Phytochemical analysis and antimicrobial activity of aqueous and methanolic extracts of the flowers of *Nelumbium speciosum*

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Abstract: In present investigation aqueous and methanolic extracts of *Nelumbium speciosum* flowers were screened for phytochemical constituents and antibacterial activity to ascertain their traditional use. Antimicrobial activity of both extracts was tested by Kirby-Bauer disc diffusion method against four Gram positive strains, viz. *Staphylococcus aureus* (ATCC25923), *Streptococcus agalactiae* (13813), *Bacillus subtilis* (ATCC 6633), *Staphylococcus epidermidis* (ATCC 12228) and four Gram negative bacterial strains *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (12453) and *Klebsiella pneumoniae* (10031). Phytochemical analysis showed the presence of tannins, saponins and alkaloids in both extracts while flavonoids and steroids were present only in methanolic extract. Methanolic extract of *Nelumbium speciosum* flower showed concentration dependent antibacterial activity against all tested strains with maximum zone of inhibition (17.3±0.3mm) against *P. aeruginosa.* Aqueous extract showed concentration dependent activity against *S. aureus, E. coli, B. subtilis* and *S. epidermidis* with maximum antibacterial activity against *E. coli* (14.3±0.3mm). MIC of methanolic and aqueous extracts was in the range of 0.015-0.251 and 0.0625-0.251 mg/mL, respectively. Results showed that methanolic extract of *Nelumbium speciosum* exhibits superior antibacterial activity than aqueous extract.

Keywords: Antibacterial activity, *Nelumbium speciosum*, phytochemical analysis.

INTRODUCTION

Plants are a source of inspiration for novel drugs because plants have made a great contribution to maintain human health. Medicinal plants have been used as remedies for many ailments since ancient times (Nostro *et al*., 2000). Plant extracts and their active constituent (s) have as source of antimicrobial agents (Saleem *et al*., 2014).

Infectious diseases are threatening the life of millions of people all over the world (Ashbo, 2004).The development of defensive mechanisms in pathogens against antibiotics have greatly affected their efficacy (Sridhar *et al*., 2014). The high cost and emergence resistance against antibiotics have led to the search for an alternative allopathic agent to alleviate and treat different diseases with few side effects (Cowan, 1999). Scientific and pharmaceutical communities have got attention of therapeutic worth of medicinal plants and research studies have been carried out on plants to validate the claims of their biological activities. Although a lot of synthetic antibiotics are available in market to treat infectious diseases, there is still need to search natural products to cope with resistance developed by microorganisms against synthetic antibiotics (Hart and Kariuki, 1998).

Nelumbium speciosum is a member of the family *Nelumbonaceae* and is native to China, Eastern Asia, Japan and has extended to India and Australia (Ivan, 2001; Staples and Herbst, 2005). In Pakistan, it is distributed in Azad Kashmir and Punjab and locally known as Kanwal (Muhammad *et al*., 2007). *Nelumbium speciosum* has been traditionally used for treatment of various diseases like piles, cholera, liver diseases, worm, fever, skin problems, dyspepsia, diarrhea, diabetes and cancer (Chopra, 1956; Muhammad *et al*., 2007). The current study was planned to evaluate its antibacterial effect against certain strains of bacteria to validate its traditional use.

MATERIALS AND METHODS

Chemicals

Nutrient broth (Oxide, USA), Mueller Hinton agar (Oxide, USA), methanol, sulfuric acid, ferric chloride, hydrochloric acid, barium chloride, dimethylsulfoxide (DMSO) (Merck Chemical Co, Darmstadt Germany), commercial antibiotics discs (Oxide, Basingstoke, Hampshire, England) were used in this study. All these chemicals were obtained from the Department of Pharmacy, University of Sargodha.

Plant material

Plant material was collected from local area of Sargodha and identified from the Department of Botany, University of Sargodha, Sargodha, Pakistan. The plant was washed

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under running tap water and then dried under shade. It was then grinded into fine powder by mechanical grinder (Sourav *et al*., 2012).

Bacterial strains

In current study different bacterial strains that were used to test antimicrobial potential of aqueous and methanolic extracts of the flowers of *Nelumbium speciosum* are *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis (*ATCC 12228), *Escherichia coli* (ATCC 8739), *Klebsiella pneumonia* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6633), *Streptococcus agalactiae* (ATCC 13813), *Proteus mirabilis* (ATCC 12453). These strains were manufactured by Liofilchem, Italy and obtained from Bio Chemika Int., Lahore, Pakistan.

