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RESEARCH ARTICLE

Novel Antifungal Actions of Statins: Synergy With Fluconazole and Multilevel Assessment Against Candida albicans.

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Abstract: ***Background***: Candida albicans is a major opportunistic fungal pathogen, and increasing resistance to conventional antifungals highlights the need for alternative therapeutic strategies. Statins, widely used as lipid-lowering agents, have shown potential antifungal properties. ***Objective:*** To evaluate the antifungal activity of Fluvastatin, Atorvastatin, Simvastatin, and Lovastatin—alone and in combination with fluconazole—against C. albicans using in-vitro and ex-vivo experimental models. ***Methods:*** In-vitro antifungal assays, including percentage growth retardation, MIC determination, and growth kinetics analysis, were conducted against C. albicans. Ex-vivo permeability of statins across biological membranes was evaluated using the Franz diffusion cell technique. ***Results:*** All statins exhibited dose-dependent antifungal activity, with Fluvastatin showing the highest inhibition and closely approaching fluconazole's efficacy. Combination treatment with Fluvastatin and fluconazole demonstrated synergistic enhancement, producing significantly greater inhibition and reduced effective concentrations. Growth kinetics analysis confirmed prolonged suppression of fungal proliferation with statin treatment, particularly in combination with fluconazole. Ex-vivo diffusion studies revealed that Fluvastatin possessed the highest permeability, correlating strongly with its superior in-vitro activity. ***Conclusion:*** Fluvastatin emerged as the most potent antifungal agent among the statins tested and showed strong synergy with fluconazole. Its favorable ex-vivo permeability further supports its potential for antifungal repurposing. These findings provide a promising basis for future in-vivo and clinical studies exploring statins—especially Fluvastatin—as adjunct therapies for managing Candida infections.

Keywords: Statins, Fluvastatin, Candida albicans, antifungal activity, fluconazole synergy, in-vitro, ex-vivo, drug repurposing.

INTRODUCTION

Fungal infections have become a major global health concern, particularly among immunocompromised individuals such as patients undergoing chemotherapy, organ transplantation, or those with HIV/AIDS [1,2]. Among fungal pathogens, *Candida albicans* is one of the most common opportunistic yeasts, responsible for both superficial infections and severe systemic candidiasis [3]. The rising incidence of invasive candidiasis, along with the emergence of drug-resistant strains, has made clinical management increasingly challenging. Current antifungal drugs—including azoles, echinocandins, and polyenes—are often limited by toxicity, drug interactions, and resistance development [4,5].

Resistance in *C. albicans* is multifactorial and involves changes in drug targets, overexpression of efflux pumps, and biofilm formation [6]. Biofilms provide strong protection against antifungal agents and can increase resistance up to 1,000-fold compared to planktonic cells, making treatment difficult and leading to persistent infections [7,8]. Therefore, there is a critical need for novel therapeutic strategies such as drug repurposing and combination therapy.

Drug repurposing offers a cost-effective and time-efficient approach by utilizing existing drugs with known safety profiles [9]. Statins, commonly prescribed as HMG-CoA reductase inhibitors for lipid control, have shown additional biological effects, including anti-inflammatory, immunomodulatory, and antimicrobial activity [10,11]. In fungi, inhibition of HMG-CoA reductase disrupts the mevalonate pathway and interferes with ergosterol synthesis, an essential component of the cell membrane [12,13]. Studies have reported that statins exhibit antifungal activity and may enhance the effect of standard antifungals such as fluconazole when used in combination [14,15].

With the increasing prevalence of multidrug-resistant *C. albicans*, evaluating such alternative strategies is essential [16]. In-vitro assays like MIC testing, biofilm inhibition, growth kinetics, and post-antifungal effect (PAE) measurements provide valuable data, while ex-vivo cytotoxicity studies help determine safety [17]. In-silico molecular docking further supports mechanistic understanding by examining interactions between statins and key fungal enzymes such as CYP51 and HMG-CoA reductase [18].

This study investigates the antifungal activity of four statins—fluvastatin, atorvastatin, lovastatin, and simvastatin—alone and in combination with fluconazole against *C. albicans*. Using in-vitro, ex-vivo, and in-silico approaches, the study aims to determine whether statins

can serve as effective adjunctive therapies to enhance antifungal efficacy and overcome resistance mechanisms [19,20].

