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In Vitro Antiviral Activity of Commercial Products of Herbal Extracts Against Highly Pathogenic Avian Influenza (H5N1) Virus

Hatem S. Abd El-Hamid[1], Hany F. Ellakany[1], Ahmed R. Elbestawy[1], Abdalla A. Seliem[2], Hazem S. Khalil[2,*]

[1] Department of Poultry and Fish diseases, Faculty of Veterinary Medicine, Damanhour university
[2] Reference laboratory for veterinary control on Poultry Production (Main branch and El-Behira branch), Animal Health Research Institute.

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Correspondence to:
Drzuma_s@yahoo.com

ABSTRACT

Three commercial products of herbal extracts (Amphiflu-Ex®, Fyto-immune Plus® and Viu-Vet®) were tested for antiviral activity against two Egyptian highly pathogenic avian influenza subtype H5N1 isolates (isolated form backyard poultry) and upon sequencing and phylogenetic analysis, they belonged to clade 2.2.1.2., The antiviral assay for the products was done by using chicken embryo fibroblast (CEF) cell culture through cytopathic effect (CPE) inhibition assay in two different layouts to elucidate post-exposure and pre-exposure antiviral activity of the products. Amphiflu-Ex® at conc. 50 µl/ml showed significant virucidal activity (100% inhibition) in post-exposure assay for both viral isolates which was further confirmed by quantitative real time RT-PCR method that showed 100% reduction in virus titers for both isolates and further investigation was done on conc. 10 and 20 µl/ml, where the virus titers were decreased by 1-1.27 and 2.72-2.8 logs (Log 10 virus titers) respectively. The maximum non-toxic concentration (MNTC) for the three products which estimated by cytotoxicity assay was 50 µl/ml and TCID50 used for both isolates was 106 copy/100 µl.

1. INTRODUCTION

Egypt has become an epicenter for highly pathogenic avian influenza (HPAI) subtype H5N1 virus activity with continuous outbreak, In addition Egyptian General Organization for Veterinary Services reported outbreaks of HPAI subtype H5N1 virus in several governorates (Pyere et al., 2009).

Egypt is one of few countries with an endemic status of clade 2.2.1, which diversified into two distinct sub-clades designated 2.2.1.1 and 2.2.1.2., Viruses of sub-clade 2.2.1.1 circulated in vaccinated poultry, including turkey, from 2007 to 2014 despite intensive blanket vaccination using over 20 diverse H5 vaccines (Aly et al., 2008, Abdel-Moneim et al., 2009 and Abdelwhab et al., 2016), Meanwhile, sub-clade 2.2.1.2 viruses were observed mainly in backyard birds, human and recently in the commercial birds, causing severe socioeconomic losses in the poultry industry and posed a serious pandemic threat because of their affinity to human type receptors (Arfa et al., 2015).

The emergence of antigenic-drift variants, improper vaccination and/or immunosuppression are commonly responsible for vaccination failure mainly in chickens (Abdelwhab et al., 2016).

The strategy to prevent and control the disease was based on increasing people awareness about the risk of the virus transmission especially in rural areas, using imported H5 poultry vaccines, depopulation of infected poultry while increasing biosafety levels and disinfection (Pyere et al., 2009).

Therefore, presence of new alternative and complementary strategies target different AIV

serotypes /subtypes/ drift variants should be encouraged. The use of chemotherapeutic agents for control of AIV in poultry was concurrently studied just after discovering their antimicrobial effects (Moses et al., 1948 and Tolba and Eskarous, 1959), Synthetic anti-viral drugs e.g. amantadine and oseltamivir are available and practiced (Gadreau et al., 1998 and Emery, 1998) but the use of these drugs is restricted due to possibility of emergence of drug resistant virus and residual effects in poultry products (Gubareva et al., 1998), As per International Livestock Regulations, amantadine is licensed only for human use; however its prophylactic use in poultry production has been recorded in Asia. Once resistance against amantadine is acquired, it can persist in the viral lineage, thus eliminating the possibility to use amantadine to treat humans H5N1 influenza cases (WHO, 2005).

