

Phytochemical, cytotoxic and antibacterial activity of two medicinal plants of Bangladesh

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Abstract

Ethanol extracts of the traditionally valuable plant *Jasminum grandiflorum* L. (Family – Oleaceae) and *Ficus religiosa* L. (Family – Moraceae) were screened for cytotoxicity and antibacterial activity as well as for some important phytochemical groups. The dried extracts of *J. grandiflorum* and *F. religiosa* were dissolved in 99.8% ethanol and qualitatively analysed for alkaloids, glycosides, steroids, gums, reducing sugars, tannins, flavonoids, and saponins. Cytotoxic potential of dimethyl sulfoxide solution of the extracts were measured by using *in vivo* brine shrimp lethality assay. LC₅₀ and LC₉₀ values were determined graphically by plotting mortality against concentrations. Agar disc diffusion method was used to determine antibacterial sensitivity of the extracts against selected 14 pathogenic bacteria. Minimum inhibitory concentration was determined by broth macro dilution assay. The percent mortality of shrimp was increased with the increase of the doses of the extracts. LC₅₀ and LC₉₀ values were found to be (2.64 & 4.42 µg/ml) and (2.7 & 4.62 µg/ml) for *J. grandiflorum* and *F. religiosa* respectively in comparison with standard vincristine sulphate (1.0 & 3.86 µg/ml). Highest zone of inhibition produced by *J. grandiflorum* was found against *Proteus vulgaris* (15 mm) and that of *F. religiosa* was found against *Enterococcus faecalis* (14 mm). The minimum inhibitory concentrations (MIC) were observed within the range of 250-500 µg/ml.

Keywords: Antibacterial activity, Cytotoxicity, *Jasminum grandiflorum* L., *Ficus religiosa* L., Minimum Inhibitory Concentration

Introduction

Cancer or tumour refers to a group of malignant diseases which may appear in many different parts of the body and spreads gradually to other areas. If untreated, it propagates and ultimately causes death^[1]. Along with other life threatening diseases, cancer causes majority of death in developed countries^[2]. Research in cancer treatment has brought some effective means include surgery, radiation and chemotherapy. However, all of the treatments are also accompanied by a number of severe side effects. The later one aims to eradicate all infected cells from the patients' body^[3].

Antibiotics are very effective to combat bacterial infections and discovery of antibiotics is greatly beneficial for the improvement of the quality of human health and wellbeing. However, the capacity of the microorganisms to become resistant is a potential threat in the treatment of infectious diseases. Many commonly used antibiotics have already become less effective against specific bacteria due to emergence of drug-resistance. Moreover, many of newly developed drugs produce toxic reactions. Hence, we are badly in need to find out newer agents without resistance and having less toxic effects. Natural products from medicinal plants can be a source of new antimicrobial agents with possibly novel mechanisms of action^[4, 5].

The effect of traditionally used plant extracts on bacteria and brine shrimps have been studied by the researchers throughout the world to evaluate cytotoxic and antibacterial potentials. As a part of our on going research two medicinal plants were studied to get scientific basis of their traditional uses.

Large scrambling or twining shrub *Jasminum grandiflorum* L. (Family- Oleaceae) is native to Himalayas and widely grows throughout India and Bangladesh. Whole plant, root, leaves and flowers of the plant are used in Ayurvedic preparations. *J. grandiflorum* is good for treatment of chronic ulcers or eruptions in mouth (leaves are chewed) as well as for skin diseases and poisoning. Fresh juice is applied to corn. It is bitter, astringent, anthelmintic, diuretic and emmenagogue. Root is used in the treatment of ringworm. Flowers and leaves are aphrodisiac and used to overcome menstrual irregularities. Flowers are applied in skin diseases, headache and weak eyes.

Flowers and leaf juice are used in various kinds of tumours. Oil prepared with juice of leaves is used in otorrhoea. Alcoholic extract of aerial parts is hypotensive and anticarcinogenic. The shrub is also used in burns. The leaves contain salicylic acid and flowers contain essential oil^[6]. In the previous studies, leaves and flower of *J. grandiflorum* are reported to heal wounds in rats^[7, 8] and to have anti-hepatitis B virus efficacy particularly of iridoid glycosides from buds^[9, 10]. It also contains secoiridoids^[11, 12], triterpenoid saponins^[13] and some other glycosides^[14]. It has antiulcer and antioxidant properties^[15] and contains angiotensin converting enzyme inhibitors^[16].

