Research Article

In Vitro Inhibitory Activity of *Justicia adhatoda* Extracts against Influenza Virus Infection and Hemagglutination

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ABSTRACT

The influenza viruses are major etiologic agents of human respiratory infections, and inflict sizable health and economic burden. The present study reports the *in vitro* antiviral effect of *Justicia adhatoda* crude extracts against influenza virus by Hemagglutination (HA) reduction in two different layouts of simultaneous and post treatment assay. The aqueous and methanolic extracts were used for antiviral activity in the non-cytotoxic range. Methanolic extract showed 100% reduction in HA in the simultaneous and post treatment assays at the concentration of 10mg/ml. The aqueous extracts at concentrations of 10mg/ml and 5mg/ml reduced the HA to 33% and 16.67%, respectively, in the simultaneous assay. These results suggest that extracts have strong anti-influenza virus activity that can inhibit viral attachment and/or viral replication, and may be used as viral prophylaxis.

Keywords: Antiviral activity, Cytotoxcity, Hemagglutination, Influenza virus, Justicia Adhatoda.

INTRODUCTION

From a pool of 200 viral respiratory pathogens know, influenza virus is considered to be one of the life-threatening infectious agents as it causes half a million death globally each year.[1] Due to the high mutagenic rate, new virulent influenza strains can arise unexpectedly to cause worldwide pandemics with markedly increased morbidity and mortality such as "avian flu" in 1997 and "swine flu" in 2009.[2] Influenza virus is an enveloped single negative-stranded RNA virus which causes acute respiratory illness and belongs to the family of *Orthomyxoviridae*. Three serotypes of influenza virus (A, B and C) are known which are based on antigenic characteristics of the nucleoprotein and matrix protein antigens. Known symptoms of influenza virus are acute febrile illness with myalgia, headache, and cough. Complications include otitis media, pneumonia, exacerbation of chronic respiratory disease, and bronchiolitis.[3]

Three classes of anti-influenza drugs which are currently being used for chemoprophylaxis and treatment of the infection are: (1) amantadine and rimantadine inhibit viral membrane protein (M2) of proton channel that is necessary for uncoating; (2) oseltamivir and zanamivir inhibit viral neuraminidase (NA) that is necessary for virion release; and (3) ribavirin inhibits enzyme activity for viral replication.[4]

Erratic cases of oseltamivir-resistant pandemic A (H1N1) influenza virus have been reported worldwide.[5] Treatment options are limited in oseltamivir-resistant strains because zanamivir is not licensed for the treatment of children under 7 years old. Recently, research has principally focused on wide-ranging antiviral drugs because of the genetic and antigenic variability of the influenza virus. The expansion in the development of antivirals has gathered pace lately on the host cell proteins which play an important role in viral replication.

The quest for natural inhibitors of virus is very ancient. The search for natural antiviral compounds from plants is a promising approach in the development of new therapeutic agents. In the past century, several scientific efforts have been directed toward identifying phytochemicals capable of inhibiting virus. Knowledge of ethnopharmacology can lead to new bioactive plant compounds suitable for drug discovery and development.[6]

As estimated by World Health Organization (WHO), 80% of population in the developed countries still relies almost on traditional medicine for their primary healthcare needs.[7] India is one of the largest producer of medicinal herbs and is known as the botanical garden of the world.[8] In recent years, the use of herbal drugs worldwide has provided an excellent step forward in India to look for therapeutic lead compounds (phytochemicals) from an ancient system of therapy, i.e. Ayurveda, which can be utilized for development of new drugs.[6]

Justicia adhatoda (L.) *Nees* (family *Acanthaceae*) is a well-known plant in Ayurvedic and Unani medicine, a shrub which is widespread throughout the tropical regions of Southeast Asia.[9] Its leaves have been used extensively for the treatment of respiratory disorders. A diverse array of phytochemical research have been conducted on *Justicia adhatoda* and some of the important activities of the plant include anti-diabetic[10], anti-phlogistic, anti-allergic[11], anti-ulcer[12], antioxidant, anti-genotoxic[13], and many more.[14-16]

