INTERNATIONAL JOURNAL OF
Clinical and Pharmaceutical Innovations
An International Peer Reviewed Open Access Journal

Research Article

ISSN (Online): 3142-8665
CODEN (USA): IJCPSM

2026, Volume 1, Issue 5, Page No: 52-64.

SYNERGISTIC ANTICANDIDAL AND ANTIBIOFILM ACTIVITY OF *MORINGA OLEIFERA* METHANOLIC EXTRACT IN COMBINATION WITH FLUCONAZOLE AGAINST *CANDIDA GLABRATA*

Poulami Shaw[1], Pooja Das[2], Banhisikha Singh[2], Aniruddha Mukherjee[2], Anirban Mukherjee[2*]

[1]Department of Microbiology, Sister Nivedita University. New Town, Kolkata – 700156.
[2]AM Educare Biotech Solutions Pvt. Ltd., Kolkata 700010, India.

Article Info: **Received:** 28 May 2026, **Revised:** 18 June 2026, **Accepted:** 08 July 2026

***Corresponding Author: Dr. Anirban Mukherjee**

AM Educare Biotech Solutions Pvt. Ltd., Kolkata 700010, India.

Citation:
Poulami Shaw[1], Pooja Das[2], Banhisikha Singh[2], Aniruddha Mukherjee[2], Anirban Mukherjee[2*]. (2026). Synergistic Anticandidal And Antibiofilm Activity Of *Moringa Oleifera* Methanolic Extract In Combination With Fluconazole Against *Candida Glabrata*. International Journal of Clinical and Pharmaceutical Innovations, 1(5), 52-64.
DOI: https://doi.org/10.5281/zenodo.21705465

ABSTRACT
Candida glabrata is an opportunistic fungal pathogen associated with increasing antifungal resistance and biofilm-mediated persistence, limiting the effectiveness of conventional therapies. The present study investigated the anticandidal and antibiofilm activities of selected medicinal plant extracts against *C. glabrata*, with particular emphasis on the methanolic extract of *Moringa oleifera*, individually and in combination with fluconazole. Following isolation and physiological characterization of the fungal isolate, antifungal activity was evaluated using agar well diffusion, broth microdilution, minimum inhibitory concentration (MIC), biofilm inhibition and eradication assays, MTT cell viability assay, and growth kinetics. Phytochemical characterization was performed using qualitative screening, thin-layer chromatography, column chromatography, and SDS–PAGE analysis. Among the tested plant extracts, M. oleifera exhibited the strongest anticandidal activity. Combination treatment with *M. oleifera* and fluconazole produced enhanced antifungal efficacy, extending inhibitory activity to higher dilutions in the MIC assay and achieving greater biofilm eradication (36%), antibiofilm inhibition (45%), and reduction in fungal cell viability (43%) than either treatment alone. Time-kill and growth kinetics studies further demonstrated sustained inhibition of fungal growth following combination treatment. Phytochemical analyses confirmed the presence of phenolics, flavonoids, alkaloids, terpenoids, glycosides, and other bioactive constituents that may contribute to the observed biological activity. Statistical analysis of triplicate experiments confirmed the reproducibility of the experimental findings ($P < 0.05$). Collectively, these results demonstrate that *M. oleifera* methanolic extract potentiates the antifungal activity of fluconazole and represents a promising natural adjunct for the management of biofilm-associated *C. glabrata* infections.

KEYWORDS: *Candida glabrata*, *Moringa oleifera*, Anticandidal activity, Biofilm inhibition, Fluconazole synergy.

1. INTRODUCTION

Fungal infections have become a significant global health concern, particularly among immunocompromised individuals, where opportunistic pathogens contribute to increased morbidity and mortality. Among these pathogens, Candida glabrata has emerged as one of the most clinically important non-*albicans* Candida species. Although it is a commensal organism of the human microbiota, *C. glabrata* is capable of causing both superficial and systemic infections, especially in individuals with weakened immune systems (Kaur et al., 2013; Kumar et al., 2020). Epidemiological studies indicate that it accounts for approximately 20–30% of Candida bloodstream infections and is currently the second leading cause of invasive candidiasis in many regions worldwide (Hassan et al., 2021; Frías-De-León et al., 2022).

A key factor contributing to the pathogenicity of *C. glabrata* is its ability to adhere to host tissues and medical devices, leading to the formation of robust biofilms. These biofilms are mediated by cell wall-associated adhesins, particularly those encoded by the EPA gene family, and are influenced by environmental factors such as pH, nutrient availability, and growth conditions (Riera et al., 2012; Valotteau et al., 2019; Kucharíková et al., 2011). Biofilm formation enhances fungal survival and significantly increases resistance to antifungal agents by limiting drug penetration and altering cellular metabolism (Seneviratne et al., 2010; Gonçalves et al., 2021). Furthermore, regulatory mechanisms, including transcription factors such as Mss11, play a role in controlling adhesion and virulence, thereby contributing to the persistence of infection (Wang et al., 2024).

The treatment of *C. glabrata* infections is particularly challenging due to its intrinsic and acquired resistance to commonly used antifungal drugs. Azole antifungals, especially Fluconazole, are widely used in clinical practice; however, resistance to these agents has increased significantly (Hitchcock et al., 1993; Bennett et al., 2004). Resistance mechanisms include overexpression of efflux pumps, mutations in target enzymes such as Erg11, and alterations in ergosterol biosynthesis pathways, which collectively reduce drug susceptibility (Costa et al., 2016; Whaley & Rogers, 2016). Additionally, prolonged antifungal exposure has been shown to increase minimum inhibitory concentrations (MICs), further complicating treatment outcomes (Parkinson et al., 1995). These challenges highlight the urgent need for alternative or adjunct therapeutic strategies.

In recent years, medicinal plants have gained considerable attention as potential sources of novel antimicrobial agents. Plants are rich in bioactive compounds such as alkaloids, flavonoids, phenolics, and essential oils, which exhibit diverse pharmacological activities, including antifungal effects. Several plant species, including neem (*Azadirachta indica*), lemon (*Citrus limon*), curry leaf (*Murraya koenigii*), and turmeric (*Curcuma longa*), have demonstrated antimicrobial properties against a range of pathogenic microorganisms (Biswas et al., 2002; Dhanavade et al., 2011; Singh et al., 2015; Edokpiawe et al., 2020). Among these, Moringa oleifera has attracted significant interest due to its wide spectrum of biological activities, including antifungal, antibacterial, antioxidant, and anti-inflammatory properties (Moyo et al., 2012; Patel et al., 2019).

