"A STUDY ON THE ANTIFUNGAL EFFICACY OF ANTIVIRAL DRUGS IN *CANDIDA ALBICANS* "

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UNDER THE FACULTY OF

INTERDISCIPLINARY STUDIES

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DECLARATION

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LIST OF PATENTS, COPYRIGHT AND PUBLICATIONS

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LIST OF ATTENDED CONFERENCES, WORKSHOP AND HANDS ON TRAINING

Poster/paper presented in conferences (03)

1. Presented paper entitled "2-Adamantylamine hydrochloride synergistically activate fluconazole and amphotericin B against *Candida albicans"* in international conference on "Emerging and re-emerging infections-2022" organized by the Department of Microbiology, D. Y. Patil Medical College, Kolhapur on 8th April 2022.

2. Presented poster entitled "Anti-morphogenic effect of Ganciclovir on human pathogenic yeast *Candida albicans*" in two-day international conference on "Emerging Trends in Applied Microbiology and Food Sciences" organized by Yashwantrao Chavan Institute of Science, Satara and held on 2nd - 3rd December 2022.

3. Presented paper entitled "*In silico* study of the binding affinity of phenylpropanoids with SARS-CoV-2 and Omicron variant (B1.1.529) spike proteins" in international conference on "Approaches towards affordable clean energy, good health and well-being" organized by the department of Life Sciences, Garden City University In association with Karnataka State Bioenergy Development Board on 2nd - 3rd March 2023.

Workshops/ hands on trainings attended (04)

1. Attended workshop and Hands on training on BIO-ATOMIC FORCE MICROSCOPY(BIO-AFM) organized by Shivaji University, Kolhapur during 4th-5th January 2022.

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3. Attended workshop on ‘3D Bioprinting Technique’ organized by Centre for Interdisciplinary Research, D. Y. Patil Education Society (Deemed to be University), Kolhapur on 6th January 2023.

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ABBREVIATIONS

2-AM	2-Adamantanamine Hydrochloride
2-DG	2-Deoxy Glucose
5-FC	5-Fluorocytosine
AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
AmB	Amphotericin B
AO	Acridine Orange
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B. mori	*Bombyx mori*
BCY1	Bypass of Cyclic AMP
C. albicans	*Candida albicans*
CAS	Caspofungin
CAUTI	Catheter-Associated Urinary Tract Infection
CD4	cluster of differentiation
CDC	Cell Division Cycle
CDR	Candida Drug Resistance Genes
CEK1	Extracellular signal-regulated kinase 1
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standard Institute
CMV	Cytomegalo Virus
COVID-19	Coronavirus Disease 2019
CPH1	Candida Pseudo Hyphal regulator 1
D/W	Distilled Water
DAPI	4',6-Diamidino-2-Phenylindole
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECE1	Extent Of Cell Elongation Protein 1
EFG1	Enhanced Filamentous Growth Protein 1
ERG	Lanosterol Demethylase Gene
EtBr	Ethidium Bromide
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovin serum
FDA	Food and Drug Administration
FIC	Fractional Inhibitory Concentration
FITC	Fluorescein isothiocyanate
FLC	Fluconazole
G1	Gap1 phase
G2/M	Gap 2 and Mitosis phase
H_2DCFDA	2,7dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen Peroxide
HBV	Hepatitis-B virus
HCV	Hepatitis-C virus
HIV	Human Immunodeficiency virus
HST7	Serine/threonine-protein kinase homolog
HSV	Herpes Simplex Virus
HWP	Hyphal wall protein 1
IC_{50}	Inhibitory concentration
ICU	Intensive Care Unit
MERS	Middle East respiratory syndrome
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
MIG1	Multicopy Inhibitor of GAL gene
MOPS	Morpholinepropanesulfonic Acid
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors
NRG1	Negative regulator of glucose-controlled gene
O.D.	Optical Density
p53	Tumor Protein P53
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDE2	Cyclic nucleotide phosphodiesterase2
PI	Propidium Iodide
PIs	Protease Inhibitors
PS	Phosphatidylserine
RAS1	Ras-like protein 1
RdRP	RNA-dependent RNA polymerase
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RPM	Rotation Per Minute
RPMI	Roswell Park Memorial Institute
SDA	Sabouraud Dextrose Agar
SEM	Scanning Electron Microscopy
S phase	Synthesis phase
SAP	Secreted Aspartyl Protease
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2

TEC1	TEA/ATTS consensus DNA binding domain
TUP1	Transcriptional repressor for hyphal growth
U.V.	Ultraviolet
VZV	Varicella zoster virus
XTT	2, 3-bis-(2-methoxy-4- nitro-5-sulfophenyl)-2H tetrazolium 5- carboxanilide
Y-H	Yeast to Hyphal transition
YPD	Yeast Peptone Dextrose

"A STUDY ON THE ANTIFUNGAL EFFICACY OF ANTIVIRAL DRUGS IN *CANDIDA ALBICANS* "

ABSTRACT

Candida albicans serves as the primary pathogenic agent responsible for infections such as invasive candidiasis. Available antifungal agents are challenged by the drug resistance, toxicity, which limits their efficiency. Drug repurposing provides an alternative approach for *de novo* method of antifungal drug development. In the current investigation, ten antiviral drugs were screened against *C. albicans.* Among them, 2-Adamantanamine hydrochloride (2-AM), 2-deoxy glucose (2-DG), Ribavirin and Vidarabine affected planktonic growth. 2-AM, 2-DG, and Ganciclovir inhibited yeast to hyphal morphogenesis.2-AM, 2-DG, Ribavirin, and Vidarabine caused disruption of the cell membrane and ROS accumulation. The fungicidal action of 2-AM and Vidarabine resulted in G2/M phase arrest and induced cell apoptosis and necrosis. Upregulation of *MIG1, TUP1,* and *NRG1* by anti-morphogenic drugs resulted in the inhibition of morphogenesis. Combination studies showed except Ribavirin all other antivirals exhibited synergism with fluconazole. Vidarabine showed synergistic activity with all the tested antifungal drugs. Anti-biofilm action of 2-AM suggest its application in catheter coating. The *in vivo* anti-*Candida* efficacy of 2-AM, 2-DG, Ganciclovir, Ribavirin and Vidarabine was confirmed in a *C. albicans*-infected *Bombyx mori* animal model. Overall, this study demonstrates the applicability of antiviral drugs in the development of therapeutic agents against *C. albicans.*

Keywords: Antiviral drugs, *Bombyx mori, Candida albicans,* Drug repurposing, Morphogenesis, ROS

CHAPTER 1

Introduction

1. Introduction

1.1. *Candida albicans:* a potential pathogenic threat

Recent research indicates that ~1.9 million individuals suffer from acute invasive fungal infections, whereas ~3 million people globally are estimated to have chronic severe fungal infections. More than ~1.6 million annual deaths are caused by all fungal diseases, many of which are life-threatening infections [1]. Fungal infections are caused by a diverse range of pathogens, including *Candida albicans,* which may adversely affect the skin and mucosal surfaces (**Fig. 1.1**). Approximately 400,000 systemic fungal infections are caused by *Candida* species and among these species, *C. albicans* is most prevalent pathogen responsible for systemic and mucosal infections, and accounting for approximately 70 % of fungal infections worldwide. Systemic candidiasis caused by *C. albicans* is responsible for majority of morbidity and mortality cases [2].

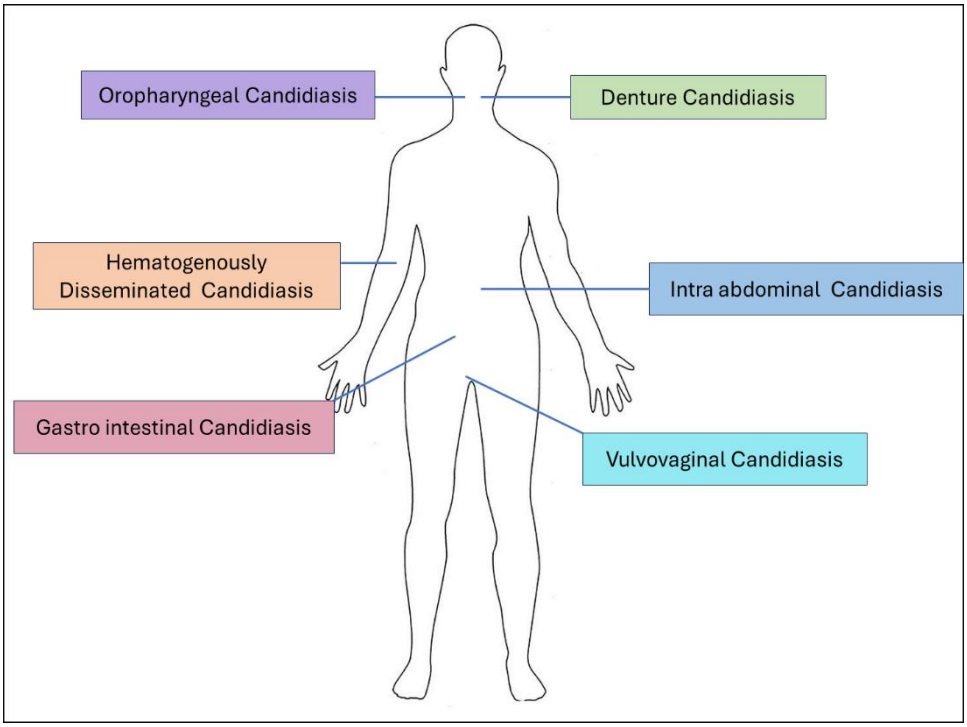

Figure 1.1. The site-specific candidiasis caused by human fungal pathogen *C. albicans.*

The prevalence of *C. albicans* infection has increased over time due to the rise in the number of immunosuppressives drugs in case of solid organ transplantation, and wide spectrum antibiotics particularly in cancer chemotherapy [3]. A serious healthcare challenge is invasive candidiasis, that is common in patients who belongs to immunosuppressed group or have experienced significant trauma [4].

Despite improvements being made to our arsenal of antifungal drugs and discovery of new therapeutic targets, the rate of drug resistance development much outpaces that of antifungal development. As fungi are eukaryotic organisms with a close evolutionary link to their human hosts, fungi pose a significant difficulty to pharmaceutical industry in developing new antifungal drugs. Evolution of drug resistance in *C. albicans* and increased incidences of mycoses in clinical settings challenged current antifungal therapeutics. It is critically necessary to increase the effectiveness of existing treatments along with developing new therapeutic approaches. To expand the use of present antifungal agents, it is worthwhile to consider novel strategies, such as combinations of currently available drugs. Anticipating a synergistic activity from a combination of drugs can indeed decrease the development of drug resistance by utilizing several mechanisms or targets [5].

Several other alternative approaches are being explored along with combination therapy such as altering host immune response, and development of new anti-microbial agent [6]. It is worthwhile to take into account novel approaches or, more simply, approaches based on carrying out antifungal therapies such as synthesis of new compounds, drug repurposing, therapeutic use of organism extracts, altering the dosage forms or modes of administration of established drugs for treating fungal diseases. Discovering combination relationships between known antifungal agents and non-antifungal agents is also serve as an effective alternative [7]. Among these, drug repurposing approach achieved much attraction.

1.2. Drug repurposing: an alternative approach

Drug repurposing or repositioning or therapeutic switching is an alternative strategy to find out new therapeutic agents from the already clinically approved drugs [8]. The aim of drug repurposing is to develop a medication outside of its original indication with an objective to gain a new regulatory approval. This will further enhance patient access, prescribing information, and treatment recommendations [9]. Drug repurposing approach based on the fundamental scientific concepts as (i) various drug targets or pathways frequently interacted with the single drugs, and (ii) distinct drugs might interact with same target or pathways [10]. Most of the findings suggested that repurposed drugs exhibit less or no link to the approved preliminary indication. One example of this approach is a compound often exhibit off-target actions that result in undesirable side effects. For certain indications, these effects may seem advantageous

in repurposed indication [11]. Comparing drug repurposing with traditional drug development procedures, there are several benefits, including reduced research durations, lower costs, and complexity. Developing a new therapeutic agent using the conventional drug discovery approach takes 10-12 years. In contrast, the drug repurposing approach is expected to require 1-3 years [8].

As in this strategy the safety and toxicity profile of the drug is already known, the initial stages of drug development processes are completed. Hence, it reduces the cost of drug development and increases probability of drug to be marketed [12].

1.3. Orientation and purpose of the current study

Increased resistance in *C. albicans* has challenged the current antifungal therapeutics hence there is a need to explore novel antifungal agents against *C. albicans*. The drug repurposing strategy has provided an alternative approach to full fill the urgent need for antifungal agents.

Literature is available on repurposed non-antifungal agents against *C. albicans* and they include drugs approved by Food and Drug administration (FDA) such as anti-bacterial, anti-cancer, anti-depressants, anti-malarial, anti-viral drugs. Moreover, there is still an opportunity for improvement in the areas such as finding of an essential target that are required for pathogen survival or any factor contributing to the pathogen virulence, and exploring probable therapeutic strategies. However, in current study, drug repurposing strategy has been used against *C. albicans* and explored the possible antifungal action of antiviral drugs that may contribute to finding a novel target or solution to the present drug targets.

From the group of antiviral drugs, Protease Inhibitors (PIs) initially approved against Human Immunodeficiency Virus (HIV) were studied against *C. albicans* and reported have significant antifungal activity either alone or in combination with other antifungal agents [13]. However, the PIs have its own side effects and restrictions that limit their use. Apart from the conventional PIs, present investigation focuses on the repurposing of other antiviral drugs for antifungal activities against *C. albicans*. By adapting methods mentioned in the Clinical & Laboratory Standards Institute (CLSI) guidelines, antiviral agents have been screened and studied for antifungal action against *C. albicans.* Selected antiviral drugs are majorly belong to the group of nucleoside/ nucleotide analogues which are altering the central dogma of the cell.

The **objectives** designed for this study,

1. To screen FDA approved antiviral drugs against the growth and virulence factors present in *C. albicans* ATCC 90028.
2. To find out synergistic combinations of effective antiviral drugs with antifungal drugs.
3. To study effective antiviral drugs for their probable mode of action against *C. albicans* and *in vivo* antifungal efficacy in *Bombyx mori*.

Table 1.1 shows the list of FDA approved antiviral drugs used for screening purpose in the study. The structures of listed antiviral drugs retrieved from PubChem database (**Fig. 1.2**).

Table 1.1. List of antiviral drugs used for screening purpose against *C. albicans*

Sr. No.	Antiviral drugs	Original approved indication	Mechanism of action	Ref.
1.	2- Adamantanamine hydrochloride (2-AM)	Influenza A	Blockage of M2 ion channel of virus	[14]
2.	2-Deoxy glucose (2-DG)	Herpes Simplex Virus (HSV)-1	Competitive inhibitor of glycolysis	[15]
3.	Acyclovir	HSV-1, 2	Acyclic nucleoside analogue	[16]
4.	Famciclovir	Herpes zoster virus	Guanosine analogue	[17]
5.	Ganciclovir	Cytomegalovirus (CMV)	Guanosine analogue	[18]
6.	Idoxuridine	HSV	Thymidine analogue	[19]
7.	Lamivudine	HIV-1	cytosine analogue	[20]
8.	Ribavirin	Hepatitis C virus (HCV)	Guanosine analogue inhibits	[21]
9.	Valganciclovir	CMV	Prodrug of ganciclovir	[22]

10.	Vidarabine	HSV, Varicella zoster virus (VZV), CMV	Purine nucleoside analogue	[23]

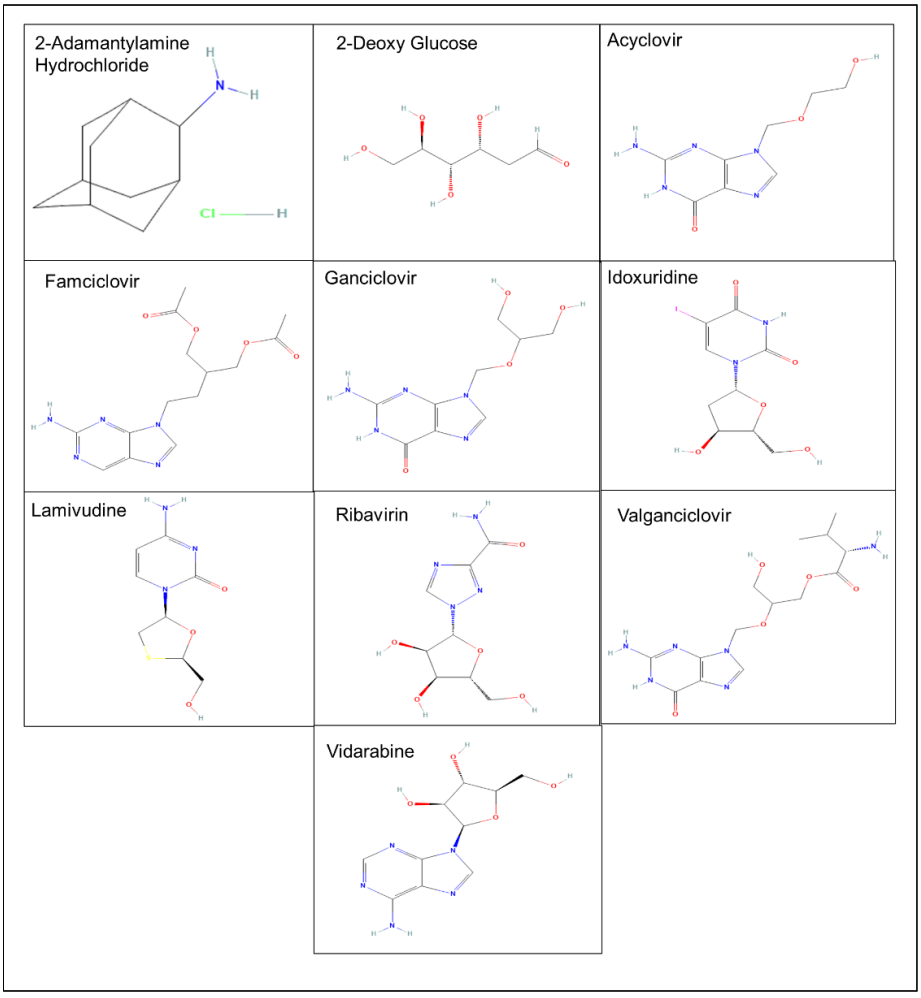

Figure 1.2. Structures of selected antiviral drugs used for screening against *C. albicans* for antifungal activities**.**

1.4. Significance of the work

The present investigation could widen the application of the drug repurposing approach for antifungal drug development process. This study offers the application of antiviral drugs as anti-*Candida* agents to manage drug resistance and increase the susceptibility of *C. albicans* to routine antifungals as well.

From the perspective of drug repurposing for fungal infection, most appealing class of drug would be that one which possess activity against a range of infectious

agents but has no proven and documented antifungal activity till date. So, this study identifies the antiviral drugs belonging to this class that can prevent *C. albicans* growth and proliferation.

From clinical perspective, nucleoside analogues belong to the diverse class of pharmacologically active drugs with medication in ribose or 2′-deoxyribose nucleosides. This class of drugs is mainly used as anti-cancer and anti-viral agents in clinical settings. Due to the ability to mimic endogenous nucleosides, these analogues get incorporated into the DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), resulting in termination of the chain elongation that leads to prevention of viral replication and in certain cases cancer proliferation also. Some literature is available that reports the ability of nucleoside analogues to act as anti-bacterial, anti-protozoal and anti-fungal agents [24].

In the treatment of fungal infections, fluorinated pyrimidines such as 5-fluorocytosine (5-FC) are widely used. Although alone 5-FC is not an active antifungal agent, but it is commonly used in combination with amphotericin B (AmB). 5-FC is a 5 -fluorouracil prodrug and uptake of 5-FC by fungal cells converts it into 5-fluorouracil and is subsequently incorporated in the DNA, and RNA leading to its antifungal action [25]. In the same way, in the current study the majority of drugs, except 2-AM and 2-DG belongs to the nucleoside/tide analogues group.

Acyclovir, and Ganciclovir are antiviral drugs approved by FDA for treatment against HSV and CMV infection. Both of these antiviral drugs are acyclic guanosine analogues that compete with the naturally occurring deoxyguanosine triphosphate and inhibit the DNA replication [18]. Valganciclovir is a prodrug of Ganciclovir with a higher bioavailability and gets metabolized in to Ganciclovir and exerts the termination effect on DNA [15]. Famciclovir is a prodrug of Penciclovir, and after activation in cells it competes with deoxyguanosine triphosphate. Its initial indication was against VZV, and HSV.

Lamivudine, a nucleoside reverse transcriptase inhibitor, is a dideoxynucleoside cytosine analogue and approved against HIV-1 and Hepatitis-B viral infections. Upon cell entry, the Lamivudine gets metabolized to form its triphosphate and mono phosphate and then inhibits DNA synthesis [22]. The very first antiviral drug approved by the United States FDA was Idoxuridine, which is a deoxy uridine and thymidine analogue and used against HSV [15]. Another purine nucleoside analogue, Ribavirin is an anti-HSV drug with a wide spectrum of activity [26]. Reports are available on antifungal

activities of Ribavirin against *C. albicans*. Being a guanosine analogue Ribavirin triphosphate competes with natural nucleosides and terminates the viral polymerase [27]. Vidarabine is an antiviral drug approved against HSV, and poxvirus. Vidarabine is an adenosine analogue that gets phosphorated in vidarabine triphosphate by cellular enzymes and incorporated in the viral and, to a lesser extent, in to the cellular DNA [25].

2-AM and 2-DG are other than the nucleoside/tide analogue group of antivirals and used in current study to identify their antifungal activities. 2-AM is an anti-influenza drug and a derivative of amantadine. Amantadine blocks proton transport of the M2 proton channel in influenza A type virus [28]. Reports on activity of 2-Adamantanamine against *C. albicans* are available. Whereas, current study deals with the anti-*Candida* action of 2-Adamantanamine salt.

Another drug, 2-DG is a glucose molecule with a replaced hydrogen in place of 2-hydroxyl group. Due to this replacement, cells are unable to convert glucose into ATP. Previous studies already reported anti-cancer, anti-viral properties of 2-DG [29]. The current investigation focused on finding newer pharmacological indication of these approved (**Table 1.1**) antiviral drugs to target *C. albicans.*

Previously available studies on the nucleoside analogues with anti-viral, anti-cancer, anti-bacterial, anti-protozoal, and to some extent anti-fungal activities encouraged to continue research on their detailing of antifungal ability. The current study is based on a drug repurposing approach to investigate the possibilities of exploring the antifungal activities of antiviral drugs. This investigation may improve the understanding of repurposing these antiviral drugs against *C. albicans* and lead to discovery of an alternative therapeutic agent. Accordingly, the study was designed to investigate potential of antiviral drugs against most common human pathogenic fungi *C. albicans.*

1.5. References

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CHAPTER 2

Review of Literature

2. Review of literature

2.1. *C. albicans*: commensal to infectious fungal agent

The human gut mycobiome is colonized by more than 66 genera and 184 fungal species. Among these fungal species, *Saccharomyces, Candida,* and *Cladosporium* fungal species are predominant [1]. Numerous yeasts belonging to the *Candida* genus form a symbiotic relationship with the human host. *C. albicans* colonizes in the early stages of life, and it resides on the mucosal surfaces, specifically in the oral cavity, gastrointestinal track, and also in the vaginal cavity [2]. *C. albicans* is the most commonly detected in nearly about 60 % of healthy individuals, and thus it is in brief described as a ubiquitous member of the human gut biome [3]. Under normal conditions, innate and acquired immunity of host and other microbiota associated with the host exert a combined effect to ensures the commensal nature of *C. albicans* in healthy individuals [4]. Besides this commensal action, *C. albicans* can infect the host and can cause different infections especially in immunocompromised or immunocompetent individuals. *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis* and *C. krusie* (now known as *Pichia kudriavzevii*) these five fungal pathogens serve as main causative agents for invasive candidiasis.

2.2. Infections caused by *C. albicans*

It is estimated that pathogenic fungi infect billions of people worldwide and they are either present in the environment or in human host as a commensal or symbiont [5]. In spite of early treatment with antifungal drugs this fungal infection has reached mortality rate up to 40 %. Among worldwide reported cases of invasive candidiasis, 50 % of infection cases were reported by alone *C. albicans* fungal species [6-7].

Again *C. albicans* stands the most common candidemia causing species with higher ability to adhere gingival epithelial cells [8]. As per previous studies, *C. albicans* was isolated from 50 % of hospitalized condition with candiduria infection. This infection become challenge and a risk factor for patients with renal transplantation [9]. The species *C. albicans* usually serves as the source of invasive candidiasis, a deadly infection that can cause sepsis, multiorgan failure, and septic shock [10].

Most of studies reported that extended Intensive care unit (ICU) admissions, prolong use of broad-spectrum antibiotics, mucosal colonization, chemotherapy, medical implants (catheters and heart valves), parenteral nutrition, gastrointestinal tract

surgeries, and renal failure are significant risk factors for *C. albicans* infection [11]. In addition, *C. albicans* infection is prevalent in ICU acquired polymicrobial Catheter-associated urinary tract infection (CAUTI) [12]. *C. albicans* forms complex biofilm matrix on the indwelling medical devices such as valves, catheters, medical implants and results in the catheter associated infections.

2.3. Virulence traits in *C. albicans*

Due to presence of virulence traits in *C. albicans*, it actively participates in the pathophysiology of infection onset and disease progression. These traits include polymorphism (pseudo hyphae, hyphae, and blastospores), phenotypic transition between white and opaque cells, thigmotropism, production of hydrolytic enzymes, synthesis of proteins required for adhesion and invasion, and biofilm formation (**Fig. 2.1)**. Due to these virulence traits *C. albicans* establishes and progress disease condition in human body [9].

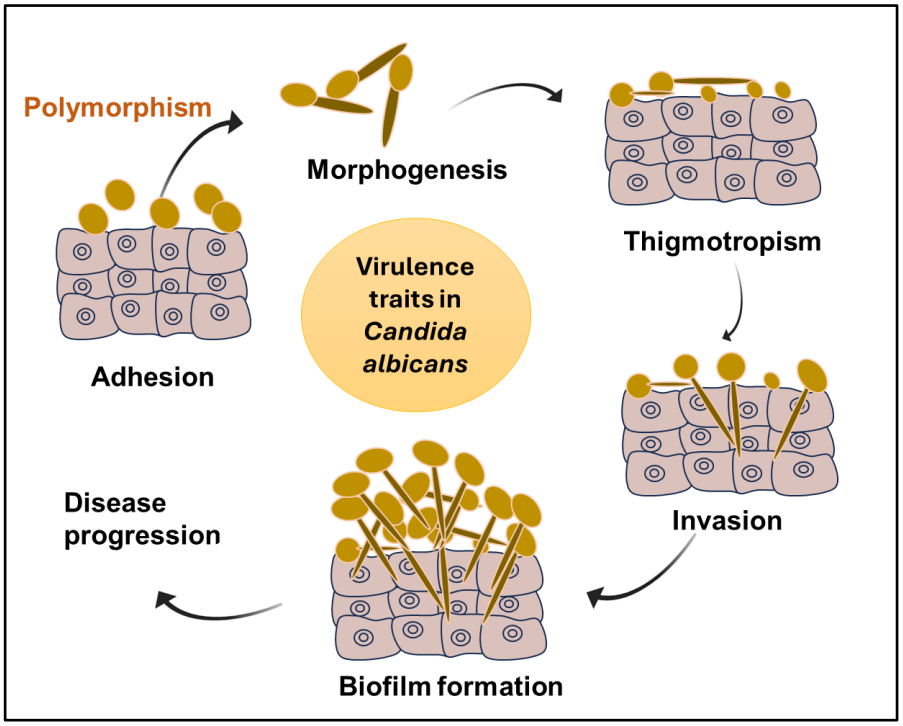

Figure 2.1. Virulence traits present in *C. albicans* imparting the pathogenicity for disease progression.

Morphogenic switching between yeast to hyphal form serves as an important virulence trait which plays an important role in pathogenesis of *C. albicans*. The yeast phase of *C. albicans* has ability to form superficial colonization whereas hyphal form has an ability to penetrate the host barriers and allows a deep-seated tissue infection [13].

Other important virulence factors of *C. albicans* are adhesion, invasion and colonization onto various surfaces such as human mucosal tissue or to medical devices including catheters, and implants [14]. Various types of specialized proteins called adhesins (Agglutinin-like sequence), Hyphal wall protein (HWP)-1, Enhanced adherence to polystyrene (Eap1), cell wall proteins and extracellular proteins, Secreted aspartyl protease (SAP) 9, SAP10 are involved in this adhesion and invasion.

The prime virulence factor which is related to the increased pathogenesis of *C. albicans* is the ability to form biofilm on biotic and non-biotic surfaces. This is a sequential process where cells of *C. albicans* initially adhere to surfaces forms a basal layer. After these, subsequent phases of cell proliferation and hyphal formation, the biofilm formation continues to the maturation phase. The extracellular matrix containing polysaccharides get accumulated and in last phase the non-adherent cells dispersed and may proceed for beginning of new biofilm formation. Attachment to the host cells, rupturing the host cell membrane, invasion in to mucosal membrane, and blood vessels facilitated by the hydrolytic enzymes secreted by *C. albicans* itself [15].

SAPs, phospholipase and hemolysin are the different types of hydrolytic enzymes present in *C. albicans.* These enzymes enhance immune escape and thus pathogenesis of *C. albicans* [9,16]. In addition, contact sensing and thigmotropism enhances the pathogenicity of *C. albicans.* By contact sensing ability, *C. albicans* can adapt changes to the environment that will in turn induce formation of invasive hyphae, trigger biofilm formation, and thus disease proliferation occurs. Adaption to the specific surface morphology is achieved through thigmotropism by repositioning of the hyphae [17]. But these virulence factors were less studied as compared to biofilm formation which remains major concern for all researchers.

2.4. Current antifungal therapeutics

There is a limited variety of antifungal drugs currently used as primary treatments for potentially fatal fungal infections. Few classes of antifungal drugs such as azoles, polyenes, allylamines and echinocandin are approved for treatment of fungal infections in human. The action of these antifungal drugs is based on the structural differences between pathogenic fungi and normal cells.

2.4.1. Azoles

A major class of antifungal drugs called azoles reduces fungal infections by inhibiting the lanosterol 14α-demethylase enzyme (*ERG11*), which is necessary for

ergosterol biosynthesis. These drugs cause demethylation of C-14 of lanosterol and leads to substitution of sterol in fungal cell membrane. This class includes original azoles developed in mid-1970-90 such as miconazole, clotrimazole, econazole, ketoconazole, tioconazole and sulconazole. In addition some drugs emerged in 1990 i.e. terconazole, fluconazole (FLC), itraconazole, voriconazole, posaconazole, efinaconazole and isovucanozonium are also counted in azole group [18]. The efficacy of azole group is challenged by the overexpression of the cell membrane efflux pump that causes high level of azole resistance. ATP-binding cassette pumps and the major facilitator transporters reduce the intracellular accumulation of azoles. In addition, alterations in *ERG11* gene contribute to the resistance development [19].

2.4.2. Polyenes

Another class of antifungal drugs called polyenes. It has heterocyclic amphipathic molecules, with one hydrophilic charged side and one hydrophobic at uncharged side. Polyenes bind to ergosterol present in fungal membrane by entering into the lipid bilayers, forming pores which affects the integrity of the plasma membrane. So, it causes cell death by enabling small particles penetrate into the membrane. Polyenes class of antifungal drugs includes Nystatin and AmB. Although having broad spectrum of activity, due to nephrotoxicity and expensive formulations limits their use in second line or salvage therapy. Resistance to this group in *C. albicans* is rare and mainly contributed by C5, 6-desaturase (*ERG3*) gene mutation and increased catalase activity, alteration in ergosterol synthesis or changed membrane sterols [20].

2.4.3. Pyrimidine analogues

The only member of this group is 5-FC which intercalate into DNA and RNA and resulted into disturbed cellular activity of *C. albicans* cell. This class has been reported with high frequency of resistance in *C. albicans* due to mutations in uracil phosphoribosyl transferase.

2.4.4. Echinocandins

Echinocandins blocks β-(1,3) glucan synthase presents in the fungal plasma membrane. It represents first line therapy against *C. albicans* infection and included agents are caspofungin (CAS), micafungin and anidulafungin. Reported resistance

reasons to this group are mutations in 1,3-β-D glucan synthase (FKS1, FKS2) genes and prolong exposure with drugs [21].

2.5. Limitation of current antifungal therapeutics

Only a limited number of antifungal drugs are available to treat the invasive fungal infection, but these are also facing challenges. Treatment of fungal infections failed due to various factors such as bioavailability of antifungal drugs, host immune response, pharmacokinetics of drugs, infection severity and side-effects, fungal biofilm formation, and antifungal drug resistance.

