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

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Abstract
Numerous numbers of biologically active agents have been identified for their diverse therapeutic functions. Detailed investigations of phytochemicals for antiviral activities have assumed greater importance in the last few decades. A wide variety of active phytochemicals including the flavonoids, terpenoids, organosulfur compounds, limonoids, lignans, sulphides, polyphenolics, coumarins, saponins, chlorophyllins, furyl compounds, alkaloids, polyines, thiophenes, proteins and peptides have been found to have therapeutic applications against different genetically and functionally diverse viruses. The antiviral mechanism of these agents may be explained on basis of their antioxidant activities, scavenging capacities, inhibiting DNA, RNA synthesis, inhibition of the viral entry, or inhibiting the viral reproduction etc.

Large number candidate substances such as phytochemicals and their synthetic derivatives have been identified by a combination of in vitro and in vivo studies in different biological assays. In this study we have made attempts to extensively review and provide comprehensive description of different phyto-antiviral agents. We have examined the recent developments in the field of plant derived antiviral agents. The major advances in the field of viral interactions in various biological assays have been summarized.

In addition, sources of origin, major viral studies mechanistic action and phase trials of various phytoantiviral agents have been included.

Keywords : Arctigenin, Phytochemical, IRF3.

Introduction

Newcastle Disease, caused by Newcastle Disease Virus (NDV), is a serious threat to the global poultry industry due to its high mortality rate. This is why outbreaks of virulent NDV require an immediate notification to the Office of International Epizootes (OIE)[2]. NDV is a negative-sense, single stranded RNA virus of the family Paramyxoviridae. It is classified as an avian paramyxovirus 1 (APMV-1)[14]. The NDV genome contains six major genes: Nucleocapsid, Phosphoprotein, Matrix (M), Fusion (F), Hemagglutinin-neuraminidase and RNA polymerase. NDV isolates are classified into one of three pathotypes based on their pathogenicity: Lentogenic, mesogenic and velogenic[2]. This classification is confirmed by the presence of a cleavage site between amino acids 112-117 of NDV fusion protein.

However, recent data suggest that this classification needs to be conducted not only by virus sequence analysis, but also by *in vivo* pathogenicity tests. These tests are required to demonstrate the clinical virulence of NDV as measured by the intracerebral pathogenicity index and the mean death time[15]. Vaccination is the main protector against NDV infection.

Different strategies are needed to either prevent the replication of NDV or to decrease its drastic effects on an infected flock[25]. One of those strategies is investigating the antiviral activity of medicinal plant extracts against NDV infection. In fact, farmers mix medicinal plants with the poultry feed to increase the possibility of obtaining healthy chickens. In this study, we have selected medicinal plants to test their antiviral activity against NDV[21]. The blessed thistle (*Cnicus benedictus*) is one of the most important medicinal plant and it belongs to family Asteraceae (Compositae) which is considered of big family in plat kingdom. Most of memebers of this family are bisexual, female flowers is radial and corella has two form: tubular or ligulate, fruit Achene[6]. It is 60 cm in hight, leaves 30 cm with hairs and small spines at the margin[30].

Flowers are yellowish placed on head inflorcence (Capitulum head) 3 – 4 cm. in diameter[12]. This family has about 1120 genus and 125000 species distributed all over the world, In Iraq there are 342 wild species and 58 cultivated[6]. Arctigenin is a natural lignan compound that is extracted from the seeds of *Cnicus benedictus* and possesses various pharmacological properties including antiproliferative, anti-inflammatory, antioxidant, antiviral, immunomodulation, neuroprotective and antidiabetic effects[42].

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[20,27].

Cytotoxicity assay of arctigenin on the cells: It was achieved by using 96-well flat-bottomed microtitration plates to measure the cytotoxic effect of arctigenin and NDV alone on cancer cells, this procedure includes three stages as described by Maliheh et al[23].