Previous studies have demonstrated that extracts of *M. oleifera* possess significant antifungal activity against Candida species, including *Candida albicans*, with some reports indicating greater efficacy than conventional antifungal agents (Abbas & Atiyah, 2018; Yoshimatsu et al., 2023). The antifungal properties of *M. oleifera* are attributed to its rich phytochemical composition, including flavonoids, alkaloids, and bioactive proteins such as Mo-CBP3, which disrupt fungal metabolism and growth (Indrastiti et al., 2025; Gani et al., 2023). Despite these promising findings, limited studies have investigated its effectiveness against drug-resistant *C. glabrata*, particularly in relation to biofilm inhibition and synergistic interactions with antifungal drugs.

Combination therapy has emerged as a promising strategy to overcome antifungal resistance. The use of plant extracts in combination with conventional antifungal agents may enhance therapeutic efficacy, reduce required drug doses, and minimize adverse effects. Synergistic interactions between natural compounds and antifungal drugs have been reported in several studies, suggesting their potential role in combating resistant fungal pathogens (Yassin et al., 2022). However, further research is needed to evaluate such combinations against *C. glabrata* and to understand their mechanisms of action.

Therefore, the present study aims to evaluate the anticandidal and antibiofilm activities of selected plant extracts against drug-resistant *C. glabrata*, with particular emphasis on the methanolic extract of *Moringa oleifera*. In addition, the study investigates the synergistic effects of *M. oleifera* extract in combination with fluconazole using various microbiological assays, including disc diffusion, minimum inhibitory concentration (MIC), biofilm inhibition assays, and cell viability analysis. This study seeks to provide insights into the potential application of plant-based therapeutics as effective alternatives or adjuncts to conventional antifungal treatments.

2. MATERIALS AND METHODS

2.1 Isolation and Maintenance of *Candida glabrata*

Water samples were aseptically collected from Subhas Sarobar Lake, Kolkata, India, using sterile sampling containers and transported immediately to the laboratory for microbiological analysis. Aliquots of the samples

were inoculated onto HiCrome Candida Differential Agar (HiMedia Laboratories, India) and incubated at 37°C for 24 h to facilitate the selective isolation of *Candida* species. Morphologically distinct colonies presumptively identified as *Candida glabrata* based on colony characteristics on the chromogenic medium were selected and purified by repeated streaking to obtain axenic cultures.

A single well-isolated colony was subsequently inoculated into sterile Sabouraud Dextrose Broth (SDB) and incubated at 37°C for 24 h under shaking conditions to obtain actively growing cultures. The resulting pure culture served as the inoculum for all subsequent physiological, antifungal, phytochemical, and biofilm-related experiments.

2.2 Optimization of Growth Conditions

The growth characteristics of the isolated *Candida glabrata* were evaluated under different environmental conditions to determine the optimum parameters for subsequent experiments. Temperature optimization was performed by incubating inoculated Sabouraud Dextrose Broth at 4°C, room temperature, 37°C, and 70°C. The influence of pH was assessed using culture media adjusted to pH 3, 5, 7, 9, and 11, while salinity tolerance was evaluated using media supplemented with 0.1%, 1%, 2%, and 4% (w/v) NaCl.

Following incubation for 24 h, fungal growth was quantified by measuring the optical density (OD) at 630 nm using a UV–Visible spectrophotometer. The environmental condition exhibiting the highest optical density was considered optimal for fungal growth and was employed in subsequent experiments.

2.3 Morphological Characterization

Morphological characterization of the isolated fungal strain was carried out by assessing both colony morphology and microscopic characteristics. Colony morphology was recorded after growth on HiCrome Candida Differential Agar (Himedia, India), including colony colour, shape, margin, elevation, surface texture, opacity, and growth pattern.

Microscopic examination was performed by simple staining using crystal violet. A thin smear of the fungal culture was prepared on a clean glass slide, heat-fixed, stained with crystal violet, washed with distilled water, air-dried, and observed under an oil immersion objective (100×). Cellular morphology, budding pattern, and the presence or absence of hyphal structures were recorded.

2.4 Antifungal Susceptibility Testing

The antifungal susceptibility profile of the isolated *Candida glabrata* was evaluated using the agar disc diffusion method. Briefly, an actively growing fungal suspension was uniformly spread onto Sabouraud Dextrose Agar (SDA) plates using a sterile cotton swab. Commercial antifungal discs containing fluconazole, miconazole, clotrimazole, ketoconazole, and itraconazole were aseptically placed on the inoculated plates. Following incubation at 37°C for 24 h, the diameters of the zones of inhibition (ZOI) were measured in millimetres. The relative antifungal susceptibility of the isolate was determined by comparing the inhibition zones produced by the different antifungal agents.

2.5 Selection of Medicinal Plants and Preparation of Extracts

Six medicinal plants, namely *Moringa oleifera*, *Azadirachta indica*, *Adhatoda vasica*, *Murraya koenigii*, *Citrus limon*, and *Curcuma longa*, were selected based on their reported antimicrobial properties. Fresh plant materials were washed thoroughly with distilled water, shade-dried at room temperature, and ground into fine powder using a laboratory grinder. The powdered materials were stored in sterile airtight containers until further use. Plant extracts were prepared by solvent extraction, with methanol used as the extraction solvent for *Moringa oleifera*. The extracts were filtered to remove insoluble materials, concentrated, and stored under sterile conditions until further evaluation of their anticandidal activity.

2.6 Screening of Anticandidal Activity

The anticandidal activity of the selected medicinal plant extracts against *Candida glabrata* was initially evaluated using the agar well diffusion assay. Sabouraud Dextrose Agar (Himedia, India) plates were inoculated with an actively growing fungal suspension, and wells were aseptically prepared using a sterile cork borer. Equal volumes of the respective plant extracts were introduced into the wells, and the plates were incubated at 37°C for 24 h. Antifungal activity was assessed by measuring the diameter of the zones of inhibition surrounding each well.

The inhibitory activity of the extracts was further quantified using the broth microdilution method in sterile 96-well microtitre plates. Briefly, fungal inoculum and plant extracts were dispensed into the wells and incubated at 37°C for 24 h. Fungal growth was determined spectrophotometrically by measuring the optical density at 630 nm using a microplate reader. Lower optical density values were considered indicative of greater antifungal activity.

2.7 Phytochemical Characterization of *Moringa oleifera* Methanolic Extract

2.7.1 Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) was performed to obtain a preliminary phytochemical profile of the methanolic extract of *Moringa oleifera*. Aliquots of the extract were applied onto silica gel TLC plates and developed using appropriate solvent systems for the separation of phenolic compounds, flavonoids, and alkaloids. Following solvent migration, the chromatograms were visualized under ultraviolet illumination, and the retention factor (Rf) values of the

resolved bands were calculated to facilitate the preliminary identification of major phytochemical constituents.

