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ANTIVIRAL ACTIVITY AND ITS PROSPECTIVE MECHANISM OF ACTION ON NEWCASTLE DISEASE VIRUS USING CRUDE EXTRACT OF FOUR MEDICINAL PLANTS

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ABSTRACT

Viral problems have been in focused of the scientists due to their high metabolic rate, drug resistance and unique nature of pathological mechanism. The failure of novel synthetic allopathic antiviral drugs impels the scientists to investigate other sources of alternative antiviral agents. Herbal extracts has various inhibitory effects against avian viruses particularly Newcastle disease virus (NDV). The current study was under taken to evaluate in-vitro NDV potential of crude extracts of different medicinal plants by means of Haemagglutination (HA) titer in vero cell line culture. To study the potential NDV activity, Vero cell line were treated with different doses (25µl, 50µl, 75µl and 100µl) of solvent free crude extracts of G. glabra, P. emblica, A. sativum and A. indica and interacted with 1000 $TCID_{50}$ of the lasota virus during infection at different time periods. Minimum dose (25µg/ml) of crude ethanolic extract of A. indica when inoculated on to vero cell line before NDV exposure showed significantly higher (48 HA titer) anti-NDV response as compare to A. sativum (64 HA), but significantly lower than P. emblica (20 HA titer). Similarly, minimum dose (25µg/ml) of crude ethanolic extract of P. emblica after one hour post NDV exposure showed significantly higher (Mean NDV HA Titer 24) anti-NDV response as compare to A. indica (48 HA Titer) and glycyrrhiza, but similar to A. sativum (48 HA). Whereas, maximum dose (100µg/ml) of P. emblica showed significantly similar antiviral (3HA) response on vero cell infected NDV when treated one hour before with A.

indica, A. sativum (3 HA), but significantly higher than G. glabra (128 HA) in terms of mean HA titer. Maximum tested dose (100µg/ml) of P. emblica post one hour NDV exposure showed significantly similar antiviral (2.5 HA) response on vero cell to A. indica (6HA) and A. sativum (4 HA), but significantly higher than G. glabra (192). However, either of the selected plants did not show any cell toxicity by means of cytopathic effect (CPE) and Haemagglutination activity alone or synergistically even at 100µg/ml. It is evaluated that simultaneous treatment (25µg/ml) with crude extract of A. sativum to expose NDV ($EID_{50}=10^5$) in 90% saturated vero cell line showed substianal antiNDV activity.

KEYWORDS: Antiviral activity, Herbal extracts, Glycyrrhiza glabra, Phyllanthus emblica, Allium sativum, Azadirchata indica.

INTRODUCTION

Viral diseases are life threatening all over the world because they are not controlled easily due to their distinctive metabolic properties and fast emerging drug resistance. These are problematic due to their rapid adaptation and emergence of new hybrid viruses. Immunocompetent and immunocompromised individuals are given antiviral medication to overcome the intensity and duration of the viral disease (Perera *et al.*, 2012).

New castle disease is a viral problem of commercial poultry and wild birds reported as cause of severe morbidity and substantial mortality up to 100%. It is one of the major disease of poultry instigating significant economic losses to the commercial poultry farmers in many parts of the world especially in developing countries (Awan *et al.*, 1994: Alexander *et al*., 1997). The causative agent is enveloped, negative-stranded RNA viruses that encode two transmembrane glycoproteins an attachment protein (HN) and a fusion protein (FP). The viral attachment protein H recognizes and binds to sialic acid-containing molecules such as glycoproteins and glycolipids on the surface of host cell membrane (Ferreira *et al.*, 2004). NDV belongs to the genus Avulavirus of family Paramyxoviridae, subfamily Paramyxovirinae, in the order Mononegavirales and is designated avian paramyxovirus-1 (APMV-1). Nine avian paramyxovirus serotypes (APMV-1 to APMV-9), of which APMV-1 is the most economically important, have been identified among these virus types (Alexander *et al*., 1993).

