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RESEARCH ARTICLE

ISOLATION OF AVIAN INFLUENZA A (H5N2) FROM FREE-GRAZING DUCKS IN THAILAND AND ANTIVIRAL EFFECTS OF TEA EXTRACTS

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ABSTRACT

During the surveillance of avian influenza, an H5N2 influenza A virus was isolated from a cloacal swab sample of an apparently healthy free-grazing duck in Banglane district, Nakhon Pathom province, Thailand in July 2007. It has been previously reported that tea extracts inhibit the growth of influenza virus by polyphenolic compounds in the leaves of *Camellia sinensis*. In this study, we found that dried tea leaves extract and green tea extract inhibited hemagglutination caused by H5N2 influenza A virus and viral propagation in embryonated chicken eggs. Total phenolic contents were recorded for dried tea leaves and green tea extracts (491 and 470 mg/GAE/g respectively), the total phenolic contents correlated with antiviral propagation. The cytotoxicity of dried tea leaves extract and green tea extract on HEK-293 cells was found to be low toxicity with IC_{50} values of 283.35 and 1765.25 mg/ml, respectively. These results are expected to provide guides for rational design of tea extracts as an antiviral substances to prevent influenza A virus infection, especially in pandemic area of avian influenza A viruses.

Key words: Antiviral activity, Avian influenza virus, Free-grazing ducks, H5N2, Tea extracts

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INTRODUCTION

Influenza is transmitted by inhalation of infectious droplets and droplet nuclei, by direct contact, and perhaps, by indirect (fomite) contact, with self-inoculation onto the upper respiratory tract or conjunctival mucosa (Bridges *et al*., 2003). In 1997, exposure to live poultry within a week before the onset of illness was associated with disease in humans with influenza A (H5N1) virus (Mounts *et al*., 1999). Plucking and preparing of diseased birds, handling fighting cocks; playing with poultry, particularly asymptomatic infected ducks, and consumption of duck's blood or possibly undercooked poultry have all been implicated (Beigel *et al*., 2005). Free-grazing ducks are known influenza A virus reservoirs and can spread viruses through frequent movements in habitats and may be significant in influenza A virus transmissions (Gilbert *et al*., 2006). Recently, influenza A virus subtypes H4N6 and H3N8 were isolated from free-grazing ducks with clinical signs of depression and ocular discharge in Phichit and Phisanulok provinces, Thailand (Boonyapisitsopa *et al*., 2016). Currently, the United States Food and Drug Administration lists two types of antiviral drugs that are approved for prevention and treatment of influenza virus; these are M ion-channel inhibitors (amantadine and remantadine) and neuraminidase inhibitors (oseltamivir, zanamivir and paramivir). However, the drug resistant influenza virus has become widespread (Hurt *et al*., 2012). This reason has motivated scientists to explore novel antiviral drugs for activity against influenza virus, including natural products (Zu *et al*., 2012). Tea leaves extracts (*Camella sinensis*) consisted of a group of relatively small polyphenols, mainly consisting of catechins, flavonols, proanthocyanidins, and theaflavins. Tea catechins, including (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechingallate (ECG), (-)-epicatechin (EC), (-)-catechin, and (+)-catechin, have been found to have antiviral property (Suganuma *et al*., 2011). EGCG is the major catechin found in tea extract, which accounts for approximately 50% of the total catechins. This edible nature compound has demonstrable benefits including antitumor, anti-oxidative, and antiviral effects (Yang *et al*., 2002; Cabrera *et al*., 2006). EGCG is multipotent in terms of its broad-spectrum antiviral efficacy *in vitro*, with inhibitory effects on human immunodeficiency virus (HIV) (Kawai *et al*., 2003; Hauber *et al*., 2009; Li *et al*., 2011), herpes simplex virus (HSV) (Lyu *et al*., 2005; Isaacs *et al*., 2008), hepatitis C virus (HCV) (Ciesek *et al*., 2011; Calland *et al*., 2012; Chen *et al*., 2012), and influenza virus (Nakayama *et al*., 1993; Song *et al*., 2005). In July 2007, we isolated H5N2 avian influenza A virus from healthy free-grazing ducks in Banglane district, Nakhon Pathom province, Thailand.

