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# Antifungal Potential of *Sophora flavescens* Root Extract: HR-LCMS Analysis and Molecular Docking of Bioactive Compounds Against Nosocomial Fungal Pathogens

Mostafa Mohammed Atiyah , Teena Merlin  and Smitha Vijayan\* 

School of Biosciences, Mar Athanasios College for Advanced Studies Tiruvalla (MACFAST),  
Pathanamthitta, Kerala, India.

## Abstract

The increasing prevalence of multidrug-resistant (MDR) *Candida* infections emphasises the urgent need for novel antifungal agents. This study evaluates the antifungal properties of *Sophora flavescens* (*S. flavescens*) root extract against five MDR *Candida* species. A total of 120 bioactive compounds, including flavonoids and alkaloids with known antimicrobial properties, were identified using high-resolution liquid chromatography-mass spectrometry. The antifungal efficacy of the extract was assessed through agar well diffusion, minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC) assays. The extract exhibited strong antifungal activity, with inhibition zone diameters ranging from 13.33 to 19.66 mm against all tested strains. The MIC values ranged from 6.25 to 25 mg/mL across all tested *Candida* species, with corresponding MFCs falling within the same range for *C. krusei*, *C. parapsilosis*, *C. albicans*, and *C. glabrata*. In contrast, *C. auris* exhibited slightly higher MFC values, ranging from 12.5 to 25 mg/mL. Biofilm inhibition was assessed using crystal violet staining, while morphological alterations in treated *Candida* cells were visualised using scanning electron microscopy. Molecular docking studies targeting fungal lanosterol 14 $\alpha$ -demethylase (CYP51) of *C. albicans* revealed that ginkgetin (-11.0 kcal/mol) and roxburghine B (-10.7 kcal/mol) displayed stronger binding affinities than the standard antifungal drug Itraconazole (-9.3 kcal/mol). These findings underscore the potential of *S. flavescens* root extract as a natural and effective antifungal candidate against hospital-acquired *Candida* infections.

**Keywords:** *Sophora flavescens*, *Candida* Species, Antifungal Activity, Bioactive Compounds, Molecular Docking, Nosocomial Infections, Drug-resistant Fungi

\*Correspondence: smitha@macfast.org

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## INTRODUCTION

Nosocomial fungal infections caused by *Candida* species represent a significant threat to immunocompromised patients, with mortality rates from invasive candidiasis reaching up to 70% in severe cases.<sup>1</sup> The rise of resistant strains and the declining effectiveness of conventional antifungal agents, including azoles and echinocandins, underscore the urgent need for novel therapeutic options.<sup>2</sup> *C. albicans* remains the primary cause of biofilm-related infections on medical devices and human tissues; however, multidrug-resistant (MDR) species, such as *C. auris* and *C. glabrata*, pose increasing challenges within clinical settings.<sup>3</sup> The excessive use of antifungal drugs, extended hospitalisation, and the frequent application of invasive healthcare instruments, including catheters and ventilators, have collectively accelerated the development of antifungal resistance.<sup>4</sup>

Medicinal plants and other natural products have recently regained prominence as valuable sources of bioactive compounds, offering structural diversity and naturally evolved defence mechanisms against microbes.<sup>5</sup> *Sophora flavescens* (*S. flavescens*, Fabaceae), commonly known as 'Kushen' in China, is native to East Asia, as well as regions of Russia and India. Its roots have traditionally been utilised in Chinese healing practices for managing various conditions, such as cancer, inflammation, skin disorders, dysentery, and parasitic infections.<sup>6</sup> *S. flavescens* displays diverse pharmacological activities, including antibacterial, antipyretic, antiarrhythmic, antiasthmatic, anti-ulcer, anti-HBV, and antineoplastic effects.<sup>7</sup> Phytochemical investigations have revealed flavonoids and alkaloids as the major bioactive compounds of *S. flavescens*. Notably, quinolizidine alkaloids such as matrine and oxymatrine, along with prenylated flavonoids like kurarinone and *S. flavanone* G, have shown potent antimicrobial activity against bacteria and viruses.<sup>8,9</sup>

*In silico* molecular docking predicts the binding affinity of bioactive compounds, providing insights into structure-activity relationships (SAR), protein-ligand interactions, and potential antifungal mechanisms.<sup>10</sup> This computational

approach is crucial in structure-based drug discovery by simulating compound interactions with biological targets to identify potential drug candidates.<sup>11</sup> Additionally, the evaluation of physicochemical properties provides essential information during the early stages of drug development, supporting the optimisation of compounds for therapeutic use.<sup>12</sup> However, its antifungal properties against nosocomial *Candida* infections remain underexplored.

This study bridges this gap by integrating high-resolution liquid chromatography-mass spectrometry (HR-LCMS) phytochemical profiling with molecular docking to evaluate the potential of *S. flavescens* as an alternative antifungal agent. By combining *in vitro* antifungal assays with computational docking studies, the bioactive compounds responsible for antifungal activity and their interactions with fungal *C. albicans* (lanosterol 14 $\alpha$ -demethylase (CYP51)), a key enzyme in ergosterol biosynthesis, are elucidated. This approach not only deepens understanding of *S. flavescens* as a prospective antifungal candidate but also contributes to the advancement of plant-derived antifungal treatments to combat drug-resistant *Candida* species.

