Antiviral activity of limonin against Newcastle Disease virus *in vitro*

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Abstract
In the past few years, numerous plant-derived phytochemicals were tested for their anti-viral effect in order to find effective drug to control viral infections. Triterpenoids such as limonin showed promising antiviral properties. Our current work examined the possible anti-Newcastle disease virus (NDV) proliferation of limonin. NDV is an extremely contagious and economically vital poultry virus. For this aim, limonin ability to reduce NDV cytotoxicity on human rhabdomyosarcoma, human cervical carcinoma and Vero monkey kidney cell line in culture are described. Viral gene and protein expressions of two important structural proteins, matrix (M) mRNA and haemagglutinin-neuraminidase (HN) protein of NDV were studied. Matrix mRNA were analyzed using qRT-PCR.

HN protein was measured using the immunofluorescent assay. IRF3 protein level was investigated as a mechanism of action to explain the antiviral activity, but the results showed no relation between limonin anti-NDV effect and IRF3 level. The results showed that limonin reduces NDV replication in all cell lines used. Furthermore, it down-regulates the NDV HN and matrix genes which confirm the antiviral nature of this phytochemical. These in-vitro results together are promising for clinical testing of this phytochemical as anti-NDV therapy in poultry.

Keywords: Phytochemical, IRF3, Matrix protein.

Introduction

Newcastle disease is a substantial infectious disease of poultry, which is caused by Newcastle disease virus (NDV). NDV is a worldwide contagious and disseminated disease that can cause significant economic losses in the poultry industry due to repeated outbreaks in vaccinated and unvaccinated avian species[16]. It is also a main problem to the poultry industry in Iraq[7]. The clinical signs and severity of ND infection are attributed to many factors such as strain, virulence, and virus tropism, and host factors like immune status, species, age, coexisting infections, route of infection, and environmental stress[3]. The disease usually affects the respiratory tract, intestinal tract and nervous system. Poultry shows different clinical signs from a mild to very severe depression, egg production decrease[27], tachypnea, diarrhea and more signs if the birds survive such as downfall and chronic nervous signs like twisted necks**.**

Purified natural products and herbal medicines deliver a precious resource for developing new antiviral drug. Antiviral mechanisms identification of natural products are important to know where they interact with NDV life cycle like viral replication and assembly, besides virus–host-specific interactions targeting[14]. Nowadays, there are limited number of successful antiviral drugs for the treatment of viral diseases. The discovery of a novel compounds with not only intracellular but also extracellular antiviral features is a necessity[19]. The inhibition of cytopathic effects, the inhibition of plaque formation, and the reduction in the virus yield, are the methods that are usually used for the assessment of in vitro antiviral activities of synthetic and natural *substances* (8).

Majority of viral diseases are self-limited and do not require antiviral treatment. Besides the therapies against human immunodeficiency virus (HIV), the available antiviral drugs in the clinical application target three groups of viruses—influenza, hepatitis, and herpes viruses[8]. Antiviral therapeutics are of three major types; ribavirin for the treatment of respiratory syncytial virus (RSV), cidofovir for the treatment of cytomegalovirus (CMV) and other DNA viral infections, and nucleos(t)ide analogs to treat chronic hepatitis B (CHB), which can additionally work as treatment for HIV[32] .

Limonin is a phytochemical that is a bitter principle of citrus fruits. It is a triterpenoid aglycone (C26H30O8). It inhibits cancer cells proliferation and induces apoptosis in cancer cells. Limonin interferes with HIV-1 replication in cultures of mononuclear cells through HIV-1 protease activity inhibition[18].

Limonin is originated from *Citrus limon*, which is a small tree from the citrus family named Rutaceae that mostly arisen from India and Pakistan or Asia in general. *Citrus limon* is grown worldwide including the Mediterranean region (11). *C. limon* is thought to have originated as a hybrid between other Citrus species[12,25].

Plant extracts and natural products provide limitless source for developing new novel antiviral agents including infectious diseases. Communicable viral diseases are critical worldwide problem[17]. The potential of Limonin as anti-viral agent and presence of NDV are viral threat to Iraqi economy; our study aimed to find if there is possibility of anti-NDV replication by limonin.

