Evaluation of Antibacterial and Antifungal Potency of Methanolic Extracts From *Nelumbo nucifera*

Irfana Assad[1] , Sami Ullah Bhat[1*] and Adil Gani[2]

[1]Department of Environmental Science, University of Kashmir, Srinagar J&K, India, 190006
[2]Department of Food Science and Technology, University of Kashmir, Srinagar J&K, India, 190006

Corresponding author Email: samiullahbhat11@gmail.com

ABSTRACT

This research explores the antimicrobial activity of methanolic extract of *Nelumbo nucifera* against an array of bacterial and fungal species. The methanolic extract of the plant demonstrated significant antibacterial activity, against *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli,* and *Bacillus subtilis,* with highest inhibition zone of 18.6 ± 0.57 mm in *Pseudomonas aeruginosa* at 100mg/ml. Furthermore, the extract showed remarkable activity against *Acinetobacter* at higher concentrations. ANOVA analysis confirmed significant variations in antibacterial effectiveness across different bacterial species ($p < 0.0001$). The extract also exhibited significant antifungal activity against *Candida albicans* and *Candida glabrata,* even at lower concentrations. Inhibition zones for *C. albicans* and *C. glabrata* ranged from 14.6 ± 0.5 mm to 5.7 ± 0.6 mm and 17.6 ± 0.5 mm to 2 ± 1.4 mm, respectively. These findings suggest that the methanolic extract of *N. nucifera* possesses potent antimicrobial properties, highlighting its potential for therapeutic applications.

Keywords: *Nelumbo nucifera*, *antibacterial, antifungal, medicinal, bioactive*

INTRODUCTION

Nelumbo nucifera Gaertn., commonly known as lotus, Indian lotus, sacred lotus or Chinese water lily, is a widely recognized dietary and medicinal plant (Bishayee *et al*., 2022; Kakkar *et al.,* 2023). China is the leading country for lotus cultivation due to its significant traditional medicinal value (Pokhrel *et al*., 2022). In South Asia and China, the seeds and rhizomes of the lotus are commonly consumed as vegetables (Pokhrel *et al.,* 2022). It is estimated that over 330,000 hectares in China and 4,000 hectares in Japan are dedicated to lotus cultivation (Kurashita *et al*., 2021). This plant also thrives in various water bodies across India, ranging from high altitudes (in Kashmir, North India) to low altitudes (Kanyakumari, Southern India) (Hussain *et al*., 2016) (Fig. 1).

Various parts of *N. nucifera* have been utilized for medicinal purposes across different systems of medicine, including folk medicine, Chinese traditional medicine, Ayurveda, and oriental medicine (Bangar *et al*., 2022; Paudel and Panth, 2015). In ancient China, *N. nucifera* leaves were considered herbal medicine for treating sunstroke, diarrhoea, thirst and fever (Ye *et al*., 2016). Indigenous communities have widely used lotus leaves as diuretics, cardiotonic, and vasodilators to treat

obesity, insomnia, diarrhoea, hyperglycemia, nervous disorders, and skin diseases (Zhang *et al*., 2022). Nearly all parts of the lotus can be utilized as food or herbs (Wang *et al*., 2023). Recently, the scientific community has shown increased interest in lotus, leading to a surge in research publications that have unravelled the mysteries of this species (Lin *et al*., 2019; Ren *et al.,* 2024; Sahu *et al*., 2024; Thongphichai *et al.,* 2023).

These medicinal applications are attributed to the plant's high content of health-promoting compounds, such as phenolic acids and flavonoids (Temviriyanukul *et al*.,2020). Currently, the plant has gained significant attention due to its rich content of bioactive secondary metabolites like flavanols and anthocyanins in the flower (Deng *et al*.,2013; Wang *et al*., 2021).

The primary edible parts of the lotus are the rhizome and seeds, contributing significantly to agricultural output. In China, approximately 45,000 tons of lotus seeds and 9 million tons of lotus rhizomes are produced annually, making it a valuable economic crop (Dhull *et al*.,2023). Despite this, the lotus leaf, with an annual production exceeding 800,000 tons, is often goes underutilized and is discarded as waste by the lotus food production (Huang *et al*., 2010). Utilizing this waste to produce value-added products is a novel approach to its sustainable utilization (Bangar *et al*., 2022).

In many developing nations, traditional medicines remains the primary approach to healthcare system, while developed countries also show an increase in the sales of plant-based health products (Van Wyk and Prinsloo, 2020). Research indicates that individuals often turn to plant-based medicines due to familial traditions, positioning them as a primary therapeutic option for many individuals experiencing mild to moderate illnesses or those with multiple conditions (Welz *et al*., 2018; Bachtel and Israni-Winger).

Antimicrobial resistance (AMR) has emerged as a critical public health threat, significantly impacting the successful prevention and treatment of persistent diseases (Dadgostar, 2019). The rise of resistance among both bacterial and fungal pathogens has diminished the effectiveness of conventional antibiotics and antifungals, challenging their lifesaving potential (AlSheikh *et al*., 2020). This issue is exacerbated by the limited development of new antimicrobial agents, leading to higher mortality rates. Recent advancements in the study of plant-derived natural products have uncovered numerous phytochemicals capable of combating drug resistance through various mechanisms, offering a

potential research avenue for addressing multidrug-resistant (MDR) bacterial and fungal pathogens (Dassanayake *et al*., 2021; Marquez and Quave, 2020).