1. Cell seeding stage:

- Cells of different cell lines (Hela, RD, Vero) were detached from their flasks when they reached to sub confluent monolayer by trypsinization as described above previously. Then 20ml of culture medium with 10٪ serum was added to the flasks and mixed gently to prepare cell suspension.
- The cell suspension in culture flask was poured aseptically to sterile beaker, 4-5 x 10^4 of cell suspension was transferred by using micropipette to each well in microtitration plate, then the plate was covered with a sterile adhesive film, lid placed on, shacked gently and incubated in CO_2 incubator at 37°C for 24hrs. To allow cell attachment, proliferation and confluent monolayer are achieved.

2. Exposure stage:

A. Exposure to phytochemical (arctigenin): Series of dilution 340, 170, 85, 42.5, 21.25, 10.62, 5.31, 2.65 µg/ml of phytochemical drug, diluted with (RPMI) 1640 medium, were added to confluent monolayer cells. In addition, negative controls (PBS) were included for each experiment to ensure validity of the assay. There were three replicates for each tested concentration. The cells were incubated for 72 hrs. at 37°C under humidified atmosphere containing 5% CO_2.

Newcastle disease virus (NDV) propagation: The Newcastle disease virus (Iraqi strain) was kindly provided by the Experimental Therapy Department / ICCMGR[7]. 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[29].

Propagation of NDV in Chicken embryo: Embryonated chicken eggs were incubated in a conventional incubator at 37°C and suitable humidity. The eggs were turned twice a day. After one day of incubation, the eggs were transilluminated with an egg candling lamp to insure the viability of the embryo. All non- fertile 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) was injected into each 9-days embryonated chicken egg allantoic fluid with a fine needle, the puncture hole in the egg was covered with paraffin.

Eggs were incubated at 37 °C in a humidified incubator and checked daily for embryo viability. Immediately after the death of the embryo, it was transferred to the refrigerator (4°C). After 12 hours, the allantoic fluid was collected by sterile syringe, purified from debris and erythrocytes as previously described. Then it was dispensed into small tubes and stored at -20°C and stored for long time at -85°C. The virus was further passaged in embryonated eggs in which high titers of virus can be recorded from the infected eggs until it reached 10 HAU/ ml[16].

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

The drug – pretreated cell free viruses are added at the same time to host cells; thus, infection starts at the same time. When intracellular viruses are drug – treated, the infection had started simultaneously. All non – infecting virus particles had been washed off and all intracellular viruses are in the same phase of replication[11].

Absorbency was determined on a microplate reader at 492 nm (test wavelength); the assay was performed in triplicate. Endpoint parameters that are calculated for each individual cell line included. The inhibiting rate of cell growth (the percentage of cytotoxicity) 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)[17].

Phytochemical Arctigenin: A stock solution was made by adding 4.3mg. in 100µl DMSO to 10 ml of serum free media (2.2mg/ml), then serial of two-fold dilutions were made and used in cytotoxicity and anti-viral assays.

Sample collection for PCR and ELISA reaction: 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, and then redistributed into tissue culture flasks (25 cm^2): 16 flasks for Hela and 16 for RD at concentration 1000,000 cell in each flask and incubated at 37°C for 24 hrs.

After the incubation, the medium was removed from the flasks, virus infection was done at MOI of 10 (MOI=10). The

infected cells treated by arctigenin (conc.=340µg/ml) control flasks were treated with serum free media only. Afterwards the flasks were re-incubated at 37ºC for 24,48 hrs (each time contain 4 flasks, one for the NDV, one for arctigenin treats infected (NDV+arctigenin) and one as control.

The cells were washed and harvested with PBS by cell scraper after the culture media was completely removed and the suspension of cells was collected from each flask into 2 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 for (PCR) study were centrifugated for 10 min at 10000rpm 4°C and stored at -80ºC.

The suspension of cells in Eppendorf tubes used for estimation of the expression for IRF3 proteins was subjected to two freeze-thaw cycles to further break the cell membranes. After that the Eppendorf tubes were centrifuged for 15 min at 5000 rpm, the supernatant was collected and stored at -80ºC for next determination and analysis of the expression of IRF3 protein.

