Preparation and in-vitro evaluation of *Grevillea robusta* extract against fungal infection

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Abstract

Objective: fungal infections are a growing health concern due to rising resistance to antifungal drugs. This study aimed to evaluate the in vitro antifungal activity of methanolic leaf extracts of *Grevillea robusta* against *Candida albicans* and *Aspergillus niger.*

Methodology: Leaves of *Grevillea robusta* were collected, shade-dried, and powdered. Methanol extraction was done using a Soxhlet apparatus. The extract was tested using the Kirby-Bauer disk diffusion method on Sabouraud Dextrose Agar. Discs with 0–1000 µg of extract were tested. Amphotericin B was the positive control; Dimethyl Sulfoxide served as the negative control.

Result: The results demonstrated that the methanol extract of *Grevillea robusta* did not exhibit any significant zone of inhibition against either *Candida albicans or Aspergillus niger* across all tested concentrations. In contrast, the positive control (Amphotericin B) produced measurable zones of inhibition: 12 mm for *Candida albicans* and 18 mm for *Aspergillus niger*, confirming the assay's reliability. This lack of antifungal activity under the current experimental conditions suggests either the absence of effective antifungal constituents in the methanol extract or the need for further purification and concentration of the active principles.

Conclusion: In conclusion, although traditional knowledge supports the medicinal use of *Grevillea robusta*, this study highlights the need for comprehensive phytochemical investigations, bioactivity-guided fractionation, and toxicity profiling. Future research should explore other extraction solvents, isolate specific bioactive compounds, and examine their efficacy against a broader range of fungal pathogens. This would contribute to the ongoing search for alternative plant-based antifungal therapies.

Keywords- *Grevillea robusta*, Antifungal activity, Methanolic extract, *Candida albicans*, *Aspergillus niger*, Disc diffusion method, Medicinal plants.

1. Introduction

The terms antimicrobial, antibiotic, and anti-infective encompass a broad range of pharmaceutical agents, including antibacterial, antifungal, antiviral, and antiparasitic medications[1]. In recent years, significant attention has been directed toward the exploration and development of novel antimicrobial agents from diverse sources, aiming to address the escalating challenge of microbial resistance so there are various bioassay techniques, including disk diffusion or agar dilution methods, are widely recognized and routinely employed for antimicrobial evaluation and also Alternative methods, such as the poisoned food technique, are commonly employed for evaluating antifungal activity[2]. The following mechanisms of action of antimicrobial agents can be categorized such as they interfering with the synthesis of cell wall[3]. Various medicinal plant parts are utilized to produce different rasayanas, each possessing unique therapeutic properties effective against a range of microbes, including clove, oregano, thyme, cinnamon, and cumin, have demonstrated notable antibacterial and antifungal

properties against food spoilage organisms such as Bacillus subtilis disease-causing bacteria like *Staphylococcus aureus* and toxic fungi such as *Aspergillus flavus*[4].The advancement of antimicrobial agents, especially antibiotics, has significantly contributed to the decline in morbidity and mortality associated with numerous infectious diseases but the recent reports highlight the widespread inappropriate and irrational use of antimicrobial agents in treating these infections, significantly contributing to the rise in antimicrobial resistance[5,6]. Between 2017 and 2023, total antibiotic consumption in inpatient departments ranged from 60.22 to 102.42 Defined Daily Doses (DDD) per 100 bed-days, with the highest usage observed in 2018 and the lowest in 2017[7].

