# "REPOSITIONING OF ANTIMALARIAL DRUGS AS ANTI - *CANDIDA ALBICANS* AGENTS"

A THESIS SUBMITTED TO

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

(Declared u/s 3 of the UGC Act 1956)

FOR THE AWARD OF
**DOCTOR OF PHILOSOPHY**
**IN**
**MICROBIOLOGY**

**UNDER THE FACULTY OF**
**INTERDISCIPLINARY STUDIES**


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## DECLARATION

I am Sargun Tushar Basrani, hereby declares that the thesis entitled **"Repositioning of Antimalarial Drugs as Anti-*Candida albicans* Agents"** submitted here for the award of **Doctor of Philosophy** in the **Microbiology,** Faculty of **Interdisciplinary Studies,** under the guidance of **Dr. Ashwini K. Jadhav** Assistant Professor, **Department of Stem Cell and Regenerative Medicine and Medical Biotechnology, Centre for Interdisciplinary Research (CIR), D. Y. Patil Education Society (Deemed to be University), Kolhapur** is completed and written by me, has not previously formed the basis for the degree or diploma or other similar title of this or any other university or examine body.

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# ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to each and every one whom I mentioned below.

I give my sincere thanks to my supervisor, **Dr. Ashwini K. Jadhav,** Assistant Professor, Department of Stem Cell and Regenerative Medicine and Medical Biotechnology for her continuous support, trust, and assistance at every stage of the research work. I am very much grateful to **Prof. Dr. S. Mohan Karuppayil**, former Head of department of Department of Stem Cell & Regenerative Medicine and Medical Biotechnology for his constant support and guidance for my Ph.D. I extended my gratitude to **Prof. Dr. C. D. Lokhande**, Dean and Research Director, Centre for interdisciplinary Research (CIR) for his valuable suggestions and support. I am thankful to Prof. **Dr. M. G. Joshi**, Head of the Department of Stem Cell & Regenerative Medicine and Medical Biotechnology for his support and suggestions.

I am grateful to **Dr. R. K. Mudgal**, Hon'ble Vice Chanceller and **Dr. V. V Bhosale**, Registrar of our institute for their support. I am grateful to **Mr. Nandkumar Kadam and Mr. Dhairyasheel Yadav,** Director of iSERA Biological Pvt Ltd., MIDC Shirala, Dist., Sangli, Maharashtra 41540, India for his guidance and help in my study. I specially thanks to **Dr. Deepak Sawant** for providing support in RT -PCR lab studies.

I am deeply grateful to **Dr. Mansingh Nimbalkar**, Department of Botany, Shivaji University Kolhapur, Maharashtra, India for providing SEM facility. I specially thanks to my research colleagues, **Tanjila Gavandi, Shivani Patil, Sayali Chougule, Anuja Vibhuti, Tejaswini Patil, Rutuja Gambhir, Pranoti Kamble and Shahabaj Mujawar** for helping me in my experiments, writing skills and providing the friendly environment in laboratory. I thank all the teaching and non-teaching staff of CIR.

I would like to express my gratitude to my parents, **Mr. Naresh gaganani** and **Mrs. Deepika gaganani my younger brother Mr. Om and Lalit Gaganani** for their support. I sincerely thankful to my husband **Mr. Tushar Basrani** and my father-in-law **Mr. Prakash Basrani,** mother-in-law **Mrs. Jaya Basrani** to their constant encouragement and support through my PhD journey. I am thankful to my brother-in-law and sister-in-law **Mr. Raj Basrani, Mrs. Bhumi Basrani, Mrs. Samisha Jasuja and Radhika Kalyani** for their support.

I very much to my daughter **Panaya Basrani** and my nephew **Thiya Basrani** for my support. I am deeply grateful to everyone who has supported me on this academic journey.

**Sargun Tushar Basrani**

## LIST OF PUBLICATIONS

**Copyrights: Granted (02)**

1) **Mode of action of antimalarial drug on fungi**.
   **Name of authors- - Dr. Ashwini Khanderao Jadhav, Ms. Sargun Basrani**
   **Registration number - L-150387/2024.**
   **Publication Date: 02/07/2024.**

2) **Targets of Hydroxychloroquine in *Candida albicans***
   **Name of authors- - Dr. Ashwini Khanderao Jadhav, Ms. Sargun Basrani**
   **Registration number - L-150596/2024.**
   **Publication Date: 05/07/2024.**

**Research Articles published in International Journals: (09)**

1) **Basrani ST**, Gavandi TC, Patil SB, Kadam NS, Yadav DV, Chougule SA, Karuppayil SM, Jadhav AK. **Hydroxychloroquine an Antimalarial Drug, Exhibits Potent Antifungal Efficacy Against *Candida albicans* Through Multitargeting.** Journal of Microbiology. 2024 Apr 8:1-1, 381-391. (IF- 3.0)

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**Conferences and workshops attended: (06)**

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2. Presented poster at International Conference on **"Nanotechnology Addressing the Convergence of Materials Science, Biotechnology and Medical Science (IC-NACMBM-2024)"** held at the Centre for Interdisciplinary Research, D. Y. Patil Education Society (Deemed to be University), Kolhapur, Maharashtra, India during 12$^{th}$ to 14$^{th}$ February 2024.

3. Presented poster at International Conference on "**Biology Beyond Boundaries"** organized by the Department of Biotechnology at Savitribai Phule Pune University during 29$^{th}$ – 31$^{st}$ January, 2024.

4. **Attended Hands on workshop on Fluorescence Activated Cell Sorter in association with DST-FLST, Govt. of India,** held at SRM university, Chennai on 15$^{th}$ – 17$^{th}$ December, 2022.

5. Presented poster at international conferences on **"Emerging Trends in Applied Microbiology and Food science (ETAMFS) at Yashavantrao Chavan Institute of Science Satara, Maharashtra, India- 2022.**

6. Presented poster at International Conference on **Stem Cells and Regenerative Medicine - Academic and Industrial Outlooks** at Department of Stem cell and Regenerative Medicine, Centre for Interdisciplinary Research, DY Patil Education Society, (Institution Deemed to be University), Kolhapur, Maharashtra, India -2021.

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# ABSTRACT

*Candida albicans* causing approximately 70 % infections among all type fungal infections. The growing resistance to current antifungal drugs shows the urgent need for new treatment options. Drug repurposing offers significant advantages over developing new medications, including reduced costs and shorter development timeline.

Antimalarial drugs, in particular, have shown a wide range of biological activities, including antibacterial, anticancer, antifungal, and antiviral effects. In this study, Eleven antimalarial drugs; includes Chloroquine, Quinine, Mefloquine, Hydroxychloroquine, Artesunate, Piperaquine, Sulfadoxine, Pyrimethamine, Atovaquone, Artether, and Amodiaquine were screened for their antifungal activity against *C. albicans* ATCC 90028. The antifungal potential was evaluated through various assays, including the determination of Minimum Inhibitory Concentration (MIC) for planktonic growth, Minimum Fungicidal Concentration (MFC), Yeast-to-Hyphal Morphogenesis Assay, Scanning Electron Microscopy (SEM), Adhesion Assay, and Biofilm Assays. Additional studies such as ergosterol assay, ROS production assays, Propidium Iodide (PI) uptake, and RT-PCR were conducted to elucidate the mechanisms of action of these drugs.

The results revealed that Quinine, Mefloquine, and Hydroxychloroquine exhibited significant antifungal activity against *C. albicans*, particularly in terms of biofilm inhibition, interference with yeast-to-hyphal morphogenesis, and reduction in virulence factors. These drugs were shown effect on *C. albicans* adhesion to polystyrene surfaces and displayed promising activity in reducing the pathogen's virulence. Additionally, *In-vivo* antifungal efficacy of these drugs was tested using a silkworm infection model.

In conclusion, Quinine, Mefloquine, and Hydroxychloroquine demonstrate potential as new antifungal agents against *C. albicans*, offering a promising direction for future antifungal drug development through repurposing approach.

**Keywords-** Antimalarial drug, *Candida albicans*, Cell cycle, Drug repurposing, Ergosterol synthesis, q RT-PCR.

# ABBREVIATIONS

**AmpB**- Amphotericin B

**ATCC**- American Type Culture collection

***C.albicans***- *Candida albicans*

**DNA**- Complementary DNA

**ºC** - Degree Celsius

**CLSI**- Clinical and Laboratory Standards Institute

**DMSO**- Dimethyl sulfoxide

**D/W**- Distilled water

**FACS**- Fluorescence Activated Cell sorting

**FBS**- Fetal bovine serum

**FLC-** Fluconazole

**$H_2O_2$**- Hydrogen Peroxide

**$H_2$DCFDA**- 2′, 7′-dichlorodihydrofluorescein diacetates

**$LD_{50}$- Lethal dose 50**

**MIC**- Minimum Inhibitory Concentration

**MFC**- Minimum fungicidal concentration

**MOPS**- Morpholinepropanesulfonic acid

**MBC**- Minimum bactericidal concentration

**NCTC**- National of type culture collection

**NZW-** New Zealand white rabbit

**PBS -** Phosphate-buffered saline

**PCR**- Polymerase Chain Reaction

**PI** - Propidium Iodide

**RPMI-** Roswell Park Memorial Institute

**RPM-** Rotation per Minute

**ROS-** Reactive Oxygen Species

**RNA** - Ribose Nucleic Acid

**RPMI 1640** - Roswell Park Memorial Institute 1640

**SEM-** Scanning Electron Microscopy

**SDA-** Sabouraud Dextrose Agar

**XTT**- 2, 3 – bis-(2- methoxy- 4- nitro-5- sulfophenyl)- 2H- tetrazolium – 5- carboxanilide

**Y-H -** Yeast to hyphal

**%** - Percentage

**µg** - Microgram

**µg/ml** - Microgram per millilitre

**µl** - Microlitre

**µm**-Micrometre

# CHAPTER I

# INTRODUCTION

## 1.1. Background

Every year, approximately 1.5 million infections are caused by fungi, which negatively impacts human health, particularly in patients of Intensive Care Unit or immunocompromised individuals [1]. The development of new antifungal therapies is necessary to overcome the problem of drug resistance, toxicity and high cost. Among the pathogenic fungi, the *Candida* genus is responsible for 50 % of fatalities in systemic fungal infections [2]. The fungal pathogen *Candida albicans* causes mucosal infections, approximately 200,000 deaths occur annually due to invasive and disseminated diseases in susceptible populations [3]. Healthy people's skin, gastrointestinal, and genitourinary systems are all regularly colonized by *Candida* spp. as commensals, which cause little to no harm to the host. Saliva in the mouth cavity and neutrophils in the blood are two examples of natural anatomical barriers and innate defensive systems that are usually enough to help sustain the commensal status [4]. Overgrowth of *C. albicans* can result in harm to host and the creation of the opportunistic infection when the usual microbiota balance is disturbed or immunity is weakened. The survival rate of severely ill patients has increased dramatically over the past few decades as a result of significant advancements in a number of medically related procedures and therapies [5].

## 1.2. Pathogenicity of *C. albicans*

The human pathogen *C. albicans* is capable of colonizing a number of biotic as well as abiotic surfaces like prosthetic devices. Biofilm formation is a major threat in medicine since colonized on prosthetics materials which act as a permanent source of infection in human body. In hospitalized patients, colonization of *C. albicans* on catheters is a severe issue. Both the human immune system and antifungal medicines cannot effectively treat *C. albicans*. Biofilms are self- adhering microbial colonies embedded in an extracellular polymeric matrix attached to a surface [6]. It has long been known that biofilms serve as a reservoir for pathogenic organisms and that their creation requires numerous stages, including the attachment to biotic or abiotic surfaces, maturity, and dissemination of mature biofilm. Polystyrene surface is used for colonization studies of *C. albicans* like adhesion, early biofilm and mature biofilm. As a result, biofilms have the potential to lead to septicemia

and develop into invasive systemic infections of the organs and tissues [6]. With the fast evolution of antimicrobial resistance in pathogenic fungi and bacteria, the multifactorial character of biofilms resulting from mixed fungal-bacterial infections provides significant hurdles for the use of conventional antimicrobials and further restricts therapy options. Based on factors like the host's immune state, the site of the infection, the features of the infection and the drug's pharmacokinetic properties, the best antifungal therapy and drug choice should be made [7].

### 1.3. Types of antifungal drugs

Three types of antifungals drugs mainly used to treat *C. albicans* infection. Azole, Polyene, Echinocandin. Polyenes, like amphotericin B, function as a fungicidal "sterol sponge" by forming additional membranous aggregates that extract ergosterol from lipid bilayers. Azoles, on the other hand, exert fungistatic activity by inhibiting lanosterol 14-α demethylase (encoded by ERG11), resulting in a halt in ergosterol synthesis and the accumulation of toxic sterol intermediates, such as 14-α-methyl-3,6-diol produced by Erg3.

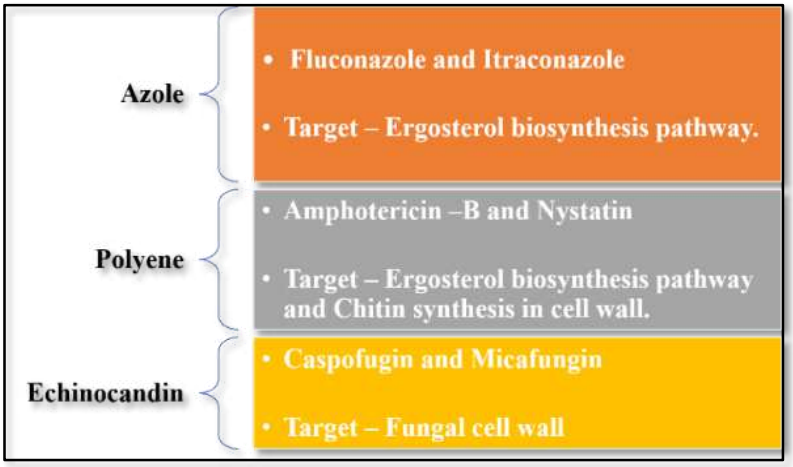


**Fig. 1.1.** Types of antifungal drugs and their targets.

Fungal cell walls consist of (1,3)-β-D-glucan covalently linked to (1,6)-β-D-glucan, in addition to chitin and mannan. Echinocandins prevent the synthesis of (1,3)-β-D-glucan by inhibiting (1,3)-β-D-glucan synthase.

## 1.4. Resistance of available antifungal

*C. albicans* has developed resistance for the frequently used of antifungal agents. Antifungal drug resistance has been linked to a number of adaptive mechanisms, such as drug target overexpression, upregulation of multidrug transporters, and stress response activation [8]. Antifungal resistance is a significant issue because there are so few treatment targets. While multidrug resistance may make it impossible to treat fungal infections, resistance to any one class of medications can considerably reduce the range of available treatments. The widespread use of a limited number of antifungal agents, azoles drugs, has led to the development of drug resistance in the treatment of *C. albicans* infections [9]. The ergosterol biosynthetic pathway is target of azole drug. Ergosterol is sterol component in cell membrane of fungi and it allow accumulation of 14-α-methyl sterol component which alter membrane permeability. Reason of resistance of azole is mutation in gene for target enzyme [10]. Polyenes, such as Amphotericin B (AmB), targets plasma membrane of fungal cells forming channels that allow potassium ions to leak out and disrupts the proton gradient [1]. Additionally, polyenes inhibit chitin synthase, an enzyme involved in cell wall synthesis, which is also membrane-bound. Resistance to polyenes is often associated with significant alterations in the lipid composition of the plasma membrane, reducing AmB's affinity for the membrane, possibly due to the absence of a suitable binding site [11]. Another factor contributing to AmB resistance may be changes in the β-1,3 glucan content of the fungal cell wall, which enhances wall stability and impedes the access of large molecules like AmB to the membrane [12]. Echinocandin resistance primarily occurs through the action of multi-drug transporters. Minor mutations in the FKS subunits of glucan synthase can lead to echinocandin resistance [13]. Drug tolerance, which allows a greater number of cells to survive drug exposure, may serve as an intermediate stage in the development of FKS-mediated resistance, providing a pool of cells that can evolve resistance over time [14].

## 1.5. Repurposing of antimalarial drug

Drug repurposing provides various advantages over the making of new medications, most notably a lower cost and less time consuming. The failure rate associated with safety or toxicity concerns is under 50 %, and the time required for developing new medications can be reduced by 5 to 7 years on average. Thorough and innovative clinical research into pharmacological pathways is essential for drug repurposing, which involves identifying new therapeutic applications for existing medications [15]. One of the merits of drug repurposing is that the mechanisms of action, cellular targets, toxicity profile, or safety of the commercial drugs have already been identified, which enables expedited regulatory approval [16]. The systematic drug repurposing pipeline primarily uses two types of approaches: "experimental testing" approaches, which include microtiter plate-based high-throughput screenings, and "in silico/computational" approaches, which make use of currently available data to identify potential new drugs to treat diseases [17]. For instance, aspirin, originally developed as an analgesic and antipyretic, is now widely used in the prevention and treatment of various conditions, including cardiovascular diseases, stroke, and certain digestive tract cancers [18]. Similarly, metformin, which was initially prescribed for diabetes management, is now also employed in the treatment and prevention of cancer, cardiovascular diseases, and mental health disorders [19].

## 1.6. Rationale for selecting antimalarial drugs

Current antifungal agents have limited efficiency in treating fungal pathogens, particularly against drug resistant *C. albicans* [20]. Drug repositioning is an alternative intervention strategy, whereby various marketed; drugs can be used for novel purpose. The current study is aimed to repurpose the antimalarial drugs against *C. albicans*. The malarial parasites and *Candida albicans* are eukaryotic organisms that depend on some similar metabolic pathways. Antimalarial drugs target specific enzymes which is important for the parasite's survival and these targets can sometimes be shared by *C. albicans*. Some antimalarial drugs inhibit enzymes involved in nucleotide synthesis or mitochondrial function and *C. albicans* shows on similar targets and inhibit its growth. Some antimalarial drugs show antimicrobial activities by inhibiting *C. albicans* growth.

## 1.7. Orientation and Purpose of thesis based on following objectives

1. To study the antifungal efficacy of antimalarial drugs in *C. albicans.*

2. To compare antifungal efficacy of antimalarial drugs with standard antifungal antibiotic in *C. albicans.*

3. To study the mechanism of action of repositioned antimalarial drugs in *C. albicans.*

The antimalarial drugs, Chloroquine, Quinine, Mefloquine, Hydroxychloroquine, Artesunate, Piperaquine, Sulfadoxine, Pyrimethamine, Atovaquone, Artether and Amodiaquine screened for its antifungal activity against *C. albicans*. The antifungal potential of antimalarial drugs were find out by using yeast to hyphal morphogenesis assay, adhesion assay, biofilm assay, cell cycle studies, ergosterol assay, ROS assay, SEM, PI uptake assay and qRT-PCR studies.

## 1.8. References

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# CHAPTER II

## REVIEW OF LITERATURE

## 2.1. Introduction

Aspergillosis, Candidiasis, and Cryptococcosis are fungal infections caused by *Aspergillus*, *Candida*, and *Cryptococcus* species. Some species are resistant to available antifungal drugs [1]. Fungal biofilm-related infections cause over 5,00,000 deaths annually and pose serious clinical problems [2]. The three major classes of antifungal drugs are used for treating systemic fungal diseases: polyenes, azoles, and echinocandins. Emerging resistance to these drugs is a global health concern, necessitating new and more effective antifungal drugs [3]. Recently, *Candida* species have become a major cause of hospital-acquired infections which show highest morbidity and mortality rate. *C. albicans* causes both life-threatening invasive and superficial infections. Candidemia is the most common invasive infection, with at least 8 % of hospital-acquired bloodstream infections caused by *Candida* species. *C. albicans* is found in the human mucosal areas, oral cavity, gastrointestinal tract, and urinary tract. It primarily affects immunosuppressed individuals, such as those with HIV, cancer, or those with catheters [4]. Frequent and prophylactic use of antifungal agents has led to resistance in *C. albicans*. Numerous adaptive mechanisms, such as altered or overexpressed drug targets, heightened activity of multidrug transporters, and cellular stress response pathways, are linked to antifungal drug resistance [5]. The ergosterol biosynthetic pathway is targeted by azole drugs. Ergosterol is a key component of the fungal cell membrane, allowing the accumulation of 14-α-methyl sterols, which changes membrane permeability. Lanosterol 14-α-demethylase (Erg11), a cytochrome P450-dependent enzyme, is specifically targeted by azoles [3]. Azoles bind to the ferric iron moiety of the heme-binding site, blocking the enzyme's natural substrate, lanosterol, and effects on biosynthetic pathway [6]. Amphotericin B (AmB) is effective against *Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans*, *Zygomycetes*, dimorphic fungi, and some dematiaceous fungi. Polyenes act by inhibiting chitin synthase, an enzyme crucial for cell wall synthesis in fungi. When amphotericin B (AmB) interacts with cholesterol in human cell membranes, it can cause toxic side effects. However, resistance to polyenes remains relatively rare. Polyene resistance is due to significant changes in the lipid composition of the plasma membrane, reducing AmB's binding affinity, likely due to a lack of binding sites. Echinocandin

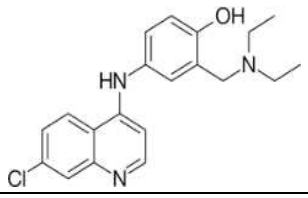
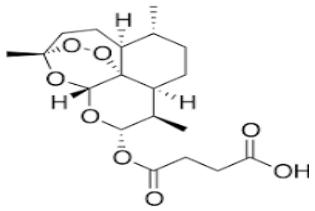
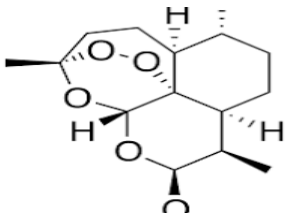
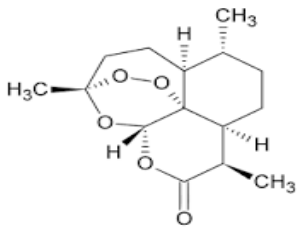
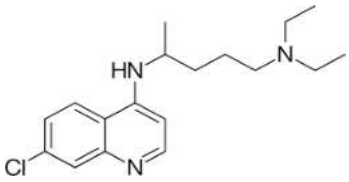
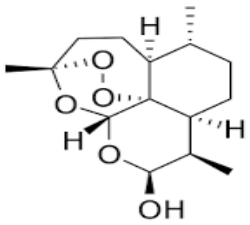
resistance is largely due to multidrug transporters. Resistance is conferred by limited amino acid substitutions in FKS subunits of glucan synthase [7]. In *C. albicans* and most other *Candida spp*., resistance mutations occur in two highly conserved "hot-spot" regions of FKS1, covering residues Phe 641, Pro 649 and Arg 1361 in *C. albicans* [8]. To address this problem, alternative drugs for fungal infections are needed. Finding new clinical uses for FDA-approved medications through new indications or commercial uses is known as "drug repurposing" [9]. Drug repurposing is based on two principles: many drugs have hidden biological activities, and different diseases share common molecular pathways and genetic factors [10]. Repurposing existing drugs is more cost-effective and innovative than developing new drugs from scratch [2]. Many reports are available on drug repurposing. Antimalarial drugs have been found to possess antibacterial, anticancer, and antiviral activities. This current review of literature focused on the repurposing of antimalarial drugs.

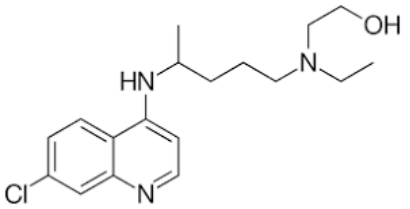
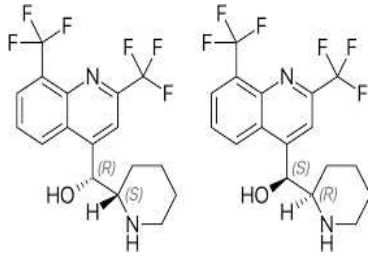
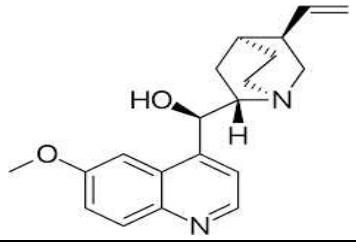
## 2.2. Antimalarial drugs

i) Aminoquinolines are the derivatives of quinolines. Aminoquinolines group divided into two types; 4-aminoquinolines and 8-aminoquinoline. 4-aminoquinoline are schizonticide and gametocytocidal in nature. 8-aminoquinoline enlist chloroquine, amodiaquine also piperaquine and primaquine, all this act against hypnozoites [1].

ii) Arylaminoalcohol one of antimalarial compound group that have bioactivity in inhibit the formation of hemozoin in the cycle of malaria. The presence of amine and hydroxyl functional group is one of the factors that caused antimalarial activity. It includes quinine and mefloquine. iii) Antimalarial artemisinin derivatives are a new class of antimalarial drugs used as initial treatment for severe malaria. These drugs inhibit growth and eventually kill the parasite. It enlists artemisinin, Dihydroartemisini, artemether and artesunate.

**Table 1. Structure of antimalarial drugs**

| Sr. No | Antimalarial drugs | Structure Of Drugs |
|---|---|---|
| 1. | Amodiaquine |  |
| 2. | Artesunate |  |
| 3. | Artemether |  |
| 4. | Artemisinin |  |
| 5. | Chloroquine |  |
| 6. | Dihydroartemisinin |  |

| | | |
|---|---|---|
| 7. | Hydroxychloroquine |  |
| 8. | Mefloquine |  |
| 9. | Quinine |  |

## 2.3. Repurposing of antimalarial drugs as antibacterial, anticancer and antiviral agent.

Repurposing is the process of finding new clinical uses for approved drugs, either through new indications or commercial opportunities [11]. Drug repurposing is based on two principles. First, many drugs have hidden biological activities and side effects. Second, different diseases share common molecular pathways and genetic factors [10]. Repurposing of known drugs reduces effort, time, and cost, making it easier to develop new treatments [2]. In this section antimicrobial activity of antimalarial drugs are mentioned.

