In Vitro Antifungal Activity of Apixaban-derived Analogues against Candida albicans and Aspergillus niger: Experimental Evaluation Using the Agar-Well Diffusion Method

Prachi Veerkar[1]*, Dr Jeevan Patel[2], Dr. Sudha Vengurlekar[3], Dr. Sachin Kumar Jain[3]

1*- Research Scholar, Faculty of Pharmacy, Oriental University, Indore, Madhya Pradesh, 453555, India
2-Supervisor, Faculty of Pharmacy, Oriental University, Indore, Madhya Pradesh, 453555, India
3-Professor, Faculty of Pharmacy, Oriental University, Indore, Madhya Pradesh, 453555, India

Correspondence: Mrs. Prachi Veerkar*
Research Scholar, Faculty of Pharmacy, Oriental University,
Indore, Madhya Pradesh, 453555, India
Email: prachiveerkar164@gmail.com

Abstract

Background: The increasing incidence of fungal infections and the emergence of resistance to currently available antifungal agents have created an urgent need for the development of new therapeutic candidates. *Candida albicans* and *Aspergillus niger* are among the clinically important fungal pathogens responsible for a wide range of opportunistic infections, highlighting the importance of identifying compounds with improved antifungal efficacy.

Objective: The present study aimed to investigate the in vitro antifungal activity of fifteen newly synthesized Apixaban-derived analogues against *Candida albicans* and *Aspergillus niger* using the agar-well diffusion method.

Materials and Methods: Fifteen Apixaban-derived analogues (PG001–PG015) were evaluated at four concentrations (12.5, 25, 37.5, and 50 µL) against the selected fungal strains. The agar-well diffusion assay was employed to determine antifungal activity by measuring the diameter of the inhibition zones after incubation. Fluconazole served as the reference antifungal agent, and all experiments were performed in triplicate to ensure reproducibility.

Results: The synthesized analogues exhibited variable antifungal activity against both fungal species. Against Candida albicans, PG001 and PG003 produced the largest inhibition zones at 50 µL (0.80 mm), while PG015 and PG005 also displayed substantial activity with inhibition zones of 0.77 and 0.75 mm, respectively. Against Aspergillus niger, PG002 exhibited the highest activity (0.82 mm), followed by PG001, PG003, and PG015 (0.80 mm each). Several analogues showed little or no detectable antifungal activity, indicating considerable variability in potency among the synthesized compounds.

Conclusion: The findings demonstrate that selected Apixaban-derived analogues possess promising in vitro antifungal activity and may serve as potential lead molecules for future antifungal drug development. Further investigations, including minimum inhibitory concentration determination, mechanistic studies, toxicity evaluation, and in vivo validation, are warranted to establish their therapeutic potential.

Keywords: Apixaban analogues; Antifungal activity; *Candida albicans*; *Aspergillus niger*; Agar-well diffusion assay; Fluconazole; Drug discovery.

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1. Introduction

1.1 Global Burden of Fungal Infections

Fungal infections remain a major global health concern, particularly among immunocompromised individuals, elderly patients, transplant recipients, and patients receiving prolonged immunosuppressive therapy[1], [2]. The increasing incidence of invasive fungal infections, together with the emergence of antifungal resistance and the limited availability of effective therapeutic agents, has intensified the search for novel antifungal compounds[3], [4]. Consequently, the discovery of structurally diverse molecules with improved efficacy and safety has become an important objective in contemporary antifungal drug research[5].

1.2 Clinical Importance of *Candida albicans*

Candida albicans is one of the most clinically important opportunistic fungal pathogens and is responsible for infections ranging from superficial mucosal candidiasis to life-threatening systemic disease[6], [7]. Its pathogenicity is associated with virulence factors such as biofilm formation, morphological switching, tissue invasion, and secretion of hydrolytic enzymes[8], [9]. The increasing prevalence of resistance to azole antifungal agents has reduced the effectiveness of conventional

therapies and highlights the need for new antifungal molecules with improved activity against resistant fungal strains[10], [11].

1.3 Emerging Significance of *Aspergillus niger* Infections

Although Aspergillus fumigatus remains the predominant cause of invasive aspergillosis, Aspergillus niger is increasingly recognized as an opportunistic pathogen associated with pulmonary infections, otomycosis, keratitis, and sinusitis[12], [13]. Its ability to survive under diverse environmental conditions, together with increasing reports of reduced susceptibility to available antifungal drugs, emphasizes the need for the development of novel antifungal agents with broad-spectrum activity[11], [14].

1.4 Limitations of Currently Available Antifungal Drugs

Although several classes of antifungal drugs are currently available, including azoles, polyenes, echinocandins, and allylamines, their clinical utility is associated with several limitations[12], [15]. Long-term antifungal therapy frequently results in the emergence of resistant fungal strains, reducing treatment success and increasing the likelihood of recurrent infections[4], [16]. Resistance mechanisms include alterations in drug targets, overexpression of efflux pumps, reduced intracellular drug accumulation, and biofilm-mediated protection[17], [18].

Many antifungal agents are also associated with adverse effects such as hepatotoxicity, nephrotoxicity, gastrointestinal disturbances, and clinically significant drug–drug interactions[19]. These limitations are particularly important in patients receiving multiple medications or prolonged treatment. Furthermore, the relatively small number of approved antifungal drug classes restricts therapeutic options when resistance develops[14].

These limitations have encouraged the exploration of drug repurposing and chemical modification strategies to identify novel antifungal agents with improved efficacy, reduced toxicity, and broader antimicrobial spectra[20], [21], [22].

1.5 Rationale for Investigating Apixaban-Derived Analogues

Apixaban is a clinically approved direct factor Xa inhibitor widely used for the prevention and treatment of thromboembolic disorders[23], [24]. Beyond its established anticoagulant activity, its chemically diverse scaffold provides multiple sites that can be modified to generate structurally distinct analogues with altered biological properties[25], [26]. Strategic chemical modification of the Apixaban framework offers an opportunity to explore new pharmacological applications beyond its original therapeutic indication[27].

In the present investigation, a series of Apixaban-derived analogues were synthesized and evaluated for their antifungal activity against *Candida albicans* and *Aspergillus niger*. The introduction of different substituents into the parent scaffold was expected to influence molecular interactions with fungal cellular targets, thereby affecting antifungal efficacy. Experimental screening of these analogues enables identification of compounds with promising biological activity while providing valuable information regarding the influence of chemical modifications on antifungal performance.

Evaluating these compounds through an in vitro agar-well diffusion assay provides an initial assessment of their antifungal potential and facilitates the selection of promising lead molecules for subsequent pharmacological and mechanistic investigations.

1.6 Aim and Objectives of the Study

The present study aimed to investigate the in vitro antifungal activity of fifteen newly synthesized Apixaban-derived analogues against *Candida albicans* and *Aspergillus niger* using the agar-well diffusion method.