The factors contributing to the pharmacokinetics of drugs play an important role in the contribution of drug efficacy against infection. Metabolism, absorption, and distribution of drug in the body serve as determining factors in drug efficacy. In addition, the efficacy of antifungal drugs also depends on the fungal population and severity of infection to the host, which further limits the applicability of drug. As per the previous reports, the administration of certain antifungals showed side effects such as the use of 5-FC, which caused bone marrow suppression, and colitis like substantial effects. AmB is frequently associated with the nephrotoxicity [22]. While the group of antifungals, namely azole and echinocandins has shown minimum side effects.

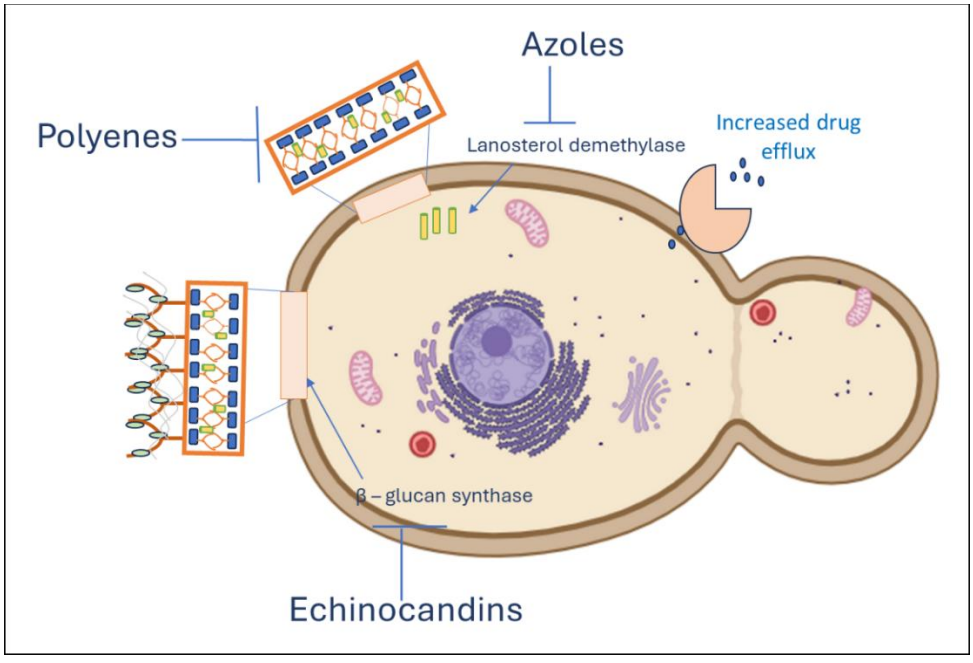

Figure 2.2. Presentation of developed drug resistance in *C. albicans* for the routine antifungal agents with probable mechanism of resistance.

Further, antifungal drug resistance contributed by over-expression of membrane transporters, altered ergosterol biosynthesis, alteration in sterol imports, acquired resistance due to prolong use of drugs, and aneuploidy [23]. **Fig. 2.2** represents the probable mechanism of antifungal drug resistance developed in *C. albicans.* As per the available literature genomic variations caused development of resistance such as upregulation or overexpression of *ERG11* gene resulted in increased resistance for azole class of antifungals. In addition to this, mutation in Candida drug resistance genes (CDR) *CDR1, CDR2* and multi drug resistance gene *MDR1* serves as another reasons for antifungal drug resistance [24].

These issues present with the current antifungal drugs compelling reasons for developing new antifungal therapeutics to improve outcomes in patients with invasive fungal infections. However, the major issue faced by the pharmaceutical industry to develop new antifungal agent is the close evolutionary link of fungi and human being as both belongs to the eukaryotes [5]. This causes a challenge to the antifungal drug targeting the fungal cells without affecting and exerting toxic effects on the host cells.

2.6. Alternative antifungal therapeutics

To tackle the current scenario of increasing fungal infections and limitations of the available antifungal therapeutics, varieties of approaches are employed. In this, active natural products stand as the potential adjuvants to the current antifungals. The source of these natural products may vary from the microorganisms such as bacteria, fungus and reach up to the sponges, plants, insects to higher animals. Antifungal activities of plant molecules like thymol, carvacrol, citral, eugenol, turmeric, olive, cinnamon, basil, and clove oil against *C. albicans* is already reported in the literature [25–27].

Antimicrobial peptides and PIs, including RNase-7, lysozyme, anti-leukoprotease, histatins, cathelicidins have shown broad antifungal activity against *C. albicans* by targeting various targets, including SAP, and adhesion activity. Defensins exhibited fungicidal activity against *C. albicans* [28].

Certain alkyl imidazolium ionic liquids belong to the class of molten salts and are made of the imidazolium cations and alkyl chains combinations affect *C. albicans* growth. Studies on these molten salts shown their role as anti-fungal and anti-biofilm [29]. In addition to this approach of nanoparticles, provide an additional strategy against biofilm formation. Certain metal, silver, and bismuth nanoparticles have shown anti-

biofilm activity against *C. albicans.* But in the case of systemic infections, this therapy has limitations [30].

Besides these, three classes of antifungal drugs (azole, polyenes and echinocandins), novel antifungals such as cyclic peptides, depsi peptides, tetrazoles, siderophores, and aryl amidines are in clinical setting procedures. While some are in the pre-clinical stage, such as phloeodictine analogues, biphenylthylaminoacetamides, thiozoyl guanidine derivatives [31,32]. Other approaches, including drug repurposing and immunotherapy provides alternative strategies to boost the antifungal drug development process. Repurposing of already approved non-antifungal drugs for fungal infections is an effective therapy that accelerates process of the antifungal drug development.

2.7. Drug repurposing approach

Among these approaches, drug repurposing (repositioning) strategy drawn much attention. This is an alternative approach to the traditional/*de novo* drug development process where new indications of already FDA approved drugs or under instigation drugs are identified [33]. In between 2007-09, FDA of United States approved new drugs and biologics in which 30-40 % where belongs to the repurposed products. During the Coronavirus Disease 2019 outbreak, repurposing has received more attention since those drugs are already FDA approved and the emergency use authorization (EUA) of multiple repurposed drugs to treat the disease can be directly implemented [34].

As per most of the investigations, drug repurposing process is less time consuming, less costly and has a higher success rate compared to the traditional drug discovery process (**Fig. 2.3**). The traditional drug discovery process includes five stages, including discovery of lead compounds, preclinical studies, safety profile studies, clinical trials, FDA approval, and post market safety monitoring. In contrast, drug repurposing has four stages: lead compound identification, acquisition and drug development, and post market safety monitoring.

As the drug repurposing approach directly uses previously approved drugs, preclinical studies on animal models are already detailed, and obtained results can be proceed with clinical applications, phase II trials [35]. Due to this repurposing/ reprofiling or repositioning of already approved non antifungal agents stands as more appealing strategy for fulfilling urgent need of antifungal drug development against *C. albicans* [36].

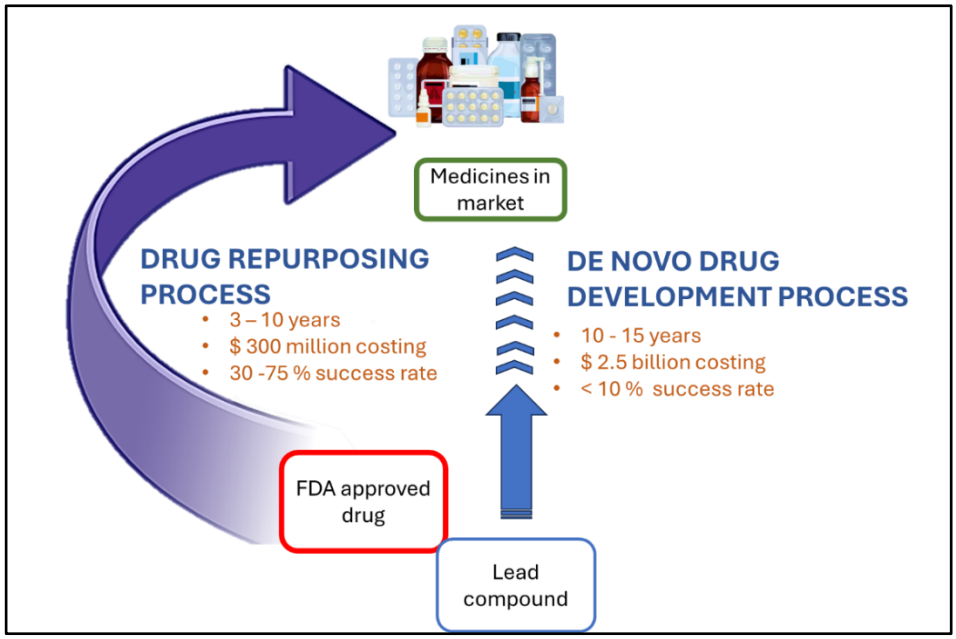

Figure 2.3. The schematic presentation of difference between *de novo* and drug repurposing approche for antifungal drug development.

In order to proceed with the repurposed drug into the drug development pipeline, the candidate drug has to pass the steps such as generation hypothesis, which means determining a promising drug candidate for a particular new indication. Then study of the mechanistic activity of the drug in a preclinical setting and lastly to identify the phase II clinical trial efficacy. Among these, step I which is the selection of a candidate drug for newer indications, is a more critical step and requires a high degree of confidence. The approach of selection is either computational based or experiment based [37].

Various non-antifungal agents were already repurposed against *C. albicans* and include antibacterial agents, immunosuppressants, statins, antiarrhythmic drugs, antidepressant drugs, antipsychotic drugs, non-steroidal anti-inflammatory drugs, antiviral drugs, and antitumor agents [36]. Several antibiotics such as gentamicin, clarithromycin, levofloxin, norfloxacin, moxifloxacin, ciprofloxacin, trovafloxacin, and linezolid were found to exhibit anti-*Candida* activity.

As per study performed by Ku et al., (2010) on gram-positive anti-bacterial agents particularly azithromycin, vancomycin and tigecycline which showed antifungal activity against *C. albicans.* Among these agents, tigecycline was able to disturb mature biofilm but at the higher concentrations [38]. Studies demonstrated the synergistic combinations of antibiotics with antifungal agents along with the probable mechanism of actions such as reduced phospholipase activity, and inhibition of DNA replication

against *C. albicans* [39]. Antidepressants such as cyclosporine were studied up to *in vivo* and revealed that cyclosporine inhibits morphogenesis in *C. albicans* by altering hyphal specific genes. In addition, rapamycin, moribana, dexamethasone, and budesonide also exerted antifungal effects either alone and/or in combination.

Certain antiarrhythmic drugs, like verapamil, block hyphal morphogenesis, increase oxidative stress, and strain susceptibility along with inhibition of efflux pump. In addition, other drugs such as micardipine, diltiazem, and mifedipine have also shown antifungal effects against *C. albicans.* Targeting calcium haemostasis, amiodarone has shown fungicidal action against *C. albicans* [40,41].

In addition, antidepressants were repurposed against *C. albicans* and among them fluoxetine named antidepressants were found to affect phospholipase activity and proceed to the fungicidal action. Another antidepressant, sertraline had antifungal action in mice model by reducing the fungal burden and tissue damage [42]. Various drugs from non-steroidal anti-inflammatory group were studied for the antifungal activity against *C. albicans.* Drugs including aspirin, celecoxib, ibuprofen, diclofenac, and etodolac affected the biosynthesis of prostaglandins involved in hyphal morphogenesis and biofilm formation in *C. albicans* [43].

Chloroquine, an anti-malarial drug, was found to affect ergosterol biosynthesis in *C. albicans* resulting in the inhibition of fungal morphogenesis. In addition to these drugs, various lipid lowering drugs like statins, and anti-parasitic drugs such as oxyclozanide were also reported to have antifungal activity against *C. albicans* [36]. The current investigation focuses on the repurposing of antiviral drugs for antifungal indication, as already a lot of research reported applications of antiviral drugs against varieties of non-antiviral indications such as anti-cancer, anti-bacterial, and anti-parasite infections.

2.8. Repurposing antiviral drugs

2.8.1. Antiviral drugs repurposing against cancer

Cancer in the metastasis phase or resemble stem cells have challenged currently available anti-cancer therapeutics [44]. Certain anti-viral drugs have been repurposed against various types of cancer such as prostrate, cervical, hepatic, and colon. Efavirenz was initially developed for HIV infection but also reported to have anti-cancer action. It has been studied for anticancer activity using pancreatic cancer cell line BxPC-3 and found that Efavirenz induced necrosis in cancerous cells. Efavirenz causes increased

phosphorylation of tumor suppressor protein p53 leading to its activation and G1 phase arrest in the cell cycle. As per reports, Efavirenz caused a selectively toxic effect on tumor cell lines compared to normal tissue cell lines [45].

Maraviroc is another anti-HIV drug repurposed against gastric cancer. As per a histopathology study, Maraviroc treatment resulted in raised intertumoral necrosis in cell lines resulting in reduced proliferation of cancer cells in the body. Maraviroc downregulated FAT1 gene a homolog of atypical cadherin gene FAT, and altered many genes like IL-10RB, MET, FAT1, NME1, and LTB. As per this study, gastric cell lines highly express CCR5 receptors, and Maraviroc acts as antagonist to CCR5 and hence it reduced tissue burden and proliferation of cancer cells [46]. Combination of maraviroc with trabectedin has reduced tumor growth, monocyte infiltration, and angiogenesis in ovarian cancer tumor xenografts [47].

Idoxuridine, a thymidine analogue used against HSV was combined with ID D1694. ID D1694 is a folate-based thymidylate synthase inhibitor against MGH-U1 bladder cancer and HCT-8 colon cancer cell lines. Another protease inhibitor, Ritonavir used in HIV infection used in chemotherapy and radiotherapy. Treatment with Ritonavir observed affect glioblastoma. The study revealed that Ritonavir exhibited cytostatic, anti-migratory, and chemo sensitizing effects. Further, Ritonavir reduced E6 and E7 oncoprotein levels in oral and genital cancer resulting in inhibition of proteasomal degradation of p53 mediated by E6. It could be the proposed in adjuvant therapy for cancer treatment. The presence of Nelfinavir increases Reactive oxygen species (ROS) level, apoptosis and tumor suppression in cervical cancer and makes cancer cells more susceptible to chemoradiation [48].

Saquinavir, is an approved HIV-PI and had a synergist effect on prostate cancer cell line PC3 when administered with Fluorouracil. Also, Saquinavir affects Kaposis sarcoma cells by targeting the growth of spindle cells and angiogenesis. Lamivudine is a cytidine and cytosine analogues approved for HIV-1 and HBV infection found to lessen the expression of MMP-9, HBsAg, and HBeAg in hepatoma cells, and also stops the spread of the HBV infection. In patients with breast cancer who are receiving adjuvant chemotherapy, lamivudine prophylaxis has been noticed to lessen hepatic side effects [49,50].

An antiviral drug cidofovir is an acyclic nucleoside analogue used in the treatment of cytomegalovirus retinitis, displayed anticancer activity against cervical cancer. Cidofovir declines E6 protein expression and activates p53 protein, causing cell

cycle arrest and apoptosis. Furthermore, cidofovir improved the cytotoxic effects of radiotherapy in glioblastoma cells, indicating its potential in anti-glioma therapy. Human papilloma virus-positive cervical tumors were affected by a combination of cidofovir and cetuximab, an anti-epidermal growth factor receptor antibody [51–53].

Nelfinavir is another effective HIV-PI and several research studies indicated that nelfinavir has positive anti-cancer effects [54,55]. Nelfinavir treatment increases apoptosis and tumor suppression in cervical cancer by making cancer cells more susceptible to chemoradiation [56,57]. In addition, research conducted on rectal cancer patients revealed that, nelfinavir and radiotherapy together might improve tumor perfusion and regression in comparison to radiotherapy alone. Nelfinavir has also been shown to cause apoptosis and necrosis in breast cancer cells, reducing their viability without affecting healthy breast cells [58,59]. Nelfinavir is under Phase I trial against solid tumor treatment.

Abacavir, is a 2-deoxyguanosine nucleoside analogue, clinically approved for HIV treatment in adults and children [60]. Abacavir in combination with antiviral reverse transcriptase inhibitors like azidothymidine has significantly shorten telomeres, accumulate H2AX, phosphorylate p53, and caused cell apoptosis in tumor cell lines [61]. Abacavir was found to activate Yin Yang 1 mediated transcription which is a therapeutic target for malignancy treatment along with gastric cancer cells [62].

In general, various antiviral drugs have been profoundly studied for their anticancer effect on different types of cancer cell lines, indicating the possibility of antiviral drugs as anticancer agents in future therapeutics.

2.8.2. Antivirals repurposing against Alzheimer's disease (AD)

An epidemiological disease called AD is a neurological disorder responsible for a great percentage of dementia cases. According to *in vitro* studies, HSV infection can cause excessive tau phosphorylation and promote the accumulation of amyloid; antiviral drugs can lessen the effects of Alzheimer's disease. As an alternative therapy, antiviral drugs also target the root cause of disease such as reduction in Aβ burden on the brain.

Acyclovir is an acyclic guanosine analogue approved against HSV-1 which targets viral replication. A study on the combination of acyclovir with dexamethasone was found to reduce the injuries caused by spatial cognition and levels of tumor necrosis factor alpha and Interleukin-6 which are neuro inflammation markers [63].

Penciclovir is another antiviral used against HSV-1, found to exhibit more

affinity towards DNA polymerase and causes blockage of the DNA elongation process. Penciclovir treatment reduced HSV-1-induced P-tau-related enzymes such as protein kinase A and glycogen synthase kinase 3b. Wozniak et al., (2011) found that foscarnet, which is a DNA polymerase inhibitor was less effective compared to acyclovir and penciclovir [63]. Foscarnet reduced Aβ accumulation at 200 μM concentration, which is higher than the other two acyclovir and penciclovir. Due to reduced efficacy of foscarnet compared with acyclovir and penciclovir, it has not carried forward for further study [63–65]. Valacyclovir, a prodrug converts, to acyclovir which is more effective in the treatment of HSV-1, 2, chicken pox, and herpes zoster viral infection. In a case study, schizophrenia-suffering patients were supplemented with valacyclovir in addition to antipsychotic drugs and found improvement in verbal and working memory and visual object learning. Due to safe consumption parameters, valacyclovir was tested in a phase II clinical trial to propose a potential agent against AD [66,67].

BAY 57–1293 belongs to a new class of potent inhibitors against HSV-1 and was found to inhibit DNA replication by targeting the helicase-primase complex. BAY 57–1293 was found to inhibit the accumulation of Aβ and P-tau production along with a reduction in the size of (African green monkey kidney) Vero cells cluster [68].

2.8.3. Antivirals repurposing against parasitic infections

About 241 million cases and 627,000 deaths due to malaria were reported in 2020, in which 77 % of the deaths occurring in children under five age [69-71]. The emergence of parasite resistance to antimalarials is a serious issue in the treatment of malaria [72]. Many antiviral drugs are repurposed against various types of Plasmodium species and proposed that antivirals may exhibit different modes of action against Plasmodium spp. than existing antimalarial drugs.

Efavirenz, Etravirine, and Nevirapine are already approved non-nucleoside reverse transcriptase inhibitors (NNRTIs) for the treatment of HIV-1 infection. It has been reported that pregnant women suffering from HIV showed reduced placental or maternal malaria on treatment with Efavirenz [69]. Incubation of live cells with Efavirenz resulted in oxidative stress and mitochondria-mediated apoptosis further leads to the production of ROS. This mechanism of action serves as a probable mode of action of NNRTIs against oxidant stress-sensitive *Plasmodium* species, though NNRTIs were found to have less impact on the parasitic load in liver [73].

PIs group of antivirals prevents the growth of malaria parasites by targeting

plasmepsins in their food vacuoles, reducing the plasmodium count. Lopinavir and Saquinavir reduced the number and size of exoerythrocytic forms; specifically, Lopinavir was acted against parasite development in the intrahepatic stage. PIs treatment prevented *in vitro* oocyst formation and development [74,75]. Trimethoprim-sulfamethoxazol is another drug used in the treatment of HIV infection and active against the *in vivo* parasitemia caused by *Plasmodium yoelii*. The exoerythrocytic form of *P. yoelii* is a rapid division stage of parasites and was found to be arrested by this combination [76].

Another parasite, *Toxoplasma gondii* is a causative agent of acute toxoplasmic encephalitis and may cause death in patients suffering from HIV or in individuals receiving immunosuppressive chemotherapy. In a study carried out by Wang et al., (2019) among 44 anti-retroviral agents, 14 were found to exhibit anti-parasitic activities. Those anti-HIV drugs belonging to the PIs group were more significant against *T. gondii* compared to other drugs [77,78].

Nelfinavir showed an inhibitory concentration (IC_{50}) of 1.18 ± 2.21 μM and exhibited a potent effect against the growth of *T. gondii* tachyzoites. While other PIs such as Saquinavir, Lopinavir, Ritonavir, and Tipranavir, were reported with 3.76 ±1.02 μM, 6.70 ± 1.10 μM, 8.04 ± 1.02 μM, and 9.69 ± 0.91 μM, IC_{50} values respectively. The severity of Toxoplasmic encephalitis was reduced with anti-retroviral therapy [77]. Cryptosporidiosis and microsporidiosis are two diseases linked to HIV that cause persistent diarrhoea in patients with reduced CD4c lymphocyte counts [78]. Indinavir, Nelfinavir, and Ritonavir inhibits parasitic growth when are used in combination with paromomycin compared to paromomycin alone. Those individuals in the double antiretroviral therapy group had excellent therapeutic responses with an increased CD4c count [79]. These findings claim that antiretroviral combinational therapy significantly altered the dosage course of microsporidiosis and cryptosporidiosis in HIV-1 positive patients [80].

Parkinson's disease suffering patients receive long-term levodopa therapy, which caused levodopa-induced dyskinesia. This is a frequently occurring motor complication. Studies confirmed that both dopaminergic and non-dopaminergic systems are affected by amantadine, an anti-influenza drug [81,82]. It works by inhibiting dopamine reuptake and enhancing dopamine release pre-synaptically. According to some previous research, Amantadine's anti-dyskinetic effects may result from its secondary effect of inhibiting N-Methyl-D-aspartic acid receptors [83,84].

2.8.4. Antivirals repurposing against bacterial infections

Trifluridine is a nucleoside analogue which is recommended as an anti-herpesvirus agent and used in monotherapy. According to the literature, trifluridine is effective against *Enterococcus faecium* P5014 [85]. As per a report by Peyclit et al., (2018) [86], Zidovudine affects Enterobacteriaceae strains containing 16 *Escherichia coli* (12 colistin-resistant isolates, 3 carbapenem-resistant isolates, and 1 susceptible isolate) and 22 strains of *Klebsiella pneumoniae* (11 isolates were colistin-resistant, 9 carbapenem-resistant isolates, 1 colistin-and carbapenem-resistant isolate, and 1 susceptible isolate). All the strains showed susceptibility towards Zidovudine. The data highlights the wide applications of antiviral drugs against bacterial infections. This approach may be employed to prevent the spread of antimicrobial drug resistance.

2.8.5. Antiviral drug repurposing for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

Apart from the non-viral indications, antiviral drugs were also repurposed for other viral indications than the proposed one. Literature is available on repositioning of anti-viral drugs for COVID-19 with *in vitro* and clinical studies. Varieties of the antiviral drugs were already repurposed against SARS-CoV-2. A literature briefing on the repurposing of Remdesivir, Molnupiravir, Favipiravir, Ribavirin, and Sofosbuvir in COVID-19 medication is available.

Remdesivir is a prodrug and structural analogue of adenosine and was originally developed for the treatment of ebola virus by Gilead Sciences [87]. RNA-dependent RNA polymerase (RdRP) is the preliminary target of the remdesivir drug against viral replication. Remdesivir administered on day 5 of SARS-CoV-2 infection showed a significant decrease in viral load and increased genomic RNA cycle threshold (Ct) values [88].

Molnupiravir is another oral drug repurposed as an anti-SARS-CoV-2 drug. It converts into the ribonucleoside analogue β-D-N4-hydroxycytidine (NHC) by the host's esterases in plasma [89]. This conversion leads to the synthesis of NHC-Triphosphate, which is then incorporated into viral RNA by the RdRP, causing errors in the viral genome and replication inhibition [70]. In 1,433 patients having mild to moderate COVID-19 supplemented with Molnupiravir for 5 days with 800 mg after every 12 h, reduced risk of hospitalization or death [88,90].

The meta-analysis performed by Taibe et al., (2022) concluded that Favipiravir

treatment resulted in fast viral clearance with improved clinical outcomes in hospitalized patients. Favipiravir transformed into an active phosphoribosyl form inside the cells, known as favipiravir-ribofuranosyl-5' triphosphate. This product act as substrate for the RdRP and inhibits its activity, including that of SARS-CoV-2 [70]. Ribavirin targets inosine monophosphate dehydrogenase, RdRP, and thus inhibits the viral replication process of SARS-CoV-2. Combined Ribavirin and interferon (rIFN-α2a, rIFN-α2b, or rIFN-β1a) were found to be efficient in the fast clearance of Middle East Respiratory Syndrome (MERS)-CoV-2 RNA. As compared to Ribavirin monotherapy, the combination therapy with Lopinavir and Ritonavir reduced the risk of acute respiratory distress syndrome and death in patients. As per the reports, treatment with Ribavirin alone or with interferon was not effective in *in vivo* and thus Ribavirin was discontinued from the suggestion list for COVID-19 treatment medications [91].

Both Lopinavir and Ritonavir are structurally related PIs and they are used in the treatment of AIDS and proposed for COVID-19 treatment. Lopinavir and Ritonavir found to exhibit an effect on viral 3CL protease in SARS-CoV-2. It was suggested that Lopinavir, together with other inhibitors of HIV protease like Darunavir, be used to target the primary protease of SARS-CoV-2. However, clinical trials using Lopinavir/Ritonavir have yielded disappointing results, with similar outcomes in the solidarity and together trials. The World Health Organization has declared the end of the Lopinavir/Ritonavir regimen for hospitalized patients [88].

Sofosbuvir, an anti-HCV drug, has been proposed as a possible drug in COVID-19 treatment. Sofosbuvir has significant activity against dengue, zika, yellow fever, and chikungunya viruses. The results of *in vitro* studies and clinical trials confirm the effectiveness of Sofosbuvir-based treatment against SARS-CoV-2 infection. Sofosbuvir treatment had a higher recovery rate and lower mortality rate [92].

2.9. Repurposing antiviral drugs as antifungal agents

Antiviral drugs are developing rapidly and have widespread use. As per the previously reported and published data, antiviral drugs are already routinely screened for non-viral indications. Antiviral drugs are repurposed against COVID-19, bacterial infection, cancer, Alzheimer's, Parkinson's diseases, parasitic, and fungal infections. In addition, literature is available on the repurposing of antiviral drugs alone or in combination with antifungal drugs against various pathogenic fungi including *C. albicans.* The field of antifungal therapy is seeing an increasing frontier in research on

antiviral drugs for the treatment of serious mycoses. Originally developed for treatment of viral infections, antiviral medications are now being investigated for their potential to treat pathogenic fungi as part of a wider search for novel therapeutic approaches [93].

As per the screening study performed by Kim et al., (2015) out of 1,581 small molecules 15 drugs were already approved drug and repurposed against C. *albicans* had effective anti-*Candida* activity [39]. Various HIV-PI were repurposed against *C. albicans* as SAP produced by *C. albicans* belongs to the same group of HIV protease. Due to this, most of the PIs showed inhibitory action on *C. albicans* [94].

As per the reports of Calugi et al., (2012) HIV-1 protease had a structural similarity with the SAP-2 present in *C. albicans* indicating the applicability of a single drug against both fungal and viral pathogenic targets [95-96]. Amprenavir, another PI used for HIV treatment had demonstrated *in vitro* inhibition of biofilm produced by *C. albicans*. One more experimental study suggests that amprenavir blocked the SAP activity of *C. albicans* [97]. The biofilm forming ability of *C. albicans* was inhibited by drug combinations such as Lopinavir-CAS, Ritonavir, Saquinavir with CAS, and AmB, and Nelfinavir with FLC, CAS and AmB. Drug combinations of HIV-1 PIs (Indinavir, Atazanavir, Nelfinavir, Lopinavir, Ritonavir, Saquinavir) synergistically disrupted the mature biofilm formed by *C. albicans* [98,99].

Fenley et al., (2022) identified the anti-planktonic, anti-morphogenic, and anti-biofilm activities of Atazanavir, and Darunavir against *C. albicans* with downregulation of BRC1 and SAP1, and SAP2 genes [98]. Lopinavir, a HIV PI had exhibited anti-morphogenic, anti-biofilm, and anti-adhesion activity against *C. albicans* along with inhibitory action on SAPs, neutral lipids, and esterase. The inhibitory action was further confirmed by *in vivo* experimentation on cyclophosphamide-immunosuppressed BALB/c mice [97]. A recent study demonstrated the action of Ritonavir resulted in *C.albicans* growth inhibition by targeting SAP though there was no synergism with FLC [100]. According to Cassone et al., (1999) Indinavir and Ritonavir have an efficacy similar to FLC in inhibiting the proliferation and SAP production in *C. albicans* in rats. Treatment with Indinavir and Ritonavir prevents the development of vaginal candidiasis [101]. In addition, *in vitro* studies showed Saquinavir and Indinavir treatment caused dose dependent effect on SAPs of *C. albicans*. Further Indinavir alone had inhibitory action on SAP-2, and adhesion ability of *C. albicans* to the epithelial cells. This study evidenced the significant antifungal activity of Indinavir compared to Saquinavir and Ritonavir [102]. Ribavirin a guanosine analogue approved against HCV was shown to

have broad spectrum, effective antifungal action on a of *Candida* spp. Ribavirin affected the growth of multidrug-resistant *C. albicans* by vacuole disruption. In addition, Ribavirin in combination with the azole group of antifungals acted synergistically and inhibited the growth of *C. albicans* [96,103,104].

2-adamantanamine is a structural analogue of the amantadine antiviral drug targeting influenza virus. As per the research, 2-adamantanamine potentiated the antifungal activity of Miconazole and FLC against biofilm-forming *C. albicans. In vivo* study on a guinea pig model with cutaneous candidiasis, 2-Adamantanamine was to target ergosterol, hyphal filamentation, and reduced fungal burden. 2-adamantanamine is found to be an effective and potentially repurposed drug against *C. albicans* [105].

Raltegravir, an integrase inhibitor, exerted antifungal activity on *C. albicans* and *C. glabrata* with minimum inhibitory concentration (MIC) of 128 μg/ml and 256 μg/ml, respectively. In the systemic Para coccidioidomycosis *in vivo* mice model Raltegravir was found effective against *Paracoccidioides brasiliensis.* This study demonstrates that Raltegravir significantly reduced the fungal burden on the lungs of *P. brasiliensis*-infected mice compared to the untreated group [106]. Raltegravir had antifungal activities against a wide range of fungal infections.

The majority of antivirals used to treat HIV are also used to repurpose against *C. albicans*; however, purine nucleoside analogues including Ribavirin, Galidesivir, Sofosbuvir, and Remdesivir were also repurposed to have broad-spectrum efficacy against a variety of RNA and DNA viruses, including SARS-CoV-2 and HCV [107].

Literature on the repurposing of HIV-aspartyl protease inhibitors against *C. albicans* highlighted the potential of aspartic protease and central metabolic pathways to use as novel drug targets. In the same regard, screening of FDA-approved antiviral drugs for other therapeutic indications may widen the coverage of drug targets, probably reduce the selection of resistant strains, and provide an urgent alternative to the limited antifungal drugs.

2.10. References

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CHAPTER 3

Screening of antiviral drugs for antifungal activity against *Candida albicans*

3. Screening of antiviral drugs for antifungal activity

3.1. Background

Repurposing approach involves the process of developing alternative pharmaceutical applications for drugs that have already been approved, or are advanced clinical candidates, or that already have targets identified [1]. With significant data support and rationale, a drug that has been approved by regulatory bodies and has demonstrated safety and toxicity in prior clinical trials could skip clinical trials and ultimately will enhance drug development process [2]. After 2010, studies on the concept of drug repurposing or reprofiling is increased tremendously as this approach has advantages of less costing, shorter time duration, and higher success rate compared to the traditional de novo drug development process [3].

