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Virucidal potential of saponin extricated from *Emblica officinalis* and *Ficus religiosa*

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A B S T R A C T

This study was conducted in order to determine the importance of crude saponins from the leaves of various medicinal plants viz. *Emblica officinalis* and *Ficus religiosa* for the treatment of various infectious diseases particularly intracellular or extracellular pathogens. However, the role of saponin is less explored especially as an virucidal agent. The objective of our proposed study is to determine its virucidal activity of crude saponin extracted from *Emblica officinalis* and *Ficus religiosa* against New castle disease virus (NDV) which is determined in animal (Swiss mice) model studies. Swiss mice (n = 5) were immunized subcutaneously on day 0 and day 7 with variable doses of saponins (50, 100 and 200 µg) along with NDV (1:100 dilution) and determined its proliferation assay, CD4/CD8 estimation and Th1 (IFN-gamma and TNF alpha) type of cytokines from cell culture supernatant by Elisa. The results showed that saponin (200 µg) at higher doses showed drastically decreased in proliferation, CD4/CD8 estimation and Th1 (IFN-gamma and TNF alpha) type of cytokines from cell culture supernatant. NDV (1:100 dilution) used as standard for these studies and the results showed that enhancement of lymphocyte proliferation, CD4/CD8 estimation and Th1 (IFN-gamma and TNF alpha) type of cytokines from cell culture supernatant. Overall, the results showed that saponin from *Emblica officinalis* and *Ficus religiosa* showed virucidal activity.

Keywords: *Emblica officinalis*; *Ficus religiosa*; saponin; virucidal.

A R T I C L E I N F O

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1. Introduction

Saponins are one of the classes of natural plant based products that are traditionally used as natural detergent. These saponins are commonly present in our foodstuffs and herbal preparations and also showed various medicinal properties e.g. anti-inflammatory, vaccine adjuvants, immunostimulatory [1-3]. In addition, saponin (triterpenoid and steroidal) have special attention because of number of industrial as well as commercial applications along with its use as main source of raw materials for the production of hormones especially steroid in the pharmaceutical industry [4- 6]. The present study focused on the presence of virucidal compounds that are present in medicinal plants and number of bioassays has already been developed or investigated.

Emblica officinalis (Indian gooseberry or amla; family *Euphorbiaceae*), medicinal plant are used traditionally for the treatment of various diseases i.e. respiratory disorders, diabetes, heart and eye disorder, scurvy [7-9]. In contrast, this medicinal plant also showed anti-bacterial, immunomodulatory, adjuvant; anti-inflammatory and astringent properties [8, 9].

Ficus religiosa (Peepal, family *Moraceae*), medicinal plant grows throughout India and widely present in temples. In Ayurveda, *Ficus religiosa* traditionally used in India for different purposes especially to cure some of the diseases like asthma, diabetes, epilepsy, gastric problems, inflammatory disorders [10-12]. In order to achieve this objective, our group focused on saponin extracted from the leaves of two medicinal plants viz. *Emblica officinalis* and *Ficus religiosa* and determined its virucidal against Newcastle disease virus (NDV).

2. Materials and Methods

Plant material

Fresh and mature plant leaves of *Emblica officinalis* and *Ficus religiosa* were collected from the udyan of Vidya Pratishthan's School of Biotechnology, Baramati, District Pune, Maharashtra, India.

Preparation of crude saponin extracts

First of all, leaves of three medicinal plants were sun dried and test protocol (liquid-liquid extraction) was carried out in order to isolate the crude saponins. To prepare the aqueous leaves extract, the dried plant materials (8 g) were macerated in liquid nitrogen and dissolved in phosphate buffered saline (80 ml, pH 7.2). Thereafter, extracted thrice with diethyl ether (20 ml), incubate for 5 min. Later on, diethyl ether layer was discarded and the retained aqueous layer settled at the bottom further with 30 ml n-butanol (four times). Finally, extracts (n-butanol) were bulked together and washed two to three times using 10 ml of 5% NaCl. The washed extract was concentrated at < 70 °C in an oven and air dried at room temperature to yield mg of crude saponin residue [6]. The residue was screened for saponin using the foaming test. The powder is dissolved in phosphate buffered saline and filtered through a Whatman filter paper. To identify the presence of saponin in the extract using Frothing test (1 ml of extract mixed with double the amount of distilled water in tube, formation of stable foams indicates the presence of saponins).