Ficus religiosa L. (Family – Oleaceae) is found in Himalayan forests, Bengal and Madhya Pradesh of India and planted throughout the Indian subcontinent. The bark, fruit, seeds, leaf buds and latex of the plant are used in Ayurvedic preparations. Different parts of *F. religiosa* are traditionally used in suppurative otitis media, mouth sores, atrophy, emaciation or cachexy, rheumatism, smallpox, carbuncle, rinderpest, mucus in urine, spermatorrhoea, gravel, cholera, etc. Leaves are aborticide. Leaves and young shoots are purgative. Bark is astringent and is found effective in gonorrhoea (decoction). Pulverised bark is applied externally on unhealthy ulcer or wounds to promote granulation. Infusion of bark is given internally in scabies, ulcers and skin diseases. It is aphrodisiac and good for lumbago. Fruits are mild laxative and digestive. Seeds are used for cooling and as laxative or alterative. Powder of seeds is taken for three days during menses sterilizes women for long time^[17]. The saponins from roots of the plant are reported to have anticonvulsant effect ^[18, 19], leaves as analgesic and anti-inflammatory^[20], anti-asthmatic^[21], and can improve renal injury induced by hypercholesterolaemia^[22] Bark is anti-diabetic and can inhibit acetylcholinesterase^[23].

Materials and methods

Plant materials

The plants materials were collected from different regions of Bangladesh – *Jasminum grandiflorum* from Jessore in July and *Ficus religiosa* were collected from Khulna in August with the help of a local traditional practitioner. Then, they were taxonomically identified by the taxonomist and

botanist of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh and the specimen accession numbers are DACB-31259 and DACB-31264 for *R. J. grandiflorum* and *F. religiosa* respectively. One sample for each specimen is preserved as a reference, in Bangladesh National Herbarium.

Chemicals

Ethanol ($\geq 99.8\%$; Reagent grade, Merck KGaA, Darmstadt, Germany) was used as solvent in maceration of the plant material. In antibacterial screening ethanol ($\geq 49.75\%$) was used for the preparation of the extract solutions. In antibacterial test ciprofloxacin (Ciprocin, Tab. 500 mg) manufactured by Square Pharmaceuticals Ltd., Bangladesh was used as reference standard. Dimethyl sulfoxide ($\geq 99.9\%$, BioReagent, for molecular biology; Sigma-Aldrich, India) was used as solvent for the preparation of sample solutions. Vincristine sulphate, used as a standard drug in the cytotoxic assay was collected from the Techno Drugs Limited, Bangladesh.

Bacteria and shrimp eggs

For antimicrobial sensitivity assay, *Enterococcus faecalis*, *Hafnia alvei*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Plesiomonas shigelloides*, *Staphylococcus pidermidis*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Salmonella typhi*, *Staphylococcus pyogenes*, *Shigella boydii*, *Shigella flexneri*, *Shigella sonnie* and *Shigella dysenteriae* were collected from Animal Research Branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR,B). Their identity was confirmed by Microbiologist, ICDDR,B following biochemical and serological tests. Then the organisms were transferred and preserved in the Microbiology Laboratory, Pharmacy Discipline, Khulna University.

Media

Nutrient agar (NA), Nutrient broth (NB) media used in antibacterial sensitivity assay were procured from HiMedia Laboratories Ltd., India.

Preparation of extract

The dried plant materials were ground into coarse powder and 800 g of which were subjected to

maceration in a properly cleaned and dried glass jar in ethanol. The jars were kept in a dark place at a temperature of $25 \pm 2^\circ\text{C}$ for seven days and with agitation each day. Extracts were filtered using Whatman-41 filter paper and the extraction procedure was repeated three times. The filtrates were combined and the plant residue was brought to dryness at normal room temperature using an electric fan facilitating evaporation of the solvent^[24]. The yield value was calculated by using the equation: $\% \text{ yield} = (W_e/W_p) \times 100$; where, W_e = weight of dried extract and W_p = weight of dried powder. The yields of the extracts were found to be 21.2%, and 20.5% w/w for *J. grandiflorum* and *F. religiosa* respectively. All the extracts were preserved in a refrigerator till further use.

Growth and maintenance of test microorganism for antimicrobial studies

Nutrient broth medium was used to maintain the growth of bacteria strains at 37°C . Then the stock culture of each organism was sub-cultured onto appropriate selective media. Colonies of the pure organisms were cultured in 10 ml of broth medium in sterile test tubes and incubated at 37°C overnight. The cultures were adjusted to a suspension density equal to 0.5 McFarland turbidity standards, which has an approximate cell density of 1.5×10^6 cfu/ml^[25].

