases*, *serine proteinases*, *proteases*, *lipases*, *phospholipases, esterase's* and *phosphatases* present at hyphal tip helps in invasion. It also contains toxins like glycoprotein which are lethal, pyrogenic and also induce anaphylactic shock. Notwithstanding that the majority of antifungal medications are unable to eradicate *Candida* biofilms, certain relatively recent medication formulations provide promise **[32].** This group includes lipid formulations of polyene antifungals such as amphotericin B and lipid liposomal amphotericin B complex. MICs obtained with planktonic cells against *C. albicans* biofilms produced in a bioprosthetic model are comparable to those found in the results **[33].** Additionally, in the same scenario, the

echinocandins micafungin and capsofungin effectively inhibit the biofilms of *C. albicans* and *C. parapsilosis*. *C. albicans* has C3b Complement receptors which binds to complement, and fibrinogen helps to neutrophils for phagocytosis. *C. albicans* also has capacity to phenotypic switching like yeast to hyphal form morphogenesis, smooth to rough and fuzzy colony morphology. *C. albicans* has plasticity and adaptation ability according to various human body anatomical sites in. It can evade the host's defense system **[34].**

Azole groups of antifungal antibiotics like *Fluconazole, Itraconazole, Posoconazole, Voriconazole,* are widely used in patients with compromised immune system. Similar to *fluconazole, azoles* are fungistatic by nature and might cause negative effects with continued usage. The triazole category of medications is most commonly used to treat gynecological infections, such as recurrent vulvo vaginitis brought about by *C. albicans* as well as non-albicans species **[35].** Spread of resistance to azoles is reported among gynaecological isolates of *Candida*. Some of the non albicans which are found to be emerging are intrinsically resistant to *Fluconazole*. *C. albicans* can form drug tolerant biofilms on prosthetic devices. The prosthesis that has become colonized could serve as a constant source of bloodstream infection. Due to the drugs' negative effects, it can be essential to increase the dosages of the medications if one is drug tolerant. Since biofilms are difficult to remove, physically removing the devices can be the only option **[36].**

In addition, colonised devices like pacemakers may malfunction. Clogging of he catheters by *Candida* is a problem in diabetic patients or immune-compromised persons where the central venous system catheters or urinary catheters may get clogged requiring replacement. Frequent removal or replacement of prosthetics is expensive, uncomfortable for the patients and may require frequent visits to the doctor as well as hospitals contributing to the expenses **[37].** Many times, mixed biofilms involving bacteria as well as fungi further complicate the situation. Another antifungal drug used in terminal ill patients is Amphotericin B. Even though very widely used and effective, Amphotericin B can cause nephrotoxicity and side effects like chills and seizers. Finding medications that can target drug-tolerant biofilms and substitute existing medications without having negative effects on patients is essential **[38].**

Many researchers have suggested that bioactive compounds derived from natural sources could be used as antifungal medicines. Natural products serve as a major reservoir for antimicrobial agents **[39].** The constituents of essential oils, specifically

terpenoids, phenolic acids, phenyl propanoids, and sesquiterpene alcohols, have great promise. It has been demonstrated that terpenoids and phenyl propanoids suppress *C. albicans* virulence factors **[40].**

Patchouli alcohol (PA) is the major active constituent of Chinese herbal plant Pogostemon cablin (Guanghuoxiang in Chinese). Qiulei Zhang et al **[41].** demonstrated that PA can inhibit the growth of multiple *C. albicans* strains, as well as four other *Candida* species, with MICs of 64 μg/mL and MFCs from 64 to 128 μg/mL. The biofilm formation and development, adhesion, yeast-to-hyphal transition and extracellular polysaccharide of *C. albicans* can be inhibited by PA in a concentration-dependent manner. However, they also concluded that the PA is not biofilm specific. The anti-*Candida* action of the PA may be due to the overproduction of the Reactive oxygen species (ROS), which result in the damage of the cell membranes.

Drug repositioning studies have identified various cancer drugs as potential antifungals. *Isothiocyanate* derivatives are potential molecules worth exploring as anti-*Candida* agents. These molecules are not explored seriously as anti-*Candida* agents. Broccoli, cauliflower, cabbage, and mustard are vegetables that are high in the glucosinulate group of chemicals. Upon cutting or chewing these vegetables, these molecules transform into a variety of beneficial substances. Furthermore, gut bacteria are capable in transforming glucosinulates into a wide range of chemicals **[42].** *Isothiocynates* derived from glucosinulates are very rich in bioactive properties. According to epidemiological research, eating these veggies may significantly lower the risk of developing cancer. Research that has extracted *Isothiocynates* from cruciferous plants and investigated their potential medicinal uses have been motivated by epidemiological research. The concentrated phytochemicals and plant extracts have an extensive of beneficial qualities, like antimicrobial, anti-tumor, anti-carcinogenic, anti-inflammatory, anti-oxidative, anti-platelet, anti-mutagenic, and insecticidal effects **[43].** The enzyme myrosinase found in plants is responsible for the derivatization of glucosinosulates to sothiocyanates. The cruciferous veggies' strong flavor and taste are caused by these chemicals. Furthermore, these compounds offer the plants significant defense against harmful bacteria, fungus, and pests **[44].**

Some of the *Isothiocynates* like *Allyl Isothiocyanate*, *Phenyl Isothiocyanate*, *Benzyl Isothiocyanate*are reported to inhibit the growth and toxin production by enterohemerogenic Escherichia coli. A variety of bacteria including multiple drug resistant human pathogenic bacteria are inhibited by *Isothiocynates*. The cell membranes

and metabolic processes of bacteria are believed to be targeted by *Isothiocyanates*. *Allyl Isothiocyanate* has been shown to inhibit at least one dozen human pathogenic bacteria **[45].** The *Allyl Isothiocyanate*, Sulfuraphane, Isopropyl Isothiocynate, Isopropyl Isothiocynate and Benzyl Isothiocynate possess antibacterial properties. Sulfurophane, a derivative of *Isothiocynates* has been shown to inhibit the ulcerogenic bacterium, *Helicobacter pylori*. Insecticidal properties of *Isothiocynates* and extracts of cruciferous plants are widely reported. There have been reports of anticancer effects of cruciferous vegetable extracts and isolated *Isothiocynates* against a range of malignancies, including breast, bladder, pancreatic, blood, prostate, ovarian, and skin cancers **[46].** The *Isothiocynates* with prominent anti-cancer activities include, *Allyl Isothiocyanate*s, *Benzyl Isothiocyanate* , *Phenylethyl Isothiocynates*. These compounds have been shown to suppress the growth of several cancer cell lines and the development of tumors in animal models, as have extracts from plants high in *Isothiocyanate*. These investigations have enlightened us on the therapeutic and preventative qualities of *Isothiocynates* **[31, 47].**

The molecular targets of Isothiocynates as anticancer agents and their potential targets are reviewed by Mitsiogianni et al **[48].** The potential targets of the *Isothiocynates* are detoxication of xenobiotics, carcinogens by activation metabolic enzymes, anti-inflammatory properties, inhibition of angiogenesis, inhibition of metastasis, inhibition of cell cycle, and epigenetic regulation. The widely studied *Isothiocynates* exhibiting anti-cancer activity are *Allyl Isothiocyanate*, *Benzyl Isothiocyanate* , *Phenyl Isothiocyanate*. The widely studied *Isothiocynates* have been shown to inhibit cell cycle, affect mitochondrial function, induce apoptosis, inhibit mitosis, and ultimately inhibit proliferation. Inhibition of signal transduction and metastasis through inhibition of metalloproteases are reported. Data on the four to five well studied *Isothiocynates* suggest that *Isothiocynates* could be excellent candidates for anti-cancer drug development, and in clinical practice. These molecules may provide considerable insights into anticancer drug development and identification of targets. There are few studies on the synergistic activation of *Isothiocynates* against certain cancers when the *Isothiocynates* are used in combination. Some of the *Isothiocynates* are known to regulate epigenetic processes like methylation of gene promoters of cancer associated genes **[49].** Histone deacetylase inhibition is exhibited by *Isothiocynates*. Individual examples of *Isothiocynates* are not narrated here. Most of the *Isothiocynates* exhibit similar targets in cancer **[50].**

Isothiocynates are excellent inhibitors of plant pathogenic fungi. The fungicidal properties of the *Isothiocynates* are reviewed by Khameneh et al **[51].** Human pathogenic dermatophytic fungi like Microsporum canis, Trichophyton rubrum, Epidermophyton are reported to be inhibited by *Isothiocynates*. The antimicrobial activity of ITCs against foodborne and plant pathogens has been well documented, but little is known about their antimicrobial properties against human pathogens. Romeo et al reviewed the antimicrobial activity of *Isothiocynates* against human infections for which the current therapeutic solutions are deficient **[52]**. The review's objective was to assess if ITCs could take the place of or supplement conventional antibiotics. They found that these ITCs exert a broad spectrum of action against Gram-positive and Gram-negative bacteria. However, none of the studies reviewed by them studied the effect of ITCs on the *Candida* species. The literature search also reveals that there are very few studies on human pathogenic yeast like *C. albicans.* A recent study on the efficacy of *Allyl Isothiocyanate* was published by Raut et al. **[53].** This study found that the proliferation and biofilm formation by *C. albicans* were suppressed by *Allyl Isothiocyanate*s both by themselves and in conjunction with fluconazole. The mode of action of *Allyl Isothiocyanate* against *C. albicans* is unknown and this necessitates further study. Toxicity data is available on the most widely used *Isothiocynates*. These molecules do not exhibit considerable toxicity at low concentrations as shown by animal studies. The molecules we propose to take up for this study, *Allyl Isothiocyanate*, *Benzyl Isothiocyanate* , and *Phenylethyl Isothiocyanate* are commonly found in vegetables and may not pose toxicity issues **[54].** Fungi like *C. albicans* share a common eukaryotic heritage with humans. Drugs targeting human metabolic processes may also inhibit fungi. Studies on *Isothiocynates* and targets identified may considerably help in our effort to identify the targets of these molecules in fungi. We expect that these molecules may exert their antifungal activity through multitargeting. As such, the chances for resistance against these molecules are little **[55].**

Isothiocynates have demonstrated antifungal properties, making them potentially useful in antifungal therapy **[56].** Glucosinolates (GSLs) have been reported in 16 families of flowering plants, mainly from the Brassicaceae family. More than 100 GSLs have been reported thus far. An enzyme called myrosinase (thioglucoside glucohydrolase) breaks down GSL-rich vegetable compounds into nitriles, thiocyanates, epithionitriles, and *Isothiocynates*` (ITCs) when they are chopped or chewed. Consuming vegetables high in GSLs has been shown in various epidemiological studies,

to substantially lower the incidence of several cancer types in these populations. These compounds have diverse pharmacological effects through their reaction with nucleophiles. ITCs are used in several agricultural and food-related industrial applications. Antifungal properties of ITCs were discovered as early as 1966. Since then, a lot of scientific research has been done to study and examine the antifungal potential of various ITCs against various fungi **[54-58].**

The primary antifungal byproducts of cruciferous plants' well-researched chemical defense system are ITCs. The antifungal activity and mechanism of action of ITCs has been studied. For instance, it has been observed that 2-(4-methoxyphenyl) ethyl ITC exhibits antifungal action against *Aspergillus niger* **[59].** Allyl,4-(methylthio) butyl and *Phenylethyl* ITCs also have antifungal activity against *Cochliobolus heterostrophus*. These compounds target genes involved in energy metabolism, oxidoreductase activity, melanin formation, and cell wall-degrading enzymes, according to investigations on their mode of action **[60].** The natural substance benzyl ITC (BITC) has been shown to prevent postharvest grey mold on strawberry fruit and significantly reduce natural decay by disrupting the integrity of the plasma membrane of Botrytis cinerea spores **[61].** Additionally, ITCs may be utilized as antimicrobial packaging to extend the fresh poultry's shelf life **[62].**

2.6 Antifungal properties of ITCs

Numerous research conducted in vitro have demonstrated the ability of cruciferous plant extracts, isolated GSL derivatives, and their combinations to prevent the growth of several plant diseases. Vig et al. provide a thorough overview of the many GSL derivatives and their fungicidal effectiveness against a range of fungi **[63].** GSLs from cabbage are reported to have toxicity against fungi such as *Peronospora parasitica, Pythium ultimum* and *Sclerotium rolfsii* **[64].** Most filamentous fungal pathogens are susceptible to GSLs, with at least 40 known to be inhibited by GSL derivatives. Effective molecules include allyl ITC (AITC), BITC, butenyl ITC, phenyl ITC (PITC) and *Phenylethyl* ITC (PEITC). AITC, BITC, PITC and PEITC have widely reported fungicidal activity. Additional investigations have also reported that ITCs have antifungal capabilities against additional 21 filamentous plant diseases. Brassica fungal pathogens are inhibited by GSL-derived ITCs **[54,64-66].** Choi et al. **[67]** have reported the antifungal activity of ITCs against human dermatophytes. Aliphatic ITCs have been shown by Smolinska et al. **[68]** to have greater antifungal activity than aromatic ITCs.

Mutants of Arabidopsis thaliana with lower levels of aliphatic GSL are less able to defend against necrotrophic fungus. Because GSL derivatives limit the growth of bacteria and fungi, they prolong the shelf life of food products and keep them from spoiling **[54, 69].** At a dosage of 1 mg/ml, AITC has been shown to inhibit the development and virulence factors of *C. albicans* [**70].**

Additionally, fluconazole at 0.004 mg/ml and AITC at 0.125 mg/ml together reduce biofilm formation, demonstrating how the two compounds work synergistically to enhance fluconazole action. Furthermore, at the biofilm inhibitory concentrations of AITC or AITC–fluconazole, no hemolytic activity has been observed, ruling out any cytotoxic effects. Brown mustard oil contains 85 and 10 percent of AITC and butenyl ITC, respectively. At two parts per million, AITC inhibits the growth of Penicillium roqueforti, Pencillium corylophilum, Eurotium repens, Aspergillus flavus, and Endomyces fibuliger in conserved rye bread. Two fungal diseases of Brassica, Alternaria brassicae and *Sclerotinia scletoriorum*, have been shown to be inhibited by 17 GSLs and GSL hydrolysis products; the effectiveness of the chemicals studied was influenced by the strain of the pathogenic organism **[54, 71].**

It was found that *B. cinerea* is sensitive to GSLs and their derivatives, whereas *A. brassicicola* was inhibited by the aliphatic form of GSLs. In addition, GSL hydrolysis products were used in the defense against *B. cinerea.* Additionally, it was demonstrated that at effective concentrations of 100–200 μg/ml, ITCs inhibited the growth of human pathogenic dermatophytes: *Trichophyton rubrum*, *Trichophyton mentagrophytes,* and *Microsporum canis.* It was looked at how effective GSLs and their derivatives were against *Alternaria brassicicola* and *B. cinerea* **[67-72].** This study postulates that GSLs from horseradish could be effective antifungal agents against dermatophytic fungi.

2.7 Mode of action of ITCs

ITCs cause the yeast Saccharomyces cerevisiae to absorb less oxygen by disabling oxidative phosphorylation in the mitochondria **[73].** In *A. brassicicola,* a pathogen of Brassica, exposure to ITCs results in a reduction in oxygen consumption and mitochondrial depolarization. The transcription factor ABAP1, MAPK and HOG1 are all activated by ITCs, which also boost the expression of numerous oxidative response genes in *A. brassiscola*. Because ABHOG1 and ABAP1 mutants are extremely sensitive to ITCs, *A. brassicicola* becomes less aggressive when these genes are absent. Redox sensors and oxidative response gene expression regulators may be ABAP1-like

genes. Oxidative response genes are activated after 20 min of exposure of *A. brasccicola* to AITC, BITC and PITC, which significantly reduce the development of *A. brassicicola*; AITC is more efficient than PITC and BITC **[74].** On exposure to AITC, more than one-third of the genes related to oxidative stress are induced in *A. brassicicola* **[75].**

One important postulation for fungal defense against GSLs may be the increased expression of genes involved in the response to the oxidative stress. PEITC inhibits *Alternaria alternata*, which causes black spot rot in pear fruits. Fumigation with PEITC at 1.22mM prevents the development of black spot rot **[76].** Treatment with PEITC inhibits the development of toxins (alternariol, tentoxin) by *A. alternata* **[77].**

One study found that the GSL compounds in horseradish essential oil cause oxidative stress in *C. albicans*. **[78].** The oil treatment raised the amount of superoxide and also enhanced the levels of glutathione reductase, glutathione peroxidase, catalase, and superoxide dismutase. A high concentration of horseradish essential oil therapy decreased the GSH pool, increased the production of superoxide, and eliminated *C. albicans* cells. Synergism prevented *C. albicans* from growing when horseradish essential oil and 1-chloro-2,4-dinitrobenzene, a substance that depletes the GSH pool, were combined. GSH metabolism seems to protect *C. albicans* biofilms from GSLs.

In a different investigation, AITC in its gaseous state inhibited the growth of *Fusarium verticillioides* and *Aspergillus parasiticus*. The ability of these fungi to produce mycotoxins was also suppressed by AITC treatment. Aflatoxin and fumonisin are two of the mycotoxins that have been studied. AITC may provide protection against microbial contamination and mycotoxin formation **[79].**

The YVC1 channel is reported to regulate vacuolar pressure in certain fungi **[80-81].** TRPP2 in animals is homologous to the YVC1 channel. The plant pathogenic fungus *Magnaporthe oryzae* has a calcium-permeable channel called YVC1 (MGG09828.5), which is important in development **[82].** Transient receptor potential (TRP) channels are essential for maintaining ion homeostasis in eukaryotes. It has been discovered that S. cerevisiae and A. thaliana possess TRP calcium-permeable channels. This study shows that YVC1 is essential for morphogenesis, polarized development, stress response, and *C. albicans* survival in host tissues. Several signaling mechanisms are used by *C. albicans* to recognize and react to oxidative stress. The TRP channel YVC1 is essential for oxidative stress tolerance **[83].** Nevertheless, it was unclear how components linked to calcium signaling impacted *C. albicans* pathogenicity. The YVC1 mutant was found

to have decreased capacity for stress response, poor morphogenesis, and decreased pathogenicity. Additionally, a YVC1 mutant exhibited a dampened response to stress and elevated sensitivity to SDS. The invigoration of the genes which are responsible for the oxidative stress response, the potassium and calcium dependent stability of the mitochondria or vacuoles, depend on this channel. In *C. albicans* under oxidative stress, ion transport is mediated by vacuoles, mitochondria, and the oxidative stress response, of which the TRP channel is a crucial component **[84].** It is known that AITC inhibits, and its mechanism of action is also known. Hyphal deformation and electrolyte leakage is found to be induced by AITC Reactive oxygen species increased and susceptibility to AITC increased with loss of FSYVC1. After receiving 4.8 g/ml AITC, FSYVC1 expression rose 12-to 30-fold in comparison to the control. The authors hypothesized that FSYVC1, a novel molecular target for medication development, might exist.

Sclerotinia sclerotiorum-induced white mold is a significant disease of *Brassica* crops. The growth and development of *S. sclerotiorum* are inhibited by GSLs and ITCs, which are the major products of GSL breakdown. AITC treatment changed the expression of 2012 genes in *S. sclerotiorum* (1156 upregulated and 1056 downregulated) **[82, 85].** 654 genes were impacted by indole-3-carbinol treatment; 149 of the genes showed upregulation and 505 showed downregulation. There was an upregulation of genes related to eliminating reactive oxygen species from the organism and guarding against oxidative damage. After extended exposure to ITCs, *S. sclerotiorum's* oxidative stress was reduced in part due to amino acid homeostasis. Genes involved in the creation of proline were upregulated after treatment with AITC, whereas the production of serine was stimulated after treatment with indole-3-carbinol. Proline has osmo-protective, thermotolerant and possible reactive oxygen species-quenching qualities. It can also stabilize proteins and membranes. Serine supports the methionine cycle and the glutathione detoxification route, which are both involved in reducing oxidative stress.

ITCs have been shown in multiple studies to be effective at inhibiting fungal development in food packaging systems. Because ITCs prevent the creation of mycotoxin, they often result in a significant extension of a product's shelf life. It was demonstrated that mustard oil, at AITC gas phase MIC values of 1.8–3.5 g/l, suppressed the development of *A. flavus*, *E. fibuliger*, *Penicillium commune* and *P. roqueforti* on bread **[82-86].** Additionally, studies have shown that either mustard flour or AITC alone can successfully prevent *P. expansum* from synthesizing patulin on wheat tortillas **[87].** In sliced mozzarella, AITC and mustard oil-containing meals shown decreased

development of *P. digitatum* and *A. parasiticus* and increased food shelf life **[88].** The use of AITC also prevented the formation of aflatoxin B1. AITC was also effective against fungi, such as *P. nalgiovense*, *Debaryomyces hansenii*, *A. flavus*, *P. commune*, and *P. roqueforti* that spoil the cheese **[89].**

Thus, it can be concluded that GSLs are a unique class of bioactive substances with a diverse range of bioactivities. GSLs serve as a significant defense system for plants, but they are also crucial to humans in many ways. Lot of interest has been generating in the recent years regarding the potential health benefits of ITCs, which are found in great levels in cruciferous vegetables. Numerous investigations have proven their beneficial effects. However, more research is required before we fully understand the features of various ITCs. ITCs offer a great choice for a far-reaching potential application, especially due to their natural origin and biodegradability, as evidenced by numerous studies (e.g., increasing the shelf life of various foods or suppressing fungal pathogenesis, growth, and/or toxin production in a range of stored plants, cereals, and fruits in the postharvest stage).

Furthermore, as several studies have shown, the drop in degradation often causes no obvious changes in a range of quality indices. Given the advantages of ITCs, a significant rise in interest and demand for such applications is predicted. More in-depth research is required in this field to assess safety and risk patterns. It makes practical sense to investigate ITCs as potential antifungal drugs against human pathogenic fungi, given their enormous potential. The effects of ITCs on human pathogenic fungi and their possible mechanisms of action have not received much attention so far. Further research is necessary to determine the in vitro antifungal efficacy of ITCs. These compounds have the potential to be innovative treatments for fungi. The need for new antifungals arises because of the ability of *C. albicans* to form stubborn biofilms on medical devices.

Following a comprehensive review of literature on *C. albicans* infections, this study aims to explore the potential of *Isothiocynates* as novel antifungal agents. *Isothiocynates*, abundant in cruciferous vegetables, have emerged as promising candidates due to their antimicrobial properties. The study seeks to elucidate the inhibitory activity of *Isothiocynates* against *C. albicans* growth and its virulence factors. Key objectives include establishing minimum inhibitory concentration and minimum fungicidal concentration values of various *Isothiocyanate* compounds against *C. albicans*. Additionally, the study aims to investigate the architectural changes in *C. albicans* biofilms in response to *Isothiocyanate* treatment using advanced microscopy

techniques. This research endeavors to contribute to the development of effective antifungal interventions, addressing the challenges posed by drug-resistant strains of *C. albicans.*

2.8 References:

1 Morad, H.O.J.; Wild, A.-M.; Wiehr, S.; Davies, G.; Maurer, A.; Pichler, B.J.; Thornton, C.R. Pre-clinical Imaging of Invasive Candidiasis Using ImmunoPET/MR. Front. Microbiol. 2018; 1: 1996:1-15.

2 Selvarangan R, Bui U, Limaye AP, Cookson BT. Rapid identification of commonly encountered *Candida* species directly from blood culture bottles. Journal of clinical microbiology. 2003; 41:5660-64.

3 Pattnaik S, Maharana L, Sethi M. Pathogenicity Mechanism of *C. albicans*. Advances in *C. albicans*. 2021; 5: 99737-59.

4 Barnett JA. A history of research on yeasts 12: medical yeasts part 1, *C. albicans*. Yeast. 2008;25: 385-417.

5 Rosati, D.; Bruno, M.; Jaeger, M.; Ten Oever, J.; Netea, M.G. Recurrent Vulvovaginal Candidiasis: An Immunological Perspective. Microorganisms 2020, 8: 144:1-14.

6 Zheng NX, Wang Y, Hu DD, Yan L, Jiang YY. The role of pattern recognition receptors in the innate recognition of *C. albicans*. Virulence. 2015; 19;6: 347-61.

7 Al-mayah RS, Abboud ZH. Molecular detection of biofilm genes and some virulence factors in *C. albicans* isolated from the oral cavity. kerbala journal of pharmaceutical sciences. 2023;2: 132-143.

8 De Bernardis F, Mühlschlegel FA, Cassone A, Fonzi WA. The pH of the host niche controls gene expression in and virulence of *C. albicans*. Infection and immunity. 1998; 1;66: 3317-25.

9 Mayer FL, Wilson D, Hube B. *C. albicans* pathogenicity mechanisms. Virulence. 2013; 15; 4:119-28.

10 Talapko J, Juzbašić M, Matijević T, Pustijanac E, Bekić S, Kotris I, Škrlec I. *C. albicans*—the virulence factors and clinical manifestations of infection. Journal of Fungi. 2021; 22;7: 79:1-19.

11 Brand A. Hyphal growth in human fungal pathogens and its role in virulence. International journal of microbiology. 2012; 517529-41.

12 Lo HJ, Köhler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR. Nonfilamentous *C. albicans* mutants are avirulent. Cell. 1997;5; 90:939-49.

13 d'Enfert C, Kaune AK, Alaban LR, Chakraborty S, Cole N, Delavy M, Kosmala D, Marsaux B, Frois-Martins R, Morelli M, Rosati D. The impact of the Fungus-Host-Microbiota interplay upon *C. albicans* infections: current knowledge and new perspectives. FEMS microbiology reviews. 2021; 45:1-22.

14 Mohammadi F, Hemmat N, Bajalan Z, Javadi A. Analysis of biofilm-related genes and antifungal susceptibility pattern of vaginal *C. albicans* and non-*C. albicans* species. BioMed Research International. 2021;29; 5598907-17.

15 Jacobsen ID, Wilson D, Wächtler B, Brunke S, Naglik JR, Hube B. *C. albicans* dimorphism as a therapeutic target. Expert review of anti-infective therapy. 2012;1; 10:85-93.

16 Djordjevic JT. Role of phospholipases in fungal fitness, pathogenicity, and drug development–lessons from Cryptococcus neoformans. Frontiers in microbiology. 2010;11; 1:125-38.

17 Chaffin WL, Lopez-Ribot JL, Casanova M, Gozalbo D, Martinez JP. Cell wall and secreted proteins of *C. albicans*: identification, function, and expression. Microbiology and molecular biology reviews. 1998 ;1; 62:130-80.

18 Sanglard D, Hube B, Monod M, Odds FC, Gow NA. A triple deletion of the secreted aspartyl proteinase genes SAP4, SAP5, and SAP6 of *C. albicans* causes attenuated virulence. Infection and immunity. 1997; 65:3539-46.

19 Navarro-García F, Sánchez M, Nombela C, Pla J. Virulence genes in the pathogenic yeast *C. albicans*. FEMS microbiology reviews. 2001;1; 25:245-68.

20 Naglik JR, Challacombe SJ, Hube B. *C. albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiology and molecular biology reviews. 2003;67(3):400-28.

21 Nouraei H, Jahromi MG, Jahromi LR, Zomorodian K, Pakshir K. Potential pathogenicity of *Candida* species isolated from oral cavity of patients with diabetes mellitus. BioMed Research International. 2021; 26; 2021:1-6.

22 Uppuluri P, Lopez Ribot JL. *C. albicans* biofilms. *C. albicans*: Cellular and molecular biology. 2017;63-75.

23 Firacative C. Invasive fungal disease in humans: are we aware of the real impact?. Memórias do Instituto Oswaldo Cruz. 2020;9;115: 200430-38.

24 Atriwal T, Azeem K, Husain FM, Hussain A, Khan MN, Alajmi MF, Abid M. Mechanistic understanding of *C. albicans* biofilm formation and approaches for its inhibition. Frontiers in Microbiology. 2021; 30; 12:638609-43.

25 Kumar D, Kumar A. Molecular Determinants Involved in *C. albicans* Biofilm Formation and Regulation. Molecular Biotechnology. 2023; 6:1-20.

26 Roy R, Tiwari M, Donelli G, Tiwari V. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence. 2018; 9:522-54.

27 Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen *C. albicans*: development, architecture, and drug resistance. Journal of bacteriology. 2001; 15; 183:5385-94.

28 Cavalheiro M, Teixeira MC. *Candida* biofilms: threats, challenges, and promising strategies. Frontiers in medicine. 2018; 13; 5:28:1-15.

29 Starkey M, Gray KA, Chang SI, Parsek MR. A sticky business: the extracellular polymeric substance matrix of bacterial biofilms. Microbial biofilms. 2004; 16:174-91.

30 Mirghani R, Saba T, Khaliq H, Mitchell J, Do L, Chambi L, Diaz K, Kennedy T, Alkassab K, Huynh T, Elmi M. Biofilms: Formation, drug resistance and alternatives to conventional approaches. AIMS microbiology. 2022;8: 239–277.

31 Taff HT, Mitchell KF, Edward JA, Andes DR. Mechanisms of *Candida* biofilm drug resistance. Future microbiology. 2013;8(10):1325-37.

32 Marinho VT, Dos Reis AC, da Costa Valente ML. Efficacy of Antimicrobial Agents in Dentifrices: A Systematic Review. Antibiotics. 2022; 14;11: 1413:1-18.

33 Aydemir D, Gecili F, Özdemir N, Ulusu NN. Synthesis and characterization of a triple enzyme-inorganic hybrid nanoflower (TrpE@ ihNF) as a combination of three pancreatic digestive enzymes amylase, protease and lipase. Journal of bioscience and bioengineering. 2020; 1; 129:679-86.

34 Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrobial agents and chemotherapy. 2002; 46:1773-80.

35 Harpf V, Rambach G, Würzner R, Lass-Flörl C, Speth C. *Candida* and complement: New aspects in an old battle. Frontiers in Immunology. 2020; 14;11: 1471:1-13.

36 Partha AD, Widodo AD, Endraswari PD. Evaluation of fluconazole, itraconazole, and voriconazole activity on *C. albicans*: A case control study. Annals of Medicine and Surgery. 2022;1;84: 104882:1-5.

37 Berkow EL, Lockhart SR. Fluconazole resistance in *Candida* species: a current perspective. Infection and drug resistance. 2017; 31:237-45.

38 Lebeaux D, Ghigo JM, Beloin C. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiology and Molecular Biology Reviews. 2014; 78:510-43.

39 Moloney MG. Natural products as a source for novel antibiotics. Trends in pharmacological sciences. 2016 Aug 1; 37:689-701.

40 Lemke A, Kiderlen AF, Kayser O. Amphotericin b. Applied microbiology and biotechnology. 2005; 68:151-62.

41 Zhang Q, Zhang J, Zhang Y, Sui Y, Du Y, Yang L, Yin Y. Antifungal and anti-biofilm activities of patchouli alcohol against *C. albicans*. International Journal of Medical Microbiology. 2024;314: 151596:1-9.

42 Raut JS, Shinde RB, Chauhan NM, Mohan Karuppayil S. Terpenoids of plant origin inhibit morphogenesis, adhesion, and biofilm formation by *C. albicans*. Biofouling. 2013;1; 29:87-96.

43 Connolly EL, Sim M, Travica N, Marx W, Beasy G, Lynch GS, Bondonno CP, Lewis JR, Hodgson JM, Blekkenhorst LC. Glucosinolates from cruciferous vegetables and their potential role in chronic disease: Investigating the preclinical and clinical evidence. Frontiers in pharmacology. 2021; 26;12: 767975:1-12.

44 Soundararajan P, Kim JS. Anti-carcinogenic glucosinolates in cruciferous vegetables and their antagonistic effects on prevention of cancers. Molecules. 2018; 15; 23:2983-3004.

45 Barba FJ, Nikmaram N, Roohinejad S, Khelfa A, Zhu Z, Koubaa M. Bioavailability of glucosinolates and their breakdown products: impact of processing. Frontiers in nutrition. 2016;16; 3:24-36.

46 Wu X, Zhou QH, Xu K. Are *Isothiocynates* potential anti-cancer drugs? Acta Pharmacologica Sinica. 2009; 30:501-12.

47 Xiao D, Srivastava SK, Lew KL, Zeng Y, Hershberger P, Johnson CS, Trump DL, Singh SV. *Allyl Isothiocyanate*, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G 2/M arrest and inducing apoptosis. Carcinogenesis. 2003; 1; 24:891-7.

48 Mitsiogianni M, Koutsidis G, Mavroudis N, Trafalis DT, Botaitis S, Franco R, Zoumpourlis V, Amery T, Galanis A, Pappa A, Panayiotidis MI. The role of

Isothiocynates as cancer chemo-preventive, chemo-therapeutic and anti-melanoma agents. Antioxidants. 2019; 8:106-142.

49 Kumar B, Singh S, Skvortsova I, Kumar V. Promising targets in anti-cancer drug development: recent updates. Current medicinal chemistry. 2017;1; 24:4729-52.

50 Nian H, Delage B, Ho E, Dashwood RH. Modulation of histone deacetylase activity by dietary *Isothiocynates* and allyl sulfides: studies with sulforaphane and garlic organosulfur compounds. Environmental and molecular mutagenesis. 2009; 50:213-21.

51 Khameneh B, Iranshahy M, Soheili V, Fazly Bazzaz BS. Review on plant antimicrobials: a mechanistic viewpoint. Antimicrobial Resistance & Infection Control. 2019; 8:1-28.

52 Romeo L, Iori R, Rollin P, Bramanti P, Mazzon E. *Isothiocynates*: An overview of their antimicrobial activity against human infections. Molecules. 2018 Mar 9; 23:624-643.

53 Raut JS, Bansode BS, Jadhav AK, Karuppayil SM. Activity of *Allyl Isothiocyanate* and its synergy with fluconazole against *C. albicans* biofilms. Journal of Microbiology Biotechnology. 2017; 28; 27:685-93.

54 Plaszkó T, Szűcs Z, Vasas G, Gonda S. Effects of glucosinolate-derived *Isothiocynates* on fungi: A comprehensive review on direct effects, mechanisms, structure-activity relationship data and possible agricultural applications. Journal of Fungi. 2021; 7:539-578.

55 Zgadzay Y, Kolosova O, Stetsenko A, Wu C, Bruchlen D, Usachev K, Validov S, Jenner L, Rogachev A, Yusupova G, Sachs MS. E-site drug specificity of the human pathogen *C. albicans* ribosome. Science Advances. 2022 ;25; 8:1062-69.

56 Kurt Ş, Güneş U, Soylu EM. In vitro and in vivo antifungal activity of synthetic pure *Isothiocynates* against Sclerotinia sclerotiorum. Pest Management Science. 2011; 67:869-75..

57 Plaszkó T, Szűcs Z, Vasas G, Gonda S. Interactions of fungi with non-*Isothiocyanate* products of the plant glucosinolate pathway: A review on product formation, antifungal activity, mode of action and biotransformation. Phytochemistry. 2022; 1;200: 113245-275.

58 Manyes L, Luciano FB, Mañes J, Meca G. In vitro antifungal activity of *Allyl Isothiocyanate* (AITC) against Aspergillus parasiticus and Penicillium expansum

and evaluation of the AITC estimated daily intake. Food and Chemical Toxicology. 2015 Sep 1;83: 293-9.

59 Wu TL, Hu YM, Sun Y, Zhang ZJ, Wu ZR, Zhao WB, Tang C, Du SS, He YH, Ma Y, Yang CJ. Insights into the mode of action of 2-(4-methoxyphenyl) *Ethyl Isothiocyanate* on Aspergillus niger. Food Control. 2022; 1;136: 108871-89.

60 Yu H, Jia W, Zhao M, Li L, Liu J, Chen J, Pan H, Zhang X. Antifungal mechanism of *Isothiocynates* against Cochliobolus heterostrophus. Pest Management Science. 2022; 78:5133-41.

61 Sun Y, Wang Y, Xu Y, Chen T, Li B, Zhang Z, Tian S. Application and mechanism of benzyl-*Isothiocyanate*, a natural antimicrobial agent from cruciferous vegetables, in controlling postharvest decay of strawberry. Postharvest Biology and Technology. 2021 ;1;180: 111604-623.

62 Moller A, Leone C, Kataria J, Sidhu G, Rama EN, Kroft B, Thippareddi H, Singh M. Effect of a carrageenan/chitosan coating with *Allyl Isothiocyanate* on microbial spoilage and quality of chicken breast. Poultry Science. 2023 ;1; 102:102442-450.

63 Vig AP, Rampal G, Thind TS, Arora S. Bio-protective effects of glucosinolates–A review. LWT-Food Science and Technology. 2009 ;1; 42:1561-72.

64 Gamliel A, Stapleton JJ. Characterization of antifungal volatile compounds evolved from solarized soil amended with cabbage residues. Phytopathology. 1993; 1; 83:899-905.

65 Asif M, Kala C, Gilani SJ, Imam SS, Mohamad T, Naaz F, Rahat I, Khan NA. Protective effects of *Isothiocynates* against alzheimer's disease. Current Traditional Medicine. 2022 ;1; 8:1-10.

66 Chen J, Ullah C, Reichelt M, Beran F, Yang ZL, Gershenzon J, Hammerbacher A, Vassão DG. The phytopathogenic fungus Sclerotinia sclerotiorum detoxifies plant glucosinolate hydrolysis products via an *Isothiocyanate* hydrolase. Nature Communications. 2020 ;18; 11:3090-102.

67 Choi KD, Kim HY, Shin IS. Antifungal activity of *Isothiocynates* extracted from horseradish (Armoracia rusticana) root against pathogenic dermal fungi. Food science and biotechnology. 2017 ;26:847-52.

68 Smolinska U, Morra MJ, Knudsen GR, James RL. *Isothiocynates* produced by Brassicaceae species as inhibitors of Fusarium oxysporum. Plant disease. 2003 ; 87:407-12.

69 Melrose J. The glucosinolates: a sulphur glucoside family of mustard anti-tumour and antimicrobial phytochemicals of potential therapeutic application. Biomedicines. 2019 ;19; 7:62-91.

70 Sotelo T, Lema M, Soengas P, Cartea ME, Velasco P. In vitro activity of glucosinolates and their degradation products against brassica-pathogenic bacteria and fungi. Applied and Environmental Microbiology. 2015;1; 81:432-40.

71 Buxdorf K, Yaffe H, Barda O, Levy M. The effects of glucosinolates and their breakdown products on necrotrophic fungi. PloS one. 2013; 8:70771-781.

72 Kojima M. Studies on the effects of *Isothiocynates* and their analogues on microorganisms. I. Effect of *Isothiocynates* on the oxygen uptake of yeasts. J. Ferment. Technol. 1971 ;49:740-6.

73 Plaszkó T, Szűcs Z, Vasas G, Gonda S. Effects of glucosinolate-derived *Isothiocynates* on fungi: A comprehensive review on direct effects, mechanisms, structure-activity relationship data and possible agricultural applications. Journal of Fungi. 2021 ;7:539-71.

74 Coscueta ER, Sousa AS, Reis CA, Pintado MM. *Phenylethyl Isothiocyanate*: A bioactive agent for gastrointestinal health. Molecules. 2022 ;25; 27:794-807.

75 Zhang M, Li Y, Bi Y, Wang T, Dong Y, Yang Q, Zhang T. 2-*Phenylethyl Isothiocyanate* exerts antifungal activity against Alternaria alternata by affecting membrane integrity and mycotoxin production. Toxins. 2020 ;15; 12:124-37.

76 Bertóti R, Vasas G, Gonda S, Nguyen NM, Szőke É, Jakab Á, Pócsi I, Emri T. Glutathione protects *C. albicans* against horseradish volatile oil. Journal of basic microbiology. 2016 ;56:1071-9.

77 Nazareth TM, Corrêa JA, Pinto AC, Palma JB, Meca G, Bordin K, Luciano FB. Evaluation of gaseous *Allyl Isothiocyanate* against the growth of mycotoxigenic fungi and mycotoxin production in corn stored for 6 months. Journal of the Science of Food and Agriculture. 2018 ;98:5235-41.

78 Chang Y, Schlenstedt G, Flockerzi V, Beck A. Properties of the intracellular transient receptor potential (TRP) channel in yeast, Yvc1. FEBS letters. 2010; 17; 584:2028-32.