Isolation of Newcastle disease virus (NDV) samples

Specific pathogen free chicken eggs (Venkys India Ltd; allantoic cavity route of 9-11 day old) were used for isolation and propagation of NDV from field samples in Baramati region, District Pune, Maharashtra [13, 14]. For inoculation, only bigger sized embryos (air cell and the area without blood vessels) were selected observed through candling. Supernatant (0.2 ml) was inoculated at 45^0 angle in chicken eggs after disinfection of egg shell with spirit. Through candling, embryo motility was observed every 4 h, if the death of embryos will occur, harvested the amnio-allantoic fluid and identified as well as determined the presence of virus through hemagglutination test (128 HA unit).

Animals

Briefly, Swiss mice (female) were distributed into eight groups consisting of six animals. (Group I) control, received PBS; (Group II) NDV; (Group III, IV and V) 0.05 mg, 0.1mg and 0.2 mg of saponin from *Emblica officinalis* including (Group VI, VII and VIII) 0.05 mg, 0.1mg and 0.2 mg of saponin from *Ficus religiosa*. All the animals were properly maintained as per ethical guidelines. Mice were immunized with NDV on day 0 and 7 along with or without variable doses of saponin (50, 100 and 200 µg). The dose volume was 0.2 ml injected subcutaneously along with NDV. On day 12, EDTA blood samples were collected from treated as well as non treated animals from retro-orbital plexus for the estimation of cell surface marker CD4 and CD8 using flow cytometry and also estimates its proliferation (peritoneal macrophages) and Th1 (IFN-gamma and TNF alpha) type of cytokines from cell culture supernatant by Elisa. All these studies are conducted as per ethical regulations on animal research; all animals (Swiss mice) used in experimental work received humane care.

Macrophage collection and proliferation assay

On day 12, mice were injected intraperitoneally (10 ml) with ice cold phosphate buffered saline. Thereafter, peritoneal cells were collected from the abdominal cavity and washed the cells twice with phosphate buffered saline and finally suspended at 2 x 10^6 cells/ml in RPMI medium containing 10 % FCS (heat inactivated). 100 µl cell suspensions of immunized mice of variable doses of saponin (50, 100 and 200 µg) were added in each 96 well plate and then add again exposure of saponin (50, 100 and 200 µg) along with NDV. Incubate the plate for 48 h at 37°C in CO_2 incubator and then centrifuge the plate for the collection of Th1 type of cytokines from cell culture supernatant. After supernatant collection, add fresh medium along with MTT solution (5 mg/ml, 10 µl). Incubate the plate for another 4 h and centrifuged at 1800 rpm for 5 minutes and the supernatant was discarded. Add 100 µl of DMSO solution to the formazon crystals and OD or absorbance was evaluated in an ELISA reader (Perkin Elmer) at 570 nm [15, 16].

Flow cytometric analysis in Whole blood

In this study, EDTA whole blood (100 µl) samples of treated as well as non treated mouse were collected in eppendorf tube and stained with 3 µl of FITC labeled CD8 and PE labeled CD4 monoclonal antibody. Incubate the

samples for 30 min in dark at room temperature. Later on, FACS lysis solution/red cell lysis buffer (incubate for 10 min) was added. After centrifuging, the supernatant was aspirated and washed two times with phosphate buffered saline. Finally, pellet dissolved in PBS and observed the cells through flow cytometer (FACS Calibur). Data acquisition of 10000 events of CD4/CD8 surface markers representing different phenotypes analyzed using cell quest software [15, 16].

Estimation of Th1 (IFN-gamma and TNF alpha) cytokines in serum by Elisa

Cytokine concentrations in the serum were determined by ELISA kits that were specific against mouse cytokines. The levels of Th1 (IFN-gamma and TNF alpha) cytokines were estimated using ELISA (BD optia, ELISA kit). Assays were performed according to the manufacturer's instructions [16].

3. Results and discussions

Proliferation assay (Macrophages)

The results obtained from these studies that both the saponins from *Emblica officinalis* and *Ficus religiosa* along with NDV showed inhibitory effect with respect to proliferation (activation of macrophages) at higher doses as compared to control. NDV used as standard and showed the enhancement of proliferation (macrophage activation). Overall, the results showed that saponins from *Emblica officinalis* and *Ficus religiosa* observed its virucidal activity.