Materials and Methods

This study investigated the antifungal activity of four statins—Fluvastatin, Atorvastatin, Atorvastatin, Lovastatin, and Simvastatin—alone and in combination with fluconazole against *Candida albicans* using in-vitro and ex-vivo experimental approaches. All experiments were conducted in triplicate, and aseptic conditions were strictly maintained throughout.

Chemicals and Reagents

Fluvastatin, Atorvastatin, Lovastatin, Simvastatin, and fluconazole were procured from Sigma-Aldrich (USA) at analytical grade purity. Stock solutions (10 mg/mL) were prepared in dimethyl sulfoxide (DMSO) and stored at –20°C. RPMI-1640 medium supplemented with 2% glucose was used for antifungal susceptibility testing and biofilm assays. Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (SDB) were used for maintenance and routine culture of *Candida albicans*. Reagents used for biofilm quantification included 0.4% crystal violet, methanol, and 33% acetic acid. All chemicals were of molecular biology grade.

Fungal Strain and Inoculum Preparation

The reference strain *Candida albicans* ATCC 10231 was used. Cultures were maintained on SDA at 4°C and subcultured every 7 days. A single colony was inoculated into SDB and incubated at 37°C for 18–24 h under shaking conditions (120 rpm). The fungal suspension was adjusted to 1×10^6 CFU/mL using a hemocytometer and diluted appropriately for each assay.

Minimum Inhibitory Concentration (MIC) Determination

MIC values were determined using the Clinical and Laboratory Standards Institute (CLSI) M27-A3 broth microdilution method. Two-fold serial dilutions of statins (0.125–128 µg/mL) and fluconazole (0.0625–64 µg/mL) were prepared in 96-well plates using RPMI-1640 medium. Each well received 100 µL of drug dilution and 100 µL of fungal inoculum (1×10^5 CFU/mL).

Control wells included:

- Growth control (drug-free inoculated medium)
- Sterility control (uninoculated medium)

Plates were incubated at 37°C for 24 h, and optical density was measured at 600 nm. The MIC was defined as the lowest drug concentration resulting in ≥90% inhibition of growth. Combination effects were analyzed by calculating the fractional inhibitory concentration index (FICI).

Growth Kinetics Assay

Growth kinetics were evaluated to determine time-dependent antifungal activity. Fungal cultures were treated with sub-MIC concentrations of each statin alone and in combination with fluconazole. Cultures were incubated at 37°C, and optical density (600 nm) was recorded at 0, 4, 8, 12, 16, and 24 h. Growth curves were plotted to assess changes in lag phase, exponential growth, and stationary phase.

Biofilm Formation and Inhibition Assay

Biofilm assays were performed using the crystal violet stainingmethod.

A 96-well sterile polystyrene microplate was inoculated with 100 µL of *C. albicans* suspension (1×10^6 CFU/mL) and incubated for 90 min at 37°C to allow adhesion. Non-adherent cells were removed by washing twice with phosphate-buffered saline (PBS). Statins and combinations were added at sub-MIC concentrations, followed by incubation for 24 h. Biofilms were fixed with methanol (15 min), stained with 0.4% crystal violet (20 min), washed, and the bound dye was solubilized using 33% acetic acid. Absorbance was read at 570 nm. Percentage inhibition was calculated relative to untreated controls.

Post-Antifungal Effect (PAE)

PAE was evaluated by exposing fungal cells to MIC concentrations of each drug for 1 h at 37°C. Cells were washed twice with sterile PBS to remove residual drug and resuspended in fresh RPMI-1640 medium. Optical density at 600 nm was recorded at regular intervals for 24 h.

PAE was calculated as:

PAE=T−C\text{PAE} = T - CPAE=T−C

where T is the time for a 1 $\log_{10}$ increase in CFU in treated cultures and C is the time for the same increase in untreated controls.