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The present study compared the antiviral activities of green tea and black tea extracts on viral propagation in embryonated chicken eggs. This study aims to determine both of tea extracts inhibited viral propagation. Instead, green tea and black tea extracts specifically targets viral cell entry into reticuloendothelial cells and also exerted inhibitory effect on hemagglutination, where affected influenza virus adsorption. In conclusion, green tea and black tea extracts blocked virus penetration into cells by physically damaging the viral integrity. These findings may explain the general antiviral mechanism of tea extract against infections with influenza virus and possibly other enveloped viruses.

MATERIALS AND METHODS

Sample collection and virus isolation

During August 2006 - July 2007, two hundred forty samples were collected from healthy free-grazing ducks in Banglane district, Nakhon Pathom province, Thailand, during this time, avian influenza virus outbreaks were reported in domestic poultry in Thialand. Collected cloacal swabs were placed in 2 ml phosphate buffered saline (PBS, pH 7.2) supplemented with penicillin G 100 U/ml, streptomycin 100 µg/ml, and kept on ice. The samples were filtered through 0.22 µm Millipore membrane. Then 0.2 ml were inoculated into 9-11-day-old specific-pathogen-free embryonated chicken egg. Eggs were incubated at 37^{o}C for 4-5 days. The hemagglutination (HA) assay with chicken erythrocytes was used to detect avian influenza virus in allantoic fluid (Brauer and Chen, 2015). In brief, serial 2-fold dilutions of allantoic fluid were mixed with 1% chicken erythrocyte suspension. After incubation at 4^{o}C for 30 min, sample with hem agglutination were interpreted as positive and the highest dilution of completed hemagglutination was considered for HA titers. For typing avian influenza A virus, Fujirebio Espline Influenza A&B-N (Fujirebio; Japan) was carried out for its ability to detect influenza antigen by following the manufacturer's protocols.

For subtyping of avian influenza A virus, hemagglutinin and neuraminidase genes of the avian influenza A virus were extracted using the RNeasy mini kit (Qiagen) following the manufacture's protocol and amplified with gene-specific primers (Table 1) using the One-Strep RT-PCR kit (Qiagen) as previously described (Hoffmann *et al*., 2001). One-Strep RT-PCR system was used. The 25 µl mixture of each PCR reaction contained 1X Qiagen Onestep RT-PCR buffer, 1 µl Qiagen Onestep RT-PCR enzyme mix, 0.5 µM of primer, 1 µl of RNA, 0.1 mM dNTPs and 15 µl of distilled water. RT-PCR was performed with the conditions of reverse transcription at 50^{o}C for 30 min, initial denaturation at 95^{o}C for 15 min, another denaturation for 35 cycles at 95^{o}C for 30 s and annealing at 42-52^{o}C for 30 s, extension at 72^{o}C for 1 min, and final extension at 72^{o}C for 10 min. PCR products were examined for subtype identification using gel electrophoresis. Positive sample of avian influenza A virus by Fujirebio Espline Influenza A&B-N testing and RT-PCR was negatively stained with 1.5% phosphotungstic acid (PTA) pH 6.8 and examined immediately in a Transmission Electron Microscope (JEOL2010LaB6 TEM, USA).

Preparation of tea crude extracts: Dried tea leaves (Three horses Co. Ltd, Thailand) and green tea powder (T Shi Jia Co. Ltd, China) was purchased from the supermarket.

