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Research Article

Antiviral activity of edible bird's nest extract on highly pathogenic avian influenza H5N1 viral infection in vitro

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Abstract. Objective. This research aimed to explain the antiviral activity of edible bird's nest (EBN) extract against the HPAI H5N1 virus clade 3.2.3 in vitro. Material and methods. This study was preceded by measuring 50% cytotoxic concentration (CC_{50}) of EBN extract and measuring sialic acid content in EBN by spectrophotometric method. Hemagglutination inhibition test (HI test) and viral neutralization test were used to measure antiviral activity of EBN extract with various concentration (1–15 mg mL^{-1}) against H5N1 virus in Vero cell. Results. The results of this study showed that EBN extract could inhibited hemagglutination activity of H5N1 virus in chicken erythrocytes at 12 µg mL^{-1} EBN concentration. A viral neutralization test showed that EBN extract could neutralize H5N1 virus infection until the third day and decreased the viral titer after treatment. There was a significant difference ($p<0.05$) between 1–5 mg mL^{-1} EBN concentration and 15 mg mL^{-1} in inhibition of H5N1 virus infection on third day, but no difference found between 6–10 mg mL^{-1} EBN concentration and 15 mg mL^{-1}. This study revealed that the mechanism of action of EBN to inhibits H5N1 virus infection is by inhibiting the attachment of the virus to the cellular receptor. Conclusion. EBN could inhibited infection of H5N1 virus, so that could be used as an alternative in prevention of H5N1 virus infection.

Key Words: Antiviral, EBN, H5N1, Neutralization, Vero cell

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Introduction

Edible bird's nest (EBN) is a nest made from saliva of swiftlet *Collocalia fuciphaga* (George Robert Gray, 1854). This bird nest has a high taste and as a traditional medicine that was popular among Chinese society. Chinese society has been consuming EBN since ancient Chinese dynasties. Edible bird's nest has been believed to be effective in dissolving phlegm, relieving gastric disorder, aiding renal function, raising libido, enhancing complexion, alleviating asthma, suppressing cough, curing tuberculosis, strengthening the immune system, curing hematemesis, nourishing the lungs, speeding recovery from illness and surgery, increasing child growth, increasing energy and metabolism, and improving concentration (Francis 1987), but the molecular mechanisms of the EBN therapeutic effect are unclear. Research on the biological activity of EBN has begun in the last few years. Haghani et al (2017) found that EBN could inhibit influenza A virus (IAV) infections as efficient as commercial antivirals such as amantadine and oseltamivir. The antiviral activity was related to sialic acid content or N-acetylneuraminic acid (Neu5Ac) in EBN (Haghani et al 2017).

Dominant sialic acid species found on EBN is Neu5Acα2-3Gal (Guo et al 2006). This compound inhibits IAV infection, but until now its mechanism of action is still debated. Guo et al (2006) found that sialic acid acts as a main inhibitor of IAV infection, that inhibits viral adhesion to the surface of target cells. Mechanism of action as described above is as receptor competitors of the target cell. Sialic acid will attach to the hemagglutinin (HA) spike of influenza virus before the HA virus attaches to the target cell receptor, so that the virus could not attach to the target cell receptor and the infection did not occur (Wibawan and Soejoedono 2013). Haghani et al (2017) found that the other mechanism of action of EBN unrelated to the attachment of the virus to target cells responsible to inhibit viral infection might be relevant to the process of virus releases from cell membranes after replication. This was because the efficacy of EBN was higher if the treatment of EBN was performed on cells that have been infected with influenza virus (post-treatment) compared with EBN and virus together to the cell (simultaneously).

Virus subtypes used in the two studies above, Guo et al (2006) and Haghani et al (2017) were H3N2 and H1N1 belonging to the

low pathogenic avian influenza (LPAI) group. In recent years, the highly pathogenic avian influenza (HPAI) H5N1 virus is responsible for panzootic events that occur among domestic birds and other birds worldwide (Dubey et al 2009). The case of direct transmission of HPAI H5N1 virus from poultry to humans has occurred in Hong Kong in 1997 and has caused death and disease in domestic poultry and migratory birds and susceptible humans (Subbarao and Matsuoka 2013). Between 2003 and 2017, 859 cases of H5N1 infections in humans and 453 of deaths were confirmed (WHO 2017). In Indonesia until 2017 was recorded 200 people infected and 168 deaths (Kemenkes 2017). Therefore this study aims to analyze the ability of EBN extract in inhibiting HPAI H5N1 virus infection so that EBN can be used as an alternative in prevention of H5N1 virus infection.