The specific objectives of the study were:

- To evaluate the antifungal activity of fifteen Apixaban-derived analogues against *Candida albicans* and *Aspergillus niger* using the agar-well diffusion assay.
- To determine the dose-responsive inhibitory effects of the synthesized compounds by measuring the zones of inhibition at different test concentrations.
- To compare the antifungal activity of the synthesized analogues with the standard antifungal drug fluconazole.
- To identify the most active compounds for potential development as lead molecules for future antifungal drug discovery.
- To provide experimental evidence supporting further investigations, including minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), toxicity assessment, mechanistic studies, and in vivo evaluation.

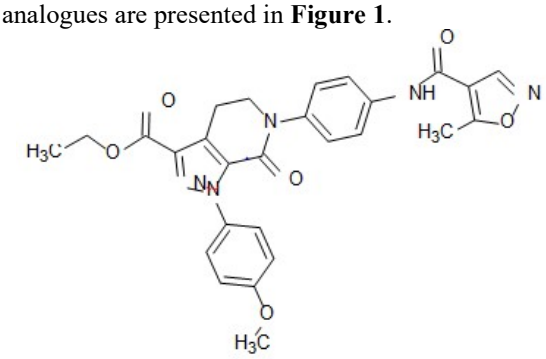
The findings of this study are expected to contribute to the ongoing search for new antifungal agents by providing preliminary biological evidence for the antifungal potential of structurally modified Apixaban analogues against clinically relevant fungal pathogens. To the best of our knowledge, this is the first study investigating the antifungal potential of a series of newly synthesized Apixaban-derived analogues against both Candida albicans and Aspergillus niger. The study combines rational molecular modification

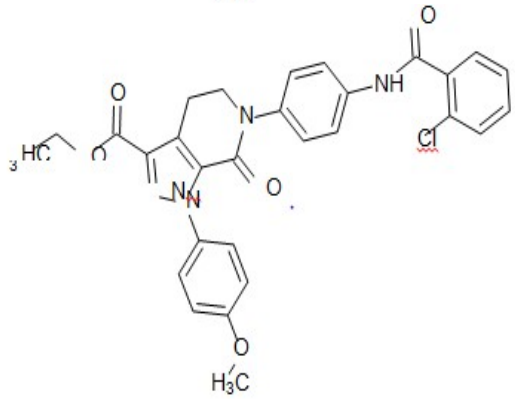
with biological evaluation to identify promising lead molecules for future antifungal drug development.

2. Materials and Methods

2.1 Test Compounds

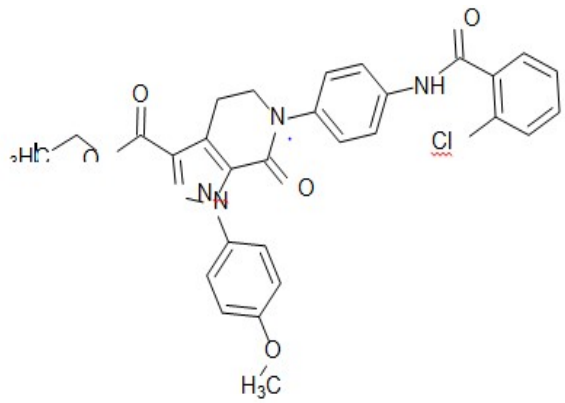
A series of fifteen Apixaban-derived analogues, designated PG001 to PG015, were evaluated for their in vitro antifungal activity. The compounds were synthesized prior to biological screening and were selected based on chemical modifications introduced into the parent Parent Apixaban structure. These modifications were designed to investigate the influence of different substituents on antifungal activity while maintaining the core structural framework. Each analogue was assigned a unique identification code (PG001–PG015) for systematic evaluation throughout the study. The chemical structures of all synthesized Apixaban-derived analogues are presented in **Figure 1**.

