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The effect of *Echinacea purpurea* and *Sambucus nigra* L. on H9N2 avian influenza virus in infected chicken embryo

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ABSTRACT

Early studies have revealed some *Echinacea* and elderberry preparations possess significant antiviral effect against human influenza viruses subtypes H1, H2, H3, highly pathogenic H5 and H7, and human influenza B in cell culture assay. We investigated the anti-viral effect of ethanol extract of *Echinacea purpurea* (Echinaforce®) and aqueous extract of *Sambucus nigra* L. (black elderberry; Sambucol®) in comparison to amantadine on a titer of H9N2 avian influenza virus in the allantoic fluid of infected chicken embryo using hemagglutination (HA) and quantitative Real Time/Polymerase Chain Reaction (qRT/PCR) tests. The study was performed in two steps. First, the neutralization index (NI) based on a standard embryo infective dose 50 (EID_{50}) method was used to evaluate the efficacy of the extracts/amantadine. Serial dilution of the virus was treated with a steady amount of the diluted extracts prior to inoculation of the embryonated eggs. The inoculated eggs then were incubated for 48 hours at 37 °C. In the next step, an equivalent of 500 EID_{50} of the virus was treated with a serial dilution of the extracts before inoculation. The titer of the virus in the allantoic fluid was evaluated and quantified using HA and qRT/PCR tests. The results confirmed the significant virucidal effects of both extracts against the virus. There was also a direct correlation between the results of the HA and qRT/PCR tests in the second step. In conclusion, the results confirm the considerable virucidal effect of both extracts against the H9N2 avian influenza virus.

Key words: *Echinacea purpurea* (purple coneflower), black elderberry, *Sambucus nigra*, H9N2 avian influenza, quantitative real-time PCR

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Introduction

Avian influenza caused by H9N2 has been endemic recently in poultry across the whole of Asia (ALEXANDER, 2007). The virus can infect humans and, in some cases, even show reassortment with the highly pathogenic influenzas H5 and H7 (BUTT et al., 2005; DONG et al., 2011; IQBAL et al., 2009), which shows it is a potentially serious public health threat. A few synthetic medicines are available against human influenza; however, no antiviral treatment is available for livestock. In addition, due to the rapid and high rate of antigenic shift and drift in influenza viruses, as well as the short rearing time of commercial broiler chickens, available vaccines are determined to be only partially effective and practical.

Alternative herbal remedies, due to their multivalent functions, are less likely to encourage resistant viruses and, in some cases, affect both the virus and symptoms of influenza (HUDSON, 2009). According to international livestock regulations, due to the likelihood of resistant mutants, the available synthetic anti-influenza drugs are certified only for human use. So, herbal remedies would be a choice for the prevention and treatment of viral infections in domesticated animals raised to produce food for human. Today, the most recognized herbal remedy for the prevention and treatment of colds and flu is probably *Echinacea*. Another remedy, *Sambucus nigra*, is a member of the Caprifoliaceae family (berries), primarily used as an antiviral agent for colds, influenza and Herpes virus infections (ROXAS and JURENKA, 2007).

A variety of antiviral, antibacterial, antifungal, antioxidant and immune-modulatory activities have been described in studies *in vitro* and *in vivo* for *Echinacea* preparations (HUDSON, 2012). Marker compounds such as caffeic acid derivatives, alkylamides, and polysaccharides are known in *Echinacea* preparations, and have been claimed to contribute to these activities (HUDSON et al., 2005; VIMALANATHAN et al., 2005; WOELKART and BAUER, 2007).

Concerning elderberry extract, the antioxidant, antiviral, anti-inflammatory and antibacterial effects of this extract *in vitro* and *in vivo* have been reported (KRAWITZ et al., 2011; VLACHOJANNIS et al., 2010). Elderberries are identified as being rich in phenolic compounds, such as phenolic acids, flavonoids, catechins, and proanthocyanidins (DE PASCUAL-TERESA et al., 2000; HAKKINEN et al., 1999). In addition, some specific elderberry flavonoids have been shown to significantly block influenza virions *in vitro* (ROSCHEK et al., 2009). *Sambucus nigra* contains some toxic compounds such as lecitins and cyanoglycosides (e.g. sambunigrin), found especially in unripe berries, leaves or stems (VLACHOJANNIS, et al., 2010). No similar toxic compounds have been reported in *Echinacea*.

