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Original Research Article

Antifungal Properties of Ethanolic Extract of *Holarrhena Pubescens* against *Candida* Species

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Abstract: Antimicrobial resistance is a global problem that jeopardizes the efficient use of modern medicine and the state of the world's health. The effectiveness of traditional antifungals used to treat fungal infections has declined, increasing mortality. Following exposure to antifungal medications, acquired mechanisms can lead to the development of resistance to currently available antifungal medicines. Invasive fungal infections have few treatment choices, and patients who are most at risk frequently have other co-morbid conditions, such as immunosuppression. This problem is made worse by the paucity of novel antifungals now under research. One potential research avenue for tackling the problem of MDR fungal pathogens is plants that are used in traditional medicine. When used to treat infectious diseases, antimicrobials derived from plants have a great deal of therapeutic promise and have less adverse effects than their synthetic counterparts. In this study, the possible antifungal action of ethanolic extract of *Holarrhena pubescens* was tested against American Type Culture Collection (ATCC) and Multidrug resistant (MDR) strains of pathogenic fungi, *Candida* species. Antifungal activities of ethanolic extract of *Holarrhena pubescens* were studied by determining the minimum inhibitory concentration (MIC) value which was done by serial dilution in Mueller Hinton broth.The ethanolic extract of *Holarrhena pubescens*showedantifungal activities exhibiting MIC values varying between 1.5625-3.125 mg/mL in both ATCC and MDR fungal pathogenic strains of *Candida*. Ethanolic extract of *Holarrhena pubescens*was found to have effective antifungal activity against *Candida* species andcould be the cure to invasive candidiasis which is a global health threat.

Keywords: *Holarrhena pubescens*, MDR fungi, ATCC fungi, antifungal activity, MIC value.

INTRODUCTION

The use of therapeutic plants for healing is as old as humanity itself. Medicinal plants are fundamentally infinitely valuable to human livelihoods. In most medical traditions, plants serve as the primary source of medicinal compounds. The usage of phytomedicine has steadily decreased since the turn away from natural to synthetic pharmaceuticals towards the end of the 19th century. Despite the widespread use of synthetic medications, several traditional medical practices such as Ayurveda still exist. They frequently employ medicinal plants because they serve as a source for identifying chemical components and using them to cure various illnesses (Zahara *et al*., 2020).

Holarrhena pubescens is a member of the *Apocynaceae* family. It is a significant medicinal plant that is found throughout the Indian subcontinent (Sharma *et al*., 2018). It is a deciduous tree with white blossoms along with oblong, elliptical leaves that can be found in India's arid and deciduous forests. Fruits have white markings and have long, terete follicles. The seeds are linear-oblong and glabrous. Since ancient times, various plant parts have been utilized in indigenous medicine. The plant is widely employed in Ayurveda, traditional Chinese medicine, and other conventional medical systems without clearly manifesting any negative effects. Aside from significant advancements in this plant's

biological and phytochemical evaluation during the past several years, thorough analyses of *H. pubescens* have limited scope. As a result of the widespread use of its seeds as antidiabetic and anthelminthic, it is economically significant. Its bark reportedly has antidiarrheal qualities. It is used to treat cholera, anemia, jaundice, dysentery, stomachaches, diarrhea, and epilepsy in Ayurvedic medicine (Kadir *et al*., 2013). Flowers are used in Ayurvedic medicine to cure leukoderma, blood, and spleen illnesses, as well as anthelmintic and antidiarrheal conditions. It is said that the roots are both aphrodisiac and abortifacient. Additionally, they are used to treat severe abscesses, gonorrhoea, ascariasis, malaria, and venereal diseases (Basha *et al*., 2012). Additionally, despite many reports of the plant's traditional applications by African and Asian people, neither in vitro nor in vivo research has been done to support its use in treating any specific diseases (Zahara *et al*., 2020).