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:

- 100ul antigen solution diluted in buffer was added to appropriate numbers of microtiter wells.
- 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.
- Microtiter wells were emptied, and the plate was inverted to tap out excess liquid onto a clean tissue.
- Blocking Step
- 200-300ul of blocking solution was added to each well.
- Wells were incubated for 1-2 hours at RT and the plate was emptied to tap out excess fluid onto a clean tissue.
- Wells were washed three times with PBS-T.
- Primary Antibody Incubation
- 100ul of primary antibody solution diluted in blocking buffer was added to each well.
- 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).
- The plate was inverted to tap out excess liquid onto a clean tissue.
- Wells were washed three times with PBS-T.
- Secondary Antibody Incubation
- 100ul of the secondary antibody diluted in blocking buffer was added to each well.
- Incubation step was performed for 1-2 hours at RT with gentle agitation.
- The plate was inverted, and excess liquid was tapped out onto a clean tissue.
- Washing of Microtiter Wells
- Each well was filled with washing solution (PBS-T) with agitation for 5 min at RT.
- The plate was inverted, and residual fluid was tapped out onto a clean tissue.
- Washing step was repeated for 3 times.
- 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 frozen 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[5].

Results were analyzed for each sample with relative quantification comparing the difference between sample and control. The mean CT values of the genes were calculated for each individual sample (as duplicate replication for each sample) and used to normalize expression levels using the ΔΔCT method described previously[9].

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 significance comparison among means in this study (36).

Results and Discussion

Propagation of the virus in embryonated chicken eggs: The result showed that the Iraqi strain of Newcastle disease

virus (NDV) has the ability to kill the chicken embryos from 48 to 72 hrs after the inoculation of the chicken embryonated eggs. This indicates the virulence level of the strain which was velogeneic type and was able to kill embryos after 40-72hrs. This result agrees with result of Livak et al[22] who isolated the Iraqi local strain that we used in this study, and these results confirm that the strain is still virulent and holds their characteristics[4]. Embryonic death within 24 hrs of inoculation was considered non-specific, and such eggs were discarded. The virulent local strain of NDV was referred as virulent viscerotropic strain[8]. 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 so the Velogenic strains kill embryos in less than 60 hrs, mesogenic strains in 60–90 hrs[39] and lentogenic strains in more than 90 hrs.

Hemorrage was clearly observed in the infected embryos when compared with the control (figure 4.1). The virus was purified and quantified by hemagglutination test showing a positive result as a typical hemagglutination mesh pattern of chicken red blood cell 128 HAU, further passages of the virus in embryonated chicken eggs observed that the titer was raised to 256 HAU in the second passage, while in the next passage the titer reached to 512 HAU[41].

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.

Arctigenin Cytotoxicity on Hela, Vero and RD cells lines: Hela tumor cell line were seeded as 1×10^4 cells / well in 96 well plats and after 24h, when the cells become confluent monolayer, they were exposed to the arctigenin at 340,170,85,42.5,21.25,10.62,5.31,2.65 ug/ml and incubated in 37℃ for 72hr, then stained by MTT stain and calculated the inhibition % .

The results showed that the *arctigenin* had cytotoxic inhibition % higher than the *arctigenin* in some conc. 0.271±0.002 ,0.257±0.001 respectively 0.316 ± 0.001, 0.316 ± 0.002, 0.315 ± 0.001, 0.306 ± 0.001, there was no significant variance ($P<0.05$) among different conc.

Antiviral cytopathic effect by arctigenin: This study aimed to determine the anti-viral effects of arctigenin. The results showed that the inhibitory effect of 340ug/ml arctigenin on NDV replication was effective by reducing the NDV cytotoxicity to 33%, 32% and 28.5% on Hela cells table 2.

Furthermore, 340ug/ml arctigenin inhibitory effect on NDV replication reduced the NDV cytotoxicity to 23%, 17%, 14% on Vero cells table 3. Moreover, 340ug/ml arctigenin inhibitory effect on NDV replication reduced the NDV cytotoxicity to 31%, 29%, 27% on RD cells as in table 4.

mRNA expression of M-gene in Hela and RD after infection with NDV: According to the mRNA level of the Materx gene for the Newcastle virus where the readings showed a decrease in the gene expression of the virus after exposure to arctigenin within 24 hours, the reading showed that the virus was not affected by exposure of the material within 48 hours as in fig. 1.