Drug repurposing is considered as booming strategy for drug development in infectious and non-infectious, rare, orphan, novel, resistant diseases such as HIV, cancer, fungal and bacterial infections, gastrointestinal, respiratory, psychiatric, Alzheimer's and Parkinson's diseases [4]. Among these diseases, fungal infections caused by *C. albicans* are life threating and leads to nearly 42-65 % mortality. Drug repurposing against *C. albicans* provide as effective alternative for the urgent need of antifungal drug development. In this study, ten FDA approved antiviral drugs mentioned in chapter 1 (**Table 1.1**) are screened to investigate the antifungal activities. The screening is carried out on the basis of their ability to target the preliminary virulence traits present in *C. albicans* which required for onset of infection in human. As per Clinical and Laboratory Standards Institute (CLSI) guidelines the susceptibility of *C. albicans* to the antivirals is studied and generalize methodology is shown in **Fig. 3.1.**

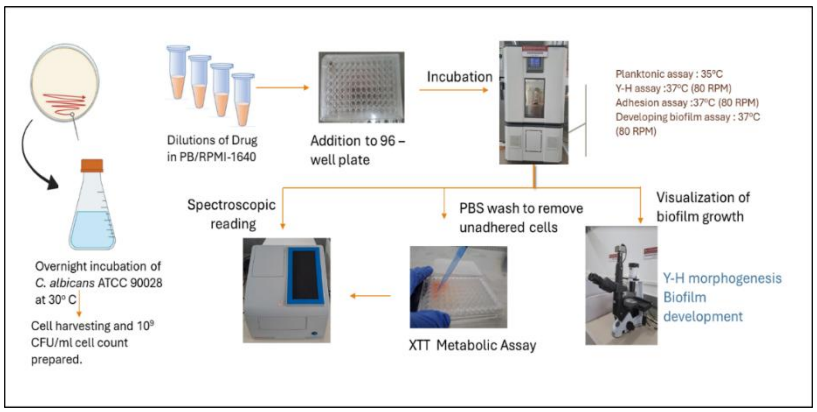

Figure 3.1. Experimental methodology performed for screening of antiviral drugs for antifungal activities.

It is significant to implement preventive measures that target planktonic cells to further inhibit the early formation of biofilms. As planktonic phase cells are less complex compared to the biofilm cells and more sensitive to the antifungal agents. So, eradication of the initial planktonic phase of *C. albicans* will inhibit the disease establishment. Fully established matured biofilms are more difficult to treat and eradicate, targeting planktonic phase cells remains first screen test of antiviral drugs for their antifungal activity determination [5]. Targeting planktonic phase may strengthen the ability of drug to inhibit biofilm formation and thus disease progression [6].

Another virulence factor of *C. albicans* is ability of dimorphism. Y-H morphogenesis has a key role in the pathogenesis in *C. albicans* in which both yeast and hyphal formation plays essential part in infection. The characteristic of *C. albicans* through which budding yeast is morphologically switched to hyphae, further imparts additional virulence to *C. albicans.* By this virulence attribute drug resistance in *C. albicans* increases and proceeds to disease establishment. This transition thus serves as the key target for various anti-fungal agents to prevent disease proliferation, host tissue invasion and tissue damage [7].

Hyphal morphogenesis is also coupled with the ability of *C. albicans* to adhere biotic or abiotic surfaces, formation of long hyphal structures and proceed to form biofilm. In clinical settings it has been observed that the medical implants such as catheter, and heart valves provide a niche to *C. albicans* adherence and the adhesion is complimented by the presence of blood, urine, saliva like body fluids [5]. Adhesion of *C. albicans* further leads in complex biofilm formation.

Furthermore, biofilm formation and the infections associated with biofilm formations on such biomaterials are increasing in alarming rate and the situation demands the development of anti-biofilm agents and/or novel fungal targets pointing the initial adherence and biofilm as well [8]. In this chapter ten antiviral drugs are screened for their anti-biofilm activity against *C. albicans.*

3.2 Materials and methods

3.2.1. Chemicals, growth medium, fluorescent dyes

All chemicals and growth mediums including Yeast Extract Peptone Dextrose (YPD) medium, Roswell Park Memorial Institute (RPMI-1640) without glutamine medium and sodium bicarbonate, 3-(N-Morpholino) propane sulphonic acid (MOPS) buffer, Fetal bovine serum (FBS), Sabouraud dextrose agar (SDA) were purchased from

HiMedia Laboratories Private Limited, India. **Table 3.1** shows the antiviral drugs used in the current study for screening purpose.

Table 3.1. Antiviral drugs with the solubility medium, stock concentration and range of concentrations used in study.

Sr. No.	Name of the antiviral drugs	Solubility in medium	Stock concentrations of drugs (mg/ml)	Range of concentrations (mg/ml)
1.	2-AM	Distilled water (D/W)	50	4 to 0.125
2.	2-DG	D/W	100	4 to 0.125
3.	Acyclovir	Dimethyl sulfoxide (DMSO)	2	0.5 to 0.015
4.	Famciclovir	DMSO	10	0.25 to 0.078
5.	Ganciclovir	D/W	50	4 to 0.125
6.	Idoxuridine	PBS	1	0.25 to 0.078
7.	Lamivudine	D/W	40	4 to 0.125
8.	Ribavirin	D/W	1	0.05 to 0.0015
9.	Valganciclovir	D/W	40	4 to 0.125
10.	Vidarabine	DMSO	50	0.6 to 0.018

Antiviral drugs used for the study were purchased from different sources such as, 2-Adamantylamine hydrochloride (2-AM), 2-deoxy glucoses (2-DG), Ribavirin were purchased from Sigma Aldrich Chemicals Co. (Germany). Vidarabine, Idoxuridine, Lamivudine antiviral drugs were purchased from Tokyo Chemical Industry India Pvt. Ltd. Acyclovir, Valganciclovir, Ganciclovir and Famciclovir were purchased from local pharma store in located in Kolhapur, Maharashtra. Fluorescent dyes such as Acridine orange (AO), Ethidium bromide (EtBr), 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), 4-6-diamidino-2-phenylindole (DAPI), Propidium Iodide (PI), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H tetrazolium 5-carboxanilide (XTT) were purchased

from HiMedia Laboratories Private Limited, India. For RNA extraction, RNeasy Mini Kit was procured from (QIAGEN, Valencia, CA, USA). Primers used in real time polymerase chain reaction (RT-PCR) were purchased from geneOmbio Technology Pvt. Ltd. Pune, India. Annexin V-FITC Apoptosis detection kit was purchased from Miltenyi Biotech Bergisch Gladbach; Germany. As per the solubility of antiviral drugs in respective medium the range of concentrations were prepared and studied for the antifungal action.

3.2.2. Fungal strain, culture and growth conditions

The biofilm forming standard strain of *C. albicans* was American Type Culture Collection ATCC 90028 procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was thawed and maintained on a YPD broth and agar medium. A single colony of *C. albicans* from YPD agar medium was inoculated in YPD broth. The broth was incubated overnight at optimal temperature of 30°C with 80 rpm agitation in orbital shaker. After the incubation, cells were harvested by centrifugation, washed with sterile phosphate buffer saline (PBS) and prior to each experiment cell count was estimated by using Neubauer chamber. For the purpose of cell counting cells were diluted to 10^{-3}/ml and 10 µl of this suspension poured in Neubauer chamber. Cell counting was done using light microscope and average cell count was determined. Accordingly, cell suspension of 1×10^{9} CFU/ml density was prepared and used for further assays. RPMI-1640 medium was used as optimal medium for cell growth.

3.2.3. Susceptibility testing of antiviral drugs to planktonic growth

Broth microdilution method was performed as per the protocol of CLSI guidelines M27-A3 to investigate the effect of antiviral drugs on the planktonic growth of *C. albicans* ATCC 90028. Briefly, 2×10^{3} CFU/ml were treated with varying concentration of antiviral drugs shown in (**Table 3.1**) prepared in RPMI-1640. Whereas cells without treatment of antiviral drugs and suspended in medium were considered as control group. 100 µl of cells suspension and 100 µl of drug dilution were added in to the 96-well flat bottom multitier plate. The 96 well microplate was incubated at 35°C for 48 h. After incubation, absorbance was taken for cell density evaluation using an UV spectrometer (Thermo Scientific Multiskan Sky Microplate Spectrophotometer) at 620 nm wavelength [9]. The concentration of antiviral drugs inhibiting minimum 50 % of yeast growth referred to as MIC of the drug and the same concentration is continued for further studies. The experiment was performed in triplicates.

3.2.4. Determination of fungicidal/static action

Previously reported protocol was referred to determine the fungicidal action of antiviral drugs with MIC against *C. albicans* [10,11]. Briefly, 10 µl of aliquots of *C. albicans* cells treated with antiviral drugs at MIC and 2 × MIC concentrations were spread plated on sterile YPD agar plates. Cells without any drug treatment were considered as control. Plates were incubated at 35°C for 24 h for development of colonies. After the incubation period the number of colonies were counted to determine the fungicidal or fungistatic nature of drug against *C. albicans.* The lowest concentration drug where it showed approximately 99 % to above killing action was referred as MFC of the respective drug against *C. albicans.* The (MFC/MIC $\geq$ 4) ratio presents fungistatic activity and (MFC/MIC $\leq$ 4) implies fungicidal action of antiviral drug against *C. albicans.*

3.2.5. Yeast to hyphal (Y-H) morphological transition study

Transition of Y-H form was studied under the influence of antiviral drugs through serum-induced morphogenesis as per the described protocol with some minor modifications [12]. FBS was diluted by 20 % using sterile D/W and filter sterilized using the 0.22-micron syringe filter. In serum, serial dilutions of antiviral drugs with mentioned concentration range (**Table 3.1**) were prepared and added into wells of non-treated tissue culture 96-well plate. *C. albicans* cells of density 1×10^6 CFU/ml were supplemented with diluted FBS and 100 µl was inoculated in each well containing drug dilutions. Hyphal formation or inhibition in the presence of antiviral drugs were observed after an incubation period of 90 min at 37°C with 80 rpm agitation. Cells without the drug treatment were referred to as control. Observation of hyphal or yeast form was assessed using a Magnus inverted microscope with magnification of 40x.

3.2.6. Surface adhesion assay

For evaluating action of antiviral drugs against adhesion ability of *C. albicans*, 100 µl of 1×10^7 CFU/ml cells were allowed to adhere tissue culture-treated flat-bottomed 96-well plate. Typical biofilm forming conditions such as incubation at 37 °C for 90 min with 100 rpm agitation speed were maintained. After the incubation period, gentle PBS wash was given to remove non adhered cells. *C. albicans* cells were further suspended in PBS. XTT metabolic assay was performed to determine the quantitative effect of antiviral drugs on the surface adherence ability of *C. albicans* [13].

3.2.7. Developing biofilm assay

Developing biofilm assay was performed to determine the effect of antiviral drugs on biofilm formation. The assay was performed in 96-well tissue culture treated plates. In brief, 1×10^7 CFU/ml cells of *C. albicans* prepared in sterile PBS were inoculated in wells of the plate and incubated for 90 min at 37°C with 80 rpm agitation. After the incubation period, the non-adhered, loosely bounded cells were rinsed by sterile PBS. Series of drug concentration as per **Table 3.1** were prepared in RPMI-1640 medium and volume of 100 µl was added in to each well of the plate. Wells containing only *C. albicans* cells without drug treatment were considered as control. The volume of wells was equilibrated by addition of 100 µl of RPMI-1640. Plate was incubated at 37°C for 48 h in static condition. After the incubation period the planktonic or loosely bound cells were removed by gentle wash using PBS and biofilm formation or inhibition was visualized by using inverted microscope. XTT metabolic assay was performed to quantify the metabolic activity of developing biofilm after the drug treatment [14].

3.2.8. XTT/ Menadione assay

Surface adherence and biofilm formation of *C. albicans* ability was quantified using XTT metabolic assay. XTT-menadione solution was prepared in PBS using 1 mg/ml stock solution of XTT and 0.4 mM menadione solution in acetone. This solution was added in to the plates containing prewashed adhesion and biofilm containing wells (methodology section 2.2.6 and 2.2.7) and a negative control was maintained with sterile PBS. The plate was further incubated at 37°C for next 2 h in dark. After the incubation 80 µl was transferred to another plate and absorbance was measured at 450 nm wavelength using U.V. spectrophotometer. The obtained absorbance values were subtracted from the negative control (blank) and the percentage of biofilm activity was calculated using the following formula [15],

$$\%\ \text{of biofilm biomass} = \frac{\text{mean OD (450 nm) of sample}}{\text{mean OD (450 nm) of untreated control}} \times 100$$

3.2.9. Statistical analysis

Each experiment was performed in triplicates and results of the experiments were expressed as mean with ± standard deviation. The results were analysed using an unpaired, one-tailed Student's t-test to determine statistical significance. P value ≤ 0.05,

0.01 and 0.001were considered statistically significant. Where, * indicates P ≤ 0.05, ** indicates ≤ 0.01 and *** corresponds to ≤ 0.001.

3.3. Results

3.3.1. Susceptibility of *C. albicans* planktonic growth to antiviral drugs

Broth microdilution method used to investigate the susceptibility of planktonic phase *C. albicans* to the selected anti-viral drugs. Experimental results are presented in the form of percentage of planktonic growth of *C. albicans* in presence or absence of enlisted antiviral drugs (**Table 3.1**).

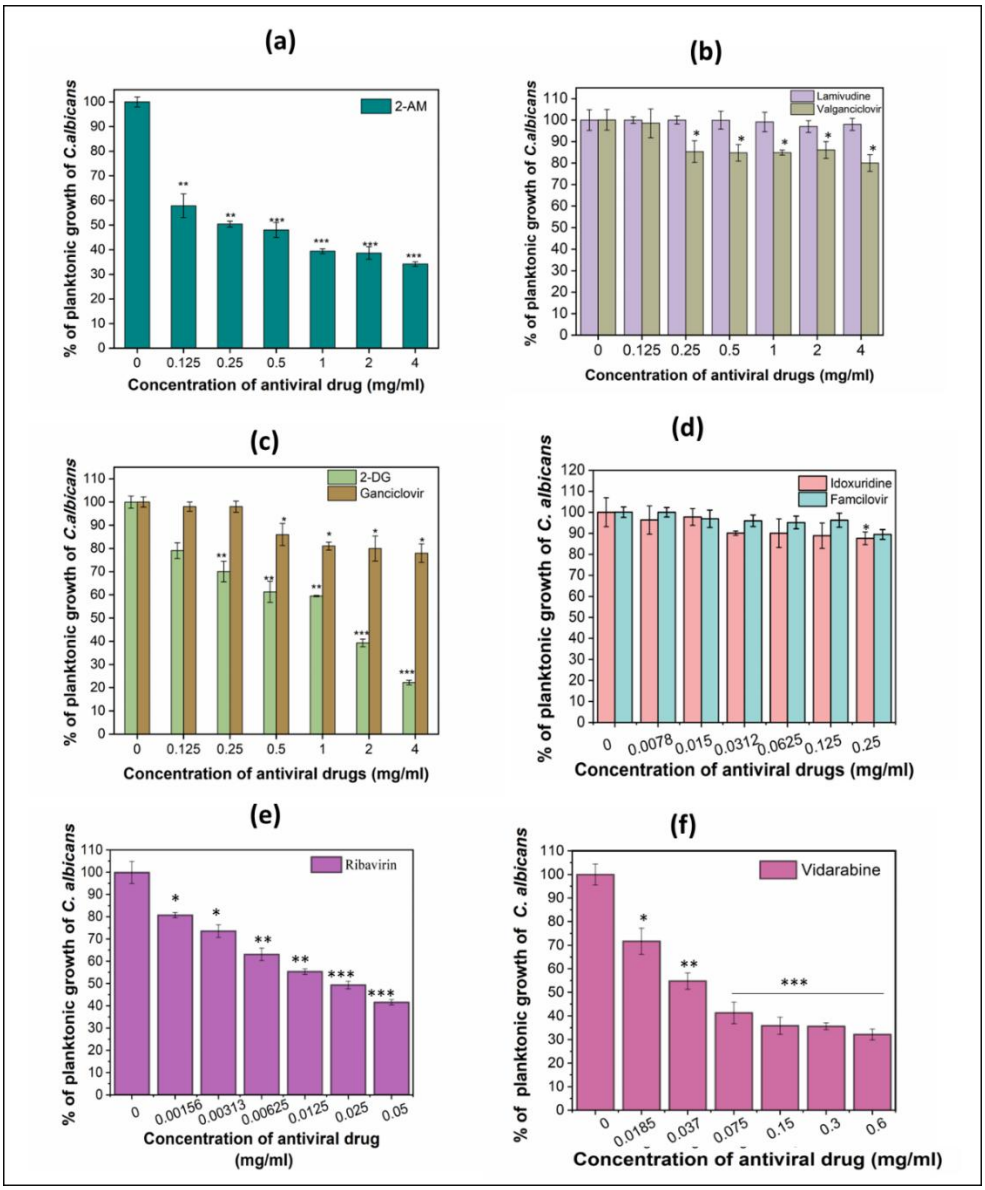

Figure 3.2. Susceptibility testing of planktonic growth of *C. albicans* ATCC 90028 after treatment of (a) 2-AM, (b) Lamivudine, Valganciclovir, (c) 2-DG, Ganciclovir, (d) Idoxuridine,

Famciclovir, (e) Ribavirin, and (f) Vidarabine with different concentration ranges. (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$).

The results revealed that among ten antiviral drugs, four drugs had affected the planktonic phase with at least 50 % inhibition of growth. The least concentration of antiviral drug resulting in minimum 50 % of growth inhibition is referred as minimum inhibitory concentration (MIC) of that drug. Effect of these four drugs namely, 2-AM, 2-DG, Ribavirin and Vidarabine against *C. albicans* cells is in concentration dependent manner as shown in **Fig. 3.2**.

2-AM, 2-DG shows antifungal effect with MIC of 0.25 mg/ml and 2 mg/ml, respectively. Whereas Ribavirin and Vidarabine exerted antifungal effect against *C. albicans* at lesser concentrations compared to the 2-AM and 2-DG with values of 0.025 mg/ml and 0.15 mg/ml, respectively (**Fig. 3.2 e, f**). The same concentrations were used for further study. Ribavirin at 0.025 mg/ml exhibit least MIC against *C. albicans* ATCC 90028 by inhibiting 49 ± 1.64 % of planktonic growth whereas Vidarabine inhibited 41 ± 4.5 % growth at 0.15 mg/ml. Antiviral drug 2-AM inhibited nearly 50 % growth at 0.25 mg/ml while 2-DG at higher concentration of 2 mg/ml showed 39 ± 1.65 % inhibitory antifungal activity against planktonic growth.

Further antiviral drugs namely Ganciclovir, Lamivudine, Valganciclovir, Idoxuridine, Famciclovir and Acyclovir were unable to arrest minimum 50 % the growth in *C. albicans* even at their respective highest concentrations (**Fig. 3.2 b, d**). However, the results of the broth microdilutions showed that the growth of *C. albicans* reduces in dose dependent manner after treatment of these drugs.

As per the obtained MIC values, the antiviral drugs potentially affecting planktonic growth are in the following order; Ribavirin > Vidarabine > 2-AM > 2-DG and through these results 2-AM, 2-DG, Ribavirin and Vidarabine were continued for the further exploring antifungal activities in detail.

3.3.2. Static/cidal nature of antiviral drugs

In accordance with the results of antifungal susceptibility assay, fungicidal or fungistatic nature of antiviral drugs was determined by the YPD agar plate-based assay and results are shown in **Fig. 3.3.** In this, antiviral drugs affecting planktonic phase of *C. albicans* were investigated for their static (growth inhibitory) and cidal (lethal) mode of activity against *C. albicans.* 2-AM, 2-DG, Ribavirin and Vidarabine with MIC, and 2 × MIC values were studied. As the concentration of antiviral drugs increases from MIC

to higher concentration the colony count on the agar plate is reduced. The results implies that 2-AM at 0.5 mg/ml and Vidarabine at 0.3 mg/ml concentration showed no colony count of *C. albicans* on agar plate after re inoculation and 24 h of incubation. As per the calculated ratios of MFC/MIC ≤ 4, 2-AM and Vidarabine had fungicidal effect. Though 2-DG and Ribavirin exhibited 50 % inhibitory action on the planktonic phase of *C. albicans*, on YPD agar method these drugs are unable to inhibit the colony formation. Colony count of *C. albicans* after treatment with 2-DG and Ribavirin was not completely inhibited indicating their fungistatic effect on the *in vitro* growth of *C. albicans.*

Figure 3.3. *In vitro* fungicidal/ static action determination of antiviral drugs on *C. albicans*. *C. albicans* cells treated with MIC and 2 × MIC of antiviral drugs namely 2-AM, 2-DG, Ribavirin and Vidarabine, and plated on YPD agar plates.

3.3.3. Effect of antiviral drugs on Y-H morphogenic transitions

One of the key steps in developing biofilms is the transition ability of *C. albicans* from yeast to hyphal form. Hyphal development was induced in *C. albicans* by addition of the 20 % FBS as the inducer. The results of this experiment revealed that among ten antiviral drugs only 2-AM, 2-DG, Ganciclovir had anti-morphogenic activity. **Fig. 3.4**

represents the inverted microscopic observations that shows inhibitory action of 2-AM, 2-DG and Ganciclovir drugs on morphogenic transition in *C. albicans*. In untreated control of *C. albicans* cells, significant hyphal growth is observed after 90 min of incubation at 37°C whereas, treatment with 2-AM, 2-DG, Ganciclovir antiviral drugs exhibited concentration dependent reduction of Y-H morphogenic transition. At 0.25, 1 and 0.5 mg/ml 2-AM, 2-DG and Ganciclovir found to reduce the hyphal development and same concentrations were considered as MIC for morphogenesis. Whereas, total suppression of hyphal development was seen at 0.50 mg/ml, 2 mg/ml and 1 mg/ml concentrations of 2-AM, 2-DG and Ganciclovir.

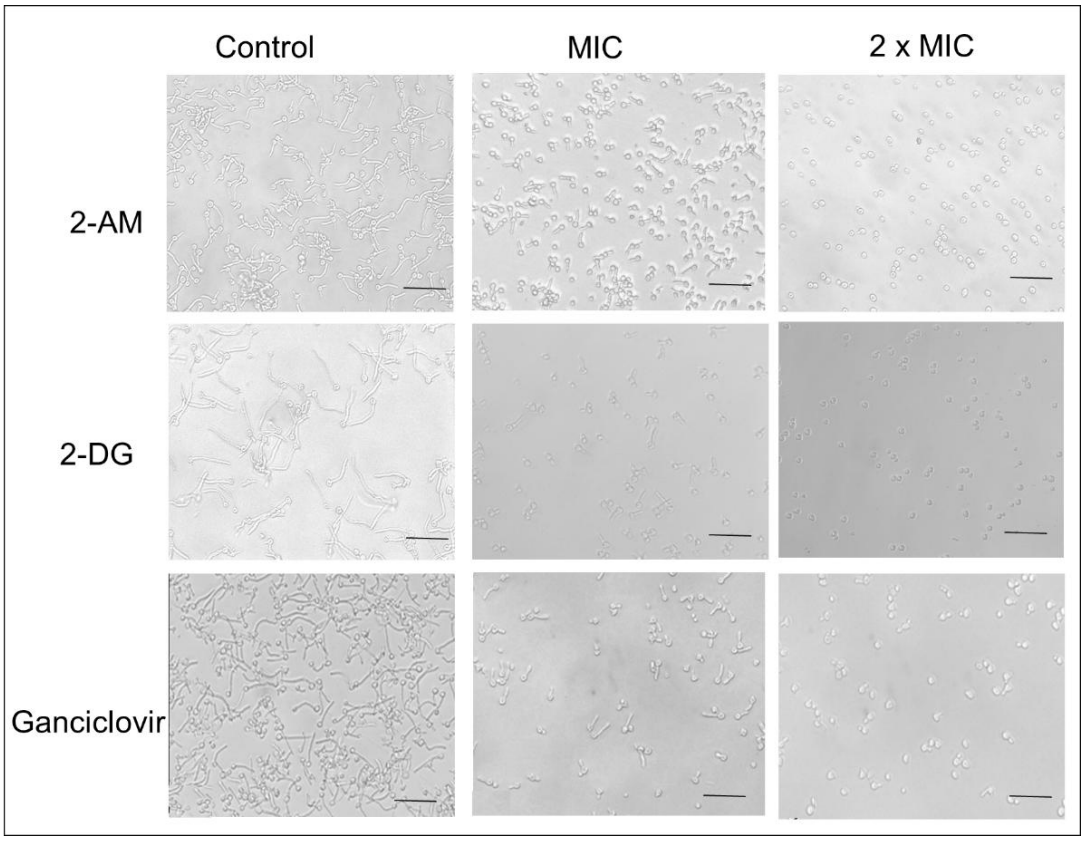

Figure 3.4. Inverted microscopic images of Y-H morphogenic switching in *C. albicans.* 2-AM, 2-DG and Ganciclovir showed MIC at 0.25, 1 and 0.5 mg/ml and inhibited morphogenesis at 0.5, 2 and 1 mg/ml (Magnification of 40x). Indicated scale is 50 µm.

However, other antiviral drugs were unable to arrest this morphogenic shift even at respective higher concentrations mentioned in **Table 3.1**. Treatment with 2-AM, 2-DG and Ganciclovir resulted in arrest of morphogenic transition in *C. albicans* to the less virulent yeast phase from more virulent hyphal phase. Thus, further studies on action of these antiviral drugs assessed to explore their antifungal potential and mode of action

against *C. albicans.* As per the MIC values of Y- H inhibition order of anti-morphogenic activity of antiviral drugs is 2-AM > Ganciclovir > 2-DG.

3.3.4. Effect of antiviral drugs on surface adhesion

The ability of *C. albicans* to adhere solid surface was studied using the tissue culture treated 96-well plate version adhesion assay method. Among ten antiviral drugs, 2-AM seems to be the only antiviral drug that affected the surface adherence ability of *C. albicans* significantly**.**

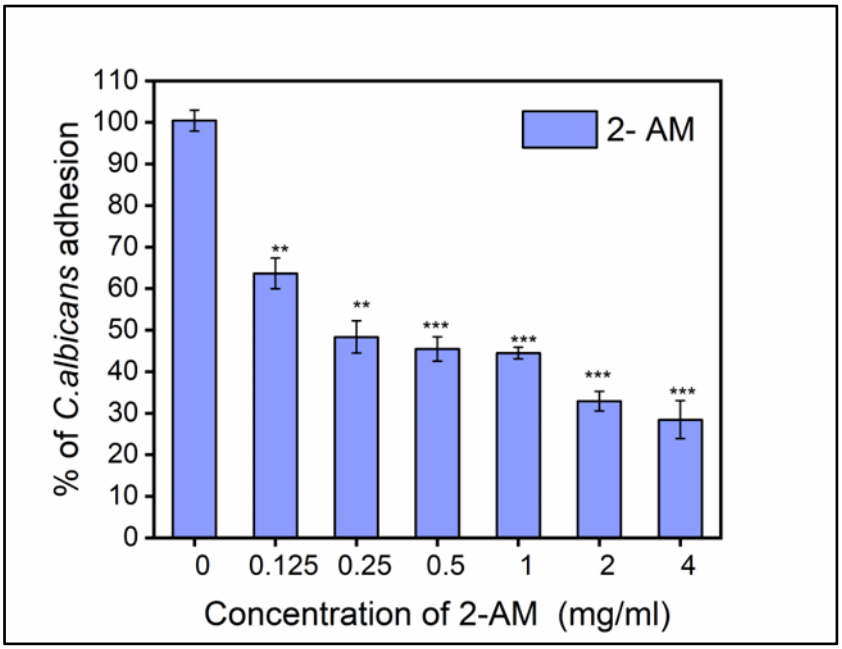

Figure 3.5. XTT metabolic assay for cell adhesion ability testing. Surface adherence ability of *C. albicans* treated with different concentration of 2-AM and evaluated by XTT metabolic assay. (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$).

According to the results of *C. albicans* cell adhesion assay shown in **Fig. 3.5**, 2-AM with concentration range of 4 to 0.125 mg/ml significantly reduced the adhered cell count compared to the untreated control *C. albicans* cells. 2-AM acts as an anti-adhesion compound that inhibits the initial phases of *C. albicans* pathogenesis.

XTT assay performed for *C. albicans* adhesion in presence of 2-DG, Acyclovir, Famciclovir, Ganciclovir, Idoxuridine, Lamivudine, Ribavirin, and Vidarabine. Although 2-DG, Ganciclovir, Idoxuridine, Famciclovir, and Valganciclovir showed dose-dependent anti-adhesion abilities against *C. albicans*, the inhibition did not exceed 50 %.

Fig. 3.6 indicates that, these antiviral drugs unable achieve MIC against adhesion

of *C. albicans* to the polystyrene surface even at their respective higher concentrations. So, it can be concluded that these antiviral drugs were less active against the adhesion of *C. albicans* cells. As 2-AM, is the only antiviral drugs which inhibits surface adherence ability of *C. albicans.* This result can be concluded as 2-AM affected the genes required for successful adhesion and hence reduced cell adhesion to the 96-polystyrene plate.

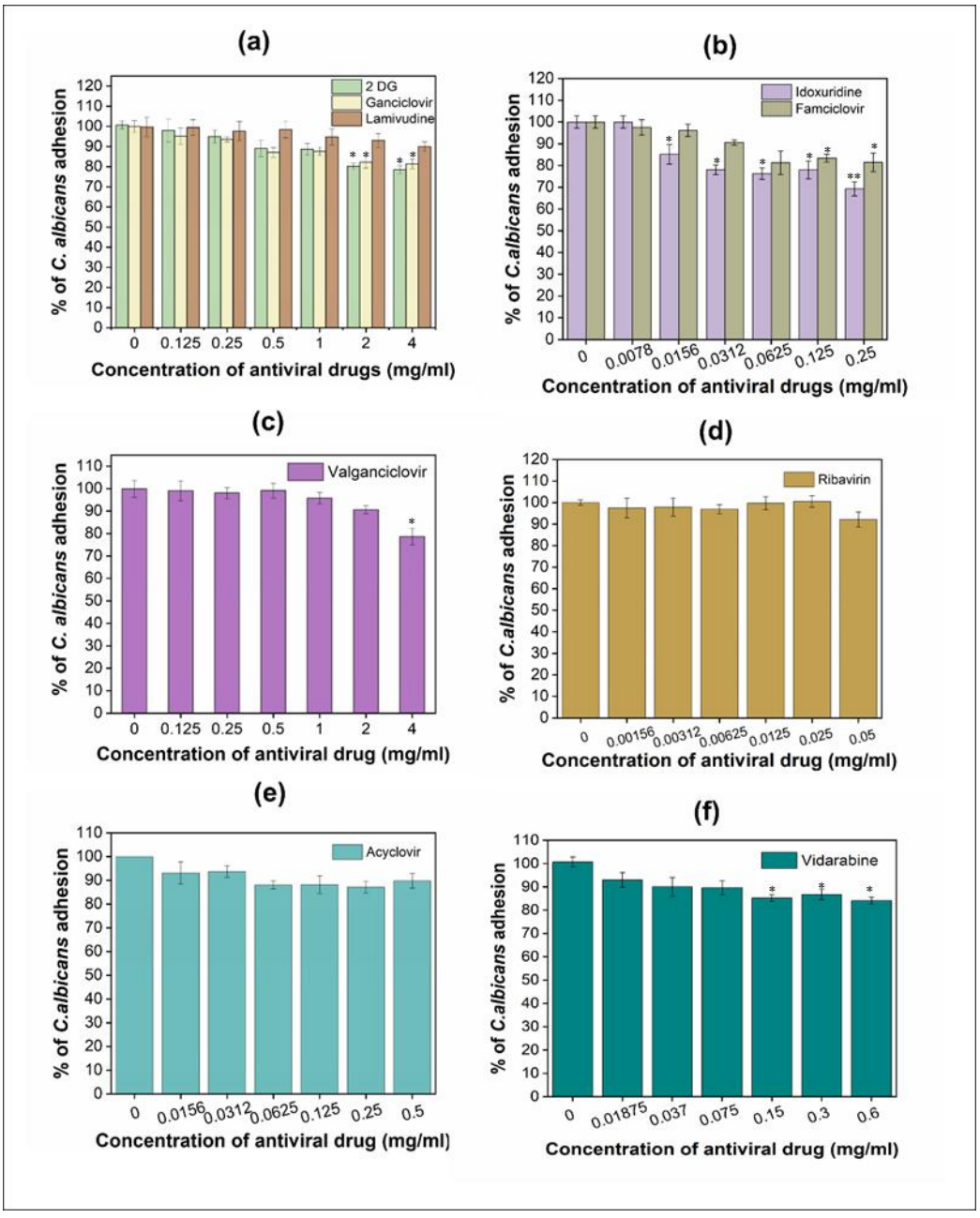

Figure 3.6. XTT metabolic assay for adhesion ability of *C. albicans.* Surface adherence ability of *C. albicans* in presence antiviral drugs at different concentrations of (a) 2-DG, Ganciclovir, and Lamivudine, (b) Idoxuridine, Famciclovir, (c) Valganciclovir (d) Ribavirin, (e) Acyclovir, and (f) Vidarabine. (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$).

3.3.5. Effect of antiviral drugs on developing biofilm

Antibiofilm activity of antiviral drugs against *C. albicans* was quantitatively assessed using the XTT metabolic assay. Anti-adhesion action of 2-AM proceeds to inhibit biofilm formation as well. As shown in **Fig. 3.7**, 2-AM has concentrations dependent activity on biofilm formation. With increase in the drug concentration of 2-AM the metabolic activity of developing biofilm is reduced. XTT assay further evidenced that treatment with 0.25 mg/ml of 2-AM reduced 50 % biofilm formation in *C. albicans*.