The demise of numerous life forms and significant agricultural loss are both attributed to fungi, which are among the most destructive eukaryotic creatures on Earth (Rolling *et al*., 2020). Commensal fungal species can transform into invasive pathogens when the immune system is compromised, moving around repeatedly to cause invasive fungal infections (IFIs), which damage a variety of organs and organ systems (Arastehfar *et al*., 2020). More recent lines of research have connected the colonization of particular fungal species with the evolution of pancreatic cancer and alcoholic cirrhosis, demonstrating that the effects of fungi on human health extend beyond acute and chronic infections.

Among such clinically relevant IFI causing pathogens, *Candida* species are the second most pathogenicfungi, the first being *Cryptococcus neoformans*, which pose a threat to human health, causing life-threateninginfections every year. It causes invasive infections in individuals with low immunity or those who went through severe clinical procedures or suffered from major trauma. It has a mortality rate of 40% which sometimes even exceeds 50% (Brown *et al*., 2012). They are the fourth most common fungal pathogen to cause hospital-acquired bloodstream infections (Wong *et al*., 2014). 98% of central venous catheter-related fungemias in cancer patients were caused by *Candida* spp. The most common fungal pathogen found to cause human diseases is *Candida albicans*. Other fungal pathogens include *Candida parapsilosis, Candida krusei, Candida glabrata*, and *Candida tropicalis*.

The CDC's 2019 study identified drug-resistant *Candida* species such as *Candida albicans*, *Candida parapsilosis, Candida glabrata*, *Candida tropicalis,* and *Candida krusei* as a severe danger with more than 34,000 cases and 1700 deaths each year. A serious problem posed by drug resistance is highlighted by the alarming cases of cross-resistance to azoles and echinocandins that have been identified among *Candida spp*. Acquired resistance can also emerge following antifungal exposure (Marquez *et al*., 2020).

C. albicans, the main fungal pathogen, lives as a symbiotic fungus on the skin, in the oral cavity, in the urogenital tract, and in the mucosa of the gut. The innate and adaptive immune system of the host consisting of macrophages, epithelial cells, dendritic cells, and neutrophils, act as a defense against *C. albicans*. Oral thrush and vaginitis are some of the mucocutaneous infections that develop in hosts who have weakened immune systems as a result of HIV infection, organ transplantation, cancer treatment, or antibiotic use that has upset the microflora. It also causes invasive candidiasis which includes both candidemia and deep-seated tissue candidiasis (Gulshan *et al*., 2007).

One of the critical concerns that had just recently surfaced was the particularly resilient and persistent fungal pathogen, *Candida auris*which is associated with nosocomial infections. Worldwide outbreaks in medical facilities have been brought about by *C. auris*. The frequency of *C. auris* infections recorded in 2018 increased by 318% when compared to rates from 2015 to 2017. Invasive bloodstream infections result from 58–76% of *C. auris* infections, according to numerous research evaluating cases from hospitals in India, Colombia, and New York.

The third most common cause of candidemia in the world is *Candida parapsilosis* (Pfaller *et al*., 2019). Recent studies in countries like India, the USA, South Africa, South Korea, Brazil, Italy, and Turkey have shown an increase in clonal expansion of fluconazole-resistant *Candida parapsilosis*. In the past twenty years, *Candida parapsilosis* has become a major factor in catheter-related bloodstream infections (CRBSI) (Yamin *et al*., 2021). In critical care units (ICUs), *C. parapsilosis* is also the second- or third-most often isolated *Candida* species (Magobo *et al*., 2017; Asadzadeh *et al*., 2019). Another distinctive feature of *C. parapsilosis* is horizontal transmission, which enables the species to spread through tainted medical supplies and healthcare personnel in the clinic, resulting in patient-to-patient infections (Vaz *et al*., 2011).

To effectively control illnesses brought on by these organisms, it is particularly important to address antifungal resistance in pathogenic fungi.