Unlimited herbal products contain polyphenols, flavonoids, alkaloids or lignans, mostly from traditional Chinese medicine, offer promise as adjuncts or alternatives to the current anti-influenza chemotherapy (Kitazato et al., 2007), In poultry, antiviral and immune adjuvant effects of several plants and/or its derivatives had been investigated, In addition to its antiviral activity, these extracts often have anti-bacterial, anti-fungal, anti-inflammatory, anti-oxidant and/or analgesic properties which may provide alternative natural broad-spectrum way for control of AIV in poultry farms (Garozzo et al., 2009 and Sood et al., 2012).

2. MATERIALS AND METHODS.

2.1.H5N1 Virus

The HPAI subtype H5N1 virus isolates used were A/chicken/Egypt/SD95/2015 and A/duck/Egypt/CAD90/2015 which isolated from backyard birds from Elbehira governorate causing 100% mortality in chickens and ducks in the field.

2.2.Real-time Reverse Transcription Polymerase Chain Reaction.

The real–time RT-PCR for AIV was done using Quantitect probes RT-PCR kit with VLA modified protocols **(**VLA, 2008). The RNA from swab samples was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA) and the real time RT-PCR was done using Qiagen one step RT-PCR Kit (Qiagen, Germany).

2.3.Virus isolation.

Samples that showed a positive reaction in the M gene and H5 gene by real time RT-PCR were inoculated in 10-day-old specific pathogen free embryonated chicken eggs through allantoic sac route.

2.4.Haemagglutination (HA) test.

This test was done according to (OIE, 2012) using v-shape bottomed microtiter plate and 1% chicken RBCs against 4 HA unit.

2.5.Sequencing and phylogenetic analysis.

The extracted RNAs of 2 positive AI samples were submitted to the gene analysis unit of the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, for sequencing and genetic analysis.

2.6.Commercial products.

A. Amphiflu-Ex® (AM Pharma, USA).

Ingrediants: (Vitamin B1= 5gm/L; Vitamin B6= 5gm/L; Zinc Methionine= 3gm/L; Glycine= 4.92gm/L; Lysine= 30gm/L; Sodium Butyrate= 20gm/L; BHT (butyl hydroxy toluene) = 25gm/L; Pidotimod (PDMD); Xylo-Oligosacharides (XOS); Taurine; Gamma Amino Butyric acid (GABA) and other propriety micro ingredients including RH 40, cremopher, propylene glycol, Deionized water and excipients)

B. Fyto-immune Plus® (Ropapharm International, Netherland).

Ingrediants: (Oregano oil (Carvacol - Thymol)= 100gm/L; Capsicum oil (Capsaicim)= 30gm/L; Cinnamon oil (Cinnamonaldhyde)= 20gm/L).

C. Viu-Vet® (Madrid, Spain).

Ingrediants: (Monoammonium Glycyrrihizinate= 4.6gm/L; Glucosamine= 92gm/L; Malic Acid= 92g/L; Arginine= 83gm/L; Glycine= 47gm/L; Ascorbic Acid= 23gm/L; Pyriodoxine hydrochloride= 4.5gm/L; Zinc Sulphate= 2.3gm/L; Potassium Sorbate= 2gm/L; Calcium pantothenate= 2.3gm/L; Folic Acid=1gm/L; Cyanocobalamine= 0.1mg/L).

2.7.CEF Culture.

CEF were generated from 10-day-old chicken embryos. The CEFs were grown overnight in Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 g/ml of streptomycin at 37◦C in 5% CO2 (A laboratory manual for the isolation and identification of avian pathogen, 2008).

2.8.Tissue Culture Infective Dose 50% (TCID50).