NDV HN protein determination: In the table above, there is a significant difference in the effect of treatment where the density of the virus in the cell decreased for Hela cells which explains the presence of significant difference in the case of RD cells at 24 and 48 hours not showing significant differences as in table 5.

The aim of this study was to determine the anti-viral effects of a arctigenin. So, the results showed that arctigenin inhibitory effect on NDV (Virus) replication was effective by reducing the NDV cytotoxicity to 33 %, 32%, 28.5% on Hela cells by using 340µg of arctigenin as in table 2.

Furthermore, 340µg arctigenin inhibitory effect on NDV virus replication reduced the NDV cytotoxicity to 23%, 17%, 14% on Vero cells table[3]. Moreover, 340 µg arctigenin inhibitory effect on NDV virus replication reduced the NDV cytotoxicity to 31%, 29%, 27% on RD cells table[4].

The results showed an effect at the 2nd concentration on Hela, Vero and RD cell lines which could be due to phytotherapy-induced early apoptosis or other cellular alterations that directly inhibit the virus life cycle or virus replication as described by Alexander et al[3]. In our experiment other concentrations showed activity on Hela, Vero and RD cell lines, which is the combined effect that is significantly greater than the expected (additive) effect[35]. When two or more drugs are given, the effect may be super additive, that is, they may demonstrate action that is above what is expected from their individual potencies and efficacies. When the effect is consistent with the individual

drug potencies, it means that the interaction is simply additive[34].

Aghi et al[1] reported that many different viral-based therapies are under investigation to facilitate an interaction between therapies that would permit lower doses of each agent to be used to minimize both cost and toxicity[33].

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[35]. 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 arctigenin (*Cnicus benedictus* 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)[1].

The active influences of the arctigenin against some species of virus are due to containing these growing some microorganism. This is consistent with inhibition due to the glycosides[24], in addition to the influence of the other active groups as alkaloids by which the *Cnicus benedictus* is distinguished, especially the alkaloids dissolved in the ethyl alcohol and has a capacity of solution superior to the solution in the water[19].

The importance of this plant is containing the active tannins in inhibiting the viruses by their capacity of inciting the Phagocyte cells as well as having an activity in destroying the protein and other compounds[32] into the virus cell all because the virus used them to attach and the terpenes activated to tear the cellular membranes by the Lipophilic compounds. The alkaloids can make interference with the RNA of the virus cells and lead to kill them[10].

The phenol compounds are distinguished by their properties being worked anti-viral by debarring proton motive oxidative phosphorylation and coagulating the cytoplasm components[18].

The phenol compounds have also a role in inhibiting the growth of virus by inhibiting the responsible metabolic reactions by interference of non-specialized with the proteins[28]. This leads to protein denaturation. The resins and the substantial oils make the aqueous botanic extracts activated towards the selective microorganisms[38]. The difference of impact of these auxiliary metabolic products in their influence is due to the difference of types of these active materials and their amounts[31].

We conclude there is a bio-activity for the arctigenin of the *Cnicus benedictus* working against virus. This gives an opportunity to be used it as an alternative from the traditional anti-virus because numerous species of viruses become assistants to them[13].

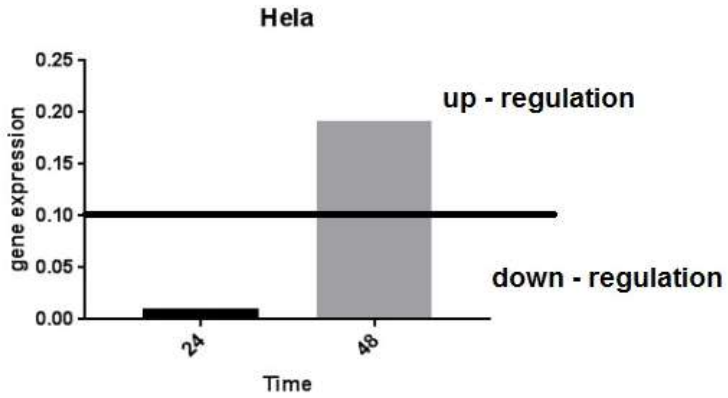

Figure 1: Relative gene expression of M gene after infected with NDV virus on Hela cell.