In this study, ATCC and MDR strains of *Candida* species were used to investigate the potential antifungal activity of an ethanolic extract of *Holarrhena pubescens*. The minimum inhibitory concentration (MIC) value of the ethanolic extract of *Holarrhena pubescens* was determined in order to study its antifungal properties.

Fig 1: Leaf of *H. pubescens*

MATERIALS AND METHODS

Procuring of the plant material

The leaves of the plant, *Holarrhena pubescens* were collected on 19th December 2022 from Basadera forest near Dharagiri Falls, Ghatshila, Jharkhand which is located at 22.6792° N and 86.5004° E.

Preparation of Mueller Hinton Broth

100 ml of Mueller Hinton Broth is prepared by dissolving 2.1 grams in 100 ml of distilled water in a sterile glass conical flask. The solution is heated to boiling to dissolve the medium completely. Then it is sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes.

Preparation of plant extract

Ethanolic extract of *Holarrhena pubescens* is prepared by dissolving 1gm dried leaves in 5 ml ethanol. The mixture was then vortexed.

Microorganisms to be tested

The strains of *Candida albicans* (ATCC 10231), *Candida parapsilosis* (MDR strain, Table 1), and *Candida auris* (MDR strain, Table 2) were taken for thisexperiment. Tables 1 and 2 display their antifungal sensitivity. These strains were obtained from the Department of Microbiology, Peerless Hospitex Hospital & Research Centre Limited, Kolkata, West Bengal, India.

Table 1: *Candida parapsilosis* (MDR) antifungal sensitivity test results using the automated VITEK 2 system

Selected Organism: *Candida parapsilosis*					
Source: Blood					
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Fluconazole	0.5	S	Micafungin	<=0.06	S
Voriconazole	1	R	Amphotericin B	0.5	S
Caspofungin	0.25	S	Flucytosine	>=64	R

R= Resistant, S= Sensitive

Table 2: *Candida auris* (MDR) antifungal sensitivity test results using the automated VITEK 2 system

Selected Organism: *Candida auris*					
Source: Blood					
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Fluconazole	32	R	Micafungin	<=0.06	S
Voriconazole	1	R	Amphotericin B	0.5	S
Caspofungin	0.25	S	Flucytosine	>=64	R

R= Resistant, S= Sensitive

Micro-dilution Test

A 96-well cell culture plate is taken. Alternate rows are filled with 100 microlitres of MHA broth from H to A. The rows in between which were left empty are filled with 100 microlitres of alcohol. They are taken as control.100 microlitres of plant extract were added to the H column of every alternate row filled with MH broth. No extract is put in the wells with alcohol. After proper mixing of the plant extract and the MH broth with the pipette, it is serially diluted across the row from H to A, each well containing 100 microlitres of plant extract and HM broth. Two rows are taken for each microorganism. One row is for the test and the other is a control. 3 fungal strains are taken, namely, *Candida albicans* (ATCC10231), *Candida parapsilosis* (MDR), and *Candida auris (*MDR). The microbial extract for each organism was prepared separately by taking a small amount of culture from the microbial culture plate with an inoculation loop and then mixed with sterile saline water taken in an Eppendorf.10 microlitres of each microbial extract are then pipetted into the test and control wells in the cell culture plate assigned for that particular microorganism. Then the optical density (OD) is taken at 0 hours. Then the plate is incubated at 37°C for 24 hours and then the optical density is measured again. Observations are noted and the graphs are plotted to depict the effect of ethanolic extract of *Holarrhena pubescens* on each microorganism.The lowest concentration of the ethanolic leaf extract of *H. pubescens*in the MH broth medium that prevents the test microorganism from growing was identified as the MIC value.

