



In vitro antiviral activity of *AegleMarmelos* against Influenza A (H1N1) Pdm09

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ABSTRACT

Due to the high prevalence of viral infections having no specific treatment and the constant appearance of resistant viral strains, the development of novel antiviral agents is essential. The aim of this study was to evaluate the antiviral activity of aqueous and ethanolic extracts of *Aeglemarmelos* against Influenza A (H1N1) pdm09. The extract was subjected to cytotoxicity as well as antiviral activity on MDCK cells. The concentration range from 10 to 100µg/ml in MDCK cell line was conducted. It showed that the treated cells with drug was nontoxic to the cells at the concentration of 50µg/ml for ethanolic and 60µg/ml for aqueous extract at 48 hrs to 72 hrs and was comparable to that of controls. Hence crude extract of both ethanolic and aqueous up to 50-60µg/ml (CC50) could be used to test the antiviral activity without affecting much of the cell viability. In simultaneous anti viral assay, 80% viral inhibition was observed at the concentration of 50µg/ml in aqueous extract whereas 100 % viral inhibition was observed in ethanolic extract at concentration of 60µg/ml. In the post treatment assay, the aqueous extract did not show viral inhibition, whereas the ethanolic extract showed 100% reduction at the concentrations of 60µg/ml. This data suggest that ethanolic extract inhibit the Influenza virus infection by blockage of viral attachment by inhibition of viral HA protein, this is consistent with previous studies.

Keywords: *Aeglemarmelos*, cytotoxicity, Antiviral Activity, Influenza A.

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INTRODUCTION

Influenza viruses are extremely infective agent to cause frequent epidemics and pandemics in humans. Yearly about 10% of population were infected and the number of deaths occurs (about 250,000) worldwide. The Influenza virus comes under *Orthomyxoviridae* and further classified into 3 types (Influenza A, B and C), Influenza A and B viruses spread all over the world and causes epidemics and pandemics through antigenic drift and shift. Due to the elevated mutagenic rate, novel virulent influenza strains can occur unpredictably to cause global pandemics with noticeable increase in morbidity and mortality such as

avian Influenza virus (H5N1) in 1997 and Influenza A (H1N1) pdm09 in 2009. Annual vaccination is the primary strategy to prevent the influenza virus infection, but it needs continues surveillance to make vaccines based on the circulating virus strains.^[1] Antiviral drugs are representing the first line of defense to pandemic infection with a novel strain. Currently existing Influenza drugs are to block the viral replication and viral spread. Adamantanes referred as first generation drugs to Influenza virus and act on the viral M2 protein by blockers the ion channels.

Adamantanes side effects associated with the gastrointestinal tract, central nervous system and also develop the resistant during treatment and it act on Influenza A viruses only. Neuraminidase inhibitors (NAI) are the second generation drugs to Influenza virus, these are oseltamivir and zanamavir. Zanamavir is a sialic acid analogue, and oseltamivir inhibit the sialidase activity of the viral neuraminidase by binding to active site of NA. Side effects of Neuraminidase inhibitors are vomiting, nausea, diarrhea, abdominal pain, headache, dizziness and sinusitis, additionally Influenza A (H1N1) pdm09 virus was resistant to oseltamivir, have been reported worldwide . This data suggest that necessity of next generation (third generation) drugs to treat the Influenza virus with a diverse mode of action. Natural products, especially obtained from plant source can be identified as next generation antiviral.^[2]

At present, plant resources are relatively unlimited for antiviral herbal medicine, but these resources are

decreasing quickly due to deforestation and industrialization. Although a number of studies have been executed the use of purified plant parts, only a few studies have answered the antiviral activities of crude plant extracts.

As estimated by WHO, 80% of population in the developed countries still relies almost on traditional medicine for their primary healthcare needs. India is one of the largest producer of medicinal herbs and is known as the botanical garden of the world. A number of plants have been shown to possess antiviral activities. *Aeglemarmelos* (L.) whose leaves, flowers, fruits and roots are widely used for medicinal value compounds against cold, cough, whooping cough, chronic bronchitis and asthma as narcotic, expectorant and antispasmodic as conventional medicines. In the present study, we have focused on the fruits of *Aeglemarmelos* (L.) crude extract, which were screened for antiviral activity against Influenza A (H1N1) pdm09.

Cell lines

Madin Darby Canine Kidney (MDCK) cell lines were obtained from National Centre for Cell Science (NCCS) Pune, India and were grown. For all the experiments, controls were included (cell control, virus control and positive control).

Influenza Virus and Titration

The Influenza A (H1N1) pdm09 strain A/India/Pune 153793/2015(H1N1) was obtained from National Institute of Virology (NIV) and it was included as a positive control and Chennai strain A/India/Che147506/2015(H1N1) confirmed in NIV was used for antiviral activity. These strains were grown in MDCK cells. After CPE appear, supernatants were collected and virus titer (TCID₅₀) was determined using with standard protocol.

