www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(4): 1461-1469
© 2023 TPI
www.thepharmajournal.com
Received: 01-02-2023
Accepted: 04-03-2023

Nagajothi S
Veterinary Assistant Surgeon, Veterinary Dispensary, Nagardhanai, Sholur Post, Sholur, Tamil Nadu, India

Mekala P
Department of Veterinary Pharmacology and Toxicology, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India

Raja A
Education Cell, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India

Raja MJ
Department of Veterinary Pharmacology and Toxicology, Veterinary College and Research Institute, Orathanadu, Tamil Nadu, India

Senthil Kumar P
Department of Veterinary Pharmacology and Toxicology, Veterinary College and Research Institute, Thirunelveli, Tamil Nadu, India

Thirumalaisamy G
Livestock Farm Complex, Veterinary College and Research Institute, Theni, Tamil Nadu, India

Corresponding Author:
Mekala P
Department of Veterinary Pharmacology and Toxicology, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India

Antiviral activity of *Andrographis paniculata* against NDV2K35 strain of Newcastle disease virus in chicken embryo fibroblast cells

Nagajothi S, Mekala P, Raja A, Raja MJ, Senthil Kumar P and Thirumalaisamy G

Abstract
Newcastle disease remains a constant threat to poultry sector especially backyard chicken due to practical difficulties in following the vaccination protocol. *Andrographis paniculata* was reported to possess antiviral activity against several viruses affecting human and animal. The present study was designed to investigate the antiviral activity of *Andrographis paniculata* against mesogenic strain of Newcastle disease virus NDV2K35. Antiviral activity of aqueous, ethanolic extracts of *Andrographis paniculata,* andrographolide standard each @1.0, 2.5, 5 µg/ml were compared with standard antiviral drug ribavirin @ 500 µg/ml in chicken embryo fibroblast cells. The antiviral activity was confirmed by assessing the cell viability, HA titre and expression of F gene by RT-PCR in cell culture fluid. Aqueous extract and andrographolide standard exhibited antiviral activity at all doses and was on par with standard antiviral drug ribavirin. The ethanolic extract was unable to completely inhibit the virus which was evident from HA titre and expression of F gene.

Keywords: *Andrographis paniculata*, andrographolide, antiviral activity, chicken embryo fibroblast, Newcastle disease virus, NDV2K35, RT-PCR

Introduction
Newcastle disease (ND) caused by avian paramyxovirus-1 in the genus Avulavirus is ranked as the fourth most important disease in terms of the number of livestock units lost for poultry species, behind highly pathogenic avian influenza, infectious bronchitis, and lowly pathogenic avian influenza [1]. Based on pathogenic studies ND is categorized into three groups: lentogenic (low virulence), mesogenic (moderate virulence) and velogenic (highly virulent). Low virulent NDV typically produces subclinical disease with some morbidity, whereas virulent isolates can result in rapid, high mortality in birds [2]. Virulent NDV are listed pathogens that require immediate notification of the OIE and outbreak typically results in trade embargos [3]. Stringent biosecurity measures and appropriate vaccination must be adopted to contain the spread of the virus as there is no treatment for ND. Even in vaccinated birds the production performance will be affected when infected with virulent strains [4]. Unlike commercial poultry morbidity and mortality is high in backyard chicken infected with NDV due to practical difficulties in vaccinating these birds. Hence, developing new control strategies and intervention program is highly imperative to contain the virus.
Natural products have proved to be an important source of lead molecules and many extracts and compounds of plant origin with antiviral activity have been reported. *Andrographis paniculata* (Burm. f.) Nees, commonly known as King of bitters, has been used as traditional medicine for treatment of various infectious and degenerative diseases in Asian countries such as India, China, Vietnam, Malaysia and Indonesia [5].
Among the several active chemical constituents, andrographolide, neoandrographolide and dehydroneoandrographolide are the most important bioprotectants with wide range of therapeutic applications. Of these, andrographolide, a diterpene lactone has been reported to have broad spectrum antiviral activity against several viruses including the recent SARS-CoV-2 virus [6]. Andrographolide has also shown immunomodulatory effect by effectively enhancing cytotoxic T cells, phagocytosis by natural killer cells, and antibody-dependent cell-mediated cytotoxicity [7]. Further, a polyherbal preparation used in Siddha medicine containing *Andrographis paniculata* as one of the ingredients was shown to improve immunity against Newcastle disease virus in backyard poultry [8].

Perusal of literature revealed few reports on the *in ovo* activity of *Andrographis paniculata*, against NDV. Hence, an attempt was made to explore the antiviral property of this potential herb against NDV in chicken embryo fibroblast cells.

Materials and Methods

Andrographis paniculata

The whole plant of *Andrographis paniculata* collected from local area was authenticated by Botanical Survey of India, Coimbatore, Tamil Nadu, India. The reference herbarium specimen was authenticated under the voucher number BSI/SRC/5/23/2017/Tech/1696. The collected leaves were shade dried, powdered and ten% aqueous and ethanolic extracts were prepared and stored in airtight container in a refrigerator until further use [9].

Newcastle disease virus

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

Experimental design for *in vitro* antiviral study

Embryonated chicken eggs of 9 to 11 days old were used throughout the study for preparing Chicken Embryo Fibroblast (CEF) and maintained in 5% CO_2 incubator. The experimental design for *in vitro* trial is as below:

The experimental design for *in vitro* trial is as below

Group	Treatment
T_1	Cell control (CEF)
T_2	Virus control (NDV2K35)
T_3	Positive control (Virus pretreated with Ribavirin 500 µg/mL)
T_4-T_6	Virus pretreated with different doses of aqueous extract of *A. paniculata* (1.0, 2.5, 5.0 µg/mL)
T_7-T_9	Virus pretreated with different doses of ethanolic extract of *A. paniculata* (1.0, 2.5, 5.0 µg/mL)
T_{10}-T_{12}	Virus pretreated with different doses of andrographolide standard (1.0, 2.5, 5.0 µg/mL)

Cultivation of NDV2K35 strain by allantoic route [10]

Briefly, 9 to 11 days old embryonated chicken eggs were candled, sterilised, and injected with 0.1mL of NDV2K35 strain and 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 and centrifuged at 5000rpm for 10 min at 4 °C. The supernatant was collected in sterile vials and stored at -20 °C until further use. The titre of NDV in freshly harvested allantoic fluid was identified by HA test.

