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In ovo antiviral activity of *Andrographis paniculata* against NDV2K35 strain of Newcastle disease virus

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Abstract

The antiviral activity of *Andrographis paniculata* was assessed in aqueous extract, ethanolic extract, andrographolide isolated from ethanolic extract and andrographolide standard against NDV2K35 strain of Newcastle disease virus by *in ovo* technique. The results were compared with standard antiviral drug ribavirin. All the embryo survived in treatment groups during the experiment. Haemagglutination was absent in all the groups except virus control and in groups treated with ethanolic extract. The absence of virus in these groups was further confirmed by RT-PCR for F gene. Thus, the present study confirms the antiviral activity for aqueous extract of *Andrographis paniculata*, andrographolide isolated from ethanolic extract and andrographolide standard against NDV2K35 strain of Newcastle disease virus.

Keywords: *Andrographis paniculata*, Andrographolide, *In ovo* antiviral, NDV2K35, Newcastle disease virus

Introduction

Newcastle disease (ND) caused by virulent strains of avian paramyxovirus-1 remains a constant threat to poultry producers worldwide despite the advances made in diagnosis of and vaccination for the virus [1]. At present, there is no specific treatment for ND and the only commercial control measure so far available is vaccination, which does not always confer 100 per cent immunity in birds. Moreover, in backyard chicken morbidity and mortality due to NDV is enormous as these birds are not protected by vaccination.

Natural products have proved to be an important source of lead molecules and many extracts and compounds of plant origin have been reported to possess antiviral activity. *Andrographis paniculata*, a member of the family Acanthaceae, commonly known as Kalmegh (King of bitters) is found throughout Asia. This plant has been widely used for treating sore throat, flu, and upper respiratory tract infections in China, India, Thailand, and Malaysia [2]. The active principle andrographolide was shown to exhibit immunomodulatory effect by effectively enhancing cytotoxic T cells, phagocytosis by natural killer cells, and antibody-dependent cell-mediated cytotoxicity [3].

The antiviral activity of *Andrographis paniculata* against herpes simplex virus 1, HIV, Ebstein-Barr virus, Simian retrovirus and Dengue virus was reported in various cell lines [5-8]. Hence, an attempt was made to explore the antiviral property of this potential herb against NDV by *in ovo* assay.

Materials and Methods***Andrographis paniculata***

The leaves of *Andrographis paniculata* were collected from Vellankovil village of Erode district, Tamil Nadu and authenticated by Botanical Survey of India, Coimbatore. The collected leaves were shade dried, powdered and used for extract preparation.

Preparation of *Andrographis paniculata* extracts

Ten per cent aqueous and ethanolic extracts of *A. paniculata* leaves were prepared by adding ten grams of dry powder to 100mL of distilled water and 70 per cent alcohol, respectively. It was kept in a rotary shaker for 48hrs, filtered and then incubated at 37°C for 48hrs to evaporate the solvent. The collected extract was stored in airtight container until further evaluation.

Isolation and quantification of andrographolide

Andrographolide was isolated from ethanolic extract by column chromatography [9]. Briefly, slurry of silica gel (mesh size-60) in chloroform was packed in a column as a stationary phase and dried ethanolic extract was dissolved in ethanol and applied at top of prepared column as a mobile phase. Fractions were collected by adding different gradient ratio of chloroform and

methanol (90:10, 80:20, 60:40, 40:60 and 20:80). Totally five fractions were collected and the presence of andrographolide in the fraction was confirmed by observing the λ_{max} (200-240nm) using UV- VIS spectrophotometry. The fraction containing andrographolide was concentrated in vacuum evaporator and stored at 4°C until further use. It was quantified by High Performance Thin Layer Chromatography (HPTLC) at Siddha Central Research Institute, Chennai.

Newcastle disease virus

Whole genome sequenced Newcastle disease virus isolate (NDV2K35) was procured from Department of Animal Biotechnology, Madras Veterinary College, Chennai.