Cytotoxicity Assay

Cytotoxicity assay was performed on MDCK cell lines to determine the drug (ethanolic extract and aqueous extract) 50% Cytotoxic Concentrations (CC₅₀) using with standard protocol. Briefly, after forming a confluent monolayer, the media were removed and by the addition of 100 µl of plant extract (dilutions ranging from 10-100µg/ml) and incubate 37°C with 5% CO₂ for 72 hours. After 72 hours, MTT assay was performed (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) (MTT, HiMedia, RM1131) and optical density (OD) was measured at 620 nm using an ELISA micro plate reader (Thermo Multikan EX, USA) and calculate the 50% Cytotoxic Concentrations (CC₅₀).^[3]

Antiviral assay

Antiviral activity was carried out by simultaneous and post treatment assays.

Virus binding or Simultaneous Treatment assay

Simultaneous anti influenza treatment assay was performed to identify, whether extracts block the viral adsorption to cells, using a standard protocol. In briefly, different concentrations of ethanolic (50, 55 and 60µg/ml), aqueous extracts (40, 45 and 50µg/ml) were mixed with virus (1TCID₅₀) and incubated for one hour at 4°C. The mixture was added to the MDCK cell line in triplicates along with cell control, drug control and virus control and wait for one hour with irregular shaking⁴. After one hour, remove the solution, add MEM media with 2µg/ml TPCK and incubate for 72 hours at 37°C under 5% CO₂ and every day observed cells under light microscope. After 72 hours to estimate the cell viability by performing the MTT assay.

To verify the effect of extracts on virus adsorption, Haemagglutination inhibition (HAI) test was performed. Before performing the HAI, plant extracts were treated with Receptor Destroying Enzyme (RDE), the purpose of this treatment was to remove the compounds that may having sialic acid like structures which mimic the receptors of RBC and compete for haemagglutinin.

Penetration or Post Treatment assay

Post Treatment assay was performed to identify the antiinfluenza activity after virus infection, using a standard protocol. In briefly, Influenza virus (1TCID₅₀) was added to the MDCK cell line and wait for one hour with irregular shaking. After one hour, the media was removed and substituted with MEM media containing different dilutions of drug with 2µg/ml TPCK were added in triplicates along with cell control, drug control and virus control. Incubate for 72 hours at 37°C under 5% CO₂ and every day observed cells under light microscope. After 72 hours to estimate the cell viability by performing the MTT assay.^[5]

Statistical analysis

Statistical Analysis was carried out by using the SPSS software version 15.0 (SPSS Inc., Chicago, USA). For data analysis, Chi-square test was used, where $P < 0.05$ was considered statistically significant.

Anti viral activity

Aqueous and ethanolic extracts of *Aeglemarmelos* screened for Influenza A (H1N1) pdm09 (A/India/Che147506/2015(H1N1) strain) virucidal activity. These extracts were subjected to cytotoxicity as well as antiviral activity on MDCK cells.

Cytotoxicity studies of plant extracts

The studies on cytotoxicity of ethanolic and aqueous extract of *Aeglemarmelos* at the concentration range from 10 to 100µg/ml in MDCK cell line were conducted. It showed that the treated cells with drug were nontoxic to the cells at the concentration of 50µg/ml for ethanolic and 60µg/ml for aqueous extract at 48 hrs to 72 hrs and was comparable to that

of controls (untreated and DMSO treated cells). However cells treated with 50 μ g/ml of the ethanolic extract and 60 μ g/ml of aqueous concentration and above exhibited toxicity as morphological changes observed as loss of monolayer granulation and vacuolation in the cytoplasm and cell damage when compared to wells treated with 50 μ g/ml and less. (Figure 1, Figure 2, Figure 3) Hence crude extract of both ethanolic and aqueous up to 50-60 μ g/ml (CC50) could be used to test the antiviral activity without affecting much of the cell viability.

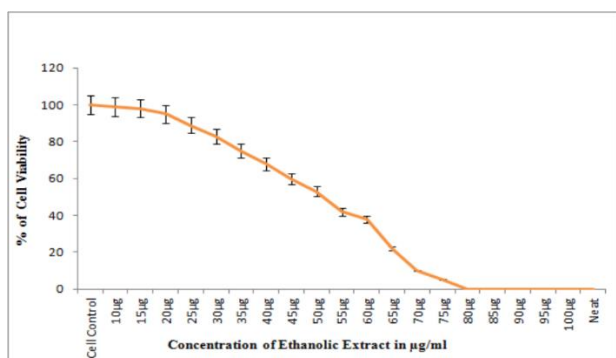


Figure 1: CC50 Concentration of Ethanolic Extract

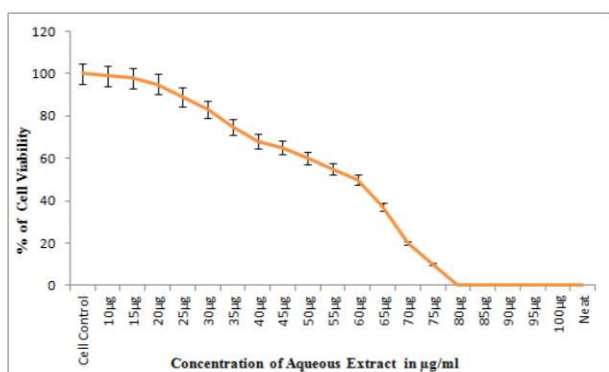


Figure 2: CC50 Concentration of Aqueous Extract



Figure 3: Cytotoxicity Effect of *Aeglemarmelos* extracts on MDCK cell Line

Measurement of Tissue Culture Influenza Virus Infectious Dose (TCID50)