79 Kim HS, Kim JE, Frailey D, Nohe A, Duncan R, Czymmek KJ, Kang S. Roles of three Fusarium oxysporum calcium ion (Ca2+) channels in generating Ca2+ signatures and controlling growth. Fungal Genetics and Biology. 2015 ;1;82: 145-57.

80 Nguyen QB, Kadotani N, Kasahara S, Tosa Y, Mayama S, Nakayashiki H. Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, Magnaporthe oryzae, using a high-throughput RNA-silencing system. Molecular microbiology. 2008; 6:1348-65.

81 Li Y, Liu Y, Zhang Z, Cao Y, Li J, Luo L. *Allyl Isothiocyanate* (AITC) triggered toxicity and FsYvc1 (a STRPC family member) responded sense in Fusarium solani. Frontiers in Microbiology. 2020; 12; 11:870-81.

82 Madloo P, Lema Márquez M, Tortosa Viqueira M, Bakhtiarizadeh MR, Soengas Fernández MD. Transcriptional analysis of the response of Sclerotinia sclerotiorum after exposure to aliphatic and indolic *Isothiocynates* (ITCs):2018:68-74.

83 Yu Q, Wang F, Zhao Q, Chen J, Zhang B, Ding X, Wang H, Yang B, Lu G, Zhang B, Li M. A novel role of the vacuolar calcium channel Yvc1 in stress response, morphogenesis and pathogenicity of *C. albicans*. International Journal of Medical Microbiology. 2014 ;1; 304:339-50.

84 Nielsen PV, Rios R. Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oil. International journal of food microbiology. 2000; 25; 60:219-29.

85 Saladino F, Manyes L, Luciano FB, Mañes J, Fernandez-Franzon M, Meca G. Bioactive compounds from mustard flours for the control of patulin production in wheat tortillas. LWT-Food Science and Technology. 2016; 1;66: 101-7.

86 Tracz BL, Bordin K, Bocate KC, Hara RV, Luz C, Macedo RE, Meca G, Luciano FB. Devices containing *Allyl Isothiocyanate* against the growth of spoilage and mycotoxigenic fungi in mozzarella cheese. Journal of Food Processing and Preservation. 2018; 42:13779-85.

87 Winther M, Nielsen PV. Active packaging of cheese with *Allyl Isothiocyanate*, an alternative to modified atmosphere packaging. Journal of Food Protection. 2006; 1; 69:2430-5.

88 Naglik JR, Gaffen SL, Hube B. *Candida* lysin: discovery and function in Candida albicans infections. Current opinion in microbiology. 2019; 52:100-9.

89 Olivier FA, Hilsenstein V, Weerasinghe H, Weir A, Hughes S, Crawford S, Vince JE, Hickey MJ, Traven A. The escape of *Candida albicans* from macrophages is

enabled by the fungal toxin *Candida* lysin and two host cell death pathways. Cell Reports. 2022; 40:111374-370.

CHAPTER – III

Materials and Methods

CHAPTER – III

Materials and Methods

3. Materials and Methods

Currently, the M27-A4 standard of the North American Clinical and Laboratory Standards Institute (CLSI) guides measurement and interpretation of the minimum inhibitory concentrations (MIC) of important antifungal drugs which are used against different yeast species like *Candida* spp. The protocol recommends the plaque microdilution method. Thereby, colony suspensions in pH 7.0 buffered RPMI-1640 broth with MOPS (3- (N-morpholino) propanesulfonic acid) are analyzed with 10 concentrations of each test drug. After incubation at 35 °C for 24 hours for *Candida* species, visual assessment of MIC is performed in comparison with a control well containing antifungal-free inoculum to support fungal growth **[1].**

3.1.1 Hypothesis, aim and objectives of the study:

Hypothesis:

Isothiocynates may show inhibitory activity against *Candida albicans* growth and its virulence factors.

Aim of the study:

To identify the activity of *Isothiocynates* as antifungals against *Candida albicans* growth and its virulence factors.

Objectives of the study:

1. To establish:

- Minimum inhibitory concentration (MIC)
- Minimum fungicidal concentration (MFC) values of *Allyl Isothiocyanate, Ethyl Isothiocyanate*, *Benzyl Isothiocyanate* , and *Phenylethyl Isothiocyanate* against one standard strain of *Candida albicans*.

2. To study:

- The architecture of the biofilms in the presence and absence of drugs using SEM and TEM.

3.1.2 Culture, Growth conditions, Media:

C. albicans strain ATCC 90028 was obtained from the Institute of Microbial Technology (IMTECH) in Chandigarh, India. The organism was sub-cultured on Yeast Extract Peptone Dextrose (YPD) agar plates and slants, then stored at 4 °*C. albicans* colonies were inoculated into a flask containing 50 ml of YPD broth and incubated at 30 °C in an

incubator shaker at 120 rpm for 24 hours. After 24 hours, cells were collected by centrifugation at a speed of 2000 rpm for 2 minutes. Subsequently, the cells were washed with phosphate-buffered saline (PBS) twice and used for various assays.

3.2 Chemicals used:

Allyl Isothiocyanate (AITC), *Ethyl Isothiocyanate* (EITC), *Benzyl Isothiocyanate* (BITC), and *Phenylethyl Isothiocyanate* (PEITC) drugs were procured from Sigma Aldrich Chemical Ltd., Mumbai, India. The *AITC, EITC, BITC, and PEITC*s were dissolved in DMSO to create a stock solution with a concentration of 2 mg/ml. This stock solution was subsequently diluted and used for in vitro studies.

3.3 Minimum Inhibitory Concentration (MIC_{50}) determination for Planktonic growth of *C. albicans:*

The recommendations of the Clinical Laboratory Standards Institute (CLSI) were followed to study how *AITC, EITC, BITC, and PEITC*s affect the floating (planktonic) growth of *C. albicans.* The method is also called as broth micro dilution is used to carry out this investigation. Various concentrations of *AITC, EITC, BITC, and PEITC*s, ranging from 0.0039 to 2 mg/ml, were prepared in RPMI-1640 with L-glutamine and without sodium bicarbonate, and added to a 96-well plate. Wells without test molecules served as controls. The plates were then incubated for 48 hours at 35 °C. Growth was assessed by measuring absorbance at 620 nm using a microtiter plate reader (Multiskan Ex, Thermo Electron Corp., USA). The concentration of *AITC, BITC, EITC and PEITC*s causing a 50 % reduction in growth compared to the control was considered the minimum inhibitory concentration for the growth of *C. albicans* **[2].**

3.4 Minimum Fungicidal Concentration (MFC) assay:

After conducting a test to see how the fungus grows in a liquid environment (planktonic growth assay) under sterile conditions, 10 µl sample of cells was taken from the concentration that inhibits 50 % of growth (MIC_{50}) and higher concentrations. The samples were spread on YPD agar, a type of growth medium. The agar plates were then incubated for 48 hours at 30 °C. After 48 hours colonies were observed, and the concentration of *AITC, EITC, BITC, and PEITC*s at which the molecule showed no growth was considered the Minimum Fungicidal Concentration (MFC) **[3].**

3.5 Yeast to Hyphal Morphogenesis (Y-H Morphogenesis) assay:

The induction of yeast-to-hyphal morphogenesis in *C. albicans* by fetal bovine serum was examined through a microplate-based assay. *AITC, EITC, BITC, and PEITC*s concentrations, ranging from 0.0039 to 2 mg/ml, were prepared in 20 % serum. In both the control and test wells of the microtiter plate, 1×10^6 cells/ml were inoculated, and the final assay volume was maintained at 200 µl. The plates were then incubated at 37 °C for 2 hours on an orbital shaker at 200 rpm. The formation of germ tubes by the cells was observed using an Inverted Microscope, and the count of yeast cells and hyphae was conducted with the assistance of a microscope **[4].**

3.6 Scanning Electron Microscopy (SEM):

To observe the yeast-to-hyphal morphogenesis of *C. albicans* cells on polystyrene discs (1×10^7 cells/ml), a process known as Y-H morphogenesis, experiments was conducted in 12-well plates at 37 °C and 50 rpm for 90 minutes. For SEM analysis, samples were fixed in a 2.5 % glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2) for 24 hours at 4 °C. Subsequently, the samples were post-fixed in a 2 % aqueous solution of osmium tetroxide for 4 hours and dehydrated using a series of graded alcohols. The prepared samples were then mounted on stubs, and gold coating was applied using an automated gold coater. Finally, images were captured using a scanning electron microscope **[5].**

3.7 Adhesion Assay:

The check the anti-adhesion property of *AITC, EITC, BITC, and PEITC* against *C. albicans* microplate-based test was used. The different concentrations of *AITC, EITC, BITC, and PEITC* were prepared in PBS (from 0.0039 to 2 mg/ml) and added 50 µl of cell suspension (with about 1×10^7 cells/ml) to each microplate well, keeping the final volume at 100 µl. The plates were left to sit for 90 minutes at 100 rpm on a shaker at 37 °C to allow cells to attach to the surface. After this, we washed the wells with PBS to remove cells that didn't stick, and we checked how many cells stuck in each well by looking at their metabolic activity using the XTT assay. The concentration where there was a 50 % reduction compared to the control was considered the MIC_{50} concentration for adhesion **[6].**

3.8 Biofilm Assays:

3.8.1 Early Biofilm assay:

To investigate the initial stages of biofilm formation, tissue culture-treated 96-well polystyrene plates were used. In each well, 100 µl of a cell suspension (1×10^7 cells/ml)introduced and cells were allowedto adhere on the solid surfaces of polystyrene plate for 90 minutes at 37 °C and 100 rpm. Following this, we removed non-adherent cells by filling the wells with PBS. Subsequently, we added RPMI-1640 medium (200 µl) containing various concentrations of *AITC, EITC, BITC, and PEITC* (ranging from 0.0039 to 2 mg/ml) and incubated the plates for 48 hours at 37 °C. Post-incubation, we washed the wells with PBS and employed the XTT metabolic assay to evaluate the impact of *AITC, EITC, BITC, and PEITC* on early biofilm growth **[7].**

3.8.2 Mature Biofilm assay:

For mature biofilms, we prepared a 24-hour-old biofilm on tissue culture-treated 96-well polystyrene plates. *AITC, EITC, BITC, and PEITC* concentrations within the range of 0.0039 to 2 mg/ml were prepared in RPMI 1640 medium and added to the wells. Using an inverted light microscope, we examined the wells to observe the effects of *AITC, EITC, BITC, and PEITC* on the pre-existing biofilm. The XTT metabolic assay was then employed to analyze the growth of the mature biofilm **[7].**

3.8.3 XTT Assay for Quantification of Biofilm:

In order to quantify biofilm growth, we utilized the XTT metabolic test. After eliminating non-adherent cells by washing the wells with PBS, we added 100 µl of XTT-Menadione solution and incubated it in the dark at 37 °C for 5 hours. Subsequently, a microplate reader was used to measure the coloration produced by the water-soluble formazan product at 450 nm **[7].**

3.9 Hemolytic assay:

To assess the toxicity of *AITC, EITC, BITC, and PEITC* , we conducted a hemolytic assay using human Red Blood Cells (RBC_S). Human blood, obtained from the blood bank, was collected and stored in tubes containing EDTA. The collected blood was then centrifuged at 2000 rpm for 10 minutes at 20 °C. The resulting RBC_S pellet was suspended in PBS (10 % v/v). Before use, the RBC_S suspension was diluted in PBS at a 1:10 ratio. For the assay, 100µl aliquots from the RBC_S suspension were added to 100µl of different concentrations of *AITC, EITC, BITC, and PEITC* in the same buffer, using

Eppendorf tubes. Total hemolysis was induced using 1 % Triton X-100. After incubating for 1 hour at 37 °C, the mixture was centrifuged for 10 minutes at 2000 rpm at 20 °C. The optical density was measured at 450 nm after transferring 150 µl of the supernatant to a microtiter plate with a flat bottom. All experiments were conducted in triplicates **[8].**

3.9.1 Calculation of Hemolysis Percentage:

Fig. 3.1 The percentage of hemolysis was determined using the following formula:

$$\%\ of\ Haemolysis = \frac{[A450\ of\ test\ compound\ treated\ Sample - A450\ of\ buffer\ treated\ sample]}{[A450\ of\ 1\%TritonX\ 100\ treated\ sample - A450\ of\ buffer\ treated\ sample]}\ x\ 100 \quad \ldots\ldots (3.1)$$

This formula compares the absorbance values of the test compound-treated sample with those of the buffer-treated sample and 1 % Triton X 100-treated sample. The result is expressed as a percentage of hemolysis, providing insights into the impact of the test compound on red blood cells **[8].**

3.10 References

1 De Sousa ES, Cortez AC, de Souza Carvalho Melhem M, Frickmann H, de Souza JV. Factors influencing susceptibility testing of antifungal drugs: a critical review of document M27-A4 from the Clinical and Laboratory Standards Institute (CLSI). Brazilian Journal of Microbiology. 2020; 51:1791-800.

2 Pfaller MA, Diekema D. Progress in antifungal susceptibility testing of *Candida* spp. by use of Clinical and Laboratory Standards Institute broth microdilution methods, 2010 to 2012. Journal of clinical microbiology. 2012; 50:2846-56.

3 Thakre A, Zore G, Kodgire S, Kazi R, Mulange S, Patil R, Shelar A, Santhakumari B, Kulkarni M, Kharat K, Karuppayil SM. Limonene inhibits *C. albicans* growth by inducing apoptosis. Medical Mycology. 2018;1; 56:565-78.

4 Thakre A, Jadhav V, Kazi R, Shelar A, Patil R, Kharat K, Zore G, Karuppayil SM. Oxidative stress induced by piperine leads to apoptosis in *C. albicans*. Medical Mycology. 2021; 59:366-78.

5 Vila T, Romo JA, Pierce CG, McHardy SF, Saville SP, Lopez-Ribot JL Targeting *C. albicans* filamentation for antifungal drug development. Virulence 2017; 8:150-158.

6 Raut JS, Bansode BS, Jadhav AK, Karuppayil SM Activity of *Allyl Isothiocyanate* and its synergy with fluconazole against *C. albicans* biofilms. J Microbiology Biotechnology 2017; 27:685-693.

7 Patil SB, Sharma RK, Gavandi TC, Basrani ST, Chougule SA, Yankanchi SR, Jadhav AK, Karuppayil SM. *Ethyl Isothiocyanate* as a Novel Antifungal Agent Against *C. albicans*. Current Microbiology. 2024; 81:29-43.

8 Raut JS, Shinde RB, Chauhan NM, Karuppayil SM. Phenylpropanoids of plant origin as inhibitors of biofilm formation by *C. albicans*. Journal of Microbiology and Biotechnology. 2014; 24:1216-25.

CHAPTER – IV

Study of Allyl Isothiocyanate (AITC)

on *Candida albicans*

CHAPTER – IV

Study of Allyl Isothiocyanate (AITC)

on *Candida albicans*

4. 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. Fungal infection mainly occurs in immunocompromised individuals **[1, 2].** Among these, fungal infections caused by *C. albicans* are the most common threat to human beings **[3, 4].** *C. albicans* is a pleiomorphic fungal pathogen that can produce biofilms on the cell surfaces of mammals as well as implanted medical devices **[5, 6].** It can 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 kneecaps. 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 **[7].**

Therefore, the treatment of biofilm-related infections has become a major challenge to clinicians **[8].** 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 increased side effects of the drugs. Since biofilms are hard to eradicate, the only available alternative may be the physical removal of the devices. In addition, colonized devices, such as pacemakers, may malfunction. Clogging of the catheters by *C. albicans* is a serious problem, especially in diabetic or immunocompromised persons since the catheters require replacement. Removal or replacement of prosthetics is expensive and uncomfortable for the patients and may require frequent visits to the doctor as well as hospitals contributing to the additional expenses, mortality, and morbidity **[9].** This necessitates an alternative treatment modality to counter the low efficacy, significant side effects, and emergence of multidrug-resistant *C. albicans* strains. Biofilms can adhere and accumulate on numerous surfaces. The new antifungal drug strategy should concentrate on the development of drugs that prevent and remove biofilms. Numerous plant-derived substances have been shown to possess potential anti-*Candida* activities through a variety of mechanisms, including inhibition of the yeast-to-hyphae transition, prevention of the formation of biofilms, impairment of cell metabolism, cell wall integrity, cell membrane fluidity, and apoptosis **[6, 10].**

Allyl Isothiocyanate (AITC) is a natural compound derived from cruciferous vegetables. An earlier study performed by Raut et al. **[11]** has suggested that AITC alone and in combination with the standard antifungal, fluconazole (FLC), successfully inhibits the growth and virulence factors of *C. albicans.* Along with this, AITC at its planktonic and biofilm inhibitory concentration was non-hemolytic. Hence, it can be used as an alternative therapeutic option for the treatment of candidiasis. The present study is aimed to verify inhibitory activity of AITC against *C. albicans.* In this regard, the antifungal properties of AITC were demonstrated in planktonic and biofilm forms of *C. albicans.*

4.1 Introduction of *Allyl Isothiocyanate* (AITC)

Allyl Isothiocyanate (AITC) is a naturally occurring unsaturated *Isothiocyanate*. This colorless oil is responsible for the pungent taste of cruciferous vegetables such as mustard and radish. It is slightly soluble in water but more soluble in most organic solvents. *Allyl Isothiocyanate* is used as a flavoring agent, in medicine as a rubefacient (counterirritant), as a fumigant, in ointments, in mustard plasters, as an adjuvant, as a fungicide, as a repellent for cats and dogs, and as a preservative in animal feed. Information on basic physical properties are shown in (**Table 4.1) [12].**

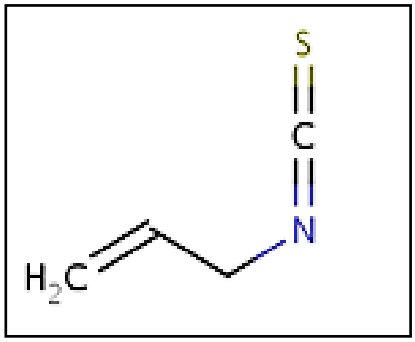


Image 4. 1. Molecular structure of *Allyl Isothiocyanate*.

Molecular Formula: C_4H_5NS **Molecular weight:** 99.15 g/mol

Table 4. 1. Properties of *Allyl Isothiocyanate*

Sr. No.	Particulars	Observation
1	Physical state	Very refractive liquid
2	Colour	Colourless or pale-yellow,
3	Odour	Very pungent, irritating odour and acrid taste
4	Melting point	-80°C (lit.)
5	Boiling point	152°C (lit.)
6	Density	1.0126 g/cm^3 at 20°C (lit.)
7	Solubility	Sparingly soluble in water; very soluble in benzene, ethyl ether and ethanol; miscible with most organic solvents

4.1.1 Biosynthesis and biological functions:

*Allyl Isothiocyanate*can be obtained from the seeds of black mustard (*Brassica nigra*) or brown Indian mustard (*Brassica juncea*). When these mustard seeds are broken, the enzyme myrosinase is released and acts on a glucosinolate known as sinigrin to give *Allyl Isothiocyanate*. This serves the plant as a defense against herbivores; since it is harmful to the plant itself it is stored in the harmless form of the glucosinolate, separate from the myrosinase enzyme. When an animal chews the plant, the *Allyl Isothiocyanate* is released, repelling the animal **[13,14].**

4.1.2 Commercial and other applications:

*Allyl Isothiocyanate*is produced commercially by the reaction of allyl chloride and potassium thiocyanate.

$CH_2=CHCH_2Cl + KSCN \rightarrow CH_2=CHCH_2NCS + KCl$ …… **(4.1)**

The product obtained in this fashion is sometimes known as synthetic mustard oil. *Allyl Isothiocyanate*can also be liberated by dry distillation of the seeds. The product obtained in this fashion is known as the volatile oil of mustard and is usually around 92 % pure. It is used principally as a flavouring agent in foods. Synthetic *Allyl Isothiocyanate* is used as an insecticide, as an anti-mold agent bactericide, and

nematicide, and is used in certain cases for crop protection. It is also used in fire alarms for the deaf **[15,16].**

4.2.1 Materials and Methods

Materials and methods followed are given in Chapter 3.

4.3 Results

4.3.1 Antifungal activity of AITC on *C. albicans* Planktonic Growth

The inhibitory effect on planktonic growth of *C. albicans* was assessed by AITC **(Fig. 4.3.1A).** The MIC_{50} of AITC for *C. albicans* was found to be 0.125 mg/ml. The minimum fungicidal concentration of AITC was assessed by spread plate technique on YPD plate with the help MIC_{50} concentration and results indicate that AITC is fungicidal at its MIC_{50} and above concentrations **(Fig. 4.3.1B).**

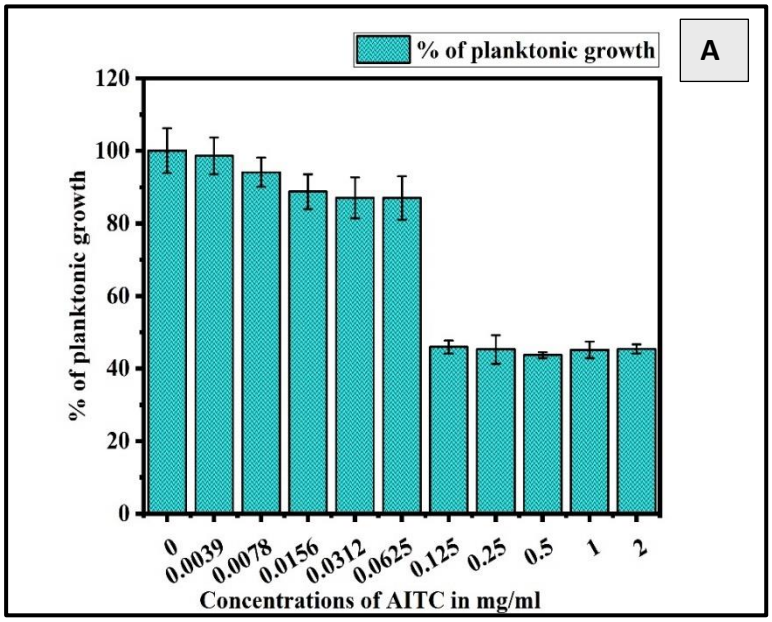


Fig. 4.3.1A: Inhibition of *C. albicans* planktonic growth by *Allyl Isothiocyanate*. The inhibitory effects of series of concentrations ranges from 0.0039 to 2 mg/ml were evaluated on planktonic growth after 48 h incubation.

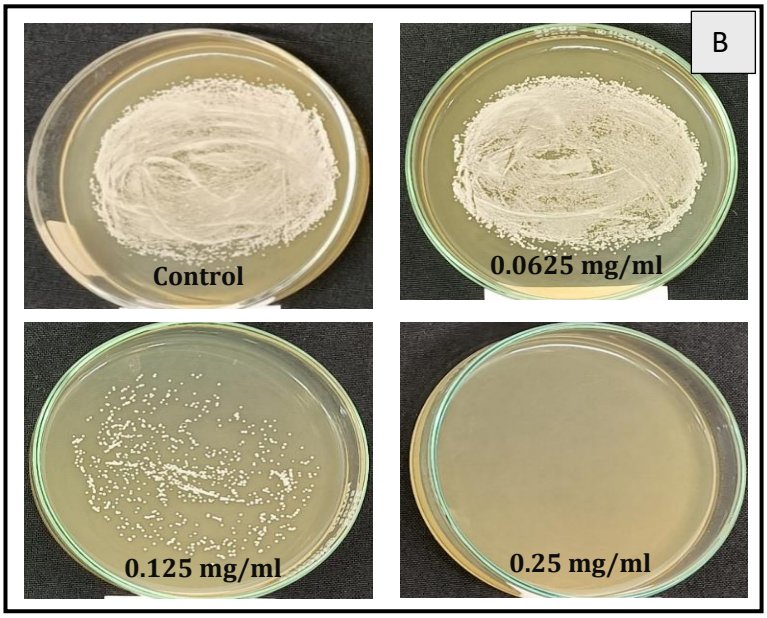


Fig. 4.3.1B: Plate based bioassay to determine minimum fungicidal concentration (MFC) in *C. albicans* 90028 strain. Application of *Allyl Isothiocyanate* (0.25 mg/ml) completely inhibited colony survival.

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

In this study, we analyzed the effect of AITC on germ tube formation of *C. albicans* using fetal bovine serum (FBS) growth media. AITC inhibited Y-H from morphogenesis 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. The morphological analysis of *C. albicans* cells was further assessed for germ tube inhibition by Scanning Electron Microscopy (SEM) **(Fig. 4.3.2).**

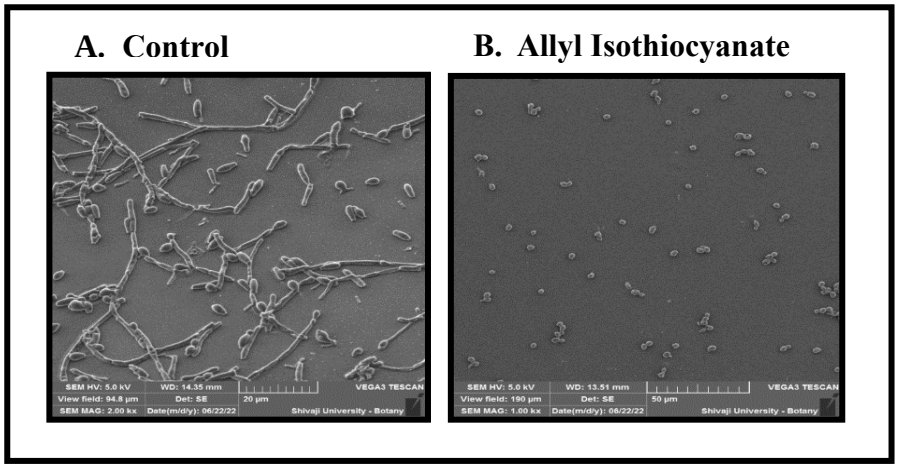


Fig. 4.3.2: Morphological characterization by scanning electron microscopy (SEM) of yeast to hyphal (Y-H) morphogenesis of *C. albicans*; (A.) *C. albicans* cells without treatment of drug at 2.00 kx magnification and (B.) *C. albicans* cells treated with 0.125 mg/ml of *Allyl Isothiocyanate* at 1.00 kx magnification.

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

Adhesion plays a vital role in biofilm formation and infection in *C. albicans*. The inhibitory effect of AITC on *C. albicans* to polystyrene surface was quantified by XTT metabolic assay. AITC inhibits adhesion to polystyrene surface to an extent of 50 % 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. 4.3.3).**

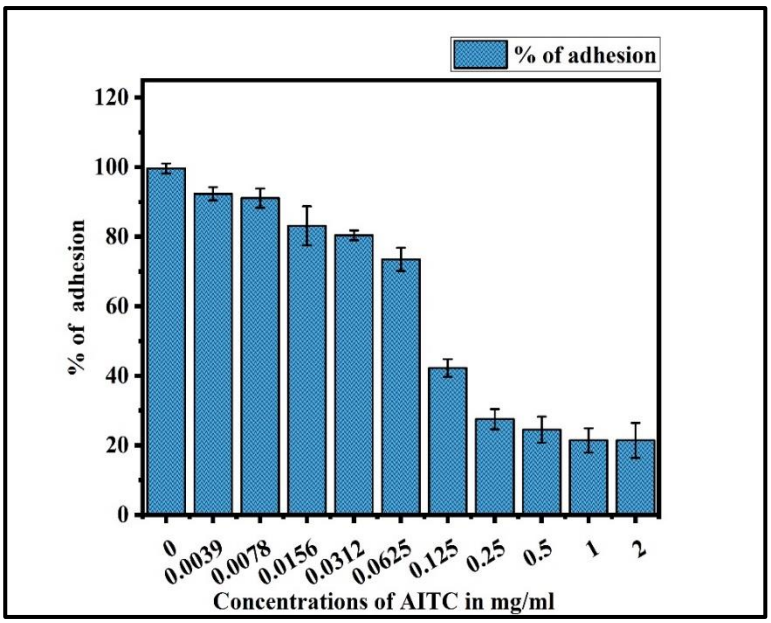


Fig. 4.3.3: Inhibition of *C. albicans* adhesion by the treatment of *Allyl Isothiocyanate* to an abiotic surface (polystyrene). *C. albicans* cells were incubated in phosphate buffered saline (PBS) in polystyrene wells for 90 min. and then washed with PBS to remove unattached cells. The attached cells were detected by measuring the reduction of XTT against *C. albicans.*

4.3.4 Effect of AITC on biofilm formation (Early and Mature biofilm)

AITC anti-biofilm activity was evaluated against the *C. albicans* ATCC 90028 strain. At a dose of 0.5 mg/ml, AITC suppresses the early or emerging biofilm and at the same concentration, AITC suppresses mature biofilm as shown by the XTT metabolic assay **(Fig. 4.3.4A, 4.3.4B).**

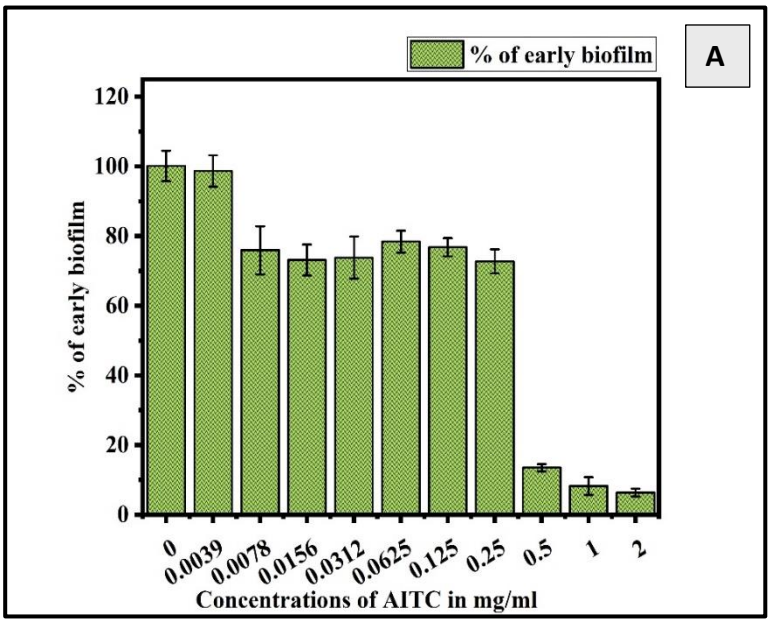


Fig. 4.3.4A: Inhibition of *C. albicans* early biofilm formation by the treatment of *Allyl Isothiocyanate*. The inhibitory effect of *Allyl Isothiocyanate* dilutions was evaluated on biofilms after a 48 h incubation with XTT metabolic.

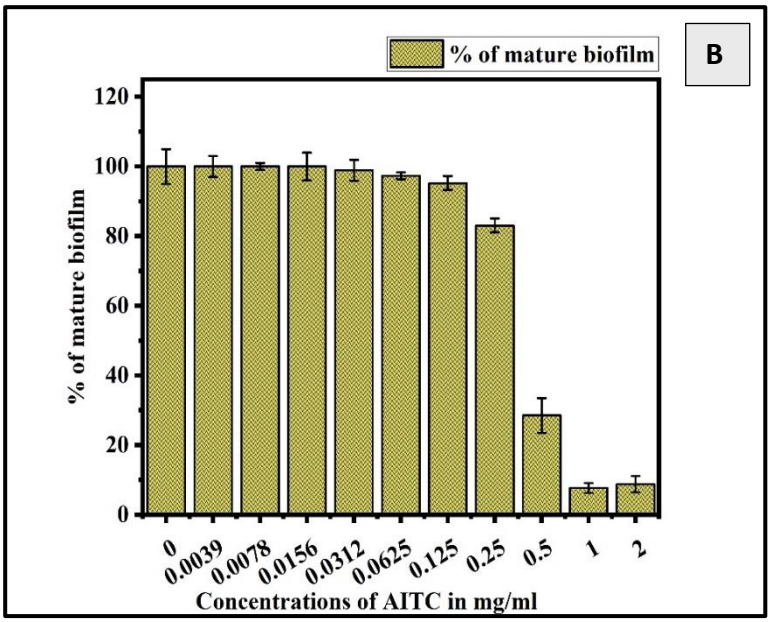


Fig. 4.3.4B: Inhibition of *C. albicans* mature biofilm formation by the treatment of *Allyl Isothiocyanate*. The inhibitory effect of *Allyl Isothiocyanate* dilutions was evaluated on biofilms after a 72 h incubation with XTT metabolic.

4.3.5 Toxicity effect of AITC on human red blood cells (RBCSs)

The toxicity of AITC was analyzed by *in vitro* hemolytic activity on human RBCSs. It was observed that AITC was non-hemolytic in a concentration range from 0.0039 to 2 mg/ml **(Fig. 4.3.5).**

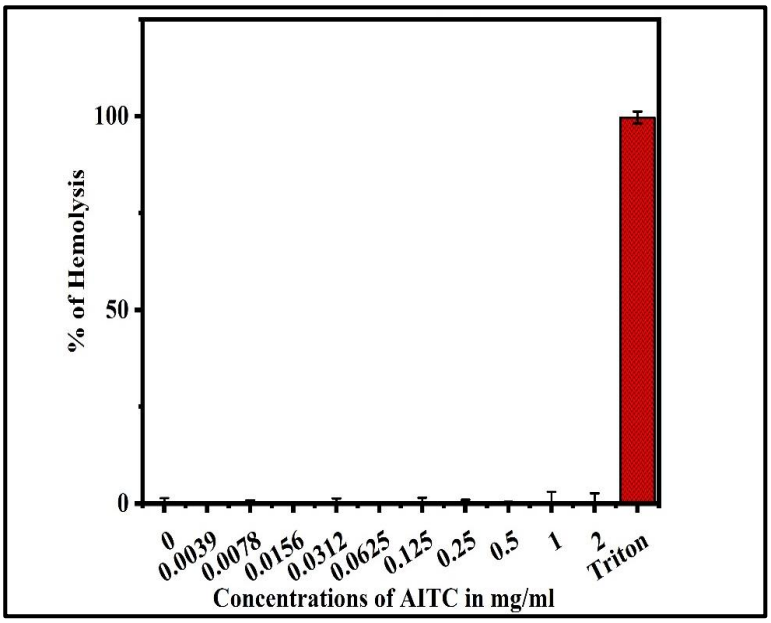


Fig. 4.3.5: Hemolysis assay of *Allyl Isothiocyanate*, including triton 100 X as positive control and 0 as negative control. The relative rate of hemolysis in human erythrocytes was measured following a 1h incubation with concentrations ranging from 0.0039 to 2 mg/ml of *Allyl Isothiocyanate* at 37°C.

4.4 Discussion:

Isothiocynates are well-known antimicrobial substances used to fight bacteria and potentially harmful fungi. It is surprising that little research has been performed on how ITCs affect yeasts, like *C. albicans.* Very few studies have shown the effect of *Isothiocyanate* on *C. albicans.* Anti-*Candida* activity of AITC alone and in combination with FLC was explored in 2017 **[11, 17].** The activity of AITC against *C. albicans* pathogenicity and planktonic growth was concentration dependent. At 1 mg/ml, the biofilm was significantly ($P \leq 0.05$) inhibited by AITC. Notably, the biofilm was not formed when 0.004 mg/ml of FLC and 0.125 mg/ml of AITC were combined. The AITC-FLC combination also significantly ($P \leq 0.05$) suppressed the developed biofilms. The fractional inhibitory concentration indices, which ranged from 0.132 to 0.312, showed that AITC and FLC worked together to prevent the development of both early

and mature biofilms. Toxicity study analysis has suggested that AITC alone and in combination with FLC causes no hemolysis **[18, 19].**

In order to find out the antifungal effect of AITC against *C. albicans*, we have performed various methods of drug susceptibility assays namely minimum inhibitory concentration (MIC_{50}) using broth micro dilution method and minimum fungicidal concentration (MFC) assay. Both micro dilution assays confirmed that AITC show the antifungal effect against *C. albicans* at MIC of 0.125 mg/ml **(Fig. 4.3.1A).** The results of MFC confirmed that AITC was fungicidal in nature **(Fig. 4.3.1B).**

C. albicans has the ability to colonise tissues, implant devices, and build biofilms which are essential for pathogenesis and medication resistance. One of the main factors in the development of biofilm and the related yeast infections is the transformation of yeast into hyphal cells. It has been observed that a variety of natural substances, including phytochemicals, prevent *C. albicans* from forming biofilm, mostly by preventing hypha production. The antifungal activity of plant molecule AITC was further assessed against virulence factors of *C. albicans* like yeast to hyphal (Y-H) morphogenesis, adhesion assay and biofilm formation and we found that all the tested strains displayed susceptibility to AITC. AITC at 0.125 mg/ml concentration inhibits Y-H morphogenesis **(Fig. 4.3.2).** AITC at 0.125 mg/ml concentration inhibits adhesion **(Fig.4.3.3).** At 0.5 mg/ml concentration AITC inhibits early and mature biofilm **(Fig. 4.3.4a and 4.3.4b).** Thus, all the drug susceptibility testing results clearly indicate that AITC is inhibitory against *C. albicans* and it may be used as alternative therapeutic agent for candidiasis after *in vivo* study.

4.5 Conclusions:

The anti-*C. albicans* capability of AITC is reported in this work. This suggests that AITC may be used to treat *C. albicans* by acting as an anti-biofilm agent. AITC inhibited Y–H morphogenesis at 0.125 mg/ml concentration, adherence to the polystyrene surface at 0.125 mg/ml concentration, and biofilm formation like early and mature at 0.5 mg/ml concentration.

4.6 References

1 Ward RA, Aghaeepour N, Bhattacharyya RP, Clish CB, Gaudillière B, Hacohen N, Mansour MK, Mudd PA, Pasupneti S, Presti RM, Rhee EP. Harnessing the potential of multiomics studies for precision medicine in infectious disease. InOpen Forum Infectious Diseases. US: Oxford University Press. 2021: 11:483-92.

2 Atriwal T, Azeem K, Husain FM, Hussain A, Khan MN, Alajmi MF, Abid M. Mechanistic Understanding of *C. albicans* Biofilm Formation and Approaches for Its Inhibition. Front Microbiol. 2021;12: 638609-643.

3 Liu X, Zhong L, Ma Z, Sui Y, Xie JN, Ma T, Yang L. Antifungal effects of alantolactone on *C. albicans*: An in vitro study. BioRxiv. 2021:01-15.

4 Macias-Paz IU, Pérez-Hernández S, Tavera-Tapia A, Luna-Arias JP, Guerra-Cárdenas JE, Reyna-Beltrán E. *C. albicans* the main opportunistic pathogenic fungus in humans. Rev Argent Microbiol. 2023; 55:189-198.

5 Yan Y, Tan F, Miao H, Wang H, Cao Y. Effect of Shikonin Against *C. albicans* Biofilms. Front Microbiol. 2019; 10:1085-96.

6 Feldman M, Sionov RV, Mechoulam R, Steinberg D. Anti-biofilm activity of cannabidiol against *C. albicans*. Microorganisms. 2021; 9:441-58.

7 Pereira R, dos Santos Fontenelle RO, De Brito EH, De Morais SM. Biofilm of *C. albicans*: formation, regulation and resistance. J Appl Microbiol. 2021; 131:11-22.

8 Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJS. *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol. 2013; 62:10-24.

9 Pereira R, dos Santos Fontenelle RO, de Brito EHS, de Morais SM. Biofilm of *C. albicans*: formation, regulation and resistance. J Appl Microbiol. 2021; 131:11–22.

10 Zhang K, Li X, Yu C, Wang Y. Promising therapeutic strategies against microbial biofilm challenges. Front. Cell. Infect. Microbiol. 2020; 10:359-65.

11 Raut JS, Bansode BS, Jadhav AK, Karuppayil SM. Activity of *Allyl Isothiocyanate* and its synergy with fluconazole against *C. albicans* biofilms. Journal of Microbiology Biotechnology. 2017; 28; 27:685-93.

12 Zhang Y. *Allyl Isothiocyanate* as a cancer chemopreventive phytochemical. Molecular nutrition & food research. 2010;54(1):127-35.