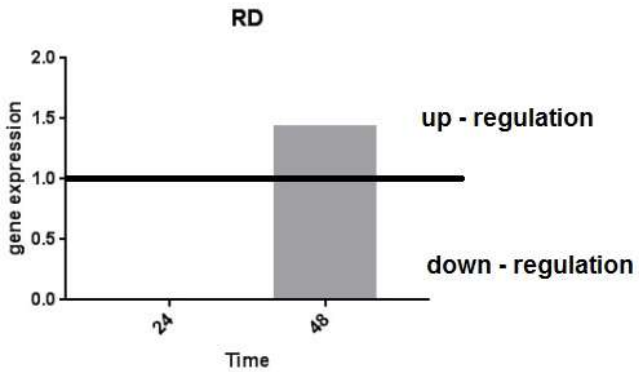

Figure 2: Relative gene expression of M gene after infected with NDV virus on RD cell

Table 1
Growth inhibition% of arctigenin on Hela, Vero and RD cells lines after 72 hr. of exposure

Conc. (µg/ml)	G.I %	Growth Inhibition effect Arctigenin on cancer cell (Hela) Mean (Time 24 hr.)
340	38%	0.211 ± 0.001 d
170	36%	0.217 ± 0.002 d
85	36%	0.218 ± 0.001 d
42.5	32%	0.231 ± 0.002 cd
21.25	25.7%	0.254 ± 0.022 abc
10.62	28%	0.245 ± 0.002 bc
5.31	24.8%	0.257 ± 0.001 ab
2.65	20%	0.271 ± 0.002 a
LSD: 0.05		Optical density (OD) 0.0241 *

Conc. (µg/ml)	G.I%	Growth Inhibition effect Arctigenin on cancer cell (Vero) Mean (Time 24 hr.)
340	12%	0.285 ± 0.003 d
170	9%	0.294 ± 0.001 c
85	4%	0.311 ± 0.001 ab
42.5	4%	0.310 ± 0.002 b
21.25	3%	0.313 ± 0.002 ab
10.62	3%	0.314 ± 0.001 ab
5.31	2%	0.316 ± 0.002 a
2.65	2%	0.316 ± 0.001 a
LSD: 0.05		Optical density (OD) 0.0053 *

Conc. (µg/ml)	G.I %	Growth Inhibition effect Arctigenin on cancer cell (RD) Mean (Time 24 hr.)
340	20%	0.222 ± 0.002 f
170	20%	0.289 ± 0.001 e
85	18%	0.298 ± 0.001 d
42.5	18%	0.299 ± 0.001 cd
21.25	18%	0.298 ± 0.002 d
10.62	17%	0.302 ± 0.002 c
5.31	16%	0.306 ± 0.001 b
2.65	13%	0.315 ± 0.001 a
LSD: 0.05		Optical density (OD) 0.0039 *

Table 2
Demonstrated that arctigenin has a antiviral effect against NDV in (Hela) effect after an exposure period of 72 hrs.

Dose of NDV (TCID50)	Dose of arctigenin µg (Hela)	Virus cytotoxicity %	NDV	Chi-Square
		Arctigenin treatment	No - arctigenin	
100000 (MOI 10)	340	33%	49.4%	5.27 *
50000 (MOI 5)	340	32%	49%	5.29 *
10000 (MOI 1)	340	28.5%	48.5%	7.75 **
100000 (MOI 10)	170	38%	49.4%	4.53 *
50000 (MOI 5)	170	35.7%	49%	5.61 *
10000 (MOI 1)	170	33.8%	48.5%	5.48 *
Chi-Square	---	4.48 *	0.437 NS	---
* (P<0.05), ** (P<0.01).				

