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Flow cytometric evaluation of anti-viral activity of *Aegle marmelos* against Newcastle disease virus

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A B S T R A C T

In the present study, *Aegle marmelos,* medicinal plant was screened for antiviral activity against Newcastle Disease Virus (NDV). The results showed that aqueous leaves extract of *Aegle marmelos* showed inhibitory activity in forward (shape and size) and side scatter (granularity of the cell) at higher doses (10 mg) in chicken lysed whole blood which is confirmed through flow cytometer. In addition, leaves aqueous extract showed rapidly decline in the proliferative response at higher doses as compared to NDV. Results indicated that *Aegle marmelos* leaves aqueous extract has antiviral property against NDV.

Keywords: *Aegle marmelos,* flow cytometer, Newcastle disease virus

A R T I C L E I N F O

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1. Introduction

Medicinal plant products are a source of many traditional medicines and even some synthetic herbal medicines [1]. These medicinal plants are generally or commonly used to treat number of diseases e.g. virus, bacteria, fungi etc

especially in some parts of the world including India. Now a day, modern herbal medicines have been widely used all over the world [2, 3]. Most of the industries especially pharmaceutical still relied on the diversity of primary and secondary metabolites present in the extract e.g. aqueous, alcoholic, chloroform, methanolic etc [4,5]. These metabolites have the capability to synthesize aromatic substances and these are used by medicinal plants which act as defensive weapon against number of harmful pathogens [5]. In general, most of the medicinal plants showed number of immunopharmacological activities such as immunomodulatory [6], anti-inflammatory [7], adjuvant activity [8] etc. In order to determine the anti-viral activity of medicinal plants, several plants have been tested for its efficiency as anti- viral agents, partly based on information concerning plants that have traditionally been used as medication to treat various human diseases. The selection of medicinal plants gives better criteria for screening its anti-viral properties against number of infectious micro-organisms or pathogens [9].

Newcastle disease virus (NDV, single stranded RNA; helical capsid), which belongs to the family *Paramyxoviridae* and genus Aulavirus. This disease showed serious burdens in the poultry industry because of its contagious and mortality records [10, 11]. Thus there is a need to search for those medicinal plants to inhibit NDV; among that *Aegle marmelos* (commonly known as bael) is one which belongs to the family *Rutacae*. The most commonly phytoconstituents isolated from various part of the *Aegle marmelos* i.e. leaves (citral, lupeol, cineol etc); stem bark (Fagarine, marmin etc) and fruits (tannin, marmelide etc). These medicinal plant products are generally used for various therapeutic purposes i.e. asthma, anemia, healing of wounds, jaundice, typhoid etc [12, 13, 14]. In addition, the leaves of this medicinal plant are generally used to treat inflammation, asthma, hypoglycemia, febrifuge, hepatitis and analgesic [14]. Recently, the strategy for research and *in vitro* evaluation of immunopharmacological activity of medicinal plant products has changed in the last few years. One of the recent development i.e. flow cytometer is the highly automated bioassay screening method for the evaluation of immunopharmacological studies of leaves aqueous extract of *Aegle marmelos*. The present investigation is to estimate the anti-viral activity of leaves aqueous extract of *Aegle marmelos* on chicken lysed whole blood against NDV using flow cytometer.

2. Materials and Methods

2.1. Plant collection

Fresh leaves of plant *Aegle marmelos* were collected from the garden of Vidya Pratishthan's School of Biotechnology, Baramati, District Pune, Maharashtra.

2.2. Preparation of aqueous extract

Weigh 10 g of fresh plant leaves obtained from the plant *Aegle marmelos* were dried in a shady area for 8 h and then macerated with liquid nitrogen to prepared fine powder. The powdered plant material was dissolved in 100 ml of phosphate buffered saline (PBS) to a final concentration of 100 mg/ml with continuously stirring. Afterwards, the aqueous extract sample was centrifuged at 15,000 rpm for 10 minutes at 4 °C. After centrifuging, the supernatant was collected and stored at -20 °C, until used for various immunopharmacological assays for anti-viral studies.