Cultivation of virus by allantoic route

The NDV2K35 strain was cultivated in allantoic route as per the method of Alexander ^[10]. Briefly, 9 to 11 day old embryonated chicken eggs were candled, and a spot was marked at 0.5cm from the base of the air cell and opposite to the eye spot of embryo. The shell was disinfected with 70 per cent alcohol and punctured at marked spot with egg driller. A syringe was used for injecting 0.1mL of NDV2K35 strain through the punctured spot. The needle was removed slowly, and the hole was sealed with melted wax. The eggs were incubated at 37°C and candled twice daily and dead embryos were discarded. After 72hrs, the embryos were chilled at 4°C for 1hr. The allantoic fluid was aspirated aseptically, centrifuged at 5000rpm for 10min at 4°C and the supernatant was collected in sterile vials and stored at -20°C. The titre of NDV in freshly harvested allantoic fluid was identified by HA test.

Experimental design for *in ovo* study

Based on the results of *in vitro* study the dose of various treatments for *in ovo* assay was fixed and the experiment was conducted as given below:

| Group | Treatment |
|----------------|--|
| T ₁ | Control |
| T ₂ | Virus control(NDV) |
| T ₃ | NDV + 0.1% DMSO |
| T ₄ | NDV +Ribavirin (500µg/mL) |
| T ₅ | NDV + Aqueous extract of <i>A. paniculata</i> (2.5µg/mL) |
| T ₆ | NDV + Ethanolic extract of <i>A. paniculata</i> (2.5µg/mL) |
| T ₇ | NDV + Andrographolide isolated/ AGI(2.5µg/mL) |
| T ₈ | NDV + Andrographolide standard/ AGS (2.5µg/mL) |

Embryo Infective Dose 50 (EID₅₀)

Embryo Infective Dose 50 (EID₅₀) was calculated by the method of Takase *et al.* (2000). The virus (NDV2K35) was serially diluted tenfold in PBS (10⁻¹ to 10⁻⁸) and 0.1mL of each dilution was injected into the allantoic cavity of ten-day old embryonated chicken eggs (four eggs/group). The eggs were incubated at 37°C for three days and examined twice daily for survivability. At the end of 72hrs the eggs were chilled at 4°C for three hours, broken for examination and recorded as unaffected/affected by recording the lesions. The dilution of virus causing EID₅₀ was calculated by Reed and Muench formula ^[11] and used for *in ovo* assay.

In ovo antiviral assay

In ovo antiviral activity of plant extracts and ribavirin was evaluated using 10-day old ECEs as per the experimental design mentioned above ^[12]. Briefly, 0.1ml of virus (EID₅₀) was mixed with 0.1mL of various treatments, incubated for

1hr at 37°C and then inoculated (0.2mL) into four eggs per group through allantoic route. The inoculated ECEs were incubated for three days and examined twice daily for survivability. At the end of incubation period the ECEs were removed from the incubator, chilled at 4°C for 3hrs, the allantoic fluid was harvested aseptically and subjected to HA test. Another aliquot of allantoic fluid was stored at -20°C for confirmation of virus by RT-PCR. The eggs were broken for examination and recorded as unaffected/affected by examining the lesions.

Confirmation of viral inhibition

Haemagglutination test

Sterile Alsever's solution and normal saline were prepared, and the test was conducted as per the standard protocol described by FAO. The harvested allantoic fluid from *in ovo* assay was subjected to haemagglutination test with 1% chicken RBCs suspension. The reduction in HA activity of virus was used as indicator of antiviral activity of *A. paniculata* extracts and active principles.

RT- PCR

The following primer sequence was used for amplifying F gene of NDV (in allantoic fluid) whose product size was 535bp.

