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Journal of the Chinese Medical Association 81 (2018) 458–468

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Original Article

Anti-pandemic influenza A (H1N1) virus potential of catechin and gallic acid

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Received July 5, 2017; accepted September 1, 2017

Abstract

Background: The pandemic influenza A (H1N1) virus has spread worldwide and infected a large proportion of the human population. Discovery of new and effective drugs for the treatment of influenza is a crucial issue for the global medical community. According to our previous study, TSL-1, a fraction of the aqueous extract from the tender leaf of *Toonasinensis,* has demonstrated antiviral activities against pandemic influenza A (H1N1) through the down-regulation of adhesion molecules and chemokine to prevent viral attachment.
Methods: The aim of the present study was to identify the active compounds in TSL-1 which exert anti-influenza A (H1N1) virus effects. XTT assay was used to detect the cell viability. Meanwhile, the inhibitory effect on the pandemic influenza A (H1N1) virus was analyzed by observing plaque formation, qRT-PCR, neuraminidase activity, and immunofluorescence staining of influenza A-specific glycoprotein.
Results: Both catechin and gallic acid were found to be potent inhibitors in terms of influenza virus mRNA replication and MDCK plaque formation. Additionally, both compounds inhibited neuraminidase activities and viral glycoprotein. The 50% effective inhibition concentration (EC_{50}) of catechin and gallic acid for the influenza A (H1N1) virus were 18.4 μg/mL and 2.6 μg/mL, respectively; whereas the 50% cytotoxic concentrations (CC_{50}) of catechin and gallic acid were >100 μg/mL and 22.1 μg/mL, respectively. Thus, the selectivity indexes (SI) of catechin and gallic acid were >5.6 and 22.1, respectively.
Conclusion: The present study demonstrates that catechin might be a safe reagent for long-term use to prevent influenza A (H1N1) virus infection; whereas gallic acid might be a sensitive reagent to inhibit influenza virus infection. We conclude that these two phyto-chemicals in TSL-1 are responsible for exerting anti-pandemic influenza A (H1N1) virus effects.

Keywords: Catechin; Gallic acid; Pandemic influenza A (H1N1) virus; *Toonascinensis*; TSL-1

1. Introduction

In April 2009, the novel pandemic influenza A (H1N1) virus was identified in Mexico and quickly spread throughout the world with extensive morbidity and unacceptable mortality rates.[1] The influenza virus presents a global health threat, associated with a high risk of severe complications since the virus circulates not only within but also between numerous host

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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https://doi.org/10.1016/j.jcma.2017.11.007

populations. The virus is transmitted mainly through coughing, sneezing, and close bodily contact. Thus far, vaccination is the major resolution strategy to control seasonal influenza viral pandemics. At present, two major classes of antiviral drugs, oseltamivir and amantadine, are commonly used as the prophylaxis and for the treatment of influenza virus infection.[2] Oseltamivir, a neuraminidase inhibitor, inhibits the spread of the virus and the release of virus progeny from infected cells. While amantadine blocks the M2 protein in order to inhibit viral uncoating from the infected cells. These drugs target on a highly variable sequence, as a consequence, the virus can easily acquire resistance by mutating the drug targets. Recently, increasing resistance of influenza A viruses to amantadine and oseltamivir has been reported.[3] The discovery of new and effective drugs for preventing and/or treating influenza infections is a crucial challenge for the global medical community.

Natural products, derived from traditional Chinese medicine, have been found to exert antiviral effects against the influenza virus.[4] The tender leaves of *Toonasinensis* (TS) are commonly regarded as a nutritional supplement, or a vegetable common for consumption in Taiwan. As reported,[5-8] TS extracts have been used to treat various diseases and have been shown to have a variety of effects, including glycemic control, inhibition of lipid accumulation, antimicrobial activity and anti-cancer. Many compounds, including retinoids, catechin, gallic acid, kaempferol, methyl gallate, quercetin, afzelin, quercitrin, isoquercitrin, and rutin have been isolated from the leaves of TS.[6] According to our previous study, TSL-1 has been verified to inhibit attachment activity towards pandemic influenza A (H1N1) through down-regulation of adhesion molecules to prevent virus infection.[9] As of yet, it is unclear which major compounds in TSL-1 execute the anti-influenza A (H1N1) virus activity. The aim of this study was to evaluate those chemical compounds present in TSL-1, including catechin, gallic acid, kaempferol, quercetin, and rutin, to examine the inhibition of pandemic influenza A(H1N1) virus replication in a cell model. Herein, we have further characterized the anti-influenza viral activities of two major compounds present in TSL-1, catechin and gallic acid.

2. Methods

2.1. Chemicals

The catechin and rutin trihydrate were purchased from Fluka (St. Louis, MO, USA). Three compounds, including gallic acid, kaempferol, and quercetin hydrate were purchased from Sigma–Aldrich Chemical Company (St. louis, MO, USA). The chemicals were dissolved in DMSO to a final stock concentration of 100 mM and stored at −80 °C. Each compound was serially diluted to various concentrations by using complete MEM.

2.2. Cells and virus preparation

The Madin Darby canine kidney (MDCK) cells and human lung carcinoma (A549) cells were purchased from ATCC (Manassas, VA, USA). Both cell lines were propagated in MEM medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heated-inactivated FBS, 250 ng/mL amphotericin B, 100 μg/mL penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). The pandemic influenza A (H1N1) virus (A/California/07/2009) was isolated from the 2009 outbreak and obtained at the Clinical Virology Laboratory, Kaohsiung Chang Gung Memorial Hospital, Taiwan. The virus were propagated in MDCK cells and assessed by plaque titration. The viral stocks were prepared as described elsewhere.[9] Virus titer was determined by cytopathic effect (CPE) and expressed as TCID50 (50% tissue culture infective dose).

2.3. Cytotoxicity assay

The cytotoxic effects of TSL-1 and chemical compounds on the proliferation of A549 cells were determined in 96-well plates using XTT assay (tetrazolium hydroxide salt) according to the manufacturer's instructions (Roche Molecular Diagnostics, Germany). Briefly, cells (1×10^4 cells/well) were plated for an incubation period of 24 h. Then, various concentrations of TSL-1(0–100 μg/mL) or chemical compounds were supplemented immediately. After incubation at 35 °C with 5% CO_2 for 3 days, XTT reagent was added and incubated for 4 h. The absorbance of the resulting solution was measured spectrophotometrically at A450 nm (Sunrise, TECAN) with a reference of A620 nm. Each experiment was carried out in triplicate and performed at least thrice separately. The cytotoxic concentration of TSL-1 or chemical compounds which reduced the cells' viability by 50% (CC_{50}) was calculated by regression analysis of the dose response curves generated from these data. The untreated control was set as 100%.