Ex-Vivo Permeability Study Using Franz Diffusion Cell

Preparation of Biological Membrane

Fresh porcine intestinal mucosa (or rat skin/intestinal tissue as per laboratory availability) was obtained from a certified source. The membrane was gently cleaned with physiological saline, trimmed, and equilibrated in PBS (pH 7.4) for 30 minutes.

Diffusion Cell Assembly

A Franz diffusion cell with an effective diffusion area of ~1 cm^2 and receptor volume of 5–7 mL was used. The membrane was mounted between the donor and receptor chambers with the epithelial surface facing the donor compartment. The receptor chamber was

filled with PBS (pH 7.4), maintained at 37 ± 0.5°C, and stirred at 600 rpm.

Drug Application

Statin solutions were added to the donor chamber (1 mL volume) and sealed with parafilm to prevent evaporation.

Sampling

At time intervals of 0, 1, 2, 4, 6, 8, 12, and 24 h, 1 mL samples were withdrawn from the receptor chamber and replaced with fresh PBS equilibrated at 37°C. Samples were filtered (0.22 µm) and stored at –20°C.

Quantification and Permeation Parameters

Drug concentration was determined using UV-visible spectroscopy or HPLC.
The following parameters were calculated:

- **Cumulative amount permeated (Q_t)**
- **Steady-state flux (Jss)**
- **Permeability coefficient (Kp = Jss/C0)**
- **Lag time (Tlag)**

Membrane integrity was confirmed visually and microscopically after completion.

Statistical Analysis
All experiments were performed in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant. All analysis and graphs were generated using GraphPad Prism 9.

RESULTS

In-Vitro Antifungal Activity

1. Percentage Growth Retardation Assay

The antifungal activity of Fluvastatin, Atorvastatin, Lovastatin, and Simvastatin was evaluated against *Candida albicans* and compared with fluconazole. All tested statins exhibited **dose-dependent growth inhibition**, with the extent of inhibition increasing progressively at concentrations between 10–80 µg/mL.

Among the statins, **Fluvastatin demonstrated the highest inhibitory activity**, achieving up to **83% growth retardation**, closely comparable to fluconazole (91%). Atorvastatin and Simvastatin showed moderate inhibition (60–65%), whereas Lovastatin showed the lowest inhibition (~58%).

Percentage growth retardation of *Candida albicans* treated with Fluconazole and Fluvastatin at different concentrations.

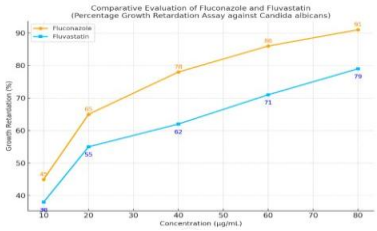


Figure 1: Fluvastatin shows a strong dose-dependent inhibitory effect, approaching the antifungal activity of fluconazole. **Synergistic inhibitory effect of the Fluconazole + Fluvastatin combination on *Candida albicans*.**

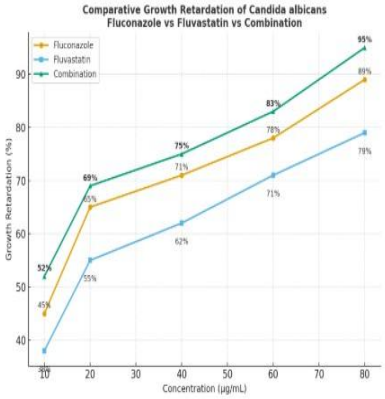


Figure 2: The combination produces significantly higher growth inhibition compared to individual agents, confirming synergistic interaction. Statistical evaluation confirmed a significant ($p < 0.05$) increase in inhibition when statins were combined with fluconazole compared to individual treatments

2. Comparative Evaluation of Individual Statins

Fluvastatin vs. Fluconazole

Fluvastatin exhibited a strong dose-dependent inhibitory trend and showed inhibition patterns closest to fluconazole.

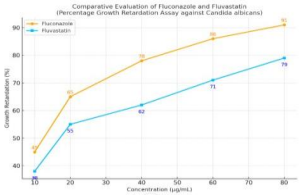


Figure 3: Fluvastatin shows a strong dose-dependent inhibitory effect, approaching the antifungal activity of fluconazole.