Materials and Methods

Edible Bird's Nest Extract

Edible bird's nest used in this study were a bird's nest has been cleaning. The EBN extract were prepared based on method of Guo et al (2006) with some modifications. Briefly, bird's nests were dried at 70 °C for 16 h grounded and filtered with a 600 µM in pore size mesh (30 mesh). Later, 30 g birds' nests were suspended in 200 ml of aquabides (150 mg mL^{-1}) for 16 h at 5 °C, heated at 60 °C for 1 h, filtered using filter paper and stored at -20 °C for further use.

Virus and Cell Culture

Virus used in this study was H5N1 HPAI virus clade 3.2.3 A/DK/BNY/F.2014P1 isolated from infected ducks in Indonesia. The infective dose of H5N1 virus isolate was 10^6 TCID50 0.1 mL^{-1} with a 2^6 or 64 HAU. Virus stock was propagated in 9 to 11 day old specific antibody negative (SAN) chicken embryonic eggs. Confluent monolayer Vero cell (African Green Monkey Kidney Cells) in 96 well plates was used to observe the EBN extract antiviral activity. Virus titer and virus infective dose were measured by tissue culture infective dose 50% (TCID50) (Villegas 1998) with hemagglutination test (OIE 2015). The infective dose was calculated using Spearman and Karber formulas (Villegas 1998). Vero cells were cultured on growth medium of Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing of 10% heat -inactivated fetal bovine serum (FBS, Gibco, USA) at 56 °C for 30 min, Hepes 1%, α Glutamine 2.92% and 100 unit mL^{-1} penicillin and 100 µg mL^{-1} streptomycin and incubated at 37 °C, 5% CO2.

Sialic Acid Content in EBN

Sialic acid content of EBN was measured using the spectrophotometric method (Li et al 2011). Briefly, EBN sample dried at 105 oC for 1 h, grounded and filtered with a 100 mesh filter. EBN powder 0.12–0.13 g in 20 mL of 50% acetic acid, heated under reflux for 10 min, cooled and flushed with aquabides to 100 mL. As much as 10 mL solution was added with 1.2 g of ammonium sulfate, dissolved, shaken and centrifuged at 3000 rpm for 10 min. Supernatant of 2 mL, was added with 2 mL of ninhydrin indicator (2.5 g of ninhydrin in 60 mL glacial acetic acid and 40 mL of HCl) and 2 mL of glacial acetic acid, stirred and heated at a water bath at 100°C for 10 min and the tube was cooled in running water immediately. Supernatant was inserted into cuvette and measured its absorbance by a visible light spectrophotometer at a wavelength of 470 nm.

Cytotoxicity Test on Edible Bird's Nest

To determine the possible cytotoxic effects of EBN extract, monolayer Vero cells were exposed with EBN extract at different concentrations, observed for 96 h with five replications. Cytotoxic evaluation was observed with inverted microscope to see the morphological changes of cells induced by EBN extracts such as cell shrinkage, blebbing membranes, ballooning cell, chromatin condensation, and cytoplasmic vacuolation (Vijayarathna and Sasidharan 2012). Briefly, EBN extract stock was dissolved with 2% DMEM starting from concentration 5–50 mg mL^{-1}. Vero cell were added into a flat bottom 96 well microplate, incubated for 24 h at 37 °C and 5% CO_2 to obtain a confluent monolayer Vero cell and washed 2 times with phosphate buffered saline (PBS) containing 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. As much as 100 µL DMEM 2% was added to each microplate well followed by 100 µL of EBN extract starting from 5 to 50 mg mL^{-1} with at 5 replications. Microplates were incubated at 37 °C, 5% CO2 for 4 days. Negative control (cell control) and cytotoxic positive control were used as comparison. Cytotoxic activity is characterized by changes in cell morphology. The EBN concentration causing cytotoxicity in 50% of Vero cells is expressed as 50% cytotoxic concentration (CC_{50}).

