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Evaluation of *In vitro* Antioxidant Activity of *Nelumbo nucifera* Leaf Extract and its Potential Application as Antibacterial Agent against Fish Pathogens

Mudasir Maqsood Hakim[1*], Nazir Ahmad Ganai[2], Syed Mudasir Ahmad[1], Oyas Ahmad Asimi[3], Tariq Raja[2], Feroz Ahmad Shah[4], Jalal-ul-Din Parrah[5] and Riaz Ahmad Shah[1]

[1]*Division of Animal Biotechnology, Faculty of Veterinary Sciences & Animal Husbandry, SKUAST-Kashmir, India*
[2]*Division of Animal Genetics and Breeding, Faculty of Veterinary Sciences & Animal Husbandry, SKUAST-Kashmir, India*
[3]*Division of Fish Nutrition and Biochemistry, Faculty of Fisheries, SKUAST-Kashmir, India*
[4]*Division of Aquatic Animal Health & Management, Faculty of Fisheries, SKUAST-Kashmir, India*
[5]*Mountain Livestock Research Institute, SKAUST-Kashmir, India*

**Corresponding author*

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A B S T R A C T

The aim of this study was to determine the antioxidant and antibacterial property of *Nelumbo nucifers* (lotus) leaf extract. Total phenolic content and DPPH (2,2-diphenyl-1-picrylhydrozyl) scavenging methods were used to evaluate the antioxidant property of crude extract. DPPH scavenging capacity of extract varied significantly ($p<0.05$) depending on the concentration, except 5.5mg/ml and 7 mg/ml concentrations. The maximum concentration (10mg/ml) of the extract showed the highest scavenging effect (57.75%), whereas the lowest concentration (0.5mg/ml) of the extract showed the least scavenging capacity (9.30 %). The phenolic contents exhibited a similar trend to that of DPPH. Total phenolic compounds increased with the increasing concentration of *Nelumbo nucifera* leaf extract. Disc diffusion and broth micro-dilution methods showed bactericidal property of lotus leaf extract. Water, acetone-water and ethanol-water based extracts were tested against selected gram-positive (*Staphylococcus aureus*) and gram-negative (*Aeromonas hydrophila*, *Pseudomonas fluorescens*) fish bacterial pathogens. The broth micro-dilution method with TTC (2,3,5-triphenyl tetrazolium chloride) to indicate the viability of aerobic bacteria was found to be the best alternative method.

Introduction

Resistance of microorganisms to existing antibiotics is evolving and there is an escalating requirement for new antibiotics not only in human but also in veterinary medicine. Antimicrobial defence strategies have evolved in aquatic ecosystem in

response to competition for space and nutrients. Therefore, aquatic plants, offer a rich source of prospective new drugs. *Nelumbo nucifera* (Family: Nelumbonaceae) commonly known as lotus or sacred lotus is an aquatic perennial plant. The plant grows up to a height of about 1.5 meters and a spreads horizontally up to 3 meters. Lotus plant remains embedded in mud of the water body. Leaves measuring approximately 60 cm in diameter, arise directly from the rhizome and can either be floating on the water or raised 30 to 46 cm (1 to 1.5 ft) above the water. The floral part arising from stem above leaves, grows up to 20 cm in diameter. Seeds and rhizome are used for propagating the plant (Sayre, 2004). Plant has been used in conventional therapies for a long time and finds it relevance in both human and veterinary medicine. There are ample reports of the plant being used in different medical conditions (anti-diabetic, anti-cancer, anti-depressant, anti-inflammatory, anti-bacterial, anti-oxidant, immunomodulatory, and anti-viral etc) (Sheikh, 2014). There are studies of lotus extracts being used to treat cancer, tissue inflammation, antiemetic, obesity and skin diseases (Ling *et al.,* 2005; Liu *et al.,* 2015; Mehta *et al.,* 2013; Ono *et al.,* 2006). However, the use of *Nelumbo nucifera* in veterinary medicine is new and no such studies are available for aquaculture species.