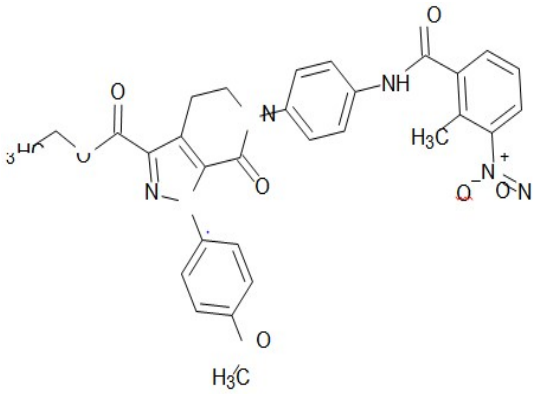




PG001

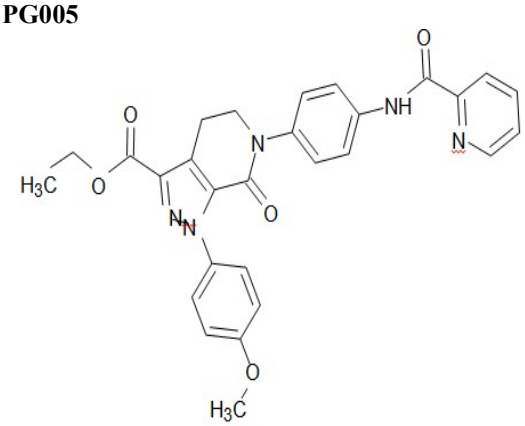
PG002

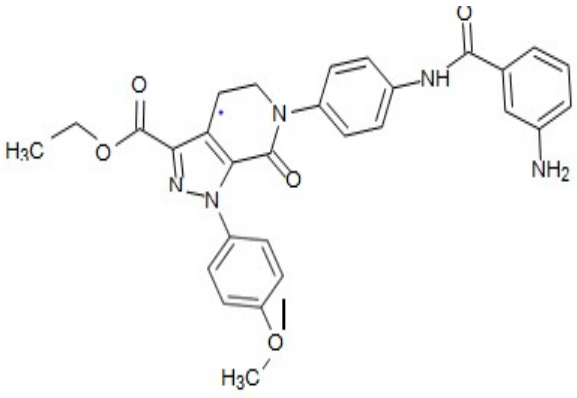




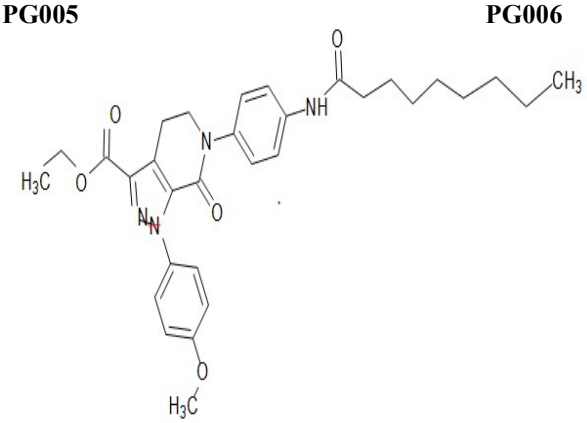
PG004

PG005

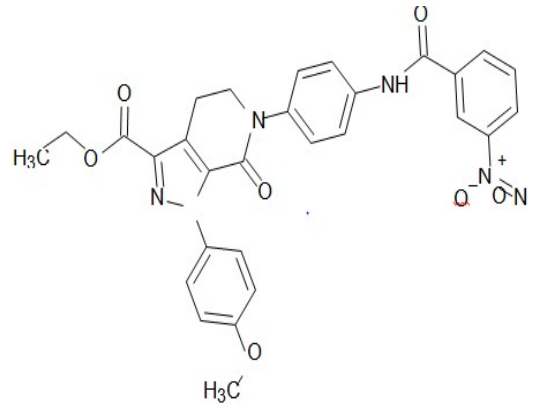




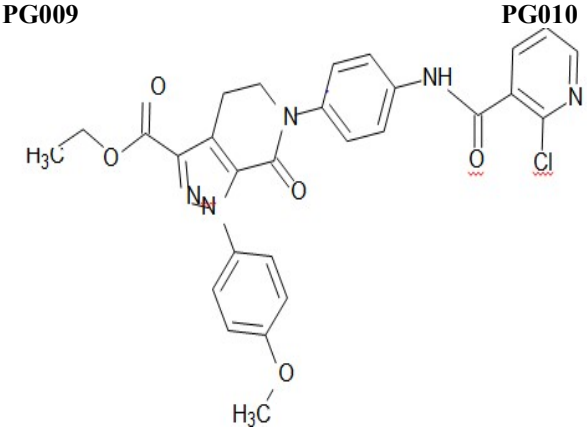
PG005



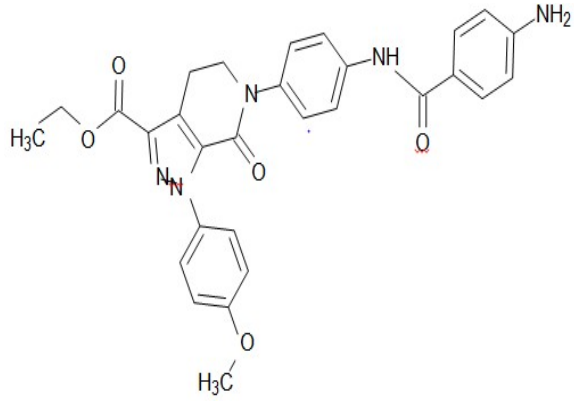
PG006



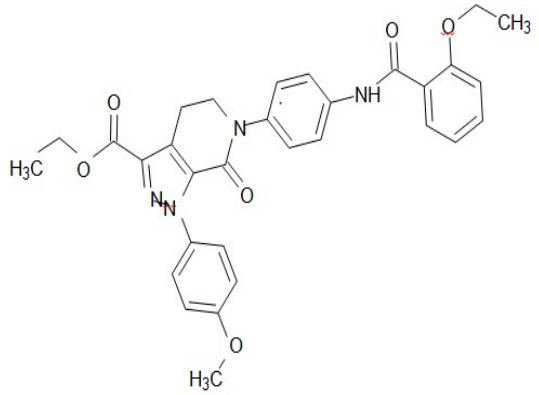
PG009



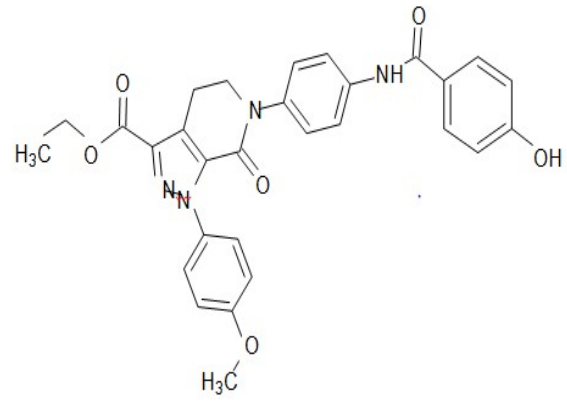
PG010



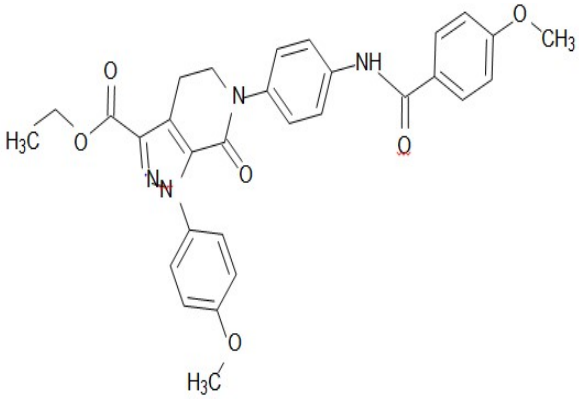
PG007



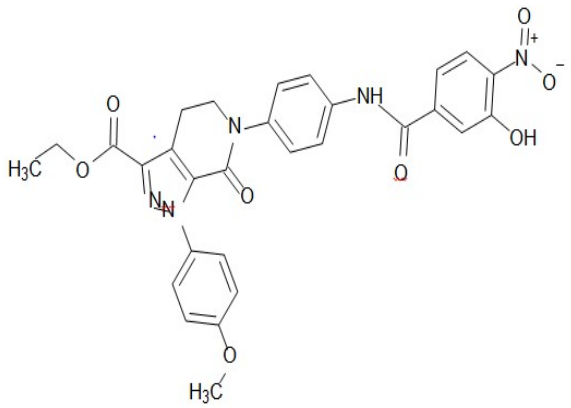
PG008



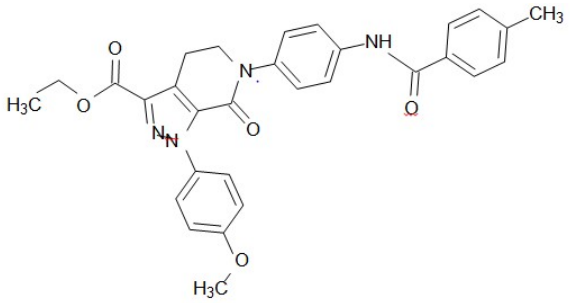
PG011



PG012



PG013 **PG014**



PG015

Figure 1: Chemical structures of the fifteen Apixaban-derived analogues

Figure 1 Chemical structures of the fifteen synthesized Apixaban-derived analogues evaluated for in vitro antifungal activity against *Candida albicans* and *Aspergillus niger*. Chemical modifications were introduced into the parent scaffold to investigate their influence on antifungal activity.

2.2 Fungal Strains

The antifungal activity of the synthesized compounds was evaluated against two clinically relevant fungal pathogens, *Candida albicans* and *Aspergillus niger*. These microorganisms were selected because of their clinical significance and their frequent association with opportunistic fungal infections in immunocompromised individuals[28]. Pure cultures of both fungal strains were maintained under appropriate laboratory conditions and were subcultured before each experiment to ensure optimal viability and reproducibility. Freshly grown cultures were used for all antifungal assays.

