The effect of the extracts of *Echinacea purpurea* and *Sambucus nigra* (black elderberry) on virus shedding in H9N2 avian influenza infected chickens

Karimi, S.[1]; Dadras, H.[2*] and Mohammadi, A.[3]

[1]Ph.D. Student, Department of Avian Medicine, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Department of Avian Medicine, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [3]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

*Correspondence: H. Dadras, Department of Avian Medicine, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: dadras@shirazu.ac.ir

Summary

Previous studies have shown antiviral effect of *Echinacea* and elderberry preparations against human influenza viruses *in vitro*. To investigate the *in vivo* antiviral effect of these herbs on avian H9N2 influenza virus, amantadine and two standardized commercial extracts of *Echinacea purpurea* (EF) and *Sambucus nigra* (SAM) were used in broiler chickens infected with H9N2 strain of the virus. EF, SAM and amantadine were added to drinking water of chickens in different groups for 7 days starting 8 h after intranasal inoculation of the challenged virus and in prophylaxis group of EF for 10 days starting 5 days before the challenge time. During post infection (PI) days, titer of the virus in tracheal mucosa and faeces were quantified using quantitative real-time PCR. The untreated challenged group showed the highest faecal and tracheal titer of the virus on day 3 PI. The virus was not detected in negative control group during the course of the experiment. Prophylaxis administration of EF considerably reduced the faecal titer of the virus in all days PI. Although overall titer of the virus in the tracheal samples was low, treatment with amantadine and SAM apparently reduced quantity of tracheal positive samples in comparison to untreated and EF-treated groups. This study indicates that using *Echinacea* and elderberry extracts in chicken can reduce H9N2 virus shedding from trachea and faeces.

Key words: *Echinacea purpurea*, *Sambucus nigra*, H9N2 avian influenza virus, Chicken, quantitative real-time PCR

Introduction

Since the 1990s, H9N2 avian influenza (AI) virus has become endemic in poultry in many countries across the whole of Asia. Unlike highly pathogenic (HP) AI viruses (H5 and H7) which are rarely isolated from wild birds, there are extremely high isolation rates for H9N2 (Alexander, 2007). The outbreak of avian influenza subtype H9N2 was first reported from Iran in 2002 and classified as LPAI (Vasfi Marandi and Bozorgmehri Fard, 2002). The infection has caused serious problems for commercial chicken flocks in Iran (Nili and Asasi, 2003). Some studies have shown novel reassortant H9N2 infection in chicken containing internal genes of human and chicken isolated H5N1 from China and HPAI H5N1 or H7N3 from Pakistan (Iqbal *et al*., 2009; Zhang *et al*., 2009). Genetic diversity of early and recent isolated H9N2 avian influenza viruses from Iran was reported (Bashashati *et al*., 2013). These novel viruses increase risk of problems by H9N2 to poultry flocks. In addition, human infection with avian H9N2 and similarity of internal genes in these human infecting H9N2 viruses with those of the H5N1 viruses, pose a serious public health threat (Peiris *et al*., 1999; Lin *et al*., 2000; Butt *et al*., 2005).

There are four synthetic drugs available for treatment and prophylaxis of human influenza; the M2 inhibitors (amantadine and rimantadine) and the neuraminidase inhibitors (Oseltamivir and Zanamivir). Incidence of viral resistance against both groups even for the most recent ones has raised concerns about control of an influenza pandemic by such medication (Kiso *et al*., 2004; Alexander *et al*., 2007; Townsend *et al*., 2008). However, according to international livestock regulations, due to likelihood of resistant mutants, available synthetic anti-influenza drugs are certified only for human use. Herbal remedies as an alternative because of multivalent functions are less likely to develop resistant viruses and in some cases affect both the virus and symptoms of influenza (Hudson, 2009).

Echinacea is popular in many parts of the world as an herbal medicine for colds and flu. Several clinical trials, although not definitively, have reported effects of *Echinacea* preparations in the prevention and treatment of upper respiratory tract infections, compared with placebo (Barnes *et al*., 2005). Immune modulation, direct viricidal and selective anti-bacterial properties of some *Echinacea*-derived preparations have also been reported (Hudson, 2012). In veterinary medicine, effect of *Echinacea* preparations has been investigated in chicken on number of lymphocytes, phagocytosis rate, NDV antibody titer and lesion scores caused by protozoan parasite *Coccidia*. In addition, the effect of *Echinacea* has been studied in pigs which were challenged with porcine reproductive and respiratory syndrome virus (PRRS virus) and in canine on severity of typical clinical

symptoms (Allen, 2003; Hermann *et al*., 2003; Reichling *et al*., 2003; Böhmer *et al*., 2009). However, there are relatively few reports of basic studies and controlled trial in animals in comparison to those described for human diseases (Hudson, 2012).