Previous studies have shown that some *Echinacea* and elderberry preparations have a significant antiviral effect against human influenza A subtype H1, H2, H3,

highly pathogenic H5 and H7, and human influenza B in cell culture assay (BINNS et al., 2002; HUDSON et al., 2005; KRAWITZ et al., 2011; PLESCHKA et al., 2009; ROSCHEK et al., 2009; SHARMA et al., 2009; VIMALANATHAN et al., 2005; ZAKAY-RONES et al., 1995)

To our knowledge, no reports have been published on the antiviral effect of *Echinacea* and elderberry preparations against the H9N2 avian influenza virus. The current study was therefore undertaken to determine the direct virucidal effects of two standardized herbal extracts, *Echinacea purpurea* (Echinaforce®[EF]) and *Sambucus nigra* (Sambucol®[SAM]) against H9N2 avian influenza virus *in vitro* when treated prior to inoculation in embryonated chicken eggs. Furthermore, a highly sensitive and specific qRT/PCR was used to quantify a titer of the virus in allantoic fluid.

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 EF used in this study contained 83.5 mg/mL dry mass/vol (herb 79 mg+4.5 mg root). The original contains standardized *Sambucus nigra L.* (black elderberry) extract. The original Sambucol® (SAM) syrup (PharmaCare Europe Ltd., West Sussex, UK, batch no. 014) contains 1.9 grams of standardized liquid *Sambucus nigra L.* (black elderberry) extracts (2:1) per 5 mL of syrup. Amantadine hydrochloride (Manufactured by Northeast General Pharmaceutical Factory (NE GPF), P.R.China, batch no. DY 04910014 and delivered by Amin Pharmaceutical Company, Iran) is a well-known synthetic anti influenza drug.

Chicken embryos. Commercial broiler fertile eggs were incubated at 37 °C for eight days and then used for inoculation.

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 eight-day old embryonated chicken eggs.

Neutralizing index (NI). A neutralizing index (NI) of the virus inactivation was used to assess the efficacy of each extract/drug. This method was previously described for evaluation of antiviral detergents and chemicals against avian influenza virus and is a modified classical avian serologic virus-neutralization test (ALPHIN et al., 2009; LOMBARDI et al., 2008). The NI of virus inactivation (equation [1]) is calculated by subtracting the log10 titer of recovered virus from the inoculated eggs with extracts/drug treated virus (t_a), from the titer of recovered virus from the inoculated eggs with untreated control virus (t_{pc}). The titers of virus obtained using Embryo Infective Dose $_{50}$ per one milliliter (EID_{50}/mL) of allantoic fluid via the method of REED and MUENCH (1938). Inactivation of the virus was considered effective when NI ≥2.8 and the positive control titer was ≥4.0 (equation [1]):

$NI = t_{pc}\text{-}t_a$ [1]

Experimental design. The study was performed in three steps. Initially (step A), the toxicity of the extracts and drug was assessed by the inoculation of serially diluted extracts and amantadine into the allantoic fluid of 8-day old embryonated chicken eggs, which were incubated at 37 °C. At 72 hours post-inoculation, the size of the embryos and any possible lesion of each dilution were compared with the control to determine any toxic effect on their growth.

In step B, a stock of allantoic fluid containing a high dose of the virus was used to prepare 4 ten-fold serial dilutions with 0.6mL sterile normal saline (NS) in 1.5mL sterile micro-tubes. Then, 0.6 mL of the diluted extracts or amantadine (maximum non-toxic concentration) was added to each tube of serially diluted virus. The final concentrations of the extracts and amantadine in each tube were as follows: EF, 20.9 mg/mL dry mass/vol; SAM, 95 mg/mL black elderberry extract/vol and amantadine, 12.5 mg/mL. The serial dilution of the virus with or without treatment was incubated at 22 °C for 60 min to allow the treatments to interact with the virus (SHARMA et al., 2009). This was followed by injection of 0.2 mL of the treated mixture and 0.1 mL of the untreated virus into the allantoic cavity of 8-day old embryos (each dilution to 5 embryos). The allantoic fluids were collected after 48 hours incubation at 37 °C and were immediately used for an HA test in a 96-well tray to obtain EID_{50} and NI for the extracts and amantadine as previously described in this article.