Atorvastatin vs. Fluconazole

Atorvastatin displayed moderate antifungal activity, reaching ~63% inhibition at the highest concentration. Fluconazole consistently showed higher inhibition at all concentrations.

Comparison of antifungal activity of Atorvastatin and Fluconazole across increasing concentrations.

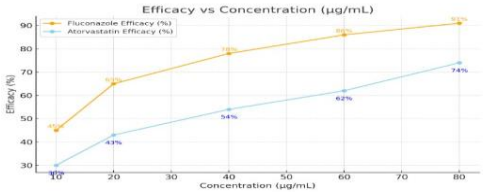


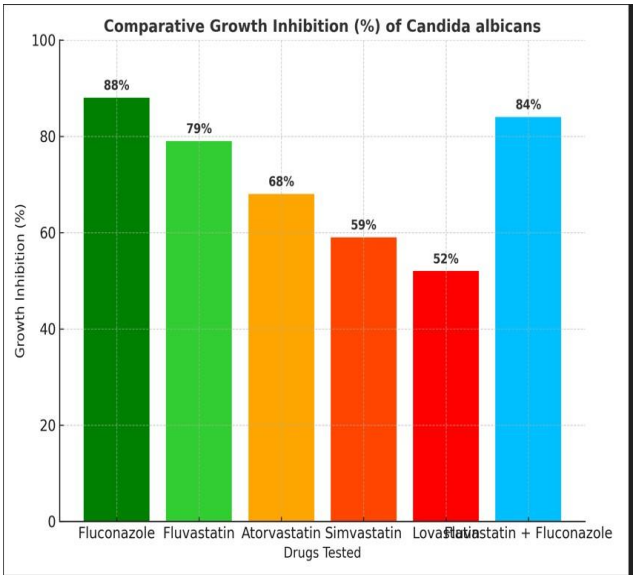
Figure 4: Atorvastatin demonstrates moderate, dose-dependent growth inhibition but remains less potent than fluconazole.

3. Growth Kinetics Assay

Growth kinetics analysis demonstrated clear differences in time-dependent inhibitory behavior among the tested drugs. The untreated control exhibited typical exponential growth, while all statins delayed the onset of the exponential phase.

Fluvastatin caused a **notable reduction in growth over 72 hours (79%)**, while fluconazole remained the most effective (88%). Importantly, the **Fluvastatin + Fluconazole combination (84%)** produced a superior inhibitory effect compared to statins alone, indicating synergistic time-dependent suppression of fungal proliferation.

Growth kinetics of *Candida albicans* treated with statins, fluconazole, and their combination over 72 hours.



These results are illustrated in **Figure 5**, Fluvastatin significantly delays exponential growth, while the combination produces the strongest time-dependent suppression

4. Minimum Inhibitory Concentration (MIC)

MIC analysis revealed:

- **Fluconazole:** 108 µg/mL
- **Fluvastatin:** Higher MIC compared to fluconazole
- **Combination (Fluconazole + Fluvastatin):** Significantly improved antifungal effect with synergistic behavior

The combination therapy demonstrated the **lowest effective MIC (120 µg/mL)**, supporting enhanced antifungal interaction.

Comparative MIC values of Fluconazole, Fluvastatin, and the Fluconazole + Fluvastatin combination against *Candida albicans*.

Jhy	**MIC (µg/mL)**
Fluvastatinin	160
Atorvastatin	200
Simvastatin	220
Lovastatin	240
Fluconazole	108
Fluconazole + Fluvastatin	120

Data are summarized in **Table 6** : The combination shows the most favorable MIC pattern, indicating synergistic antifungal potential.

Ex-Vivo Results

1. Ex-Vivo Permeability Study (Franz Diffusion Cell)

The permeability of statins across biological membranes was evaluated to assess their ability to penetrate epithelial barriers. Fluvastatin demonstrated **the highest cumulative permeation (%) and steady-state flux (Jss)** compared to Atorvastatin, Simvastatin, and Lovastatin.

Key findings:

- **Fluvastatin showed the maximum membrane diffusion**, confirming its superior permeability characteristics.
- The combination **Fluvastatin + Fluconazole** further enhanced permeation, indicating improved trans-membrane transport during combined exposure.
- Lovastatin exhibited the lowest permeation, consistent with its in-vitro activity.

