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PHYTOCHEMICAL COMPOSITION AND POTENT BIOLOGICAL ACTIVITIES OF *FICUS BENJAMINA VAR. COMOSA* LEAVES EXTRACT

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Abstract

Present work has been commenced to evaluate the possible antioxidant, antibacterial, hemolytic and antiviral properties of ethanolic extract of leaves of *Ficus benjamina var. comosa.* Total phenolic and flavonoid have also been estimated. DPPH free radicals scavenging method was used to assess antioxidant activity while anti-biofilm activity of the ethanolic leaves extracts was done by microtitre-plate assay against *Pseudomonas aerugonisa, Escherichia coli* and *Bacillus cerus*. For hemolytic activity the ethanolic extract toxicity was observed against human erythrocytes *In vitro.* In addition, SPF embryonated chicken eggs were used to check the antiviral activity against Newcastle disease virus. The values of total phenolic and flavonoid components were 4.50 ± 1.1 mg (GAE gm) and 11.20 ± 8.9 (QE mg) respectively. The results indicate that leaves have good antioxidant activity of 68.27 % ±1.08 with respect to ascorbic acid equivalence and biofilm inhibition against all the tested bacterial strains. Hemolytic activity is 2.14 % ±0.41 however at low concentration there was not any significant change observed on erythrocyte membrane. In antiviral *In ovo* activity 60 µg/mL showed complete inhibition of NDV growth while 120 µg/mL dose is lethal for embryo.

Key words: *Ficus benjamina* variety *comosa*, Antioxidant, Biofilm inhibition, Hemolytic, Antiviral activity, Newcastle disease virus.

Introduction

The plants produce secondary metabolites to defend themselves against microbes and insects and these secondary metabolites are also beneficial in the treatment of diabetes (Jamila *et al*., 2020) and in the cure of many viral or other diseases (Pedras, 2008; Öztürk & Hakeem, 2018). Plants also produce compounds/ metabolites having antioxidant and antimicrobial properties which are helpful for their better development, growth and protect themselves against their adversaries (González *et al*., 2009). These metabolites may have ability to combat with other viruses of animals and humans (Yarmolinsky *et al*., 2010). In order to reduce the viruses' effect, research efforts have been devoted to the discovery of new antiviral natural products (Perera & Efferth, 2012). It is the need of the time to discover new antiviral agents to fight against viral diseases like Newcastle disease, as alternatives to expensive drugs.

Ficus benjamina var. *comosa* (weeping fig) belongs to family Moraceae, a species native to Asia and Australia. It is popular ornamental plant growing at mild temperatures but is able to tolerate both low and high temperature conditions (Kim *et al*., 2008). Wild animals like to eat this plant as a food source and it contains many medicinally potent substances such as flavonoids, sugars, phenols, enzymes and vitamins A, C, K. (Berg *et al*., 2005). Its leaves, bark and fruits contain stigmasterol, lactose, quercetin acid, cinnamic, and caffeic acid (Sirisha *et al*., 2010). The *F. benjamina* variety *comosa* leaves extract has shown antioxidant, antibacterial, antiviral activity against Virus Herpes Simplex 1 and 2 (Parveen *et al*., 2009; Yarmolinsky *et al*., 2012), antinociceptive and analgesic activities (Parveen *et al*., 2009). Caffeic acid extracted from *F. benjamina* variety *comosa* has shown cytotoxic activity against T-lymphoblastic leukemic cell (Dafalla *et al*., 2003). The researchers have discovered that flavonoids (especially quercetin and quercetin 3-rhamnoside) present in plants play an active role to fight against several viruses (Choi *et al*., 2009). Based on the literature survey, this plant may possess potent biological activities. So, this study was planned to assess the efficacy of ethanolic extract of *F. benjamina* leaves as antioxidant, antibacterial, hemolytic and antiviral agent.

Materials and Methods

Sample collection and identification: The leaves of *F. benjamina* var. *comosa* were collected from Botanical Garden of Government College University Faisalabad (GCUF) and standard keys and descriptions at the field were utilized for the identification (Dalziel, 1955).