Preparation of chicken embryo fibroblast cell culture

Primary CEF cultures were prepared from chicken embryos of 9 to 11 days old[11]. A cell suspension of 0.5 mL was diluted with an equal amount of trypan blue solution and the cells were counted in haemocytometer. Concentration of the cells was adjusted to one million cells / mL of growth medium and dispensed into tissue culture flasks and incubated for 48 hrs at 37 °C in 5% CO_2 incubator and 75% humidity to obtain confluent monolayer.

Cultivation of NDV2K35 strain in CEF

The NDV2K35 strain was cultivated in chicken embryo fibroblast cells as per standard protocol [12]. After formation of CEF monolayer, 200 µL of antibiotic (Penicillin-streptomycin (5000U/ml) treated NDV2K35 strain was inoculated into each of the 25 cm flasks. The flasks were incubated for one hour at 37 °C in 5% CO_2 incubator and media was decanted. Freshly prepared maintenance media (2% FBS) was added. The cells were observed daily under inverted microscope for the appearance of cytopathic effect (CPE). At the end of 48 hrs the culture fluid was harvested after five cycles of freeze thaw and collected in sterile vials and stored at -20 °C.

Tissue Culture Infective Dose 50 ($TCID_{50}$)

Tissue Culture Infective Dose 50 was estimated as per standard protocol [13]. Briefly, 1.5×10^4 cells/ well were seeded into 96 well plate and incubated at 37 °C with 5 percent CO_2 for 24hrs. After incubation, total volume of 100 µL of ten-fold serially diluted NDV2K35 was inoculated into each well with six replicates for each dilution. The plates were incubated at 37 °C for 72hrs and observed for CPE under inverted microscope. The proportion of wells with CPE in each serial dilution of virus was examined under inverted microscope and $TCID_{50}$ was estimated by Reed and Muench formula [14].

Maximum Non-Toxic Dose (MNTD) [13]

The assay for calculating maximum nontoxic dose of *A. paniculata* extracts was initiated by seeding 1.5×10^4 cells/ well into each well of 96 well flat bottom plate and incubated at 37 °C with 5 percent CO_2 for 24hrs. After 24hrs the cells were treated with diluted stock solution of aqueous and ethanolic extracts, andrographolide purified and andrographolide standard at the concentrations ranging from 0.5 to 25 µg/mL and ribavirin (25 to 100 µg/mL) and incubated. After 48 hrs, 10 µL of MTT [3-(4-5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide] dye was added into each well and incubated for 4hrs and the supernatant was removed. A total of 100 µL of dimethyl sulphoxide was added followed by continuous shaking for 10 min to dissolve the purple-blue MTT formazan precipitate. The absorbance of each cell was measured using micro plate reader at 570 nm and percentage of cell viability was calculated.

In vitro antiviral assay

In vitro antiviral activity was assessed as per the experimental design mentioned above [13]. The assay was initiated by seeding 1.5×10^4 cells/ well into each well of 6 well flat bottom plate and incubated at 37 °C with 5% CO_2 for 24hrs. After incubation, the medium was removed and treated with the plant extracts and ribavirin at specified concentrations (100 µL) which were previously mixed with 0.1MOI of NDV2K35 strain (100 µL) for 1 hr at 37 °C. The plates were incubated for 24, 48, 72hrs and CPE of the cells were

observed. At the end of 24, 48 and 72hrs the cell culture fluid was harvested after five cycles of freeze thaw and subjected to HA test. The remaining fluid was stored at -20 °C in sterile vials for confirmation of antiviral activity by RT-PCR. All the experiments were done in triplicate.

Confirmation of viral inhibition

Haemagglutination test

The harvested cell culture fluid from *in vitro* assay was subjected to haemagglutination test with 1% chicken RBCs suspension. Sterile Alsever's solution and normal saline were prepared, and the test was conducted as per the standard protocol described by FAO [15]. The reduction in HA activity of virus was used as an indicator of antiviral activity of *A. paniculata* extracts and active principles.

RT- PCR

The cell culture fluid from various groups were subjected to RT-PCR for amplification of F gene as described by the same author[9]. The following primer sequence was used for amplifying F gene of NDV 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'

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

$TCID_{50}$ for NDV2K35 strain in CEF

The cytopathic effect (CPE) in CEF cells infected with NDV2K35 was observed at 48 hrs post infection. The CPE was characterized by cell rounding, cell death, vacuolation, clumping or aggregation of the cells and detachment of cells from the surface. The number of wells showing CPE in each serial dilution was recorded and $TCID_{50}$ was estimated to be 10^{-7}/0.1mL as shown in Table 1. The $TCID_{50}$/ml were $10^{7.9}$ for nonvirulent strain and $10^{8.4}$ for mesogenic strain of NDV tagged with green fluorescence protein[16] and in yet another study it was $10^{5.5}$ /100 µl for both live and UV-irradiated NDV[17].

Table 1: Estimation of $TCID_{50}$ for NDV2K35 in CEF cells

Dilution	No of wells	No of wells showing CPE	No of wells showing non CPE	Accumulated value		% of cytopathogenic
				CPE	Non CPE	
10^{-1}	6	6	0	40	0	100
10^{-2}	6	6	0	34	0	100
10^{-3}	6	6	0	28	0	100
10^{-4}	6	6	0	22	0	100
10^{-5}	6	6	0	16	0	100
10^{-6}	6	5	1	10	1	91
10^{-7}*	6	3	3	5	4	56
10^{-8}	6	2	4	2	8	20

* $TCID_{50}$

MNTD for *A. paniculata* extracts and andrographolide

The percentage cell viability for different concentrations (0.5 to 25.0 µg/mL) of aqueous and ethanolic extracts of *A. paniculata* and its active principle were determined in CEF cells by MTT assay at 48 hrs and presented in Table 2. The MNTD for both the extracts and its active principles was found to be 5.0 µg/mL hence, three doses at and below MNTD (1.0, 2.5 and 5.0 µg/mL) were used for exploring antiviral activity. The methanolic extract of *A. paniculata* was reported to be safe and effective when used at 50 µg/mL in Vero cells infected with dengue virus [18]. The ethanolic extract was found nontoxic (IC_{50} 124.30 µg/mL) to A549 cells and used as treatment for A549 cells transfected with Simian Retro Virus [19]. In yet another study, the MNTD of aqueous extract was reported to be 31.25 µg/mL and ethanolic extract was found to be 9.36 µg/mL in Vero cells however, the plant was found ineffective against Chikungunya virus [20]. The MNTD reported in the present study is less than the earlier reports probably due to variation in the cells.