Natural products of the plants origin have been conventionally used widely to treat variety of diseases all over the world (Parekh and Chanda, 2007). *A. sativum* (Garlic) belongs to family

Alliaceae used as traditional remedy for many of the humans physiological disorders and has been showed remarkable effect on the microorganisms including fungi, viruses and bacteria (Benkeblia *et al*., 2004). Whereas, *G. glabra* is reflected as another important member of medicinal plants family showing some notable properties of antiviral activity against human cytomegalo viruses, herpes simplex viruses-1, varicella zoster virus, NDV, Influenza A virus, H5N1 virus, and H1N1 virus. It contains many bio active compounds such as Glycyrrhizin, biochanin B, 18 beta glycyrrhetinic acid and Gaboroneetc showing powerful antiviral activity against many viruses (Anagha *et al.*, 2015). Nevertheless, *P. emblica* belongs to family Euphorbiaceous has been showing effective response alongside viruses and bacteria. All parts of the plant such as shoots, flowers, roots, bark, leaves and fruit are individually or synergistically showed much promising anti-microbial activity. One of the important components of the crude extract is vitamin c which helps to resist against flu infections (Avanishah *et al.*, 2013). *A. indica* is tall, woody and evergreen plant of the family maliacae. To date three important compounds have been confirmed from oil extracts includes nimbin, nimbinine and nimbidin showing antiviral activity (Ganguly, 1994).

In the present study anti NDV potential was investigated in terms of its association with optimum non cytotoxic dose of crude extracts of *G. glabra*, *A. indica, A. sativum* and *P. emblica* by adapting Lasota strain of NDV on in vitro cultured vero cell line and its possible antiviral mechanism of action.

MATERIAL AND METHOD

Fine powder of medicinal plants having reported antiviral activity such as *G. glabra, P. emblica, A. sativum* and *A. indicia* were purchased from local market. The powder of each plant was weighed and shifted to separate labelled plastic container. Ethanol and distill water was used as solvent for the crude extraction in the following ratio *(*Powdered Material=150g, Ethanol= 160ml, Distilled water= 40ml).

Purification

Each of the ethanolic powder suspension was passed through 0.5µm pore size of paper filter (Whatman-Germany) followed by rotary vaccume processing at 50°C to make it free from solvent.

Freeze drying

Five cryo-vials each containing one ml of crude extract of the every plant were prepared and shifted to -80°C ultra low temperature freezer (WiseCryo-Korea) and kept there for six hours which is subsequently transferred to Lyophilizer (Freezed Dried) for 24 hours at -40°C. The resulted pellet formation in bottom of each cryo-vial was used throughout the study.

Cell Line

Vero cell line used in the study were obtained from the cell and tissue culture department, Centre for Research in Molecular Medicine, The University of Lahore, Pakistan.

Virus Strain

Vaccinal strain Lasota of NDV (Chicken/A/Op-KR1/LAS/07) adapted to grow in Vero cell line was provided by Ottoman Pharma (Immuno Division) Raiwind road Lahore, Pakistan.

Culture Media and Chemicals

Dulbeco Modified Eagles's Media (DMEM), Fetal Bovine serum and antibiotic (Penstrep) were supplied by Invitrogen (Glasgow, UK). Whereas, 75 cm^2 tissue culture disposable flasks were purchased from Amersham Biosciences (Uppsala, Sweden). All other chemicals were obtained from Sigma (St.Louis, USA).

Growth in Static Culture

Vero cells were grown in serum containing medium as described by Freshney. Seeding density of 1×10^5 cells/cm^2 (85%) cultures of vero cells were maintained at 37°C with constant supply of 5% concentration of CO_2 in incubator (Lab Line Instruments-Korea).

Haemagglutination (HA) Test

The haemagglutination (HA) test was performed as described by Grimes... year. 50 µl of normal saline (0.9%, pH=7.2) was dispensed in 96-well U-shaped microtiter plate from wells 1-12. One hundred microlitres of virus sample was added to well 1 and made serial dilution up to well 11 followed by addition of fifty microlitres of 1% washed chicken red blood cell (RBC) to all wells. Agglutination of RBC was observed in wells containing the virus while clumping of RBC was seen in wells containing no virus. Dilution of the last well showing agglutination gave the titre of the virus sample. Haemagglutination units thus, expressed per fifty microliter as HAU/50ul.

Statistical Analysis

Data were analyzed using General Linear Model procedure of statistical package for social sciences (SPSS) 15th version and comparison of means tested using Bonferroni test and significance was considered at $P<0.05$.