Fifty grams of dried tea leaves or powdered green tea were extracted with 1000 ml of 95% ethanol for 24 h, followed by filtration. The extraction procedure was repeated 2 times and the extract was pooled and then taken to dryness under rotary evaporation to give a dark brown solid (1.854 mg) from dried tea leaves and dark green solid from green tea powder (2.562 mg). The extracts were dissolved and diluted with PBS to the tested concentrations.

Virus propagation inhibition assay

Virus propagation inhibition assay was carried out through embryonated chicken egg inoculation. One ml of dried tea leaves extract (5, 10, and 35 mg/ml) and green tea extract (100, 200, and 400 mg/ml) was incubated with 1 ml of virus suspension (2.86 x 10^{8} virus particles/ml) at 37^{o}C for 30 min and then 100 µl of the mixture was inoculated into each embryonated chicken egg and incubated at 37^{o}C for 4-5 days. The allantoic fluid was tested by HA test as previously described (Brauer and Chen, 2015).

Hemagglutination inhibition assay

Hemagglutination inhibition assay was employed to test the effect of tea extracts in virus adsorption to target cells. The tea extract solutions (25 µl) with 2-fold serial dilution with PBS were mixed with equal volume of influenza virus solution (200 HAU/25 µl). After incubation at room temperature for 30 min, 50 µl of the solution was mixed with equal volume of 1% chicken erythrocyte suspension and incubated at 4^{o}C for 30 min.

Total phenolic assay

Total polymeric phenol content was determined by the Folin-Ciocalteu method. Twenty microliters of 2-fold serial dilution of 30 mg/ml of dried tea leaves extract and 400 mg/ml of green tea extract was placed into 96-well plate and then mixed with 100 µl of diluted Folin-Ciocalteu reagent (1N). After 3 min of reaction, 80 µl of 10% Na_2CO_3 was added, and the mixture was incubated for 60 min at room temperature. The absorbance was measured at 760 nm with a Packard SpectraCount BS10000 microtiter plate reader (Hewlett Packard, USA) against a blank (20 µl distilled water, plus reagent). Gallic acid was used as the standard (r = 0.9979) (Kähkönen *et al*., 1999).

Cytotoxicity test by MTT assay

The effect of tea extracts on proliferation of HEK-293 cells was determined in 96-well plates (Nunc, USA) by MTT assay (Mosmann, 1983). Briefly, confluent cells in a 96-well plate were exposed to 50 µl/well of DMEM containing 2-fold serial dilution of 10 mg/ml of dried tea leaves extract and 200 mg/ml of green tea extract for 24 h in a CO_2 incubator. The culture medium was removed and 20 µl of 5 mg/ml MTT, 3-(4,5-dimethylthiozol-2-yl)-3,5-dipheryl tetrazolium bromide (Sigma, USA) solution was added to each well and incubated at 37^{o}C for 5 h. After removal of supernatant, 100 µl of DMSO was added for solubilization of formazan crystals and incubated for 30 min. The optical absorbance at 540 nm was measured by using a Packard SpectraCount BS10000 microtiter plate reader (Hewlett Packard, USA). Cell viability was estimated by comparing values of tea extracts with that of DMEM without tea extracts.

RESULTS AND DISCUSSION

We isolated avian influenza virus from the cloacal swabs of free-grazing ducks in Banglane district, Nakhon Pathom province, Thailand, in August 2007. Based on the results in immunoassay by Fujirebio Espline Influenza A&B-N, and RT-PCR, this avian influenza virus was identified as H5N2 influenza A virus (designated A/Free-Grazing-duck/Nakhon-Pathom/Thailand/1/07 (H5N2)).