Material and Methods

Cell lines: Rhabdomyosarcoma (RD) and Vero Cell Lines were cultured in RPMI- 1640 medium (US Biological, USA) supplemented with 10% fetal bovine serum (FBS) (Capricorn-Scientific, Germany), 100 unit's/mL penicillin, and 100 µg/mL streptomycin (Capricorn-Scientific, Germany) and incubated at 37°C. Human epithelial carcinoma (Hela) cell line was cultured in MEM medium (US Biological, USA) with 10% fetal bovine serum (FBS), 100 unit's/mL penicillin, and 100 µg/mL streptomycin and incubated at 37°C. All the cell lines were supplied by the Cell bank Unit, Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), Mustansiriyah University[5].

Newcastle disease virus (NDV) propagation: The Newcastle disease virus (Iraqi strain) was kindly provided by the Experimental Therapy Department / ICCMGR. It was directly thawed and then antibiotics were added to the virus sample: Ampicillin (100µg/ml) and Streptomycin (100µg/ml). The sample was centrifuged at 3000 rpm for 30 min at 4°C. This would initially remove any debris and large particulate matter. The supernatant was taken, and the virus sample was injected into 10 days embryonated chicken eggs. The allantoic fluid was harvested, distributed in a small tube and stored at -86 °C[2].

Propagation of NDV in Chicken embryo: Embryonated chicken eggs were incubated at 37°C with humidity. The eggs were turned twice a day. After one day of incubation, the eggs were trans-illuminated with an egg candling lamp to ensure the viability of the embryo. All infertile eggs and those containing a dead embryo were discarded.

The second candling before inoculation was made so that the air sac is made marked off and a suitable site of injection was picked on the egg shell where no important blood vessels were running away from the embryo. The egg was rinsed with 70٪ ethanol to sterilize outer surface, then the egg shell was sterilized with iodine at the inoculation site where a small hole is drilled about 0.4 cm above the air sac border.

After that 0.1ml of diluted NDV was injected into the 9-days embryonated chicken egg allantoic fluid area using a fine needle. The puncture hole was covered using paraffin and incubated at 37 °C in a humidified incubator and the embryo was observed daily for viability. After the death of the embryo, it was transferred immediately to the refrigerator (4°C). After 12 hrs at 4^0C, allantoic fluids were collected and purified by centrifugation and stored at -85°C[2].

Cytotoxicity of Limonin: Limonin diluted in 2-fold different concentrations (440 to 3.43 µg/ml) in RPMI and 100 µl of the dilutions were exposed on the Hela, Vero, or RD cells. It was done in quadruplicates. Untreated negative control was exposed to serum free media only. All plates were cultured for 72 h at 37°C. The cytopathic effect was determined using MTT assay according to AI-shammari et al[6].

Antiviral cytopathic effect by Limonin: The effects of the components against NDV were also tested during the replication period by the addition of drugs to the overlay medium after cell infection, as typically performed in antiviral susceptibility studies. Each assay was run in three replicates. Plaque reduction assays were carried out, and the percentage of cytotoxicity of drug-treated cells and viruses were compared to untreated controls. Wells containing DMSO with no drug were also included on each plate as controls.

The drug-pretreated, cell-free viruses were added at the same time to host cells so that the infection starts at the same time. When intracellular viruses are drug-treated, the infection should had started simultaneously. All non – infecting virus particles have been washed off and all intracellular viruses are in the same phase of replication.

Absorbency was determined on a microplate reader at 492 nm test wavelength; the assay was performed in triplicate[10]. The endpoint parameter that was calculated for each cell line included the inhibiting rate of cell growth (the percentage of cytotoxicity) which was calculated as:

$$(G.I) = (A-B)/Ax100$$

where A is the mean optical density of untreated wells, and B is the optical density of treated wells[15].

Sample collection for PCR and ELISA reactions: The grown Hela cells represented at passage 40 and the grown RD cells represented at passage 33 were detached by trypsinization to obtain cell suspension. The cells were then redistributed into 25 cm² tissue culture flasks (16 flasks for Hella and 16 for RD) at concentration 1000,000 cell in each flask and then incubated at 37°C for 24 hrs.

After incubation, the medium was removed from the flasks and cells infected with NDV (MOI = 10) in serum-free media were added to the flasks. After 2 hours, the drug (limonin) (conc. = 440 µg/ml) was added to the infected and noninfected flasks. Negative control flasks were treated with Serum Free Media only. Afterward, the flasks were re-incubated at 37°C for 24 and 48 hrs. Each time point contained four flasks, one for the cell infected with NDV and not treated, one for non-infected cells but treated with limonin, one for limonin treated NDV-infected cells, and one as control.