2.3. Qualitative and quantitative (HPTLC, high performance thin layer chromatography) analysis

To determine the presence of secondary metabolites in the leaves aqueous extract of *Aegle marmelos*, different tests were performed. During qualitative based assay, the leaves aqueous extract showed the presence of terpenoids (Absolute Alcohol extraction); flavonoids (Methanolic extraction) and phenolics (lead acetate test). In addition, quantitative based assay is estimated through HPTLC (upgraded test of TLC, thin layer chromatography).The retardation factor of terpenoids in leaves aqueous extract is 0.92.

2.4. Sample collection of Newcastle disease virus (NDV)

The samples of NDV suspected birds were collected under surveillance of diseases caused in animals in Baramati taluka programme of "BIO-VILLAGE" scheme of Vidya Pratishthan's School of Biotechnology. The collection of swabs (oro-pharyngeal and cloacal) from live ailing birds and also collects the organs (i.e. liver, trachea, brain) of dead birds and stored in phosphate buffered saline (pH, 7.2 – 7.4) including antibiotics [9]. The samples of suspected birds were pooled or mixed together and centrifuged at 10,000 rpm for 10 minutes at 4 °C. The supernatant of tissue extract were collected and stored in antibiotic solution and used for inoculation of specific pathogen free (SPF) embryonated chicken eggs.

2.5. Isolation and propagation of NDV in embryonated chicken eggs (ECE).

The SPF embryonated chicken eggs (9-11 day old, allantoic cavity route; purchased from Venkys India Ltd) were used for isolation and propagation of NDV from field samples. After several observations of SPF eggs using candle, only bigger sized embryos selected for inoculation of virus samples. The criteria for inoculation below the air cell in the absence of blood vessels (3-4 mm below the air cell). After disinfection of egg shell with spirit, 200 µl of supernatant was inoculated at 45^0 angle into embryonated chicken eggs. Embryo motility was observed every 8 - 10 hours by candling. After the death of embryos, amnio-allantoic fluid was harvested and checked for presence of virus.

2.6. Titration of virus

For the confirmation of virus present in the amnio-allantoic fluid, hemagglutination test was performed. The titre observed was 64 HA unit. The virus is aliquoted into 2 ml vials and stored at - 40^0 C. The working dilutions of virus namely 1:10, 1:100, 1:300 and 1:500 were made in 1X sterile PBS and used further for immunopharmacological studies.

2.7. Chicken blood samples

In order to determine the anti-viral activity of *Aegle marmelos*, non-infected EDTA blood samples (collected from poultry in the Baramati region, Maharashtra) of chicken have been used as experimental animals for

immunopharmacological studies against Newcastle disease virus (NDV).

2.8. Flow cytometric analysis in whole blood of chicken

The *in vitro* inhibitory potential of leaves aqueous extract of *Aegle marmelos* was evaluated in chicken whole blood and was incubated with serial dilutions of NDV. After getting the optimized dose of NDV, the blood samples of chicken were treated with variable doses of leaves aqueous extract i.e. 0.312 – 10 mg of *Aegle marmelos*.

To evaluate the effect of leaves aqueous extract of *Aegle marmelos* on chicken whole blood, lysed the whole blood with 1 - 2 ml of FACS lysing solution/red cell lysis buffer/ACK lysing solution by centrifuging for 5 minutes at 3000 rpm at 4 °C. The supernatant was removed and washed 2-3 times with phosphate buffered saline (PBS). Experiments were performed in duplicates (firstly, flow cytometer and secondly for cytotoxicity assay), Cell suspension of chicken lysed whole blood (10^6 cells/ml, 100 µl) was pipetted into 96 well plates along with serial dilutions of aqueous extract of *Aegle marmelos* (0.3 - 10 mg) containing NDV. For these studies, NDV (1:100 dilution) was used as standard for these immunopharmacological studies. Incubate the plates for 24 h at 37 °C, 5 % carbon dioxide incubator.

The numbers of lymphocytes, monocytes and granulocytes count in chicken lysed whole blood and these samples were analyzed through flow cytometer (FACS Calibur). Similar experiments were performed to measure the forward (shape and size) and side (granularity of the cell) and these studies were analyzed through flow cytometer [6, 7, 8]. In another set of experiment, add MTT solution (5 mg/ml, 10 µl) on chicken lysed whole blood along with aqueous extract of *Aegle marmelos* (0.3 - 10 mg) containing NDV and incubated for 4 h. The plates were centrifuged at 1800 rpm for 10 minutes and then the supernatant was discarded. Add 100 µl of DMSO solution to the formazon crystals and the absorbance was evaluated in an ELISA reader at 570 nm [9].