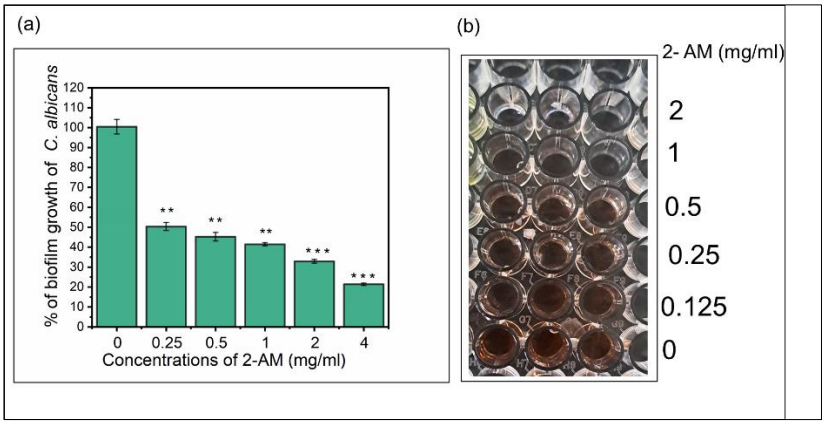

Figure 3.7. Effect of 2-AM on developing biofilm of *C. albicans*. (a) XTT metabolic assay for developing biofilm of *C. albicans* treated with different concentrations of 2-AM, and (b) Polystyrene plate representing the XTT metabolic assay. (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$).

Investigation of various antiviral drugs at concentrations outlined in **Table 3.1** was conducted to assess their antibiofilm potential using the XTT assay. **Fig. 3.8** demonstrates that antiviral agents are ineffective in suppressing *C. albicans* biofilm growth, even when administered at higher doses. Although higher concentrations of 2-DG, Ganciclovir, Lamivudine, Idoxuridine, Famciclovir and Vidarabine shows statistically significant difference between treated and untreated group of *C. albicans* cells notably, none of the tested compounds inhibited minimum 50 % of biofilm inhibition in *C. albicans*. In brief, among all screened antiviral drugs, only 2-AM inhibited developing biofilm of *C. albicans.*

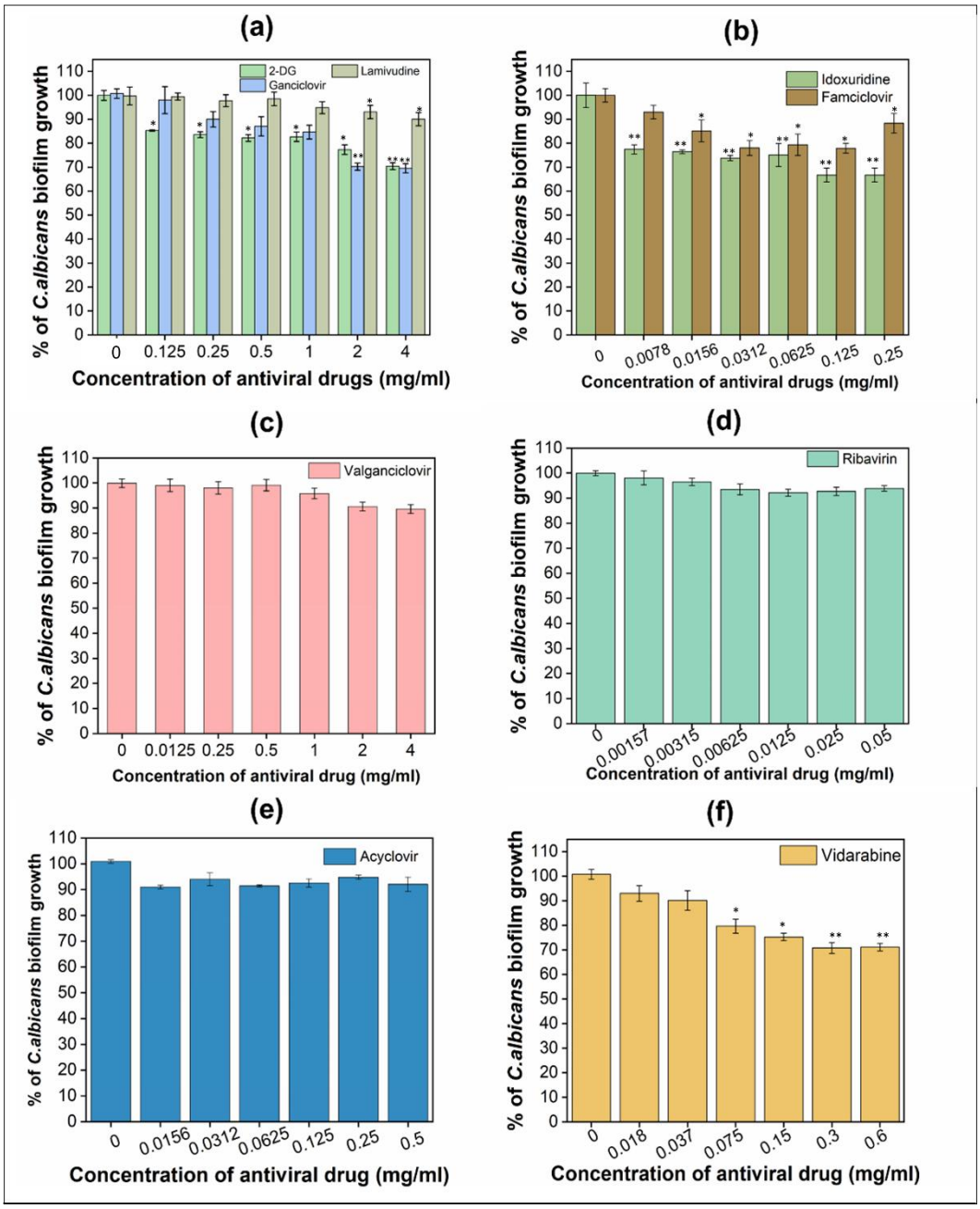

Figure 3.8. XTT metabolic assay for developing biofilm of *C. albicans*. Percentage of biofilm reduction in *C. albicans* treated with the different concentration of (a) 2-DG, Ganciclovir, and Lamivudine, (b) Idoxuridine, Famciclovir, (c) Valganciclovir, (d) Ribavirin, (e) Acyclovir, and (f) Vidarabine. (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$).

3.4. Discussion

C. albicans poses a threat to global health due to its high level of resistance to conventional antifungals. Modern medicinal therapies may find solutions for the search for therapeutic substitutes, which may include repurposing non-antifungal agents and their combination with routine antifungal agents [16]. In this study, we screened antiviral

drugs for their non-prescribed antifungal activities. The summary results are presented in **Table 3.2**.

Table 3.2. Summative results of MICs of antiviral drugs for *C. albicans* obtained in the primary screening.

Antiviral drugs	Planktonic growth (mg/ml)	Static/cidal action (mg/ml)	Morphogenic transition (mg/ml)	Adhesion assay (mg/ml)	Early biofilm development (mg/ml)
2-AM	**0.25**	**0.5**	**0.25**	**0.25**	**0.25**
2-DG	**2**	N.A.	**1**	N.A.	N.A.
Acyclovir	N.A.	N.A.	N.A.	N.A.	N.A.
Famciclovir	N.A.	N.A.	N.A.	N.A.	N.A.
Ganciclovir	N.A.	N.A.	**0.5**	N.A.	N.A.
Idoxuridine	N.A.	N.A.	N.A.	N.A.	N.A.
Lamivudine	N.A.	N.A.	N.A.	N.A.	N.A.
Ribavirin	**0.025**	N.A.	N.A.	N.A.	N.A.
Valganciclovir	N.A.	N.A.	N.A.	N.A.	N.A.
Vidarabine	**0.15**	**0.3**	N.A.	N.A.	N.A.

N. A.: Not Achieved

Screening methodology targets the preliminary and important virulence traits present in *C. albicans* which are required for establishment of disease. As per the CLSI guidelines, the broth microdilution assay was performed to find the antifungal susceptibility of these antivirals against the planktonic growth of *C. albicans*.

According to the results, *C. albicans* ATCC 90028 was susceptible to four antiviral drugs namely, 2-AM, 2-DG, Ribavirin and Vidarabine. Treatment to planktonic phase cells of *C. albicans* with 2-AM, 2-DG, Ribavirin, and Vidarabine reduced growth in dependence with the concentration. Ribavirin, Vidarabine drugs affected 50 % growth at 0.025 and 0.15 mg/ml, respectively indicating lesser concentrations required for inhibition as compared to the 2-AM and 2-DG with MIC of 0.25 mg/ml and 2 mg/ml, respectively. As per this data, Ribavirin stands more active drug against the planktonic growth of *C. albicans.*

In addition, in process of development of antifungal agent fungicidal activity serve as the desirable quality compared to the static as this ability enables antifungal to eradicate the fungal growth from tissues [17]. The majority of antifungals now in use or research are therefore in search of cidal class. In same concern, preliminary antifungal susceptibility study was proceeded to determine the fungicidal or static action of these

antiviral drugs against *C. albicans.* Notably, it is reassuring to observe that antiviral drugs namely, 2-AM and Vidarabine had MFC/MIC ≤ 4 ratio indicating their fungicidal nature. 0.5 and 0.3 mg/ml concentrations of 2-AM and Vidarabine were considered as MFC of respective drugs. Pharmaceutical formulations such as topical appliances made up with these antiviral drugs in combination with antifungal drugs are possibly useful as a widely prescribed FLC is facing a challenge due to its fungistatic mode of action [18].

In consideration of the significance of the Y-H transition for *C. albicans* pathogenicity, prophylactic and/or therapeutic approaches for candidiasis have suggested to focusing on morphogenesis. Several chemicals that disrupt hyphal development have been identified and described including lithium, propranolol, azoles, actin antagonists, rapamycin, propranolol, geldanamycin, hydroxyurea, nocodazole [18]. The morphogenetic transition of *C. albicans* from Y-H is a virulence factor that targeted for the treatment of infections in the mucosa and systemic circulation [7]. Here, to find inhibitors of hyphal development, current research used a "drug repurposing" screen on antiviral drugs. Ability of antiviral drug to inhibit the morphogenic swift in *C. albicans* was assessed specifically in presence of hyphal inducing environment.

In *C. albicans*, presence of serum act as an efficient inducer of hyphal development. Yeast-form cells cultured in YPD media at 37°C will start the hyphal formation minutes after being exposed to serum. The breakdown of serum glycoproteins produces N-acetylglucosamine and proline, both of which can independently promote filamentation. Further elevated temperature such as 37-39°C induces hyphal formation in *C. albicans* [19, 20].

The findings from this experiment demonstrated that out of all tested antiviral drugs, 2-AM, 2-DG, and Ganciclovir effectively suppressed the morphogenic transition. The extent of hyphal inhibition appeared to correlate positively with the concentration of these antiviral agents. Utilizing these drugs to target the Y-H morphogenic switch has been suggested as a potential alternative approach for candidiasis treatment. In contrast, other antiviral medications, such as Ribavirin and Vidarabine, did not show any inhibitory effect on this transition.

This finding additionally proved that 2-AM, 2-DG, and Ganciclovir retained their efficacy, even in the presence of serum, which acts as a hyphal inducer. This finding is significant from the viewpoint of drug development. However, Ganciclovir significantly inhibited the morphogenic transition but did not show any planktonic growth-inhibiting effect on *C. albicans*. This seems that Ganciclovir even at higher

concentration unable to inhibit the cell viability by least 50 % instead target the virulence trait of hyphal development in *C. albicans*.

Further, fungal colonisations and subsequently biofilm formation is based on the ability of *C. albicans* cells to adhere biotic or abiotic surfaces. Even though the cell adhesion assay has a lower throughput than the typical optical density assay, it is still worth using this assay to reveal more indirect biofilm defects that would otherwise remain unnoticed in other biofilm formation assays. Targeting the adhesion ability of *C. albicans* is another promising step in the development of antifungal agents. Therefore, ten antiviral drugs were screened for anti-adhesion action on polystyrene plates and quantified according to the XTT metabolic assay. Among the screened antiviral drugs, only 2-AM reduced the surface adherence ability of *C. albicans.* Further screening of antiviral drugs process continues up to the inhibitory action on biofilm formation ability of *C. albicans.* The anti-biofilm inhibitory activity of antiviral drugs was investigated using the most accurate and reproducible quantitative XTT metabolic assay [21,22]. Among all the screened antiviral drugs, 2-AM showed significant inhibitory action on biofilm development with inhibitory concentration at 0.25 mg/ml.

Inhibition of pathogen proliferation or targeting a particular microbial protein present in pathogen for inhibition are two criteria employed for searching new antifungal drugs. Therefore, antivirals with antifungal actions were continued with same approach. This data suggests that five antivirals 2-AM, 2-DG, Ganciclovir, Ribavirin, and Vidarabine (**Fig. 3.9**) could be employed for repurposed approach as promising antifungal agents.

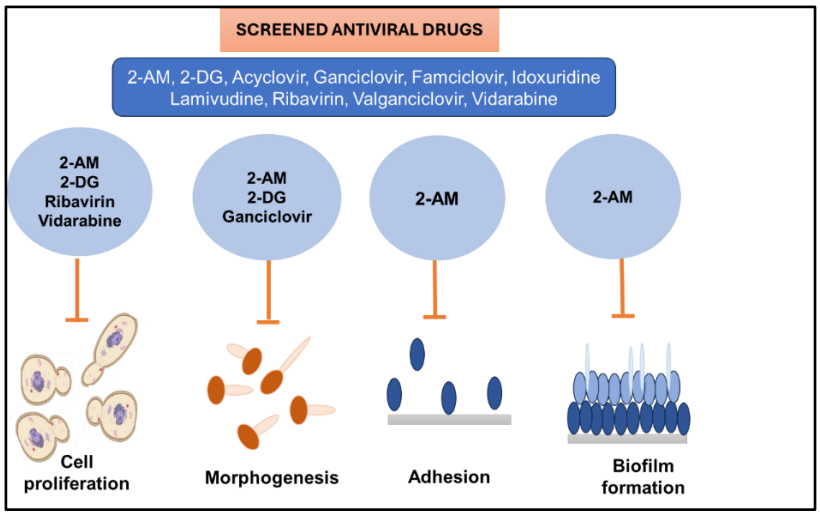

Figure 3.9. Graphical presentation of antifungal effects of screened antiviral drugs affecting the preliminary growth and morphogenesis in *C. albicans.*

Detailing on antifungal activities of antiviral drugs with probable mode of actions may widen the applicability of these antiviral drugs.

3.5. Conclusions

In conclusion, ten antiviral drugs namely 2-AM, 2-DG, Acyclovir, Famciclovir, Ganciclovir, Idoxuridine, Lamivudine, Ribavirin, Valganciclovir, and Vidarabine were screened for their inhibitory action on *C. albicans.* Among them 2-AM (0.25 mg/ml), 2-DG (2 mg/ml), Ribavirin (0.025 mg/ml), and Vidarabine (0.3 mg/ml) reduced cell growth of *C. albicans* ATCC 90028. 2-AM, Vidarabine exhibited fungicidal action against *C. albicans* at 0.5 and 0.3 mg/ml, respectively. Ganciclovir treatment did not inhibit planktonic growth but showed anti-morphogenic action similar to 2-AM and 2-DG against Y-H morphogenic transition. 2-AM, 2-DG and Ganciclovir reduced hyphal morphogenesis at 0.25, 1 and 0.5 mg/ml concentrations, respectively. Among the screened antiviral drugs, 2-AM had a potent effect on surface adherence and biofilm-forming ability of *C. albicans* with MIC at 0.25 mg/ml for biofilm development. In brief, out of ten antiviral drugs five drugs namely, 2-AM, 2-DG, Ribavirin, Vidarabine and Ganciclovir achieved MIC against growth and primary virulence factors present in *C. albicans.* The antifungal activities of these drug claims further exploration for the various virulence attributes of *C. albicans.*

3.6. References

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CHAPTER 4

Exploring antiviral drugs for antifungal activity against *Candida albicans*

4. Exploring antiviral drugs for antifungal activity

4.1. Background

In chapter 3, antiviral drugs were screened against *C. albicans* for their antifungal activity. Screening of antiviral drugs was based on the ability of the drugs to target the preliminary growth and important virulence factors present in *C. albicans*. Prior to investigating the probable mode of action of these antiviral drugs against *C. albicans*, their further antifungal activities on various virulence traits needed to be detailed. This chapter deals with the effect of screened antiviral drugs on different virulence attributes present in *C. albicans*.

Concentration of drug inhibiting at least 50 % planktonic growth or morphogenic transition of *C. albicans* was considered as MIC of that drug. Same MIC values of antiviral drugs are used to treat *C. albicans* for further study. In accordance this, antiviral drugs namely, 2-AM, 2-DG, Ganciclovir, Ribavirin, and Vidarabine are accessed in this chapter, and study outline is briefed in **Fig. 4.1**.

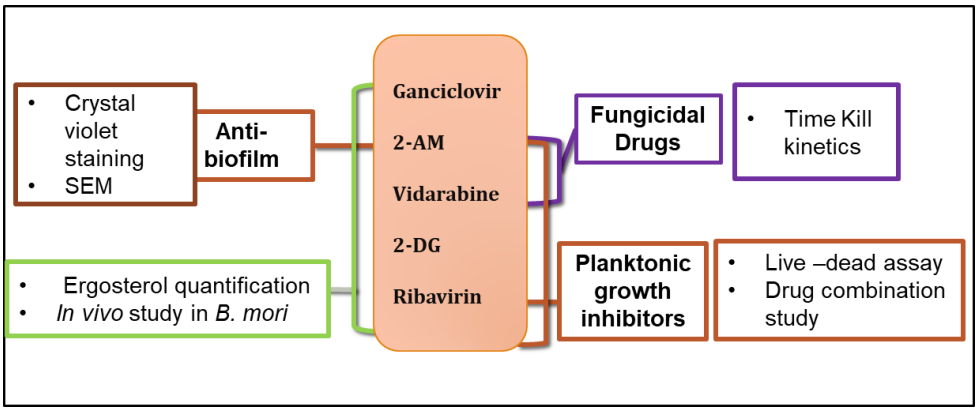

Figure 4.1. Outline of the assays referred for exploring antiviral drugs for antifungal activity against *C. albicans.*

Fungicidal activity of antiviral drugs is further explored with time-kill kinetics, in which the killing time of a susceptible pathogen by the antifungal agent is revealed. This data could improve the therapeutics based on antifungal agents for management of various infections [1]. The time-kill kinetics study is based on the assumption that the fungicidal agent possesses time dependent activity. This data provides the rate of fungicidal action and pharmacodynamic characteristics of the antifungal agents [2]. In this chapter, antiviral drugs with cidal nature are tested for a time kill study that validated the fungicidal action of antiviral drugs and confirms the significant inhibitory action on *C. albicans.*

Anti-*Candida* action of antiviral drugs on the planktonic phase of *C. albicans* is further evaluated by using the live-dead cells staining method. Fluorescence microscopic images of stained cells enables qualitative study on the altered cell population after the therapeutic applications of antiviral drugs [3].

Ergosterol is a primary fundamental constituent of *C. albicans* cell membrane that maintains cell fluidity, thickness, and permeability. Ergosterol serves as a potential target for the majority of antifungal drugs to inhibit *C. albicans* cells, as this component is absent in mammals [4]. Compounds interfering with any step of ergosterol biosynthesis or directly complexing the ergosterol, act as antifungal agents that lead to *C. albicans* inhibition. In the current chapter, antiviral drugs are evaluated for ergosterol quantification to determine their effect on this fungal cell membrane component [5].

Central venous catheters, urinary catheters, and other medical implants, including heart valves, and cardioverter defibrillators, are implanted in patients for medicinal purposes. These medical devices provide the niche for various types of pathogenic organisms, including *C. albicans.* Due to the ability of surface adherence and biofilm formation, *C. albicans* attached to the catheter surface and led to the consequences of catheter related infections. Exploring the anti-biofilm ability of newer non-antifungal drugs may accelerate the process of treatment for catheter related infections. 2-AM shown the anti-biofilm activity as mentioned in chapter 3 is further studied for its anti-biofilm ability on urinary catheter.

In this chapter, the efficacy of antiviral drugs namely, 2-AM, 2-DG, Ganciclovir, Ribavirin and Vidarabine are further checked in an *in vivo* model to explore their antifungal activity, and toxicity. The infection of *C. albicans* is given to *Bombyx mori* and used as an *in vivo* animal model [6]. Silkworms have various advantages over mammals as an *in vivo* experimental animal, including lower raring costs, the capacity to maintain large numbers of animals in a compact space, and fewer or no ethical concerns with their usage in research. The silkworm *B. mori,* thus serves as an excellent experimental animal for assessing the pathogenicity of fungi in systemic infections, as well as the efficacy of antifungal drugs against fungal infections [7]. Thus, the *in vivo* effectiveness of selected antiviral drugs in *C. albicans* infected *B. mori* silkworm animal model is evaluated. Furthermore, in addition to the monotherapy (single drug), drug combination-based therapy also brought much attention, as combined therapy was found to reduce drug toxicity, increase efficacy, and improve prognosis rates in patients [8–10]. In this chapter investigation of combinations of antiviral drugs with antifungals

(FLC, AmB, and CAS) are studied and obtained results propose that these combinations may restore the susceptibility of *C. albicans* to the routine antifungal drugs.

4.2. Materials and methods

4.2.1. Time-Kill study

Screened antiviral drugs with an ability to affect the planktonic growth of *C. albicans* with fungicidal effect were further characterized by the time-kill kinetics study. The experiment was performed as per previously reported protocol for 2-AM and Vidarabine with MFC [11]. In brief, cells harvested from overnight incubated YPD broth are used as the starting inoculum with density of 1×10^6 CFU/ml. Cells were resuspended in the RPMI-1640 medium containing MFC of antiviral drugs. The medium was further incubated at 35°C for next 48 h in slight agitation condition. At the sampling time interval of 0, 1, 2, 4, 6, 8, 10, 12, 18 and 24 h of incubation, 10 µl of aliquots of medium was collected in the sterile condition and spread inoculated on YPD agar plates. Then agar plates were incubated at 35°C for 24 h. Cells without drug treatment were considered as control for the experiment. The curve of time-kill assay was plotted by counting the log of CFU per plate.

4.2.2. Live and dead cell detection assay

To differentiate between *C. albicans* viable and dead cells after antiviral drug treatment dual staining with DNA-binding dyes was performed [12,13]. For this Acridine Orange (AO)-Ethidium Bromide (EtBr) both dyes were used. Fluorescence microscopy exhibits a distinct fluorescence pattern in the dual stained cells, which is dependent on membrane integrity and viability. For testing the same, antiviral drugs affecting planktonic growth of *C. albicans* with MIC were studied. 1×10^6 cells suspended in RPMI-1640, were exposed to the inhibitory concentrations of 2-DG, 2-AM, Ribavirin and Vidarabine, and mixture was incubated for 3 h at 30°C. After the incubation period, cells were washed in sterile PBS and proceed for staining. To prepare AO-EtBr dual fluorescent stains, 100 µg/ml concentration solutions of AO and EtBr each were mixed 1:1 (v/v). 10 µl of dual stain was applied to *C. albicans* cells and incubated for 15-20 min in dark condition. Stained cell pellet was washed with sterile PBS and proceed for fluorescence imaging. Using a Nikon Eclipse fluoroscence microscope with a 40x objective, imaging was carried out.

4.2.3. Ergosterol extraction and quantification

Quantification of ergosterol provides a significant step to evaluate the effect of antiviral drugs on *C. albicans.* Antiviral drugs active against planktonic growth and Y-H morphogenic shift namely, 2-AM, 2-DG,Ganciclovir, Ribavirin and Ganciclovir were selected for ergosterol assay. Briefly, a single colony of *C. albicans* from Sabouraud agar plate was inoculated into 50 ml of Sabouraud broths containing different concentrations antiviral drugs. Broth containing *C. albicans* cells suspended in vehicle solvents, D/W and DMSO and without any drug treatment was referred to as control. All broths were incubated at 30°C for 16 h in shaking condition with 100 rpm. After incubation cells were harvested using a centrifuge at 2700 rpm for five min in pre-weight centrifuge tubes. The harvested cells were treated with a 25 % alcoholic potassium hydroxide solution of 3 ml volume and mixed thoroughly. The solution is transferred to the glass bottles and further incubated at 85°C for 1 h. After the incubation period tubes were cooled to room temperature. For sterol extraction, 1 ml of distilled water and 3 ml of n-heptane were added to each tube, and the mixture was vortexed for 3 min. Detection of ergosterol samples was done in fivefold diluted solution prepared in absolute alcohol and then analysed by spectrophotometrically between the wavelength 200 to 300 nm. The content of ergosterol in presence and absence of antiviral drugs was determined by using following equations:

$$\%\ \text{Ergosterol} + \%\ 24(28)\text{DHE} = \frac{\frac{A(281.5)}{290} \text{ x F}}{\text{Pellet weight}}$$

$$\%\ 24(28)\text{DHE} = \frac{\frac{A(230)}{518} \text{ x F}}{\text{Pellet weight}}$$

$$\%\ \text{Ergosterol} = (\%\ \text{Ergosterol} + \%\ 24(28)\ \text{DHE}) - \%\ 24(28)\ \text{DHE} \qquad [14]$$

Where % 24 (28) DHE is the late sterol intermediate, 290 and 518 are percent extinction coefficient values (in percentages per centimeter) determined for crystalline ergosterol, and 24 (28) DHE in absolute alcohol, respectively, F is Dilution factor of hexane extract present in ethanol, and A (281.5) and A (230) are the absorbance at 281.5 and 230 nm, respectively [15].

4.2.4. Crystal violet staining

Quantitation of biofilm inhibition was further performed by the crystal violet staining with little modifications [16]. The assay was performed for 2-AM as it is the only antiviral drug inhibiting the developing biofilm. For this, different concentration

grades of 2-AM (0.125-2 mg/ml) were prepared in RPMI-1640 medium, and the assay was performed as mentioned in chapter 3 (methodology section 2.2.7). After the incubation period, wells were washed with 200 µl of PBS and dried for 45 min. Cells were stained with crystal violet (0.4 % D/W diluted) with a volume of 110 µl and then plate was kept undisturbed for 45 min. After the incubation period, wells with stained cells were washed four times with sterile D/W. For destaining purpose, 200 µl of 95 % ethanol was added to the wells and kept for 45 min. Afterwards, the destained solution of 100 µl was added to the wells of the new plate, and the absorbance was measured at 595 nm on microtiter plate reader (Thermo Scientific Multiskan Sky Microplate Spectrophotometer).

4.2.5. Scanning electron microscopy (SEM)

The SEM was used for morphological study of hyphal and biofilm inhibition in *C. albicans* after the treatment of antiviral drugs. Silicon based urinary catheters were used as the abiotic surface for *C. albicans* SEM analysis. The hyphal induction and biofilm formation assays were performed as per described methods (methodology section 2.2.5 and 2.2.7) on the urinary catheters. For the assay, hyphal and biofilm inhibiting antiviral drugs were selected only. Catheters containing antiviral drugs treated *C. albicans* cells were considered as treated catheters and catheter pieces containing cells without drug treatment referred as control. After the treatment, catheters were gently washed to remove non-adhered cells with sterile PBS. Catheters were dipped in 2.5 % glutaraldehyde for overnight at 4°C for the purpose of cell fixation and then immersed in 1 % solution of osmium tetra oxide solution for 4 h followed by dehydration in alcohol gradient (10 %, 30 %, 60 %, and 90 %) made up in D/W. Samples were then air dried for overnight [17]. After gold coat spraying, samples were proceeded for visualization under SEM (VEGA3 TESCAN Microscope) with magnifications of 1000x and 2500x.

4.2.6. Drug combination study

Two-dimensional checkerboard microbroth dilution assay was performed to study the combination of two drugs in *in vitro* conditions. Antiviral drugs affecting the planktonic growth of *C. albicans* were used for the combinational study with antifungal drugs. As per the CLSI guidelines microbroth dilution assay was modified for checkerboard assay. In this, inoculum of size 2×10^3 CFU/ml of *C. albicans* was used and the four-fold higher concentrations of MIC of antiviral and antifungal drugs were prepared in the RPMI-1640 medium. 50 µl of antifungal dilutions were added in to the

column from 1 to 11 and 50 µl the antiviral drug dilutions were added to the rows from A to G in 96-well microtiter plate. 100 µl of cells were added to the each well and incubated at 35°C for 48 h. The growth of the cells was measured in terms of absorbance at 620 nm using spectrophotometer. The drug combination pattern was analysed based on non-parametric approach by using fractional inhibitory concentration (FIC) calculations [18]. In this, FIC index ≥ 4 is indicates antagonism, ≤ 0.5 considered as synergistic combinations and index value between 1 to 4 referred as indifference. The FIC index values are determined by following formula,

FICI index = FIC A+ FIC B where,

FIC A drug = $\frac{\text{MIC A}^{\text{combination}}}{\text{MIC A}^{\text{Alone}}}$ and FIC B drug = $\frac{\text{MIC B}^{\text{combination}}}{\text{MIC B}^{\text{Alone}}}$ [19].

Where, A and B are two different drugs

4.2.7. *Bombyx mori* survival assay

Using *B. mori* silkworm larvae as an *in vivo* model organism, the effectiveness of antiviral drugs as a therapeutic agent against the pathogenicity of *C. albicans* was investigated. As previously reported, *C. albicans* infected *B. mori* model was developed with slight modifications. The Department of Zoology at Shivaji University in Kolhapur provided larvae in their fourth instar, which then underwent rearing as per the guidelines provided by Krishnaswami [20]. For reproducibility of the work silkworm larvae weighing in between 0.8-1 g with uniform colour, clear and no dark spots were selected for the survival study. *C. albicans* infected *B. mori* model for antiviral drug efficacy was used as per the **Table 4.1** and each group contained 5 larvae in number.

Table 4.1. Grouping of silkworms *Bombyx mori* larvae for study of *in vivo* efficacy of antiviral drugs against *C. albicans* infection

Group I	Group II	Group III	Group IV
Positive control	Negative control	Vehicle control	Test
B. mori infected with 1×10^6 *C. albicans* /larvae and FLC with MIC	*B. mori* infected with 1×10^6 *C. albicans* /larvae	*B. mori* infected with 1×10^6 *C. albicans* / larvae and 1 % DMSO	*B. mori* infected with 1×10^6 *C. albicans* / larvae and MIC of antiviral drugs

In brief, the volume of 50 µl containing suspension of 1 × 10^6 CFU/ml *C. albicans* cells along with MIC of antiviral drug was injected into the haemolymph of silkworm larvae. For infection an insulin syringe of 1 ml volume capacity was used and injected through the pro leg of larvae [21]. Then larvae were placed in petri dishes with rearing diet and observed for survival period for the next three days at 27°C. Validation of larval death was visually confirmed by development of melanization and larval reaction to physical stimuli by lightly touching them. Results of silkworm survival was built by using GraphPad prism 7.0. version as per the Kaplan–Meier method.

4.3. Results

4.3.1. Studies of time-kill curves

Antifungal performance of fungicidal antiviral drugs (2-AM and Vidarabine) was further characterized by time-kill kinetics parameter. In this, 2-AM and Vidarabine at their MFC values of 0.5 and 0.3 mg/ml, respectively were used to determine the time dependant killing pattern. Antiviral drug treated *C. albicans* cells were spread plated on YPD agar plate and noted for colony count. *C. albicans* cells without any treatment were considered as control. The time-kill plot of *C. albicans* cells treated with to 2-AM and Vidarabine shown in **Fig. 4.2**.

The killing curve was plotted by counting the fungal colonies grown on the YPD agar plates for predefined time duration. The cells from control group survived, grew in exponential stationary phase, and formed more colonies on agar plate compared to the antiviral drug treated cells.

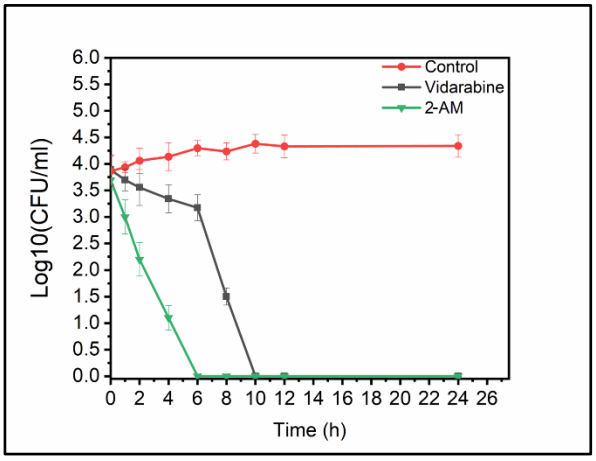

Figure 4.2. Representative time-kill kinetics curves for *C. albicans* after the exposure of fungicidal antiviral drugs, 2-AM (0.5 mg/ml) and Vidarabine (0.3 mg/ml). Aliquots of treated and un-treated *C. albicans* assessed for $\log_{10}$ CFUs.