Preparation of extracts

Aqueous extract

Aqueous extract was prepared by boiling 500g of dried powder of flowers of *Nelumbium speciosum* in distilled water for 6h. It was then filtered through 8 layers muslin cloth, centrifuged at 3000 rpm for 15 minutes, supernatant was collected, concentrated to one fourth of its original volume and stored at 4°C for further use.

Methanolic extract

Methanolic extract was prepared by taking 500g of dried powder of flowers in 100mL of methanol in round bottom flask. It was kept on rotary shaker (Bibby Sterilin, UK. CAT No: RE003/MS) for 24h at 199-220 rpm then it was filtered using 8 layers muslin cloth and centrifuged for 15 minutes. The supernatant was collected and evaporated on rotary evaporator to one fourth of original volume. It was stored in airtight bottles at 4°C for further use (Nair *et al*., 2005).

Phytochemical screening of plant extracts

Aqueous and methanolic extracts of *Nelumbium speciosum*, were tested for tannins, saponins, flavonoids, alkaloids, terpenoids, steroids, reducing sugar, cardiac glycoside, anthraquinones by using already described methods (Yaday and Munin, 2011; Abdullahi and lawal, 2010).

Test for tannins

Plant extract (100 mg) was taken in test tube and added 2-3 mL of FeCl3 solution (2% w/v). The presence of tannins in the extract is indicated by appearance of bluish green or black color (Yaday and Munin, 2011).

Test for saponins

Crude extract (100mg) was mixed with 3mL water. The presence of saponins is indicated if stable foam is formed in test tube on vigorous shaking (Yaday and Munin, 2011).

Test for flavonoids

Magnesium ribbon was added to crude extract of plant (100mg) and added few drops of concentrated HCl. The appearance of pink scarlet color indicated presence of flavonoids (Yaday and Munin, 2011).

Test for alkaloids

2-3mL of 1% HCl was added to crude extract (100mg) and heated gently. Wagners and Mayers reagents were added. The appearance of precipitates indicated the presence of alkaloids (Yaday and Munin, 2011).

Test for terpinoids

2-3mL of chloroform was mixed with crude extract and mixture was evaporated to dryness. To this 2-3mL of concentrated H2SO4 was added and heating was carried out for 2-3 minutes. The appearance of grey color indicated the presence of terpinoids.

Test for steroids

Chloroform (2mL) was added to crude extract and few drops of concentrated H2SO4 to mixture. The appearance of red color in the bottom of chloroform layer indicated the presence of steroids.

Test for anthraquinones

Crude extract 100mg was boiled with 3mL of H_2SO_4. Filtration of mixture was carried out using whatman filter paper No. 1. To the filtrate 1mL of chloroform was added and vigorous shaking was carried out. The mixture was allowed to stand for some time and chloroform layer was pipetted out in another test tube. Ammonia solution (10%) was added to the test tube and color change was noticed for the presence of anthraquinones (Abdullahi and Lawal, 2010).

Test for reducing sugar

To the crude extract (100 mg) of plant equal volumes of Fehling A and Fehling B reagents (1mL each) were added and solution was heated to boiling. Appearance of brick red color precipitates indicated the presence of reducing sugar (Yaday and Munin, 2011).

Test for cardiac glycosides

Crude extract (200mg) was diluted with water (3mL). Glacial acetic acid (1mL), premixed with 2-3 drops of FeCl3 solution was added to the mixture. In this mixture concentrated H_2SO_4 (1mL) was added. Appearance of brown ring at interface indicated the presence of cardiac glycosides or the formation of violet ring below brown ring is an indication of cardiac glycosides (Abdullahi and Lawal, 2010).

Preparation of bacterial growth media

Mueller hinton agar media

36g of Mueller Hinton Agar powder was suspended in 1 liter of distilled or deionized water and kept stirring with continuous agitation until complete dissolution was attained. It was transferred into flask and autoclaved at 115°C (at 15p.s.i.) for 30 minutes then it was cooled and

poured into pre- sterilized Petri plates by keeping thickness up to 4mm approx. and allowed to solidify in an incubator to avoid water bubbles on the agar surface (Mueller and Hinton, 1941).

