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(RESEARCH ARTICLE)

In-vitro evaluation of antimicrobial properties of *Nelumbo nucifera*

Madhu Rajput *, Arvind Singh Jadon and Poonam Bhadauriya

Gurukul Institute of Pharmaceutical Science and Research, Gwalior, Madhya Pradesh, India- 464001

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Abstract

Antimicrobial property of *Nelumbo nucifera*was evaluated. Flower and seed pod of *Nelumbo nucifera*was collected, and identified. Morphologically, flowers were pink colored with smooth texture and seeds were dark brown. Powdered plant material was extracted by maceration method in ethanol. Ethanolic Extract of Flowers and seeds were dark brown, semisolid and percentage yields were 15.52 and 36.75% respectively. Various phytochemical tests were revealed that the carbohydrates and glycosides, proteins and free amino acids, fixed oils and fats, steroids and triterpenoids were present in both extracts. Antioxidant activity was determined by DPPH assay, the IC50 were calculated. Antimicrobial testing were performed against Gram positive (Staphylococcus Aureus) and Gram negative bacteria (*Pseudomonas Aeruginosa*) and strain of fungus (*C. Albicans* and *A. Niger*). Antimicrobial activities were estimated by disk diffusion method. It was observed that extract of flowers was more effective but extract of Seeds was less effective against *Staphylococcus Aureus* and *Pseudomonas Aeruginosa*, Cefotaxime was used as standard. Extract of flowers was equally effective against *candida albicans* and *Aspergilusniger* but extract of seeds was not effective against both fungal strains.

Keywords: Antimicrobial*; Nelumbo nucifera;* Ethanolic Extract*;* Flowers*;* Seeds

1. Introduction

It is estimated that there are 250,000 to 500,000 species ofplants on Earth [1]. Relatively small percentages (1 to 10%) of these are used as foods by both humans and other animalspecies. It is possible that even more are used for medicinalpurposes [2]. North America's history of plant medicinal use follows twostrands—their use by indigenous cultures (Native Americans),dating from prehistory [3] and an "alternative" movementamong Americans of European origin, beginning in the 19thcentury.Plants have an almost limitless ability to synthesize aromaticsubstances, most of which are phenols or their oxygen-substituted derivatives[4]. Most are secondary metabolites, ofwhich at least 12,000 have been isolated, a number estimatedto be less than 10% of the total [5]. In many cases, thesesubstances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores [6]. Some, such asterpenoids, give plants their odors; others (quinones and tannins) are responsible for plant pigment [7]. After the revolution in the"golden era", when almost all groupsof important antibiotics (tetracyclines, cephalosporins, aminoglycosides and macrolides) were discovered and the main problemsof chemotherapy were solved in the 1960s, the history repeatsitself nowadays and these exciting compounds are in danger oflosing their efficacy because of the increase in microbial resistance [8]. Currently, its impact is considerable with treatment failuresassociated with multidrug-resistant bacteria and it has become aglobal concern to public health.For this reason, discovery of new antibiotics is an exclusivelyimportant objective [9]. Natural products are still one of the majorsources of new drug molecules today[10]. They are derived fromprokaryotic bacteria, eukaryotic microorganisms, plants andvarious animal organisms [11]. Microbial and plant products occupythe major part of the antimicrobial compounds discovered until now [12].

*Corresponding author: Madhu Rajput
Gurukul Institute of Pharmaceutical Science and Research, Gwalior, Madhya Pradesh, India- 464001.

Owing to the new attraction to the properties of new antimicrobial products like combating multidrug-resistant bacteria, itis important to develop a better understanding of the currentmethods available for screening and/or quantifying the antimicrobial effect of an extract or a pure compound for its applications in human health, agriculture and environment[13].

2. Material and methods

2.1. Collection and identification of plant material

Flower and seedpod of *Nelumbo nucifera*was collected from a local market Gwalior, India. The plantmaterialwas identified and authenticated by Department of Botany, Saifia Science College, Bhopal. The plant materials flowers and seed pod were cleaned, dried in shade and grinded to fine powder with the help of a mixer grinder.