### 2.3.1. Antimalarial agent act as antibacterial agent

#### 2.3.1.1. Chloroquine as an antibacterial agent

Chloroquine is the derivative of 8-aminoquinoline. It was first synthesized by German scientists Resochin in 1934. This drug is primarily used to treat malaria caused by *Plasmodium falciparum* and is orally or parenterally administrated at a dose of 500 mg or 10 mg/kg.

Chloroquine is also widely used to treat multiple diseases. Chloroquine in combination with artemisinin used to develop masks with antibacterial functions. These masks show inhibition of *Staphylococcus aureus* and *Escherichia coli* [12]. Chloroquine phosphate has been found to diminish the efficacy of ampicillin trihydrate against Staphylococcus aureus NCTC 6571. The study employed various physicochemical techniques, including thin-layer chromatography and ultraviolet spectrophotometry, alongside microbiological methods such as agar diffusion and kill kinetics assays to evaluate this interaction. While no chemical interaction was detected between ampicillin trihydrate and chloroquine phosphate in vitro, a pharmacodynamic antagonism was observed, leading to reduced antibacterial activity of ampicillin in the presence of chloroquine. Further investigation is required to clarify this interaction. Clinicians should be cautious when co-administering these drugs to treat malaria and bacterial infections, as the combination may compromise ampicillin's therapeutic effectiveness [13].

### 2.3.1.2. Amodiaquine as an antibacterial agent

Amodiaquine has also been reported to have antibacterial activity. It inhibits growth *Bacillus anthracis* by acting as a host-oriented inhibitor of anthrax toxin endocytosis. In a study with NZW rabbits, amodiaquine was tested for efficacy against aerosolized *B. anthracis*. The $LD_{50}$ value of aerosol spores was measured with and without amodiaquine. Animals' respiration was monitored to ensure aerosol deposition of $2 \times 10^7$ spores. After the challenge, animals received 0, 5, or 20 mg/kg of amodiaquine. A dose of 5 mg/kg, given twice daily for 5 days, was effective. *In vivo*, amodiaquine enhances the effectiveness of levofloxacin, reducing bacteremia in plasma and lungs and preventing bacteremia. The study shows that amodiaquine protects hosts from anthrax through both antitoxin and antibacterial mechanisms [14].

### 2.3.1.3. Hydroxychloroquine as an antibacterial agent

The hydroxychloroquine in combination with solid lipid nanoparticles (SLN) to deliver streptomycin (STR) and hydroxychloroquine for treating intracellular *Brucella abortus* infection has shown promising results. The methodology involved using SLNs for the targeted delivery of streptomycin and hydroxychloroquine, with

the double emulsion technique employed for nanoparticle synthesis. The study assessed the physicochemical properties, *in vitro* antibacterial activity, and compared the healing effects in Wistar rats. STR/ hydroxychloroquine -SLN formulation demonstrated better inhibitory effects against chronic *B. abortus* infection compared to STR-SLN. Nano drug carriers can effectively inhibit intracellular *B. abortus* infection, indicating their potential in improving treatment outcomes. Nanoplatforms, particularly STR/ hydroxychloroquine -SLN, show promise in enhancing the efficacy of conventional anti-brucellosis drugs. Nanoplatforms have the potential to enhance the efficacy of conventional anti-brucellosis drugs, showing promising and safe results [15].

### 2.3.1.4. Quinine as an antibacterial agent

Quinine has antibacterial activity when combined with erythromycin. Erythromycin is produced by *Streptococcus erythreus*, with the chemical formula $C_{37}H_{67}O_{13}$. It has a prokinetic effect and reverses gastrostatic action. Erythromycin contains a 14-membered lactone ring with ten asymmetric centres and two sugars, L-cladinose and D-desosamine. The inhibition zone produced by erythromycin alone ranges from 13 ± 1.0 mm to 31 ± 1.0 mm from 100 µl of 1000 µg/ml. The combination of erythromycin and quinine shows concentration-dependent inhibition. High concentrations of the drug combination inhibited tested organisms like *S. aureus*, *E. coli*, *S. marcescens*, *M. luteus, S. flexneri*, *K. pneumoniae*, *P. aeruginosa*, and *E. faecalis*, which are resistant to erythromycin alone and its combination with 17.5 and 8.75 µg/ml quinine. The MIC of erythromycin against all isolates ranged between 25 µg/ml and 50 µg/ml, while the MBC ranged between 50 µg/ml and 100 µg/ml. Quinine alone does not show antibacterial activity. However, when combined with erythromycin, the MIC of erythromycin ranged between 12.5 µg/ml and 50 µg/ml. This indicates that the combination of erythromycin and quinine has synergistic and antagonistic interactions at 200 µg/ml [16].

### 2.3.1.5. Mefloquine as an antibacterial agent

Mefloquine has been reported as an antibacterial agent against Tuberculosis (TB). TB, caused by *Mycobacterium tuberculosis*, is a serious global health problem, causing thousands of deaths and primarily affecting the lungs. It is transmitted

through the air. Mefloquine shows MIC values of 9 to 392 μM in treatment. It inhibits DNA gyrase, an enzyme involved in DNA metabolism, which is absent in eukaryotic cells and is a potential target. Mefloquine also inhibits ATP synthase, essential for generating ATP, leading to bacterial death [17]. Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) is a significant concern due to multidrug resistance, necessitating new treatments. AS101 and mefloquine are potential synergistic antibacterial agents against CRPA. The methodology included collecting CRPA strains, determining sequence types, conducting antimicrobial susceptibility testing, determining MIC, performing antibacterial activity assays, and assessing the mechanism of AS101 and mefloquine against CRPA. The combination of AS101 and mefloquine showed a substantial synergistic antibacterial effect against CRPA *in vitro*. It effectively suppressed biofilm formation, disrupted the bacterial cell wall, enhanced cell membrane permeability, hindered bacterial growth, and significantly reduced bacterial load in a mice model of CRPA-3 peritoneal infection. The global spread and high mortality of CRPA highlight the need for new treatments. The combination of AS101 and mefloquine shows promise as a new treatment strategy with synergistic effects against multidrug-resistant *P. aeruginosa* strains [18].

#### 2.3.1.6. Artemisinin derivates act as an antibacterial agent

In 2022, Seid Mohammed et al., reported the extraction of 7 mg (0.004%) of artemisinin from *Artemisia annua* leaves using a rapid fractionation method involving n-hexane [19]. Various extracts from different *Artemisia* species were tested for their antibacterial properties, revealing differing levels of effectiveness. Specifically, the n-hexane extract of *A. abyssinica*, the ethyl acetate extract of *A. absinthium*, and the n-hexane extract of *A. annua* were evaluated against bacterial strains including *Staphylococcus aureus* ATCC 25923, *Salmonella enteritidis* ATCC 13076, *Klebsiella pneumoniae* ATCC 1053, *Enterobacter cloacae* ATCC 1233, *Escherichia coli* ATCC 25922, and hospital-acquired *Acinetobacter baumannii*. The antibacterial activity was observed for ethyl acetate extract of *A. absinthium*, which produces 35 mm of inhibition zone against *A. baumannii*. In contrast, the n-hexane extract of *A. annua* exhibited minimal activity, with an inhibition zone of less than 3 mm against *K. pneumoniae* ATCC 1053. Overall, the

ethyl acetate extract of *A. absinthium* was found to be the most potent, with inhibition zones ranging from 5 to 35 mm [20]. The antibacterial effectiveness of dihydroartemisinin (DHA) combined with cefuroxime (CFX) or ampicillin against *Escherichia coli* was also investigated. Using the broth microdilution and 2,3,5-triphenyltetrazolium chloride (TTC) methods, the minimum inhibitory concentration of DHA, cefuroxime, ampicillin was found to be 300 µmol/L, 25 µmol/L, and 25 µmol/L, respectively. The MBC was 25 µmol/L for cefuroxime and above 600 µmol/L for DHA. The checkerboard microdilution assay revealed a FICI of 0.375 for DHA combined with cefuroxime and 0.75 for DHA combined with ampicillin, indicating a synergistic or additive effect of the drug combinations [19].

### 2.3.2. Antimalarial agent act as anticancer agent

#### 2.3.2.1. Chloroquine as an anticancer agent

The antimalarial drug Chloroquine (CQ) has broad bioactivities. Chloroquine shows effect on HepG2 cells, CQ inhibited cell viability. CQ treatment decreased level of ATP concentration in HepG2 cells. qRT-PCR result showed that CQ at 1.5 mg/kg downregulated the expression of K19 and sox9 gene [20].

#### 2.3.2.2. Hydroxychloroquine as an anticancer agent

Hydroxychloroquine is commonly prescribed for treating uncomplicated malaria, rheumatoid arthritis, chronic discoid lupus erythematosus, and systemic lupus erythematosus. Additionally, hydroxychloroquine is used for malaria prophylaxis in areas where chloroquine resistance is unlikely. Hydroxychloroquine has the ability to induce apoptosis by activation of cellular stress response pathways, increased activity of multidrug transporters, and changes or overexpression of drug targets are some of the adaptive mechanisms linked to antifungal drug resistance. PPAR is most important receptor member of the steroid hormone which consists of three major subtypes PPARα, PPARβ, PPARγ. Autophagy is the mechanism in which organelles or macromolecular proteins are wrapped into autophagosomes; small molecules are generated in the lysosomes from the decomposed organelles for recycling by the eukaryotic cells. One essential aspect of autophagy is autophagic flux. To maintain cellular homeostasis, autophagy can control differentiation, growth, and proliferation. The role of autophagy in tumours cell

growth and death is unclear, some researchers believe it has protective role by enhancing apoptosis or inducing autophagic cell death. Mao et al., (2021), reported that, the 786-O renal cell line treated with a combination of GW6471 and hydroxychloroquine showed reduced viability compared to those treated with GW 6471 or hydroxychloroquine alone. Additionally, GW6471 induced integral autophagy in 786-O renal cell line and increased the ratio of apoptotic cells in 786-O renal cell line that received this cotreatment. A somewhat alkaline medication called hydroxychloroquine can mildly raise the pH of the lysosome, prevent autophagosome-lysosome fusion, and create autophagy independent Golgi-endo lysosomal system disarray that could hamper fusion [4].

### 2.3.2.3. Amodiaquine as an anticancer agent

To investigate the therapeutic potential of amodiaquine analogues, new compounds were synthesized through nucleophilic substitution at the 4-amino position and characterized using NMR and FTIR techniques. Antibacterial and cytotoxic evaluations showed that the high efficacy of these compounds. Analogue AS1 exhibited a 34.3 ± 0.18 mm zone of inhibition against *Pseudomonas aeruginosa*, while analogue AS2 showed strong antifungal activity against *C. albicans* with a 39.6 ± 0.23 mm zone of inhibition. Additionally, analogue AS1 displayed a significant cytotoxic effect on the HeLa cell line with an IC50 of 4.2 µg/ml. In docking studies, AS1 showed binding energies of -8.32688 kcal/mol with 5GWK, -6.4780 kcal/mol with 1PFK, and -6.5279 kcal/mol. These findings indicate that these analogues possess potent antimicrobial and cytotoxic properties [5]. The global burden of cancer and the need for new, low-cost, and effective cancer treatments, and introduces aminoquinoline compounds as a potential strategic class of molecules for developing new cancer therapies. Aminoquinolines have similar mechanisms of action in different tumor types, although their pharmacological potencies may vary. Aminoquinolines can be used as adjuvant chemotherapy and radiotherapy sensitizers against solid tumors, acting as lysosomal alkalinizers. Continuous pharmacovigilance is necessary for aminoquinolines due to their multiple unspecific modes of action, narrow therapeutic windows, adverse effects, and potential for self-treatment poisoning [21].

### 2.3.2.4. Quinine as an anticancer agent

Quinine is reported to have anticancer activity. In HeLa and HepG2 cell lines, quinine alkaloids have significantly increased the amount of DOX-induced cell death. Cell viability has been analyzed using the MTT assay. Western blotting analysis has been used to explore the ability of the alkaloids to enhance doxorubicin-induced apoptosis. Flow cytometry has been used to analyze cell fractions in different cell cycle phases. The quinoline alkaloids cinchonine, cinchonidine, quinine, and quinidine have been able to enhance the cell-killing effects of the chemotherapy drug doxorubicin in cervical and liver cancer cell lines. This enhancement has been due to the induction of apoptosis, as indicated by the cleavage of caspase-3 and PARP. The alkaloids have also increased the proportion of cells in the sub-G0/G1 phase of the cell cycle, which is indicative of apoptosis. The quinoline alkaloids cinchonine, cinchonidine, quinine, and quinidine have been found to enhance doxorubicin-induced apoptotic cell death. [6].

### 2.3.2.5. Mefloquine as an anticancer agent

The effect of mefloquine has been tested on glioblastoma in mice. Glioblastoma, a type of brain cancer, is characterized by angiogenesis, making it an appealing target for treatment, and there is increasing interest in repurposing the anti-malarial drug mefloquine for treating glioblastoma due to its known anti-cancer properties. Isolated glioblastoma microvascular endothelial cells (GMECs) from glioblastoma patients. Mefloquine effects of GMECS differentiation, capillary network formation, adhesion, growth, and survival. Examined the effects of mefloquine on glioblastoma growth and angiogenesis in mice. The mechanism of mefloquine's effects, showing it disrupted lysosomal integrity and function in GMECs. Mefloquine inhibits glioblastoma angiogenesis by disrupting the function and integrity of glioblastoma microvascular endothelial cells. Mefloquine also causes glioblastoma cells to undergo apoptosis and stops their growth. The mechanism of action involves mefloquine disrupting the lysosomal function in glioblastoma microvascular endothelial cells. Mefloquine is a dual inhibitor oglioblastoma angiogenesis and disrupting lysosomal function [22]. The NF-κB signaling pathway is activated in colorectal cancer and plays a critical role in cancer progression, and that the study found the antimalarial drug mefloquine inhibits the NF-κB pathway

in colorectal cancer cells. The antimalarial drug mefloquine inhibits the NF-κB signaling pathway, leading to apoptosis in colorectal cancer cells, and acts synergistically with the chemotherapeutic drug doxorubicin, suggesting mefloquine could potentially be repurposed as a chemotherapeutic agent for the treatment of colorectal cancer. the methodology includes cell culture, plasmid/siRNA transfections, NF-κB reporter assays, immunoblotting, qRT-PCR, *in vitro* kinase assays, *in-vivo* xenograft studies, and confocal microscopy. Mefloquine, an antimalarial drug, inhibits the NF-κB signaling pathway by blocking IKK activation, leading to apoptosis in colorectal cancer cells. Mefloquine can be used as a single agent or in combination with other anticancer drugs, such as doxorubicin, to enhance the cytotoxic effect on colorectal cancer cells. Expressing a constitutively active IKK can attenuate the inhibitory effects of mefloquine on the NF-κB pathway and apoptosis, indicating that the inhibition of IKK activation is a critical mechanism of action for mefloquine [7].

### 2.3.2.6. Artemisinin derivatives as an anticancer agent

Zhang et al., (2020) reported that, artemisinin has anticancer activity; the potential of artemisinin-derived dimers as anticancer agents, focusing on how the linker between the two artemisinin moieties shows the anticancer activity and providing insights into the structure-activity relationships and mechanisms of action to guide the rational design of more effective artemisinin-derived dimers for cancer treatment [8]. The primary components of chemotherapy for malaria are artemisinin and its semisynthetic derivatives. Additionally, molecules generated from artemisinin, particularly dimers, have strong anticancer action both *in vitro* and *in vivo*. According to the structure-activity relationship (SAR), the linker between the two artemisinin moieties greatly affected the anticancer activity; as a result, the linker's well-thought-out design may offer a useful therapeutic intervention for the treatment of cancer. The linker between the two artemisinin moieties affects the anticancer action, according to the structure-activity relationship (SAR). Rational design of the linker may provide valuable therapeutic intervention for cancer treatment. The artemisinin act as an anti-tumor agent, and the use of nanostructured drug delivery systems to improve its therapeutic efficacy and reduce toxicity. Artemisinin has shown anticancer properties by inducing cell cycle arrest,

promoting ferroptosis and autophagy, and inhibiting cell metastasis. However, artemisinin has also been reported to cause neurotoxicity and cardiotoxicity in animal models [8].

### 2.3.3. Antimalarial agent act as antiviral agent

#### 2.3.3.1. Amodiaquine as an antiviral agent

Amodiaquine has demonstrated antiviral properties against the dengue virus, by inhibiting NS2B-NS3 protease activity and suppresses replication and infectivity. Studies using BHK-21 and Vero/WNV replicon cells revealed that amodiaquine achieved 76.31 ± 1.60% and 96.30 ± 0.39% inhibition of DENV2 and WNV replicon replication, respectively. The reported EC50 and CC50 values in BHK-21/DENV2, Vero/DENV4, and Vero/WNV replicon cells indicated a therapeutic index (TI) of 7.03 and an EC50 of 7.41 ± 1.09 µM. Boonyasuppayakorn et al. (2014) observed that while amodiaquine exhibited cytotoxicity to Vero cells, it did not interfere with Rluc enzyme activity in BHK-21/DENV2 replicon cell lysates. At a concentration of 5 µM, amodiaquine reduced both intracellular and extracellular DENV2 RNA levels and virus infectivity in BHK-21 cells, as determined through qRT-PCR and plaque assays [10]. African countries have seen a slower spread of the virus compared to other regions, and outlines several hypotheses that may explain this, including the potential role of antimalarial drugs. *In vitro* antiviral activity of several antimalarial drugs against SARS-CoV-2 and suggests that certain antimalarial drug combinations may be associated with fewer COVID-19 cases and deaths in countries that commonly use them, but cautions that further *in vivo* evaluation is needed to confirm the clinical efficacy of these antimalarial drugs against COVID-19 [23].

#### 2.3.3.2. Hydroxychloroquine as an antiviral agent

Hydroxychloroquine has been explored as an antiviral agent, particularly against COVID-19, which is caused by enveloped viruses belonging to the Nidovirales order. These viruses are classified into four genera based on their phylogeny and possess virions approximately 120–160 nm in diameter. Their genome is a linear, positive-sense, single-stranded RNA of about 26- 32 kilobases, encoding 16 non-structural proteins (nsp1 to nsp16) and four or five structural proteins, including

spike (S), envelope (E), membrane (M), nucleocapsid (N), and, in the case of HCoV-OC43 and HCoV-HKU1, hemagglutinin (HE). Hydroxychloroquine administered orally at a dose of 400 mg, hydroxychloroquine reaches a maximum blood concentration (Cmax) of 1.22 µM and accumulates in the lungs at concentrations 30 times higher than in the blood. In vitro studies on Vero E6 cells infected with the virus demonstrated that hydroxychloroquine exhibited an EC50 value ranging from 1.5 µM to 17.3 µM, while in Vero cells, the range was 0.7 to 6.3 µM concentrations that were also observed in human plasma and lungs. Additionally, hydroxychloroquine has been shown to reduce plasma levels of HIV-1 RNA and interleukin-6 (IL-6) in HIV patients, thereby lowering the plasma HIV-1 RNA levels [11].

#### 2.3.3.3. Quinine as an antiviral agent

Quinine inhibits the production of progeny virions completely blocked in COVID-19. The quinine inhibits the SARS-CoV-2 PRI in Vero B4 cells. Vero B4 cells were infected with SARS-CoV-2 PRI and treated with quinine. After 3 days post infection cell supernatants were harvested and virus production was analyzed at 10µM. The checking on Vero B4 cells after that human cell system was checked for the quinine activity. Calu-3 lung cells, expressing ACE2 and TMPRSS2 endogenously. In Calu-3, infections with SARS-CoV-2 were completely blocked by quinine [12].

#### 2.3.3.4. Mefloquine as an antiviral agent

Mefloquine has demonstrated antiviral activity against several viruses, including Zika virus, SARS-CoV-2, and feline calicivirus. Mefloquine has shown the ability to reduce the infection rate of the Zika virus. In studies involving Vero E6 cells, the EC50 values of mefloquine against SARS-CoV-2 were 1.8 µM but it exhibited a low selectivity index (SI) of 2.3 which indicating a narrow therapeutic window. Mefloquine concentration of 10 µM, completely inhibited the cytopathic effects of SARS-CoV-2 in Vero E6 cells. Additionally, mefloquine displayed antiviral activity against feline calicivirus, a virus responsible for upper respiratory tract symptoms in cats. At a concentration of 10 µM, mefloquine inhibited 88.6 % of the virus and reduced viral progeny in a concentration-dependent manner [24].

## Table no. 2.2. Antifungal activity of antimalarial drugs

| Drug name | Fungal species | Mode of action | Ref. |
|---|---|---|---|
| Chloroquine | *Cryptococcus neoformans* | Inhibit the growth. | [26] |
| | *Aspergillus fumigatus* | Invasive fungal infection at ≥ 0.25 mM concentration. | [28] |
| | *C. albicans* | Inhibited planktonic growth and virulence factor. | [29] |
| Hydroxychloroquine | *Pneumocystis jirovecii* | Cumulative dose of ≥ 14 g reduce *Pneumocystis pneumonia.* | [30] |
| Quinine | *Sclerotinia sclerotiorum* | Increase in cell membrane permeability, and inhibition of fungal spore germination. | [31] |
| | *C. albicans* | Inactivation of planktonic *C. albicans.* | [33] |
| Mefloquine | *C.albicans* | Inhibit the lysosomal/vacuole formation and disruptor of the mitochondrial proton motive force. | [34] |
| Artemisinin | *Sclerotinia sclerotiorum* | Inhibit the conidial germination and germ tube elongation | [35] |
| Artemether | *C. albicans* | Inhibited the growth | [36] |
| Artesunate | Basidiobolomycosis | Inhibited the growth | [39] |
| Amodiaquines | *C. albicans* | Zone of inhibition | [40] |

### 2.3.4. Antimalarial drugs act as antifungal drug

#### 2.3.4.1. Chloroquine as antifungal agent

Inhibition of opportunistic fungal pathogens such as *Cryptococcus neoformans* and *Histoplasma capsulatum, C. albicans* reported to has antifungal activity. The mechanisms of inhibition by chloroquine may differ between fungal species. The growth of *C. neoformans* was inhibited by Chloroquine by changes in phagolysosome pH that produced an environment that was too alkaline for this pathogen to growth [26]. Chloroquine kills the yeast cells by limiting the iron availability [27]. Chloroquine also able to inhibit the growth of *Aspergillus fumigatus* at ≥ 0.25 mM concentration.

In presence of chloroquine decreases of TNF n and IL-1β release by healthy and CGD PBMCs in *A. fumigatus* and *A. nidulans*. Chloroquine as lysosomotropism and anisotropic agent its effect in the presence of healthy and CGD PMNs. The addition of 100 μM Chloroquine increased the antifungal activity of healthy PMNs against *A. fumigatus* by 75 % [28]. Chloroquine also inhibited planktonic growth of *C. albicans* a dose dependent manner. Chloroquine inhibited virulence factor morphogenesis at very low concentration in *C. albicans.* Chloroquine also found to disrupt ergosterol production and alter the membrane permeability and typical membrane activities. Cells were cultured with varied doses of chloroquine in order to perform ergosterol profiling in order to examine this potential. Ergosterol levels in cells dropped in *C. albicans* with concentration dependent manner [29].

#### 2.3.4.2. Amodiaquine as an antifungal agent

Amodiaquine is one of the a 4-aminoquinoline type compounds. Amodiaquine is used for the treatment of uncomplicated malaria caused by *P. falciparum.* It is commonly sold under the trade name Pamaquine® or Coarsucam. This drug acts on the parasite's food vacuole and forming a complex with heme, which prevents crystallization in food vacuole. Amodiaquine mixed with Paraminobenzoic acid form complexes in ratio 1:1:1 in using different metal ions. The prepared complexes and their ligands have been screened *in vitro* for their antifungal activity against *C. albicans using* broth dilution techniques. The solution of the synthesized complexes has been prepared in dimethyl sulfoxide at different concentrations 10, 20, 30, 40

µg/ml. The solutions of the parent free ligands (Amodiaquine and Paraminobenzoic acid) were also prepared in DMSO. Alone amodiaquine showed 3.07±0.35 zone of inhibition also PABA alone showed 6.21±0.52 zone of inhibition. When amodiaquine and PABA coordinate with metal complex and showed 20.39±0.51 to 27.57±0.84 zone of inhibition against *C. albicans* growth [30].

### 2.3.4.3. Hydroxychloroquine as an antifungal agent

Hydroxychloroquine is immunomodulatory agent used for the treatment of *Pneumocystis pneumonia*, caused by *Pneumocystis jirovecii* which is life-threatening infection and is increasingly diagnosed in the immunocompromised patient and autoimmune diseases. In this study three months with cumulative dose of hydroxychloroquine ≥ 14 g is given to reduce the risk of *Pneumocystis pneumonia* [31].

### 2.3.4.4. Quinine as an antifungal agent

In 17$^{th}$ century the active ingredient 'quinine' is derived from the bark of the cinchona tree and used for treating fevers. Quinine is used as treatment for uncomplicated and severe malaria in many different therapeutic regimens. Quinine has less affinity for heme and weaker base than chloroquine. For the action of these drugs requires ion transport into the food vacuole and heme-drug interactions. Quinine acts similar way of chloroquine but it has some differences like chloroquine causes clumping of the malaria pigment, while quinine antagonizes the process. It also inhibits heme polymerization.