Despite the widespread use of various lotus parts, there are limitations in the pharmacological data and applications of the petal waste (Laoung-on *et al*., 2021). Industrial applications of *Nelumbo nucifera* leaves as food (Je *et al*., 2015), dairy products (Kim *et al*., 2019), beverages, meat products, pasta dishes, fruit preservation, rice and meat zong, food packaging membranes, and health products have been discussed (Lu *et al.,* 2022). Utilizing lotus waste will expand the market and ensure the sustainable growth of lotus food industry (Lu *et al.,* 2022). However, its therapeutic efficacy and applications remain unresolved; thus, more studies and long-term clinical trials are recommended to gain in-depth insights into its safety, physicochemical mechanisms, and bio-effects (Wang *et al*., 2021). Keeping in view the abovementioned, this study aims to investigate the antimicrobial activity of methanolic extract of *N. nucifera* against bacterial and fungal species.

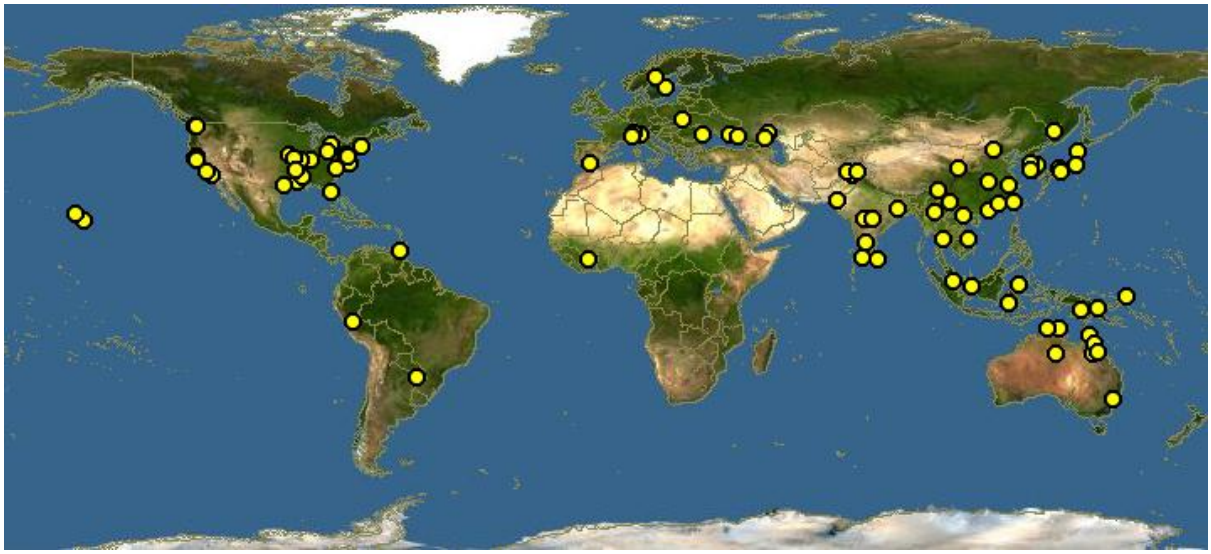

Fig. 1 Global distribution of *N. nucifera*

MATERIALS AND METHODS

The flow chart of the methodological steps is illustrated in Fig 2.