## MATERIALS AND METHODS

### Plant collection and authentication

The roots of *S. flavescens* were harvested from Baghdad, Iraq. Plant identification and verification were conducted by Dr. Jalal Hameed Ali, Assistant Professor at the College of Agriculture, University of Sumer, Thi-Qar, Iraq. Comprehensive morphological documentation and photographic records were maintained for reference (Supplementary data Figure S1).

### Preparation of ethanol extract

A total of 150 g of dried *S. flavescens* root powder was extracted with 1000 mL of ethanol at 50-60 °C using a Soxhlet apparatus until the extract appeared clear. The resulting filtrate was concentrated under reduced pressure at 40 °C using a rotary evaporator and subsequently stored at 4 °C for further use.<sup>13,14</sup> The percentage yield of the extract was calculated using the following formula:

$$\text{Percentage Yield} = \frac{\text{Weight of Dried Extract}}{\text{Weight of Plant Material}} \times 100$$

### HR-LCMS analysis for bioactive compound profiling

Secondary metabolites present in the crude extract of *S. flavescens* root were identified using HR-LCMS performed on an Agilent 1200 LC system. This analysis was conducted at the Sophisticated Analytical Instrument Facility (SAIF) at the Indian Institute of Technology (IIT), Bombay, India. The HR-LCMS system operated over a mass detection range of 50-3200 amu, featuring a high resolution of 40,000 full width at half maximum, which ensured exceptional mass accuracy within one part per million (ppm). The system demonstrated high sensitivity with a signal-to-noise ratio (S/N) of 100:1 for one picogram of reserpine, thereby enabling the detection of trace-level compounds. Atmospheric pressure ionisation was applied using both electrospray ionisation and atmospheric pressure chemical ionisation modes, operating in positive and negative ionisation polarities. Mass analysis was performed via direct infusion in MS and MS/MS modes, allowing detailed fragmentation patterns for accurate compound identification. A UHPLC-PDA mass spectrometer was utilised to obtain a comprehensive profile of the secondary metabolites present in the *S. flavescens* root extract. Compounds were identified by comparing their *m/z* values with standard databases, such as METLIN and MassBank, and further confirmed through MS/MS fragmentation patterns.

### Fungal strains and identification

The antifungal activity of *S. flavescens* root extract was evaluated against five clinically relevant opportunistic fungal pathogens commonly associated with nosocomial infections: *C. auris*, *C. krusei*, *C. parapsilosis*, *C. albicans*, and *C. glabrata*. These clinical isolates were collected from the Clinical Microbiology Laboratory at the Pushpagiri Institute of Medical Sciences and Research Centre, Tiruvalla, Kerala, India. The fungal cultures were maintained on Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar plates and incubated at 37 °C for 48 h to yield well-developed colonies suitable for experimental analysis.

The *Candida* species, initially identified by the Clinical Microbiology Laboratory at Pushpagiri Institute, was subsequently confirmed using HiCrome™ *Candida* Differential Agar (HiMedia Laboratories, India). This chromogenic medium enabled species-level differentiation of *Candida* by producing distinct colony pigmentation resulting from species-specific enzymatic activities. Each isolate was streaked onto designated segments of the agar plate and incubated at 37 °C for 48 h. Colony morphology and chromogenic profiles were interpreted in accordance with the manufacturer's guidelines and corroborated with established literature.<sup>15-17</sup>

### Antifungal activity of plant extract

#### Well diffusion Assay

The antifungal activity of *S. flavescens* root extract was evaluated using the well diffusion method. The extract residue (50 mg) was re-dissolved in 5 mL of dimethyl sulfoxide (DMSO) and used for the assay. Sterile SDA plates were seeded with freshly prepared fungal cultures adjusted to an optical density (1 OD). Wells with a diameter of 6 mm were punched into the agar using a sterile cork borer, and each well was filled with 200 µL of the plant extract solution, corresponding to a final concentration of 10 mg/mL. Itraconazole (1% w/v) was used as the positive control, while DMSO served as the negative control. The plates were incubated at 37 °C for 48 hours. Antifungal activity was determined by measuring the diameter of the zone of inhibition around each well using a Vernier caliper.<sup>14,18</sup>

### MIC determination

The minimum inhibitory concentrations (MICs) of *S. flavescens* root extract against fungal pathogens were determined using a 96-well microtiter plate-based antifungal assay employing the resazurin dye method. Each well in columns 1 to 12 of a sterile 96-well plate was initially filled with 100 µL of Sabouraud Dextrose Broth. Subsequently, 100 µL of the plant extract (25 mg/mL) was added to the first well of column 1 and subjected to twofold serial dilutions across columns 1 to 10, with column 1 containing the highest extract concentration and column 10 the lowest. Column 11 served as the positive control

(medium with fungal inoculum, without extract), whereas column 12 was designated as the negative control (medium only, without inoculum). A 20 µL aliquot of fungal suspension (1 OD) was added to all wells except those in column 12.