2.4. Virus infection

Monolayer of A549 cells at a concentration of 3×10^5 cells/mL were infected with the pandemic influenza A (H1N1) virus at 1.5 multiplicity of infection (MOI). After 1 h, the solution was removed; the cells were washed twice with phosphate buffer saline (PBS) and supplemented with growth media containing the five chemical compounds at different concentrations. Amantadine was used as a positive control. Cells were harvested at 72 h after-infection, and the viral yield was estimated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), neuraminidase activity assay and plaque assay on MDCK cells. As a vehicle control, the infected cells incubated in chemical-free medium were included throughout the experiment.

2.5. Plaque assay

Monolayer of A549 cells at a concentration of 3×10^5 cells/mL were infected with the pandemic influenza A (H1N1) virus at 1.5 MOI for 1 h, and then replaced with medium containing TSL-1 or chemical compounds of various concentrations. Viruses were harvested at 72 h post-infection using a process through

three cycles of freezing and thawing, and clarified by low speed centrifugation (500 g for 10 min). Virus yields in the culture supernatants were assayed by the standard plaque assay in MDCK cells to determine the actual infectious viral titer. Briefly, confluent monolayer MDCK cells cultured in a 6-well tissue culture plate (2×10^5 cells/cm^2) were infected with the supernatants. After 60 min for virus incubation, the inoculums were removed and replaced with overlaying medium (MEM containing 2 μg/ml trypsin, 5% low melting agarose, without serum) that contained TSL-1 or chemical compounds at different concentrations. After incubating cultures for 72 h at 35 °C with 5% CO_2, monolayer was fixed with 4% formaldehyde solution for 60 min. The agarose was then removed by flowing water and stained with 1% (w/v) crystal violet solution. Assays were performed in 6-well plates in triplicate. Viral titers were determined by counting the number of plaques. Virus plaques were counted by Quantity One version 4.6.5 (Bio-Rad Laboratories) examination and percentage of plaque inhibition was calculated as relative to the untreated control. A required concentration to reduce the 50% plaque number (EC_{50}) was calculated by regression analysis of the dose–response curves generated from these data.

2.6. Quantification of pandemic influenza A (H1N1) virus

Monolayer of A549 cells at a concentration of 3×10^5 cells/mL were infected with the pandemic influenza A (H1N1) virus at 1.5 MOI. After 1 h, the solution was removed; the cells were washed twice with PBS and supplemented with growth media containing TSL-1 or chemical compounds at different concentrations. Amantadine was used as a positive control. Cells were harvested at 72 h post-infection, and the viral yield was estimated by qRT-PCR. As a vehicle control, the infected cells incubated in chemical-free medium were included throughout the experiment. Total nucleic acids were extracted using the High Pure RNA kit (Roche Molecular Diagnostics, Germany) following the manufacturer's instructions. All qRT-PCR assays were performed using standard precaution to avoid contamination. Quantification RT-PCR was performed using the TaqMan® Fast 1-Step Kit (Invitrogen, San Diego, CA) with primer, as previously described.[9]

2.7. Neuraminidase activity (NA) assay

Following the treatment procedures of the above experiments, the NA activity assay was performed according to the manufacturer's instructions using the Neuraminidase activity Assay kit (Abcam, CA, USA). The reaction was monitored by using a spectrometer in the kinetic mode at an excitation wavelength of 535 nm and emission wavelength of 590 nm.

2.8. Immunofluorescence staining of influenza A specific glycoprotein

A549 cells (3×10^5 cells/mL) grown on a tissue culture slide were cultured at 37 °C for 24 h. The cells were washed with PBS and infected with pandemic influenza A (H1N1) virus at 1.5 MOI. After 1 h, the solution was removed; then the cells were washed twice with PBS and supplemented with growth media contained either catechin or gallic acid at different concentrations. After incubating cultures for 72 h at 35 °C with 5% CO_2, cells were washed with PBS three times and fixed with acetone. After blocking, fixed cells were incubated with IMAGEN™ FITC conjugated anti-influenza A monoclonal antibody in conjunction with Evans blue contrast stain (OXOID, Hampshire, UK). Cell count was performed using an Olympus-BX50 fluorescence microscope (Olympus, Tokyo, Japan).

2.9. Statistical analysis

The data were presented as the means ± standard deviation (SD). The data were statistically evaluated using either Student's *t*-test or analysis of variance (ANOVA). A level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of five chemical compounds against pandemic influenza A (H1N1) virus infection

The cytotoxic effect of pandemic influenza A (H1N1) virus with five chemical compounds was screened by XTT method. The viability was shown in percent compared to control cells without treatment. A549 cells were infected with the virus at an MOI of 1.5 with media in the absence or presence of the chemicals for a period of 3 days. Cell viability was measured after treatment with XTT for 4 h. Amantadine (10 μg/mL) was used as a positive control. As shown in Fig. 1, the CC_{50} of those five compounds were as below. The CC_{50} of catechin (Fig. 1A), kaempferol (Fig. 1C), quercetin hydrate (Fig. 1D) and rutintrihydrate (Fig. 1E) were more than 100 μg/mL. The CC_{50} of gallic acid (Fig. 1B) was at 22.1 ± 0.6 μg/mL. The EC_{50} of catechin might be around 10–25 μg/mL (Fig. 1A). The EC_{50} of gallic acid will be around 1–5 μg/mL (Fig. 1B). Our results demonstrated that catechin and gallic acid exerted protective effects against pandemic influenza A (H1N1) virus infection with a dose-dependent manner under 100 μg/mL and 10 μg/mL treatment, respectively. The other three compounds also had a protective effect against the pandemic influenza A (H1N1) virus, but not as prominent or dose-dependent, in comparison with catechin and gallic acid. Based on the above findings, we selected catechin and gallic acid for further investigation.

