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Inhibitory effect of *Ephedrae herba*, an oriental traditional medicine, on the growth of influenza A/PR/8 virus in MDCK cells

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Abstract

Using several herbal extracts, we investigated whether certain Kampo medicines exert an inhibitory effect on the acidification of intracellular compartments such as endosomes and lysosomes (referred to as ELS), and thereby inhibit the growth of influenza A virus in Madin-Darby canine kidney cells. The vital fluorescence microscopic study showed that the extract of *Ephedrae herba* (EHext) among five herbal extracts inhibited acidification of endosomes and lysosomes in a concentration-dependent manner (100–400 μg/ml). Moreover the growth of influenza A/PR/8/34 (H1N1) (PR8) virus was inhibited when the cells were treated with EHext for 1 h immediately after infection, or treated as early as 5–10 min after infection. Conversely, virus growth resumed concomitantly with the reappearance of acidified ELS after removal of EHext. The fact that the inhibitory effect of EHext was completely or partially reversed by $FeCl_3$, a tannin-reactive agent, strongly suggests that tannin is one of the active components in the extract.

Keywords: Influenza virus; *Ephedrae herba*; Tannin; Acid-catalyzed fusion reaction

1. Introduction

Kampo (Japanese herbal) medicine is an oriental traditional medical treatment frequently used in Japan. Patients with influenza as well as common cold are currently treated with this medicine in Japan. However, little has been clarified as to how Kampo medicine affects these virus infections.

We have previously reported that Bafilomycin A1, a specific inhibitor for vacuolar-type proton

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($V\text{-}H^+$) pump in endosomes and lysosomes (referred to as ELS in this study), inhibits the growth of influenza A and B viruses in Madin-Darby canine kidney (MDCK) cells. The compound is targeted at an early phase of infection (i.e. uncoating) when an acid-catalyzed fusion reaction occurs in acidified ELS (Ochiai et al., 1995). Projecting this finding into the field of Kampo medicine, we investigated whether certain Kampo medicines could inhibit the acidification of ELS and thereby inhibit the growth of influenza virus in MDCK cells. We found that the extract of *Ephedrae herba* (EHext) inhibits the growth of influenza A PR8 virus in a transient association with the disappearance of acidified ELS at an early phase of infection.

2. Materials and methods

2.1. Drug

A total of five herbs (*Puerariae radix*, *Bupleuri radix*, *Coptidis rhizoma*, *Ginseng radix* and *Ephedrae herba*) were purchased from Tochimoto (Osaka, Japan). Water extracts of herbs were prepared by boiling the herbs (50 g) in 500 ml of distilled water for 30 min followed by freezing and drying. The lyophilized powders were dissolved in Eagle's minimum essential medium (MEM) at appropriate concentrations. $FeCl_3.6H_2O$ (Wako, Osaka, Japan) was dissolved in MEM at a concentration of 4 mM. The dissolved drugs were sterilized with Millipore filter before use.

2.2. Virus and cells

Influenza A/PR/8/34 (H1N1) (PR8) virus was propagated for 3 days at 35°C in chorio-allantoic cavities of 10-day old embryonated hen eggs. The infected allantoic fluids were clarified by centrifugation at $1000 \times g$ for 20 min and stored in small portions at -80°C as the virus stock solution. Madin-Darby canine kidney (MDCK) cells were cultured as monolayers in MEM supplemented with 8% fetal bovine serum.

2.3. Vital fluorescence staining of acidified ELS

MDCK cells on cover slips in 24-well plates were washed once with serum-free MEM and then cultured in 0.5 ml of serum-free MEM in the absence or presence of drugs at various concentrations at 37°C for 1 h. Thereafter, the cells were processed for vital fluorescence staining (Yoshimori et al., 1991) for 20 min with acridine orange, which is taken up by living cells and changes its color from green at low concentrations to orange when accumulated in ELS (Allison and Young, 1969; Holtzman, 1989), and observed with the aid of a fluorescence microscope.

2.4. Virus growth assay

Confluent monolayers of MDCK cells in 24-well plates were washed once with phosphate-buffered saline and then infected with PR8 virus at a multiplicity of infection (MOI) of 5 plaque forming units (PFU)/cell for 45 min at room temperature in drug-free conditions. Thereafter, the cultures were warmed to 37°C (0 h post infection) and received a 1-h drug treatment initiated either at 0, 1 or 2 h post infection (p.i.). Alternatively, the target stage of infection was examined in more detail in a strict time-related study in which drug treatment was initiated at various times p.i., within 30 min and lasting until 3 h p.i. The cells were then further incubated in a drug-free medium. After incubation for a total of 10 h, the cultures were processed through three cycles of freezing and thawing, and clarified by low speed centrifugation ($500 \times g$ for 10 min) to determine virus yield in the supernatants by plaque titration on MDCK cells as described previously (Tobita, 1975). To examine the reversibility of the drug effect on virus growth, the culture period was prolonged up to 15 h p.i. after the removal of drug. As a control, the infected cells were incubated in drug-free medium throughout the experiment.