Table 2: Estimation of MNTD for *A. paniculata* extracts and andrographolide standard in CEF cells

S. No	Dose (µg/mL)	Cell viability (%)		
		Aqueous extract	Ethanolic extract	Andrographolide Standard
1	0.5	100	100	100
2	1.0	100	100	100
3	2.5	100	100	100
4	5.0	100	100	100
5	10.0	86	84	88
6	15.0	80	81	86
7	20.0	78	79	83
8	25.0	75	76	80

Each value is mean of three observations

Effect of treatment on cell morphology

The cell morphology observed under inverted microscope in different groups at 24, 48 and 72 hrs are presented in Plates 1-12.

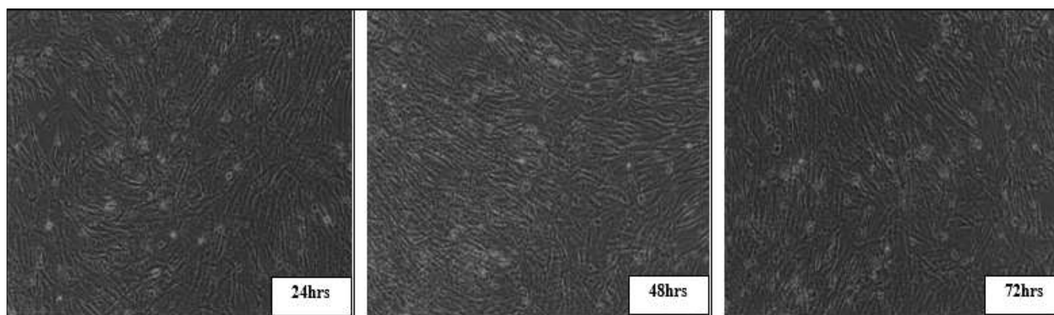

Plate 1: Uninfected chick embryo fibroblast cells

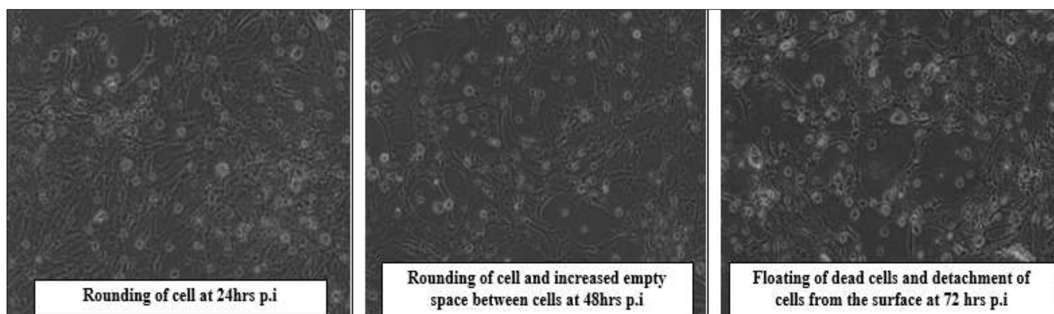

Plate 2: Chick embryo fibroblast cells infected with NDV2K35 strain of Newcastle disease virus

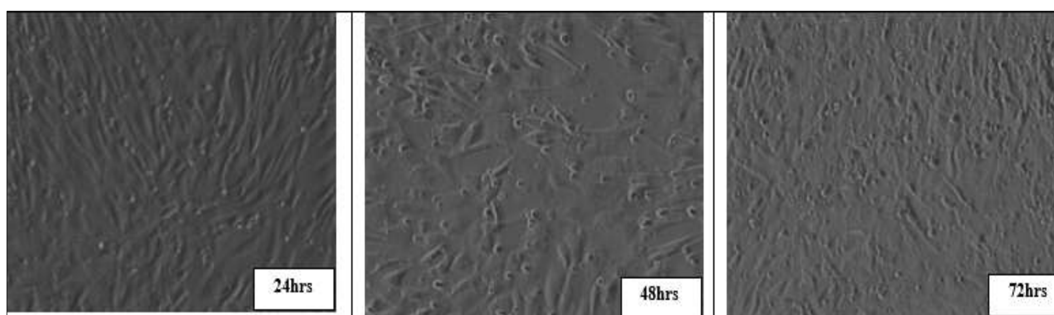

Plate 3: Chick embryo fibroblast cells infected with NDV2K35 preincubated with ribavirin

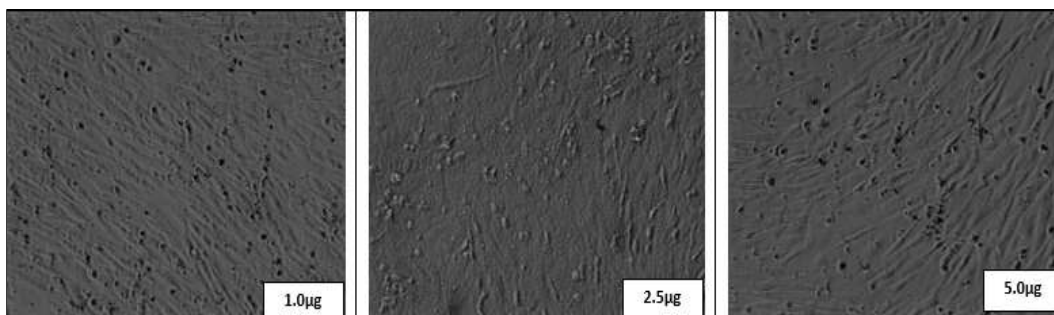

Plate 4: Chick embryo fibroblast cells infected with NDV2K35 preincubated with aqueous extract of *A. paniculata* at 24hrs

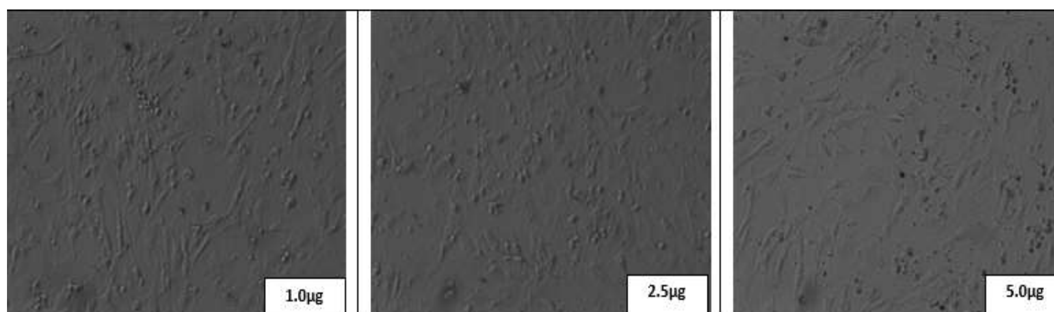

Plate 5: Chick embryo fibroblast cells infected with NDV2K35 preincubated with aqueous extract of *A. paniculata* at 48 hrs