Step C was implemented to achieve an inhibitory dose of the extracts, as follows: The extracts (1:4 dilution) of EF, corresponding to a concentration of 20.9 mg/mL dry mass/vol (herb 19.8 mg+1.1 mg root); 1:4 dilution of SAM, (corresponding to a concentration of 95 mg/mL black elderberry extract/vol) were serially diluted two-fold across 1.5mL sterile microtubes in 0.6 mL of sterile NS. The virus (500 EID_{50}/0.1 mL in total volume of 0.6 mL) was added to each tube. The mixtures were then incubated at 22 °C for 60 min. As control, two tubes containing 0.6 mL of the virus (500 EID_{50}/0.1 mL) were prepared. One was kept on ice until inoculation (control 1) and the other incubated at 22 °C along with the mixtures (control 2). The contents of each tube were injected into the allantoic cavities of five embryonated eggs, 0.2 mL/egg of the mixtures and 0.1 mL/egg of untreated virus. After incubation for 48 hours at 37 °C, an HA test was performed and 1mL of allantoic fluid collected and stored at -70 °C for quantification of the virus titers. In step C, 48 hours post-inoculation, the eggs were transferred to a refrigerator and kept at 4 °C for 24 hours. Then the allantoic fluids were collected and stored at -70 °C until used.

RNA extraction. Total RNA extracted from 200µL of allantoic fluid using the RNX™ (-plus) Kit (CinnaGen Inc, Iran), according to the manufacturer's protocol, was based on the phenol-thiocyonate method. After the first centrifugation, an equal volume of

600µL supernatant was transferred for extraction in all cases. Finally, an RNA pellet was dissolved in 50µL of 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, 5µL of isolated total RNA, 10pmol random hexamer and 10pmol of primer specific to a highly conserved region of matrix protein gene of influenza A virus were used for cDNA preparation (Table 1). 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. The first, real-time PCR was performed as a screening test to detect the presence of influenza virus genome in the allantoic fluid samples in step C. Then, quantitative real-time PCR was used to quantify the titer of the virus per 1µL of allantoic fluid in positive samples for the virus genome. The assays were performed on a 48-well microtitre plate of the BIO-RAD MiniOpticonTM System. The reaction mixture contained 5µL of target cDNA, 10pmol of each primer, 6 pmol TaqMan probe, 6mM of MgCl2, 0.2mM of dNTP mix, 1 U Taq DNA polymerase, 2 µL of x10 PCR buffer and 7.4 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 (WARD et al., 2004). All primers and the TaqMan probe used in this study were previously described (WARD et al., 2004) (Table 1). The program included 40 two-step cycles (15 sec at 95 ºC for denaturation of DNA, 1 min at 60 ºC for primer annealing and extension). The standard calibration curve was obtained by stepwise 10-fold dilution of plasmid standard with a known copy number.

Table 1. RT-PCR and real time-PCR primer and probe sequences used to detect and quantify the H9N2 avian influenza genome in allantoic fluid.

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 TTT GTG TTC ACG CTC ACC GT TAMRA 3´5

FAM = 6-carboxy fluorescein; TAMRA = 6-carboxy tetramethylrhodamine

Standard preparation. The standard recombinant plasmid containing the quantitative real-time PCR amplicon was previously cloned in *E. coli* strain XL1-blue with MOSLEH et al. (2009) in the Laboratory of Virology, School of Veterinary Medicine; Shiraz University. It was purified using BIONEER Accuprep Plasmid Extraction Kit. The concentration of the plasmid DNA was determined using Eppendorf Biophotometer Spectrophometer UV/VIS.

Statistics. One-way ANOVA was carried out using SPSS 19.0 software to compare quantitative real-time PCR titers of the virus in step C. P values<0.05 were considered statistically significant.

Results

Neutralizing index (NI). The antiviral efficacy of the extracts and amantadine were initially determined using NI method. A summary of the results is shown in Table 2. In this experiment, the control titer (t_{pc}) was 8.7 EID_{50}/mL, much more than the minimum ≥4.0 required. The results showed that both extracts were considerably effective against the virus with NI>7.7 at the same concentration (maximum non-toxic concentration, equal to 1:4 dilution of the original stocks of both standard extracts). It was equivalent to 20.9 mg/mL dry mass/vol for EF and 95 mg liquid extract (2:1) for SAM in virus-extract mixtures. But amantadine was considered to have no direct antiviral effect against the virus with NI = 2.2, which is less than the minimum (NI≥2.8) required to be considered effective.