The titers of both isolates were determined in CEF cells and expressed TCID50 (A laboratory manual for the isolation and identification of avian pathogen, 2008). The titrated virus was stored at -80°C until further use.

2.9.Cytotoxicity assays.

The maximum non-toxic concentration (MNTC) of each product was determined based on cellular morphologic changes on CEF tissue culture. Concentration exhibiting toxicity to cells was omitted and the antiviral activity was studied at MNTC of each extract. In brief, serial dilutions of each extract (10 µl/ml till 100 µl/ml) were incubated in contact with confluent monolayer of CEF cells in quadr-replicates in 96-well plates while at column 12 in each plate kept as negative control for 4 days and the cells were observed under microscope every 24 h for visible morphological changes. The highest concentration of the products without any cellular morphologic change up to 4 days in all the four replicates was considered as their MNTC (Repetto et al., 2008).

2.10. CPE inhibition assay.

The CPE inhibition assay was used as a screening test to identify the products which showed antiviral effect against the HPAI virus, as the maximum non-toxic concentration (MNTC) of each product tested against both isolates. An additional investigation was done on Amphiflu-Ex® with the concentration of 10 and 20µl/ml. The CPE inhibition assay was carried out in two different layouts, the pre-exposure and post exposure antiviral assay (Elkosy et al., 2005).

a. Pre-exposure antiviral activity.

The confluent monolayer of CEF cells in 24-wells plate were pre-incubated with the three products at their MNTCs in triplicates for 1 h, three wells were kept as cell controls, the cells were washed once then incubated for 1h at 37°C with both viral isolates (TCID50; 10^6 copy/100 µl) then overlaid with plain medium and incubated at 37°C in 5% CO2 for 72 h and CPE scores were noted every 24 h intervals.

b. Post-exposure antiviral activity.

Confluent monolayer of CEF cells in 24-wells plate were incubated for 1 h with both viral isolates (TCID50;10^6 copy/100 µl) except in the 3 wells for cell control and 3 wells for virus control, products at their MNTC in plain medium added in triplicate to the wells and incubated at 37°C in 5% CO2 for 72 hrs., CPE scores were noted at 24, 48 and 72 hrs post-infection. The virus control was as virus + plain medium and the cell control was as uninfected cells in plain medium.

2.11. **Antiviral activity analysis by Quantitative real time RT-PCR**.

Using the quantitative PCR for evaluation the antiviral activity of three commercial products by quantification the virus yield of afore-mentioned two layouts (pre-exposure and post-exposure), viral RNA extraction using QIAamp, Qiagen, Germany according to the manufacturer's instructions of the kit and detection and quantitation of influenza virus subtype H5 by Real- Time RT-PCR according to manufacture of Quantitect kit-Qiagen.

3. RESULTS

3.1.**Molecular detection of avian influenza for matrix and H5 genes by real time RT-PCR**.

Testing of the collected 2 samples by real time RT-PCR revealed positive for matrix gene and subtype H5 avian influenza primers **(Table 1).**

3.2.**Virus isolation**.

The two samples were inoculated into fertile SPF eggs and had HA titer log2 ranged from 7 to 8.

3.3.Sequence and phylogenetic analysis.

The HA gene of both isolates sequenced, compared with H5N1 strains of Egypt from 2006 to 2015 and vaccine strains (Hong Kong H5N1-1994 and Mexican H5N2-1994), both isolates found to be have an identity with other isolates in 2015 by 98.1% - 99.2%, with isolates of 2012 -2014 by 96.2% - 97.3%, with 2006 isolates by 96.1% - 96.3%, with Hong Kong H5N1-1994 by 90.9% to 91.1% and Mexican H5N2-1994 by 78.2% - 78.5%, so the obtained results revealed that both isolates belonged to variant clade 2.2.1.2. (Fig. 1&2).

3.3.Tissue Culture Infective Dose (50%).