Plant extract preparation: Plant leaves were washed with tap water to remove debris and shade dried for 20-25 days in lab. The leaves were then grinded and stored in cool dry room by putting in airtight bags. Extraction was performed by simple maceration method described by Parekh & Chanda (2006). Powdered plant leaf, weighing 500 g was drenched in a conical flask in 99.8% v/v of ethanol (1 liter), and kept for 72 h at room temperature in a dark place. After filtration, filtrate was concentrated using a rotary evaporator at 50°C to evaporate all the ethanol. Evaporated extract was dissolved in dimethylsulphoxide (DMSO) and kept at 4°C until used.

Total phenolic contents: The quantification of total phenolic contents of ethanolic leaf extract was carried out by Folin Ciocalteau reagent with respect to standard curve of gallic acid (Kumar *et al*., 2013). The crude ethanolic

extract was diluted to make different concentrations (125 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL). In 1 mL of each extract added 2.5 mL of Foiln reagent and 2m L of sodium bicarbonate (Na_2Co_3 7.5%). After 20 mints the absorbance was checked at 765nm by taking Na_2CO_3 as blank. The results were expressed in equivalence to gallic acid (µg).

Total flavonoid contents: Flavonoids were determined by Aluminium chloride method with different dilutions (125 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL) of crude ethanolic extracts mixed with 1 mL of aluminium chloride solution (2% ethanol). After 15-20 mints, absorbance was recorded at 420 nm by taking $AlCl_3$ solution (2% $AlCl_3$ in 1 mL water) as blank. Results were reported as Quercetin equivalence (QE) in µg (Ordonez *et al*., 2006).

Antioxidant assay: The antioxidant activity of *F. benjamina* was determined following the method developed by Ashraf *et al*., (2015) and Ali *et al*., (2016) by scavenging of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). Different concentrations (10µg/mL, 15µg/mL, 20 µg/mL, 25 µg/mL and 30 µg/mL) of ethanolic extract of *F. benjamina* leaves were prepared by diluting the extract with distilled water and added 1mL methanolic solution of 1,1-diphenyl-2-picrylhydrazyl radical (0.2 mmol) in 2mL of each dilution of extract in a separate test tube and make the volume upto 4mL with methanol. After 60 min putting in dark, recorded absorbance at 517 nm by taking methanol as blank.

$$\text{Free radical scavenging activity} = [A_0 - A_1/ A_0] \times 100$$

A_o= absorbance of control solution, A_1= Absorbance of tested solution (extract). Lowered absorbance of mixture specifies stronger DPPH radical scavenging activity.

Hemolytic studies towards human erythrocytes: Hemolytic activity of the plant ethanolic extract was performed by the technique described by Anjum *et al*., (2013) and Shahid *et al*., (2015). For each assay, human erythrocytes were upheld @ 10^8 cells per ml. These cells were mixed separately with 100 µl of each compound. With gentle agitation after 10 min, samples were incubated at 37ºC for 35 min and then for 5 min kept on ice. Samples were immediately centrifuged at 1000x*g* for 5 min. From each tube, 100 µl supernatant was mixed with chilled (4ºC) PBS having pH 7.4 diluted them 10 times. Same process was performed with PBS (-ve control) and 0.1% v/v Triton X-100 (+ve control). For measuring % RBCs lysis for each sample, the absorbance at 576 nm was observed using µQuant (Bioteck, USA).

Biofilm inhibition assay: A 100 µl of nutrient broth with 100 µl testing sample and 20 µl of bacterial suspension (*Escherichia coli, Bacillus cerus* and *Pseudomonas aeruginosa*) was inoculated in flat bottomed 96 sterile well tissue culture plates while the wells containing only nutrient broth served as –ve control. After three times washing with 220 µl of sterile phosphate buffer, 220 µl of methanol was used for the fixation of bacteria and plates were emptied after 15 min. For staining 50 % crystal violet for 5 minutes for these dried plates (Rubab *et al*., 2015; Hussain *et al*., 2015). In air dried plates glacial acetic acid (33%, v/v) per well was added for resolubilization of dye bound adherent cells. The microplate reader (BioTek, USA) was used for measuring OD of each well at 630 nm using. Inhibition of bacterial growth (INH%) was determined as follows:

$$INH\% = 100 - (OD_{630\ sample} \times 100)/ OD_{630\ control}$$

Antiviral activity: Specific pathogen free (SPF) chicken eggs were collected from a local poultry breeder and incubated for 9 days at 37°C while the NDV Lasota strain was purchased from local market, Pakistan. Fifty percent (50%) embryo infectious dose (EID_{50}) of the virus was recorded by following the protocol of Young *et al*., (2002). By adopting Reed and Muench formula index the infection rate immediately above 50 percent was analyzed (i.e. $10^{-3.5}$).