Table 2. Results of hemagglutination testing on the allantoic fluid of embryonated chicken eggs inoculated with H9N2 avian influenza virus

	Virus dilutions									
Treatments	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	EID_{50}/mL	NI
Echinaforce®	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	(t_a)>1	>7.7
Sambucol®	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	(t_a)>1	>7.7
Amantadine	5/5	5/5	5/5	4/5	3/5	2/5	1/5	0/5	(t_a) 6.5	2.2
Untreated control	5/5	5/5	5/5	5/5	5/5	5/5	4/5	2/5	(t_{pc}) 8.7	-

NI = Neutralizing Index (NI = t_{pc}-t_a), t_a = $\log_{10}$ of EID_{50} of extracts/drug treated virus; t_{pc} = $\log_{10}$ of EID_{50} of untreated control virus

Viral quantification. The results of HA and qRT/PCR are shown in Table 3. The results of the HA test were exactly identical to the high/low titers of the viral genome detected and quantified with qRT/PCR. The avian influenza H9N2 virus with the dose equivalent to 500 EID_{50}/0.1 mL was considerably neutralized with an equal volume of the extracts up to 1:64 dilution of EF and SAM original stocks (it was equivalent to 1.3 mg/mL dry mass/vol for EF and 5.9 mg liquid extract [2:1] for SAM in virus-extract mixtures) (Fig. 1). The dilution ≥1:128 of the extracts had no inhibitory effect against the virus, determined with positive HA results and high titers of the viral genome, similar to the untreated controls. Both untreated positive controls showed the same HA results and similar high titers of the virus. The statistical significant difference was evident for low titers (up to 1:64 dilution for both extracts) and high titers (dilution ≥1:128 of both extracts). But no statistical

difference was obtained between the high titers and the control. In addition, the low/high titers of EF and SAM showed no difference ($P<0.05$) (Table 3).

Table 3. Results of hemagglutination and qRT-PCR tests on the allantoic fluid of embryonated chicken eggs inoculated with 500 EID_{50} of H9N2 avian influenza virus

Dilution of the extracts	Treatment			
	Echinaforce®		Sambucol®	
	HA	Titer	HA	Titer
2^{-2}	0/5	166[a]	0/5	93[a]
2^{-3}	0/5	116[a]	0/5	211[a]
2^{-4}	0/5	1161[a]	0/5	64[a]
2^{-5}	0/5	56[a]	0/5	45[a]
2^{-6}	0/5	226[a]	0/5	121[a]
2^{-7}	3/5	3.60×10^{6b}	4/5	3.05×10^{7b}
2^{-8}	5/5	5.79×10^{6b}	5/5	7.16×10^{6b}
2^{-9}	5/5	9.73×10^{6b}	5/5	$9.44\times10^{6}_{b}$
2^{-10}	5/5	9.27×10^{6b}	5/5	4.79×10^{6b}
2^{-11}	5/5	1.03×10^{7b}	5/5	6.55×10^{6b}
Control 1	5/5	9.57×10^{6b}		
Control 2	5/5	8.96×10^{6b}		

HA = Number of positives out of 5 samples using a hemagglutination test. Titer = Mean viral genome copy number of two samples per 1 microliter of allantoic fluids. 500 EID_{50} of H9N2 avian influenza virus was treated with two-fold dilutions of Echinaforce® or Sambucol® prior to inoculation into the allantoic cavity of embryonated chicken eggs. Results are presented as the average of two samples per group and dilution of extracts. The same superscript alphabets indicate that differences between means were not statistically significant in the ANOVA test ($P<0.05$). Control 1 = Untreated control virus incubated at 22 ºC for 60 min prior to inoculation (Same as treated inoculums). Control 2 = Untreated control virus incubated on ice prior to inoculation.

Discussion

The avian influenza H9N2 virus used in this study was significantly neutralized by EF and SAM when treated with the extracts before inoculation into chicken embryos, determined with the NI test ($NI>7.7$). $NI\geq2.8$ was considered as positive result, which confirms the efficacy of the extracts or drug. Unlike the extracts, amantadine showed no antiviral effect against the virus, with NI = 2.2. The NI test was previously used to evaluate the inactivation of avian influenza virus using detergents and chemicals (ALPHIN et al., 2009; LOMBARDI et al., 2008).