The H5N1 virus was titrated using CEF cells in 96 wells plate, the TCID50 was $10^{7.25}$/100 µl for the isolate A/chicken/Egypt/SD95/2015, $10^{6.5}$/100µl for the isolate A/duck/Egypt/CAD90/2015 and 10^{6} copy/100 µl TCID50 of both isolates were used for the following antiviral assays.

3.4.Cytotoxicity assay.

The obtained results revealed that the highest concentration from the three products showed no CPE in CEF cells considered as a safe was 50 µl/ml and this concentration used in the further antiviral assays (Fig. 3 a & b).

3.5. CPE Inhibition assay and virucidal activity of commercial products.

The obtained results revealed that the Amphiflu-Ex® at conc. 50µL/ml had no cytopathic effect when used 1hour post infection of CEF cell culture with H5N1 virus isolates (post-exposure) as shown (Fig. 3 d & e), While Viu-Vet® and Fyto-immune Plus® had no anti-cytopathic effect neither in pre-exposure assay (treatment of CEF cell culture before the infection with H5N1 virus isolates) nor in post-exposure assay (treatment of CEF cell culture after the infection with virus as shown (Fig. 3 c, f, g, h & i).

3.6.Antiviral activity analysis by Quantitative real time RT-PCR.

a. Pre-exposure assay.

in which the products were added first, followed by incubation for one hour then the virus were added, the results revealed that the products were not effective against H5N1 HPAI virus (Table 2).

b. Post-exposure assay.

In which the virus was added firstly to the cells, incubated for one hour (allowing the virus to penetrate the cells) then the products were added.

The obtained results revealed that Viu-Vet® and Fyto-immune Plus® were not effective against H5N1 HPAI virus but the Amphiflu-Ex® has complete virucidal effect at conc. 50µl/ml and the virus titers decreased by 2.72 to 2.8 logs in case of using conc. 20µl/ml and decreased by 1 to 1.27 logs by using conc. 10µl/ml for isolates No. 1 and 2 respectively (Table 3).

Table 1: Results for real time RT-PCR for matrix and H5 genes:

Sample no.	Results of M gene		Results of H5 gene	
	Result	CT value	Result	CT value
Neg. control	Neg.	No CT.	Neg.	No CT.
1	Pos.	25.35	Pos.	26.31
2	Pos.	20.87	Pos.	21.31

1: A/chicken/Egypt/SD95/2015 2: A/duck/Egypt/CAD90/2015 CT: Cycle Threshold

Table 2: Viral titers by RRT-PCR (Log 10) in CEF culture treated with 3 commercial products with fixed Conc. 50µl/ml then infected with viruses after 1 hr. of incubation.

	H5N1 AIV Titers (log 10) in CEF			
H5N1 Isolates	Viu-Vet® 50µl/ml	Fyto-immune Plus® 50µl/ml	Amphiflu-Ex® 50µl/ml	Virus only.
1	8.47	8.41	8.38	8.48
2	8.58	8.42	8.34	8.38