It is plant pathogenic fungi and one of the most damaging plant parasitic organisms and can cause serious diseases and adverse yield losses in crops. Fungi can produce various toxins that can seriously compromise food safety. Quinine affects the permeability of the cell membrane of *S. sclerotiorum*. It increases the cell membrane permeability, and inhibition of fungal spore germination and resulting in cell death [32]. Quinine has been shown to inhibit serotonin synthesis in both yeast and human cells. Serotonin synthesis begins with the amino acid tryptophan and involves two key enzymatic reactions. The rate-limiting step is mediated by tryptophan hydroxylase (TPH), which converts tryptophan into 5-hydroxytryptophan. In the central nervous system, this step is performed by tryptophan hydroxylase-2 (TPH2), while in peripheral tissues, it is carried out by

the isoform tryptophan hydroxylase-1 (TPH1). The second reaction, converting 5-hydroxytryptophan to serotonin, is catalyzed by aromatic amino acid decarboxylase. Serotonin production is influenced by tryptophan availability, which is regulated partly by its transport across the blood-brain barrier. Islahudin et al. (2014) reported that quinine can inhibit both serotonin production and its function in yeast and mammalian cells [33].

Quinine and tryptophan compete for cellular uptake in yeast, where the tryptophan derivative serotonin can promote yeast growth. Growth yields of *C. albicans* and *S. cerevisiae* were significantly enhanced in the presence of 1 mM serotonin, increasing by 1.7-fold and 1.4-fold, respectively. The effect of quinine on serotonin production was examined in the rat raphe RN46A cell line, which exhibits TPH2 activity and naturally synthesizes serotonin. As a positive control, the TPH2 inhibitor p-chlorophenyl alanine (pCPA) significantly reduced serotonin levels at concentrations above 10 mM. When RN46A cells were treated with 2 mM quinine, serotonin levels decreased to 65% of those in control cells, with some inhibition of cell growth observed at this concentration [33].

The combination of antimicrobial light (108 J/cm) with quinine (1 mg/mL) inactivation of planktonic *C. albicans* (strain CEC 749) cells and also effective at inactivating 48 h biofilms. Transmission electron microscopy study showed that quinine + antimicrobial light damages the cells of *C. albicans* [34].

**2.3.4.5. Mefloquine as an antifungal agent**

Mefloquine has shown antifungal activity against *C. albicans*. It can inhibit hyphae formation in *C. albicans*, a key virulence factor of this organism, in a dose-dependent manner. Mode of action studies revealed that mefloquine can inhibit the lysosomal/vacuole formation and disrupts the mitochondrial proton motive force [35].

**2.3.4.6. Artemisinin derivatives as an antifungal agent**

In China, sweet wormwood, or Artemisia annua, often known as "qinghao," has been used for almost 2,000 years to treat fevers. Chinese scientists identified the active component of this plant in 1970. Since most quinoline-containing medications and all antifolate medications cause high levels of resistance, artemisinin and its derivatives artesunate, artemether, and artether—have been used widely in China and Southeast Asia [36]. The natural extract of artemisinin or its

semi-synthetic derivatives (Dihydroartemisinin, artesunate, and artemether) are used now a days [36]. Artemisinin used against *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Phytophthora infestans* and *Verticillium dahliae*. Minimum fungicidal concentrations of the volatile phase of the essential oil for *S. sclerotiorum*, *B. cinerea*, *P. infestans* and *V. dahliae* were 1.6, 2.4, 2.4 and 4.4 µg/ml air, respectively. 2.4 and 51.2 µg/ml concentrations, were found to completely inhibit the conidial germination and germ tube elongation of both fungal pathogens tested [37]. *Cryptococcus neoformans* is the cause of the most common life-threatening opportunistic fungal infections in patients with HIV/AIDS. Cryptococcosis caused by *C. neoformans* involves infection of the central nervous system, which is often manifested as meningitis, and is now seen most often in patients with AIDS, of whom 2-20 % develop this development. Observed inhibit growth of *Cryptococcus neoformans* at 2 mg/L polymorphonuclear leukocytes kill *C. neoformans*, at least in part via generation of fungicidal oxidants [38]. Artemisinin was found to have no impact on the growth of *C. albicans* but exhibited significant inhibition of its hyphal development, including clinical isolates resistant to azole drugs. This inhibition resulted in reduced damage to oral epithelial cells, with artemether demonstrating the strongest activity among the artemisinin tested. Transcriptome analysis indicated that artemether could disrupt the energy metabolism of *C. albicans*. Further investigation revealed that seven artemisinin significantly inhibited the production of ATP and cAMP, while also reducing hyphal inhibition in a strain overexpressing *RAS1*. This suggests that artemisinin regulate the Ras1-cAMP-Efg1 pathway to inhibit hyphal development in *C. albicans*. Importantly, artemether significantly reduced fungal burden and infections in murine models of oropharyngeal candidiasis caused by both fluconazole-sensitive and resistant strains, without causing systemic toxicity [39]. One of the most serious fungi infections that can kill an HIV/AIDS patient is candidiasis, which is brought on by *C. albicans*. With a MIC 50 of 2.52 mg/l artemisinin partially suppressed the formation of *C. albicans* hyphal colonies in both YEPD containing 10% serum medium and Spider medium, which are both adequate for inducing it. Inhibition of hyphal development is observed when artemisinin and fluconazole are combined (40). With its 86.3 % and 91.6 %, artemether slowed the growth of clinical isolates of *C. albicans* that were resistant to FLC. Artemether is able to restore the sensitivity of these clinical isolates, as demonstrated by the larger inhibitory impact

that the combination of artemether and FLC produced when compared to either FLC or artemether alone. Comparing the pdr5 mutant to WT and other mutants, FLC inhibited 82.4% of growth, while less than 10% did so in the former case. Intriguingly, treatment with artemether also caused a more severe growth suppression in the pdr5 mutant, as cells lacking pdr5 showed a growth inhibition of 70.2 %, as opposed to the WT and other ABC transporter mutants, which only showed a growth inhibition of 20 -40 % [40]. Basidiobolomycosis, which is caused by pathogenic Basidiobolus species, is a rare fungal infection affecting the skin and gastrointestinal tract, which is mainly reported from tropical and subtropical regions. Diagnosis of Gastrointestinal bleeding (GIB) based on clinical suspicion is challenging and requires histopathological and mycological confirmation. Similarly, treatment is difficult given the nature of the disease. Artesunate sensitive to *Basidiobolus ranarum*, and showing 22mm.

**2.4. Conclusion** In this review of literature, repurposing work of antimalarial drugs has been summarized. The enlisted antimalarial drugs are reported as antibacterial, antiviral, anticancer and antifungal activity. Drug repurposing is one of the promising strategies for the fungal infections. This strategy is less time consuming and cost effective. The use of antimalarial drugs as anti-*Candida albicans* agent can be one of the future strategies to treat fungal infections.

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# CHAPTER III

# MATERIALS AND METHODS

### 3.1.Cultures, media and chemicals

*Candida albicans* ATCC 90028 was taken from the Institute of Microbial Technology in Chandigarh, India. The culture was stored on YPD agar slants at 4°C. A single colony was transferred from the YPD agar plates to a 250 ml flask containing 50 ml of YPD broth, then incubated overnight at 30°C with shaking at 120 rpm. Cells from the activated culture were collected by centrifugation at 2000 g for 5 minutes, washed three times, and resuspended in PBS and RPMI-1640 medium (containing L-glutamine, without sodium bicarbonate), adjusted to pH 7 with 165 mM MOPS (3-[N-morpholine] propane sulfonic acid) and filter-sterilized with 0.22 µm filters. Antimalarial drug solutions were prepared in RPMI-1640 medium by serial double dilution. Fluconazole (Forcan, Cipla Pvt. Ltd., Mumbai, India) served as the standard antifungal. XTT and menadione were sourced from Sigma Aldrich Chem. Ltd., Mumbai, India.

### 3.2. Antimalarial drugs

Chloroquine, Quinine, Mefloquine, Artesunate, Piperaquine, Sulfadoxine, Pyrimethamine, Atovaquone, Artether and Amodiaquine were purchased form TCI chemicals Pvt. Ltd., India. Hydroxychloroquine 500 mg tablet was purchased from local medical shop.

### 3.3. Planktonic assay

To assess the growth kinetics of *Candida albicans* following antimalarial drug treatment, a planktonic growth assay was conducted according to CLSI guidelines. Eleven different antimalarial drugs in concentrations ranging from 0.031 to 4 mg/ml were prepared in RPMI-1640 medium and distributed into 96-well plates (Hi Media, Mumbai, Maharashtra, India). Wells containing only *C. albicans* cells were included as controls, with fluconazole (FLC) serving as the standard antifungal agent. Into each well, 100 µl of *C. albicans* suspension at a density of $1 \times 10^3$ cells/ml, along with RPMI-1640 medium and graded concentrations of the drugs (0.031 to 4 mg/ml), were added. The plates were incubated at 35°C for 48 hours, after which absorbance was measured at 620 nm using a microplate reader (Multiskan EX, Thermo Electron Corp., USA) to evaluate growth. The minimum inhibitory concentration (MIC) was determined as the lowest drug concentration

that achieved a 50% reduction in absorbance relative to the control, indicating inhibition of *C. albicans* planktonic growth [1].

### 3.4. Determination of Minimum fungicidal concertation (MFC)

The minimum inhibitory concentration (MIC) represents the lowest concentration of an antifungal drug needed to inhibit the growth of fungal cells, while the minimum fungicidal concentration (MFC) identifies the minimum concentration required to kill the cells. Antimalarial drugs with MIC values in the range of 0.031 to 4 mg/ml were chosen for MFC assessment. To establish the MFC, cells from the MIC well and those at higher concentrations were tested. A 10 µl sample from each selected well was spread onto YPD agar plates, which were incubated at 30 °C for 48 hours. After incubation, the plates were checked for the presence of *C. albicans* colonies. The absence of colonies indicated a fungicidal effect, and the lowest concentration at which no colonies appeared was recorded as the MFC [2].

### 3.5. Kill time assay

Kill time assays provide quantitative data on how quickly a drug kill *C. albicans* cells. It is essential for determining the efficacy of the of an effective antimalarial drug. MFC was considered the lowest concentration killing 99 % of cells. $10^3$ cells/ml were treated with MFC concentration of antimalarial drugs. 0.5 ml cell suspension was taken at various time intervals 0 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 10 h, 12 h, 18 h, 24 h in 10 ml RPMI broth and inoculated on YPD agar plate. The plates were incubated at 30 °C for 48 h. The number of colonies grown on the plate was counted and reported [3].

### 3.6. Yeast to hyphal formation

Serum induced yeast to hyphal formation in *C. albicans* was studied on a 96 well non- treated micro plate assay. Various concentrations of antimalarial drugs were prepared in 20 % bovine serum and deionized distilled water. A 100 µl of $1 \times 10^6$ *C. albicans* cells and 100 µl of various drug concentrations in each well were added. The plates were incubated at 37 °C with shaking at 200 rpm on an orbital shaker for 90 minutes. Following incubation, the plates were examined under a microscope to observe the formation of hyphae. The concentration that resulted in a 50% reduction

in hyphae formation compared to the control was recorded as the MIC for morphogenesis inhibition [4].

### 3.7. Adhesion assay

Adhesion is the first step in the establishment of *Candida* infections, allowing the fungus to colonize and invade host tissues. To study the mechanisms underlying fungal adhesion and identify factors that promote or inhibit the adhesion, this assay was performed.

Effect of antimalarial drugs on adherence of *C. albicans* to a solid surface (i. e. polystyrene) was studied by using micro plate-based assay. A range of concentrations (0.031 to 4 mg/ml) of each antimalarial drug was prepared using PBS. Wells without antimalarial drugs served as controls, while FLC was included as the standard antifungal agent. Each well received 100 µl of cell suspension along with the drug dilutions, maintaining a final assay volume of 200 µl per well. The plates were incubated at 37 °C for 90 minutes with shaking at 100 rpm in an orbital shaker to facilitate cell attachment to the plate surface. After incubation, the wells were rinsed with PBS to eliminate non-adherent cells. The adherence level in each well was then assessed using the XTT assay. The experiment was performed in triplicates [5].

### 3.8. Biofilm formation assay

To study effect of antimalarial drugs on biofilm formation of *C. albicans* biofilm assay was performed. *C. albicans* biofilms were established on the polystyrene surface of a 96-well plate. A cell suspension of 1 x $10^7$ cells/ml in PBS was prepared, and 100 µl was added to each well. During the adhesion phase, plates were incubated at 37 °C with shaking at 100 rpm for 90 minutes to promote cell attachment to the solid surface. After incubation, non-adherent cells were removed by washing the wells with sterile PBS. Subsequently, 100 µl of RPMI-1640 medium and 100 µl of different drug concentrations in RPMI-1640 were added to each well, and the plates were incubated at 37 °C for 48 hours to support biofilm formation. Biofilm development was observed using an inverted microscope and confirmed with the XTT metabolic assay. Biofilm growth was quantified using XTT metabolic assay. The wells containing biofilms were washed with PBS to remove non-adhered cells and incubated with 100 µl of XTT Menadione solution in dark, at 37 °C for 5

h. Color formation by the water-soluble formazan product was measured at 450 nm using a microplate reader (Multiskan EX, Thermo Electron Corp. USA) [6].

### 3.9. Scanning electron microscopy (SEM)

To see the effect of selected anti-malarial drugs on architecture of developing biofilm SEM was performed. $1 \times 10^7$ cells of *C. albicans* adhered on a piece of catheter model at 37 °C for 90 min at 80 rpm. After adhesion, the catheter was dipped in 2 ml of RPMI-1640 medium containing MIC concentration of drug, and without drug was used as control. Biofilm on samples were fixed by 2.5 % of glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C. After that samples were treated with 2 % aqueous solution osmium tetroxide for 4 h and then dehydrated in a series of alcohol. The gold coating of the sample was done using an automated gold coater. Images were obtained by SEM (TESCAN Model-VEGA3) [7].

### 3.10. Propidium iodide (PI) uptake assay

To study the effect of effective molecules on plasma membrane of *C. albicans*. PI a membrane-impermeable red fluorescent dye was employed. The impact of effective drug on the membrane of *C. albicans* was investigated through a series of steps. *C. albicans* cells were cultured in the presence of effective drug after treatment, cells in their exponential phase were collected by centrifugation. The cells were resuspended in 50 µl of PBS and incubated with PI at a concentration of 10 µg/ml in the dark for at least 20-30 minutes. Stained cells were then examined under a fluorescence microscope at a magnification of 40 X (ECLIPSE T1 SAM, Japan) to observe any changes indicative of plasma membrane damage caused by the treatment of effective drug. The experiment was conducted in triplicates [8].

### 3.11. Effect of selected molecules on cell cycle

To study the effects of selective molecules on *C. albicans* cell cycle progression and identify molecular targets within the cell cycle. A single colony of *C. albicans* was inoculated in 50 ml YPD broth and incubated for 24 h. The *C. albicans* cells were pooled and washed with PBS, followed by 1 h of starvation. After that, $1\times10^7$ cells/ml were added in a flask containing RPMI-1640 with MIC concentration of effective drug and without drug serve as control. The flasks were incubated at 35 ºC for 4 h. The cell suspensions were centrifuged at 6000 rpm for 3 min and washed

with chilled PBS post-fixed by chilled ethanol. Cells treating RNase and Propidium iodide and analysis using FACS. The experiment was done in triplicates [9].

### 3.12. Effect of antimalarial drugs on ergosterol synthesis

Quantifying ergosterol in *C. albicans* provides valuable insights into fungal cell membrane dynamics, antifungal drug susceptibility, mechanisms of drug resistance, and potential targets for antifungal therapy. A single colony of *C. albicans* from the Sabouraud dextrose agar plate was inoculated in 50 ml of SDA broth. The culture was incubated for 16 h and harvested by centrifugation at 2700 RPM for 5 min. The net weight of the cell pellet was determined, and 3 ml of 25 % alcoholic potassium hydroxide solution was added to each pellet and vortex mixed for 1 min. These suspensions were transferred to sterile borosilicate glass screw cap tubes and incubated at 85 °C in a water bath for one hour, and after incubation, the tubes were cooled. Sterol was extracted by adding one ml of sterile distilled water and 3 ml of n-heptane followed by 3 min mixing. The extracted sterol was confirmed by spectrophotometry by measuring O. D. for a 230 - 300 nm range. Ergosterol content was calculated by using the following formula = % ergosterol + % 24(28) DHE = [(A281.5 / 290) × F] /pellet weight, % 24(28) DHE = [(A230/518) ×F] /cell pellet weight, % ergosterol = [% ergosterol + % 24(28) DHE] - % 24(28) DHE, where F is the factor for dilution in ethanol and 290 and 518 are the E values. The experiment was done in triplicates [10].

### 3.13. Reactive oxygen species (ROS) generation analysis

To quantify the ability of *C. albicans* to produce ROS in response to environmental stresses, including exposure of antimalarial drugs host immune cells, and oxidative conditions. The amount of intracellular generated ROS was measured using fluorescent dye 2′,7′ Dichlorodihydrofluorescein diacetate ($H_2$DCFH). Cells treated with fluorescent dye and hydrogen peroxide served as positive control. The cells without any treatment were served as negative control. To measure ROS level in planktonic cells, *C. albicans* cells were treated with planktonic MIC concentration of drug incubate for 4 h at 30 °C. Cells were harvested and washed with sterile PBS. For measuring the ROS level in biofilm formation using MIC concentration. The cells were incubated with sterile PBS for 90 min at 37 °C. After that the incubation cells were washed with PBS and added RPMI-1640 medium with $MIC_{50}$ concentration and washed with PBS. Then 40 µg/ml of $H_2$DCFH was added and

incubated in dark for 30 mins. The fluorescent intensity of the cells was measured with spectrofluorometer at 486 nm excitation and 525 nm emission wavelengths [11].

### 3.14. Gene expression study by qRT-PCR

To study the effect of selective molecules on gene expression in *C. albicans* qRT-PCR was performed *C. albicans* ($1\times10^3$ cells/ml) cells were inoculated in RPMI-1640 containing $MIC_{50}$ of drug concentration and incubated for 4 hours at 35 °C, whereas as genes involved in biofilm were accessed by inoculating ($1\times10^7$ cells/ml) cells were inoculated in RPMI-1640 containing effective molecules and incubated for 24 hours at 37°C, cells without drugs served as controls. Total RNA was extracted using the RNeasy® Mini Kit (QIAGEN, USA) and subsequently converted to cDNA with the Superscript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies, USA). PCR reactions were performed with the KAPA SYBR® Fast qPCR Master Mix (2x) on a Bio-Rad Real-Time PCR machine. Reactions were set up in 96-well PCR plates and began with an initial denaturation step at 95 °C for 3 minutes, followed by 32 cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 20 seconds, and primer extension at 72 °C for 30 seconds (CFX96 Real-Time System, Bio-Rad, USA). The transcript levels of selected genes were calculated using the formula $2^{-\Delta\Delta CT}$ [12].

### 3.15. *In vivo* antifungal efficacy in silkworm animal model

To check the antifungal efficacy of Quinine, Mefloquine and Hydroxychloroquine against *C. albicans* pathogenesis *Bombyx mori* silkworm model was used. Silkworms were procured from the Department of Zoology, Shivaji University Kolhapur. $1\times10^6$ *C. albicans* cells/ml were injected on the first day of the $5^{th}$ instar phase of silkworm through the dorsal surface. MIC concentration of QN, MQ and HCQ was injected immediately after injecting *C. albicans* cells at the dorsal surface. After injection, silkworms were kept at 27 ºC. Silkworms were observed for survival [13].

### 3.16. Statistical analysis

All experiments were carried out in triplicate and standard deviation from the mean was calculated.

## 3.17. References

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# CHAPTER IV

# ANTIFUNGAL ACTIVITY OF QUININE ON *CANDIDA ALBICANS*

## 4.1. Introduction

The human pathogen *Candida albicans* is capable of colonizing several biotic as well as abiotic surfaces [1]. In hospitalized patients, colonization of *C. albicans* on catheters is a serious issue. Many of the antifungal medications that are currently in the market have side effects and cause resistance. It is necessary to search for effective and less toxic antifungal agents [2]. The development of new antifungal drugs is a major challenge for the pharmaceutical industry. Drug repurposing can be a therapeutic option for fungal infections [4]. The antimalarial medicine quinine in presence blue light reported to have photodynamic activity against *C. albicans* pathogenesis. It causes inactivation of the *C. albicans* planktonic cells *in vitro* and inhibitory activity against cutaneous candidiasis *in vivo* in mice [3]. The present study, focused on the mode of action of quinine alone against *C. albicans* through ergosterol biosynthesis, ROS generation, PI uptake assay, inhibition of virulence factors studies. Furthermore, *in vivo* antifungal efficacy of quinine tested in silkworm animal model.

## 4.2. Materials and Methods

### Preparation of drug solution

Quinine was purchased from Sigma Aldrich Mumbai and stock solution prepared in 2 % DMSO. 0.003 mg/ml to 4 mg/ml concentrations series was used for experiments. Methodology was followed as mentioned in chapter III from page no. 33-38.

## 4.3. Results

### 4.3.1. Quinine inhibited planktonic growth of *C. albicans*

To check inhibitory effect on planktonic growth of *C. albicans* broth microdilution method is used. *C. albicans* treated with 0.003 mg/ml to 4 mg/ml concentration range of quinine and without treatment served as control. The MIC of quinine for the planktonic growth of *C. albicans* is observed at 0.5 mg/ml (Fig. 4.1 A).

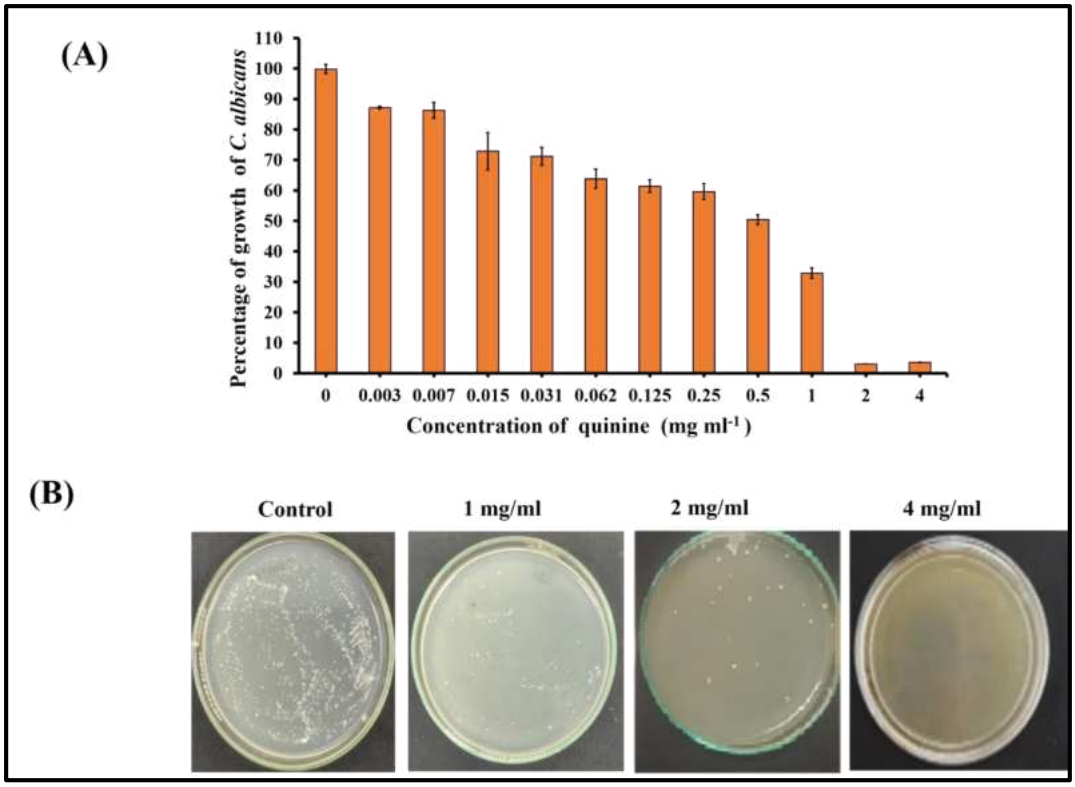


**Fig. 4.1.** Effect of quinine on planktonic growth of *C. albicans* (A) Concentration dependent inhibition of *C. albicans* planktonic growth by treatment of quinine. (B) Concentration dependent fungicidal nature of quinine.

### 4.3.2. Determination of MFC

The MFC of quinine is determined by spread plate technique on a YPD plate with the help of MIC and above concentrations of quinine and the results indicated that quinine is fungicidal in nature at 4 mg/ml while 2 mg/ml and 1 mg/ml concentrations showing less numbers of colonies (Fig. 4.1 B).

### 4.3.3. Time-dependent killing assay

Based on MFC value, time dependent killing assay has been carried out. The killing effect is observed for the exposure time intervals of 0, 0.25, 0.5, 1, 4, 8, 10, 12, 16 h. Time-dependent killing assay revealed that quinine kills 99 % of *C. albicans* cells at 4 mg/ml within 6 h of exposure (Fig. 4.2).

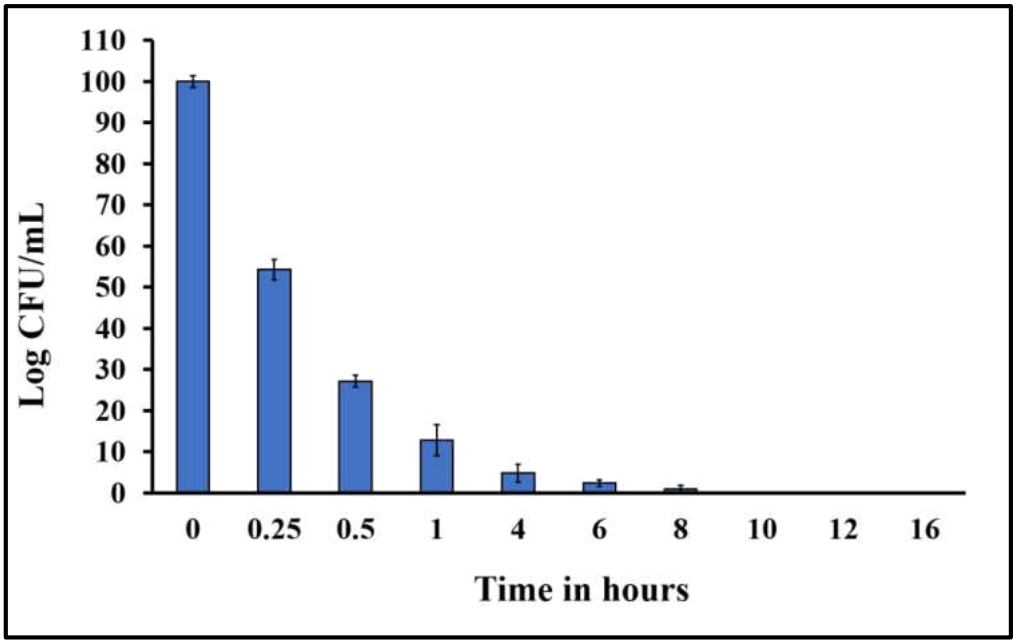


**Fig. 4.2.** Time dependent killing of *C. albicans* by the treatment of quinine.

### 4.3.4 Quinine inhibited Yeast to hyphal transformation

*C. albicans* strain ATCC 90028 is exposed 0.031 mg/ml to 4 mg/ml concentrations of quinine. After the treatment of quinine, inhibition of serum induced yeast to hyphal formation in *C. albicans* is observed in a dose-dependent manner (Fig. 4.3). The MIC of yeast to hyphal formation was at 0.125 mg/ml.