The majority of important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, phenolic compounds, and

many more compounds.[17] The alkaloids from *Justicia adhatoda* have reported excellent antibacterial activity against the most resistant bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and the highly pathogenic bacteria like *Salmonella typhi.*[18]

Recent studies have indicated that several plant alkaloids have anti-influenza virus activities.[19] In the previous study, we reported that the methanolic extract of *Justicia adhatoda* was the most active antiviral agent against Herpes Simplex Virus-2 (HSV-2) and aqueous extract against HSV-1.[20] These methanolic and aqueous extracts may contain alkaloids and its derivatives as potent molecular targets against the HSV. Therefore, in this paper we investigate anti-influenza activity of methanolic and aqueous crude extracts obtained from *Justicia adhatoda* and show a comparison between their activities in vitro.

MATERIALS AND METHODS

Plant Material

The plant *Justicia adhatoda* was collected from R.A.Podar Ayurvedic College, Mumbai. The plant was authenticated by comparing with corresponding herbarium specimen at Blatter Herbarium, St. Xavier's College, Mumbai **(Blatter Herbaium specimen no.1503 of H.Santapau)**. Leaves were washed with distilled water, shade dried and powdered.

Preparation of the extract

Thirty grams of the powdered sample was subjected to successive solvent extraction separately with 300ml each of hexane, dichloromethane, methanol, and water at room temperature for 24 hours. The solvent extract obtained was evaporated to dryness in a rotary evaporator in vacuum. Hexane and dichloromethane solvents were used to wash and free the extracts from lipids, fats and waxes. The aqueous and methanolic extracts were used for further tests. The extracts were filtered with Whatman No 1 filter paper and concentrated and reconstituted at 100mg/ml in the Minimum Essential Medium (MEM).

Reagents

All extraction reagents such as dichloromethane, methanol, n-hexane were Analytical reagent (AR) grade. Reagents for cell culture, such as MEM, Trypsin-Ethylenediaminetetraacetic acid (EDTA), and sodium bicarbonate were purchased from Life Technologies (C.A.USA).

Cell Line and Viruses

Madin-Darby Canine Kidney (MDCK) cell lines were procured from Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGI, Lucknow) and were grown in MEM with L-glutamine (2mM), penicillin (100IU/ml), streptomycin (100µg/ml) and gentamicin (10µg/ml), and supplemented with 10% Foetal Bovine Serum (FBS). The standard strain of influenza virus was obtained from the Department of Microbiology, SGPGI, Lucknow.

Confluent MDCK cell monolayers in 96-well tissue culture plates were washed once with serum free MEM before use. Serial 10-fold dilutions of virus in serum-free MEM containing 0.3% bovine serum albumin (BSA) and 1µg/ml L-(toslyamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) were incubated in replicate wells (200µl/well) for 2 to 3 days at 37°C temperature with 5% CO_2. Wells positive for virus growth were identified by the presence of hemagglutinating (HAg) activity in the supernatant, and hemagglutination units (HAU) were calculated. The virus stocks were stored at -80˚C temperature for further use.

Phytochemical analysis of the extract using HPTLC

Phytochemical analysis was performed using a Camag HPTLC system equipped with sample applicator and a Camag TLC scanner at 254nm and 366nm wavelength and data filtering by Savitsky-Goyal 7 in Anchrom Test Lab Pvt. Ltd. (Mumbai). The 3 conditions for sample application through Camag automatic TLC sampler were: Spray gas: Nitrogen (N_2), Sample solvent type: methanol and filling speeds: 15µl/second. Pre-coated silica gel 60G F254 TLC aluminium plates (10x10cm, 0.2mm thick) were obtained from E. Merck Ltd. (Mumbai). Analytical grade toluene, ethyl acetate, methanol, chloroform, glacial acetic acid, diethyl amine and formic acid were obtained from SD Fine Chem Ltd (Mumbai). The Table 1 illustrates the various phytochemicals screened with the respective solvent system and derivatizing agent.