Several articles reported useful effect of aqueous elderberry extract SAM in treatment of viral influenza infections. *In vitro* studies have shown antioxidant, antiviral, anti-inflammatory, antibacterial and anti-proliferative effects of elderberry fruit. But *in vivo* studies are scanty (Vlachojannis *et al*., 2010).

Although *in vitro* studies show that *Echinacea purpurea* and *Sambucus nigra* extracts inhibit the ability of human influenza virus to infect host cells (Pleschka *et al*., 2009; Roschek *et al*., 2009; Sharma *et al*., 2009) there is no data related to *in vivo* effects of them in poultry industry.

To control influenza in a community, decreasing the "virus load" is a valuable assist (Hudson, 2009). Intranasal treatment of mice infected with influenza H1N1 with extract of *Echinacea purpurea* for 5 days did not alter lung viral titers, in comparison to untreated mice. But, this treatment modulated immune responses (Fusco *et al*., 2010). Bodinet *et al*. (2002) showed slight reduction in lung viral titer of H1N1 influenza virus after oral administration of a mixture of four herbal extracts including two *Echinacea* species in mice. There was an increase in survival rate and time and decrease of lung consolidation. No published work was found which investigated the effects of herbal extracts on virus shedding titer in the poultry.

In this study, two commercial herbal extracts of *Echinacea purpurea* and *Sambucus nigra* and a synthetic drug amantadine hydrochloride were used to investigate their effects on viral shedding in broiler chicken that had been infected with avian H9N2 influenza virus.

Materials and Methods

Extracts and drug

Echinaforce[®] (A. Vogel Bioforce AG, Roggwil, Switzerland, batch No.: 029462A) is a preparation derived by ethanol extraction of *Echinacea purpurea* aerial parts and root (95:5). Each 1 ml of the EF used in this study contained 83.5 mg/ml dry mass/vol (herb 79 mg + 4.5 mg root). Sambucol[®] (Pharma Care Europe Ltd., West Sussex, UK, batch No. 014) is an aqueous extract of ripe fruits of *Sambucus nigra* (black elderberry). 5 ml of the syrup contains 1.9 g standardized *Sambucus nigra L.* (black elderberry) extracts. Amantadine hydrochloride manufactured by Northeast General Pharmaceutical Factory (NE GPF), China, batch No. DY 04910014 and delivered by Amin Pharma-ceutical Co., Iran. It is a well-known synthetic anti influenza drug.

Birds

One hundred and sixty two commercial Cobb broiler chicks were purchased from a local hatchery farm. They were randomly divided into 6 equal groups with three replicates in each group. Each group was kept in an isolated room and each replicate was reared in a separate cage. Feed and water was available *ad. lib*.

Virus

Avian influenza, A/chicken/Iran/772/1998(H9N2), which was obtained from Razi Vaccine and Serum Research Institute (Iran) was passaged three times in 7 to 9 day-old embryonated chicken eggs. The embryo infective dose (EID_{50}) of infected allantoic fluid was calculated based on the Reed and Muench formula. A concentration of $10^{6.5}$ EID_{50}/100 µL of the virus was used to inoculate the individual chicks.

Experimental design

On days 1, 10 and 20, blood samples of five birds were collected. The samples were tested using hemagglutination inhibition test (HI) for antibodies to influenza H9 antigen (Burleson *et al*., 1992). At the age of 28 days, birds were considered to be susceptible for challenge.

In groups 1, 2, 3, 4, and 6 all birds were challenged intranasally (IN) with 100 µL diluted allantoic fluid containing the virus. The birds in group 5 were kept as unchallenged negative control.

The treatments were performed in different groups using either the extracts or amantadine via drinking water (Table 1). The chicks in groups 1, 2 and 3 received their medications for 7 days, beginning 8 h PI. Group 4 was considered as positive control and did not receive any treatment. Group 6 (as a prophylaxis group) was treated with Echinaforce[®] for 10 days starting 5 days before virus infection with 8 h no-treatment interval before and after challenge time.