Eggs inoculation: Eight groups (having five embryonated eggs in each group) were formed after 9 days of eggs incubation and labeled them regarding for inoculated concentration of extract in them. 0.1 ml of the inoculum was inoculated through a hole made in egg shell just above the air sac. Group G1 has uninoculated eggs (-ve control), G2 has eggs with virus suspension only (+ve control), G3 has extract control and G4 eggs injected with DMSO + virus (VS+). From five to eight groups were inoculated with 0.2 ml of *F. benjamina* leave ethanolic extract/virus at final concentration of 30µg/ml, 60µg/ml, 90µg/ml, 120µg/ml and sealed the inoculation hole with paraffin wax. Kept all the eggs at 37°C for 4 days. The growth and movement of embryos were observed daily. At 5th day, the eggs were chilled and allantoic fluid was collected from treated eggs to detect NDV by performing haemagglutination test (HA) as documented by Murakawa *et al*., (2003). The antiviral activity of plant extract with its different concentrations was assessed on embryo survival rate and virus concentration was recorded by HA.

Statistical analysis: All the tests were performed as triplet. The data is presented as mean ± deviation (SD). The result were analyzed by using MS Excel and ANOVA (one way) to determine significant level (p<0.05) between data sets.

Results

Antioxidant assay: The percentage radical scavenging of ethanolic leave extract of *F. benjamina* is 68.27 ±1.08 µg/mL and this activity increase with increase in time and concentration of dose. The results are expressed as percentage inhibition of DPPH (n=3). The results of DPPH radical scavenging activity are represented in ascorbic acid equivalence and reported in as depicted in Fig. 1.

Total phenolic and flavonoid contents: In this study phenolic and flavonoid content showed dose dependent pattern. The TPC value measured by Folin Ciocalteau reagent procedure with respect to standard curve of gallic acid was 4.50 ± 1.1 mg (GAE gm) in dose dependent pattern compare to Gallic acid equivalence in µg. The results are summarized in Fig. 2. The value of flavonoid for ethanolic leave extract of *F. benjamina* was 11.20 ± 8.9 mg with respect to 2% aluminium chloride solution, results are expressed as Quercetin equivalence (QE) in µg shown in Fig. 3.

Hemolysis towards human erythrocytes: Hemolytic activities of the *F. benjamina* leave is reported as mean ± SD of all the replicates presented in Table 1. No hemolysis (0%) was obtained with buffer and Triton X-100 (0.1%) showed total hemolysis. The yield of positive control Triton X-100 was 96.58±1.72, negative control PBS was 0.63±0.19 and extraction of leave of *F. benjamina* was 2.14±0.41. The leaf extract of *F. benjamina* displayed very low hemolytic effect toward human erythrocytes.

Microtitre-plate method of biofilm inhibition assay: The results of biofilm inhibition activity obtained by microtitre-plate method (Table 2) showed that the ethanolic extract of leaves of *F. benjamina* yielded the inhibition against all bacterial strains namely as *Bacillus cerus*, *Pseudomonas aeruginosa* and *E. coli.*

Antiviral activity: The results of *In ovo* antiviral activity (Table 3), revealed that eggs of group 4 were died within 24 to 48 h of post inoculation with positive HA test values. The *F. benjamina* ethanolic leaf extract concentrations 30, 90 and 120µg/mL partially inhibit the virus growth and showed the mortality rate of 20%, 20% and 60% respectively. Sixty (60) µg/mL concentration of leaves extract showed the virus growth 0% and stop progression of virus completely. At 90 and 30 µg/mL the growth is very low as only one embryo dead after 48 & 72 h but 120 µg/mL dose itself become toxic for embryo because of high concentration.

Table 1. Hemolytic activity of the ethanolic extract of *F. benjamina* leaves.

***Ficus benjamina* leaves extract**	**Hemolytic activity (%)**
Leave	2.14% ± 0.41
PBS	0.63% ± 0.19
Triton X-100	96.58% ± 1.72

Table 2. Antibiofilm activity of *F. benjamina* by microtitre-plate method.

