# *In vivo* antiviral potential of *Glycyrrhiza glabra* extract against Newcastle disease virus

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**Abstract**: Newcastle disease is highly infectious viral disease causing huge economic losses worldwide. These losses can be prevented by control of viral diseases. Medicinal plants have been traditionally used for treatment of different diseases since long. In this study the effect of extracts from *Glycyrrhiza glabra* leaves are investigated against Newcastle disease virus (NDV) by an *in-vivo* assay. Seven groups of nine-day-old embryonated chicken eggs were inoculated with various treatments of different plant extracts. All the groups except uninoculated negative control group were inoculated with velogenic NDV strain; five groups received different concentrations of the three extracts. Daily observe the rate of embryo survival. Allantoic fluid from treated eggs was collected for hem agglutination test. Results showed that embryo survival rate was higher 300µg/mL treated group as all the extracts showed antiviral activity. Similarly, the plant extracts effectively control virus as no viruses were identified in the allantoic fluids of all groups treated with low doses of plant. The current results have clearly verified that all the extracts especially that of methanol 300µg/mL from leaves of *Glycyrrhiza glabra* have strong antiviral activity against NDV *in vivo.*

**Keywords**: Newcastle disease, *Glycyrrhiza glabra*, Haemagglutination test, *in-vivo*, poultry.

## INTRODUCTION

Newcastle disease (ND) is a contagious bird disease affecting many domestic and wild avian species (Omer *et al*., 2014). ND is commonly known as Ranikhait disease in India (Narayanan *et al*., 2010) and also in Pakistan, the causative agent is Newcastle disease virus (NDV). The commercial poultry farmers from around the world suffer massive economic losses due to ND. In 2012, rigorous outburst of ND reasoned by APMV 1 serotype, ensued in Jallo Wildlife Park, Lahore. Approximately 190 peacocks died within seven days with a death rate 100% and loss of the susceptible birds was 50%. NDV depreciate the quality of eggs, impairs growth and decreases the performance of birds (Yan *et al*., 2011). In acute cases, death occurs immediately without appearing any signs (Ashraf & Shah, 2014). Currently, many live and inactivated ND vaccines are accessible just about all the world (Xiao *et al*., 2013) but still the outbreak due to ND occurs. Due to mutations within viral strains, NDV can become resistant and difficult to control, and there is a need to search for alternative measures. A variety of diseases are caused by viruses, yet only a a small number of antiviral drugs have been developed, there is a need to discover new antiviral compounds that are safe, effective, less toxic and over comes resistance (Nogon *et al*., 2011).

Plants can serve as a superior source of antiviral drugs. The plants do not have mobility and they have the only possibility of protection is by producing chemicals against pathogens such as viruses. Similary, these chemicals could have the ability to protect animals and humans against different viruses as well (Si Yuan *et al*., 2014). Many plants native to Pakistan promises immense possibilities for the discovery of new compounds with important medicinal applications in strengthening the immune system and combating infection. The demand and consumption of medically important plants is being taken up by many countries because of easy availability, low cost, good antimicrobial nature, varied functions in convalescing growth rate, feed conversion rate and weight gain in birds (Sajid *et al*., 2011).

The utilization of medicinal plants is as primitive as mankind itself and from the entire world reported higher plant species; about 10% has proven their capacity to heal ailing population (Shinwari, 2010). Numerous present medicinal systems such as Homeopathy, Ayurveda, Naturopathy, Unani, Sidha etc have developed plants as efficient medicines to fight against many damaging diseases (Prasad *et al*., 2011).

Throughout history, plants have used as a rich source of medicine and pharmaceuticals (Schmidt *et al*., 2008).

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Herbal preparations are frequently used in management of poultry diseases in rural areas (Musa, 2008). The traditional basis for the utilization of *Glycyrrhiza glabra* species as an herbal medicine is reported in ancient manuscripts from India, China and Greece. Number of researchers has reported the use of this plant against many viral respiratory tract infections. A compound "glycyrrhizin," along with its derivatives demonstrated less damage in chronic cases of hepatitis B & C infected patients (Cristina, 2007). Glycyrrhizin CD $4^+$ T-cell tumor necrosis factor-mediated cytotoxicity (Yoshikawa, 1997). The current study was designed to investigate the *in vivo* efficacy of plant leaf extract as an anti viral agent against the NDV.

## MATERIALS AND METHODS

***Identification and collection of samples***

The test plant species were collected from botanical garden at Govt. College University, Faisalabad and identified as *Glycyrrhiza glabra* in field using standard keys and descriptions. Its botanical identity was confirmed and authenticated from Dr. Naeem Iqbal, Department of Botany Government College University, Faisalabad. The voucher specimen was prepared and preserved in the Department of Botany.