The cells were washed and harvested with PBS by cell scraper after the culture media were completely removed. The cell suspensions were next collected from each flask into two Eppendorf tubes, one for mRNA detection of the matrix gene level and one for estimation of the expression of IRF3 (Interferon regulatory factor 3) proteins. Cells used for

genetic detection by PCR were centrifugated for 10 min at 10000rpm 4°C and stored at -80ºC. The cell suspensions in Eppendorf tubes used for estimation of the expression for IRF3 proteins were subjected to two freeze-thaw cycles to further break cell membranes. After that the Eppendorf tubes were centrifugated for 15 min at 5000rpm and the supernatant was collected and stored at -80ºC for next determination and analysis of the expression of IRF3 protein.

One-Step EvaGreen qRT-PCR Kit

Protocol: RT-PCR was added in a nuclease-free environment. The RNA sample was synthesized, the reaction mixture was synthesized; PCR and subsequent reaction analysis were analyzed in separate regions. "Clean" pipettes designed for PCR and anti-aerosol barrier were used.

Primers: The concentration of Primers 100 pmol/µl was kept as a stock in -20ºC. To prepare 10µM concentration as a work primer, 10 µl of 100 pmol/µl primer was re-suspended in 90 µl of deionized water to reach a final concentration of 10µM.

NDV HN protein detection by simple indirect immunofluorescence assay: Materials used were; Primary monoclonal antibody – (anti-NDV, HN) (Santacruz Biotechnology, USA). Conjugated secondary antibody FITC) (Santacruz Biotechnology, USA). Coating buffer: 50 mM sodium carbonate, pH 9.5. 1X PBS (Phosphate buffered saline): 8.0g sodium chloride, 1.3g dibasic sodium phosphate, 0.2g monobasic sodium phosphate in 1.0 liter distilled water, pH (7.4).1X PBS-T (Phosphate buffered saline-Tween 20 solutions (PBS-T): PBS containing 0.05% Tween-20.Blocking buffer: PBS-T, 5% Bovine Serum Albumin ELISA 96 well microtiter plate or 8 well strips.

Antigen Application:

1. 100ul antigen solution diluted in buffer was added to appropriate numbers of microtiter wells.
2. Wells were incubated at 4°C overnight in a humid environment i.e. they were covered by a glass plate or in a sealed box with a dampened paper towel inside.
3. Microtiter wells were emptied, and the plate was inverted to tap out excess liquid onto a clean tissue.

Blocking Step:

1. 300-200ul of blocking solution was added to each well.
2. Wells were incubated for 1-2 hours at RT and the plate was emptied to tap out excess fluid onto a clean tissue.
3. Wells were washed three times with PBS-T.

Primary Antibody Incubation:

1. 100ul of primary antibody solution diluted in blocking buffer was added to each well.
2. Incubation step was performed for 1-2 hours at RT (or 4 hours at 4°C) with gentle agitation (on a rocker plate, for example).
3. The plate was inverted to tap out excess liquid onto a clean tissue.
4. Wells were washed three times with PBS-T.

Secondary Antibody Incubation:

1. 100ul of the secondary antibody diluted in blocking buffer was added to each well.
2. Incubation step was performed for 1-2 hours at RT with gentle agitation.
3. The plate was inverted, and excess liquid was tapped out onto a clean tissue.

Washing of Microtiter Wells:

1. Each well was filled with washing solution (PBS-T) with agitation for 5 min at RT.
2. The plate was inverted, and residual fluid was tapped out onto a clean tissue.
3. Washing step was repeated for 3 times.
4. Absorbance was read at 580 nm with microplate fluorescent reader.

Measurement of Matrix gene expression: Quantification of NDV matrix mRNA level, the cell lysate of treated cells and untreated control cells were collected at regular intervals (24 and 48 hours), and they froze at -86°C until used. NDV matrix mRNA level was determined by using a QPCR assay. Total RNA from cell lysate was isolated with a Magnesia® total RNA extraction Kit (Anatolia Geneworks, Turkey) according to the manufacturer's protocol. The extraction method was fully automated using automated Magnesia Extraction machine (Anatolia Geneworks, Turkey). The yield was quantified using Biodrop machine (Biochrom, UK).