Nutrient broth

8g of nutrient broth media (containing 5g of peptone and 3g of beef extract) was dissolved in one liter of distilled water and mixed thoroughly. It was autoclaved at 115°C for 30 minutes (Ibrahim *et al*., 2005).

McFarland preparation

0.5 McFarland turbidity standard was prepared by mixing 99.5mL of 0.36 N of sulfuric acid and 0.5mL of 0.048 N Barium Chloride (Nakade, 2012).

Inoculum preparation

The inoculum was prepared by taking four or five colonies with the help of loop from overnight inoculated agar in Nutrient broth and incubated at 37°C for 24h. Its turbidity was adjusted to 0.5 McFarland standards. It was done by visual comparison of turbidity of inoculum taken in same size tubes and standard McFarland in good light (EUCAST, 2003).

Preparation of stock solution of extracts

Stock solutions of extracts were prepared by dissolving 500mg and 250 mg of both aqueous and methanolic extracts of the flowers of *Nelumbium speciosum* in 5mL of DMSO in eppendorf tube with concentration of 100 mg/mL and 50 mg/mL respectively, stored in refrigerator and were removed one hour prior to use (Sourav *et al*., 2012).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was conducted by disc diffusion method (Ergene *et al*., 2006). Filter paper discs of 6 mm diameter were impregnated with the solution of aqueous and methanolic extracts of plant having 1 and 2 mg of dried extract per disc and allowed to dry (Nishanta *et al*., 2002). The cotton swab was dipped in standard inoculum, spread on the surface of media (Taylor *et al*., 1995) and with flamed forceps filter paper discs were placed on the surface of media and incubated for 24h at 37ºC (Muhammad *et al*., 2012). DMSO was used as negative control throughout the experiment (Sourav *et al*., 2012). Commercial antibiotics discs of ciprofloxacin were used as a positive control. After incubation, antibacterial activity was assessed by the measurement of zone of inhibitions (Muhammad *et al*., 2012).

Minimum inhibitory concentration of methanolic and aqueous extracts of Nelumbium speciosum

Minimum inhibitory concentration of methanolic and aqueous extracts of the flowers of *Nelumbium speciosum* was conducted by disc diffusion method. Extracts (methanolic and aqueous) were serially diluted with respective solvents from a concentration ranging 2-0.0075mg/mL. Discs impregnated with 10µL of each dilution were allowed to dry and placed on to petri plates on which inoculum was grown. Zone of inhibitions were recorded after 24h of incubation at 37°C. The lowest concentration at which no zone of inhibition was noticed was marked as MIC (Cock, 2009).

RESULTS

Phytochemical screening

Phytochemical analysis of methanolic and aqueous extracts of the plant revealed the presence of tannins, saponins and alkaloids. Steroids and flavonoids were present only in methanolic extract. The terpenoids, reducing sugars, anthraquinones and cardiac glycosides were found to be absent in both the extracts (table 1).

Antimicrobial activity

The methanolic extract showed concentration dependent antimicrobial activity against all the tested strains as shown in table 2. Maximum activity was shown against *Pseudomonas aeruginosa* with zone of inhibition (17.3± 0.3mm). It also showed activity against *Streptococcus agalactiae* with zone of inhibition (16.3±0.3mm),

Staphylococcus epidermidis (15.7±0.3mm), *Staphylococcus aureus* (15.6±0.3mm). Aqueous extract of plant also showed concentration dependent antimicrobial activity as shown in table 2. Maximum antimicrobial activity of aqueous extract of flowers of *N. speciosum* was observed against *Escherichia coli* with inhibition zone (14.3±0.3mm). However, aqueous extract exhibited minimum antimicrobial activity against *Bacillus subtilis* where, zone of inhibition (7.0±0.1mm) was observed. It also showed activity against *Staphylococcus aureus* with the inhibition zone (12.7±0.3mm) and *Staphylococcus epidermidis* (11.7±0.3mm*).* However, it didn't showed activity against *Pseudomonas aeruginosa, Streptococcus agalactiae, Proteus mirbilis* and *Klebsiella pneumonia.*