Table 1: List of phytochemicals, solvent systems and derivatizing agents used in HPTLC analysis

Phyto chemicals	Solvent system	Derivatizing agent
Tannins	Toluene:ethyl acetate:formic acid (6:4:0.3)	Ferric chloride
Saponins	Chloroform:acetic acid:methanol: water(6.4:3.2:1.2:0.8)	Anisaldehyde, Sulfuric acid
Flavanoids	Ethyl acetate:formic acid:glacial acetic acid:water (10:0.5:0.5:1.3)	Anisaldehyde solution
Alkaloids	Toluene:ethylacetate:diethylamine(7:2:1)	Dragendroffs reagent

Cytotoxicity Assessment

The evaluation of cytotoxic activity of plant extracts (CC_{50}) was carried out using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MDCK cells were cultured onto 96 well plate at the density of 1.0 x 10^5 cells/ml. Different concentrations (10mg/ml to 0.01mg/ml) of aqueous and methanolic crude extract were added to each culture wells at a final volume of 100µl, in triplicate, adding dimethyl sulfoxide (DMSO) as a negative control. After incubation at 37˚C temperature with 5% CO_2 for 16 to 18 hours, 10% of 5mg/ml MTT (100µl) was added to each well. After 4 hours of further

incubation at 37˚C temperature, the formazan was solubilised by adding DMSO to each well and the absorbance was read at 550nm by an ELISA reader.[21]

Percent cytotoxicity was calculated using following formula:

Percent Cytotoxicity = 100 – Percent Cell Survival.

Percent Cell Survival = {(At- Ab) / (Ac-Ab)} x 100

Where,

Absorbance value of test compound - At

Absorbance value of blank - Ab

Absorbance value of control - Ac

Antiviral Assays

a) Simultaneous Treatment assay

In simultaneous treatment assay, 50µl of virus (64 HAU) was first exposed to 50µl of different dilutions of plant extract prepared in MEM without phenol red (aqueous and methanolic extracts) and was incubated at 37°C temperature for 1 hour. Following incubation, 100µl of the above mixture was added to the 96 wells plate containing confluent monolayer of MDCK cell line (1×10^5cells/well). After 1 hour of incubation at 37°C temperature, the supernatants were removed and the cells were washed with MEM. After washing, the media was discarded and then 100µl of virus growth medium was added and the plate was kept at 37ºC temperature in 5% CO_2 incubator. [22]

b) Post Treatment assay

In post treatment assay, confluent monolayer of MDCK cell line was washed twice with 50µl of virus growth medium and then the medium was removed and 100µl of virus (64 HAU) was added to the 96 wells plate. The virus was allowed to adsorb for 1 hour at 37ºC temperature in the 5% CO_2 incubator. After incubation, the virus was removed from each well by washing with MEM. The media was then removed and 100µl of different dilutions of plant extracts prepared in virus growth medium was added to the monolayer and the plate was kept at 37ºC temperature in 5% CO_2 incubator. [22]

Hemagglutination assay

For carrying out hemagglutination assay, 'V bottom' 96 well microtiter plate was used and 50µl phosphate buffer saline (pH=7.2) was added as a diluent in each well by using a multichannel auto pipette. A 50µl of sample (cell free supernatant of simultaneous and post treatment assay) was added in the first well of each row. Two fold dilutions of the sample were made by transferring 50µl suspension from the first well of each column to the next well by using a multichannel auto pipette. This procedure was repeated till the last column of the 96 well microtiter plate. After serially diluting the sample, 50µl of 0.75% guinea pig RBCs was added to each well and the plate was incubated at 4ºC temperature for 1 hour. After incubation, cell control was checked for complete settling of RBCs and results of hemagglutination assay i.e. the virus titer were recorded as hemagglutination units (HAU). [23]

The HAU was calculated using the following formula

Percent (%) $\log_2$HAU reduction= (1 - A / B) X 100

Where,

A - $\log_2$HAU titer of virus control

B - $\log_2$HAU titer of sample.