Table 1: Plan of the experiment

Groups	Type of treatment	Dosage/ (Kg BW daily[a])	Duration
1	Echinaforce[®]	0.2 ml[b] (≡16.7 mg dry mass)	7 days PI
2	Amantadine HCL	4 mg	7 days PI
3	Sambucol[®]	0.4 ml (≡304 mg dry mass)	7 days PI
4	Positive control	-	-
5	Negative control	-	-
6	Echinaforce[®]	0.2 ml	10 days[c]

[a] Kilogram body weight daily. [b] The commercial medicines were added daily to drinking water of chickens. All birds in groups 1, 2, 3, 4 and 6 were challenged with H9N2 avian influenza virus intranasally ($10^{6.5}$ EID 50/100 µL/bird). Control groups 5 and 6 did not receive any treatment. [c] Starting 5 days before challenge

Sample collection

Two hours prior to challenge and on days 3, 6, 9, 12, 15, 18, 21, and 24 PI, three chicks from each group (one bird of each replicate) were randomly selected and samples of sera, trachea and faeces were separately collected. First blood samples were taken and then the birds were euthanized and samples of trachea and faeces (contents of rectum) were collected for virus detection and titration. Sera were separated at the same days and were kept at -20°C until used in HI test. The samples of trachea and faeces were kept on ice from time of collection and stored at -70°C until used.

Serology

Hemagglutination inhibition (HI) test was used to test presence of antibodies to the challenge virus antigen in serum samples. Diluted H9N2 influenza virus antigen equivalent to four hemagglutinating units (HA) was used to evaluate HI activity of two-fold serially diluted test serums in 96-well microtiter plate (Burleson *et al.*, 1992).

RNA extraction

Mucosal surface of trachea was scratched and total RNA was extracted using the RNX™ (-plus) Kit (CinnaGen Inc., Iran), according to manufacturer's protocol based on phenol-thiocyonate method. Faeces first were suspended in sterile distilled water (1/10 w/v) and centrifuged (1500 g for 10 min at 4°C). RNA was extracted from 200 μL of supernatants using the same protocol. Finally, RNA pellet was suspended in 50 μL sterile distilled water and stored at -70°C until used.

Reverse transcription

The cDNA was synthesized using AccuPowder® RT PreMix kit (BioNeer Corporation, South Korea) according to the manufacturer's protocol. In brief, 5 μl of solved total RNA, 10 pmol random hexamer and 10 pmol of primer that was specific to highly conserved region of matrix protein gene of influenza A virus were used for cDNA preparation (Table 2). The cDNA synthesis was performed at 42°C for 60 min, heated to

95°C for 5 min, cooled to 4°C then stored at -20°C until used.

Real-Time PCR

Real-time PCR was performed as a screening test to detect the presence of influenza virus genome in the samples. Total RNA of all positive samples was normalized after spectrophotometry. The cDNA was synthesized and tested by quantitative real-time PCR (qRT/PCR) to quantify titer of the virus.

The assays were performed on a 48-well microtiter plate of BIO-RAD Mini Opticon TM System. The reaction mixture contained 5 μl of target cDNA, 10 pmol of each primer and TaqMan probe, 10 μl of commercial qPCR Probes Master (Jena Bioscience, Lot 110.798) and 2 μl of PCR grade water (Jena Bioscience, Lot No. 110.745) in a final volume of 20 μl. The primers amplified a 104 bp fragment in the M1 gene of influenza A. The probe annealed to the part of the sequence amplified by two primers (Table 2). All primers and TaqMan probe used in this study were described previously (Ward *et al.*, 2004). The program included 40 two-step cycles (15 sec at 95°C for denaturation of DNA, 1 min at 60°C for primer annealing and extension). The standard calibration curve was obtained by stepwise 10 fold dilution of plasmid standard with known copy number.

Standard preparation

Recombinant plasmid previously cloned in *E. coli* strain XL1-blue was purified using BIONEER Accuprep Plasmid Extraction kit (Mosleh *et al.*, 2009). The concentration of the plasmid DNA was determined with Eppendorf Biophotometer spectrophotometer UV/VIS.