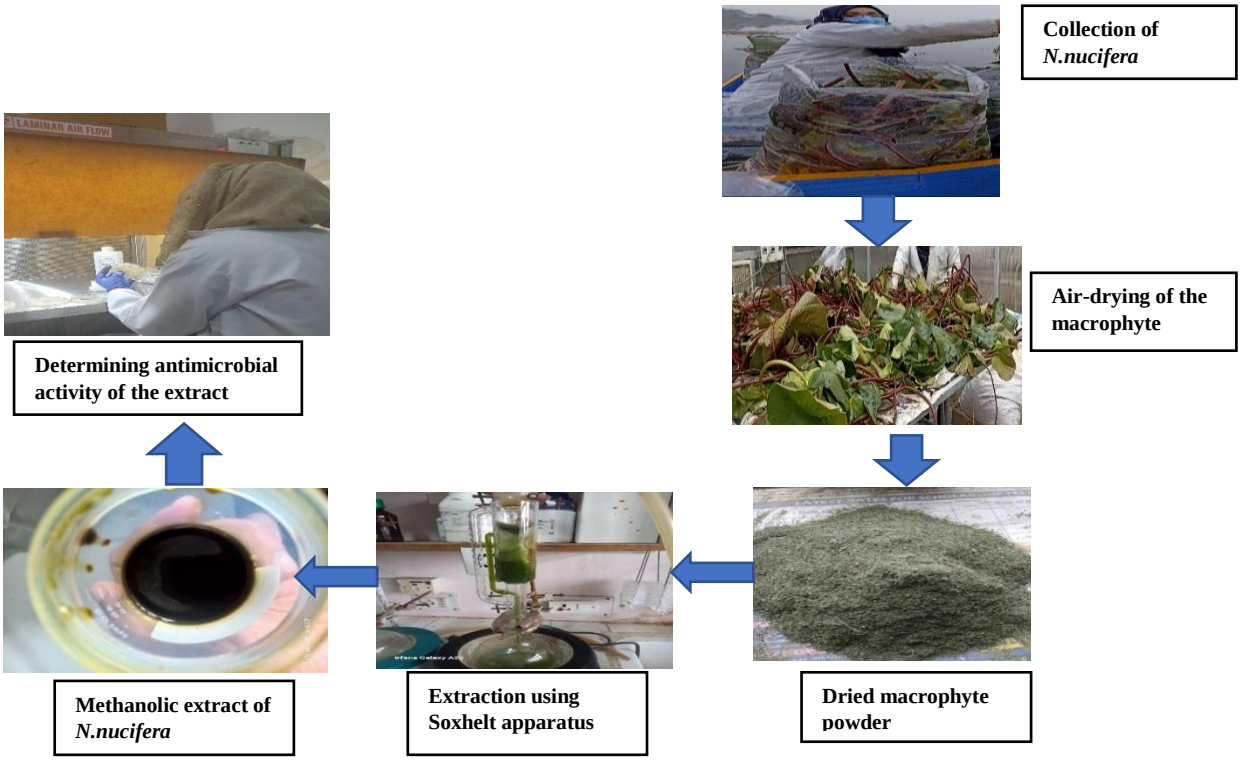

Fig.2. Flow chart of the methodology used in the study

Collection and preparation of the extract

The plant material of *N. nucifera* was collected in the month of October, from the Dal Lake of Kashmir valley. Identification of the plant was conducted at Centre for Biodiversity and Taxonomy, University of Kashmir, Srinagar. The voucher specimen No. 2860-*(KASH)* was deposited for future reference**.**

The freshly obtained macrophyte was washed to remove debris and then air-dried in the shade for one week. The dried plant material (leaves with stalk) was subsequently chopped into small pieces and pulverized into a fine powder using an electric grinder. The powdered plant material was subjected to extraction using a Soxhlet apparatus (Borosil) with 100%methanol as the solvent for 48 hours. The resulting extract was filtered through Whatman filter paper No. 1 (pore size, 11µm, CAT. No.1001 125), to obtain the supernatant fraction. The solvent was removed from the supernatant using a rotary vacuum evaporator, yielding a viscous concentrate. The percentage yield of the extract was 14.26±0.23%. This extract

was then labelled and stored at 4°C in storage vials for future experimental use.

Antimicrobial activity

Stock slants of bacterial culture were prepared using Mueller-Hinton agar. From these cultures, a small colony of the pathogenic bacteria were inoculated into 20ml of selective broths to prepare inoculum. Trypticase soy broth was used for *S. aureus*, nutrient broth for *E. coli* and Mueller Hinton broth for *B. subtilus, Acinetobacter* sp*, P. aeruginosa* and *K. pneumonia*) and turbidity of each broth was adjusted to 0.5 McFarland Scale.

A list consisting of six bacterial strains encompassing both Gram positive and Gram negative as detailed in Table.1) was employed to test the antibacterial efficacy of the selected macrophyte

Table 1. List of bacterial species used for screening of antibacterial activity

S. No	Test bacteria	Type	Source	Antibiotics used as positive control	Culture broth used
1	*Staphylococcus aureus*	Gram positive	ATCC-25923	Gentamycin	Trypticase soy broth
2	*Escherichia coli*	Gram negative	ATCC-25922	Gentamycin	Nutrient broth
3	*Klebsiella pneumoniae*	Gram negative	GMC	Gentamycin	Mueller Hinton broth
4	*Acinetobacter sp.*	Gram negative	GMC	Ciprofloxin	Mueller Hinton broth
5	*Pseudomonas aeruginosa*	Gram negative	GMC	Gentamycin	Mueller Hinton broth
6	*Bacillus subtilis*	Gram positive	MTCC-736	Gentamycin	Mueller Hinton broth

In Vitro Antibacterial Assay Using Agar Well Diffusion Method

The antibacterial efficacy of *N. nucifera* was evaluated using the agar well diffusion method, a standardized technique established by Perez *et al*. (1990) and widely used in clinical laboratories for routine antimicrobial susceptibility testing. Various concentrations of the plant extract (100, 50, 25, 12.5, 6.25) mg/ml were prepared by dissolving in 10% DMSO (Dimethyl sulfoxide). The agar core (4 mm) was removed from the solidified Mueller-Hinton agar in Petri dishes at six peripheral positions. The wells were aseptically filled with 20 microliters of the plant extracts from each concentration into their respective holes Gentamycin, (ciprofloxin in case of *Acinetobacter*) were used as positive controls while DMSO served as negative control in the experiment. The Petri-plates

were sealed and kept in a flat position for an hour, before incubating for 24 hours at 37°C to allow bacterial cultures to grow. clear zones around the wells were measured, and results were expressed as mean ± SD from three independent experiments.

Antifungal Assay (Disc diffusion method)

The disc diffusion method as developed by Bauer *et al*. (1966), was used to assess the antifungal activity of *N. nucifera* extract against *Candida albicans* (ATCC-24433) and *Candida glabrata* (ATCC-2001). Mueller-Hinton agar supplemented with 2% glucose, maintained at a pH of 7.2 to 7.4, was used for the test. Once solidified, lawn cultures of the test organisms were prepared on the agar, and sterile discs were impregnated with the *N. nucifera* extract at five different concentrations. After air-drying, the discs were placed on the fungal lawn cultures along with a known antibiotic sensitive to the test organism. The Petri dishes were incubated at 30-35°C for 24 hours, though some strains required longer incubation for adequate growth. Itraconazole and DMSO (Dimethyl sulfoxide) served as positive and negative reference standards respectively. The inhibition zones were measured to categorize the test isolates as susceptible, non-susceptible, or resistant according to CSLI (2009) guidelines.