RESULTS

Different doses of crude extract such as 25µl, 50µl, 75µl and 100µl were interacted with Lasota strain of the NDV (EID_{50}= 1×10^5, HA= $I0^5$) in 90% saturated vero cell line with constant supply of CO_2 at 37°C. It is evaluated that one hour pre-exposure, simultaneous and post exposure of *G. glabra* to ND-infected vero cell line showed mean HA titer 192±73.9, 96±36.9, 256±.00 respectively after 48 hours of incubation. Whereas, one hour pre-exposure, simultaneous and post exposure of *P. emblica* to ND-infected vero cell line showed 20±8.0, 64±45.2, 24±9.2 mean HA titer respectively. One hour pre-exposure, simultaneous and post exposure of *A. sativum* to ND-infected vero cell line showed 64±00, 32±00, 48±18.4 mean HA titer respectively. Moreover, non-medicated control group inoculated with the same dose of NDV provided with same conditions showed 256+.00 mean NDV-HA antigen titer. Thus, it is concluded that minimum dose (25µg/ml) of crude ethanolic extract of *A. indica* (48 HA Titer) when inoculated on to vero cell line before NDV exposure showed significantly higher (48 HA titer) anti-NDV response as compare to *A. sativum (64 HA)*, but significantly lower than *P. emblica* (20 HA titer). Similarly, minimum dose (25µg/ml) of crude ethanolic extract of *P. emblica* when inoculated on to vero cell line after one hour post NDV exposure showed significantly higher (Mean NDV HA Titer 24) anti-NDV response as compare to *Azadirchata indica (48 HA Titer) and glycyrrhiza*, but similar to *A. sativum* (48 HA). Whereas, maximum dose (100µg/ml) of *P. emblica* showed significantly similar antiviral (3HA) response on vero cell infected NDV when treated one hour before with *A. indica*, *A. sativum (3 HA)*, but significantly higher than *G. glabra* (128 HA) in terms of mean HA titer. Maximum tested dose (100µg/ml) of *P. emblica* post one hour NDV exposure showed significantly similar antiviral (2.5 HA) response on vero cell to *A. indica (6HA) and A. sativum* (4 HA), but significantly higher than *G. glabra* (192) in terms of mean HA titer. However, either of the selected plants did not show any cell toxicity by means of cytopathic effect (CPE) and Haemagglutination activity alone or synergistically even at 100µg/ml.

DISCUSSION

By improving the purification technique of plant extracts such as *P. emblica, A. sativum* and *A. indica* could be the candidate future plants for the elimination of new castle disease viruses from the host. Results based on the current study would help us to suggest that use of *P. emblica* crude extracts as prevention while, crude extracts of *A. sativum* as treatment may give best results in control of NDV outbreaks keeping in mind the probable mechanism of action. The present study provides evidence that compounds present in the ethanolic extracts of *P. emblica, A. sativum* and *A. indica* has antiviral activity against ND virus. On the basis of results it is indicated that minimal dose (25µg/ml) of ethanolic extract of *A. sativum, P. emblica* and *A. indica* treated vero cell line when exposed with Newcastle disease virus incubated at 37^{o}C for 48 hours in CO_2 supplied incubator showed promising anti NDV activity. It may be assumed from results of the experiment that the extract blocked the receptors site of virus on the surface of vero cell line which enables the virus to penetrate into the cell. This makes virus ineffective during attachment process. A sialylated molecule that can block virus attachment to cellular receptors might act to limit the initial stages of virus infection, compared to NA inhibition that is believed to act largely through preventing release of new virions from virus-infected cells. Moreover, NAI must be administered in the early stages of infection as they are less efficient during the later phases (Chang *et al*., 2011). Whereas, up to 100 µg/ml of ethanolic crude extracts of *G. glabra* did not show any significant inhibition of new castle disease virus in vero cell line culture. Therefore, *A. sativum*, *P. emblica* and *A. indica* extracts could be considered as potential antiviral agent against infections caused by multi drug resistant viruses showing rapid mutations in adverse conditions. Commonly used cell lines to replicate NDV are rabbit, pig, calf, chicken, monkey kidney cells, chicken embryo fibroblast (CEF), chicken embryo kidney (CEK), baby hamster kidney (BHK-21), Hela cells, KB cells.