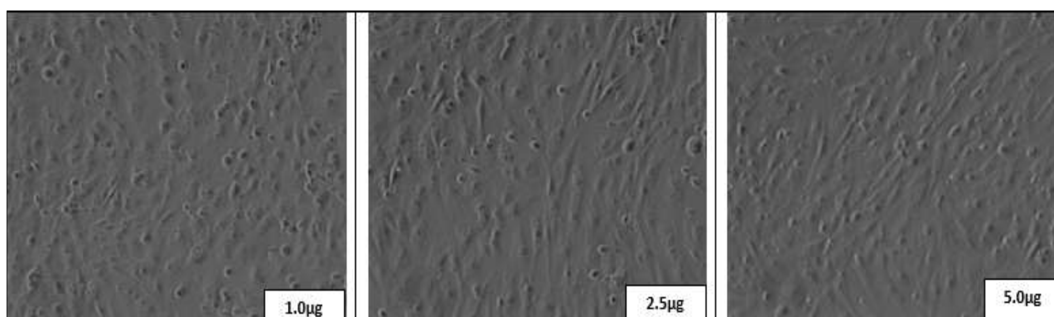

Plate 6: Chick embryo fibroblast cells infected with NDV2K35 preincubated with aqueous extract of *A. paniculata* at 72hrs

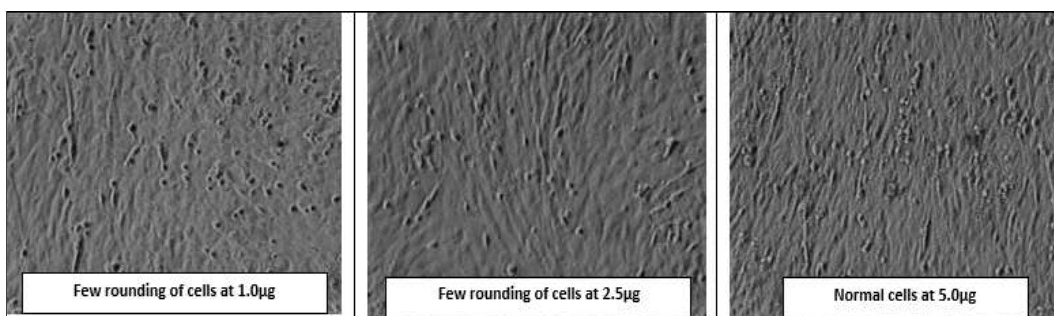

Plate 7: Chick embryo fibroblast cells infected with NDV2K35 preincubated with ethanolic extract of *A. paniculata* at 24hrs

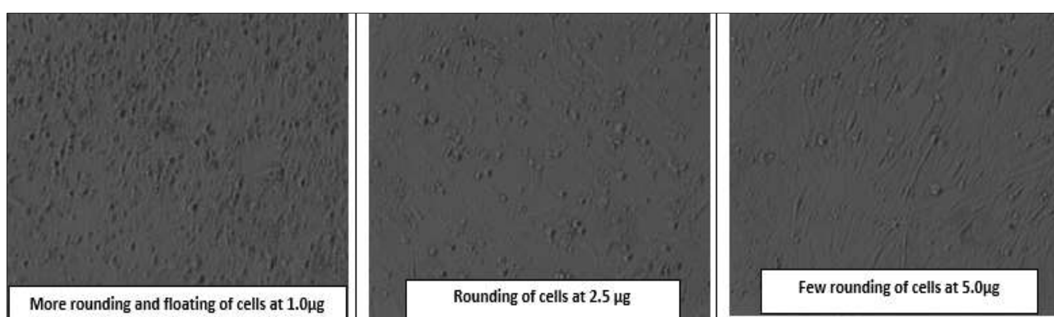

Plate 8: Chick embryo fibroblast cells infected with NDV2K35 preincubated with ethanolic extract of *A. paniculata* at 48 hrs

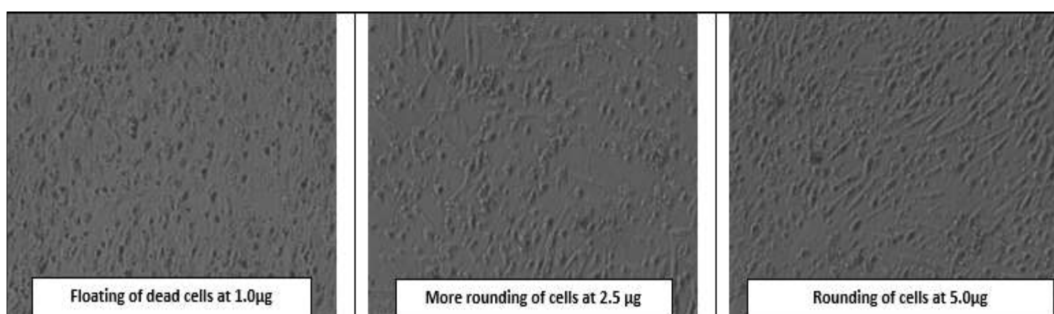

Plate 9: Chick embryo fibroblast cells infected with NDV2K35 preincubated with ethanolic extract of *A. paniculata* at 72hrs

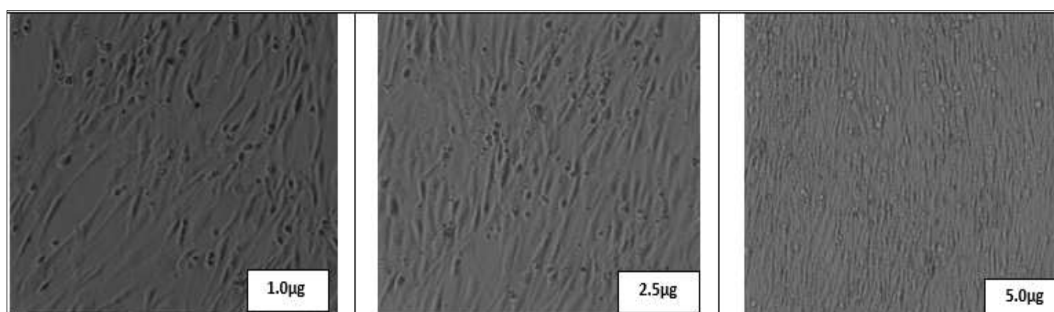

Plate 10: Chick embryo fibroblast cells infected with NDV2K35 preincubated with andrographolide standard at 24hrs