2.7.2 Column Chromatography

Fractionation of the methanolic extract of *Moringa oleifera* was carried out using silica gel column chromatography. The extract was loaded onto a silica gel column and eluted with a solvent system consisting of toluene : ethyl acetate : acetic acid (25:20:5). Eluted fractions were collected sequentially and analysed by thin-layer chromatography to monitor the separation of phytochemical constituents. Fractions exhibiting similar TLC profiles were grouped for subsequent phytochemical evaluation.

2.7.3 Qualitative Phytochemical Screening

The methanolic extract of *Moringa oleifera* was subjected to qualitative phytochemical screening using standard colorimetric assays to detect the presence of major classes of secondary metabolites. The extract was examined for saponins (foam test), alkaloids (Dragendorff's test), terpenoids (Salkowski test), phenolic compounds and tannins (ferric chloride test), glycosides (Keller–Killiani test), carbohydrates (Molisch's and Benedict's tests)**,** and proteins (Biuret test). The presence or absence of each phytochemical group was determined based on the characteristic colour changes or precipitates produced during the respective reactions.

2.8 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the methanolic extract of *Moringa oleifera*, fluconazole, and their combination against *Candida glabrata* was determined using the broth microdilution method. Two-fold serial dilutions ranging from 1:2 to 1:64 were prepared in sterile 96-well microtitre plates. Each well received fungal inoculum together with the respective treatment and was incubated at 37°C for 24 h. Fungal growth was evaluated spectrophotometrically, and the lowest dilution that completely inhibited visible growth was recorded as the MIC.

2.9 Biofilm Assays

2.9.1 Biofilm Eradication Assay

The ability of the treatments to eradicate preformed biofilms of *Candida glabrata* was evaluated using the microtitre plate assay. Mature biofilms were established by incubating fungal cultures in sterile 96-well plates for 48 h. Following biofilm formation, planktonic cells were removed, and the wells were treated with the respective plant extract, fluconazole, or their combination. Residual biofilm biomass was quantified by crystal violet staining, and absorbance was measured spectrophotometrically to determine the percentage of biofilm eradication.

2.9.2 Biofilm Inhibition Assay

The inhibitory effect of the treatments on biofilm formation was evaluated by incubating fungal cells in the presence of the respective treatments during biofilm development. Following incubation, non-adherent cells were removed, and the attached biofilms were stained with crystal violet. The stained biofilms were quantified spectrophotometrically, and the percentage inhibition of biofilm formation was calculated relative to the untreated control.

2.10 Cell Viability Assay (MTT)

The effect of the methanolic extract of *Moringa oleifera*, fluconazole, and their combination on the viability of *Candida glabrata* cells was evaluated using the MTT colorimetric assay. Following treatment under the respective experimental conditions, MTT reagent was added to each well and incubated to allow the formation of intracellular formazan crystals by metabolically active cells. The crystals were subsequently dissolved in dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as the percentage reduction relative to the untreated control.

2.11 Growth Kinetics and Time-Kill Assay

The effect of *Moringa oleifera* methanolic extract, fluconazole, and their combination on the growth dynamics of *Candida glabrata* was investigated using growth kinetics and time-kill assays. Fungal cultures were inoculated into Sabouraud Dextrose Broth containing the respective treatments and incubated under optimized growth conditions. Growth was monitored at predetermined time intervals by measuring the optical density at 630 nm. Growth curves were constructed to compare fungal proliferation among the untreated control, individual treatments, and combination therapy, thereby evaluating the time-dependent antifungal activity of each treatment.

2.12 SDS–PAGE Analysis

Protein profiling of *Candida glabrata* was carried out using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Whole-cell and extracellular protein fractions were prepared from untreated and treated fungal cultures. Briefly, fungal cells were harvested by centrifugation, washed with acetone to remove residual medium components, and processed for protein extraction. Extracellular proteins were recovered separately from the corresponding culture supernatants.

Protein samples were mixed with SDS–PAGE loading buffer and heated at 95°C for 5 min to ensure complete protein denaturation before electrophoresis. The denatured proteins were resolved on polyacrylamide gels under standard electrophoretic conditions and subsequently stained for visualization. Comparative analysis of protein banding patterns was performed to

evaluate changes in protein expression following treatment.

2.13 Statistical Analysis

All experiments were performed independently in triplicate, and the results are expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 10.0 (GraphPad Software Inc., San Diego, CA, USA). Differences among experimental groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Growth kinetics data were analyzed using two-way ANOVA with Tukey's post hoc test to evaluate the effects of treatment and incubation time. Statistical significance was established at $P < 0.05$.

3. RESULTS

3.1 Isolation and Morphological Characterization of *Candida glabrata*

The fungal isolate was successfully recovered from the collected pond water samples following cultivation on HiCrome Candida Differential Agar (Figure 1). The colonies were circular with smooth entire margins, convex elevation, and a glistening opaque surface. Colony pigmentation ranged from cream to off-white, while the texture was characteristically buttery to pasty. Small, discrete colonies developed uniformly along the streak lines, indicating successful isolation of a pure yeast culture (Table 1). Subsequent inoculation into Sabouraud Dextrose Broth produced uniform turbidity after 24 h of incubation at 37°C (Figure 2), confirming active growth of the isolate without visible contamination.

Figure 1: Colony Morphology of Candida glabrata.

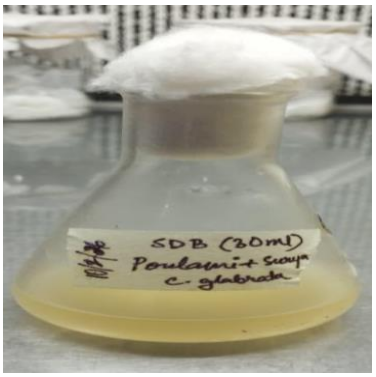

Figure 2: Pure Culture of Candida glabrata.

Table 1: Colony Morphological Characteristics of Candida glabrata Isolated on HiCrome Candida Differential Agar.