Table 1. Primers used in this study

Target	Primer sequence (5' to 3')	Melting temperature (°C)	Amplicon size (bp)
H1	Forward primer : AAC AAY AAR GRG AAA GAA GT	46.69	467
	Reverse primer : GGG ACD TTY CTT ART CCT GT	52.17	
H2	Forward primer : GAG AAA RTW AAG ATT CTG CC	46.44	622
	Reverse primer : CCA AAC AAY CCY CTT GAY TC	52.27	
H3	Forward primer : CAR AAT GAR GTG ACH AAT GC	49.67	722
	Reverse primer : GGT GCA TCT GAY CTC ATT A	49.86	
H4	Forward primer : GCA GGG GAA ACA ATG CTA TC	53.92	770
	Reverse primer : CCW GGY TCT ACA ATW GTC C	50.96	
H5	Forward primer : ACA CAT GCY CAR GAC ATA CT	53.25	545
	Reverse primer : CTY TGR TTY AGT GTT GAT GT	48.01	
H6	Forward primer : AGC ATG AAT TTT GCC AAG AG	50.71	302
	Reverse primer : GGR CAT TCT CCT ATC CAC AG	53.65	
H7	Forward primer : GGG ATA CAA AAT GAA YAC TC	46.18	634
	Reverse primer : CCA TAB ARY YTR GTC TGY TC	49.99	
H8	Forward primer : GTG GAA ACA GAG AAA CAT	46	432
	Reverse primer : CCA TAA GAA RAT GAT GTC T	43.87	
H9	Forward primer : CTY CAC ACA GAR CAC AAT GG	53.81	488
	Reverse primer : GTC ACA CTT GTT GTT GTR TC	49.93	
H10	Forward primer : GGA CAA AAY TTC CCT CAG AC	48.36	412
	Reverse primer : GRA AAG GGA GCT TTG TAT TT	51.95	
H11	Forward primer : TGY TCM TTT GCT GGR TGG AT	55.52	450
	Reverse primer : CTC TGA ACC CAC TGC TAC AT	54.18	
H12	Forward primer : AGG GGT CAC AAT GGA AAA A	51.13	421
	Reverse primer : GGT GAA ATC AAA CAT CTT CA	47.11	
H13	Forward primer : CCA CAC AGG AAC ATA YTG TTC	52.06	231
	Reverse primer : CTA CTG AAW GAY CTG ATT CC	48.02	
H14	Forward primer : TCA TCG CCG AAC AAT TCA CC	55.72	543
	Reverse primer : GCA GTT TCC TAT AGC AAT CC	50.42	
H15	Forward primer : GTG CGT GTA AGA GAA CAG TG	53.54	383
	Reverse primer : ATT AGA GCG GAG AAA GGT GG	54.23	
N1	Forward primer : TTG CTT GGT CAG CAA GTG CA	57.94	615
	Reverse primer : TCT GTC CAT CCA TTA GGA TCC	53.33	
N2	Forward primer : ATG GTC CAG CTC AAG TTG TCA	56.33	434
	Reverse primer : TCC AGT TAT GTG TGC TCA GG	54.42	

The virus particles seen by negative stain electronmicroscopy in allantoic fluid of embryonated chicken egg inoculation had the characteristic appearances of influenza virus (Fig. 1). Viruses of the same subtype have been found among avian species in several countries, including the United States (Lee *et al*., 2004), Mexico (Garcia *et al*., 1996), Italy (Donatelli *et al*., 2001), Nigeria (Gaidet *et al*., 2008), China (Duan *et al*., 2007), Taiwan (Cheng *et al*., 2010; Soda *et al*., 2011; Lee *et al*., 2014) and Japan (Okamatsu *et al*., 2007). However, This virus was also isolated from swine in South Korea (Lee *et al*., 2009). It is presently believed that only strains with H5 or H7 subtype hemagglutinins become highly pathogenic avian influenza viruses (HPAIVs) during extensive infections in chicken populations (Ito *et al*., 1998). H5N2 HPAIVs have caused three large outbreaks in poultry: in Pennsylvania in 1983 (Capua *et al*., 2003; Kishida *et al*., 2004), in Mexico from 1994 to 1995 (Horimoto *et al*., 1995; Garcia *et al*, 1996) and Italy from 1997 to 1998 (Donatelli *et al*., 2001; Capua *et al*., 2003). However, some strains of H5N2 have been reported as low pathogenic avian influenza viruses (LPAIVs). H5N2 LPAI Vs have become endemic in Central America since 1994, despite eradication programs in combination with vaccination (Lee *et al*., 2004; Nguyen *et al*., 2005). LPAIVs, A/chicken/Taiwan/1209/2003 (H5N2) (Taiwan03) and A/chicken/Taiwan/K703-1/2008 (H5N2) (Taiwan08), were isolated from apparently healthy chickens during routine surveillance in Taiwan (Cheng *et al*., 2010). At the end of May 2005, LPAIV, A/chicken/Ibaraki/1/2005 (H5N2) (Ibaraki05), was isolated from chicken in Japan (Okamatsu *et al*., 2007). In this study, we reported another LPAIV, A/Free-Grazing-duck/Nakhon-Pathom/Thailand/1/07 (H5N2) which was isolated from healthy free-grazing duck for the first time in Thailand.

