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EXPLORING THE POTENT ANTIBACTERIAL ATTRIBUTES OF NYMPHAEA ALBA AND NYMPHAEA RUBRA: NOVEL STRATEGIES AGAINST MULTIDRUG-RESISTANT PATHOGENS

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ABSTRACT The increasing prevalence of drug-resistant pathogens, there is an urgent necessity to promptly identify and isolate novel bioactive compounds from medicinal plants. Compounds derived from medicinal plants hold the potential to offer innovative and direct strategies against pathogenic bacteria. This study is dedicated to exploring the antimicrobial attributes of plants widely used in traditional medicine. Our investigation centers on the antibacterial capabilities of *Nymphaea alba* and *Nymphaea rubra* against multidrug-resistant isolates of six genera. Both ethanol and water extracts exhibited a diverse range of phytochemicals, with the ethanol extract particularly showcasing a significant presence. Notably, the ethanol extract of *Nymphaea alba* displayed superior antibacterial activity. Amidst the six bacterial isolates, the gram-positive *S. aureus* strain was profoundly suppressed by both plant species. The experiment conclusively validated the effectiveness of specific plant extracts as natural antimicrobials and proposed their potential utilization in drug development for treating infectious diseases attributed to the tested microorganisms

KEYWORDS : MDR, *Nymphaea rubra,* Antimicrobial activity, Phytochemical analysis

INTRODUCTION

Bacterial infections and the growing threat of antibiotic resistance have spurred the development of comprehensive global strategies in healthcare. However, accurately understanding the frequency, complications, and mortality tied to these infections remains complex. Developing nations face a critical challenge due to expensive antibiotics being inaccessible to most patients, compounded by unregulated antibiotic use driving antimicrobial resistance. Rapidly spreading antimicrobial-resistant organisms further strain healthcare systems amid rising infectious disease rates (Muhie, 2019).

The World Health Organization identifies antimicrobial resistance (AMR) as a top-ten global health threat. Widespread drug-resistant strains are rendering antibiotics less effective, complicating the treatment of common bacterial infections like urinary tract infections, sepsis, sexually transmitted infections, and certain types of diarrhea. This poses a significant challenge for healthcare facilities running low on effective antibiotics (WHO, 2018).

Growing concern is evident in numerous clinical scenarios where limited treatment choices compromise the fight against common infections. Bacterial resistance to initial drugs varies widely, from none to nearly 100%, with instances where resistance to second and third-line drugs seriously hampers treatment efficacy. This complexity of drug resistance necessitates international efforts to address AMR urgently (Hailemariam *et al*., 2021).

The substantial rise in antibiotic resistance, driven by persistent resistant bacteria, poses a global health threat. Compounded by dwindling drug production since the late 1960s and lengthy drug approval processes, alternatives to antibiotics for microbial infections are sought. Natural botanicals, rich in compounds like alkaloids, terpenoids, and flavonoids, hold promise as alternatives or complements to antibiotics, potentially sidestepping resistance (AlSheikh *et al*., 2020).

The potential synergy between natural compounds and therapeutic drugs presents a crucial strategy against infections. We examined the antimicrobial properties of *Nymphaea alba* (*N. alba*) and *Nymphaea rubra*, members of the Nymphaeaceae family of aquatic plants with traditional medicinal use. Notably, *N. alba* species are rich in antioxidant polyphenols and flavonoids (Cudalbeanu *et al*., 2019).

Nymphaea rubra (*N. rubra*) is a rare perennial aquatic herb. Several components have shown efficacy in addressing bleeding disorders, and powdered rhizomes combined with honey have been used for various treatments. The plant exhibits antihelmintic, immunomodulatory, antiinsulin resistant, and antihyperglycemic properties. Moreover, it displays anti-inflammatory, antipyretic, hepatoprotective qualities, and free radical scavenging. Phytochemical analysis of *N. rubra* flowers reveals the presence of polyphenolic compounds (Marufa *et al*., 2023). Despite its potential, limited knowledge has kept *Nymphaea rubra* relatively unexplored. Our study assessed the antibacterial properties of these plants against multidrug-resistant bacteria.