2.2. Extraction of plant material

The commonly employed technique for separation of active substance from crude drug is called as 'Extraction' which involves the use of different solvents. The plant material used for extraction should be properly authenticated or identified. The choice of the plant material for extraction depends upon its nature and the components required being isolated. The dried powdered plant material is commonly used for extraction. The solvent used for extraction is called menstrum and the residue is known as marc.About 100 grams of coarsely powdered plant material was extracted by maceration method using 90%ethanol for 48 hour. All the extracts thus obtained were stored in air-tight bottles at 4ºC for further experiments.

2.3. Phytochemical Screening

The various phytochemical tests were performed using standard methods.

2.3.1. Anti-oxidant activity

The antioxidant activity of the extracts was determined using the DPPH free radical scavenging assay. Briefly, the universal bottle was contained 50 µL of *Nelumbo nucifera*extracts in concentrations from 1 to 5 mg/ml and 5 ml 0.004% (w/v) solution of DPPH was added. The obtained mixture was vortexed, incubated for 30 min in room temperature in a relatively dark place and then was read using spectrophotometer at 517 nm. The blank was 80% (v/v) methanol. Ascorbic acid (Vitamin C) was used for comparison. Measurements were taken in triplicate. DPPH scavenging effect was calculated using the following equation:

$$DPPHScavenging(\%) = \frac{A0 - A}{A0} X\ 100$$

Where, A0 is the absorbance of negative control (0.004% DPPH solution) and A is the absorbance in presence of extract. The results were reported as IC_{50} values and ascorbic acid equivalents (AAE, mg/g) of *Nelumbo nucifera*extracts.

2.3.2. Antimicrobial Susceptibility Testing

Disk Diffusion Assay

The antibacterial susceptibility was initially assayed by the agar disk diffusion method. Three concentrations (crude extract, 10-fold dilution, and 100-fold dilution) of each plant extract were prepared in 10% DMSO. Bacteria cell suspensions were adjusted to 0.5 McFarland turbidity standards to prepare 1×108 bacterial/ml inoculum. Each bacterial suspension was inoculated on Mueller-Hinton agar plates and the plates were then allowed to dry for 5 minutes. The sterile filter paper disks (Whatman No. 1, diameter = 6 mm) were soaked in 10µl of each plant extract. The extract-soaked filter paper disks were then placed on the inoculated Mueller-Hinton agar plates. Cefotaxime (30µg) disk was used as the positive control, and 10% DMSO-soaked filter paper disk was used as the negative control. Plates were incubated for 18 hr at 35 ± 2°C. After incubation, the zones of inhibition were recorded as the diameter of the growth-free zones measured in mm using a Vernier caliper.

Minimum Inhibitory Concentration (MIC)

Plant extracts that gave a positive result for the disk diffusion assay were used to determine MIC using the microplate dilution method. Serial 5-fold dilutions of the plant extracts were prepared in the 10% DMSO, yielding seven serial dilutions of the original extract. Inoculum of organism was prepared in Mueller-Hinton broth, and the turbidity was

adjusted to approximately 0.5 McFarland turbidity standard to prepare 1 × 108 bacterial/ml. 150 µl of plant extract was added to each well of the 96-well microplate. 50 µl of bacterial suspension was added to each well except the negative controls. Cefotaxime IV drug was used as the positive control. 10% DMSO and plant extracts without bacterial suspension were used as the negative controls. Microtiter plates were incubated at 35 ± 2°C for 24 hr. Antimicrobial activity was assessed by measuring absorbance at 630 nm of wave length.

2.3.3. Antifungal Susceptibility Testing

Culture Media

To test the biological activity of the extract, Sabouraud dextrose broth (SDB) and Sabouraud dextrose agar (SDA) were purchased from Hi-Media Laboratories and agar-cornmeal from HiMédia Laboratories (Mumbai, India), and RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, São Paulo, SP, Brazil) culture media were used. They were prepared and used according to the manufacturers' instructions.