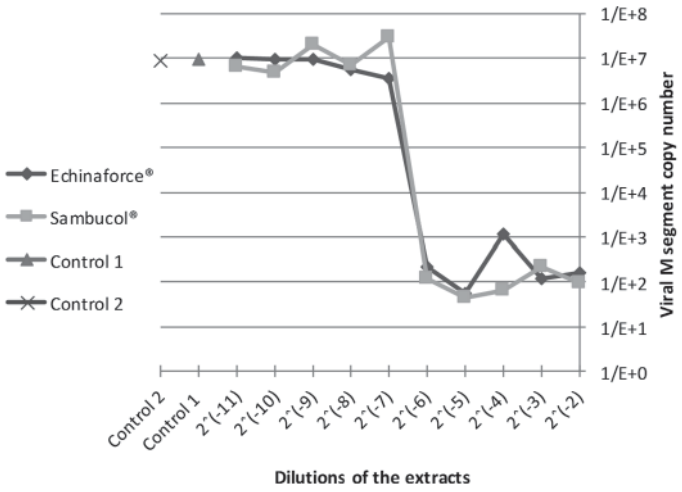

Fig. 1. Neutralization of H9N2 avian influenza virus by Echinaforce® (EF) and Sambucol® (SAM) in the allantoic fluid of embryonated chicken eggs virus was neutralized with Echinaforce® (EF) and Sambucol® (SAM) up to 2^{-6} dilutions of the original stocks (equivalent to 1.3 mg/mL dry mass/vol for EF and 5.9 mg liquid extract (2:1) for SAM in virus-extract mixtures) in step C of the experiment. The virus was treated with the extracts prior to inoculation into embryonated eggs and the viral genome copy numbers were quantified per 1 microliter of allantoic fluids using qRT-PCR.

In addition, a sensitive and specific qRT/PCR test was used along with the HA test to determine the dose-dependent inhibitory effect of the extracts against the virus. The results confirm the powerful antiviral effect that was initially obtained with the NI test and was remarkably the same for both extracts. Considerable virucidal effects were observed using 0.6 mL of both extracts (up to 1:64 vol of main stocks) against 0.6 mL of the virus (500 EID_{50}/0.1 mL). Other research has reported the inhibitory effects of *Echinacea* and elderberry extracts against human influenza viruses A subtype H1, H2, H3 and highly pathogenic H5 and H7 and human influenza B *in vitro* using cell culture media (BINNS et al., 2002; HUDSON et al., 2005; KRAWITZ et al., 2011; PLESCHKA et al., 2009; ROSCHEK and ALBERTE, 2008; SHARMA et al., 2009; VIMALANATHAN, et al., 2005; ZAKAY-RONES et al., 1995). In all of the above studies, the extracts effectively blocked the virions when the virus was treated before inoculation into cell culture media. But no significant antiviral effect was observed when infection occurred prior to treatment with *Echinacea,* or when *Echinacea*-treated cells were infected. These results suggest that the replication and spread of the virus are not affected by the antiviral components of the extract, once the virus has entered the cells (PLESCHKA et al., 2009). Although another study reported a

reduction in the number of foci and titer of the virus in cell culture media pre-treated with elderberry extract (KRAWITZ et al., 2011), it suggested that the elderberry extract had blocked some factors on the cell surface which were needed for infection with the virus.

The dose-dependent antiviral effects of the extracts using the HA test and qRT/PCR test have been shown. A simple PCR test is not suitable for the purpose, as there are few copy numbers of the viral genome in all samples with negative HA results, which cannot be differentiated from the high copy numbers (with positive HA) when simple PCR is used.

Two control untreated viruses were used in step C. Control 1 was treated at 22 ºC for 60 min, the same as the mixtures. But control 2 was kept on ice until inoculation. Similar results were obtained for controls 1 and 2. So, it was confirmed that incubation of the untreated virus at 22 ºC for 60 min had no effect on the infectivity of the virus.