Preparation of inoculums

The sub-cultures of the test bacteria were taken in a rotary shaker at 37°C , centrifuged at 10,000 rpm for 5 min, pellets were suspended in double distilled water and the cell density was standardized spectrophotometrically (A 610 nm) to obtain a final concentration of approximately $1-2 \times 10^7$ cfu/ml^[25].

Phytochemical group tests

The dried extracts of *Jasminum grandiflorum* and *Ficus religiosa* were dissolved in 99.8% ethanol and phytochemical groups present in the solutions were screened by using standard test procedures outlined by Trease and Evans^[26]. The extracts were screened for the presence of alkaloids, glycosides, steroids, gums, reducing sugars, tannins, flavonoids, and saponins. The reagents were first tested by using standard drugs of corresponding groups available in market. The resulting data are

summarized in the Table 1.

Determination of cytotoxic activity by brine shrimp lethality assay

Cytotoxic potential of the plant extracts were measured by using *in vivo* brine shrimp lethality assay^[26]. At first, the shrimp eggs were hatched in simulated seawater (3.8% w/v sea salt in distilled water) at 24°C–28°C in front of a lamp.

Eggs were left for 48 h to produce large number of larvae (nauplii). Then, test solutions were prepared in different concentrations (10, 20, 40, 80 and 160 mg/ml) by dissolving the extracts in dimethyl sulfoxide(DMSO). For each sample four test tubes were used and in each test tube 10 shrimps were taken and the prepared extract solutions were applied in it. Finally, volume of liquid was adjusted by saline water. Ten shrimps were taken in a test tube (control) containing saline water and DMSO and were kept for observation under the same conditions with the test sample. The test tubes were kept for 24 h, and the surviving nauplii were counted. To calculate the lethal concentrations (LC₅₀ and LC₉₀), percent mortality and concentrations of the extract were plotted which produced an approximate linear correlation of concentration and mortality on graph. The 50% and 90% lethal concentrations (LC₅₀ and LC₉₀) were determined from the graph (Figure 1). The results are summarized in Table 3.

Antibacterial activity test

Disc diffusion method, described by Khan *et al.* was used to determine antibacterial sensitivity of the extracts^[27]. First, nutrient agar medium was prepared by dissolving the mixture of agar medium in dematerialized water. Then, the prepared medium and the necessary apparatus were sterilized by autoclave. On the other hand, measured amount of the test samples were dissolved in definite volume of ethanol to prepare the solutions of 500 µg/µl. Then, sterile filter paper discs were impregnated with known amount of test substances using micropipette and dried. Positive control (ciprofloxacin 50 µg/µl) and negative control (ethanol) discs were prepared and dried. These discs were then placed in petridishes (96 mm in diameter) containing nutrient agar seeded with the test organisms

using sterile transfer loop for antibacterial screening. The plates were inverted and kept at 4°C for facilitating maximum diffusion. After 24 h, the petridishes of all groups were then transferred into an incubator and left for 18 h at 37°C. Then, zone of inhibition (mm) were measured. The experiments were repeated in case of discrete growth and uneven inhibition of the bacteria. The results of antibacterial sensitivity test are summarized in Table 3.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration was determined by broth macro dilution assay with some modifications^[28, 29]. In brief, selected bacterial strains were cultured on nutrient agar media (HiMedia Laboratories Ltd, India.) at 37 °C for overnight. The bacterial colony was suspended in sterile 0.9% NaCl solution in such a way to get an absorbance of 0.1 at 620 nm (1 × 10⁸ CFU/ml). Aliquot of 100 µl of this bacterial suspension was then mixed with 9.9 ml of Mueller Hinton broth (HiMedia Laboratories Ltd., India.) to get the inoculum (1 × 10⁶ CFU/ml). LCLE was mixed with Mueller Hinton broth with the assistance of DMSO to get a concentration of 4 mg/ml (DMSO concentration < 5%). The extract (1 ml) was then serially diluted in sterile capped tubes containing 1 ml of media in each followed by the addition of bacterial inoculum (1 ml) in each tube. The same procedure was also followed for the antibiotic ciprofloxacin with the starting concentration adjusted to 16 µg/ml. The tubes were then incubated for 18 h. MIC values were recorded as the lowest extract concentration with no bacterial growth. The MIC values were further confirmed with the addition of 40 µl resazurin solution (0.01% in sterile distilled water) (Sigma-Aldrich Co. LLC, Missouri, United States). Pink color or discolouration of resazurin indicated bacterial growth.