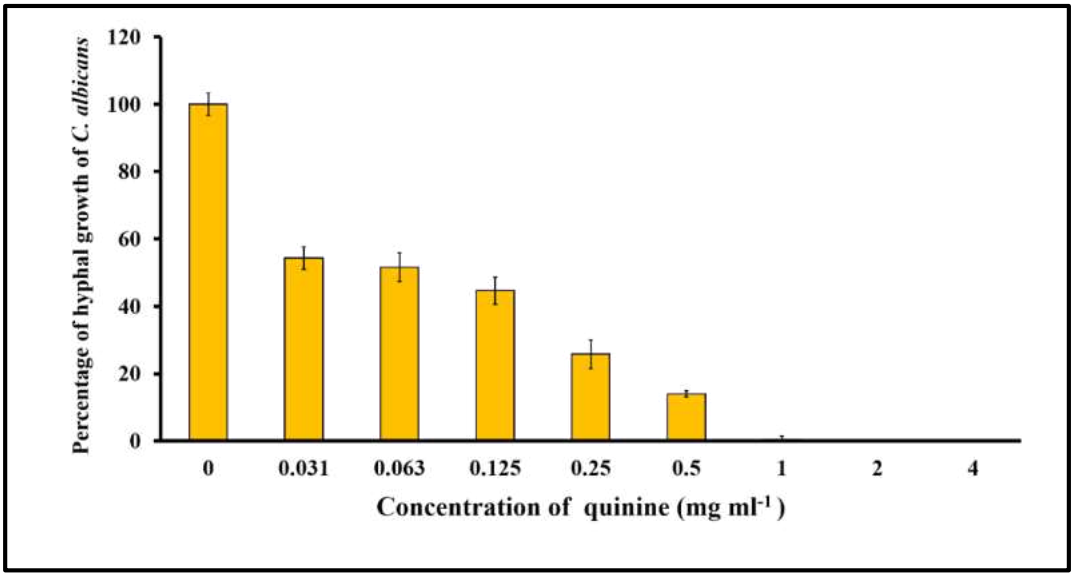


**Fig. 4.3.** Effect of quinine on hyphal growth of *C. albicans*.

### 4.3.5. Quinine inhibited adhesion to a polystyrene surface

Adhesion is one of the important virulence factors in *C. albicans***.** The adhesion of *C. albicans* cells to polystyrene surface was examined in the presence of quinine concentration (0.003 mg/ml to 4 mg/ml), The $MIC_{50}$ of quinine for *C. albicans* adhesion was found at 0.125 mg/ml (Fig. 4.4)

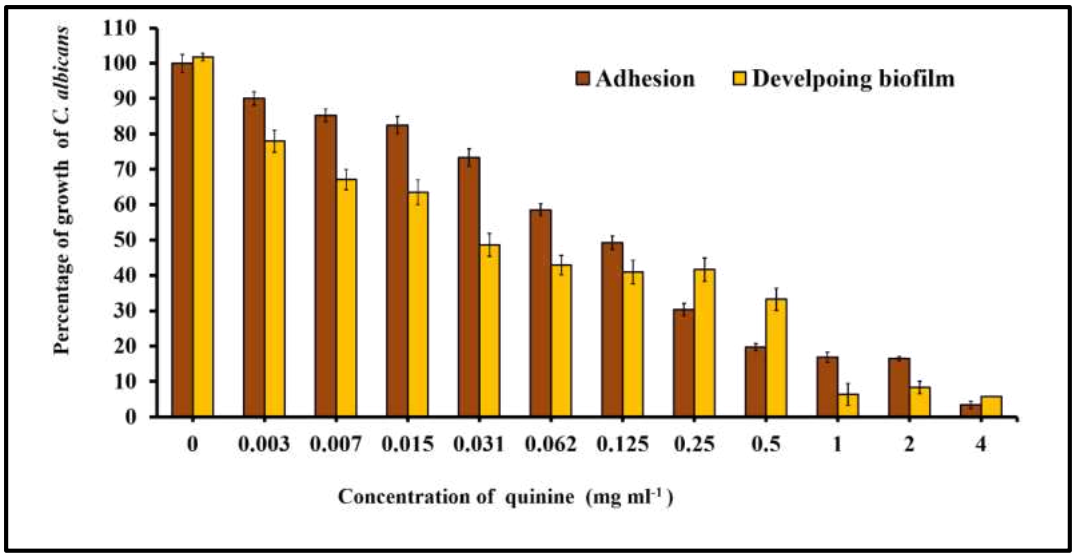


**Fig. 4.4.** Effect of quinine on adhesion and developing biofilm of *C. albicans*. Concentration dependent inhibition of adhesion and developing biofilm of *C. albicans.*

### 4.3.6. Quinine inhibits developing biofilm

The anti-biofilm ability of quinine at different concentration ranges between 0.003 to 4 mg/ml was tested by XTT metabolic assay. The concentration dependent inhibition of biofilm formation has showed in Fig. 4.4. Quinine inhibited 50 % developing biofilm at 0.031 mg/ml of *C. albicans.*

### 4.3.7. SEM analysis

SEM micrographs showing a difference in architecture of *C. albicans* biofilm of control and quinine-treated cells (Fig. 4.5). Biofilm formation is inhibited and few yeast cells is present at 2 mg/ml, while the control of *C. albicans* displayed a dynamic and structurally sophisticated biofilm network that included both yeast and hyphal cells.

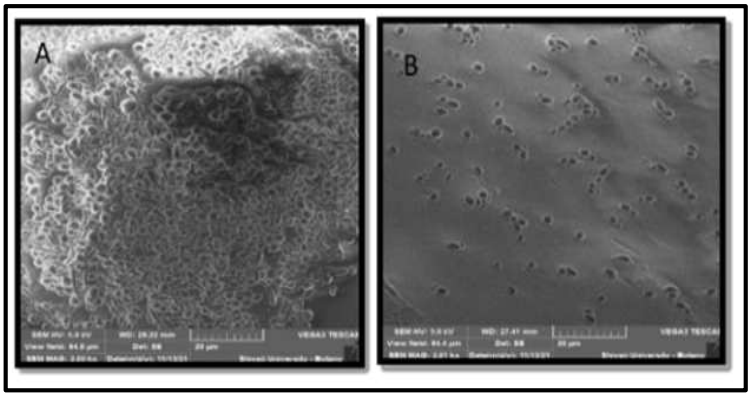


**Fig. 4.5.** SEM images of *C. albicans* biofilm, A: *C. albicans* biofilm without any treatment. B: No biofilm formation by the treatment of quinine.

### 4.3.8. Quinine damages cell membrane of *C. albicans*

The investigation into membrane integrity using the membrane-specific fluorescence dye PI revealed red fluorescence is observed as increasing concentration of quinine. Notably, control cells exhibited no PI accumulation, unlike treated cells subjected to higher concentrations (1 mg/ml) of quinine and MIC values.

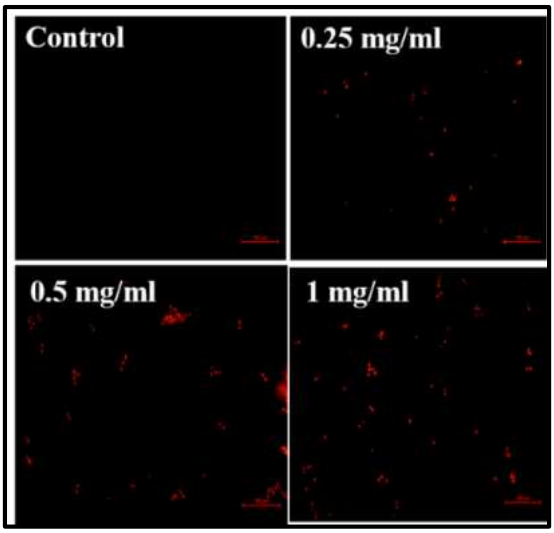


**Fig. 4.6.** Fluorescent microscopic image of PI stained of *C. albicans* cells treated with different concentration of quinine. Control does not show any fluorescence while 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml show increasing in fluorescence.

### 4.3.9. Quinine inhibited ergosterol biosynthesis

Quinine has found to inhibit the ergosterol biosynthesis in *C. albicans*. A dose-dependent decrease in ergosterol content has been shown in Fig. 4.7. Total

ergosterol content is determined for the treatment at 2 mg/ml to 0.0625 mg/ml concentrations of quinine (Fig.4.7).

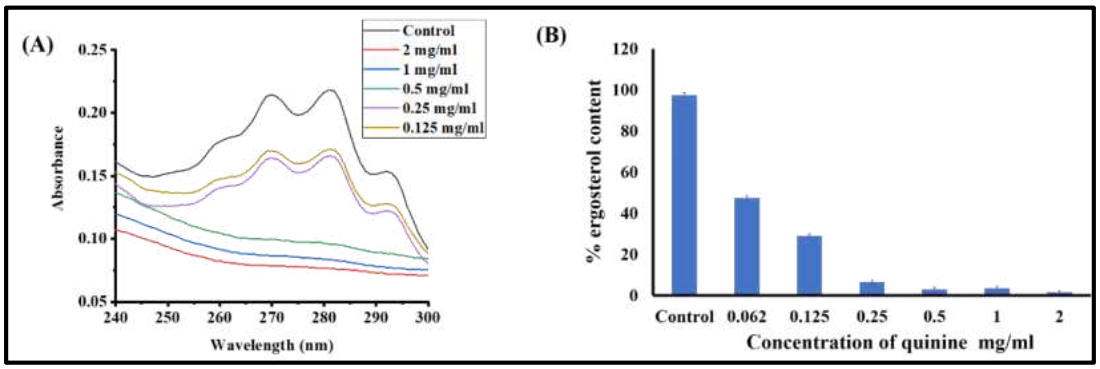


**Fig. 4.7.** Effect of quinine on ergosterol biosynthesis. (A) The sterol content reduced in quinine treated cells as shown in plot. (B) Bar graph revealed the reduction of ergosterol content in quinine treated (0.062 mg/ml to 2 mg/ml) *C. albicans* as compared with control.

### 4.3.10. Quinine affected ROS generation in *C. albicans*

ROS accumulation plays important role in pathogenicity and key adaptive response in *C. albicans,* helping it survive in hostile environments. ROS accumulation study showed the enhanced intracellular ROS generation with quinine treatment. Quinine enhanced the generation of ROS at 0.5 mg /ml for planktonic and 0.03 mg/ml in biofilm *C. albicans* and it was quantified using $H_2$DCFH fluorescent dye (Fig. 4.8). Based on this result, we concluded that both planktonic and biofilm cells of *C. albicans* produced ROS in response to quinine treatment. This may lead to lipid peroxidation of the cell membrane, directly damaging phospholipids, and the inhibition of biofilm may also function as a signal of cell death.

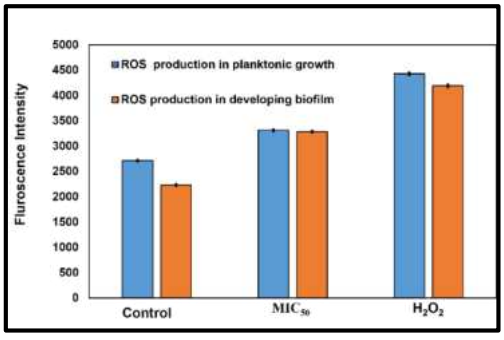


**Fig. 4.8.** Effect of quinine on ROS production. The graph denotes ROS generation in *C. albicans* in presence of quinine, planktonic cells are treated with 0.5 mg/ml and biofilm cells were treated with 0.03 mg/ml.

### 4.3.11 Quinine inhibited of cell division

Quinine was found to affect the G2/M phase of cell cycle of *C. albicans* at 0.5 mg/ml. 41 % of cells are arrested in the G2/M phase and 28 % in the S phase after the treatment of quinine at 0.5 mg/ml concentration whereas 32 % in the G2/M phase and 9 % in the S phase in control cells (Fig. 4.9).

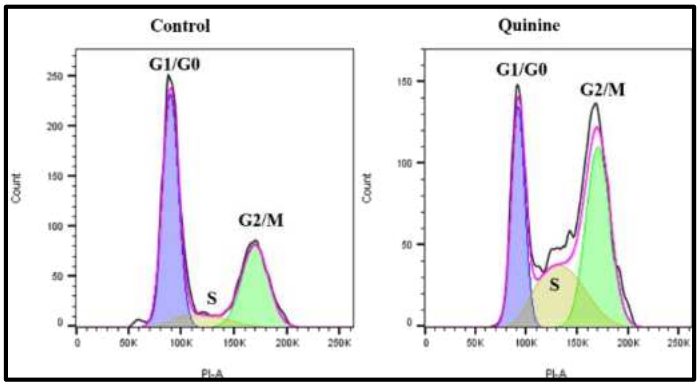


**Fig. 4.9.** Effect of quinine on cell cycle progression of *C. albicans*.

### 4.3.12. Gene expression analysis in *C. albicans* planktonic growth

qRT-PCR studies were performed to check the effect of quinine on the gene expression in *C. albicans*. There was upregulation of *SOD1* and *SOD2* gene expression by 2 and 5 fold ,respectively was observed after the treatment of quinine at 0.5 mg/ml. The other genes like *KRE9* and *MCA1* *ALS1*, *ALS3*, and *CAT1* were also upregulated by 1.4, 2, 2, 6, and 1.2 fold ,respectively after the treatment of quinine (Fig. 4.10).

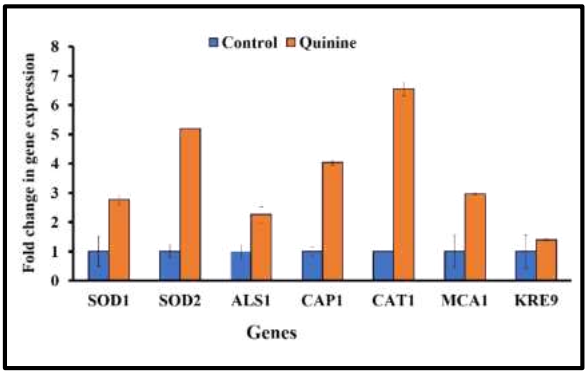


**Fig. 4.10.** Effect of quinine on gene expression. 0.25 mg/ml quinine treated with *C. albicans* cells show upregulation of *SOD1*, *SOD2*, *ALS1*, *CAP1*, *CAT1*, *MCA1* and *KRE9* gene.

### 4.3.13 Therapeutic effects of quinine in the silkworm animal model

The antifungal efficacy of quinine against *C. albicans* has been studied in the silkworm animal model. The infection model is developed by injecting $1\times10^6$ cells/ml through the $6^{th}$ leg of the $5^{th}$ instar silkworm. Infected silkworms treated with 0.5 mg/ml quinine survived up to 72 h, whereas silkworm without any treatment died within 24 h (Fig. 4.11). Standard drug FLC treated silkworm survived upto 72 h and velicle contol served as DMSO treated silkworm.

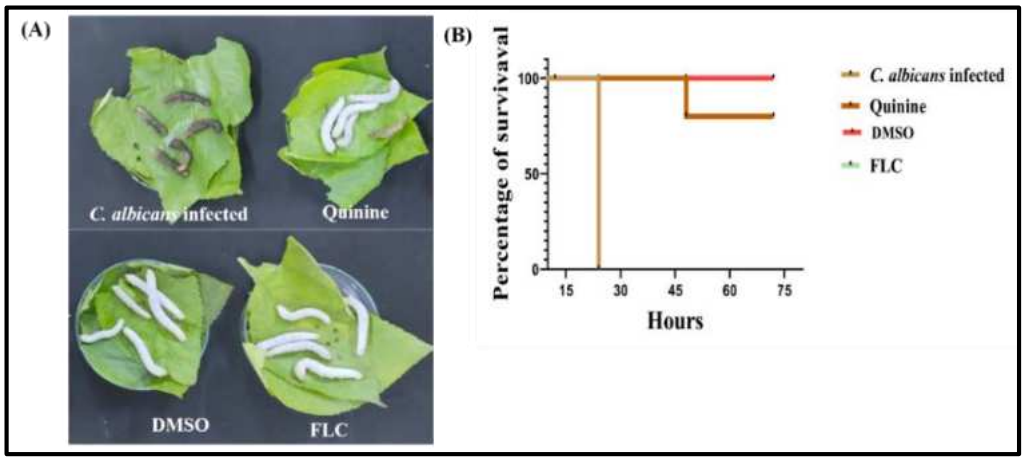


**Fig. 4.11.** Effects of quinine on *C. albicans* infected silkworm. (A) Images of treated silkworm. (B) The graph indicating probability of survival of silkworm.

## 4.4. Discussion

The significant increase of *Candida* spp. infections associated to the frequent failure of traditional antifungal therapy makes the study of more therapeutic options [4]. A recent study reported that *C. albicans* shows a 50 % fluconazole and itraconazole resistance, two antifungal drugs currently used to treat candidiasis, to supporting the need to find novel treatments as alternatives or adjuvants to the traditional ones [5]. Due to resistance in *C. albicans* to available standard antifungal drugs, there is a need for alternative options for treatment.

Drug repurposing is one of the alternative options to develop new antifungal drugs. Generally, Repurposing has focused on identifying already FDA-approved drugs that can be used without modification of structure [6]. Antimalarial drugs have already been reported to have antibacterial, anticancer, and antifungal activities [7,

8]. The previous study reported that the antimalaria drug chloroquine shows inhibitory activity against *C. albicans* growth, morphogenesis, and ergosterol biosynthesis in a dose-dependent manner [9]. Mefloquine is another antimalarial drug which is found to inhibit morphogenesis and disrupt mitochondrial function in *C. albicans* [10].

The antimalarial quinine can inactivate malarial parasites and digest hemoglobin. It is reported to have anticancer activity and anti-proliferative properties in Hela cells [11]. Quinine has antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* [12]. A previous study reported that the combination of antimicrobial light (108 J/cm$^2$) with quinine (1 mg/mL) inactivates planktonic growth *C. albicans* (strain CEC 749) cells and inactivates 48 h mature biofilms growth. A transmission electron microscopy study showed that quinine + antimicrobial light damages the cell. first time, we report that quinine alone inhibits planktonic growth and virulence factors in the *C. albicans.* Quinine inhibited planktonic growth at 0.5 mg/ml concentration.

4 mg/ml concentration absence of colonies indicated that quinine is fungicidal. The quinine exposure time was log 10 cells/ml 6 h at 2 mg/ml concentration showed fungicidal action. *C. albicans* cells treated with quinine inhibited the virulence traits including, yeast to hyphal form morphogenesis, biofilm formation, and adhesion, quinine was found to inhibit yeast to hyphal formation.

The *C. albicans* biofilm extracellular matrix plays an essential role in the development of resistance as a physical barrier against drug penetration. The first stage of *Candida* biofilm formation involves the attachment of the cells to the hyphae surface. The hyphal cells then proliferate to form microcolonies forming a biofilm base layer [13]. Biofilm formation poses a serious threat to physicians, drug developers as well as patients. Colonized catheters can get clogged obstructing the urine flow and requiring the frequent removal of the catheters [14]. Polystyrene surface was used to investigate the effect of quinine on adherence and biofilm formation by *C. albicans.* Quinine also inhibited the virulence factors, adhesion, biofilm development at 1 mg/ml, 0.031 mg/ml. Biofilm formation, the phenotypic variability of fungi could play role in the clinical outcome of therapeutic interventions like drug repurposing. However, the *C. albicans* SEM experiment visualized the biofilm structure generated by quinine treatments, growth was

inhibited and few yeast cells were found to be dispersed, whereas complex biofilm structure was observed for control. All these changes involved in membrane and cytoplasmic content, on the basis of this we chose to study cellular changes by cell cycle and changes in cell membrane by PI uptake, ergosterol biosynthesis, to understand the possible mechanism of action of quinine. It was shown that quinine cause damage to *C. albicans* cells via PI uptake quinine can compromise the integrity of the fungal cell membrane and cause membrane permeabilization as the concentration of rises. The present investigation of the PI uptake assay was enhanced permeability of *C. albicans* cellular membrane caused by quinine exposure could be due to contact with negatively charged components of the fungal surface. In addition, Ergosterol synthesis is one of the important targets for antifungal drugs against *C. albicans*. To investigate the effect of quinine on the ergosterol synthesis pathway in *C. albicans* ergosterol content was quantified and compared with fluconazole treatment for the same. The Ergosterol profile analysis revealed that quinine inhibits ergosterol biosynthesis significantly in a concentration dependent manner. ROS induction plays an important role in the potential mechanisms of antifungal drugs. ROS production is a multifaceted and critical aspect of *C. albicans* pathobiology.

It enables the fungus to evade the host immune system, biofilms, switch between different morphological forms, acquire essential nutrients, and express virulence factors. ROS accumulation assay revealed that quinine increases intracellular ROS accumulation in *C. albicans*. The overproduction of ROS may lead to cell damage and death. ROS enhanced accumulation can be another mode of action quinine in *C. albicans.* Additionally, the cell cycle studies revealed that quinine arrested the cell cycle of *C. albicans* at the G2/M phase and S phase, which eventually leads to the inhibition of growth. All these changes indicate that during the S phase chromatin progressively condenses and suggests that the condensation is associated with the nucleus. Further, the effect of quinine on the expression of genes such as *SOD1, SOD2, ALS1, CAP1, CAT1, MCA1 and KRE9* in *C. albicans* planktonic cells was studied by qRT PCR.

Quinine treatment to *C. albicans* significantly upregulated the expression of both the *SOD1* and *SOD2*. These genes involved in the destruction of a toxic radical and reduce oxidative stress. The gene expression of the *SOD1*, *SOD2* and *CAT1* genes

in *C. albicans* were upregulated by 2-fold, 5-fold and 6-fold change after the treatment of quinine. *ALS1* gene encodes cell surface glycoprotein implicated in adhesion to host surfaces [8]. *ALS1* gene expression was upregulated 2-fold after treatment of quinine. The *MCA1* gene in *C. albicans* is an apoptosis regulator at the transcriptional level [15]. They were upregulated by 2.97-fold in response to quinine. Quinine significantly upregulated expression of the cell wall synthesis gene *KRE9*.

To check the efficacy of quinine *in vivo*, we used a silkworm model. The antifungal efficacy of quinine against *C. albicans* was arrested in a silkworm animal model. The infection model was developed by injecting $1\times10^6$ cells/ml through the $5^{th}$ instar larva treated with 0.5 mg/ml quinine has survived up to the pupa phase.

In summary, multitarget mechanism of action of quinine strongly support its development as an antifungal agent against *C. albicans.*

### 4.5. Conclusions

This chapter concludes that, Quinine has capacity to inhibit growth of virulence factors. The MIC of quinine against planktonic growth at 0.5 mg/ml concentration. Quinine shows fungicidal activity at 2 mg/ml and within 6 h exposure kills *C. albicans*. Quinine treatment inhibited virulence factors such as yeast to hyphal formation and biofilm formation at 0.25 mg/ml and 1 mg/ml in *C. albicans*. The study on PI accumulation indicates the damaged membrane integrity of *C. albicans* after quinine exposure. Quinine also inhibited ergosterol biosynthesis in *C. albicans*. Quinine enhanced intracellular ROS production in *C. albicans*. Quinine traded cells were arrested in G2/M and S phase. The qRT PCR study revealed that *MCA1, SOD1, SOD2, and ALS3* gene expressions were upregulated after the treatment of quinine. The anti-*C. albicans* efficacy was confirmed in the silkworm model. Quinine can be a good therapeutic option for the treatment of candidiasis and could be repurposed against *C. albicans* infection.

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# CHAPTER V

# ANTIFUNGAL ACTIVITY OF HYDROXYCHLOROQUINE ON *CANDIDA ALBICANS*

## 5.1. Introduction

The virulence traits of the opportunistic fungus *C. albicans* enhances its capacity to survive under drastic environmental conditions and its pathogenicity. The virulent characteristics of *C. albicans* include the expression of host-recognizing proteins, the synthesis of lipolytic and proteolytic enzymes, the creation of biofilms, and morphological alterations between yeast and filamentous forms. One crucial aspect of *C. albicans* pathogenicity is the production of biofilms [1]. *C. albicans* biofilms are resistant to various antifungal drugs [2]. 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. Drug repurposing strategy has been thoroughly investigated in antifungal drug research [3]. Hydroxychloroquine is an antimalarial medication marketed as Plaquenil. It is used to treat autoimmune illnesses by modulating the immune system. Hydroxychloroquine is used for the treatment of systemic lupus erythematosus (SLE) and Rheumatoid arthritis at 5 mg/kg/day of actual body weight [4]. Hydroxychloroquine is a derivative of chloroquine; it shows the difference in hydroxyl group (-OH) added in the structure of chloroquine (1983). This study explores the antifungal efficacy and probable mode of action of hydroxychloroquine in *C. albicans* by performing yeast to hyphal transformation, adhesion, biofilm formation, ergosterol synthesis, cell cycle progression and *in vivo* studies.