In relation to human influenza, other human membranous viruses such as respiratory syncytial virus, coronavirus, herpes simplex virus and retrovirus are very susceptible to direct contact with *Echinacea* preparations, although viruses without membranes such as poliovirus, rhinovirus and feline calcivirus are more resistant (BIRT et al., 2008; HUDSON et al., 2005; PLESCHKA et al., 2009; VIMALANATHAN et al., 2005). Thus, it is possible that some other important avian membranous viruses such as Newcastle disease virus (paramyxovirus) and avian infectious bronchitis virus (coronavirus), which cause great losses in commercial poultry flocks, could be inhibited using *Echinacea* preparations.

Influenza virus H9N2 is classified as low pathogenic avian influenza virus (LPAI) with much higher shedding from the GI tract in comparison to other organs such as the respiratory tract (MOSLEH et al., 2009; SWAYNE and HALVORSON, 2008; TAVAKKOLI et al., 2011). There is, however, a question of whether the virucidal components of the herbal extracts could be absorbed via the GI tract and show their direct antiviral effects in other organs, especially the upper respiratory tract. Meanwhile, inhibition of the virus in the GI tract of chickens could have some potential benefits in controlling infective virus shedding and its environmental load. However, the possible direct antiviral effect of such herbal preparations along the GI tract could be affected by the stability of their active virucidal ingredients in different pH, and the likelihood of their reaction with other molecules before contact with the target pathogens.

There appears to be more than one antiviral compound among the recognized marker compounds of *Echinacea* preparations, that is, caffeic acids, polysaccharides, and alkylamides, and no correlation exists between the composition or purified fractions of these biomarkers and the antiviral activity of *Echinacea* (BINNS et al., 2002; HUDSON et al., 2005; VIMALANATHAN et al., 2005). However, the EF used in this study is essentially free of polysaccharides (SHARMA et al., 2009).

It was shown that *Echinacea purpurea* extract, over a range of concentrations, inhibited the receptor-binding activity of influenza A viruses using hemagglutination assays. This suggests that *Echinacea* interfered with viral entry into the cells (PLESCHKA et al., 2009). It was also suggested that the active compounds of *Echinacea* could block influenza virus entry via at least two virion targets, hemaglutinin (HA) and neuraminidase (NA). However, due to the susceptibility of other viruses, which do not rely on HA or NA functions, there must be additional molecular targets (HUDSON, 2012).

Flavonoids from the elderberry extract show virucidal effect against human influenza H1N1 virions by blocking the ability of the viruses to infect host cells *in vitro*. This antiviral effect could be favorably compared with the known anti-influenza activities of oseltamivir (Tamiflu®; 0.32 μM) and amantadine (27μM), which were shown using focus reduction assay in cell culture media (ROSCHEK et al., 2009). However, the direct antiviral effect of amantadine against H9N2 avian influenza was not shown using the NI method in the present study.

Conclusion

Both the standardized *Echinacea* and black elderberry extract used in the current study showed significant dose-dependent direct antiviral effects against H9N2 avian influenza virus *in vitro* when treated prior to inoculation into embryonated chicken eggs, evaluated with HA and qRT/PCR tests. But amantadine possessed no direct virucidal effect against the virus. Furthermore, there was a correlation between HA and qRT/PCR results. It seems that administration of these two extracts via drinking water in poultry could potentially have some beneficial effects in the prophylaxis and treatment of avian influenza and probably other viral infections, although *in vivo* testing is required to confirm such efficacy.

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References

ALEXANDER, D. J. (2007): Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002-2006. Avian Dis. 51, 161-166.

ALPHIN, R. L., K. J. JOHNSON, B. S. LADMAN, E. R. BENSON (2009): Inactivation of avian influenza virus using four common chemicals and one detergent. Poultry Sci. 88, 1181-1185.

BINNS, S. E., J. HUDSON, S. MERALI, J. T. ARNASON (2002): Antiviral activity of characterized extracts from *Echinacea* spp. (Heliantheae: Asteraceae) against *herpes simplex* virus (HSV-I). Planta Med. 68, 780-783.

BIRT, D. F., M. P. WIDRLECHNER, C. A. LALONE, L. WU, J. BAE, A. K. S. SOLCO, G. A. KRAUS, P.A. MURPHY, E.S. WURTELE, Q. LENG (2008): *Echinacea* in infection. Am. J. Clin. Nutr. 87, 488-492.