13 Blažević I, Montaut S, Burčul F, Olsen CE, Burow M, Rollin P, Agerbirk N. Glucosinolate structural diversity, identification, chemical synthesis and metabolism in plants. Phytochemistry. 2020;1; 169:112100-57.

14 Varelis P, Melton L, Shahidi F. Encyclopedia of food chemistry. Reference Module in Food Science. Elsevier: Food Standards Australia, New Zealand, Canberra; 2018; 1: 720-739.

15 Vilela AD, Faroni LR, Gomes JL, Sousa AH, Cecon PR. *Allyl Isothiocyanate* as a fumigant in the cowpea and its effect on the physical properties of the grain. Revista Ciência Agronômica. 2021;27;52-59.

16 Masuda S, Masuda H, Shimamura Y, Sugiyama C, Takabayashi F. Improvement effects of wasabi (Wasabia japonica) leaves and *Allyl Isothiocyanate* on stomach lesions of mongolian gerbils infected with Helicobacter pylori. Natural Product Communications. 2017; 12:594-598.

17 Soliman S, Alnajdy D, El-Keblawy AA, Mosa KA, Khoder G, Noreddin AM. Plants' Natural Products as Alternative Promising Anti-*Candida* Drugs. Pharmacogn Review. 2017; 11:104-122.

18 Matsumoto H, Nagao J, Cho T, Kodama J. Evaluation of pathogenicity of *C. albicans* in germination-ready states using a silkworm infection model. Journal of Medical Mycology. 2013; 54:131-40.

19 De Zoysa GH, Glossop HD, Sarojini V. Unexplored antifungal activity of linear battacin lipopeptides against planktonic and mature biofilms of *C. albicans.* European Journal of Medicinal Chemistry. 2018; 146:344-353.

CHAPTER – V

Study of Ethyl Isothiocyanate (EITC)

on *Candida albicans*

CHAPTER – V

Study of Ethyl Isothiocyanate (EITC) on *Candida albicans*

5. Introduction:

There are around 8.7 million eukaryotic species present on Earth. According to a recent estimate, approximately 611,000 are fungal species, and about 600 species are pathogenic to humans **[1,2].** *Candida* is a part of the natural flora, and it can become pathogenic in immune compromised patients. *C. albicans* has the ability to form biofilms on medical devices **[3]**. Biofilm-related infections are becoming difficult to treat in hospitalized patients as well as in patients who has recently gone through surgery as they exhibit drug resistance **[4].** To treat this fluconazole and amphotericin B are widely prescribed but they exhibit side effects due to high doses **[5].** It has become important to find alternatives to the current drugs and find drugs which can target drug tolerant biofilms without causing side effects to the patients **[6, 7].**

The potential of using bioactive molecules of natural origin as antifungal agents is proposed by various workers as they inhibit biofilm formation **[8, 9].** Recent research revealed that the *Brassicaceae* family of crops, including cabbage, broccoli, cauliflower, and radish, are rich of nutrients that can benefit human health, including carotenoids, polyphenols, flavonoids, and glucosinolates (GLS) as well as *Isothiocynates* (ITCs). The breakdown products of GLS are ITCs. Their numerous pharmacological qualities, including their antibacterial, antifungal, antiprotozoal, anti-inflammatory, and chemo protective actions, make them useful in the pharmaceutical business as well **[10].** It has been observed that ITC compounds exhibit antifungal properties against soil-borne fungal infections **[11, 12].** Along with this a few reports on the antifungal activities of ITCs against postharvest plant pathogenic fungi such as *Botrytis cinerea, Penicillium expansum, Alternaria alternata* and *Monilinia laxa* are known **[13].** In current study we have explored the anti-*C. albicans* activity of *Ethyl Isothiocyanate* (EITC). EITC inhibits *C. albicans* planktonic growth and virulence factors like, yeast to hyphal form transition, adhesion to polystyrene surface, developing biofilm and mature biofilm.

5.1 Introduction to *Ethyl Isothiocyanate*

Synonym: Isothiocyanic Acid Ethyl Ester

An *Isothiocyanate* has an Ethyl group attached to nitrogen.

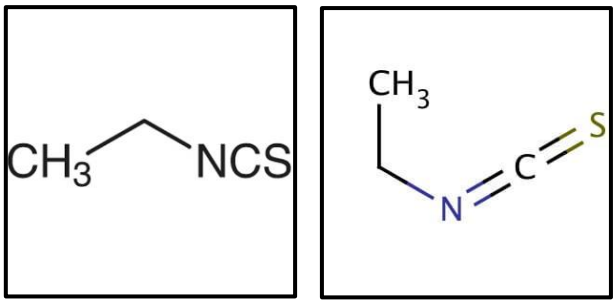


Image 5. 1. Molecular structure of *Ethyl Isothiocyanate*.

Formula: C_3H_5NS **Molecular weight:** 87,14 g/mol

Table 5.1. Physical properties of *Ethyl Isothiocyanate*

Sr. No	Particulars	Observations
1	Physical state	Clear, liquid
2	Colour	Light yellow
3	Odour	Sharp mustard-like aroma
4	Melting point	-6 °C (lit.)
5	Boiling point	130 - 132 °C (lit.)
6	Density	0.995 g/cm3 at 25 °C (lit.)
7	Solubility	Insolubility in water. Miscible with alcohol, ether.

Also known as Iso-thio-cyanato-ethane, *Ethyl Isothiocyanate* (EITC) is a naturally occurring chemical compound found in various plants. Due to its unique characteristics, EITC has found extensive applications in scientific research, spanning synthesis, biochemistry, and physiology. *Ethyl Isothiocyanate* demonstrates broad utility in scientific research across multiple domains. In organic synthesis, it serves as a highly reactive nucleophile, facilitating the synthesis of diverse compounds. In the field of biochemistry, EITC is employed to investigate enzyme kinetics and protein-ligand interactions. The Basic physical properties shown in **(Table 5.1).**

5.2 Materials and Methods

Material and methods followed are given in Chapter 3.

5.3.12 Statistical Analysis

The values mentioned were the means 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.

5.4 Results

5.4.1. EITC inhibited planktonic growth in *C. albicans.*

EITC inhibited Planktonic growth of *C. albicans* Initially, we studied the effect of EITC on *C. albicans* ATCC 90028 strain using the micro broth dilution method. For this purpose, we have exposed *C. albicans* cells to a series of concentrations of EITC in the range of 0.0039 to 2 mg/ml for 48hrs. After 48 hrs. 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 respectively were seen **(Fig. 5.4.1A).** Exposure to EITC affected the viability of *C. albicans* cells. EITC was fungicidal at 2 mg/ml concentration. 99 % of the killing of *C. albicans* cells was observed on the YPD plate **(Fig. 5.4.1B).**

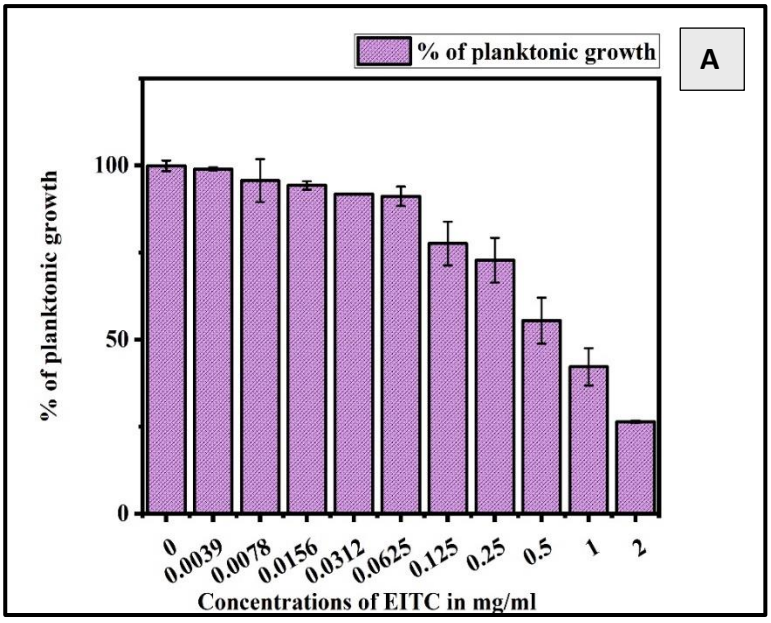


Fig. 5.4.1A: Antifungal activity of *Ethyl Isothiocyanate* against planktonic growth of *C. albicans.* the planktonic growth of *C. albicans* 90028 strain treated by various concentrations of *Ethyl Isothiocyanate* (0.0039 to 2 mg/ml) and the growth of *C. albicans* measured by spectrophotometer.

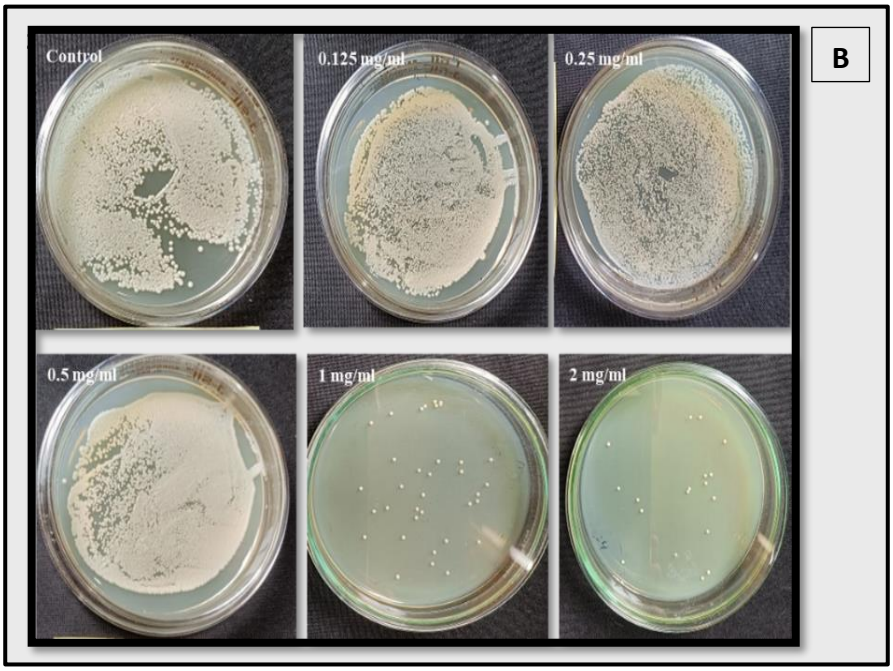


Fig. 5.4.1B: Candidacidal activity of *Ethyl Isothiocyanate* using a minimum fungicidal concentration (MFC) assay. The *C. albicans* 90028 strain was treated with different concentrations of *Ethyl Isothiocyanate*. The significant Candidacidal activity of *Ethyl Isothiocyanate* was seen at 2 mg/ml concentration.

5.4.2. EITC inhibited Yeast to Hyphal Morphogenesis in *C. albicans.*

The yeast-to-hyphal form morphogenesis plays a vital role in the pathogenesis of *C. albicans* infections and is considered as an important virulent trait of *C. albicans.* Therefore, we have assessed the effects of EITC on yeast-hyphal morphogenesis by using a 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. Scanning Electron Microscopy (SEM) analysis was performed to confirm the inhibition of Y-H morphology of *C. albicans* cells by the treatment of EITC. SEM analysis revealed that

EITC inhibited the yeast-hyphal morphogenesis in *C. albicans* cells at 0.0312 mg/ml **(Fig. 5.4.2.).**

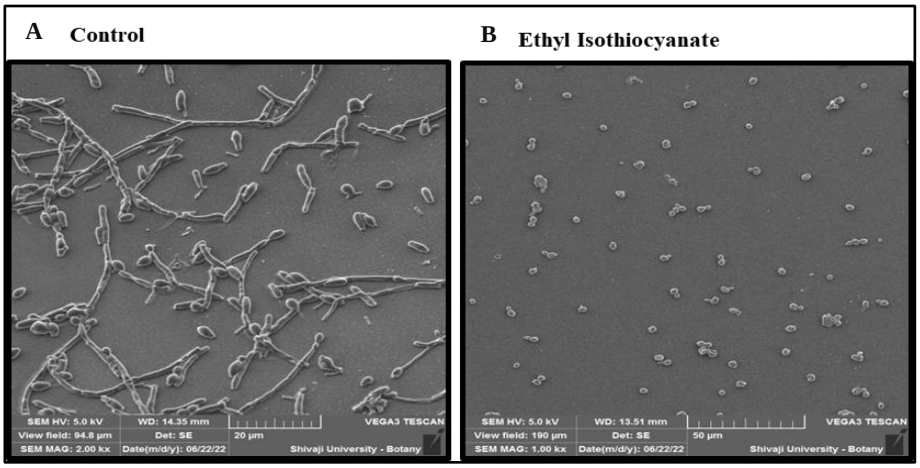


Fig. 5.4.2: The treatment of *Ethyl Isothiocyanate* significantly inhibits yeast to hyphal morphogenesis in *C. albicans* analysed with the help of scanning electron microscopy (SEM) **(A.)** *C. albicans* cells without treatment of *Ethyl Isothiocyanate* at 2.00 kx magnification, and **(B.)** *C. albicans* cells with the treatment of 0.0312 mg/ml of *Ethyl Isothiocyanate* at 1.00 kx magnification.

5.4.3. Inhibition of Adhesion by EITC

EITC inhibited of adhesion *C. albicans* cells to polystyrene surface We examined the effect of EITC on the adhesion of *C. albicans* to the 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 50 % viability of adhered cells to the polystyrene surface **(Fig. 5.4.3.).** Treatment above 0.0312 mg/ml concentration inhibited approximately 60 - 75 % of the adhesion compared to the non-treated control groups. From the above observation, it is concluded that EITC inhibits the adhesion of *C. albicans* cells to the polystyrene surface.

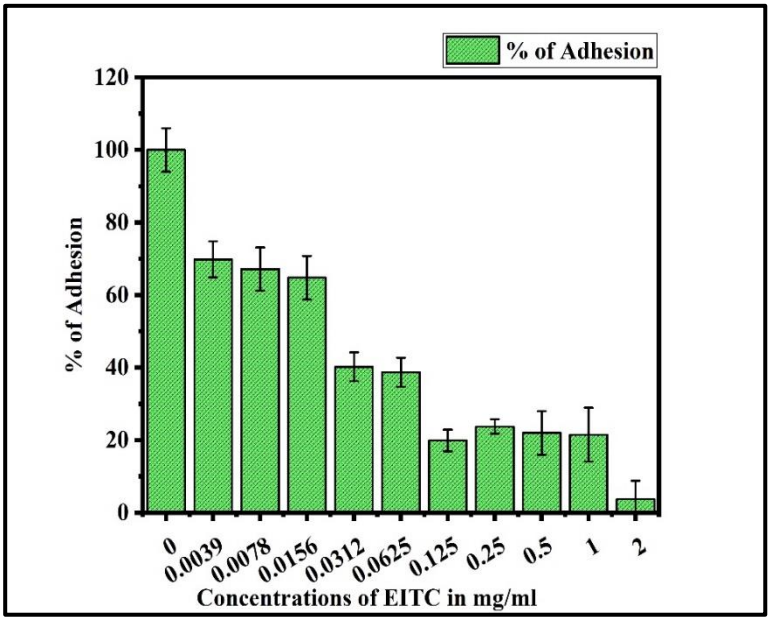


Fig. 5.4.3: XTT metabolic analysis-based polystyrene adhesion assay to evaluate the effect of *Ethyl Isothiocyanate* on *C. albicans* adherence. *C. albicans* cells were exposed to 0.0039 to 2 mg/ml concentrations of *Ethyl Isothiocyanate* for 90 min. at 37 °C. Control bar (0) indicates untreated cells, accepted as 100 % growth.

5.4.4. Inhibition of early and mature biofilm of *C. albicans* by EITC

The anti-biofilm ability of EITC at different concentration ranges between 0.0039 to 2 mg/ml was analyzed by XTT metabolic assay. As shown in **(Fig. 5.4.4A)** and **(Fig. 5.4.4B),** formation of early and mature biofilm was reduced by 50% at 2 mg/ml and 0.5 mg/ml concentrations respectively as compared to non-treated control.

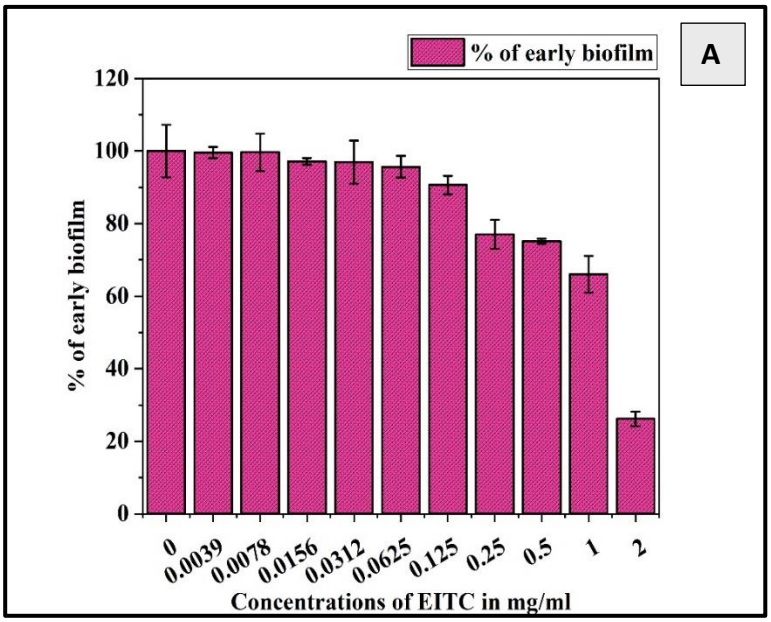


Fig. 5.4.4A: *C. albicans* cells were treated with 0.0039 to 2 mg/ml concentration of *Ethyl Isothiocyanate* during early biofilm, has shown concentration dependent inhibition of biofilm development quantified by XTT assay.

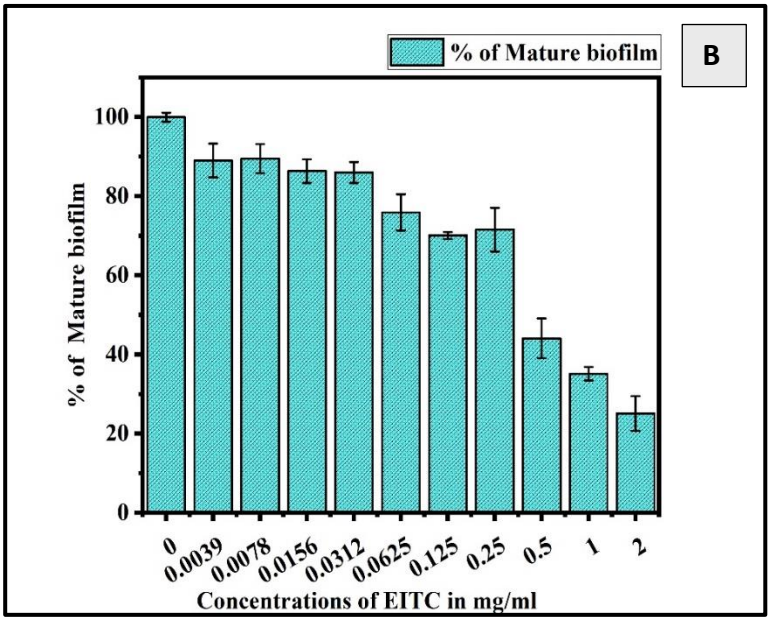


Fig. 5.4.4B: The inhibitory effect of *Ethyl Isothiocyanate* against mature biofilm formation of *C. albicans*. Cells were treated with concentrations ranging from 0.0039 to 2 mg/ml of EITC during biofilm development, revealing concentration-dependent inhibition of biofilm formation quantified by XTT assay.

5.4.5. Hemolytic activity of EITC

A hemolytic analysis study was performed to assess the toxicity of EITC on human RBCSs. EITC exhibited 19 % hemolysis at 0.5 mg/ml concentration and at 0.0156, 0.0078, and 0.0039 mg/ml concentrations it showed 8 %, 5 %, and 5 % hemolysis, respectively **(Fig. 5.4.5).** This indicates that EITC may be less toxic at lower concentrations and more toxic at higher concentration.

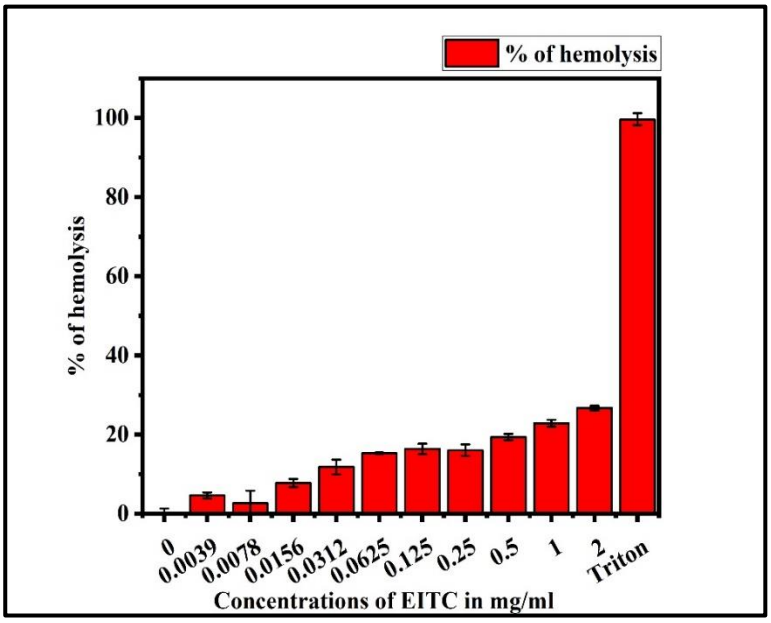


Fig. 5.4.5: Hemolysis assay of *Ethyl Isothiocyanate*, including triton 100 X as positive control and 0 as negative control. The relative rate of hemolysis in human erythrocytes was measured following a 1h incubation with concentrations ranging from 0.0039 to 2 mg/ml of *Ethyl Isothiocyanate* at 37°C.

5.5 Discussion

Antifungal activity of EITC is reported against *Fusarium oxysporum, Sclerotinia sclerotorium, Geotrichum citriaurantii, Aspergillus niger, Rizoctonia solani, Botrytis cinerea and Penicillium expansum*. In the present study, we have examined the antifungal efficacy of EITC against *C. albicans* growth and virulence factors. EITC inhibited planktonic growth at 0.5 mg/ml concentration **(Fig. 5.4.1A).** Also, it was found that EITC was fungicidal at 2 mg/ml **(Fig. 5.4.1B).** Yeast to hyphal (Y–H) morphogenesis plays an important role in virulence of *C. albicans* as it plays major role in tissue invasion and systematic infection. Present study showed that EITC inhibited Y–H morphogenesis at concentration i.e. 0.0312 mg/ml. The scanning electron microscopy confirmed that EITC inhibits yeast to hyphal form morphogenesis of *C. albicans* **(Fig. 5.4.2.).** 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 **[14].** Adhesion plays important role in colonization, biofilm formation and multidrug resistance inhibiting adhesion ultimately leads to prevention of biofilm formation. EITC inhibits adhesion to

polystyrene surface at 0.0312 mg/ml concentration **(Fig. 5.4.3).** *C. albicans* biofilm-related infections are became difficult to treat as it shows resistance to standard antifungals as well as they show side effects on prolonged use so now a day's researchers focus on natural products that specifically inhibits biofilm formation **[15,16].** EITC inhibited early and mature biofilm formed by *C. albicans* at 2 mg/ml and 0.5 mg/ml concentrations, respectively **(Fig. 5.4.4A and 5.4.4B)** Haemolytic activity of EITC was analysed for further therapeutic application. EITC shows 19% haemolysis at 0.5 mg/ml concentration while at lower concentrations it is less haemolytic **(Fig. 5.4.5).**

5.6 Conclusions

The 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 at 0.5 mg/ml concentration and virulence factors like Y–H morphogenesis (at 0.0312 mg/ml), adhesion (0.0312 mg/ml) to polystyrene surface, and biofilm formation (early biofilm at 2 mg/ml and mature biofilm at 0.5 mg/ml concentration).

5.7 References

1. Mayer FL, Wilson D, Hube B. *C. albicans* pathogenicity mechanisms. Virulence. 2013; 4:119-28.
2. Talapko J, Juzbašić M, Matijević T, Pustijanac E, Bekić S, Kotris I, Škrlec I. *C. albicans*—the virulence factors and clinical manifestations of infection. Journal of Fungi. 2021; 7:79-86.
3. Kojic EM, Darouiche RO. *Candida* infections of medical devices. Clinical microbiology reviews. 2004 :255-67.
4. Privett BJ, Nutz ST, Schoenfisch MH. Efficacy of surface-generated nitric oxide against *C. albicans* adhesion and biofilm formation. Biofouling. 2010; 26:973-83.
5. Campbell BC, Chan KL, Kim JH. Chemosensitization as a means to augment commercial antifungal agents. Frontiers in microbiology. 2012; 29; 3:79-99.
6. Scorzoni L, de Paula e Silva AC, Marcos CM, Assato PA, de Melo WC, de Oliveira HC, Costa-Orlandi CB, Mendes-Giannini MJ, Fusco-Almeida AM. Antifungal therapy: new advances in the understanding and treatment of mycosis. Frontiers in microbiology. 2017; 8:36-59.
7. Van Daele R, Spriet I, Wauters J, Maertens J, Mercier T, Van Hecke S, Brüggemann R. Antifungal drugs: what brings the future?. Medical Mycology. 2019; 57:328-43.
8. Thakre A, Zore G, Kodgire S, Kazi R, Mulange S, Patil R, Shelar A, Santhakumari B, Kulkarni M, Kharat K, Karuppayil SM. Limonene inhibits *C. albicans* growth by inducing apoptosis. Medical Mycology. 2018; 56:565-78.
9. Singla RK, Dubey AK. Molecules and metabolites from natural products as inhibitors of biofilm in *Candida* spp. pathogens. Current Topics in Medicinal Chemistry. 2019; 19:2567-78.
10. Le TN, Chiu CH, Hsieh PC. Bioactive compounds and bioactivities of Brassica oleracea L. var. italica sprouts and microgreens: An updated overview from a nutraceutical perspective. Plants. 2020; 9:946-70.
11. Kurt Ş, Güneş U, Soylu EM. In vitro and in vivo antifungal activity of synthetic pure *Isothiocynates* against Sclerotinia sclerotiorum. Pest Management Science. 2011; 67:869-75.

12. Smolinska U, Morra MJ, Knudsen GR, James RL. *Isothiocynates* produced by Brassicaceae species as inhibitors of Fusarium oxysporum. Plant disease. 2003; 87:407-12.
13. Plaszkó T, Szűcs Z, Vasas G, Gonda S. Effects of glucosinolate-derived *Isothiocynates* on fungi: A comprehensive review on direct effects, mechanisms, structure-activity relationship data and possible agricultural applications. Journal of Fungi. 2021; 7:539-78.
14. Park YK, Shin J, Lee HY, Kim HD, Kim J. Inhibition of Ras1-MAPK pathways for hypha formation by novel drug Candidates in *C. albicans*. bioRxiv.org:2021:1-44.
15. Shinde RB, Raut JS, Karuppayil MS. Biofilm formation by *C. albicans* on various prosthetic materials and its fluconazole sensitivity: a kinetic study. Mycoscience. 2012; 53:220-6.
16. Cavalheiro M, Teixeira MC. *Candida* biofilms: threats, challenges, and promising strategies. Frontiers in medicine. 2018; 5:28-42.

CHAPTER – VI

Study of Benzyl Isothiocyanate (BITC) on *Candida albicans*

CHAPTER – VI

Study of Benzyl Isothiocyanate (BITC) on *Candida albicans*

6. Introduction:

Candida albicans, a commensal fungus residing in the human microbiota, can turn pathogenic, causing a range of infections from mucosal to life-threatening systemic diseases, particularly in immunocompromised individuals **[1].** With the escalating incidence of candidiasis and the emergence of drug-resistant strains, there is a pressing need to explore novel antifungal agents with enhanced efficacy and reduced risk of resistance development **[2].** The surge in antifungal resistance and the limitations of existing treatments underscore the urgency to explore novel therapeutic strategies.

In this context, *Benzyl Isothiocyanate* (BITC), a naturally occurring compound found in cruciferous vegetables, has garnered attention for its potential in combating *Candida* infections. In this context, *Benzyl Isothiocyanate* (BITC), a naturally occurring compound derived from cruciferous vegetables, has emerged as a promising Candidate due to its multifaceted anti-*Candida* properties.

BITC has been extensively studied for its broad-spectrum antimicrobial activity, exhibiting inhibitory effects against various bacterial and fungal pathogens **[3].**

Recent research has shed light on the specific anti-*Candida* potential of BITC, particularly in inhibiting *C. albicans* morphogenesis, a key virulence factor. Notably, BITC demonstrates efficacy at concentrations lower than those required for growth inhibition, showcasing its potential to modulate virulence without exerting significant selective pressure. The ability of *C. albicans* to transition between yeast and filamentous forms is integral to its pathogenicity. BITC's capacity to hinder this morphological shift holds promise in restricting the pathogen and mitigating the risk of drug-resistant microbial populations. Beyond morphogenesis, BITC exhibits a remarkable ability to disrupt key virulence factors associated with biofilm formation, a critical aspect of *C. albicans* pathogenicity **[4,5].**

Biofilm formation is a protective strategy employed by *C. albicans*, rendering traditional antifungal drugs, such as fluconazole, less effective **[6].** The insidious nature of biofilms contributes to persistent infections and increases the likelihood of resistance development. In this context, BITC presents a compelling solution by significantly reducing the adherence of *C. albicans* cells to surfaces and inhibiting biofilm formation in a concentration-dependent manner **[7].**

The rising prevalence of antifungal resistance and the limitations of current therapeutic options necessitate the exploration of alternative compounds with diverse mechanisms of action. BITCs ability to target multiple facets of *C. albicans* pathogenicity positions it as a promising Candidate for further study. Understanding the underlying mechanisms of BITCs anti-*Candida* activity is crucial for developing targeted and effective antifungal strategies. Moreover, BITC's impact on the yeast-to-filamentous conversion, pivotal in biofilm network establishment, highlights its potential as a disruptive force against mature biofilms. The observation that mature biofilms of *C. albicans* exhibit heightened sensitivity to BITC underscores its efficacy in combating established infections **[8].**

This chapter provides a glimpse into the multifaceted anti-*Candida* properties of BITC, emphasizing its potential to address crucial aspects of *C. albicans* pathogenicity. It will delve into the mechanistic insights, experimental findings, and implications of BITC as a novel antifungal agent, with a focus on its ability to modulate virulence factors and counteract biofilm formation.

6.1 *Benzyl Isothiocyanate*

Benzyl Isothiocyanate (BITC) is an *Isothiocyanate* and a member of benzenes. It has a role as an antibacterial drug. It can be found in *Alliaria petiolata*, pilu oil, and papaya seeds where it is the main product of the glucotropaeolin breakdown by the enzyme myrosinase. BITC, and other *Isothiocynates* in general, were found to be protective against pancreatic carcinogenesis *in vitro* via expression of the *p21/WAF1* gene. A recently published study showed its restraining impact on obesity, fatty liver, and insulin resistance in diet-induced obesity mouse model **[9].** The physical properties are shown in **(Table 6.1).**

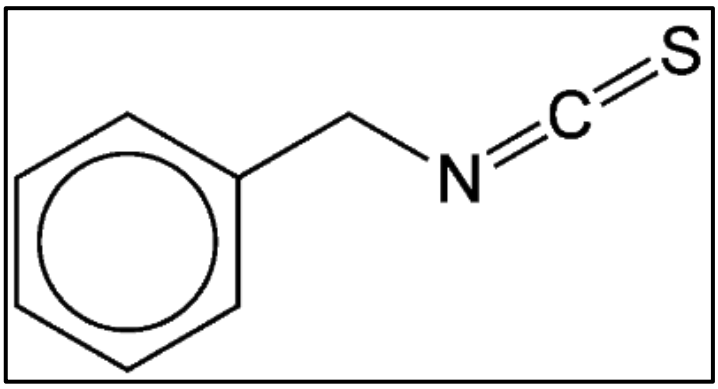


Image 6. 1. Molecular structure of *Benzyl Isothiocyanate*.

Formula: C_8H_7NS **Molecular weight:** 149.21 g/mol

Table 6.1: Physical properties of *Benzyl Isothiocyanate*

Sr.	Particulars	Observations
1	Physical state	Colorless to pale yellow liquid
2	Colour	Colorless
3	Odour	Watercress-like odor
4	Melting point	Melting point 41 °C (lit.)
5	Boiling point	242-243 °C (lit.)
6	Initial boiling point	242-243 °C (lit.)
7	Density	1.125 g/mL at 25 °C (lit.)
8	Solubility	Insoluble in water & Soluble in DMSO, and Ethanol

6.1.1 Biosynthesis and biological functions:

BITC is a natural product found in the Eastern Hemisphere mustard plant *Alliaria petiolata*, seeds of the pilu tree (*Salvadora persica*), and papaya (*Carica papaya*) seeds. It is a colorless to pale yellow liquid with a boiling point of 242–243°C and a characteristic watercress-like odor. In 2006, there was a report that *Isothiocynates*, including *Benzyl Isothiocyanate*, protected against pancreatic carcinogenesis in vitro. *Benzyl Isothiocyanate* is a naturally occurring constituent of cruciferous vegetables. It has antibacterial properties and its metabolism in man has been investigated. It inhibits chemically induced cancer in animal models. *Benzyl Isothiocyanate*has demonstrated anti-cancer properties, inhibiting the growth of various cancer cell lines **[10].** Studies have indicated that BITC possesses anti-inflammatory properties, suggesting its potential use in pharmaceuticals **[11].**

6.1.2 Commercial and other applications:

BITC is a compound that has been studied for various commercial and medicinal applications. Here are some commercial and other applications of *Benzyl Isothiocyanate* :

1) Food Preservation:
   BITC has shown potential as a natural food preservative due to its antimicrobial properties **[12].**
2) Insecticidal Properties:
   BITC has been investigated for its insecticidal activity, suggesting its potential as a natural insecticide **[13].**
3) Flavor and Fragrance Industry:
   BITC may have applications in the flavor and fragrance industry due to its distinctive odor and taste **[14].**

6.2 Materials and Methods

The material and methods followed are given in Chapter 3.

6.3 Results

6.3.1 Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration of *Benzyl Isothiocyanate* (BITC)

In the experiment, *C. albicans* cells were treated with a series of concentrations (0.0039 to 2 mg/ml) of BITC. *Benzyl Isothiocyanate* produced a strong and dose-dependent inhibitory effect on *C. albicans* with MIC_{50} and MFC values of 0.125 mg/ml and 0.25 mg/ml, respectively **(Fig 6.3.1A and 6.3.2B).**

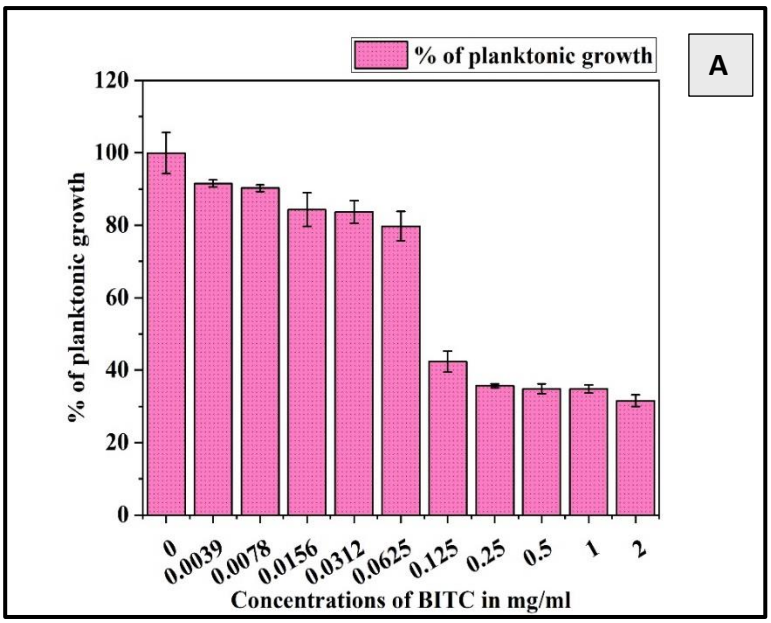


Fig 6.3.1A: Growth curve of *C. albicans* planktonic cells treated with *Benzyl Isothiocyanate* . Planktonic cells were co-incubated with various concentrations (0.0039 to 2 mg/ml) of *Benzyl Isothiocyanate* for 48 hrs. The graph shows that *Benzyl Isothiocyanate* (0.125 mg/ml) significantly inhibits the growth of *C. albicans*.

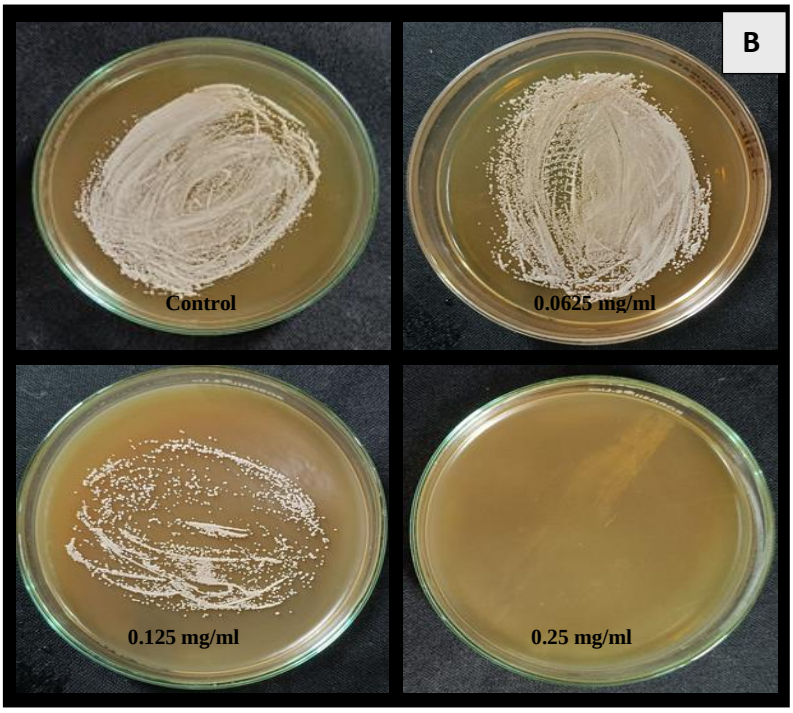


Fig. 6.3.1B: Minimum Fungicidal Concentration of *Benzyl Isothiocyanate* against *C. albicans* obtained by counting the colonies grown on YPD agar plate after performing planktonic growth assay. Minimum fungicidal concentration is achieved at 0.25 mg/ml concentration.

6.3.2 Inhibitory activity of *Benzyl Isothiocyanate* 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 *Benzyl Isothiocyanate* at a low concentration of 0.0312 mg/ml effectively inhibit transition from yeast to hyphae.

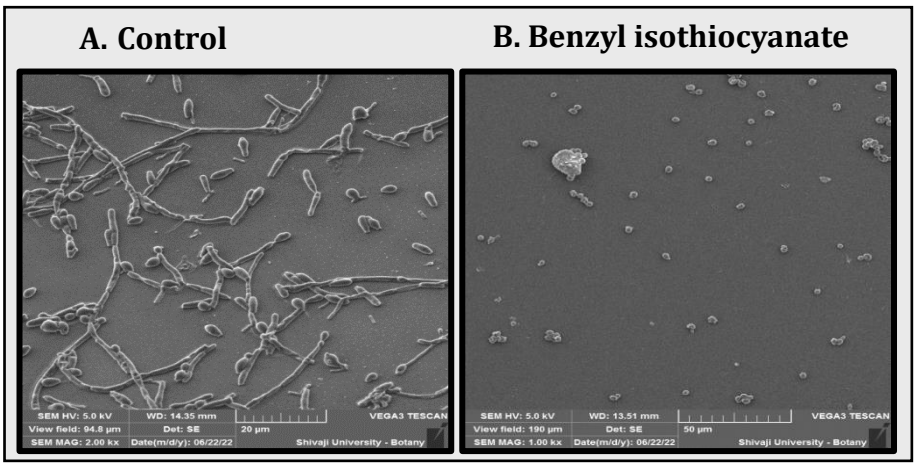


Fig. 6.3.2: The treatment of *Benzyl Isothiocyanate* inhibits yeast to hyphal morphogenesis in *C. albicans* analysed with the help of Scanning Electron Microscopy, A) *C. albicans* cells without treatment of *Benzyl Isothiocyanate*; B*) C. albicans* cells with the treatment of 0.0312 mg/ml *Benzyl Isothiocyanate*.

