



Research Article
**International Journal of Current Trends in
Pharmaceutical Research**

IJCTPR, 2014, Vol. 2(6): 715-722
www.pharmaresearchlibrary.com/ijctpr



**Anti-viral activity of *Azadirachta indica* leaves against Newcastle disease virus:
A study by *in vitro* and *in vivo* immunological approach**

Amit Gupta¹, Ramesh B Jagtap¹ and Sushama R Chaphalkar^{1,2}

¹Department of Immunology and Virology, Vidya Pratishthan's School of Biotechnology (VSBT) Vidyanagari, Baramati, District Pune, India

²Director, Vidya Pratishthan's School of Biotechnology (VSBT) Vidyanagari, Baramati, District Pune, India
Received: 20 September 2014, Accepted: 29 October 2014, Published Online: 15 November 2014

Abstract

The objective of our study is to present a reliable method for the determination of anti-viral activity of leaf of *Azadirachta indica* against new castle disease virus (NDV). To achieve this objective, aqueous extract of leaf of *Azadirachta indica* were subjected to qualitative estimation of metabolites and also estimate the Azadirachtin content using HPTLC. To determine the anti-viral activity, variable doses of aqueous extract against NDV *in vitro* as well as *in vivo* in mice to observe the effect on spleen cells by proliferation assay and estimate the cell surface markers i.e. CD3/CD4/CD8 population, Th1 (IFN-gamma, TNF alpha) type of cytokines in cell culture supernatant and also observed the effect of variable doses of aqueous effect on human PBMC and chicken whole blood against optimized dose of NDV. The results showed that *Azadirachta indica* leaf showed dose-dependently and significantly decreased the *in vitro* and *in vivo* lymphocyte population of spleen cells with serial dilutions of *Azadirachta indica* leaf along with NDV in mice. Furthermore, variable doses of aqueous extract increased the CD3, CD4/CD8 count and decreased the Th1 (IFN-gamma, TNF alpha) cytokines at higher doses in mice. On the other hand, variable doses of aqueous extract showed anti-viral activity in human PBMC, mice and chicken whole blood as compared to optimized dose of NDV and control. The results showed that at higher concentration of aqueous extract in mice, human and chicken against NDV showed antiviral activity.

Keywords: *Azadirachta indica*, Newcastle disease virus, aqueous extract

Contents

1. Introduction	716
2. Materials and Methods	716
3. Results and Discussion	718
4. Conclusion	721
5. Acknowledgement.	721
6. References	721

***Corresponding author**

Dr. Sushama R Chaphalkar

Director,
Vidya Pratishthan's School of Biotechnology
Vidyanagari, Baramati, Pune, India
Manuscript ID: IJCTPR2317



PAPER-QR CODE

1. Introduction

Medicinal plant products have been used for a long time as folk remedies and are generally used for different kinds of ailments including viral diseases [1]. So, there is an urgent requirement to search for new plant based formulations for the treatment of various viral infections since there is an increasing resistance to antiviral drugs [2, 3, 4, 5]. Recently, a number of medicinal plant products have been shown to have antiviral activity [6,7]. Traditional plant extracts having anti-infective properties and has been screened for their antiviral activity [8].

Antivirals (other than a virus or virus containing specific vaccine antigen or antibody) are those substances which is able to produce either a protective or therapeutic effect on the virus infected host cell. For the last so many years, the researchers focused on antiviral agents isolated or purified from medicinal plants but this was directed mainly showing by chance, with little or no scientific basis. Now a day, the researchers achieved the success for the screening of plants for antibacterial as well as antiviral actions. The use of plants or plant parts i.e. root, stem and leaf, traditionally used as antiviral agents is relatively wider than their use in modern medicine. Number of antiviral substances has so far been isolated from higher plants including algae as well as lichens. There are number of suitable methods for evaluating antiviral properties of plants and their extracts include use of animal models studies, egg inoculation studies and cell culture methods [9].