Inoculum Preparation

The suspensions were prepared from recent C. albicans cultures, plated on SDA, and incubated at 35°C for 24–48 h. After incubation, we transferred roughly 4-5 yeast colonies (with a sterile loop) to test tubes containing 5 ml of saline solution 0.9%. The resulting suspensions were stirred for 15 seconds with the aid of a Vortex apparatus. The turbidity of the final inoculum was standardized using a barium sulfate suspension (tube 0.5 on the McFarland scale). The final concentration obtained was about 1–5 × 106 colony forming units per milliliter (CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The determination of the products' MIC on the ten strains used in the biological assays was determined by the broth microdilution method. One hundred milliliters (100 µL) of liquid medium RPMI-1640 was transferred into the wells of a 96-well microdilution plate with a "U" shaped bottom (Alamar, Diadema, SP, Brazil). Then, 100 µL of citral emulsion was inoculated in the first horizontal row of the plate wells. Doubled serial dilutions, where a 100 µL aliquot removed from the most concentrated well went to the next well, and yielded concentrations of 1024-1 µg/mL. Finally, 10 µL of *C. albicans* inoculums suspension was added to each well of the plate, where each column represented a yeast strain. In parallel, controls were made for yeast viability and for susceptibility with the standard antifungal amphotericin B. The plates were incubated at 35°C for 24–48 h. After the appropriate incubation time, the presence (or absence) of growth was observed visually. The formation of cell clusters or "buttons" in the plate wells was considered. The MIC was defined as the lowest citral concentration that produced visible inhibition of yeast growth.To determine the MFC, we subcultured 1 µL aliquots of MIC, MIC × 2, and MIC × 4 of the citral products, amphotericin B, and the control yeast growth onto Petri dishes containing SDA. After 24–48 hours of incubation at 35°C, a reading was made to evaluate the MFC, as based on the growth of the controls. The MFC was defined as the lowest product concentration that inhibited growth of the yeast or permitted less than three CFUs to occur, resulting thus in 99.9% fungicidal activity.

3. Result and discussion

3.1. Preliminary study

Flower and seedpod of *Nelumbo nucifera*was collected from a local market of Gwalior, India. The plantmaterialwas identified and authenticated.Theplantmaterials flowers and seed pod were cleaned, dried in shade and grinded to fine powder with the help of a mixer grinder. Morphologically, flowers were pink colored, characteristic odor, cone shape of 10-12 cm long, 15-20 cm in diameter in size with smooth texture and seeds were dark brown in color, odorless, oval shaped, 1-2 cm in size with smooth texture. Coarsely powdered plant material was extracted by maceration method using 90%ethanol. All the extracts thus obtained were stored in air-tight bottles at 4ºC for further experiments. Ethanolic Extract of Flowers and seeds were dark brown, semisolid and percentage yields were 15.52 and 36.75% respectively. Various phytochemical tests were revealed that the carbohydrates and glycosides, proteins and free amino acids, fixed oils and fats, steroids and triterpenoids were present in both extracts, tannins, flavonoids, mucilage and gums were absent and Alkaloids present in seeds extract and absent in flowers.

3.2. Antioxidant activity

Antioxidant activity was determined by DPPH assay, the IC_{50} of Ethanolic Extract of Flower, Ethanolic Extract of Seed were found 400 and 300µg/ml respectively while IC_{50} of Ascorbic acid was 100 µg/ml.

Table 1 Antioxidant activity by DPPH assay

S. No.	Sample Name	IC 50 (µg/ml)
01	Standard (Ascorbic acid)	100
02	Ethanolic Extract of Flower	400
03	Ethanolic Extract of Seed	300

3.3. Antimicrobial activity

Table 2 Antibacterial activity of*Nelumbo nucifera*extracts

Name of Sample	Diameter of zone of inhibition (mm)	
	Staphylococcus Aureus	Pseudomonas Aeruginosa
Extract of Flowers 300 (µg/ml) (C1)	20 (6.25)	14(6.25)
Extract of Seeds 300 (µg/ml) (C2)	12(6.25)	10(6.25)
Control (N)	-	-
Cefotaxime (30µg) (P)	36	35

Values in brackets are MIC values (µg ml^{-1})