METHODOLOGY

Plant material collection and extract preparation

In this study, we utilized flowers of *Nymphaea rubra* and *Nymphaea alba* collected from Dharmapuri District, Tamil Nadu, India. After cleaning and air-drying, the flowers were ground to create a powder. Extraction was carried out using a soxhlet extractor with 200 mL of ethanol and water as solvents. The extraction process continued until obtaining colorless extracts from the top of the extractor. Each extract was then concentrated separately under reduced pressure. After complete evaporation, the dry extracts were weighed and used for subsequent investigations. The extracts were stored at temperatures ranging from 2°C to 8°C for further studies (Mohana *et al*., 2008).

Phytochemical studies

Phytochemical analyses were conducted following the method outlined by Solomon *et al*., 2013 on the flower extracts of *Nymphaea rubra* and *Nymphaea alba* to determine the presence of different phytochemical constituents such as carbohydrates, alkaloids, phenols, quinols, saponins, and proteins.

Pathogens under examination

Clinical strains of *E.coli, Proteus mirabilis, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Enterococcus faecalis* were obtained from Coimbatore's Microtech Microbiology Laboratory for the study. The strains were cultured on Chromogenic agar media, and colony morphology was observed after 24 hours of incubation to validate the isolates.

Antibiotic susceptibility testing

Antibiotic susceptibility screening followed the National Committee for Clinical Laboratory Standards (NCCLS) guidelines. The Kirby-Bauer disc diffusion technique was used for antibiogram testing. Antibiotic discs and Mueller-Hinton Agar were procured from Hi-Media, Mumbai. Prepared plates were checked for sterility through overnight incubation at 37°C. Antibiotic discs were equilibrated at room temperature for an hour. Agar plates were overlaid with bacterial isolates matching a 0.5 McFarland standard turbidity. After 24 hours of incubation, zone of inhibition sizes were measured and compared to a standard chart.

Antimicrobial activity of flower extracts (Jahir *et al*., 2011)

Extracts from the soxhlet apparatus were dried and dissolved using the same solvent after vacuum treatment. The antibacterial activity of *Nymphaea rubra* and *Nymphaea alba* flower extracts was assessed using the agar well diffusion method against bacterial strains. Sterile Muller-Hinton agar plates were prepared and swabbed with 24-hour-old bacterial cultures. Using a sterile cork borer, 8 mm diameter wells

were created. Different concentrations of flower extracts were placed in labeled wells, alongside Ampicillin (5µg/ml) as a positive control and DMSO as a negative control. Plates were incubated upright at 37°C for 24 hours, and the resulting zones of inhibition were measured.

RESULTS AND DISCUSSION

Plant extracts and phytochemicals with established antimicrobial properties are crucial for therapeutic treatments. Recent global studies support their effectiveness. Many plants derive their antimicrobial abilities from secondary metabolism compounds. This research evaluates solvent extracts from different parts of *Nymphaea rubra* and *Nymphaea alba* for their antimicrobial potential.

In phytochemical tests, *Nymphaea alba* ethanol flower extract contained alkaloids, carbohydrates, phenols, tannins, terpenoids, and sterols, while flavonoids, saponins, quinines, and proteins were absent. The aqueous flower extract showed carbohydrates, phenols, tannins, and quinines. *Nymphaea rubra* ethanol flower extract exhibited the highest phytochemical diversity, detecting all compounds except proteins, while its aqueous flower extract was positive for alkaloids, flavonoids, phenols, tannins, saponins, and quinines, but lacked carbohydrates, terpenoids, sterols, and proteins. Protein was undetected in both plants. Similar studies on Nymphaea species, particularly from flowers, have revealed the presence of sterols, alkaloids, tannins, saponins, flavonoids, contributing to antimicrobial properties and traditional uses for conditions like diabetes, inflammation, urinary and liver disorders, indigestion, and menstruation (Marufa et al., 2023; Agnihotri et al., 2020; Raja et al., 2010; Ugbogu et al., 2021).

Preliminary investigations of Nymphaea species extracts revealed valuable secondary metabolites. Phytochemical analysis confirmed diverse medicinally important phyto constituents. Both ethanol and water extracts displayed multiple phytochemicals, with ethanol extract showing a notable presence.