3.2. Inhibitory effects of catechin and gallic acid on plaque formation by pandemic influenza A (H1N1) virus in MDCK cells

To further evaluate the anti-pandemic influenza A (H1N1) virus activity, we employed the plaque assay to test the in vitro effect of catechin and gallic acid on viral replication. We found that these two compounds exhibited inhibitory effects

against pandemic influenza A (H1N1) viral infection with dose-dependent manner. The results of the plaque assays showed an approximately 50% reduction of pandemic influenza A (H1N1) viral production upon treatment with 25 μg/mL of catechin (EC_{50} = 18.4 ± 0.7 μg/mL) and 2.5 μg/mL of gallic acid (EC_{50} = 2.6 ± 0.07 μg/mL), as shown in Fig. 2.

3.3. Inhibitory effects of catechin and gallic acid on pandemic influenza A (H1N1) virus replication in A549 cells

The A549 cells were infected with pandemic influenza A(H1N1) virus and incubated for 72 h in the presence of various concentrations of catechin and gallic acid. Total RNAs were isolated from infected A549 cells and qRT-PCR analysis was performed using specific primers for viral M gene RNA. The inhibitory effect on viral genome load in infected A549 was shown by log10 copy number decrement in treatments that were calculated through absolute quantification. As illustrated in Fig. 3, catechin and gallic acid significantly reduced the number of infected A549 cells at an MOI of 1.5. The qRT-PCR results demonstrated significant inhibition of viral RNA synthesis in infected A549 cells at concentrations greater than 5 μg/mL of catechin (Fig. 3A) and 1 μg/mL of gallic acid (Fig. 3B) treatments, in comparison with control group.

3.4. Inhibitory effects of catechin and gallic acid on NA activity

NA is a key viral protein responsible for releasing newly produced virus particles and the recognition and cleavage of target cell receptor sialic acid moieties (N-acetylneuraminic acid) on infected cells.[10] We performed NA assay to explore whether catechin or gallic acid inhibited virus replication through targeting NA activity. The virus-only control showed NA activity prominently. However, the NA activity was significantly reduced in the presence of these two chemicals, compared to the vehicle control (Fig. 4A and B). The results suggest that catechin and gallic acid had inhibitory effects on the releasing step of the pandemic influenza A (H1N1) virus through NA inactivation.

3.5. Inhibitory effects of catechin and gallic acid on virus infection

To investigate whether catechin and gallic acid could affect infection of cells by the virus, an immunofluorescence staining assay on influenza A virus glycoprotein was performed. Normal cells showed red color (Figs. 5A and 6A), while extensive specifically green fluorescence was observed in the virus control cells (Figs. 5B and 6B). However, green fluorescence was decreased in a dose-dependent manner when cells were treated with catechin (Fig. 5C–G) and gallic acid (Fig. 6C–G). Overall, catechin and gallic acid exhibited obvious anti-influenza A virus activity, as shown in Figs. 5H and 6H.

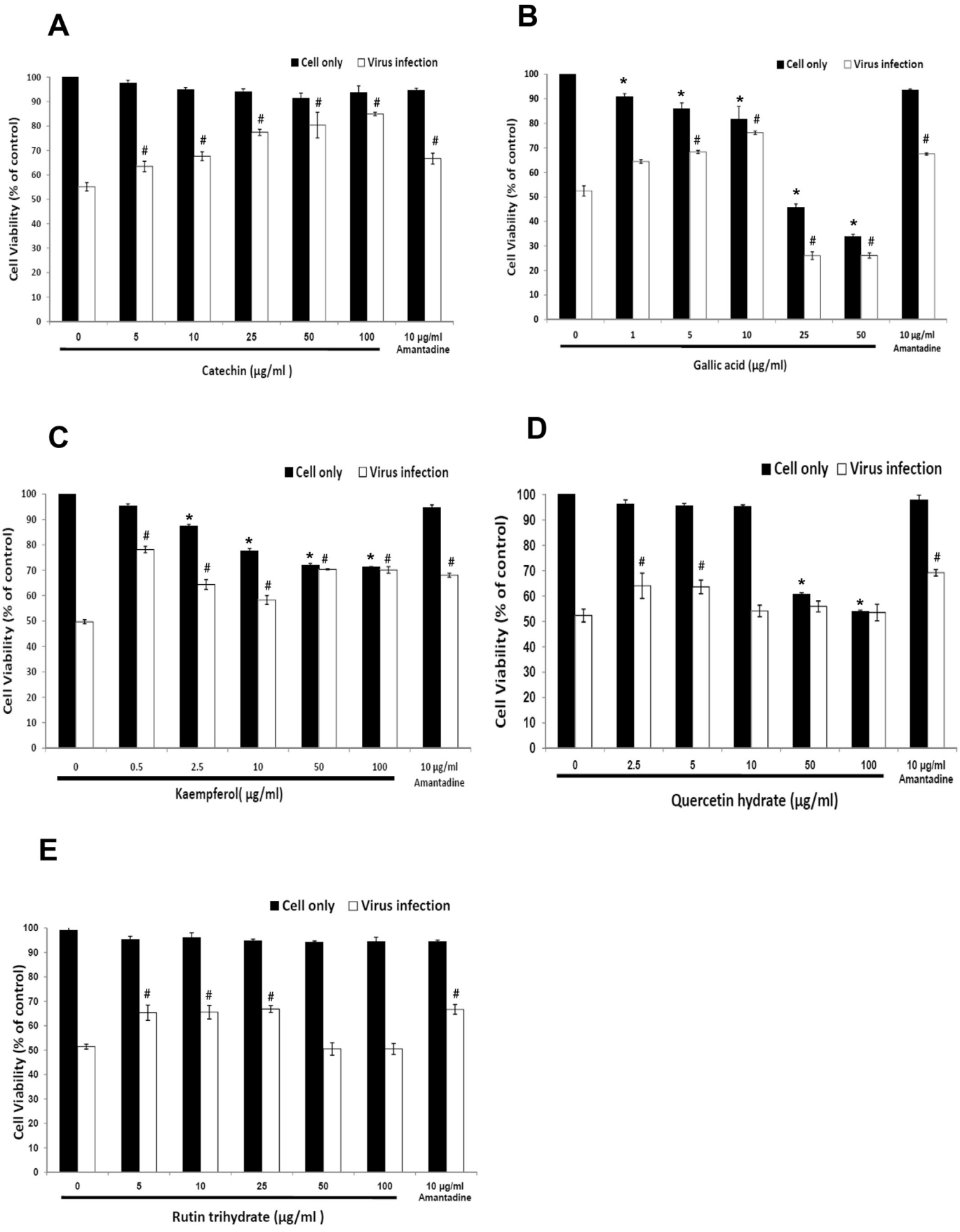
3.6. Selectivity index against pandemic influenza A (H1N1) virus of five chemicals