Azadirachta indica (commonly known as neem) belongs to the family *Meliaceae* [10] and is generally used in ayurveda, unani and homoeopathic medicine. *Azadirachta indica* is well known for its various medicinal properties and has great impact on Indian traditional medicine. For the last so many years, there are number of research articles which is published related to the different parts of the plant i.e. leaves, stem and root showed immunomodulatory [11], anti-inflammatory [12], anti-hyperglycemic [13], antiulcer [14], antimalarial [15], antibacterial [16] as well as antioxidant [17] properties. These plant parts especially leaves, roots and stem of *Azadirachta indica* have been extensively used for treating inflammation, bacterial infections, fever, skin related diseases and dental disorders [18]. Generally, the chemical constituents of *Azadirachta indica* contains many biologically active compounds that can be extracted i.e. alkaloids, flavonoids, triterpenoids and phenolic compounds [19]. The number of compounds have been isolated from the different parts of the plant i.e. roots, stem and leaves and also published number of review articles related to synthesis and structural diversity of these compounds [20]. Keeping this view in mind, the present studies were to investigate the anti-viral activity of leaves of *Azadirachta indica* in mice, human and chicken against new castle disease virus.

2. Materials and Methods

2.1. Plant material

The plants used in this study were collected in the Vidya Pratishthan's School of Biotechnology, Baramati, District Pune, Maharashtra, India

2.2. Preparation of aqueous extract

Known weight of fresh leaves of *Azadirachta indica* was collected, washed with distilled water and then sun dried. For aqueous extract preparation, sample was macerated to powder form and was used for the immunological studies. The aqueous extraction was done in 50 ml phosphate buffered saline and crushed in a grinder and the extract was centrifuged at 10,000 rpm at 4 °C for 10 minutes. The supernatant was collected and was used within four hours for various immunological *in vitro* as well as *in vivo* assays.

2.3. HPTLC and determination of Azadirachtin content

The aqueous extract of leaf of *Azadirachta indica* was subjected to qualitative and quantitative investigation of metabolites by HPTLC to determine the metabolites. The solvents and other purified reagents, HPTLC plates (10 x 10 cm) were purchased from Qualigens and Merck. The solvent system used in mobile phase and detect its wavelength at 366 nm. The stock solution of *Azadirachta indica* was prepared for HPTLC studies and dissolved the 5 g of weighed compound of leaf in phosphate buffered saline or with different solvents in a final volume of 50 ml. Further dilutions were made to obtain the working standards solution. The aqueous extract of leaf of *Azadirachta indica* showed the presence of saponin, terpenoids, flavonoids and phenolics in the phytochemical profile of *Azadirachta indica*. The percentage of Azadirachtin in the sample was found to be 2.2% and retardation factor (Rf) value of terpenoid was 0.96 where as saponin was found in the peak value between 490-665 and the Rf value of saponin was 0.38. These results are indicative of the excellent reliability, reproducibility, accuracy and precision of the method. On the other hand, the aqueous extract of leaf showed the bio-inorganic fingerprinting i.e. presence of metal concentration in ppm i.e. Cu (HCl) - 0.10; Fe (H₂O)- 0.55; Mn (HCl)- 4.98; Mg (H₂O)- 0.08; Ca (HCl)- 0.48 and Zn (H₂O)- 0.08.

2.4. Collection of samples

The samples of Newcastle disease (NDV) suspected birds were collected under Animal disease surveillance programme of "BIO-VILLAGE" scheme of Vidya Pratishthan's School of Biotechnology. The oro-pharyngeal

swabs, cloacal swabs from live ailing birds and intestine with its contents, liver, trachea, brain, etc. from dead birds were collected in isotonic PBS (pH 7.0-7.4) with antibiotics. The tissue samples were pooled together, triturated and briefly centrifuged. The supernatant of tissue extract and fecal sample as 20% suspension in antibiotic solution were 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 chicken eggs were purchased from Venkys India Ltd. The allantoic cavity route of 9-11 day old SPF embryonated chicken eggs was used for isolation and propagation of NDV from field samples. SPF eggs were candled and bigger sized embryos selected for inoculation. The air cell and the area without blood vessels, 3-4 mm below the air cell was marked for inoculation. After disinfection of egg shell with spirit, 0.2 ml of supernatant was inoculated at 45° angle into embryonated chicken eggs. Embryo motility was observed every 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

Hemagglutination test was performed for confirming presence and titration of virus in amnio-allantoic fluid. The titre observed was 128 HA unit. The virus is aliquoted into 1.5 ml vials and stored at -40° C. The working dilutions of virus namely 1:10, 1:30, 1:100 and 1:500 were made in 1X sterile PBS and used further for infection of mice.