***Desiccation and maceration***

After collection the leaves of the plant was pulverized to clean and dried for two weeks under shady places then ground in the grinder to form a powder. The powder was passed through a sieve size of 0.1mm and then kept in airtight bags in a cool dry room until use. Aqueous, methanolic and ethanolic extracts were made separately. For this purpose, 500g of *G. glabra* leaves powder was mixed separately in 1L of distilled water or methanol or ethanol and kept for one week at a room temperature. These suspensions were filtered by What Mann's paper no. 1 and then concentrated by using a rotary evaporator at $50^0$C to clear the solvent and stored in refrigerator for further use in the *in-vivo* assay. After weighing, different aqueous concentrations (0µg/mL as control, 300µg/mL, 400µg/mL, 500µg/mL and 600µg/mL) of *G. glabra* were formulated to evaluate its antiviral activity.

***Sterility test***

The sterility test was performed to check the bacterial contamination in each extract. For sterility test nutrient agar plates were used and 0.1mL of each extract was poured on it and incubated it at 37°C. The bacterial growth was observed after 24 hrs.

***Determination of median embryo infectious dose ($EID_{50}$) of the virus***

The $EID_{50}$ of the virus was determined using method of Young *et al*. (2002). 4.5mL each of PBS was dispensed into 4 sterile McCartney bottles, labeled $10^{-1}$ to $10^{-10}$. 0.2mL of NDV viral stock was added to bottle1 labeled $10^{-1}$ and mixed thoroughly. 0.2mL of dilution in bottle 1 was transferred into bottle 2 and mixed thoroughly. The same dilution was done across the bottles until bottle 10 to make 10 fold serial dilutions so these diluents solutions were prepared as ($10^{-1}$ to $10^{-10}$). Nine- day- old embryonated chicken eggs, obtained from healthy flock of local poultry farm, were candled, labeled and swabbed with 70% ethanol. Five Eggs in each group were inoculated with 0.2mL of respective dilution. Eggs were candled after every 24 hours to confirm viability and also after 48 hours. Results were calculated appropriately. $EID_{50}$ was calculated according to the method of Young *et al*. (2002).

Dilution= % Mortality at dilution just above 50% - 50%
% Mortality at dilution just above 50%-% Mortality at dilution just below 50%

The above formula provided $1EID_{50}$/ 0.2ml

From this, $100EID_{50}$/0.2ml of the virus stock was calculated for the experiment

***Antiviral assay***

The antiviral assay was performed by adopting the method described by Sally (2002) using NDV Lasota strain and non infectious 9-day-old embryonated chicken eggs. The eggs were grouped in a competely randomized pattern into seven groups. To prepare 10% w/v working stock solutions, 2g of aqueous, ethanolic and methanolic extracts were dissolved in 20mL dimethylsulphoxide (DMSO). From these working solutions, four different concentrations 300, 400, 500, 600µg/mL was prepared by serial dilution method. The different prepared inoculums were injected to the embryonated eggs according to the procedure of Chollom *et al*. (2012). A volume of 0.1mL of virus suspension and 0.9mL of different plant extract from the stock solution at different concentrations was mixed and kept for one hour at room temperature to react and then inoculated in eggs. The eggs were kept in incubator at $37^o$C for 72 hrs. The eggs with virus suspension without extract dole out as positive controls while uninoculated eggs dole out as negative controls.

The eggs were set in incubator for 24 hours with frequent candling after every 24 hours. Observations were made on the embryo movements, blood vessels and time of embryo death. After 72 hours the embryos were chilled at 4°C and allantoic fluids from the treated eggs were collected for spot Hem agglutination test to detect the NDV in the eggs by following the procedure of Murakawa *et al*., 2003. The protocol of Young *et al*., 2002 was adopted to perform Hem agglutination test to confirm the presence of NDV.

## RESULTS

***Median percent (%) embryo infectious dose ($EID_{50}$)***

The concentration of NDV in a suspension is expressed as an infectivity titre. The infectivity titre is established by performing a titration. The results of titration are shown in table 1.