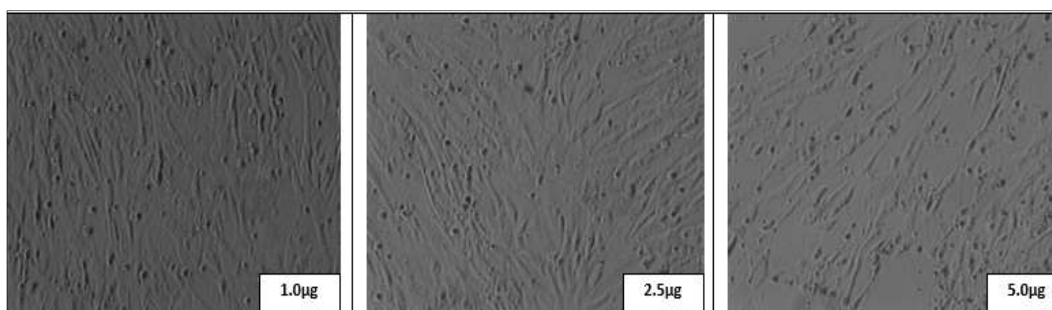

Plate 11: Chick embryo fibroblast cells infected with NDV2K35 preincubated with andrographolide standard at 48 hrs

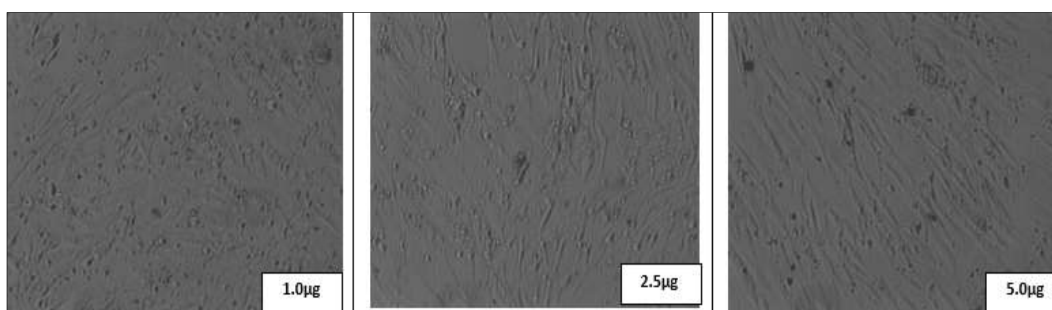

Plate 12: Chick embryo fibroblast cells infected with NDV2K35 preincubated with andrographolide standard at 72hrs

The uninfected CEF (T_1) retained normal morphology up to 72hrs. The cells infected with NDV2K35 strain showed morphological changes like increased granularity, cell rounding, vacuolation, cell death and floating of dead cells in a time dependant manner. Velogenic and mesogenic strains of NDV were reported to cause rounding of the cells, syncytia formation and cell death in CEF [21]. The strain used in this study was reported to be mesogenic [22] and the morphological changes noticed correlate with the earlier report.

The cells treated with different doses of aqueous extract, ethanolic extract, and andrographolide standard (drug control) at 1.0, 2.5 and 5.0 µg/mL retained normal morphology at the end of 24, 48 and 72hrs which indicates the nontoxic effect of the extracts. Earlier study had shown that *A. paniculata* extracts at 20.48 and 25.00 µg/mL was nontoxic to chicken embryo fibroblast and Vero cell lines, respectively [23, 13].

The cells infected with NDV2K35 preincubated with ribavirin showed normal morphology up to 72hrs which indicates the antiviral potential of the drug. Ribavirin, a unique guanosine analogue is known for broad spectrum antiviral activity against RNA and DNA viruses. By inhibiting inosine monophosphate dehydrogenase, it depletes the intracellular GTP resulting in inhibition of mRNA capping, impacts host cell gene expression, inhibit viral RNA dependent RNA polymerase as well induce viral mutagenesis at high dose [24]. Ribavirin was proven to be highly effective against picorna virus when added prior to infection in HeLa cells [25]. The replication of borna virus in duck embryonic fibroblast cells was inhibited by ribavirin at 25µg/mL and Chikungunya virus at 250 µg/mL [25, 20].

The CEF cells infected with NDV2K35 pretreated with aqueous extract of *A. paniculata* (T_4 - T_6) and andrographolide standard (T_{10} - T_{12}) retained normal cell morphology which reflects better antiviral activity when compared to ethanolic extract. The cells infected with NDV2K35 preincubated with ethanolic extract at 1.0 and 2.5 (T_7 and T_8) showed few rounding of cells whereas at 5.0 µg/mL (T_9) the cells were normal at 24hrs. The intensity of cell damage increased at 48 hrs with more rounding in lower dose and few rounding of cells in higher dose. At 72 hrs, dead cells were seen floating in lower dose (T_7) and rounding was noticed in remaining two doses (T_8 and T_9). Several *in silico* studies predicted that andrographolide and its analogs bind to several antiviral targets of SARS-CoV-2 that are important for attachment to host cells and replication. The analogs, especially neoandrographolide and bisandrographolide were predicted to show better binding affinities than andrographolide regardless of target receptor[26]. Hence, it is presumed that better synergism was exhibited by the components of the aqueous extract than ethanolic extract as evidenced by the antiviral activity.

Effect of treatment on cell viability

The cell viability (%) of different groups was estimated by MTT assay at 48 and 72hrs and the results shown in Table 3.

At the end of 48 and 72hrs cells treated with virus showed a significant ($p<0.05$) decrease in cell viability when compared to all the groups. The groups treated with ethanolic extract at 1.0, 2.5 and 5.0 µg/mL showed significant decrease in cell viability when compared to control but was significantly higher than virus control. There was no significant difference in cell viability in the groups treated with different doses of aqueous extract, andrographolide standard and ribavirin.

The cell viability (%) in groups infected with NDV2K35 preincubated with aqueous extract, andrographolide standard and ribavirin were similar to the control whereas that of ethanolic extract showed reduced cell viability but was more than 80% at all the doses. The antiviral property of the plant extract and its active principle is obvious as the cell viability in virus control was only 18.00 and 5.00% at 48 and 72hrs, respectively.