MIC of methanolic extract of the flowers of *N. speciosum* for *Staphylococcus aureus* (ATCC 25923), *E. coli* (ATCC 8739), *Staphylococcus epidermidis (*ATCC 12228) and *Proteus mirabilis (*ATCC 12453) was ascertained to be 0.0625mg/mL. Whereas, MIC of methanolic extract of flowers for *Pseudomonas aeruginosa* (ATCC 27853) was observed to be 0.25mg/mL and for *Bacillus subtilis* (ATCC 6633) it was observed to be 0.031mg/mL (table 3). In case of the aqueous extract of flowers, the MIC was found to be 0.0625 for *Staphylococcus aureus* (ATCC 25923) and *E. coli* (ATCC 8739). Whereas, MIC for *Bacillus subtillis* (ATCC 6633) and *Staphylococcus epidermidis (*ATCC 12228) was noted to be 0.125 mg/mL (table 4).

Table 1: Phytochemical screening of *Nelumbium speciosum* flower extracts

Constituents	Tests	Methanolic extract	Aqueous extract
Tannin	Ferric chloride test	+	+
Saponins	Frothing test	+	+
Flavonoids	HCl test	+	_
Alkaloids	Dragendroff's reagent test	+	+
Terpenoides	Salkowski test	_	_
Steroids	Test for steroids	+	_
Anthraquinones	Test for Anthraquinone	_	_
Reducing sugar	Fehling's test	_	_
Cardiac glycoside	Keller-kiliani test	_	_

Table 2: Antimicrobial activity of *Nelumbium speciosum*

Strains	Methanolic extract (mg/mL)	Average inhibition zone (mm) ± S.E.M	Aqueous extract (mg/mL)	Average inhibition zone (mm) ± S.E.M
S. aureus	1	13.3±0.6	1	9.4±0.4
	2	15.6±0.3	2	12.7±0.3
E. coli	1	12.0±0.0	1	10.6±0.6
	2	14.7±0. 3	2	14.3±0.3
P. aeruginosa	1	12.0±0.0	1	Nil
	2	17.3±0.3	2	Nil
B. subtillis	1	11.7±0.3	1	7.0±0.0
	2	14.0±0.5	2	9.6±0.3
S. epidermidis	1	14.6±0.3	1	10.5±0.2
	2	15.7±0.3	2	11.7±0.3
S. agalactiae	1	12.0±0.0	1	Nil
	2	16.3±0.3	2	Nil
P. mirabilis	1	12.5±0.2	1	Nil
	2	13.6±0.3	2	Nil
K. pneumoniae	1	13.0±0.0	1	Nil
	2	15.2±0.4	2	Nil

Table 3: Minimum inhibitory concentration of methanolic extract of *Nelumbium speciosum*

	Average zone of inhibitions (mm) ± S.E.M							
Conc. (mg/mL)	*S. aureus*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*P. mirabilis*	*S. epidermidis*	*S. agalactiae*	*B. subtillis*
2	15.6±0.3	14.7±0.3	17.6± 0.6	15.2 ± 0.4	15.3 ±0.3	14.6 ± 0.5	16.3 ±0.3	17.5 ± 0.2
1	13.3±0.6	12.0±0.6	12.3± 0.2	13.0 ± 0.5	13.5 ±0.5	10.5 ± 0.3	12.6 ±0.3	14.6 ± 0.3
0.5	10.0±0.0	10.2±0.2	9.3 ±0.3	10.0 ± 0.5	10.6 ±0.3	11.1 ± 0.2	9.8 ± 0.4	11.1 ± 0.6
0.25	9.1±0.1	9.0 ±0.1	7.3 ±0.3	7.50 ± 0.3	10.0 ±0.2	10.2 ± 0.2	8.3 ± 0.3	10.2 ± 0.6
0.125	8.0 ±0.1	7.9 ±0.1	Nil	6.8 ± 0.2	8.3 ± 0.3	9.0 ± 0.0	7.5 ± 0.3	9.0 ± 0.2
0.06	6.8 ±0.2	6.8 ±0.2	Nil	Nil	7.2 ± 0.2	7.8 ± 0.2	7.2 ± 0.2	7.3 ± 0.3
0.031	Nil	Nil	Nil	Nil	Nil	Nil	7.0 ± 0.5	6.5 ± 0.1
0.015	Nil	Nil	Nil	Nil	Nil	Nil	6.3 ± 0.2	Nil
0.0075	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