Results

HI test results

As shown in Table 3, the HI antibody titer was increased at day 9 PI in all infected groups and continued to rise until day 24 PI. The HI titer of the negative

Table 2: RT-PCR and real-time PCR primer and probe sequences

Specificity	Primer/Probe	Sequence
cDNA synthesis	Forward	´TCT AAC CGA GGT CGA AAC GTA 3´5
Real-time PCR	Forward	5´AAG ACC AAT CCT GTC ACC TCT GA 3´
Real-time PCR	Reverse	´CAA AGC GTC TAC GCT GCA GTC C 3´5
Real-time PCR	Probe	´FAM[a] TTT GTG TTC ACG CTC ACC GT TAMRA[b] 3´5

[a] FAM, 6-carboxy fluorescein [b]TAMRA, 6-carboxy tetramethylrhodamine

Table 3: Mean HI antibody titer of the chicken sera against H9 antigen after challenge with H9N2 virus in different treatment groups

	Treatment drug	Days post inoculation (DPI)								
		0	3	6	9	12	15	18	21	24
Groups	Echinaforce®	2.3±0.58ᵃ	2.3±0.58ᵃ	2±0.00ᵃ	4.3±0.58ᵇ	4.7±0.58ᵇ	4±0.00ᵃ	4.3±1.15ᵇ	4.3±0.58ᵇ	4.7±0.58ᵇ
	Amantadine	2.3±0.58ᵃ	2±0.00ᵃ	2.7±0.58ᵃ	3±1ᵃ	3.7±1.15ᵃ	4.3±1.53ᵃ	4.7±1.15ᵇ	5.3±1.15ᵇ	4.3±0.58ᵇ
	Sambucol®	2.3±0.58ᵃ	2.3±0.58ᵃ	2.3±0.58ᵃ	3.3±0.58ᵃ	4.3±1.53ᵃ	5±1ᵇ	4±1ᵃ	5±0.00ᵇ	3.7±0.58ᶜ
	Positive control	2±1ᵃ	2.3±0.58ᵃ	2.7±0.58ᵃ	4±0.00ᵇ	4.3±0.58ᵃ	4±1ᵃ	4.7±0.58ᵇ	5±1ᵇ	5±1ᵇ
	Negative control	2.3±0.58ᵃ	2.7±0.58ᵃ	2.3±0.58ᵃ	2±0.00ᵃ	2.3±0.58ᵃ	2±0.00ᵃ	2±0.00ᵃ	2.3±0.58ᵃ	2±0.00ᵃ
	Echinaforce® prophylaxis	2±1ᵃ	2.3±0.58ᵃ	2±0.00ᵃ	4±0.00ᵇ	4.3±0.58ᵃ	5±1ᵇ	4.7±1.15ᵃ	5.3±0.58ᵇ	6±1ᵇ

Different superscript letters (ᵃ, ᵇ and ᶜ) in each row or column indicate significant differences (P<0.05)

control group remained unchanged throughout the experiment. Mean HI titer of all treated birds along days 9-24 was significantly higher than negative control group. The treated groups did not show a significant difference with positive control group in HI antibody titer.

Virus titration

To study the effect of different treatments on viral replication, the mean copies of the virus genome per 1 μg of total RNA in the trachea and faeces were measured using qRT-PCR in treated and untreated control groups on days 3, 6, 9, 12 post-infection. Figures 1 and 2 show mean copies of the virus genome for the different treatment groups at each time point examined. The virus was detected in both tracheal and faecal samples of the infected birds, in variable manner. No virus was detected in any samples of the uninfected control group on tested days.
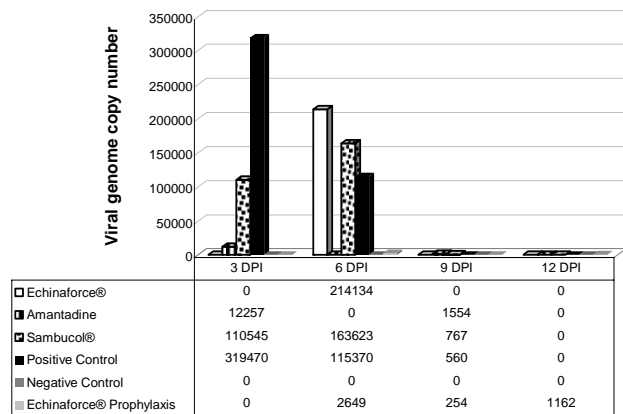

	3 DPI	6 DPI	9 DPI	12 DPI
☐ Echinaforce®	0	214134	0	0
▣ Amantadine	12257	0	1554	0
▨ Sambucol®	110545	163623	767	0
■ Positive Control	319470	115370	560	0
▨ Negative Control	0	0	0	0
▨ Echinaforce® Prophylaxis	0	2649	254	1162