Aquaculture production has witnessed a remarkable increase since last decade. Increasing demand for animal protein has made fish culture vulnerable on many levels. Increasing mortality due to disease incidence is the prime cause of low productivity, which ultimately affects the income (Figueiredo *et al.,* 2006, Hatha *et al.,* 2005). Fish are susceptible to a number of bacterial infections, primarily when stocked in high densities. In order to prevent the disease outbreak, antibiotics are used as one of the prophylactic measures. However, indiscriminate use of such disease management practices exposes the fish to a range of potential problems. Evolving resistance is one of the major concerns of using antibiotics in aquaculture. The practice not only puts fish species at risk but also becomes a potential source of resistance development in other animal and human pathogens (Serrano, 2005). Some bacterial fish pathogens are also associated to human diseases, making the aquaculture products a likely risk to the consumer's health (Yanong and Francis-Floyd, 2006).

Aeromonas hydrophila is responsible for cases of skin infections, septicemia and gastroenteritis in fish and human (Yu *et al.,* 2007). This bacterium causes haemorrhagic septicaemia, infectious abdominal dropsy in a verity of fish species and has been observed occasionally in marine fish species, amphibians, reptiles, cattle and humans throughout the world (Bullock *et al.,* 1971; Egusa, 1978; Schäperclaus *et al.,* 1992); Khardori and Fainstein, 1988). The bacterium is distributed widely in fresh water and bottom sediments containing organic material, as well as in the intestinal tract of fish (Egusa, 1978; Hazen *et al.,* 1978). *Aeromonas hydrophila* is typically recognised as an opportunistic pathogen or secondary invader (Austin and Austin, 1987). Conversely, there have been reports of *A. hydrophila* acting as a primary pathogen in fish. Isolates differ greatly in their pathogenicity with some strains being highly virulent and others non-virulent. Most cultured and wild freshwater fish species are susceptible to *Aeromonas hydrophila* infection. However, cold-water fish, including brown trout (*Salmo trutta*), and rainbow trout (*Oncorhynchus mykiss*) are more prone to diseases due to this bacterial pathogen (Bullock *et al.,* 1971; Egusa, 1978).

Pseudomonas fluorescens is a common gram-negative, rod-shaped bacterium, recognised as one of the bacterial species that are frequently associated with fish diseases (Bullock, 1964).

Pseudomonas infection in fish leads to the development of haemorrhagic septicaemia, so-called red skin disease, a condition called pseudomonasis, which occur throughout the year particularly when fish is in stress either because of inappropriate handling or during transportation. The prevailing lacunae in terms of disease management often lead to higher mortality, resulting in economic losses. *Staphylococcus aureus* is a gram-positive round shaped bacteria affecting various aquaculture fish species globally. The affected fishes exhibit distended abdomen, erratic swimming, melanosis, exophthalmia, haemorrhages, peri-anal edema, similar to the symptom by *Edwardsiella tarda* infection (Lin *et al.*, 2007; Pressley *et al.*, 2005).

Although, there are policies devised by Food and Agricultural Organisation (FAO) and other regulatory authorities to check the indiscriminate use of antibiotics in aquaculture.

In order to address the problems of microbial resistance in a more responsible way, there is an urgent need to find alternatives; the discovery of new phyto-chemicals and unconventional therapies to control bacterial diseases is one of the promising areas to explore**.**

Owing the ability to synthesise many different compounds, the plants are one of the potential sources of new drugs (Antunes *et al.*, 2006, Cowan, 1999). The aim of this study was to find out the in vitro antioxidant activity and antibacterial activity of leaf extract of *Nelumbo nucifera* (NNLE) against important fish pathogens, which affect the commercial aquaculture throughout the world. NNLE showed species specific activity in inhibiting the growth of three virulent bacteria pathogenic to fish viz., *Aeromonas hydrophila, Pseudomonas fluorescens, and Staphylococcus auras.*

Materials and Methods

Plant material

Fresh, disease free *Nelumbo nucifera* leaves were collected during vegetative phase (May-June, 2018) from Mansbal Lake, Safapora Ganderbal, Jammu & Kashmir. The leaves were thoroughly washed with tap water to remove any debris and dirt. After chopping, the plant material was dried in hot air oven at 60°C for 12 hours (Arjun *et al.*, 2012). The dried leaves were made into fine power by using a grinder (Philips Hl1645 750-watt), and the powder was subsequently sieved through a 20 mesh (0.74 mm gap size) and stored at 4°C until further use.