2.7. Determination of antiviral activity

2.7.1. In vitro virus inhibition assay in human PBMC, mice and chicken whole blood

The *in vitro* inhibitory potential of leaf of *Azadirachta indica* was evaluated in human PBMC, mice and chicken whole blood and was incubated with serial dilutions of NDV. After getting the optimized dose of NDV, the blood samples of human, mice and chicken were treated with variable doses of aqueous extract i.e. 0.625 – 2.5 mg of leaf of *Azadirachta indica*.

The human peripheral blood mononuclear cells, mice and chicken whole blood were placed in PBS buffer stored on ice. The peripheral blood mononuclear cells, mice and chicken whole blood were lysed with red blood cell lysing buffer. The cell suspensions were stored in RPMI medium containing 10 % fetal bovine serum. For each sample, 2×10^6 cells were incubated for 48 – 72h with or without NDV along with variable doses of aqueous extract at 37 °C and 5 % carbon dioxide incubator. Add 10 µl of MTT solution (5 mg/ml) were added to each well 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 (Gupta et al, 2011).

2.7.2. In vivo virus inhibition assay in mice

The *in vivo* inhibitory potential of leaf of *Azadirachta indica* against NDV was evaluated in Swiss mice. Briefly, the serial dilutions of aqueous extract (i.e. 50 µl containing different concentration 0.625 – 2.5 mg) and NDV (1:100 dilution) were mixed together in a final volume of 0.2 ml. These samples were incubated for 1 h at room temperature. After incubation, 0.2 ml of the formulation of aqueous extract containing NDV was injected intraperitoneally on day 0 and 7 in mice along with and healthy mice control. The mice were then observed daily for the clinical signs viz., weight loss, flaccid paralysis followed by death. On 10th day, the mice spleen cells were collected and proceed for proliferation assay, estimation of CD3, CD4/CD8 count and determined its Th1 (IFN-gamma and TNF alpha) cytokines.

On day 10, mouse was sacrificed by carbon dioxide anesthesia. The spleen was excised aseptically and lymphocytes isolated. Briefly, single cell suspensions of spleen were prepared by teasing the tissue between two glass slides and the cells were centrifuged at $400 \times g$ for 10 min at 4 °C. Erythrocytes present were lysed with red cell lysis buffer for 5 min. Lymphocytes obtained were then washed thrice with PBS. Cell number was counted with a haemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95 % . To evaluate the *in vivo* effect of leaf of *Azadirachta indica* on the proliferation of splenic lymphocytes, spleen cell suspension (2×10^6 cell/ml) was pipetted into 96 well plates (200 µl/well) in the presence of leaf (0.625 – 2.5 mg) and again exposure of NDV (1:100) cultured at 37 °C for 48 h, the plates were centrifuged at $1400 \times g$, 5 min and the supernatant was collected for the estimation of cytokines and add fresh 100 µl fresh complete media in 96 well plate and again incubate for 24 h and then add 10 µl of MTT solution (5 mg/ml) were added to each well 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 [21].

In lymphocyte proliferation assay, spleen cells were again co-cultured with serial dilutions of leaf of *Azadirachta indica* along with NDV for flow cytometric analysis. 100 µl of spleen cells (10^6 cells/ml) was taken in each tube. FITC labeled CD8, CD3 and PE labeled CD4+ monoclonal antibody were added directly to 100 µl of cells. Tubes were incubated in dark for 30 min at room temperature. Subsequently, 2 ml of 1× FACS lysis solution was added at room temperature with gentle mixing followed by incubation for 10 min [21]. The samples were spun ($300 - 400 \times g$) and the supernatant was aspirated and sample was given three washings of PBS (pH 7.4). The resulting stained cell pellet was resuspended in 500 µl of PBS and was run on a flow cytometer. The forward and side scatter gating

applied for data acquisition on 10,000 events and fraction of cell populations representing different phenotypes analyzed using cell quest software.