The fungicidal time-kill curves of antiviral drugs show that after treatment the colony count of *C. albicans* declines with the time duration. After the exposure of 2-AM and Vidarabine for 6 h and 8 h, respectively viable colony count of *C. albicans* was found to be reduced up to 99.9 %. As per the results, it can be concluded that 2-AM and Vidarabine has time dependent antifungal activity against *C. albicans.*

4.3.2. Live-dead cell analysis

Differentiation of live and dead cells after the antiviral drug treatment studied using fluorescence microscopy. *C. albicans* cells were dual stained with AO and EtBr, and then their specific fluorescence was studied. Among these two dyes, AO is vital and permissible dye cross the cell membrane while EtBr is unable to cross the cell membrane barrier of live cells. Fluorescence microscopic images in **Fig. 4.3** shows that after 4 h of incubation, untreated *C. albicans* cells are highly stained by AO and emits green fluorescence whereas cells from antiviral drug treated experimental setup intensely stained by EtBr dye and produces orange red fluorescence.

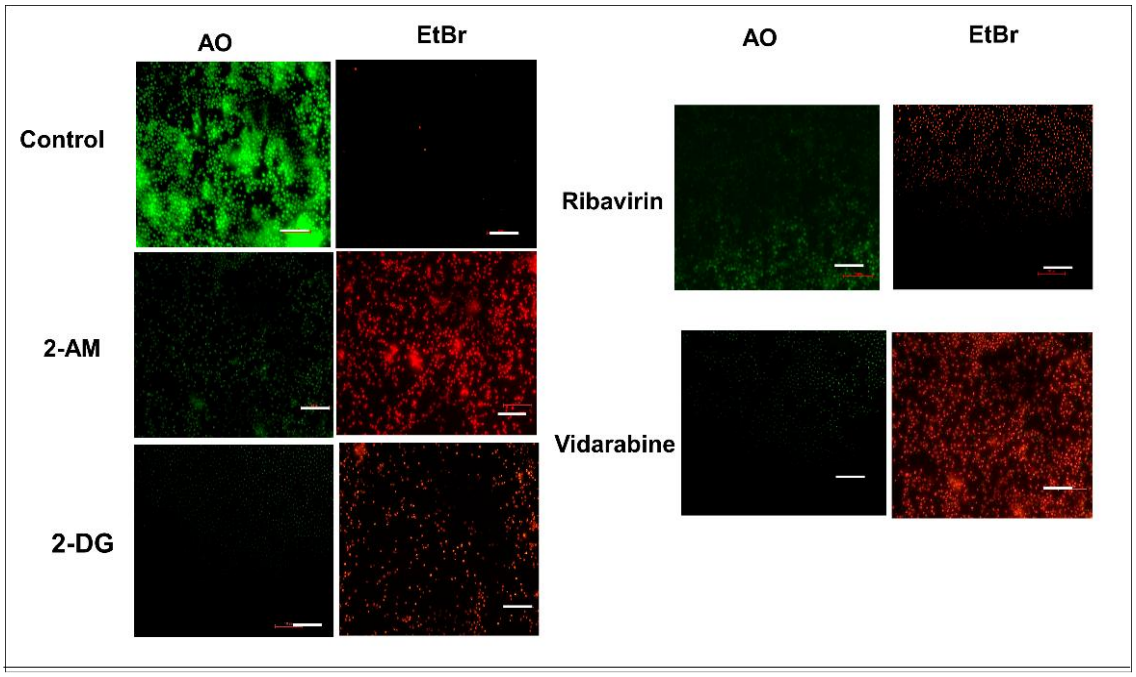

Figure 4.3. Fluorescence microscopic images of AO-EtBr-stained *C. albicans*. *C. albicans* cells treated with antiviral drugs 2-AM, 2-DG, Ribavirin and Vidarabine at 0.5, 4,0.05 and 0.3 mg/ml, respective concentrations were visualized under fluorescence microscope. The indicated scale is 50 µm.

C. albicans cells treated with 2-AM, 2-DG, Ribavirin and Vidarabine at 2 × MIC values 0.5, 4, 0.05 and 0.3 mg/ml, respectively showed intense orange red fluorescence compared to the green fluorescence. This data reflects the higher non-viable *C. albicans*

cells after antiviral drug treatment whereas the control set showed higher AO-stained green fluorescence emitting *C. albicans.* In control sample of *C. albicans* cells less orange red fluorescence was observed pointing the lesser dead cell count. The results of the study suggest that cells from control group were viable and grown in RPMI-1640 medium, whereas cells from antiviral treated group were dead and unable to proliferate. Dual staining confirmed that treatment of 2-AM, 2-DG, Ribavirin and Vidarabine inhibited *C. albicans* planktonic growth and increased cell dead count (**Fig. 4.3)**.

4.3.3. Quantification of ergosterol content

The total intracellular sterol from antiviral drug treated *C. albicans* cells was extracted to determine the effect of antiviral drug on the ergosterol content. Using UV/VIS spectrophotometer the absorption spectra of sterol was scanned between 200-300 nm wavelength. The spectral absorption pattern showed characteristic four peaks and that confirmed the presence of ergosterol in extracted sample (**Fig. 4.4**).

Antiviral drugs with concentration range from 2 × MIC to lower were selected for studies on the effect of concentrations on ergosterol content. In this 0.5, 4, 1, 0.05 and 0.3 mg/ml were 2 × MIC values of 2-AM, 2-DG, Ganciclovir, Ribavirin and Vidarabine, respectively used in this study. As compared to the control, treatment with 2-AM, Ribavirin, Vidarabine, 2-DG and Ganciclovir antiviral drugs reduced ergosterol content and was plotted in the form of the percentage of ergosterol inhibition (**Fig. 4.5)**.

Treatment with antiviral drugs to *C. albicans* resulted in concentration dependent reduction in ergosterol content. The data of ergosterol biosynthesis in presence and absence of antiviral agents is summarized in **Fig. 4.5**. The control group of cells contains untreated cells in all experimental set up and showed ergosterol presence and considered as 100 % synthesis while there was a reduction of ergosterol content after exposure of antiviral drugs.

Specifically, treatment to *C. albicans* cells with MIC values of 2-AM, Ribavirin, Vidarabine, 2-DG and Ganciclovir reduced ergosterol by 86, 81,78, 68 and 52 %, respectively. In addition, treatment with higher concentrations of antivirals that is with 2 × MIC drastically altered the sterol profiles and impaired biosynthesis process that leads to reduction in ergosterol content by 92, 82, 91, 90 and 90 %, respectively.

Results of this assay indicates that MIC of 2-AM has significantly reduced biosynthesis of ergosterol compared to the other antiviral drugs. Flat lines at dosage of

2 × MIC of antiviral drugs signify that no detectable content of ergosterol was found in antiviral drug treated *C. albicans* cells.

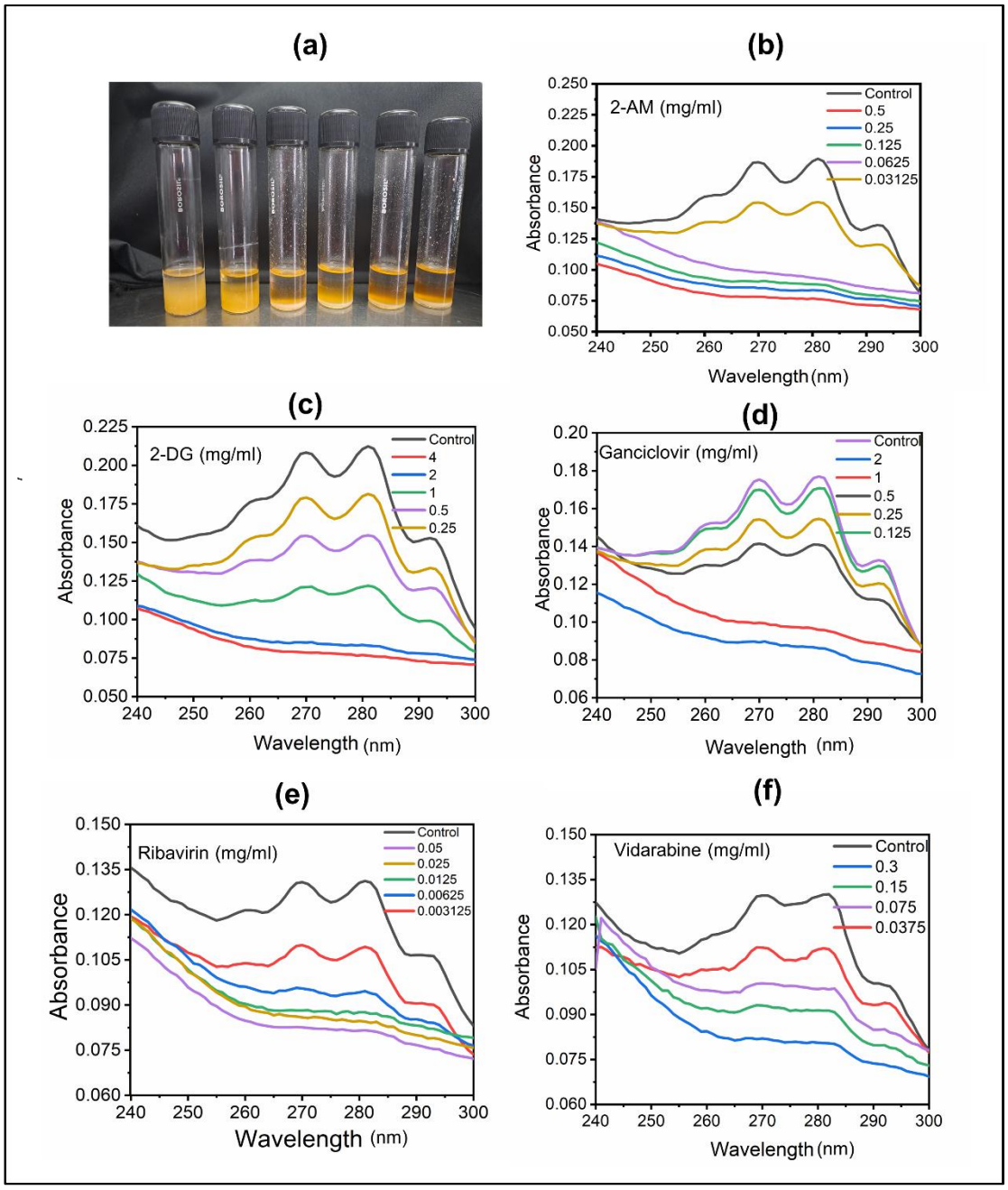

Figure 4.4. Spectrophotometric ergosterol quantifications in *C. albicans* (a) visual appearance of separated sterol from *C. albicans* after treatment with antiviral drug. Spectral profiles of extracted ergosterol in *C. albicans* treated with various concentrations of (b) 2-AM, (c) 2-DG, (d) Ganciclovir, (e) Ribavirin, and (f) Vidarabine.

In addition to the dose dependent reduction in ergosterol content, study further proposed that antiviral drugs may have a probable target in ergosterol biosynthesis process and therefore exhibited antifungal effect against *C. albicans.*

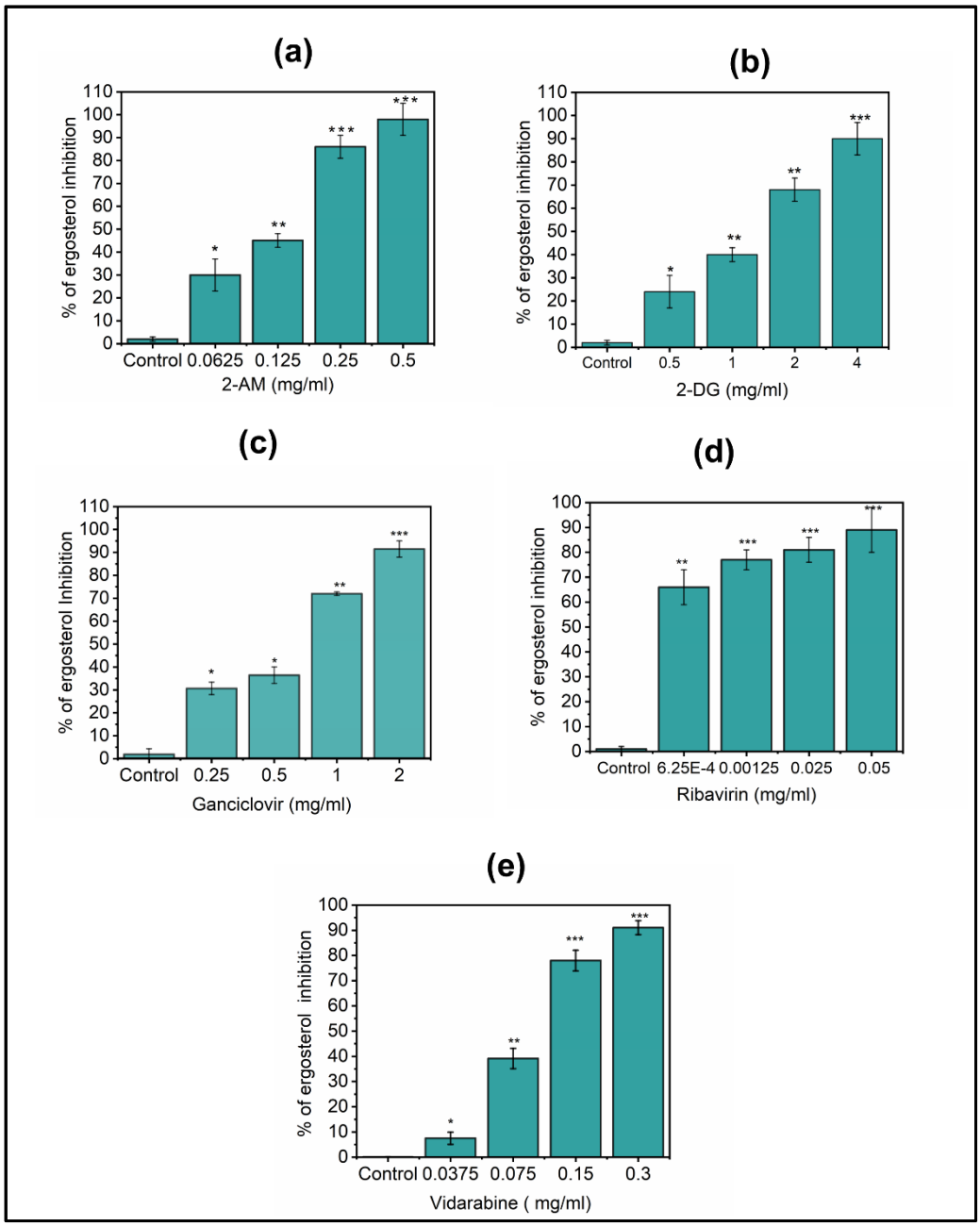

Figure 4.5. Plots depict the percentage of ergosterol inhibition after treatment with different concentrations of (a) 2-AM, (b) 2-DG, (c) Ganciclovir, (d) Ribavirin, and (e) Vidarabine to *C. albicans* and compared with control cells. (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$).

4.3.4. Crystal violet-based fungal biomass detection

Crystal violet staining is used to quantify the total developing biofilm biomass of *C. albicans* in presence of an antibiofilm agent 2-AM.

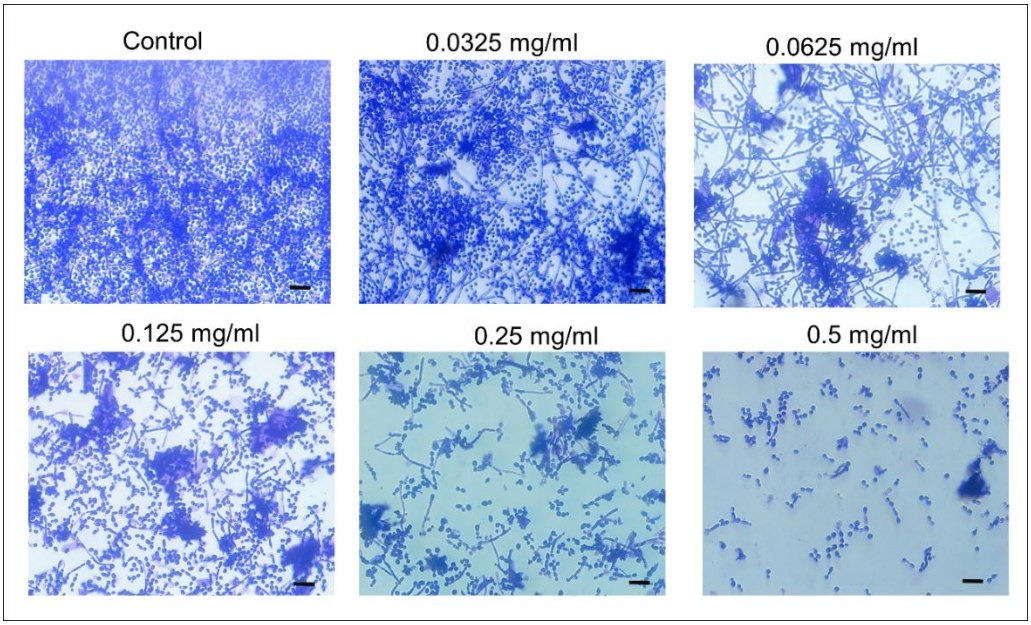

Figure 4.6. Inverted microscopic images of crystal violet stained biofilm of *C. albicans* in presence of different concentrations of 2-AM. Indicated scale is 50 µm.

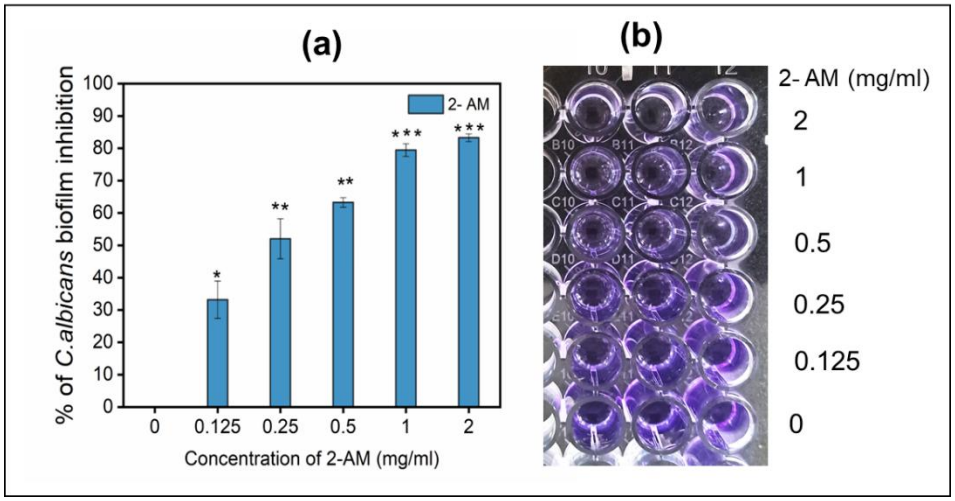

Figure 4.7. Quantification of biofilm biomass using crystal violet staining (a) Percentage reduction in biofilm biomass of *C. albicans* after treatment of concentration of 2-AM, and (b) Crystal violet-stained wells in 96-well plate after treatment of 2-AM to *C. albicans*. (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$).

The inverted microscopic images of crystal violet stained *C. albicans* biofilm (**Fig. 4.6**) show that the higher hyphal growth and complex biofilm structure in control group. However, as the concentration of 2-AM increases, the hyphal growth and fungal biomass is reduced and at higher concentration more yeast cells appeared in the field. Considerable biofilm was reduced at 0.5 mg/ml of 2-AM as compared to the untreated *C. albicans* cells. Results of quantitative reduction of biofilm biomass shows that as concentration of 2-AM increases fungal biomass decrease. Treatment with 2 mg/ml concentration of 2-AM to developing biofilm of *C. albicans* resulted in 83 % of biofilm biomass reduction compared to the control cells (**Fig. 7 a).**

4.3.5. Visualization of Y-H morphogenesis and biofilm inhibition using SEM

The SEM was used to visualize the anti-morphogenic and anti-biofilm activity of screened antiviral drugs on the silicon-based urinary catheter. 2-AM, 2-DG and Ganciclovir at concentrations of 0.5 mg/ml, 2 mg/ml and 1 mg/ml, respectively suppressed hyphal development in *C. albicans.* The inhibitory action was also continued on the catheter pieces. The SEM images confirmed that 2-AM, 2-DG and Ganciclovir substantially arrested *C. albicans* cells in the yeast form whereas cells from drug free group able to switch the Y-H morphogenic transition and able to form hyphae on silicon based urinary catheters (**Fig. 4.8**).

Visualization of biofilm formation in absence and presence of 2-AM was also done using the SEM. It was found that 2-AM appear to target both surface adherence and biofilm formation in *C. albicans* on the surface of urinary catheter (**Fig. 4.9**). 2-AM treated *C. albicans* biofilms were deprived of hyphal growth, and consisting of blastopores. After 48 h of incubation, non-treated *C. albicans* cells with typical true hyphae formed a well-developed, stable and complex biofilm structure. This ability of 2-AM suggests the strong inhibitory action of 2-AM on Y-H transition and biofilm development on both polystyrene surface and silicon based urinary catheter.

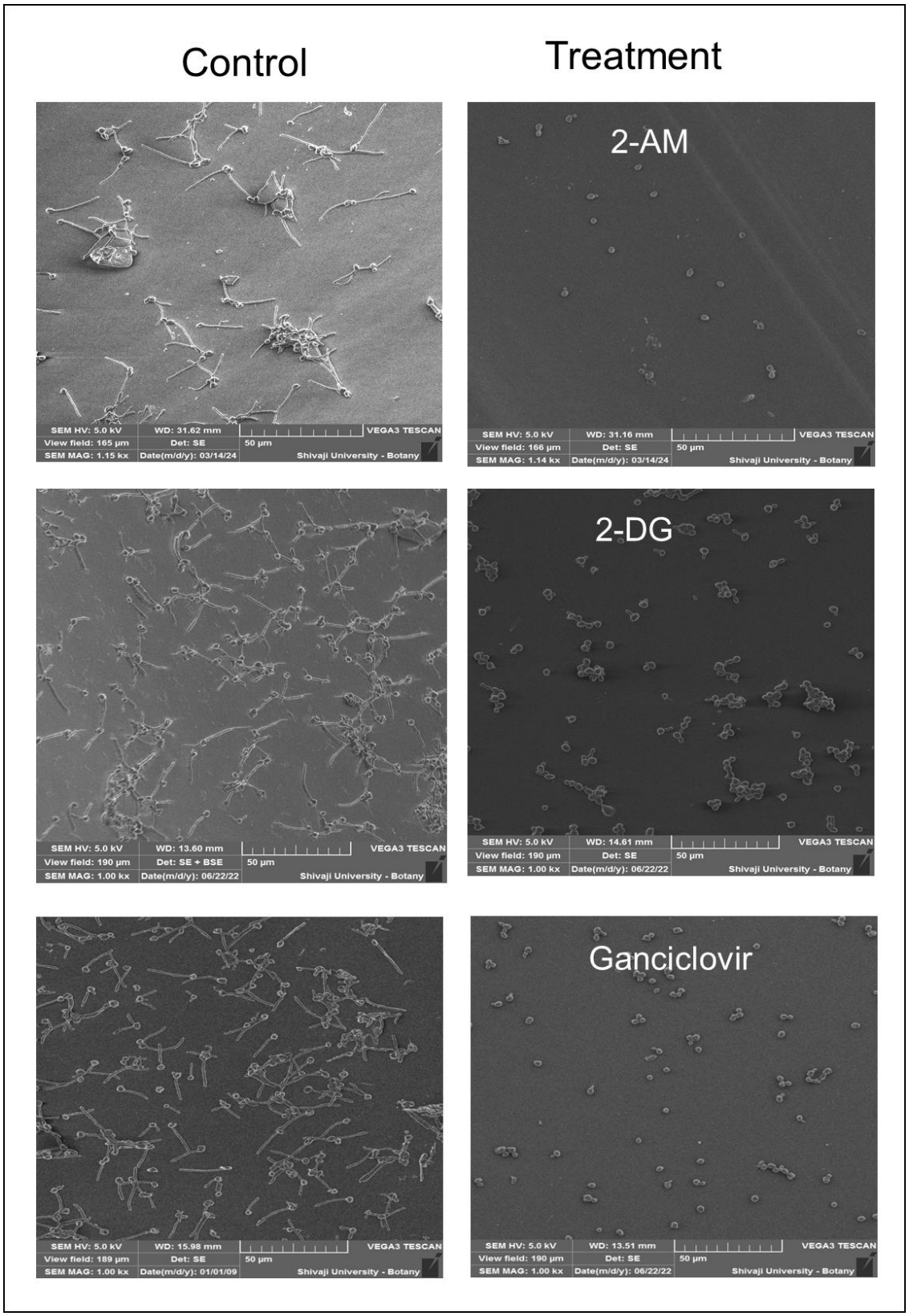

Figure 4.8. SEM images of *C. albicans* morphogenesis after the treatment of 2-AM (0.5 mg/ml), 2-DG (2 mg/ml), and Ganciclovir (1 mg/ml) on silicon based urinary catheter. *C. albicans* cells without treatment of antiviral drugs referred to control. Magnification of 1500x and scale is 50 µm.

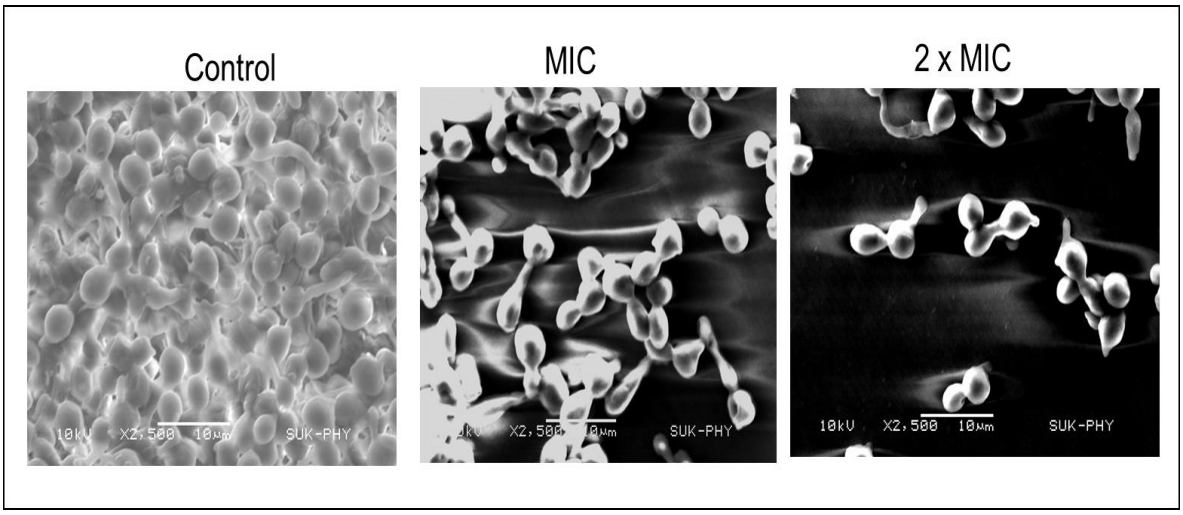

Figure 4.9. SEM images of *C. albicans* biofilm development in presence of 2-AM (0.25 and 0.5 mg/ml). Cells without 2-AM treatment are considered as control. (Magnification 2500x and scale is 10 µm).

4.3.6. Drug combination studies

Antiviral drugs in combination with antifungal agents FLC, AmB and CAS were studied. Checkerboard assay with FIC index was used to identify the synergistic drug combinations of antifungal and antiviral drugs. Antiviral drugs capable of inhibiting planktonic growth of *C. albicans* were selected for the study.

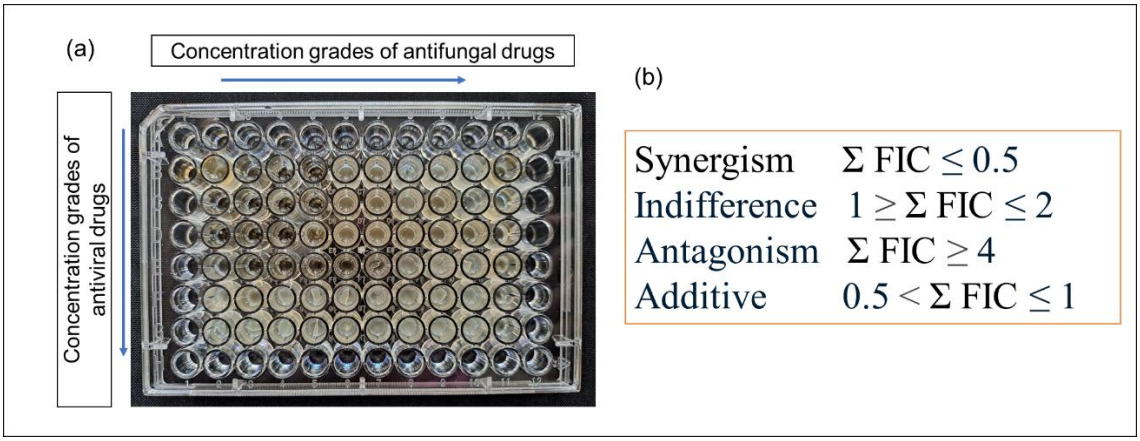

Figure 4.10. (a) Checkerboard microtiter plate assay for antiviral and antifungal drug combination in *C. albicans* exhibiting the reduced turbidity in synergistic combination, and (b) types of antiviral-antifungals drug combination depending upon the FIC index.

In this study, antiviral drugs affecting the planktonic phase (2-AM, 2-DG, Ribavirin and Vidarabine) were combined with antifungal drugs. The obtained combinations are analysed as per the FIC values shown in **Fig. 4.10**. Results of *in vitro* drug combinations of antiviral drugs with antifungal drugs are reported in **Table 4.2**.

Table 4.2. MIC values of antiviral drugs alone and in combination with antifungal drugs FLC, CAS, and AmB against planktonic growth of *C. albicans* with FIC index

Sr. No.	**MIC**				**FIC index**	**Remarks for combination analysis**
	Alone		**Combination**			
	Antiviral drugs (mg/ml)	Antifungal drugs (µg/ml)	Antiviral drugs (mg/ml)	Antifungal drugs (µg/ml)		
1.	**2-AM**	**FLC**	**2-AM**	**FLC**	**0.50**	Synergistic
	0.25	1.5	0.015	0.75		
2.	**2-AM**	**AmB**	**2-AM**	**AmB**	**2.0**	Indifference
	0.25	0.78	0.25	0.78		
3.	**2-AM**	**CAS**	**2-AM**	**CAS**	**0.38**	Synergistic
	0.25	0.5	0.062	0.12		
4.	**2-DG**	**FLC**	**2-DG**	**FLC**	**0.50**	Synergistic
	2	1.5	0.062	0.75		
5.	**2-DG**	**AmB**	**2-DG**	**AmB**	**0.50**	Synergistic
	2	0.78	0.5	0.19		
6.	**2-DG**	**CAS**	**2-DG**	**CAS**	**0.63**	Indifference
	2	0.5	0.25	0.25		
7.	**Vidarabine**	**FLC**	**Vidarabine**	**FLC**	**0.37**	Synergistic
	0.15	1.5	0.037	0.18		
8.	**Vidarabine**	**AmB**	**Vidarabine**	**AmB**	**0.50**	Synergistic
	0.15	0.78	0.037	0.1		
9.	**Vidarabine**	**CAS**	**Vidarabine**	**CAS**	**0.50**	Synergistic
	0.15	0.5	0.037	0.12		
10.	**Ribavirin**	**FLC**	**Ribavirin**	**FLC**	**1.50**	Indifference
	0.025	1.5	0.0125	1.5		
11.	**Ribavirin**	**AmB**	**Ribavirin**	**AmB**	**1.25**	Indifference
	0.025	0.78	0.00625	0.78		
12.	**Ribavirin**	**CAS**	**Ribavirin**	**CAS**	**0.75**	Indifference
	0.025	0.5	0.00625	0.25		

In this study, 12 types of antivirals-antifungal drug combinations are analysed which includes combinations of 2-AM, 2-DG, Ribavirin, and Vidarabine with FLC, AmB, and CAS. As per calculated FIC index, all tested antiviral drugs except Ribavirin

had synergistic combination of with FLC. According to the classic checkerboard method of drug combination, 2-AM, Vidarabine, and 2-DG reduced MIC value of FLC. In addition, MIC of 2-AM, Vidarabine and 2-DG also reduced from 0.25, 0.15 and 2 mg/ml to 0.015 mg/ml, 0.037 mg/ml and 0.062 mg/ml, respectively. As MIC of both FLC and antiviral drugs are reduced, indicating the FIC index < 0.5 that further corresponds to the synergistic type of combination. **Table 4.2** shows the reduced MIC of the antiviral, and antifungal drugs in combinations compared to the alone thus exhibiting a synergistic activity.