The isolated RNAs were reversed transcribed to produce double-stranded cDNA using reverse transcriptase polymerase enzyme using bright green FAST One-Step qRT-PCR universal kit (Applied Biological materials, Canada) and measured with real-time PCR using the MX3005 Real-Time PCR machine (Agilent Technologies, USA). Specific primers[4]. Results were analyzed for each sample with relative quantification comparing the difference between sample and control. Mean CT values of the genes was calculated for each individual sample (as duplicate replication for each sample) and used to normalize expression levels using the ΔΔCT method described previously[20].

Statistical Analysis: The Statistical Analysis program - SAS (2012) was used to study the effect of different factors on study parameters. Chi-square test was used for significant comparison among percentages. Least significant difference –LSD test was used for significant comparison among means in this study.

Results and Discussion

Propagation of the virus in embryonated chicken eggs: The results showed that the Iraqi strain of NDV has the

ability to kill the chicken embryos starting from 48 to 72 hrs after the inoculation of the chicken embryonated eggs. This indicates that the virulence level of the strain which was velogenic type was able to kill embryos after 40-72hrs. This result agrees with findings from other researchers[4] who isolated the same Iraqi local strain that we used in this study. Our results also confirm that the strain is still virulent and holding its characteristics[30]. Considering that eggs with embryonic death within 24 hrs of inoculation is described as non-specific, such eggs were discarded. The virulent local strain of NDV was previously described as virulent viscerotropic strain[19].

The virulent viruses are tested by the mean death time (MDT) test which is based on the experience that virulent viruses kill embryos quicker than those with lower virulence. Thus, the Velogenic strains kill embryos in less than 60 hrs, mesogenic strains in 60–90 hrs and lentogenic strains in more than 90 hrs[2]. Hemorrhage was clearly observed in the infected embryos when compared with the control. The virus was purified and quantified by hemagglutination test and showed a positive result as a typical hemagglutination mesh pattern of chicken red blood cell at 128 HAU. For further passages of the virus in embryonated chicken eggs, it was observed that the titer was raised to 256 HAU in the second passage while in the next passage the titer reached to 512 HAU[26].

Titration of the virus in cell culture: The allantoic fluid was collected from the infected fertilized eggs and filtered in Millipore filter (0.45um), then the virus titer was measured by TCID50. The results showed that the titer of the isolated virus in primary chicken embryo fibroblast cells was 2TCID50/ 0.1ml while the titer in Hela cell line was 2TCID50 / 0.1ml.

Tissue culture infective dose (TCID50): The titer of NDV virus was measured on RD cells for the determination of TCID50.

The (NDV)-RD system was chosen to study the multiplicity of infection; it was observed that with much virulence, which was sufficient to infect all cells in the culture, the time at which antigen first appeared and the amount present at subsequent intervals were dependent on the multiplicity of infection. It seemed probable that the time at which alterations in cell structure and functions would appear might also depend on the multiplicity. When RD cells were infected with NDV at low multiplicity of infection, the yield of progeny virus was higher, and the cytopathic changes were more extensive than those in control non-infected RD cells.

Limonin Cytotoxicity on Hela, Vero and RD cells lines: Hela tumor cell line was seeded as 1 Cells / well in 96 well plates and after 24h, when the cells formed a confluent monolayer, they were exposed to the limonin at 440, 220, 110, 55, 27.5, 13.75, 6.87, or 3.43 ug/ml and incubated in 37 for 72hr. Cells were then stained by MTT stain and the inhibition percentage was calculated. The results showed that the *limonin* had no cytotoxic inhibition at the concentrations tested.

Antiviral cytopathic effect by Limonin: This study aimed to determine the anti-viral effects of limonin. The results showed that the inhibitory effect of 440ug/ml limonin on NDV replication was effective by reducing the NDV cytotoxicity to 31%, 16% and 8% on Hela cells (figure4A). Furthermore, 440ug/ml limonin inhibitory effect on NDV replication reduced the NDV cytotoxicity to 30%, 26%, 15% on Vero cells (figure4C).