## 5.2. Materials and Methods

Hydroxychloroquine was purchased form Sigma Aldrich Ltd., Mumbai, India. For current study 5 mg/ml stock of hydroxychloroquine was prepared by dissolving it in D/W. Methodology was followed as per mentioned in chapter III; page no. 33-38.

## 5.3. Results

### 5.3.1. Hydroxychloroquine inhibited *C. albicans* planktonic growth

The MIC of hydroxychloroquine on planktonic growth of *C. albicans* was determined by broth microdilution method.

In concentration-dependent manner, hydroxychloroquine is inhibited *C. albicans* planktonic growth and 50 % growth inhibited at 0.5 mg/ml concentration (Fig. 5.1).

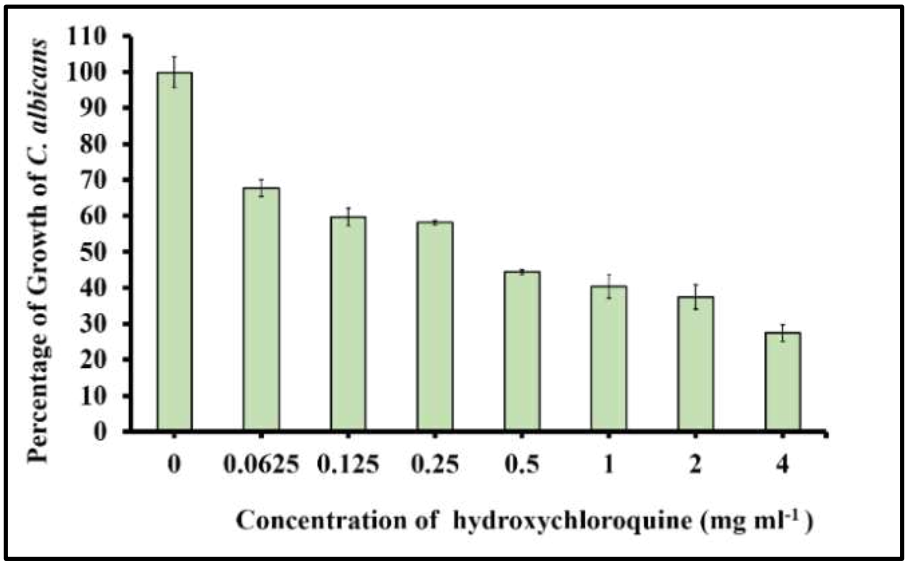


**Fig. 5.1.** Effect of hydroxychloroquine on planktonic growth of *C. albicans*.

### 5.3.2. Hydroxychloroquine inhibited Yeast to hyphal formation

*C. albicans* was exposed to 0.0625 mg/ml to 4 mg/ml concentration range of hydroxychloroquine. 50 % inhibition of serum induced yeast to hyphae formation was observed at 0.5 mg/ml by the treatment of hydroxychloroquine (Fig. 5.2).

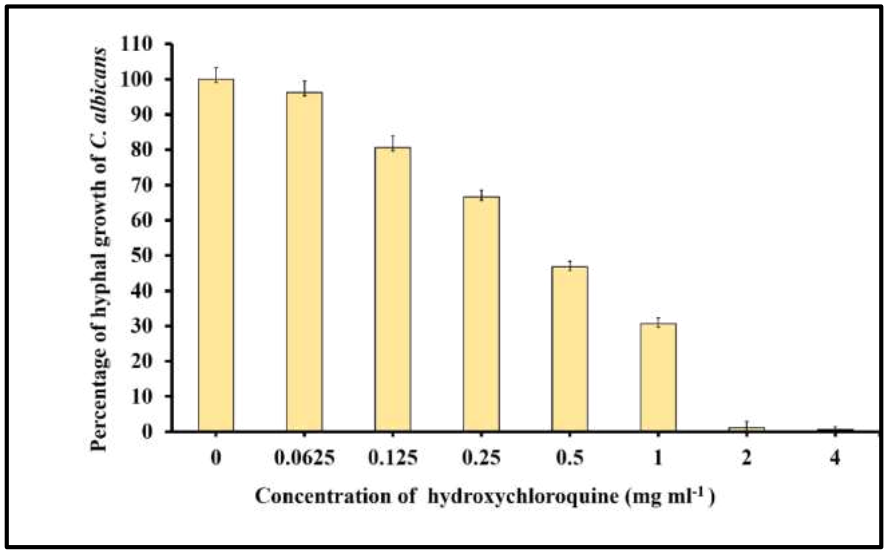


**Fig. 5.2.** Effect of hydroxychloroquine on yeast to hyphal formation of *C. albicans*. The MIC concentration for yeast to hyphal is observed at 0.5 mg/ml.

### 5.3.3. Hydroxychloroquine inhibited adhesion and developing biofilm formation in *C. albicans*

The MIC of hydroxychloroquine for adhesion was found at 2 mg/ml (Fig. 5.3). hydroxychloroquine at 0.25 mg/ml concentration significantly inhibits biofilm formation of *C. albicans*. The effect of hydroxychloroquine on developing biofilm observed by using inverted microscope and biofilm growth was quantified by XTT metabolic assay. The concentration dependent inhibition of adhesion and developing biofilm has been shown in Fig. 5.3.

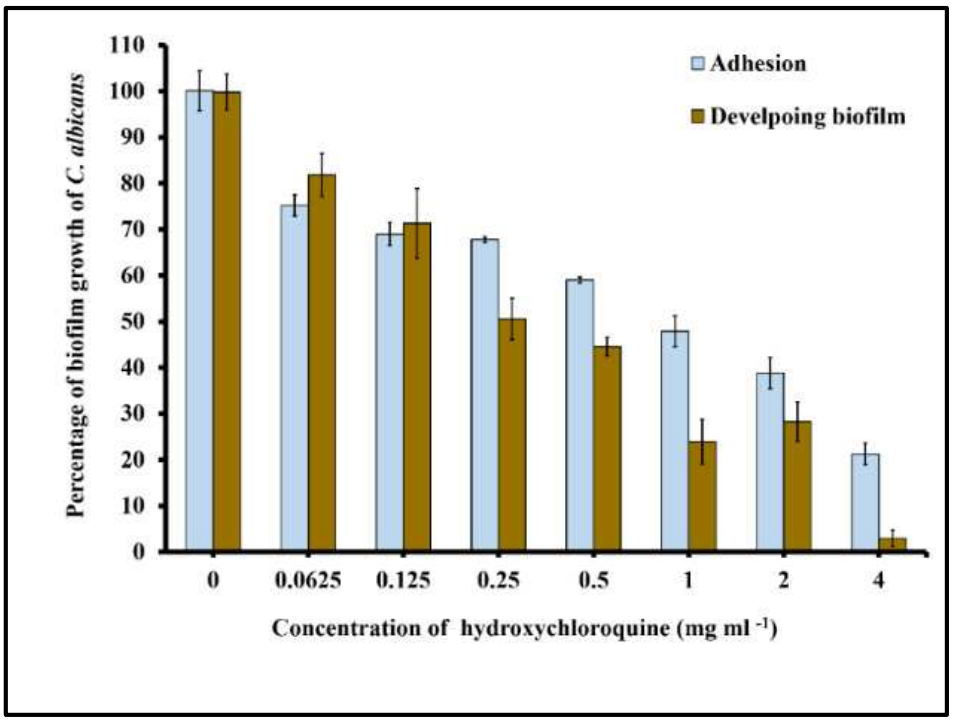


**Fig. 5.3**. Effect of hydroxychloroquine on adhesion and developing biofilm of *C. albicans*.

### 5.3.4. SEM of biofilm architecture

SEM analysis performed to check the effect of hydroxychloroquine on biofilm architecture in *C. albicans*. It was found that, in presence of hydroxychloroquine (0.25 mg/ml) only yeast cells adhered whereas in control without any treatment of hydroxychloroquine displayed astructurally complex biofilm network containing yeast cells, hyphal cells and extracellular matrix (Fig. 5.4)

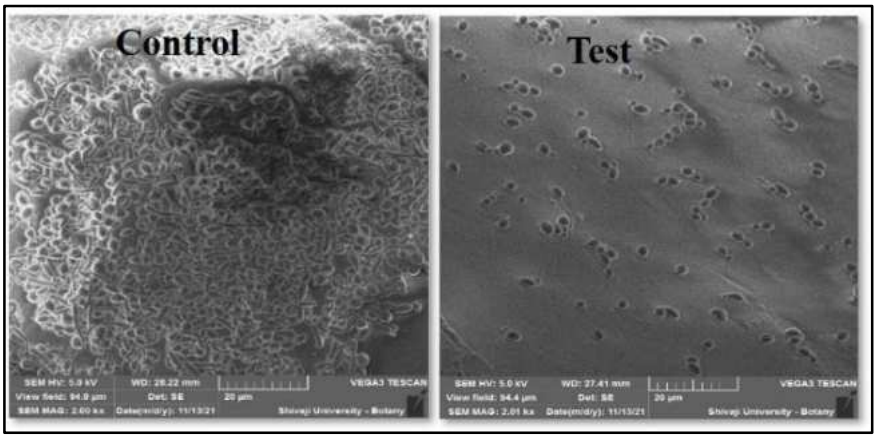


**Fig. 5.4.** Scanning electron microscopic images of *C. albicans* biofilm; Control: Biofilm without treatment; Test: Inhibition of biofilm formation at 0.25 mg/ml concentration of hydroxychloroquine.

### 5.3.5. Hydroxychloroquine damages cell membrane of *C. albicans*

The membrane integrity of *C. albicans* was assessed using the membrane-specific fluorescent dye PI. Fluorescence microscopy images showed a concentration-dependent increase in red fluorescence in hydroxychloroquine -treated 1 mg/ml, 0.25 mg/ml and 0.5 mg/ml of C. *albicans* cells, while control cells exhibited no fluorescence (Fig. 5.5). These results indicates that PI penetrates the cell membrane and binds with DNA, confirming that hydroxychloroquine induces membrane damage in *C. albicans.*

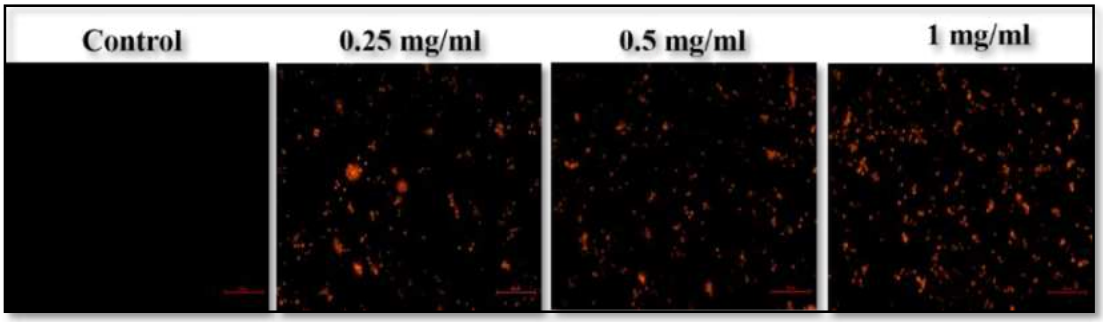


**Fig. 5.5.** Fluorescent microscopic images of PI-stained *C. albicans* cells; Control: *C. albicans* cells showing no fluorescence, whereas *C. albicans* cells treated with 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml concentrations of hydroxychloroquine showing concentration-dependent increase in fluorescence.

### 5.3.6. Hydroxychloroquine inhibited ergosterol synthesis in *C. albicans*

A key target for the development of antifungal medications is ergosterol synthesis. Ergosterol content in the membranes of *C. albicans* cells treated with hydroxychloroquine has been determined. The dose-dependent rapid drop in sterol content was observed in the UV spectrophotometric profile, whereas the untreated control cells showed a characteristic peak of ergosterol (Fig. 5.6 A, B). The percentage of ergosterol content in control *C. albicans* cells has been compared with hydroxychloroquine treated cells and represented in Fig. 5.6 B.

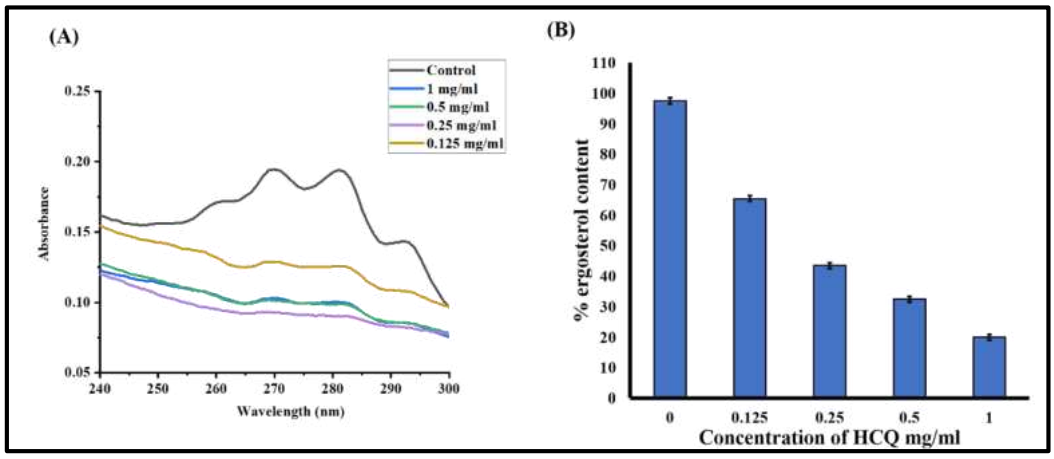


**Fig. 5.6.** Effect of hydroxychloroquine on ergosterol biosynthesis. (A) The sterol content reduced in hydroxychloroquine treated cells as shown in plot. (B) The Bar graph show the reduction of ergosterol content in hydroxychloroquine treated (0.125 mg/ml to 1 mg/ml) *C. albicans* as compared with control.

### 5.3.7. Hydroxychloroquine arrested cell cycle in *C. albicans*

The flow cytometry data represents that, hydroxychloroquine at 0.5 mg/ml increased the populations of *C. albicans* cells in the G0/G1 phase as compared to control cells. 25 % *C. albicans* cells were present in G0/G1 phase in control sample while 36 % hydroxychloroquine treated *C. albicans* cells were present in G0/G1 phase (Fig. 5.7).

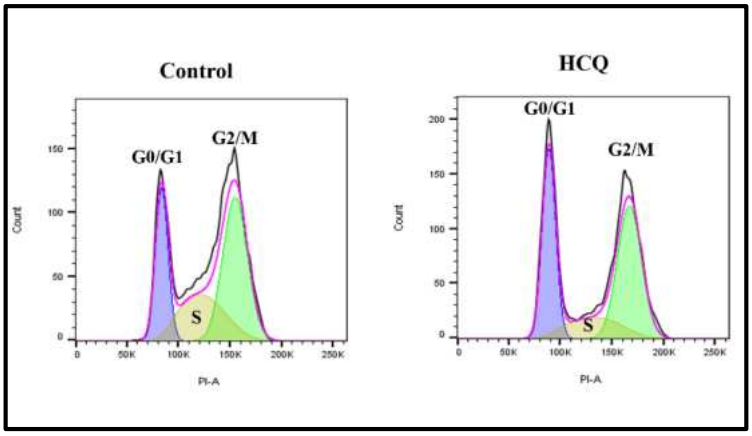


**Fig. 5.7.** Effect of hydroxychloroquine on cell cycle progression of *C. albicans;* (A) Control: Cell cycles phases of *C. albicans* cells without any drug treatment; Test: hydroxychloroquine treated cells (0.5 mg/ml).

### 5.3.8. Hydroxychloroquine affected on ROS generation in *C. albicans*

ROS accumulation has important role in pathogenicity and key adaptive response in *C. albicans,* helping it survive in hostile environments. ROS accumulation study showed the enhanced intracellular ROS generation in *C. albicans* increased with treatment of hydroxychloroquine. Hydroxychloroquine enhanced the generation of ROS at 0.5 mg /ml in planktonic and 0.25 mg/ml in biofilm *C. albicans* cells and it was quantified using $H_2$DCFH fluorescent dye. The data was analyzed and compared with control (Fig. 5.8).

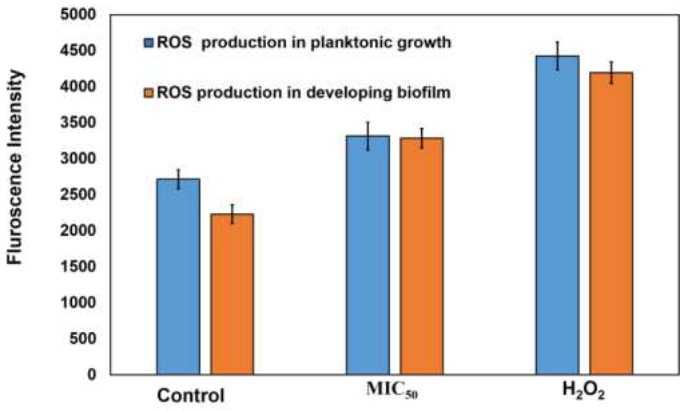


**Fig. 5.8**. Effect of hydroxychloroquine on ROS generation in *C. albicans*. The graph represents, enhancement of ROS level in planktonic cells treated with 0.5 mg/ml and biofilm cells treated with 0.25 mg/ml of hydroxychloroquine.

### 5.3.9. Gene expression study

Gene expression analysis was performed by using qRT-PCR to determine the effect of hydroxychloroquine on the gene expression in *C. albicans* planktonic growth. The genes inculding *SOD1*, *SOD2*, *ALS1*, *CAP1, CAT1, MCA1,* and *KRE9* were found to be upregulated. The *SOD1* and *SOD2* gene expression upregulated by 2 and 6 fold respectively was observed after the treatment of hydroxychloroquine at 0.5 mg/ml. The other genes like *ALS1, KRE9, MCA1*, *CAP1* and *CAT1* were also upregulated by 1.8, 6, 2, 1 and 4 fold, respectively.

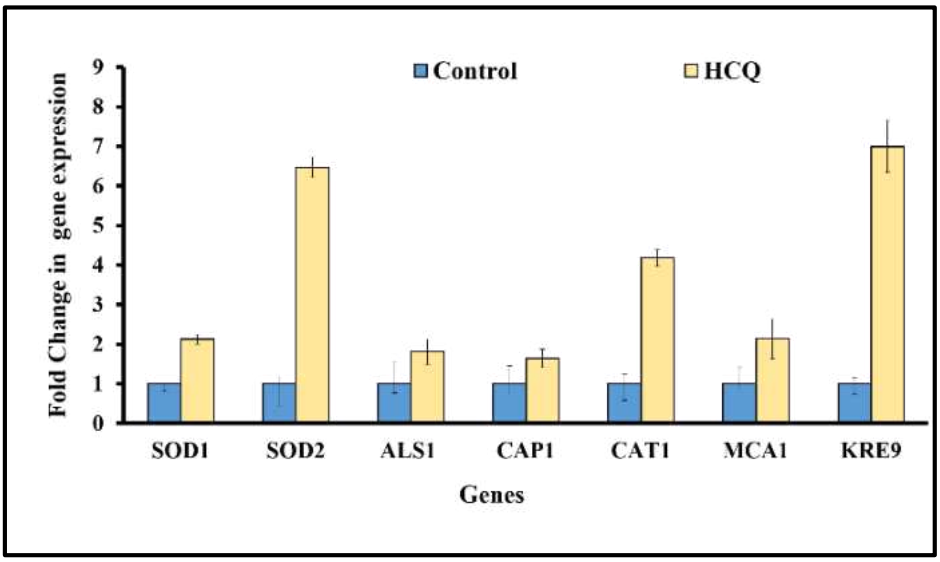


**Fig. 5.9.** Gene expression studies by qRT-PCR. 0.5 mg/ml hydroxychloroquine treated *C. albicans* cells shows upregulation of *SOD1*, *SOD2*, *ALS1*, *CAP1, CAT1, MCA1,* and *KRE9.*

### 5.3.10. Therapeutic effects of hydroxychloroquine in the silkworm

The antifungal efficacy of hydroxychloroquine against *C. albicans* was studied in the silkworm. The infection model was developed by injecting $1\times10^6$ cells/ml through the 6$^{th}$ leg of the 5$^{th}$ instar silkworm. Infected silkworms treated with 0.5 mg/ml hydroxychloroquine drug, FLC treated positive control and DMSO vehicle control group survived and completed its life cycle whereas silkworm larvae with infected with *C. albicans* died within 24 h (Fig. 5.10).

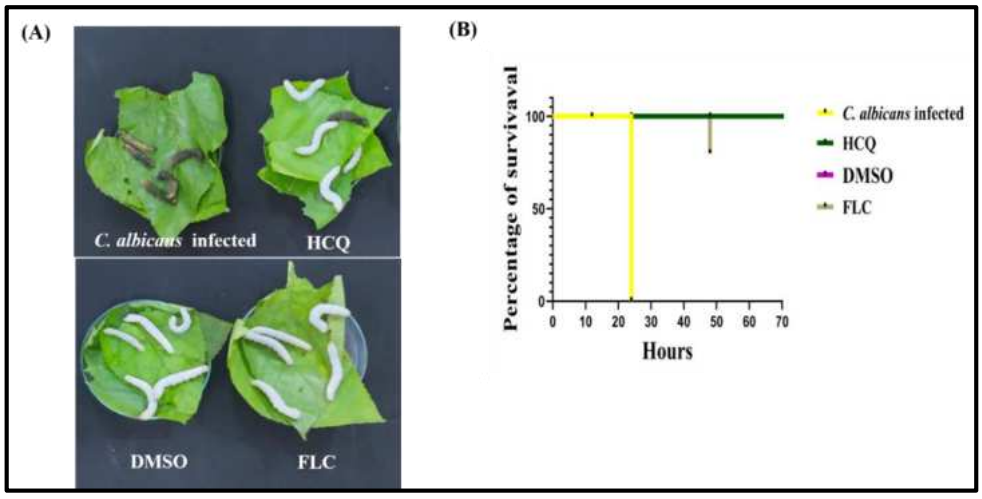


**Fig. 5.10.** Effects of hydroxychloroquine on *C. albicans* infected silkworm. (A) Images of treated silkworm. (B) The graph indicating percentage of silkworm survived.

## 5.4. Discussion

Invasive candidiasis (IC) is a fungal infection caused by different types of *Candida* species and it is associated with considerable morbidity and mortality rate across the globe. Among the *Candida* species, *C. albicans* is the most frequently isolated fungal species [5]. The azole drugs are widely used to treat IC. Their widespread use is constrained by their harmful side effects, which include nephrotoxicity, hepatotoxicity, and neurotoxicity, and abusive use of azole drug has caused resistance in *C. albicans* [6]. Discovering new antifungal agents against drug-resistant *C. albicans* is highly desirable. Drug repurposing is one of the alternative strategies that can be used against *Candidiasis.* This method is cost-effective and less time-consuming [7]. Some previous repurposing studies revealed that hydroxychloroquine has antibacterial, antiviral, anticancer, and anti-antifungal activities [8–10]. Hydroxychloroquine reduces the risk of *Pneumocystis pneumonia* in lupus patients [11]. Also it has antiviral activity against SARS-CoV-2 [12]. However, for the first time, the antifungal activity of Hydroxychloroquine with its mode of action has been reported in the current study. Hydroxychloroquine is found to affect planktonic growth, yeast to hyphal formation, adhesion, biofilm formation, ergosterol biosynthesis and cell cycle progression in *C. albicans*.

In previous study it is reported that, the antimalarial drug Chloroquine has capacity to inhibit the growth at 1 mg/ml. Chloroquine inhibits virulence factors, Yeast-hyphal formation, biofilm formation, ergosterol biosynthesis pathway of *C. albicans* in concentration dependent manner. Hydroxychloroquine, a derivative of chloroquine affected the planktonic growth at 0.5 mg/ml (Fig. 5.1). Hydroxychloroquine inhibited the key virulence traits which are involved in pathogenicity. Hydroxychloroquine significantly inhibited the morphological transitions like yeast to hyphal morphogenesis of *C. albicans*. These morphological forms play essential roles in disease progression, invasion, adhesion, and host tissue damage. For *C. albicans* to be virulent, it must possess the ability to go through morphogenesis. Treating *C. albicans* cells with 0.5 mg/ml of hydroxychloroquine. It was found to reduce 50 % of hyphal induction (Fig. 5.2). Hyphae development and biofilm formation are closely interlinked in *C. albicans*. Biofilm formation is the primary virulence trait associated with *C. albicans* infection. An essential therapeutic aim is to decrease the survival of biofilms that have developedon the surface of medical devices while avoiding biofilm development might stop infections brought on by device-associated biofilm. The XTT assay was used to examine *C. albicans* metabolic activity while exposed to hydroxychloroquine concentrations. Biofilm generation was seen to be inhibited at 0.25 mg/ml (Fig. 5.3). To study the effect of hydroxychloroquine on biofilm architecture of *C. albicans* was observed through scanning electron microscopy (SEM). SEM images depicted that, at 0.25 mg/ml concentration of hydroxychloroquine, *C. albicans* biofilm growth was inhibited and few yeast cells were found to be dispersed (Fig. 5.4), whereas complex biofilm structure was observed for control. To validate this data, gene expression study is performed in *C. albicans* biofilm system. Ergosterol is an essential structural component in fungal cell membranes, and it plays an important role in maintaining cell integrity. Sterol 14-α-demethylase (CYP51) is one of the most important targets for developing antifungal drugs to inhibit ergosterol biosynthesis [13]. Hydroxychloroquine had an effect on ergosterol synthesis in *C. albicans* and was investigated by sterol profile. This study revealed that ergosterol biosynthesis is inhibited by hydroxychloroquine in a concentration-dependent manner (Fig. 5.5).