Forward primer: 5'- ATG GGC TCC AGA CCT TCT ACCA-3'
Reverse primer: 5'- CTG CCA CTG CTA GTT GTG ATA ATCC -3'

Method of RNA extraction

Total RNA was extracted using TRIZOL LR reagent ^[13]. Briefly, 200µL of allantoic fluid was mixed with 600µL TRIZOL LR reagent and incubated for 20min at room temperature. To the mixture, 200µL of chloroform was added, mixed gently for about 15sec and incubated at room temperature for 15min. The mixtures were phase separated by micro centrifugation at 12,000rpm for 20min at 4° C. The RNA was then precipitated by adding 500µL of isopropanol to the aqueous phase and left at -20°C for 30 min. The precipitated RNA was micro centrifuged at 12,000 rpm for 20min and the pellet obtained was washed once with nuclease free water. The pellet was air dried and dissolved in nuclease free water, the RNA concentration was determined by Nanodrop method and stored at -20°C for further use.

c-DNA synthesis

The extracted RNA was used for the cDNA synthesis by using cDNA Synthesis Kit. The reaction mixture was carried out in 20µL volume according to the manufacturer's protocol as described below:

| Reagents | Volume |
|---|----------|
| 5x Reaction Buffer | : 4.0µL |
| dNTP (Deoxyribonucleotide triphosphate) | : 1.0µL |
| Random Hexamer | : 2.0µL |
| Reverse Transcriptase | : 1.0µL |
| RNase inhibitor | : 1.0µL |
| RNA (1µg) | : 1.0µg |
| Nuclease Free Water add to | : 20.0µL |

The contents were mixed gently and kept in a thermal cycler (BioRad, USA) with the cyclic condition as mentioned below:

| | |
|--------|------------------|
| Step 1 | : 25°C for 5min |
| Step 2 | : 42°C for 60min |
| Step 3 | : 85°C for 5min |

Step 4 : hold at 4°C

The synthesized cDNA was stored at -20°C for long term use.

RT-PCR to detect F gene of NDV

The RT-PCR was carried out in a final volume of 20µL using 200µL capacity thin wall PCR tubes. The reaction mixture was prepared according to the manufacturer's protocol as described below:

| Components | Quantity(µL) |
|---|--------------|
| Taq master mix red (2x) (Ampliqon, USA) | : 10.0 |
| Forward primers (Nd) | : 1.0 |
| Reverse primers (Nx) | : 1.0 |
| Template cDNA | : 2.0 |
| Nuclease free water add to | : 20.0 |

Negative control (nuclease free water) was used in each PCR cycle without template DNA. PCR tubes containing the mixture were tapped gently and spun briefly. The PCR tubes with all the components were transferred to thermal cycler with the cyclic condition as mentioned below:

| S. No. | Steps | Temperature | Time |
|--------------------------------------|----------------------|-------------|-------|
| Step 1 | Initial denaturation | 94°C | 5min |
| Step 2 | Denaturation | 94°C | 30sec |
| Step 3 | Annealing | 60°C | 45sec |
| Step 4 | Extension | 72°C | 45sec |
| Step 2-4 were repeated for 35 cycles | | | |
| Step 5 | Final extension | 72°C | 10min |
| Step 6 | Holding temperature | 4 °C | Hold |

Electrophoresis of PCR product

- The agarose gel was prepared with 1.5 per cent agarose containing one per cent ethidium bromide @0.5µL/100mL in 1X Tris Acetic acid EDTA (TAE) buffer.
- Gel was casted with comb inserted.
- After polymerization, comb was carefully removed without disturbing the wells.
- 5µL of PCR products were loaded in each well.
- 5µL of 100bp DNA was loaded in one well to compare the amplified products.
- The electrophoresis was carried out in 1X TAE buffer at 80V for 40min.
- The gel was examined in Gel doc™ system (Bio Rad, USA) and documented.

- The expected size of PCR products for F gene was 535bp.

Statistical Analysis

The data collected were analyzed by one-way ANOVA procedure using SPSS® 20.0 software package for Windows and level of significance was tested by Duncan's multiple range test.