2.9. Statistical analysis

Values are expressed as Mean ± S.E. The difference between the control and treated groups is determined through One way Anova test i.e. Boniferroni multiple comparison test

3. Results and Discussion

3.1. Determination of optimized dose of NDV on chicken whole blood

The effect of variable doses of NDV (1:10, 1:50; 1:100; 1:500 and 1:1000) on chicken whole blood as shown in **Fig.1**. To determine the optimized dose of NDV, the flow cytometric results showed that NDV at a concentration 1:100 showed markedly increased in the number of monocytes as well as granulocytes count as compared to control.

3.2. Flow cytometric analysis (Forward and side scatter)

The effect of leaves aqueous extract on forward (shape and size) and side scatter (granularity of the cell) as shown in **Fig.2**. The results showed that the leaves aqueous extract of *Aegle marmelos* showed rapidly decline in the count of forward as well as side scatter at higher doses. NDV (1:100 dilution) used as standard for these studies and results showed that there is enormous increased in forward and side scatter as compared to control. In comparison with NDV, *Aegle marmelos* at higher doses showed anti-viral activity.

3.3. Cytotoxicity assay

The effect of variable doses of leaves aqueous extract of *Aegle marmelos* (0.312 – 10 mg) on chicken lysed whole blood containing NDV (1:100) as shown in **Fig .3**. The results showed that the leaves aqueous extract of *Aegle marmelos* at higher doses showed significantly decline in the proliferative response (which is determined through MTT) as compared to control. NDV (1:100 dilution) used as standard for these studies and the results showed that there is significant increase in proliferative response as compared to control. Overall, the data suggests that the leaves aqueous extract of Aegle *marmelos* at higher doses showed anti-viral activity.

Discussion

In this study, our group focused on the anti-viral activity of leaves aqueous extract of *Aegle marmelos* and the results were presented or depicted in the respective figures. The preliminary investigation studies of *Aegle marmelos* were performed with respect to qualitative and quantitative tests in order to determine the presence of secondary metabolites present in the aqueous extract. The results showed that the *Aegle marmelos* aqueous extract leaves showed qualitatively and quantitatively the presence of flavonoids, terpenoids and phenolics which is determined through HPTLC. This technique is very important in order to obtain the reliable information about the pharmacologically active components of the medicinal plant products and also standardized the medicinal drug preparation for the proper identification of medicinal plants. In this study, influence of leaves aqueous extract of *Aegle marmelos* that have shown anti-viral activity against NDV. The results obtained from this preliminary study indicate that the aqueous extraction of *Aegle marmelos* exerted an antiviral effect against NDV on the *in vitro* proliferation chicken lysed whole blood with a dosage-dependent relationship which is determined through flow cytometer. Flow cytometry is one of the major as well as crucial immunological methods for the assessment of normal blood counts in chicken/human/animal whole blood which gives the information related to immune system in case of healthy animals and human [15, 16]. Now a day, flow cytometry is routinely used in clinical or research laboratories for the assessment of blood counts, surface markers, cell cycle analysis etc [15, 16]. As per the principle of flow cytometer with respect to forward (shape and size) and side scatter (granularity of the cell) is concerned, dead cells have lower forward scatter and higher side scatter in comparison to live cells where as debris can be easily differentiated from single cells in terms of size which is measured through forward scatter. Meanwhile, in this flow cytometric results showed that the leaves aqueous extract of *Aegle marmelos* showed suddenly decline in forward scatter (inhibited the population of infected cells) and side scatter as compared to

control. In addition, NDV used as standard for these immunopharmacological studies and the results showed that there is drastic increase in forward and side scatter as compared to normal control group. On the other hand, chicken lysed whole blood were incubated with serial dilutions of aqueous extract containing NDV and the results showed that there is drastically reduction of proliferation response at higher doses as compared to control group. Overall, the data suggests that the aqueous extract of *Aegle marmelos* showed anti-viral activity.