To summarize the anti-virus effects of these five compounds on the growth of influenza virus particles, the estimated doses that reduced cell viability by about 50% are shown in Table 1. The 50% cytotoxic concentrations (CC_{50}) of the chemical compounds were calculated. The selectivity index (SI) was defined as the ratio of CC_{50}: EC_{50} to indicate therapeutic safety. The results of the analysis showed that of the five chemicals analyzed, catechin, kaemperol, quercetin and rutin had the lowest toxicity. However, only catechin demonstrated anti-viral infection effects with EC_{50} of 18.4 ± 0.7 μg/mL, while the other three chemicals were not applicable. As for gallic acid, it also exerted effects against pandemic influenza A(H1N1) viral infection, with EC_{50} of 2.6 ± 0.07 μg/mL. The CC_{50} of gallic acid was 22.1 ± 0.6 μg/mL, indicating that gallic acid had the highest toxicity of MDCK cells infected by pandemic influenza A (H1N1) virus, with SI 8.6 ± 0.5.

4. Discussion

Influenza viruses generally infect approximately 10% of the global population annually, and are associated with approximately 250,000 deaths each year.[11] The influenza A (H1N1) virus is a novel and highly contagious pathogen for humans, associated with a high risk of severe complications, and first identified in Mexico in 2009, causing seasonal epidemics and pandemics. Oseltamivir (Tamiflu) and zanamivir (Relenza) are approved for use against Type A and Type B influenza infections. However, the development of drug resistance due to the use of insufficient doses or inadequate courses of therapy, may limit the future clinical applications of these drugs.[12] Based on the limitations of health care resources and the high cost of current antiviral drugs, in addition to the development of drug resistance, the need to develop new anti-influenza treatments is of utmost importance to the global community. Chinese herbs are traditionally used for preventing and treating influenza in Taiwan, especially in rural regions. Recently, *Isatisindigotica* (Ban-Lan-Gen) has been reported to effectly inhibit the attachment ability of influenza A and B viruses by interfering with the viral particles of the host cell surface.[5,13] *Paeonialactiflora* has also been demonstrated to inhibit influenza A/WSN/33 (H1N1) infection, both in vitro and in vivo, through viral hemagglutination with viral binding and penetration into host cells.[14] Furthermore, *Mosladianthera* has exhibited therapeutic effects in influenza A virus-infected mice by suppressing the viral replication and inflammatory mediators, and by boosting the antioxidant potential to prevent subsequent pneumonia occurrence.[15]

TSL-1 is a fraction of the aqueous extract from the tender leaf of TS, a vegetable commonly consumed by Taiwanese in daily life. Our previous study found that TSL-1 had the effect of inhibiting the coronavirus, the life-threatening and highly contagious virus which induces severe acute respiratory syndrome (SARS).[16] Meanwhile, TSL-1 inhibits influenza A


A
Cell only
Virus infection
Cell Viability (% of control)
0 5 10 25 50 100 10 μg/ml Amantadine
Catechin (μg/ml)
B
Cell only
Virus infection
Cell Viability (% of control)
0 1 5 10 25 50 10 μg/ml Amantadine
Gallic acid (μg/ml)
C
Cell only
Virus infection
Cell Viability (% of control)
0 0.5 2.5 10 50 100 10 μg/ml Amantadine
Kaempferol(μg/ml)
D
Cell only
Virus infection
Cell Viability (% of control)
0 2.5 5 10 50 100 10 μg/ml Amantadine
Quercetin hydrate (μg/ml)
E
Cell only
Virus infection
Cell Viability (% of control)
0 5 10 25 50 100 10 μg/ml Amantadine
Rutin trihydrate (μg/ml)