Results and Discussion

Isolation and Quantification of Andrographolide

Andrographolide was successfully separated from ethanolic extract of *A. paniculata* by column chromatography using different gradients of chloroform and methanol. As per the earlier reports, the fractions showing maximum absorbance at 220-240nm was assumed to contain andrographolide [9, 14, 15]. In the present study, the first fraction showed maximum absorbance at 220nm and on quantification by HPTLC was found to contain 1.752 per cent.

The andrographolide content in leaf powder detected by HPLC was found to vary from 0.98-1.15% w/w [14]. In whole plant, the maximum concentration (2.02%) was observed at 110 days of plantation [16]. Andrographolide is the major diterpenoid in *A. paniculata* making up about 4, 0.8-1.2 and 0.5-6% in dried whole plant, stem and leaf extracts, respectively [17]. The concentration of andrographolide obtained in the study was almost equal to the level obtained by combining reflux extraction with column chromatography where in maximum extraction was 1.9 per cent [18]. The leaf used in the present study was collected from plants over 120 days and the quantity identified is on par with the previous reports which reflect the medicinal potential of the collected plant.

EID₅₀ for NDV2K35 strain

Embryo Infective Dose 50 (EID₅₀) for the virus was calculated using 10⁻¹ to 10⁻⁸ dilution of stock virus and the result is presented in Table 1 and Plate 1. At 72hrs the eggs were chilled, opened and observed for the lesions such as congestion and haemorrhage in extremities and compared with control. It was observed that the lesions gradually reduced as the dilution of the virus increased. Based on the lesion score the EID₅₀ titre of NDV2K35 strain was calculated to be 10⁻⁵/0.1mL.

Table 1: Estimation of EID₅₀ for NDV2K35 in embryonated chicken eggs

| Dilution | No of eggs inoculated | No of eggs with lesion | No of eggs without lesion | Accumulated value | | % of eggs with lesion |
|-------------------|-----------------------|------------------------|---------------------------|-------------------|----------------|-----------------------|
| | | | | With lesion | Without lesion | |
| 10 ⁻¹ | 4 | 4 | 0 | 18 | 0 | 100 |
| 10 ⁻² | 4 | 4 | 0 | 14 | 0 | 100 |
| 10 ⁻³ | 4 | 4 | 0 | 10 | 0 | 100 |
| 10 ⁻⁴ | 4 | 3 | 1 | 6 | 1 | 86 |
| 10 ^{-5*} | 4 | 2 | 2 | 3 | 3 | 50 |
| 10 ⁻⁶ | 4 | 1 | 3 | 1 | 6 | 14 |
| 10 ⁻⁷ | 4 | 0 | 4 | 0 | 10 | 0 |
| 10 ⁻⁸ | 4 | 0 | 4 | 0 | 14 | 0 |