Table 3
Demonstrated that arctigenin has a antiviral effect against NDV in (Vero) effect after an exposure period of 72 hrs.

Dose of NDV (TCID50)	Dose of arctigenin µg (Vero)	Virus cytotoxicity %	NDV	Chi-Square
		Arctigenin treatment	No - arctigenin	
100000 (MOI 10)	340	23%	30.4%	3.96 *
50000 (MOI 5)	340	17%	28.5%	5.17 *
10000 (MOI 1)	340	14%	27.5%	5.47 *
100000 (MOI 10)	170	25%	30.4%	2.51 NS
50000 (MOI 5)	170	24%	28.5%	1.46 NS
10000 (MOI 1)	170	21%	27.5%	2.72 NS
* (P<0.05), NS : Non – significant				

Table 4
Demonstrated that arctigenin has a antiviral effect against NDV in (RD) effect after an exposure period of 72 hrs.

Dose of NDV (TCID50)	Dose of arctigenin µg (RD)	Virus cytotoxicity %	NDV	Chi-Square
		Arctigenin treatment	No - arctigenin	
100000 (MOI 10)	340	31%	41%	4.39 *
50000 (MOI 5)	340	29%	39%	4.39 *
10000 (MOI 1)	340	27%	38%	4.52 *
100000 (MOI 10)	170	20%	41%	8.09 **
50000 (MOI 5)	170	19%	39%	7.75 **
10000 (MOI 1)	170	15%	38%	8.61 **
Chi-Square	---	6.31 **	0.946 NS	---
* (P<0.05), ** (P<0.01).				

Table 5
Exposure cell line 24 and 48 hr by Antibody kit.

Cell line	Exposure material	Exposure material 24 hr. & 48 hr.	Reading Mean
Hela	Virus	24 hr.	49023
Hela	Arctigenin – V	24 hr.	39834
T-Test: 0.05	---	---	251.82 *
Hela	Virus	48 hr.	42319
Hela	Arctigenin – V	48 hr.	40722
T-Test: 0.05	---	---	2894.51 NS
RD	Virus	24 hr.	50273
RD	Arctigenin – V	24 hr.	49713
T-Test: 0.05	---	---	2189.72 NS
RD	Virus	48 hr.	43971
RD	Arctigenin – V	48 hr.	42083
T-Test: 0.05	---	---	1988.35 NS
* (P<0.05), NS: Non-Significant			

References

1. Aghi M., Rabkin S. and Martuza R.L., Effect of chemotherapy-induced DNA repair on oncolytic herpes simplex viral replication, *J Natl Cancer Inst*., **98(1)**, 38-50 **(2006)**

2. Alexander D.J., Newcastle disease and other avian paramyxoviruses, *Revue Scientifique et Technique-Office International des Epizooties*, **19(2)**, 443-455 **(2000)**

3. Alexander D.J. and Senne D.A., Newcastle disease, other avian paramyxoviruses and pneumovirus infections, Diseases of poultry, 12th ed., Blackwell Publishing, Ames, IA, 75–98 **(2008)**

4. Alexander D.J., Newcastle disease, other avian paramyxoviruses and pneumovirus infections, *J. Diseases Poult.*, **11**, 63-99 **(2003)**

5. Al-Habeeb M.A., Mohamed M.H.A. and Sharawi S., Detection and characterization of Newcastle disease virus in clinical samples using real time RT-PCR and melting curve analysis based on

matrix and fusion genes amplification, *Vet. World*, **6(5)**, 239-243 **(2013)**

6. Al-Katib Yousef Mansour, Classification of seed plants, Ministry of Higher Education, University of Baghdad **(2000)**

7. Al-Shammari A.M. and Yaseen N.Y., In Vitro Synergistic enhancement of Newcastle Disease Virus to Methotrexate cytotoxicity against tumor cells, *Al-Anbar J. Vet. Sci.*, **5(2)**, 102-109 **(2012)**