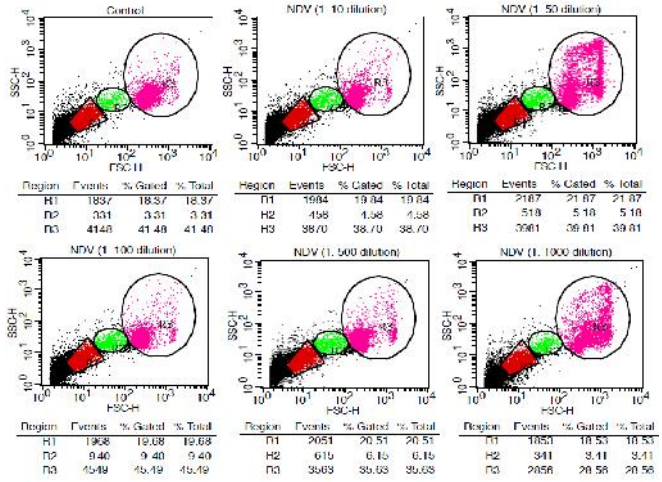

Figure 1: Effect of leaves aqueous extract of *Aegle marmelos* on lymphocytes, monocytes and granulocytes count in chicken lysed whole blood. Data acquisition of 10000 events and fraction or separation of cell populations representing different phenotypes analyzed using cell quest software.

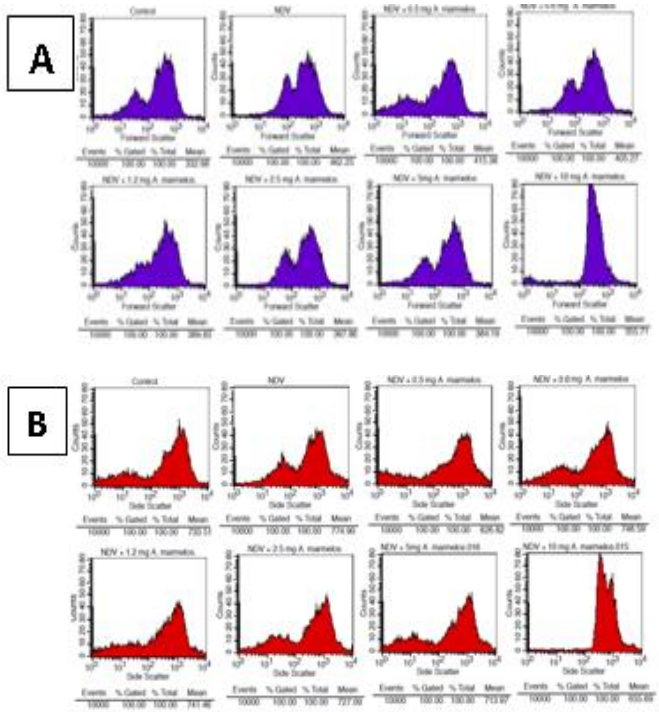

Figure 2: Effect of leaves aqueous extract of *Aegle marmelos* on the parameters of flow cytometer (forward and side scatter) in chicken lysed whole blood. Data acquisition of 10000 events and fraction or separation of cell populations representing forward and side scatter using cell quest software.

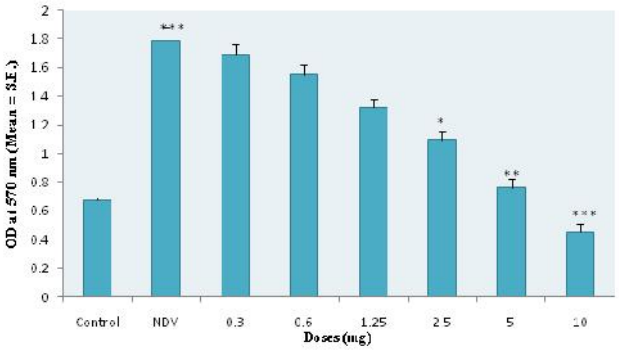

Figure 3: Effect of leaves aqueous extract of *Aegle marmelos* on NDV in chicken lysed whole blood. Values are expressed as Mean± S.E. The absorbance was evaluated in an ELISA reader at 570 nm

4. Conclusion

The result showed that *Aegle marmelos* leaves aqueous extract was safe at higher doses because of declining of forward scatter, side scatter and also inhibits NDV proliferative response in comparison to NDV standard group. Antiviral activity of *Aegle marmelos* leaves aqueous extract indicated that it showed strong antiviral activity at higher concentrations.

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