* EID₅₀

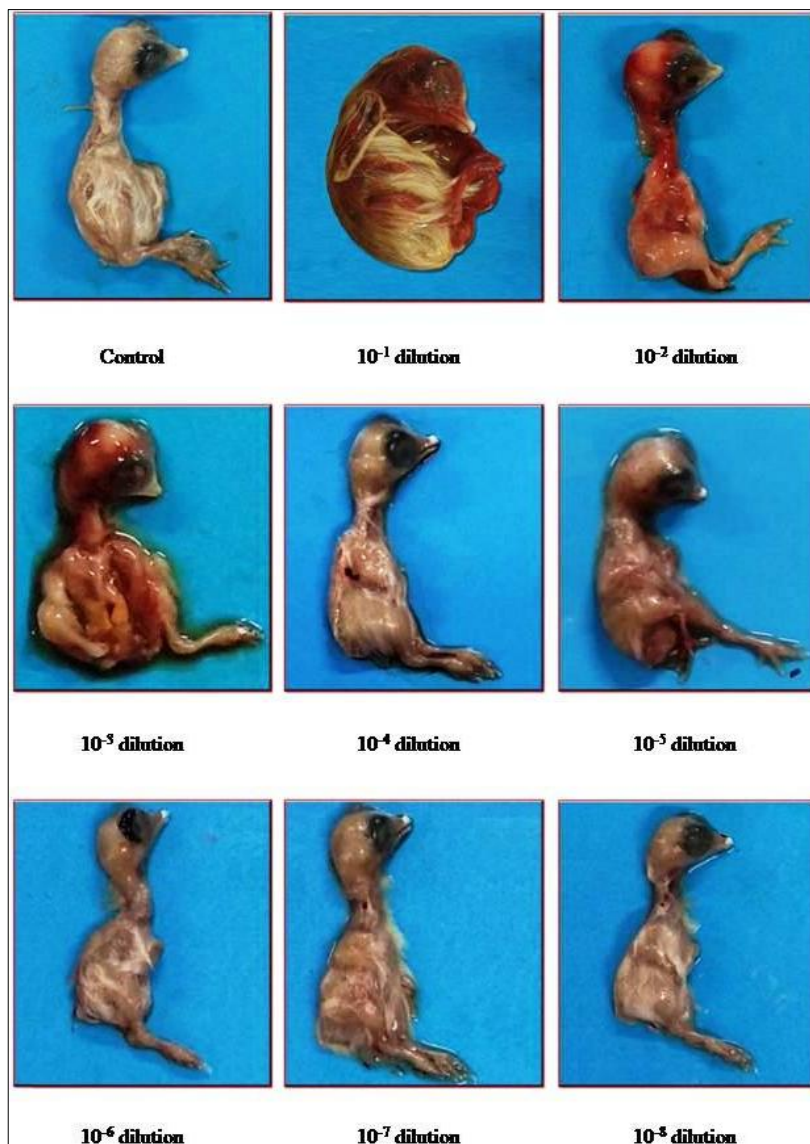


Plate 1: Estimation of EID₅₀ for NDV2K35 strain of Newcastle disease virus

***In ovo* Antiviral Activity**

Effect of treatment on embryo survivability

For *in ovo* assay 100EID₅₀ of the virus was used and the eggs in all treatment groups were observed daily for survivability. At the end of 72hrs, 50 per cent embryo death was noticed in virus control whereas all the embryos survived in the remaining groups (Table 2 and Plate 2). The strain was

originally isolated from Japanese quail by Bhuvanewari^[19] and based on mean death time of chicken embryo (67hrs) it was classified as mesogenic. However, in the present study only 50 per cent mortality was noticed at the end of 72hrs but the movement was sluggish in the remaining embryos. Thus, the strain confirms to be mesogenic (mean death time 60-90hrs) as per the classification of Hanson and Brandly^[20].

Table 2: Effect of *A. paniculata* extracts, andrographolide isolated and standard on embryo survivability (%) and HA titre (Mean±SELog₂) against NDV antigen in allantoic fluid

| S. No | Treatment groups | Embryo survivability (%) | HA titre (Log ₂) |
|-------|-----------------------------------|--------------------------|------------------------------|
| 1 | Control | 100 | 0.00 ^a ±0.00 |
| 2 | NDV alone | 50 | 6.66 ^c ±0.54 |
| 3 | DMSO alone | 100 | 0.00 ^a ±0.00 |
| 4 | Ribavirin (500µg/mL) | 100 | 0.00 ^a ±0.00 |
| 5 | NDV+ Aqueous extract (2.5µg/mL) | 100 | 0.00 ^a ±0.00 |
| 6 | NDV+ Ethanolic extract (2.5µg/mL) | 100 | 2.11 ^b ±0.58 |
| 7 | NDV+ AGI (2.5µg/mL) | 100 | 0.00 ^a ±0.00 |
| 8 | NDV+ AGS (2.5µg/mL) | 100 | 0.00 ^a ±0.00 |

Each value is mean of four observations

Means bearing different superscripts within a column differ significantly ($p < 0.05$)