Crude extracts of *P. emblica* restrict virus replication when used at 25ug/ml which is minimal level of inhibitory dose used in the experiment upto the 100 µg/ml and did not show any kind of cell cytotoxicity in controlled (non-infected) vero cell line. Pre treated dose of 100 µg/ml showed significant high anti-NDV response (<2HA units) followed by 75ug/ml and 50ug /ml and 25µg/ml as shown in fig: 01. Dose of 100 µg/ml of the extract when inoculated simultaneously with NDV virus in vero cell line showed significantly high anti-NDV response (<2HA units) followed by 75µg/ml and 50µg /ml and 25µg/ml as shown in fig: 01. Post treated dose of 100 µg/ml showed significant high anti-NDV response (<2HA units)

followed by 75µg/ml and 50µg /ml and 25µg/ml as shown in fig: 01. The anti- NDV response of *P. emblica* in comparison with dose regime showed that 25ug/ml is the suggested minimal anti NDV effective dose found in in vitro cultivation of the virus on vero cell line. Whereas, the results showed that prevention (pre treated) against NDV with high dose of 100µg/ml showed significantly higher anti NDV effect (<2HA units) followed by treatment after exposure (post treated) and entrance of NDV and injection of extract at the same time (simultaneous) (fig: 01). *P. emblica*. (Euphorbiaceae) is a plant of tropical and subtropical areas of Indonesia, China, Malay Peninsula and India. The roots leaves and barks of the plant has used conventionally used for the treatment of wart, headache, diarrhea and eczema (Xiang *et al*., 2010). Our data is also in line with the data obtained by Mahmood *et al*., on the study about inhibition of HIV infection by medicinal plant extracts. The data also supports the observation of (Estari *et al*., 2012) who established that different medicinal plant extracts inhibit HIV reverse transcriptase in a non-specific manner. Crude extracts of *A. sativum* restrict virus republication when used at 25ug/ml which is minimal level of inhibitory dose used in the experiment upto the 100 µg/ml and did not show any kind cell cytotoxicity in controlled (non-infected) vero cell line. *A. sativum* Pre treated dose of 100 µg/ml showed significant high anti-NDV response (<2HA units) followed by 75ug/ml and 50ug /ml and 25µg/ml as shown in fig: 03. Whereas, dose of 100 µg/ml of the extract when inoculated simultaneously with NDV virus in vero cell line showed significantly high anti-NDV response (<2HA units) followed by 75µg/ml and 50µg /ml and 25µg/ml as shown in fig: 03. Moreover, Post treated dose of 100 µg/ml showed significant high anti-NDV response (<2HA units) followed by 75µg/ml and 50µg /ml and 25µg/ml as shown in fig: 03. The anti- NDV response of *A. sativum* in comparison with dose regime showed that 25ug/ml is the suggested minimal anti NDV effective dose found in invitro cultivation of the virus on vero cell line. Whereas, the results showed that prevention (pre treated) against NDV with high dose of 100µg/ml showed significantly higher anti NDV effect (<2HA units) followed by treatment after exposure (post treated) and entrance of NDV and injection of extract at the same time (simultaneous) (Table: III, fig: 03).

Crude extracts of *A. indica* restrict virus republication when used at 25ug/ml which is minimal level of inhibitory dose used in the experiment upto the 100 µg/ml and did not show any kind cell cytotoxicity in controlled (non-infected) vero cell line. Pre treated dose of 100 µg/ml showed significant high anti-NDV response (<2HA units) followed by 75ug/ml and 50ug /ml and 25µg/ml as shown in table: 04, Fig: IV. Dose of 100 µg/ml of the extract when

inoculated simultaneously with NDV virus in vero cell line showed significantly high anti-NDV response (<2HA units) followed by 75µg/ml and 50µg /ml and 25µg/ml as shown in Fig: 04 Post treated dose of 100 µg/ml showed significant high anti-NDV response (<2HA units) followed by 75µg/ml and 50µg /ml and 25µg/ml as shown in table: IV, Fig: 04. The anti- NDV response of *A. indica* in comparison with dose regime showed that 25ug/ml is the suggested minimal anti NDV effective dose found in invitro cultivation of the virus on vero cell line. Whereas, the results showed that prevention (pre treated) against NDV with high dose of 100µg/ml showed significantly higher anti NDV effect (<2HA units) followed by treatment after exposure (post treated) and entrance of NDV and injection of extract at the same time (simultaneous) (table: IV, fig: 04). Aqueous extract of neem leaves a friction of neem oil (NIM-76) have also ability to suppress to HIV and polio viruses respectively (Sai Ram *et al*., 2000). Aqueous extract of neem leaves and pour neem compound (Azadirachtin) has inhibitory potential on the replication of dengue virus type-2 (Parida *et al*., 2002). Haemagglutinin protein present on the surface of virus mediate attachment to the glycoprotein and glycolipids on the surface of host cell which leads to penetration of virus into the cell (Chang *et al*., 2011). Three compounds are extracted from neem oil are nimbin, nimbinine and nimbidin (Ganguly, 1994) are a complex secondary metabolites yield Beta sitosterol have antiviral potential activity (Parida *et al*., 2002).