6.3.3 Inhibitory activity of *Benzyl Isothiocyanate* against adhesion to the polystyrene surface

The adherence of *C. albicans* cells to polystyrene was influenced by *Benzyl Isothiocyanate*. *C. albicans* cells were exposed to series of concentration of *Benzyl Isothiocyanate* in the range of 0.0039 to 2 mg/ml. Analyzing the density of adhered cells with XTT assay showed up to 65 % decrease in adhesion was seen at 0.5 mg/ml concentration. *Benzyl Isothiocynate s*ignificantly inhibited adhesion of cells to the solid surface **(Fig. 6.3.3).**

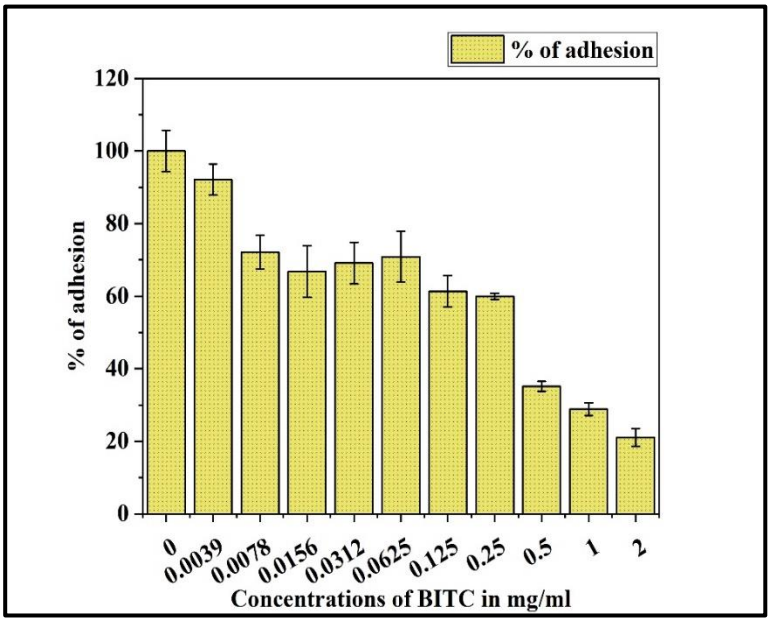


Fig. 6.3.3: The effect of *Benzyl Isothiocyanate* on the adhesion of *C. albicans* to polystyrene plates. *C. albicans* in PBS with 0.0039 to 2 mg/ml concentrations of *Benzyl Isothiocyanate* were added to 96-well plate and incubated at 37 °C for 90 min., followed by an XTT assay to assess the adhesion rate of the cells compared to the control group.

6.3.4 *Benzyl Isothiocyanate*potentially inhibits developing and mature biofilm.

Analysis of biofilm growth by using XTT-metabolic assay showed that the addition of *Benzyl Isothiocyanate* at a concentration range from 0.0039 to 2 mg/ml concentration prevented developing and mature biofilm formation by *C. albicans.* Treatment with a 2 mg/ml concentration of *Benzyl Isothiocyanate*caused a 61 % decrease in the developing biofilm growth of strain ATCC 90028 and at 0.25 mg/ml concentration, *Benzyl Isothiocyanate* inhibits mature biofilm, which was evident from notable (> 50 %) lowering in relative metabolic activity (RMA) analyzed by XTT assay, compared to that of control **(Fig.6.3.4A and Fig.6.3.4B).**

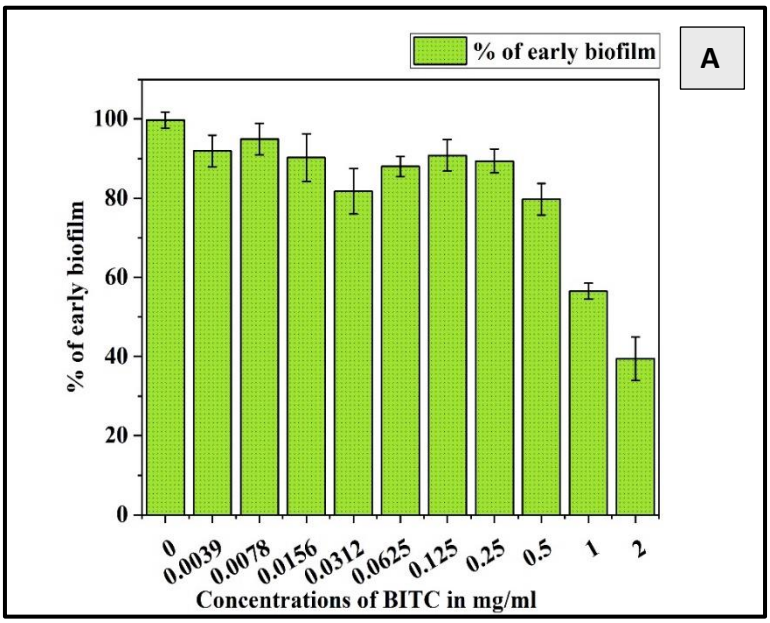


Fig. 6.3.4A: Anti-biofilm activity of *Benzyl Isothiocyanate* against early biofilm of *C. albicans*. Early biofilm of *C. albicans* were treated with *Benzyl Isothiocyanate* in 0.0039 to 2 mg/ml concentrations range and incubated at 48 h in 96 well polystyrene plate.

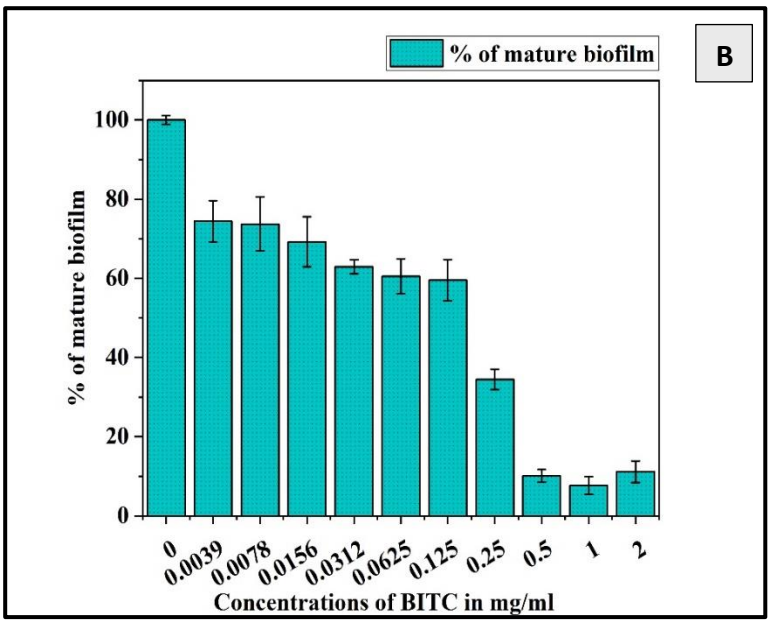


Fig. 6.3.4B: Anti-biofilm activity of *Benzyl Isothiocyanate* against mature biofilm of *C. albicans*. Mature biofilms of *C. albicans* were treated with *Benzyl Isothiocyanate* in 0.0039 to 2 mg/ml concentrations range for 48 h in 96 well polystyrene plate.

6.3.5 Toxicity analysis of *Benzyl Isothiocyanate*

The toxicity of *Benzyl Isothiocyanate* was analyzed by *in vitro* haemolytic activity on human RBCSs. It was observed that *Benzyl Isothiocyanate* was haemolytic in a concentration range from 0.0039 to 2 mg/ml. *Benzyl Isothiocynate* shows 13 % hemolysis at 2 mg/ml concentration.

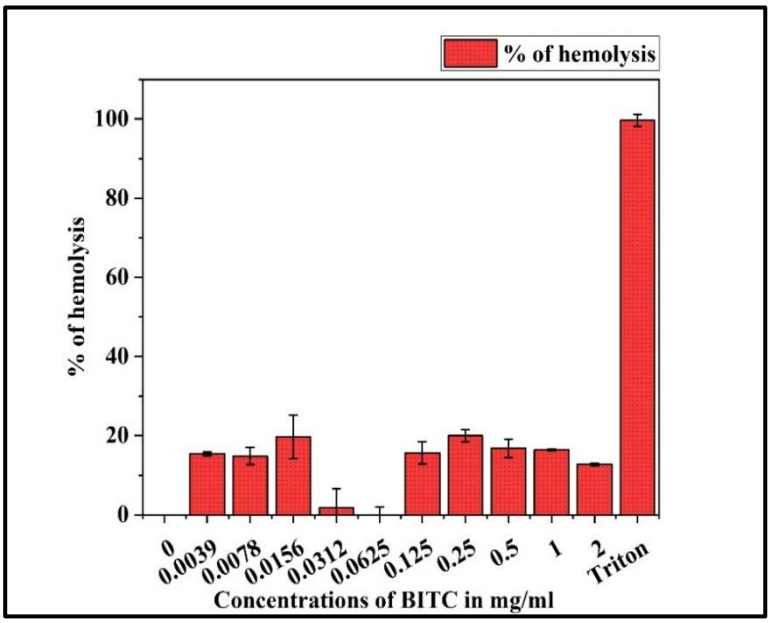


Fig. 6.3.5: Hemolysis assay of *Benzyl Isothiocyanate*, including triton 100 X as positive control and 0 as negative control. The relative rate of hemolysis in human erythrocytes was measured following a 1h incubation with concentrations ranging from 0.0039 to 2 mg/ml of *Benzyl Isothiocyanate* at 37°C.

6.4 Discussion

Benzyl Isothiocyanate results showed that it possesses significant anti-*Candida* activity at low concentrations. *Benzyl Isothiocyanate* was found to inhibit *C. albicans* morphogenesis, at concentrations lower than growth inhibitory concentrations. Our study elucidates BITC's proficiency in inhibiting *C. albicans* morphogenesis, a pivotal virulence factor associated with the transition from a commensal to a pathogenic state. The ability to inhibit virulence factors like morphogenesis may be used to restrict the pathogen so that natural selection and thereby emergence of drug-resistant population of microbes can be avoided. Adhesion of cells to solid surface and biofilm forms were found well tolerant to the most commonly prescribed drug, fluconazole. *Benzyl Isothiocyanate* reduced the number of cells adhering to the surface by 80 % at 2 mg/ml concentration. Biofilm formation is a central strategy employed by *C. albicans* for survival and evasion of host defenses. Our findings underscore BITC's potency in disrupting this protective shield. The compound significantly reduces cell adhesion to surfaces, a critical initial step in biofilm establishment. Furthermore, BITC exhibits concentration-dependent disruption of mature biofilms, presenting a dual-action potential in preventing biofilm formation and eradicating established biofilms. This may be due to the *Benzyl Isothiocyanate*-mediated inhibition of yeast to filamentous conversion which is important in the formation of a heterogeneous biofilm network. Mature biofilms of *C. albicans* were comparatively more sensitive to *Benzyl Isothiocyanate* .

A noteworthy aspect of our study is the observed efficacy of BITC against biofilms that exhibit tolerance to fluconazole, a commonly prescribed antifungal drug. This highlights BITC's potential as a valuable candidate for combination therapies, offering a solution to the challenges posed by drug-resistant strains and expanding the therapeutic arsenal against *Candida* infections. Our work gives insight into the development of *Benzyl Isothiocyanate* as a therapeutic strategy against *C. albicans*, especially biofilm-associated infections.

As we navigate the intricate landscape of antifungal research, the promising attributes of BITC beckon further exploration. Beyond the laboratory setting, clinical trials are warranted to validate the translational potential of BITC in human subjects.

6.5 Conclusions:

In conclusion our study demonstrates that *Benzyl Isothiocyanate* acts as a good antifungal agent and may be used as alternative therapeutic option for the treatment of candidiasis. *Benzyl Isothiocyanate* inhibit planktonic growth and is fungicidal in nature. Along with this *Benzyl Isothiocyanate* inhibits virulence factors like yeast to hyphal morphogenesis, adhesion to abiotic surface, early and mature biofilm.

6.6 References

1. Mayer FL, Wilson D, Hube B. *C. albicans* pathogenicity mechanisms. Virulence. 2013;15; 4:119-28.
2. Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. The global problem of antifungal resistance: prevalence, mechanisms, and management. The Lancet infectious diseases. 2017;1;17: 383-92.
3. Chen D, Chen T, Chen Y, Zhang Z, Li B, Tian S. Bio-source substances against postharvest diseases of fruits: mechanisms, applications and perspectives. Postharvest Biology and Technology. 2023; 198:112240-53.
4. Guo T, Noble W, Hanger DP. Roles of tau protein in health and disease. Acta neuropathologica. 2017; 133:665-704.
5. Pierce CG, Vila T, Romo JA, et al. The *C. albicans* biofilm matrix: composition, structure and function. J Fungi (Basel). 2015; 1:265-279.
6. Seneviratne CJ, Samaranayake LP, Jin L. Biofilm lifestyle of *Candida*: a mini review. Oral Dis. 2017; 23:841-844.
7. Almadiy AA, Nenaah GE, Shawer DM. Facile synthesis of silver nanoparticles using harmala alkaloids and their insecticidal and growth inhibitory activities against the khapra beetle. Journal of Pest Science. 2018; 91:727-37.
8. Desai JV, Mitchell AP. *C. albicans* biofilm development and its genetic control. Microbial Biofilms. 2015:99-114.
9. Alsanea S, Liu D. BITC and S-carvone restrain high-fat diet-induced obesity and ameliorate hepatic steatosis and insulin resistance. Pharmaceutical research. 2017;34: 2241-9.
10. Gupta P, Srivastava SK. Antitumor activity of *Benzyl Isothiocyanate*in anaplastic large cell lymphoma. Biochemical Pharmacology. 2012; 83:1376-1384.
11. Hamsa TP, Kuttan G. Anti-inflammatory activity of *Benzyl Isothiocyanate*in a murine model of peritonitis. Inflammation. 2013; 36:1304-1311.
12. Mau JL, Lin HC, Chen CC. Antimicrobial effect of extracts from Chinese chive, cinnamon, and corni fructus against foodborne pathogens. Journal of Agricultural and Food Chemistry. 2001; 49:183-188.

13. Nenaah G. Chemical composition, toxicity and growth inhibitory activities of essential oils of three Achillea species and their nanoemulsions against Tribolium castaneum (Herbst). Industrial Crops and Products. 2018; 122:612-623.
14. Parveen A, Subedi L, Kim HW, Khan Z, Zahra Z, Farooqi MQ, Kim SY. Phytochemicals targeting VEGF and VEGF-related multifactors as anticancer therapy. Journal of clinical medicine. 2019; 8:350-89.

CHAPTER – VII

Study of Phenylethyl Isothiocyanate (PEITC) on *Candida albicans*

CHAPTER – VII

Study of Phenylethyl Isothiocyanate (PEITC) on *Candida albicans*

7. Introduction:

C. albicans is a prominent opportunistic fungal pathogen responsible for a range of infections, from mucocutaneous to systemic diseases, particularly in immunocompromised individuals and those with underlying medical conditions **[1].** With the increasing prevalence of antifungal resistance and the limited armamentarium of effective antifungal drugs, there is a pressing need for the development of novel therapeutic strategies to combat *C. albicans* infections **[2].**

One promising avenue for antifungal drug development involves exploring natural compounds with potential antifungal properties. *Phenylethyl Isothiocyanate* (PEITC) is a natural compound derived from cruciferous vegetables such as watercress, broccoli, and Brussels sprouts **[3].** PEITC has garnered attention for its diverse pharmacological activities, including anticancer, antimicrobial, and anti-inflammatory effects **[4].** However, its potential as an antifungal agent against *C. albicans* remains relatively unexplored.

The pathogenicity of *C. albicans* is attributed to its ability to transition between yeast and hyphal forms, which facilitates tissue invasion, immune evasion, and biofilm formation **[5].** Hyphal development is a critical virulence factor associated with the pathogenesis of *C. albicans* infections, making it an attractive target for antifungal therapy **[6].** Additionally, adhesion to host surfaces and biofilm formation are pivotal in *C. albicans* colonization and persistence within host tissues, contributing to its virulence and resistance to antifungal therapy **[7].**

Given the multifactorial nature of *C. albicans* pathogenicity, targeting multiple virulence factors simultaneously presents a promising approach for antifungal drug development **[8].** Therefore, in this study, we aimed to investigate the antifungal activity of PEITC against *C. albicans* and its effects on key virulence factors, including hyphal formation, adhesion, and biofilm development.

The assessment of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) is crucial for determining the antifungal efficacy of PEITC against *C. albicans*. MIC represents the lowest concentration of an antifungal agent that inhibits visible fungal growth, while MFC denotes the lowest concentration that results in fungal

death **[9].** Furthermore, the inhibition of hyphal formation by PEITC could impede *C. albicans* virulence and pathogenesis, thereby attenuating its ability to cause invasive infections.

Adhesion to host surfaces is a prerequisite for *C. albicans* colonization and establishment of infection **[10].** Therefore, assessing the effect of PEITC on *C. albicans* adhesion could provide insights into its potential as an antifungal agent. Additionally, biofilm formation is a key virulence factor contributing to *C. albicans* persistence and resistance to antifungal therapy **[11].** Thus, evaluating the impact of PEITC on biofilm formation could offer valuable information regarding its therapeutic potential against biofilm-related infections.

While the antifungal activity of PEITC against *C. albicans* is of interest, it is essential to consider its potential cytotoxic effects on mammalian cells. Hemolytic activity assays provide insights into the cytotoxicity of PEITC towards human red blood cells (RBCSs), which could have implications for its safety profile and clinical utility **[12].**

This study aims to investigate the antifungal activity of PEITC against *C. albicans* and its effects on key virulence factors, including hyphal formation, adhesion, and biofilm development. Understanding the mechanisms underlying PEITC's antifungal activity and its impact on *C. albicans* pathogenicity could pave the way for the development of novel therapeutic strategies against *C. albicans* infections.

7.1 Introduction to *Phenylethyl Isothiocyanate* (PEITC)

Phenylethyl Isothiocyanate has been used in trials studying the prevention and treatment of leukemia, lung cancer, tobacco use disorder, and lymphoproliferative disorders.

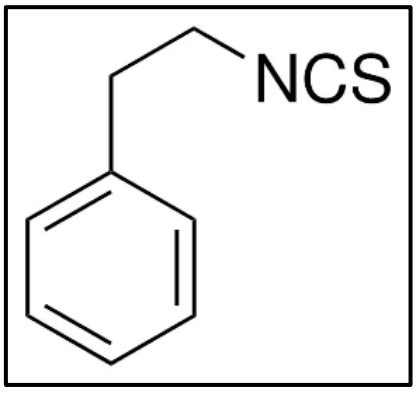


Image 7. 1. Molecular structure of *Phenylethyl Isothiocyanate*.

Formula: C_9H_9NS **Molecular weight:** 163.24 g/mol

Table 7.1: Physical properties of *Phenylethyl Isothiocyanate*

Sr. No	Particulars	Observations
1	Physical state	Liquid
2	Colour	Light yellow
3	Odour	Odourless
4	Melting point	61-63 °C (lit.)
5	Boiling point	217-219 °C (lit.)
6	Density	1.100 (lit.)
7	Solubility	Insoluble in water

PEITC is a bioactive compound involved in several biological mechanisms that are naturally related to protecting the plant against external factors. The plant produces PEITC in response to specific stress situations, since it presents biocidal activity against various pathogens, such as bacteria, fungi, insects, and other biotic stressors. However, the physiological properties of PEITC are not limited to those exerted at the source of origin. In this way, PEITC can act in humans, combining a series of biological properties with antioxidant, anti-inflammatory, and anti-cancer action. PEITC's activity on the organism is justified by different bioactive mechanisms, namely, the generation of free radicals, reducing inflammation, and blocking the stages of carcinogenesis. PEITC is also known to inhibit cell proliferation arrest the cell cycle, reduce the expression of carcinogenesis, or even tumor suppression via apoptosis and autophagy induction. Since 2000, PEITC has been one of the main pure glucosinolate derivatives (9.1 %) used in clinical trials, particularly to study its anti-cancer effects.

Phenylethyl Isothiocyanate is an *Isothiocyanate* having a phenethyl group attached to nitrogen. It is a naturally occurring compound found in some cruciferous vegetables (e.g. watercress) and is known to possess anticancer properties.

7.2 Materials and Methods

Materials and methods followed are given in Chapter 3.

7.2.12 Statistical Analysis

The values mentioned were the means 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.

7.3 Results

7.3.1 Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration of *Phenylethyl Isothiocyanate* (PEITC)

Impact of PEITC on the growth of *C. albicans* was assessed using micro broth dilution method according to the guidelines of Clinical and Laboratory Standards Institute. The MIC_{50} of PEITC for *C. albicans* was found to be 1 mg/ml **(Fig. 7.3.1A).** The minimum fungicidal concentration of PEITC was assessed by spread plate technique on YPD plate with the help MIC_{50} concentration and results indicate that PEITC was fungicidal at its MIC_{50} and above concentrations **(Fig. 7.3.1B).**

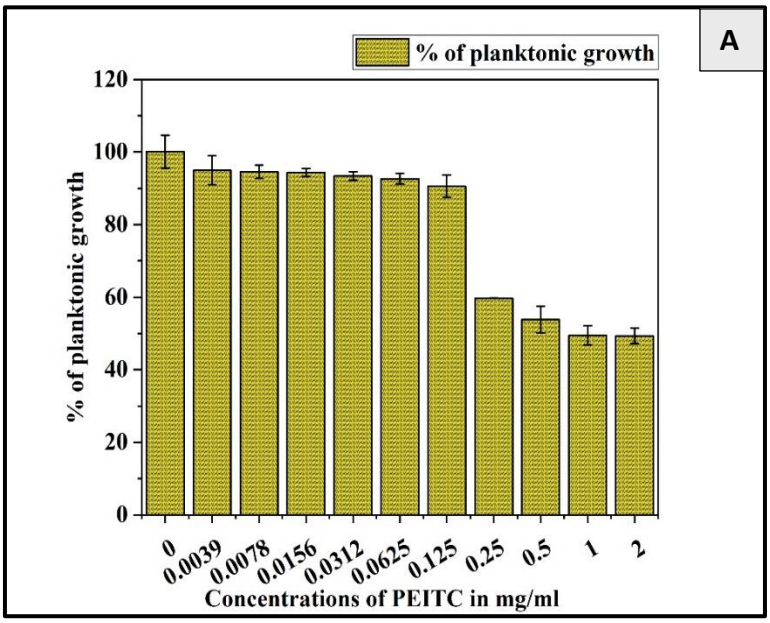


Fig. 7.3.1A: The inhibitory effects of series of concentrations ranges from 0.0039 to 2 mg/ml were evaluated on planktonic growth after 48 h incubation. Inhibition of *C. albicans* planktonic growth by *Phenylethyl Isothiocyanate* was observed at 1 mg/ml concentration.

..

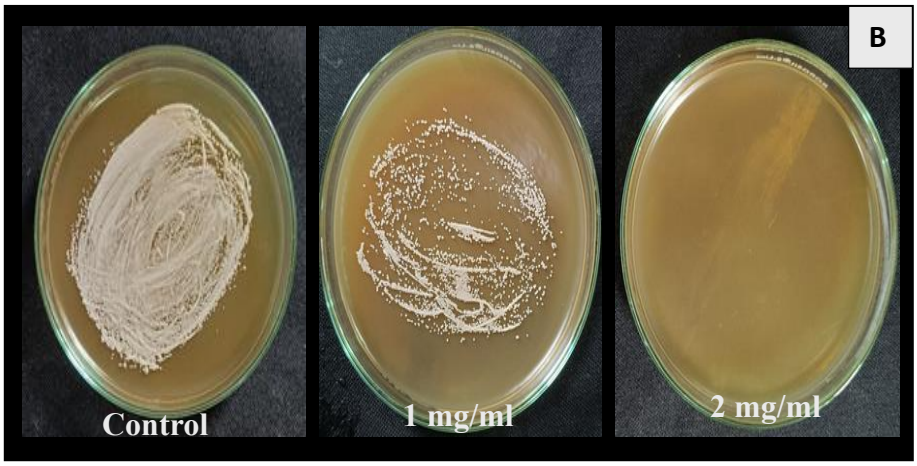


Fig. 7.3.1B: Minimum fungicidal concentration against *C. albicans* growth after the treatment of *Phenylethyl Isothiocyanate*. Plate based bioassay to determine minimum fungicidal concentration (MFC) in *C. albicans* 90028 strains.

7.3.2 Inhibitory activity of *Phenylethyl Isothiocyanate* against the transition of *C. albicans* from yeast to hyphae

Since hyphal development is the main pathogenic factor of *C. albicans.* We examined the effect of PEITC on hyphal formation *in vitro* by microscopy. PEITC effectively inhibited the morphological transformation from yeast to hyphae. In the present study, we observed that PEITC at a low concentration of 0.0312 mg/ml effectively inhibits transition from yeast to hyphae **(Fig. 7.3.2).**

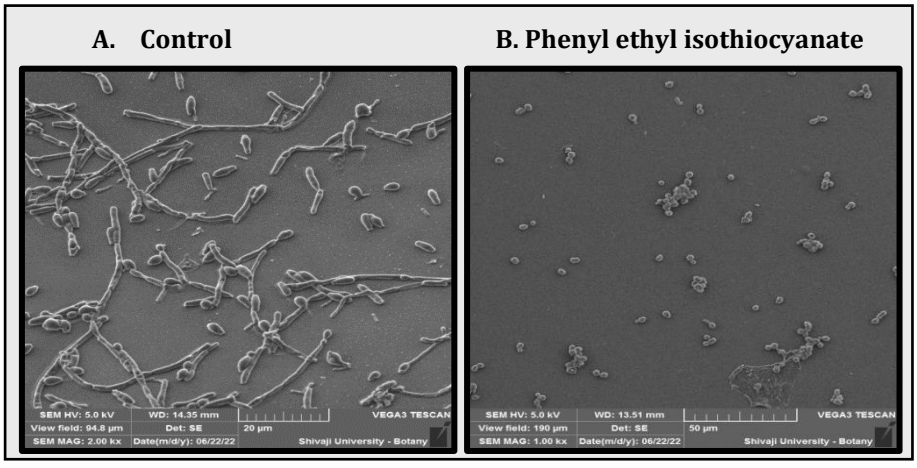


Fig. 7.3.2: The treatment of *Phenylethyl Isothiocyanate* inhibits yeast to hyphal morphogenesis in *C. albicans* analyzed with the help of Scanning Electron Microscopy, A) *C. albicans* cells without treatment of *Phenylethyl Isothiocyanate*; B) *C. albicans* cells with the treatment of 0.0312 mg/ml *Phenylethyl Isothiocyanate*.

7.3.3 Inhibitory activity of *Phenylethyl Isothiocyanate* against adhesion to the polystyrene surface

Adhesion ability is one of the primary independent factors contributing to the virulence of *C. albicans*. The effect of PEITC on the adhesion of *C. albicans* at the bottom of the 96-well plates was tested. 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. Analyzing the density of adhered cells with XTT assay showed up to 52 % decrease in adhesion was seen at 0.125 mg/ml concentration **(Fig. 7.3.3).**

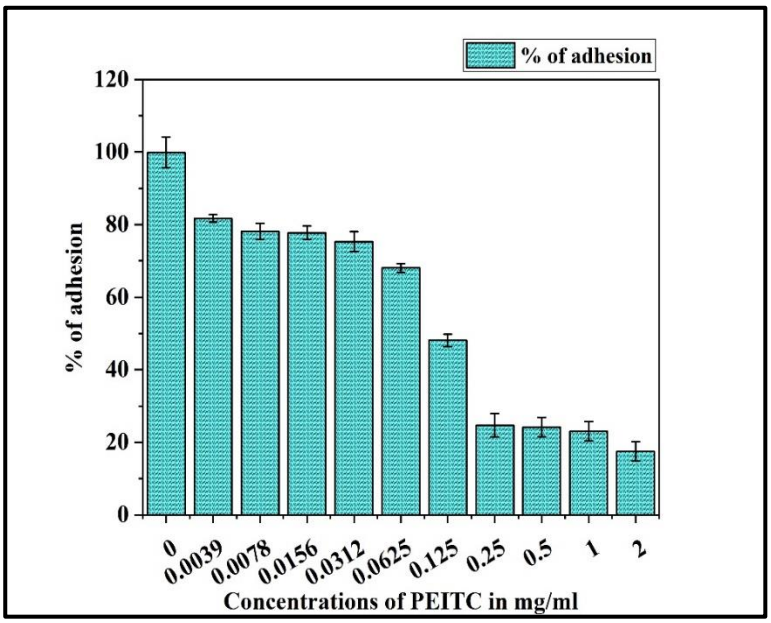


Fig. 7.3.3: Effect of *Phenylethyl Isothiocyanate* on adhesion of *C. albicans*. Cell adhesion to a polystyrene surface. *C. albicans* cells were incubated in phosphate buffered saline (PBS) in polystyrene wells for 90 min and then cells were detected by measuring the reduction of XTT against *C. albicans*.

7.3.4 *Phenylethyl Isothiocyanate* potentially inhibit early 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 examine how PEITC affects the production of biofilms growth by using XTT-metabolic assay showed that addition of PEITC at a concentration range from 0.0039 to 2 mg/ml concentration prevented

developing and mature biofilm formation by *C. albicans*. Treatment with 0.0312 mg/ml concentration of PEITC caused 50 % decrease in developing biofilm growth **(Fig.7.3.4)** of strain ATCC 90028 and at 2 mg/ml concentration of PEITC inhibits mature biofilm compared to that of control **(Fig. 7.3.5.).**

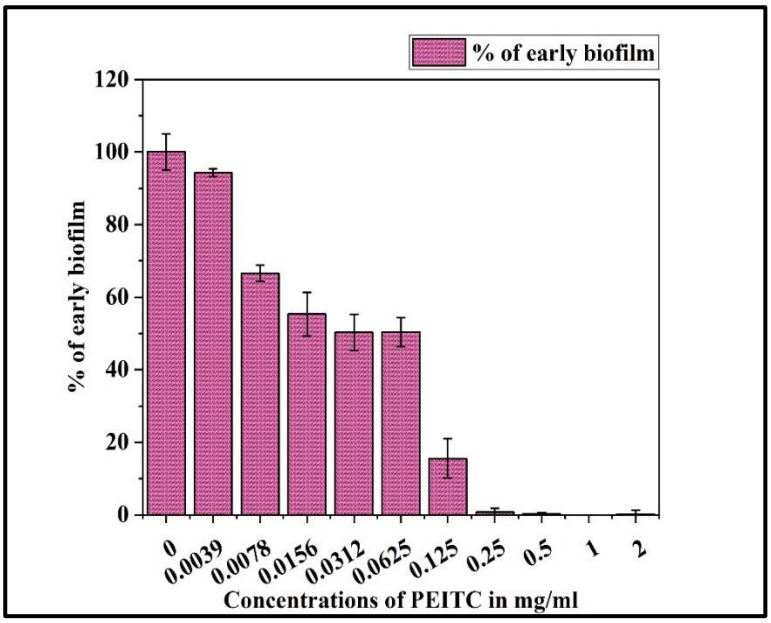


Fig. 7.3.4: Effect of *Phenylethyl Isothiocyanate* on metabolic activity of early biofilm. The inhibitory effects of *Phenylethyl Isothiocyanate* dilutions were evaluated on biofilms after a 48 h incubation with XTT metabolic effect of *Phenylethyl Isothiocyanate* on metabolic activity of early biofilm. The inhibitory effects of *Phenylethyl Isothiocyanate* dilutions were evaluated on biofilms after a 48 h incubation with XTT.

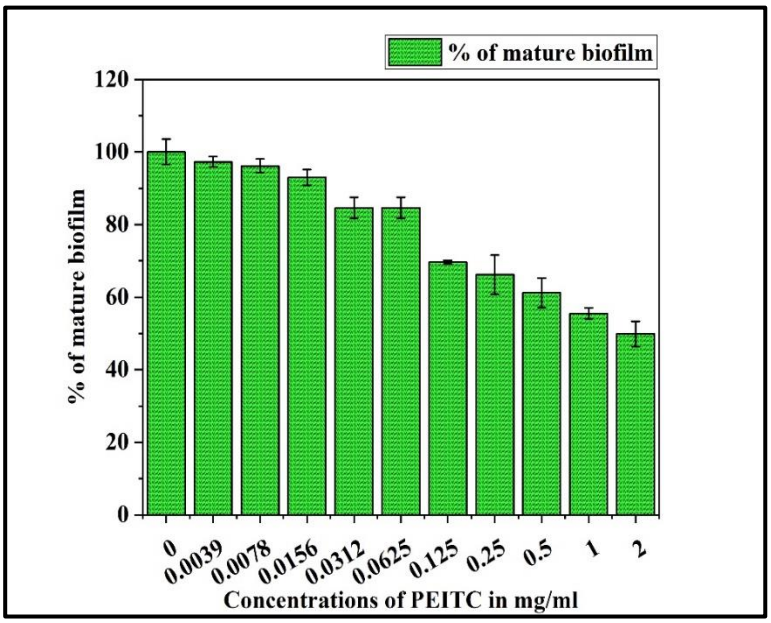


Fig. 7.3.5: Effect of *Phenylethyl Isothiocyanate* on metabolic activity of mature biofilm. The inhibitory effects of *Phenylethyl Isothiocyanate* dilutions were evaluated on biofilms after a 48 h incubation with XTT metabolic.

7.3.5 Toxicity analysis of *Phenylethyl Isothiocyanate*

The toxicity of PEITC was analysed by *in vitro* haemolytic activity on human RBCSs. It was observed that PEITC was haemolytic in nature in a concentration range from 0.0039 to 2 mg/ml **(Fig.7.3.6).**

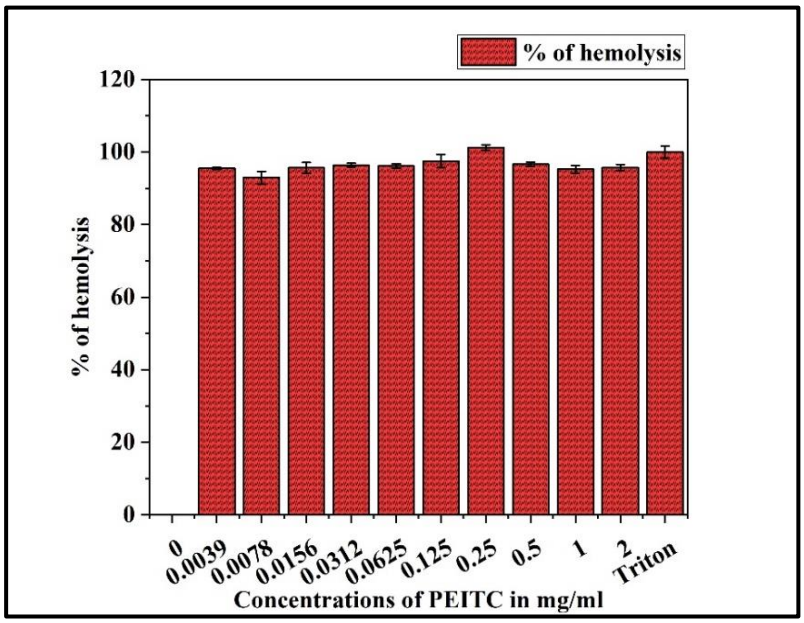


Fig. 7.3.6: Hemolysis assay of *Phenylethyl Isothiocyanate*, including triton 100 X as positive control and 0 as negative control. The relative rate of hemolysis in human erythrocytes was measured following a 1h incubation with concentrations ranging from 0.0039 to 2 mg/ml of *Phenylethyl Isothiocyanate* at 37°C.

7.4 Discussion

The impact of *Phenylethyl Isothiocyanate* (PEITC) on *C. albicans*, a significant opportunistic fungal pathogen, was investigated through a series of experiments. The MIC_{50} of PEITC against *C. albicans* was found to be 2 mg/ml, indicating its potent antifungal activity **(Fig. 7.3.1).** Moreover, the minimum fungicidal concentration of PEITC was determined to be at its MIC_{50} and above concentrations, affirming its fungicidal potential. Furthermore, PEITC effectively inhibited hyphal formation, a crucial pathogenic factor of *C. albicans*, even at low concentrations (0.0312 mg/ml).

This inhibitory effect on hyphal development could impede the virulence and pathogenesis of *C. albicans.*

Additionally, PEITC demonstrated significant effects on *C. albicans* adhesion, a key virulence determinant, with up to a 50 % reduction observed at 0.125 mg/ml concentration. This reduction in adhesion ability could potentially hinder the establishment of *C. albicans* infections. Furthermore, PEITC exhibited inhibitory effects on biofilm formation, a crucial virulence factor contributing to drug resistance and immune evasion. The prevention of both developing and mature biofilm formation, even at low concentrations, suggests the potential of PEITC as a therapeutic agent against biofilm-related infections.

However, it's worth noting that PEITC displayed haemolytic activity on human red blood cells (RBCSs) across the concentration range tested (0.0039 to 2 mg/ml). This hemolytic nature could pose a safety concern for its therapeutic use and warrants further investigation into its potential side effects. The findings of this study underscore the promising antifungal properties of PEITC against *C. albicans,* including inhibition of growth, hyphal formation, adhesion, and biofilm formation. However, further research is necessary to assess its safety profile and potential clinical applications.

7.5 Conclusions

In conclusion, the present study elucidates the multifaceted impact of *Phenylethyl Isothiocyanate* (PEITC) on *C. albicans*, a significant fungal pathogen implicated in various infections. The results demonstrate PEITC's potent antifungal activity, as evidenced by its low MIC_{50} and fungicidal effects against *C. albicans at* 1 mg/ml and 2 mg/ml concentrations respectively. Additionally, PEITC effectively inhibits key virulence factors of *C. albicans,* including hyphal formation (0.0312 mg/ml), adhesion (0.125 mg/ml), and biofilm (Early biofilm at 0.0312 mg/ml and mature biofilm at 2 mg/ml concentrations), thus attenuating its pathogenicity. However, the observed haemolytic activity on human red blood cells raises concerns regarding PEITC's safety profile for potential therapeutic use. Further investigations are warranted to assess the toxicity and side effects of PEITC, as well as its efficacy *in vivo*.

Overall, the findings suggest that PEITC holds promise as a Candidate for the development of novel antifungal agents targeting *C. albicans* infections. However, careful evaluation of its safety and efficacy in clinical settings is essential before its translation into therapeutic applications. Further research exploring the mechanistic basis of PEITC's antifungal activity and its potential synergistic effects with existing antifungal agents could provide valuable insights into its therapeutic utility against *C. albicans* and other fungal pathogens. This study contributes to our understanding of PEITC as a potential antifungal agent and highlights avenues for future research aimed at harnessing its therapeutic potential while mitigating potential risks.