Hemagglutination-Inhibition Test (HI Test)

Thirteen samples of EBN extract from 12 swiftlet houses were tested for their ability to inhibit hemagglutination. The HI test was performed by diluting EBN extract with PBS two fold dilutions on a 96 well microplate. The virus stock 4 HA of 25 µL (the lowest amount of virus particles able to agglutinate the chicken red blood cells) was added to all wells and incubated at room temperature for 45 min. Around 25 µL chicken red blood cells (RBC) were added to all wells, homogenized and incubated at room temperature for 1 h and read (OIE 2015).

EBN Extracts Antiviral Activity on H5N1 HPAI Virus

Assessment of antiviral activity of EBN extract using a virus neutralizing principle refer to Guo et al (2006) and Haghani et al (2017) with some modifications. The test were conducted in two step, the first step was virus 10^2 TCID50 0.1 mL^{-1} and EBN at different concentrations incubated at 37 oC, 5% CO_2 for 30 min, then cultured on Vero cells and observed for 96 h. The second step was after 96 h, Vero cells was harvested and cultured on Vero cells for measured of viral titer that un-neutralized in the first step. Briefly, EBN extracts of 1–10 mg mL^{-1} and 15 mg mL^{-1} were dissolved in 2% DMEM. Virus isolates (10^6 $TCID_{50}$ 0.1 mL^{-1}) were dissolved to 10^3 $TCID_{50}$ 0.1 mL^{-1} with PBS containing 100 unit mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. As much 0.2 mL of isolate virus (103) in microplate 12 wells added 1.8 mL of EBN extract 1 mg mL^{-1}. And so on for EBN extract 2, 3 to 15 mg mL^{-1}. The microplate homogenized with a shaker on a scale of 4 (500 rpm) for 30 min at 37 °C, 5% CO_2. Monolayer Vero cell on microplate (after incubation for 24 h at 37 °C, 5% CO_2) was washed with 2% DMEM as 2 time. Furthermore, 0.1 mL of 2% DMEM added to all microplate wells and 0.1 mL mixtures of EBN extract and H5N1

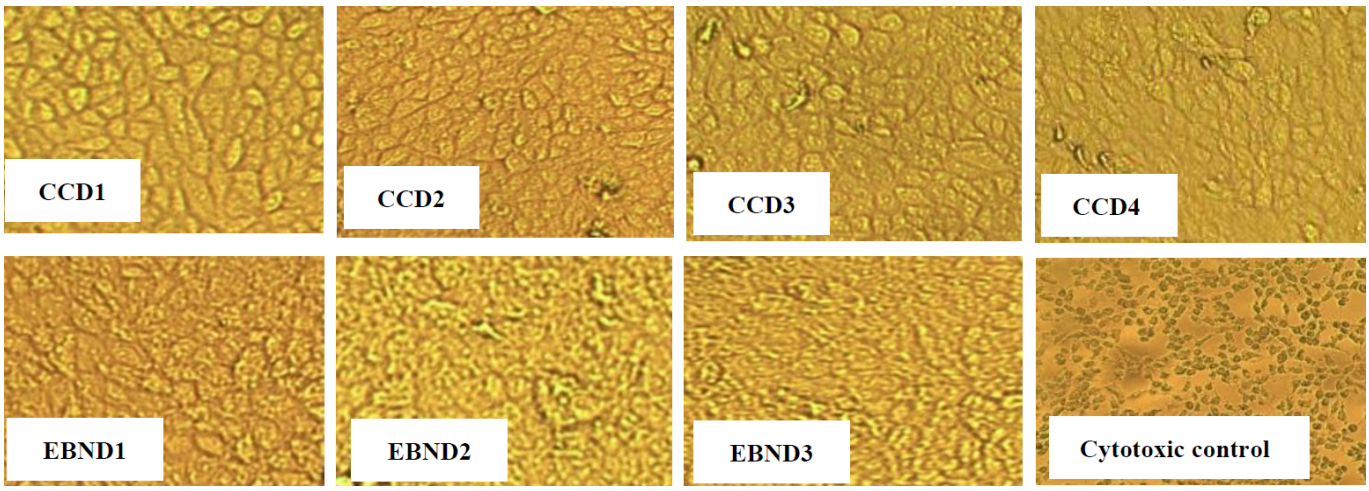

Fig. 1. Cytotoxicity test of EBN extract on Vero cells. Above is the control of Vero cell first day to day 4 and bottom is EBN extract at 50 mg mL^{-1} first day to day 3 and cytotoxic positive control. Until the fourth day, cell culture is normal, there is no cytotoxic signs but there is little morphology change of cell culture.

virus at least 4 replications. Treatment were accompanied by EBN control, cell control (negative control) and positive control of H5N1 virus to compared antiviral activity of each treatment. Microplates were incubated at 37 °C, 5% CO_2 for 96 h and observed for morphological changes in cell with inverted microscope.