8. Al-Shammary A.M., Al-Nassrawei H.A. and Kadhim A.M.A., Isolation and sero-diagnosis of Newcastle Disease Virus Infection in Human and Chicken Poultry Flocks in Three cities of Middle Euphrates, *Kufa Journal of Veterinary Medical Sciences*, **5(1)**, 16-21 **(2014)**

9. Aursnes I.A., Rishovd A.L., Karlsen H.E. and Gjøen T., Validation of reference genes for quantitative RT-qPCR studies of gene expression in Atlanticcod (Gadus morhua l.) during temperature stress, *BMC Research Notes*, **4**, 104 **(2011)**

10. Zouari Ahmed Rachida, Ouahrani M. Ridha, Laouini S. Eddine and Meneceur Souhaila, Screening of phenolic compounds from Abelmoschus esculentus L extract fruits and in vitro evaluation of antioxidant and antibacterial activities, *Res. J. Chem. Environ.*, **22(2)**, 37-42 **(2018)**

11. Betancur-Galvis L.A., Morales G.E., Forero J.E. and Roldan J., Cytotoxic and Antiviral Activity of Colombian Medicinal Plant Extract of the Euphorbia Genus, *Mem. Inst. Oswaldo Cruz, Rio de Janeiro*, **97(4)**, 541-546 **(2002)**

12. Chukwudi O.E., Chukwuemeka E.D. and Mary U., Newcastle disease virus shedding among healthy commercial chickens and its epidemiological importance, *Pakistan Veterinary Journal*, **32(3)**, 354-356 **(2012)**

13. Cowan M.M., Plant products as Antimicrobial agents, *Clinical Microbiology Reviews*, **12(4)**, 564-582 **(1999)**

14. Nasri Nawel, Maatallah Samira, Saïdi Issam and Lachaâl Mokhtar, In Vitro properties of acid phosphatase from lettuce seedlings, *Res. J. Chem. Environ.*, **22(11)**, 68-72 **(2018)**

15. Diel D.G., da Silva L.H., Liu H., Wang Z., Miller P.J. and Afonso C.L., Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes, *Infect. Genet. Evol.*, **12(8)**, 1770-9 **(2012)**

16. Flint S.J., Enqist W. and Racaniello V.R. and Skalka A.M., Virological Methods (Principles) of virology **(2009)**

17. Freshney R.I., Culture of animal cells: A manual for basic technique, 5th ed., Wiley-liss, John Wiley and Sons, Inc. Publication, New York **(2005)**

18. Gerl R. and Vaux D.L., Apoptosis in the development and treatment of cancer, *Carcinogenesis*, **26**, 263-70 **(2005)**

19. Kodamala Kranthi Raj, Kaki Sowjanya, Mathi Pardhasaradhi and Dittakavi Ramachandran, Synthesis, In Vitro Antibacterial and In Silico Evaluation of Indole Based Molecules as Staphylococcus Aureus Sortase A Inhibitors, *Res. J. Chem. Environ.*, **22(6)**, 72-79 **(2018)**

20. Kashi V.P., Hatley M.E. and Galindo R.L., Probing for a deeper understanding of rhabdomyosarcoma: insights from complementary model systems, *Nature Reviews Cancer*, **15(7)**, 426 **(2015)**

21. Lam H.Y., Yeap S.K., Rasoli M., Omar A.R., Yusoff K., Suraini A.A. and Banu Alitheen N., Safety and clinical usage of newcastle disease virus in cancer therapy, *J. BioMed Biotechnol*, doi: 10.1155/2011/718710 **(2011)**

22. Livak K.J. and Schmittgen T.D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods*, **25(4)**, 402-8 **(2001)**

23. Maliheh E., Ahmad M., Fathollah F., Sedigheh M., Mehrdad H. and Abdolreza A.L., Antimutagenicity and anticancer effects of Citrus medica fruit juice, *Acta Medica Iranica*, **5(47)**, 373-377 **(2009)**