Feature	Observation
Shape	Circular
Margin	Entire (Smooth edge)
Elevation	Convex
Surface	Smooth and glistening
Color	Cream to off-white
Texture	Buttery or pasty
Opacity	Opaque
Growth Pattern	Small colonies scattered along streak lines

3.2 Optimization of Growth Condition

The growth characteristics of the isolated *Candida glabrata* were evaluated under different environmental conditions by measuring optical density at 630 nm (Charts 1–3). Maximum fungal growth was observed at room temperature and 37°C, whereas substantially lower growth occurred at 4°C and 70°C. Salinity studies showed optimum growth at 0.1% NaCl, followed by a progressive decline in growth with increasing salt concentration. Similarly, pH significantly influenced fungal proliferation, with maximum growth recorded at pH 7, followed by pH 5, while markedly reduced growth was observed under highly acidic (pH 3) and alkaline (pH 11) conditions. The differences observed among the tested environmental conditions were statistically significant ($P < 0.05$).

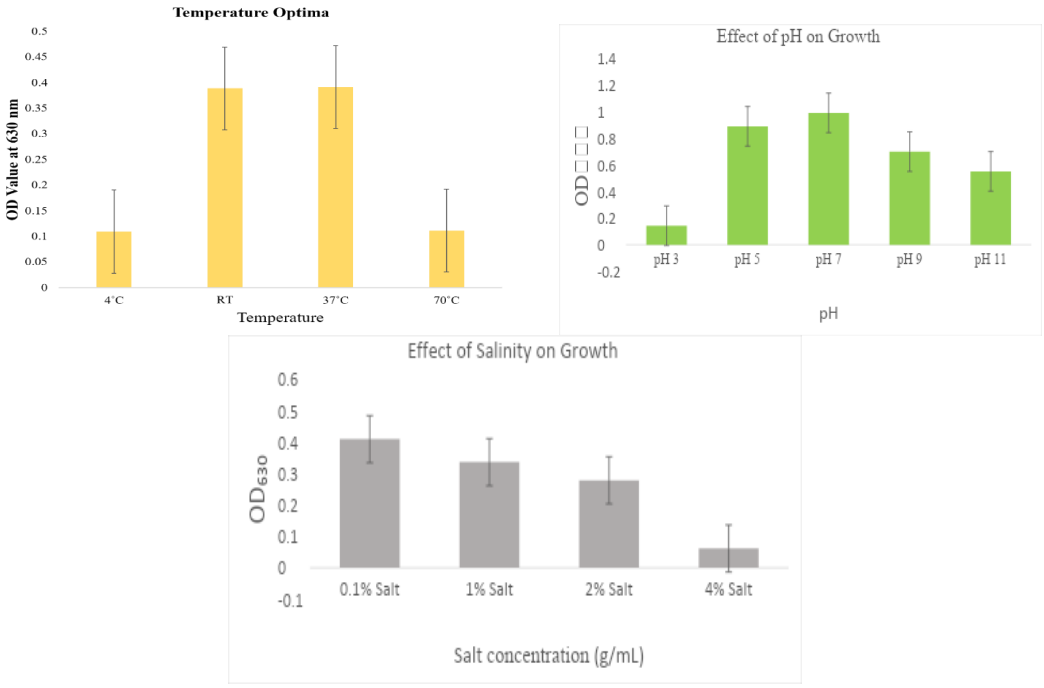

Figure 3: Comparative evaluation of the effects of temperature, salinity, and pH on the growth of Candida glabrata.

3.3 Microscopic Characterization of the Isolated Fungus

Microscopic examination of the purified isolate following crystal violet staining revealed oval to spherical yeast cells occurring predominantly as single cells and budding forms (Figure 4). The cells exhibited a uniform morphology with distinct budding characteristics and lacked true hyphae or pseudohyphal structures under the experimental conditions employed. The microscopic observations were consistent throughout the culture and confirmed the morphological uniformity of the purified isolate.

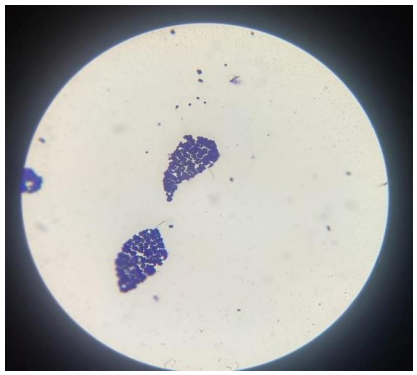

Figure 4: Microscopic appearance of crystal violet-stained Candida glabrata showing oval to spherical budding.

Table 2: Microscopic Characteristics of the Isolated Yeast Observed by Simple Staining.

Feature	Observation
Stain Used	Crystal Violet
Cell Shape	Oval to spherical
Cell Arrangement	Clusters & single cells
Cell Size	1–5 µm (typical for C. glabrata)
Staining Pattern	Uniform deep blue/violet staining
Hyphae	Absent (no true hyphae – confirms C. glabrata)
Budding	Budding cells visible

3.4 Antifungal Susceptibility Profile

The antifungal susceptibility profile of the isolated *Candida glabrata* was evaluated against five commonly used antifungal agents using the disc diffusion method (Table 2; Figure 5). Among the antifungal drugs tested, fluconazole produced the largest zone of inhibition, indicating the highest antifungal activity against the isolate. Miconazole and clotrimazole also exhibited inhibitory activity but produced comparatively smaller inhibition zones. Ketoconazole demonstrated moderate antifungal activity, whereas itraconazole showed the least inhibitory effect. Based on these findings, fluconazole was selected as the reference antifungal agent for subsequent combination studies with the plant extract.

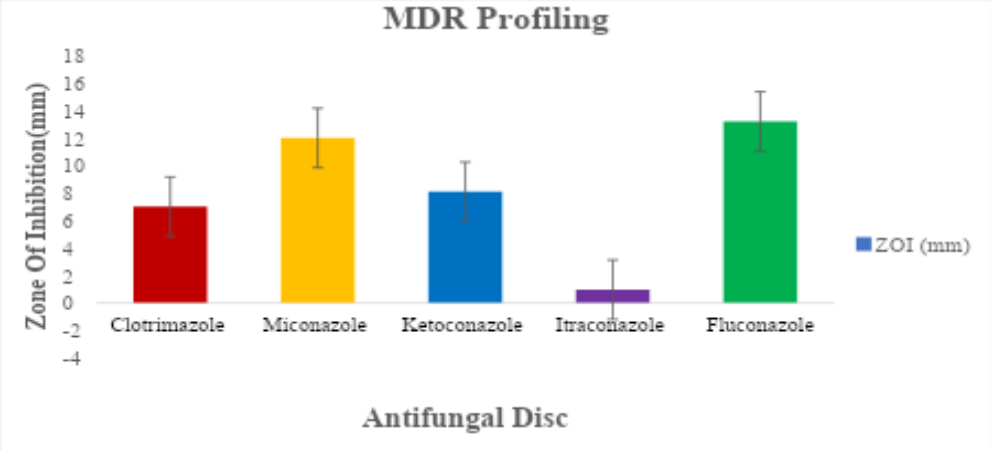

Figure 5: Antifungal Susceptibility Profile of Candida glabrata by Agar Disc Diffusion Method.