7.6 References:

1. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. Hidden killers: human fungal infections. Science Translational Medicine. 2012; 4:165-78.
2. Perfect JR. The antifungal pipeline: a reality check. Nat Rev Drug Discovery. 2017; 16:603-616.
3. Gupta P, Wright SE, Kim SH, Srivastava SK. Phen*Ethyl Isothiocyanate*: a comprehensive review of anti-cancer mechanisms. Biochimica et Biophysica Acta Reviews on Cancer. 2014;1846(2):405-424.
4. Houghton CA, Fassett RG, Coombes JS. Sulforaphane: translational research from laboratory bench to Clinical Nutrition Review 2013; 71:709-726.
5. Sudbery PE. Growth of *C. albicans* hyphae. Nature Reviews Microbiology. 2011; 9:737-748.
6. Gow NA, Hube B. Importance of the *C. albicans* cell wall during commensalism and infection. Current Opinion in Microbiology. 2012; 15:406-412.
7. Nobile CJ, Johnson AD. *C. albicans* biofilms and human disease. Annual Review of Microbiology. 2015; 69:71-92.
8. Rybtke M, Hultqvist LD, Givskov M, Tolker-Nielsen T. Pseudomonas aeruginosa biofilm infections: community structure, antimicrobial tolerance and immune response. Journal of Molecular Biology. 2015;427 :3628-3645.
9. Espinel-Ingroff A, Diekema DJ, Fothergill A, et al. Wild-type MIC distributions and epidemiological cutoff values for amphotericin B, flucytosine, and itraconazole and *Candida* spp. as determined by CLSI broth microdilution. Journal of Clinical Microbiology. 2010; 48:2140-2146.
10. Fanning S, Mitchell AP. Fungal biofilms. PLOS Pathogens. 2012; 8:1002585-590.
11. Douglas LJ. *Candida* biofilms and their role in infection. Trends Microbiology. 2003; 11:30-36.
12. Ames BN, Dubin DT. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. Journal of Biological Chemistry. 1960; 235:769-775.

CHAPTER – VIII

Summary

CHAPTER – VIII

Summary

8. Summary:

The emergence of drug-resistant fungal pathogens poses a significant challenge to global public health, necessitating the exploration of novel antifungal agents with diverse mechanisms of action. In this study, we investigated the antifungal activity of *Isothiocynates*, specifically *Phenylethyl Isothiocyanate* (PEITC), *Benzyl Isothiocyanate* (BITC), *Ethyl Isothiocyanate* (EITC), and *Allyl Isothiocyanate* (AITC), against *C. albicans*, a prominent opportunistic fungal pathogen. Our findings underscore the potential of *Isothiocynates* as promising candidates for the development of alternative antifungal therapies. AITC exhibited concentration-dependent inhibition of *C. albicans* planktonic growth and biofilm formation. Notably, the combination of AITC with fluconazole (FLC) resulted in synergistic inhibition of biofilm formation, highlighting the therapeutic potential of combination therapy in combating fungal infections. Furthermore, AITC demonstrated no hemolytic activity, suggesting its safety for potential therapeutic use.

Similarly, EITC exhibited significant antifungal activity against *C. albicans*, inhibiting both planktonic growth and biofilm formation. The inhibitory effect of EITC on yeast-to-hyphal morphogenesis, a crucial virulence factor of *C. albicans*, underscores its potential as a therapeutic agent against invasive fungal infections. Moreover, EITC displayed minimal hemolytic activity, further supporting its *Candida*cy for therapeutic development. PEITC, another *Isothiocyanate* investigated in this study, demonstrated potent antifungal activity against *C. albicans*, inhibiting hyphal formation, adhesion, and biofilm development. The observed fungicidal activity of PEITC underscores its potential as an effective antifungal agent against *C. albicans* infections. However, the hemolytic activity of PEITC raises concerns regarding its safety profile, highlighting the need for further investigation into its potential adverse effects. *Benzyl Isothiocyanate* (BITC) showed strong antifungal activity against *C. albicans*, inhibiting both planktonic growth and biofilm formation. It also suppressed yeast-to-hyphal morphogenesis, a key virulence factor, with minimal hemolytic activity, suggesting its potential as a therapeutic agent.

The multifaceted antifungal mechanisms of *Isothiocynates* offer several advantages for therapeutic development. Their ability to inhibit key virulence factors of *C. albicans*, including hyphal formation, adhesion, and biofilm development, could

potentially attenuate the pathogenicity of this fungal pathogen and enhance the efficacy of existing antifungal therapies. Additionally, the synergistic effects observed with combination therapy highlight the potential of *Isothiocynates* as adjunctive agents in antifungal treatment regimens. However, several challenges and considerations must be addressed in the development of *Isothiocynates* as antifungal agents. Firstly, further investigations are warranted to elucidate the mechanisms underlying the antifungal activity of *Isothiocynates* and their potential interactions with existing antifungal drugs.

Additionally, comprehensive safety assessments, including in vivo studies and evaluation of potential side effects, are essential to determine the suitability of *Isothiocynates* for clinical use.

Sr. No.	Molecules	Planktonic Growth (mg/ml)	MFC Conc. (mg/ml)	Adhesion Assay (mg/ml)	Early biofilm (mg/ml)	Mature biofilm (mg/ml)	Hemolytic Assay
1.	***Allyl Isothiocyanate***	0.125	Fungicidal in nature at 0.25 mg/ml	0.125	0.5	0.5	No hemolysis
2.	***Ethyl Isothiocyanate***	0.5	Fungicidal in nature 1 mg/ml	0.0312	2	0.5	Hemolytic in nature
3.	***Benzyl Isothiocyanate***	0.125	Fungicidal in nature 0.25 mg/ml	0.5	1	0.25	Hemolytic in nature
4.	***Phenylethyl Isothiocyanate***	1	Fungicidal at 2 mg/ml	0.125	1	2	Hemolytic in nature

Table 8.1: Inhibitory Concentrations and Hemolytic Activities of *Isothiocyanate* Molecules.

In **Table 8.1** AITC exhibited higher activity as an anti-biofilm agent by inhibiting both early and mature biofilm formation at a concentration of 0.5 mg/ml, while EITC, BITC, and PEITC all showed a similar effect on Y-H morphogenesis in *C. albicans.* The natural compounds such as AITC and BITC show promising potential for the development of transformative antifungal interventions.

Moreover, the development of drug delivery systems to enhance the bioavailability and efficacy of *Isothiocynates* represents a promising avenue for future research. Nano formulations and encapsulation strategies could overcome the limitations associated with the poor solubility and stability of *Isothiocynates*, thereby facilitating their clinical translation. Our study highlights the potential of *Isothiocynates* as novel

antifungal agents against *C. albicans* infections. AITC, BITC, EITC, and PEITC demonstrate significant antifungal activity, inhibiting key.

Further investigation is imperative to unravel the mechanistic underpinnings, refine therapeutic formulations, and conduct comprehensive safety and efficacy assessments of *Isothiocynates*, notably including *Benzyl Isothiocyanate* (BITC), within clinical contexts. With sustained exploration and advancement, *Allyl Isothiocyanate*s emerge as promising alternative therapeutic modalities for combatting fungal infections, addressing the pressing demand for innovative antifungal strategies in clinical practice.

CHAPTER – IX

Conclusions

CHAPTER – IX

Conclusions

Conclusions:

The comprehensive exploration conducted across chapters 4 to 7 underscores the potential of *Isothiocynates* (ITCs) as promising candidates for combatting *C. albicans* infections. Through rigorous investigation into the antifungal activity of various ITCs, including *Ethyl Isothiocyanate* (EITC), *Allyl Isothiocyanate* (AITC), *Benzyl Isothiocyanate* (BITC) and *Phenylethyl Isothiocyanate* (PEITC), significant efficacy against *C. albicans* planktonic growth, virulence factors, and biofilm formation has been demonstrated. These findings contribute to the growing body of evidence supporting the therapeutic utility of ITCs in addressing the challenge of *C. albicans* infections.

AITC demonstrated concentration-dependent inhibition of *C. albicans* biofilm formation, with significant suppression observed at 1 mg/ml concentration. Moreover, the combination of AITC with fluconazole (FLC) exhibited synergistic effects in preventing both early and mature biofilm development, as evidenced by the fractional inhibitory concentration indices. Notably, AITC displayed no hemolytic activity alone or in combination with FLC, suggesting its safety profile for potential therapeutic applications. The MIC and MFC assays further confirmed AITC's antifungal activity, with a MIC of 0.125 mg/ml and fungicidal nature against *C. albicans.*

BITC looks promising as an antifungal against *C. albicans.* It stops the fungus from growing freely, reduces its harmful effects, and breaks down the sticky layers it forms. Even at low doses, BITC can tackle the fungus, including strains that don't respond well to typical treatments like fluconazole. This suggests BITC could be used alongside other treatments to fight stubborn fungal infections. But before that, we need *in vivo* studies and then clinical trials to confirm its antifungal efficacy.

Similarly, EITC demonstrated potent antifungal activity against *C. albicans,* inhibiting planktonic growth at 0.5 mg/ml concentration and exhibiting fungicidal effects at 2 mg/ml. Additionally, EITC effectively inhibited yeast to hyphal (Y–H) morphogenesis, a crucial virulence factor implicated in tissue invasion and systemic infection. Notably, EITC also hindered adhesion to polystyrene surfaces and inhibited early and mature biofilm formation by *C. albicans,* highlighting its potential as a multifaceted antifungal agent. However, hemolytic activity analysis revealed some

degree of toxicity at higher concentrations, suggesting the need for careful dose optimization.

Furthermore, PEITC demonstrated potent antifungal activity against *C. albicans*, with a MIC_{50} of 2 mg/ml and fungicidal effects at its MIC_{50} and above concentrations. PEITC effectively inhibited hyphal formation, adhesion, and biofilm formation by *C. albicans*, indicating its potential as a therapeutic agent against biofilm-related infections. However, the observed hemolytic activity across a range of concentrations raises concerns regarding its safety profile for clinical use, necessitating further investigation into potential side effects.

Collectively, these findings highlight the multifaceted antifungal properties of ITCs against *C. albicans*, targeting various stages of fungal growth, virulence, and biofilm formation. The synergistic effects observed with FLC underscore the potential for combination therapy to enhance antifungal efficacy and overcome resistance. However, further research is warranted to elucidate the underlying mechanisms of action, optimize dosing regimens, and assess safety profiles *in vivo*.

In conclusion, ITCs represent promising candidates for the development of novel antifungal agents targeting *C. albicans* infections. Their multifaceted mode of action, synergistic effects, and relatively low toxicity profiles make them attractive candidates for further exploration in preclinical and clinical studies. However, *Isothiocynates* (ITCs), like AITC, BITC, EITC, and PEITC, have strong abilities to inhibit the growth and virulence factors of *C. albicans.* AITC can inhibit biofilm formation when it is used with fluconazole, a common antifungal medicine. BITC works even against *C. albicans* that resist fluconazole. EITC and PEITC also inhibit growth and adhesion capacity of *C. albicans*. When these ITCs are combined with fluconazole, they work together even better. However, one of the ITCs, *Phenethyl Isothiocyanate*, might cause problems at certain levels, so more research is needed to be sure it's safe to use. Overall, the study suggests that these compounds could help fight fungal infections, but we still need to learn more about how they work and how to use them safely in real-life treatments.

CHAPTER – X

Recommendations

CHAPTER – X

Recommendations

Recommendations:

The comprehensive findings presented in this study provide valuable insights into the potential of *Isothiocynates* (ITCs), specifically the Anti-*Candida* activity of *Allyl Isothiocyanate*s (AITC), *Ethyl Isothiocyanate* (EITC), and *Phenylethyl Isothiocyanate* (PEITC), as promising Candidates for combating *C. albicans* infections. Building upon these findings, several recommendations emerge that could guide future research and clinical applications in the field of antifungal therapy.

1. Mechanistic Studies and comparison with standard antifungal drugs:

Further elucidation of the underlying mechanisms of action of ITCs against *C. albicans* is essential to enhance our understanding of their antifungal activity. Investigating the molecular targets and pathways modulated by ITCs could provide insights into their mode of action, synergistic interactions with conventional antifungal agents, and potential mechanisms of resistance. Techniques such as transcriptomics, proteomics, and metabolomics could be employed to unravel the complex interactions between ITCs and *C. albicans.* Further, antifungal efficacy can be compared with standard antifungal drugs like fluconazole and Amphotericin B.

2. Optimization of Formulations:

Optimization of ITC formulations, including encapsulation strategies, nanoformulations, and prodrug approaches, could enhance their stability, bioavailability, and efficacy. Encapsulation of ITCs within biocompatible carriers such as liposomes, nanoparticles, or micelles could improve their solubility, targeted delivery, and pharmacokinetic profiles, thereby enhancing their therapeutic potential and reducing systemic toxicity.

3. Combination Therapy:

Exploring the synergistic interactions between ITCs and conventional antifungal agents, such as azoles, echinocandins, and polyenes, holds promise for overcoming drug resistance and improving treatment outcomes. Further investigations into the optimal combinations, dosing regimens, and mechanisms underlying synergism could inform the development of effective combination therapies against *C. albicans* infections.

4. Preclinical and Clinical Studies:

Translation of preclinical findings into clinical applications requires rigorous evaluation of the safety, efficacy, and pharmacokinetics of ITCs in relevant animal models and human subjects. Conducting well-designed preclinical studies, including pharmacokinetic, pharmacodynamic, and toxicity assessments, is crucial to inform dose selection, treatment regimens, and potential adverse effects in clinical trials.

5. Assessment of Safety Profiles:

Comprehensive evaluation of the safety profiles of ITCs, including acute and chronic toxicity, genotoxicity, and immunotoxicity, is imperative to assess their suitability for clinical use. Conducting in vivo studies in animal models and ex vivo assays using human cells and tissues can provide valuable insights into the potential adverse effects and safety margins of ITC-based therapies.

6. Exploration of Structure-Activity Relationships:

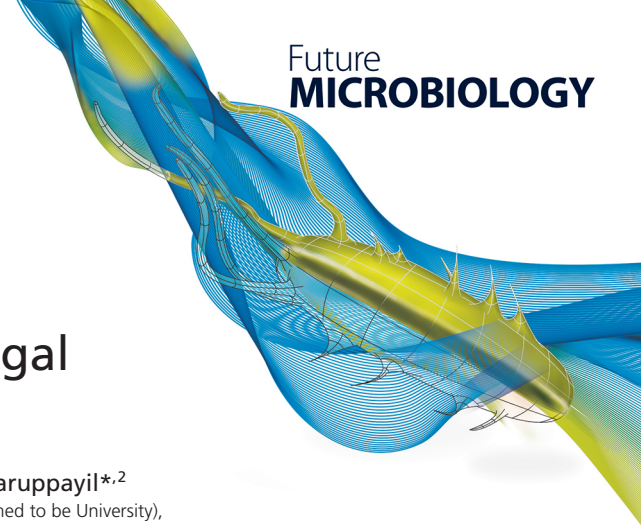
Investigating structure-activity relationships of ITC derivatives could facilitate the design and synthesis of novel analogs with improved potency, selectivity, and pharmacokinetic properties. Structure-based drug design, molecular modeling, and computational chemistry approaches could aid in the rational design of ITC-based antifungal agents with enhanced efficacy and reduced toxicity.

In summary, the recommendations outlined above aim to guide future research and clinical efforts in harnessing the therapeutic potential of *Isothiocynates* for the management of *C. albicans* infections. By addressing key knowledge gaps, optimizing formulation strategies, and conducting rigorous preclinical and clinical studies, ITC-based therapies have the potential to revolutionize antifungal treatment paradigms and improve patient outcomes in the fight against fungal infections.

Publications

Special Report

For reprint orders, please contact: reprints@futuremedicine.com



Future MICROBIOLOGY

Isothiocyanates as potential antifungal agents: a mini-review

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

[1]Department of Obstetrics & Gynecology, DY Patil Medical College, DY Patil Education Society (Deemed to be University), Kolhapur, Kasaba Bawada, Maharashtra, 416006, India

[2]Department of Stem Cell and Regenerative Medicine, Center for Interdisciplinary Research, DY Patil Education Society (Deemed to be University), Kolhapur, Kasaba Bawada, Maharashtra, 416006, India

*Author for correspondence: Tel.: +91 976 438 6253; prof.karuppayil@gmail.com

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.

First draft submitted: 29 October 2022; Accepted for publication: 26 April 2023; Published online: 31 July 2023

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

Future Medicine

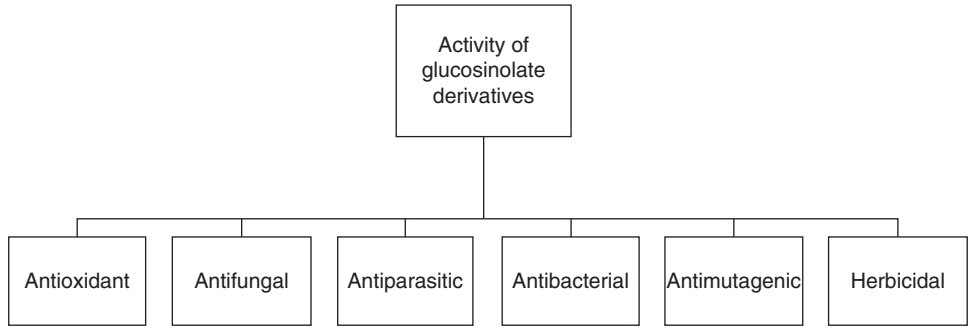


Figure 1. Biological activity of glucosinolate derivatives.

research has produced a large number of studies discussing the antifungal activity of different ITCs against diverse fungi [2]. ITCs are the main antifungal byproducts of a chemical defense system of cruciferous plant that has been widely studied. The current review focuses on the antifungal activity of ITCs and their mode of action. For example, 2-(4-methoxyphenyl)ethyl ITC is reported to have antifungal activity against *Aspergillus niger* [3]. Allyl, 4-(methylthio)butyl and phenylethyl ITCs also have antifungal activity against *Cochliobolus heterostrophus*. The mode of action studies revealed that these molecules targets gens involved in energy metabolism, oxidoreductase activity, melanin biosynthesis and cell wall-degrading enzymes [4]. The natural substance benzyl ITC (BITC) has been shown to prevent postharvest gray mold on strawberry fruit and significantly reduce natural decay by disrupting the integrity of the plasma membrane of *Botrytis cinerea* spores [5]. ITCs also have the potential to be used as antimicrobial packaging to increase the shelf life of fresh poultry [6].

Antifungal properties of ITCs

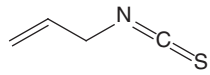
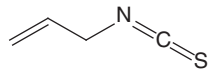
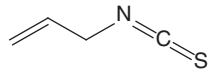
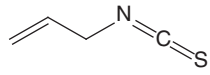
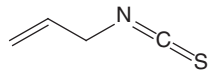
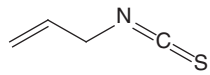
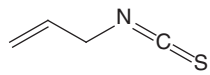
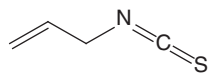
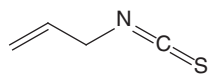
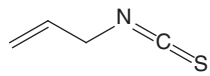
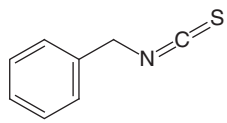
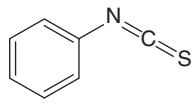
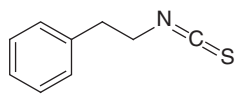
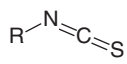
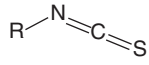
Extracts of cruciferous plants, isolated GSL derivatives and their combinations have been shown by a number of *in vitro* studies to inhibit the growth of many plant pathogens. A comprehensive list of different GSL derivatives and their fungicidal activity against a variety of fungi are reviewed by Vig *et al.* [7]. GSLs from cabbage are reported to have toxicity against fungi such as *Peronospora parasitica*, *Pythium ultimum* and *Sclerotium rolfsii* [8]. Most filamentous fungal pathogens are susceptible to GSLs, with at least 40 known to be inhibited by GSL derivatives [2]. Effective molecules include allyl ITC (AITC), BITC, butenyl ITC, phenyl ITC (PITC) and phenylethyl ITC (PEITC). AITC, BITC, PITC and PEITC have widely reported fungicidal activity [9]. Some of the antifungal properties of ITCs are listed in Table 1.

AITC has been found to inhibit growth and virulence factors of *Candida albicans* as well as *C. albicans* biofilms at a concentration of 1 mg/ml [21]. AITC also synergistically potentiates fluconazole activity, with biofilm formation inhibited by a combination of fluconazole at 0.004 mg/ml and AITC at 0.125 mg/ml. Moreover, there is no hemolytic activity at the biofilm inhibitory concentrations of AITC or AITC–fluconazole, which rules out any cytotoxic consequences.

The antifungal activity of ITCs against human dermatophytes has been reported by Choi *et al.* [10]. The antifungal activity of ITCs against 21 other filamentous plant pathogens has been reported in other studies as well [7,19]. The growth of several fungal pathogens of *Brassica* is inhibited by GSL-derived ITCs [8]. Smolinska *et al.* observed that aliphatic ITCs exhibit more antifungal action than aromatic ITCs [11]. *Arabidopsis thaliana* mutants with reduced aliphatic GSL content have less ability to protect themselves against necrotrophic fungi. GSL derivatives are inhibitors of bacterial and fungal growth hence it prevents spoilage of food products as well as increase shelf life of food products [20]. The percentages of AITC and butenyl ITC in brown mustard oil are 85 and 10%, respectively. The growth of *Penicillium roqueforti*, *Pencillium corylophilum*, *Eurotium repens*, *Aspergillus flavus* and *Endomyces fibuliger* in preserved rye bread is prevented by AITC at 2 ppm [2]. Two fungal diseases of *Brassica*, *Alternaria brassicae* and *Sclerotinia scletoriorum*, have been shown to be inhibited by 17 GSLs and GSL hydrolysis products; the effectiveness of the chemicals studied was influenced by the strain of the pathogenic organism [22].

The efficacy of GSLs and their derivatives against *B. cinerea* and *Alternaria brassicicola* was investigated [12]. It was found that *B. cinerea* was sensitive to GSLs and their derivatives, whereas *A. brassicicola* was inhibited by the aliphatic form of GSLs. In addition, GSL hydrolysis products were used in the defense against *B. cinerea*. ITCs were also shown to inhibit the growth of four human pathogenic dermatophytes, including *Trichophyton rubrum*, *Trichophyton*

Table 1. Antifungal activity of isothiocyanates against various fungi.

S. no.	Molecule	Structure	Antifungal activity	Ref.
1	AITC		*Candida albicans*	[5]
2	AITC		*Penicillium roqueforti*, *Penicillium corylophilum*, *Eurotium repens*, *Aspergillus flavus*, *Endomyces fibuliger*	[2]
3	AITC		*Alternaria brassicicola*	[10,11]
4	AITC		*Aspergillus parasiticus*, *Fusarium verticillioides*	[12]
5	AITC		*Fusarium solani*	[13]
6	AITC		*Sclerotinia sclerotiorum*	[14]
7	AITC		*Penicillium commune*, *Penicillium roqueforti*, *Aspergillus flavus*, *Endomyces fibuliger*	[15]
8	AITC		*Penicillium digitatum*, *Aspergillus parasiticus*	[16]
9	AITC		*Penicillium commune*, *Penicillium roqueforti*, *Penicillium nalgiovense*, *Debaryomyces hansenii*, *Aspergillus flavus*	[17]
10	AITC		*Penicillium expansum*	[18]
11	BITC		*Alternaria brassicicola*	[10]
12	PITC		*Alternaria brassicicola*	[19]
13	PEITC		*Alternaria alternata*	[20]
14	ITC from horseradish		*Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis*	[6]
15	ITC		*Saccharomyces cerevisiae*	[10]

AITC: Allyl isothiocyanate; BITC: Benzyl isothiocyanate; ITC: Isothiocyanate; PEITC: Phenylethyl isothiocyanate; PITC: Phenyl isothiocyanate; S. no.: Serial number.

10.2217/fmb-2022-0251

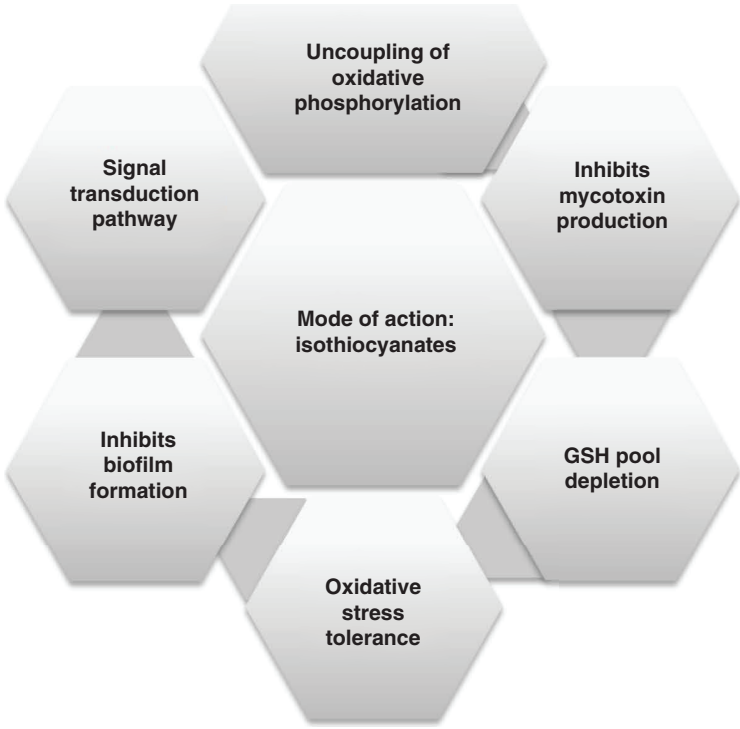


Figure 2. Mode of action of isothiocyanates.

mentagrophytes and *Microsporum canis*, with effective concentrations in the range of 100–200 μg/ml [10]. This study postulates that GSLs from horseradish could be effective antifungal agents against dermatophytic fungi.

Mode of action of ITCs

ITCs inhibit oxygen uptake by the yeast *Saccharomyces cerevisiae* through the uncoupling of oxidative phosphorylation in mitochondria [23]. In *A. brassicicola*, a pathogen of *Brassica*, exposure to ITCs results in a reduction in oxygen consumption and mitochondrial depolarization. The transcription factor ABAP1, MAPK and HOG1 are all activated by ITCs, which also increase the expression of many oxidative response genes in *A. brassiscola*. *ABHOG1* and *ABAP1* mutants are hypersensitive to ITCs, and loss of these genes results in loss of aggressiveness in *A. brassicicola*. *ABAP1*-like genes may act as redox sensors and regulate the expression of oxidative response genes. Oxidative response genes are activated after 20 min of exposure of *A. brasccicola* to AITC, BITC and PITC, which significantly reduce the development of *A. brassicicola*; AITC is more efficient than PITC and BITC [24]. On exposure to AITC, more than one-third of the genes related to oxidative stress are induced in *A. brassicicola* [25]. Increased expression of genes involved in the response to oxidative stress may serve as a major mechanism for fungal defense against GSLs.

PEITC inhibits *Alternaria alternata*, which causes black spot rot in pear fruits. Fumigation with PEITC at 1.22 mM prevents the development of black spot rot [26]. Treatment with PEITC inhibits the development of toxins (alternariol, tentoxin) by *A. alternata*.

Oxidative stress in *C. albicans* was shown in one study to be triggered by horseradish essential oil, which contains GSL derivatives [13]. Glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase were all elevated as a result of the oil treatment, which also increased the amount of superoxide. A high horseradish essential oil treatment concentration reduced the GSH pool, boosted superoxide generation and killed *Candida* cells. When horseradish essential oil and 1-chloro-2,4-dinitrobenzene, a GSH pool-depleting chemical, were combined, *C. albicans* was killed as a result of synergism. It appears that GSH metabolism guards *C. albicans* biofilms against GSLs.

In another study, the growth of *Aspergillus parasiticus* and *Fusarium verticillioides* was hindered by AITC in its gaseous state [14]. The ability of these fungi to produce mycotoxins was also suppressed by AITC treatment at 50 μl^{-1}. Aflatoxin and fumonisin are two of the mycotoxins that have been studied. AITC may provide protection against microbial contamination and mycotoxin formation (Figure 2) [14].

The YVC1 channel is reported to regulate vacuolar pressure in certain fungi [15,18]. TRPP2 in animals is homologous to the YVC1 channel. The plant pathogenic fungus *Magnaporthe oryzae* has a calcium-permeable channel called YVC1 (MGG09828.5), which is important in development [16]. In eukaryotes, transient receptor potential (TRP) channels play a key role in the ion homeostasis. TRP calcium-permeable channels were discovered in *S. cerevisiae* and *A. thaliana* [17]. However, the mechanisms by which calcium signaling-associated elements affected the pathogenicity of *C. albicans* were not so clear. In addition, a *yvc1* mutant showed a dampened response to stress and enhanced sensitivity to SDS, and it was discovered that the *yvc1*Δ/Δ mutant demonstrated reduced capacity for a stress response, impaired morphogenesis and diminished pathogenicity [27]. According to this study, YVC1 is crucial for *C. albicans* survival in host tissues, stress response, morphogenesis and polarized growth. Several signaling mechanisms are used by *C. albicans* to recognize and react to oxidative stress. According to Yu *et al.*, the TRP channel YVC1 is essential for oxidative stress tolerance [17]. This channel is necessary for the oxidative stress response genes to be activated and for the mitochondria and vacuoles to remain stable in a potassium- and calcium-dependent manner. The oxidative stress response, vacuoles and mitochondria all work together to mediate ion transport in *C. albicans* under oxidative stress, and the TRP channel is a key player for this response. *Fusarium solani's* YVC homolog, *FSYVC1*, is linked to pathogenicity and growth [27]. It is known that AITC inhibits *F. solani*, and its mechanism of action is also known. Hyphal deformation and electrolyte leakage is found to be induced by AITC. Loss of *FSYVC1* increased sensitivity to AITC and led to an increase in reactive oxygen species. The expression of *FSYVC1* increased 12- to 30-fold after treatment with 4.8 g/ml AITC compared with the control. The authors suggested that a new molecular target for drug development (*FSYVC*1) may exist.

Sclerotinia sclerotiorum-induced white mold is a significant disease of *Brassica* crops. The growth and development of *S. sclerotiorum* are inhibited by GSLs and ITCs, which are the major products of GSL breakdown. AITC treatment changed the expression of 2012 genes in *S. sclerotiorum* (1156 upregulated and 1056 downregulated) [28]. Indole-3-carbinol therapy affected 654 genes, of which 149 were upregulated and 505 were downregulated. The genes involved in removing reactive oxygen species from the body and preventing oxidative damage were upregulated. Homeostasis of amino acids had a role in the reduction of oxidative stress in *S. sclerotiorum* following prolonged exposure to ITCs. Genes involved in the creation of proline were upregulated after treatment with AITC, whereas the production of serine was stimulated after treatment with indole-3-carbinol. Proline has osmoprotective, thermotolerant and possible reactive oxygen species-quenching qualities. It can also stabilize proteins and membranes. Serine supports the methionine cycle and the glutathione detoxification route, which are both involved in reducing oxidative stress.

According to several studies, ITCs can be used in food packaging system to inhibit fungal growth. ITCs frequently leads to considerable extension of a product's shelf life by inhibiting mycotoxin production. The growth of *Penicillium commune*, *P. roqueforti*, *A. flavus* and *E. fibuliger* on bread was shown to be inhibited by mustard oil at AITC gas phase MIC values of 1.8–3.5 g/l [29]. Researchers have also demonstrated that mustard flour or AITC alone effectively suppresses the synthesis of patulin by *Penicillium expansum* on wheat tortillas [30]. AITC and mustard oil containing meal showed reduced growth of *Penicillium digitatum* and *A. parasiticus* in sliced mozzarella and increase in the shelf life of food [31]. Aflatoxin B1 synthesis was also inhibited by the treatment of AITC. It was discovered that AITC was found to be effective against cheese spoilage causing fungi like *P. commune*, *P. roqueforti*, *Penicillium nalgiovense*, *Debaryomyces hansenii* and *A. flavus* [32].

Conclusion

GSLs are a unique class of bioactive substances with a diverse range of bioactivities. GSLs serve as a significant defense system for plants, but they are also crucial to humans in many ways. The propensity of ITCs, which are present in large quantities in cruciferous vegetables, to have a positive impact on human health has attracted a lot of interest in recent years. Their positive effects have been demonstrated by numerous investigations. However, in detailed characteristics of ITCs need to be explored by researchers. Various studies have demonstrated how ITCs' natural origin and biodegradability make them a strong choice for a broad range of potential applications (e.g., increasing the shelf life of various foods or suppressing fungal growth, pathogenesis and/or toxin production in a range of stored plants, cereals and fruits in the postharvest stage). Furthermore, as multiple studies have shown, the decline in degradation is typically accompanied by no visible changes in a variety of quality indicators. Although more extensive research will be needed in this area to evaluate safety concerns, given the benefits of ITCs, it is anticipated that there will be a large increase in interest and demand for such applications.

10.2217/fmb-2022-0251

Future perspective

Considering the huge potential of ITCs, exploration of this group of molecules as antifungal agents against human pathogenic fungi is worthwhile. There is a small amount of research on the impact of ITCs on human pathogenic fungi and their potential modes of action. ITCs need to be studied in more detail for *in vitro* antifungal efficacy. These molecules can be used as novel therapeutics against fungal infections.

Executive summary

Background

- Glucosinolates and their derivatives, including isothiocyanates (ITCs), are present in cruciferous vegetables.
- ITCs have various antifungal, antibacterial, antiparasitic, antioxidant, herbicidal, antimutagenic and anticarcinogenic properties.

Antifungal properties of ITCs

- ITC molecules have antifungal properties against a variety of fungi.

Mode of action of ITCs

- The probable mode of action of ITCs is the induction of oxidative stress.

Conclusion

- In recent years, there has been a lot of interest in the possibility that ITCs, which are abundant in cruciferous vegetables, may have a beneficial effect on human health.
- Many investigations have shown the beneficial effects of ITCs. Several studies have shown how the natural origin and biodegradability of ITCs make them good candidates for a wide range of uses.
- More in-depth research will be required in this field to address safety concerns.

Financial & competing interests disclosure

The DY Patil Education Society (Deemed to be University) Kolhapur provided project funding. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Plaszkó T, Szűcs Z, Vasas G, Gonda S. Interactions of fungi with non-isothiocyanate products of the plant glucosinolate pathway: a review on product formation, antifungal activity, mode of action and biotransformation. *Phytochemistry* 200, 113245 (2022).

•• **Discusses the mode of action of non-isothiocyanates.**

2. Plaszkó T, Szűcs Z, Vasas G, Gonda S. Effects of glucosinolate-derived isothiocyanates on fungi: a comprehensive review on direct effects, mechanisms, structure–activity relationship data and possible agricultural applications. *J. Fungi (Basel)* 7(7), 539 (2021).

•• **Discusses the mode of action of isothiocyanates (ITCs).**

3. Wu TL, Hu YM, Sun Y *et al.* Insights into the mode of action of 2-(4-methoxyphenyl)ethyl isothiocyanate on *Aspergillus niger*. *Food Control* 136, 108871 (2022).

4. Yu H, Jia W, Zhao M *et al.* Antifungal mechanism of isothiocyanates against *Cochliobolus heterostrophus*. *Pest Manag. Sci.* 78(12), 5133–5141 (2022).

5. Sun Y, Wang Y, Xu Y *et al.* Application and mechanism of benzyl-isothiocyanate, a natural antimicrobial agent from cruciferous vegetables, in controlling postharvest decay of strawberry. *Postharvest Biol. Technol.* 180, 111604 (2021).

6. Moller A, Leone C, Kataria J *et al.* Effect of a carrageenan/chitosan coating with allyl isothiocyanate on microbial spoilage and quality of chicken breast. *Poult. Sci.* 102(3), 102442 (2023).

7. Vig AP, Rampal G, Thind TS, Arora S. Bio-protective effects of glucosinolates – a review. *LWT* 42(10), 1561–1572 (2009).

• **Discusses the various bioprotective effects of glucosinolates (GSLs).**

8. Gamliel A. Characterization of antifungal volatile compounds evolved from solarized soil amended with cabbage residues (1993).

9. Kala C, Ali SS, Ahmad N, Gilani SJ, Khan NA. Isothiocyanates: a review. *Res. J. Pharmacogn.* 5(2), 71–89 (2018).

10. Choi KD, Kim HY, Shin IS. Antifungal activity of isothiocyanates extracted from horseradish (*Armoracia rusticana*) root against pathogenic dermal fungi. *Food Sci. Biotechnol.* 26(3), 847–852 (2017).

•• **Discusses the antifungal activity of ITCs against four dermal fungi.**

11. Smolinska U, Morra MJ, Knudsen GR, James RL. Isothiocyanates produced by Brassicaceae species as inhibitors of *Fusarium oxysporum*. *Plant Dis.* 87(4), 407–412 (2003).

12. Buxdorf K, Yaffe H, Barda O, Levy M. The effects of glucosinolates and their breakdown products on necrotrophic fungi. *PLOS ONE* 8(8), (2013).
13. Bertóti R, Vasas G, Gonda S *et al.* Glutathione protects *Candida albicans* against horseradish volatile oil. *J. Basic Microbiol.* 56(10), 1071–1079 (2016).
14. Nazareth TM, Corrêa JAF, Pinto ACSM *et al.* Evaluation of gaseous allyl isothiocyanate against the growth of mycotoxigenic fungi and mycotoxin production in corn stored for 6 months. *J. Sci. Food Agric.* 98(14), 5235–5241 (2018).
15. Chang Y, Schlenstedt G, Flockerzi V, Beck A. Properties of the intracellular transient receptor potential (TRP) channel in yeast, Yvc1. *FEBS Lett.* 584(10), 2028–2032 (2010).
16. Nguyen QB, Kadotani N, Kasahara S, Tosa Y, Mayama S, Nakayashiki H. Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, *Magnaporthe oryzae*, using a high-throughput RNA-silencing system. *Mol. Microbiol.* 68(6), 1348–1365 (2008).
17. Yu Q, Wang F, Zhao Q *et al.* A novel role of the vacuolar calcium channel Yvc1 in stress response, morphogenesis and pathogenicity of *Candida albicans*. *Int. J. Med. Microbiol.* 304(3–4), 339–350 (2014).
18. Kim HS, Kim JE, Frailey D *et al.* Roles of three *Fusarium oxysporum* calcium ion (Ca2+) channels in generating Ca2+ signatures and controlling growth. *Fungal Genet. Biol.* 82, 145–157 (2015).
19. Chen J, Ullah C, Reichelt M *et al.* The phytopathogenic fungus *Sclerotinia sclerotiorum* detoxifies plant glucosinolate hydrolysis products via an isothiocyanate hydrolase. *Nat. Commun.* 11(1), 1–12 (2020).
20. Melrose J. The glucosinolates: a sulphur glucoside family of mustard anti-tumour and antimicrobial phytochemicals of potential therapeutic application. *Biomedicines* 7(3), 1–28 (2019).

• **Discusses the bioactive poperties of GSLs.**

21. Raut JS, Bansode BS, Jadhav AK, Karuppayil SM. Activity of allyl isothiocyanate and its synergy with fluconazole against *Candida albicans* biofilms. *J. Microbiol. Biotechnol.* 27(4), 685–693 (2017).
22. Sotelo T, Lema M, Soengas P, Cartea ME, Velasco P. *In vitro* activity of glucosinolates and their degradation products against brassica-pathogenic bacteria and fungi. *Appl. Environ. Microbiol.* 81(1), 432–440 (2015).
23. Kojima M. Studies on the effects of isothiocyanates and their analogues on microorganisms. I. Effect of isothiocyanates on the oxygen uptake of yeasts. https://scholar.google.co.in/scholar?hl=en&as_sdt=0%2C5&q=%5B12%5D.%09Kojima+M.+Studies+on+the+effects+of+isothiocyanates+and+their+analogues+6.&btnG=
24. Calmes B, N'Guyen G, Dumur J *et al.* Glucosinolate-derived isothiocyanates impact mitochondrial function in fungal cells and elicit an oxidative stress response necessary for growth recovery. *Front. Plant Sci.* 6, 414 (2015).

•• **Discusses the molecular mode of action of ITCs.**

25. Coscueta ER, Sousa AS, Reis CA, Pintado MM. Phenylethyl isothiocyanate: a bioactive agent for gastrointestinal health. *Molecules* 27(3), 794 (2022).
26. Zhang M, Li Y, Bi Y *et al.* 2-phenylethyl isothiocyanate exerts antifungal activity against *Alternaria alternata* by affecting membrane integrity and mycotoxin production. *Toxins (Basel)* 12(2), 124 (2020).
27. Li Y, Liu Y, Zhang Z, Cao Y, Li J, Luo L. Allyl isothiocyanate (AITC) triggered toxicity and FsYvc1 (a STRPC family member) responded sense in *Fusarium solani*. *Front. Microbiol.* 11, 870 (2020).