The decrease in ergosterol content in the cell membrane may create pores in fungal cell membranes and a consequent loss of essential ions such as potassium and protons and other molecules, ultimately killing the fungal cells [14]. The ergosterol biosynthesis inhibition is one of the modes of action of hydroxychloroquine in *C. albicans*. The literature survey suggests that chloroquine had inhibiting action against *C. albicans* by targeting ergosterol and hyphal transition alone or in combination with stand antifungal drugs. Further, *C.albicans* forms filamentous growth in several environmental conditions without blocking cell cycle progression. To study the effect of hydroxychloroquine on *C. albicans* cell cycle, flow cytometry technique was used, and it was observed that hydroxychloroquine -treated cells arrested in G0/G1 phase as compared with control (Fig. 5.6). It may be responsible for inhibiting the filamentous growth of *C. albicans* [15]. In addition to ergosterol biosynthesis, ROS induction plays an important role in the potential mechanisms of antifungal drugs. Based on this result, it was concluded that hydroxychloroquine treatment caused enhancement in production of ROS in *C. albicans* both planktonic and biofilm cells, which cause lipid peroxidation of the cell membrane, which damages phospholipids directly and inhibition biofilm lead to cell death. ROS production is a multifaceted and critical aspect of *C. albicans* pathobiology (Fig. 5.7). The overproduction of ROS may lead to cell damage and death. The ROS species induce oxidative damage to proteins, lipids, and DNA. Failure to suppress this damage is connected with cell death. Hydroxychloroquine significantly increases ROS production. ROS enhanced accumulation can be another mode of action hydroxychloroquine in *C. albicans.* To validate this data, we performed gene expression study by q RTPCR. Hydroxychloroquine treatment to *C. albicans* upregulated the expression of *SOD1* and *SOD2*. These genes involved in the destruction of a toxic radical and reduce oxidative stress. This hypothesis supports ROS production experiment. The gene expression of the *SOD1* and *SOD2* genes in *C. albicans* were upregulated by 2-fold and 6-fold change respectively after the treatment of hydroxychloroquine, it was noted that the *CAT* gene, which helps to protects *C. albicans* cells from oxidative stress, was upregulated by 4-fold.

Based on this data it was revealed that, the effect on hydroxychloroquine on ROS production and gene expression to be one of the mode of actions against *C. albicans* [16]. In summary, the multitarget mechanism of action of hydroxychloroquine strongly supports the development and optimization of the antifungal activity against *C. albicans*.

## 5.5. Conclusions

The present study is the first report of the antifungal potential of hydroxychloroquine against *C. albicans*. Hydroxychloroquine significantly inhibited planktonic growth and virulence factors such as yeast to hyphal formation, adhesion and biofilm formation at 0.5 mg/ml, 2 mg/ml and 0.25 mg/ml *in C. albicans*. Hydroxychloroquine found to inhibit ergosterol biosynthesis and cell cycle progression at the G0/G1 phase in *C. albicans.* Ergosterol biosynthesis and cell cycle are proposed targets of hydroxychloroquine responsible for inhibition of the growth of *C. albicans*. Hydroxychloroquine upregulates *SOD1*, *SOD2*, *MCA1*, *CAT1 and CAP1* gene expressions in *C. albicans*. Since hydroxychloroquine is found to have multiple targets in *C. albicans*, the chances of developing drug resistance are less. Preclinical and clinical studies are necessary to confirm the antifungal efficacy for *C. albicans* infections.

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# CHAPTER VI

# ANTIFUNGAL ACTIVITY OF MEFLOQUINE ON *CANDIDA ALBICANS*

## 6.1. Introduction

Mefloquine has antifungal activity for *C. albicancs* by targeting the vacuole, morphogenesis and disrupting mitochondrial proton motive force [2]. It also exhibits antimicrobial activity against *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli* and *Mycobacterium avium* [3]. The current work explores the antifungal efficacy and probable mode of action of mefloquine in *C. albicans* through inhibition of germ tube formation, adhesion, biofilm, ergosterol synthesis, ROS production, cell cycle arrest, and biofilm formation experiments.

## 6.2. Materials and Methods

Mefloquine was purchased form TCI chemicals Pvt. Ltd., India and media components were purchased form Sigma Aldrich Ltd., Mumbai, India. Mefloquine was dissolved in DMSO (2%) solution and 10 mg/ml stock was made for further studies. 0.062 mg/ml to 4 mg/ml concentration series was used for experiments. Methods were followed as per chapter III, page no 33-38.

## 6.3. Results

### 6.3.1. Mefloquine inhibited *C. albicans* planktonic growth

The concentration series of mefloquine ranged from 0.062 mg/ml to 4 mg/ml prepared and *C. albicans* cells were treated for 48 h. The MIC of mefloquine against *C. albicans* planktonic growth was observed at 0.25 mg/ml. The effect of mefloquine on planktonic growth of *C. albicans* ATCC 90028 was observed concentration dependent manner (Fig. 6.1 A). Mefloquine inhibited 99 % planktonic growth at 4 mg/ml and 80 % at 2 mg/ml. Fungicidal nature of mefloquine was confirmed by MFC assay; at 4 mg/ml concertation no colonies were observed on YPD plate, while 2 mg/ml concertation showed a smaller number of colonies as compared to control. Hence 4 mg/ml was considered as MFC of mefloquine against *C. albicans* (Fig. 6.1. B).

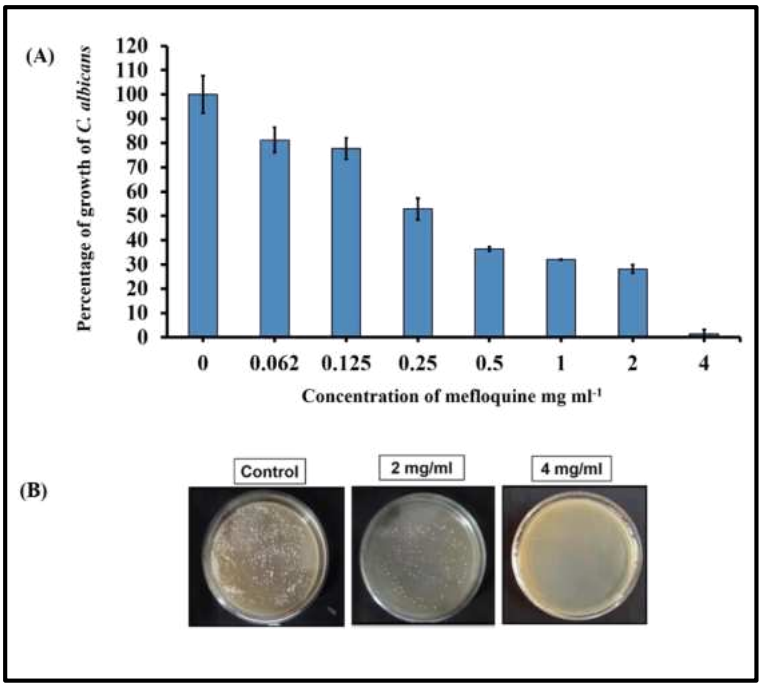


**Fig. 6.1.** Antifungal activity of mefloquine on growth of *C. albicans;* (A) concentration dependent inhibition of planktonic growth of *C. albicans* (B) 4 mg/ml is the MFC of mefloquine against *C. albicans.*

### 6.3.2. Mefloquine inhibited yeast to hyphal formation in *C. albicans*

*C. albicans* cells were exposed to 0.062 mg/ml to 4 mg/ml concentration range of mefloquine prepared in fetal bovine serum for 90 min. The significant inhibition of serum-induced germ tube formation was observed at 0.125 mg/ml and above concentrations (Fig. 6.2).

### 6.3.3. Mefloquine inhibited adhesion of *C. albicans*

Adhesion has crucial role of infection and biofilm formation by *C. albicans*. The impact of mefloquine on the adhesion of *C. albicans* was investigated on polystyrene surfaces of 96 well plate. The findings indicated that the treatment of mefloquine at a concentration of 0.25 mg/ml resulted in 50 % decrease in the adhesion by *C. albicans* on the polystyrene surface (Fig. 6.2).

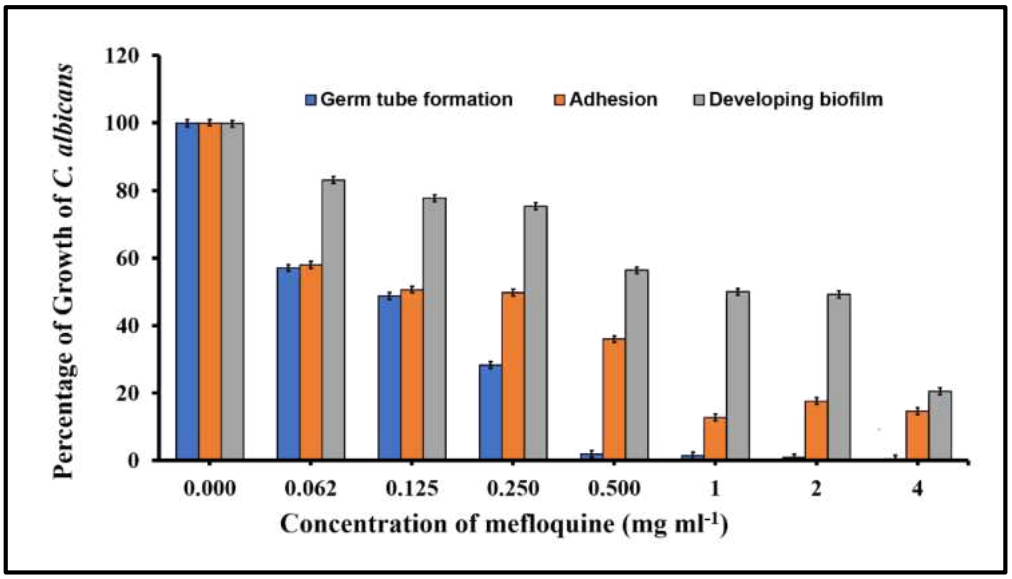


**Fig. 6.2.** Concentration-dependent inhibitory activity of mefloquine on yeast to hyphal formation, adhesion and developing biofilm against *C. albicans*

### 6.3.4. Mefloquine inhibited developing biofilm of *C. albicans*

The biofilm formation is a virulence factor and plays important role in the pathogenesis of *C. albicans*. Mefloquine at 1 mg/ml concentration inhibited biofilm formation in *C. albicans*. The mefloquine affected on developing biofilm observed by using inverted microscope and the biofilm growth was quantified by XTT metabolic assay (Fig. 6.2).

### 6.3.5. Inhibition of biofilm formation confirmed by SEM

Scanning electron microscopic images depicted the complete inhibition of biofilm formation on catheter pieces in presence of mefloquine at 1 mg/ml concentration. The biofilm of *C. albicans* was observed on control catheter piece (without treatment of mefloquine) while in presence of mefloquine at 1 mg/ml concentration only yeast cells were observed on catheter surface (Fig. 6.3).

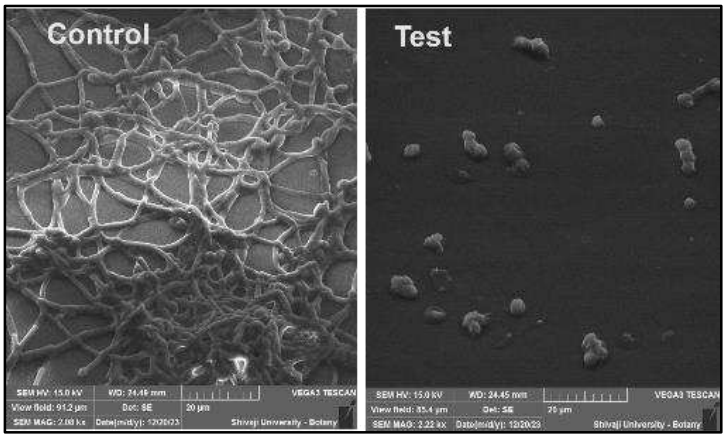


**Fig. 6.3.** Scanning electron micrograph of *C. albicans* biofilm. Control: without mefloquine treatment; Test: inhibition of biofilm formation by the treatment of 1 mg/ml mefloquine.

### 6.3.6. Mefloquine damages the cell membrane of *C. albicans*

The membrane integrity of *C. albicans* studied using the membrane-specific fluorescence dye propidium iodide (PI). The fluorescence microscopy images depicted that a concentration-dependent increase in the red fluorescence was observed in mefloquine treated *C. albicans* cells while control cells did not show red fluorescence (Fig. 6.4). These findings confirmed that PI penetrates the cell membrane and binds with DNA in *C. albicans* cells treated with mefloquine which proved that mefloquine causes membrane damage of *C. albicans*.

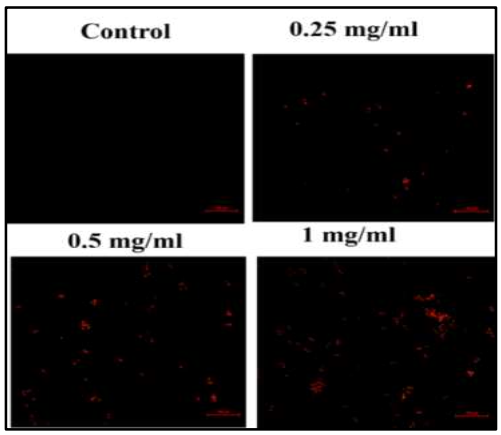


**Fig. 6.4.** Fluorescent microscopic images of PI stained of *C. albicans* cells with and without treatment of mefloquine at different concertation.

### 6.3.7. Mefloquine inhibits ergosterol biosynthesis

To determine the ergosterol content in *C. albicans* cells treated with mefloquine, UV spectrophotometric-based sterol quantification method was used. The characteristic curve for sterol content is observed at 0.125 mg/ml, 0.06 mg/ml, 0.031mg/ml, and control while the characteristic curve is not observed at 0.5 mg/ml and 0.25 mg/ml of mefloquine. This proved that mefloquine treatment inhibits the ergosterol synthesis in a concentration-dependent manner (Fig. 6.5 A, B).

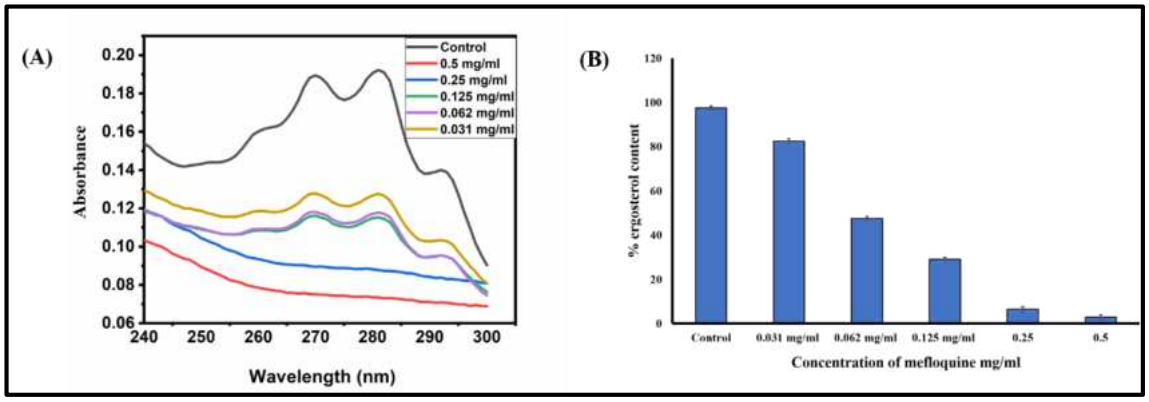


**Fig. 6.5.** Effect of mefloquine on ergosterol biosynthesis. (A) Sterol spectral profiles of *C. albicans* and Fluorescent microscopic images of PI stained of *C. albicans* cells with and without treatment of mefloquine at different concertation. (0.031 mg/ml to 0.5 mg/ml) treated cells (B) The concertation dependent reduction of ergosterol content in mefloquine treated (0.031 mg/ml - 2 mg/ml) *C. albicans* as compared with control.

### 6.3.8. Mefloquine increases ROS level in *C. albicans*

Reactive oxygen species (ROS) accumulation is a pivotal factor in the pathogenicity and adaptive responses of *C. albicans,* facilitating its survival in the host. A study on ROS accumulation revealed heightened intracellular ROS levels upon mefloquine treatment to *C. albicans* log phase cells. Specifically, mefloquine increased ROS generation at concentrations of 0.25 mg/ml in planktonic cells and 1 mg/ml in biofilm-forming *C. albicans* cells, quantified using the $H_2$DCFH fluorescent dye. Consequently, mefloquine treatment increased the ROS production in both planktonic and biofilm states of *C. albicans* (Fig. 6.6).

This induction of ROS may lead to lipid peroxidation of the cell membrane, directly damaging phospholipids. Additionally, the observed inhibition of biofilm formation has been proposed as a potential target in *C. albicans*.

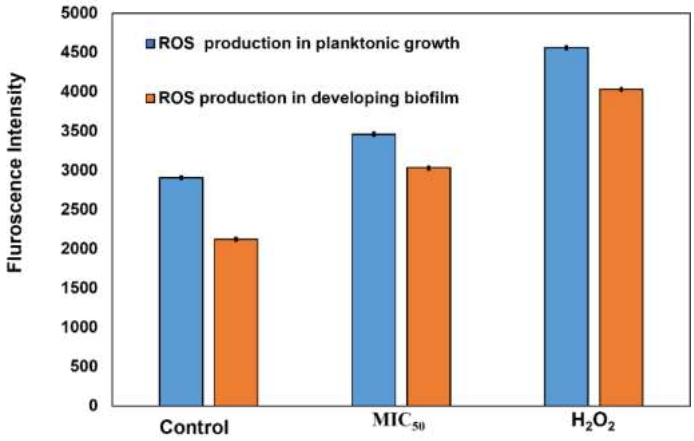


**Fig. 6.6.** Effect of mefloquine on ROS generation of *C. albicans.*

### 6.3.9. Mefloquine inhibits cell division in *C. albicans*

The flow cytometry analysis showed that mefloquine arrests *C. albicans* cell division at different phases of the cell cycle. In this study, control (without mefloquine treatment) showed 56.3 % cells in G1/G0 phase, 9 % in G2/M phase and 32 % in S phase whereas in mefloquine treated samples showed 26 % in G1/G0 phase, 29 % G2/M phase and 43 % in S phase. These results confirmed that mefloquine arrests the cell cycle of *C. albicans* in G2/M phase and S phase by the treatment at a concentration of 0.25 mg/ml (Fig. 6.7).

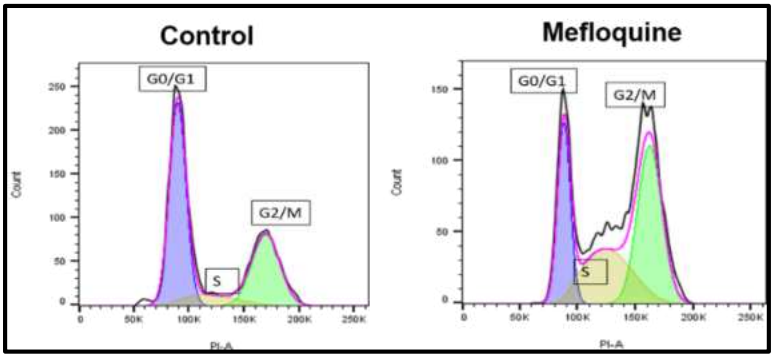


**Fig. 6.7.** Effect of mefloquine on cell cycle progression of *C. albicans*. *C. albicans* cells treated with 0.25 mg/ml concentration of mefloquine are arrested at G2/M phase and S phase compared with control (without treated with mefloquine).

### 6.3.10. Gene expression analysis in *C. albicans* planktonic growth

qRT-PCR experiment was performed to study the effect of mefloquine on the gene expression level in *C. albicans* planktonic cells. qRT-PCR study revealed that treatment of mefloquine at 0.25 mg/ml to *C. albicans* showed upregulation of *SOD1, SOD2, KRE9* gene expression by 3.7, 3.5, and 3.1 fold and *MCA1, ALS1* and *CAP1* gene expressions by 4.6, 2 and 2.6 fold, respectively (Fig. 6.8). The change in the gene expression level may be responsible for inhibition of *C. albicans* growth.

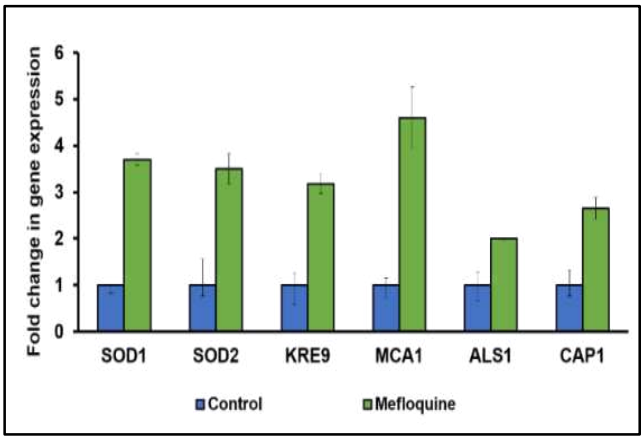


**Fig. 6.8.** Effect of mefloquine on gene expression of *C. albicans.* Mefloquine (0.25 mg/ml) treated *C. albicans* cells showing upregulation of gene expression of *SOD1*, *SOD2*, *MCA1*, *ALS1* and *CAP1* genes.

### 6.3.11. *In vivo* efficacy study of mefloquine in silkworm model

The antifungal efficacy of mefloquine against *C. albicans* pathogenesis was studied in the silkworm animal model. The infection model was developed by injecting $1\times10^6$ cells/ml through the $6^{th}$ leg of the $5^{th}$ instar larvae and silkworm. Infected silkworms (n=6) treated with 0.5 mg/ml mefloquine survived and completed it is life cycle, whereas silkworm larvae with any treatment died within 24 h (Fig. 6.9). Silkworm injected with DMSO was considered as a DMSO vehicle control while *C. albicans* infected silkworm treated with fluconazole served as positive control. These two groups also were survived up to the 72 h and beyond. The results has been shown in Fig. 6.9.

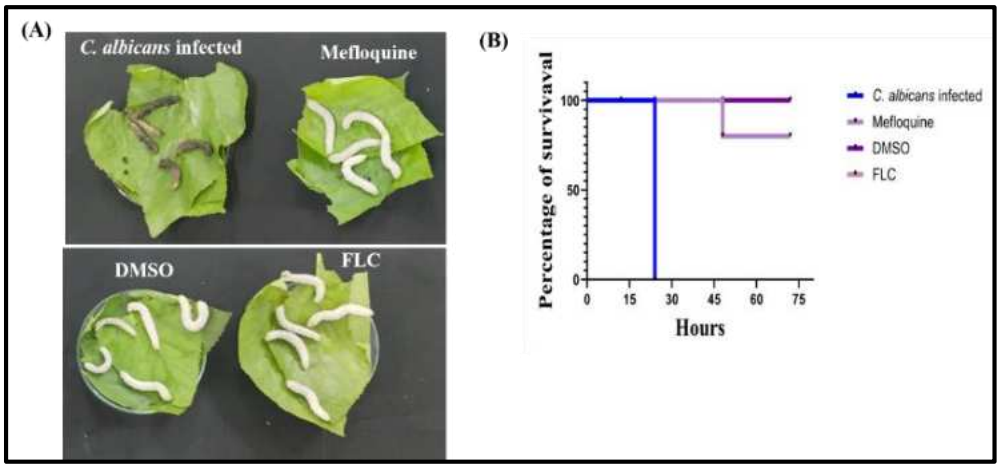


**Fig. 6.9.** (A) *in vivo* experiment in silkworms (B) The graph indicating percentage of silkworm survived.

### 6.4. Discussion

Fungal infections pose a significant health threat, particularly to those with weakened immune systems and *C. albicans* play inherent resistance to medications [4]. Nowadays drug-resistant *C. albicans* strains are evolving and this requires new interventions or strategies for the treatment. Repurposing initiatives have typically concentrated on finding of medicines that can be directly used in new clinical use without alteration of the structure or formulation [5]. Mefloquine is an antimalarial drug but it has shown antiviral activity at 10 µM against feline calicivirus found in cats and is causative of respiratory tract infections. It reduces the virus load in a concentration-dependent manner [6]. Mefloquine is reported to have antifungal activity against *C. albicans* strain SC5314 by targeting mitochondrial and vacuoles function [2]. The current study has brought new insights into the mechanism of action of mefloquine against *C. albicans*. Mefloquine inhibited the planktonic growth in a concertation-dependent manner and showed significant fungicidal activity at 4 mg/ml concentration. Mefloquine restricted the yeast to hyphal formation at 0.5 mg/ml concentration. Biofilm formation and yeast to hyphal formation are major virulence traits towards *C. albicans* pathogenicity. Biofilms are microbial cell populations encased in an extracellular polysaccharide matrix and less sensitive to antifungal medications for superficial and systematic infection caused by *C. albicans* [7].

Mefloquine also was found to inhibit biofilm of *C. albicans* at 1 mg/ml. Further, scanning electron microscopic and confocal microscopic analysis confirmed that mefloquine inhibits the virulence factors including yeast to hyphal, biofilm formation, and adhesion in *C. albicans*, which is essential for the success of an antifungal therapy. Besides cell growth and virulence traits, other probable targets of mefloquine in *C. albicans* are proposed by observing the results of PI uptake assay, ergosterol quantification, and cell cycle studies. All these changes are involved in membrane and cytoplasmic content. On this basis, cellular changes by cell cycle and changes in cell membrane by PI uptake and ergosterol biosynthesis, to understand the possible mechanism of action of mefloquine. It was shown that mefloquine causes damage to *C. albicans* cell membrane. Treated *C. albicans* cells uptake more due to disruption of cell membrane integrity in a concentration-dependent manner. The present investigation of the PI uptake assay was observed with 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml of mefloquine, the enhanced permeability of *C. albicans* cellular membrane caused by mefloquine exposure could be due to contact with negatively charged components of the fungal surface. A decrease in plasma membrane ergosterol content. The primary mode of action of azole antifungals occurs through inhibiting the 14-α-demethylase enzyme, which inhibits the synthesis of ergosterol, the main sterol component of the fungal cell membrane, and maintains cellular function and integrity [8]. In current study ergosterol synthesis assay revealed that mefloquine also inhibits ergosterol synthesis in cell membrane of *C. albicans*. In addition to influencing ergosterol biosynthesis, the induction of reactive oxygen species (ROS) plays a crucial role in the potential mechanisms of antifungal drugs. ROS production stands out as a multifaceted and vital aspect of *C. albicans* pathobiology, contributing to the fungus's ability to evade the host immune system, adapt to various stressors, form biofilms, undergo morphological transitions, acquire essential nutrients, and express virulence factors. The ROS accumulation assay has unveiled that mefloquine has a discernible impact on intracellular ROS levels in *C. albicans*.