3.5 Screening of Medicinal Plant Extracts for Anticandidal Activity

The anticandidal activity of six medicinal plant extracts was evaluated against *Candida glabrata* using the agar well diffusion assay (Table 3; Figures 6 and 7). Among the extracts tested, the methanolic extract of *Moringa oleifera* exhibited the highest antifungal activity, producing the largest zone of inhibition against the fungal isolate. *Azadirachta indica* and *Adhatoda vasica* demonstrated moderate inhibitory activity, whereas *Murraya koenigii*, *Citrus limon*, and *Curcuma longa produced* comparatively smaller zones of inhibition.

Figure 6: Selected medicinal plant powders used in the study and methanol employed as the extraction solvent.

3.6 In Vitro Anticandidal Activity of Selected Medicinal Plant Extracts

The antifungal activities of the extracts were further quantified using the broth microdilution assay (Table 4). Consistent with the agar well diffusion results, the methanolic extract of *Moringa oleifera* exhibited the greatest reduction in fungal growth, as indicated by the lowest optical density values. The remaining plant extracts showed variable inhibitory effects, with comparatively higher optical density values indicating reduced antifungal activity. Based on these observations, the methanolic extract of *Moringa oleifera* was selected for all subsequent phytochemical characterization and combination studies with fluconazole.

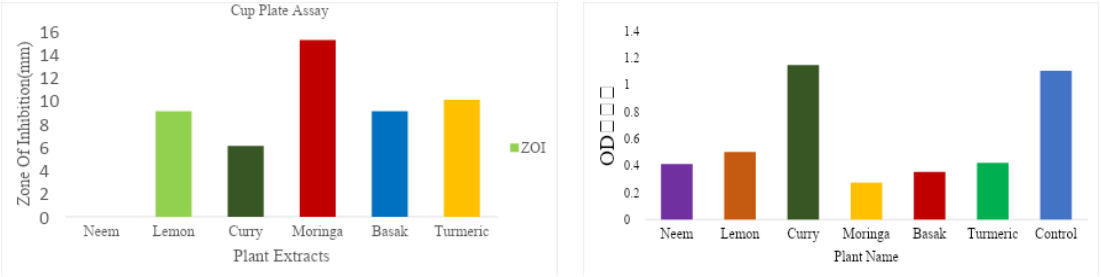

Figure 7: Comparative evaluation of the anticandidal activity of selected medicinal plant extracts against Candida glabrata using agar well diffusion and broth microdilution methods.

3.7 Phytochemical Characterization of *Moringa oleifera* Methanolic Extract

The phytochemical profile of the methanolic extract of *Moringa oleifera* was investigated using thin-layer chromatography (TLC), column chromatography, and qualitative phytochemical screening. Thin-layer chromatographic analysis resolved the extract into multiple distinct bands with different retention factor (Rf) values, indicating the presence of several phytochemical constituents with varying polarities (Figure 8). The chromatographic separation demonstrated a heterogeneous phytochemical composition of the extract. Further fractionation by silica gel column chromatography yielded multiple fractions based on differential elution with the selected solvent system. Fractions exhibiting similar TLC profiles were pooled for subsequent phytochemical evaluation (Figure 9).

Qualitative phytochemical screening confirmed the presence of several bioactive secondary metabolites in the methanolic extract (Table 5). The extract tested positive for phenolic compounds, flavonoids, alkaloids, terpenoids, glycosides, tannins, carbohydrates, proteins, and saponins, while the remaining phytochemical groups were absent under the experimental conditions employed.

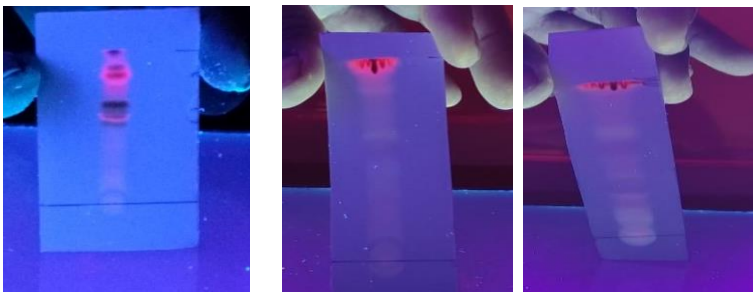

Figure 8: Thin Layer Chromatography (TLC) Profile of Bioactive Compounds in Moringa oleifera Methanolic Extract (a) Phenolic (b) Flavonoids (c) Alkaloids.

Table 3: Rf values of phytochemical constituents identified in the methanolic extract of Moringa oleifera by thin layer chromatography (TLC)

Compound	Rf Value Range
Phenolic	0.10 – 0.88
Flavonoids	0.26 – 0.83
Alkaloids	0.21 – 0.89

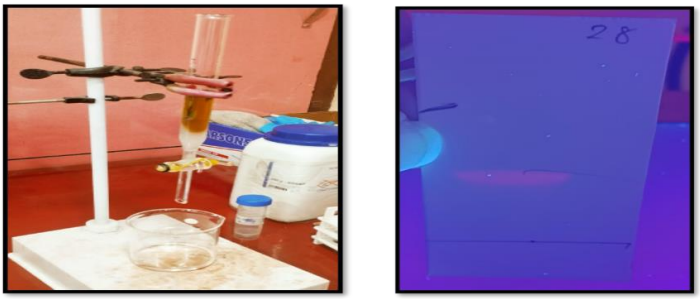

Figure 9: Silica gel column chromatography of the methanolic extract of Moringa oleifera and TLC analysis of the collected fractions.

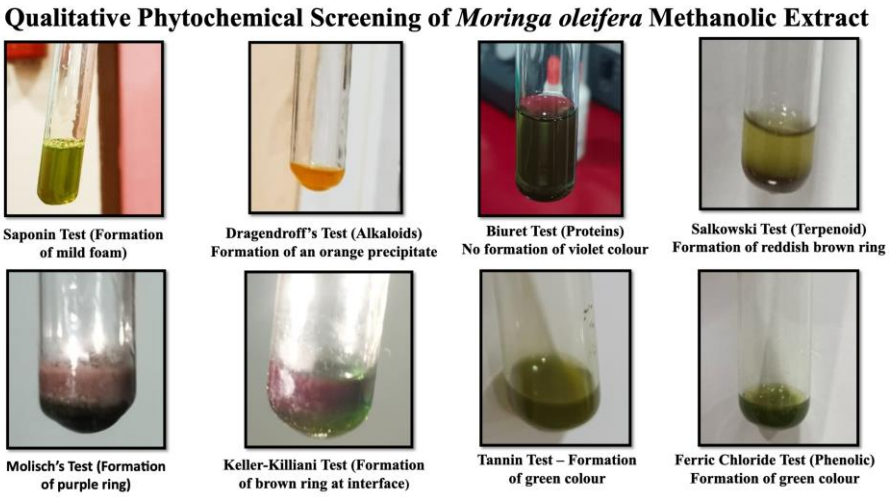

Figure 10: Qualitative phytochemical screening of the methanolic extract of Moringa oleifera showing the presence of major phytochemical constituents.