In drug combination, reduced MIC of antiviral drugs Vidarabine and 2-DG confirms their individual synergistic action with AmB. Whereas indifference was observed for combination of 2-AM and AmB with no reduction in MIC values. 2-AM and Vidarabine, showed synergistic type of interaction with CAS drug. In this combination the MIC values of both antiviral drugs and CAS are reduced. Among all the antiviral drugs only Vidarabine found to exhibit the synergistic combination with all tested antifungal drugs against *C. albicans* growth. Interestingly, in these 12 combinations, none of the drug combinations showed antagonism that potentiate the concept of combinational drug based antifungal therapeutic development.

4.3.7. Survival of silkworm larvae

In order to investigate the *in vivo* effectiveness of antiviral drugs with *in vitro* antifungal activities, *B. mori* and *C. albicans* infection animal model was studied. In this, the survival period of larval group as per **Table 4.1** was recorded up to 3 days of post *C. albicans* infection. The data of silkworm survival is used to plot the Kaplan-Meier curves.

Silkworms with FLC administration serves as positive control group. Larvae without infection referred as negative control whereas infected larvae with 1 % DMSO are considered as vehicle control of the set. MIC at 0.25, 2, 0.5, 0.025 and 0.15 mg/ml of 2-AM, 2-DG, Ganciclovir, Ribavirin and Vidarabine, respectively inoculated in *C. albicans* infected larvae as a test. Infection of *C. albicans* to the silkworm larvae induces mortality within 24 h of infections whereas larval sets treated with MIC of 2-AM, 2-DG, Ganciclovir, Ribavirin and Vidarabine had similar effect to that of antifungal drug FLC.

Fig. 4.11 represents the *B. mori* larval set consisting healthy larvae, process of inoculation and the groups tested in the study. Treatment with antiviral drugs promoted the survival of *B. mori* infected with *C. albicans* that further leads to the cocoon phase

of silkworm larvae life cycle. Results of *in vivo* studies signifies that tested antiviral drugs might affected the *in vivo* proliferation of *C. albicans* that resulted in increased survival span as compared to untreated *C. albicans* infected *B. mori.*

Figure 4.11. Studies on antifungal efficacy testing of selected antiviral drugs in *B. mori* silkworm larvae. Sets of larvae infected with or without *C. albicans* establish *in vivo* model and observed for survival. 2-AM, 2-DG, Ganciclovir, Ribavirin, and Vidarabine at MIC values of 0.25, 2, 0.5, 0.025 and 0.15 mg/ml, respectively used as test group of larvae.

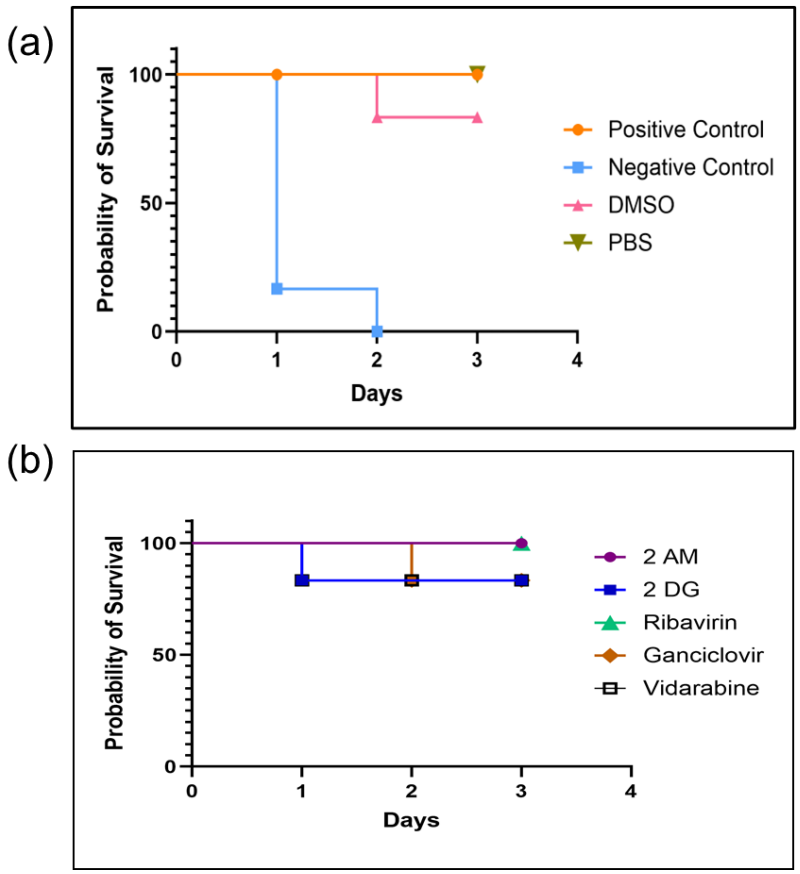

Figure 4.12. Kaplan-Meier survival curves of *C. albicans* infected *B. mori* silkworm larvae. (a) Plot depicts the average survival of 5 larvae per group with experimental controls and (b) administered with antiviral drugs and monitored for 3 days after the infection.

Kaplan-Meier curve further implies that the vehicle control, solvents used in this study did not hamper the survival of *B. mori*. However, 80 % larvae infected with *C. albicans* died within 24 h after infection and dead larvae were confirmed by gentle tapping. Administration of antiviral drugs resulted in improved larval survival compared to untreated *C. albicans* infected *B. mori*. 2-AM and Ribavirin treated *C. albicans* infected *B. mori* showed 100 % survival whereas, Vidarabine showed 60 % survival. 2-DG and Ganciclovir treatment improved the survival of infected larvae by 80 %. These observations concludes that antiviral drug, 2-AM and Ribavirin has significant anti-*Candida* potential *in vivo* conditions (**Fig. 4.12)**.

4.4. Discussion

For the last decade, researchers have focused on the issue of fungal infections and drug resistance to antifungals. The growing prevalence of AIDS and cancer can be

linked, to this focus. Patients with AIDS or cancer with already compromised immunity are exposed to prolonged dosages of antibiotics, which leads to the eradication of natural flora, and this status ultimately increases the colonization rate of *C. albicans* in the body. Consequently, the development of novel antifungal compounds is necessary. A wide range of antimicrobials and natural products were reported for their anti-microbial studies, but limited reports are available on antifungal effects. Repurposing with non-antifungal drugs accelerates the antifungal drug development process through the screening of various already approved agents [21].

In this chapter, antiviral agents were explored to characterize their antifungal action and highlight their antifungal potential against human fungal pathogen *C. albicans*. The fungicidal action of 2-AM and Vidarabine was further confirmed in the time-kill kinetic assay. The time-kill assay for antiviral drugs highlighted the time required for killing was in the duration of 6 and 8 h, respectively after the treatment. In assessing these results, it has been found that treatment with fungicidal concentrations of 2-AM and Vidarabine was consistent with growth inhibition and continued in complete inhibition of *C. albicans* growth after 6 and 8 h of incubation, respectively.

Dual fluorescence staining with AO-EtBr dye was used to discriminate between live and dead cells after the treatment. This study evidenced the massive dead cell count after treatment of those antiviral drugs affecting *in vitro* planktonic growth compared to the drug-free control cells. The dual staining concluded that the antiviral drugs had dose-depended and significant antifungal activity. The results of this study were consistent with the finding that the effects of fungicidal antivirals resulted in a reduced viable count.

Ergosterol biosynthesis is another and most targeted site of many pharmaceutical agents that are currently in the market. As ergosterol is a key and differentiating component present in fungi and absent in bacterial cells or plant or animal cells. Ergosterol plays a major role in cell integrity maintenance and cell fluidity. In the same regard, the antiviral drugs were also studied for ergosterol quantification and found to reduce the ergosterol content. The decrease in ergosterol content in *C. albicans* treated with 2-AM, 2-DG, Vidarabine, Ribavirin, and Ganciclovir was significant compared to the respective non-treated controls. As with most antifungals such as FLC, itraconazole, terbinafine, and AmB, these antivirals also shared the same target of ergosterol biosynthesis pathway. Results demonstrate that these antiviral drugs have route of action is the suppression of ergosterol biosynthesis and their potential antifungal properties.

Further, another important virulence traits such as morphogenesis and biofilm formation were studied using SEM. The nature of the substrate governs the adhesion and biofilm formation by *C. albicans.* It is well documented that silicon, elastomer, and latex materials are suitable for biofilm development of *C. albicans*. This ability further poses a major issue in hospitals. The SEM analysis for the morphogenesis and biofilm of *C. albicans* was performed on silicon based urinary catheter pieces and studied for the action of 2-AM, 2-DG, and Ganciclovir.

The SEM images confirmed anti-morphogenic ability of 2-AM, 2-DG, and Ganciclovir against *C. albicans* in both liquid media and on silicon-based catheters. 2-AM, 2-DG, and Ganciclovir had anti-morphogenic action even in a hyphal inducing environmental condition, and this poses the additional advantage of being used in antifungal therapeutic development.

In addition, a qualitative assessment of biofilm inhibition in *C. albicans* was performed by SEM. SEM revealed the morphology and architecture of *C. albicans* and demonstrated the biofilm inhibitory action of 2-AM. Results of SEM image analysis showed that 2-AM exhibited concentration-dependent biofilm inhibitory action by targeting the true hyphal development. Control cells showed the presence of complex structures consisting of elongated hyphae and pseudo hyphae, whereas 2-AM treated *C. albicans* displayed round yeast cells with small or no hyphal structure. As per reports, catheter associated *Candida* bloodstream infections have higher mortality, especially in patients in ICUs, and *C. albicans* serves as the most common biofilm producer on these catheters [22].

However, the severe infections associated with catheters are treated with high antifungal drug dosages or removal of catheters, but these procedures are complicated and costly. Studies on catheter coating with antifungal agents have drawn attention to biomaterial innovations as another strategy to limit catheter-associated biofilm infections [23]. Inhibition of biofilm formation on catheters by 2-AM provides additional therapy along with photodynamic inactivation, catheter coating, and natural peptide products to limit *Candida* infections.

The anti-biofilm activity of 2-AM may be further explored as an anti-biofilm repurposed agent to inhibit the biofilm formation on silicon based urinary catheters. It acts as a marker of the compound's efficiency in controlling biofilm-related issues by preventing biofilm growth [24].

Combination treatments offer a possible way of boosting antifungal effectiveness

and preventing the development of new resistance. The combination of drugs with different targets or mechanisms of action suggests another antifungal therapeutic strategy [25]. As a result, current study evaluated the drug combination efficiency of routine antifungals FLC, AmB, and CAS with screened antiviral drugs. Results in the form of the FIC index obtained by checkerboard assay showed that drugs, except for Ribavirin, all other antivirals, 2-AM, 2-DG, and Vidarabine, exhibited synergistic activities in combination with FLC. These combinations will propose to revert the drug resistance developed in *C. albicans* against azoles. Whereas, 2-AM and Vidarabine showed synergistic interactions with CAS. FICI results of Vidarabine and 2-DG combination with AmB exhibited synergistic action. Combinations of antifungals with Ribavirin seem to have less impact on producing the synergistic drug combinations. However, strong synergistic combinations of Vidarabine with all three tested antifungal agents provide an amazing result for combination based antifungal therapeutic development.

Moreover, it is reported that the synergy arises from combination therapy becomes even more significant, when it takes into account the drugs under investigation lack of efficacy if employed alone [26]. So, this synergy may improve the combinational therapy against *C. albicans* by broadening the antifungal spectrum. In situations such as monotherapy failure, biofilm-forming infection, and site of infection, combination therapy may widen the drug activity. This combinational approach improves the antifungal effect by lowering the drug dosage, probable side effects and toxicity along with delayed emergence of drug resistance in *C. albicans.* Based on the investigation of the checkerboard assay, most of the antiviral drugs have paired effectively with the FLC and may act as azole-resensitizing agents against *C. albicans. In vitro,* synergistic combinations of FLC with antiviral drugs indicate the possible potential use to limit the initial phases of disease establishment and progression by inhibiting the proliferation of cells.

Further, the *in vivo* antifungal efficacy and toxicity of antiviral drugs were evaluated by using silkworm *B. mori* as an *in vivo* animal model. *B. mori* silkworm larvae have been an established model system for fungal infections. In comparison with the mammalian model, the silkworm study is cheap and requires a small space for rearing. Initial screening of novel therapeutic compounds by using the *B. mori* infection model identified therapeutic effectiveness, which was further found in a mouse model as well [27].

Studies on silkworms suggest that larvae possess similar functioning organs to those of mammals that deal with the pharmacokinetics of compounds and their toxicity. In addition to this, the compound absorption in the intestinal tract of the silkworm mimics that of the mammal [6,28]. Based on the analysis of antifungal activities of antiviral drugs *in vitro* studies, effectiveness against *C. albicans* was studied in a silkworm animal model.

As shown in the Kaplan-Meier survival curve (**Fig. 4.11)**, *B. mori* larvae non-infected with *C. albicans* survives up to 3 days, indicating proper maintenance environment to the larval growth. Infection of *C. albicans* with *B. mori* resulted in killing within 24 h of infection; however, the vehicle and positive control injected resulted in no alteration in the survival period of larvae, and larvae continued up to the cocoon phase. The findings suggest that administration with antiviral drugs 2-AM, 2-DG, Ganciclovir, Vidarabine, and Ribavirin to *C. albicans*-infected larvae had an enhanced survival period similar to the FLC-inoculated control larval set. As per the survival curve, 2-AM and Ribavirin had interesting results indicating effective *in vivo* activity for *C. albicans*. Overall, this chapter highlights the potential of antiviral drugs to affect the growth and virulence factors present in *C. albicans* and proposes the applicability in the process of antifungal drug development.

4.5. Conclusions

This chapter further explores the effects of antiviral drugs on planktonic growth and Y-H morphogenesis in *C. albicans* for their antifungal potential. The inhibitory action of antiviral drugs, namely 2-AM, 2-DG, Ribavirin, and Vidarabine with MIC values 0.25, 2, 0.025 and 0.15 mg/ml, respectively was identified. Using the dual staining method, and the massive dead count of *C. albicans* after antiviral drug treatment was visualized under fluorescence microscope. Checkerboard experimentation for combination studies demonstrated the synergetic action of antiviral drugs 2-AM, 2-DG, and Vidarabine with FLC, suggesting their beneficial role in the combinational treatment of fungal infections. The synergistic combination of Vidarabine with FLC, CAS, and AmB indicates its potential as an antifungal candidate. The SEM image analysis confirmed the anti-biofilm activity of 2-AM at 0.5 mg/ml on a silicon-based urinary catheter, which further recommends 2-AM as an interesting candidate for coating medical devices. Further, an *in vivo* study in the *B. mori* silkworm animal model evaluated the therapeutic effectiveness of antiviral drugs at MIC against *C. albicans*.

4.6. References

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CHAPTER 5

Insights on mechanism of action of antiviral drugs against *Candida albicans*

5. Insights on mode of action of antiviral drugs against *C. albicans*

5.1. Background

Chapters 3 and 4 covered the screening and exploring of antifungal activities of antiviral drugs where 2-AM, 2-DG, Ganciclovir, Ribavirin, and Vidarabine affected key virulence attributes present in *C. albicans.* These chapters highlighted antiviral drug action against *C. albicans* such as planktonic growth, hyphal development, ergosterol biosynthesis, cell adhesion, and biofilm formation. In addition, antiviral drugs have also been studied for combinational approaches with antifungal medications. Antifungal efficacy in a *C. albicans*-infected *B. mori* silkworm model confirmed inhibitory action of antiviral drugs. Although antiviral drugs have shown antifungal activities against *C. albicans*, some insights into their mechanism of action need to be explored. The current chapter details the probable mode of action of these five antiviral drugs on *C. albicans* targets, and **Fig. 5.1** represents the outline of the chapter.

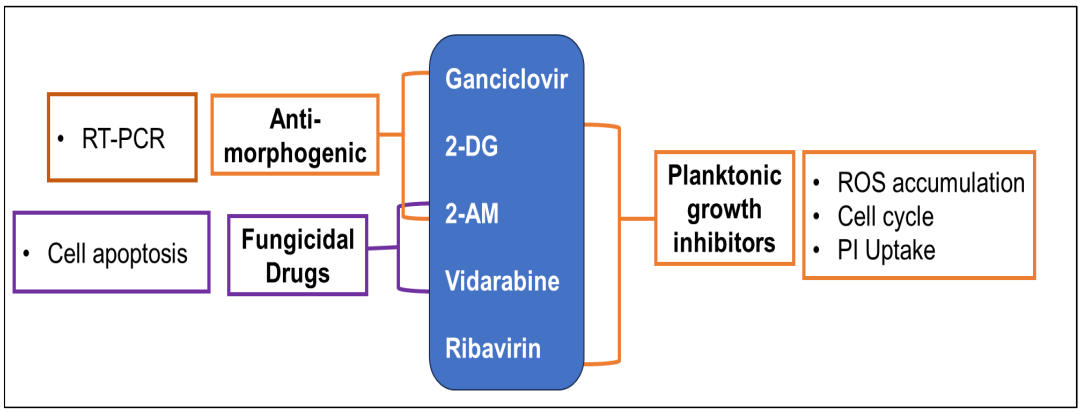

Figure 5.1. Outline of study to investigate the probable mode of action of antiviral drugs on *C. albicans.*

Due to the close evolutionary linkage of fungi with human host, antifungal drug development poses a therapeutic challenge to the pharmaceutical industry. It reduces the possibility of exploring new targets to eradicate fungal infections. For this reason, antiviral drugs were further explored to specify their antifungal activity with a probable target. The study was characterized by assays such as Reactive oxygen species (ROS) accumulation, cell cycle progression, drug uptake, and gene expressions.

ROS generation is thought to be one of the indicators of apoptotic cell death. Normal cells have an antioxidant system made up of antioxidant enzymes that balance the formation of ROS. However, the process of antioxidant detoxification is triggered by external factors such as antifungal agents, and oxidative stress develops, which causes

cellular death [1]. So, the accumulation of ROS serves as a target for the inhibition of *C. albicans.*

The cell cycle process in *C. albicans* is a complex biological process that regulates several cellular functions, including chromosome segregation during cell division, DNA replication, and DNA damage repair. It is crucial for preserving genome stability [2]. Any alteration in this process leads to the accumulation of cells at any of the checkpoints, resulting in no participation of cells in cell division and it is termed as cell cycle arrest at specific phase [3]. This chapter briefs arrest in cell cycle caused by the treatment of antiviral drugs. Cell membrane integrity is another important target of *C. albicans* for antifungal therapeutic development [4]. The propidium iodide (PI) dye uptake method was used to investigate the altered cell membrane integrity after exposure of antiviral drugs. In this chapter, disturbed cell membrane by antiviral drug treatment have been studied and visualized under fluorescence microscope. Cells affecting the planktonic phase of *C. albicans* were characterized by assays including PI uptake, FACS analysis, and ROS generation.

In addition, the molecular targets of anti-morphogenic antiviral drugs is studied and discussed in this chapter. Hyphal development is mainly controlled by hyphal-specific genes and regulators. In response to any internal or external factor, if the transcriptional level of these genes is altered, then it results in a halting of Y-H morphogenic transition [5]. Studies on gene expression profiles in the presence of inhibitors might highlight the base of mechanistic action against the hyphal development. Thus, in the same regard, the transcription analysis of hyphal-specific genes in *C. albicans* after anti-morphogenic antiviral drug treatment was studied in this chapter. In brief, this chapter highlights the probable mechanistic insights of the antifungal activities of active antiviral drugs against *C. albicans*.

5. 2. Materials and methods

5.2.1. Intracellular ROS accumulation assay

In *C. albicans*, intracellular ROS accumulation was measured with a 2,7–dichlorodihydrofluroscein diacetate (H_2DCFDA) ROS-sensitive probe. Antiviral drugs affecting the planktonic growth of *C. albicans* namely, 2-AM, 2-DG, Ribavirin, and Vidarabine with MIC and 2 × MIC were studied in this assay. Briefly, 1×10^6 CFU/ml cells of logarithmically grown *C. albicans* were treated with antiviral drugs for 4 h at 30°C. After incubation, cells were extracted by centrifugation at 6000 rpm for 2 min.

The cell pellets were exposed to 10 µM H_2DCFDA for 1 h to analyse the accumulated endogenous ROS. Negative control cells were not exposed to any antiviral drug, whereas positive control cells were treated with 5 mM H_2O_2 [6]. Prior to analysis, samples were washed with PBS, and then fluorescence intensity was quantified by a Spectro fluorophotometer (Jasco FP8300, Japan) with an excitation wavelength of 486 nm and an emission wavelength of 525 nm.

5.2.2. Cell cycle progression study

Effect of antiviral drugs on the cell cycle progression of *C. albicans* was detected according to the previously published protocol [7]. Antiviral drugs affecting the planktonic growth which includes 2-AM, 2-DG, Ribavirin and Vidarabine were studied in this assay. The overnight-grown culture of *C. albicans* in logarithmic phase was harvested using centrifugation. 1×10^6 CFU/ml cells were resuspended in RPMI-1640 medium for an incubation period of 4 h at 30°C. Treatment of antiviral drugs at MIC were given to *C. albicans* cells. Cells were collected in ice-cold PBS using centrifugation at 6000 rpm for 2 min. After cell harvesting, process proceed for cell fixation in ice cold ethanol. Alcohol-fixed cells were treated with 25 µl of 10 mg/ml RNase A and then stained by PI fluorescent dye of 50 µg/ml concentration and kept 30 min. in dark for incubation. A cell cycle progression study was performed using FACS Calibur flow cytometer.

5.2.3. Apoptosis assay

For the study of cell apoptosis, an Annexin V-FITC and PI kit based staining method was performed. Apoptosis induced in *C. albicans* after the treatment of fungicidal drugs, namely 2-AM and Vidarabine, was studied in this assay. In brief, *C. albicans* cells in mid-exponential phase (1×10^3 CFU/ml) were treated with MFC values of 2-AM and Vidarabine for 4 h at 30 °C. Cells were centrifuged and washed with sterile PBS. In 0.1 M potassium phosphate buffer (PPB), cells were resuspended. PPB contains 0.02 mg/ml zymolyse and sorbitol and a pH of 7.2. To investigate the phosphatidylserine (PS) externalization after antiviral drug treatment an apoptosis detection kit was used [8]. An apoptosis detection kit was procured from Miltenyi Biotech Bergisch Gladbach, Germany. According to the manufacturer's instructions, the procedure was performed and stained cells were analysed using MACS QUANT 10 flow cytometry and FlowJo software. The generated data displays the percentage of Annexin V- FITC and PI-stained population.

5.2.4. PI uptake study

Fluorescence microscopy for the PI uptake assay was used to visualize the permeabilization of the cell membrane in *C. albicans* treated with antiviral drugs. For this assay antiviral drugs affecting planktonic growth at MIC were used. PI is a nuclear, non-vital fluorescence stain used for analysing membrane integrity. *C. albicans* (1×10^6 CFU/ml) cells were cultured for 4 h at 30°C with 2-AM, 2-DG, Ribavirin, and Vidarabine. Following incubation, cells were rinsed and then resuspended in PBS. After the treatment, cells were stained with 6 µM PI, and incubated for 5 minutes at room temperature in the dark [9,10]. Fluorescence microscope with the objective of 40x was used to visualize the permeabilization of PI (red-fluorescence) into *C. albicans* cells after the treatment of active antiviral drugs. The fluorescence images were taken on a Nikon Eclipse fluorescent microscope at an excitation wavelength of 525 nm.

5.2.5. Gene expression study

Using quantitative real time polymerase chain reaction (qRT-PCR), expressions of Y-H transition controlling genes in signal transduction of *C. albicans* were measured. The FBS induced morphogenesis assay mentioned in Chapter 3 (Methodology section 3.2.5) was performed for *C. albicans.* For this assay cells were treated with anti-morphogenic drugs, 2-AM, 2-DG, and Ganciclovir at MIC for 90 min at 37°C. In this, 1×10^6 CFU/ml cells were used to extract total RNA using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). After the incubation period, RNA extraction procedure proceeded. RNA extracted from antiviral treated cells was referred to as test RNA, whereas RNA from untreated cells was considered control. Reverse transcription of RNA to the first stand of cDNA was carried out using SuperScript III (Bio-Rad Laboratories India Pvt. Ltd., India). Primers used in this study are listed in **Table 5.1** and procured from GeneOmebio Technology Pvt. Ltd., Pune. 96-well Bio-Rad plates were used for PCR, where 10 µl of reaction mixture prepared as per the instructions provided by the UNISYBR GREEN SUPER mix manufacturer, and was added in each well. Actine was referred to as a housekeeping gene for the study of altered gene expressions. The cycling profile consisted of 35 amplification cycles of 95°C for 60 sec, 60°C for 60 sec, and then 72°C for 45 sec. Real-time PCR system was used for data collection and analysis. By calculating $2^{-\Delta\Delta CT}$ the level of relative gene expression was determined [11,12].

Table 5.1 List of primers used in the study with the respective 5→3 sequences

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′GGGCTGCAGTTAATGACCAGCAGTTGGGT 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'

5.3. Results

5.3.1. Antiviral drugs increased ROS generation

The effect of antiviral drugs on the generation of intracellular ROS in *C. albicans* was measured in this assay. Intracellularly generated ROS can oxidise the H_2DCFDA fluorescence probe to the green fluorescence emitting DCF, and this emitted fluorescence is used to detect the presence of ROS in the cell.

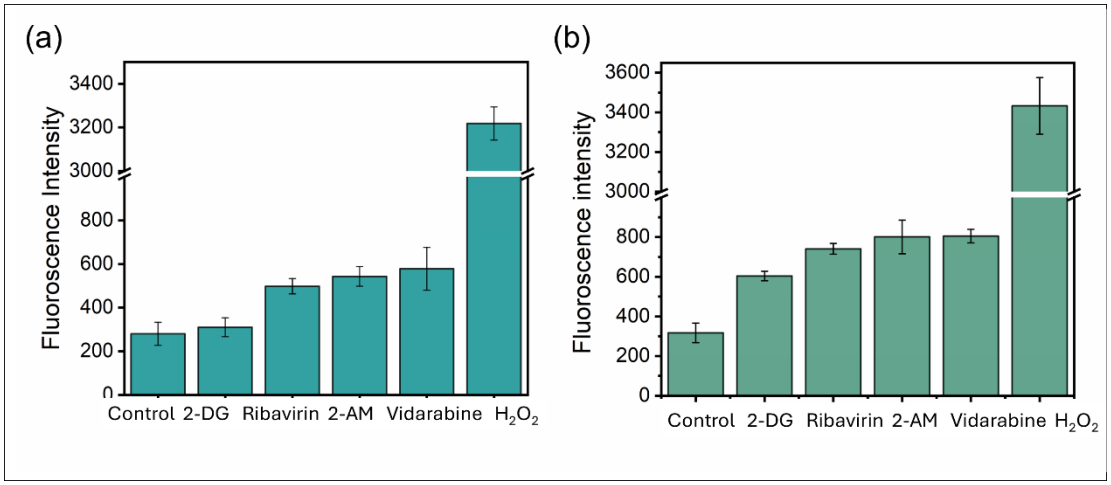

Figure 5.2. Fluorescence intensity of H_2DCFDA pointing the intracellular ROS generation in *C. albicans* after treatment with 2-DG, Ribavirin, 2-AM and Vidarabine at MICs values 2, 0.025, 0.25 and 0.15 mg/ml, respectively and (b) 2 × MIC. Treatment with H_2O_2 was considered as positive control.

The fluorescence intensity of DCF is directly proportional to the cellular ROS generated within *C. albicans* cells. As shown in **Fig. 5.2**, fluorescence intensity increases in 2-AM, 2-DG, Ribavirin, and Vidarabine treated *C. albicans* cells compared to the control cells. The fluorescence intensity gradually increases with the increase in the antiviral drug concentrations but it is less compared to the positive control, indicating the ROS-inducing ability of antiviral drugs in *C. albicans.* As shown in **Fig. 5.2** Vidarabine, 2-AM, and Ribavirin at respective MICs of 0.15, 0.25, and 0.025 mg/ml increases ROS accumulation by 2, 1.9, and 1.7-fold, respectively. Whereas, treatment with 2 × MIC of 2-AM, Vidarabine, Ribavirin, and 2-DG significantly increased accumulation of ROS by 2.5, 2.5, 2.3, and 1.9-fold, respectively, as compared to the drug-free cells. More intense fluorescence absorbance was obtained in H_2O_2-treated *C. albicans* cells that were considered as positive control for the experiment. Treatment with 2-AM, Vidarabine, and Ribavirin induced more than 1-fold ROS in *C. albicans* cells, that implies antiviral drugs induced ROS generation in *C. albicans* which may

further cause oxidative stress and damage to the cell membrane and may induce apoptosis [13].

5.3.2. Antiviral drugs arrested cell cycle progression

Cell cycle progression study was quantitatively determined by the PI-stained DNA of antiviral drug treated *C. albicans* cells. MICs of 2-AM, 2-DG, Ribavirin, and Vidarabine at 0.25, 2, 0.025, and 0.15 mg/ml, respectively were used for *C. albicans* treatment.

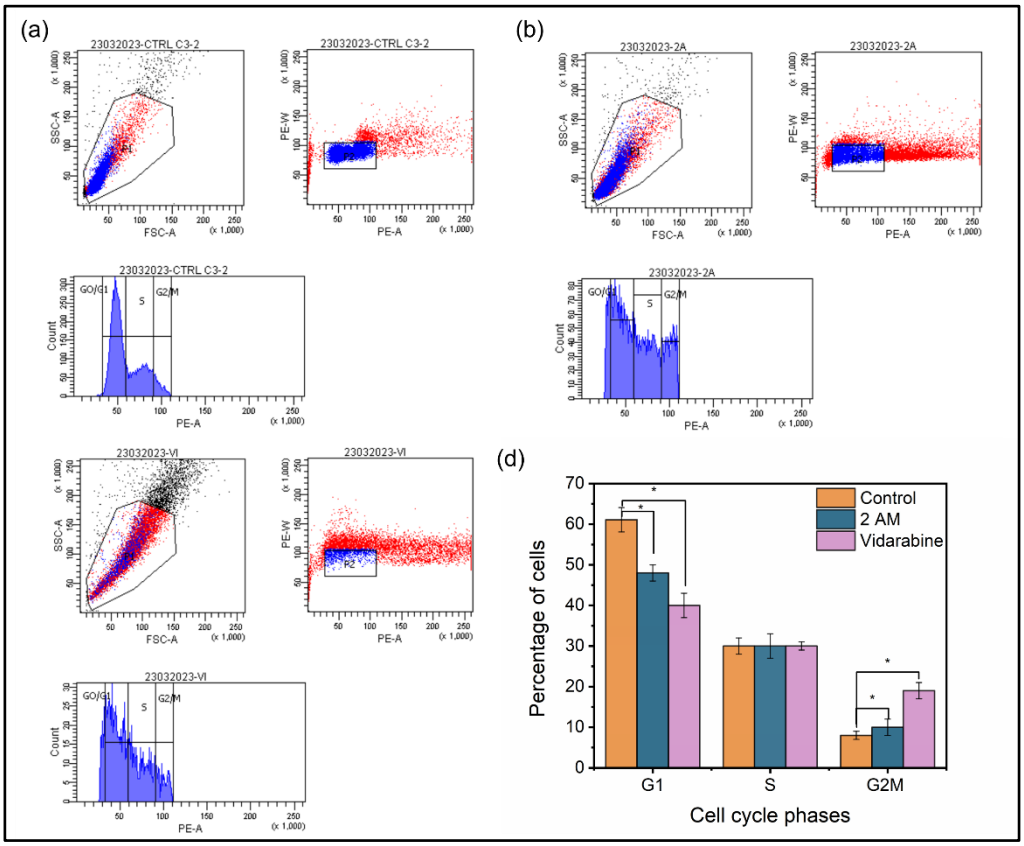

Figure 5.3. Analysis of cell cycle progression by flow cytometry. Dot plots of *C. albicans* cells (a) control, and (b) 2-AM (0.25 mg/ml), (c) Vidarabine (0.15 mg/ml) treated *C. albicans* cells whereas, (d) Percentage of *C. albicans* cells in G0/G1, S and G2/M phase of cell cycle after antiviral drug treatment. (* indicate P<0.05 statistically significant difference).

Antiviral drugs affecting the planktonic growth seems to alter the cell cycle phases of *C. albicans*, as shown in **Fig. 5.3** and **Fig. 5.4**. FACS analysis indicates that G0/G1 phase of untreated control sample contains 61 % of cells, whereas S phase and G2/M has 30 and 8 % of cells, respectively. This cell count was altered after 2-AM treatment, where 40, 30, and 19 % of cells present in G0/G1, S phase, and G2/M,

respectively. In addition, Vidarabine treatment resulted in 48, 30, and 10 % of cells in G0/G1, S and G2/M phase, respectively (**Fig. 5.3)**.