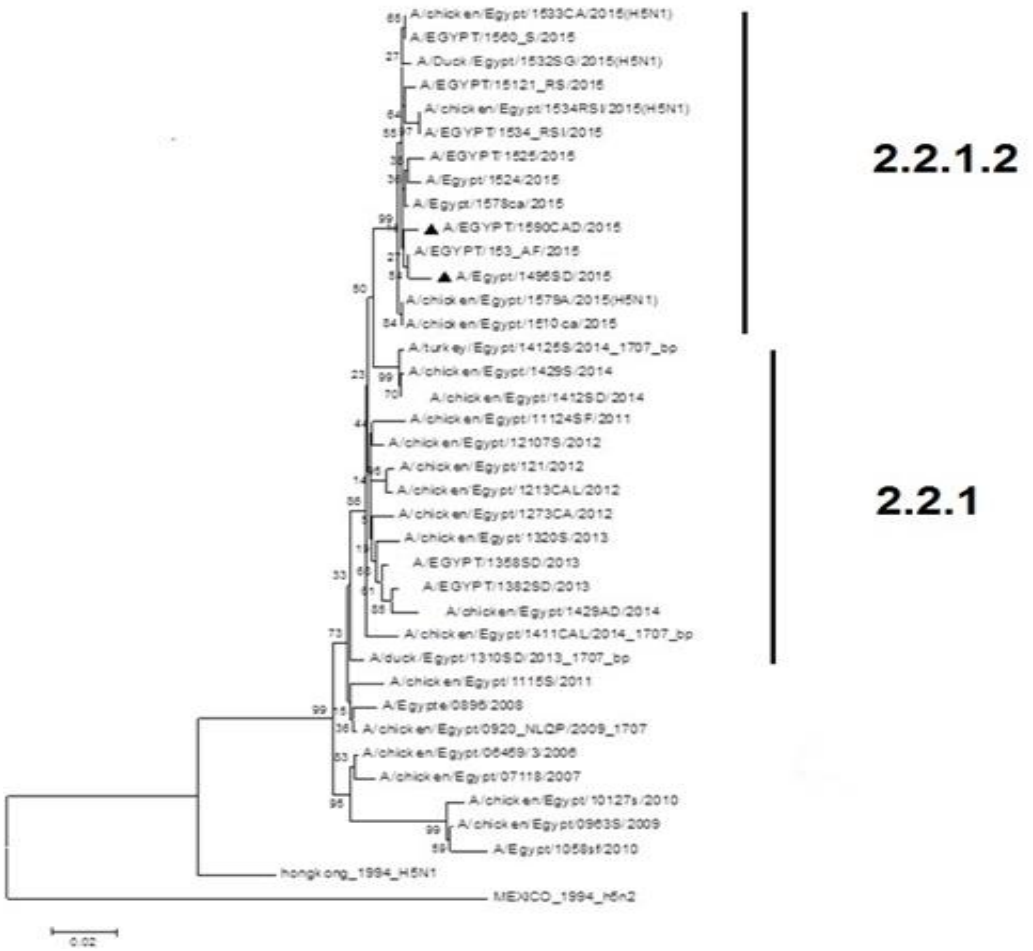
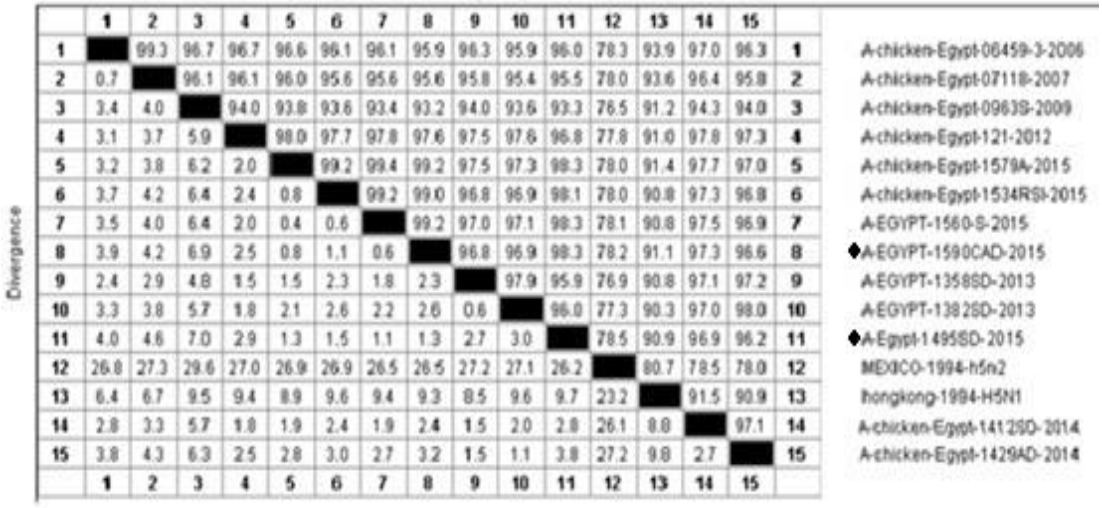