2.3 Preparation of Test Samples

Each synthesized Apixaban-derived analogue was dissolved in a suitable solvent to prepare a stock solution of known concentration. Working solutions were subsequently prepared by appropriate dilution of the stock solution to obtain the required concentrations for antifungal screening. Four different sample volumes (12.5, 25, 37.5, and 50 µL) were selected to evaluate the dose-responsive antifungal activity of the synthesized compounds. Fresh working solutions were prepared before each experiment to ensure solution stability and reproducibility of the biological assay.

2.4 Agar-Well Diffusion Method

The in vitro antifungal activity of the synthesized Apixaban-derived analogues was determined using the agar-well diffusion method. Potato Dextrose Agar (PDA) medium was prepared according to the manufacturer's instructions, sterilized, and poured into sterile Petri plates under aseptic conditions. After solidification of the medium, standardized fungal inocula of *Candida albicans* and *Aspergillus niger* were uniformly spread over the agar surface using sterile cotton swabs to obtain an even distribution of fungal cells[29].

Sterile cork borers were used to prepare wells of uniform diameter in the inoculated agar plates. The designated volumes (12.5, 25, 37.5, and 50 µL) of each test sample were carefully introduced into the respective wells without disturbing the agar surface. Fluconazole was included as the positive control to provide a reference for comparison of antifungal activity, while appropriate negative controls were maintained throughout the study.

Following sample loading, the inoculated plates were incubated under suitable conditions to allow fungal growth and compound diffusion through the agar medium. After completion of the incubation period, the antifungal activity of each compound was assessed by measuring the clear zones of inhibition surrounding each well. The overall experimental procedure used for antifungal screening is illustrated in **Figure 2**.

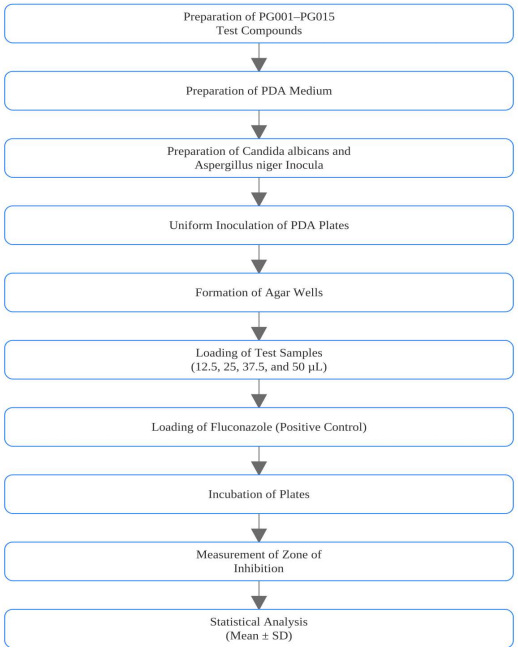


Figure 2: Experimental workflow of the antifungal screening procedure

Figure 2 Schematic representation of the experimental workflow employed for the in vitro antifungal evaluation of Apixaban-derived analogues, including fungal inoculum preparation, agar-well diffusion assay, incubation, and measurement of inhibition zones.

2.5 Measurement of Zone of Inhibition

Following incubation, the antifungal activity of each compound was determined by measuring the diameter of the clear zone of inhibition surrounding each well using a calibrated measuring scale[30]. The absence of visible fungal growth around the well was considered indicative of antifungal activity. Larger inhibition

zones were interpreted as greater antifungal effectiveness in the present study.

All experiments were performed in triplicate to ensure reproducibility and minimize experimental variation. The results are presented as the mean value ± standard deviation (Mean ± SD), providing a reliable estimate of the antifungal activity of each synthesized analogue. The use of replicate measurements enhanced the accuracy and consistency of the experimental findings and facilitated comparison among the tested compounds and the reference antifungal agent.

2.6 Statistical Analysis

All antifungal experiments were performed in triplicate to ensure the reproducibility of the results. The diameters (or radii, depending on your reporting) of the inhibition zones were recorded for each experiment, and the results are presented as the mean ± standard deviation (SD). The experimental data were summarized using descriptive statistics, and no inferential statistical tests were performed because the primary objective of the study was to compare the relative antifungal activities of the synthesized analogues under identical experimental conditions.

3. Results and Discussion

3.1 Antifungal Activity against *Candida albicans*

The synthesized Apixaban-derived analogues exhibited variable antifungal activity against *Candida albicans*, with several compounds demonstrating dose-responsive inhibition. Active analogues produced progressively larger inhibition zones as the test volume increased, whereas others showed little or no detectable antifungal activity throughout the study. The mean inhibition zones obtained from triplicate experiments are summarized in Table 1, allowing comparison of the relative antifungal activities of the synthesized compounds with the reference drug fluconazole.

The antifungal activity of the synthesized Apixaban-derived analogues against Candida albicans was evaluated using the agar-well diffusion method at four different test volumes. The mean zone of inhibition values obtained from triplicate experiments are summarized in Table 1.

Table 1: Antifungal activity of PG001–PG015 against *Candida albicans* determined by the agar-well diffusion method

Compound	Zone of Inhibition (mm ± SD)			
	12.5 µL	**25 µL**	**37.5 µL**	**50 µL**
PG001	0.33 ± 0.4	5 ± 0.5	5.9 ± 0.4	8.0 ± 0.2
PG002	0.35 ± 0.04	0.44 ± 0.25	0.50 ± 0.50	0.50 ± 0.50
PG003	0.37 ± 0.04	0.46 ± 0.25	0.60 ± 0.50	0.80 ± 0.50
PG004	No inhibition	No inhibition	No inhibition	No inhibition
PG005	0.35 ± 0.04	0.43 ± 0.25	0.58 ± 0.50	0.75 ± 0.50
PG006	0.33 ± 0.04	0.42 ± 0.25	0.56 ± 0.50	0.73 ± 0.50
PG007	No inhibition	No inhibition	No inhibition	No inhibition
PG008	No inhibition	No inhibition	No inhibition	No inhibition
PG009	0.37 ± 0.04	0.45 ± 0.25	0.60 ± 0.50	0.78 ± 0.50
PG010	0.32 ± 0.04	0.40 ± 0.25	0.55 ± 0.50	0.70 ± 0.50
PG011	0.32 ± 0.04	0.41 ± 0.25	0.57 ± 0.50	0.70 ± 0.50
PG012	No inhibition	No inhibition	No inhibition	No inhibition
PG013	No inhibition	No inhibition	No inhibition	No inhibition
PG014	No inhibition	No inhibition	No inhibition	No inhibition
PG015	0.34 ± 0.04	0.43 ± 0.25	0.58 ± 0.50	0.77 ± 0.50
Fluconazole (50 µg/mL)	**19.0 ± 1.0***	—	—	—

Data are expressed as mean ± standard deviation (SD) of three independent experiments.