Extract preparation

10 grams of *Nelumbo nucifera* leaf powder was macerated first with 100 ml of distilled water followed by 100 ml of 75% ethanol for 36 hours with continuous starring. The suspension was filtered through Whatman no. 1 filter paper. The filtrate was dried in a rotary evaporator (Singla Scientific Glass Industries, India). Similar procedure was followed when acetone was used as solvent instead of ethanol. A separate crude extract of lotus leaf was prepared by using only water as solvent. The final yield of *Nelumbo nucifera* leaf extract (NNLE) was expressed in mg/gram (table 2) based on dried leaf weight. The NNLE was stored at 4° C until further use.

Methods of evaluating antioxidant properties

Estimation of DPPH scavenging

Antioxidant activity of lotus crude extract was determined by following MacDonald *et al.*, 2006 with slight modification. The lotus leaf extract samples with different concentrations were taken and then 2 ml of 0.06 M methanolic DPPH (procured from Sigma-

Aldrich, USA) was added. After through mixing and incubating in dark for 30 min at room temperature, the radical scavenging activity was determined by measuring the optical density (OD) value at 517 nm using UV-Visible light spectrophotometer (Evolution 201, Thermo Scientific™) against the reagent blank. The control containing no lotus leaf extract was also run along with the samples.

Estimation of total phenolic contents

Total phenolic content in the crude leaf extract was estimated by the method of Singleton & Rosy (1965). 30 µL of lotus leaf was taken in a test tube and the volume was made up to 3 ml with distilled water. 0.5 ml of Folin-Ciocalteau reagent was added followed by 2 ml of 20% of sodium carbonate after 3 min. The tubes were then placed in boiling water for 1 minute and the absorbance was taken at 650 nm against the reagent blank. Gallic acid was used as the standard and the standard curve of absorbance against different concentrations was prepared. The total phenolic content was expressed in mg phenols/100g sample.

Bacterial strains

All the three fish bacterial pathogens were procured from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India (Table1).

Antimicrobial activity

Minimum Inhibitory Concentration (MIC) of NNLE was determined by using disk diffusion method and broth micro-dilution methods as described by Klancnik *et al.,* 2010and Irith *et al.,* 2008 with slight modification. The bacterial strains were maintained in nutrient broth (sigma) under culture conditions at 37 °C.

Disk diffusion method

For the disk diffusion assay (NARMS, 2002)1 mL of each bacterial suspension (10^4 CFU mL^{-1}) was uniformly spread on a Miller Hinton agar in a petri dish. Five millimetre (diameter) discs prepared from Whatman no. 4 filter paper. Different concentrations of 250µg/ml, 125µg/ml, 62.5µg/ml, and 31.25µg/ml of NNLE were prepared by dissolving the extract in DMSO. The discs incorporated with respective concentration of NNLE and were left to dry for 1 hour under sterile conditions and placed on cultured pathogenic bacteria on MHA plates incubated at 37° C. Antibacterial activity as MIC was determined as the lowest concentration of plant extract, which produced an inhibition zone around a disk following the 24 h incubation (Valgas *et al.,* 2007). Discs impregnated with sterile distilled water and DMSO served as negative controls, and a disk with an antibiotic (Chloramphenicol 25 mcg procured from HiMedia) served as a positive control. Replicas at each concentration were performed.