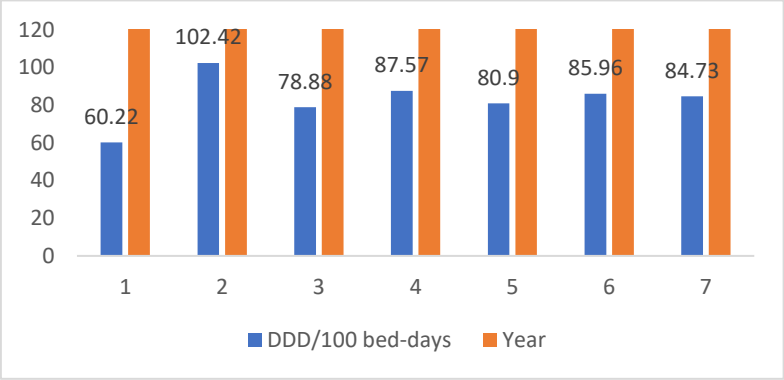

Figure 1: Annual antibiotic utilization, expressed in DDD per 100 bed-days, over 2017 to 2023 years

Fungal infections are caused by eukaryotic organisms, making them harder to detect and treat effectively than bacterial infections[8]. Substances like essential oils (including thymol, eugenol, citral, and cineole), along with vanillin, sodium hypochlorite, acetic acid, potassium sorbate, and hydrogen peroxide, were tested for their effectiveness[9].

As of Friday, December 20, 2024, India's population stands at 1,457,001,793, making it the most populous country globally and accounting for 17.78% of the world's total population. Approximately 4.4% of the Indian population is affected by serious fungal infections[10.] Resistance to antifungal medications has emerged as a significant challenge in managing various infectious diseases today[11].

Grevillea robusta A. Cunn. ex R.Br. (Synonyms: *Grevillea venusta A. Cunn. ex Meisn., Stylurus robustus A. Cunn. O. Deg.;* Bengali name: Rupasi) is a rapidly growing, evergreen tree from the Proteaceae family, commonly referred to as the southern silky oak or Australian silver oak[12]. It is a vertically growing, single-trunked tree that typically attains a height of 20 to 30 meters and a stem diameter of approximately 80 centimeters[13]. It is widely cultivated in South and Central America, South Asia, and parts of Africa. It is commonly used as a shade tree in tea and coffee plantations also important in agroforestry for fuelwood and timber additionally, it serves as an attractive ornamental tree[14]. *Grevillea robusta* also used as traditional medicine in Northeast India and Kenya. Its bark and leaves help relieve headaches and dizziness in India. Kenyan communities use it to treat respiratory and ear-related ailments.

It is valued for its broad therapeutic applications in folk medicine and also has anti-inflammatory activity[15]. It also has both Gram-negative and Gram-positive bacterial species, as well as certain fungal strains activities of *Grevillea robusta* leaf and flower extracts, has been scientifically investigated[16]. Seven phenolic compounds were successfully isolated from the methanolic extract of Grevillea robusta leaves such as Grevirobstol A, Grevirobstol C, Robustaside A and Robustaside D etc[17].

The crude methanol extract of *Grevillea robusta* leaves from Bangladesh, along with its organic and aqueous soluble fractions, has been investigated for various bioactivities. These include cytotoxic, thrombolytic, membrane stabilizing, and antimicrobial properties[12].

2. Material and Method:

2.1 Plant material: The *Grevillea robusta* plant was chosen for the study, and its leaves were collected from the college campus.

2.2 Soxhlet Extraction Process[18,19,20,21,22]**:** Soxhlet extraction is a continuous extraction technique used to extract bioactive compounds from plant materials using a methanol solvent. In this process, 20 grams of shade-dried powdered leaves of *Grevillea robusta* were placed in a thimble and loaded into the Soxhlet apparatus. The extraction was carried out using 250 mL of methanol as the solvent. The solvent extractions were carried out at the respective boiling points of the solvents. Following extraction, the solvents were removed from the solute mixtures under water bath. To ensure complete drying of the extracts, the residues were transferred to glass bottles. Subsequently, the dried extracts were stored at 4°C until further evaluation of their antimicrobial activities.

2.3 Microorganism[23]**:** The fungal strains – *Aspergillus niger* and *Candida albicans* were used. (Moses ikegbunam)

2.4 Anti-Fungal-Zone Inhibition Test[24,25]

For *Aspergillus niger and Candida albicans*

2.4.1 Requirements:

The following materials and reagents were used in the study:

- Sabouraud Dextrose Agar (SDA) plates (SRL Chemicals, Catalog No. 19427).
- Fungal strain: *Candida albicans* (MTCC 854) and *Aspergillus niger* (MTCC 281), obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh.
- Filter paper discs: Whatman No. 1, 5 mm diameter.
- Solvent (vehicle control): Dimethyl Sulfoxide (DMSO) (SRL Chemicals, Catalog No. 28580).