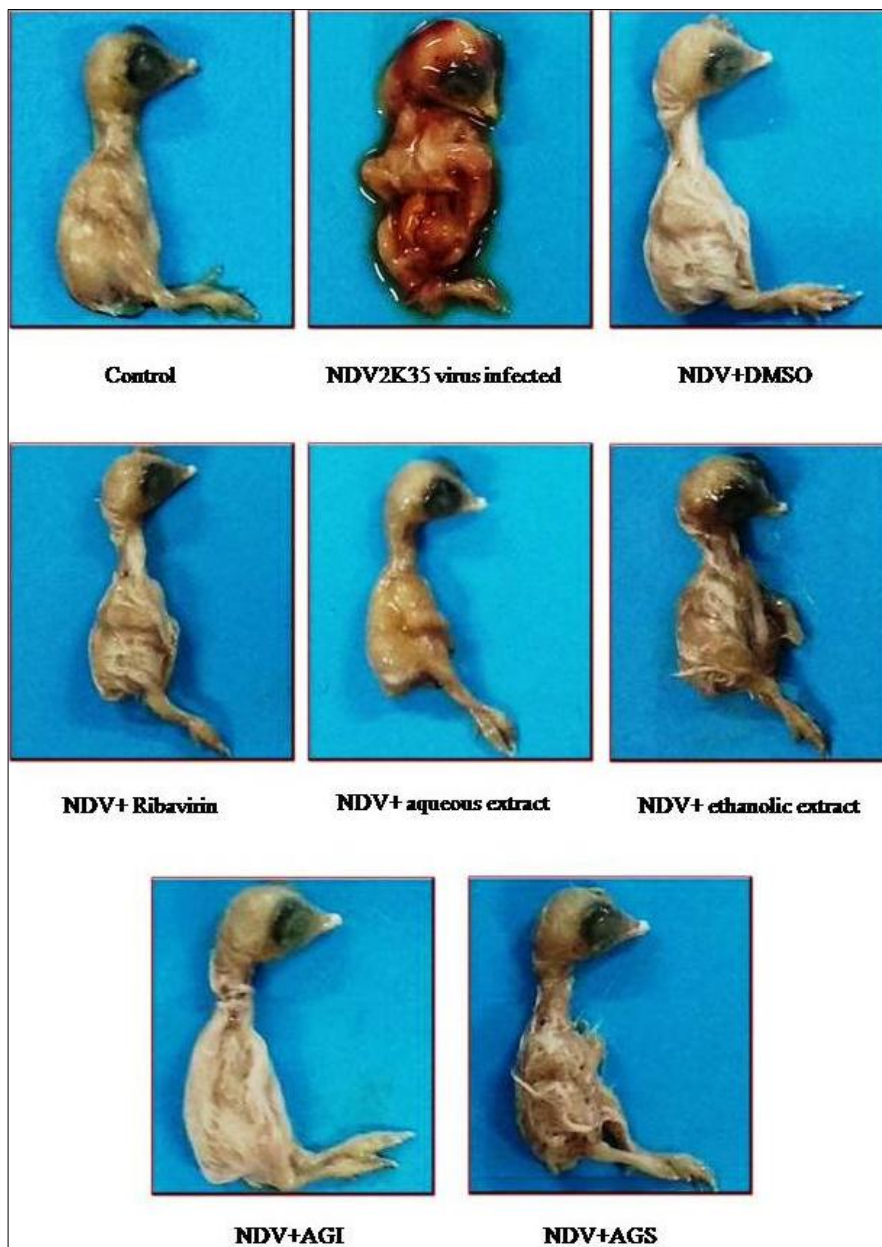


Plate 2: *In Ovo* antiviral activity of *Andrographis paniculata*

Effect of treatment on HA titre

The HA titre against NDV was detected in the allantoic fluid collected at the end of 72hrs (Table 2). The titre was significantly higher ($p < 0.05$) in virus control (6.66 ± 0.54) when compared to virus preincubated with ethanolic extract (2.11 ± 0.58) which indicate the replication of the virus. The other treatment groups did not cause haemagglutination which confirm the antiviral activity. In yet another report, ethanolic extract of *A. paniculata* at $5.0 \mu\text{g/mL}$ was found effective against NDV by *in ovo* method [21]. Hence, it is recommended to explore the antiviral activity of ethanolic extract in doses higher than the one tried in this study.