Results

Phytochemical tests

In the present study, the sample solutions of *Jasminum grandiflorum* L. and *Ficus religiosa* L. were tested to determine whether steroidal compounds, tannins, gums and reducing sugars, alkaloids, flavonoids, gums and saponins were

present or not. *J. grandiflorum* extract showed the negative interference in alkaloid tests as well as in the gums. It was found that *F. religiosa* contains steroidal compounds, tannins, gums, reducing sugars, alkaloids as well as flavonoids. Saponins were found to be absent in all samples.

Brine shrimp lethality bioassay

The percent mortality of larvae was increased with the increase of the doses of the extracts. LC₅₀ and LC₉₀ values were found to be (2.64 & 4.42 µg/ml), (2.7 & 4.62 µg/ml) and (1.0 & 3.86 µg/ml) for

J. grandiflorum, *F. religiosa* respectively and vincristine sulphate (Figure 1).

Antibacterial sensitivity test

The extracts showed a moderate zone of inhibition against most of the Gram positive and Gram negative bacteria (Table 3). *J. grandiflorum* showed highest zone of inhibition against *Proteus vulgaris* (14 mm) and *F. religiosa* against *Enterococcus faecalis*. However, *J. grandiflorum* was inactive against *E. faecalis*, *H. alvei*, *P. vulgaris*, *P. shigelloides*, *S. typhi* and *F. religiosa* against *P. aeruginosa*, *P. shigelloides*, *S. epidermidis*, *S. aureus*, *S. typhi*, and *S. boydii*.

Results of minimum inhibitory concentration (MIC)

In the broth macro dilution assay, the extracts inhibited the growth of all the microorganisms tested except *S. dysentery*. The obtained MICs were between 250 and 500 µg/ml (Table 4).

Discussion

Plants that belong to family – Oleaceae (*J. grandiflorum* L.) and Moraceae (*F. religiosa* L.) are rich sources of various biologically active substances with strong pharmacological activity. However, the identity of highly active constituents and their mechanisms of action are not clear. These species contains very important compounds like alkaloids, flavonoids, tannins and so on. The secondary plant metabolites steroids, alkaloids, flavonoids, tannins were reported to have cytotoxicity in different cell lines^[30-34]. In the present report, all of the plant species showed good brine shrimp lethality. However, further studies are required for isolation and identification of bioactive constituents and to

observe their effects on human cell line. Moreover, in many instances flavonoids, steroids, alkaloids and tannins isolated from medicinal plant extracts were reported to have antibacterial activity^[35-37]. All of our extracts showed significant but various grade antibacterial activities against the selected strains. The constituents present in the extract may produce bactericidal or bacteriostatic effects against the growth of tested strains. In the present study, scientific rationale of the traditional uses of the plants is established for their cytotoxic and antimicrobial properties. This depicts that these plants can be a source of new or even noble anticancer, pesticidal as well as anti-inflammatory agents. However, further research should be continued for isolation and identification of individual compounds and to determine their specific activity.

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Table 1: Result of Phytochemical test

Test for phytochemical group	Reagent/test	Ethanol extract of <i>Jasminum grandiflorum</i> L.	Ethanol extract of <i>Ficus religiosa</i> L.
Reducing sugar	Fehling's test	+	+
	Benedict's test	+	+
Alkaloid	Mayer's test	-	+
	Dragendorff's test	-	+
	Hager's test	+	+
	Wagner's test	-	+
	Salkowski's test	+	+
Steroid and terpenoid	Libermann-Burchard reagent	+	+
	Ferric chloride test	+	+
Tannin	Ferric chloride test	+	+
Gum	Molisch's test	-	+
Flavonoid	Shinoda test	+	+
	Alkaline reagent test	+	+
Saponin	Frothing test	-	-

“+” indicates presence and “-” indicates absence.