Moreover, 440ug/ml limonin inhibitory effect on NDV replication reduced the NDV cytotoxicity to 30%, 21%, 12 on RD cells as in figure4.

mRNA expression of M-gene in Hela and RD after infection with NDV: Regarding mRNA level of the matrix gene expression for the Newcastle virus, the results showed a decrease in the gene expression of the virus infecting Hela cells after exposure to Limonin within 24 hours. However, the downregulation effect did not last for 48h indicating that limonin needs to be added every 24h.

The results show that limonin affects the reproduction of the virus within 24 hours in Hela cells, but RD cells need 48 hours to exert their effects on virus replication. This indicates that limonin needs to be added every 24 hours on Hela cells, possibly due to cell activity and rapid cell metabolism accompanying high cell proliferation.

NDV HN protein determination: Results showed a significant effect of limonin treatment on NDV replication represented by decreased NDV virus HN protein density in infected and treated Hela and RD cells in both exposure times 24 and 48h (figure6).

This study aimed to determine the anti-viral effects of limonin. The results showed that 440ug/ml limonin inhibitory effect on NDV replication was effective by reducing the NDV cytotoxicity to 31%, 16%, and 8% on Hela cells (table1). Furthermore, 440ug/ml limonin inhibitory effect on NDV replication reduced the NDV cytotoxicity to 30%, 26%, 15% on Vero cells (table 1).

Moreover, 440ug/ml limonin inhibitory effect on NDV replication reduced the NDV cytotoxicity to 30%, 21%, 12 on RD cells (table 1). Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new antiviral drugs since the chemical diversity provides unmatched availability[21]. Besides small molecules from medicinal chemistry, natural products are still major sources of innovative therapeutic agents for various conditions including infectious diseases[23].

Infectious viral diseases remain an important worldwide problem since many viruses have resisted prophylaxis or therapy longer than other microorganisms. At the moment, only a few effective antiviral drugs are available for the treatment of viral diseases[33]. There is a need to find new substances with not only intracellular but also extracellular antiviral properties. The methods commonly used for the evaluation of *in vitro* antiviral activities of synthetic and natural substances are based mainly on the inhibition of cytopathic effects, the reduction or inhibition of plaque formation, and the reduction in the virus yield, but also on other viral functions in selected host cell cultures.

There is considerable evidence emerging from *in vitro* studies and controlled trials of the potential of plant-derived phyto-antiviral agents for the treatment of human viral infections[24]. Many essential oils were investigated towards their antiviral activity. Most of them were tested against enveloped RNA and DNA viruses such as NDV, dengue virus type 2, Junin virus, and influenza virus (RNA viruses) as well as herpes simplex virus type 1 and type 2 (DNA viruses). Nonetheless, only few natural products such as limonin (*Citrus limon* L.) and clove (*Syzygium aromaticum*) oil were also tested against non-enveloped RNA and DNA viruses such as adenovirus type 3 (DNA virus), poliovirus and coxsackievirus B1 (RNA viruses)[34].

The best candidates as clinically useful antiviral drugs are substances which act on specific steps of viral biosynthesis. They inhibit specific processes in the viral replication cycle so that little or no viral progeny is produced[25]. These antiviral drugs should act at low concentrations and should not influence the host cell machinery, prevent the spread of viruses, and ultimately cure infected cells. On the other hand, virucidal drugs denature viral structural proteins or glycoproteins. Thus, the infectivity of virus particles is completely lost[31]. To learn more about the antiviral mechanism of limonin on enveloped viruses, we investigated exemplarily the antiviral activity of limonin (*Citrus limon)*. The replication cycle of NDV virus is characterized by a complex sequence of different steps which offers opportunities to antiviral agents to intervene. Inhibition of NDV replication was measured by a plaque reduction assay as described previously[28].

In this assay, the number of plaques of drug-treated viruses was expressed in percent of the untreated control (number of plaques formed by viruses in the absence of limonin). In all assays, the maximum noncytotoxic concentrations of the limonin tested were used. According to our findings, pretreatment of cells with essential oils for 1 h prior to virus infection did not reduce the virus plaque formation indicating that essential oils did not affect the adsorption of viruses to cell surface and did not interfere with virus binding by blocking cellular receptors[29].