The antiviral activity might be due to the presence of polyphenols which act by binding to the virus or host cell protein thus preventing the adsorption of the virus [22]. The promising antiviral activity of terpenoids and polyphenols in other herbs were reported against H9N2 virus as evidenced by reduction in virus titre, measured by haemagglutination test and real time quantitative reverse transcription polymerase chain reaction [23]. *A. paniculata* used in this study was found to be rich in alkaloids, phenols, flavonoids, tannins and terpenoids whose synergistic effect would have resulted in

better antiviral activity. The quantity of these active principles was reported higher in ethanolic extract than aqueous extract except terpenoid [24]. Since andrographolide is a terpenoid, the highest concentration in aqueous extract would be the probable reason for the difference in antiviral activity between the two extracts.

Detection of NDV by RT-PCR

The allantoic fluid collected from various groups was subjected to RT-PCR to confirm the antiviral activity and the results are presented in Plate 3. The virus control and virus pre incubated with ethanolic extract showed amplification of F gene at 535bp whereas there was no amplification in the remaining groups which confirm their antiviral activity.

In a field outbreak of NDV, the F gene of the virus was found to amplify at 535bp and HN gene amplified at 1922bp [25]. In other reports the specific primers used for F gene of NDV amplified at 254bp/232bp, 356bp and 374bp, respectively which confirmed the presence of virus [26-28]. Thus, in this study, RT-PCR for F gene further confirms the antiviral activity of plant extracts and its active principle.

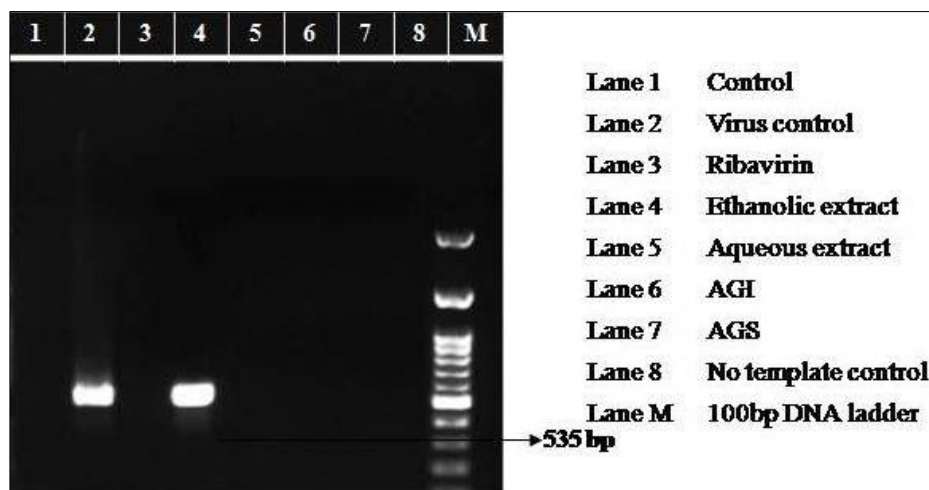


Plate 3: RT-PCR for F gene of NDV in allantoic fluid – 72hrs

Conclusion

The results of *in ovo* analysis confirm the antiviral activity of *A. paniculata* against NDV. The aqueous extract was on par with andrographolide standard which were similar to ribavirin, the standard antiviral drug as confirmed by RT-PCR. Though alcoholic extract and andrographolide isolated were unable to totally inhibit the virus, they too exhibited antiviral activity. Thus, *Andrographis paniculata* can be recommended for prevention of ND in poultry.

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