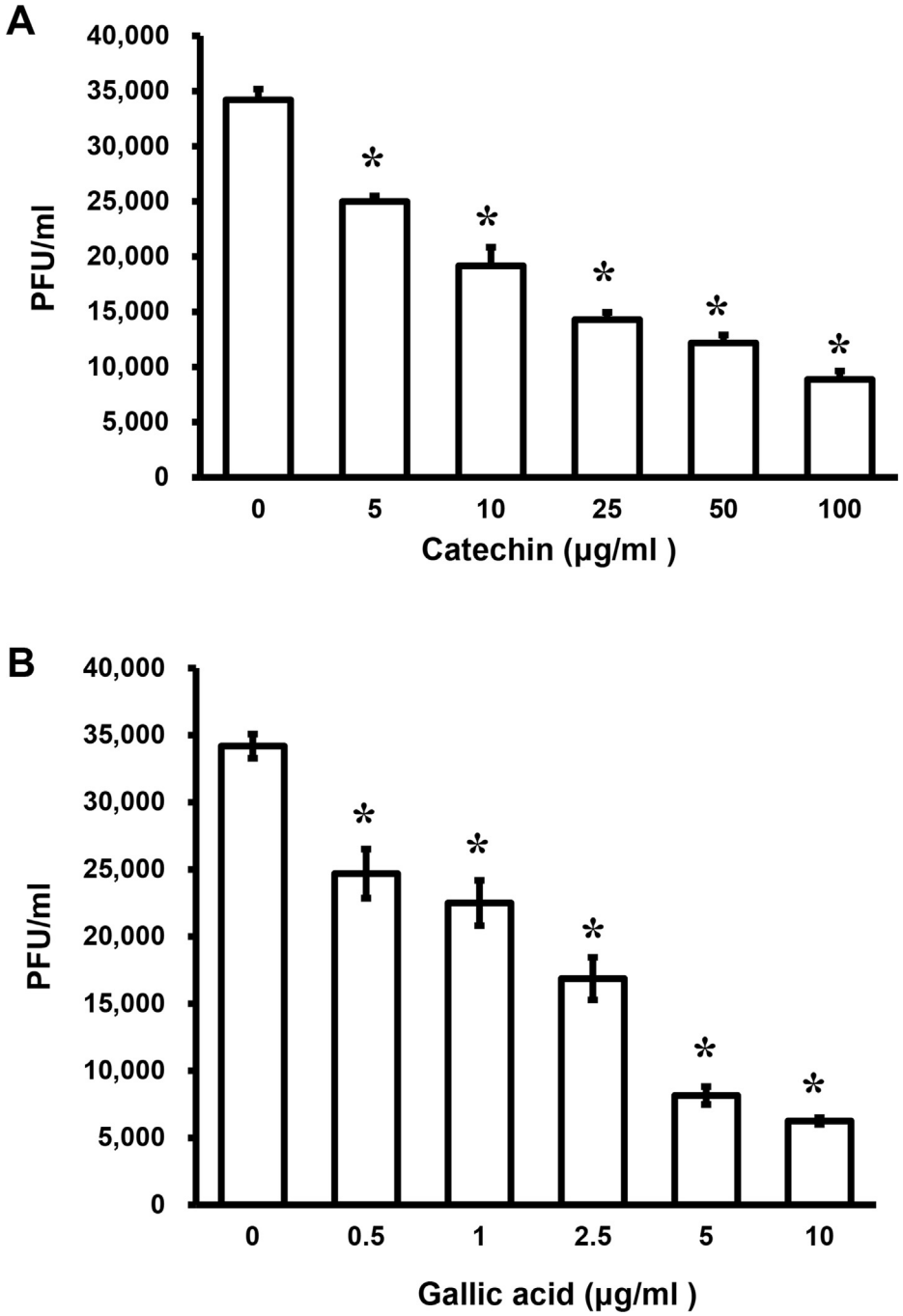


Fig. 2. Catechin and gallic acid inhibited influenza virus propagation. Pandemic influenza A(H1N1) virus at an MOI of 1.5 was inoculated in A549 cells. After 1 h, viruses were removed. A549 cells were treated with (A) catechin (5−100 μg/mL), (B) gallic acid (0.5−10 μg/mL) individually. The cultures were incubated for 3 days at 37 °C under 5% CO_2. The yield of progeny viruses in A549 cells was determined by plaque titrations assay on MDCK cells. Each concentration of chemicals was analyzed in triplicate. The results represented the mean ± SD of three independent assays. Asterisk (*) indicates significant difference (p <0 .05) compared to the vehicle control respectively.

(H1N1) virus attachment through significant down-regulation of adhesion molecules and chemokines, including VCAM-1, ICAM-1, E-selectin, IL-8, and fractalkine.[9] This aqueous extract of TS was characterized by HPLC. Nine compounds present in TS, gallic acid, methyl gallate, catechin, kaempferol, kaempferol 3-O-β-D-glucoside, quercetin, quercitrin,

Fig. 1. Cytotoxicity assay of five chemical compounds. A549 cells were infected with or without virus at an MOI of 1.5 at various concentrations of each chemical compound. Cell viability was measured after 3 days by using XTT viability assay. Five chemical compounds included (A) catechin, (B) gallic acid, (C) kaempferol, (D) quercetin, and (E) rutin. To normalize, the relative viability in untreated cells was set as 100%. The results represented the mean ± SD of three independent assays. Asterisk and pound (*, #) indicate significant difference ($p < 0.05$) compared to the control group without or with the treatment of virus infection, respectively.