Fig. 1: Mean titer of viral genome copy number of the challenged virus per 1 μg of total RNA for three faecal samples in different groups and different days post infection (DPI) using quantitative real-time PCR (qRT/PCR)
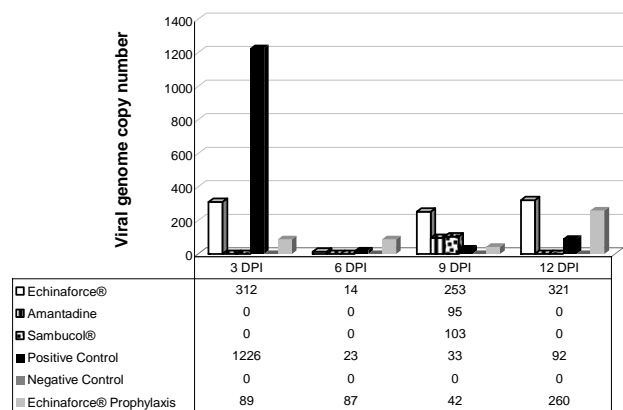

	3 DPI	6 DPI	9 DPI	12 DPI
☐ Echinaforce®	312	14	253	321
▣ Amantadine	0	0	95	0
▨ Sambucol®	0	0	103	0
■ Positive Control	1226	23	33	92
▨ Negative Control	0	0	0	0
▨ Echinaforce® Prophylaxis	89	87	42	260

Fig. 2: Mean titer of viral genome copy number of the challenge virus per 1 μg of total RNA for three tracheal samples in different groups and different days post infection (DPI) using quantitative real-time PCR (qRT/PCR)

Faeces

The viral load was much higher in faeces in comparison to trachea. The highest rate of shedding was

observed in positive control group on day 3 PI that was considerably decreased in the following days. Among the treated groups, the birds in group 6 (prophylaxis) showed much less viral titers on all days PI. On day 3 PI, EF-treated birds in groups 1 and 6 had no titer. But high titer of the virus was detected for some birds from positive control group, amantadine-treated and SAM-treated groups. Low titer was detected on day 9 PI in groups 2, 3 and 4 which finally disappeared on day 12 PI. Only one chick in group 6 still showed low titer on day 12 PI.

Trachea

The results are shown in Fig. 2. Generally, very low titers of the virus were detected in trachea of all infected groups. The highest viral titer was observed in the positive control group on day 3 PI, which decreased steadily in the following days. In addition, groups 2 and 3 had lower rate of shedding in comparison to groups 1, 4 and 6 in days 3 PI to 12 PI (Table 4). Virus detection was more persistent in echinaforce treated and positive control groups and it was continued up to 12 days PI. The virus was not detected in amantadine and sambucol treated groups except day 9 PI.

Table 4: Comparison of viral detection in the trachea of chickens in different groups during days post inoculation

No.	Groups	Days post infection			
		3	6	9	12
1	Echinaforce®	1/2[*]	1/3	3/3	1/3
2	Amantadine	0/3	0/3	1/3	0/3
3	Sambucol®	0/3	0/3	2/3	0/3
4	Positive control	2/3	1/3	1/3	1/3
5	Negative control	0/3	0/3	0/3	0/3
6	Echinaforce® prophylaxis	1/3	2/3	2/3	1/3

[*] Number of positive samples/total tracheal samples tested

Discussion

Caffeic acid derivatives, alkyl amides, and polysaccharides in *Echinacea* preparations and flavonoids in elderberry extracts are reported as active veridical, immunoenhancer and immunomodulator components. Content of these biomarkers are sub-stantially different in a variety of preparations, especially for *Echinacea*. This happens mainly because of the plant parts used for extraction (aerial and root) and method of extraction (usually ethanol or aqueous) in case of *Echinacea purpurea* (Roschek *et al*., 2009; Hudson, 2012). It is expected that interactions between *Echinacea purpurea* and cells *in vivo* will likely be affected by the nature and location of the cell type and composition of the extract (Hudson, 2012). Nevertheless, most studies have been done *in vitro* and there are few reports of *in vivo* researches. In order to avoid uncertainty in the identity of principal bioactive ingredients, to reflect some partial comparison and repeatability of the study, the standardized commercial extracts of *Echinacea purpurea* and *Sambucus nigra* were used in this *in vivo* study.