Bacterial strains	**Biofilm inhibition (%) of leaves of *F. benjamina***
Bacillus cerus	62.70% ± 0.31
Pseudomonas aeruginosa	59.75% ± 0.14
Escherichia coli	63.73% ± 0.78

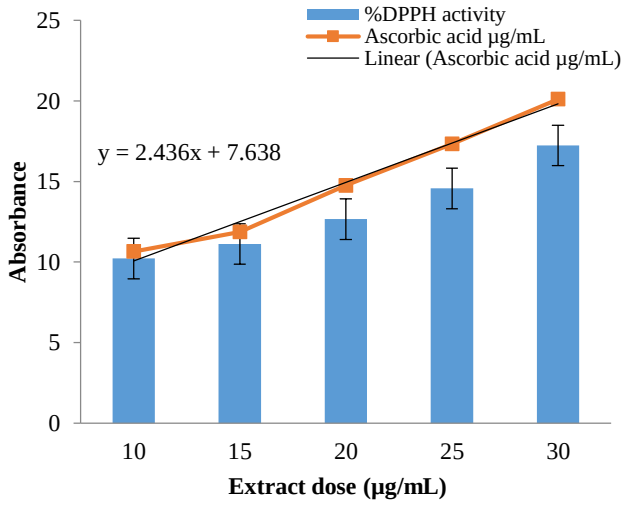

Fig. 1. DPPH free radical scavenging assay of different concentrations of ethanolic extract of *Ficus benjamina* leaves compared with ascorbic acid equivalence (µg/ml).

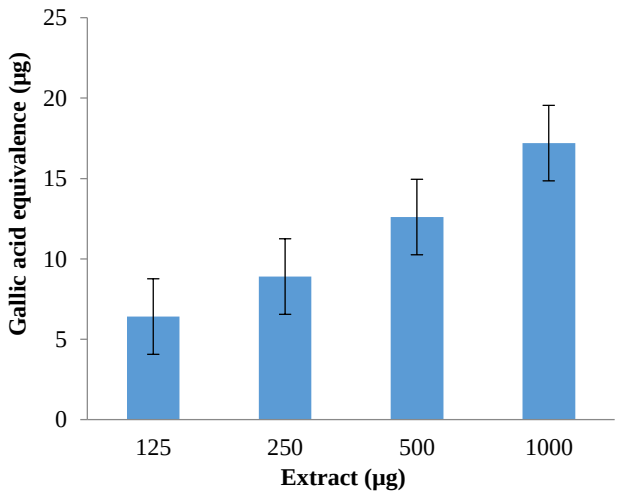

Fig. 2. Total phenolic contents of ethanolic extract of *F. benjamina* leaves. Data is expressesed Mean ± SE with gallic acid equivalence (µg).

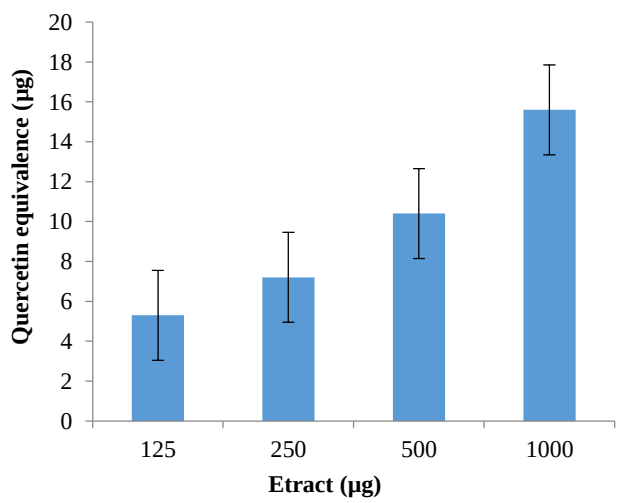

Fig. 3. Total flavonoid contents of ethanolic extract of *F. benjamina* leaves. Results present the data with Mean ± SE in Quercetin equivalence (µg).

Table 3. Ethanolic extract of leaves for Mortality (%) and HA assay of *Ficus benjamina*.