Statistical Analysis

Sampling proceeded on three independent replication (n=3) for each test. Data were subjected to Graph Pad Prism *v*5.04 and *v*6.0 [24] and the HAUs were calculated by two-tailed t-test with $p<0.05$ as significance.

RESULTS

i. Phytochemical Analysis

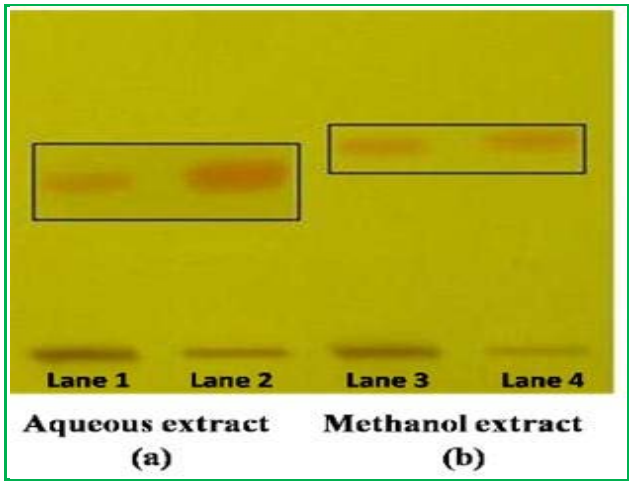

Figure 1: Quantification of Alkaloids in both Aqueous and Methanolic Extracts

The Figure 1 shows the plate after derivatization with ferric chloride. The plates were viewed under White Light. Lane 1 (10µl) and Lane 2 (20µl) were of aqueous extract and Lane 3 (10µl) and Lane 4 (20µl) of methanolic extract.

Calculation of alkaloid vasicine by comparing the extracts to the standard vasicine hydrochloride as per HPTLC graphs obtained from Anchrom Test Lab Pvt. Ltd:

Concentration of sample used: 100mg/ml

Application volume: 20µl

Concentration of standard used: 0.5mg

Area of standard: 12991

Area in aqueous sample: 12049.8

Therefore 0.023% of the extract is alkaloid vasicine

Area in methanolic sample: 13997.5

Therefore 0.026% of the extract is alkaloid vasicine.

ii. Cytotoxicity

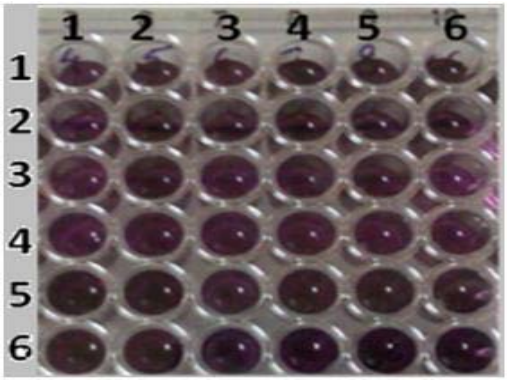

Figure 2: Cytotoxicity Assessment of Plant Extracts by MTT

The Figure 2 shows concentration of the plant extracts in the range of 10mg/ml to 0.1mg/ml. These concentrations were found to be non-cytotoxic and thus CC50 could not be calculated. Column 1 is the cell control; columns 2 to 6 contain 10mg/ml to 0.1mg/ml; rows 1 and 2 contain methanolic extract in duplicates; rows 3 and 4 are cell controls and rows 5 and 6 contains aqueous extract.