DISCUSSION

Many studies have been conducted with the aim of determining the antimicrobial activity and phytochemical constituents of plants used to treat topical and systemic infections caused by microorganisms as alternatives to synthetic antibiotics against which microorganisms have developed resistance (Akinpelu and Onakoya, 2006). Antimicrobial activity of many plants throughout the world has been reported by researchers (Sudharameshwari and Radhika., 2007; Omer *et al.*, 2000 and Bonjar, 2004). *Nelumbium speciosum flowers and stem* have been used to treat certain bacterial diseases, i.e. diarrhea and leprosy on empirical basis (Onishi *et al.*, 1984). The present study

Table 4: Minimum inhibitory concentration of aqueous extract of *Nelumbium speciosum*

Average zone of inhibitions (mm) ± S.E.M				
Conc. (mg/mL)	*S. aureus*	*E. coli*	*S. epidermidis*	*B. subtillis*
2	12.7±0.3	14.3±0.3	11.7±0.3	9.6 ± 0.3
1	9.4±0.4	10.6±0.0	10.5±0.2	7.0 ± 0.0
0.5	8.0±0.0	8.1±0.1	8.50±0.0	6.0 ± 0.5
0.25	7.1±0.1	7.0±0.0	6.20±0.2	4.1 ± 0.6
0.125	4.1±0.0	5.9±0.0	4.6±0.0	3.6 ± 0.0
0.0625	3.2±0.1	3.8±0.1	Nil	Nil
0.031	Nil	Nil	Nil	Nil
0.015	Nil	Nil	Nil	Nil
0.0075	Nil	Nil	Nil	Nil

entails extensive research to authenticate the claim of antimicrobial properties of the flowers of *Nelumbium speciosum*. In the current study methanolic and aqueous extracts of the flowers showed the presence of different secondary metabolites as well as potency against pathogenic microorganisms. Previously antimicrobial potential of aqueous extract of pulp of *Tamarindus indica* has been reported, suggesting the viability of aqueous plant extracts against bacteria (Abubakar *et al*., 2010). Methanolic extract of *Nelumbium speciosum* was active against all tested organisams viz. *Staphylococcus aureus*, *Escherica coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Klebsiella pneumoniae*, *Proteus mirabilis* with maximum zone of inhibition (17.3±0.33mm) against *Pseudomonas aeruginosa* and minimum inhibitory concentration of methanolic extract was found to be in the range 0.015 to 0.251mg/mL whereas, in case of aqueous extract MIC was found to be in the range of 0.0625-0.251mg/mL. The antibacterial activity of *Nelumbium speciosum* may be attributed to various active constituent (s) in the methanolic and aqueous extracts. The phytochmical analysis revealed that aqueous and methanolic extracts of the flowers of *Nelumbium speciosum* contained various bioactive compounds; flavonoids, tannins, saponins and alkaloids. Previously it has been reported that the tannins, saponins and alkaloids from various plants demonstrated the antibacterial activity (Stray, 1998; Okwu, 2004; Sala *et al*., 2002) and these results agree with the present investigations. The medicinal importance of these phytochemical constituents has been established in the treatment of various diseases (Yadav and Munin, 2011).

The results of current study showed that antibacterial activity of the aqueous and methanolic extracts of the flowers of *Nelumbium speciosum* was concentration dependent. Methanolic extract of the flowers was more potent against tested bacterial strains as compared to aqueous extract (table 2). This indicates that the active constituent(s) showing the antibacterial activity were more concentrated in the methanolic extract. The flavonoids were present only in the methanolic extract which may exert additional antibacterial activity in methanolic extract than the aqueous extract which does not have flavonoids. The findings of this study justify the traditional use of *Nelumbium speciosum* in various infectious diseases in local communities of Pakistan. The present investigation calls for further activity directed fractionation studies of the *Nelumbium speciosum* and to isolate the active phytochemical constituent (s) and elucidate exact antibacterial mechanism of action.

CONCLUSION

Antimicrobial potential of aqueous and methanolic extracts of *Nelumbium speciosum* flowers was successfully evaluated against pathogenic bacterial strains. Phytochemical screening of *N. speciosum* flowers extracts revealed rich phytochemical makeup of the flower's extract. Current study also indemnifies the traditional use of *N. speciosum* flowers for the treatment of various disorders. However, there is potential thrust to isolate phytochemical constituents that endure antimicrobial activity to flowers extract that will open new corridors in the field of dosage form development as an alternate to traditional antibiotics.

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