In conclusion, our results indicate that free viruses, in particular, are very sensitive to limonin. The possibility that limonin interferes with the Hemagglutinin–neuraminidase (HN), sites necessary for attachment and penetration of the virion into the living cell, is very likely. Newcastle disease virus can grow within different animal cells including primary cell culture and established cell line as referred by previous studies[1] that mentioned that NDV have the ability to replicate in and kill human tumor cells. Plaque assays were used to determine the cytolytic activity of NDV. NDV formed plaques on all tumor cells tested as well as on chick embryo cells (CEC), the native host for NDV. Plaques did not form on any of the normal fibroblast lines. To detect NDV replication, virus yield assays were performed which measured virus particles in infected cell culture lysate.

Figure 1: The lesions on dead embryos of chicken eggs that inoculated with virulent Newcastle virus (A) compared to the control group (B) after 48 hours

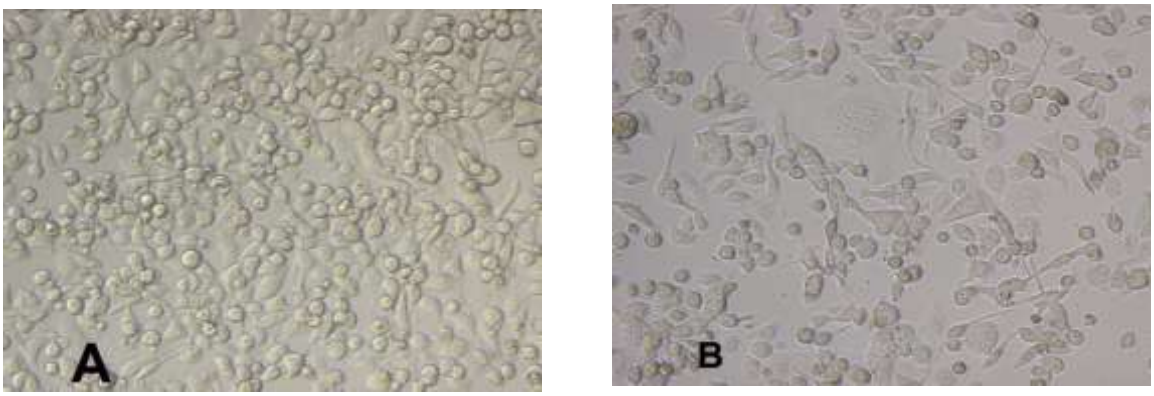

Figure 2: (A): Control RD cell line not infected with NDV, (B): RD cell line infected with NDV