The Influenza A/India/Che147506/2015(H1N1) strain was used to quantify the viral titer by TCID50 on MDCK cell lines for antiviral activity. CPE was expressed with a "+". No CPE was observed at high dilutions in all the wells and CPE was observed at low dilutions in all the wells (Table 1). At particular virus dilution (10⁻⁵ dilution) half of the cell cultures showed CPE. This is the end point which was defined as the dilution of virus at which 50% of the cell cultures are infected. Estimate the cell viability by performing the MTT assay. This number can be calculated and expressed as 50% infectious dose (ID50) per milliliter.

RESULTS AND DISCUSSION

Antiviral activity

In the present study, both ethanolic and aqueous extracts were non cytotoxic in the concentration range of 10-60 μ g/ml (Figure 1 and Figure 2). This indicated that below the concentration of the extract could be used for further antiviral assay.

Inhibitory activity of *Aeglemarmelos* on Influenza virus binding to cell receptors

In order to test the ability of the ethanolic and aqueous extracts to preventing the attachment of influenza virus to MDCK cells, we used and analysed the simultaneous treatment assays. The results showed that 80% viral inhibition was observed at a concentration of 60 μ g/ml in aqueous extract whereas 100% viral inhibition was observed in ethanol extract at concentration of 50 μ g/ml as showed in Fig 4. As the concentration decreased, the viral inhibition also decreased.

Inhibitory activity of *Aeglemarmelos* on Influenza virus Replication

Post treatment assay carryout to estimate the anti-influenza activity after virus infection. In the post treatment assay, the aqueous extract did not show any inhibition whereas the ethanol extract showed 100% viral inhibition at concentration of 50 μ g/ml showed in Fig 5. As the concentration decreased, the viral inhibition also decreased.

Influenza virus carryon to emerging remerging and remains a most important public health concern. As a substitute to chemically synthesized antivirals such as amantadine or oseltamivir. Many plant extracts and purified substance phytochemicals have been tested and reported to have selective antiviral activity inhibiting Influenza viruses.^[6] In a similar way within the reach for identifying new antiviral substances of plant origin, the antiviral potential of crude extract of *Aeglemarmelos* was tested against Influenza virus in the present study. The results of the phytochemical analysis revealed that alkaloids, phenols, tannins, saponins, anthraquinones, amino acids, flavonoids, and reducing sugars are present in the

fruits of *Aeglemarmelos*. In the present study, both ethanolic and aqueous extracts were non-cytotoxic in the concentration range of 10-50µg/ml his indicated that above range of concentration of the extract could be used for further antiviral assay.^[7]

The Influenza virus replication cycle can be divided into 5 steps: 1. Adsorption 2. Endocytosis and fusion, 3. Uncoating, 4. Packaging and budding and 5. Release. In the current study, antiviral activity was carried out by simultaneous and post treatment assays.^[8] Simultaneous anti-influenza treatment assay was performed to identify, whether *Aeglemarmelos* extracts block the viral adsorption to cells.^[9] In simultaneous assay, 80% viral inhibition was observed at the concentration of 50µg/ml in aqueous extract whereas 100 % viral inhibition was observed in ethanolic extract at concentration of 60µg/ml. These data suggest that aqueous and ethanolic extracts may directly interfere with viral envelope protein and not with the SA (sialic acid) receptor on the cell surface. To evaluate the antiinfluenza activity after virus infection, we performed the post treatment assay¹⁰. In the post treatment assay, the aqueous extract did not show viral inhibition, whereas the ethanolic extract showed 100% reduction at the concentrations of 60µg/ml. This data suggest that Ethanolic extract inhibit the Influenza virus infection by blockage of viral attachment by inhibition of viral HA protein, this is consistent with previous studies.

CONCLUSION

Conclusion of the study revealed the *Aeglemarmelos* fruit crude extract could be a potential resource for novel antiviral drugs. The plant extracts investigated could serve as capable candidates for the development of third generation anti influenza drugs, thus challenging the neuraminidase drug resistant viruses in an attempt to safeguard human health and the global economy. Treatment with synergistically active antiviral compound that have diverse mechanism of action may provide several advantages such as greater potency, fewer side effects 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 *Aeglemarmelos* and to extend this study to other respiratory viruses.

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