Spleens cells were cultured in 96-well plates at 2×10^6 cells/ml and cytokines was then measured from the supernatants by ELISA. The assay was performed according to the manufacturer's instruction with multipoint analysis [22]. Briefly, 100 μ l of diluted capture antibody was added to each well in a 96 well plate and was allowed to adhere overnight for 4 °C. The Elisa plates were washed and then blocked with 1X PBS supplemented with 10 % FBS for 1 h at room temperature. After washing the plates, the spleen cell culture supernatant samples were added into the plates and also prepared and added the serial dilutions of the standard into the plates and then incubated for 2 h at room temperature. Then, plates were washed and then add detector antibody and avidin–horse radish peroxidase reagent (1:100 dilution) into each well. Again, the plates were incubated for 1 h at room temperature. After washing, 100 μ l of tri-methyl benzidine (TMB) substrate was added into each well. Stop solution was finally added after incubation in the dark for 30 min at room temperature. The absorbance was read at 450 nm. The result was analyzed using softmax program and values determined against the standard provided by the manufacturer.

2.8. Statistical analysis

Values are expressed as Mean \pm 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

Results

3.1. Effect of aqueous extract of leaf of *Azadirachta indica* on in vitro virus inhibition assay in human PBMC, mice and chicken whole blood

The effect of variable doses of NDV on human PBMC, mice and chicken whole blood as shown in **Fig.1 and Fig.2.** In human PBMC, the optimized dose of NDV was observed at 1:30 dilution where as in mice and chicken whole blood, at 1:100 dilution and 1:500 dilution. In addition, after getting the optimized dose of NDV on human PBMC, mice and chicken whole blood, the samples of three different species were incubated with variable doses of aqueous extract of leaf of *Azadirachta indica*. The results showed that at 2.5 mg showed anti-viral activity as compared to NDV control and control. With the increasing doses of aqueous extract, the aqueous extract showed dose dependently increase in the anti-viral activity as compared to control.

3.2. Effect of leaf aqueous extract of *Azadirachta indica* on in vivo virus inhibition assay in mice

The effect of variable doses of aqueous extract of leaf of *Azadirachta indica* on NDV stimulated splenocyte proliferation in mice immunized with leaf aqueous extract are shown in **Fig. 3.** The results showed that the aqueous extract significantly reduced the proliferation assay as compared to the NDV control group. The leaf aqueous extract at 2.5 mg significantly reduced proliferative response to NDV was observed of the splenocytes isolated from the mice immunized with NDV alone. The results indicated that the leaf aqueous extract significantly reduced the splenocyte proliferation assay as compared with NDV control group.

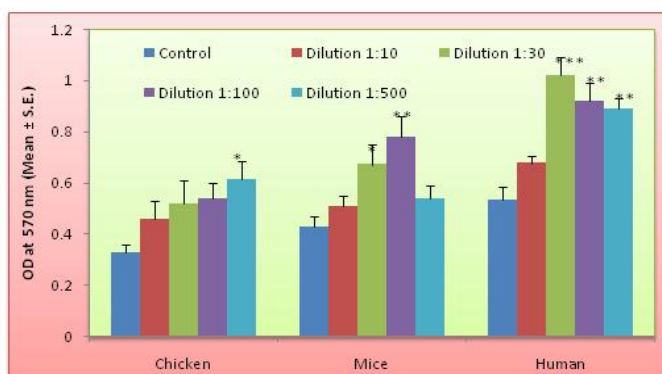
The effect of variable doses of aqueous extract of *Azadirachta indica* on CD3, CD4/CD8 count in mice as shown in **Fig.4.** Although the proportions of CD4+ and CD8+ T cells in the spleen cells from the mice immunized with 2.5 mg of *Azadirachta indica* along with NDV were higher than those from the NDV control group. In addition, NDV used as standard and there is significant increase in the CD4 population but there is no enhancement in CD3 and CD8 population. But the proportion of CD3+ and CD8+ T cells was significantly changed by 2.5 mg of *Azadirachta indica* along with NDV. The effect of variable doses of aqueous extract of *Azadirachta indica* on Th1 (IFN-gamma and TNF alpha) cytokines in mice as shown in **Fig.5.** The leaf aqueous extract significantly inhibited the production of IFN-gamma and TNF alpha was found in the cell cultures of the mice immunized with variable doses of aqueous extract. NDV used as positive control, there is significant increase in Th1 (IFN-gamma and TNF alpha) cytokines. The results showed that the leaf aqueous extract showed anti-viral activity.