The plates were incubated at 37 °C for 24-48 h. After incubation, 50 µL of 0.01% resazurin solution was introduced to each well, followed by a further 4 h incubation. Fungal viability was assessed by monitoring the colour change of the resazurin dye: a shift to pink indicated viable fungal growth, while no colour change (blue or purple) indicated growth inhibition. The MIC was defined as the lowest extract concentration that completely inhibited visible fungal growth, as evidenced by the absence of colour change.<sup>19</sup>

#### MFC determination

To evaluate the minimum fungicidal concentration (MFC), streaks were taken from the four lowest concentrations of *S. flavescentis* root extract that showed no visible growth on the MIC plates. These were then subcultured onto sterile SDA plates. The plates were maintained at 37 °C for 48 h and examined for fungal growth at each concentration. The MFC was defined as the lowest concentration of *S. flavescentis* root extract that prevented fungal growth on the freshly inoculated agar plates, thereby confirming its fungicidal activity.<sup>20</sup>

#### Antibiofilm assay by test tube method

The antibiofilm activity of *S. flavescentis* root extract was evaluated using the ring formation method at concentrations of 25, 12.5, 6.25, 3.125, and 1.56 mg/mL. For each assay, 2 mL of yeast extract-peptone-dextrose broth (pH 6.5) was dispensed into sterile test tubes, followed by the addition of 500 µL of the plant extract dissolved in DMSO at 25 mg/mL. Subsequently, 2 mL of fungal cell suspension, adjusted to a 1 OD at 600 nm, was added. The tubes were incubated under static conditions at 37 °C for 72 h to facilitate biofilm formation. After incubation, the liquid contents were gently decanted, and the tubes were rinsed with phosphate-buffered saline (PBS, pH 7.2) to remove non-adherent fungal cells. The tubes were then air-dried at room temperature. Adherent biofilms were stained with 0.1% (w/v) crystal violet

for 1 min, followed by rinsing with PBS to remove excess dye.

To quantify the biofilm, the retained stain was solubilised using glacial acetic acid, and the absorbance was measured at 595 nm using a UV-visible spectrophotometer (Orion AquaMate 8100, Thermo Scientific, USA). A reduction in optical density compared to the untreated control indicated inhibition of biofilm formation by the extract.<sup>21</sup> The following formula was used to estimate biofilm inhibition:

$$\text{Inhibition (\%)} = \frac{\text{OD}_{\text{Control}} - \text{OD}_{\text{Treated}}}{\text{OD}_{\text{Control}}} \times 100$$

where  $\text{OD}_{\text{Control}}$  denotes the absorbance of the positive control (untreated biofilm), while  $\text{OD}_{\text{Treated}}$  represents the absorbance of the sample treated with *S. flavescentis* root extract.

#### SEM analysis

The antifungal mechanism of *S. flavescentis* root extract was examined through scanning electron microscopy (SEM). Fresh cultures of clinical isolates, including *C. albicans*, were grown on SDA and adjusted to a 1 McFarland optical density standard. A 100 µL aliquot of *C. albicans* culture was mixed with 500 µL of the crude extract (10 mg/mL) in 1 mL of SDA. The mixture was kept at 37 °C for 12 h with constant shaking. A control sample, prepared under identical conditions but without the extract, was used for comparison. After incubation, the samples were centrifuged at 10,000 rpm for 10 min to pellet the fungal cells. The collected pellets were washed with sterile Milli-Q water, air-dried, and mounted onto glass slides, sputter-coated with gold using a vacuum evaporator, and examined by SEM (JEOL JSM-6390) to observe surface morphological alterations induced by the extract.<sup>22</sup>

#### Molecular docking

Molecular docking studies were conducted using PyRx software, which utilises AutoDock Vina, to investigate the binding interactions between bioactive compounds from *S. flavescentis* root extract and CYP51,<sup>23</sup> a key enzyme involved in fungal ergosterol biosynthesis. The docking protocol was validated by calculating the root mean square deviation (RMSD) to ensure

accuracy. Ligand flexibility was considered during docking to enhance the reliability of the results. Protein preparation and post-docking interaction analyses were performed using Discovery Studio Visualizer.

### Protein preparation

The three-dimensional (3D) crystal structure of CYP51 from *C. albicans* was retrieved from the Protein Data Bank (PDB ID: 5V5Z, <https://www.rcsb.org>). Protein preparation was carried out utilising Discovery Studio 2021, wherein all water molecules, heteroatoms, and non-essential ligands were removed to eliminate steric interference (Figure 1). Furthermore, hydrogen atoms were added to optimise the structure, followed by energy minimisation using the CHARMM force field to improve docking accuracy. The active site region was identified based on the co-crystallised ligand and supported by previous literature reports,<sup>24</sup> ensuring precise docking simulations.

### Ligand preparation

Bioactive compounds identified through HR-LCMS analysis were chosen for docking studies. Their two-dimensional molecular structures were retrieved from the PubChem database, converted into 3D structures, and then optimised using Open Babel within the PyRx platform. To obtain the most stable conformations, energy minimisation was performed utilising the universal force field in PyRx, ensuring that the ligands adopted biologically relevant poses.<sup>25</sup>

### Molecular docking analysis

Docking simulations were performed utilising AutoDock Vina 4.2 integrated within the PyRx platform to evaluate the binding affinity and interaction mechanisms of bioactive compounds with CYP51. The docking grid was positioned at the enzyme's active site, ensuring the inclusion of all essential binding residues within the defined docking area. The grid box dimensions were set at 40 × 40 × 40 Å, providing ample space for ligand flexibility and interactions within the binding pocket. To maintain the balance between computational speed and docking accuracy, the exhaustiveness parameter was adjusted to eight.<sup>26</sup>

### Docking validation and RMSD calculation

To validate the docking protocol, itraconazole was utilised as a reference standard.<sup>27</sup> The co-crystallised ligand from the crystal structure of CYP51 was re-docked into its native binding site using PyRx. The RMSD between the docked pose and the crystallographic conformation was calculated with Discovery Studio. An RMSD value below 2 Å was considered indicative of a reliable docking methodology, thereby confirming the accuracy of the simulation.<sup>28</sup>