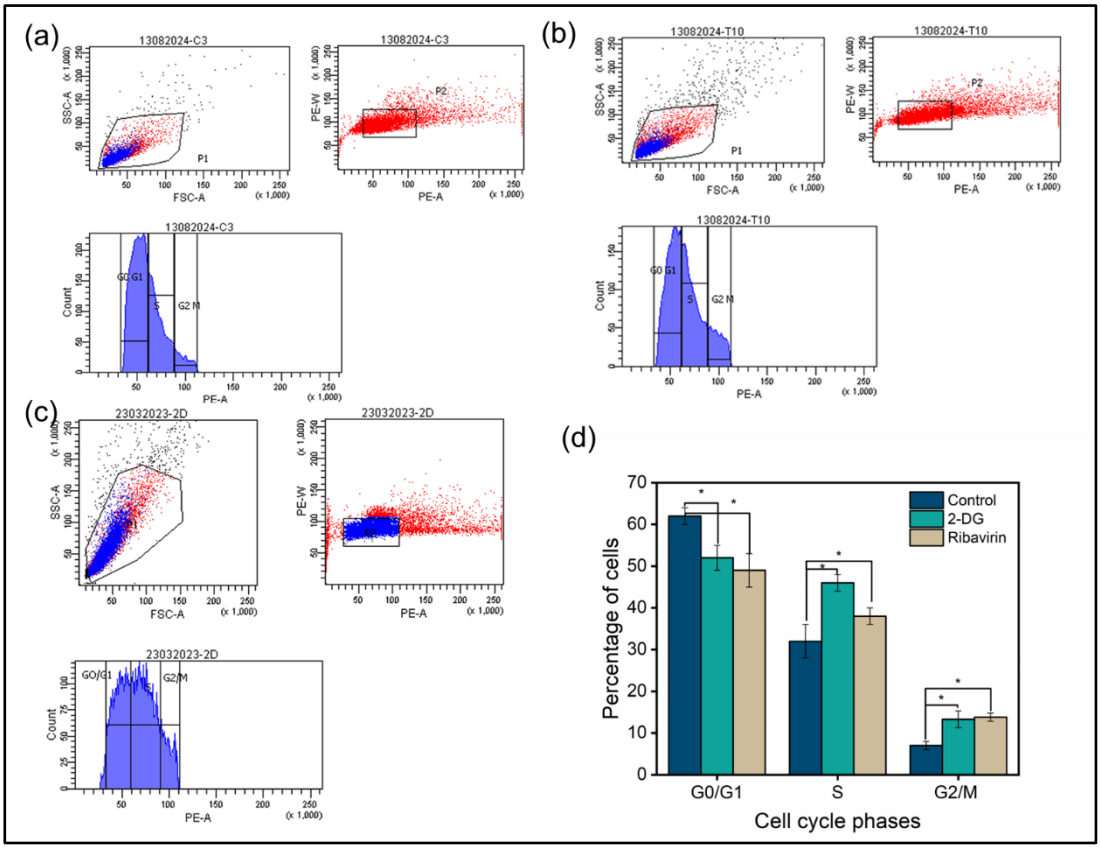

Figure 5.4. Analysis of cell cycle progression by flow cytometry. Dot plots of *C. albicans* cells (a) control and (b) 2-DG (2 mg/ml), (c) Ribavirin (0.025 mg/ml) treated *C. albicans* cells whereas, (d) Percentage of *C. albicans* cells in G0/G1, S and G2/M phase of cell cycle after antiviral drug treatment. (* indicate P<0.05 statistically significant difference).

Results suggest that 2-AM and Vidarabine treatment caused more cell accumulation in the G2/M phase compared to the untreated cells, indicating cell cycle arrest at the G2/M phase.

Fig. 5.4 shows plots of cell cycle analysis in the presence of Ribavirin and 2-DG, and the graph represents respective cell percentages in the cell cycle phases. FACS analysis showed that in control *C. albicans* cell sample G0/G1, S and G2/M phases counted about 62, 32 and 7 % of cells, respectively. 13.8 % of cells were arrested in G2/M phase after Ribavirin treatment. *C. albicans* cell percentages after treatment of 2-DG where 52, 46 and 13 % in G0/G1, S and G2/M phase, respectively indicating arrest in S phase of cell cycle. Among the effective antiviral drugs, only 2-DG arrested *C. albicans* cells in the S-phase of the cell cycle.

5.3.3. Apoptosis induced by antiviral drugs

Further to investigate the fungicidal action of antiviral drugs, Annexin V-FITC and PI staining-based apoptosis assay was studied. This method also validates the ROS-induced escort for apoptosis or necrosis. In this experiment antiviral drugs with MFC against the planktonic growth were used. This assay validates cell apoptosis by the treatment of 2-AM and Vidarabine at 0.5 mg/ml and 0.3 mg/ml concentrations, respectively. The principle of Annexin V-FITC apoptosis detection kit is that, in the early phases of apoptosis, PS present in the inner leaflet gets translocated to the outer side of the plasma membrane, and analysed by flow cytometry.

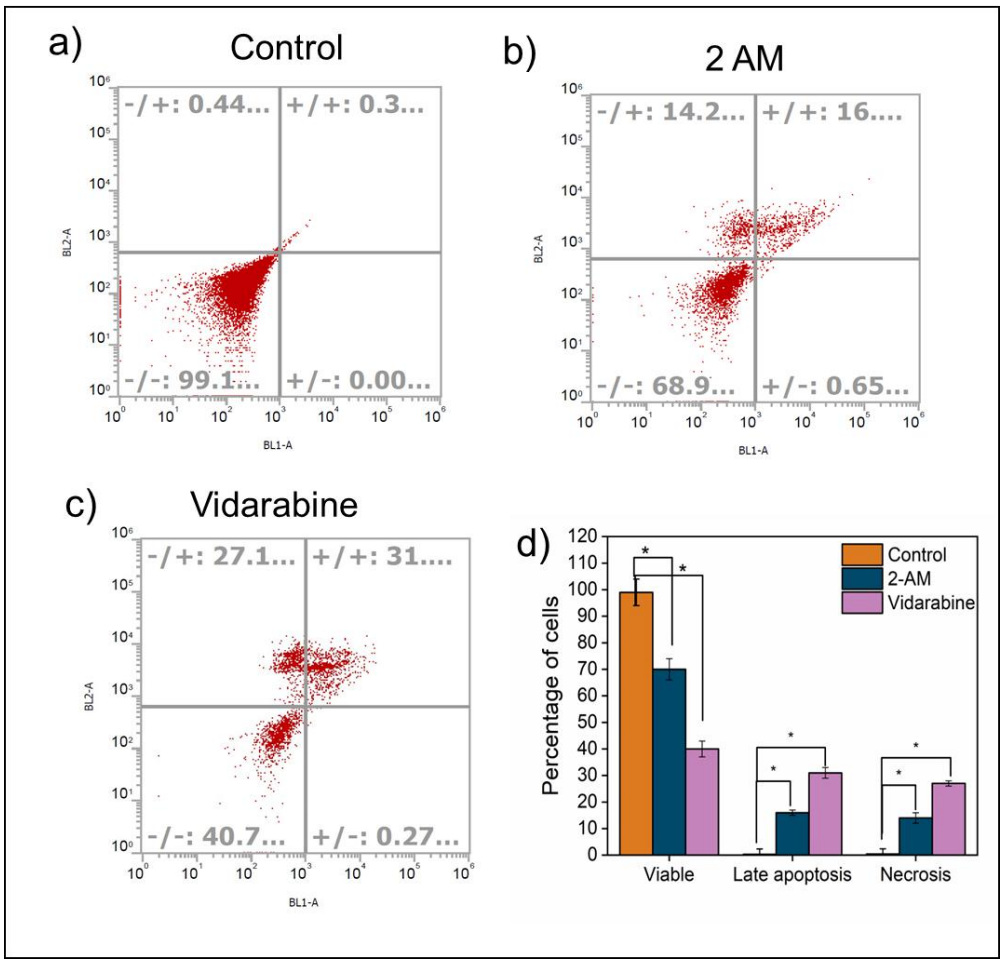

Figure 5.5. Dot plots of flow cytometry analysis of apoptosis in *C. albicans* after the antiviral drug treatments. Annexin V-FITC/PI-stained cells were analysed for apoptosis induced by (b) 2-AM (0.5 mg/ml), and (c) Vidarabine (0.3 mg/ml) compared to untreated cells whereas (d) percentage of *C. albicans* cells in quadrants of apoptosis analysis. (*indicate $P<0.05$ statistically significant difference).

Results of this experiment shows that compared to the untreated *C. albicans* cells, Annexin V-FITC-stained cells were higher in 2-AM and Vidarabine treated sample. Data shows the induction of apoptosis by 2-AM and Vidarabine at 0.5 and 0.3 mg/ml. In comparison with control cells, 16 % of 2-AM treated *C. albicans* cells were observed in the late apoptosis quadrant, whereas nearly 14 % of the cell population was in the necrotic phase. Further, **Fig**. **5.5** depicts that treatment with Vidarabine leads to an increased cell percentage in late apoptosis and necrotic phase by 31 % and 27 %, respectively. In addition, the viable cell count was also observed to be significantly reduced in the treatment group as compared to the control. In brief, 2-AM and Vidarabine drug treatment to *C. albicans* could induce cell apoptosis.

5.3.4. Antiviral drugs induced PI uptake

The fluorescent dye PI can intercalate DNA and RNA but cannot penetrate intact cells due to the higher molecular weight. However, PI cross the cell membrane only if the cell is compromised, reaches the cytoplasm, attaches to DNA, and emits red fluorescence. Thus, the integrity of the cell membrane can be studied with this fluorescent probe [14].

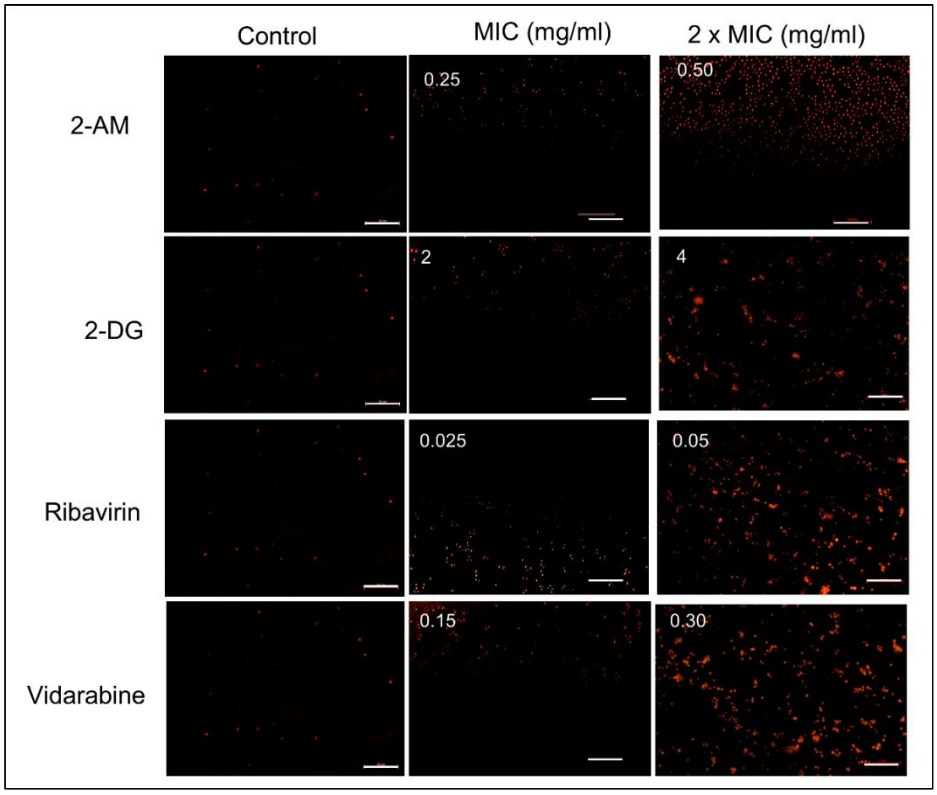

Figure 5.6. Fluorescence microscopic images of PI-stained *C. albicans* after treatment of 2-AM, 2-DG, Ribavirin and Vidarabine at MIC and 2×MIC. Indicated scale is 100 µm.

2-AM, 2-DG, Ribavirin, and Vidarabine at MIC and 2×MIC values were used to treat *C. albicans* planktonic growth. 0.25, 2, 0.025, and 0.15 mg/ml are MIC values of 2-AM, 2-DG, Ribavirin, and Vidarabine, respectively were used. Microscopic images in **Fig. 5.6** shows higher count of red fluorescence emitting antiviral drugs treated *C. albicans* cells compared to untreated cells. PI-stained, non-treated *C. albicans* cells signifies the undamaged cell membrane, whereas orange-red emitting cells evidenced damaged cell membrane after treatment with 2-AM, 2-DG, Ribavirin and Vidarabine. Fluorescence microscopic image shows that as the concentration of antiviral drugs increases, the count of red fluorescence emitting *C. albicans* cells also increases. Impairment of *C. albicans* cell membrane was initiated at MIC of antiviral drugs and treatment at 2 × MIC leads to detectable damage to the cell membrane. The fluorescence microscopic analysis confirmed that treatment with antiviral drugs disrupted cell wall integrity, which further leads to cell membrane injury.

5.3.5. Gene expression analysis

To acquire an additional understanding of the molecular mechanisms behind the suppression of *C. albicans* hyphal growth by antiviral drugs, expression patterns of significant genes involved in the hyphal growth were examined using qRT-PCR. Anti-morphogenic antiviral drugs, namely 2-AM, 2-DG, and Ganciclovir at 0.25, 1 and 0.5 mg/ml concentrations, respectively were studied for altered gene expression in *C. albicans* morphogenic transition. For both antiviral treated and untreated cells, the expression levels of each gene were normalized using the housekeeping gene (ACT1), and the results were presented as a relative expression fold change. The graphical representation of altered gene expressions is shown in **Fig. 5.7**.

Fig. 5.7 shows the effect of 2-AM on hyphal gene expressions in *C. albicans.* Gene expressions of hyphal growth key regulators such as *MIG1, TUP1,* and *NRG1* genes are upregulated by 3.4, 3.8, and 3.5-fold, respectively. Whereas the *CEK1* gene down regulated by 2.5-fold. However, the expression of hyphal development inducing *HWP1* is upregulated by 3.5-fold and *RAS1* seems to be upregulated by 3.2-fold. Significant downregulation of *BCY1,* and *ECE1* is observed in *C. albicans* cells after 2-AM treatment.

In the Y-H morphogenic transition of *C. albicans* in the presence of 2-DG, gene expressions of the negative regulators *MIG1, TUP1,* and *NRG1* were upregulated by 2.4, 1.7 and 1.9-fold, respectively. The expressions of *PDE2* and *CEK1* genes were

downregulated by 1.3 and 1 folds. 1.7-fold of *HWP1* upregulation was also noted after 2-DG treatment (**Fig. 5.7 b**).

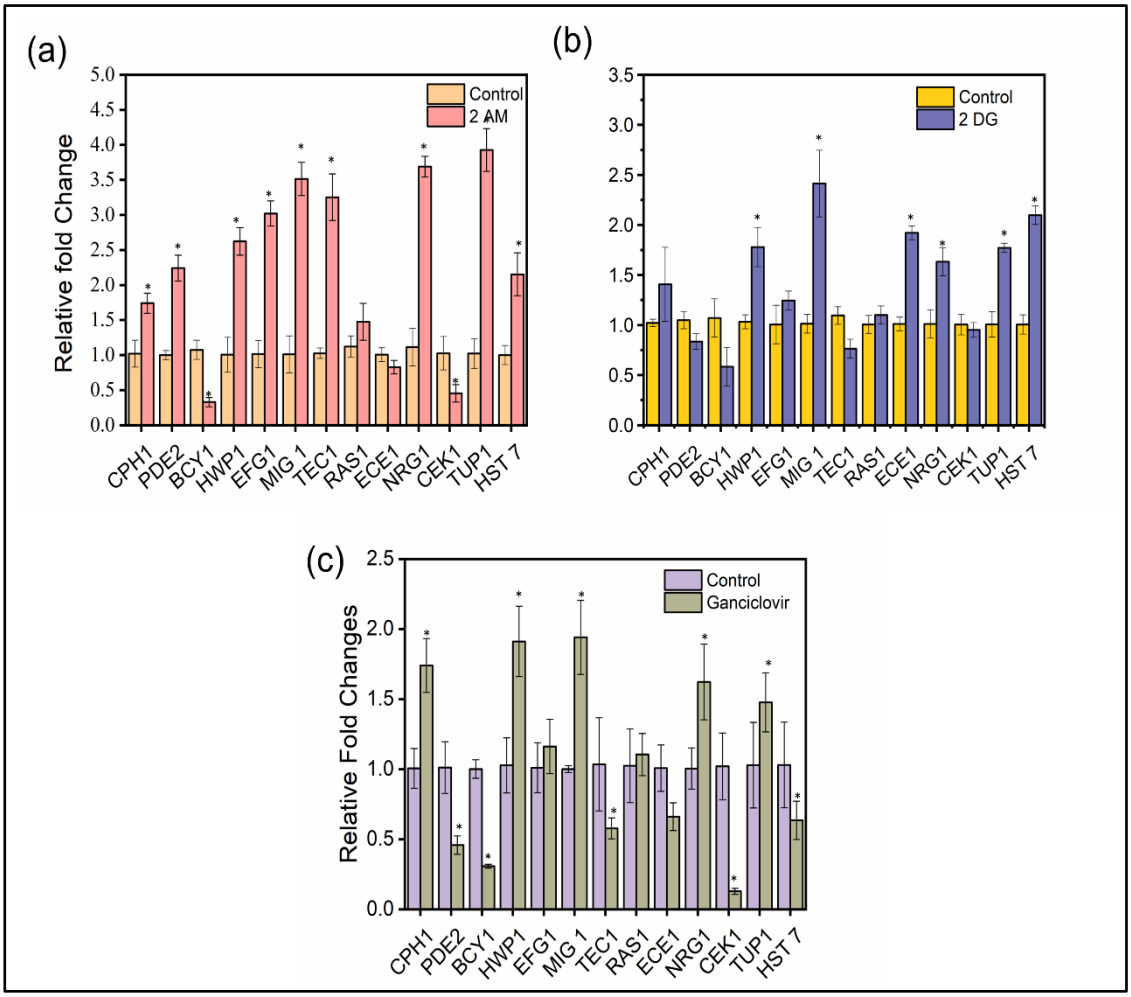

Figure 5.7. Relative fold expressions of hyphal specific genes in *C albicans.* Altered gene expressions of *C. albicans* after treatment of anti-morphogenic antivirals (a) 2-AM (0.25 mg/ml), (b) 2-DG (1 mg/ml), and (c) Ganciclovir (0.5 mg/ml) were quantified and compared with control cells.

The anti-morphogenic Ganciclovir drug also resulted in a similar expression alteration as that of 2-AM and 2-DG. Gene expressions of negative regulators *MIG1*, *TUP1*, and *NRG1* was found to be upregulated by 1.9, 1.4, and 1.6-fold, respectively. However, gene expressions of *CEK1* and *PDE2* were down regulated significantly. Although the gene expression of *HWP1* was upregulated by 1.8-fold, hyphal development was suppressed in *C. albicans* (**Fig. 5.7 c**). Treatment with anti-morphogenic antiviral drugs also hampered other genes involved in the hyphal induction

pathway. Collectively, the upregulation of hyphal growth negative regulators potentially affected the morphogenesis in *C. albicans.*

5.4. Discussion

Currently, antifungal agents with different and multiple ways of action have been used in antifungal therapy. Searches for novel antifungal drugs can be based on one of two strategies: (i) looking for compounds that exhibit antifungal activity by interfering with a particular target on the fungal cell, or (ii) identifying compounds that can enhance the activity of a known antifungal agent. Understanding the modes of action of antifungals is crucial for both the development of safer and more effective compounds and for alerting medical professionals to the rise of antifungal resistance [15].

Commercial antifungal medications primarily act by interfering with the cell wall, cell membrane, and biosynthesis process of cell wall / and membrane components. The inhibition of fungal virulence factors (such as morphogenic transition, surface adhesion to host cells, or biofilm formation), intracellular ROS accumulation, and disturbance in mitochondrial activity are additional mechanisms of action that increase the antifungal activity of drugs which boosts their main effect on fungal cells [16]. So, in the same regard, the current chapter aims to investigate the probable mode of action of effective antiviral drugs against C. *albicans* ATCC 90028. Among the screened antiviral drugs (from chapters 3 and 4), those antivirals affecting the virulence traits of *C. albicans* were selected for further detailed analysis.

In *C. albicans,* ROS is generated as a byproduct of cellular metabolism, and the primarily site of generation is mitochondria. It is generally hypothesized that any antifungal agent causing enhanced ROS accumulation has indirectly damaged mitochondria [17]. Thus, antiviral drugs were investigated for the ROS accumulation using H_2DCFDA fluorescence dye. The obtained results of the fluorescence spectrophotometer-based ROS study in *C. albicans,* imply that after treatment with 2-AM, 2-DG, Ribavirin and Vidarabine, ROS had been accumulated in treated cells. Among these, 2-DG has less, whereas 2-AM has the highest ROS accumulation. In brief, antiviral drugs under the study exhibited antifungal action by the production of ROS in *C. albicans.* Intracellular studies on the ROS suggested that its accumulation can activate and control cellular apoptosis in *C. albicans* and this mode of action has been well adapted by several antifungal drugs, including azoles [18].

In an attempt to uncover targeted mechanism of action of antifungal drugs, cell

cycle progression study was performed using FACS. Results of the study showed the detrimental effect of antiviral drugs over the cell cycle of *C. albicans*. Accordingly, if the antiviral drug treatment caused percentage of cells that are distorted and deviates from the normal healthy and typical trend, indicates that antiviral drugs are having an impact on the cell cycle, which may lead to cell cycle arrest at various stages [19]. The findings on the fluorescence intensity of PI labelled DNA were collected and quantitatively estimated to distinguish the cell percentage in different phases. The obtained results suggest that treatment with 2-AM, Ribavirin, and Vidarabine blocked cell cycle progression in the G2/M phase of *C. albicans*. This study will deepen the understanding of antifungal action by antiviral drugs. Treatment with 2-DG arrest cells in the S-phase indicates damaged DNA by the action of 2-DG. Anti-*candida* potential of these antiviral drugs was further improved by the modulating impact on the cell cycle progression of *C. albicans* ATCC 90028.

The study of cell cycle progression proceeded up to the apoptosis study using the Annexin V-FITC apoptosis detection kit. The principle of this apoptosis kit is based on the fact that the plasma membrane serves as a prime site of damage in cells. Damage to this membrane makes cells susceptible to necrosis and further continues to the loss of cell membrane integrity and cell lysis [20]. The externalization of PS to the outer plasma membrane is a marker of apoptosis as in normal and viable cells it has been distributed asymmetrically in the lipid bilayer of plasma membrane of cells. Exposure to the outer surface of PS serves as an indication of the early apoptosis stage [21]. Annexin V-FITC assay detects externalization of PS after the treatment of antiviral drugs in *C. albicans.*

In the quadrants of the flow cytometry-based apoptosis differentiation of viable, early apoptotic, late apoptotic, and necrotic cells was analysis. *C. albicans* cells present in Annexin V and PI negative quadrants represent viable cells; Annexin V positive and PI negative show early apoptosis; the upper left quadrant contains dead cells, where cells were Annexin V negative and PI positive. Late apoptotic quadrants contain both positive Annexin V and PI-stained cells [22]. 2-AM and Vidarabine treated cells were both Annexin V and PI positive, indicating the cell population were in late apoptotic or necrotic phase.

The Annexin V-FITC based apoptosis assay showed that antiviral drugs, 2-AM and Vidarabine at MFC values induced cellular apoptosis and necrosis. The results of the current investigation coincide with the previous studies on various drugs that induce G2/M phase mediated cell apoptosis.

Further, it is reported that the enhanced accumulation of ROS is linked with the oxidation of macromolecules, and further hampered activity of cell membrane [23]. Studies demonstrate the correlation between the higher ROS accumulation and the damaged cell membrane of *C. albicans* after antiviral drug treatment. Chapter 4 already detailed ergosterol quantification in presence of antiviral drugs. The PI permeability assay further validates effect on *C. albicans* growth by targeting the cell membrane damage.

PI is a nucleic acid stain that is impermeable to the cell membrane of healthy *C. albicans.* PI detects non-viable cells with altered cell membrane as it can only penetrate the cells and nucleolus membrane which are altered than normal. PI passes through the altered cell membrane and results in red fluorescence [24]. Red fluorescence of *C. albicans* cells has been increased after treatment of 2-AM, Ribavirin, and Vidarabine at 2 × MIC. It proves that antiviral drug treatment can hamper the permeability of plasma cell membrane, and nucleolus and thus PI can penetrate the membrane and stain the cells [25]. The PI uptake assay confirmed that exposure to fungicidal antiviral drugs has damaged cell membrane integrity and ultimately caused apoptosis and necrosis. The effectiveness of antiviral drugs on *C. albicans* via ROS-mediated cell membrane damage was confirmed using the PI-fluorescent dye-based method.

Further, the molecular base of the anti-morphogenic action of 2-AM, 2-DG, and Ganciclovir was studied using qRT-PCR. This study demonstrated comprehension of the impact of the anti-morphogenic antivirals on the morphogenesis of *C. albicans*. This study assessed the expression of genes linked to hyphal formation in conditions that induced hyphal growth, such as the presence of serum and temperature. Gene expression analysis suggests that *HWP1* was found to be upregulated in antiviral drug treated *C. albicans* cells. The inhibition of hyphal formation might be the combined effect of the upregulation of negative regulators of hyphal formation such as *MIG1*, *NRG1*, and *TUP1*. As per the available literature, *NRG1* and *TUP1* are the transcriptional negative regulators limiting the filamentation and hyphal development [26].

Upregulation of these genes by the antiviral drug treatment might restrict serum induced Y-H morphogenesis in *C. albicans.* Studies on the gene expressions during Y-H conversion already showed that in response to environmental stimuli, *TUP1* in combination with the *MIG1*, *NRG1* represses the expressions of genes required for morphological transition [27].

Table 5.2 List of gene along with their respective role in *C. albicans*

Gene	**Function of genes in *C. albicans***	**Ref.**
MIG1	Retards hyphal formation	[28]
ACTIN	Involved in cell division	[29]
RAS1	Involved in cell adhesion, hyphal formation and filamentation	[30]
PDE 2	Resistance of planktonic phase cell to antifungals	[31]
BCY 1	Involved in white-opaque switching, cell differentiation	[32]
EFG 1	Essential gene in biofilm formation and white-opaque cell transition	[33]
TEC 1	Regulate the hyphal formation and virulence	[34]
ECE 1	Candida lysin, a hyphal specific protein	[35]
CEK 1	Cell wall formation	[36]
HST 7	Required for white biofilm formation and opaque mating	[37]
CPH1	Involved in pseudo hyphal development	[38]
CDC 35	Involved in azole resistance	[39]
HWP1	Induces hyphal and biofilm development	[40]
NRG1	Negative regulator of hyphal formation	[26]
TUP1	Transcriptional regulator involved in repression of hyphal development and regulates morphological switching	[41]

Table 5.2 details the roles of genes and transcription factors involved in Y-H morphogenesis and point out that the upregulation of *MIG1* is another striking feature of anti-morphogenic agents. The antiviral drugs with anti-morphogenic activity against *C. albicans* upregulated transcription level of *MIG1*. Previous studies reported that collective upregulation of *NRG1*, *TUP1*, and *MIG1* causes the retardation of Y-H morphogenesis in *C. albicans* [28]. Some of the transcription factors, such as *HWP1*, and *RAS1*, are required for hyphal growth at least in certain circumstances, but their unusual expressions are insufficient to cause complete hyphal gene expression profiles of proper hyphal morphogenesis. Another gene, *CEK1*, is directly linked to *C. albicans* pathogenicity as it is involved in cell wall formation and invasive growth of *C. albicans*. This gene was found to be downregulated in Ganciclovir and 2-AM treated *C. albicans.* Studies on hyphal-specific gene expressions provide a surprising conclusion that Y-H

morphogenic inhibition is linked with the increased transcriptive expressions of hyphal development regulating genes. Results of the gene expression study confirmed that 2-AM, 2-DG, and Ganciclovir may be proposed as the anti-morphogenic drug candidates that arrested the morphogenic transition in *C. albicans.* Based on screening and exploration of antifungal activities of antiviral drugs, the obtained results are summarized in graphical form and presented in figures **Fig 5.8, 5.9,** and **5.10.**

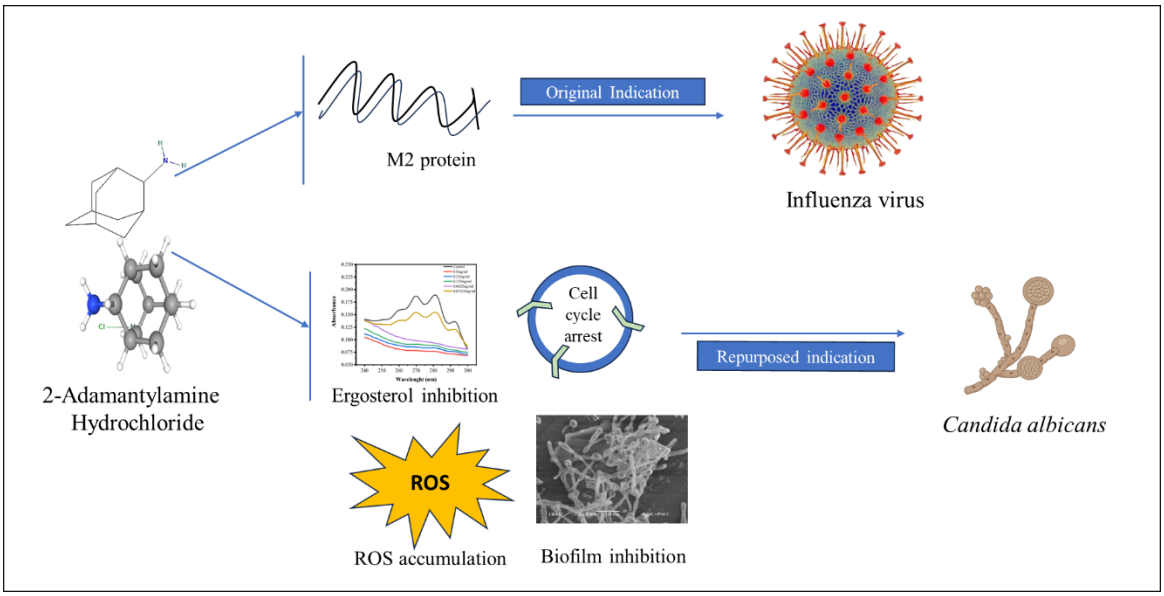

Figure 5.8. Presentation of probable mode of action of 2-AM as an antifungal agent against *C. albicans.*

Fig. 5.8. graphically briefs the antifungal activity of the antiviral drug 2-AM against both the planktonic and biofilm phases of *C. albicans.* 2-AM stands as the only drug with anti-proliferative, anti-morphogenic, anti-adhesion, and anti-biofilm action against *C. albicans.* Based on the results of studies on mechanistic insight, it was proposed that 2-AM treatment may alter cell stability via inhibition of ergosterol biosynthesis, which further continued to increase the accumulation of ROS. The accumulated ROS resulting in damage to the cell membrane is visualized under fluorescence microscope. ROS induced apoptosis with G2/M cell cycle phase arrest highlights the possible mode of action of 2-AM. In the future, this study shows the probable application of 2-AM as a therapeutic repurposed antifungal candidate against *C. albicans* alone or in combination with routine antifungal drugs available in market.

Fig. 5.9. briefs the anti-proliferative action of Vidarabine against the planktonic phase of C. *albicans*. Furthermore, Vidarabine in combination with FLC, AmB, and CAS showed a synergistic effect, and boosting the susceptibility of *C. albicans* cells to

these common antifungal medications. According to mechanistic understanding, Vidarabine targets *C. albicans* ergosterol production pathway, increases intracellular ROS accumulation, and ultimately triggers cell apoptosis (**Fig. 5.9**).