Fig.1. Phylogenetic analysis of the HA gene segment of A/chicken/Egypt/SD95/2015 and A/duck/Egypt/CAD90/2015 to other related H5N1 viruses in poultry, phylogenetic analysis of the HA genes of Egyptian viruses showing different genetic clades. Viruses in 2015 from Egypt are clustered in clade 2.2.1.2 and A/chicken/Egypt/SD95/2015, A/duck/Egypt/CAD90/2015 were located in clade 2.2.1.2 with recent viruses isolated in 2015 in poultry in Egypt. (Tree was generated using Megaligne module of the DNASTAR programe, the sequences obtained in this study was labelled with black triangles).



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1		99.3	96.7	96.7	96.6	96.1	96.1	95.9	96.3	95.9	96.0	78.3	93.9	97.0	96.3	1
2	0.7		96.1	96.1	96.0	95.6	95.6	95.6	95.8	95.4	95.5	78.0	93.6	96.4	95.8	2
3	3.4	4.0		94.0	93.8	93.6	93.4	93.2	94.0	93.6	93.3	76.5	91.2	94.3	94.0	3
4	3.1	3.7	5.9		98.0	97.7	97.8	97.6	97.5	97.6	96.8	77.8	91.0	97.8	97.3	4
5	3.2	3.8	6.2	2.0		99.2	99.4	99.2	97.5	97.3	98.3	78.0	91.4	97.7	97.0	5
6	3.7	4.2	6.4	2.4	0.8		99.2	99.0	96.8	96.9	98.1	78.0	90.8	97.3	96.8	6
7	3.5	4.0	6.4	2.0	0.4	0.6		99.2	97.0	97.1	98.3	78.1	90.8	97.5	96.9	7
8	3.9	4.2	6.9	2.5	0.8	1.1	0.6		96.8	96.9	98.3	78.2	91.1	97.3	96.6	8
9	2.4	2.9	4.8	1.5	1.5	2.3	1.8	2.3		97.9	95.9	76.9	90.8	97.1	97.2	9
10	3.3	3.8	5.7	1.8	2.1	2.6	2.2	2.6	0.6		96.0	77.3	90.3	97.0	98.0	10
11	4.0	4.6	7.0	2.9	1.3	1.5	1.1	1.3	2.7	3.0		78.5	90.9	96.9	96.2	11
12	26.8	27.3	29.6	27.0	26.9	26.9	26.5	26.5	27.2	27.1	26.2		80.7	78.5	78.0	12
13	6.4	6.7	9.5	9.4	8.9	9.6	9.4	9.3	8.5	9.6	9.7	23.2		91.5	90.9	13
14	2.8	3.3	5.7	1.8	1.9	2.4	1.9	2.4	1.5	2.0	2.8	26.1	8.8		97.1	14
15	3.8	4.3	6.3	2.5	2.8	3.0	2.7	3.2	1.5	1.1	3.8	27.2	9.8	2.7		15
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	

Fig.2: H5 nucleotide identities and divergence of the isolates in this study and Egyptian H5N1strains in comparison with A/chicken/Egypt/06459-3-NLQP/2006strain.