Antiviral activity is characterized by the no CPE in cell culture. Cytopathic effect was morphological changes observed in cell culture due to viral infection. Avian influenza virus H5N1 infection generates rounding cell, destroyes cells, fusion with adjacent cell to form large cell with four nuclei called syncytia and appearance of cytoplasmic inclusion bodies (Suchman and Blair 2012). The percentage of infection inhibition (or protectivity) was calculated with formula described by Lu et al (2003) : %Protection = Number of microplate well no CPE/total of microplate well inoculated x100%.

Back Titration of Treated Virus

On the last day of treatment, Vero cells were harvested feezed and thawed 3 times. Vero cells with positive and negative CPE were harvested separately, and centrifuged 10.000 rpm for 10 min. Vral titer was measured by HA test (OIE 2015) and virus titration (Villegas 1998). Virus titers were calculated using Spearman and Karber formulas (Villegas 1998).

Statistical analysis

Chi-square was used to evaluate the difference between the concentration of EBN extract and the observation day in infection inhibit by using XLSTAT 2014.5.03 program.

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Results

Quantification of sialic acid by spectrophotometric method based on sialic acid reaction with ninhydrin reagent in acid solution yields stable color with maximum absorbance of 470 nm. This reaction is specific to sialic acid and was not influenced by other sugars that did not contain sialic acid. Ninhydrin also reacts to amino acids such as cysteine, cystine, proline and tryptophan but its maximum absorbance differs from sialic acid (Yao et al 1987). Based on the results of spectrophotometric test, sialic acid content in EBN used in this study was 10.14% (w/w).

EBN cytotoxicity test showed that up to 50 mg mL^{-1} did not generate cytotoxic in cell culture (Figure 1). The healthy cell condition was seen until day 4. Thus, the 50% cytotoxic concentration (CC_{50}) of EBN extract was detected at more than 50 mg mL^{-1}. To measure the biological response of EBN extract, HI test and neutralization test were performed. Some viruses including HPAI H5N1 virus could agglutinate RBC and these properties could be inhibited by certain antibodies or certain compounds. The expected biological responses of the EBN extract to the H5N1 virus was hemagglutination reaction inhibited by the presence of EBN extract, as evidenced by HI test. A total of 13 EBN samples from 12 swiftlet houses with different concentrations (25–150 mg mL^{-1}) showed varying HI titers ranged from 7 log 2 to 11 log 2 (128–2048 HIU) and EBN concentrations begin to show HI activity at 12 µg mL^{-1}, as in Figure 2. EBN with low concentration (25 mg mL^{-1}) showed a high HI titers compared with EBN of higher concentration (35 mg mL^{-1}). Suggesting that HI activity is not correlated with EBN concentration but related to sialic acid content in EBN.

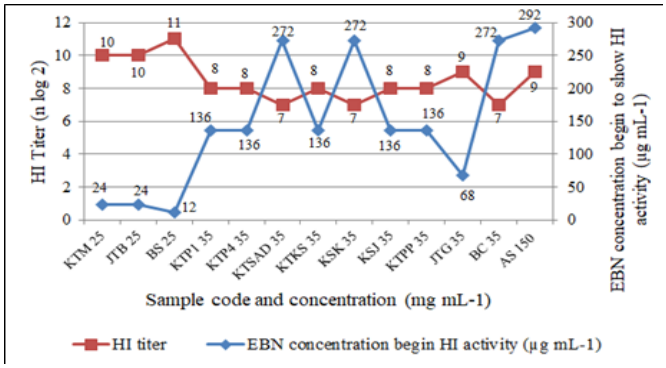

Fig. 2. EBN concentration and HI activity to H5N1 HPAI virus and also EBN concentrations begin to show HI activity.