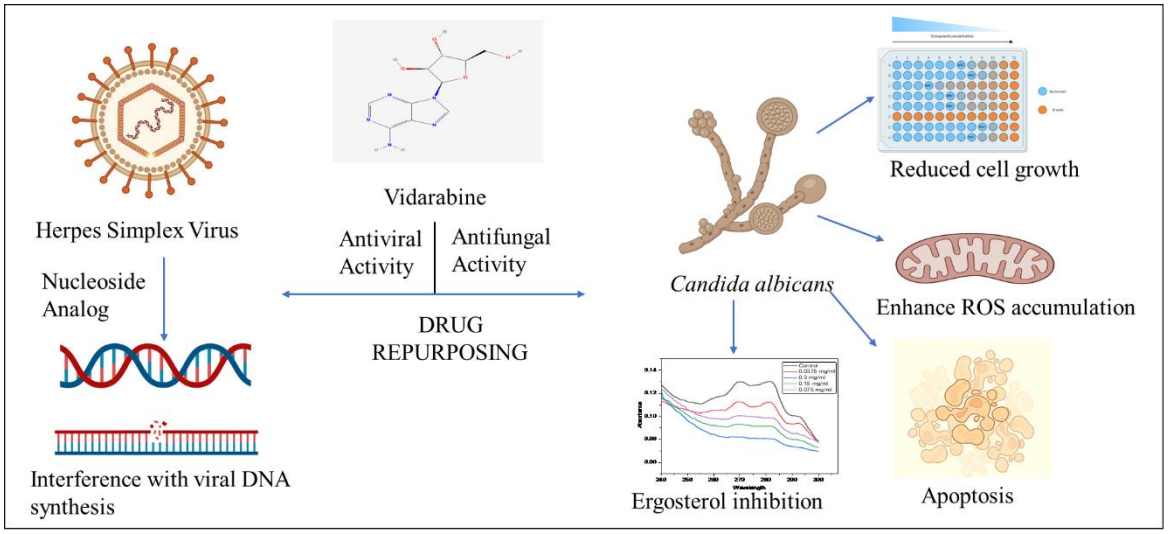

Figure 5.9. Graphical presentation of repurposing Vidarabine as an antifungal drug with probable mode of actions against *C. albicans.*

This study reported the antifungal activity of Vidarabine both *in vitro* and *in vivo*, as well as possible mechanisms of action. These findings could help to clarify Vidarabine's possible therapeutic application as an antifungal agent or as an addition to traditional antifungal drugs in the future.

A glucose analogue, 2-DG, also showed effective antifungal action against *C. albicans*. 2-DG shown less antifungal activity as compared to that of 2-AM, Ribavirin, and Vidarabine. However, 2-DG induced ROS accumulation and was found to arrest cells in S-phase. Upregulation of hyphal suppressor genes *MIG1*, *TUP1*, and *NRG1* by 2-DG treatment presents the molecular base of its anti-morphogenic action on *C. albicans*. Intriguing outcomes of the anti-morphogenic and anti-proliferative action of 2-DG emphasize its possible ability as an anti-*Candida* agent in therapeutic development (**Fig. 5.10**)

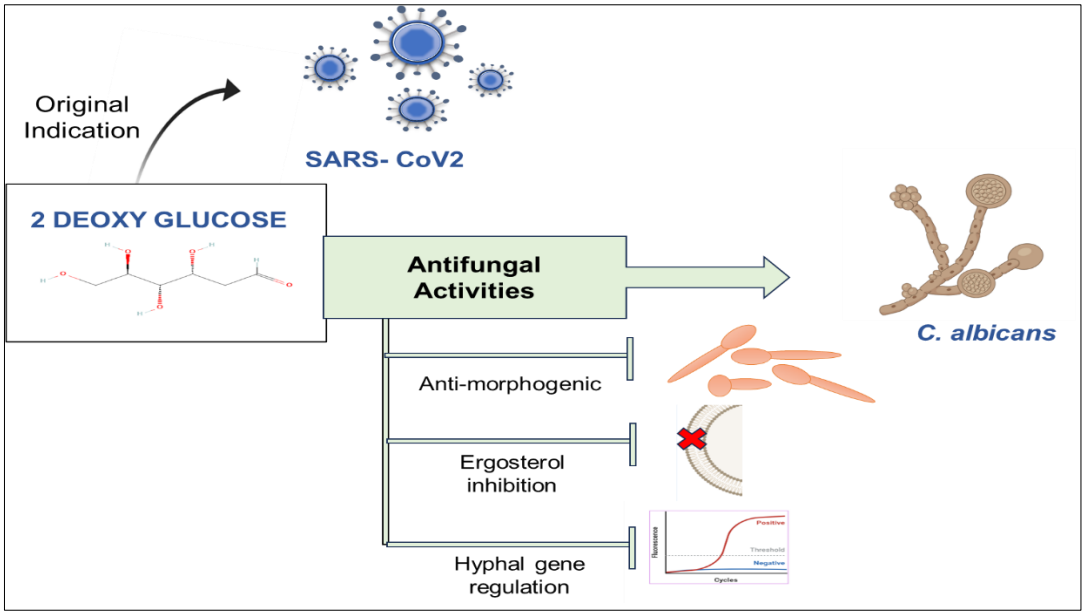

Figure 5.10. Illustration of antifungal activities of 2-DG against human fungal pathogen *C. albicans.*

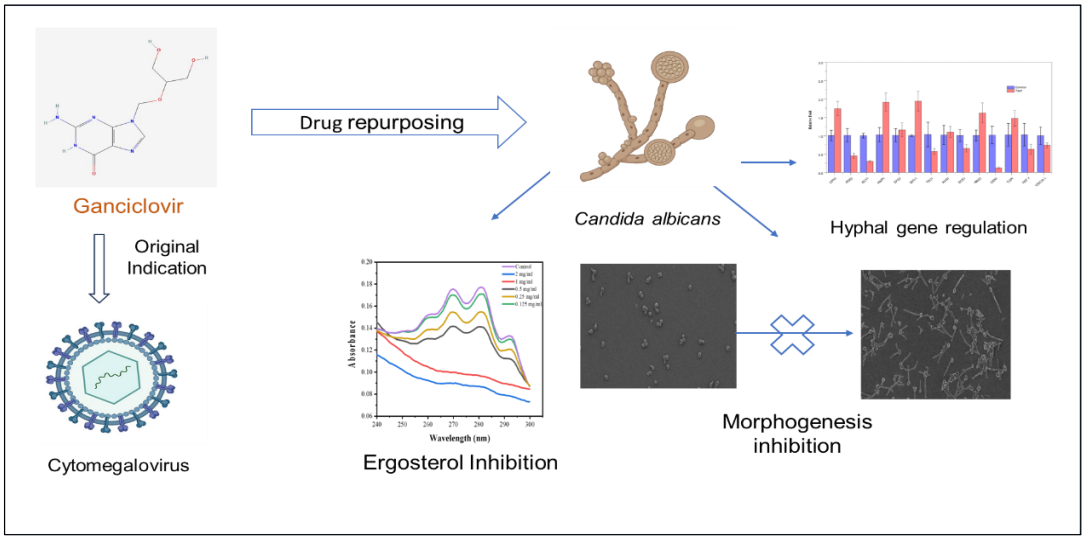

Figure 5.11. Graphical presentation of original and proposed indication of an antiviral drug Ganciclovir against *C. albicans*.

In summary, **Fig. 5.11** presents the anti-morphogenic properties of the antiviral drug Ganciclovir against *C. albicans.* Apart from its hyphal inhibitory effect, Ganciclovir exhibited a dose dependant reduction of ergosterol biosynthesis in *C. albicans*. Negative regulators of hyphal growth, *NRG1* and *MIG1* markedly increased their expressions by treatment of Ganciclovir. Furthermore, the anti-morphogenic action of Ganciclovir on urinary catheters provides a new perspective for its use in antifungal therapeutics. Altogether, Ganciclovir can be proposed as an antifungal agent that targets

one of the key virulence traits in *C. albicans.*

Another antiviral drug namely, Ribavirin with fungicidal action targeted the initial planktonic growth of *C. albicans.* Ribavirin induced G2/M mediated cell cycle arrest. Though Ribavirin is unable affect the virulence traits in *C. albicans*, it's strong anti-proliferative action could be recommended for antifungal therapeutic development.

In brief, antiviral drugs namely, 2-AM, Vidarabine, and Ribavirin had anti-proliferative activity and were further confirmed by apoptosis assay, whereas 2-DG, 2-AM, and Ganciclovir regulated hyphal specific gene expressions required for inhibition of morphogenic switch in *C. albicans*.

5.5. Conclusions

Overall, this chapter deals with the probable mode of action of antiviral drugs, namely 2-AM, 2-DG, Ganciclovir, Ribavirin, and Vidarabine against *C. albicans.* Cells are treated with MIC of 2-AM, 2-DG, Ribavirin, and Vidarabine at 0.25, 2, 0.025 and 0.15 mg/ml concentrations, respectively. In response to the fungicidal drugs, 2-AM and Vidarabine, *C. albicans* cells accumulated significant levels of ROS, which further led to cell cycle arrest at the G2/M phase, resulting in cell apoptosis and necrosis. Oxidative stress induced by 2-AM and Vidarabine disturbed cell membrane permeability in *C. albicans*. Although fungistatic drugs, namely 2-DG and Ribavirin, caused less ROS accumulation than fungicidal drugs, these drugs increased PI uptake and arrested the cell cycle at the S and G2/M phases, confirming their anti-*Candida* action. Anti-morphogenic drugs, namely, 2-AM, 2-DG, and Ganciclovir, were further studied to determine the molecular basis of morphogenesis inhibition at MIC of 0.25, 1 and 0.5 mg/ml, respectively. Significant upregulation of negative regulators of hyphal growth, *MIG1*, *TUP1*, and *NRG1* leads to suppression of hyphal morphogenesis. Although the study is a preliminary step, the obtained results are more appealing for drug repurposing.

5.6. References

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CHAPTER 6

Summary and conclusions

6. Summary and conclusions

6.1. Summary

Nearly 15 million deaths were reported annually due to the infectious diseases caused by *C. albicans*, that created a huge burden on public healthcare. Development of drug resistance in *C. albicans* has further challenged current antifungal therapeutics and thus demanded an urgent development of effective antifungal therapeutic agents. Repurposing of already FDA approved drugs for a newer indication than the original indication provides a less time and cost consuming alternative strategy to the traditional *de novo* drug development process, with a higher success rate.

In the current study, FDA approved ten antiviral drugs were screened against the preliminary phases of *C. albicans* virulence, including planktonic growth, Y-H morphological transition, surface adhesion, and biofilm formation. Experimentation was performed as per CLSI guidelines and found that 2-AM, 2-DG, Ribavirin, and Vidarabine affected *C. albicans* planktonic growth and survival negatively. 2-AM, 2-DG, Ribavirin, and Vidarabine reduced growth of *C. albicans* by nearly 50 % at 0.25, 2, 0.025, and 0.15 mg/ml, respectively, same concentrations were referred as MICs of drugs. Further, 2-AM and Vidarabine at 0.5 and 0.3 mg/ml concentrations exhibited strong fungicidal action on *C. albicans*, which was confirmed by the agar plate method.

Fluorescence microscopic images of AO-EtBr dual dye-stained *C. albicans* evidenced the massive dead count after treatment of 2-AM, 2-DG, Ribavirin and Vidarabine at MIC to cells. Damage of *C. albicans* cell membrane by antiviral drugs was proved by ergosterol quantification. For the same, sterols were extracted from *C. albicans* cells after drug treatment; ergosterol was characterized and quantified using a spectrophotometer and then compared with the untreated control. Reduced biosynthesis of ergosterol by the action of antiviral drugs proved that ergosterol serves as a possible drug target in *C. albicans*, resulting in altered cell membrane integrity.

Further, 2-AM, 2-DG, Ribavirin and Vidarabine treated *C. albicans* cells on PI staining showed increased fluorescence compared to the untreated cells. This data suggests that, the antiviral drug treatment remarkably increases the cell membrane permeability, and resulting in the altered cell integrity. Overall data of dual staining, PI-staining, and ergosterol quantification experiments suggests that antiviral drugs actively damage the *C. albicans* cell membrane that alters membrane integrity, stability, and permeability.

Investigation for the base of antiviral drug action against *C. albicans* using ROS showed that treatment with 2-AM, 2-DG, Ribavirin, and Vidarabine induces ROS accumulation, which subsequently damages the cell membrane, its permeability, and may causes mitochondrial dysfunction. This data prompted to highlight of the pattern of cell killing using cell cycle and apoptosis studies. Results of this studies concluded that, treatment of 2-AM, 2-DG, Ribavirin and Vidarabine altered the normal cell cycle progression of *C. albicans*. Specifically, treatment with 2-AM, Ribavirin and Vidarabine at MIC values 0.25, 0.025 and 0.3 mg/ml, respectively arrested cells in G2/M phase while, 2-DG treatment arrested cells in S-phase of cell cycle. Externalisation of PS on cell membrane was detected using Annexin V-FITC based apoptosis detection kit that confirms the apoptotic nature of 2-AM and Vidarabine against *C. albicans*.

In brief, treatment with fungicidal drugs namely, 2-AM and Vidarabine causes accumulation of ROS, which generates oxidative stress that eventually hampers cell membrane integrity. In addition, their treatment to *C. albicans* leads to cell cycle arrest in the G2/M phase, ultimately culminating in programmed cell death.

The drug repurposing strategy was coupled with the drug combination approach. Results of checkerboard assay for antiviral-antifungal drug combinations showed the synergistic combinations of 2-AM, 2-DG, and Vidarabine with FLC. This synergy provides a combinational approach to overcome drug resistance in *C. albicans* and may restore the azole drug susceptibility in *C. albicans*, as tested antivirals also share the common target of ergosterol biosynthesis akin to FLC. In addition to this, synergism was also observed in combinations of AmB-Vidarabine, AmB-2-DG, and 2-AM-CAS. Reduced drug dosage obtained in these synergistic combinations may improve the antifungal drug action with fewer side effects compared to alone.

In addition to the planktonic phase, other virulence contributing factors in *C. albicans* such as hyphal morphogenesis, surface adherence, and biofilm formation were also studied for antiviral drug action. Antiviral drugs specifically 2-AM, 2-DG and Ganciclovir possess significant anti-morphogenic action against *C. albicans* even in the presence of an inducer. In the serum induced morphogenesis assay, 2-AM, 2-DG and Ganciclovir treated *C. albicans* cells were restricted from hyphal development and arrested in less pathogenic yeast phase. Concentration for hyphal inhibition was less for 2-AM (0.5 mg/ml) compared to the 2-DG and Ganciclovir at 2 and 1 mg/ml, respectively indicating that 2-AM has significant anti-morphogenic action against *C. albicans.* Further, SEM visualization confirmed the anti-morphogenic action of 2-AM, 2-DG and

Ganciclovir on silicon-based urinary catheters posing the therapeutic application of these drugs to target initial phases of biofilm development. In this, Ganciclovir completely inhibited hyphal development and ergosterol biosynthesis without hampering the growth of *C. albicans* pointing its action on the virulence attributes only.

The ability of antiviral drugs to target the adhesion which is initial phase of biofilm formation, was applied in a screening of antiviral drug repurposing. As per the obtained data of the XTT metabolic assay, among all screened antiviral drugs, 2-AM was only drug that significantly inhibited surface adherence ability of *C. albicans* on polystyrene and catheter surfaces as well. In addition, SEM further evidenced the inhibition of biofilm by the treatment of 2-AM on the surface of silicon based urinary catheter model and showed potential of 2-AM as an anti-biofilm agent for treating the issues of catheter linked fungal biofilm infections in clinical settings.

Anti-morphogenic activities of 2-AM, 2-DG and Ganciclovir were investigated up to transcription analysis and pointed out that treatment with these drugs on *C. albicans* cells showed significant alterations in gene expressions of several hyphae-specific genes. Inhibition of Y-H morphological transition by 2-AM, 2-DG and Ganciclovir was mainly contributed by the upregulation of hyphal repressor genes such as *NRG1, MIG1,* and *TUP1*.

In vivo efficacy of selected effective antiviral drugs namely, 2-AM, 2-DG, Ganciclovir, Ribavirin and Vidarabine at MIC was studied in *B. mori* silkworm larvae. As per the obtained results it can be concluded that these antiviral drugs exhibit anti-*Candida* activity in *B. mori* infected animal model.

In summary, this work demonstrates that, antiviral drugs damaged the cell membrane permeability, altered ergosterol biosynthesis, triggered intracellular ROS accumulation in *C. albicans* and led to ROS induced apoptosis. Additionally, as these antivirals found to have more therapeutic potential against *C. albicans*, more insights into all possible mechanisms of action will lead to the development of these repurposed antiviral drugs as antifungal agents.

This study presents relatively obscure activities of antiviral drugs with the characterization such as anti-proliferative, anti-morphogenic and anti-biofilm against *C. albicans.* This work proposed anti-morphogenic activities of 2-DG and Ganciclovir, and anti-proliferative action of Vidarabine against *C. albicans* for the first time. Based on the obtained results, the study highlights the superiority of 2-AM over other antivirals by exerting a significant inhibitory effect on planktonic growth, Y-H morphogenesis,

surface adherence, biofilm formation, and cell membrane integrity. 2-AM is proposed as the potential therapeutic candidate in the pipeline of antifungal drug development against *C. albicans*.

Major conclusions of the study are:

1. Among the selected antiviral drugs (mentioned in **Table 3.1** chapter 3) 2-AM, 2-DG, Ribavirin and Vidarabine are able to inhibit nearly 50 % *C. albicans* proliferation at 0.25, 2, 0.025 and 0.15 mg/ml, respectively. In which 2-AM and Vidarabine are fungicidal in nature at 0.5 and 0.3 mg/ml, respectively.
2. 2-AM, 2-DG, Ribavirin and Vidarabine capable to disturb ergosterol biosynthesis, ROS level, and thus cell membrane integrity. This further affected the normal progression of cell cycle in *C. albicans.* Apoptosis induced by 2-AM (0.5 mg/ml) and Vidarabine (0.3 mg/ml) drug treatment proves the fungicidal nature of these drugs against *C. albicans.*
3. Among all screened antiviral drugs, only 2-AM at 0.5 mg/ml inhibits surface adhesion and early biofilm development in *C. albicans* and its inhibitory action is validated on silicon based urinary catheter using SEM.
4. 2-AM (0.5 mg/ml), 2-DG (2 mg/ml) and Ganciclovir (1 mg/ml) inhibited Y-H morphogenic transition by causing upregulation of negative regulators of hyphal growth, *MIG1*, *NRG1*, and *TUP1* in *C. albicans.*
5. Synergistic combination of 2-AM, 2-DG and Vidarabine with FLC along with ergosterol inhibitory action can be claimed as antifungal activities of antiviral drugs are alike azole group.
6. Considering the anti-morphogenic, anti-biofilm and ROS induced fungicidal potential, 2-AM may be claimed as an active antifungal drug candidate against *C. albicans* either alone or in combination with antifungal drugs.

Recommendations

Recommendations

Repurposing of already approved non-antifungal drugs as antifungal agents provides a promising strategy to treat fungal infections. This strategy has accelerated antifungal drug development process. Current study focused on repurposing of FDA approved antiviral drugs against human fungal pathogen *C. albicans.* Results obtained in this study showed that among the screened antiviral drugs, five antivirals namely 2-AM, 2-DG, Ganciclovir, Ribavirin and Vidarabine significantly affected growth and/or preliminary virulence factors in present in *C. albicans.* In this, 2-AM, 2-DG, Ribavirin and Vidarabine hampered *C. albicans* cell proliferation by altering cell cycle progression. Further, Y-H morphogenic shift inhibition by 2-AM, 2-DG and Ganciclovir prompted to investigate the molecular basis of inhibition using RT-PCR. Subsequently, these antiviral drugs found to upregulate gene expressions of negative regulators resulting in hyphal inhibition. Current investigation provide that antiviral drugs are capable to interfere the ergosterol biosynthesis, ROS generation, cell membrane integrity. Overall, findings of the study suggest the applicability of antiviral drugs for therapeutic purpose against *C. albicans.*

Although current research will contribute to the approach of antiviral drug repurposing against *C. albicans* and establishes the anti-*Candida* potential of the non-antifungal drugs namely, 2-AM, 2-DG, Ganciclovir, Ribavirin and Vidarabine. Further, in detail some challenges need to be addressed along with the molecular basis of drug actions prior to the clinical setting.

The present study can be extended in the following areas,

- Anti-morphogenic, anti-proliferative and anti-biofilm activities of 2-AM are reported in this study. These results open up new opportunities on detailing the exact mode of action and *in vivo* efficacy of 2-AM in animal model such as mice and rat.
- Further studies on the reduction of 2-AM drug dosage by drug combination approach will lessens the dose related challenges in clinical settings. In addition, synergistic drug combinations of 2-AM with FLC and CAS could be used to investigate their mode of action against *C. albicans.*
- Anti-biofilm action of 2-AM open up the aspect of development of drug coated catheter to counter the catheter linked urinary infections. Urinary catheters coated with 2-AM could be proceed as the future perspective for targeting the CAUTI.

- Anti-morphogenic formulations containing 2-AM as an active component can be prepared. Formulations in the form of oral adhesives, toffy and oral gels provide a new and effective way to cure oral candidiasis caused by *C. albicans.* The oral and topical formulations can be processed for detailed studies such as spread ability, pharmacokinetic properties and then to the clinical trial.
- Detailing on the fungicidal action of 2-AM up to the gene expression level probably point the presence of new drug target in *C. albicans.*
- In addition, the anti-proliferative and fungicidal action of 2-AM can be used in topical antifungal formulations either alone or in combination with FLC that may overcome the drug resistance developed in *C. albicans.*

Publications

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BACTERIAL AND FUNGAL PATHOGENESIS - RESEARCH PAPER

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

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

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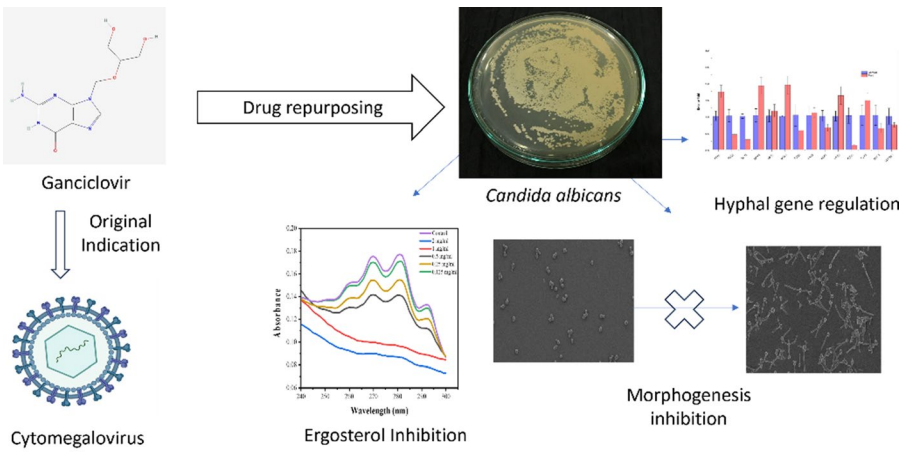
Abstract

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

Graphical abstract



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

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RESEARCH

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

Tanjila C. Gavandi[1] · Sargun T. Basrani[1] · Sayali A. Chougule[1] · Shivani B. Patil[1] · Omkar S. Nille[2] · Govind B. Kolekar[2] · Shivanand R. Yankanchi[3] · S. Mohan Karuppayil[1] · Ashwini K. Jadhav[1]

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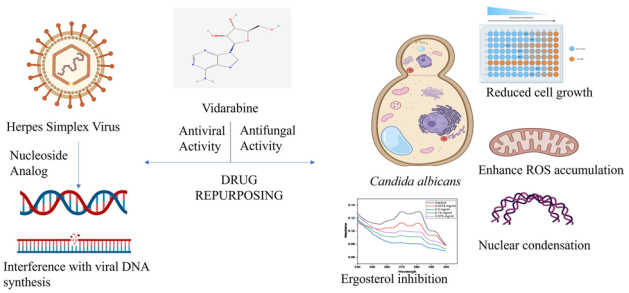
Abstract

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

Graphical Abstract



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

Introduction

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

Extended author information available on the last page of the article

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RESEARCH ARTICLE

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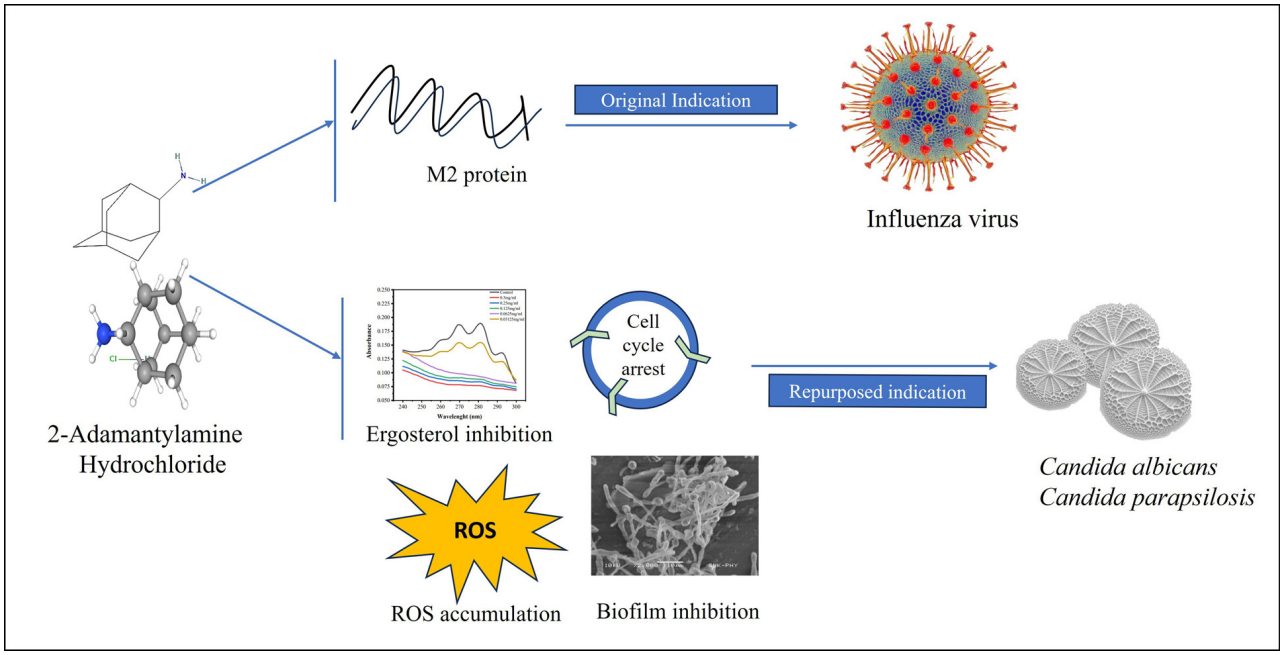
MICROBIOLOGY SOCIETY

Antifungal activity of 2-adamantylamine hydrochloride on *Candida albicans* and *Candida parapsilosis*

Tanjila C. Gavandi, Sayali A. Chougule, Shivani B. Patil, Sargun T. Basrani, S. Mohan Karuppayil* and Ashwini K. Jadhav*



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Impact statement

Increased drug resistance in *Candida* species has challenged current antifungal therapeutics, leading to an increased rate of morbidity and mortality. The application of existing non-fungal antifungal agents has expanded the pipeline of antifungal drug development. A large amount of literature has already reported on repurposing antiviral drugs, especially protease inhibitors, against *Candida* species. 2 adamantylamine hydrochloride (2-AM) has been investigated for its anti-*Candida* action for the first time, and research findings suggest that 2-AM effectively arrested the proliferation of *C. albicans* and *C. parapsilosis*. The antibiofilm action of 2-AM against *C. albicans* supports the concept of drug repurposing for the development of antifungal therapeutics.

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Keywords: 2-adamantylamine hydrochloride; apoptosis; *Candida*; drug repurposing; ergosterol; fungal biofilm; ROS.

Abbreviations: 2-AM, 2-adamantylamine hydrochloride; Amp B, amphotericin B; AO, acridine orange; BIC, biofilm inhibitory concentration; CAS, caspofungin; c.f.u., Colony Forming Unit; DCFDA, 2′,7′-dichlorofluorescin diacetates; EtBr, ethidium bromide; FACS, fluorescence-activated cell sorting; FBS, foetal bovine serum; FIC, fractional inhibitory concentration; LA, Loewe Additivity; MFC, minimum fungicidal concentration; MIC, Minimum Inhibitory Concentration; PI, propidium iodide; PS, phosphatidylserine; SEM, scanning electron microscopy; YPD, yeast extract peptone dextrose.

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MICROBIAL PATHOGENESIS AND HOST-MICROBE INTERACTION

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

Sargun Tushar Basrani[1] · Tanjila Chandsaheb Gavandi[1] · Shivani Balasaheb Patil[1] · Nandkumar Subhash Kadam[1,2] · Dhairyasheel Vasantrao Yadav[1,2] · Sayali Ashok Chougule[1] · Sankunny Mohan Karuppayil[1] · Ashwini Khanderao Jadhav[1]

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Abstract

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

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

Introduction

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

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WILEY

REVIEW

Glucosinolate derivatives as antifungals: A review

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Abstract

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

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

1 | INTRODUCTION

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

Shivani Patil was first author.

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ORIGINAL PAPER

Manganese Iron Oxide Nanoparticles for Magnetic Hyperthermia, Antibacterial and ROS Generation Performance

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Abstract
The preparation of manganese substituted iron oxide magnetic nanoparticles by polyol synthesis route. Due to the unique properties, diethylene glycol (DEG) and tri-ethylene glycol (TEG) used as a solvent in synthesis method with different volumetric variations. The structural, morphological and hyperthermic properties of prepared samples are investigated. Formation of single-phase cubic spinel lattice for all compositions confirmed by X-ray diffraction and crystallite size was found to be decreased from 20.6 ± 1.3 to 15.2 ± 1.7 nm with varying ratio of DEG/TEG. Transmission electron microscopy (TEM) analysis displayed spherical grains with an agglomeration of the $MnFe_2O_4$ magnetic nanoparticles (MNPs). Heating ability of MNPs studied with an induction heating system under different magnetic field strengths at 20 kA/m and 26.6 kA/m by varying nanoparticle concentrations at fixed frequency of 278 kHz. Antimicrobial activity on *E. coli* and antifungal activity on *C. albicans* showed effectiveness of MNPs at 10 mg/mL for such activities. Additionally, ROS induction in presence of MNPs illustrates probable action against *E. coli* and *C. albicans* and as antibacterial and antifungal agent in the medical field due to ROS generation ability.It has been shown that these optimized MNPs will play multifaceted roles for magnetic hyperthermia therapy as heat mediators, and antibacterial/antifungal agents owing to their magnetic induction heating properties and biological activities.

Keywords Nanoparticles · Bio-materials · Magnetic hyperthermia · Magnetic nanoparticles · Surface chemistry · $MnFe_2O_4$

Introduction

Magnetic nanoparticles have been deeply investigated in recent decades because of their versatile physiochemical and biomedical properties in biomedical applications, ranging from diagnostic to therapeutic fields. In particular, iron-oxide based magnetic nanoparticles (MNPs) are widely investigated as contrast agents for magnetic resonance imaging (MRI) applications [1] or magnetic hyperthermia treatments [2] due to their magnetic and structural characteristics.It have been also investigate as drug carrier in targeted drug delivery [3] and antibacterial agent [4] etc. Some physio-chemical methods are used to synthesis of magnetic nanoparticles (MNPs), such as co-precipitation, combustion, sol-gel, microwave, hydrothermal, polyol, solvothermal, and micro-emulsion method [5]. Multi advantageous one-step polyol synthesis technique responsible to control effective factors such as (i) shape and sizes of nanoparticles (NPs) by varying some flexible parameters like applied temperature as well as pH of prepared solution, (ii) structure, and (iii) amount of production [6]. In the polyol technique a single event of nucleation at multiple sites is involved and inhibits activation of growth mechanism [7]. The high temperature required, nearly about 200 to 300 °C for ferritization, and prepared particles are significantly small compared to another synthesis routs [8].

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

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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.

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ORIGINAL PAPER

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

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

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

Abstract

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

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

Introduction

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

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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*

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A B S T R A C T

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

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

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

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

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

➢ **How to cite this paper**
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Introduction

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

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

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CME CODE NO: MMC/WEB/2022/C-015391

This is to certify that

Mr/Ms./Dr. *Tanjila Gavandi*

has participated as Delegate / presented a Poster / Oral presentation in the International Conference on **"EMERGING AND RE-EMERGING INFECTIONS (ICERI) – 2022"** held on 8th April, 2022.

The Maharashtra Medical Council (MMC) has granted 2 (TWO) Credit points for the delegates.

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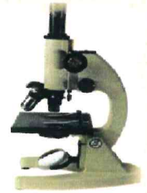

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has presented the paper titled **"In silico study of the binding affinity of phenylpropanoids with SARS - CoV2 and Omicron variant (B1.1.529) spike proteins"** in two days International Conference on "**Approaches towards affordable clean energy, good health and well-being**" Organized by The Department of Life Sciences, Garden City University In association with **Karnataka State Bioenergy Development Board** on 2nd and 3rd March 2023.

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of Participation

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