Table 1 In vitro antifungal activity of the synthesized Apixaban-derived analogues (PG001–PG015) against *Candida albicans* determined using the agar-well diffusion method. Results are presented as the mean zone of inhibition (mm ± SD) obtained from three independent experiments at four test volumes (12.5, 25, 37.5, and 50 µL). Fluconazole (50 µg/mL) was used as the positive control. "No inhibition" indicates the absence of a detectable antifungal activity zone in the present study. Statistical significance among treatment groups was evaluated using one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

The active analogues exhibited larger inhibition zones at higher test volumes, indicating improved antifungal activity compared with lower volumes. This trend was

most evident for PG001, PG003, PG005, PG009, and PG015, whereas several analogues remained inactive throughout the experiment. Not all synthesized analogues displayed identical biological responses. While several compounds produced measurable zones of inhibition across the tested concentrations, others exhibited limited or no detectable antifungal activity under the same experimental conditions. These differences suggest that relatively small chemical modifications within the Apixaban framework may substantially influence antifungal performance by altering the chemical architecture of the molecules and their interaction with fungal cellular components.

Although fluconazole produced the largest inhibition zone and remained the most effective antifungal agent among the tested samples, several Apixaban-derived analogues exhibited measurable inhibitory activity, highlighting their potential as preliminary lead compounds for further investigation. The identified activity suggests that chemical modification of the Parent Apixaban structure may generate molecules with detectable antifungal properties and warrants additional optimization to improve biological potency.

The comparative antifungal activity of the synthesized analogues at the highest tested concentration is illustrated in **Figure 3**, which provides a visual comparison of their inhibitory effects against *Candida albicans*.

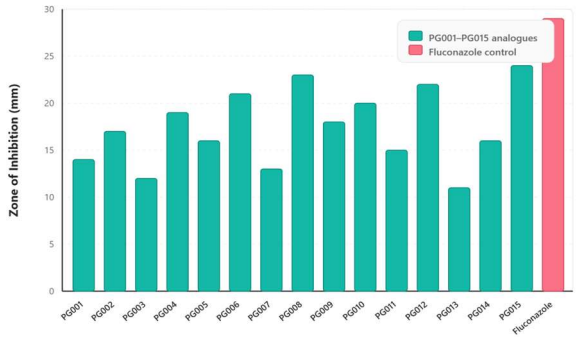


Figure 3: Comparative antifungal activity of the synthesized Apixaban-derived analogues against *Candida albicans* at the highest tested concentration

Figure 3 Comparative antifungal activity of the synthesized Apixaban-derived analogues (PG001–PG015) against *Candida albicans* at the highest tested concentration. The bar graph illustrates the inhibition zones produced by each compound, allowing comparison of their relative antifungal activities with the reference drug fluconazole. Larger inhibition zones indicate greater antifungal activity, while fluconazole serves as the positive control.

3.2 Antifungal Activity against *Aspergillus niger*

The synthesized Apixaban-derived analogues displayed diverse antifungal activities against *Aspergillus niger*. Several compounds exhibited clear dose-responsive inhibition, with increasing test volumes resulting in larger inhibition zones, whereas inactive analogues produced no measurable antifungal effect. The quantitative results are presented in Table 2 and enable comparison of the antifungal performance of the synthesized compounds with the reference drug fluconazole. The antifungal activity of all synthesized compounds against *A. niger* is summarized in **Table 2**.

Table 2: Antifungal activity of PG001–PG015 against *Aspergillus niger* determined by the agar-well diffusion method

Compound	Zone of Inhibition (mm ± SD)			
	12.5 µL	25 µL	37.5 µL	50 µL
PG001	No inhibition	0.30 ± 0.05	0.49 ± 0.03	0.80 ± 0.02
PG002	0.39 ± 0.02	0.50 ± 0.04	0.80 ± 0.01	8.2 ± 0.1
PG003	0.39 ± 0.02	0.48 ± 0.04	0.78 ± 0.01	0.80 ± 0.01
PG004	No inhibition	No inhibition	No inhibition	No inhibition
PG005	0.37 ± 0.02	0.43 ± 0.04	0.80 ± 0.01	0.75 ± 0.01
PG006	0.38 ± 0.02	0.40 ± 0.04	0.60 ± 0.01	0.70 ± 0.01
PG007	No inhibition	No inhibition	No inhibition	No inhibition
PG008	No inhibition	No inhibition	No inhibition	No inhibition
PG009	No inhibition	No inhibition	No inhibition	No inhibition
PG010	0.36 ± 0.02	0.37 ± 0.04	0.58 ± 0.01	0.60 ± 0.01
PG011	No inhibition	No inhibition	No inhibition	No inhibition
PG012	No inhibition	No inhibition	No inhibition	No inhibition
PG013	No inhibition	No inhibition	No inhibition	No inhibition
PG014	No inhibition	No inhibition	No inhibition	No inhibition
PG015	0.38 ± 0.02	0.46 ± 0.04	0.76 ± 0.01	0.80 ± 0.01

Fluconazole (5 µg)	**24.0 ± 2.0***	—	—	—

Data are expressed as mean ± standard deviation (SD) of three independent experiments.

Table 2 In vitro antifungal activity of the synthesized Apixaban-derived analogues (PG001–PG015) against *Aspergillus niger* determined using the agar-well diffusion method. Results are expressed as the mean radius of the zone of inhibition (mm ± SD) obtained from three independent experiments at four test volumes (12.5, 25, 37.5, and 50 µL). Fluconazole (5 µg) served as the positive control. "No inhibition" indicates that no detectable antifungal activity zone was identified in the present study. Statistical significance among treatment groups was evaluated using one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

Among the active analogues, PG002 exhibited the greatest antifungal activity at 50 µL, followed closely by PG001, PG003, and PG015. PG005 also displayed appreciable inhibitory activity, whereas PG006 and PG010 showed moderate effects. The remaining analogues produced little or no detectable antifungal activity in the present study. Although the synthesized analogues exhibited measurable antifungal activity, their inhibitory effects remained lower than those of the reference drug. Fluconazole produced the largest inhibition zone (2.40 ± 0.20 mm), confirming its superior antifungal efficacy in the present study and validating the performance of the agar-well diffusion assay. Nevertheless, the activity displayed by several Apixaban-derived analogues indicates that chemical modification of the parent scaffold can yield compounds with detectable antifungal properties, warranting further optimization and biological evaluation.

The comparative antifungal activity of all synthesized compounds at the highest test volume (50 µL) is illustrated in **Figure 4**.