BUTT, K. M., G. J. D. SMITH, H. CHEN, L. J. ZHANG, Y. H. C. LEUNG, K. M. XU, W. LIM, R. G. WEBSTER, K. Y. YUEN, J. S. M. PEIRIS (2005): Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J. Clin. Microbiol. 43, 5760-5767.

DE PASCUAL-TERESA, S., C. SANTOS-BUELGA, J. C. RIVAS-GONZALO (2000): Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. J. Agr. Food Chem. 48, 5331-5337.

DONG, G., C. XU, C. WANG, B. WU, J. LU, H. ZHANG, D. L. NOLTE, T. J. DELIBERTO, M. DUAN, G. JI, H. HE (2011): Reassortant H9N2 influenza viruses containing H5N1-like PB1 genes isolated from black-billed magpies in southern China. PLoS One 6, e17212.

HAKKINEN, S. H., S. O. KARENLAMPI, I. M. HEINONEN, H. M. MYKKANEN, A. R. TORRONEN (1999): Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. J. Agr. Food Chem. 47, 2274-2279.

HUDSON, J., S. VIMALANATHAN, L. KANG, V. T. AMIGUET, J. LIVESEY, J. T. ARNASON (2005): Characterization of antiviral activities in *Echinacea*. root preparations. Pharm. Biol. 43, 790-796.

HUDSON, J. B. (2009): The use of herbal extracts in the control of influenza. J. Med. Plants Res. 3, 1189-1195.

HUDSON, J. B. (2012): Applications of the phytomedicine *Echinacea purpurea* (Purple Coneflower) in infectious diseases. J. Biom. Biotechnol. 2012, 1-16.

IQBAL, M., T. YAQUB, K. REDDY, J. W. MCCAULEY (2009): Novel genotypes of H9N2 influenza A viruses isolated from poultry in Pakistan containing NS genes similar to highly pathogenic H7N3 and H5N1 viruses. PLoS One 4, e5788.

KRAWITZ, C., M. A. MRAHEIL, M. STEIN, C. IMIRZALIOGLU, E. DOMANN, S. PLESCHKA, T. HAIN (2011): Inhibitory activity of a standardized elderberry liquid extract against clinically-relevant human respiratory bacterial pathogens and influenza A and B viruses. BMC Complem. Altern. Med. 11, 16-21.

LOMBARDI, M. E., B. S. LADMAN, R. L. ALPHIN, E. R. BENSON (2008): Inactivation of avian influenza virus using common detergents and chemicals. Avian Dis. 52, 118-123.

MOSLEH, N., H. DADRAS, A. MOHAMMADI (2009): Molecular quantitation of H9N2 avian influenza virus in various organs of broiler chickens using TaqMan real time PCR. J. Mol. Genet. Med. 3, 152-157.

PLESCHKA, S., M. STEIN, R. SCHOOP, J. B. HUDSON (2009): Anti-viral properties and mode of action of standardized *Echinacea purpurea* extract against highly pathogenic avian influenza virus (H5N1, H7N7) and swine-origin H1N1 (S-OIV). Virol. J. 6, 197-205.

REED, L. J., H. MUENCH (1938): A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27, 493-497.

ROSCHEK JR, B. R. S. ALBERTE (2008): Pharmacokinetics of cyanidin and anti-influenza phytonutrients in an elderberry extract determined by LC-MS and DART TOF-MS. Online J. Pharmacol. Kinetics 4, 1-17.

ROSCHEK, B. Jr., R. C. FINK, M. D. McMICHAEL, D. LI, R. S. ALBERTE (2009): Elderberry flavonoids bind to and prevent H1N1 infection *in vitro*. Phytochemistry 70, 1255-1261.

ROXAS, M., J. JURENKA (2007): Colds and influenza: a review of diagnosis and conventional, botanical, and nutritional considerations. Alternat Med. Rev. 12, 25-48.

SHARMA, M., S. A. ANDERSON, R. SCHOOP, J. B. HUDSON (2009): Induction of multiple pro-inflammatory cytokines by respiratory viruses and reversal by standardized *Echinacea*, a potent antiviral herbal extract. Antivir. Res. 83, 165-170.

SWAYNE, D. E., D. A. HALVORSON (2008): Influenza. In: Disease of Poultry. (Saif Y. M., A. M. Fadly, J. R. Glisson, L. R. Macdougald, L. K. Nolan, D. E. Swayne, Eds.), Iowa State University Press, Ames, Iowa, pp. 153-184.