Discussion

Drug screening is essential for the discovery of antiviral compounds. Diverse *in vitro* as well as *in vivo* antiviral assays exist and most of them are cell based assay including MTT assay (measurement of cell variability). One of the assays i.e. ELISA is frequently used to detect the presence of protein for cytotoxicity study of the drug. These antiviral assays are already standardized and time -consuming and therefore, other new methods are also used for drug screening [9]. The present study was carried out to test the *in vitro* and *in vivo* antiviral activity of leaf of *Azadirachta indica* in mice against new castle disease virus (NDV) using cytotoxicity assay, cell surface markers and cytokine estimation. In addition, antiviral activity is also been reported in human and chicken using virus infectivity i.e. NDV and estimate the optimized mice of NDV on human PBMC and chicken blood. The findings of the preliminary phytochemical investigations of the aqueous extract and the results of antiviral activity were depicted in the respective figures. The preliminary phytochemical tests performed were of qualitative type and from

the phytochemical investigations it was observed that alkaloids, tannins, flavonoids, terpenoids, saponins and glycoside were present in the aqueous extract. This study focused on the influence of aqueous extract of leaf of *Azadirachta indica* that have shown anti-viral activity against NDV. The results obtained from this study indicated that aqueous extraction of *Azadirachta indica* exerted an antiviral effect against NDV on the *in vitro* proliferation of spleen cells of mice, human PBMCs and chicken blood with a dosage-dependent relationship.

Generally, NDV showed the phenomenon of antineoplastic activity i.e. induction of TNF alpha secretion and the enhancement of the sensitivity of neoplastic cells to the cytolytic effects of TNF alpha [23, 24]. On the basis of these factors, the results showed that NDV along with variable doses of leaf aqueous extract showed the reduction of IFN-gamma and TNF alpha at higher doses and showed anti-viral activity. Furthermore, the aqueous extract of leaf of *Azadirachta indica* inhibited the *in vivo* secretion of the pro-inflammatory cytokine IFN-gamma and TNF alpha and increase in the CD3 and CD4/CD8 marker at higher concentration as compared to control. The results indicated that leaf of *Azadirachta indica* could significantly increase the activation potential of CD4 and CD8 count in NDV-immunized mice. NDV along with the aqueous extract of leaf of *Azadirachta indica* had a significant effect on CD4+ and CD8+ T cells, thereby confirming its general effect on the cell-mediated immune response. Meanwhile, cytokine measurement also revealed that leaf of *Azadirachta indica* at higher concentration significantly inhibited the production of the Th1 (IFN-gamma and TNF alpha) cytokine in NDV immunized mice. These results suggested that leaf of *Azadirachta indica*, be able to simultaneously inhibit the Th1 type of immune response. It should be noted that the inhibitory effects at higher concentration observed in this study could not be considered as the toxic effect of the plants, because in each case the viability of cells was determined, and in all of the experiments the cells showed a high viability. Finally, the aqueous extract of leaf of *Azadirachta indica* at higher concentration showed antiviral activity.



Effect of variable doses of NDV in human PBMC, mice and chicken whole blood. The peripheral blood mononuclear cells, mice and chicken whole blood were lysed with red blood cell lysing buffer and were incubated with serial dilutions of NDV. After 3 days, proliferation was measured by MTT assay. The results are presented as Mean \pm S.E. P values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ as compared to control.