•• **Discusses the antifungal activity of allyl ITC (AITC) with mode of action against *Fusarium solani*.**

28. Madloo P, Márquez ML, Viqueira MT. Transcriptional analysis of the response of *Sclerotinia sclerotiorum* after exposure to aliphatic and indolic isothiocyanates (ITCs) (2018).

• **Discusses the effect of aliphatic and indolic GSLs on *Sclerotinia sclerotiorum*.**

29. Nielsen PV, Rios R. Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oil. *Int. J. Food Microbiol.* 60(2–3), 219–229 (2000).
30. Saladino F, Manyes L, Luciano FB, Mañes J, Fernandez-Franzon M, Meca G. Bioactive compounds from mustard flours for the control of patulin production in wheat tortillas. *LWT* 66, 101–107 (2016).
31. Tracz BL, Bordin K, Bocate KCP *et al.* Devices containing allyl isothiocyanate against the growth of spoilage and mycotoxigenic fungi in mozzarella cheese. *J. Food Process. Preserv.* 42(11), e13779 (2018).
32. Winther M, Nielsen PV. Active packaging of cheese with allyl isothiocyanate, an alternative to modified atmosphere packaging. *J. Food Prot.* 69(10), 2430–2435 (2006).

Current Microbiology (2024) 81:29
https://doi.org/10.1007/s00284-023-03542-3

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]

Received: 9 August 2023 / Accepted: 25 October 2023

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.

✉ Ashwini Khanderao Jadhav
ashujadhav09@gmail.com

✉ Sankunny Mohan Karuppayil
prof.karuppayil@gmail.com

Shivani Balasaheb Patil
patilshivani007@gmail.com

Rakesh Kumar Sharma
drrksharmaz@gmail.com

Tanjila Chandsaheb Gavandi
tanjilagavandi@gmail.com

Sargun Tushar Basrani
pg21780@gmail.com

Sayali Ashok Chougule
sayalichougule777@gmail.com

Shivanand Ramappa Yankanchi
dryankanchi@gmail.com

[1] Department of Stem Cell and Regenerative Medicine, Medical Biotechnology, Centre for Interdisciplinary Research, D. Y. Patil Education Society (Deemed to be University), Kolhapur, Maharashtra 416003, India

[2] Department of Obstetrics and Gynaecology, D. Y. Patil Medical College Hospital and Research Institute, Kadamwadi, Kolhapur, Maharashtra 416003, India

[3] Department of Zoology, Shivaji University, Kolhapur, Maharashtra 416004, India

Published online: 05 December 2023

Introduction

There are around 8.7 million eukaryotic species present on earth. According to a recent estimate among that 611,000 are fungal species and approximately 600 species are pathogenic to humans [1, 2]. Candida is a part of the natural flora and it can become pathogenic in immune compromised patients. *C. albicans* has the ability to form biofilms on medical devices [3] biofilm-related infections are becoming difficult to treat in hospitalised patients as well as in patients who has recently gone through surgery as they exhibit drug resistance [4]. To treat this Fluconazole and Amphotericin B are widely prescribed but they exhibit side effects due to high doses [5]. It has become important to find alternatives to the current drugs and find drugs which can target drug tolerant biofilms without causing side effects to the patients [6, 7].

The potential of using bioactive molecules of natural origin as antifungal agents is proposed by various workers as they inhibit biofilm formation [8, 9]. Recent research revealed that the *Brassicaceae* family of crops, including cabbage, broccoli, cauliflower, and radish, are full of nutrients that can benefit human health, including carotenoids, polyphenols, flavonoids, and glucosinolates (GLS) as well as isothiocyanates (ITCs). The breakdown products of GLS are ITCs. Their numerous pharmacological qualities, including their antibacterial, antifungal, antiprotozoal, anti-inflammatory, and chemo protective actions, make them useful in the pharmaceutical business as well [10]. It has been observed that ITC compounds exhibit antifungal properties against soil-borne fungal infections [11, 12]. Along with this a few reports on the antifungal activities of ITC's against postharvest plant pathogenic fungi such as *Botrytis cinerea, Penicillium expansum, Alternaria alternata* and *Monilinia laxa* are known [13]. There are very few studies present which focuses the effect of ITC on *C. albicans* among that, a study by Pereira et al., reported the activity of benzyl isothiocyanate (BITC) against *C. albicans*. The described study examines the ability of ITCs to combat oral isolates of *C. albicans*. Allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), and phenyl ethyl isothiocyanate (PEITC) were used in a preliminary susceptibility disc diffusion test at a concentration range of 0.001–0.1 M. Since *C. albicans* isolates were more vulnerable to these aromatic isothiocyanates like BITC and PEITC, effect of these compounds on cell size and germ tube formation (GTF) were investigated. Further research was conducted on the most promising compound, BITC produces oxidative stress which may responsible for alterations in the cells ultrastructure by interfering the structure of the cell wall. Research shown that aromatic ITCs have the capacity to affect *C. albicans* cells in a variety of ways, including size, shape, and GTF, oxidative stress, and ultrastructure. Overall findings imply that BITC may be effectively used against *C. albicans* invasive potential [14].

In current study we have explored the anti-*C. albicans* activity and mechanism of action of ethyl isothiocyanate (EITC). EITC is an aliphatic isothiocyanate and it inhibits its *C. albicans* planktonic growth and virulence factor like, yeast to hyphal form transition, adhesion to polystyrene surface, developing biofilm and mature biofilm. Additionally, it demonstrated anti-*C. albicans* activity through inhibiting ergosterol biosynthesis, EITC alters the expression of genes involved in yeast to hyphal morphogenesis signal transduction pathway, cell cycle arrest, reactive oxygen species (ROS) production, DNA condensation and in vivo study in silkworm model. These studies provide insight into how EITC works to combat *C. albicans* infection.

Materials and Methods

Culture, Growth Condition, Media and Chemicals

Candida albicans strain ATCC 90028 was received from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The organism was sub cultured on Yeast Extract Peptone Dextrose (YPD) agar plates and slants and stored at 4 °C. *C. albicans* colonies were inoculated in flask containing 50 ml of YPD broth and incubated at 30 °C on an incubator shaker at 120 rpm, for 24 h. After 24 h cells were collected by centrifugation at 2000 × g speed for 2 min followed by washing with phosphate buffer saline (PBS) twice and used for different of assays in this study.

Chemicals

EITC drug was purchased from Sigma Aldrich Chemical Ltd., Mumbai, India. EITC was dissolved in DMSO to make stock solution of 2 mg/ml. This stock further diluted used for in vitro and in vivo study.

Minimum Inhibitory Concentration (MIC_{50}) Determination for Planktonic Growth of *C. albicans*

As per Clinical Laboratory Standards Institute (CLSI) guideline effect of EITC on the planktonic growth of *C. albicans* were studied by following the micro broth dilution method. Various concentrations of EITC ranging from 0.0039 to 2 mg/ml prepared in RPMI-1640 (with L-glutamine and without sodium bicarbonate) and were added into 96 well plate. Wells without EITC was considered as control. The plates were incubated for 48 h at 35 °C. To check the growth, absorbance was taken at 620 nm using microtiter plate reader (Multiskan Ex, Thermo Electron Corp., USA). The concentration of EITC which causes 50% reduction in growth as compared with control was considered as minimum inhibitory concentration for

growth of *C. albicans* [15]. The experiment was conducted in triplicates.

Minimum Fungicidal Concentration (MFC) Determination

After performing planktonic growth assay at sterile condition 10 µl of aliquot of cells from the MIC_{50} and concentration above were used and spread on YPD agar plates. The agar plates were incubated for 48 h at 30 °C. After 48 h colonies were observed, the concentration at which molecule didn't shows any growth was considered as MFC concentration [16]. The experiment was conducted in triplicates.

Yeast to Hyphal Morphogenesis (Y–H Morphogenesis) Inhibition Assay

Fetal bovine serum induced Y–H morphogenesis of *C. albicans* was studied with the help of Microplate based assay. Concentrations of EITC ranging from 0.0039 to 2 mg/ml were prepared in 20% serum. In control and test well of microtiter plate 1×10^6 cells/ml were inoculated and final volume of the assay was kept 200 µl. The plates were incubated at 37 °C for 2 h on orbital shaker at 200 rpm. The formation of germ tubes by the cells was observed by using Inverted Microscope. Number of yeast cells and hyphae were counted with the help of Microscope. The experiment was conducted in triplicates [14].

Adhesion Assay

Effect of EITC on adherence of *C. albicans* to polystyrene surface was studied by using microplate based assay. Concentrations of EITC ranging from 0.0039 to 2 mg/ml were prepared in PBS. To achieve 1×10^7 cells/ml cell number 50 µl of cell suspension added to each well of microplate the final volume of each well was kept 100 µl. The plates were incubated for 90 min at 100 rpm on orbital shaker for attachment of cell on solid surface and at 37 °C. After incubations wells were washed with PBS to remove non-adhered cells the density of adherence of cells in each well was examined by relative metabolic activity using XTT assay. The concentration at which 50% reduction was measured as compare to control was considered as MIC concentration for adhesion. The experiment was conducted in triplicates [16].

Biofilm Assay

Early Biofilm

Tissue culture-treated 96 well polystyrene plates used for *C. albicans* biofilm development. 100 µl of cell suspension of (1×10^7 cells/ml) was added to each well, and the cells were then allowed to adhere to the solid surface at 37 °C for 90 min at 100 rpm. The wells were filled with PBS to remove non-adhered cells. RPMI-1640 medium (200 µl) along with various concentrations of EITC ranging from 0.0039 to 2 mg/ml was prepared added in the wells and incubate for 48 h at 37 °C then wells washed with PBS. XTT metabolic assay was performed to measure the effect of EITC on early biofilm growth. The experiment was conducted in triplicates [17].

Mature Biofilm

To check the activity of EITC against mature biofilm, 24 h old biofilm was prepared on tissue culture-treated 96 well polystyrene plate. Concentrations of EITC with range 0.0039 to 2 mg/ml were prepared in RPMI-1640 medium and was added in to the wells. The wells were investigated with an inverted light microscope. XTT metabolic assay was used to analyse biofilm growth. The experiment was conducted in triplicates [17].

XTT Assay for Quantification of Biofilm

By employing XTT metabolic test the growth of the biofilm was measured. The wells containing biofilms was filled with PBS to remove non-adhered cells and incubated with 100 µl of XTT-Menadione solution in dark, at 37 °C for 5 h. A microplate reader was used to measure the coloration produced by the water-soluble formazan product at 450 nm. The experiment was conducted in triplicates [17].

Haemolytic Assay

The toxicity of EITC was observed by using human Red Blood Cells (RBC's). Human blood collected from blood bank. The collected blood stored in tube containing EDTA was centrifuged at 2000 rpm for 10 min at 20 °C. The pellet of RBC suspended in PBS (10% v/v). RBC suspension was diluted in PBS 1:10 proportion before use. Aliquots of 100 µl from suspension added in to 100 µl of a different concentration of EITC in the same buffer in eppendorf tubes. 1% Triton X 100 used for total haemolysis. After incubation for 1 h at 37 °C it will be then centrifuged for 10 min at 2000 rpm and 20 °C. Optical density was obtained at 450 nm after 150 µl of supernatant was transferred to a microtiter plate with a flat bottom. The experiment was done in triplicates [18].

The haemolysis percentage was calculated by following formula:

% of Haemolysis = [[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.

Gene Expression Study with Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

To study the gene expressions profile in *C. albicans* during Y–H morphogenesis of signal transduction genes, RNA was extracted from *C. albicans* culture. 1×10^6 cells were incubated for 90 min at 37 °C with constant shaking in presence and absence of EITC at its morphogenesis inhibitory concentration. After 90 min incubation RNA was isolated 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 gneOmbiome Technologies Pvt. Ltd., Pune (primer sequences mentioned in Table 1) was added in SYBR Green Super mix in predetermined ratio. Expression of gene were analysed with the help of thermal cycler (Real Time System Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiment was done in triplicates [19].

Assessment of Intracellular Reactive Oxygen Species (ROS) Production

ROS levels of *C. albicans* were detected by an oxidant-sensitive fluorescent dye 2′,7′ dichlorofluorescein diacetate (DCFH-DA) (Table 2). The cells were grown in the presence and absence of planktonic MIC_{50} concentration of EITC, 1.5 mM/l of hydrogen peroxide for 4 h at 30 °C. The cells were then harvested, followed by washing with PBS buffer twice to remove the media. Cells were re-suspended in 3 ml of PBS. The fluorescent dye DCFH-DA (final concentration 10 µM) was added to each cell suspension and incubated at 30 °C for 1 h. Fluorescence intensity was measured by using a fluorescent spectrophotometer. The experiment was done in triplicates [20].

Table 1 Gene specific primers used for real time polymerase chain reaction

Primes	Sequence(5′–3′)
ACTIN-F	5′ATGGACGGTGAAGAAGTTGC 3′
ACTIN-R	5′ACCTCTTTTGGATTGGGCTTCA 3′
RAS1-F	5′GGCCATGAGAGAACAATATA 3′
RAS1-R	5′GTCTTTCCATTTCTAAATCAC 3′
PDE 2-F	5′ ACCACCACCACTACTACTAC 3′
PDE 2-R	5′ AAAATGAGTTGTTCCTGTCC 3′
BCY 1-F	5′ CCC AAGCTTATGTCTAATCCTCAACAGCA 3′
BCY 1-R	5′ GGG CTGCAGTTAATGACCAGCAGTTGGGT 3′
EFG 1-F	5′ TATGCCCCAGCAAACAACTG 3′
EFG 1-R	5′ TTGTTGTCCTGCTGTCTGTC3′
TEC 1-F	5′ AGGTTCCCTGGTTTAAGTG 3′
TEC 1-R	5′ ACTGGTATGTGTGGGTGAT 3′
ECE 1-F	5′-CCCTCAACTTGCTCCTTCACC-3′
ECE 1-R	5′-GATCACTTGTGGGATGTTGGTAA-3′
CEK 1-F	5′ AGCTATACAACGACCAATTAA 3′
CEK 1-R	5′ CATTAGCTGA ATGCATAGCT 3′
HST 7-F	5′ ACTCCAACATCCAATATAACA 3′
HST 7-R	5′ TTGATTGACGTTCAATGAAGA 3′
CPH1-F	5′ATGCAACACTATTTATACCTC 3′
CPH2-R	5′CGGATATTGTTGATGATGATA 3′
CDC35-F	5′TTCATCAGGGGTTATTTCAC 3′
CDC35-R	5′CTCTATCAACCCGCCATTTC 3′
HWP1-F	5′TGGTGCTATTACTATTCCGG 3′
HWP1-R	5′CAATAATAGCAGCACCGAAG 3′
MIG1-F	5′CTTCAACTAGCCTATATTCCGATGG 3′
MIG1-R	5′-CTTTCT GTAGGTACCAACAACTAC 3′
NRG1-F	5′CACCTCACTTGCAACCCC 3′
NRG1-R	5′GCCCTGGAGATGGTCTGA 3′
Tup1-F	5′ GAGGATCCCATGTATCCCCAACGCACCCAG 3′
Tup1-R	5′GGCGACGCGTCGTTTTTTGGTCCATTTCCAAATTCTG 3′

Table 2 Specification of groups involved in in vivo silkworm animal model experiments

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_{50} concentration (0.5 mg/ml) of EITC drug
Group IV	DMSO	Silkworm injected with DMSO (1%)
Group V	Standard drug fluconazole	Silkworm injected with *C. albicans* and fluconazole MIC_{50} concentration (0.15 µg/ml)

Effect of EITC on Nuclear Condensation or Fragmentation in *C. albicans* Biofilm

4-6-Diamidino-2-phenylindole (DAPI), a DNA-specific fluorescent dye, was used to observe nuclear condensation/fragmentation. Treatment of EITC (2 mg/ml) given to *C. albicans* cells at 37 °C and 120 rpm for 24 h. Following incubation, cells were collected and suspended in PBS. The washed cells incubated with DAPI (1 g/ml) at 30 °C in the dark condition. Cells were examined on glass slide under Zeiss LSM 880 confocal laser scanning microscope to detect nuclear staining [21].

Cell Cycle Analysis

50 ml YPD broth was inoculated with single colony of *C. albicans* from Yeast dextrose agar plate and incubated for 24 h. *C. albicans* cells were washed and starved. 2×10^7 cell/ml added in RPMI-1640 with EITC at its planktonic inhibitory concentration and without EITC treatment as control and incubated flask at 30 °C for 4 h. Cells were centrifuged at 6000 rpm for 3 min and washed with chilled PBS and fixed by using chilled absolute alcohol and kept at 4 °C. Next day cells were washed with PBS and incubated with 10 µg RNaseA, following RNaseA treatment 50 µg/ml Propidium iodide was added. After 30 min of incubation at 4 °C, the cells were analysed using FACS (FACS Diva Version 6.1.3) [22].

Ergosterol Assay

A single colony of *C. albicans* from Sabouraud dextrose agar plate was used to inoculate 50 ml of Sabouraud dextrose broth for control and for various concentrations of molecules. After 16 h of incubation, the cultures were harvested at 2700 rpm (856 g) for five minutes. The cell pellet's net weight was calculated. 3 ml of 25% alcoholic potassium hydroxide solution was added to each pellet and vortex mixed for one min. Cell suspensions were transferred to sterile borosilicate glass screw-cap tubes and were incubated in an 85 °C water bath for one hour. After incubation, the tubes were kept for cooling. Extraction of sterol was done by addition of 1 ml of sterile distilled water and 3 ml of n-heptane by continuous mixing for 3 min. The heptane layer was transferred to a clean borosilicate glass screw-cap tube and stored at − 20 °C. For analysis, 0.6 ml aliquot of sterol extract was diluted five-fold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm with a spectrophotometer. The extracted material has a characteristic four-peaked curve due to the presence of ergosterol. The absence of ergosterol in extracts was indicated by a flat line [23].

Scanning Electron Microscopy

To study the effect of EITC (0.0312 mg/ml) on Y–H morphogenesis in *C. albicans* cells adhered on polystyrene discs SEM analysis was performed. *C. albicans* (1×10^7 cells/ml) cells were added in RPMI and incubated at 37 °C and 50 rpm for 90 min. Without EITC treatment was served as control. The samples were fixed in 2.5% of glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C. Samples were post fixed in 2% aqueous solution of osmium tetroxide for 4 h, and then dehydrated in a series of graded alcohols. The samples were mounted over stubs and gold coating was performed using an automated gold coater. Images were obtained by scanning electron microscope [24].

In Vivo Study in Silk Worm Model

To check the antifungal efficacy of EITC in vivo experiment was carried out in silk worm larvae. Third instar silkworm larvae were obtained from Department of Zoology Shivaji University Kolhapur, larvae were fed with V1 variety mulberry leaves at 25 °C and maintained until they developed to fifth-instar larvae. Then, we only chose 1.9–2.2 g of silkworm larvae for our subsequent research. *C. albicans* cells

were grown overnight in YPD broth and were washed and re-suspended in phosphate-buffered saline. The *C. albicans* (1×10^6) cells were injected into the haemolymph through the dorsal surface of a silkworm larva using an insulin syringe. To determine the activity of EITC against *C. albicans* MIC_{50} concentration of EITC was injected into the haemolymph. Silkworm's mortality was assessed at 8 h of intervals. Silkworm larvae were kept at 25 °C throughout the experiments and survival was observed. Experiments were carried out in triplicates [25].

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.

Results

EITC Inhibited Planktonic Growth of *C. albicans*

Initially we studied the effect of EITC on *C. albicans* ATCC 90028 strain using micro broth dilution method. For this purpose, we have exposed *C. albicans* cells to 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. 1a). 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 plate (Fig. 1b).

EITC Inhibited Yeast to Hyphal 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, we have assessed the effects of EITC on the yeast-hyphal 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. Scanning Electron Microscopy (SEM) analysis was performed to confirm the inhibition of Y–H morphology of *C. albicans* cells by the treatment of EITC. SEM analysis revealed that, EITC inhibited the yeast-hyphal morphogenesis in *C. albicans* cells at 0.0312 mg/ml (Fig. 2a).

EITC Treatment Modulated the Gene Expression in *C. albicans*

The effect of EITC on Y–H morphogenesis shows significant reduction in hyphal formation of *C. albicans*. EITC treatment altered the expression of genes involved in Y–H morphogenesis of *C. albicans.* qRT-PCR analysis revealed relative fold change in the gene expressions (Table 3 and Fig. 2b). EITC at 0.0312 mg/ml concentration altered the expression of genes involved in signal transduction pathway by upregulating expression of *TUP1, MIG1* and *NRG1* genes 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 Inhibited of Adhesion *C. albicans* Cells to Polystyrene Surface

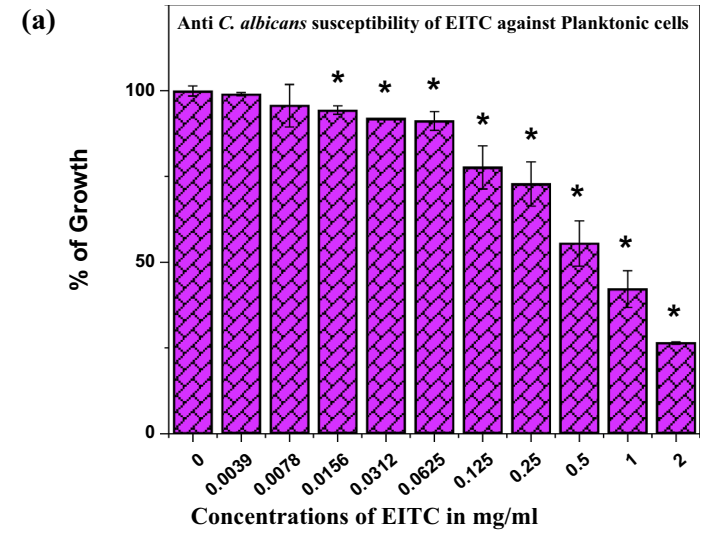
We examined the effect of EITC on the adhesion of *C. albicans* to polystyrene surface because infections and biofilm formation of *C. albicans* start from adhesion. The result suggests that EITC at 0.0312 mg/ml concentration decreased 50% viability of adhered cells to the polystyrene surface (Fig. 3a). 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.

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. 3b and Fig. 3c formation of early and mature biofilm was reduced by 50% at 2 mg/ml and 0.5 mg/ml concentration respectively compared to non-treated control.

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_{50} concentration successfully inhibited ergosterol biosynthesis (Fig. 4a). *C. albicans* cells which were not -treated with EITC (control) represent sharp peaks which indicates ergosterol biosynthesis. However, *C. albicans* cells treated with planktonic MIC_{50} 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.



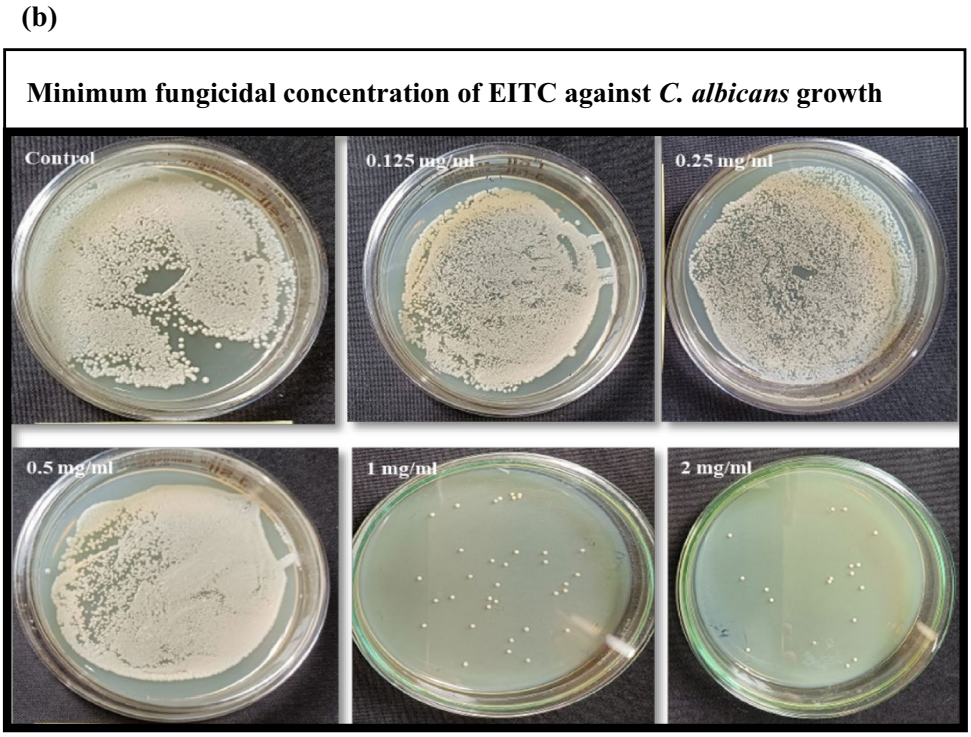


Fig. 1 **a** The antifungal activity of ethyl isothiocyanate on planktonic *C. albicans* cells. MIC of ethyl isothiocyanate against *C. albicans* was determined as per the micro broth dilution method. *C. albicans* cells were incubated with 0.0039 to 2 mg/ml concentrations of ethyl isothiocyanate for 48 h. The growth was analysed in terms of absorbance at 620 nm and percentage of growth as compared to that of the control was calculated. The experiment was performed in triplicates. Error bars indicate standard deviations. $*P < 0.05$ vs. non-treated controls. **b** minimum fungicidal concentration of ethyl isothiocyanate against *C. albicans* was obtained by counting the colonies grown on YPD agar plate after performing planktonic growth assay. Minimum fungicidal concentration was achieved at 2 mg/ml concentration

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, we have exposed *C. albicans* cells 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. 4b). From this study it was observed that EITC arrested the cells at synthetic S-phase.

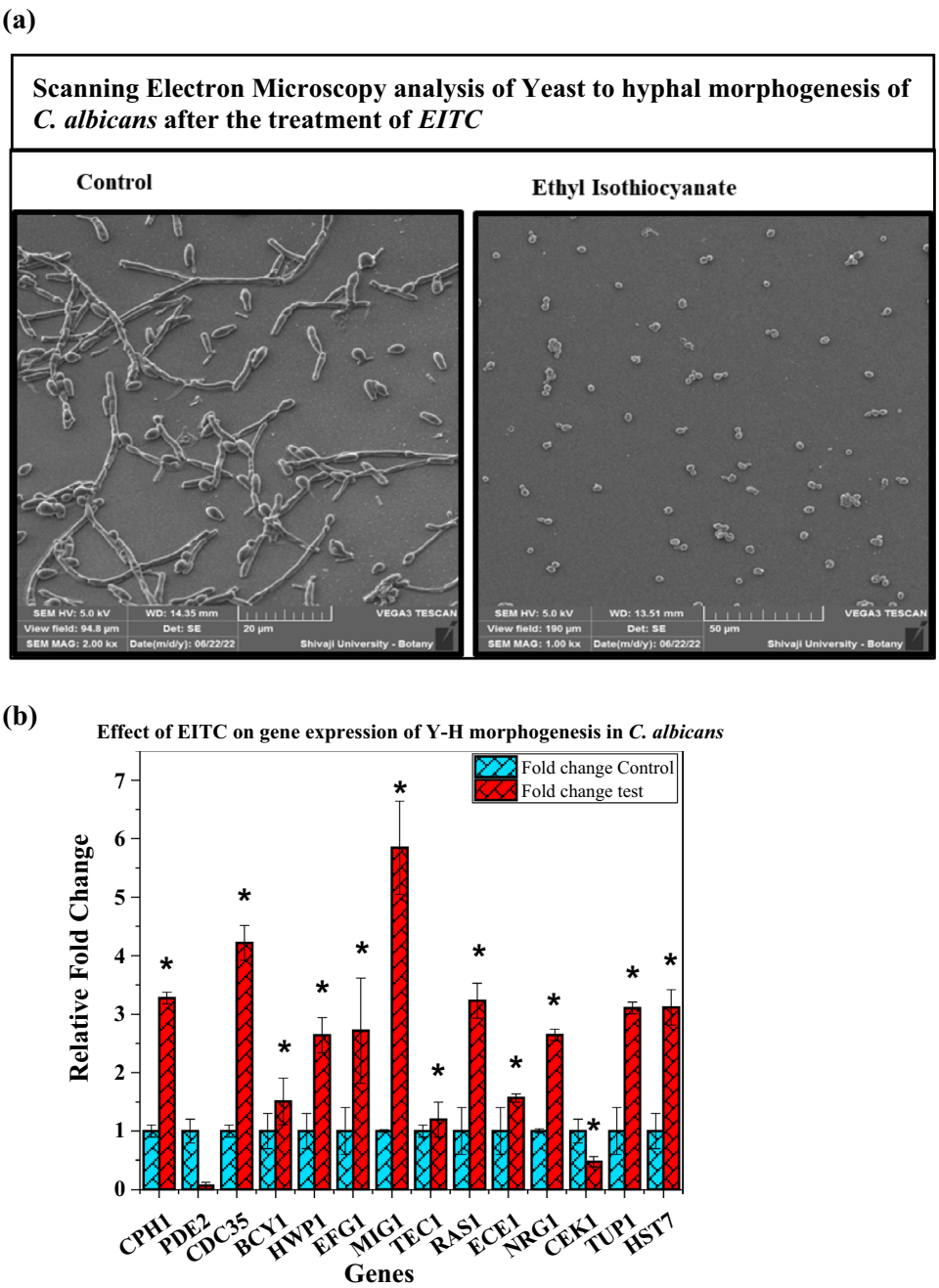


Fig. 2 **a** Effects of ethyl isothiocyanate on *C. albicans* morphology. Inhibition of fetal bovine serum induced yeast to hyphal growth was visualized by Scanning Electron Microscopy at a concentration of 0.0312 mg/ml. Two independent experiments were conducted. Control indicates non-treated *C. albicans* cells and Test indicates ethyl isothiocyanate treated *C. albicans* cells. **b** Transcriptional profiles of *C. albicans* cells treated with and without Ethyl isothiocyanate. *C. albicans* was cultivated with and without Ethyl isothiocyanate Y–H morphogenesis inhibitory concentration (0.0312 mg/ml) for 90 min. Transcriptional profiles were measured by qRT-PCR. Relative fold change represents transcriptional levels after treatment with ethyl isothiocyanate as compared to non-treated controls. Fold changes represents transcription changes in treated *C. albicans* vs. non-treated controls (value of 1.0). The experiment was performed in triplicates. Error bars indicate standard deviations. $*P < 0.05$ vs. non-treated controls

EITC Treatment Induced Intracellular Reactive Oxygen Species (ROS) Production in *C. albicans*

The ability of EITC to increase endogenous ROS production in *C. albicans* was evaluated using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) (Fig. 4c). The enhanced fluorescence intensity in Fig. 4c reveals EITC's ability to stimulate endogenous ROS generation. *C. albicans* are known to create 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,

Table 3 Relative fold change (FC) in gene expression in *Candida albicans* after the treatment of Ethyl isothiocyanate

Sr. no.	*Genes*	Fold change
1	*CPH1*	Upregulated [1.58-fold]
2	*PDE2*	Downregulated [0.95-fold]
3	*CDC35*	Upregulated [3.17-fold]
4	*BCY1*	Upregulated [0.44-fold]
5	*HWP1*	Upregulated [1.04-fold]
6	*EFG1*	Upregulated [1.57-fold]
7	*MIG1*	Upregulated [4.85-fold]
8	*TEC1*	Upregulated [0.19-fold]
9	*RAS1*	Upregulated [2.03-fold]
10	*ECE1*	Upregulated [0.57-fold]
11	*NRG1*	Upregulated [1.64-fold]
12	*CEK1*	Downregulated [0.53-fold]
13	*TUP1*	Upregulated [1.91-fold]
14	*HST7*	Upregulated [2.02-fold]

hydroxyl radicals, hydrogen peroxide, hypochlorous acid, and hydroperoxyl radicals, oxidative stress and eventual cell death can follow [26]. EITC at its planktonic growth inhibitory concentration (0.5 mg/ml) stimulates ROS production and eradicate *C. albicans* growth.

Effect of EITC on DNA Condensation in *C. albicans*

We evaluated EITCs' impact on DNA integrity in *C. albicans* cells as part of the DNA condensation investigation. DAPI was utilised in this investigation to track nuclear condensation and fragmentation linked to morphological changes in the nucleus. DAPI binds to DNA in the minor groove of A–T rich regions. EITC treated *C. albicans* have nuclear abnormalities linked to oxidative stress and apoptosis than untreated cells in the current result of nuclear condensation (Fig. 4d). In present study we have assessed the effect of EITC on cells of *C. albicans*. A DNA binding fluorescent dye DAPI was used for this study and visualised using confocal microscopy (Zeiss LSM 710). Result suggested that in non-treated cells chromatin appears as single round nuclei whereas, cells treated with biofilm inhibitory concentration (2 mg/ml) of EITC shows morphological changes in nucleus which indicates condensation of DNA.

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. 5a). This indicates that EITC may be less toxic at lower concentrations and more toxic at higher concentration.

In Vivo Study in Silkworm Animal Model

To investigate the effect of EITC, on silkworm (*Bombyx mori*) against *C. albicans* infection In vivo experiment was carried out. Silkworm was injected with *C. albicans* in presence of DMSO, fluconazole and EITC. In current study *C. albicans* considerably decreases the lifespan of positive control silkworm compared with non-treated negative control. We observed that fluconazole has increased the lifespan of the infected silkworms indicating that fluconazole protects silkworms during infection. Whereas silkworms infected with EITC were survived up to 24 h. After 24 h' silkworms were died. Decreased lifespan of silkworm in presence of EITC affect survival of silkworm after 24 h. Survival graph of silkworm was determined by Kaplan–Meier survival curve (Fig. 5b). Therefore, by decreasing the concentration of EITC we can increase the survival of silkworm and protects silkworm against *C. albicans* infection.

Discussion

Antifungal activity of EITC is reported against *Fusarium oxysporum, Sclerotinia sclerotorium, Geotrichum citri-aurantii, Aspergillus niger, Rizoctonia solani, Botrytis cinerea* and *Penicillium expansum* [13]. In present study we have examined the antifungal efficacy of EITC against *C. albicans* growth and virulence factors. EITC inhibited planktonic growth at 0.5 mg/ml concentration (Fig. 1a). Also, it was found that, EITC was fungicidal at 2 mg/ml (Fig. 1b). To investigate mechanism of action of EITC against *C. albicans* different assays were performed. Ergosterol assay revealed that, EITC significantly inhibited biosynthesis of ergosterol in *C. albicans* at its MIC concentration (Fig. 4a). 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 [27]. Ergosterol is a component of lipid membrane and supports membrane fluidity. A decrease in its concentration after EITC treatment could cause reduction of membrane permeability, which would make cells more susceptible or potentially cause cell death [28]. Ergosterol synthesis may be one of targets of EITC in *C. albicans*.