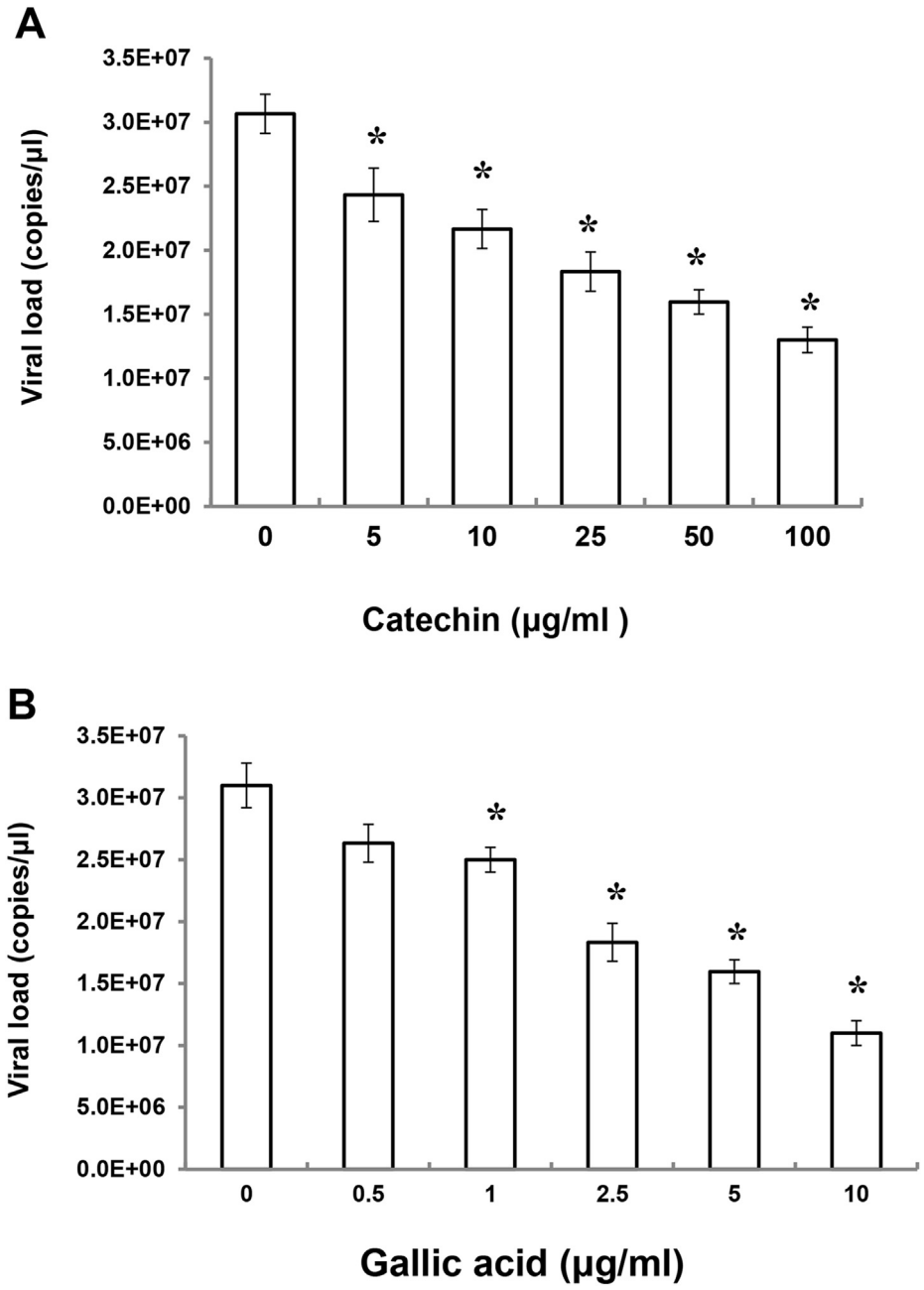


Fig. 3. Anti-viral assay on catechin and gallic acid. A549 cells were infected with Pandemic influenza A (H1N1) virus. After 1 h, viruses were removed. A549 cells were treated with (A) catechin (5−100 μg/mL) and (B) gallic acid (0.5−10 μg/mL), or untreated as a vehicle control. Total RNA extraction was performed at 3 days after influenza virus infection and the levels of intracellular influenza viral RNA replication was measured by qRT-PCR. Each concentration of chemicals was assayed in triplicate. The results represented the mean ± SD of three independent assays. Asterisk (*) indicates significant difference ($p < 0.05$) compared to the vehicle control, respectively.

quercetin 3-O-β-D-glucoside, and rutin, have previously been reported as exerting positive effects in terms of disease treatment .[17] Thus, we chose the major five compounds, catechin, gallic acid, kaempferol, quercetin, and rutin, which are all commercially available, to further investigate their anti-influenza A (H1N1) virus effects. Analyses found that both catechin and gallic acid had protective effects. The present study demonstrates that gallic acid is effective at inhibiting influenza A (H1N1) virus, but is toxic to A549 cells, in comparison with catechin. While catechin demonstrates an inhibitory effect against the influenza A (H1N1) virus, it is also more promising in terms of safety. The other three major compounds are either cytotoxic to A549 cells, or offer less protective effects against influenza A (H1N1) virus infection.

Catechin is the major chemical compound present in green tea, one of the most widely consumed beverages in the world and which provides a variety of health benefits.[18,19] Studies have reported that green tea catechins have several anti-influenza virus activities, both in vivo and in vitro. Matsumoto *et al* found that consuming catechin/theanine for 5

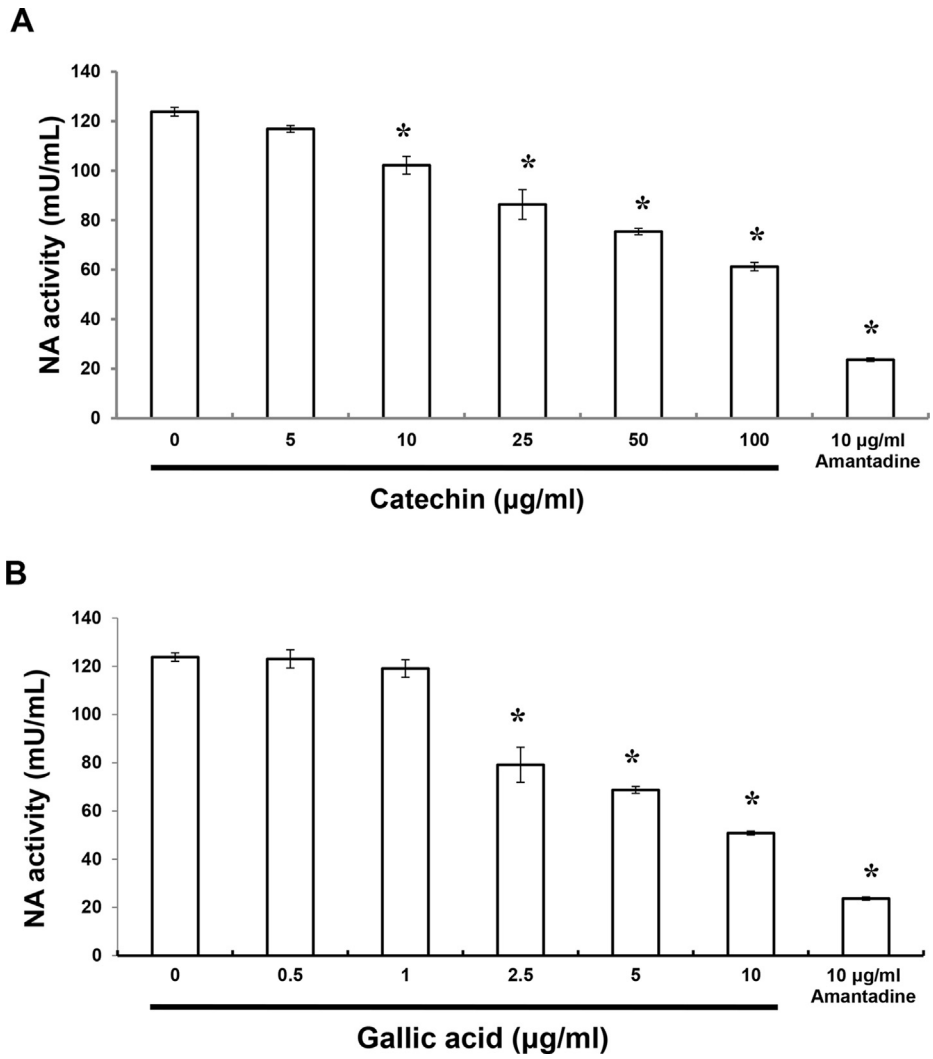


Fig. 4. Measurement of the anti-viral activity of catechin and gallic acid by using neuraminidase activity assay. A549 cells were infected with pandemic influenza A (H1N1) virus. After 1 h, viruses were removed. A549 cells were treated with (A) catechin (5–100 μg/mL) and (B) gallic acid (0.5–10 μg/mL). Fluorescence was monitored with excitation of wavelength 535 nm and emission of wavelength 590 nm. Each experiment was repeated three times independently and differences were shown at $*p < 0.05$ significantly.