Effect of treatment on HA titre

The HA titre could not be detected in cell culture fluid from control, cells infected with virus preincubated with ribavirin, aqueous extract, andrographolide standard at all doses and

ethanolic extract at 5.0 µg/mL at 24hrs whereas virus control showed highest titre which increased with time. The groups treated with different doses of ethanolic extract and andrographolide isolated showed titre at 48 and 72hrs which indicated the replication of virus (Table 4). The presence of more amount of terpenoids, tannins and saponins in aqueous extract than ethanolic extract in addition to the presence of alkaloids, flavonoids and phenols, would have acted synergistically and resulted in better antiviral activity [27]. The antiviral activity of *A. paniculata* is considered better than leaf extract *of Momordica balsamina* as it required higher concentration (20mg/mL) to inhibit NDV [28]. Since the virus and extract were preincubated before infection of cells the extract might have resulted in inactivation of the virus and prevented its attachment to the host cells.

Table 3: Effect of treatment (ribavirin, *A. paniculata* extracts and andrographolide standard) on cell viability (Mean±SE%) in CEF cells

Treatment/ Hrs	Cell viability (%)											
	Control (T_1)	Virus Control (T_2)	Ribavirin (µg/mL) 500 (T_3)	Aqueous extract (µg/mL)			Ethanolic extract (µg/mL)			Andrographolide standard (µg/mL)		
				1.0 (T_4)	2.5 (T_5)	5.0 (T_6)	1.0 (T_7)	2.5 (T_8)	5.0 (T_9)	1.0 (T_{10})	2.5 (T_{11})	5.0 (T_{12})
48 hrs	$98.33^{c}\pm0.88$	$18.00^{a}\pm2.30$	$93.33^{bc}\pm2.33$	$93.67^{bc}\pm2.02$	$92.00^{bc}\pm1.15$	$90.67^{bc}\pm1.20$	$80.33^{b}\pm1.88$	$83.67^{b}\pm3.17$	$85.66^{b}\pm1.38$	$93.33^{bc}\pm2.33$	$91.67^{bc}\pm3.84$	$90.33^{bc}\pm1.33$
72hrs	$94.33^{c}\pm2.90$	$5.00^{a}\pm1.76$	$90.67^{c}\pm1.20$	$91.33^{c}\pm1.88$	$90.67^{c}\pm1.67$	$90.00^{c}\pm1.58$	$80.02^{b}\pm1.88$	$83.62^{b}\pm1.73$	$83.67^{b}\pm1.73$	$90.00^{c}\pm2.30$	$90.33^{c}\pm1.20$	$89.97^{c}\pm1.58$

Each value is mean of three observations
Means bearing different superscripts within a row differ significantly ($p<0.05$)

Table 4: Effect of treatment (ribavirin, A. paniculata extracts and andrographolide standard) on HA titre (Mean±SE Log2) against NDV antigen

Treatment/ Hrs	HA titre (Log2)											
	Control (T_1)	Virus Control (T_2)	Ribavirin (µg/mL) 500 (T_3)	Aqueous extract (µg/mL)			Ethanolic extract (µg/mL)			Andrographolide standard (µg/mL)		
				1.0 (T_4)	2.5 (T_5)	5.0 (T_6)	1.0 (T_7)	2.5 (T_8)	5.0 (T_9)	1.0 (T_{10})	2.5 (T_{11})	5.0 (T_{12})
24hrs	$0.00^{a}\pm0.00$	$6.67^{c}\pm0.21$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$1.37^{b}\pm0.33$	$1.21^{b}\pm0.09$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$
48 hrs	$0.00^{a}\pm0.00$	$8.67^{d}\pm0.23$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$1.62^{c}\pm0.23$	$1.33^{b}\pm0.23$	$1.24^{b}\pm0.04$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$
72hrs	$0.00^{a}\pm0.00$	$8.33^{d}\pm0.30$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$1.69^{c}\pm0.21$	$1.34^{b}\pm0.02$	$1.33^{b}\pm0.21$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$

Each value is mean of three observations
Means bearing different superscripts within a row differ significantly ($p<0.01$)

Detection of NDV by RT-PCR

The collected cell culture fluid was subjected to RT- PCR to confirm the antiviral activity of extracts of *A. paniculata* and andrographolide (Plate 13-15). Agarose gel electrophoresis showed amplification at 535bp for F gene of NDV in virus control, virus preincubated with ethanolic extract at all-time intervals which confirm the presence of the virus. However, the intensity of amplification was mild in the treated groups. There was no amplification in the remaining groups which confirms the antiviral activity of the extracts. The envelope of NDV contains two glycoproteins, the haemagglutinin neuraminidase (HN) and fusion (F) proteins. The HN glycoprotein is involved in attachment and release of the virus and F glycoprotein mediated fusion of viral envelope with cellular membrane. The F protein is synthesised as precursor F0 and must be proteolytically cleaved to F1 and F2 to activate fusion. The cleavage of F protein is considered as a major determinant of virulence[29]. In this study, failure to amplify F gene is taken as a confirmation that the virus has not replicated.

Preincubation of the virus with extracts probably resulted in direct inactivation of the virus and prevented its replication when added to CEF cells [30]. The alcoholic extract of *A. paniculata* and andrographolide were found effective against Epstein – Barr virus lytic proteins, Dengue virus and Simian Retro virus [31, 32, 33]. However, in the present study ethanolic extract was not as effective as other treatments. In another observation ethanolic extract of *A. paniculata* was found ineffective against Chikungunya virus [20]. The antiviral activity of andrographolide standard was already proven against HIV, Herpes simplex, Avian influenza A, Hepatitis C and Dengue virus [33-37]. Thus, the present study confirms the antiviral activity of the aqueous extract of the plant against NDV in CEF cells based on the results of cell morphology, cell viability, HA titre correlated with RT-PCR result.