RESULTS

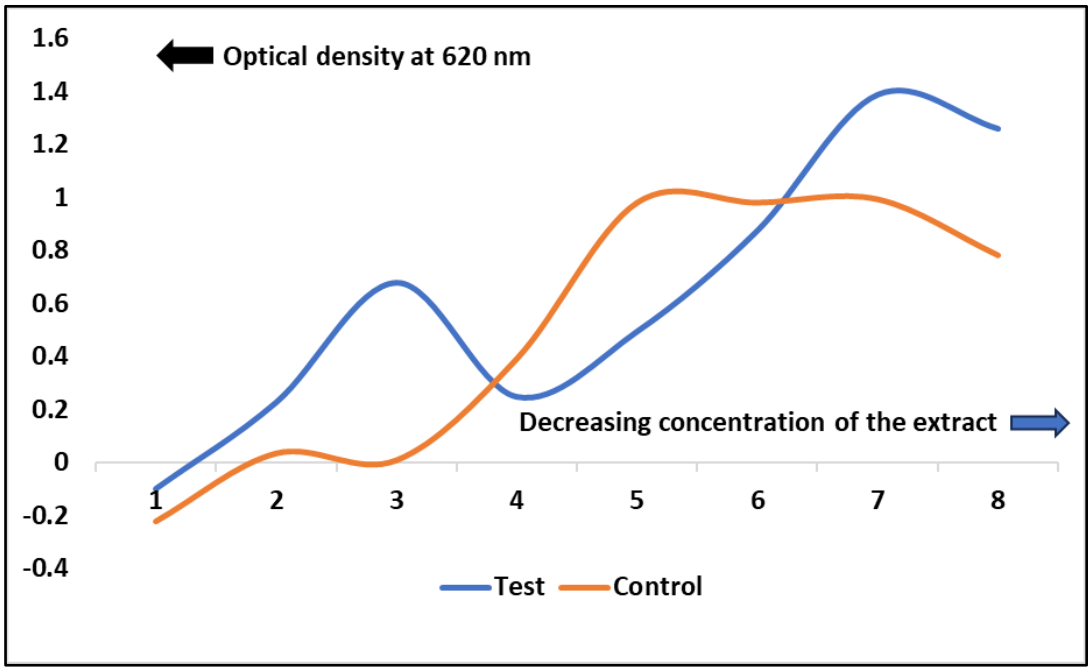

Fig 2: Effect of *Holarrhena pubescens* ethanolic extract on *Candida albicans* (ATCC 10231) showing MIC value of 3.125 mg/ml. The concentration of the extract 1: 100 mg/ml, 2: 50 mg/ml, 3: 25 mg/ml. 4: 12.5 mg/ml. 5: 6.25 mg/ml, 6: 3.125 mg/ml, 7: 1.5625 mg/ml. 8: 0.78125 mg/ml