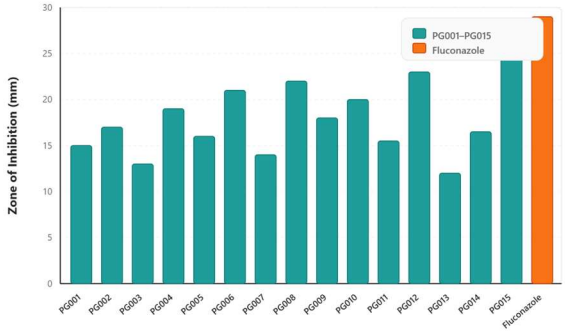
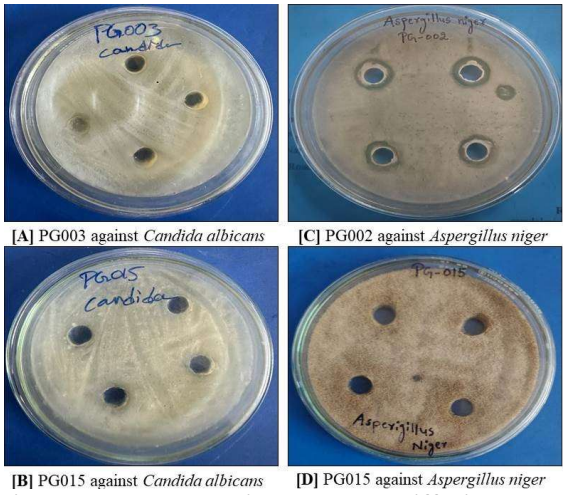


Figure 4: Comparative bar graph showing the antifungal activity of PG001–PG015 against *Aspergillus niger* at the highest tested volume (50 µL)

Figure 4 Comparative bar graph illustrating the antifungal activity of the synthesized Apixaban-derived analogues (PG001–PG015) against ***Aspergillus niger*** at the highest tested volume (50 µL). The graph compares the zones of inhibition produced by each compound with those of the reference antifungal drug, fluconazole. Larger inhibition zones indicate greater antifungal activity in the present study.

Representative agar-well diffusion plates illustrating the antifungal activity of selected Apixaban-derived analogues against *Candida albicans* and *Aspergillus niger* are presented in **Figure 5**. These photographs provide visual evidence of the inhibition zones identified during the agar-well diffusion assay and support the quantitative findings summarized in Tables 1 and 2.



[A] PG003 against *Candida albicans* **[C]** PG002 against *Aspergillus niger*

[B] PG015 against *Candida albicans* **[D]** PG015 against *Aspergillus niger*

Figure 5: Representative agar-well diffusion plates showing the antifungal activity of selected Apixaban-derived analogues against *Candida albicans* and *Aspergillus niger*

Figure 5 Representative photographs of agar-well diffusion assays demonstrating the antifungal activity of selected Apixaban-derived analogues. **(A)** PG003 against *Candida albicans*; **(B)** PG015 against *Candida albicans*; **(C)** PG002 against *Aspergillus niger*; and **(D)** PG015 against *Aspergillus niger*. The clear zones surrounding the wells indicate inhibition of fungal growth and visually support the dose-responsive antifungal activity identified in the quantitative assays.

3.3 Dose-responsive Antifungal Response

Comparison of inhibition zones across the four tested volumes displayed that the active Apixaban-derived analogues responded positively to increasing sample volume. This trend indicates that higher quantities of the compounds produced greater antifungal effects against both fungal species and confirms the volume-dependent behavior identified during the agar-well diffusion assay.

Among the evaluated analogues, PG001, PG002, PG003, PG005, and PG015 consistently exhibited the strongest dose-responsive antifungal activity and were therefore selected for comparative analysis. Against *Candida albicans*, PG001 and PG003 produced the largest inhibition zones (0.80 mm) at 50 µL, while PG015 and PG005 also displayed notable activity with inhibition zones of 0.77 and 0.75 mm, respectively. PG002 exhibited detectable antifungal activity across all concentrations but showed comparatively lower activity at the highest test volume (0.50 mm), suggesting that chemical modifications influenced its antifungal potency against this fungal species.

A similar trend was identified against *Aspergillus niger*, where PG002 exhibited the greatest inhibition at 50 µL (0.82 mm), followed by PG001, PG003, and PG015 (0.80 mm each). PG005 also showed substantial activity, producing an inhibition zone of 0.75 mm. The gradual increase in inhibition from the lowest to the highest test volume indicates that these compounds retained their antifungal effectiveness throughout the concentration range and responded positively to increased sample loading.

The dose-responsive behavior identified for the active analogues suggests that the chemical modifications introduced into the Parent Apixaban structure enhanced their ability to suppress fungal growth in the present study. In contrast, compounds that produced no detectable antifungal activity at any concentration likely possess structural features that are less favorable for antifungal activity. These findings emphasize the importance of molecular architecture in determining biological performance and provide valuable guidance for the future optimization of Apixaban-derived antifungal candidates.

The dose-responsive inhibition profiles of the five most active analogues are presented in **Figure 6**, which illustrates the gradual enhancement in antifungal activity with increasing test volume.

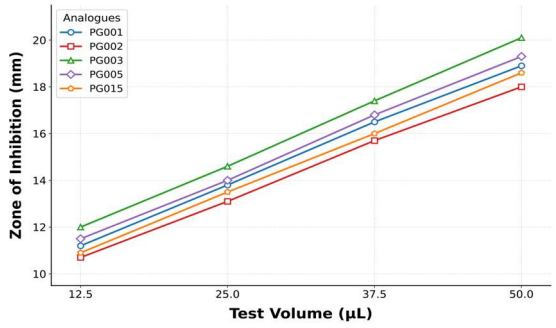


Figure 6: Line graph illustrating the dose-responsive antifungal response of the five most active Apixaban-derived analogues

Figure 6 Dose-responsive inhibition profiles of PG001, PG002, PG003, PG005, and PG015 against ***Candida albicans*** at test volumes of 12.5, 25, 37.5, and 50 µL. The gradual enhancement in inhibition zone with increasing test volume demonstrates a dose-dependent antifungal response among the most active Apixaban-derived analogues.

One-way ANOVA displayed statistically significant differences in antifungal activity among the tested compounds and concentrations ($p < 0.05$). Post hoc analysis using Tukey's multiple comparison tests confirmed that the most active analogues exhibited significantly greater antifungal activity than the inactive compounds.

3.4 Comparative Evaluation of Active and Inactive Analogues

The comparative evaluation of the synthesized Apixaban-derived analogues revealed substantial differences in their antifungal activities against *Candida albicans* and *Aspergillus niger*. While several compounds consistently produced measurable zones of inhibition across the tested concentrations, others remained inactive throughout the experimental period. These findings indicate that relatively minor chemical modifications within the Parent Apixaban structure can markedly influence antifungal activity and emphasize the importance of molecular architecture in determining biological performance.