3.8 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MICs) of the methanolic extract of *Moringa oleifera*, fluconazole, and their combination against *Candida glabrata* were determined using the broth microdilution method (Table 4). The methanolic extract and fluconazole individually inhibited fungal growth up to the 1:16 dilution, beyond which visible growth was observed. In contrast, the combination of *Moringa oleifera* extract and fluconazole inhibited fungal growth up to the 1:32 dilution, indicating enhanced antifungal activity compared with either treatment alone.

No visible fungal growth was observed in the positive inhibitory wells, whereas fungal proliferation was evident at higher dilutions where the inhibitory effect was lost. The extended inhibitory activity exhibited by the combination treatment demonstrated improved growth suppression of *C. glabrata* under the experimental conditions employed.

Table 4: Minimum Inhibitory Concentration (MIC) of Moringa oleifera Extract, Fluconazole, and Their Combination Against the Isolated Candida Species.

Dilution	Moringa	Moringa + FLC	Fluconazole
1:2	(-)	(-)	(-)
1:4	(-)	(-)	(-)
1:8	(+)	(-)	(+)
1:16	(+)	(-)	(+)
1:32	(+)	(-)	(+)
1:64	(+)	(+)	(+)

['+' indicates lesser effect of factor on cell; ' -' indicates more effect of factor on cell]

3.8.1 Biofilm Eradication Assay

The ability of the methanolic extract of *Moringa oleifera*, fluconazole, and their combination to eradicate preformed *Candida glabrata* biofilms was evaluated using the crystal violet assay (Figure 11; Table 7). Among the individual treatments, *Moringa oleifera* extract exhibited greater biofilm eradication than fluconazole. The combination treatment produced the highest antibiofilm activity, achieving 36% biofilm eradication, which exceeded that of either treatment alone. These differences among the treatment groups were statistically significant ($P < 0.05$).

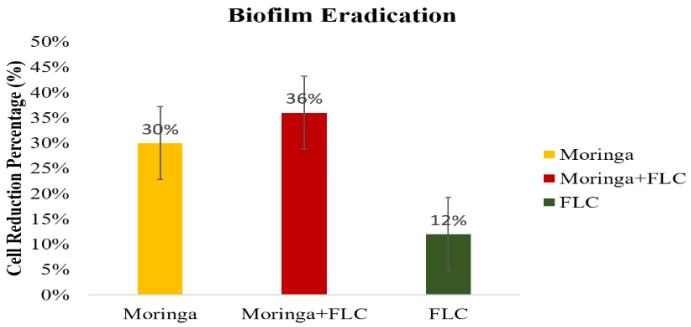

Figure 11: Quantitative Analysis of Biofilm Eradication Activity of Moringa oleifera, Fluconazole, and Their Combination.

3.8.2 Biofilm Inhibition Assay

The inhibitory effects of the treatments on biofilm formation by *Candida glabrata* are presented in Figure 12 and Table 8. The methanolic extract of *Moringa oleifera* inhibited biofilm formation more effectively than fluconazole when administered individually. The combined treatment demonstrated the greatest inhibitory activity, resulting in 45% inhibition of biofilm formation. Statistical analysis indicated significant differences among the experimental groups ($P < 0.05$).

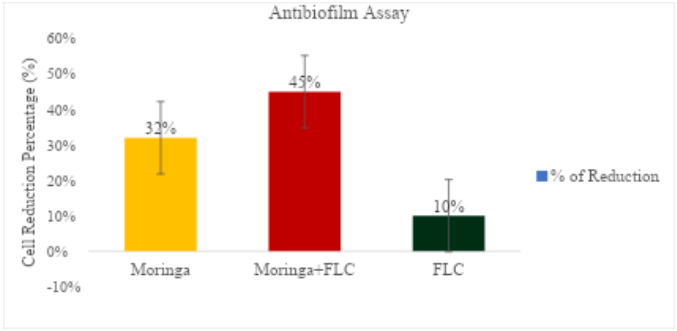

Figure 12: Antibiofilm Inhibitory Activity of Moringa oleifera, Fluconazole, and Their Combination.

3.9 Effect of *Moringa oleifera* Extract and Fluconazole on Fungal Cell Viability

The effect of the methanolic extract of *Moringa oleifera*, fluconazole, and their combination on the viability of *Candida glabrata* cells was evaluated using the MTT assay (Figure 13; Table 9). All treatments reduced fungal cell viability compared with the untreated control. Among the individual treatments, the methanolic extract exhibited a greater reduction in cell viability than fluconazole. The combination treatment produced the highest inhibitory effect, resulting in 43% reduction in viable fungal cells. Statistical analysis demonstrated significant differences among the treatment groups ($P < 0.05$).

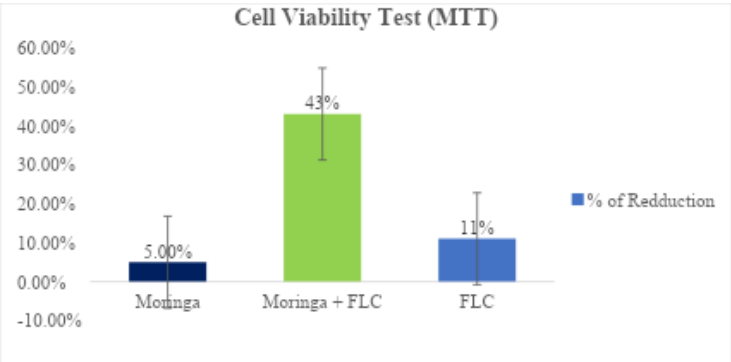

Figure 13: Cell Viability Assessment by MTT Assay for Moringa oleifera, Fluconazole, and Their Combination.