Statistical Analysis

All experiments were performed in triplicate, and the results were presented as the mean (± SD). Prior to analysis of variance (ANOVA), preliminary test (Shapiro Wilks test), ensured normality of the data. Pairwise comparisons were made using Tukey's HSD test ($p < 0.05$). The R packages used for one-way ANOVA included “tidyverse,” “ggpubr,” and “rstatix” (R Core Team 2017).

RESULTS AND DISCUSSION

The present study evaluated the antibacterial efficacy of methanolic extracts of *N. nucifera* against a range of bacterial strains, with results showing varying degrees of activity (Fig.3; Table 2). This is consistent with previous studies, such as those by Arjun *et al*. (2012) and Chen *et al*. (2015), which also reported significant antibacterial effects of *N. nucifera* extracts against various bacteria. The extract demonstrated the highest activity against *P. aeruginosa*, with inhibition zones ranging from 18.6±0.57mm at 100 mg/ml to 8.6±0.5mm at 6.25 mg/ml. This aligns with Maqsood *et al*. (2019), who found ethanol-water extracts to be particularly effective against *Aeromonas hydrophila*. Our findings extend this by showing substantial activity against *P. aeruginosa*, highlighting the

broad-spectrum efficacy of *N. nucifera* extracts.

Staphylococcus aureus, Klebsiella pneumoniae, *Escherichia coli* and *Bacillus subtilis* also showed significant sensitivity to the extract, with inhibition zones 15.8±0.76mm, 16.6±0.57mm, 15±1mm and 16.6±2mm respectively at 100mg/ml. This corroborates the findings of Chen *et al*. (2015) and Venkatesh and Dorai (2011), who reported similar antibacterial effects of *N. nucifera* extracts against these bacteria. It was also observed that the extract demonstrated almost similar inhibitory effect against *K. pneumoniae* and *B. subtilis* at all the concentrations. The extract was more potent than the positive control in inhibiting Acinetobacter even at low concentration. *Acinetobacter* exhibited zone of inhibition of 12.8±0.76mm at 100mg/ml and 4.6±0.6 at 12.5mg/ml while has the positive control displayed 2±1mm inhibition zone. The extract showed decreased activity with decrease in concentration (100mg/ml-6.25mg/ml) (Fig. 4). For instance, *S. aureus* exhibited inhibition zones ranging from 15.8 ± 0.76 mm at 100 mg/ml to 1.5 ± 0.8 mm at 6.25 mg/ml. The observed dose-dependent decrease in activity is consistent with the findings of Arjun *et al*. (2012), highlighting the importance of concentration in achieving effective antibacterial action. This dose-response relationship further validates the potential of using *N. nucifera* extracts in various applications, provided optimal concentrations are used.

Utilizing ANOVA analysis, the statistical significance of differences in antibacterial activity across different bacterial strains was examined. The results revealed significant variations in the antibacterial activity of *N. nucifera* against different bacterial species, with $p < 0.0001$ for all comparisons (Fig. 7). Notably, *N. nucifera* exhibited potent antibacterial activity against *K. pneumoniae* ($F(6,14) = 435.58$), *S. aureus* ($F(6,14) = 354.88$), *E. coli* ($F(6,14) = 89.97$), *P. aeruginosa* ($F(6,14) = 201.91$), *B. subtilis* ($F(6,14) = 25.36$), and *Acinetobacter* ($F(6,14) = 163.48$). Furthermore, when analyzing the overall antibacterial activity of *N. nucifera* against all tested bacterial strains collectively, the results still demonstrated a significant ($p < 0.0001$) antibacterial effect ($F(6,140) = 5.26$) Fig. 7. The biological activities of lotus leaves are attributed to phenolic compounds like flavonoids and phenolic acids, including hyperin, isoquercetin, kaempferol, myricetin, and catechin (kaur *et al*.,2019; Limwachiranon *et al*., 2018; Lu *et al*.2022). Polyphenols demonstrate antimicrobial activity against a wide range of bacteria (Miklasińska-Majdanik *et al*., 2018). The

mechanisms of action of phenolic compounds on bacterial cells are partially attributed to membrane damage, inhibition of virulence factors such as enzymes and toxins, and suppression of biofilm formation (Miklasińska-Majdanik *et al*., 2018). Natural phenolic compounds, with their multiple-target mechanisms, are promising candidates for combating microbial infections (Takó *et al*., 2020). With increasing knowledge of their bioactive properties, plant phenolics have attracted attention in food research as potential inhibitors of foodborne pathogenic and spoilage bacteria (Takó *et al*., 2020). Furthermore, lotus leaf extract demonstrated superior antiseptic capacity than sodium benzoate, a common food preservative, in apple juice . Therefore, lotus leaves may be used as a botanical natural food preservative against food-borne pathogens(Chen *et al*.,2015).