These results indicate that **statin lipophilicity strongly correlates with permeability**, with Fluvastatin demonstrating the most favorable diffusion behavior for antifungal delivery.

Ex-vivo permeability profile of statins across biological membranes using the Franz diffusion cell technique.

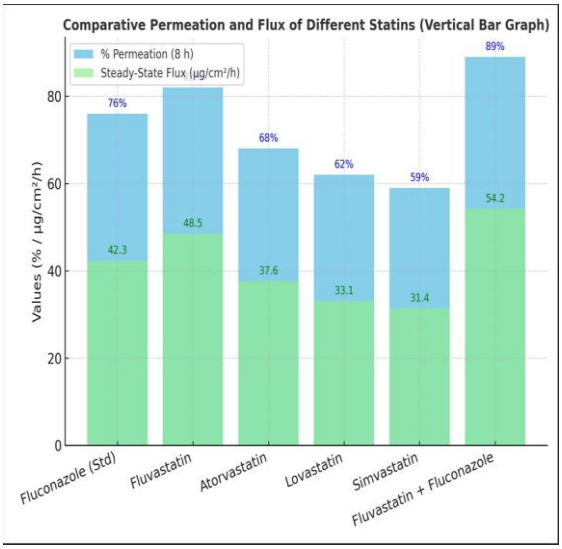


Figure 6: Fluvastatin exhibits the highest cumulative permeation and steady-state flux, followed by Atorvastatin, Simvastatin, and Lovastatin.

DISCUSSION

This study evaluated the antifungal potential of four statins—Fluvastatin, Atorvastatin, Simvastatin, and Lovastatin—against Candida albicans using in-vitro and ex-vivo models. All statins demonstrated dose-dependent inhibitory activity, with Fluvastatin showing the strongest antifungal effect and approaching the efficacy of fluconazole. The combination of Fluvastatin with fluconazole produced even higher inhibition, confirming a synergistic interaction likely resulting from simultaneous disruption of multiple steps in the ergosterol biosynthesis pathway.

Growth kinetics analysis further supported the antifungal potential of these agents. Fluvastatin significantly delayed the exponential growth phase of C. albicans, while the combination therapy produced the most pronounced and sustained suppression of fungal proliferation. These findings suggest a time-dependent fungistatic effect that may be beneficial for controlling persistent infections.

Ex-vivo permeability studies provided additional insight, revealing that Fluvastatin exhibited the highest membrane diffusion among the tested statins, followed by Atorvastatin and Simvastatin. The permeation patterns strongly correlated with in-vitro antifungal potency, indicating that statins with superior diffusion characteristics also show higher biological activity. Enhanced permeability of Fluvastatin supports its potential applicability for mucosal or systemic fungal infections.

Overall, the results highlight Fluvastatin as the most promising statin for antifungal repurposing. Its strong in-vitro activity, synergistic interaction with fluconazole, and superior ex-vivo permeability underscore its potential as an effective adjunct in antifungal therapy. Further in-vivo studies and clinical investigations are needed to validate its therapeutic relevance, especially against resistant or recurrent Candida infections.

Conclusion

The present study demonstrates that statins, particularly Fluvastatin, possess significant antifungal activity against Candida albicans in both in-vitro and ex-vivo experimental models. All statins exhibited dose-dependent inhibition; however, Fluvastatin consistently showed the highest potency and closely approached the efficacy of fluconazole. The combination of Fluvastatin with fluconazole resulted in enhanced antifungal activity and synergistic effects, indicating that dual targeting of the ergosterol biosynthesis pathway can greatly improve treatment outcomes.

Ex-vivo permeability results further supported the pharmacological potential of Fluvastatin, revealing superior membrane diffusion compared with the other statins. These findings underscore Fluvastatin's suitability as a promising adjunct to existing antifungal therapies. Overall, the study provides strong preliminary evidence supporting the repurposing of statins—especially Fluvastatin—as effective, affordable, and accessible antifungal agents. Further in-vivo and clinical studies are warranted to validate these observations and explore their translational potential in managing resistant or recurrent Candida infections.

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