Minimal dose (25µg/ml) of *A. sativum*, *P. emblica* and *A. indica* extracts were shown to be the most effective anti-Newcastle disease virus agent among all tested plants extracts as indicated by its Haemagglutination (HA) values (<2HA units). The extract also displayed substantial antiviral activity when used at the rate of 50 µg/ml, 75µg/ml and 100µg/ml (IV figure: 01, 03, and 04) without posing any kind of cell cytotoxicity (CPE) when tested alone in response to vero cell line. Hence, the plant may be consider as better source of antiviral components that inhibit viral replication even at very low concentration. Garlic extracts demonstrated higher antiviral activity as compared to *A. indica, P. emblica* and *G. glabra* indicating that amino acid sequence in the virus may be responsible for the variations in the antiviral inhibition demonstrated by the plant extracts and higher concentrations of extracts.

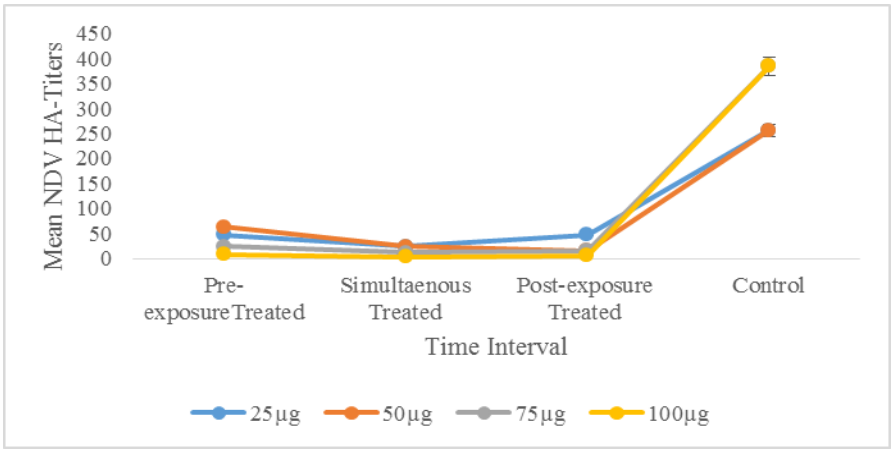

Fig. 1: Comparison of dose dependent Anti – NDV Activity of *Phyllanthus emblica* crude extracts in different time course.

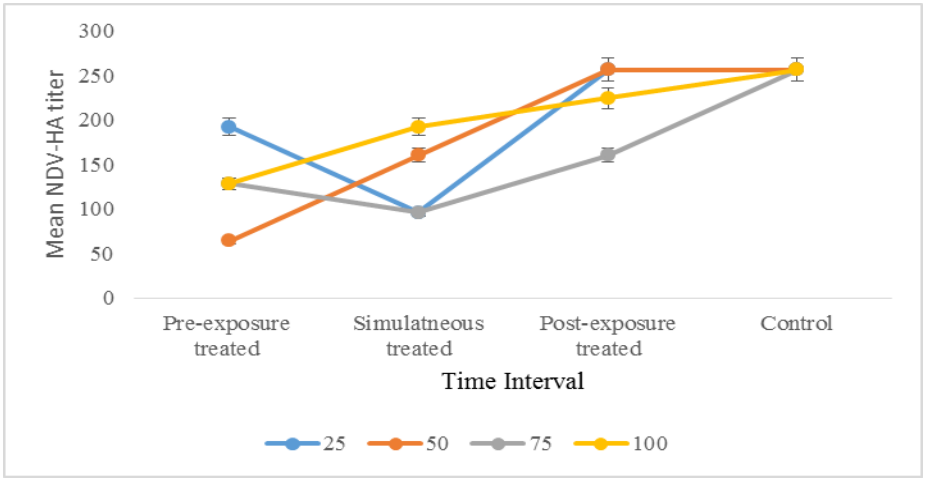

Fig. 2: Comparison of dose dependent anti-NDV activity of *Glyceriza glybra* crude extracts in different time courses.