24. Mason T.L. and Wasserman B.P., Inactivation of red beta Glucan synthase by native oxidized phenolic compounds, *Phytochemistry*, **26**, 2197 – 2202 **(1987)**

25. Miller P.J. et al, Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features, *Infection, Genetics and Evolution*, **29**, 216-29 **(2015)**

26. Nelson N.J., Scientific interest in Newcastle disease virus is reviving, *J. Natl. Cancer Inst*., **91**, 1708-10 **(1999)**

27. Ammerman Nicole C., Beier – Sexton Magda and Azad Abdu F., Growth and Maintenance of Vero Cell Lines, NIH Public Acess. Curr Protoc Microbiol, doi:10.1002 **(2008)**

28. Nostro A., Germano M.P. and Marino A., Plant antimicrobial activity, *Lett. Microbiol.*, **30(1)**, 379-38 **(2000)**

29. Nutrition Jansen S., Anticancer and health protective properties of citrus fruits components, *Asia Pacific Journal of Clinical Nutr.*, **1(11)**, 79–84 **(2002)**

30. Philipson J.D. and O´Neill M.J., New leads to the treatment of protozoal infections based on natural product molecules, *Acat Pharm. Nord.*, **1**, 131-144 **(1987)**

31. Pol J.G., Resseguier J. and Lichty B.D., Oncolytic viruses: astep into cancer immunotherapy, *Virus Adaptation and Treatment*, **4(1)**, 2110-2147 **(2011)**

32. Rahman A., Siddiqui S.A., Jakhar R. and Kang S.C., Growth inhibition of various human cancer cell lines by imperatorin and limonin from poncirus trifoliate rafin. Seeds, *Anticancer Agents Med Chem*, **15(2)**, 236 – 41 **(2015)**

33. Randhir R., Lin Y.T. and Shetty K., Phenolics, their antioxidant and antibacterial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors, *Asia Pac. J. Clin. Nutr.*, **13(3)**, 295-307 **(2004)**

34. Ravindra P.V. et al, Detection of canine adenoviral infections in urine and faeces by the polymerase chain reaction, *J. Virol. Meth*., **149**, 260-263 **(2008)**

35. Rezaeianzadeh G., Dadras H., Safar A., Ali M. and Nazemshirazi M.H., Serological and molecular study of Newcastle disease virus circulating in village chickens of Fars province, *Iran. J. Vet. Med. Anim. Health*, **3(8)**, 105-111 **(2011)**

36. Salih R., Odisho S., Al-Shammari A. and Ibrahim O., Antiviral effects of olea europaea leaves extract and interferon-beta on gene expression of newcastle disease virus, *Adv Anim Vet Sci*., **5(11)**, 436-45 **(2017)**

37. SAS, Statistical Analysis System, User's Guide. Statistical, Version 9.1th ed., SAS, Inst. Inc. Cary, N.C., USA **(2012)**

38. Schnitzler P., Schön K. and Reichling J., Antiviral activity of Australian tea tree oil and eucalyptus oil against herpes simplex virus in cell culture, P*harmazie*, **56**, 343 – 347 **(2001)**

39. Singh K., Jindal N., Gupta S.L. and Mittal D., detection of Newcastle disease virus genome from the field outbreaks in poultry by reverse transcription polymerase chain reaction, *J. Poult. Sci.*, **4(7)**, 472-475 **(2005)**

40. Vigil A., Park M.S., Martinez O., Chua M.A., Xiao S., Cros J.F., Sobrido L.M., Woo S.L.C. and Sastre A.G., Use of reverse genetics to enhance the oncolytic properties of Newcastle disease virus, *Cancer Research*, **67(17)**, 8285-8292 **(2007)**

41. Zahid A.A.H. and Bunny M.N., Immunization of broiers against Newcastle disease using inactivated oil based vaccine, *Veterinary Doctor*, **12(2)**, 1-12 **(2002)**

42. Zamarin D. and Palese P., Oncolytic Newcastle disease virus for cancer therapy: old challenges and new directions, *Future Microbiology*, **7(3)**, 347-367 **(2012)**.