Treatment	Time of embryo death (h)						HA +ve	HA -ve	% Mortality
	0h	24	48	60	72	96			
Uninoculated eggs	0	0	0	0	0	0	0/5	5/5	0
NDV alone	0	1	4	-	-	-	5/5	0/5	100
Extract control	0	0	0	0	0	0	0/5	5/5	0
DMSO + Virus	0	0	5	-	-	-	5/5	0/5	100
30 µg/mL	0	0	0	0	1	0	1/5	4/5	20
60 µg/mL	0	0	0	0	0	0	0/5	5/5	0
90 µg/mL	0	0	0	1	0	0	1/5	4/5	20
120 µg/mL	0	0	1	1	1	0	3/5	2/5	60

Discussion

The bioactive components of natural plants play dynamic role in drug development. Antiviral drugs in the form of secondary metabolites are stored in plants which act as a vital reservoir of natural chemical having antiviral potential (Babich *et al*., 2003). Multiple plant based phytochemicals comprising the flavanoids, terpenoids, polyphenols, furyl compounds, and many others have been isolated to have healing application against wide-ranging viruses. Few of these secondary metabolites or phytochemicals have been extracted from *F. benjamina*. This indicates that the plant is a rich source of phytochemicals which may account for different biological activities like antioxidative, hemolytic, biofilm inhibition and antiviral activity. Phenolic and flavonoids contents exhibit antioxidant activity and protect the animals and human cells from damaging against free radicals. In this present study phenolic and flavonoid content has shown dose dependent pattern. Phenolic compounds has health benefeciary roles because of their antioxidative activities. Various clinical ailments like cancer, AIDS, neurodegenerative and metabolic disorders are due to free radicals. These types of aliments can be treated by antioxidants having scavenging ability (Suresh *et al*., 2008). It is well established that phenolic compounds from natural plants are highly effective in their antioxidative and free radical scavenging potentials (Mohamed *et al*., 2009). The DPPH free radical scavenging procedure is generally used to estimate antioxidant activity of compounds which can reduce DPPH by hydrogen atom donation and creating DPPH-H (Cho *et al*., 2011). A number of workers have used erythrocytes as a model system to study drugs membranes interaction. Hemolytic assays are performed to check hemolytic effect of compounds before establishing their pharmacological preparations. Hemolysis is the cytotoxicity indicator to RBC's. Phytochemical present in extract could have hemolytic activity (Lakshmi *et al*., 2014). Predominantly they can change the membrane structure of erythrocytes and it can easily count the amount of hemolysis in cells (Kumar *et al*., 2011). The leaf extract of *F. benjamina* displayed very low hemolytic effect toward human erythrocytes. The medicinal value of polyphenols in the plants is due to their higher antioxidant nature. For the first time, the chicken *In ovo* technique in present study has revealed, antiviral potential of ethanolic extracts from *F. benjamina* against NDV. 60 µg/mL concentration of leave extract showed the virus growth 0% and stop progression of virus completely while the other doses showed either less inhibition of virus or exert toxicity to embryo. It can be inferred, the extract contains high concentration of phytochemicals as low dose is efficacious while high dose could be lethal to the embryo. The effects of *Aloe secundiflora* against *S. gallinarum* and ND virus in chickens has been studied by Waihenya *et al*., (2002), they observed no therapeutic or prophylactic effects against the virus. Different crude extracts of *Oputia vulgaris, Citrum limon* and *Capsicum flutescens* showed no effect against NDV in poultry (Mtambo *et al*., 1999). Whereas ethanolic extract of *N. latifolia* showed the highest antiviral effect in one hour pre-infection with 12.5 mg of extract per egg with 60% viral reduction studied by Ukamaka *et al*., 2015.

The mechanism of NDV replication inhibition by this extracts is not known and require further investigation. However, inhibition of attachment/ penetration of virus into host cells may be possible mechanisms (Sprawdow, 1999). There is need to identify the active phytochemical(s) responsible for the observed antiviral effects. Further studies need to be done to ascertain the antiviral effect of the plant in live bird. More importantly, there is need to ensure that these plants with promising antiviral activity do not go into extinction with time.

Conclusion

This novel study found the effectiveness of the *F. benjamina* as a possible source of natural antimicrobial, antioxidants, hemolysis and antiviral agents. For more detailed understanding for the distinctive inhibitory effect of *F. benjamina* extracts, it is prerequisite to design advance studies including purification and identification of the antiviral agents present in plant. Consequently, the ethanolic extract of *F. benjamina* could accept for significantly curing or controling several aliments.

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