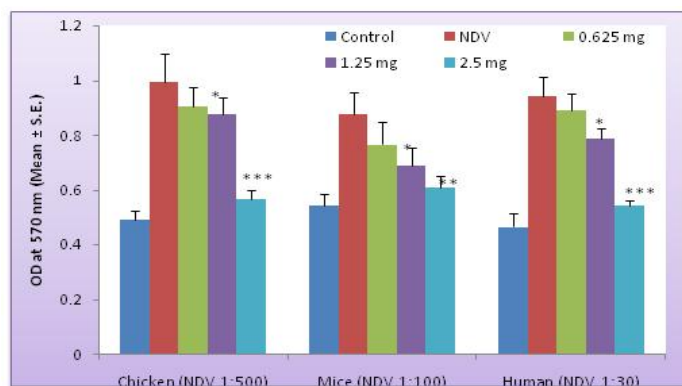


Figure 2: Effect of variable doses of aqueous extract of leaf of *Azadirachta indica* on NDV in human PBMC, mice and chicken whole blood. The peripheral blood mononuclear cells, mice and chicken whole blood were lysed with red blood cell lysing buffer and were incubated with optimized dose of NDV in different species along with variable doses of aqueous extract (0.625, 1.25 and 2.5 mg) or NDV alone. After 3 days, proliferation was measured by MTT assay. The results are presented as Mean \pm S.E. P values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ as compared to control.

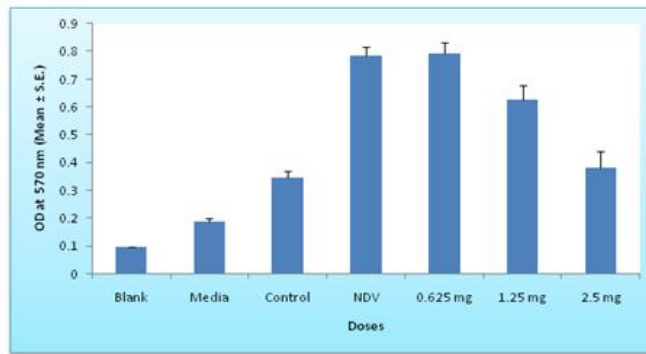


Figure 3: Effect of variable doses of aqueous extract of leaf of *Azadirachta indica* on splenocyte proliferation assay in mice using NDV. On day 10, spleen cells were collected from immunized mice and again re-exposure with NDV along with variable doses of aqueous extract (0.625, 1.25 and 2.5 mg) or NDV alone. After 3 days, proliferation was measured by MTT assay. The results are presented as Mean ± S.E. *P* values: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 as compared to control.

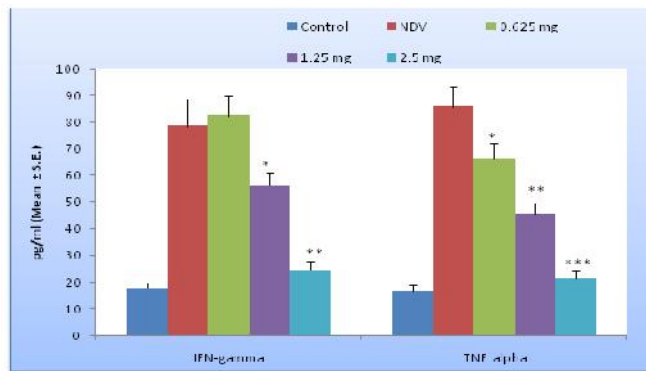


Figure 5: Effect of variable doses of aqueous extract of leaf of *Azadirachta indica* along with NDV on Th1 (IFN gamma and TNF alpha) cytokines in mouse serum. Groups received variable doses of leaf aqueous extract (0.625, 1.25 and 2.5 mg) on day 0 and 7 along with the NDV. Another five animals were not immunized but served as negative controls (control). Animals were bled on day 10 after the secondary immunization and the sera were evaluated for the estimation of Th1 (IFN-gamma, TNF alpha) cytokines. Each bar represents the group mean (n=5). Value for the concentration of cytokine expressed in pg/ml. *P* values: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 when compared to the value of control