- Positive control: Amphotericin B (Amphocare), prepared at a concentration of 10 mg/mL.
- Sample loading volume: 5 µL per disc.

2.4.2 Antifungal Activity Assay

The antifungal activity was evaluated using the Zone of Inhibition method, specifically the Kirby-Bauer disk diffusion assay, against two fungal strains: *Candida albicans* and *Aspergillus niger*. Sabouraud Dextrose Agar (SDA) plates were uniformly inoculated with 100 µL of each fungal suspension, standardized to 0.5 McFarland turbidity (approximately 1.5×10^8 CFU/mL) using Sabouraud Dextrose Broth. Sterile Whatman No. 1 filter paper discs (5 mm diameter) were impregnated with 5 µL of the test samples at varying concentrations (0–200 mg/mL) and placed on the surface of the inoculated agar. A disc containing Dimethyl Sulfoxide (DMSO) served as the vehicle control, while a disc impregnated with Amphotericin B (100 µg) was used as the positive control. Plates inoculated with *Candida albicans* were incubated at 37 °C for 24 hours, while those with *Aspergillus niger* were incubated at 37 °C for 48 hours in a controlled incubator (Basil Scientific Corp., India). After incubation, the diameters of the clear zones of inhibition formed around each disc were measured and recorded to assess the antifungal efficacy of the test samples.

3. Observation

Bioassay	Antifungal Activity	
Test Organism	*Candida albicans & Aspergillus niger*	
X Axis	Amount (µg/disc)	
Y Axis	*Zone Size (in mm)*	
Sample code	Methanol extract	
Title	Antifungal Activity-*Candida albicans* -Methanol extract	

Amount (µg/disc)	**Plate A**	**Plate B**	**Plate C**	**Average**	**SD**	**SEM**
PC	12	12	12	12	0	0
0	0	0	0	0	0	0
62.5	0	0	0	0	0	0
125	0	0	0	0	0	0
250	0	0	0	0	0	0
500	0	0	0	0	0	0
1000	0	0	0	0	0	0

Table No.1: - Antifungal Activity-*Candida albicans* -Methanol extract

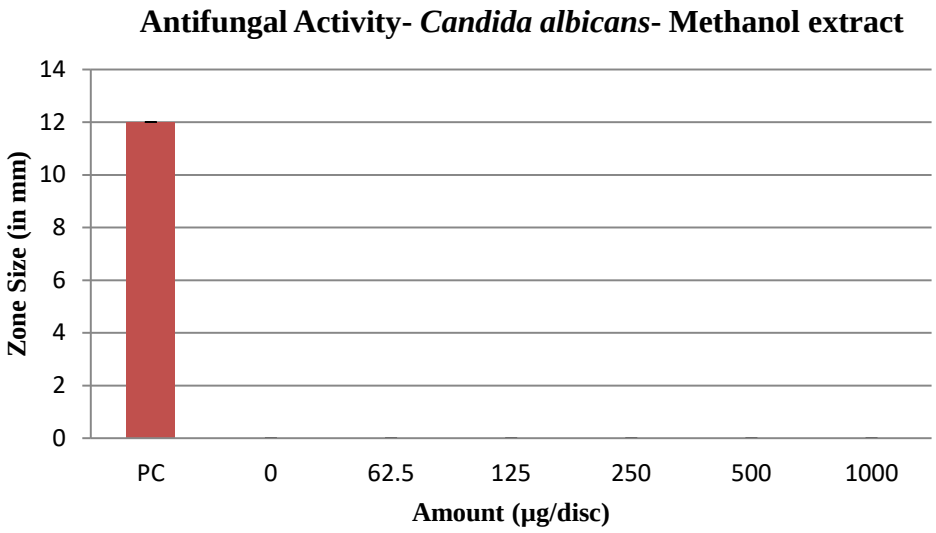

Figure 2: Zone of inhibition against *Candida albicans* graph

Amount (µg/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
PC	18	18	18	18	0	0
0	0	0	0	0	0	0
62.5	0	0	0	0	0	0
125	0	0	0	0	0	0
250	0	0	0	0	0	0
500	0	0	0	0	0	0
1000	0	0	0	0	0	0