### Post-docking analysis

The interactions within the docked protein-ligand complexes were evaluated using Discovery Studio Visualizer, focusing on essential molecular interactions, including hydrogen bonding, hydrophobic interactions, and  $\pi$ - $\pi$  stacking. Compounds exhibiting the lowest binding energy values were shortlisted as top candidates for further assessment. Their docking scores were compared against itraconazole to determine relative binding efficiency. Additionally, structural visualisations of the docked complexes were generated to highlight key ligand interactions within the active site, offering insights into their potential antifungal properties.<sup>28</sup>

### Statistical analysis

To maintain accuracy and consistency, each test was performed in triplicate. Data were analysed using Microsoft Excel, and results are presented as the mean  $\pm$  standard deviation to reflect the reliability and variability of the measurements.

## RESULTS

### Identification of bioactive compounds using HR-LCMS

#### Phytochemical composition

The ethanol extract of *S. flavescens* root was analysed using HR-LCMS, leading to the detection of 120 bioactive compounds. The predominant chemical groups detected were flavonoids and alkaloids, both known for their antibacterial and antifungal properties. Among the flavonoids, ginkgetin, kurarinone, and Sophora flavanone G were identified, each previously



reported to exhibit strong antifungal effects. These compounds are recognised to disrupt fungal cell membrane stability and inhibit ergosterol biosynthesis, a crucial pathway for fungal viability.

Additionally, the analysis confirmed the presence of alkaloids, including matrine and oxymatrine, which are associated with antifungal and immunomodulatory effects. The antifungal efficacy of *S. flavescens* is likely linked to the ability of these phytochemicals to destabilise fungal membranes, inhibit ergosterol biosynthesis, and impair vital enzymatic functions necessary for fungal survival. These findings offer valuable insights into the metabolite profile of *S. flavescens*, further reinforcing its potential as a promising source of antifungal agents. Detailed information is available in the Supplementary data (Table S1, Figure S2-S3).

#### Identification of fungal pathogens

Each isolate exhibited distinct colony colours and morphological characteristics consistent with species-specific profiles (Table 1, Figure 2a and 2b).

#### Evaluation of antifungal efficacy against nosocomial *Candida* strains

The antifungal efficacy of the ethanol extract of *S. flavescens* root (10 mg/mL) was evaluated against different *Candida* species. Comprehensive results are presented in Table 2 and Figure 3. The extract displayed considerable

antifungal effects, as reflected by the varying sizes of the inhibition zones.

#### MIC and MFC of plant extracts

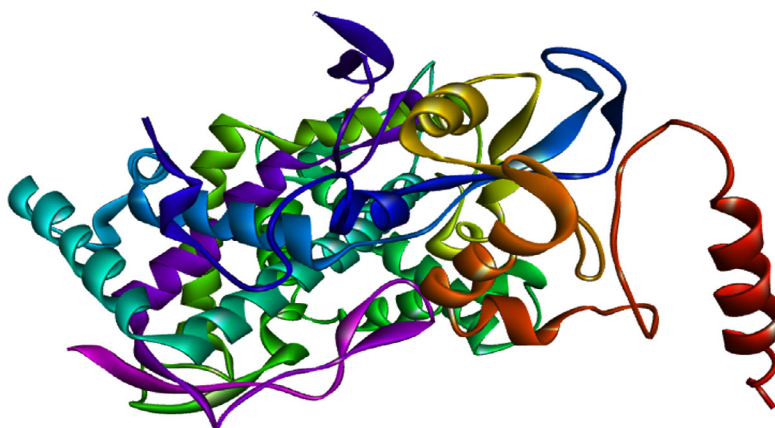
The ethanol extract of *S. flavescens* root demonstrated noticeable antifungal activity against various *Candida* species. Inhibition of fungal growth was confirmed through the broth microdilution method, while fungicidal potential was assessed by subculturing on SDA plates. The complete absence of colony growth after incubation signified potent fungicidal activity. The MIC and MFC values observed across the tested isolates are summarised in Table 3 (Figure S4).

#### Antibiofilm assay

The ethanolic extract of *S. flavescens* root expressed significant antibiofilm activity against *Candida* species, as illustrated in Figure 4 and S5.

**Table 1.** Chromogenic agar characteristics of *Candida* species

No.	<i>Candida</i> species	Colony Color	Morphology
1	<i>C. auris</i>	Cream-pale pink, bluish halo	Smooth, round
2	<i>C. krusei</i>	Purple	Fuzzy, spreading
3	<i>C. parapsilosis</i>	Cream	Smooth, convex
4	<i>C. albicans</i>	Light green	Smooth, raised
5	<i>C. glabrata</i>	Cream-white	Smooth, small, round