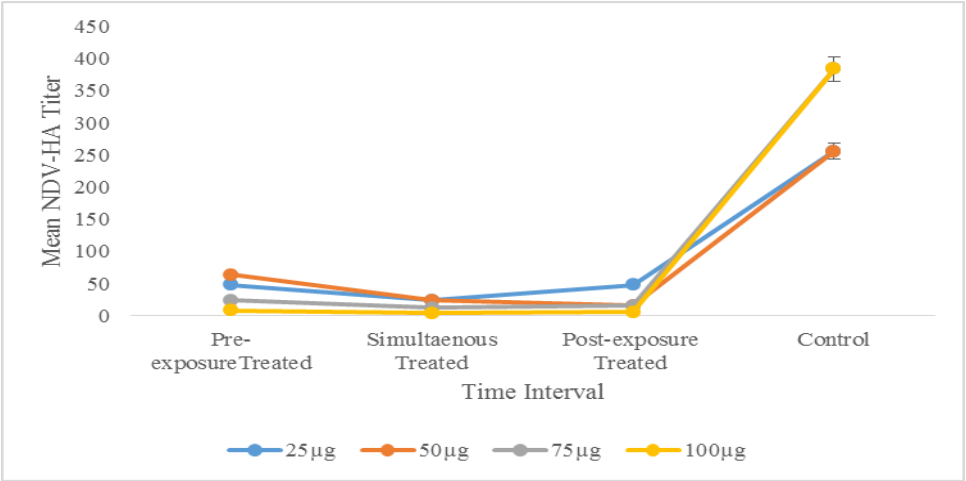

Fig. 3: Comparison of dose dependent Anti – NDV Activity of *Allium sativum* crude extracts in different time course.

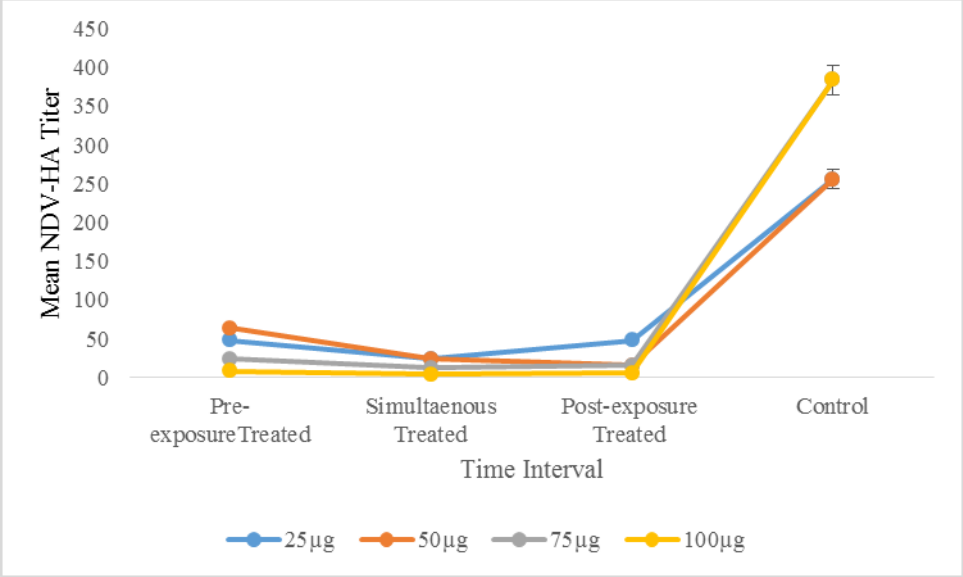

Fig. 4: Comparison of dose dependent Anti – NDV Activity of *Azadirchita indica* crude extracts in different time course.

Fig. 5: Steps Involved in Evaluation of Anti-NDV Activity on in Vitro Cultured Vero Cell line.

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