Additionally, alkaloids such as nuciferine, nerine, N-nornuciferine, roemerine, and pronuciferine found in lotus exhibit pharmacological effects (Chang *et al*., 2016). Alkaloids are emerging as a new type of natural antibiotic with a broad antibacterial spectrum, rare adverse reactions, and a low tendency to induce drug resistance (Yan *et al*., 2021). Natural alkaloids have shown potential activity against various bacteria, including methicillin-resistant Staphylococcus aureus (MRSA) (Chen *et al*., 2012). Their primary antibacterial mechanisms include inhibition of bacterial cell wall synthesis, alteration of cell membrane permeability, and inhibition of bacterial metabolism, nucleic acid, and protein synthesis (Yan *et al*., 2021). The presence of coumarins, flavonoids, saponins, sterols, tannins, terpenes, and phenolic acids in the extracts (Tano *et al*., 2018) further supports the multifaceted antimicrobial activity (Guimarães *et al*., 2019; Sahoo *et al*., 2021; Sharma *et al*.,2023) observed in our study.

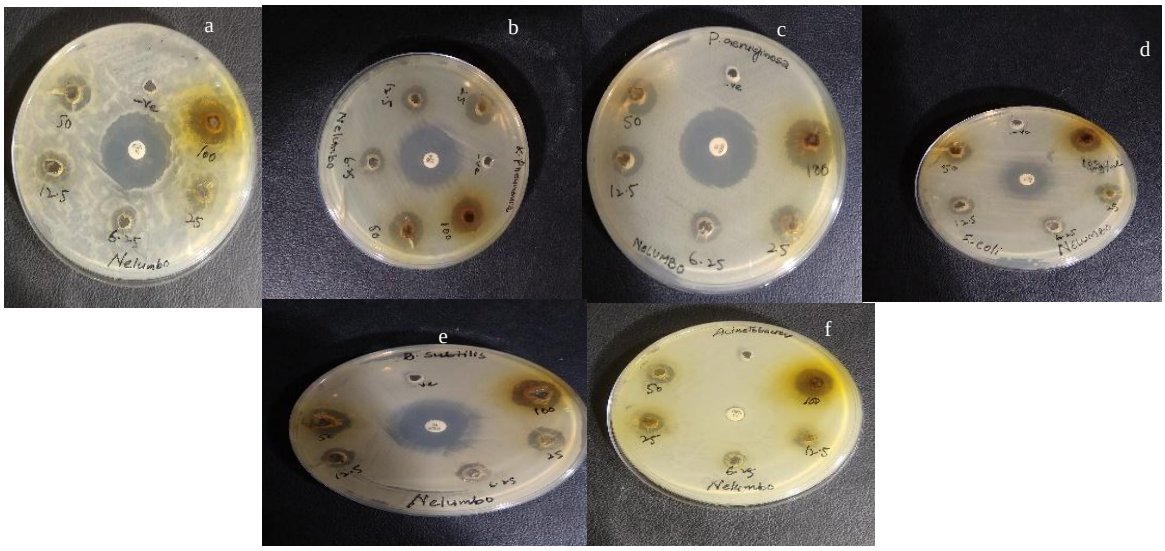

Fig. 3. Zones of inhibition by methanolic extract of *N.nucifera*

(a) against *S.aeurus* (b) against *K.pneumonia (c) against P.aeruginosa* (d) against *E.coli*

(e) against *B.subtilis* (f) against *Acinetobacter*

Table 2. Antibacterial activity of methanolic extract of *N. nucifera* against different bacterial **strains**

Bacteria	***Nelumbo nucifera* Concentration(mg/ml)**					**Positive control**	**Negative control (DMSO)**
	100	50	25	12.5	6.25		
	Inhibition zones(mm) at different concentrations						-
S.aeurus	15.8±0.76	13.3±1.1	3.5±0.9	2±1	1.5±0.8	26.5±1.2	-
K.pneumonia	16.6±0.57	14.3±0.8	13±1	11.6±0.6	11±0.4	27±2	-
P.aeruginosa	18.6±0.57	15.6±1.1	13±1.7	10±0.5	8.6±0.5	22.3±2.5	-
E.coli	15±1	13±1	11.6±0.6	10±1	8.6±1.1	18±1.7	-
B.subtilus	16.6±2	15.6±2.8	13.5±2.7	12.5±2.5	10±3.2	23.3±2	-
Acinetobacter	12.8±0.76	10.3±0.6	8±1	4.6±0.6	0±0	2±1	-

(Mean ± SD)