Evaluation of EBN antiviral activity against H5N1 HPAI virus was performed by viral neutralization. Neutralization of a virus is defined as the loss of infectivity through reaction of the virus with specific antibody or specific compound. Neutralization was seen from normal cell morphology without CPE. Effect of EBN extract inhibitory on H5N1 virus showed on Figure 3 and

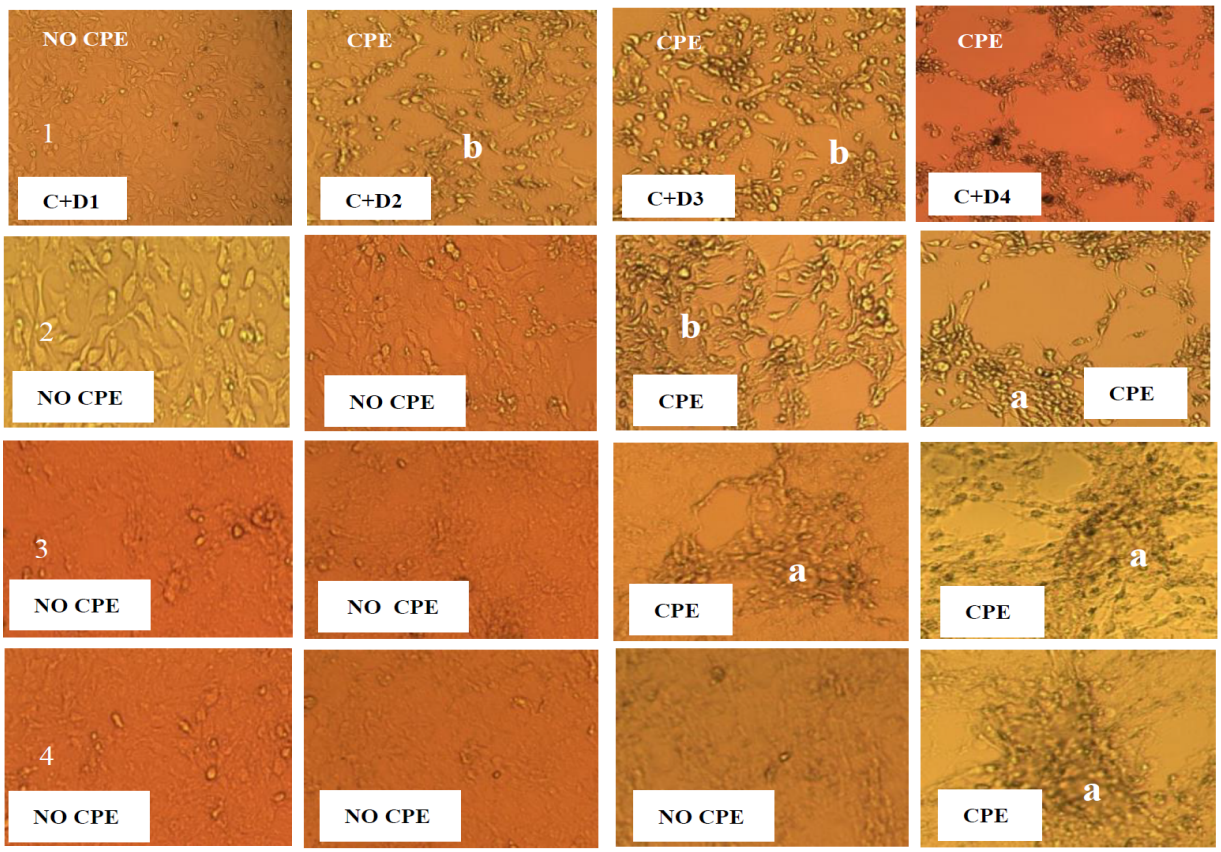

Figure 3 An overview of EBN extract inhibition of H5N1 virus infection in Vero cells from first day to day 4. 1) positive contol H5N1 on day 1 to day 4. 2, 3 and 4) were EBN concentrations of 5, 10 dan 15 mg mL^{-1}. Cell morphology changes from normal to CPE, giant cell (a), cell rounding (b) on day 3 and 4 after infection.