3.10 Growth Kinetics of *Candida glabrata* Under Different Treatments

The growth kinetics of *Candida glabrata* in the presence of the methanolic extract of *Moringa oleifera*, fluconazole, and their combination were monitored by measuring the optical density at 630 nm over the incubation period (Figure 14). The untreated control exhibited the characteristic growth pattern comprising lag, exponential, stationary, and decline phases. In contrast, all treatment groups showed reduced fungal growth throughout the incubation period.

Among the individual treatments, the methanolic extract of *Moringa oleifera* produced greater growth inhibition than fluconazole. The combination treatment consistently exhibited the lowest optical density values at all observation points, indicating the greatest suppression of fungal growth. Two-way ANOVA revealed significant effects of both treatment and incubation time on fungal growth ($P < 0.05$), with the combination treatment demonstrating the most pronounced inhibitory response.

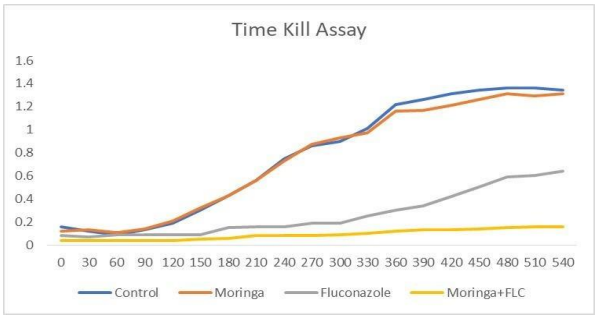

Figure 14: Growth Kinetics and Time–Kill Analysis of Candida glabrata Treated with Moringa oleifera, Fluconazole, and Their Combination.

3.11 SDS–PAGE Protein Profiling

The protein profiles of untreated and treated *Candida glabrata* cultures were analyzed by SDS–PAGE using whole-cell and extracellular protein fractions (Figure 15). Distinct protein banding patterns were observed among the experimental groups. Whole-cell protein extracts exhibited a greater number of well-resolved protein bands than the corresponding extracellular fractions. Variations in the intensity and distribution of protein bands were evident following treatment with the methanolic extract of *Moringa oleifera*, fluconazole, and their combination.

The combination treatment exhibited the most pronounced alterations in protein banding patterns compared with the untreated control, whereas comparatively moderate changes were observed following the individual treatments. These differences indicate treatment-associated modifications in the protein profile of *Candida glabrata* under the experimental conditions employed.

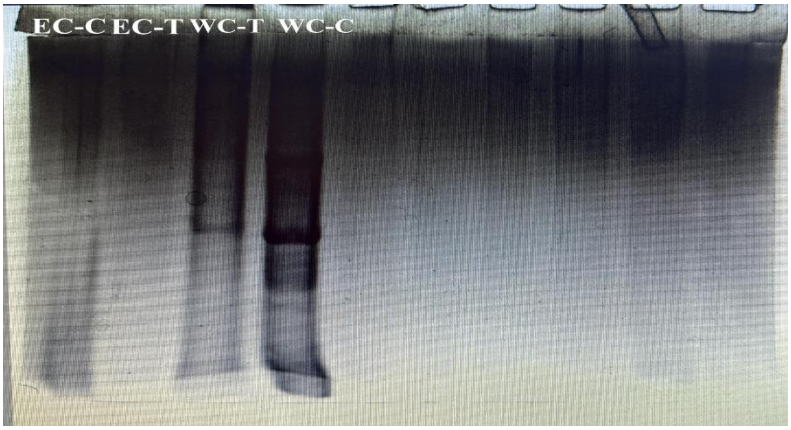

Figure 15: SDS–PAGE analysis.

3.12 Statistical Analysis of Experimental Data

Statistical analysis confirmed that the observed differences among the experimental groups were significant ($P < 0.05$). One-way ANOVA followed by Tukey's multiple comparison test demonstrated significant differences in antifungal activity, minimum inhibitory concentration, biofilm eradication, biofilm inhibition, and fungal cell viability among the untreated control, individual treatments, and combination treatment. In each of these assays, the combination of *Moringa oleifera* methanolic extract and fluconazole consistently exhibited superior antifungal efficacy compared with either treatment alone.

Analysis of the growth kinetics data using two-way ANOVA further revealed significant effects of both treatment and incubation time on fungal growth ($P < 0.05$). The combination treatment produced the greatest suppression of fungal growth throughout the incubation period, confirming the reproducibility of the observed inhibitory response. Collectively, the statistical analyses validated the consistency and reliability of the experimental findings and supported the enhanced antifungal performance of the combination treatment.

DISCUSSION

The successful isolation of *Candida glabrata* from pond water demonstrates that environmental aquatic habitats can serve as reservoirs for opportunistic Candida species. The colony characteristics observed on HiCrome Candida Differential Agar, including cream to off-white, smooth, convex, opaque colonies with an entire margin, were consistent with the typical morphological features previously reported for *C. glabrata*. Although colony morphology alone is insufficient for definitive species identification, it provides an important preliminary basis for selecting representative isolates for further physiological and biochemical characterization. The development of uniform turbidity in Sabouraud Dextrose Broth further confirmed the viability and purity of the isolate, providing a reliable culture for subsequent experimental analyses.

The present study demonstrated that environmental factors significantly influenced the growth of *Candida glabrata*. Maximum growth was observed at room temperature and 37°C, indicating the mesophilic nature of the organism and its ability to proliferate under conditions similar to the human host. Growth was markedly reduced at 4°C and 70°C, suggesting that both low and high temperatures adversely affect fungal metabolism. Similarly, maximum growth occurred at 0.1% NaCl, whereas increasing salinity progressively reduced fungal growth, indicating the inhibitory effect of osmotic stress. The isolate also exhibited optimum growth at neutral pH, with comparatively good growth at pH 5 and reduced growth under highly acidic and alkaline conditions. These observations agree with previous reports demonstrating that *C. glabrata* grows most efficiently under moderate environmental conditions while maintaining sufficient physiological adaptability to survive under fluctuating environmental stresses.

Microscopic examination further confirmed the characteristic morphology of *Candida glabrata*. Crystal violet staining revealed oval to spherical budding yeast cells arranged singly and in clusters, while hyphal and pseudohyphal structures were absent. These microscopic characteristics are consistent with the established morphology of *C. glabrata* and support the preliminary identification based on colony characteristics. The preservation of normal cellular morphology throughout cultivation also indicated that the isolate remained suitable for subsequent physiological and antifungal investigations.

The antifungal susceptibility profile demonstrated variable responses of *Candida glabrata* to different azole antifungal agents. Fluconazole exhibited the highest inhibitory activity, whereas itraconazole showed comparatively lower activity. Differences in susceptibility among azole antifungal drugs have previously been reported and are commonly associated with variations in drug uptake, affinity for the target enzyme, and intrinsic resistance mechanisms of Candida species. Establishing the antifungal susceptibility profile before evaluating plant extracts provides an important baseline for assessing the effectiveness of natural products and their potential use in combination with conventional antifungal therapy.