Table No.2: - Antifungal Activity- *Aspergillus niger*-Methanol extract

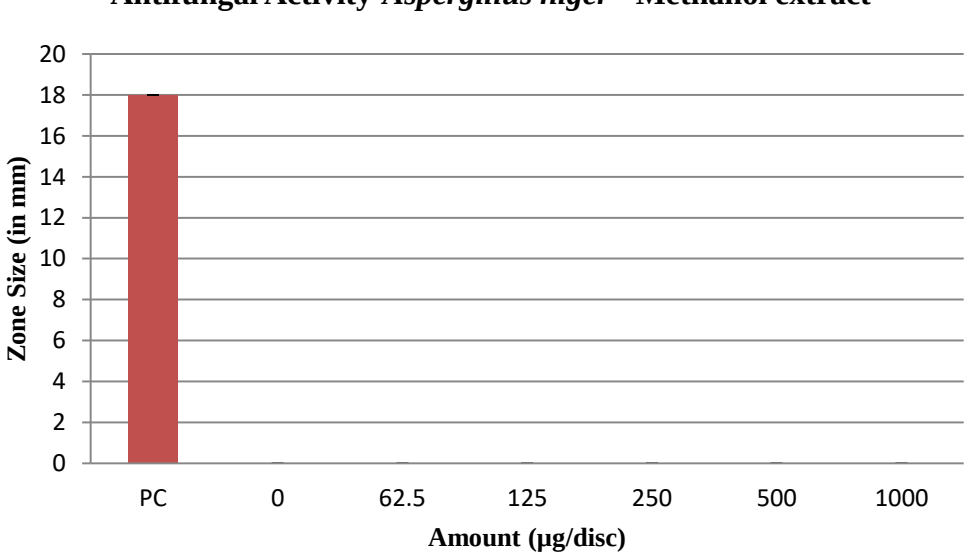

Figure 3: Zone of inhibition against *Aspergillus niger*

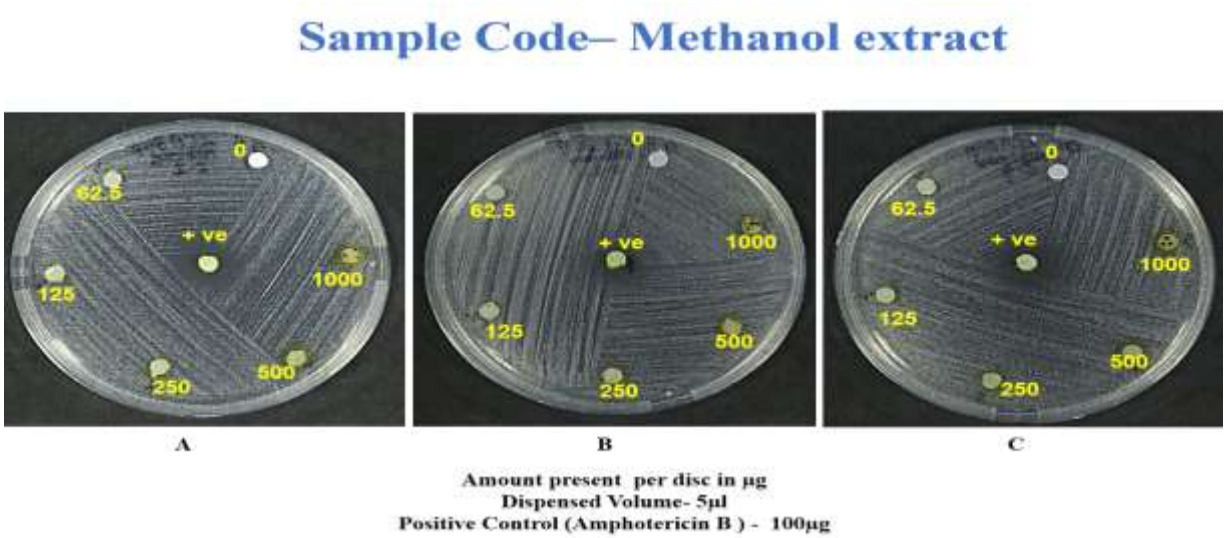

Figure 4: Antifungal Activity Against *Candida albicans* on SDA Plates (Disc Diffusion Method)

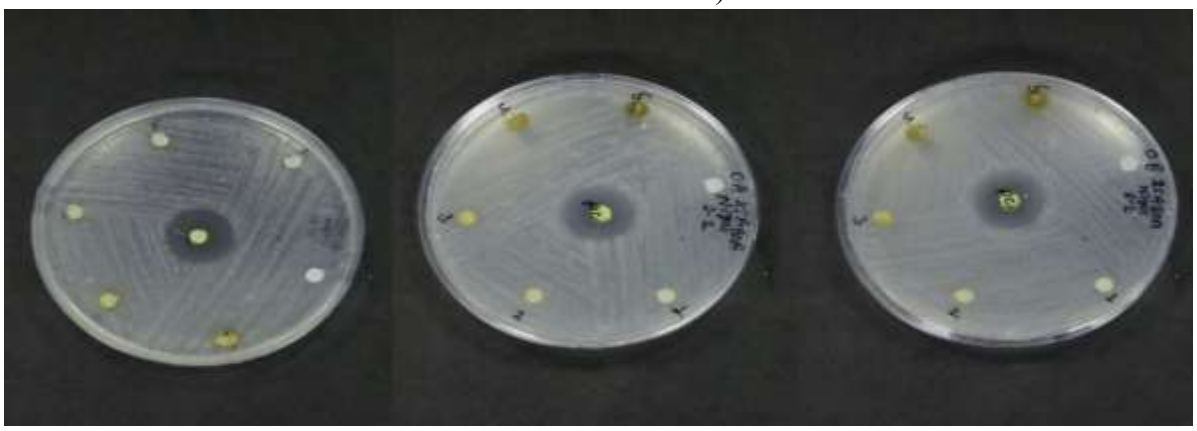

Figure 5: Antifungal Activity Against *Aspergillus niger* on SDA Plates (Disc Diffusion Method)

Abbreviations

SD- Standard Deviation
SEM- Standrad Error of the Mean

Result and Discussion

The methanol extract tested was evaluated for its antifungal activity against *Candida albicans* and *Aspergillus niger* using the Kirby-Bauer disk diffusion method. The extract was applied at concentrations up to 1000 µg/disc. No zone of inhibition was observed around the discs for either fungal strain, indicating the absence of detectable antifungal activity at the tested concentration.

S.No.	Extract	Effective Amount	Average Zone at Effective Amount (in mm)
1	Amphotericin B (PC)	100µg	12 (*Candida albicans*) &18 (*Aspergillus niger*)
2	Methanolic extract	-	-

Table No. 3: Antifungal Activity of Test Samples Compared to Positive Control (Amphotericin B)

The lack of zone formation suggests that the methanol extract prepared does not exhibit effective antifungal activity against the selected fungal strains at or below 1000 µg/disc. This may be attributed to a low concentration of active antifungal phytoconstituents in the crude extract or the possible resistance of the tested organisms to the constituents present. Methanol extracts often contain polar compounds, which may not be sufficiently potent or bioavailable in crude form. Further phytochemical screening and fractionation may help isolate any potentially active components that were not effective in crude form.

Conclusion

The methanolic extract prepared did not show antifungal activity against *Candida albicans* and *Aspergillus niger* up to a concentration of 1000 µg/disc. Further studies with higher concentrations, purified fractions, or different extraction techniques may be necessary to assess the antifungal potential of the plant material used.

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