months had a statistically significant preventive effect on clinically defined influenza infection and was well-tolerated.[20] They conclude that green tea may be an effective prophylaxis for influenza infection. Furthermore, the clinical trial by Yamada *et al* has shown that gargling with tea containing catechin extracts had the effect of preventing influenza virus infection in elderly nursing home residents.[21] Catechin has been assessed in terms of its ability to interact with influenza neuraminidase, and to significantly suppress the increased ROS level to prevent influenza infection.[22,23] Gallic acid is easy to extract from a number of plants, fruits, teas, and cloves. Gallic acid reportedly has potent and broad antiviral activity against influenza A and B type viruses, both in vitro and in vivo.[24] Theisen *et al* reported the cytotoxic and antiviral activities against H1N1 virus (H1N1 A/Puerto Rico/8/34-NS116-GFP) of gallic acid with CC_{50}, EC_{50} and SI were 770.5 μM, 50.8 μM, and 15.2 μM, respectively, which differs from the results reported from the present study.[25] This could possibly result from the different viral strains used in experimentation. In addition, the therapeutic safety of gallic acid was found to be lower than other hydrolysable tannins and pseudotannins.[25]

In this study, we found that both of these two compounds indeed exerted anti-influenza A (H1N1) virus replication via significant inhibition of viral RNA synthesis. Meanwhile, catechin and gallic acid were shown to inhibit the growth of pandemic influenza A (H1N1) with EC50 of 18.4 μg/mL and 2.6 μg/mL in MDCK cells, and SI of >5.6 and 8.6, respectively. It indicated that catechin might be a safe reagent for long-term use in the prevention of influenza A (H1N1) virus infection; whereas gallic acid might be a sensitive reagent to inhibit influenza virus infection, and a promising candidate to protect the host against pandemic influenza A (H1N1) virus. Therefore, TSL-1 may be used as a food supplement or as an adjunct therapy with current drugs to prevent influenza A (H1N1) virus infection.

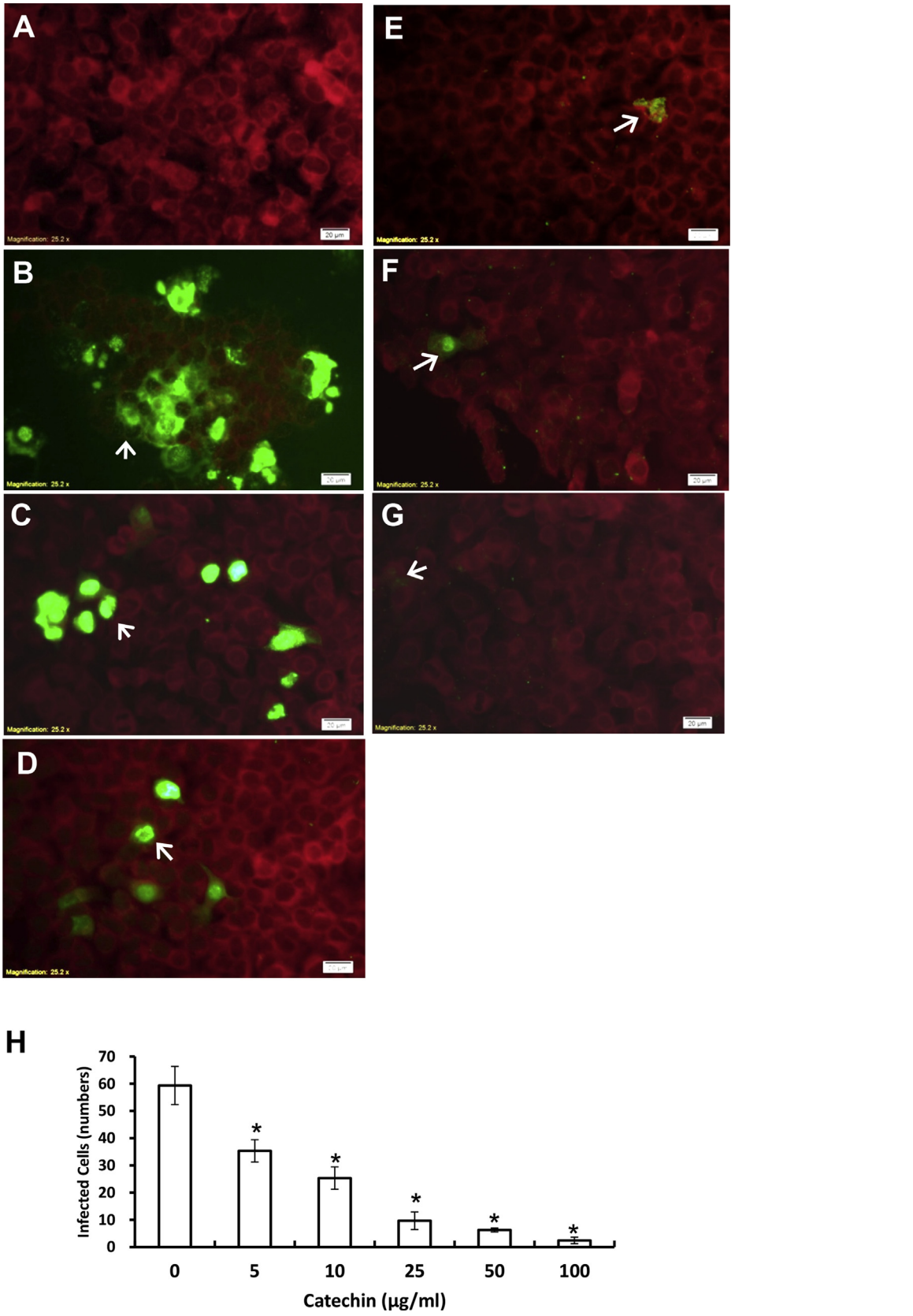


Fig. 5. Catechin inhibited pandemic influenza A (H1N1) virus infection on A549 cells, as observed by immunofluorescence staining. (A) Cells only. (B) Extensive fluorescence was observed in virus-infected control. The quantitative of fluorescence foci was decreased in dose-dependent manner when cells treated with (C) 0.5 μg/mL, (D) 1 μg/mL, (E) 2.5 μg/mL, (F) 5 μg/mL and (G) 10 μg/mL of catechin. The green foci represented viral protein (marked by arrows) but not observed in control cells. Red fluorescence due to Evans blue staining shows the cells presented in each field of view under the Olympus fluorescence microscope at × 400 magnification. The scale bar in each panel represents 20 μM. The inhibition effect of the catechin was calculated at figure H. Asterisk (*) indicates significant difference ($p < 0.05$) compared to the virus infection control, respectively.