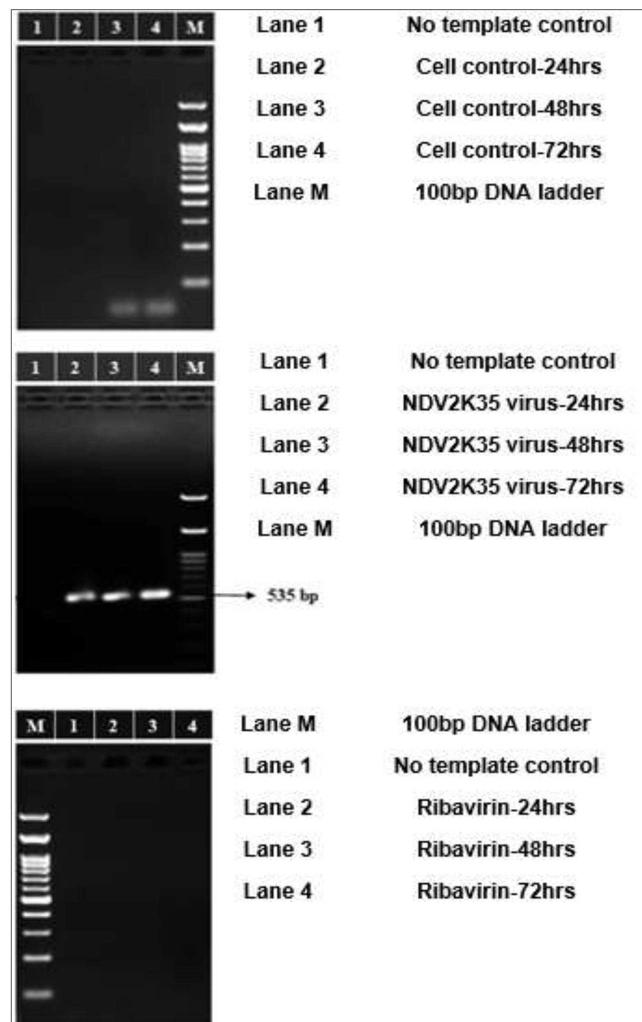

Plate 13: RT- PCR for F gene of NDV in cell culture fluid

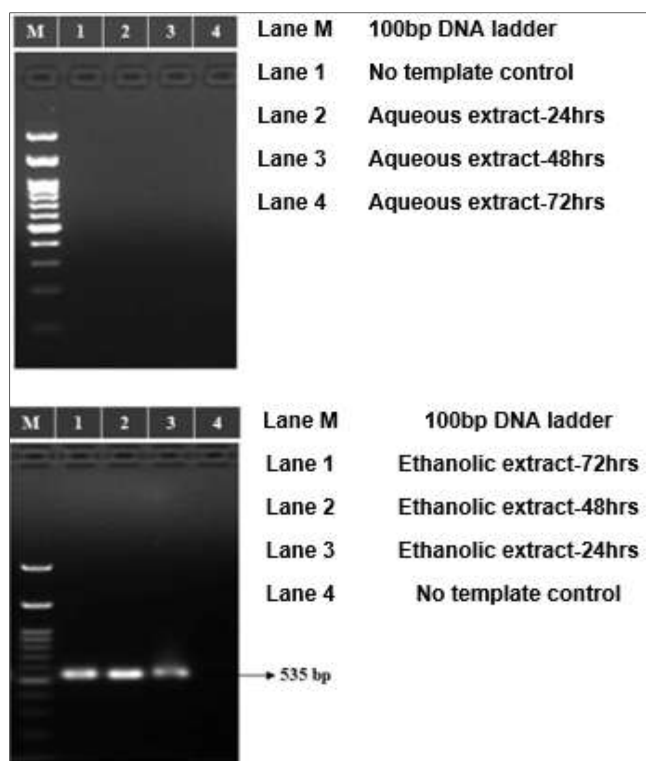

Plate 14: RT- PCR for F gene of NDV in cell culture fluid

Plate 15: RT- PCR for F gene of NDV in cell culture fluid

Conclusion

The results of *in vitro* analysis confirmed 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. The viral inhibition was further confirmed by RT-PCR. The alcoholic extract was unable to totally inhibit the virus at the concentration used in this study. Thus, the plant can be effectively used for prevention of ND in poultry. The antiviral efficacy should be further tested against velogenic strains and detailed study should be conducted to identify the molecular mechanism of action.

Acknowledgment

The authors acknowledge the financial assistance and facilities provided by Tamil Nadu Veterinary and Animal Sciences University, Chennai- 600 051 to conduct this research.

References

1. Anonymous. World livestock disease atlas: A quantitative analysis of global animal health data A (2006-2009). In: The International bank for reconstruction and development - The World Bank and The TAFS Forum, (Ed.) Washington, DC, 2011.
2. Alexander DJ. Newcastle Disease: Methods of spread in Newcastle disease. Kluwer Academic Publishers, Boston; c1998. p. 256-272.
3. Kapczynski DR, Claudio LA, Miller PJ. Immune responses of poultry to Newcastle disease virus. Developmental and Comparative Immunology. 2013;41:447-453.
4. Puro K, Sen A. Newcastle Disease in Backyard Poultry Rearing in the North-eastern States of India: Challenges and Control Strategies. Frontiers in Veterinary Science. 2022. DOI=10.3389/fvets.2022.799813.
5. Adiguna SP, Panggabean JA, Atikana A, Untari F, Izzati F, Bayu A, *et al*. Antiviral activities of Andrographolide and its derivatives: Mechanism of action and delivery system. Pharmaceuticals (Basel) 2021; 14(11):1102. doi: 10.3390/ph14111102. PMID: 34832884; PMCID: PMC8619093.
6. Sa-Ngiamsuntorn K, Suksatu A, Pewkliang Y, Thongsri P, Kanjanasirirat P, Manopwisedjaroen S, *et al*. Anti-SARS-CoV-2 Activity of *Andrographis paniculata* extract and its major component Andrographolide in human lung epithelial cells and cytotoxicity evaluation in major organ cell representatives. Journal of Natural Product. 2021;84(4):1261-1270.
doi: 10.1021/acs.jnatprod.0c01324.
7. Gupta S, Mishra KP, Ganju L. Broad-spectrum antiviral properties of andrographolide. Archves of Virology. 2017;162(3):611-623.
8. Kavinilavan R, Mekala P, Raja MJ, Arthanari Eswaran M, Thirumalaisamy G. Exploration of immunomodulatory effect of Nilavembu Kudineer Chooranam against Newcastle Disease Virus in backyard chicken. Journal of Pharmacognosy and Phytochemistry. 2017;6(6):749-751.
9. Nagajothi S, Mekala P, Raja A, Raja MJ, Senthilkumar P. *In ovo* antiviral activity of *Andrographis paniculata* against NDV2K35 strain of Newcastle disease virus. Journal of Pharmacognosy and Phytochemistry. 2010;9(4):2023-2029.
10. Alexander DJ. Newcastle disease, In: OIE Manual of diagnostic tests and vaccines for terrestrial animals (Paris). 2009, 7-8.
11. Hernandez R, Sinodis C, Brown DT. Growth and Maintenance of Chick Embryo Fibroblast. Current Protocols in Microbiology. 2010;17(1):41-48.
12. Freshney RI. Quantitation and experimental design. In Culture of animal cells. A manual of basic techniques. Fourth Edn. Wiley-LIss, Inc, New York. 2000, 267-286.
13. Tang LI, Ling AP, Koh RY, Chye SM, Voon KG. Screening of anti-dengue activity in methanolic extracts of medicinal plants. Complementary and Alternative Medicine. 2014;12(3):1-10.
14. Reed LJ, Muench H. A simple method of estimating fifty% end points. American Journal of Hygiene. 1938;27:493-497.
15. FAO. Manual for the production of Marek's disease, Gumboro disease and inactivated Newcastle disease vaccines. FAO Animal Production and Health Paper 89 (edn) V. Palya. Rome; c1991. p. 29-62.