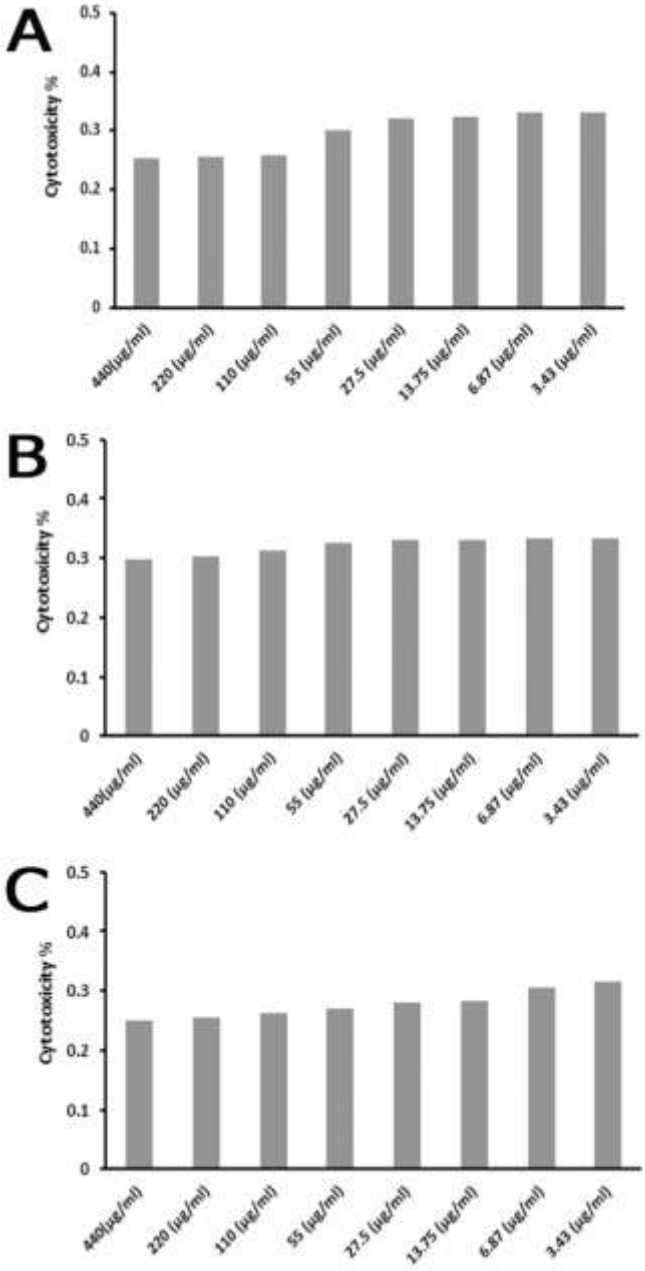

Figure 3: Growth inhibition (%) of limonin (440Mg/ml) on (A) Hela, (B) Vero and (C) RD cell lines after 72 hr of exposure

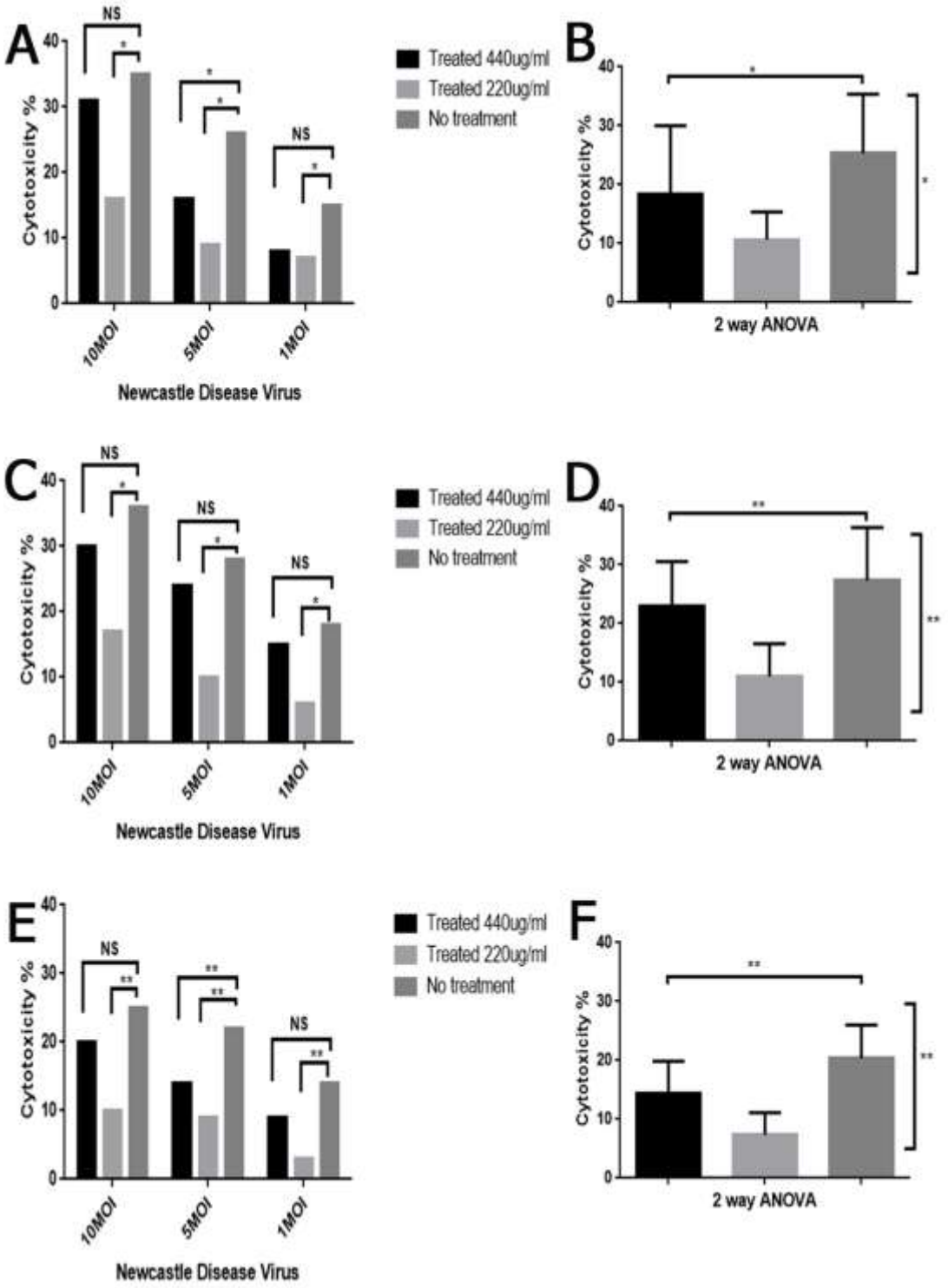

Figure 4: Antiviral cytopathic effect by Limonin, A, Hela cells. C, Vero. E, RD cells