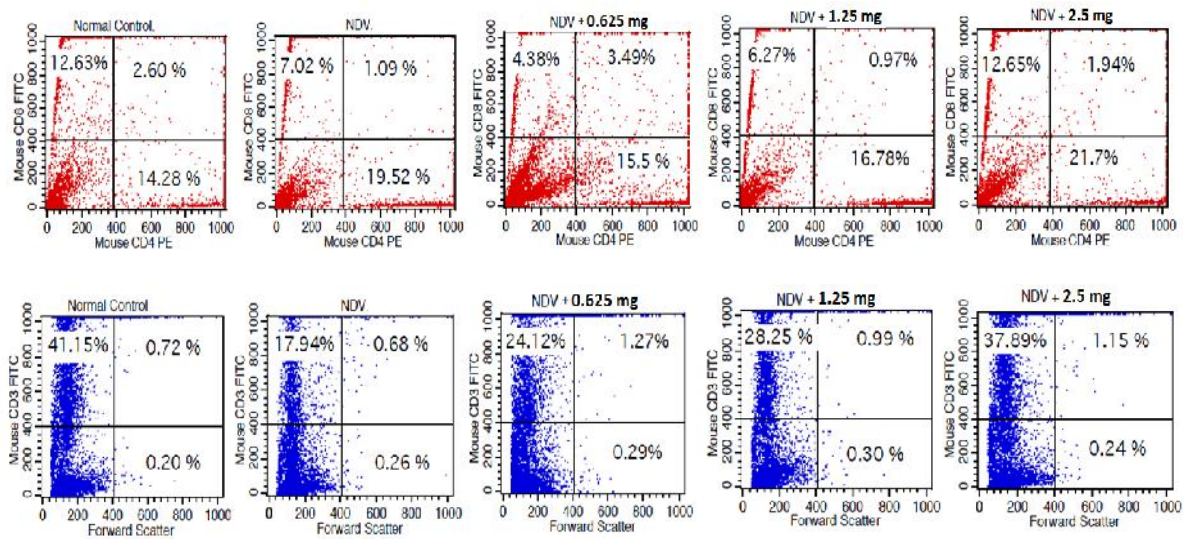


Figure 4: Flowcytometry histogram representation for CD3 and CD4/CD8+ T cell count in mice. The histogram represents the counts for mouse from group of Normal Control, NDV and variable doses of leaf aqueous extract of treated mouse. The histograms are acquired according to the standard procedures of the FACS Calibur

flow cytometer. Staining of whole blood with T cell surface marker CD3 and CD8 (FITC conjugated monoclonal antibody) and CD4 (PE-conjugated monoclonal antibody).

4. Conclusion

It may be concluded from this study that *Azadirachta indica* leaf aqueous extract has antiviral activity against NDV. It is expected that using medicinal plant products as therapeutic agents and is generally use of the plant in treating infections in traditional medicine. The medicinal plant could be a cheaper substitute for conventional drugs which is available in the market since the plant is easily obtainable and the extract can easily be made via a simple process of maceration or infusion.

5. Acknowledgement

We would also like to thanks Dr H. Pawar Veterinary Doctor for collection of NDV samples under the project of Biovillage- Sustainable development through Biotechnology.