A previous study found that free-grazing ducks and wild birds share the same habitats, which may increase risks of influenza A virus transmissions between their populations (Cappelle *et al*., 2014). Identical LPAIVs have been reported in both wild birds and domesticated ducks (Duan *et al*., 2011). It was found that many wildbird species, including little egret, open-bill stork, white-breasted waterhen, lesser-whistling duck, swallows and the others share feeding areas with free-grazing duck flocks in rice-paddy fields. Another possible source of influenza A virus transmissions, transport trucks, is possible. The free-grazing duck flocks were moved from one area to another by rented multi-level trucks that are regularly shared with other free-grazing duck flocks. Because they transport multiple free-grazing duck flocks, the rental truck may become contaminated and spread influenza A viruses from one flock to another. It has been reported that HPAIV-H5N1 infections in wildbirds in Thailand have been documented (Siengsanan *et al*., 2009). Influenza A virus subtype H12N1 was previously isolated from watercocks and lesser-whistling ducks (Wongphatcharachai *et al*., 2012), and influenza A virus subtype H3N8 and H4N6 were previously isolated from free-grazing ducks (Boonyapisitsopa *et al*., 2016), in December 2010 to April 2011, influenza A virus subtype H1N3 and H1N9 were also isolated from free-grazing ducks

(Chaiyawong *et al*., 2016). The influenza A virus infection among these avian may affect on dynamic influenza virus gene pooling, and new viruses are created by reassortment events that are very likely to occur in the field, exemplified by H5 viruses from south-eastern China (Duan *et al*., 2007).

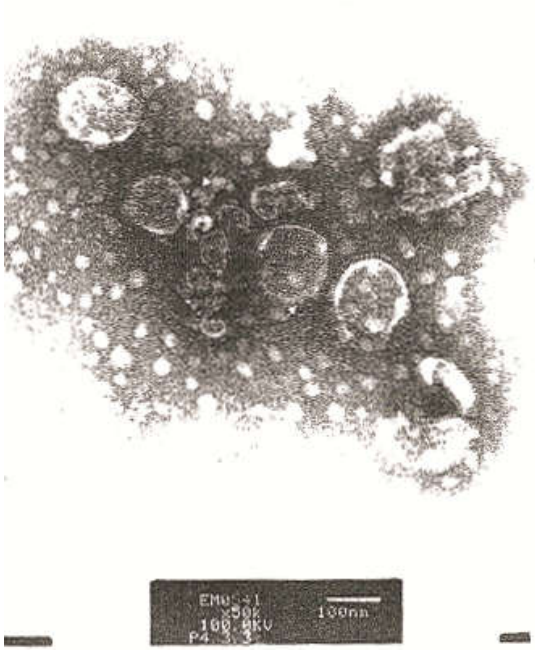

Figure 1. Electronmicrographs of avian influenza virus particles from the allantoic fluid of embryonated chicken egg inoculation showing the morphological structure of influenza virus. Arrows show the hemagglutinin and neuraminidase spikes on the envelope. Magnification x 150000. Bar = 100 nm