The overproduction of ROS induced by mefloquine may result in cellular damage and subsequent cell death, as ROS species are known to cause oxidative harm to proteins, lipids, and DNA. Failure to counteract this damage is associated with cell death, highlighting ROS as a significant player in *C. albicans* biology [9]. Moreover, mefloquine's significant enhancement of ROS production suggests that this may be an additional mode of action against *C. albicans*. To further investigate the impact of mefloquine, a gene expression study was conducted. Mefloquine treatment led to a notable upregulation in the expression of both *SOD1* and *SOD2* genes, which are involved in neutralizing toxic radicals and reducing oxidative stress. This finding aligns with the ROS production experiment, supporting the notion that mefloquine affects *C. albicans* through ROS modulation. The gene expression analysis revealed a 3.7-fold and 3.5-fold increase in the expression of *SOD1* and *SOD2*, respectively, after mefloquine treatment. The *MCA1* gene in *C. albicans* is an apoptosis regulator at the transcriptional level [10]. They were upregulated by 4.6-fold in response to mefloquine which may cause apoptosis. Mefloquine significantly upregulated the cell wall synthesis gene *KRE9* by 3.1-fold. *MCA1* gene was upregulated by 4.6-fold respectively. Furthermore, the upregulation of the *ALS1* gene encodes cell surface glycoprotein implicated in adhesion to host surfaces [11] was observed at a 2 fold increases after the treatment of mefloquine. The upregulation of the *CAP1* gene, responsible for shielding *C. albicans* cells from oxidative stress, was observed at a 2.6-fold increase following mefloquine treatment. Collectively, these findings suggest that mefloquine's impact on ROS production, coupled with the modulation of gene expression, constitutes one of its modes of action against *C. albicans*. In addition, this study showed that mefloquine arrests cell cycle of *C. albicans* at G2/M phase and S phase. However, according to reports, pro-apoptotic therapies lead to G2/M phase arrest in the cell cycle of *C. albicans* [12]. Wani et al. (2021), observed the coincidence of the DNA damage repair checkpoint activation and the G2/M phase. The induction of yeast cell death may result in DNA damage due to reactive oxygen species (ROS) production, ultimately leading to G2/M and S phase cell cycle arrest [13].

To summarize, mefloquine's extensive spectrum of activity, well-established and advantageous pharmacological properties, along with its multitarget mechanism of action, strongly endorse the pursuit of developing and optimizing its antifungal capabilities.

### 6.5. Conclusions

The current study revealed that mefloquine inhibits growth and virulence factors such as yeast to hyphal formation, biofilm formation in *C. albicans*. The mode of action studies showed that, mefloquine arrests cells at G2/M phase and S phase of cell cycle and inhibits ergosterol biosynthesis in *C. albicans*. mefloquine treatment also induces oxidative stress in *C. albicans* by increasing ROS level. The upregulation of gene expression of *SOD1, SOD2, CAT1* genes in *C. albicans* is also observed in the presence of mefloquine. Mefloquine is found to have multiple targets in *C. albicans*, so the chances of developing drug resistance are less.

## 6.6. References

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# CHAPTER VII

## EFFECT OF OTHER ANTIMALARIAL DRUGS ON CANDIDA ALBICANS GROWTH AND VIRULENCE FACTORS

## 7.1. Introduction

In the current study, eight antimalarial drugs were tested for their effects on various aspects of *Candida albicans* growth and development, including planktonic growth, yeast to hyphal transition, adhesion, and biofilm formation.

**i) Sulfadoxine:**

Mechanism: A long-acting sulphonamide that targets Plasmodium dihydropteroate synthase and dihydrofolate reductase. Sulfadoxine competes with para-aminobenzoic acid (PABA) for incorporation into folic acid, exploiting differences in folic acid metabolism between mammalian cells and other cells [1].

Uses: Treatment and prevention of malaria, and respiratory and urinary tract infections, typically in combination with other drugs.

**ii) Pyrimethamine:**

Mechanism: Inhibits dihydrofolate reductase in Plasmodium, blocking the biosynthesis of purines and pyrimidines, which are crucial for DNA synthesis and cell multiplication [2].

Uses: Prevention and treatment of toxoplasmosis and malaria.

**iii) Artesunate:**

Mechanism: An artemisinin derivative metabolized to dihydroartemisinin (DHA). The endoperoxide bridge of DHA reacts with heme, producing free radicals that inhibit protein and nucleic acid synthesis [3].

Uses: Initial treatment of severe malaria.

**iv) Piperaquine:**

Mechanism: The exact mechanism is unknown, but it is believed to inhibit the heme detoxification pathway, similar to chloroquine [4].

Uses: An antimalarial agent historically used in China; its use declined due to resistance and the availability of artemisinin derivatives.

**v) Atovaquone:**

Mechanism: A hydroxynaphthoquinone, or ubiquinone analog, that inhibits the cytochrome bc1 complex (Complex III) in Plasmodium species, affecting the mitochondrial electron transport chain and indirectly inhibiting several metabolic enzymes [5].

Uses: Prevention and treatment of Pneumocystis jirovecii pneumonia (PCP) and Plasmodium falciparum malaria.

**vi) Artemether:**

Mechanism: Administered in combination with lumefantrine, it interacts with heme to form toxic radicals, disrupting the erythrocytic stages of Plasmodium spp.[6].

Uses: Treatment of acute uncomplicated malaria, effective against chloroquine-resistant strains.

**vii) Amodiaquine:**

Mechanism: a 4-aminoquinoline that may cause hazardous free heme to accumulate by inhibiting the activity of heme polymerase. When free heme is bound by the medication, a hazardous compound is formed that impairs membrane function [7].

Uses: Antimalarial and anti-inflammatory agent, effective against some chloroquine-resistant strains

**viii) Chloroquine:**

Mechanism:

Chloroquine inhibits the action of heme polymerase in malarial trophozoites, preventing the conversion of heme to hemazo in. *Plasmodium* species continue to accumulate toxic heme, killing the parasite [8].

Uses: Antimalarial and anti-inflammatory agent.

This study investigated the potential of antimalarial drugs for antifungal effects against *C. albicans*, focusing on their ability to inhibit planktonic growth, yeast to hyphal morphological transformation, adhesion and biofilm formation.

## 7.2. Materials and methods

Sulfadoxine, Pyrimethamine, Atovaquone, Artether, Artesunate, Piperaquine, and Amodiaquine and Chloroquine were purchased form TCI chemicals Pvt. Ltd., India. Methods followed by Chapter III Page No. 33 to 38.

## 7.3. Results

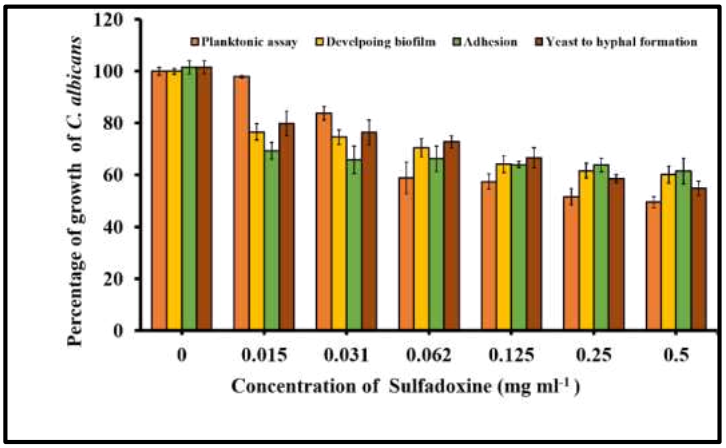
### 7.3.1. Activity of sulfadoxine on planktonic growth and virulence factors of *C. albicans*

The broth microdilution method was used to measure the inhibitory effect of sulfadoxine on the planktonic growth of *C. albicans*. Cells of *C. albicans* were treated with sulfadoxine at concentrations ranging from 0.015 mg/ml to 0.5 mg/ml; untreated cells were used as the control. The results showed that sulfadoxine has no effect on planktonic growth, developing biofilm, adhesion and yeast to hyphal formation of *C. albicans* in concentration dependent manner (Fig. 7.1 A).

### 7.3.2. Activity of pyrimethamine on planktonic growth and virulence factors of *C. albicans*

The inhibitory effect of pyrimethamine on planktonic growth of *C. albicans* was assessed by using broth microdilution method. Cells of *C. albicans* were treated with 0.007 mg/ml to 0.25 mg/ml concentration range of pyrimethamine and without treatment considered as control. The experimental data depicts that, pyrimethamine has not inhibited the planktonic growth, developing biofilm, adhesion and yeast to hyphal formation of *C. albicans* (Fig. 7.1 B).

**A)**




**B)**

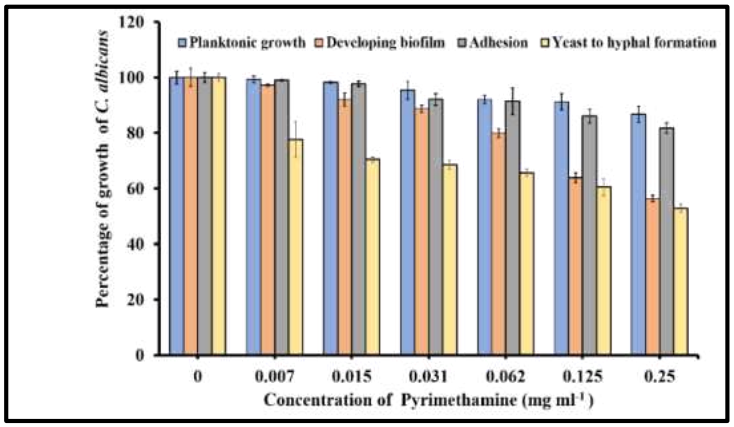



**Fig. 7.1.** A) The effect of Sulfadoxine on planktonic growth, developing biofilm, adhesion and yeast to hyphal formation B) The effect of Pyrimethamine on planktonic growth, developing biofilm, adhesion and yeast to hyphal formation in *C. albicans.*

### 7.3.3. Activity of Artesunate on planktonic growth and virulence factors of *C. albicans*

The broth microdilution method was used to measure the inhibitory effect of artesunate on the planktonic growth of *C. albicans*. Cells of *C. albicans* were treated with artesunate at concentrations ranging from 0.125 mg/ml to 4 mg/ml; untreated cells were used as the control (Fig.7.2). The results showed that artesunate had no effect on *C. albicans* planktonic development, yeast to hyphal formation, adhesion and biofilm formation.

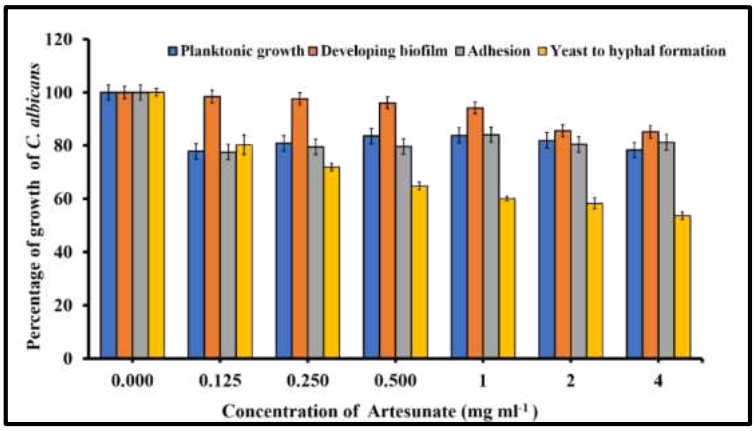



**Fig. 7.2.** The effect of Artesunate on planktonic growth, developing biofilm, adhesion and yeast to hyphal formation in *C. albicans.*

### 7.3.4. Activity of Piperaquine on Planktonic growth and virulence factors of *C. albicans*

The inhibitory effect of piperaquine on planktonic growth of *C. albicans* was assessed by using broth microdilution method. Cells of *C. albicans* were treated with 0.007 mg/ml to 0.25 mg/ml concentration range of piperaquine and without treatment considered as control. As per the experimented data was piperaquine was not affected the planktonic growth, developing biofilm, adhesion and yeast to hyphal formation of *C. albicans* (Fig. 7.3).

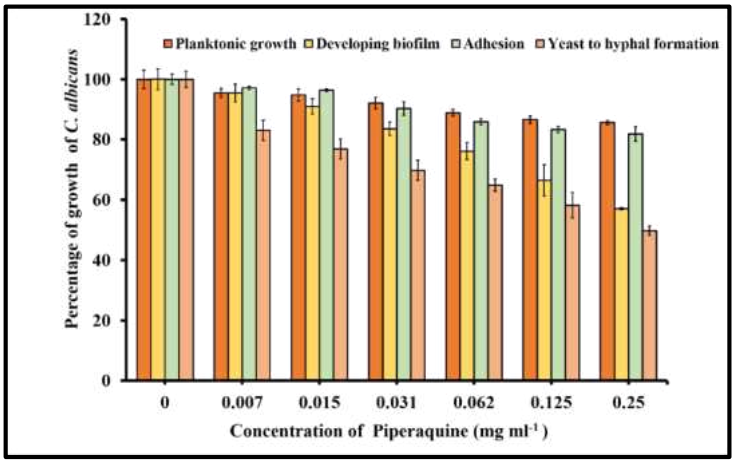



**Fig. 7.3.** The effect of Piperaquine on planktonic growth, developing biofilm, adhesion and yeast to hyphal formation in *C. albicans.*

### 7.3.5. Activity of Artemether on planktonic growth and virulence factors of *C. albicans*

The broth microdilution was used to measure the inhibitory effect of artemether on the planktonic growth of *C. albicans*. Cells of *C. albicans* were treated with sulfadoxine at concentrations ranging from 0.015 mg/ml to 0.5 mg/ml; untreated cells were used as the control. The results showed that sulfadoxine has no effect on *C. albicans* planktonic growth, developing biofilm, adhesion and yeast to hyphal formation (Fig.7.4. A).

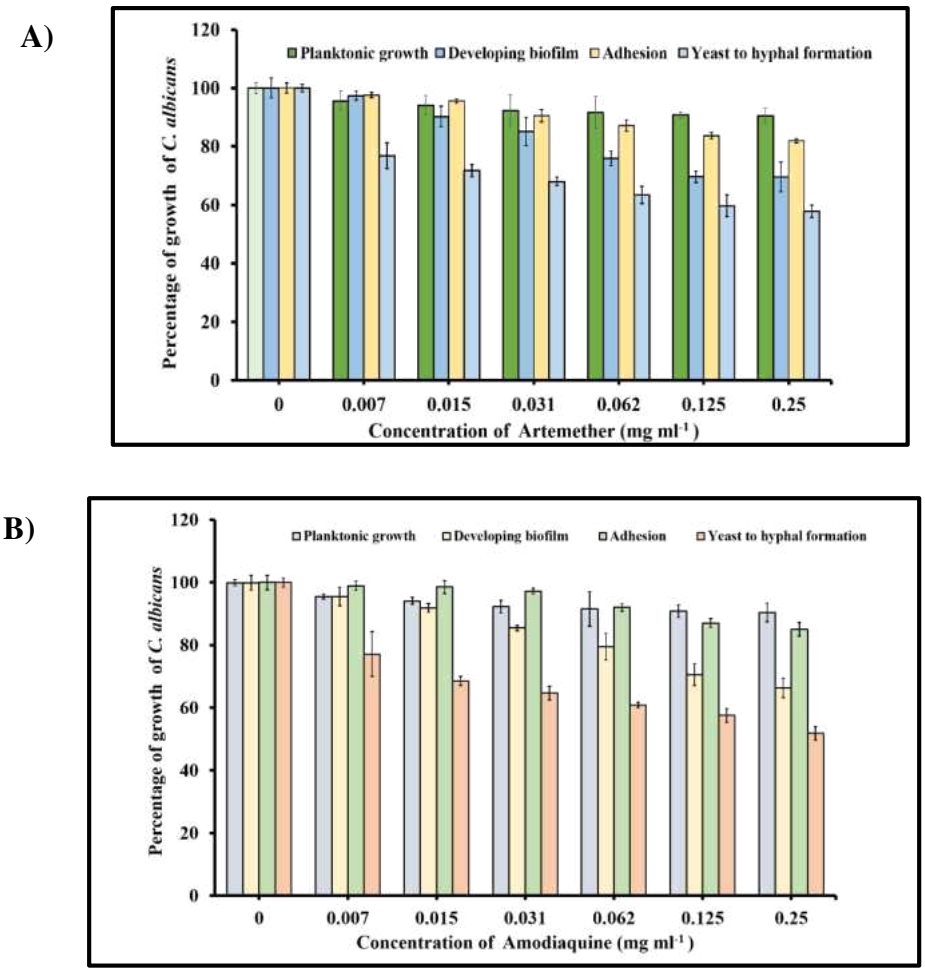



**Fig. 7.4.** A) The effect of Artemether on planktonic growth, developing biofilm, adhesion and yeast to hyphal formation. B) The effect of Amodiaquine on planktonic growth, developing biofilm, adhesion and yeast to hyphal formation in *C. albicans.*

### 7.3.6. Activity of Amodiaquine on Planktonic growth and virulence factors of *C. albicans*

The inhibitory effect of amodiaquine on planktonic growth and virulence factors of *C. albicans* was assessed by using broth microdilution method. *C. albicans* cells were treated with 0.007 mg/ml to 0.25 mg/ml concentration range of amodiaquine and without treatment considered as control. As per the experiments amodiaquine not inhibited the planktonic growth, developing biofilm, adhesion and yeast to hyphal formation of *C. albicans* (Fig.7.4 B).

### 7.3.7. Activity of Chloroquine on planktonic growth and virulence factors of *C. albicans*

The inhibitory effect of chloroquine on planktonic growth of *C. albicans* was assessed by using broth microdilution method. *C. albicans* cells were treated with 0.007 mg/ml to 0.25 mg/ml concentration range of chloroquine and without treatment considered as control. As per the experiments chloroquine was not able to inhibit planktonic growth, developing biofilm, adhesion and yeast to hyphal formation of *C. albicans* (Fig. 7.5). There is concentration dependent inhibition of adhesion, planktonic growth, and developing biofilm of *C. albicans* shown in Fig. 7.5.

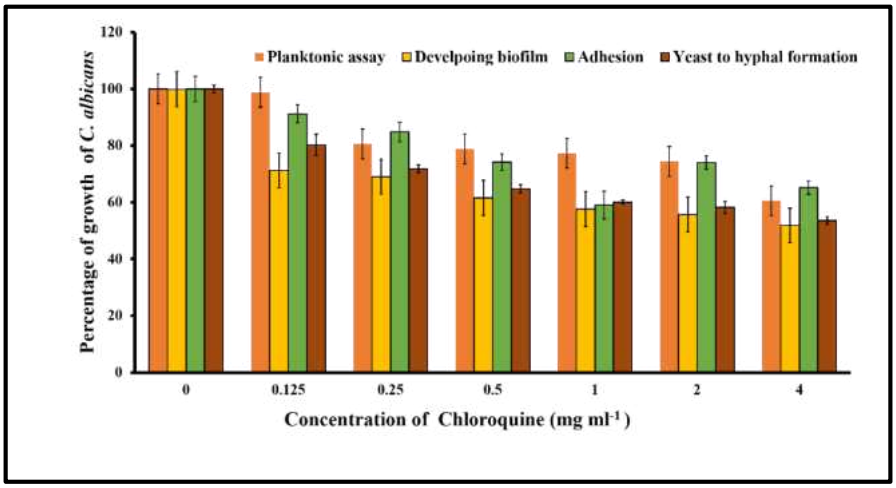



**Fig. 7.5.** The effect of Chloroquine on planktonic growth, developing biofilm, adhesion and yeast to hyphal morphogenesis formation in *C. albicans.*

## 7.4. Conclusions

The inhibitory effects of Sulfadoxine, Pyrimethamine, Artesunate, Piperaquine, Artemether, Amodiaquine and Chloroquine on the planktonic growth, yeast to hyphal formation, adhesion, and biofilm formation of *C. albicans* were assessed using the microdilution broth method. The compounds tested included sulfadoxine, pyrimethamine, artesunate, piperaquine, artemether, amodiaquine, and chloroquine, each in a concentration range of 0.007 mg/ml to 0.25 mg/ml, with untreated cells serving as the control. No inhibition was observed of planktonic growth, yeast to hyphal formation, adhesion, and biofilm formation at tested concentrations. Concentration-dependent inhibition was observed for adhesion, planktonic growth, and biofilm development. Only chloroquine inhibited yeast to hyphal formation except chloroquine, chloroquine inhibited Y-H transition. While none of the compounds tested inhibited the yeast to hyphal formation of *C. albicans*, all demonstrated a concentration-dependent inhibitory effect on adhesion, planktonic growth, and biofilm formation. These findings suggest that, despite the lack of effect on morphological switching, these compounds can significantly reduce the ability of *C. albicans* to grow, adhere, and form biofilms in a dose-dependent manner. Although none of the substances examined prevented the yeast from forming *C. albicans* hyphae, they all showed concentration-dependent inhibitory effects on adhesion, planktonic development, and biofilm formation.

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# CHAPTER VIII

## COMPARISON OF ANTIFUNGAL EFFICACY OF ANTIMALARIAL DRUGS WITH STANDARD ANTIFUNGAL DRUG

## 8.1. Introduction

The global burden of fungal diseases poses a significant challenge, affecting both developing and developed countries. The treatment of life-threatening fungal infections is often inefficient and costly. The oral cavity, gastrointestinal tract, and skin are frequently colonised by the opportunistic fungal pathogen *Candida albicans.* It's linked to high rates of morbidity and death, particularly in individuals with compromised immune systems or disrupted microbiota, where invasive growth can lead to severe infections. The antifungal drugs available for treating *C. albicans* infections are limited to the azole, polyene, and echinocandin classes. However, these drugs often fail due to multiple drug resistance mechanisms and alternative survival strategies employed by the pathogen, such as biofilm formation. Additionally, the toxicity of these drugs leads to side effects, further complicating treatment. Therefore, there is a pressing need to discover novel molecules with antifungal properties. Developing a new drug is a time-consuming and expensive process. In this context, drug repositioning finding new uses for existing drugs emerges as an attractive and cost-effective strategy to address this urgent need. In this chapter the antifungal activity of antimalarial drugs has been compared with fluconazole (FLC) and amphotericin-B (AmB) standard antifungal.

## 8.2. Materials and Methods

FLC and AmB were obtained from Sigma Aldrich Chemicals Co. (Germany). Methods were followed as per mentioned in Chapter III, page no. 33-38.

## 8.3. Result and discussion

### 8.3.1 Effect of FLC on planktonic growth and virulence factors of *C. albicans*

As per the antifungal susceptibility assay, FLC inhibited the only planktonic growth, adhesion and developing biofilm of *C. albicans* in concentration dependent manner. The MIC of FLC against *C. albicans* with 50 % reduced

planktonic growth was at 1.56 µg/ml. FLC inhibits yeast to hyphal formation and adhesion at 6.25 µg/ml and 12.5 µg/ml (Fig. 8.1).

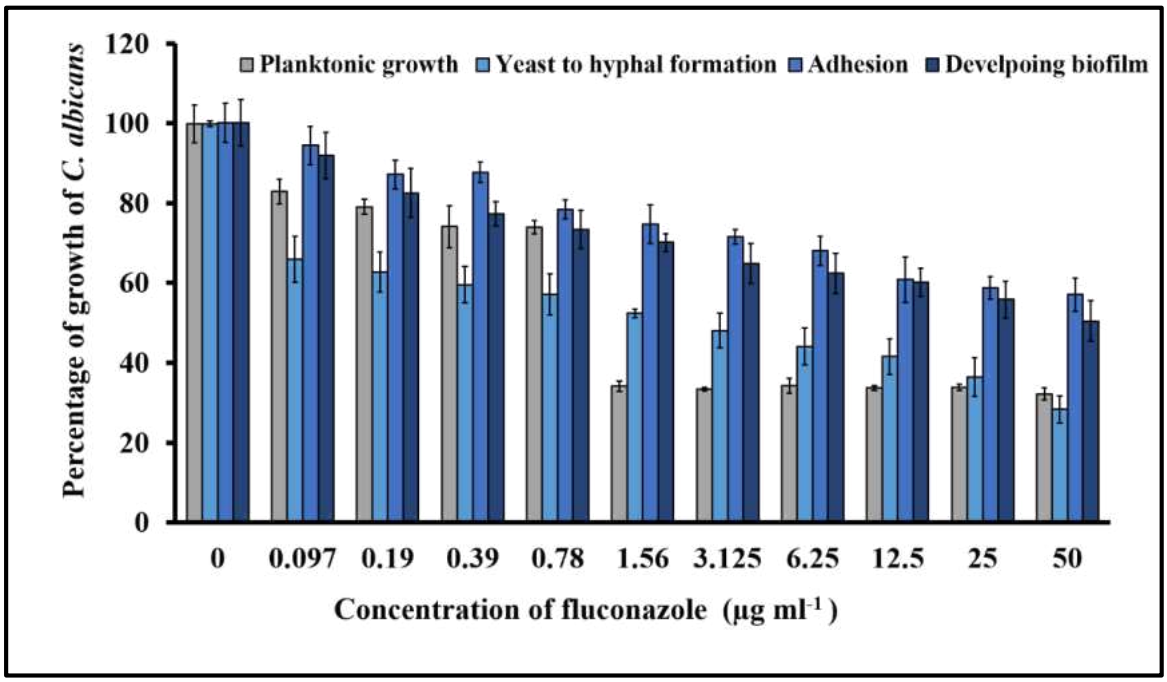


**Fig. 8.1.** Effect of FLC on planktonic growth, yeast to hyphal formation, adhesion and developing biofilm in *C. albicans*.