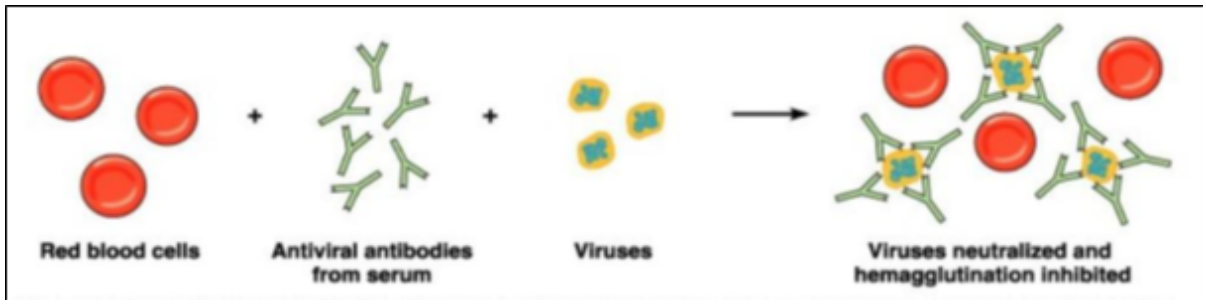

Fig. 4. Mechanism of hemagglutination inhibition by sialic acid against AI virus (Rao 2013)

Table 1. Figure 1 showed that positive control (C+) was infected with H5N1 HPAI virus on day 2, while cell culture containing EBN extract 15 mg mL^{-1}, the infection was inhibited until day 3. Even until day 4 the EBN extract was still able to protect Vero cell culture from H5N1 HPAI virus infection up to 62.5%. Statistically, there were significant differences ($p<0.05$) between EBN concentrations on day 2 (H2), H3 and H4 in inhibition of virus infection, but the Marascuito procedure only able to seen the difference in H3. There was a significant difference between EBN concentration at 1–5 mg mL^{-1} and 15 mg mL^{-1} on day 3, but no difference between 6–10 mg mL^{-1} and 15 mg mL^{-1}. The EBN concentration at 1–5 mg mL^{-1} was able to inhibit H5N1 HPAI virus infection until day 2, while 15 mg mL^{-1} was able to inhibit H5N1 HPAI virus infection until day 3. On day 4, cell were infected. Cytopathic effect such as giant cells and cell rounding was detected as in Figures 3.

Discussion

In this study found that EBN extract proven to inhibit H5N1virus infection. This inhibitory activity was seen in the HI test and the viral neutralization test. Hemagglutination inhibition activity began to be seen at 12 µg mL^{-1}. These results line with Guo et al (2006) stated that EBN from cave treated with pancreatin F enzyme, the HI activity against human influenza A virus (H3N2) began to be seen at 4 µg mL^{-1}, while EBN from swiftlet house HI activity was seen at 125 µg mL^{-1}. The HI activity occurs because the EBN extract contains compounds in sufficient quantities to make complexes with viral hemagglutinin preventing the attachment of hemagglutinin virus to RBC. This reaction causes inhibition of hemagglutination, as illustrated in Figure 4. Guo et al (2006) explained that the compound in EBN that inhibit hemagglutination of infuenza A virus was sialic acid Neu5Acα2-3Gal. As previously described, the presence of concentration difference in HI is probably due to the influence of sialic acid

Table 1. The level EBN protection in inhibition H5N1 viral infection in Vero cell

No	EBN concentration (mg mL-1)	% Protection				Virus titer post treatment		
		Day 1	Day 2	Day 3	Day 4	Rapid HA	HA Titration	$TCID_{50}$
1	**1**	100	50	0	0	Td	Nd	Nd
2	**2**	100	50	25	0	Td	Nd	Nd
3	**3**	100	62.5	25	0	Td	Nd	Nd
4	**4**	100	100	0	0	Td	Nd	Nd
5	**5**	100	100	12.5	0	Td	Nd	Nd
6	**6**	100	100	50	50	(-)	0 log 2	0 log 10
7	**7**	100	100	50	50	(-)	0 log 2	0 log 10
8	**8**	100	100	62.5	50	(-)	0 log 2	0 log 10
9	**10**	100	100	62.5	50	(-)	0 log 2	0 log 10
10	**15**	100	100	100	62.5	(-)	0 log 2	0 log 10
11	**C-Virus**	100	0	0	0	(+)	1 log 2	5 log 10
12	**C-EBN**	100	100	100	100	(-)	0 log 2	Nd
13	**C-Cell**	100	100	100	100	(-)	0 log 2	Nd

content in EBN or may be different kind of sialic acid, because Haghani et al (2016) has found other kinds of sialic acid on EBN that were Neu2,4,7,8,9 Ac6.