6. References

1. Vanden DA, Vlietinck AJ, Van Hoof DL. Plant products as potential antiviral agents. Bull Inst Pasteur 1986; 84:101- 147.
2. De Clercq E. Antiviral agents: Characteristic activity spectrum depending on the molecular target with which they interact. Adv Virus Res 1993; 42:1-55.
3. Field AK, Biron KK. The end of innocence. revisited: Resistance of herpes viruses to antiviral drugs. Clin Microbiol Rev 1994; 7: 1-13.
4. Severson JL, Tying SK. Relation between herpes simplex viruses and human immunodeficiency virus infections. Arch Dermatol 1999; 135: 1393-7.
5. Khan MT, Ather A, Thompson KD, Gambari R. Extracts and molecules from medicinal plants against herpes simplex viruses. Antiviral Res 2005; 67: 107-19.
6. Fukuchi K, Sakagami H, Okuda T, Hatano T, Tanuma S, Kitajima K, et al. Inhibition of herpes simplex virus infection by tannis and related compounds. Antiviral Res 1989; 11: 285- 97.
7. Vijayan P, Raghu C, Ashok G, Dhanaraj SA, Suresh B. Antiviral activity of medicinal plants of Nilgiris. Indian J Med Res 2004; 120:24-9.
8. Vijayan P, Vinodkumar S, Dhanaraj SA, Mukherjee PK, Suresh B. Hepatoprotective effect of the total alkaloid fraction of *Solanum pseudocapsicum* leaves. Pharm Biol 2003; 41:443-8.
9. Nirmala R, Nepolean R, Chandramohan K, Jaiganesh KP, Nithya G, Jeyavalli A, Rakhi B. Review on anti-viral activity in medicinal plants. International Journal of Pharmacy and Industrial Research 2013; 3(01): 61–63.
10. Bhambal A, Kothari S, Saxena S, Jain M. comparative effect of neemstick and tooth brush on plaque removal and gingival health- A clinical trial. Journal of Advanced oral research 2011; 2(3): 51 – 56.
11. Shah AS, Gunjal MA, Juvekar AR. Immunostimulatory activity of aqueous extract of *Azadirachta indica* flowers on specific and non specific immune response. Journal of Natural remedies 2009; 9(1): 35 – 42.
12. Manogaran S, Sulochana N, Kavimani S. An anti-inflammatory and antimicrobial activity of the root, bark and leaves of *A. indica*. Ancient Science of Life 1998; 18 (1), 29 – 34.
13. Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U. Biological activities and medicinal properties of neem (*A. indica*). Current Science 2002; 82: 1336.
14. Maity P, Chattopadhyay I, Biswas K, Banerjee RK, Bandyopadhyay U. The use of neem for controlling gastric hyperacidity and ulcer. Phytotherapy research 2009; 23 (6): 747 – 755.
15. Gianotti RL, Bomblies A, Dafalla M, Issa-Arzika I, Duchemin JB, Eltahir EAB. Efficacy of local neem extracts for sustainable malaria vector control in an African village. Malaria Journal 2008; 7:138.
16. Jain D, Jayaram L, Prabhu VM, Bhat GK. Antibacterial effect of neem (*Azadirachta indica*) oil on multidrug resistant bacteria isolated from human infections. International Journal of Biological and Medical research 2013; 4(4): 3544 – 3546.
17. Ghimeray AK, Jin CW, Ghimire BK, Cho DH. Antioxidant activity and quantitative estimation of azadirachtin and nimbin in *Azadirachta Indica* A. Juss grown in foothills of Nepal. African Journal of Biotechnology 2009; 8(13): 3084–3091.
18. Maithani A, Versha P, Geeta P, Kumar D, Dhulia I. Pharmacognostical standardization of leaves of *Azadirachta Indica*- An effective panacea for all disease. Int J Res Pharm Sci 2011; 2(4): 563 – 568.
19. Vinoth B, Manivasagaperumal, Rajaravindran. Phytochemical analysis and antibacterial activity of *Azadirachta Indica* A Juss. International Journal of Research in Plant Science 2012; 2(3): 50 – 55.

20. Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U. Biological activities and medicinal properties of neem (*Azadirachta Indica*). *Current Science* 2002; 82(11): 1336 – 1345.
21. Gupta A, Khajuria A, Singh J, Singh S, Suri KA, Qazi GN. Immunological adjuvant effect of *Boswellia serrata* (BOS 2000) on specific antibody and cellular response to ovalbumin in mice. *International Immunopharmacology* 2011, 11(8):968 – 975.
22. Malik F, Singh J, Khajuria A, Suri KA, Satti NK, Singh S, Kaul MK, Kumar A, Bhatia A, Qazi GN. A standardized root extract of *Withania somnifera* and its major constituent withanolide-A elicit humoral and cell-mediated immune responses by up regulation of Th1-dominant polarization in BALB/c mice. *Life Sciences* 2007, 80 (16): 1525 – 1538.
23. Waye MMY and Sing CW. Antiviral Drugs for Human Adenoviruses. *Pharmaceuticals* 2010; 3: 3343 - 3354.
24. Lorence RM, Rood PA, Kelley KW. Newcastle disease virus as an antineoplastic agent: induction of tumor necrosis factor-alpha and augmentation of its cytotoxicity. *J Natl Cancer Inst* 1988; 80(16):1305- 1312.