### 8.3.2. Effect of AmB on planktonic growth and virulence factors of *C. albicans*

AmB inhibited the planktonic growth of *C. albicans* in concentration dependent manner. The MIC of AmB against *C. albicans* with 50 % reduction of planktonic growth was found at 0.39 µg/ml. AmB inhibits yeast to hyphal formation at 3.12 µg/ml. Adhesion and developing biofilm was inhibited at 12.5 µg/ml and 0.39 µg/ml, respectively (Fig. 8.2)

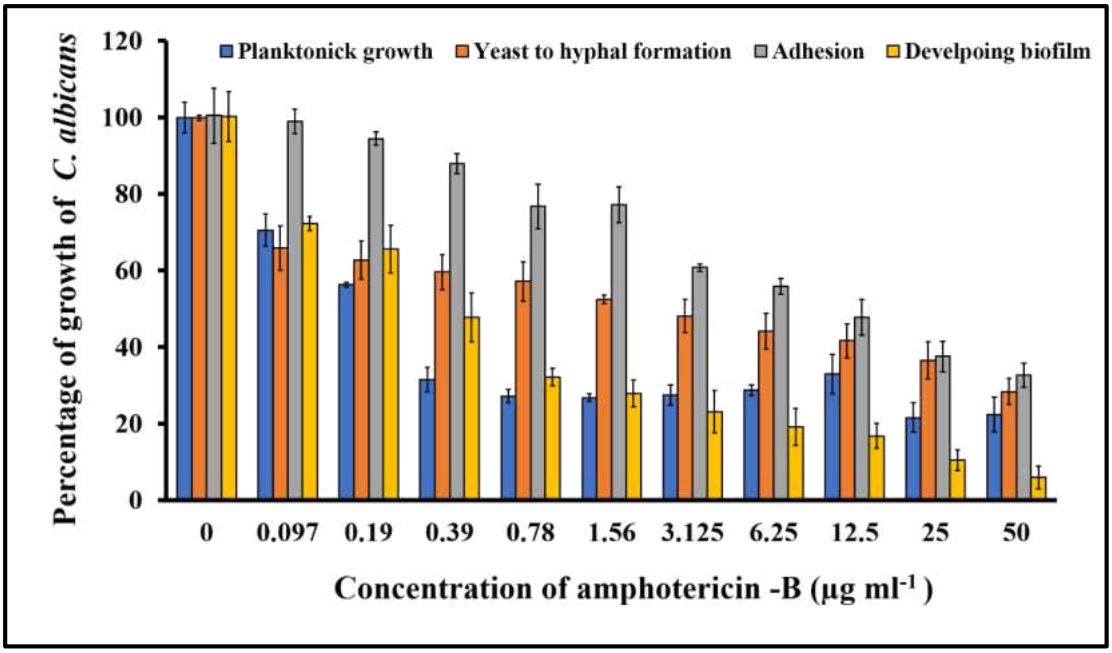


**Fig. 8.2.** Effect of AmB on planktonic growth, yeast to hyphal formation, adhesion and developing biofilm in *C. albicans*.

## 8.4.Conclusions

FLC and AmB standard antifungal drugs were used as positive controls in the study. *C. albicans* biofilm shows resistance to FLC treatment but it is sensitive to Quinine, hydroxychloroquine and mefloquine. Due to toxicity of AmB was less prescribed against *C. albicans.* Based on this study we performed the screening of antimalarial drugs against *C. albicans* and compared its antifungal efficacy with FLC and AmB. The study revealed that quinine, hydroxychloroquine and mefloquine have good antifungal activity against *C. albicans* but at higher concentrations than FLC and AmB. Since MIC values of quinine, hydroxychloroquine and mefloquine are higher than FLC and AmB, the detailed investigation is required to reduce the MIC dosage by using combination approach. In future quinine, hydroxychloroquine and mefloquine can explored as repurposed drugs against *C. albicans* infections. Out of this three drugs mefloquine is best drug for further studies.

**Table 8.1.** MIC of antifungal and antimalarial drugs of planktonic growth, Yeast to hyphal formation, Adhesion and Biofilm formation.

| Sr No. | Name of Drug | Planktonic Growth | Yeast to Hyphal | Adhesion | Biofilm formation |
|---|---|---|---|---|---|
| 1. | FLC | 1.56 µg/ml | 6.25 µg/ml | 12.5 µg/ml | NA |
| 2. | AmB | 0.39 µg/ml | 3.12 µg/ml | 12.5 µg/ml | 0.39 µg/ml |
| 3. | Quinine | 0.5 mg/ml | 0.25 mg/ml | 1 mg/ml | 0.031 mg/ml |
| 4. | Hydroxychloroquine | 0.5 mg/ml | 0.5 mg/ml | 2 mg/ml | 0.25 mg/ml |
| 5. | Mefloquine | 0.25 mg/ml | 0.125 mg/ml | 0.25 mg/ml | 1 mg/ml |
| 6. | Chloroquine | NA | 0.5 mg/ml | NA | NA |
| 7. | Sulfadoxine | NA | NA | NA | NA |
| 8. | Pyrimethamine | NA | NA | NA | NA |
| 9. | Atovaquone | NA | NA | NA | NA |
| 10. | Artesunate | NA | NA | NA | NA |
| 11. | Piperaquine | NA | NA | NA | NA |
| 12. | Artether | NA | NA | NA | NA |
| 13. | Amodiaquine | NA | NA | NA | NA |

NA – Not achieved

# CHAPTER IX

## SUMMARY AND CONCLUSIONS

Fungal infections represent a substantial health concern, especially for individuals with compromised immune systems and *C. albicans* in particular, exhibits intrinsic resistance to medications. Presently, there is a growing emergence of drug-resistant strains of *C. albicans*, necessitating the exploration of novel interventions or treatment strategies. Repurposing efforts have predominantly focused on identifying existing drugs that can be directly repurposed for new clinical applications without necessitating alterations to their structure or formulation.

**Chapter I** provides an introduction of the current thesis. The chapter contains general information about *C. albicans* and its pathogenicity. It displays types of antifungal drugs, resistance of available antifungal drugs. This chapter also explains rationale of thesis.

**Chapter II** presents the review of literature for repurposing of antimalarial drugs. Various reports are available regarding repurposing of antimalarial drugs as antibacterial, anticancer, antiviral and antifungal agents. This chapter discusses about the advantages of drug repurposing. The reported data explored that Chloroquine, Quinine, Mefloquine, Hydroxychloroquine, Artesunate and Piperaquine has antifungal activity against various fungi.

**Chapter III** deals with different methodologies to identify the antifungal activity of antimalarial drugs chloroquine, quinine, mefloquine, hydroxychloroquine artesunate, piperaquine, sulfadoxine, pyrimethamine, atovaquone, artemether and amodiaquine against *C. albicans*. For screening, planktonic growth, yeast to hyphal formation, adhesion and developing biofilm experiments were performed. The effective antimalarial drugs were selected to find out its mode of action against *C. albicans* by using cell cycle studies, ergosterol assay, ROS assay, SEM, PI uptake, cell viability assay and qRT-PCR studies.

**Chapter IV** presents studies on Quinine against *C. albicans* to find out its antifungal activity. The MIC for planktonic growth was at 0.5 mg/ml, yeast to hyphal formation at 0.25 mg/ml, adhesion and biofilm formation at 1 mg/ml and 0.031 mg/ml, respectively. Quinine has Candida cidal activity at 2 mg/ml and within 8 h kills the *C. albicans* cells. PI uptake assay showed that intensity of PI inside the cell increases with increase in quinine concentration. Hence it is proved that

quinine treatment altered cell membrane permeability in *C. albicans*. The cell cycle progression experiment revealed that, quinine arrest cell cycle at G2/M phase. Quinine also inhibited the ergosterol synthesis and enhanced the ROS generation in *C. albicans.* Gene expression study showed upregulation of *SOD1*, *SOD2*, *KRE9*, *MCA1*, *CAT1 and CAP1.* Therapeutic effects of quinine in silkworm animal model study showed quinine treated silkworm survived up to 72 h. All these findings suggest that, quinine can act as antifungal agent against *C. albicans*.

**Chapter V** presents the antifungal activity of hydroxychloroquine against *C. albicans*. The study showed that, hydroxychloroquine inhibits planktonic growth, yeast to hyphal formation, adhesion and developing biofilm of *C. albicans* at 0.5 mg/ml, 0.5 mg/ml, 2 mg/ml and 0.25 mg/ml, respectively. Hydroxychloroquine is fungistatic in nature, did not show any MFC up to 4 mg/ml. HCQ inhibits ergosterol synthesis and arrest G0/G1 phase in *C. albicans*. Hydroxychloroquine enhanced the ROS generation in *C. albicans.* The gene expression study showed upregulation of *SOD1*, *SOD2*, *MCA1*, *CAT1* and *CAP1* gene related to planktonic growth.

**Chapter VI** deals with activity of mefloquine on *C. albicans.* Mefloquine inhibits planktonic growth and virulence factors at concentrations. Mefloquine has shown MFC at concentration 4 mg/ml. The PI uptake assay showed PI penetrates insides the cell membrane and binds with DNA in *C. albicans* cells treated with mefloquine. Mefloquine inhibits ergosterol biosynthesis and enhanced ROS level in *C. albicans*. Mefloquine arrested the cell cycle of *C. albicans* at G2/M and S phase. The gene expression study revealed, the upregulation of *SOD1*, *SOD2*, *KRE9*, *MCA1*, *ALS1*, *CAP1* genes.

**Chapter VII** presents the effect of remaining antimalarial drugs Sulfadoxine, Pyrimethamine, Artesunate, Piperaquine, Atovaquone, Artemether, and Amodiaquine against *C. albicans*. All these drugs could not inhibit planktonic growth, yeast to hyphal formation, adhesion and developing biofilm of *C. albicans*.

**Chapter VIII** contains the comparison of effective antimalarial drugs with standard antifungal drugs FLC and AmB. Quinine, Mefloquine and Hydroxychloroquine compare with FLC and AmB. The MIC of Quinine, Mefloquine and Hydroxychloroquine were observed at 0.5 mg/ml, 0.25 mg/ml and 0.5 mg/ml,

respectively. The MIC of FLC and AmB was observed at 1.56 µg/ml and 0.39 µg/ml, respectively. FLC was not able to inhibit Y-H morphogenesis while QN, MQ and HCQ could inhibit Y-H morphogenesis significantly at 0.25 mg/ml, 0.5 mg/ml and 0.125 mg/ml, respectively.

Based on this comparison quinine and mefloquine are good candidates to find mode of action against *C. albicans*. Ergosterol is one of the important targets of FLC and AmB similarly quinine, mefloquine and hydroxychloroquine also has probable target in ergosterol synthesis of *C. albicans*. The effective antimalarial drugs quinine, mefloquine and hydroxychloroquine inhibits the virulence factors, ergosterol synthesis, cell viability, PI uptake assay, cell cycle and gene expression in *C. albicans*. Since mefloquine has multiple mechanism of action, chances of drug resistances are less.

# RECOMMENDATIONS

## Recommendations

Drug repurposing is promising approach in drug development process. The antimalarial drugs chloroquine, quinine, mefloquine, hydroxychloroquine artesunate, piperaquine, sulfadoxine, pyrimethamine, atovaquone, artemether and amodiaquine screened for its antifungal activity against *C. albicans.* Mefloquine inhibits growth, virulence factors and ergosterol pathway in *C. albicans* at lowest concentration. On the basis of this mefloquine renominated for further studies.

- **Synergistic Effects**: Investigate how mefloquine might work synergistically with current antifungal drugs fluconazole and amphotericin B to enhance treatment efficacy against *C. albicans*.
- **Mechanisms of Resistance**: Study of potential resistance mechanisms that *C. albicans* may develop against these repurposed agents and explore combination therapies to mitigate this issue.
- ***In vivo* Studies**: Confirm the antifungal efficacy of antimalarial drug mefloquine using larger animal models.
- **Preparation of antifungal formulations:** Mefloquine can be used to prepare topical formulations includes cream and gel for candidiasis. These formulations could be more effective to treat fungal skin infections.
- **Clinical Relevance**: Find the pharmacokinetics and safety profiles of mefloquine in relation to antifungal use, considering patient populations that may benefit from such repurposing.

  By focusing on these areas, quinine could be further developed into a promising antifungal agent.

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# D. Y. PATIL MEDICAL COLLEGE KOLHAPUR

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

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Dean & Professor (Obst. & Gyn)

Padmashree Dr. D. Y. Patil
Founder president

Dr. Sanjay D. Patil
President


No. DYPMCK/...301...../2020/IEC

Date: 20 MAR 2020

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

This is to certify that the research project titled,

**"Repositioning of Antimalarial Drugs as Anti*candida albicans agents*."**

Submitted by : **Mrs. Sargun Tushar Basrani**

Under the supervision of appointed Guide (if any): **Dr. Ashwini K. Jadhav**

Has been studied by the Institutional Ethics Committee (IEC) at its meeting held on **20/03/2020** and granted approval for the study with due effect with the following caveats:

1. If you desire any change in the protocol or standard recording document at any time, please submit the same to the IEC for information and approval before the change is implemented.
2. All serious and/or unexpected adverse events due to the drug/procedures tested in the study must be informed to the IEC within 24 hours and steps for appropriate treatment must be immediately instituted.
3. In case of injury/disability/death of any participant attributable to the drug/procedure under study, all compensation is to be made by the sponsor of the study.
4. The Chief investigator/Researcher must inform the IEC immediately if the study is terminated earlier than planned with the reasons for the same.
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(Member Secretary, IEC)

Dr. (Mrs) Shimpa Sharma
Member Secretary,
Institutional Ethics Committee
D. Y. Patil Medical College,
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This is to certify that, Mr. /Ms. /Mrs. Sazgun Tushar Baseani

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| Coordinator | Director, Students' Development | Convener | Pro-Vice-Chancellor |

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| | | |
|---|---|---|
| **Dr. Jayavant L. Gunjakar** | **Prof. Meghnad G. Joshi** | **Prof. Chandrakant D. Lokhande** |
| Convener | Convener | Chairman |

**D. Y. PATIL EDUCATION SOCIETY**
(Institution Deemed To Be University)
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Azadi Ka Amrit Mahotsav

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has participated in Industry-Academia Interaction and Workshop on Protein purification techniques in Polyclonal antibody development, organized on the occasion of **Bharat ki Azadi ka Amrut Mahotsav** by **Internal Quality Assurance Cell (IQAC), Centre for Interdisciplinary Research (CIR)**, D. Y. Patil Education Society (Deemed to be University), Kolhapur and **iSERA Biological Pvt. Ltd. Shirala** on 21st-22nd April 2022.

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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*

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

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**Article Info**

***Article type:***
Original Article

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

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

## Introduction

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

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

Archives of Microbiology (2024) 206:251
https://doi.org/10.1007/s00203-024-03983-9

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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Communicated by Nischith R.

Published online: 10 May 2024

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

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

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

Received: 9 August 2023 / Accepted: 25 October 2023


## Abstract

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

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Published online: 05 December 2023

Brazilian Journal of Microbiology
https://doi.org/10.1007/s42770-024-01344-8

BACTERIAL AND FUNGAL PATHOGENESIS - RESEARCH PAPER

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

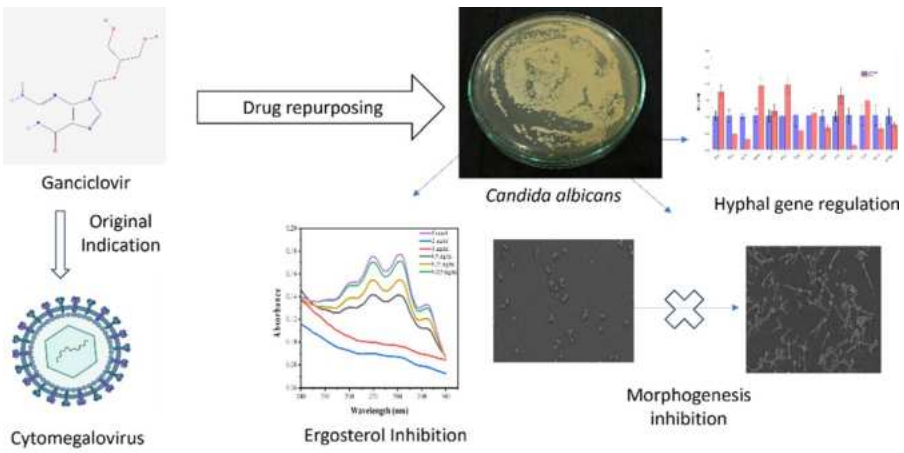
**Tanjila Gavandi[1] · Shivani Patil[1] · Sargun Basrani[1] · Shivanand Yankanchi[2] · Sayali Chougule[1] · S. Mohan Karuppayil[1] et al.** *[full author details at the end of the article]*

Received: 6 November 2023 / Accepted: 9 April 2024


## Abstract

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

## Graphical abstract




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

Responsible Editor: Rosana Puccia.

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View Journal



Cite this: DOI: 10.1039/d4na00176a

# A poly-δ-decalactone (PDL) based nanoemulgel for topical delivery of ketoconazole and eugenol against *Candida albicans*†

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This study aimed to investigate the potential of poly-δ-decalactone (PDL) and a block copolymer (methoxy-poly(ethylene glycol)-*b*-poly-δ-decalactone (mPEG-*b*-PDL)) in the topical delivery of ketoconazole (KTZ) and eugenol (EUG) against *Candida albicans*. The nanoemulsion (NE) was studied for its significant factors and was optimized using the design of experiments (DOE) methodologies. A simple robust nanoprecipitation method was employed to successfully produce a nanoemulsion (KTZ–EUG–NE). The spherical globules exhibited rough surfaces, explaining the adsorption of mPEG-*b*-PDL onto PDL. The sustained drug release effects were governed by the amorphous nature of PDL. KTZ–EUG–NE was further used to develop a 1% w/v Carbopol-940-based nanoemulgel (KTZ–EUG–NE gel). The optimal rheological and spreadability properties of the developed nanoemulgel explain the ease of topical applications. *Ex vivo* permeation and retention studies confirmed the accumulation of KTZ–EUG–NE at different layers of the skin when applied topically. The cytotoxicity of the developed NE in human keratinocyte (HaCaT) cells demonstrated the utility of this newly explored nanocarrier in reducing the cell toxicity of KTZ. The higher antifungal activities of KTZ–EUG–NE at 19.23-fold lower concentrations for planktonic growth and 4-fold lower concentrations for biofilm formation than coarse drugs explain the effectiveness of the developed NE.

Received 29th February 2024
Accepted 13th July 2024

DOI: 10.1039/d4na00176a

rsc.li/nanoscale-advances



## 1. Introduction

Globally, the frequency of fungal infections is increasing continuously, and more than thirty-five million people are currently affected by superficial fungal infections.[1] Among such, infections caused by the *Candida* species, especially *Candida albicans*, human fungal pathogens account for more of the reported worldwide deaths by fungi. It occurs at the superficial layer of the nails, skin, and hair (superficial mycosis) and can also spread to inner tissues.[2] Various drugs (azoles, amphotericin B, allylamines, and echinocandins) have been employed for the treatment of *Candida albicans*. However, repeated use of such medicines leads to the development of resistance to single antifungal agents. Combination therapy is required to produce additive or synergistic effects at lower concentrations that can restrict the development of drug resistance against *Candida albicans*.[3] Ketoconazole (KTZ) is a broad-spectrum imidazole drug with antifungal activity. Poor water solubility limits the antifungal potential of such drug, necessitating the use of drug carriers that can efficiently enhance the therapeutic potential of such agent.[4] The existing conventional topical KTZ formulations are creams, gels, and lotions. However, all such formulations either have less skin penetration (creams and gels) or less contact time with the targeted area (lotions). Advanced nanotechnology-driven KTZ formulations, including nanostructured lipid carriers (NLCs), solid lipid nanoparticles (SLNs), poly(lactic-*co*-glycolic acid nanoparticles (PLGA NPs), and nanocomplexes, have been explored by researchers.[5–8] KTZ has also been studied for combination approaches employing oils to produce nanoemulsions and microemulsion-based nanoemulgels.[9,10] Eugenol (EUG), a phenolic aromatic oil belonging to the allylbenzene class, has been reported to have antifungal properties. The development of formulations containing such essential oils as bioactive agents has been restricted owing to their volatile nature, instability, and low

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† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d4na00176a

International Microbiology
https://doi.org/10.1007/s10123-024-00565-z

RESEARCH

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

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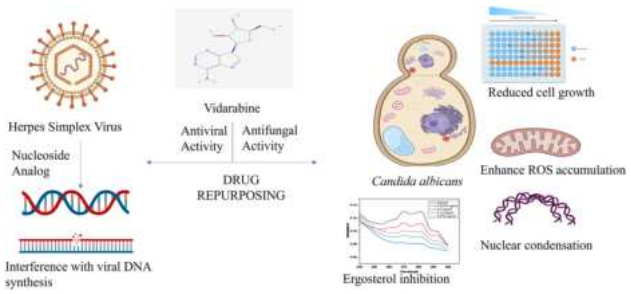
Received: 7 September 2023 / Revised: 11 May 2024 / Accepted: 31 July 2024


## 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

Published online: 10 August 2024

Journal of Microbiology (2024) 62:381–391
https://doi.org/10.1007/s12275-024-00111-6



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

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Received: 24 November 2023 / Revised: 6 January 2024 / Accepted: 9 January 2024 / Published online: 8 April 2024


## 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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Journal of Pharmaceutical Sciences 000 (2023) 1–13

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutical Nanotechnology

# Development and *In-Vitro* Evaluation of Eugenol-Based Nanostructured Lipid Carriers for Effectual Topical Treatment Against *C. albicans*

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ARTICLE INFO

*Article history:*
Received 19 July 2023
Revised 28 November 2023
Accepted 28 November 2023
Available online xxx

*Keywords:*
Candidiasis
Antifungal
Nanostructured lipid carriers
Hydrogel
Topical drug delivery

ABSTRACT

The main objective of the experiment is to develop and evaluate hydrogel-bearing nanostructured lipid carriers (NLCs) loaded with ketoconazole (KTZ) for the effective treatment of candidiasis. The eugenol was used as a liquid lipid (excipient) for the development of KTZ-loaded NLCs and was explored for anti-fungal effect. The production of NLCs involves high energy processes to generate spherical, uniform particles, having a higher percentage of entrapment efficiency (%EE) for KTZ with 89.83 ± 2.31 %. The data from differential scanning calorimeter (DSC), powder x-ray diffraction (PXRD), and attenuated total reflectance (ATR) demonstrated the KTZ dispersion in NLCs. The NLCs loaded hydrogel possessed optimum spreadability and exhibited shear thinning behavior, indicating the ease of application of the final formulation. The 6.41-fold higher transdermal flux (Jss) was governed for KTZ from KTZ-NLC than coarse-KTZ, which explains the usefulness of NLCs. The KTZ-NLCs exhibited significant 2.58 and 6.35-fold higher retention in the stratum corneum and viable epidermis of the skin. The cell cytotoxicity studies using human dermal fibroblast cell (HDFS) lines depicted the usefulness of NLCs in reducing cell toxicities for KTZ. The KTZ-NLCs were found to inhibit planktonic growth and hyphal transition and showed a larger zone of inhibition against *C. albicans* strains with a MIC-50 value of 0.39 $\mu$g/mL. The antibiofilm activity of KTZ-NLCs at lower concentrations, in contrast to plain KTZ, explained the interaction of developed NLCs with fungal membranes. The overall results depicted the effectiveness of the loading KTZ in the lipid matrix to achieve antifungal activity against *C. albicans*.


## Introduction

Every year, nearly 150 million individuals worldwide contract a fungal infection, and 1.7 million of them die.[1] There are around 600 different types of fungi that can infect individuals and cause diseases ranging from mild to fatal, such as allergies and infections of the mucous membranes, skin, hair, and nails.[2] Among all fungi, the genus *Candida* has several distinct species that cause candidiasis, which is highly common in people with impaired immune systems.[3] Antifungal medications, including azoles, amphotericin B, allylamines, and echinocandins, are available to treat candidiasis. However, repeated use of these antifungal medications caused *Candida* species to develop resistance.[4] The pathogen *Candida albicans*, which accounts for 50 % of infections among all *Candida* species and is treatable, requires greater consideration.[5] The most urgent issues in the world are microorganisms that develop multidrug resistance through various methods and produce biofilms that exhibit efflux action on pharmaceuticals, thus declining the drug concentration at the microbial site. Ketoconazole (KTZ) is a broad-spectrum triazole antifungal drug used to treat superficial skin candidiasis and systemic fungal infections. The underlying mechanism hinders the candida species' ergosterol synthesis by suppressing CYP450 14$\alpha$-demethylase.[6]

The lower aqueous solubility, ocular and systemic toxicities, and development of resistance after repeated use are challenges associated with azoles.[7] Many studies elaborated on the combination approaches by employing essential oils (EOs) and azole anti-fungal agents to synergize or potentiate the anti-fungal effect against resistant species. The KTZ has been studied with clove oil, eucalyptus oil, otacanthus azureus rose oil, and melaleuca alternifolia for effective antifungal activities.[7–9] The KTZ has been investigated alone or in

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https://doi.org/10.1016/j.xphs.2023.11.031
0022-3549/

Current Microbiology (2024) 81:213
https://doi.org/10.1007/s00284-024-03739-0

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

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Received: 23 December 2023 / Accepted: 11 May 2024


## Abstract

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

## Introduction

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

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