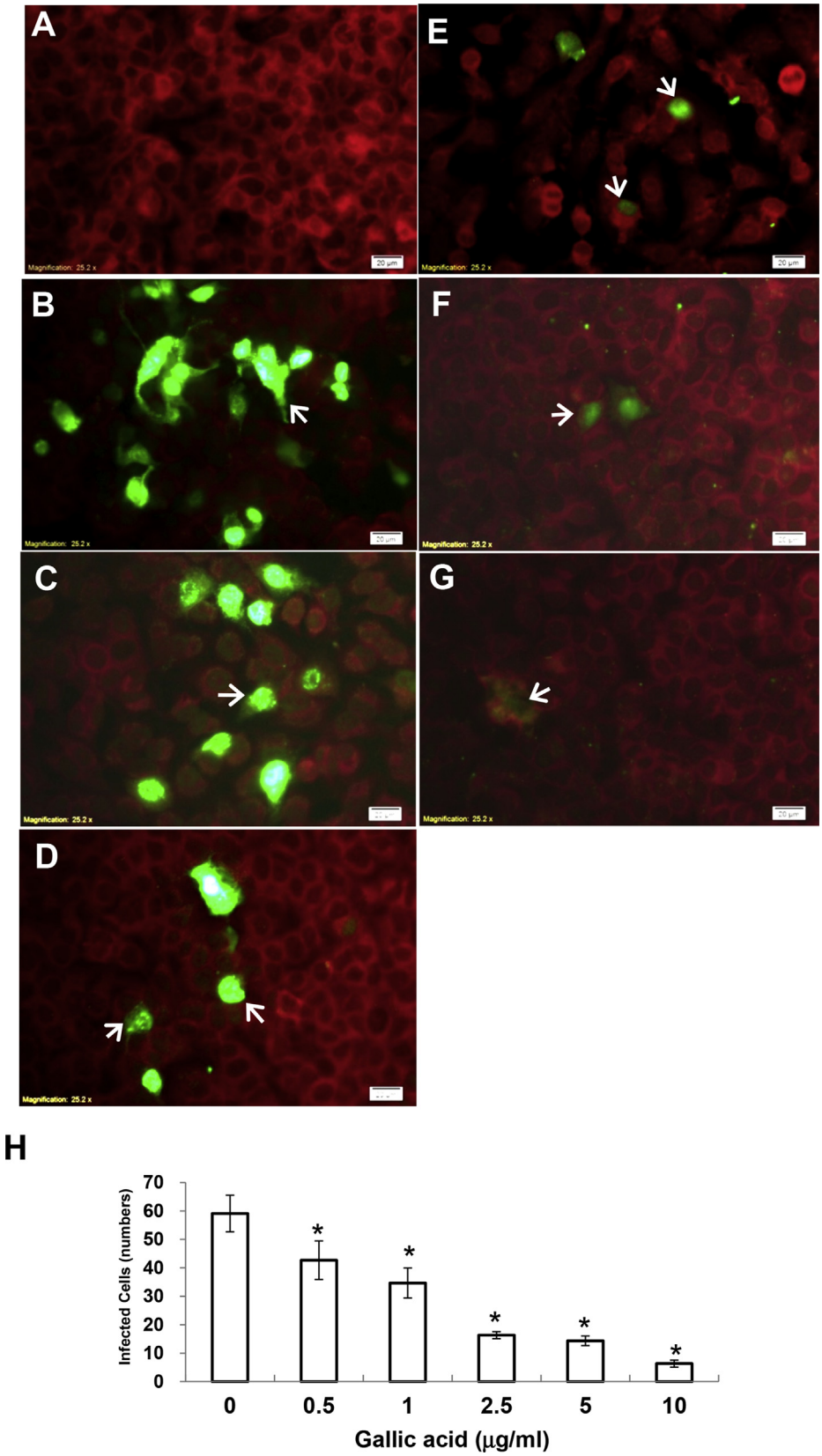


Fig. 6. Immunofluorescence assay of A549 cells infected with pandemic influenza A (H1N1) virus and inoculated with gallic acid. (A) Cells only. (B) Green fluorescence was observed in virus-infected control. The green fluorescence foci decreased in dose-dependent manner when cells treated with (C) 0.5 μg/mL, (D) 1 μg/mL, (E) 2.5 μg/mL, (F) 5 μg/mL and (G) 10 μg/mL of gallic acid. The green foci represents viral protein (marked by arrows) but not observed in control cells. Red fluorescence due to Evans blue staining showed the cells presented in each field of view of the Olympus fluorescence microscope at ×400 magnification. The scale bar in each panel represents 20 μM. The inhibition effect of the gallic acid was calculated at (H). Asterisk (*) indicates significant difference ($p < 0.05$) compared to the virus infection control, respectively.

Table 1
Cellular toxicity and selectivity index of five compounds on MDCK cells.

Compound	SI	Plaque inhibition (EC_{50}) μg/mL	XTT cytoxicity (CC_{50}) μg/mL
Catechin	>5.4±0.2	18.4±0.7	>100
Kaemperol	N/A	N/A	>100
Quercetin hydrate	N/A	N/A	>100
Rutintrihydrate	N/A	N/A	>100
Gallic acid	8.6±0.5	2.6±0.07	22.1±0.6

SI = the selectivity index (SI) was calculated as the ratio of CC_{50}: EC_{50}.
EC_{50} = concentration for a reduction in plaque number by 50% relative to control.
CC_{50} = 50% cellular toxicity compared to uninfected MDCK cells was determined by XTT assay.
Standard deviations from three replicate experiments were indicated.

In conclusion, this study provides evidence to support that catechin and gallic acid inhibit pandemic influenza A (H1N1) virus activity. As such, catechin has the promise of being a low-toxic, abundantly available treatment for the prevention of the influenza infection. Furthermore, gallic acid offers the potential of being developed into a new drug due to its significant anti-influenza virus effects, with less cytotoxic effects at low doses to the host cells. However, more specific characterization of the active compounds and mechanisms providing protective effects against influenza virus infection requires further investigation. At present, both catechin and gallic acid may provide effective alternatives as replacements or supplements for currently available anti-influenza remedies.

Acknowledgments

This work was also supported and funded by Ministry of Science and Technology with MOST 105-2320-B-039-035, China Medical University Hospital with DMR-106-005, China Medical University under the Aim for Top University Plan of the Ministry of Education, Taiwan (CHM106-7 and CHM106-8). and Chang Gung Memorial Hospital with CGMH8C1271. The authors would like to thank James Waddell for the proofreading and editing of our manuscript.

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