iii. Antiviral Assay

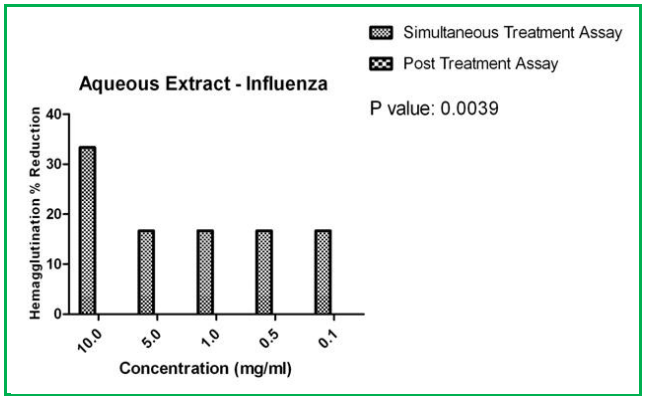

Figure 3: Hemagglutination Percent Reduction vs Concentration of Aqueous Extract

The Figure 3 shows the hemagglutination percent reduction vs concentration of aqueous extract of *Justicia adhatoda* on the simultaneous and post treatment assay.

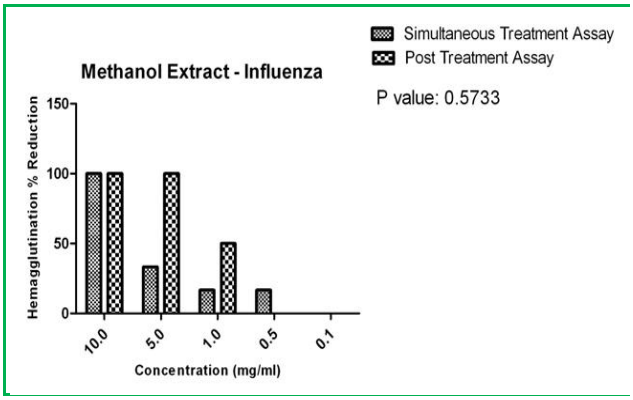

Figure 4: Hemagglutination Percent Reduction vs Concentration of Methanol Extract

The Figure 4 indicates the hemagglutination percent reduction vs concentration of methanol extract of *Justicia adhatoda* on the simultaneous and post treatment assay.

DISCUSSION

Influenza virus continues to emerge and re-emerge and remains a major public health concern.[25] As an alternative to chemically synthesized antivirals such as amantadine[26] or oseltamivir[27], many plant extracts, and purified substances like phytochemicals have been tested and reported to have selective antiviral activities inhibiting influenza viruses.[28, 29] In a similar manner within the reach for identifying novel antiviral substances of plant origin, the antiviral potential of crude extract of leaves of *Justicia adhatoda* was tested against influenza virus in the present study which seems to be the first report on antiviral activity of *Justicia adhatoda* against influenza virus.

The phytochemical analysis of *Justicia adhatoda* plant shows that phenols, tannins, alkaloids, anthraquinone, saponins, flavonoids, and reducing sugars are found in the leaves.[30] However, the pharmacologically studied chemical component in *Justicia adhatoda* is a bitter quinazoline alkaloid, vasicine (1,2,3,9-tetrahydropyrrole [2,1-b] quinozolin-3-ol, $C_{11}H_{12}N_2O$) which is found in the leaves, roots, and flowers. Besides vasicine, the leaves also contain several other alkaloids (vasicinone, vasicinol, adhatodine, adhatonine, adhvasinone, anisotine, and hydroxypeganine), betaine, steroids, and alkanes.[31,32]

In the previous study, we reported the qualitative presence of tannins, flavonoids, alkaloids, and saponins as the major phytochemicals in the plant extracts by performing HPTLC.[20] In this report, we carried out the further quantitative analysis of vasicine as it is the major alkaloid present in the leaves of *Justicia adhatoda*. Our analysis showed that 0.023% and 0.026% of the standard vasicine was present in the aqueous and methanolic extracts, respectively as shown in Figure 1.