Broth micro-dilution method

10 µL of each bacterial suspension (10^5–10^6 CFU/mL) in nutrient broth was added to the wells of a sterile 96-well micro-titre plate already containing 190 µL of two-fold serially diluted NNLE. The final volume in each well was 200 µL. Control wells were prepared with culture medium, bacterial suspension only, plant extracts only and DMSO in amounts corresponding to the highest quantity present. The contents of each well were mixed on a microplate shaker (Eppendorf, Hamburg Germany) at 900 rpm for 1 min prior to incubation for 24 h in the cultivation conditions described above. The MIC was the lowest concentration where no viability was observed after 24 h based on metabolic activity (Mourey and Canillac, 2002). To indicate respiratory activity the presence of

colour was determined after adding 10 µL/well of TTC (2,3,5- triphenyl tetrazoliumchloride, Sigma) dissolved in sterile water (TTC 20 mg/mL) and incubated under appropriate cultivation conditions for 30 min in dark (Ellof, 1998). All measurements of MIC values were repeated in triplicate.

Statistical analysis

To validate the reproducibility of results, each assay was done in triplicate. One-way analysis of variance (ANOVA) using SPSS v. 20 was performed after the data ensured normal distribution. All analyses were performed considering a level of 95% of confidence ($P < 0.05$).

Results and Discussion

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging method

The DPPH radical has a deep purple colour which is reduced by antioxidant/reducing compound to the corresponding pale yellow hydrazine. The free radical scavenging capacity of the crude leaf extract with different concentrations was tested using the stable free radical DPPH. The ability of each concentration of extract to scavenge DPPH radical are represented as percentage inhibition (%) (Table 3). The crude extract exhibited varying degrees of scavenging capacity depending on the concentration. All the concentrations of extract vary significantly ($p<0.05$), except that there was no significant different in the scavenging capacity of 5.5mg/ml and 7 mg/ml concentrations. The maximum concentration (10mg/ml) of the extract showed the highest scavenging effect (57.75%), whereas the lowest concentration (0.5mg/ml) of the extract showed the least scavenging capacity (9.30%).

Total phenolic content

The total phenolic contents of lotus leaf extract with different concentrations were significantly different ($p<0.05$) (Table 3). The phenolic contents exhibited similar trend as that of DPPH. Total phenolic compounds increased with the increasing concentration of lotus leaf extract.

Antibacterial property

Disc diffusion and broth micro-dilution methods showed bactericidal properties of lotus leaf extract. In disc diffusion test, MIC values of *Nelumbo nucifera* leaf extracts against the different bacterial strains were ranged from 31.25 ul/ml to 250 ul/ml, as shown in table 4. The maximum activity was against *Aeromonas hydrphila* with MIC value of 31.25 ul/ml using ethanol-water based solvent. The lowest inhibition of 1 mm was against *Staphylococcus aureus* using water as extraction solvent. In broth micro-dilution method, MIC values of lotus leaf extract for different fish pathogenic bacteria was 250 ul/ml (Figure 1).

Table.1 Bacterial strains procured from MTCC CSIR-Institute of Microbial Technology, Chandigarh, India

S. No.	Bacterial strain	MTCC collection acc. no.
1	*Staphylococcus aureus*	3103
2	*Pseudomonas fluorescens*	103
3	*Aeromonas hydrophila*	1739

Table.2 Final yield of dried *Nelumbo nucifera* leaf powder

	Aqueous	**Acetone-Water**	**Ethanol-Water**
Yield (mg/gram)	40	150	250

Table.3 DPPH inhibition (%) of crude lotus leaf extract at different concentration

Concentrations (mg/ml)	**DPPH (inhibition %)**	**Phenolic content (mg/100g**
0.50	9.30^{a} ±0.0	13.75^{a} ± 0.01
1.25	17.08^{c} ± 0.0	15.39^{b} ±0.01
2.50	22.68^{d} ±0.01	18.51^{c} ±0.02
4.00	33.15^{e} ±0.0	20.93^{d} ±0.02
5.00	38.56^{f} ±0.0	22.53^{e} ±0.015
5.50	41.85^{g} ±0.0	24.96^{f} ±0.02
7.0	42.47^{g} ±1.33	25.08^{f} ±.0.005
8.50	48.01^{h} ±0.0	28.60^{h} ±0.005
10.0	57.75^{i} ±0.0	37.76^{i} ±0.01