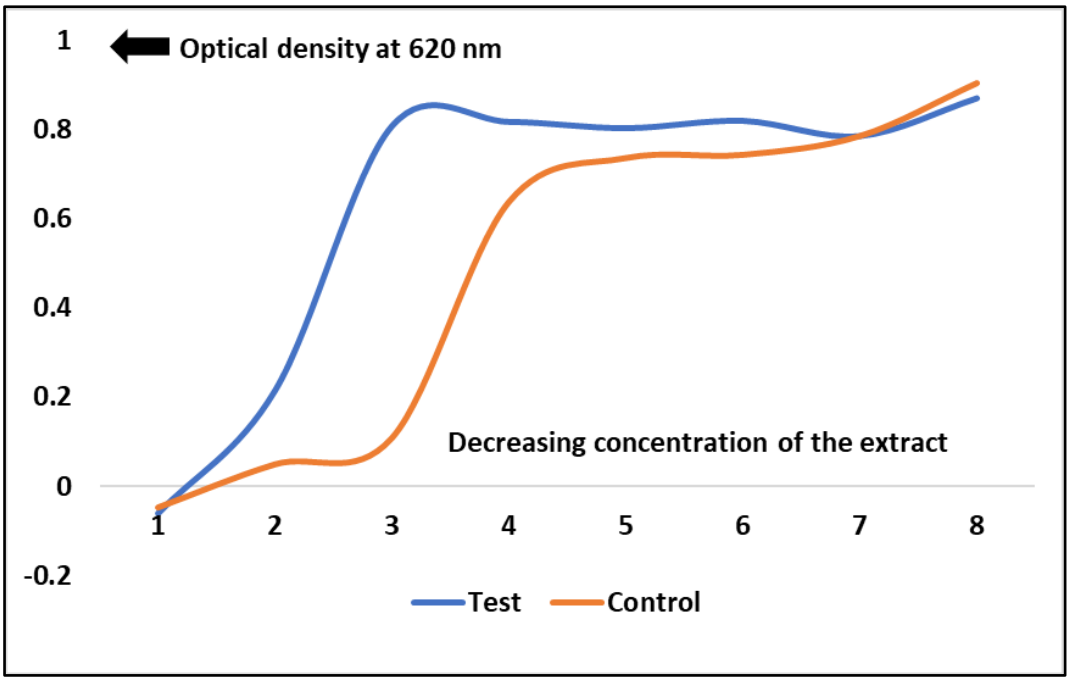

Fig 3: Effect of *Holarrhena pubescens* ethanolic extract on *Candida parapsilosis* (MDR) showing MIC value of 1.5625 mg/ml. The concentration of the extract 1: 100 mg/ml, 2: 50 mg/ml, 3: 25 mg/ml. 4: 12.5 mtg/ml. 5: 6.25 mg/ml, 6: 3.125 mg/ml, 7: 1.5625 mg/ml. 8: 0.78125 mg/ml

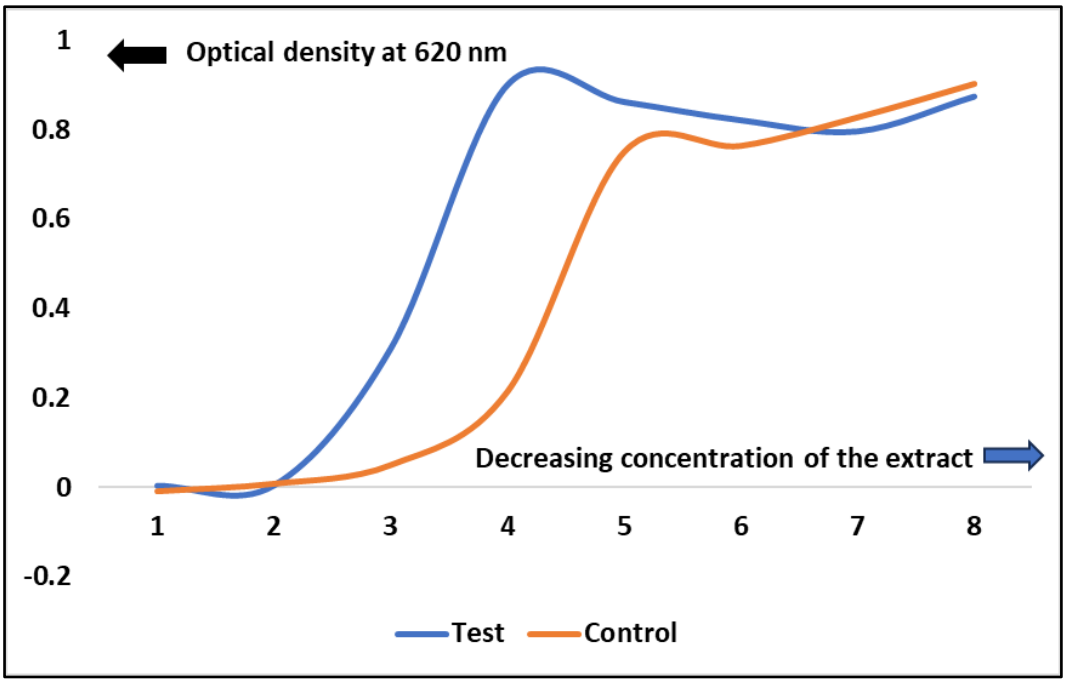

Fig 4: Effect of *Holarrhena pubescens* ethanolic extract on *Candida auris* (MDR) showingMIC value of 1.5625 mg/ml. The concentration of the extract 1: 100 mg/ml, 2: 50 mg/ml, 3: 25 mg/ml. 4: 12.5 mtg/ml. 5: 6.25 mg/ml, 6: 3.125 mg/ml, 7: 1.5625 mg/ml. 8: 0.78125 mg/ml