The obtained bacterial isolates were validated using chromogenic media. By analyzing their colony morphology, the isolates were identified as *E. coli, S. aureus, K. pneumoniae, P. mirabilis, E. faecalis,* and *P. Aeruginosa.* Antibiotic resistance arises from overuse, creating a global concern. Bacteria's resistance development after exposure is risky. Pathogens' resistance to multiple antibiotics, termed MDR, imperils humans and animals. Notably, resistance worsens with prolonged treatment (Serwecińska *et al*., 2020).

This study assessed antimicrobial resistance patterns for distinct bacterial genera. Six genera were tested against twelve antibiotics. Penicillin showed universal resistance, followed by Ampicillin and Cefotaxime at 83.3%. Cefixime, Ceftriaxone, and Kanamycin had 67% resistance, while Chloramphenicol and Amikacin were lowest at 17%. Notably, all isolates were sensitive to Ciprofloxacin and Tetracycline. *P. mirabilis* had the highest resistance (67%), followed by *K. pneumoniae* (50%) (Figure 1).

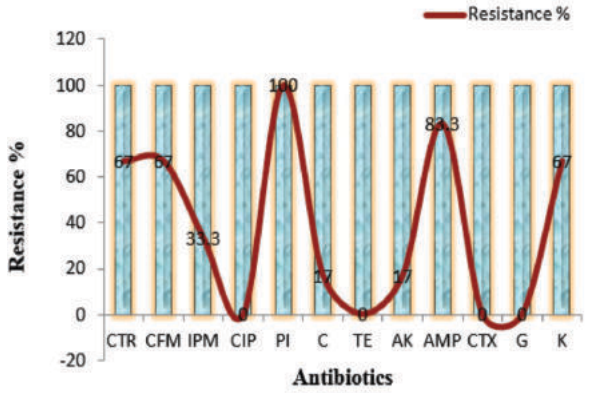

Figure 1: Antibiotic resistance % analysis

In recent years, increased multiple resistances among human pathogenic microorganisms result from the widespread use of commercial antimicrobial drugs (Nagasinduja *et al.,* 2022; Aslam *et al.,* 2018). This, combined with antibiotic side effects and emerging uncommon infections, poses a serious medical challenge. Using novel compounds unrelated to existing synthetics is a way to counter pathogenic resistance; certain medicinal plants outperform synthetic antibiotics (Vaou *et al.,* 2021). Herbal assessment moves closer to achieving natural antibiotics for infection control and treatment without promoting resistance.

Herbal sources for antimicrobials draw substantial interest, aiming to replace synthetic agents. Plants have historical health value, with growing attention to natural therapy. Global use of plant compounds in pharmaceuticals is increasing. The World Health Organization regards medicinal plants as vital for diverse drugs, used by 80% of people in developed countries. Identifying these plants is crucial for understanding their properties, safety, and efficacy (Samiha, 2022).

Antibacterial activity implies a diverse range of antibiotic compounds. Salisu and Nura (2022) emphasized the significance of bioactive constituents such as tannins, alkaloids, flavonoids, and phenolic compounds for a plant's medicinal benefits, all present in the examined aquatic plant.

The ethanolic flower extracts of *Nymphaea alba* exhibited highest activity against *S.aureus* and *P.mirabilis* while lowest activity was recorded *K.pneumoniae*. While using the aqueous extract of *Nymphaea alba* highly controlled bacteria was *S.aureus* which was suppressed even at 1mg concentration of the extract and least effective on *K.pneumoniae* which was suppressed only at 4mg concentration of the extract (Table 1).

Similar to Siva and Savithramma (2016), their study observed antimicrobial activity against diverse bacterial species. In 2017, Ghazi *et al*. noted the highest inhibitory activity using the ethanolic extract of *Nymphaea alba*.

The ethanolic flower extracts of *Nymphaea rubra* exhibited better inhibitory activity against all isolates. Highly suppressed isolate was *E.coli* and the least suppressed isolate was *P.mirabilis*. The literature review revealed that there have been no studies investigating the antimicrobial activity of *Nymphaea rubra* against bacteria (Table 1& 2).