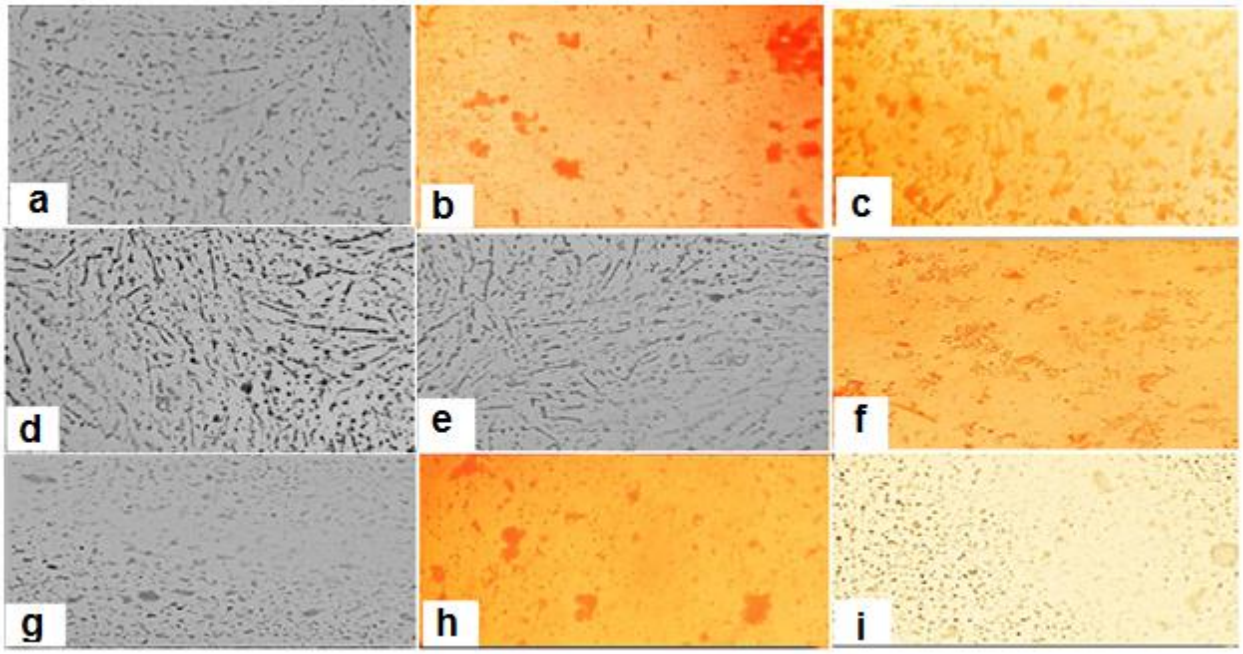


Fig.3. (3.a) CEF cells with no CPE in case of conc. 50 µl/ml from the three products (cells+ MNTCS) after 72 hrs., (3.b) CEF cells with CPE in case of conc. above 50 µl/ml from the three products after 72 hrs., (3.c) CEF cells treated with VIU-VET® then after 1h infected by H5N1 virus showing CPE 72hrs P.I, (3.d & e) CEF infected with H5N1 virus then treated after 1hr by AMPHI-FLU-EX® with Conc. 50µL/ML showing no CPE with no change in monolayer cells 72hrs P.I, (3.f) CEF cells treated with FYTO-IMMUNE PLUS ® then after 1h infected by H5N1 virus showing CPE 72 hrs P.I, (3.g) CEF cells treated with AMPHI-FLU-EX® then after 1h infected by H5N1 virus showing CPE 72hrs P.I, (3.h) CEF cells infected with H5N1 virus then after 1h treated with VIUVET® showing CPE 72hrs P.I. and (3. i) CEF cells infected with H5N1 virus then after 1h treated with FYTO-IMMUNE PLUS® showing CPE 72 hrs P.I.

Table 3: Viral titers by RRT-PCR (Log 10) in CEF cells infected with virus then treated with the 3 commercial products after 1 hr. of incubation.

H5N1 Isolates	H5N1 AIV Titers (log 10) in CEF					
	Viu-Vet® 50µl/ml	Fyto-immune Plus® 50µl/ml	Amphiflu-Ex® µl/ml			Virus only.
			10	20	50	
1	8.38	8.42	7.21	5.76	0	8.48
2	8.47	8.36	7.32	5.58	0	8.38

4. DISCUSSION

In the present study 2 suspected AIVs subtype H5N1 collected during 2015 from backyard chickens and ducks in El-Behira governorate and confirmed by real time RT-PCR.