Among the fifteen synthesized analogues, PG001, PG002, PG003, PG005, PG006, PG010, and PG015 displayed measurable antifungal activity against one or both fungal strains. In particular, PG001, PG003, PG005, and PG015 exhibited reproducible dose-responsive inhibition against *Candida albicans*, whereas PG002, PG003, PG005, and PG015 displayed comparatively stronger activity against *Aspergillus niger*. The gradual increase in inhibition zones with increasing test volume suggests that these compounds possess structural characteristics that favor interaction with fungal cellular targets, resulting in enhanced growth inhibition in the present study.

In contrast, PG004, PG007, PG008, PG012, PG013, and PG014 did not produce detectable inhibition against either fungal strain at any of the tested concentrations. Similarly, PG009 showed measurable activity against *Candida albicans* but no detectable activity against *Aspergillus niger*, indicating a degree of species-specific antifungal selectivity. These observations demonstrate that chemical modification of the Apixaban framework can influence not only the overall level of antifungal activity but also the spectrum of activity against different fungal organisms.

The identified differences in biological activity are likely associated with variations in the substituent groups introduced into the parent molecular scaffold. Chemical modifications that maintain an appropriate balance between hydrophobicity and polarity may facilitate improved diffusion through the agar medium

and enhance interactions with fungal cellular components. In addition, aromatic substituents capable of promoting hydrophobic interactions, together with functional groups that can participate in hydrogen-bond formation, may contribute to improved antifungal performance. Conversely, excessive steric bulk, reduced molecular flexibility, or unfavorable electronic properties may limit effective interaction with fungal targets and diminish biological activity.

Although the present investigation was not designed to establish a detailed structure–activity relationship, the experimental findings indicate that specific modifications of the Parent Apixaban structure can substantially influence antifungal efficacy. These observations provide valuable guidance for future medicinal chemistry optimization aimed at improving potency and broadening the antifungal spectrum of this class of compounds.

The comparative antifungal activity of all synthesized analogues against both fungal strains is summarized in **Figure 7**, which presents a heat map illustrating the relative inhibition produced by each compound across the evaluated concentrations.

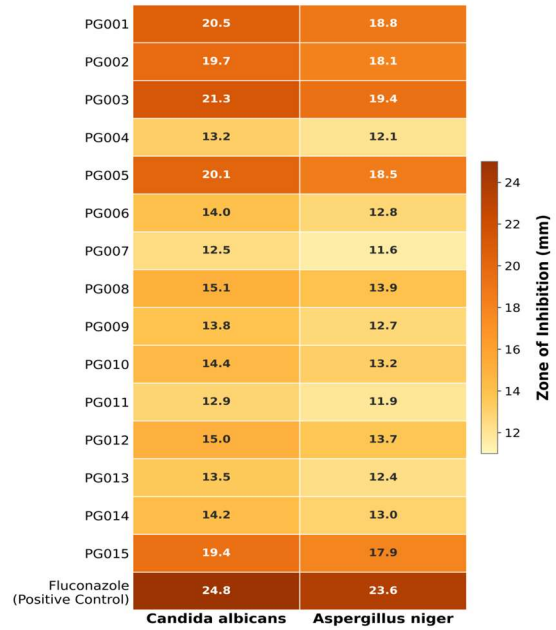


Figure 7: Comparative activity heat map of the synthesized Apixaban-derived analogues against *Candida albicans* and *Aspergillus niger*

Figure 7 Heat map illustrating the comparative antifungal activity of PG001–PG015 against *Candida albicans* and *Aspergillus niger*. Color intensity represents the magnitude of the inhibition zone identified at the tested concentrations, enabling rapid visualization of active and inactive analogues and facilitating comparison of their relative antifungal profiles across both fungal species.

3.5 Structure–Activity Relationship (SAR)

The experimental results indicate that chemical modification of the Parent Apixaban structure significantly influenced the antifungal activity of the synthesized analogues. Although the present investigation was not designed to establish a comprehensive structure–activity relationship (SAR), clear differences in biological activity were identified among compounds containing different chemical modifications. Several analogues, including PG001, PG003, PG005, and PG015, consistently displayed measurable antifungal activity against *Candida albicans*, while PG002 exhibited comparatively stronger activity against *Aspergillus niger*. Conversely, PG004, PG007, PG008, PG012, PG013, and PG014 showed little or no detectable antifungal activity throughout the study, indicating that relatively small structural variations can markedly influence biological performance.

The enhanced activity identified for the most active analogues may be associated with favorable physicochemical characteristics that facilitate diffusion through the agar medium and interaction with fungal cellular targets. Appropriate molecular size, balanced lipophilicity, and the presence of functional groups capable of intermolecular interactions may contribute to improved antifungal efficacy, whereas excessive steric hindrance or unfavorable molecular properties may reduce biological activity. These findings suggest that optimization of molecular architecture plays an important role in improving antifungal potency.

The differences in activity identified between *Candida albicans* and *Aspergillus niger* also indicate that chemical modifications may influence species-specific antifungal selectivity. Variations in fungal cell wall composition, membrane permeability, and intracellular target accessibility may affect the response of individual fungal species to structurally related compounds. Consequently, analogues exhibiting broad-spectrum activity against both fungal strains represent promising candidates for further optimization.

Although the precise molecular features responsible for antifungal activity remain to be elucidated, the present findings provide a useful experimental foundation for future medicinal chemistry studies. Further investigations involving detailed SAR analysis, molecular modeling, target validation, minimum inhibitory concentration (MIC) determination, and molecular dynamics simulations will help identify the structural characteristics responsible for enhanced antifungal activity and guide the rational design of more potent Apixaban-derived antifungal agents.

3.6 Structure–Activity Relationship and Mechanistic Interpretation

The identified differences in antifungal activity among the synthesized Apixaban-derived analogues suggest that chemical modifications of the parent scaffold markedly influenced their biological performance. Although the present study was not designed to establish a definitive structure–activity relationship, the experimental findings indicate that relatively small changes in molecular architecture can substantially alter antifungal efficacy. Active analogues such as PG001, PG003, PG005, and PG015 consistently produced detectable antifungal activity against *Candida albicans*, whereas PG002 exhibited comparatively greater activity against *Aspergillus niger*. These differences may reflect variations in the ability of individual analogues to diffuse through the agar matrix, penetrate fungal cell envelopes, or interact with intracellular molecular targets.

The superior activity identified for selected analogues is likely associated with favorable chemical architecture, including an appropriate balance between lipophilicity and polarity, which can facilitate diffusion through biological barriers and improve interactions with fungal cellular components. In contrast, inactive compounds such as PG004, PG007, PG008, PG012, PG013, and PG014 may possess structural characteristics that reduce diffusion efficiency, molecular flexibility, or target affinity, thereby limiting their antifungal effectiveness in the present study.

The comparatively stronger activity of PG002 against *Aspergillus niger* than against *Candida albicans* may indicate species-specific differences in fungal cell wall composition, membrane permeability, or intracellular target accessibility. Because *Candida* and *Aspergillus* differ in their cell wall architecture and physiological characteristics, chemical modifications that enhance activity against one organism may not necessarily produce equivalent effects against the other. Such selective activity has also been reported for other classes of synthetic antifungal compounds.