Individual plaques obtained from varying dilutions of a viral stock can be counted to determine the viral titer (PFU/ml) of a given transfection or virus stock. The condition of the cells and their even distribution over the surface of the tissue culture plate is important to the success of a plaque assay. Plaque formation can take three days[9].

The kinetics follow up of the number of infected cells after infection with serial dilutions of the virus allowed estimation of the duration of the replication cycle, and consequently, the optimal infection time. When each infected cell produces virus and eventually lyses, only the immediately adjacent cells become infected. Each group of infected cells is referred to as a plaque. Uninfected cells surround the plaques as reported previously[13]. After several infection cycles, the infected cells in the center of the plaques begin to lyse and the peripheral infected cells remain surrounded by uninfected cells[22].

In conclusion, the results showed that limonin has anti-viral properties as it has downregulation effect on NDV that infected different cell lines *in vitro*. The results suggest an *in vivo* effect which need to be tested before moving to clinical studies.

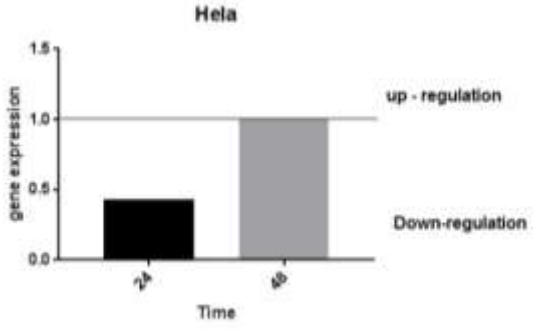

Figure 5: Relative gene expression of M gene of NDV virus in Hela cells after treatment with limonin (440µg/ml) for 24 and 48hs.

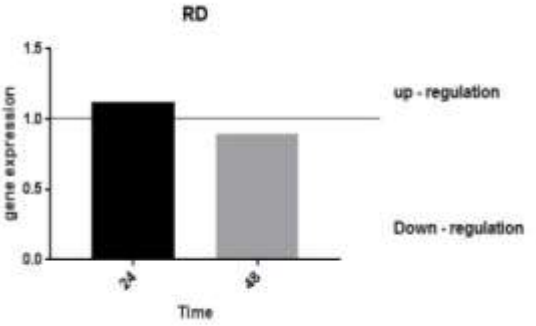

Figure 6: Relative gene expression of the M gene of NDV virus in RD cells after treatment with limonin (440µg/ml) for 24 and 48hs

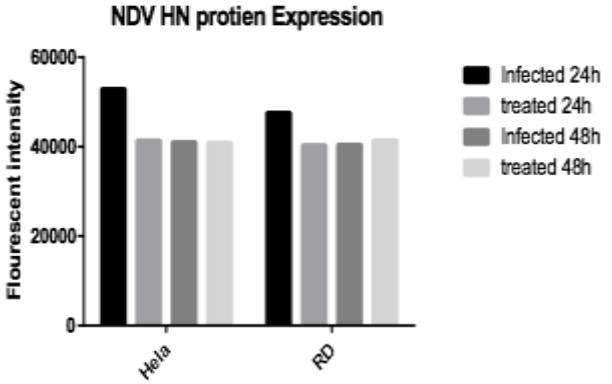

Figure 7: Significant difference in the effect of treatment, where the density of the virus proteins was decreased in Hela and RD cells after treatment in both exposure times

Table 1
Primers

Gene	Sequence
M gene F R	5'- AGTGATGTGCTCGGACCTTC-3' 5'CCTGAGGAGAGGCATTTGCTA-3
rRNA 18s F (Human) R	5'-GGAGTATGGTTGCAAAGCTGA-3' 5'-ATCTGTCAATCCTGTCCGTGT-3'

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