It also is possible that following transmission, successive infections of susceptible host was clinical or subclinical. Subsequently to successful cross-species transmission, spreading within the new host population usually requires a period of adaptation of the virus to that new host (Webster *et al*., 1992). Such features of the avian-swine H5N2 influenza A virus (Lee *et al*., 2009) could be considered a potential model for pandemic highly pathogenic avian influenza (e.g. H5N1 and H7N7) virus outbreaks, in which viruses that were previously no transmissible in a new host (e.g., human) could also gain selective advantage by genetic reassortment with other strains of different subtype due to coinfection and through accumulated gene mutations. Although there are no known clinical implications of the avian-swine reassortment virus for pathogenicity to other species, but the efficient transmissibility of the relatively avian-swine-adapted virus could facilitate virus spread, and association with disease outbreaks among avian-swine populations could also be possible. Thus, it raises concerns for continued surveillance of another atypical influenza virus in avian that may have the potential to cross host-species barriers. As early as 1949, Green *et al*. reported the antiviral activity of tea extracts against influenza virus (Green *et al*., 1949). We analyzed the effect of tea extracts on virus propagation at various concentration in embryonated chicken eggs. The virus yields were determined by hemagglutination test. As shown in Fig. 2, The virus yields were obtained only in control, while no virus was detected in any dilution of tea extract treatments. Furthermore, we investigated the effect of tea extracts on adsorption of influenza A virus to chicken erythrocytes by hemagglutination inhibition test. Interestingly, as expected both tea extracts inhibited viral binding to the cells, and green tea extract (2500 μg/ml) inhibited viral binding better than dried tea leaves extract (1250 μg/ml), as shown in Fig. 3. These data suggest that tea extracts affect the early step (viral adsorption) of influenza virus infection. In addition, we analyzed the total phenolic contents in tea extracts.

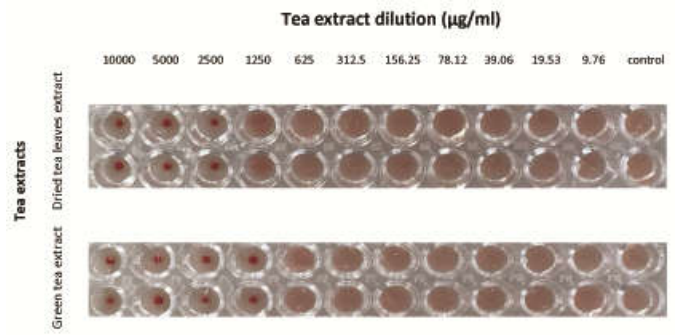

Figure 2. Inhibitory effects of tea extracts on virus propagation at various concentration in embryonated chicken eggs. The viral hemagglutination-mediated chicken erythrocyte agglutination was monitored after incubation at 4°C for 30 min. (A) Dried leaf tea extract and control. (B) Green tea extract