CD4/CD8 surface marker

The effect of variable doses of saponin (50, 100 and 200 µg) from *Emblica officinalis* and *Ficus religiosa* along with NDV on CD4/CD8 surface marker as shown in Fig.1. The results showed that both the saponins showed dose dependent decrease in CD4/CD8 estimation in whole blood as compared to control. NDV used as standard, there is drastic increased in CD4/CD8 population as compared to control.

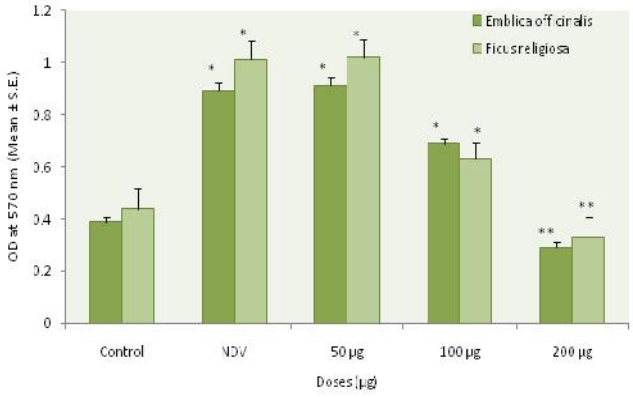

Figure 1: Effect of variable doses of saponin from Emblica officinalis and Ficus religiosa on peritoneal macrophages. Macrophages (10^6 cells/ml, 100 µl) were again exposed to variable doses of saponin (0.05, 0.1 and 0.2 mg) along with NDV (1:100, 100 µl). Incubate the plate for 48 h at 37°C in CO_2 incubator and add MTT solution. After incubation and centrifuging, supernatant discarded and add 100 µl of DMSO solution to the formazon crystals and OD or absorbance was evaluated in an ELISA reader at 570 nm. Values are expressed as Mean ± S.E. *P<0.05; **P<0.01versus control

Th1 (IFN-gamma and TNF alpha) cytokines

The effect of variable doses of saponin (50, 100 and 200 µg) along with NDV were comparable to the standard NDV alone, the results showed the inhibition of Th1 type of cytokines from cell culture supernatant as compared to control Overall the data represents the virucidal activity of saponin from *Emblica officinalis* and *Ficus religiosa*.

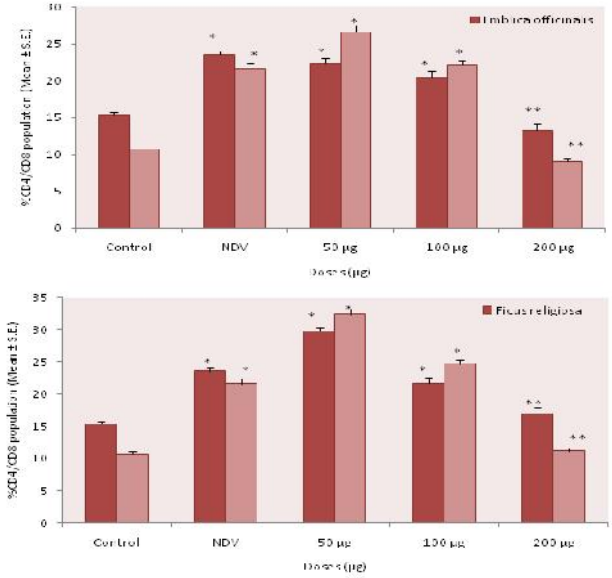

Figure 2: Effect of variable doses of saponin from Emblica officinalis and Ficus religiosa on CD4/CD8 population.

EDTA whole blood (100 µl) were collected from retro-orbital plexus of mice and stained with CD4 PE monoclonal antibody and CD8 FITC monoclonal antibody. Incubate the samples for 30 min at 37°C in CO_2 incubator. After lysing and washing, the samples were analyzed through flow cytometer (FACS calibur). Values are expressed as Mean ± S.E. *P<0.05; **P<0.01versus control