The ethanolic extract of *Holarrhena pubescens*showed antifungal activity against the respective ATCC and MDR strains of the *Candida* species. MIC value of the extract against *Candida albicans* (ATCC) was found to be 3.125 mg/ml. At the same time, the MIC value of the extract against *Candida parapsilosis* (MDR) was 1.5625 mg/ml. The extract showed an MIC value of 1.5625 mg/ml against *Candida auris* (MDR). Figures 2-4 represent the graphs of differences between the absorbance values obtained at 0 hours and 24 hours versus the concentration of dilution of the plant extract.

DISCUSSION

From this study, it is established that ethanolic leaf extract of *Holarrhena pubescens* has antifungalactivity against various pathogenic ATCC and MDR strains of *Candida* like *Candida albicans* (ATCC), *Candida parapsilosis*(MDR) and *Candida auris* (MDR).No other study reflects on the antimicrobial properties of the leaves extract of this plant.

Other plant parts of *Holarrhena pubescens* has been studied extensively for its antibacterial properties against various pathogens as shown in other studies.

One study demonstrated the antibacterial activity of the flower oil of *H. pubescens* at a concentration of 50 mg/mL, which was examined using the disc diffusion assay against tested human pathogens which included both bacteria and fungi. The outcomes were contrasted with those of 1.0 mg/mL of chloramphenicol, a reference medication. Table 3 lists *H. pubescens* flower oil's antibacterial properties (Sripahco *et al*., 2021).

In another study, the methanolic extract of the bark of *Holarrhena pubescens*, its fractions, and conessine, a steroidal alkaloid, were tested using the agar diffusion method for their ability to inhibit a variety of bacteria and fungi. It was discovered that all of them had noticeable activity against some of the tested microorganisms. A few of the studied fungus were also moderately responsive to the alkaloidal fraction and conessine. The maximum activity was shown against *Micrococcus luteus* ATCC 9341 (MIC: 15.6 mg per disc) when the minimum inhibitory concentration (MIC) value of conessine was evaluated against various microorganisms (Siddiqui *et al*., 2011).

In a study, seed ethanol extracts of *H. pubescens* exhibited concentration-dependent antibacterial activity against enteropathogenic Escherichia coli (EPEC). The petroleum ether extract of its bark similarly demonstrated inhibition of E. coli at a minimum inhibitory dose of 50 g/mL. It did, however, exhibit a moderate level of activity when compared to other plants (Siddiqui *et al*., 2012).

According to another study, with a minimum inhibitory concentration (MIC) of 15.6 g per disc, the alkaloidal fraction of *H. pubescens* demonstrated questionable antifungal efficacy. *H. pubescens* bark's methanol extract shown a substantial antifungal effect against *Candida albicans* (Raman *et al*., 2004).

In our study, the MIC values of the ethanolic extract of *Holarrhena pubescens* were 3.125 mg/ml, 1.5625 mg/ml and 1.5625 mg/ml against *Candida albicans*, *Candida parapsilosis* and *Candida auris* respectively.

CONCLUSION

This study proved that ATCC and MDR strains of the examined *Candida* species are susceptible to the antifungal effects of the ethanolic extract of the leaves of *Holarrhena pubescens*. The extract consists of various active ingredients that are responsible for its antifungal activity. So, in the future, this might be used to fight pathogenic fungal ATCC and MDR strains of *Candida*. However, more research is essential in this area.

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Authors Contribution

All the authors have contributed to this project equally.

Conflict Of Interest

There is no conflict of interest of any author in this manuscript.

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