Table 1: Efficacy of ethanol & aqueous extracts of *Nymphaea alba* flower

Isolates	Ethanol extract				Aqueous extract			
	1	2	3	4	1	2	3	4
E.coli	-	11±1.632	13±1.632	17±1.632	-	11±1.632	13±1.632	17±1.632
P.aeruginosa	12±2.054	15±1.632	17.3±2.05	18.6±1.24	12±2.054	15±1.632	17.3±2.05	18.6±1.24
S.aureus	12±1.632	15±1.632	17±1.632	18±1.632	12±1.632	15±1.632	17±1.632	18±1.632
E.faecalis	-	12.33±2.05	15±1.632	15.6±1.247	-	12.33±2.05	15±1.632	15.6±1.247
K.pneumoniae	-	-	-	14±1.632	-	-	-	14±1.632
P.mirabilis	-	13±0.816	15.6±1.247	18±1.632	-	13±0.816	15.6±1.247	18±1.632

Table 2: Efficacy of ethanol & aqueous extracts of *Nymphaea rubra* flower

Isolates	Ethanol extract				Aqueous extract			
	1	2	3	4	1	2	3	4
E.coli	12±0.816	15±1.63	19±0.81	22±1.63	-	-	10.3±1.24	13±1.63
P.aeruginosa	12±1.63	15±0.81	16.6±1.24	20±1.63	-	-	11.3±1.24	13.3±1.24
S.aureus	13±0.81	16±0.81	19±1.26	21±0.81	10±0.81	12±0.81	13.6±1.24	15±0.81
E.faecalis	12±0.81	13.5±1.43	16±1.63	18.3±1.24	-	11±0.81	13.3±1.24	16±1.63
K.pneumoniae	11.3±1.24	13.5±1.43	16.3±1.24	18.6±1.24	-	-	-	12.3±1.24
P.mirabilis	-	12±1.63	15±0.81	16.6±1.24	-	11.6±1.24	13±0.81	14±1.63

In this study, antibacterial activity was evaluated against both gram-negative and gram-positive isolates. Notably, most plant extracts effectively suppressed gram-positive *S. aureus* isolates. The varying sensitivity between these bacterial types could be attributed to

morphological differences; Gram-negative bacteria possess an outer phospholipidic membrane containing lipopolysaccharide components, rendering their cell wall less permeable to lipophilic solutes. Additionally, porins serve as selective barriers to hydrophilic solutes, with an exclusion limit around 600 Da. In contrast, the presence of only an outer peptidoglycan layer in Gram-positive bacteria makes them more susceptible due to its limited permeability barrier (Gonelimali, 2018).

Statistical analysis

By conducting an ANOVA to calculate the p-values for all the extracts (table 3), we can infer that the likelihood of randomness in the efficacy of *Nymphaea rubra* flower ethanol extract is lower compared to the other extracts. This analysis suggests that *Nymphaea rubra* flower ethanol extract exhibits more pronounced antimicrobial activity than the rest of the extracts, indicating its effectiveness in controlling the selected isolates.

Table 3: Anova p value for the *Nymphaea flower* extracts

Extract	Anova p value
Ethanol extract of Nymphaea alba	0.036844
Aqueous extract of Nymphaea alba	0.01618
Ethanol extract of Nymphaea rubra	0.001328
Aqueous extract of Nymphaea rubra	0.008506

The outcomes presented above strongly indicate that the ethanol extract derived from *Nymphaea rubra* flowers holds significant promise as an antibacterial agent against multidrug-resistant bacterial strains this may be due to the presence of highest phytochemicals in it than the other extracts. Alkaloids target drug-resistance EPs. Quinolones inhibit topoisomerase, blocking DNA replication (Tushar et al., 2021). Flavonols and phenols inhibit bacterial virulence, interact with membranes, curb biofilms, boost antibiotics, and reduce polysaccharide activity (Khameneh et al., 2021). Tannins penetrate, disrupt metabolism, and destroy bacterial cells effectively (Kaczmarek et al., 2020). Terpenoids permeate bacterial membranes, exhibiting antibacterial or bactericidal properties (Huang et al., 2022). Hence the presence of such phytochemicals in *Nymphaea rubra* flower ethanol extract makes it more potent antibacterial agent against MDR bacteria and hence these findings propose its potential as a viable alternative to the presently employed antibiotics.

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