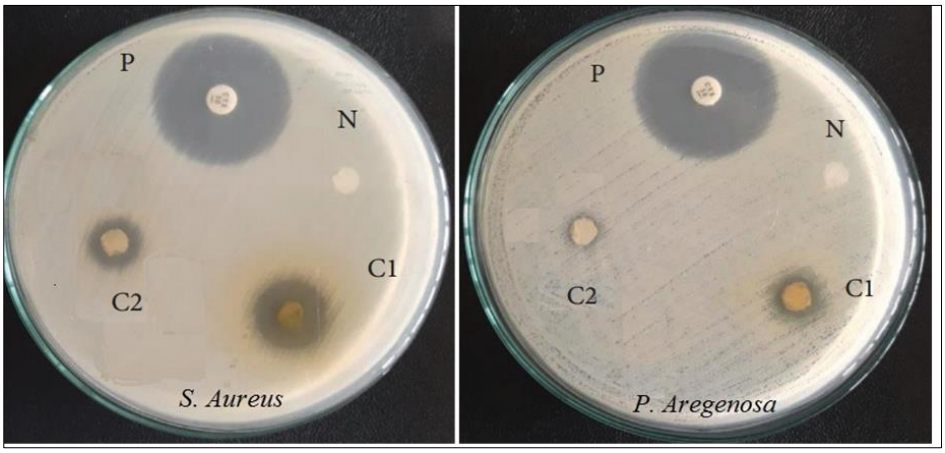

Figure 1 Antibacterial activity of *Nelumbo nucifera*

Table 3 Antifungal activity of *Nelumbo nucifera*extracts

Name of sample	Diameter of zone of inhibition (mm)	
	Candida albicans	***Aspergilus Niger***
Extract of Flower 300 (µg/ml) (C1)	16(6.25)	15(6.25)
Extract of Seed300 (µg/ml) (C2)	0.0(6.25)	0(6.25)
Control (N)	-	-
Cefotaxime (30µg) (P)	32	34

Values in brackets are MIC values (µg ml^{-1})

Antimicrobial testing were performed against Gram positive (*Staphylococcus Aureus*) and Gram negative bacteria (*Pseudomonas Aeruginosa*) and strain of fungus (*C. Albicans* and *A. Niger*). Antimicrobial activities were estimated by disk diffusion method. Firstly, minimum inhibitory concentration (MIC) values obtained. Antimicrobial activities of ethanolicextract of flowers and seeds (300µg/ml)were evaluated in the term of zone of inhibition. It was observed that

extract of flowers was more effective but extract of Seeds was less effectiveagainst *Staphylococcus Aureus* and *Pseudomonas Aeruginosa,* Cefotaxime was used as standard. Extract of flowers was equally effective against *candida albicans* and *aspergilus niger* but extract of seeds was not effective against both fungal strains.