Earlier it was reported that benzyl isothiocyanate (BITC) and Phenyl ethyl isothiocyanate (PEITC) generate oxidative stress in *C. albicans* (Pereira et al., 2020). In current study, we investigated the effect of EITC on intracellular ROS level in *C. albicans*. EITC treatment

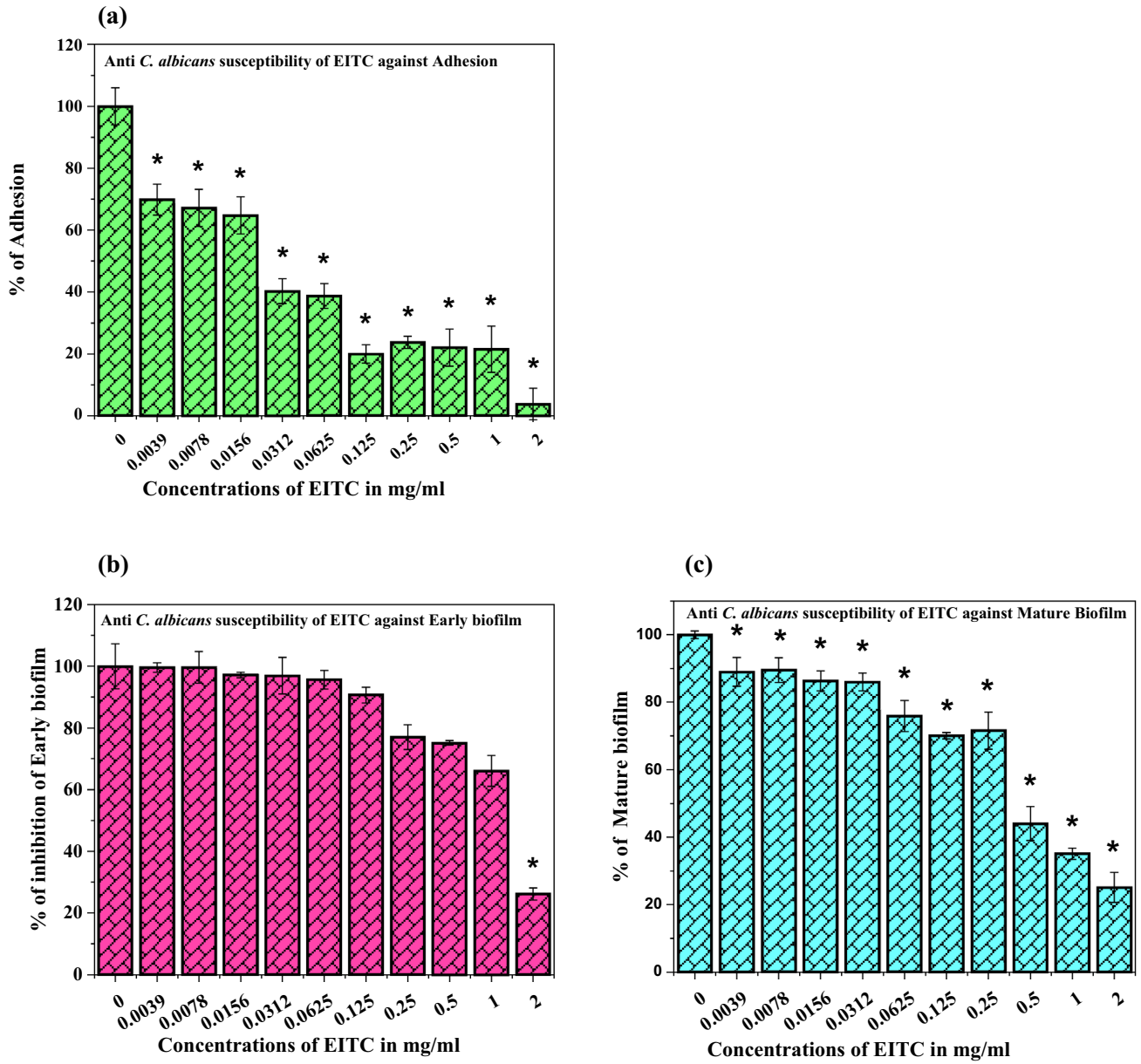


Fig. 3 **a** The effect of ethyl isothiocyanate on the adhesion of *C. albicans* to polystyrene plates. *C. albicans* in PBS medium with 0.0039 to 2 mg/ml concentrations of ethyl isothiocyanate were added to 96 well plate and incubated at 37◦C for 90 min., followed by an XTT assay to assess the adhesion rate of the cells compared to the control group. The experiment was performed in triplicates. Error bars indicate standard deviations. $*P<0.05$ vs. non-treated controls. **b** Anti-biofilm activities of ethyl isothiocyanate against *C. albicans*. Early biofilms of *C. albicans* were treated with ethyl isothiocyanate in 0.0039 to 2 mg/ml concentrations range for 48 h in 96 well polystyrene plate. Biofilm was quantified by XTT metabolic assay results are presented as mean percent of metabolic activities. The experiment was performed in triplicates. Error bars indicate standard deviations. $*P<0.05$ vs. non-treated controls. **c** Anti-biofilm activities of ethyl isothiocyanate against *C. albicans* mature biofilm. *C. albicans* cells were treated with ethyl isothiocyanate in 0.0039 to 2 mg/ml concentrations range for 72 h in 96 well polystyrene plate. Biofilm was quantified by XTT metabolic assay results are presented as mean percent of metabolic activities. The experiment was performed in triplicates. Error bars indicate standard deviations. $*P<0.05$ vs. non-treated controls

at 0.5 mg/ml concentration increases ROS level in *C. albicans* (Fig. 4c). It is widely known that the fungicidal actions of antifungal drugs like Amphotericin B against pathogenic fungi are influenced by the generation of ROS. Fungicidal medications encourage cellular death that is ROS-dependent and is regulated by a similar signalling and metabolic pathway. Furthermore, during yeast death, ROS function as a crucial intracellular messenger. Protein

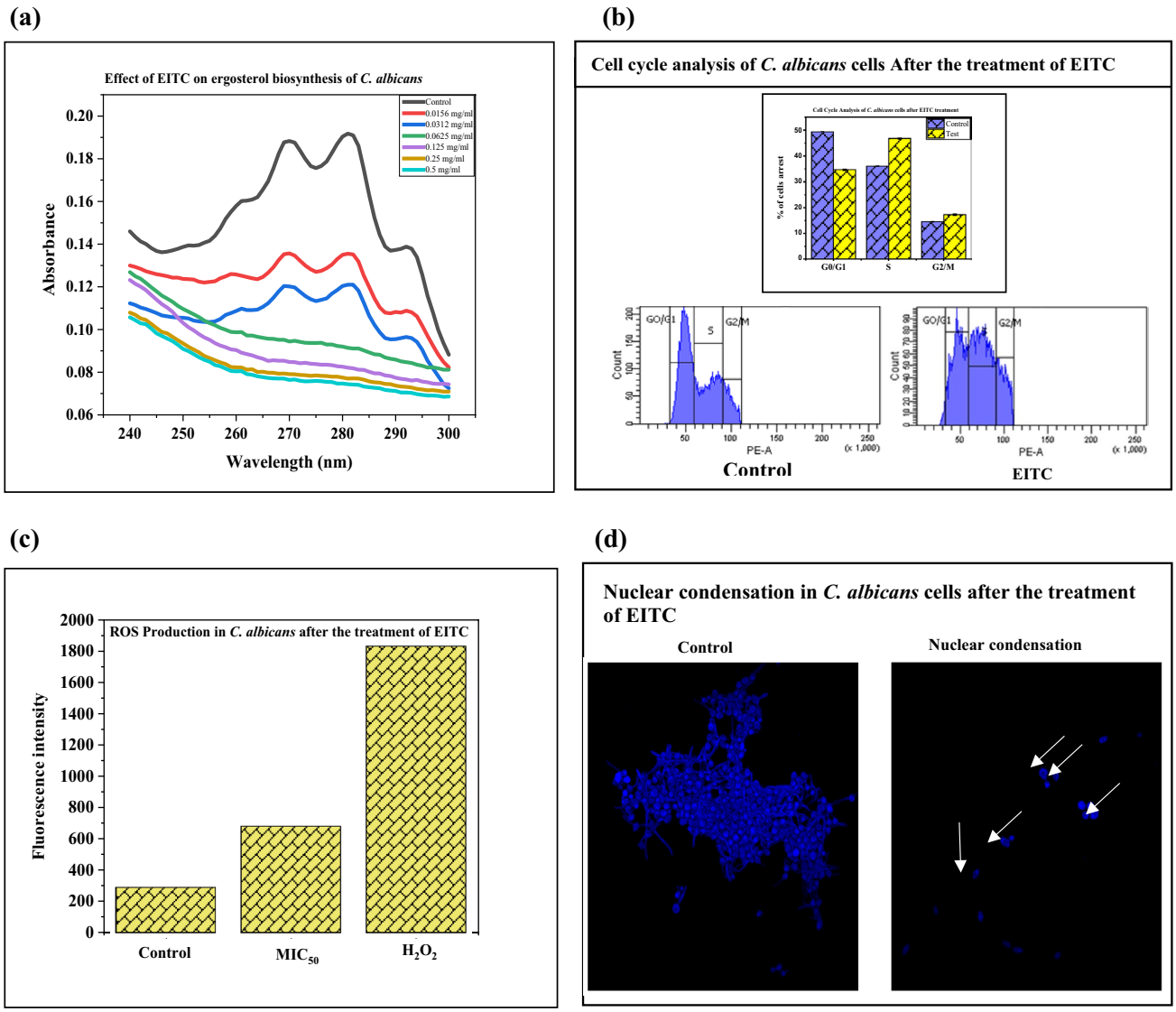


Fig. 4 **a** The effect of ethyl isothiocyanate on ergosterol biosynthesis. Ethyl isothiocyanate decreased the ergosterol content in *C. albicans*. Ergosterol was extracted from cells treated by 0.0156 to 0.5 mg/ml concentrations of ethyl isothiocyanate and measured by the spectrophotometric method. **b** Flow cytometric analysis of cell cycle 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 0.5 mg/ml test i.e. ethyl isothiocyanate treated cells (yellow bars). Ethyl isothiocyanate arrest *C. albicans* cells at S- phase. **c** Detection of ROS level using DCFDA staining involved in the planktonic inhibitory activity of ethyl isothiocyanate. Treatment of ethyl isothiocyanate increased the endogenous ROS production. The relative fluorescence intensity of treated cells stained by DCFDA was measured by fluorescent spectrophotometer. **d** Nuclear fragmentation in *C. albicans* cells treated with 2 mg/ml concentration of ethyl isothiocyanate and without ethyl isothiocyanate was detected by DAPI staining. Nuclear fragmentation was visualized with confocal microscopy and the scale bar represents 20 µm

and lipid peroxidation, decreased mitochondrial enzyme performance, DNA damage are all results of excessive ROS formation during apoptosis [26]. To investigate the effect of EITC on DNA damage, EITC treated and non-treated *C. albicans* cells stained with fluorescent dye DAPI and visualized under confocal microscope (Zeiss LSM 710). The confocal microscopy imaged showed that, non-treated *C. albicans* cells contain chromatin appears as single round nuclei whereas, cells treated with biofilm inhibitory concentration (2 mg/ml) of EITC shows morphological changes and condensation of DNA (Fig. 4d). According to the results, EITC may induces apoptosis and cell cycle arrest in *C. albicans*.

Furthermore, to understand the physiological changes caused by EITC, cell cycle analysis of EITC treated *C. albicans* cells was carried out. Cell cycle analysis revealed that, EITC arrested the *C. albicans* cells at S-phase (Fig. 4b) and

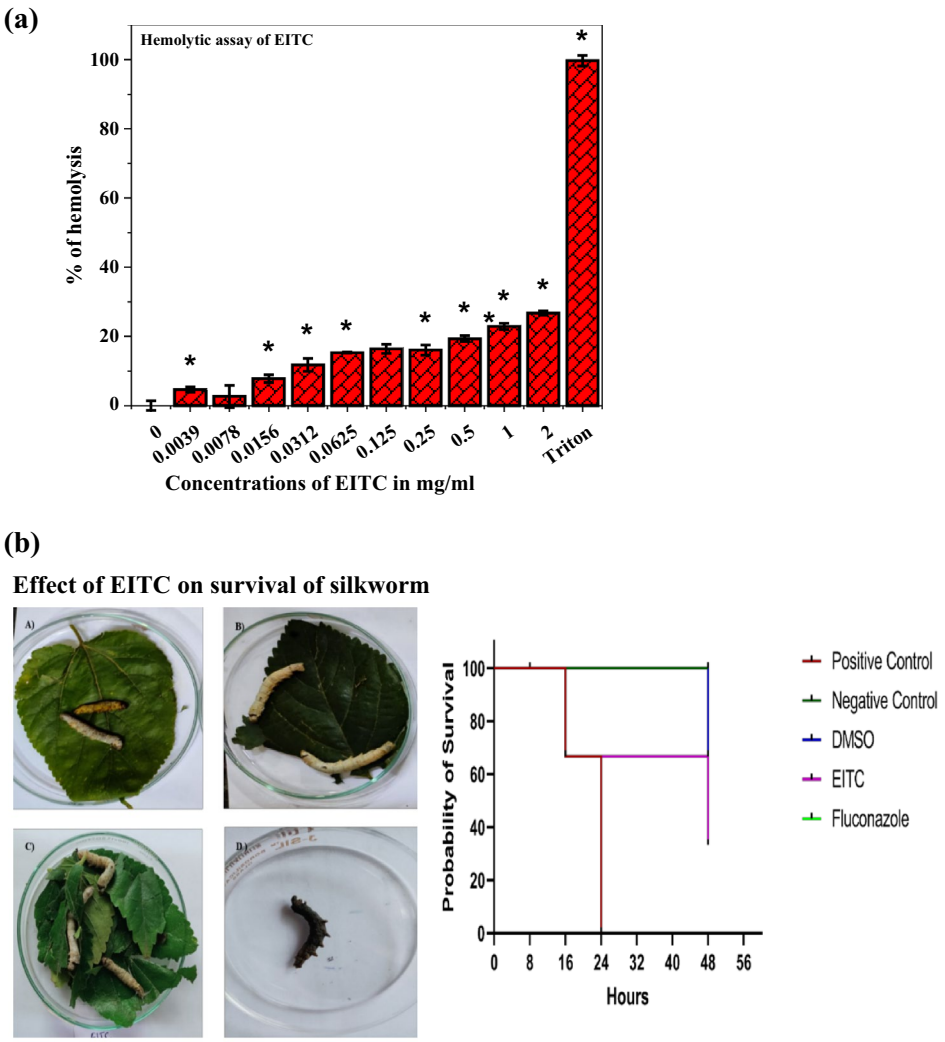


Fig. 5 **a** Effect of EITC on human Red Blood Cells. Haemolytic activity helps to analyse the effect of ethyl isothiocyanate on human Red Blood Cells (RBC). Ethyl isothiocyanate exhibited 19% haemolysis at 0.5 mg/ml concentration. Error bars indicate standard deviations. *$P < 0.05$ vs. non-treated controls. **b** Effects of ethyl isothiocyanate on *C. albicans* infected silkworm. The graph indicates percentage of worm survival after exposure of *C. albicans* for 48 h to ethyl isothiocyanate. The toxicities of ethyl isothiocyanate was studied using non-infected silkworm by determining survival rates after 48 h. Silkworm injects with *C. albicans* indicates positive control, silkworm injected with PBS was used as a negative control, whereas silkworm injected with fluconazole served as standard. The experiment was performed in triplicates

inhibit cellular processes of DNA replication which leads to apoptosis of fungal cell.

Yeast to hyphal (Y–H) morphogenesis plays important role in virulence of *C. albicans* as it plays major role in tissue invasion and systematic infection [24]. Present study showed that, EITC inhibited Y–H morphogenesis at very low concentration i.e. 0.0312 mg/ml. The Scanning Electron Microscopy confirmed that EITC inhibits yeast to hyphal form morphogenesis of *C. albicans* (Fig. 2a). RT-PCR study performed to identify molecular targets of in *C. albicans*. We examined the expression of genes involved in yeast to hyphal (Y–H) morphogenesis signal transduction pathway in *C. albicans*. Y–H signal transduction pathway comprises CEK1-MAPK pathway and RAS1-cAMP-PKA pathway [29]. EITC targets important components in Y–H transition pathway in *C. albicans*. EITC treatment upregulated the expression of *MIG, NRG1, TUP1* by 5.84-fold, 2.64-fold, and 3.10-fold, respectively (Fig. 2b). MIG1, TUP1 and, NRG1 are negative regulators having pivotal role in Y–H form transition inhibition. The expression of *PDE2* and *CEK1* genes were down regulated by 15.38-fold and 2.10-fold, respectively. *PDE2* gene has role in relation of intracellular cAMP level. Down regulation of *PDE2* activates cAMP which decrease cell wall and cell membrane thickness of fungi and drives the virulence and makes cell more susceptible to antifungal drug while up regulation of *PDE2* blocks cAMP synthesis and limited hyphal production [30]. Whereas CEK1 involved in invasive growth and filamentation [31] down regulation of *CEK1* inhibit filamentation.

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 [17]. EITC inhibits adhesion to polystyrene surface at 0.0312 mg/ml concentration (Fig. 3a).

C. albicans biofilm-related infections are became difficult to treat as it shows resistance to standard antifungals as well as they shows side effects on prolonged use so now a days researchers focuses on natural products that specifically inhibits biofilm formation [32]. EITC inhibited Early and Mature biofilm formed by *C. albicans* at 2 mg/ml and 0.5 mg/ml concentration, respectively (Fig. 3b and c).

Haemolytic activity of EITC was analysed for further therapeutic application. EITC shows 19% haemolysis at 0.5 mg/ml concentration while at lower concentrations it is less haemolytic (Fig. 5a). To confirm antifungal efficacy of EITC, the silkworm infection model was used. Silkworms are used in many studies on pathogenic microorganisms around the world. 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 [33]. In present study silkworms died after 24 h with the treatment of EITC (0.5 mg/ml) may be due to its toxicity (Fig. 5b). 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 in mice model to confirm the antifungal efficacy.

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 and nuclear or DNA fragmentation. Yeast to hyphal transition was inhibited by EITC by upregulating *MIG1, TUP1* and *NRG1* genes and downregulating *PDE2* and *CEK1* genes. Toxicity study and in vivo assay in silkworm model suggest that EITC after combination with other antifungal agents could be used as alternative therapeutic agent in candidiasis treatment.

Acknowledgements Authors are thankful to D. Y. Patil Education Society (Deemed to be University), Kolhapur 416006, India for financial support through intramural research project (Project no. DYPES/DU/R&D/2021/273). Authors are thankful to Mr. Amit S. Salunkhe, National Centre for Cell Science, Pune for FACS facility. Authors are also thankful to Dr. Mansingh Nimbalkar, Department of Botany, Shivaji University Kolhapur, Maharashtra, India for Scanning Electron Microscopy facility.

Author Contributions SP: Investigation, Methodology, writing—original draft. RKS: Data curation, Methodology. TG: Data curation, Methodology. SB: Investigation and writing. SC: Writing. SY: Interpretation of silkworm data. AJ: Data Validation**.** SMK: Conceptualisation, Supervision.

Funding Authors are thankful to D. Y. Patil Education Society (Deemed to be University), Kolhapur 416006, India for their financial support through research project no. DYPES/DU/R&D/2021/273.

Data Availability There are no data associated with this article.

Declarations

Conflict of interest There is no conflict of interest.

References

1. Mayer FL, Wilson D, Hube B (2013) *Candida albicans* pathogenicity mechanisms. Virulence 4:119–128
2. Talapko J, Juzbašić M, Matijević T, Pustijanac E, Bekić S, Kotris I, Škrlec I (2021) *Candida albicans*-the virulence factors and clinical manifestations of infection. J Fungi 7:1–19
3. Kojic EM, Darouiche RO (2004) Candida infections of medical devices. Clin Microbiol Rev 17:255–267
4. Privett BJ, Nutz ST, Schoenfisch MH (2010) Efficacy of surface-generated nitric oxide against *Candida albicans* adhesion and biofilm formation. Biofouling 26:973–983
5. Campbell BC, Chan KL, Kim JH (2012) Chemosensitization as a means to augment commercial antifungal agents. Front Microbiol 3:1–20
6. Scorzoni L, de Paula e Silva ACA, Marcos CM, Assato PA, de Melo WCMA, de Oliveira HC, Costa-Orlandi CB, Mendes-Giannini MJS, Fusco-Almeida AM (2017) Antifungal therapy: new advances in the understanding and treatment of mycosis. Front Microbiol 8:1–23
7. Van Daele R, Spriet I, Wauters J, Maertens J, Mercier T, Van Hecke S, Brüggemann R (2019) Antifungal drugs: what brings the future? Med Mycol 57:S328–S343
8. Thakre A, Zore G, Kodgire S et al (2017) OUP accepted manuscript. Med Mycol 56:565–578
9. Singla RK, Dubey AK (2019) Molecules and metabolites from natural products as inhibitors of biofilm in *Candida* spp. pathogens. Curr Top Med Chem 19:2567–2578
10. Le TN, Chiu C, Hsieh P (2020) And microgreens: an updated overview from a nutraceutical perspective. Plant Sci Today 9:1–23
11. Kurt Ş, Güneş U, Soylu EM (2011) In vitro and in vivo antifungal activity of synthetic pure isothiocyanates against *Sclerotinia sclerotiorum*. Pest Manag Sci 67:869–875
12. Smolinska U, Morra MJ, Knudsen GR, James RL (2003) Isothiocyanates produced by *Brassicaceae* species as inhibitors of *Fusarium oxysporum*. Plant Dis 87:407–412
13. Plaszkó T, Szűcs Z, Vasas G, Gonda S (2021) Effects of glucosinolate-derived isothiocyanates on fungi: A comprehensive review on direct effects, mechanisms, structure-activity relationship data and possible agricultural applications. J Fungi 7:1–38

14. Pereira C, Calado AM, Sampaio AC (2020) The effect of benzyl isothiocyanate on *Candida albicans* growth, cell size, morphogenesis, and ultrastructure. World J Microbiol Biotechnol 36:1–12
15. Raut JS, Shinde RB, Chauhan NM, Mohan Karuppayil S (2013) Terpenoids of plant origin inhibit morphogenesis, adhesion, and biofilm formation by *Candida albicans*. Biofouling 29:87–96
16. Jadhav A, Mortale S, Halbandge S, Jangid P, Patil R, Gade W, Kharat K, Karuppayil SM (2017) The dietary food components capric acid and caprylic acid inhibit virulence factors in *Candida albicans* through multitargeting. J Med Food 20:1083–1090
17. Shinde RB, Raut JS, Karuppayil MS (2012) Biofilm formation by *Candida albicans* on various prosthetic materials and its fluconazole sensitivity: A kinetic study. Mycoscience 53:220–226
18. Raut JS, Bansode BS, Jadhav AK, Karuppayil SM (2017) Activity of allyl isothiocyanate and its synergy with fluconazole against *Candida albicans* biofilms. J Microbiol Biotechnol 27:685–693
19. Priya A, Pandian SK (2020) Piperine impedes biofilm formation and hyphal morphogenesis of *Candida albicans*. Front Microbiol 11:1–18
20. Radhakrishnan VS, Dwivedi SP, Siddiqui MH, Prasad T (2018) In vitro studies on oxidative stress-independent, ag nanoparticles-induced cell toxicity of *Candida albicans*, an opportunistic pathogen. Int J Nanomedicine 13:91–96
21. Ahamad I, Bano F, Anwer R, Srivastava P, Kumar R, Fatma T (2022) Antibiofilm activities of biogenic silver nanoparticles against *Candida albicans*. Front Microbiol 12:1–13
22. Zore GB, Thakre AD, Jadhav S, Karuppayil SM (2011) Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle. Phytomedicine 18:1181–1190
23. Ahmad A, Khan A, Manzoor N, Khan LA (2010) Evolution of ergosterol biosynthesis inhibitors as fungicidal against Candida. Microb Pathog 48:35–41
24. Vila T, Romo JA, Pierce CG, McHardy SF, Saville SP, Lopez-Ribot JL (2017) Targeting *Candida albicans* filamentation for antifungal drug development. Virulence 8:150–158
25. Hasegawa S, Yamada Y, Iwanami N, Nakayama Y, Nakayama H, Iwatani S, Oura T, Kajiwara S (2019) Identification and functional characterization of *Candida albicans* mannose–ethanolamine phosphotransferase (Mcd4p). Curr Genet 65:1251–1261
26. Choi H, Lee DG (2015) Lycopene induces apoptosis in *Candida albicans* through reactive oxygen species production and mitochondrial dysfunction. Biochimie 115:108–115
27. Rajput SB, Mohan Karuppayil S (2013) Small molecules inhibit growth, viability and ergosterol biosynthesis in *Candida albicans*. Springerplus 2:1–6
28. Zorić N, Kosalec I, Tomić S, Bobnjarić I, Jug M, Vlainić T, Vlainić J (2017) Membrane of *Candida albicans* as a target of berberine. BMC Complement Altern Med 17:1–10
29. Chang W, Li Y, Zhang L, Cheng A, Lou H (2012) Retigeric acid B attenuates the virulence of *Candida albicans* via inhibiting adenylyl cyclase activity targeted by enhanced farnesol production. PLoS ONE 7:1–10
30. Zou L, Mei Z, Guan T, Zhang B, Deng Q (2021) Underlying mechanisms of the effect of minocycline against *Candida albicans* biofilms. Exp Ther Med 21:1–9
31. Park YK, Shin J, Lee H-Y, Kim HD, Kim J (2021) Inhibition of Ras1-MAPK pathways for hypha formation by novel drug candidates in. BioRxtv 1:1–44
32. Cavalheiro M, Teixeira MC (2018) Candida biofilms: threats, challenges, and promising strategies. Front Med 5:1–15
33. Garip Z, Harran FT (2021) Silkworm in pharmacology and toxicology. Int J Vet Anim Res 4:34–38

Current Medical Mycology

2023, 9(2): 29-38

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

Shivani Balasaheb Patil[1,2], Ashwini Khanderao Jadhav[1,2*], Rakesh Kumar Sharma[3], Sargun Tushar Basrani[1,2], Tanjila Chandsaheb Gavandi[1,2], Sayali Ashok Chougule[1,2], Shivanand Ramappa Yankanchi[4], Sankunny Mohan Karuppayil[1,2]

[1]Patil Education Society (Deemed to be University), Kolhapur-416-006, Maharashtra, India
[2]Department of Stem Cell and Regenerative Medicine and Medical Biotechnology Centre for Interdisciplinary Research, Maharashtra, India
[3]Patil Medical College Hospital and Research Institute, Kadamwadi-416012-, Kolhapur, Maharashtra, India
[4]Department of Zoology, Shivaji University, Kolhapur- 416004, Maharashtra, India

Article Info

Article type:
Original Article

Article History:
Received: 07 July 2023
Revised: 20 September 2023
Accepted: 25 October 2023

**** Corresponding author:***
Ashwini Khanderao Jadhav
Patil Education Society (Deemed to be University), Kolhapur-416-006, Maharashtra, India.

Email: ashujadhav09@gmail.com

A B S T R A C T

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

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

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

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

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

➢ **How to cite this paper**
Balasaheb Patil Sh, Khanderao Jadhav A, Kumar Sharma R, Tushar Basrani S, Chandsaheb Gavandi T, Ashok Chougule S, Ramappa Yankanchi Sh, Mohan Karuppayil S. Antifungal activity of Allyl isothiocyanate by targeting signal transduction pathway, ergosterol biosynthesis, and cell cycle in *Candida albicans*. Curr Med Mycol. 2023; 9(2): 29-38 DOI: 10.22034/CMM.2023.345081.1429

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

increased side effects of the drugs.

Since the biofilms are hard to eradicate, the only available alternative may be the physical removal of the devices. In addition, colonized devices, such as pacemakers, may malfunction. Clogging of the catheters by *C. albicans* is a serious problem, especially in diabetic or immuno-compromised persons since the catheters require replacement. Removal or replacement of prosthetics is expensive and uncomfortable for the patients and may require frequent visits to the doctor as well as hospitals contributing to the additional expenses, mortality, and morbidity [10].

This necessitates an alternative treatment modality to counter the low efficacy, significant side effects, and emergence of multidrug-resistant *C. albicans* strains. Biofilms have the ability to adhere and accumulate on numerous surfaces [6]. The new antifungal drug strategy should concentrate on the development of drugs that prevent and remove biofilms. Numerous plant-derived substances have been shown to possess potential anti-*Candida* activities through a variety of mechanisms, including inhibition of the yeast-to-hyphae transition, prevention of the formation of biofilms, impairment of cell metabolism, cell wall integrity, cell membrane fluidity, and apoptosis [11].

Allyl isothiocyanate (AITC) is a natural compound derived from cruciferous vegetables. An earlier study performed by Raut et al. [12] has suggested that AITC alone and in combination with the standard antifungal, fluconazole (FLC), successfully inhibits the growth and virulence factors of *C. albicans*. Along with this, AITC at its planktonic and biofilm inhibitory concentration was non-hemolytic in nature. Hence, it can be used as an alternative therapeutic option for the treatment of candidiasis. The present study aimed to verify the mode of action of AITC against *C. albicans*.

In this study, AITC was explored to better understand its activity and modes of action against *C. albicans*. In this regard, the antifungal properties of AITC were demonstrated in planktonic and biofilm forms of *C. albicans*. Furthermore, AITC exhibited anti-*C. albicans* efficacy via a variety of mechanisms, like the inhibition of ergosterol production, the effect of AITC on signal transduction gene involved in yeast to hyphal morphogenesis, cell cycle arrest, and reactive oxygen species (ROS) production. These discoveries collectively shed light on the modes of action of AITC against *C. albicans*.

Materials and Methods

Fungal strain, growth conditions, and Chemicals

In the present study, *C. albicans* strain ATCC 90028 was used, which was obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained on Yeast Extract Peptone Dextrose (YPD) Agar Plates and slants and maintained at 4ºC. *Candida albicans* cells were grown in YPD broth and incubated at 30ºC on an incubator shaker at 120 rpm for 24 h. After 24 h, the cells were collected by centrifugation, and the cell pellet was washed with phosphate buffer saline (PBS) twice and used for varieties of assays in the current study. Fetal bovine serum (FBS) was used for yeast to hyphal (Y-H) form transition of *C. albicans*. The RPMI1640 (w/L- Glutamine w/o sodium bicarbonate) medium was used for micro broth dilution assay to determine minimum inhibitory concentrations (MICs) against planktonic and biofilm growth.

The AITC was purchased from Sigma Aldrich Chemical Ltd., Mumbai, India. It was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 2 mg/ml. This stock was further diluted and used for *in vitro* and *in vivo* experiments in the current study against *C. albicans*. Moreover, FLC was used as a standard antifungal agent in the current study against *C. albicans*. Experiments were performed according to the guidelines of Clinical Laboratory Standards Institutes (CLSI) micro broth dilution method to determine MICs [13].

Determination of minimum inhibitory concentration for Candida albicans planktonic growth

According to the guideline of CLSI M27-Ed4 [14], the effect of AITC alone and in combination with FLC on the planktonic cells of *C. albicans* was assessed with the help of micro broth dilution method in a 96-well microtiter plate [13]. Various concentrations of AITC ranging from 0.0039 to 2 mg/ml and FLC from 0.097 to 50 µg/ml were prepared in RPMI-1640 and added to the 96-well plate. Cells without test molecules were considered the control cells. The plates were incubated for 48 h at 35 °C. To examine the cell density, absorbance was taken at 620 nm using a microtiter plate reader (Multiskan Ex, Thermo Electron Corp., USA). The concentration of AITC which caused a 50% reduction in growth, compared to the control, was considered the MIC_{50} for the growth of *C. albicans* [13,15].

Determination of minimum fungicidal concentration

Effect of AITC and FLC on the growth of *C. albicans* was visibly assessed using a minimum fungicidal concentration (MFC) assay after performing MIC. In total, 10 µl of cell suspension from the wells of MIC_{50} and above MIC_{50} concentrations of AITC and FLC were selected to assess MFC and spread on the YPD agar plate. The YPD agar plates were incubated at 30 ºC for 48 h. After 48 h, the plates were observed. The ones that showed less growth or no growth were selected and photographed [15].

Estimation of Ergosterol Content

In total, 50 ml of sabouraud dextrose broth was inoculated with a single colony of *C. albicans* from a sabouraud dextrose agar plate. Planktonic MIC_{50} and sub-MIC concentrations of AITC within the range of 0.0078 to 0.125 mg/ml were added into different flasks, without AITC treatment served as control. It should be mentioned that flasks were incubated for 16 h. *Candida albicans* cells were harvested by centrifugation at 2,700 rpm (856 g) for 5 min. The net weight of the cell pellet was determined. Each pellet was mixed with 3 ml of a 25% alcoholic potassium hydroxide solution and was vortexed for 1 min. The cell suspension was transferred to sterile borosilicate glass screw-cap tubes and incubated in an 85 °C water bath for 1 h. The tubes were left to cool after the incubation.

The next step was to extract the sterols by the addition of 3 ml of n-heptane and 1 ml of sterile distilled water, and vortexing for 3 min. The heptane layer was put into a sterile screw-cap borosilicate glass tube and kept at 20 °C. In order to conduct the analysis, a 0.6 ml aliquot of sterol extract was multiplied five times in 100% ethanol before being spectrophotometrically scanned between 240 and 300 nm. Due to the presence of ergosterol, the isolated material shows a recognizable four-peaked curve. A flat line demonstrated the absence of ergosterol in the extracts [16].

Measurement of reactive oxygen species production for planktonic and biofilm cells of Candida albicans

Using 2′, 7′ - dichlorofluorescein diacetate (DCFH-DA), *C. albicans* cells were assessed to find the production of ROS. Cells were harvested by centrifugation, subjected to planktonic MIC_{50} concentration of AITC and 1.5 mM/L of hydrogen peroxide for 4 h, and then washed once with PBS before being re-suspended in 0.5 mL PBS. Afterward, they were incubated at 30 °C for 30 min in the dark followed by the

addition of 10µM DCFH-DA.

The developing biofilm of AITC-treated (0.5 mg/ml) and non-treated *C. albicans* was formed in a 12-well treated microtiter plate. After 24 h, the wells were washed with PBS and exposed to 10 µM DCFH-DA. Following incubation for 1 h at 37 °C, the biofilms were washed with PBS.

A spectrofluorometer FP-8300 (Jasco) was used to determine the fluorescence intensities of re-suspended planktonic and biofilm cells of *C. albicans* [6,17].

Cell cycle analysis

Log phase *C. albicans* cells were treated with AITC in RPMI medium for about 6 h at 30 °C and then washed twice with chilled PBS (pH 7.0). After washing, the cells were fixed overnight in 70% chilled ethanol at 4 °C. The next day, the cells were washed with PBS and incubated with 10 µg RNaseA, followed by the addition of 50 µg/ml propidium iodide. After 30 min of incubation at 4°C, the cells were analyzed using FACS (FACS Diva Version 6.1.3) [18].

Germ tube formation inhibitory assay

The FBS growth media was used to assess the effect of AITC on the induction of the germ tube of *C. albicans*. In control and test wells of the 96-well plate, 1×10^6 cells/ml were inoculated and concentrations of AITC ranging from 0.0039 to 2 mg/ml and FLC from 0.097 to 50 µg/ml were prepared in 20% serum and added to a plate. The plates were incubated at 37 °C for 2 h at 200 rpm. The formation of germ tubes by the cells was observed by using Inverted Microscope. The number of yeast cells and hyphae were counted with the help of Microscope [5].

Scanning Electron Microscopy

Germ tube formation of *C. albicans* cells was allowed to form on Foley's catheter. The MIC_{50} concentration of germ tube formation of AITC and cells (1×10^6 cells/ml) was added to a 12-well plate as a test and the cells without AITC in the plate served as the control. Both test and control cells were incubated at 37 °C and 50 rpm for 90 min. The samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C for scanning electron microscopy (SEM) preparation. The samples were dehydrated in a succession of grades of alcohols after being post-fixed for 4 h in an osmium tetroxide 2% aqueous solution. Samples were mounted on stubs and coated with gold using an automated gold coater. A scanning electron microscope was used to capture images [19].

Adhesion assay

To assess the effect of AITC on the adhesion of *C. albicans*, a polystyrene tissue culture 96-well plate was used. The AITC in a concentration range of 0.0039 to 2 mg/ml and FLC from 0.097 to 50 µg/ml were prepared in PBS and added to the well plate along with 1×10^7 cells/ml. The plate was incubated for 90 min at 37 °C on an orbital shaker with 100 rpm. After incubation, the wells were washed with PBS to remove non-adhered cells. The density of the adherence of cells in each well was examined by relative metabolic activity using XTT assay. The concentration at which a 50% reduction was seen, compared to the control was considered the MIC concentration [3].

Biofilm assay

Development of biofilm and mature biofilm

To assess the effect of AITC and FLC on the developing and preformed biofilm, *C. albicans* biofilm was prepared in a 96-well polystyrene tissue culture plate. A range of AITC concentration from 0.0039 to 2 mg/ml and FLC from 0.097 to 50 µg/ml was prepared in RPMI-1640 medium and added along with 1×10^7 cells/ml into a 96-well plate. The plate was incubated for 48 h at 37 °C. After incubation, the wells were washed with PBS and XTT metabolic assay was performed to analyze biofilm growth. *Candida albicans* 1×10^7 cells/ml were allowed to mature for 24 h at 37 °C. After incubation, the wells were washed with PBS, and drug dilution prepared in RPMI-1640 medium was added to the plate and incubated for 48 h at 37°C. Finally, the biofilm was analyzed using XTT metabolic assay [20].

XTT assay for quantification of biofilm

Growth of the biofilm was measured using the XTT metabolic test. The wells containing biofilms were filled with PBS to remove the non-adhered cells and then incubated with 100 µl of XTT-Menadione solution in the dark at 37 °C for 5 h. Color formation by the water-soluble formazan product was measured at 450 nm using a microplate reader [20].

Toxicity assay

Human red blood cells (RBCs) were used to study the toxicity of AITC. 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 Ethylenediaminetetraacetic acid-containing tube at 2,000 rpm for 10 min at 20 °C. The RBC pellet was suspended in PBS at 10% volume by volume. Before use, the RBC suspension was diluted in PBS 1:10 proportion. Moreover, 100 ml aliquots from the suspension were mixed with 100 ml of AITC at a different concentration in the same buffer in Eppendorf tubes. Besides, 1% Triton X 100 was used for total hemolysis. After incubation for 1 h at 37 °C, it was centrifuged for 10 min at 2,000 rpm at 20 °C. Optical density was obtained at 450 nm after 150 µl of supernatant was transferred to a microtiter plate with a flat bottom. All the experiments were performed in triplicates [21]. The hemolysis percentage was calculated using the following formula:

$$\% \text{ of Haemolysis} = [[A_{450} \text{ of test compound treated Sample} - A_{450} \text{ of buffer treated sample}] / [A_{450} \text{ of 1\%TritonX 100 treated sample} - A_{450} \text{ of buffer treated sample}]] \times 100.$$

Gene expression study with real-time polymerase chain reaction

To study the expressions of signal transduction genes involved in germ tube formation of *C. albicans*, RNA was extracted from *C. albicans* culture from germ tube formation. The 1×10^6 cells were incubated in RPMI-1640 medium for 90 min at 37 °C with constant shaking in the presence and absence of AITC at its morphogenesis inhibitory concentration. The RNA was extracted with a RNeasy mini kit (QIAGEN, Valencia, CA, USA) and was reverse transcripted to cDNA using Super Script III (Invitrogen, Life technologies, Camarillo, CA, USA).

The PCR was carried out (Biorad Real-Time PCR Machine, 0.2 ml, 96 wells) in 96-well PCR plates with the help of UNI SYBR GREEN SUPERMIX. The quantitative PCR reaction total volume was 10 µl. Primers purchased from geneOmbiome Technologies Pvt Ltd.; Pune (Table 1) were added to SYBR GREEN SUPER MIX in a predetermined ratio. Expressions of the gene were analyzed with the help of a thermal cycler (Real Time System Bio-Rad Laboratories, Inc., Hercules, CA, USA) [13].

Table 1. Gene-specific primers used for Real-Time Polymerase Chain Reaction

Gene name	Primes	Sequence (5' → 3')
Actin	ACTIN-F	5'ATGGACGGTGAAGAAGTTGC 3'
	ACTIN-R	5'ACCTCTTTTGGATTGGGCTTCA 3'
Ras-like protein 1	RAS1-F	5'GGCCATGAGAGAACAATATA 3'
	RAS1-R	5'GTCTTTCCATTTCTAAATCAC 3'
Phosphodiesterase 2	PDE 2-F	5'ACCACCACCACTACTACTAC 3'
	PDE 2-R	5' AAAATGAGTTGTTCCTGTCC 3'
Bypass of CYclic-AMP requirement	BCY 1-F	5′ CCC AAGCTTATGTCTAATCCTCAACAGCA 3′
	BCY 1-R	5′ GGG CTGCAGTTAATGACCAGCAGTTGGGT 3′
Enhanced filamentous growth protein 1	EFG 1-F	5' TATGCCCCAGCAAACAACTG 3'
	EFG 1-R	5' TTGTTGTCCTGCTGTCTGTC3'
Transcription activator TEC1	TEC 1-F	5' AGGTTCCCTGGTTTAAGTG 3'
	TEC 1-R	5' ACTGGTATGTGTGGGTGAT 3'
Extent of cell elongation protein 1	ECE 1-F	5'-CCCTCAACTTGCTCCTTCACC-3'
	ECE 1-R	5'-GATCACTTGTGGGATGTTGGTAA-3'
Extracellular signal-regulated kinase 1	CEK 1-F	5' AGCTATACAACGACCAATTAA 3'
	CEK 1-R	5' CATTAGCTGA ATGCATAGCT 3'
Serine/threonine-protein kinase STE7 homolog	HST 7-F	5' ACTCCAACATCCAATATAACA 3'
	HST 7-R	5' TTGATTGACGTTCAATGAAGA 3'
Chlamydomonas photolyase homolog 1	CPH1-F	5'ATGCAACACTATTTATACCTC 3'
	CPH1-R	5'CGGATATTGTTGATGATGATA 3'
Cell-division-cycle 35	CDC35-F	5'TTCATCAGGGGTTATTTCAC 3'
	CDC35-R	5'CTCTATCAACCCGCCATTTC 3'
Hyphal wall protein 1	HWP1-F	5'TGGTGCTATTACTATTCCGG 3'
	HWP1-R	5'CAATAATAGCAGCACCGAAG 3'
Multicopy Inhibitor of GAL gene	MIG1-F	5'CTTCAACTAGCCTATATTCCGATGG 3'
	MIG1-R	5'CTTTCT GTAGGTACCAACAACTAC 3'
Neuregulin 1	NRG1-F	5'CACCTCACTTGCAACCCC 3'
	NRG1-R	5'GCCCTGGAGATGGTCTGA 3'
Transcriptional repressor TUP1	Tup1-F	5' GAGGATCCCATGTATCCCCAACGCACCCAG 3'
	Tup1-R	5'GGCGACGCGTCGTTTTTTGGTCCATTTCCAAATTCTG 3'

In vivo study in silkworm animal model

Department of Zoology at Shivaji University in Kolhapur, India provided third-instar silkworm larvae (*Bombyx mori*), which were fed mulberry leaves and kept alive until they reached the fifth instar. For the present study, only 1.9–2.2 g of silkworm larvae was selected. Cells of *C. albicans* were cultured in YPD broth overnight before being washed and re-dissolved in phosphate-buffered saline. An insulin syringe was used to inject 1×10^6 cells into the hemolymph through the dorsal surface of a silkworm larva.

The planktonic MIC_{50} concentration (0.125 mg/ml) of AITC was injected into the hemolymph to assess its anti-*C. albicans* effectiveness. Silkworms injected with FLC and *C. albicans* were considered the standard. Silkworm injected with DMSO. The mortality of silkworms was measured every 8 h for a duration of 48 h. Throughout the studies, silkworm larvae were maintained at 25°C, and survival was noted. It should be mentioned that experiments were carried out in triplicates [22]. Specification of groups used in *in vivo* silkworm animal model experiments is mentioned in Table 2.

Statistical analysis

All experiments were carried out in triplicates and the mentioned values were the mean values obtained from three different observations. Values in the control and treatment groups for various molecules were compared using Student's t-test. The *P*-values of < 0.05 were considered statistically significant. The *in vivo* experimental data was analyzed using the GraphPad Prism software (version 6.0, San Diego, CA).

Ethical Consideration

The University Ethics Committee registration number is ECR/738/Inst/MH/2015/RR-21.