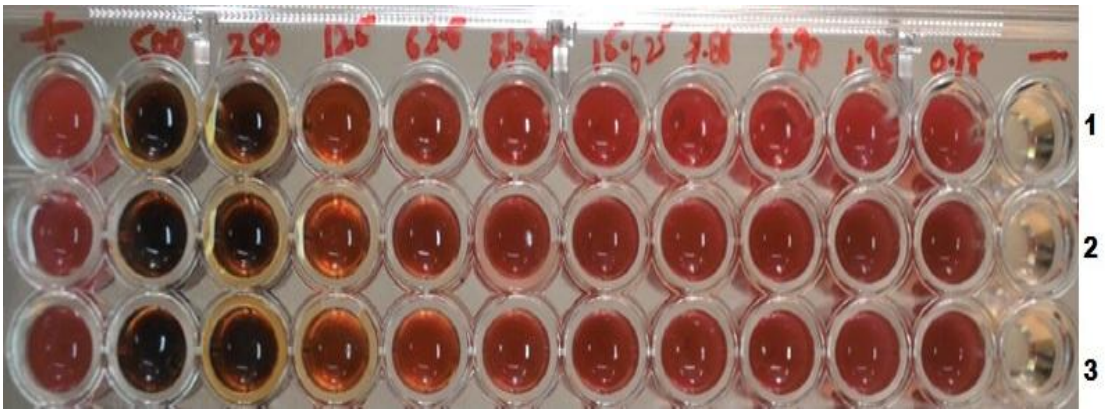
Mean values in a row with different superscript differ significantly (*P<0.05*). Data expressed as mean±S.D. n=3

Table.4 MIC *of Nelumbo nucifera* leaf extract by disc diffusion method

Extraction Medium	**Concentration of Crude Extract**	**Zone of inhibition (mm)**		
		Staphylococcus aureus	*Pseudomonas fluorescens*	*Aeromonas hydrophila*
Aqueous	250µg/ml	$3±0.0^{a}$	$2±0.1^{a}$	$4±0.0^{a}$
	125µg/ml	$1±0.1^{b}$	NI	$1±0.0^{b}$
	62.5µg/ml	NI	NI	NI
	31.25µg/ml	NI	NI	NI
Acetone-Water	250µg/ml	$6±0.1^{e}$	$7±0.1^{e}$	$9±0.21^{e}$
	125µg/ml	$3±0.11^{f}$	$5±0.0^{f}$	$7±0.1^{f}$
	62.5µg/ml	$1±0.0^{g}$	$2±0.0^{g}$	$4±0.0^{g}$
	31.25µg/ml	NI	NI	$1±0.0^{h}$
Ethanol-Water	250µg/ml	$10±1.05^{i}$	$11±0.1^{i}$	$13±2.01^{i}$
	125µg/ml	$9.9±0.2^{i}$	$8±0.0^{i}$	$8±0.12^{i}$
	62.5µg/ml	$5±0.1^{j}$	$6±0.14^{j}$	$5±0.0^{j}$
	31.25µg/ml	$4.3±0.11^{j}$	$3±0.0^{k}$	$4±0.11^{j}$

Mean values in a row with different superscript differ significantly (*P<0.05*). Data expressed as mean±S.D. n=3, NI means no inhibition

Fig.1 Minimum inhibition concentration of crude lotus leaf extract (NNLE) against different fish pathogenic bacteria using broth micro-dilution method. Rows (from top): 1-*Aeromemas hydrophila*, 2-*Pseudomonas fluorescens*, 3-*Staphylococcus aureus*. Columns (from left): 1-Positive control, 2–500 ul/ml, 3– 250 ul/ml, 4–125 ul/ml, 5–62.5 ul/ml, 6–31.25 ul/ml, 7–15.62 ul/ml, 8–7.81 ul/ml, 9–3.90 ul/ml, 10–1.95 ul/ml, 11–0.97 ul/ml, 12–Negative control



Several methods are available for the extraction of antioxidants from the plant materials (organic solvent extraction, aqueous extraction etc). The effectiveness of the extraction depends upon the method employed and the species used (Balouiri *et al.*, 2016). The results of determining the antioxidant activity can be highly variable which cannot be explained by on single method. Thus, in the present study, aqueous extraction was carried out and two different methods, each having different mechanisms of antioxidant action, were employed to check the antioxidant property of the extract.