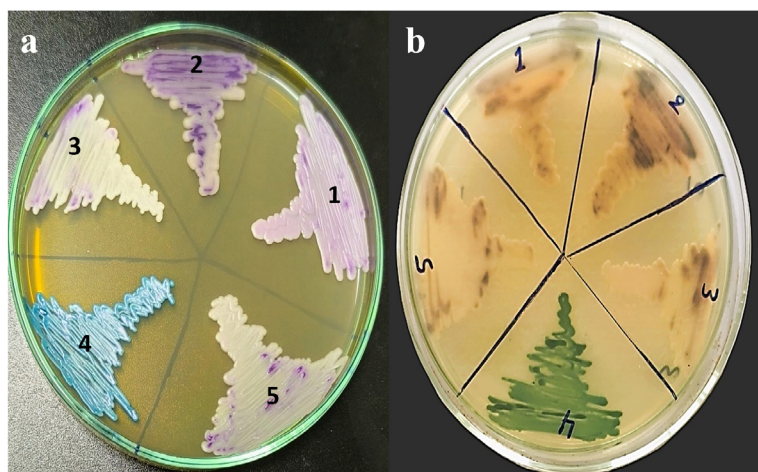
**Figure 1.** 3D structure of *C. albicans* CYP51, PDB ID: 5V5Z, prepared using Discovery Studio. The active site region is highlighted, showing key residues involved in ligand binding

Compared to the untreated control, the extract effectively reduced biofilm formation, suggesting its potential to disrupt fungal adhesion and maturation. The noted decrease in biofilm biomass indicated interference with fungal cell growth and biofilm development. The use of ethanol as a solvent likely facilitated the extraction of bioactive compounds, particularly polar phytochemicals, which may contribute to the observed antibiofilm effect.

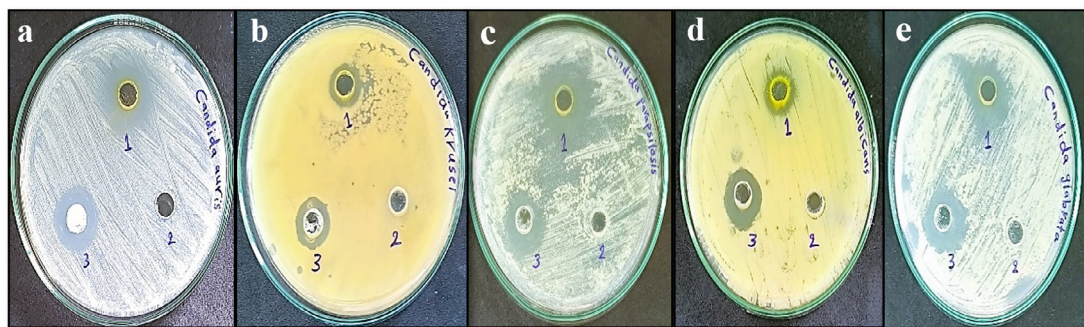
### SEM analysis

SEM analysis revealed pronounced morphological changes in *C. albicans* following treatment with the ethanol extract of *S. flavescens* root. In the untreated control, *C. albicans* appeared

as oval-shaped cells with smooth, intact surfaces and well-defined cell walls, characteristic of healthy yeast cells (Figure 5a). In contrast, cells exposed to the ethanol extract showed notable structural disruptions, including damage to the cell wall, membrane roughness, and irregular cell shapes. Treated cells showed shrivelling, surface collapse, and increased roughness, suggesting membrane disruption and potential cytoplasmic leakage (Figure 5b). Fragmentation and lysis were also observed in some cells, indicating strong antifungal activity of the extract. These morphological changes highlight the potential of *S. flavescens* root extract as an effective antifungal agent, likely targeting the cell wall integrity and membrane stability of *C. albicans*.



**Figure 2.** Identification of *Candida* species using HiChrome™ *Candida* Differential Agar. (a) Distinct colony colours of different species on agar plate; (b) Inverted view of the same plate showing colony characteristics. Species: (1) *C. auris*, (2) *C. krusei*, (3) *C. parapsilosis*, (4) *C. albicans*, and (5) *C. glabrata*