In Figure 2 showed that low EBN concentration (25 mg mL^{-1}) had a high HI titer compared to EBN of higher concentration (35 mg mL^{-1}). Suggesting that HI activity is not correlated with EBN concentration but related to sialic acid content in EBN. Sialic acid in EBN was found to be different in some swiftlets houses and sialic acid in EBN that origin from swiftlet house in Kalimantan island was found higher than Java island. Factors influencing of sialic acid content in EBN at significantly level ($p<0.05$) were the vegetation around swiftlet house, availability of plants, harvesting system, the altitude swiftlet house above sea level and diversity and also number of insects eaten by swiftlet (Helmi et al 2018). Chua et al (2014) also found that environmental variations had an impact on the composition of bioactive compound in EBN, such as differences in bioactive compound found in EBN from Malaysia, Indonesia and Thailand. Differences in the active compound content of EBN, may have an effect on the difference in antiviral activity. Marni et al (2014) found that higher concentration of sialic acid in EBN might be due to the condition of surrounding habitats, availability and abundance of food source in around of swiftlet house.

To assess the effectiveness of antivirals in inhibiting viral infections, there are two methods conducted were the exposure of EBN extract and H5N1 virus. The exposure of EBN extract and H5N1 virus to cell culture adding together (simultaneously) and post treatment method was cell culture infected with H5N1 virus and 24 h later treated with EBN extract (Kwon et al 2010) before there was cytopathic effect (CPE) and 48 h later after there were CPE. Of these two methods were seen that simultaneous methods has been better antivirus activity compared with post treatment method. This approach has been able to explain the mechanism of action of sialic acid in inhibiting influenza virus infection. In Table 1, low EBN concentration (1, 2, and 3 mg mL^{-1}) were able to inhibit H5N1 virus infection for 1 day. It might be related to number of virus hemagglutinin trimers and the amount of sialic acid added. Skehel and Wiley (2000) reported that the the influenza virion contains about 350–400 hemagglutinin trimers on its surface, and all of these spikes should be blocked by attached of sialic acid so effectively prevent viral adhesion to target cells. If the sialic acid is insufficient (low EBN concentration), the hemagglutinin virus would attach to the target cell and the virus will enter to the host cell so that replicated virus. Virus replication process occurs very quickly which is about 10 hours/cycle (Coleman 2007) that produces many progeny.

The influenza virus replication cycle could be devided in 5 steps, starting with binding of viral haemagglutinin to sialic acid receptor on host cell surface, internalization of virus by receptor-mediated endocytosis, release of viral genes into the cytoplasm, packing of viral proteins with viral genes after viral RNA replication, transcription and translation, and budding of new virus and new virus release by sialidase cleaving sialic acid receptor (Palese 2004). Effect of sialic acid inhibition on influenza virus involves at least two mechanisms. Inhibition of attachment of the virus to the receptors on the host cell surface and inhibition of virus neuraminidase to prevent release of new virus from infected cells (Guo et al 2002). In this study we were found that antiviral activity was better when EBN and viral exposure were simultaneously compared with post treatment. This indicates that mechanism of action of sialic acid on H5N1 HPAI virus infection is by inhibiting viral attachment to receptors on the host cell surface. Dissolved sialic acid will be attached to hemagglutinin spike and the virus could not attach to the cell receptor so that the infection did not occur. These results was suitable with the findings of Guo et al (2006), but opposite with findings of Haghani et al (2017) stating that EBN's mechanism of action was not related to the attachment of viruses to host cells, but might be more relevant to the virus-releasing process from infected cell membranes. This irrelevant finding is probably due to the influence of the active substances on the EBN. Kong et al (1987) stated that EBN containing epidermal growth factor (EGF) in high enough amount. This EGF compound was known to stimulate cell growth (Kong et al 1987) and promotes the process of attachment and internalization of AI virus through

activation of EGF receptors (Eierhoff 2010). Therefore, the EBN mechanism of action inhibits viral infection more precise by inhibiting viral attachment to receptors than by inhibiting the release of new viruses from infected cells. EBN could be used as an alternative in prevention of H5N1 virus infection.

Conclusion

In this study we found that EBN extract could inhibit infection H5N1 virus. Extract EBN could neutralized infection H5N1 virus in Vero cells until day 3 and decreased viral titer after treatment. Extract EBN could inhibit hemagglutination of H5N1 virus in chicken erythrocytes. Furthermore, the EBN extract acts as an antiviral by inhibiting attachment of H5N1 virus to sialic acid receptors on the host cell surface. EBN could be used as an alternative in prevention of H5N1 virus infection.

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