CONCLUSION

The present study demonstrated the successful isolation and characterization of *Candida glabrata* from pond water and established its optimum growth under neutral pH, moderate temperature, and low salinity conditions. Morphological and microscopic analyses confirmed the characteristic features of the isolate, while antifungal susceptibility testing revealed fluconazole as the most effective antifungal agent among the drugs tested. Among the medicinal plants evaluated, the methanolic extract of *Moringa oleifera* exhibited the highest anticandidal activity. Furthermore, the combination of *Moringa oleifera* extract and fluconazole produced enhanced antifungal efficacy, showing improved minimum inhibitory concentration (MIC), biofilm eradication, antibiofilm activity, reduced cell viability, and greater inhibition in the time–kill assay compared with either treatment alone. Phytochemical screening, TLC, column chromatography, and SDS–PAGE analyses confirmed the presence of bioactive constituents and distinct protein profiles associated with the extract. Collectively, these findings suggest that *Moringa oleifera* possesses promising anticandidal and antibiofilm potential and may serve as a valuable natural adjunct to conventional antifungal therapy against *Candida glabrata*. Further studies involving purification of active compounds and in vivo evaluation are warranted to validate its therapeutic applicability.

REFERENCES

1. Abbas, H. H., & Atiyah, M. M. (2018). Anti-fungal activities of aqueous and alcoholic leaf extracts of Moringa oleifera Lam. on Candida albicans isolated from diabetic foot infections. Journal of Global Pharma Technology, 10(5): 123–131.
2. Adnan, M., Umer, A., Ahmad, I., Hayat, K., & Shakeel, S. N. (2017). In vitro evaluation of biological activities of citrus leaf extracts. Sains Malaysiana, 46(8): 1345–1353.
3. Alzohairy, M. A. (2016). Therapeutics role of Azadirachta indica (Neem) and their active constituents in disease prevention and treatment. Evidence-Based Complementary and Alternative Medicine, 2016; 7382506. https://doi.org/10.1155/2016/7382506
4. Baral, P. K., Afroz, F., Begum, N., Rony, S. R., Sharmin, S., Moni, F., Akhter, S., Sultana, S., & Sohrab, M. H. (2018). Exploration of endophytic fungi and their bioactive potential isolated from the medicinal plant Adhatoda vasica. Research Journal of Pharmacognosy, 11(1): 9–18.
5. Ben-Ami, R., Zimmerman, O., Finn, T., Amit, S., Novikov, A., Wertheimer, N., Lurie-Weinberger,

M., & Berman, J. (2016). Heteroresistance to fluconazole is a continuously distributed phenotype among Candida glabrata clinical strains associated with in vivo persistence. mBio, 7(5): e00655-16. https://doi.org/10.1128/mBio.00655-16
6. Bennett, J. E., Izumikawa, K., & Marr, K. A. (2004). Mechanism of increased fluconazole resistance in Candida glabrata during prophylaxis. Antimicrobial Agents and Chemotherapy, 48(5): 1773–1777. https://doi.org/10.1128/AAC.48.5.1773-1777.2004
7. Brass, C., Galgiani, J. N., Blaschke, T. F., Defelice, R., O'Reilly, R. A., & Stevens, D. A. (1982). Disposition of ketoconazole, an oral antifungal, in humans. Antimicrobial Agents and Chemotherapy, 21(1): 151–158. https://doi.org/10.1128/AAC.21.1.151
8. Clinical and Laboratory Standards Institute. (2008). Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved standard – Third edition (M27-A3). Clinical and Laboratory Standards Institute.
9. Costa, C., Ribeiro, J., Miranda, I. M., Silva-Dias, A., Cavalheiro, M., Costa-de-Oliveira, S., Rodrigues, A. G., & Teixeira, M. C. (2016). Clotrimazole drug resistance in Candida glabrata clinical isolates correlates with increased expression of the drug:H^+ antiporters CgAqr1, CgTpo1_1, CgTpo3, and CgQdr2. Frontiers in Microbiology, 7: 526. https://doi.org/10.3389/fmicb.2016.00526
10. Farmakiotis, D., Tarrand, J. J., & Kontoyiannis, D. P. (2014). Drug-resistant Candida glabrata infection in cancer patients. Emerging Infectious Diseases, 20(11): 1949–1952. https://doi.org/10.3201/eid2011.140685
11. Fothergill, A. W. (2006). Miconazole: a historical perspective. Expert Review of Anti-infective Therapy, 4(2): 171–175. https://doi.org/10.1586/14787210.4.2.171
12. Frej-Mądrzak, M., Golec, S., Włodarczyk, K., Teryks-Wołyniec, D., Jama-Kmiecik, A., Sarowska, J., & Choroszy-Król, I. (2021). Susceptibility to clotrimazole of Candida spp. isolated from the genitourinary system—A single center study. Pathogens, 10(5): 595. https://doi.org/10.3390/pathogens10050595
13. Gani, B. A., Soraya, C., Sugiaman, V. K., & Pranata, N. (2023). Fungistatic effect of Moringa oleifera Lam. on the metabolism changes of Candida albicans. Journal of Pharmacy & Pharmacognosy Research, 11(1): 179–188.
14. Gonçalves, B., Azevedo, N., Osório, H., Henriques, M., & Silva, S. (2021). Revealing Candida glabrata biofilm matrix proteome: global characterization and pH response. Biochemical Journal, 478(4): 961–974. https://doi.org/10.1042/BCJ20200955
15. Gupta, P., Gupta, S., Sharma, M., Kumar, N., & Pruthi, V. (2018). Effectiveness of phytoactive molecules on transcriptional expression, biofilm matrix, and cell wall components of Candida glabrata and its clinical isolates. ACS Omega, 3(10): 14201–14211. https://doi.org/10.1021/acsomega.8b01856
16. Hitchcock, C. A., Pye, G. W., Troke, P. F., Johnson, E. M., & Warnock, D. W. (1993). Fluconazole resistance in Candida glabrata. Antimicrobial Agents and Chemotherapy, 37(9): 1962–1965. https://doi.org/10.1128/AAC.37.9.1962
17. Indrastiti, R. K., Rahmayanti, F., & Kurnia, D. (2025). Antifungal activities of Moringa oleifera Lam. and its defense mechanism against Candida albicans: A literature review. AIP Conference Proceedings, 3312(1): 020003. https://doi.org/10.1063/5.0251736