**Table 1**: Median percent Embryo Infectious Dose

| Median percent Embryo Infectious Dose Dilutions | No. of eggs | No. of eggs alive | No. of eggs dead | Percentage Mortality |
|---|---|---|---|---|
| $10^{-1}$ | 5 | 0 | 5 | 100% |
| $10^{-2}$ | 5 | 2 | 3 | 60% |
| $10^{-3}$ | 5 | 3 | 2 | 40% |
| $10^{-4}$ | 5 | 5 | 5 | 60% |
| $10^{-5}$ | 5 | 5 | 0 | 0% |
| $10^{-6}$ | 5 | 5 | 0 | 0% |
| $10^{-7}$ | 5 | 5 | 0 | 0% |
| $10^{-8}$ | 5 | 5 | 0 | 0% |
| $10^{-9}$ | 5 | 5 | 0 | 0% |
| $10^{-10}$ | 5 | 5 | 0 | 0% |
| Virus control | 5 | 0 | 5 | 100% |
| PBS control | 5 | 5 | 0 | 0% |

**Table 2**: Embryo deaths following inoculation of embryonated chicken eggs with Newcastle disease virus (NDV) and incubation at different concentrations of different extracts from leaves of *G. glabra*

| Treatment | Concentration (µg/mL) | Time of embryo death (h) | | | |
|---|---|---|---|---|---|
| | | 0 | 24 | 48 | 72 |
| Untreated egg | - | 0 | 0 | 0 | 0 |
| NDV alone | - | 0 | 2 | 3 | - |
| Aqueous extract + NDV(AE) | 300 | 0 | 0 | 0 | 0 |
| | 400 | 0 | 0 | 1 | 0 |
| | 500 | 0 | 0 | 1 | 2 |
| | 600 | 0 | 3 | 1 | 1 |
| Methanolic extract + NDV (ME) | 300 | 0 | 0 | 0 | 0 |
| | 400 | 0 | 0 | 0 | 2 |
| | 500 | 0 | 0 | 1 | 1 |
| | 600 | 0 | 0 | 1 | 2 |
| Ethanolic extract + NDV (EE) | 300 | 0 | 0 | 1 | 2 |
| | 400 | 0 | 0 | 0 | 0 |
| | 500 | 0 | 0 | 2 | 0 |
| | 600 | 0 | 0 | 2 | 2 |

**Table 3**: Mean hemagglutination (HA) titres in embryonated chicken eggs following inoculation with new castle disease virus (NDV) and different concentrations of different extracts from leaves of *G. glabra*

| Treatment | Concentration (µg/mL) | HA titre |
|---|---|---|
| Untreated egg | - | 0 |
| NDV alone | - | 2048 |
| Aqueous extract + NDV(AE) | 300 | 0 |
| | 400 | 4 |
| | 500 | 8 |
| | 600 | 32 |
| Methanolic extract + NDV (ME) | 300 | 2 |
| | 400 | 4 |
| | 500 | 4 |
| | 600 | 8 |
| Ethanolic extract + NDV (EE) | 300 | 8 |
| | 400 | 2 |
| | 500 | 4 |
| | 600 | 16 |

Dilution of $10^{-2}$ is % mortality at dilution just above 50%= 60 percent
Dilution of $10^{-3}$ is % mortality at dilution just below 50%= 40 percent

Dilution= % Mortality at dilution just above 50% - 50%
% Mortality at dilution just above 50% - % Mortality at dilution just below 50%
= 60%-50%
= 10%
20% = 0.5
The dilution that produced the infection rate directly above 50 percent=$10^{-3.5}$
one $EID_{50}$ unit of virus in this dilution of the virus suspension is in 0.2ml.
Ten times the reciprocal of the calculated dilution will be present in 1ml of the virus suspension.
As a result infectivity titre of virus suspension in $EID_{50}$/mL= 10 x $10^{3.5}$= $10^{4.5}$ $EID_{50}$/mL.

***In vivo antiviral activity of different extracts from G. glabra***

*Time for embryo death*

The table 2 illustrates the time of embryo deaths. Throughout the three days of experiment, the following groups did not show any embryo death: negative control (untreated eggs), aqueous and methanolic extract at 300 µg/mL and ethanolic extract at 400µg/mL. The death of first embryo was observed in the positive control group (NDV alone) 24 hrs post inoculation; the mortality rate in this group at 48 hrs was 80% and at 72h all the embryos died. This was followed by the group aqueous extract at 600µg/mL. At the highest concentration 600µg/mL of different plant extracts, embryo deaths were recorded at 24h for aqueous extract and 48h for methanolic and ethanolic extract (table 2).