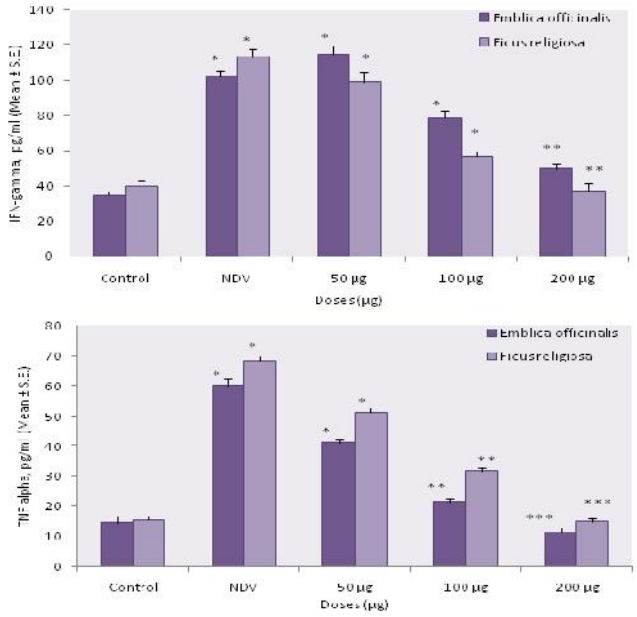

Figure 3: Effect of variable doses of saponin from Emblica officinalis and Ficus religiosa on Th1 cytokine production from cell culture supernatant.

Assays were performed according to the manufacturer's instructions (BD optia kits). Values are expressed as Mean ± S.E. *P<0.05; **P<0.01versus control.

Discussion

Infectious diseases are one of the major causes of morbidity and mortality worldwide. Inspite of these infectious diseases, researchers focused on various medicinal plants which believed to be important source for eliminating these intracellular or extracellular pathogens [13, 14]. In addition, medicinal plants contained number of phytochemicals that are present that could be used as virucidal drug. In an effort to search for novel virucidal drug from medicinal plant is of great significance for therapeutic treatment [13, 14].

The present study was carried out in order to determine the *in vivo* antiviral activity of crude saponin from *Emblica officinalis* and *Ficus religiosa* in mice against new castle disease virus (NDV, isolated from specific pathogen free embryonated chicken eggs using proliferation assay (macrophages), estimation of CD4/CD8 surface marker and Th1 (IFN-gamma and TNF alpha) type of cytokines from cell culture supernatant. The results showed both the saponins from *Emblica officinalis* and *Ficus religiosa* showed inhibitory activity (dosage-dependent relationship) against NDV with respect to proliferation assay (macrophages), Th1 (IFN-gamma and TNF alpha) cytokines from cell culture supernatant and CD4/CD8 estimation. CD4 T cells (expressing class MHC II) are activated by antigens (generally proteins or peptides) after their processing by antigen-presenting cells and differentiate into two functional subsets T helper (Th1; cytokines i.e. IFN-gamma and TNF alpha, inhibits intracellular pathogens and Th2; IL-4, extracellular pathogens). Similarly, CD8+ T cells (expressing class MHC I) are also processed through antigen presenting cells and also produce IFN-gamma and TNF alpha and kill their target cells through a direct cytolytic mechanism [17].

In the present study, crude saponin from *Emblica officinalis* and *Ficus religiosa* along with NDV inhibited the *in vivo* secretion of the pro-inflammatory cytokine IFN-gamma and TNF alpha and decrease in CD4/CD8 marker athigher concentration as compared to NDV control and returned to its normal profile. The results indicated that crude saponin from both the medicinal plantscould significantly inhibit the activation potential of CD4 and CD8 count in NDV-immunized mice. NDV along with crude saponin had a significant inhibitory effect on CD4+ and CD8+ T cells, thereby confirming its general effect on the cell-mediated immune response. Meanwhile, cytokine measurement also revealed that crude saponin from *Emblica officinalis* and *Ficus religiosa*at higher concentration significantly inhibited the production of the Th1 (IFN-gamma and TNF alpha) type of cytokines in NDV immunized mice. These results suggested that crude saponin from *Emblica officinalis* and *Ficus religiosa*, able to simultaneously inhibit the Th1 type of immune response. Overall, the results showed the inhibitory effects of saponin at higher concentration with respect to NDV and returned to its normal range as compared to control and these could not be considered as the toxic effect of saponin because in each case the viability of cells was determined, and in all of the experiments the cells showed a high viability. Finally, it may be proved that crude saponin from *Emblica officinalis* and *Ficus religiosa* showed antiviral activity.

4. Conclusion

The main conclusion of this study is that inhibition of activation of macrophages, CD4/CD8 surface marker and Th1 (IFN-gamma and TNF alpha) in mice that were treated with crude saponin along with or without NDV. This conclusion is based on the finding that Endogenous TNF alpha probably inhibits activation of macrophages and reduced its proliferation activity and Th1 cytokines from cell culture supernatant in macrophages. It is expected that using medicinal plant products (cheaper substitute for conventional drugs) as therapeutic agents and is generally use of the plant in treating infections in traditional medicine.

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