DPPH is a free radical compound that has been widely used to determine the free radical scavenging capacity of the various samples. The advantage of using DPPH assay is its stability of free radical and speed (Bozin *et al.*, 2007). The free radical scavenging activity of the lotus leaf extract was expressed as percentage inhibition. The results showed that the higher concentration of the extract had higher radical scavenging effect (52.04 ±0.00). In the present study, it was observed that the greater phenolic contents exhibited increased DPPH scavenging activity. The possible reason for this could be the increase in concentration of phenolic compounds present in the plant extract as its concentration was increased. According to Li *et al.,* (2008) boiling could be better choice for obtaining antioxidant rich extracts from the plants, which is in agreement with the present study.

The total phenolic content of the plant extract is a good indicator of the total antioxidant power of the extract (Fernandes *et al.*, 2016). Considering this, the total phenolic contents of the extract was studied by Folin-Ciocalteu method, which showed an increasing trend between the concentration of the extract and the antioxidant activity parameters. The highest concentration of the lotus leaf extract (10mg/ml) showed the presence of highest amount of total phenols 34.66±0.011mg/100g. Li *et al.,* (2008) observed a high correlation between the antioxidant capacities obtained from Ferric Reducing Antioxidant Power (FRAP) assay and the phenolic contents of 45 different plants (r^2= 0.8672). Moreover many studies have reported that the phenolic compounds are responsible the antioxidant

activity (Liu *et al.,* 2008; Rempe *et al.,* 2017; Sharifi- Rad *et al.,* 2018). Phenolic compounds (flavonoids for instance) have redox properties, which allow them to act as antioxidants. As their free radical scavenging ability is facilitated by their hydroxyl groups, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity. Flavonoids, including flavones, flavanols and condensed tannins, are plant secondary metabolites, the antioxidant activity of which depends on the presence of free OH groups, especially 3-OH. Plant flavonoids show both *in vitro* and *in vivo* antioxidant activity (Geeta, *et al.,* 2003; Shimoi, *et al.,* 1996). The crude lotus leaf extract was observed to have good total phenolic contents which indicate its potential as a natural antioxidant to prevent oxidative damage in fish.

Chen *et al.,* (2015), have evaluated the antibacterial activity of *Nulembo nucifera* leaf extract. They have reported lotus leaf extract as potential antibacterial agent against *E. coli, S. typhimurium, S. aureus and B. subtilis*. The present *in vitro* results for antibacterial study are in agreement with the findings of (Dubey *et al.,* 2012) who reported that ethanol extract of most plants had effective antimicrobial activity against all the isolated multidrug resistant bacteria. Furthermore, the extracts (ethanol and acetone) of leaves showed significant activity against Gram-negative bacteria and Gram-positive bacteria. According to some reports the presence of secondary metabolites in plants *viz.,* alkaloids (Gurudeeban *et al.,* 2013; Budeyri *et al.,* 2012) and flavones (Islam *et al.,* 2002; Li *et al.,* 2012) have significant antimicrobial activities. This may explain the efficiency of ethanol-extract for antimicrobial activity. It indicates that the alkaloids and flavones present in plant extract might have synergistic effect against bacterial growth. While some alkaloids such as colchicine, aconitine, scopolamine, strychnine are toxic even they are isolated from natural product, there is no reports about the toxicological evaluation of lotus leaves alkaloids. It is essential to perform toxicological evaluation of lotus leaves alkaloids in the future for its safe use as animal or fish feed additive.

In conclusion, the antibacterial activity of the lotus extract could be related to the presence of bioactive components like alkaloids and flavonoids. Results of present study suggest that the lotus leaf extract possess significant antioxidant activity and antibacterial compounds, which may be used as feed additives and therapeutics in fish nutrition and aquaculture industry. The antibacterial mechanism of lotus leaf extract is unclear and needs further research.

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