***NDV hem agglutination titres***

HA titres of different groups of NDV-infected chicken eggs are shown in table 3. The results signified that all experimental extracts were able to significantly lower the HA titers. NDV was not detected in the negative control and aqueous and methanolic extracts at the concentration of 300µg/mL while in case of ethanolic extract, no virus was detected in 400µg/mL. The highest HA titres value, 2,048 and 32 were recorded in eggs inoculated with NDV alone and NDV in aqueous extract at 600µg/mL, respectively (table 3).

## DISCUSSION

The poultry industry is playing important role in the economy of many countries. Birds are exposed to stress conditions when rearing on large scale. Disease development due to bad environmental conditions leads to high economic losses in the flocks. Antimicrobial agents are being used as growth promoters for poultry industry. Due to side effects of these antibiotics, manufacturers and consumers are searching for alternative of these antibiotics. Recently medicinal phytochemicals have fascinated a lot of attention for their possible role as alternative for valuable effects on the health, growth and performance of the birds by improving immune system of the body and increasing antimicrobial activity (Alloui *et al*., 2014).

The World Organization for Animal Health has placed ND in list A. ND is one of the most significant diseases of poultry birds, because it harshly affects worldwide poultry production (Fernandas *et al*., 2014). ND virus was propagated in 9 to 10 days old embryonated eggs. The ND virus was identified by serological test known as a Hem agglutination inhibition test. In this test, the HA receptors on the ND virus were neutralized by specific antibodies, due to which ND virus failed to cause the Hem agglutination of chicken $RBC_S$ . This was in an agreement with the findings of Brugh *et al*., 1980 who described that ND virus can be confirmed by Hem agglutination inhibition activity along with the means death time (MDT), interacerebral pathogenicity index (ICPI), and an intravenous pathogenicity index.

The current study has established the antiviral activity of different extracts from leaves of *G. glabra* against Newcastle disease virus by applying a chicken *in ovo* technique. In this study, within 2 days post- inoculation death of all chicken embryos with the NDV clearly showed highly virulent nature of the virus strain. However the survival time of the chicken embryos was considerably lengthened in *G. glabra* extract treated groups in a dose-dependent manner. Utilization of plant extracts have been reported to inhibit ND virus activity. Waihenya *et al*. (2002) documented the effect of *Salmonella gallinarum* and *Aloe secundiflora* against NDV and other bacterial species in chickens but no therapeutic or prophylactic effects were reported. While Mtambo *et al*., 1999 studied basic extracts of *Citrum limon, Capsicum flutescens and Oputia vulgaris* showing no effects against ND virus in chickens.

The antiviral potential of different extracts from *G. glabra* was further verified in the current study from hemagglutination (HA) test data through which amount of virus is quantified from the allantoic fluid of chicken embryos. The highly significant decrease in virus populations recommended a strong viricidal effect in test plant extract treated embryos.

Wafaa *et al*. (2007) establish that 3 to 4µg/mL neem tree extract had a robusting inhibitory action against IBDV and NDV *in ovo*. Related results were also observed by Waihenya *et al*. (2002) by applying *Aloe* spp. extract which inhibited NDV multiplication in embryonated chick eggs. Sulaiman *et al*. (2011) also observed that

methanolic root bark extract from *Adansonia digitata* can completely inhibit the growth of NDV. While Omer *et al*., 2014 evaluated the comparative antiviral efficacy and toxicity of *Glycyrrhiza glabra* aqueous extract and ribavirin against the NDV. The researchers found that 60 mg/100 ml concentration of Glycyrrhiza extract produced no toxicity in the embryonated eggs and showed anti-viral activity against the virus (Omer *et al*., 2014).

The mechanisms through which plant extract inhibit NDV multiplication *in ovo* are still unknown. Glycyrrhizic acid (GA) is considered the principal component in Glycyrrhiza spp. with a wide spectrum of antiviral activity (Wang *et al*., 2013). The Glycyrrhizic acid acts on different levels of the ND viral infection including adsorption, penetration, transcription, translation, assembly and releasing from the host cell. These findings are in line to the Pompei *et al* (1984) Baba and Shigeta (1987) Badam (1987), Utsunomiya (1997), Harada (2005) and Cristina *et al* (2007). In these studies, the researchers indicated that Glycyrrhizic acid inhibits the virus infection by the reduction of membrane fluidity, by the production of Gama interferon, inhibition of phosphorylating enzymes and reduction in viral latency.

## CONCLUSION

In conclusion, the present study has strongly verified that the different extracts of *G. glabra* acquire antiviral activity against NDV *in vivo*, while the three tested extracts showed similar efficacy with minor difference, we suggest the use of *G. glabra* to control NDV but still i*n vivo* investigations are required to authorize the utilization of this plant againt Newcastle disease in birds.

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