16. Al-Garib SO, Gielkens AL, Gruys E, Peeters BP, Koch G. Tissue tropism in the chicken embryo of non-virulent and virulent Newcastle diseases strains that express green fluorescence protein. Avian Pathology. 2003;32(6):591-596.
doi: 10.1080/03079450310001610695. PMID: 14676009.
17. Anjum FR, Rahman SU, Aslam MA, Qureshi AS. Antiviral potential and stability analysis of chicken interferon-α produced by Newcastle disease virus in chicken embryo fibroblast cells. Veterinarni Medicina-Czech. 2021;66:197-207.
18. Churiyah C, Pongtuluran OB, Rofanni E, Tarwadi T. Antiviral and immunostimulant activities of *Andrographis paniculata.* Journal of Biosciences. 2015;22:67-72.
19. Sangeetha K, Rajarajan S. *In-vitro* antiviral activity of Indian medicinal plants to Asian and East Central South African lineage of Chikungunya virus. International Journal of Pharmaceutical Sciences and Research. 2015;6(2):692.
20. Mazumder AC, Khatun S, Nooruzzaman M, Chowdhury EH, Das PM, Islam MR. Isolation and identification of Newcastle disease viruses from field outbreaks in chickens and pigeons. Bangladesh Veterinarian. 2012;29(2):41-48.
21. Bhuvaneswari S, Tirumurugaan KG, Jones JC, Kumanan K. Complete genome sequence of a Newcastle disease virus from a *Coturnix Coturnix japonica* (Japanese Quail) covey in India. Genome Announcements. 2014;2(3):1-2.
22. Suriani MN, Jamaliah H, Normah M, Goon SC, Iswadi MI, Resni M. Antiviral effect of *Andrographis paniculata* ethanolic extract against Newcastle Disease virus. PCVC6 & 27VAM Conference; c2015. p. 23-27.
23. Nystrom K, Waldenstrom J, Tang KW, Lagging M. Ribavirin: pharmacology, multiple modes of action and possible future perspectives. Future Virology. 2019;14(3):153-160.
24. Andersen DO, Murray TBK, Robins RK, North JA. *In vitro* antiviral activity of ribavirin against picorna viruses. Antiviral Chemistry and Chemotherapy. 1992;3(6):361-370.
25. Musser JM, Heatley JJ, Koinis AV, Suchodolski PF, Guo J, Candon P, *et al*. Ribavirin inhibits parrot bornavirus 4 replication in cell culture. Plos One. 2015;10(7):e0134080.
26. Xin YL, Chan JSW, Tan TYC, Teh BP, Abd Razak MRM, Mohamad S, *et al*. *Andrographis paniculata* (Burm. F.) Wall. Ex Nees, andrographolide, and andrographolide Analogues as SARS-CoV-2 Antivirals? A Rapid Review. Natural Products Communication. 2021;16(5):1-15.
27. Nagajothi S, Mekala P, Raja A, Raja MJ, Senthilkumar P. *Andrographis paniculata*: Qualitative and quantitative phytochemical analysis. Journal of Pharmacognosy and Phytochemistry. 2018;7(4):1251-1253.
28. Chollom S, Olawuyi A, Danjuma L, Nanbol L, Makinde I. Antiviral potential of aqueous extracts of some parts of *Momordica balsamina* plant against Newcastle disease virus. Journal of Advance Pharmacy Education and Research. 2012;2:82-92.
29. Scheid A, Choppin PW. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity by proteolytic cleavage of an inactive protein of Sendai virus. Virology. 1974;57:470–490.
30. Xu R, Weng J, Hu L, Peng G, Ren Z, Deng J, *et al*. Anti-NDV activity of 9-oxo10, 11-dehydroageraphorone extracted from *Eupatorium adenophorum in vitro*. Natural Products Research. 2017;7:1-4.
31. Lin TP, Chen SY, Duh PD, Chang LK, Liu YN. Inhibition of the Epstein- Barr virus lytic cycle by andrographolide. Biological and Pharmaceutical Bulletin. 2008;31(11):2018-2023.
32. Ling APK, Bee Fong KHOO, Ching Hua SEAH, Kar Yue FOO, Cheah RK, Soi Moi CHYE, *et al*. Inhibitory activities of methanol extracts of *Andrographis paniculata* and *Ocimum sanctum* against Dengue-1 virus. International Conference on Biological, Environment and Food Engineering (BEFE-2014) August 4-5, Bali (Indonesia), 2014, 47-52.
33. Calabrese C, Berman SH, Babish JG, Ma X, Shinto L, Dorr M, *et al*. A phase I trial of andrographolide in HIV positive patients and normal volunteers. Phytotherapy Research. 2000;14(5):333-338.
34. Wiart C, Kumar K, Yusof MY, Hamimah H, Fauzi ZM, Sulaiman M. Antiviral properties of ent-labdene diterpenes of *Andrographis paniculata* Nees inhibitors of herpes simplex virus type 1. Phytotherapy Research. 2005;19(12):1069-1070.
35. Chen JX, Xue HJ, Ye WC, Fang BH, Liu YH, Yuan SH. Activity of andrographolide and its derivatives against influenza virus. Biological and Pharmaceutical Bulletin. 2009;32(8):1385-1391.
36. Lee JC, Tseng C, Young K, Sun H, Wang S, Chen W, *et al*. Andrographolide exerts anti-hepatitis C virus activity by up-regulating haem oxygenase-1 *via* the P38 MAPK/Nrf2 pathway in human hepatoma cells. British Journal of Pharmacology. 2014;171:237-252.
37. Panraksa P, Ramphan S, Khongwichit S, Smith DR. Activity of andrographolide against dengue virus. Antiviral Research. 2017;139:69-78.