Table 2. Specification of groups used in *in vivo* silkworm animal model experiment

Group I	Positive control	Silkworm injected with *Candida albicans*
Group II	Negative control	Silkworm injected with PBS
Group III	Test	Silkworm injected with *C. albicans* + planktonic MIC_{50} concentration (0.125 mg/ml) of AITC drug
Group IV	DMSO	Silkworm injected with DMSO (1%)
Group V	Standard	Silkworm injected with *C. albicans* and fluconazole MIC_{50} concentration (0.15µg/ml)

PBS: Phosphate Buffer Saline, MIC_{50}: Minimum Inhibitory Concentrations, AITC: Allyl Isothiocyanate, DMSO: Dimethyl Sulfoxide

Results

Antifungal activity of AITC on C. albicans planktonic growth

The inhibitory effect on planktonic growth of *C. albicans* was assessed by AITC (Figure 1A). The MIC_{50} of AITC for *C. albicans* was found to be 0.125 mg/ml (Figure 1A). The MFC of AITC was assessed by spread plate technique on a YPD plate with the help of MIC_{50} concentration and the results indicated that AITC was fungicidal in nature at its MIC_{50} and higher concentrations (Figure 1A). Fluconazole inhibits planktonic growth of *C. albicans* at 1 µg/ml concentration.

Effect of AITC on C. albicans cell membrane

The cell membrane is the primary target for many standard antifungal drugs, like azoles and polyenes. Therefore, in the present study, we estimated total ergosterol content in *C. albicans* cells upon treatment with AITC. The impact of AITC on the cell membrane of *C. albicans* was examined using ergosterol biosynthesis. Ergosterol concentrations of AITC-treated and untreated *C. albicans* cell membranes were measured. Untreated control cells show characteristic peaks that signify ergosterol production. However, in this study,

ergosterol biosynthesis suppression in *C. albicans* cells treated with AITC at planktonic MIC_{50} concentration and below MIC_{50} concentration in the range of 0.0078 to 0.125 mg/ml was represented by a flat curve (Figure 1B).

Allyl isothiocyanate induced intracellular reactive oxygen species generation in C. albicans

Under extremely stressful conditions, *C. albicans* 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 as lipids, proteins, and nucleic acids, resulting in oxidative stress and ultimately cell death [23].

The fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate was used to assess AITCs capacity to stimulate endogenous ROS generation in *C. albicans* (Figure 1C). Figure 1C shows that AITC increased fluorescence intensity, which demonstrates the capacity of AITC to boost endogenous ROS production. The AITC promoted intracellular ROS generation at its 0.125 mg/ml planktonic inhibitory concentration. However, an increase in the production of ROS after the treatment of AITC, compared to non-treated *C. albicans* cells may be responsible for the eradication of *C. albicans* planktonic growth. Along with this, AITC treatment also increased ROS production in *C. albicans* biofilm at 0.5 mg/ml concentration. An increase in ROS production in biofilm cells of *C. albicans* may be a reason for the anti-biofilm activity of *C. albicans*.

Effect of allyl isothiocyanate on cell cycle

We 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 the growth and multiplication of a cell, 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 were exposed to a planktonic inhibitory dose of AITC (0.125 mg/ml) and compared to untreated cells. The DNA content

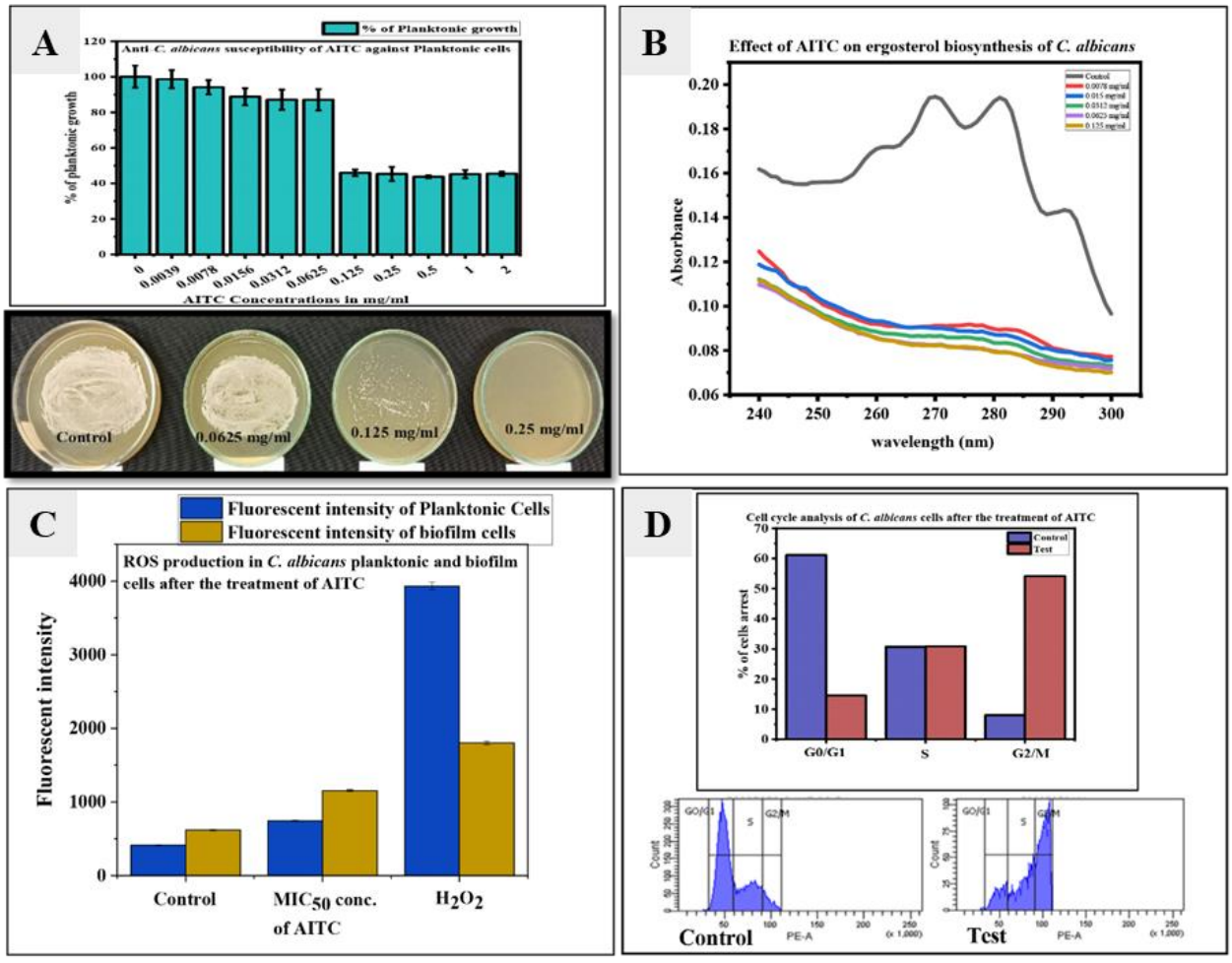


Figure 1.
A. Planktonic growth inhibitory activity fungicidal nature of allyl isothiocyanate against *Candida albicans*.
B. The effect of allyl isothiocyanate on ergosterol biosynthesis.
C. Detection of ROS level after treatment of allyl isothiocyanate using DCFDA staining involved in the planktonic and biofilm cells of *C. albicans*.
D. Flow cytometry analysis of cell cycle arrest in *C. albicans* planktonic cells with propidium iodide staining after allyl isothiocyanate treatment.

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*. Regarding the control cells, 61.1% of them were arrested in G0/G1 phase, 30.7% in the S phase, and 8.0% in the G2/M phase while regarding the test cells, 14.6% cells were arrested in G0/G1 phase, 30.9% in S phase and 54. % in G2/M phase (Figure 1D). The results suggested that AITC arrested cells in the G2/M phase of *C. albicans*.

Inhibitory effect of allyl isothiocyanate on germ tube formation of Candida albicans

In this study, the effect of AITC on germ tube formation of *C. albicans* was analyzed using FBS growth media. The AITC inhibited the 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 the germ tube formation (Figure 2A). In the current study, FLC did not inhibit germ tube formation.

Morphology analysis of Candida albicans germ tube on exposure to allyl isothiocyanate

The morphological analysis of *C. albicans* cells was further assessed for germ tube inhibition by SEM. *Candida albicans* control cells consisted of germ tube, whereas cells treated with 0.125 mg/ml concentration of AITC showed that germ tube formation was completely inhibited (Figure 2B).

Table 3. Relative fold changes in the gene expressions involved in the signal transduction pathway of *Candida albicans* after the treatment of AITC

Genes	Fold change (FC)
CPH1	Upregulated (2.43-fold)
PDE2	Downregulated (2.54-fold)
CDC35	Upregulated (1.29-fold)
BCY1	Upregulated (1.69-fold)
HWP1	Upregulated (3.99-fold)
EFG1	Upregulated (1.78-fold)
MIG1	Upregulated (9.22-fold)
TEC1	Downregulate (1.04-fold)
RAS1	Upregulated (4.08-fold)
ECE1	Upregulated (1.29-fold)
NRG1	Upregulated (3.35-fold)
CEK1	Downregulated (1.91-fold)
TUP1	Upregulated (7.80-fold)
HST7	Upregulated (1.80-fold)

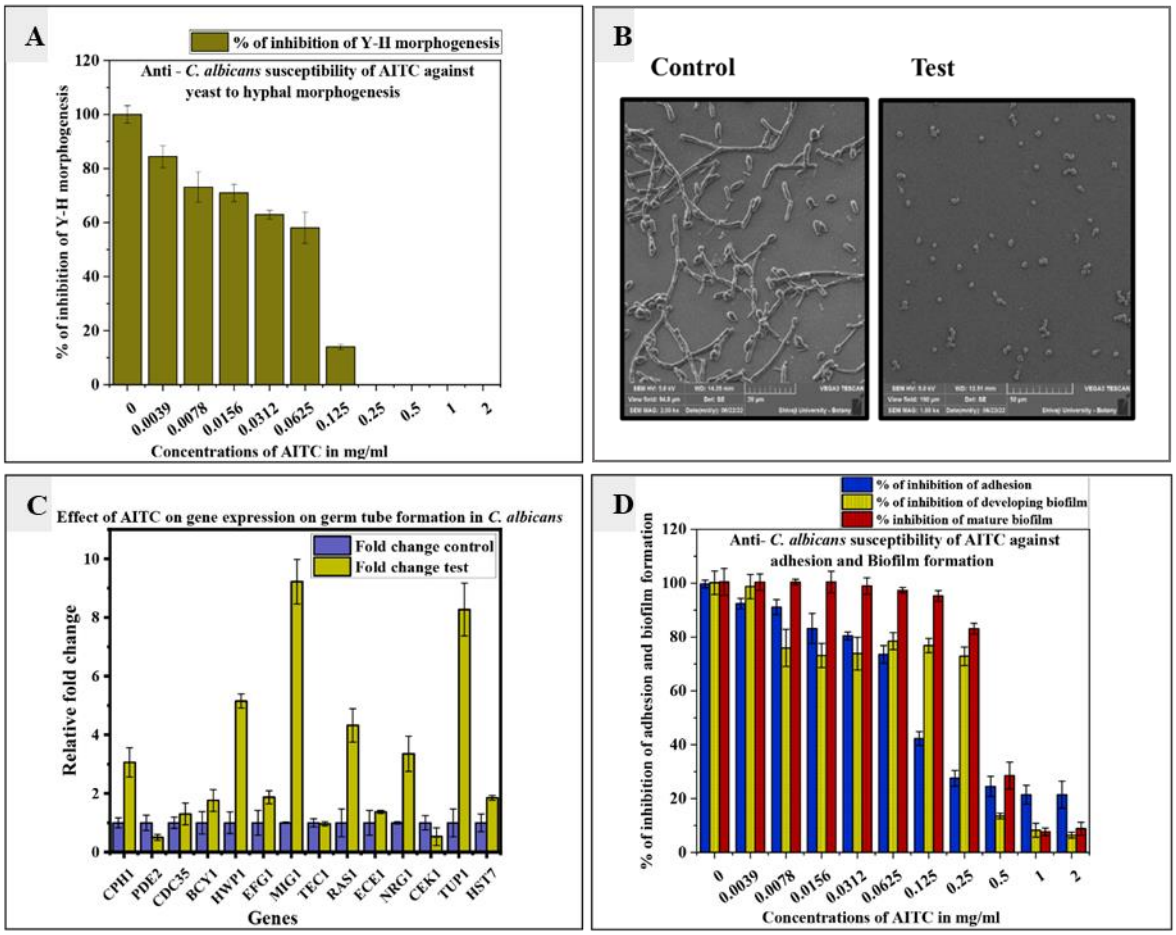


Figure 2.
A. Inhibitory effects of allyl isothiocyanate on *Candida albicans* germ tube formation in a concentration-dependent manner.
B. Scanning electron microscopy analysis to assess the effect of 0.125 mg/ml of allyl isothiocyanate on germ tube formation of *Candida albicans*.
C. Transcriptional profiles of *Candida albicans* cells on germ tube formation treated with and without allyl isothiocyanate.
D. Effect of allyl isothiocyanate on the adhesion, development of biofilm, and mature biofilm of *Candida albicans* to polystyrene plates.

Effect of allyl isothiocyanate on signal transduction gene expression involved in germ tube inhibition of Candida albicans

By usage of Qrt-PCR analysis, the impact of AITC on *C. albicans* germ tube inhibition was evaluated at the transcriptional level. The impact of 0.125 mg/ml concentration of AITC on the germ tube revealed a notable decrease in *C. albicans* hyphal development, compared to the non-treated control. Genes involved in the germ tube formation of *C. albicans* have their expression changed by the AITC treatment. The qRT-PCR analysis showed a relative fold change in the gene expressions. Allyl isothiocyanate inhibited virulence factors, like germ tube formation by downregulation of *PDE2*, *CEK1*, and *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 (Table 3, Figure 2C).

Effect of allyl isothiocyanate on adhesion of Candida albicans

Adhesion plays a vital role in biofilm formation and infection of *C. albicans*. The inhibitory effect of AITC and FLC on *C. albicans* on a polystyrene surface was quantified by XTT metabolic assay. The AITC inhibited the adhesion to the polystyrene surface to an extent of 50% at a concentration of 0.125 mg/ml. Moreover, AITC concentrations of 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, and 2 mg/ml significantly decreased adhesion to an extent of 28%, 25%, 21% and 21%, respectively (Figure 2D) while FLC was unable to inhibit the adhesion of *C. albicans* cells.

Effect of allyl isothiocyanate on biofilm formation (developing and mature biofilm)

The AITCs anti-biofilm activity was evaluated against the *C. albicans* ATCC 90028 strain. At a dose of 0.5 mg/ml, AITC suppressed the early or emerging biofilm and at the same concentration, AITC suppressed the mature biofilm as shown by the XTT metabolic assay (Figure 2D).

Toxicity effect of allyl isothiocyanate on human red blood cells

Toxicity of AITC was analyzed by *in vitro* hemolytic activity on human RBCs. It was observed that AITC was non-hemolytic in nature in a concentration range of 0.0039-2 mg/ml (Figure 3A).

In vivo study using silkworm animal model

In vivo experiment was conducted on silkworm (*Bombyx mori*) to examine the antifungal efficacy of AITC against *C. albicans*. Silkworms injected with *C. albicans* cells were considered positive control and silkworms injected only with PBS were considered negative control. Silkworms injected with AITC (0.125mg/ml) and *C. albicans* were considered the test group. Positive control silkworms died within 24 h, while negative control silkworms completed their life cycle and underwent the cocoon phase. However, silkworms injected with AITC and *C. albicans* cells survived and completed their life cycle (Figure 3B).

Discussion

Isothiocyanates are well-known antimicrobial substances used to fight bacteria and potentially harmful fungi. It is surprising that little research has been performed on how ITCs affect yeasts, like *C. albicans*. Very few studies have shown the effect of isothiocyanate on *C. albicans*. However, Pereira et al. in 2020 [24] reported that benzyl isothiocyanate (BITC) inhibits germ tube formation in *C. albicans*. They demonstrated that BITC treatment increased cell size and oxidative stress within the cell and significantly altered the ultrastructure of the cell wall. The BITC treatment causes adverse effects on the inner layer of the cell wall by interfering with the formation of glucans or the structure of the cell wall in *C. albicans*. The yeast-to-hyphal transition is a significant pathogenic factor in *C. albicans* infections. This inhibition is most likely caused by the effect on the cell wall of *C. albicans*. Overall, these effects may be able to influence *C. albicans* colonization by limiting or restraining the invasiveness of the organism and therefore, allowing host defenses to respond [24].

Anti-*Candida* activity of AITC alone and in combination with FLC was explored by Raut et al. in 2017 [12]. The activity of AITC against *C. albicans* pathogenicity and planktonic growth was concentration-dependent. At 1 mg/ml, the biofilm was significantly ($P \leq 0.05$) inhibited by AITC. Notably, the biofilm was not formed when 0.004 mg/ml of FLC and 0.125 mg/ml of AITC were combined. The AITC-FLC combination also significantly ($P \leq 0.05$) suppressed the developed biofilms. The fractional inhibitory concentration indices, which ranged from 0.132 to 0.312, showed that AITC and FLC worked together to prevent the development of both early and mature biofilms. Toxicity study analysis has suggested that AITC alone and in combination with FLC causes no hemolysis [12].

The present study brings new insight into the mechanism of action of AITC against *C. albicans*. Ergosterol is an important component of fungal cell membranes and a prime target of antifungal agents. The AITC inhibited ergosterol biosynthesis at its MIC and sub-MIC concentrations (Figure 1B). Both human and fungal cells are eukaryotic, and since antifungal medications target both of these cell types, there are fewer accessible targets for pharmacological action and significant adverse effects for patients. Many newer antifungals have been identified by appropriate studies. However, these molecules have not yet reached the clinical application levels.

Available antifungal drugs belong to various groups, such as azoles (FLC, itraconazole, ketoconazole, miconazole, and clotrimazole), polyenes (Amphotericin B, Nystatin), Allylamines, Thiocarbamates, Morpholines, 5-fluorocytosine (an analog of deoxyribonucleic acid), and Echinocandins. Azoles mainly act via targeting ergosterol biosynthesis. In the endoplasmic reticulum of the fungal cell, azoles prevent the enzyme lanosterol 14-demethylase from converting lanosterol into ergosterol, a component that is essential for the building of the plasma membrane structure of the fungus. As a result, the hazardous substances 14-methyl-3, and 6-diol will build up. As ergosterol concentration decreases, it causes changes to the cell membrane structure that prevents *C. albicans* growth [25,26]. In the current study, it was found that AITC significantly inhibited ergosterol biosynthesis in *C. albicans* (Figure 1B).

In the current study, it was found that AITC increased the ROS production in both planktonic and biofilm cells of *C. albicans*, compared to the control (Figure 1C). According to the reports, substances responsible for induction of ROS production may have promising antifungal properties. Many studies have shown that ROS-induced *C. albicans* apoptosis occurs in the presence of acetic acid, resveratrol, farnesol, and antimicrobial peptides. The ROS has apoptotic effects on a variety of cell types, including *C. albicans*. The fungicidal nature of commonly used antifungal medications, such as azoles, has been linked to their increased ROS effects in addition to their target-specific actions. Additionally, miconazole-tolerant *Candida* cells have higher activity in

inactivating ROS. Superoxide dismutase is a crucial component of *C. albicans* pathogenicity and one of the enzymatic and non-enzymatic antioxidant defense mechanisms found in *C. albicans* [27]. Therefore, the planktonic growth and virulence factor inhibiting the ability of AITC might be due to ROS production.

In addition, this study demonstrated that AITC triggered the *C. albicans* cell cycle by arresting cells at the G2/M phase (Figure 1D). 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. Wani et al. in 2021 and Phillips et al. in 2003 [28,29] reported that 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 cell cycle arrest [28,29].

SEM was used to examine how AITC affected the morphology of the *C. albicans*. The SEM analysis revealed that germ tube induction was visible in the control sample (Figure 2B), while it was completely absent in the AITC-treated sample and only smooth-walled spherical entities were present. Germ tube formation is a crucial component of the pathogenicity of *Candida*. In biofilms, the hyphae help the structure to become stable. To enhance antifungal therapy, inhibition of germ tube formation plays a crucial role [6,30].

We have investigated the expression of genes that are involved in the signal transduction pathway of germ tube formation. RAS1-cAMP-PKA and CEK1-Mitogen Activated Protein Kinase (MAPK) are two components of the germ tube formation signal transduction pathway [31]. The AITC targets a crucial element in the germ tube formation pathway. The CPH1 and EFG1 are the major transcription regulators of filamentous growth [5] which were upregulated by 1.43-fold and 1.78-fold, respectively (Figure 2C). *MIG1*, *NRG1*, and *TUP1* expressions were upregulated by AITC 9.22, 3.35, and 7.80-fold change respectively. The MIG1, TUP1, and NRG1 play crucial roles in the suppression of the Y-H form transition as they are negative regulators [32].

The expression of *PDE2*, *TEC1* and *CEK1* genes underwent 2.54-, 1.04-, and 1.91-fold decreases, respectively (Figure 2C). The PDE2, a high-affinity phosphodiesterase, was necessary for *C. albicans* hyphal growth and cell wall integrity. The downregulation of *PDE2* elevates cAMP levels, prevents normal hyphal development in a hypha-inducing liquid medium, and inhibits the formation of biofilms [33]. A transcription factor TEC1 is connected to morphogenesis and functions in controlling hyphal differentiation [34]. In *C. albicans*, four MAPK have been discovered, which play a significant role in the development of cell walls and biofilms. One of these four, the CEK1-MAPK, plays a key role in filamentous growth and is a key determinant of virulence in *C. albicans*. Downregulation of the *CEK1* gene expression can cause a reduction in germ tube or hyphal production and diminish virulence in *C. albicans* [35].

In order to stop the global spread of infections that are multi-drug resistant, there is an increasing need for new antibiotics. Silkworms are susceptible to the same pathogenic bacteria and fungi that may infect humans, and the same drugs that are used to treat human infections can also cure infected silkworms. The simplicity, low cost, and lack of ethical concerns of the silkworm as an animal model are some of its distinguishing features. The silkworm infection model is a good choice for evaluation of the therapeutic efficacy of antimicrobial medicines as it has a conserved gene sequence and similar pharmacokinetics to mammals [36,37].

In many studies it was demonstrated that the dose (ED_{50}) needed to treat 50% of fatal infections in silkworms is comparable to that needed to treat 50% of fatal infections in mice, indicating that the pharmacokinetics of these antibiotics are similar in silkworms and mammals. In many bacterial infection studies, silkworm has been used as a model to test the therapeutic potency of different antibacterial compounds.

Researchers have been able to successfully identify lysocin E, a novel antibiotic with a mode of action that involves binding to menaquinone to cause membrane damage and bactericidal activity. Other therapeutically effective novel antibiotics, including nosokomycin and ASP2397 (VL-2397), were discovered as a result of the same method used to screen *Candida*te antibiotics. This suggests that the silkworm antibiotic screening strategy is quite successful in identifying new antibiotics [36,37]. Similarly, in the current study, silkworm was used as a model organism to screen the drug. Based on the *in vivo* study, it was concluded that Planktonic MIC_{50} concentration (0.125 mg/ml) of AITC inhibited *C. albicans* infection and did not cause toxicity to the silkworm (Figure 3B). The findings of this study suggested that AITC may be a promising molecule for the development of a future antifungal drug.

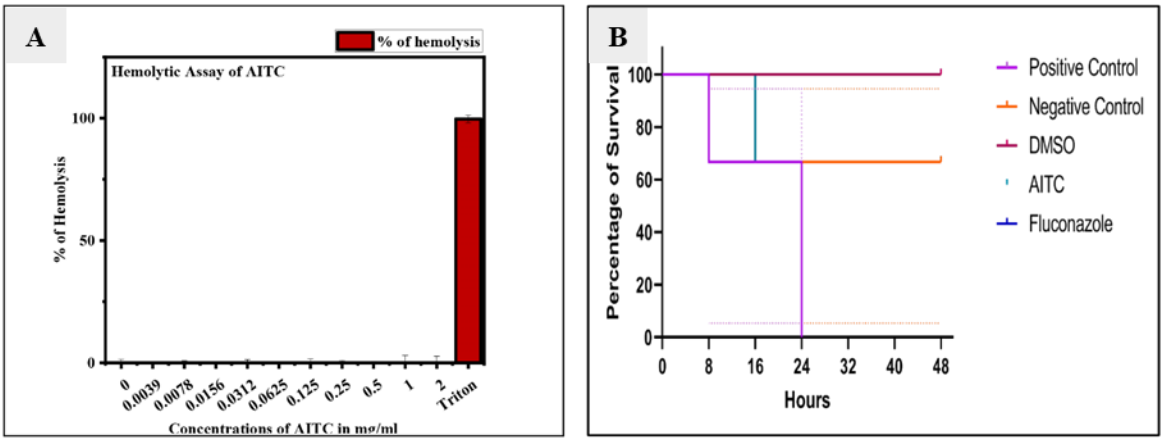


Figure 3.
A. Effect of allyl isothiocyanate on human red blood cells.
B. Effects of allyl isothiocyanate on *Candida albicans* infected silkworm. The graph indicates the percentage of worm survival after exposure of *C. albicans* to allyl isothiocyanate for 48 h.

Conclusion

The AITC is a potential inhibitor of growth and virulence factors in *C. albicans*. It alters the sterol profile and blocks ergosterol biosynthesis. Moreover, AITC produces ROS in both planktonic and biofilm cells and arrests cells at the G2/M pre-apoptic phase. The AITC alters the expression of genes involved in the signal transduction pathway which inhibits germ tube formation by downregulating *PDE2*, *CEK1*, and *TEC1* and upregulating *TUP1*, *MIG1*, and *NRG1* genes. Toxicity assay has revealed that AITC can be used as an alternate therapeutic option to treat candidiasis as it is non-toxic to human RBCs. *In vivo* study has proved that AITC also increases the survival rate of silkworms by inhibiting *C. albicans* infection. There is a need for further evaluation of AITC by performing *in vivo* studies on mice.

Acknowledgments

The authors are thankful to D. Y. Patil Education Society (Deemed to be University), Kolhapur 416 006, India for financial support through research project no. DYPES/DU/R&D/2021/273.

The authors are also thankful to Mr. Amit S. Salunkhe sir National Centre for Cell Science, Pune for the FACS facility, Dr. Mansingh Nimbalkar Department of Botany, Shivaji University Kolhapur, Maharashtra, India. For the SEM facility, and Dr. G. B. Kolekar and Mr. Omkar Nile for the Fluorescence spectrophotometer facility.

Authors' contribution

Sh. B. P.: Investigation, methodology, writing of the original draft, writing-review editing.
R. K. Sh.: Data curation and methodology.
T. G.: Data curation and methodology.
S. B.: Investigation and writing.
A. J.: Validation.
Sh. Y.: Interpretation of silkworm data.
S. M. K.: Conceptualisation and supervision.
Sh. B. P.: and R. K. Sh., both authors have equally contributed.
All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that there is no conflict of interest.

Financial disclosure

The authors are thankful to D. Y. Patil Education Society (Deemed to be University), Kolhapur 416 006, India for financial support through research project no. DYPES/DU/R&D/2021/273.

References

1. Rayens E, Norris KA. Prevalence and Healthcare Burden of Fungal Infections in the United States, 2018. Open Forum Infect Dis. 2022;9(1):ofab593.
2. Atriwal T, Azeem K, Husain FM, Hussain A, Khan MN, Alajmi MF, Abid M. Mechanistic Understanding of *Candida albicans* Biofilm Formation and Approaches for Its Inhibition. Front Microbiol. 2021;12:638609.
3. Yang L, Zhong L, Ma Z, Sui Y, Xie J, Liu X, Ma T. Antifungal effects of alantolactone on *Candida albicans*: An in vitro study. Biomed Pharmacother. 2022;149:112814.
4. Macias-Paz IU, Pérez-Hernández S, Tavera-Tapia A, Luna-Arias JP, Guerra-Cárdenas JE, Reyna-Beltrán E. *Candida albicans* the main opportunistic pathogenic fungus in humans. Rev Argent Microbiol. 2023;55(2):189-198.
5. Yan Y, Tan F, Miao H, Wang H, Cao Y. Effect of Shikonin Against *Candida albicans* Biofilms. Front Microbiol. 2019;10:1085
6. Feldman M, Sionov RV, Mechoulam R, Steinberg D. Anti-Biofilm Activity of Cannabidiol against *Candida albicans*. Microorganisms. 2021;9(2):441.
7. Pereira R, dos Santos Fontenelle RO, De Brito EH, De Morais SM. Biofilm of Candida albicans: formation, regulation and resistance. J Appl Microbiol. 2021;131(1):11-22.
8. Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJS. *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol. 2013;62(1):10-24.
9. Pereira R, dos Santos Fontenelle RO, de Brito EHS, de Morais SM. Biofilm of *Candida albicans*: formation, regulation and resistance. J Appl Microbiol. 2021;131:11–22.
10. Zhang K, Li X, Yu C, Wang Y. Promising therapeutic strategies against microbial biofilm challenges. Front. Cell. Infect. Microbiol. 2020;10:359.
11. Soliman S, Alnajdy D, El-Keblawy AA, Mosa KA, Khoder G, Noreddin AM. Plants' Natural Products as Alternative Promising Anti-*Candida* Drugs. Pharmacogn Rev. 2017;11(22):104-122.
12. Raut JS, Bansode BS, Jadhav AK, Karuppayil SM. Activity of Allyl Isothiocyanate and Its Synergy with Fluconazole against *Candida albicans* Biofilms. J Microbiol Biotechnol. 2017;27(4):685-693.
13. Priya A, Pandian SK. Piperine Impedes Biofilm Formation and Hyphal Morphogenesis of *Candida albicans*. Front Microbiol. 2020;11:756.
14. Clinical ALSI. Document M27-A4. Ref Method Broth Dilution Antifung Suscetibility Test Yeasts. 2012.
15. Peng C, Liu Y, Shui L, Zhao Z, Mao X, Liu Z. Mechanisms of Action of the Antimicrobial Peptide Cecropin in the Killing of *Candida albicans*. Life (Basel). 2022;12(10):1581.
16. Ahmad A, Khan A, Manzoor N, Khan LA. Evolution of ergosterol biosynthesis inhibitors as fungicidal against *Candida*. Microb Pathog. 2010;48(1):35-41.
17. Sun L, Liao K, Hang C, Wang D. Honokiol induces reactive oxygen species-mediated apoptosis in *Candida albicans* through mitochondrial dysfunction. PLoS One. 2017;12(2):e0172228.
18. Zore G, Thakre A, Abdulghani M, Bhosle K, Shelar A, Patil R, Kharat K, Karuppayil S. Menthol Inhibits *Candida albicans* Growth by Affecting the Membrane Integrity Followed by Apoptosis. Evid Based Complement Alternat Med. 2022;2022:1297888.
19. Sun AM, Wang YX, Hu GX, Li L, Wang RR. Polyphyllin I Effects *Candida albicans* via Inhibition of Virulence Factors. Evid Based Complement Alternat Med. 2023;2023:5645500.
20. Yang L, Liu X, Sui Y, Ma Z, Feng X, Wang F, Ma T. Lycorine Hydrochloride Inhibits the Virulence Traits of *Candida albicans*. Biomed Res Int. 2019;2019:1851740.
21. Ahmedi S, Pant P, Raj N, Manzoor N. Limonene inhibits virulence associated traits in *Candida albicans*: In-vitro and in-silico studies. Phytomedicine Plus. 2022;2(3):100285.
22. Matsumoto H, Nagao J, Cho T, Kodama J. Evaluation of pathogenicity of *Candida albicans* in germination-ready states using a silkworm infection model. Med Mycol J. 2013; 54(2):131-40.
23. De Zoysa GH, Glossop HD, Sarojini V. Unexplored antifungal activity of linear battacin lipopeptides against planktonic and mature biofilms of *C. albicans*. Eur J Med Chem. 2018;146:344-353.
24. Pereira C, Calado AM, Sampaio AC. The effect of benzyl isothiocyanate on *Candida albicans* growth, cell size, morphogenesis, and ultrastructure. World J Microbiol Biotechnol. 2020;36(10):153.
25. de Oliveira Santos GC, Vasconcelos CC, Lopes AJO, de Sousa Cartágenes MDS, Filho AKDB, do Nascimento FRF, Ramos RM, Pires ERRB, de Andrade MS, Rocha FMG, de Andrade Monteiro C. *Candida* Infections and Therapeutic Strategies: Mechanisms of Action for Traditional and Alternative Agents. Front Microbiol. 2018;9:1351.
26. Vandeputte P, Ferrari S, Coste AT. Antifungal resistance and new strategies to control fungal infections. Int J Microbiol.

2012;2012:713687.
27. Celiksoy V, Moses RL, Sloan AJ, Moseley R, Heard CM. Synergistic activity of pomegranate rind extract and Zn (II) against *Candida albicans* under planktonic and biofilm conditions, and a mechanistic insight based upon intracellular ROS induction. Sci Rep. 2022;12(1):19560.
28. Wani MY, Ahmad A, Aqlan FM, Al-Bogami AS. Citral derivative activates cell cycle arrest and apoptosis signaling pathways in *Candida albicans* by generating oxidative stress. Bioorg Chem. 2021;115:105260.
29. Phillips AJ, Sudbery I, Ramsdale M. Apoptosis induced by environmental stresses and amphotericin B in *Candida albicans*. Proc Natl Acad Sci U S A. 2003;100(24):14327-32.
30. Chow EWL, Pang LM, Wang Y. From Jekyll to Hyde: The Yeast-Hyphal Transition of *Candida albicans*. Pathogens. 2021;10(7):859.
31. Biswas S, Van Dijck P, Datta A. Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. Microbiol Mol Biol Rev. 2007;71(2):348-76.
32. Halbandge SD, Jadhav AK, Jangid PM, Shelar AV, Patil RH, Karuppayil SM. Molecular targets of biofabricated silver nanoparticles in *Candida albicans*. J Antibiot (Tokyo). 2019;72(8):640-644.
33. Chen S, Xia J, Li C, Zuo L, Wei X. The possible molecular mechanisms of farnesol on the antifungal resistance of *C. albicans* biofilms: the regulation of CYR1 and PDE2. BMC Microbiol. 2018;18:1-4.
34. Freire F, de Barros PP, Pereira CA, Junqueira JC. Photodynamic inactivation in the expression of the *Candida albicans*. Laser Med Sci. 2018;33(77): 1447-1454.
35. Roman E, Alonso-Monge R, Gong Q, Li D, Calderone R, Pla J. The Cek1 MAPK is a short-lived protein regulated by quorum sensing in the fungal pathogen *Candida albicans*.. FEMS YEAST. 2009;9(6):942-55.
36. Panthee S, Paudel A, Hamamoto H, Sekimizu K. Advantages of the Silkworm As an Animal Model for Developing Novel Antimicrobial Agents. Front Microbiol. 2017;8:373.
37. Meng X, Zhu F, Chen K. Silkworm: A Promising Model Organism in Life Science. J Insect Sci. 2017;17(5):97.

Curriculum Vitae

CONTACT

Dr. Rakesh Kumar Sharma

Mobile: +919820619211

E-mail:
drrksharmaz@gmail.com

RESEARCH AND PUBLICATIONS

Research Papers: (33)

Research Book: (1)

Dr. Rakesh Kumar Sharma

EDUCATION

Year	Summary of Qualification
1976-1981	M.B.B.S.
1988-1990	M.D. (O & G).
2016-2017	Post Graduate Diploma in Health Economics, Health Care Financing and Policy.
2018	Fellowship in Minimal Access Surgery (FMAS).
2019-2020	Post Graduate Diploma in Quality Management of Hospital & Healthcare Organization.
2019-2020	Fellowship in Minimal Access Surgery-Gynecology (FMASG)
2020-2021	Post Graduate Diploma in Medicolegal System.

EXPERIENCE

2004-2024	Professor in Obstetrics & Gynecology
2012-2024	Dean Medical Colleges (Mauritius & Kolhapur)

MEMBERSHIP of PROFESSIONAL BODIES:

1. Life member of the Indian Medical Association.
2. Member of the National Academy of Medical Sciences (India) 2019.
3. Life member of the Medical Consultants Association.
4. Member, Independent Ethics Committee for Research on Human Beings, Mumbai, India.
5. Life Member, Family Planning Association of India.
6. Life Member, Navi-Mumbai Obstetrics & Gynecological Society.
7. Member of Governing Council, National Society for Equal Opportunities for The Handicapped (NASEOH), Chembur, India.
8. Governing Body Member of Navi Mumbai Obstetrics and Gynecologists Society for 4 years from 2005-2007.
9. Life Member, Forum for Ethics Review Committees in India (FERCI).
10. Life member, Indian Association of Gynecological Endoscopist (IAGE).
11. Member, Governing Body of the Indian Council of Medical Research (ICMR).

Conference Certificates

The Fetal Medicine Foundation India

IMAGING QUEST

IMAGING QUEST

AN INITIATIVE OF IMAGING SCIENCE COMMITTEE FOGSI
IN COLLABORTION WITH FMF INDIA

Certificate of Attendance

PRESENTED TO

Dr. Rakesh Kumar Sharma

FOR ATTENDING

AN STRUCTURED ONLINE COURSE IN
BASIC OBSTETRIC ULTRASOUND **(FULL COURSE)**
FROM 26 NOVEMBER 2020 TO 7 MARCH 2020

Alpesh Gandhi	**Archana Baser**	**Jaydeep Tank**	**Anita Kaul**	**Meenu Agarwal**
President, FOGSI	Vice President, FOGSI	Secretary-General, FOGSI	President, FMF, India	Chairperson, Imaging Science Committee, FOGSI

CERTIFICATE OF PARTICIPATION

This is to certify that

Dr Rakesh Kumar Sharma

has successfully participated in the two-day National Webinar on “Attainment of Quality Benchmark in Medical Education: Responsibilities of a Health Institution” organized by IQAC, KGMU in collaboration with ECHO India on December 3rd - 4th, 2021.

Professor Divya Mehrotra
Organizing Chairperson, IQAC
King George's Medical University, U.P.

Dr. (Col) Kumud Rai
Chairman, ECHO India

This certificate is issued by KGMU using iECHO.To verify the certificate, please scan this QR code.

सत्यमेव जयते

Azadi Ka Amrit Mahotsav

राष्ट्रीय जैविक संस्थान NATIONAL INSTITUTE OF BIOLOGICALS

CERTIFICATE OF PARTICIPATION

This is to certify that

Prof./Dr./Mr./Ms.

Dr Rakesh Kumar Sharma

has participated as a Participant in the

FIRST NATIONAL CONFERENCE ON HAEMOVIGILANCE

Organized by

National Institute of Biologicals, NOIDA

Ministry of Health & Family Welfare, Government of India

Online Mode

(9th to 11th December, 2021)

DR. ANUP ANVIKAR

Director, National Institute of Biologicals

Ministry of Health & Family Welfare

Government of India

XIII NATIONAL CONFERENCE ON HEALTH PROFESSIONS EDUCATION

(Under the Aiges of Indian Association of Health Professions Education)

2nd & 3rd November 2022

Himalayan Institute of Medical Sciences
Swami Rama Himalayan University
Swami Ram Nagar, Jolly Grant, Dehradun - 248016, Uttarakhand

Certificate

This is to certify that

Dr R akesh Kumar Sharma

has participated in the

National Conference of Health Professions Education (NCHPE) *2022*

as a Delegate / Chairperson / Resource Person / Volunteer.

Uttarakhand Medical Council has awarded six credit hours for the participation.

Dr. Juhi Kalra
Organizing Secretary

Dr. Vijendra D. Chauhan
Organizing Chariman

D. Y. PATIL EDUCATION SOCIETY
(Deemed to be University), KOLHAPUR
NAAC 'A+' Grade in 3rd Cycle

Certificate

This is to certify that **Prof. Dr. Rakesh Kumar Sharma,** Dean, D. Y. Patil Medical College, Kolhapur has delivered key note address 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.

Dr. Jayavant L. Gunjakar
Convener

Prof. Meghnad G. Joshi
Convener

Prof. Chandrakant D. Lokhande
Chairman

D. Y. PATIL EDUCATION SOCIETY
(Deemed to be University), KOLHAPUR
NAAC 'A+' Grade in 3rd Cycle

Certificate

This is to certify that **Dr. Rakesh Kumar Sharma,** Dean, D. Y. Patil Medical College, Kolhapur has chaired the session 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.

Dr. Jayavant L. Gunjakar
Convener

Prof. Meghnad G. Joshi
Convener

Prof. Chandrakant D. Lokhande
Chairman