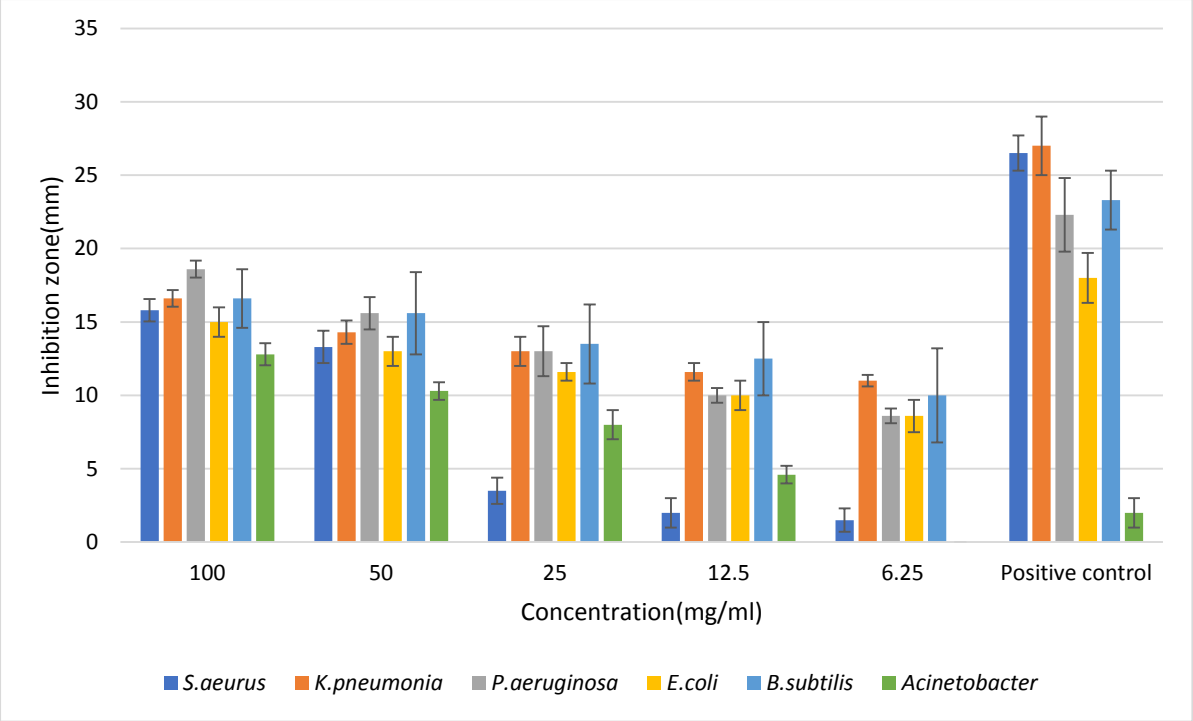

Fig. 4. Antibacterial activity of methanolic extract of *N. nucifera* against different bacterial strains

The antifungal activity of the methanolic extract of *N. nucifera* was observed against *Candida albicans* and *Candida glabrata* (Fig.5; Table 3). At higher concentrations (100mg/ml) the extract demonstrated significant antifungal activity surpassing that of the positive control (Itraconazole). For *Candida albicans*, inhibition zones ranged from 14.6 ± 0.5 mm at 100 mg/ml to 5.7 ± 0.6 mm at 6.25 mg/ml, compared to 8 ± 2 mm for Itraconazole. Similarly, *Candida glabrata* showed inhibition zones from 17.6 ± 0.5 mm at 100 mg/ml to 2 ± 1.4 mm at 6.25 mg/ml, compared to 6.5 ± 0.2 mm for Itraconazole, *N. nucifera* demonstrated potent antifungal activity against specific strains at different concentrations, notably *C. albicans* ($F(6,14) = 40.6$, $p < 0.0001$ and *C. glabrata* ($F(6,14) = 132.89$, $p < 0.0001$ (Fig. 5); yet failed to exhibit an overall significant antifungal effect when the effect was compared between the two strains ($F(6,12) = 0.19$, $p = 0.66$) (Fig.8). Overall, the antifungal activity of *N.nucifera* extract exhibited a dose-dependent response, with higher concentrations (Fig. 6), showing greater efficiency against both fungal species compared to the positive control.

The challenges in managing Candida infections are well-documented, with issues such as limited availability of effective medications, increasing drug resistance, high treatment costs, and potential adverse effects (Alyousef, 2021). This underscores

the urgent need for novel antifungal agents or adjunctive therapies that can reduce the required doses of existing antifungal drugs while minimizing toxicity (Costa *et al*., 2019).

Various extracts of *N. nucifera* have shown antifungal activity against different Candida species. For instance, hexane and acetone extracts demonstrated significant activity against *Candida tropicalis* and *C. krusei,* with the highest activity against *C. albicans* (Arjun *et al*., 2012). Additionally, the ethanolic extract of *N. nucifera* flowers was effective against *Candida albicans* and *Aspergillus niger,* whereas the seed extract was ineffective against both strains (Rajput *et al*., 2022).

Among Candida species, *C. albicans* is the most prevalent in causing human infections, often linked to biofilm formation (Simonetti *et al*., 2020). This study's findings that *N. nucifera* extract is effective against *C. albicans* align with previous research showing the efficacy of terpenoid phenols against both planktonic cells and biofilms of *Candida albicans*, which are typically resistant to many antifungal medications (Rao *et al*., 2020). Phenolic compounds in *N. nucifera* may contribute to its antifungal activity by inducing apoptotic processes in Candida (Teodoro *et al*., 2015). These compounds exhibit a strong binding affinity to molecular structures like proteins and glycoproteins, potentially disrupting cellular functions (Simonetti *et al*., 2020). Moreover, antifungal alkaloids can alter mycelium morphology, total lipid content, and cause cell content leakage in fungi such as *Botrytis cinerea* (Wang *et al*., 2023). Terpenes, a significant component of essential oils, are known to penetrate fungal cell walls and accumulate among fatty acid chains in the lipid bilayer, modifying cell membrane structures (Zida *et al*., 2017).