In the present study, both methanolic and aqueous extracts were non-cytotoxic in the concentration range of 10mg/ml to 0.01mg/ml and as these extracts were non-cytotoxic, a CC50 for the same was not calculable as shown in Figure 2. This indicated that above range of concentration of the extract could be used for further antiviral assay. Further repetition of the assay can be carried out to find out the toxic concentration above 10mg/ml.

The influenza virus replication cycle can be divided into 5 steps: 1) binding of viral hemagglutinin to sialic acid (SA) receptor on host cell surface (adsorption step), 2) internalization of virus by receptor-mediated endocytosis and fusion of viral HA2 with endosomal membranes triggered by influx of protons through M2 channel (endocytosis and fusion step), 3) release of viral genes into the cytoplasm (uncoating step), 4) packaging of viral proteins with viral genes after viral RNA replication, transcription and translation, and budding of

new viruses (packaging and budding step), and 5) release of new viruses by sialidase cleaving SA receptors (release step).[33,34]

In the present study, anti-influenza activity was carried out by simultaneous and post treatment assays. Simultaneous anti-influenza treatment was used to identify whether *Justicia adhatoda* extracts block the viral adsorption to cells. As observed in Figure 3 and Figure 4, in simultaneous assay, 33% reduction in HA was observed at the concentration of 10mg/ml in aqueous extract and further only 16.67% reduction was observed from 1mg/ml to 0.1mg/ml. Whereas, 100% reduction was observed in methanolic extract at concentration of 10mg/ml. As the concentration decreased, the percent HA reduction also decreased to 33.34% at 1mg/ml to 16.67% at 0.5mg/ml. These data suggest that aqueous and methanolic extracts may directly interfere with viral envelope protein and not with the SA receptor at the cell surface.

To evaluate the anti-influenza activity after virus infection, we employed the post treatment assay. In the post treatment assay, the aqueous extract did not show any percent inhibition in hemagglutination units, whereas the methanolic extract showed 100% reduction at the concentrations of 10mg/ml and 5mg/ml. As the concentration further decreased to only 1mg/ml, 50% HA reduction was observed as shown in Figure 4. We found that only methanolic extract inhibited influenza virus infection suggesting the possible ways of viral inhibitions by blockage of viral attachment by inhibition of viral HA protein.

A similar work previously reported in which the simultaneous exposure assays were used to identify whether the extracts blocked the viral adsorption to cells, by synergistically binding to the free virus particles or by blocking the sialic acid receptors to prevent virus entry into the cells. [35] From the post exposure treatment, they concluded that the extracts may be inhibiting the replication of influenza virus or virus budding from the infected MDCK cells.

Previous reports on alkaloids like pavaine alkaloid (–)-thalimonine (Thl), isolated from the Mongolian plant *Thalictrum simplex* markedly inhibited the reproduction of influenza virus in cell cultures.[36] One more scientific research on alkaloid as potent anti-influenza is from *Mahonia bealei* (Fort) plant in which roots are of clinical importance which contain bisbenzylisoquinoline as the chief alkaloid.[37] The research conducted on alkaloids as antiviral agents is limited except for the few mentioned above. We report that methanolic extract of *Justicia adhatoda* contains vasicine as a principle compound and has a potent antiviral activity against influenza virus

CONCLUSION

The current study explores the potential of crude extracts of *Justicia adhatoda* as valuable antiviral agent and provides the scientific basis for promising therapeutic use for influenza virus. Treatment with synergistically active antiviral compound that have diverse mechanism of action may provide several advantages such as greater potency, fewer side effect and toxicity, and better clinical studies over single compound treatment. The present findings persuade the need for clinical studies to investigate the therapeutic and prophylactic potential of extracts of *Justicia adhatoda* and to extend this study to other viruses.

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