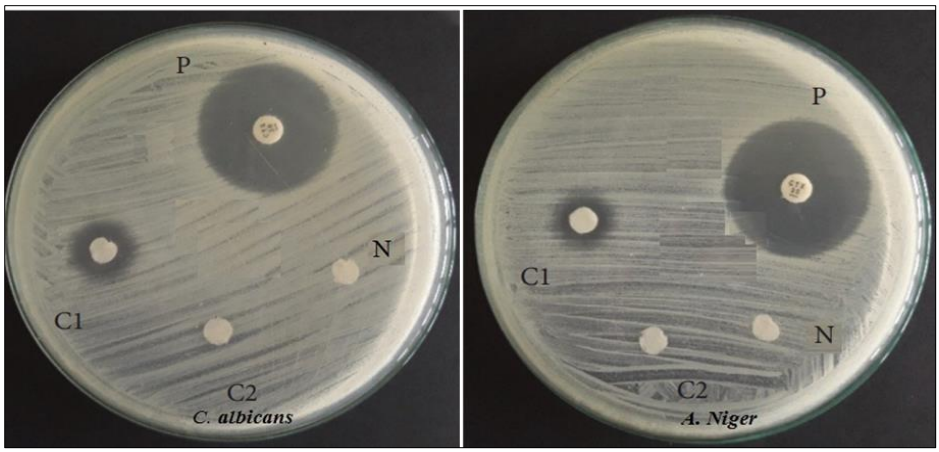

Figure 2 Antifungal activity of *Nelumbo nucifera* extract

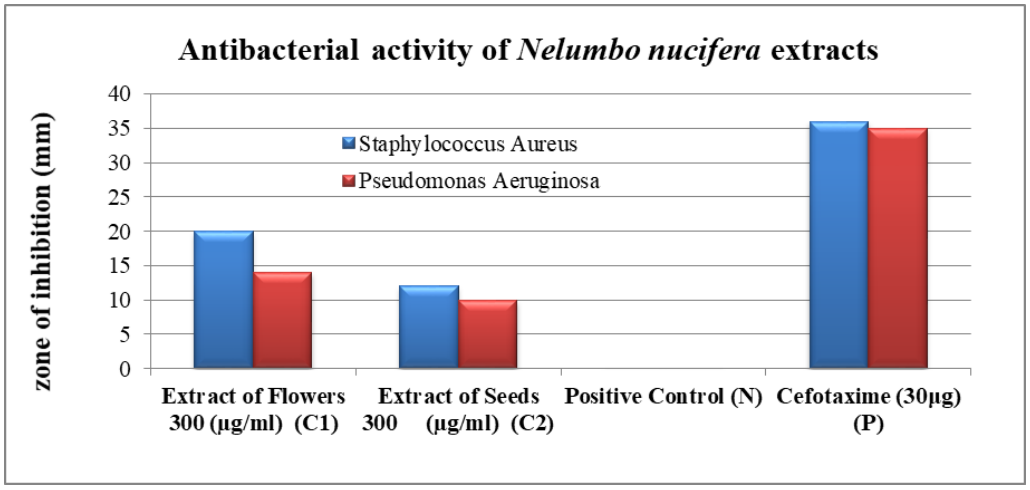

Figure 3 Comparison of Antibacterial activity

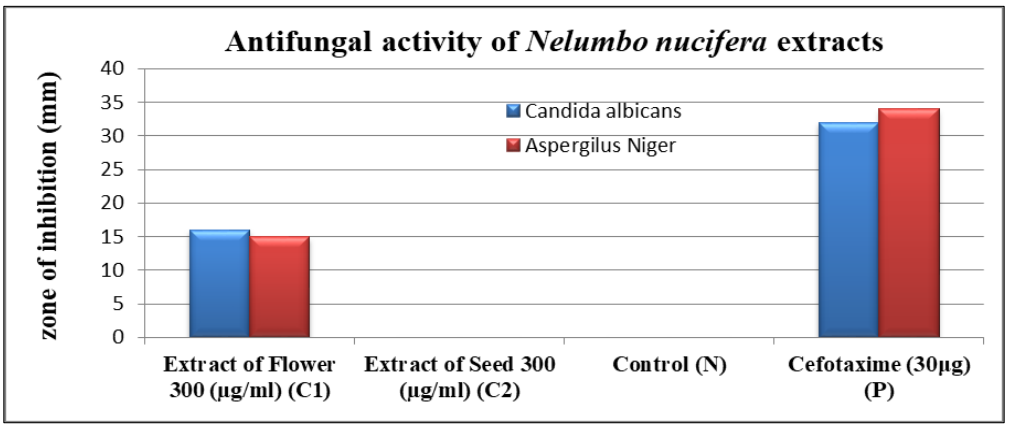

Figure 4 Comparison of Antifungal activity

4. Conclusion

In the present study, an attempt was to collect plant material of *N. nucifera* and prepare extract, characterize and evaluate for its anti-microbial activity. Two extracts of *N. nucifera* were prepared and evaluated.

The extraction of *N. nucifera*Flower and seeds were carried out in ethanol by using maceration method. The extracts were screened for the presence of various medicinally active constituents. Physicochemical studies revealed active constituent present in ethanolic extract were carbohydrates and glycosides, proteins and free amino acids, fixed oils and fats, steroids and triterpenoids were present in both extracts.

The extracts of *N. nucifera*flower and seeds had potential in antioxidant activities in the *in-vitro* system.

It was found that, the extracts of *N. nucifera*flower and seeds possessed good activity against different bacterial and fungal strains in culture.

In conclusion, *N. nucifera* extracts of flowers and seeds contained carbohydrates and glycosides, proteins and free amino acids, fixed oils and fats, steroids and triterpenoids were present in both extracts had potential in antioxidant activities in the *in-vitro* system. Extract of flowers was more effective but extract of seeds was less effectiveagainst *Staphylococcus Aureus* and *Pseudomonas Aeruginosa,* Cefotaxime was used as standard. Extract of flowers was equally effective against *candida albicans* and *aspergilus niger* but extract of seeds was not effective against both fungal strains. Overall, anti-bacterial activity was possessed by extract of flowers and seeds but anti-fungal activity was possessed by extract of flowers only.

Compliance with ethical standards

Acknowledgments

We would like to express our sincere gratitude to Department of Botany, Saifia Science College, Bhopal for providing authenticating our plant and plant parts.

Disclosure of conflict of interest

There are no conflicts of interests.

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