The antifungal properties of terpenes and terpenoids are attributed to their high lipophilicity and low molecular weight, enabling them to disrupt cell membranes, leading to cell death, and inhibit the sporulation and germination of spoilage fungi (Norma *et al*., 2023). These mechanisms likely contribute to the observed antifungal efficacy of *N. nucifera* extract against *C. albicans* and *C. glabrata.*

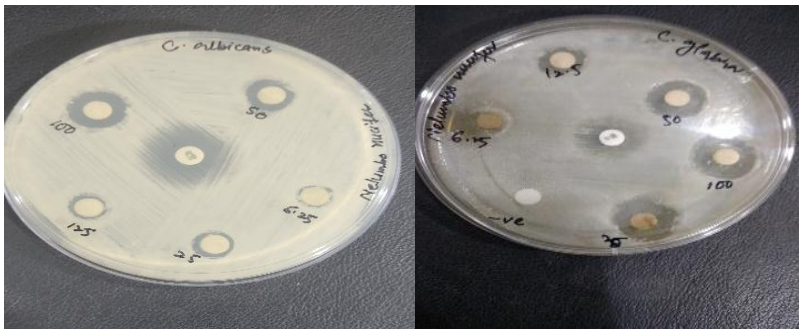

Fig. 5. Zones of inhibition by methanolic extract of *N.nucifera* against a. *Candida albicans* and b. *Candida glabrata*

Table 2. Antifungal activity of methanolic extract of *N.nucifera* (Mean± SD)

Fungus	***N.nucifera* (concentration mg/ml)**					**Positive con trol (itraconazole)**	**Negative control (DMSO)**
	100	50	25	12.5	6.25		
C.albicans	14.6±0.5	12.5±0.5	10.8±0.8	8.7±2.4	5.7±0.6	8±2	-
C.glabrata	17.6±0.5	15.6±1.5	13.3±1.1	11±1	2±1.4	6.5±0.2	-