HA gene of both isolates sequenced, compared with H5N1 strains of Egypt from 2006 to 2015 and vaccine strains (Hong Kong H5N1-1994 and Mexican H5N2-1994).

The obtained results revealed that both isolates belonged to variant clade 2.2.1.2 and the antigenic drift variance between both isolates and vaccine strains explains the vaccination failure and insufficient efficacy of the vaccines (Savill et al., 2006, Hafez et al., 2010 and El-Zoghby et al., 2012) especially when the mutations occur in the immunogenic epitopes. This drifts enabled the variant viruses to escape from the humeral immune response induced by H5 vaccines and many studies showed that vaccination of the poultry with antigenically related vaccines protected birds from the morbidity, mortality and virus excretion (Li et al., 2014 and Zeng et al., 2016).

Therefore, presence of new alternative and complementary strategies target different AIV serotypes /subtypes/ drift variants should be encouraged. The use of chemotherapeutic agents for control of AIV in poultry was concurrently studied just after discovering their

antimicrobial effects (Moses et al., 1948 and Tolba and Eskarous., 1959).

In poultry, antiviral and immune adjuvant effects of several plants and/or its derivatives have been investigated. In addition to its antiviral activity, these extracts often have anti-bacterial, anti-fungal, anti-inflammatory, anti-oxidant and/or analgesic properties which may provide alternative natural broad-spectrum way for control of AIV in poultry farms (Garozzo et al., 2009 and Sood et al., 2012).

The present in vitro study showed that Amphiflu-Ex®, Fyto-immune Plus® and Viu-Vet® had no effect on HPAI H5NI isolates in pre-exposure assay and the viruses showed CPE on the cells similar to positive control, but in post-exposure assay, Amphiflu-Ex® show marked reduction of CPE of the two isolates at concentration of 50µl/ml in comparison with Fyto-immune Plus® and Viu-Vet® and data obtained from the quantitative real time RT-PCR confirmed these results in special with Amphiflu-Ex® that showed complete virucidal effect at conc. 50µl/ml with complete inactivation of HPAI H5N1 isolates, even at low concentration (10 and 20µl/ml), Amphiflu-Ex® reduced the virus titers for both isolates.

Pre-exposure assay was used to identify whether the three products block the viral adsorption to cells. These data indicate that the three products can't directly interfere with viral envelope protein at the cell surface. Also they had no prophylactic effect on the HPAI virus and can't be used as preventive treatment before HPAI H5N1virus infection due to allow the virus binding and penetrating the cells. These results agreed with (Wen et al., 2011).

On the other hand, Amphiflu-Ex® had negative regulation of viral replication that can be attributed to apoptosis inhibition leading to limitation of cell to cell viral transmission, inhibition of viral propagation, multiplication on cells, decreasing their cytopathic and damage effect on the cell and this mechanism of action of antioxidants in viral diseases discussed previously by (Lin et al., 2016 and Liu et al., 2017) when used in post-exposure assay.

Other studies clarified the antiviral activity of taurine and BHT as (Uchio et al., 2010) who experimentally describe the in vitro antiviral effect of taurine derivative on adenoviral kerato-conjunctivitis confirmed by quantitative polymerase chain reaction methods.

Also in vitro synergistic antiviral effect between natural antioxidant (rosemary extract) and synthetic antioxidant (BHT) on replication of Herpes-1 virus (HSV-1) reported by (Mancini et al., 2009).

In conclusion, the Amphiflu-Ex® have in vitro antiviral activity as it has effect on the HPAI H5N1 virus titers in post-exposure assay and this antiviral effect may be due to its contents of natural antioxidant (Taurine) and synthetic antioxidant (BHT) through inhibits the generation of reactive oxygen species (ROS) and its bad effect of oxidative stress decreasing the replication of AIV subtype H5N1 and protection against mitochondrial damage induced by H5N1 infection.

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