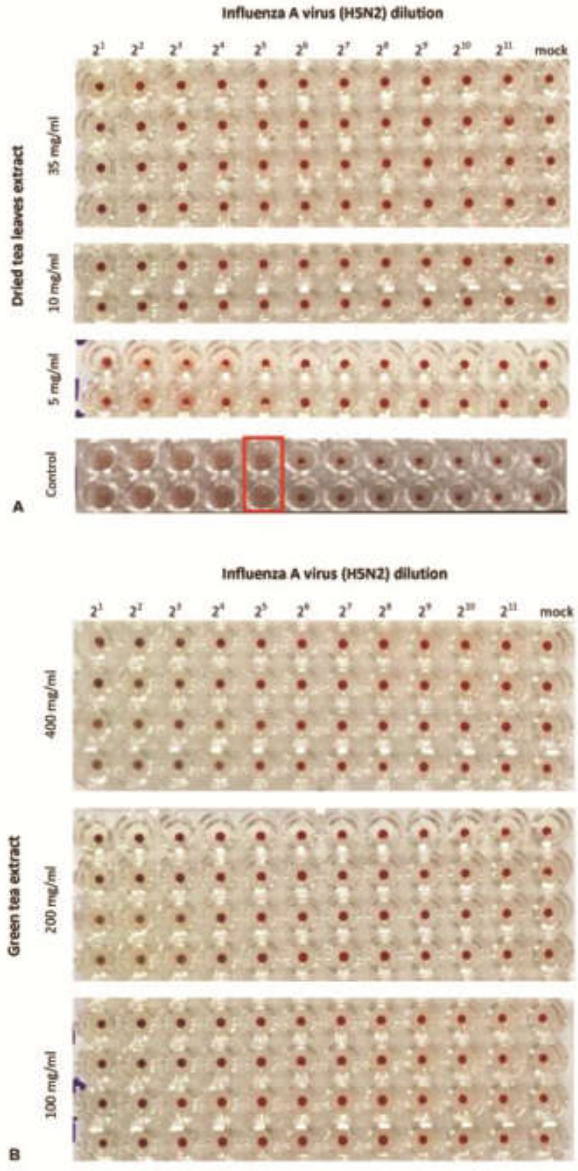

Figure 3. Inhibitory effects of tea extracts on adsorption of influenza virus to chicken erythrocytes. Two hundred-HA units of virus was incubated with an equal volume of serially diluted tea extracts in PBS for 30 min. Chicken erythrocytes were then added to each well with equal volume. The viral hemagglutination-mediated chicken erythrocyte agglutination was monitored after incubation at 4°C for 30 min

It was found that total phenolic contents in green tea extract and dried tea leaves extract was 491 and 470 mg/g of gallic acid equivalents, respectively. These results support the other reports that tea extract prevented infectivity of influenza virus by content of polyphenols (Nakayama *et al*., 1993; Imanishi *et al*., 2002; Song *et al*., 2005; Noguchi *et al*., 2008; Yang *et al*., 2014). In 1993, Nakayama's research group demonstrated the effects of EGCG against influenza A and B viruses (Nakayama *et al*., 1993). They found that the infection of both influenza A and influenza B virus was inhibited by EGCG. Moreover, EGCG exerted agglutination effects on virions and prevented the virus from absorbing onto the cell surface. Imanishi *et al*. further revealed that the anti-influenza activity of green tea extracts that included EGCG possibly arose from its inhibitory effects on the acidification of endosomes and lysosomes (Imanishi *et al*., 2002). Since, EGCG have been reported toxic to erythrocytes at concentrations above 50 μM, so its inhibitory effect on the activity of viral hemagglutination is not permissible at higher concentration (Kim *et al*., 2013). In this study, the highest concentration of both tea extracts (10000 μg/ml) was not toxic to chicken erythrocytes (Fig. 3). However, we evaluated the cytotoxicity of tea extracts by MTT assay on HEK-293 cells. The estimated doses that reduced cell viability about 50% in green tea extract and dried tea leaves extract were 1765.25 and 283.35 mg/ml, respectively. The results showed that green tea extract has lower toxicity than dried tea leaves extract. The viabilities of the all test set were at least 25% at the highest dose tested (100-400 mg/ml in green tea extract and 5-35 mg/ml in dried tea leaves extract).

Conclusion

In August 2007, we isolated H5N2 avian influenza A virus from the cloacal swabs of healthy free-grazing ducks in Banglane district, Nakhon Pathom province, Thailand. We found that tea extracts inhibited virus propagation on viral attachment of host cells and the antiviral activity of phenolic compounds in tea extracts are associated with viral adsorption stage. This inhibitor may provide a new approach to prevent influenza A virus infection, especially in pandemic area.

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