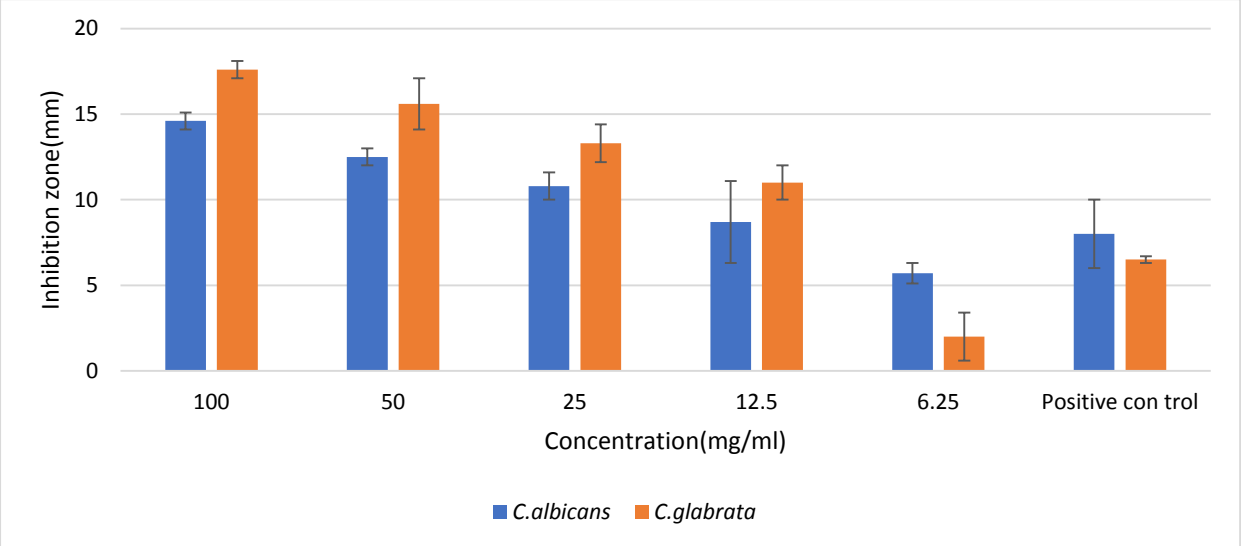

Fig. 6. Antifungal activity of methanolic extract of *N. nucifera*

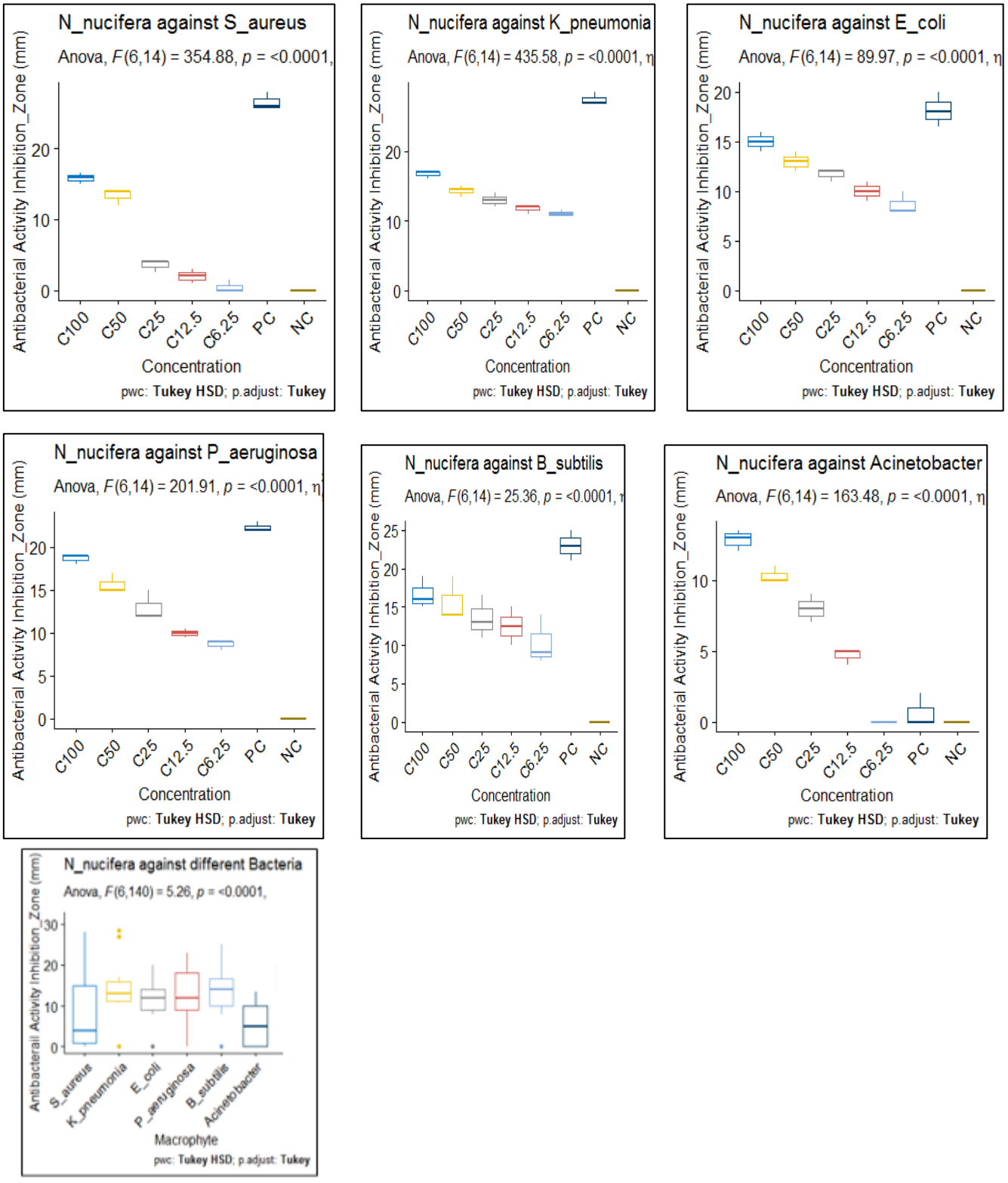

Fig. 7. ANOVA results for antibacterial activity of methanolic extract of *N.nucifera*

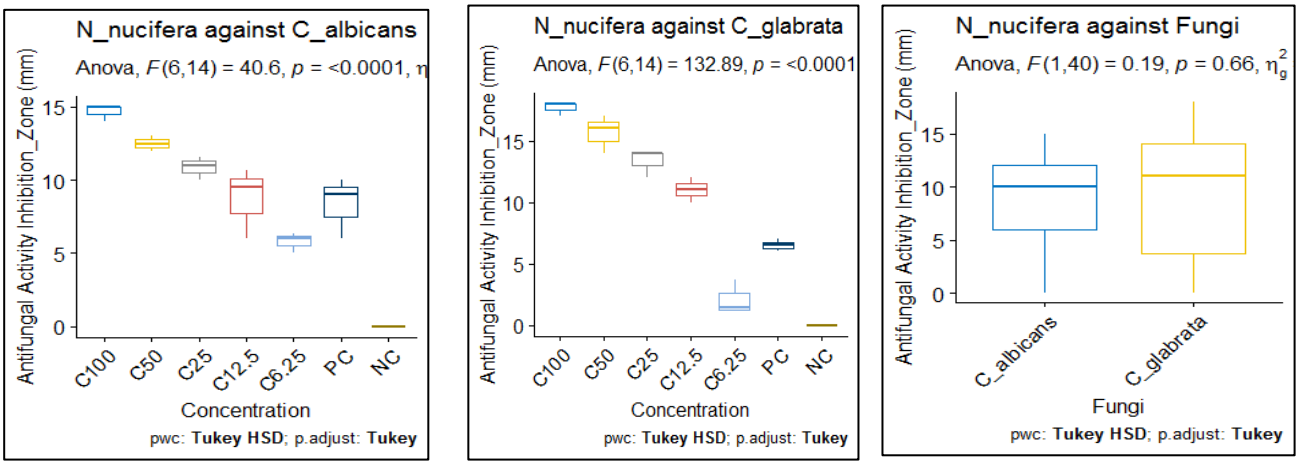

Fig. 8. ANOVA results for antifungal activity of methanolic extract of *N. nucifera*

CONCLUSION

In conclusion, the methanolic extract of *N.nucifera* exhibits significant antimicrobial activity against a variety of bacterial and fungal species. The extract also demonstrated higher efficacy against *Acinetobacter, C.albicans* and *C.glabrata*, suggesting its potential as an alternative therapeutic agent. The dose-dependent response observed in both antibacterial and antifungal activities further underscores the therapeutic potential of *N. nucifera*. However, the study has some limitations. It examined only a selected number of bacterial and fungal strains, so the findings may not be applicable to all strains or species, particularly those with different resistance patterns. Furthermore, the experiments were conducted under controlled laboratory conditions, which do not fully replicate the complexity of in vivo environments.Future research should focus on isolating and characterizing the active compounds responsible for these effects and evaluating their mechanisms of action. It is also important to conduct in vivo studies to validate the broader application and efficacy of *N. nucifera* extracts. These findings contribute to the growing body of evidence supporting the medicinal value of *N. nucifera* and its potential application in treating microbial infections.

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