TAVAKKOLI, H., K. ASASI, A. MOHAMMADI (2011): Effectiveness of two H9N2 low pathogenic avian influenza conventional inactivated oil emulsion vaccines on H9N2 viral replication and shedding in broiler chickens. Iranian J. Vet. Res. 12, 214-221.

VIMALANATHAN, S., L. KANG, V. T. AMIGUET, J. LIVESEY, J. T. ARNASON, J. HUDSON (2005): *Echinacea purpurea* aerial parts contain multiple antiviral compounds. Pharm. Biol. 43, 740-745.

VLACHOJANNIS, J. E., M. CAMERON, S. CHRUBASIK (2010): A systematic review on the sambuci fructus effect and efficacy profiles. Phytother. Res. 24, 1-8.

WARD, C. L., M. H. DEMPSEY, C. J. A. RING, R. E. KEMPSON, L. ZHANG, D. GOR, B. W. SNOWDEN, M. TISDALE (2004): Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. J. Clin. Virol. 29, 179-188.

WOELKART, K., R. BAUER (2007): The role of alkamides as an active principle of *Echinacea*. Planta Med. 73, 615-623.

ZAKAY-RONES, Z., N. VARSANO, M. ZLOTNIK, O. MANOR, L. REGEV, M. SCHLESINGER, M. MUMCUOGLU (1995): Inhibition of several strains of influenza virus in vitro and reduction of symptoms by an elderberry extract (*Sambucus nigra* L.) during an outbreak of influenza B Panama. J. Altern. Complem. Med. 1, 361-369.

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KARIMI, S., A. MOHAMMADI, H. DADRAS: Učinak iscrpka crvene pupavice (*Echinacea purpurea)* i crne bazge (*Sambucus nigra* L.) na serotip H9N2 virusa influence ptica u zaraženim kokošjim zametcima. Vet. arhiv 84, 153-165, 2014.

SAŽETAK

Prijašnja istraživanja pokazala su da pripravci nekih vrsta *Echinacea* i bobica bazge imaju značajan protuvirusni učinak na ljudske podtipove virusa influence H1, H2, H3 i na visoko patogene H5 i H7 te virus influence B u pokusima na staničnoj kulturi. U ovom je radu istražen antivirusni učinak alkoholnog iscrpka vrste *Echinacea purpurea* (Echinaforce®) (crvena pupavica) i vodenog iscrpka crne bazge *Sambucus nigra* L. (Sambucol®) u usporedbi s amantadinom. Učinak je procijenjen na osnovi određivanja virusnog titra podtipa H9N2 virusa influence ptica u alantoisnoj tekućini inficiranih kokošjih zametaka hemaglutinacijom (HA) i kvantitativnom lančanom reakcijom polimerazom u stvarnom vremenu (qRT/PCR). Istraživanje je bilo provedeno u dva koraka. Prvo je neutralizacijski indeks (NI), određen na osnovi standardne infekcijske doze za embrije (EID_{50}), bio rabljen za prosudbu učinkovitosti iscrpka i amantadina. Serijskom razrjeđenju virusa bila je dodana jednaka količina razrijeđenog iscrpka prije inokulacije kokošjih zametaka. Nakon inokulacije zametci su stavljeni na inkubaciju tijekom 48 sati pri 37 ˚C. U sljedećem koraku je 500 EID_{50} virusa bilo dodavano serijskim razrjeđenjima iscrpka prije inokulacije. Titar virusa u alantoisnoj tekućini bio je određen hemaglutinacijom (HA) i kvantitativnom lančanom reakcijom polimerazom u stvarnom vremenu (qRT/PCR). Rezultati su potvrdili značajne virucidne učinke obaju iscrpaka. Ustanovljena je i izravna korelacija između rezultata HA i qRT/PCR u drugom koraku. Zaključuje se da rezultati potvrđuju značajan virucidni učinak obaju iscrpaka na podtip H9N2 virusa influence ptica.

Ključne riječi: *Echinacea purpurea*, crvena pupavica, *Sambucus nigra*, crna bazga, H9N2, influenca ptica, kvantitativna lančana reakcija polimerazom u stvarnom vremenu