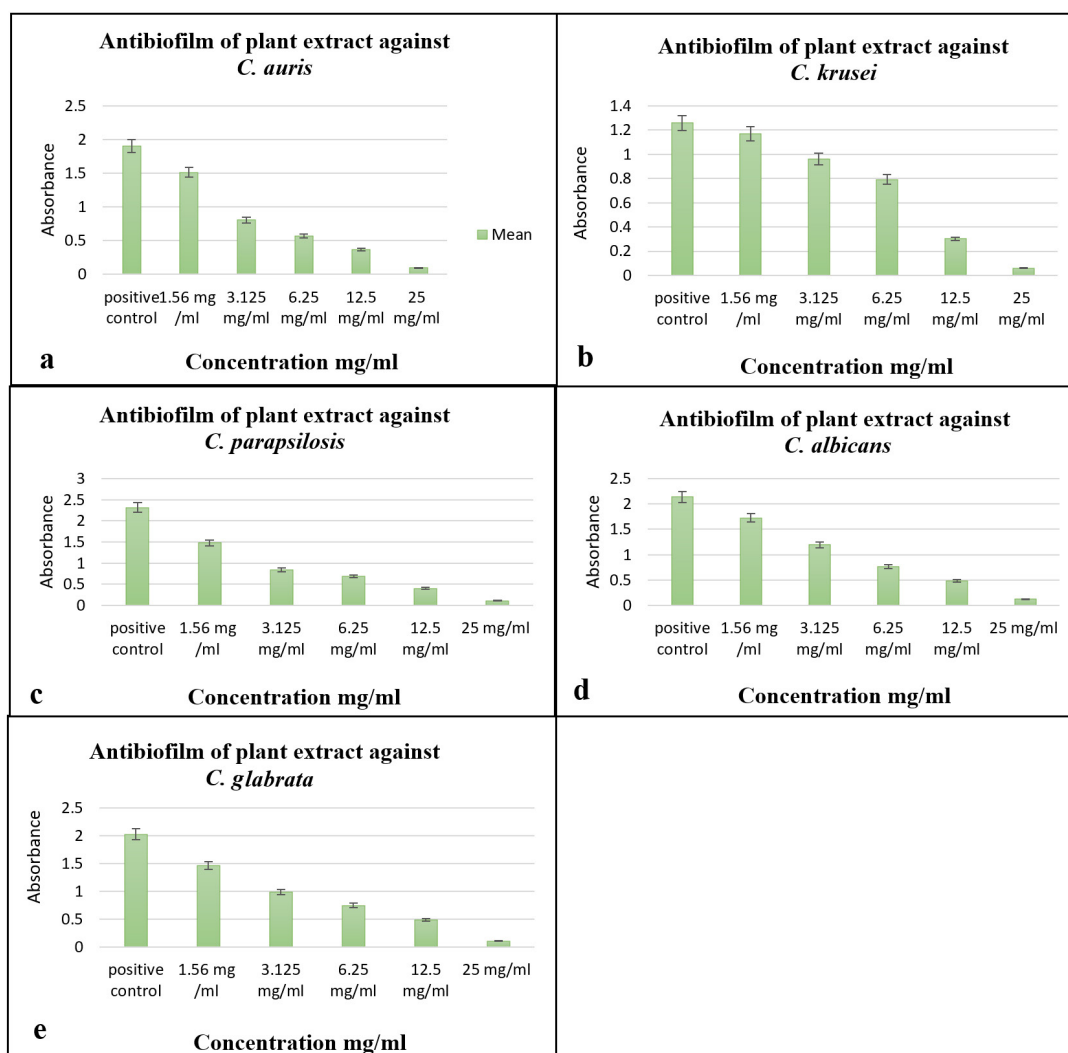


**Figure 3.** Growth inhibition of nosocomial fungal strains following treatment with (1) *S. flavescens* root extract, (2) negative control (DMSO), and (3) positive control (Itraconazole). The tested fungal species include: (a) *C. auris*, (b) *C. krusei*, (c) *C. parapsilosis*, (d) *C. albicans*, and (e) *C. glabrata*.

### Molecular docking studies of isolated compounds

Molecular docking simulations were performed to investigate the binding interactions and inhibitory potential of bioactive compounds derived from *S. flavescens* against CYP51 of *C. albicans*. A total of 120 compounds were identified through HR-LCMS analysis. Of these, 101 compounds, including 100 phytochemicals and the reference antifungal agent Itraconazole, were selected for virtual screening to evaluate their binding affinities, molecular interactions,

and SAR. Compounds exhibiting docking scores weaker than -5.0 kcal/mol were considered to have negligible binding affinity and were excluded from further analysis. Consequently, 20 compounds were not included in the final interpretation. Notably, several of the remaining phytochemicals demonstrated higher binding affinities than itraconazole, suggesting their potential as promising antifungal candidates targeting CYP51.



**Figure 4.** Antibiofilm activity of *S. flavescens* root extract against *Candida* species. Biofilm inhibition at various concentrations (1.56-25 mg/mL) against (a) *C. auris*, (b) *C. krusei*, (c) *C. parapsilosis*, (d) *C. albicans*, and (e) *C. glabrata*. A positive control was included for comparison



### Docking scores and binding affinities

Among the docked compounds, ginkgetin (-11.0 kcal/mol), roxburghine B (-10.7 kcal/mol), Mulberroside F (-10.5 kcal/mol), and Austinol (-10.5 kcal/mol) exhibited the highest binding affinities, all outperforming itraconazole (-9.3 kcal/mol), which functioned as the standard antifungal control (see Supplementary data, Table S2, Figures S6). These findings suggest that these compounds could serve as promising CYP51 inhibitors, potentially disrupting ergosterol

biosynthesis in fungal pathogens and exerting remarkable antifungal effects.

### Interaction analysis

Molecular interaction analysis provided a further understanding of the binding mechanisms of the top-ranked compounds. Ginkgetin (-11.0 kcal/mol) formed strong hydrogen bonds with Tyr118 and Phe228, two crucial residues involved in CYP51 inhibition. Similarly, Mulberroside F (-10.5 kcal/mol) established  $\pi$ - $\pi$  stacking

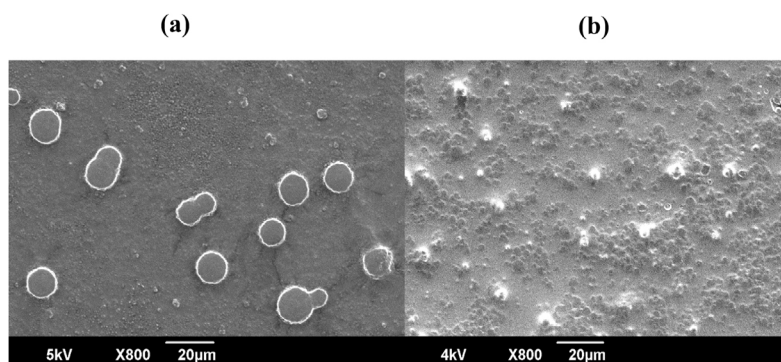
**Table 2.** Antifungal activity of ethanolic root extract of *S. flavescentis* (10 mg/ml) against fungal strains associated with nosocomial infections