Table 2: Result of Brine shrimp lethality bioassay

Test sample	Concentrations (µg/ml)					LD ₅₀ (µg/ml)	LD ₉₀ (µg/ml)
	10	20	40	80	160		
	% mortality						
Ethanol extract of <i>Jasminum grandiflorum</i>	15	30	60	85	100	2.64	4.42
Ethanol extract of <i>Ficus religiosa</i>	20	30	50	80	100	2.7	4.62
Vincristine sulphate	50	60	80	100	100	1.0	3.86

LD= Lethal dose

Table 3: In vitro anti-microbial activity of ethanol extract

Test sample	Bacterial strains													
	Efa	Hal	Pae	Pvu	Psh	Sep	Sau	Ssa	Sty	Spy	Sbo	Sfl	Sso	Sdy
Ciprofl oxacin	32	33	33	27	31	30	30	26	33	26	32	29	30	31
EJG	0	0	7	0	0	15	7	7	0	7	7	10	7	6
EFR	14	5	0	8	0	0	0	8	0	7	0	9	9	12

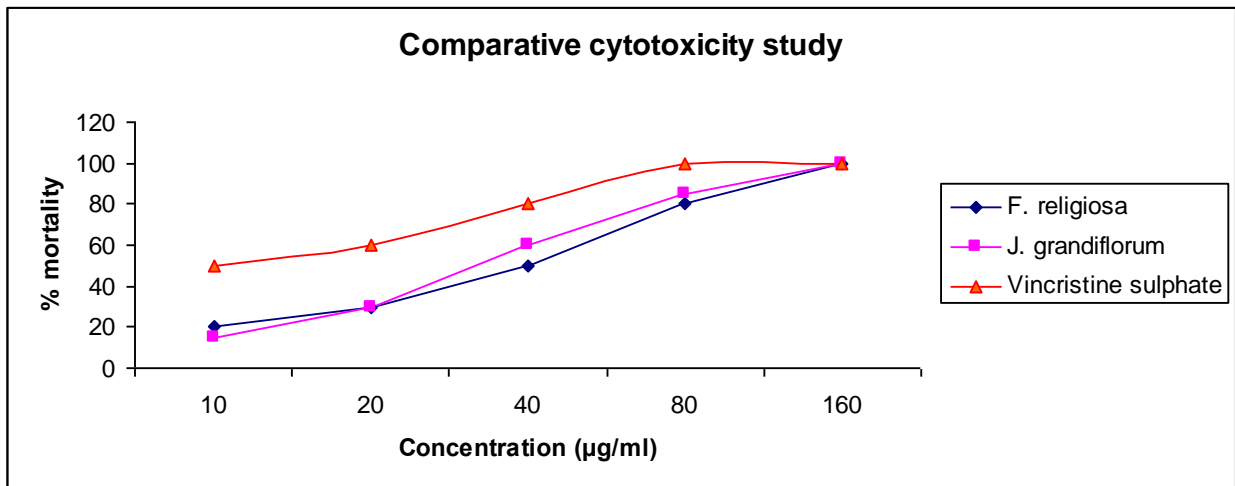
Efa = *Enterococcus faecalis*, Hal = *Hafnia alvei*, Pae = *Pseudomonas aeruginosa*, Pvu = *Proteus vulgaris*, Psh = *Plesiomonas shigelloides*, Sep = *Staphylococcus epidermidis*, Sau = *Staphylococcus aureus*, Ssa = *Staphylococcus saprophyticus*, Sty = *Salmonella typhi*, Spy = *Staphylococcus pyogenes*, Sbo = *Shigella boydii*, Sfl = *Shigella flexneri*, Sso = *Shigella sonnie* and Sdy = *Shigella dysenteriae*

EJG= Ethanol extract of *Jasminum grandiflorum* L. and EFR = Ethanol extract of *Ficus religiosa* L.

Table 4: Results of the *in vitro* broth macrodilution assay of ethanol extract

Test sample	Concentration ($\mu\text{g/ml}$)						
	Efa	Pvu	Sep	Ssa	Sfl	Sso	Sdy
Ciprofloxacin	4	4	1	3	1	1	0.5
Ethanol extract of <i>Jasminum grandiflorum</i> L.	-	-	500	-	250	-	-
Ethanol extract of <i>Ficus religiosa</i> L.	400	400	-	500	300	500	500

Efa = *Enterococcus faecalis*, Pvu = *Proteus vulgaris*, Sep = *Staphylococcus epidermidis*, Ssa = *Staphylococcus saprophyticus*, Sfl = *Shigella flexneri*, Sso = *Shigella sonnie* and Sdy = *Shigella dysenteriae*

**Figure 1:** The comparative lethality assay of ethanol extracts and vincristine sulphate on brine shrimp