Respiratory and gastrointestinal (GI) tracts of chickens are common site of infection for LPAI viruses (Swayne and Halvorson, 2008). Therefore, tracheal and

faecal samples were collected to evaluate shedding of the challenged virus from its main replication organs.

qRT-PCR was used as highly specific and sensitive test for detection of positive samples and quantification of the viral titer. There are two studies with the same virus, evaluating PI viral load in different organs including samples of trachea and faeces of challenged broiler chicken using qRT-PCR (Mosleh *et al.*, 2009; Tavakkoli *et al.*, 2010). Similar to the findings of the positive control group in the current study, both articles reported few positive tracheal samples (3 out of 10 in day 3 and day 6 or 7 PI). Likewise, the faecal titers were much higher than tracheal titers.

Concerning titer of the virus in faeces, the major decrease was observed in group 6. Every bird in all tested days PI shed low titer of the virus. In group 6, EF was administered for the prophylaxis of influenza infection for 10 days starting 5 days before challenge. Bodinet *et al.* (2002) reported similar result in mice infected with human influenza H1N1. They used a mixture of herbs including *Echinacea*.

Meanwhile, all faecal samples of EF-treated groups (groups 1 and 6) obtained on day 3 PI lacked the viral RNA. It seems EF apparently interferes with an early stage in virus replication in GI tract. But frequency of tracheal positive samples in both EF-treated groups unlike amantadine-treated group and SAM-treated group was similar to that was observed in untreated positive control group.

One of the few analogous investigations showed that PI administration of *Echinacea purpurea* extract alters the clinical course of influenza infection in mice through modulation of cytokines and not direct antiviral activity. No reduction in lung viral titers was observed administrating the extract via drinking water for 5 days starting on the day of infection, evaluated with cell culture method (Fusco *et al.*, 2010). However, the extract used in that study contains little to no alkyl amides, cichoric acid and cynarin but significant amount of polysaccharides. In contrast, cichoric acid and alkylamides are main components of EF which is essentially free of polysaccharides (Sharma *et al.*, 2009). So, it is likely the results would be influenced by different components of *Echinacea* extracts used in that study and in our study. In addition, subtype of influenza virus, infected host, tested tissue and method of detection were different.

Shedding of the virus from faeces of amantadine-treated chickens was considerable just in day 3 PI. It was decreased to zero on day 6 PI and then low titer reappeared in day 9 PI, just after ending the treatment on day 7 PI. Also, minimum rate of shedding (5%) was observed for trachea of chickens treated with amantadine. These results confirm that administration of amantadine had major effect to reduce both faecal and tracheal shedding of the virus. However, the challenge virus used in this investigation was isolated from chicken in 1998, Iran. Therefore, because of rapid prevalence of resistant influenza virus variants after administration of amantadine in human, it is illegal for use in livestock and

its eligibility and effectiveness against currently circulating H9N2 avian influenza in poultry could not be concluded.

There was no remarkable overall difference between faecal titers of SAM-treated bird in most days PI in comparison to untreated control group; except on day 3 PI when the titer in control group was much higher. As shown in Table 4 the frequency of positive tracheal samples in SAM-treated group and amantadine-treated birds was lower than positive control and both EF-treated groups. So, we hypothesized that a mixture of *Echinacea purpurea* (ethanol extract) and fruits of *Sumbucus nigra* (aqueous extract) could potentially control the influenza virus shedding from both respiratory and digestive tract, especially by prophylaxis administration.

Mean HI antibody titer of the chicken sera against H9 antigen after challenge with H9N2 virus in different treatment groups was increased but there was no serological evidence of influenza H9 infection in uninfected control group throughout the experiment. This shows that despite low virus detection, there were enough viral antigens to stimulate antibody production and from this point of view there was no difference between treated and untreated (positive control) groups.

Böhmer *et al.* (2009) and Gurbuz *et al.* (2010) reported that *Echinacea* extract could alter humoral immune response to enhance HI antibody titers in Newcastle disease virus vaccinated chickens. The results of this study also showed higher mean HI antibody titer in EF treated groups instead of considerable lower faecal titer of the virus as compared with untreated positive control.

The EF possesses acceptable potential to decrease faecal shedding of the H9N2 avian influenza virus when administered prior to infection. However, it seems post-infection administration of EF revealed mild effect on the shedding. SAM caused reduction in frequency of positive trachea in comparison to positive control and EF-treated chickens. We propose a further study to evaluate the synergistic effect of a mixture of *Echinacea* and elderberry extract on viral shedding. Despite considerable effect of amantadine on the viral titer, due to rapid development of resistant mutants, it is banned for use in livestock, and so is not suggested. This study confirms the benefits of using *Echinacea* and elderberry extracts in chicken to reduce load of the virus as valuable aid to control avian influenza in poultry fields.

Acknowledgement

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