No.	<i>Candida</i> species	Zone of inhibition (mm)		
		<i>S. flavescentis</i> root extract	Positive Control Itraconazole	Negative Control DMSO
1	<i>C. auris</i>	19.66 ± 0.57	20	0
2	<i>C. krusei</i>	13.33 ± 0.57	14	0
3	<i>C. parapsilosis</i>	17.33 ± 1.154	16	0
4	<i>C. albicans</i>	15.33 ± 0.57	18	0
5	<i>C. glabrata</i>	17.33 ± 1.154	18	0

**Table 3.** MIC and MFC values of *S. flavescentis* ethanol extract against *Candida* species

<i>Candida</i> species	MIC (mg/mL)	MFC (mg/mL)
<i>C. auris</i>	25-6.25	25-12.5
<i>C. krusei</i>	25-6.25	25-6.25
<i>C. parapsilosis</i>	25-6.25	25-6.25
<i>C. albicans</i>	25-6.25	25-6.25
<i>C. glabrata</i>	25-6.25	25-6.25

interactions with His377 and Tyr132, enhancing its stability within the active site. Additional hydrophobic interactions with Arg96 and Glu150 were also observed, further strengthening the binding affinities of these compounds (Figure S6). The high binding stability demonstrated by these compounds suggests their potential to effectively inhibit CYP51 activity, thereby interfering with fungal membrane synthesis.



**Figure 5.** SEM images of *C. albicans* fungal cells: (a) untreated control and (b) cells after treatment

### Structure-Activity Relationship (SAR) evaluation

SAR analysis highlighted that flavonoid-based compounds displayed strong interactions with the CYP51 active site, primarily attributed to the presence of hydroxyl (-OH) functional groups. Notably, kaempferol 3 (-9.3 kcal/mol) and isorhamnetin 3-O- $\beta$  (-8.7 kcal/mol) formed multiple hydrogen bonds with active site residues (Figure S6). The presence of electron-donating groups within the flavonoid structure appears to enhance binding efficiency, further reinforcing the potential of flavonoids as natural antifungal agents.

### DISCUSSION

The use of HiCrome™ Candida Differential Agar proved effective in distinguishing clinically significant *Candida* species based on their unique colony appearances. In this study, each species displayed characteristic pigmentation: *C. albicans* formed light green colonies, *C. krusei* showed rough purple colonies, *C. glabrata* produced smooth cream-white colonies, and *C. parapsilosis* appeared as smooth cream colonies, which are consistent with previous findings.<sup>15,17,29,30</sup> Notably, *C. auris* presented cream to pale colonies, a variation attributed to strain-specific morphological differences documented in recent literature.<sup>31,32</sup> These findings underscore the value of chromogenic media for the presumptive identification of *Candida* species, supporting its implementation in routine diagnostics and surveillance, particularly in settings requiring rapid differentiation of pathogenic yeasts.

The ethanolic extract of *S. flavescentis* exhibited potent antifungal effects against drug-resistant *Candida* strains, with *C. auris* showing the largest zone of inhibition ( $19.66 \pm 0.57$  mm). This aligns with prior studies highlighting the efficacy of flavonoids and alkaloids present in *S. flavescentis*, such as kurarinone and matrine, against fungal pathogens.<sup>6,9</sup> Notably, the extract showcased potent activity against fluconazole-resistant strains, with MIC values ranging from 6.25 to 25 mg/mL. While these MICs are higher than those of synthetic drugs like fluconazole (MIC >64  $\mu$ g/mL for resistant isolates),<sup>33</sup> this discrepancy likely reflects differences in purity,

as plant extracts contain complex mixtures of bioactive compounds. Nevertheless, the extract's multi-target mechanism may compensate for the need for higher concentrations by mitigating the risk of resistance.<sup>5</sup> Comparatively, extracts from *Artemisia annua* and *Glycyrrhiza glabra* have shown antifungal activity, albeit with a narrower spectrum, primarily against fluconazole-susceptible *Candida* strains, thereby underscoring the distinctive potential of *S. flavescentis* in targeting resistant fungal pathogens.<sup>34</sup> These results support its potential as a broad-spectrum antifungal agent, especially in light of the rising resistance to azoles.<sup>35</sup>

This study offers insights into the antifungal activity of compounds from *S. flavescentis* against *C. albicans* CYP51. The high binding affinities observed for ginkgetin (-11.0 kcal/mol) and roxburghine B (-10.7 kcal/mol) suggest their strong potential as inhibitors, surpassing those of standard antifungal drugs like itraconazole (-9.3 kcal/mol). These findings align with previous studies demonstrating the effectiveness of natural flavonoids and alkaloids as antifungal agents.<sup>36</sup>

HR-LCMS identified 120 bioactive compounds, including Kurarinone (binding affinity of -9.8 kcal/mol to CYP51) and *S. flavanone* G (-8.6 kcal/mol to  $\beta$ -glucan synthase). These results are consistent with earlier reports on the quinolizidine alkaloids and prenylated flavonoids of *S. flavescentis*, which have been shown to disrupt ergosterol biosynthesis.<sup>37</sup>

Meanwhile, prior studies have established that compounds exhibiting docking scores weaker than -5.0 kcal/mol generally exhibit negligible binding affinity and are typically excluded from further analysis.<sup>38</sup> In line with this criterion, 20 compounds were omitted from the final interpretation in this study.