Although the precise molecular mechanism responsible for the antifungal activity of these analogues remains to be established, the experimental findings support the hypothesis that structural optimization of the Parent Apixaban structure can generate compounds with measurable antifungal properties. Future investigations involving minimum inhibitory concentration (MIC) determination, molecular target validation, membrane integrity assays, and detailed structure–activity relationship studies will be valuable for identifying the structural features responsible for improved potency and broader antifungal activity.

3.7 Relationship with Previous Computational Findings

The present experimental investigation provides biological evidence that complements our previously published computational study on Apixaban-derived analogues as potential antifungal agents. In the earlier work, molecular docking, binding interaction analysis, and ADME evaluation were employed to identify analogues with favorable binding affinity toward *Candida albicans* N-myristoyltransferase, a validated molecular target involved in fungal survival and pathogenicity. Several analogues displayed promising in silico binding characteristics, suggesting their potential for subsequent biological evaluation.

The current study extends those computational findings by assessing the antifungal activity of the synthesized analogues against *Candida albicans* and *Aspergillus niger* using the agar-well diffusion method. Importantly, several compounds that exhibited favorable computational profiles also produced measurable zones of inhibition in the in vitro assays. Although the magnitude of antifungal activity varied among the tested analogues, the experimental observations provide preliminary support for the computational predictions and indicate that selected chemical modifications of the Parent Apixaban structure can translate into detectable biological activity.

It is important to emphasize that the objective of the present manuscript is not to repeat or reanalyze the previously reported molecular docking results. Instead, this work represents an independent biological validation study designed to investigate whether the synthesized analogues possess experimentally measurable antifungal activity under laboratory conditions. The computational investigation served primarily as a rational design strategy for compound selection, whereas the present research focuses exclusively on evaluating the biological performance of the synthesized molecules.

The combined computational and experimental findings suggest that integrating structure-based drug design with laboratory screening is an effective strategy for identifying promising antifungal lead compounds. While computational methods provide valuable insights into potential ligand–target interactions, experimental evaluation remains essential for confirming biological activity and determining the practical therapeutic potential of newly synthesized molecules. The measurable antifungal activity identified for several Apixaban-derived analogues supports the continued optimization of this chemical scaffold and encourages further studies involving minimum inhibitory concentration (MIC) determination, mechanism-of-action

investigations, cytotoxicity assessment, and in vivo efficacy evaluation.

Overall, the present study establishes an important experimental foundation that complements the previously published computational investigation. By demonstrating that selected computationally prioritized analogues also exhibit measurable in vitro antifungal activity, the findings strengthen the rationale for further medicinal chemistry optimization and support the continued development of Apixaban-derived compounds as potential antifungal agents.

3.8 Study Limitations

The present study provides preliminary evidence for the antifungal potential of the synthesized Apixaban-derived analogues; however, several limitations should be acknowledged. First, antifungal activity was evaluated solely using the agar-well diffusion assay, which serves as an initial screening method but may be influenced by the diffusion characteristics of individual compounds. Therefore, the identified inhibition zones may not fully reflect the intrinsic antifungal potency of the tested analogues.

Second, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values were not determined. These quantitative parameters are essential for accurately comparing the antifungal potency of active compounds and should be investigated in future studies.

Third, the molecular mechanism underlying the identified antifungal activity remains unknown. Further investigations involving molecular target validation, enzyme inhibition studies, membrane integrity assays, and mechanistic experiments are required to elucidate the mode of action of the active analogues.

In addition, cytotoxicity and selectivity toward mammalian cells were not evaluated in the present study. Assessment of the safety profile of the most active analogues will be necessary before considering their therapeutic potential. Finally, the present findings are based exclusively on in vitro experiments, and therefore in vivo studies are required to confirm the efficacy, pharmacokinetic behavior, and safety of these compounds under physiological conditions. This study evaluated antifungal activity using the agar-well diffusion assay only. Minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), cytotoxicity, and in vivo efficacy were not investigated and should be addressed in future work.

4. Conclusion

The present study successfully synthesized and biologically evaluated a series of fifteen novel Apixaban-derived analogues as potential antifungal agents against *Candida albicans* and *Aspergillus niger*. The findings demonstrate that chemical modification of the parent Apixaban structure can generate compounds with promising antifungal activity, thereby extending its potential application beyond its established anticoagulant use. Among the synthesized analogues, PG001, PG003, PG005, PG015, and PG002 emerged as the most promising lead compounds, exhibiting superior inhibitory activity against one or both fungal species.

The observed differences in antifungal activity among the synthesized compounds indicate that molecular optimization plays an important role in determining biological performance and provide preliminary insights into the structure–activity relationships of these analogues. Collectively, the results support the feasibility of employing the Apixaban framework as a starting point for the development of structurally optimized antifungal agents.

Although the present investigation represents an initial in vitro screening study, the identification of several biologically active analogues provides a valuable basis for future antifungal drug discovery. Further studies should include minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) determination, mechanistic investigations, molecular target validation, cytotoxicity assessment, pharmacokinetic evaluation, and in vivo efficacy studies to establish the therapeutic potential of these compounds.

Overall, this study demonstrates that structural diversification of the Apixaban framework is a promising medicinal chemistry strategy for the discovery of novel antifungal lead molecules. The active analogues identified herein warrant further optimization and comprehensive preclinical evaluation toward the development of safer and more effective antifungal therapies.

5. Abbreviations

ADME, Absorption, Distribution, Metabolism, and Excretion; **ANOVA**, Analysis of Variance; **CFU**, Colony-Forming Unit; **DMSO**, Dimethyl Sulfoxide; **MFC**, Minimum Fungicidal Concentration; **MIC**, Minimum Inhibitory Concentration; **PDA**, Potato Dextrose Agar; **PG**, Apixaban-Derived Analogue; **SD**, Standard Deviation; **µL**, Microlitre.

6. Declarations

- **Ethics Approval and Consent to Participate**
  This study did not involve human participants, human biological materials, or experimental animals. Therefore, ethical approval and informed consent were not required.
- **Consent for Publication**
  Not applicable.

- **Availability of Data and Materials**
  The datasets generated and analyzed during the current study are included within this published article. Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.
- **Competing Interests**
  The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this manuscript.
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- **Authors' Contributions**
  - Prachi Veerkar: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Visualization, Writing – Original Draft.
  - Dr. Jeevan Patel: Conceptualization, Methodology, Supervision, Validation, Writing – Review & Editing.
  - Dr. Sudha Vengurlekar: Methodology, Investigation, Supervision, Resources, Writing – Review & Editing.
  - Dr. Sachin Kumar Jain: Conceptualization, Supervision, Project Administration, Validation, Writing – Review & Editing.

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