Kurarinone demonstrated a strong affinity for CYP51 (-9.8 kcal/mol), equivalent to that of fluconazole (-8.6 kcal/mol), suggesting its potential for competitive inhibition.<sup>39</sup> Additionally, oleuropein, identified in the present study, has previously been reported to possess antifungal activity in *Olea europaea* extracts by destabilising fungal membrane integrity,<sup>40</sup> thereby supporting its possible role in fungal suppression. The structural diversity of these bioactive compounds

enables multi-pathway targeting, an approach increasingly emphasised in combating antifungal resistance.<sup>41</sup>

Several studies have documented the inhibitory effects of natural products on CYP51. Baicalein (-9.1 kcal/mol), a flavone derivative, has demonstrated antifungal activity against *C. albicans* biofilms by interfering with ergosterol biosynthesis.<sup>42</sup> For instance, a study utilised genome mining to discover biosynthetic gene clusters responsible for the production of restriction and lanomycin, both of which act as inhibitors of CYP51.<sup>43</sup>

Kean and Ramage's review emphasises the clinical consequences of antifungal resistance in *C. auris*, offering an in-depth analysis of the mechanisms responsible for resistance to azoles, polyenes, and echinocandins.<sup>44</sup> Furthermore, Berkow *et al.* report *C. auris* isolates exhibiting resistance to four major classes of antifungal agents, underscoring the pathogen's capacity to develop MDR.<sup>45</sup> Nevertheless, the extract's MFC/MIC ratio  $\leq 4$  confirms its fungicidal action, comparable to that of amphotericin B. Although natural products often necessitate higher concentrations than synthetic drugs due to their complex matrices, their multi-target mechanisms reduce the likelihood of resistance development.<sup>5</sup> For instance, thymol derived from oregano shows similar MIC ranges (15-30 mg/mL) against *C. albicans*.<sup>46</sup>

SEM micrographs revealed cell wall collapse and cytoplasmic leakage in treated *Candida*, consistent with membrane-targeting activity.<sup>47</sup> Similar structural damage has been observed for *Melaleuca alternifolia* oil, known to induce pore formation.<sup>48</sup> The extract exhibited dose-dependent biofilm inhibition ( $IC_{50}$ : 18.5 mg/mL), paralleling findings for curcumin, which disrupts *C. albicans* biofilms via agglutinin-like sequence protein 3 (Als3) suppression.<sup>49</sup> Biofilm reduction is particularly important, as approximately 60% of fungal infections involve biofilm-mediated resistance.<sup>50</sup> Notably, the anti-adhesion properties of oleuropein<sup>51</sup> may act synergistically with the membrane-disrupting effects of kurarinone, thereby enhancing biofilm penetration.

The multifaceted antifungal properties of *S. flavescentis*, largely attributed to its prenylated

flavonoids, render it a promising candidate for both topical and systemic therapies. These compounds have demonstrated potent antifungal activity, suggesting potential therapeutic benefits.<sup>52</sup> Moreover, the combination of *S. flavescentis* with fluconazole has shown enhanced antifungal efficacy. A clinical study observed that patients treated with a combination of *Sophora* gel and fluconazole capsules experienced higher cure rates and lower recurrence rates of mycotic vaginitis compared to those receiving fluconazole alone. Specifically, the combination therapy achieved a cure rate of 90.7% and a recurrence rate of 2.6%, whereas fluconazole monotherapy resulted in a cure rate of 71.4% and a recurrence rate of 20.0%.<sup>53</sup> Advancements in green extraction methods, such as subcritical water extraction, could enhance compound yield and promote sustainability.<sup>54</sup> Nanoparticle encapsulation and combinatorial therapies may improve bioavailability while reducing therapeutic doses.<sup>55</sup> The structural diversity of *S. flavescentis* compounds enables multi-target antifungal mechanisms, lowering drug resistance risks and making it a promising alternative against azole-resistant *Candida* strains.<sup>35</sup>

## CONCLUSION

This study demonstrated the strong antifungal activity of *S. flavescentis* root extract against MDR *Candida* species, highlighting its potential as a natural therapeutic agent for nosocomial fungal infections. HR-LCMS analysis identified 120 bioactive compounds, among which several flavonoids and alkaloids showed strong antifungal properties. *In vitro* assays confirmed broad-spectrum activity, with notable inhibition of biofilm formation and fungicidal effects observed across all tested *Candida* strains. SEM revealed significant structural disruption in fungal cells, corroborating the extract's membrane-targeting mechanism. Molecular docking further identified key compounds, such as ginkgetin and roxburghine B, possessing high binding affinities toward the fungal CYP51 enzyme, surpassing those of the reference antifungal itraconazole. These results support the therapeutic relevance of *S. flavescentis*, especially in the context of emerging drug-resistance. Future investigations

are warranted to explore *in vivo* efficacy, pharmacokinetics, and formulation strategies, including nanoencapsulation, to enhance its clinical applicability and bioavailability.

#### SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at <https://doi.org/10.22207/JPAM.19.3.10>

**Additional file:** Additional Table S1-S2 and Figure S1-S6.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHORS' CONTRIBUTION

MMA and SV conceptualized the study. MMA applied methodology, performed visualization, experiments, Investigation and data curation. TM performed molecular docking data analysis. SV performed supervision, project administration and data validation. MMA wrote the manuscript. SV reviewed and edited the

manuscript. All authors read and approved the final manuscript for publication.

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#### DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

#### ETHICS STATEMENT

Not applicable.

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