2.5. Treatment of herbal extract with $FeCl_3$

It has been reported that tannin, one of the major components of certain herbs used in this

study, reacts with metal ions such as $FeCl_3$, resulting in the formation of precipitates (Finar, 1956) which are easily removed by centrifugation. Thus, to prepare a tannin-deficient extract, various amounts of $FeCl_3.6H_2O$ were added to the herbal extract followed by centrifugation (1000 rpm for 5 min) to remove the precipitates. The inhibitory effects of $FeCl_3$-treated extract were assessed in comparison with the $FeCl_3$-untreated extracts both in vital fluorescence and virus growth studies.

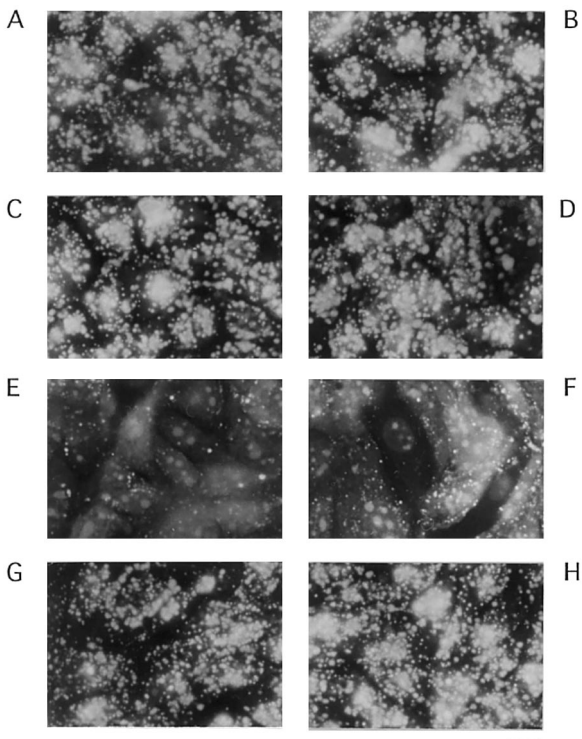

Fig. 1. Effect of herbal extracts on the acidification of ELS in MDCK cells. Confluent monolayers of MDCK cells on the cover slips in a 24-well plate were washed once with phosphate-buffered saline and then each treated with 1600 μg/ml of *P. radix* (A), *B. radix* (B), *C. rhizoma* (C) and *G. radix* (D) for 1 h at 37°C. Alternatively, the cells were treated with 400 μg/ml (E), 200 μg/ml (F) or 50 μg/ml (G) of *E. herba* or not (H) in serum-free medium for 1 h at 37°C. Thereafter, the cells were processed for vital fluorescence microscopy. Bar represents 20 μm.

3. Results

3.1. Effect of herbal extracts on the acidification of intracellular compartments

Initially, we examined the effect of five herbal extracts on the acidification of ELS (Fig. 1). The vital fluorescence microscopic study showed that numerous granular fluorescence foci with an orange color were observed in the cytoplasm of the drug-untreated control cells (Fig. 1H). However, the number of acidified ELS and the intensity of orange fluorescence decreased in the EHext-treated cells in a concentration-dependent manner. Following treatment with 400 μg/ml EHext (Fig. 1E), acidified ELS were markedly decreased to a level of faint granular fluorescence. In the cells treated with 200 μg/ml EHext (Fig. 1F), the numbers of acidified ELS were decreased slightly, whereas the amount and intensity of granular orange fluorescence in the cells treated with 50 μg/ml EHext (Fig. 1G) were similar to the control cells. The effect of the remaining four herbal extracts (Fig. 1A–D) was negligible even at a concentration of 1600 μg/ml. The inhibitory effect of EHext was exhibited only after a 1-h treatment, but not after 45 min or shorter treatment periods (data not shown).

The cytotoxic activity of EHext was examined by the MTT assay, a sensitive method for cytotoxicity based on the detection of mitochondrial enzymatic activity reacting with a chromogenic substrate, the color development of which is read at an optical density (OD) of 570 nm (Mosmann, 1983). When subconfluent cells (10^4 cells/well in a 96-well plate) were treated with various concentrations of EHext for 1 h and then cultured in drug-free medium for as long as 10 h, the OD value of culture without drug treatment (control) was 0.251 ± 0.011 (mean $\pm$ S.D. of triplicate culture), whereas the values of cultures with drug treatment at concentrations of 900, 600, 450 and 300 μg/ml of EHext were 0.082 ± 0.003, 0.247 ± 0.006, 0.265 ± 0.007 and 0.261 ± 0.013, respectively. The OD value of the culture treated with 900 μg/ml of EHext was significantly lower, but the OD value at the concentration of 600 μg/ml was not decreased as compared with that of the

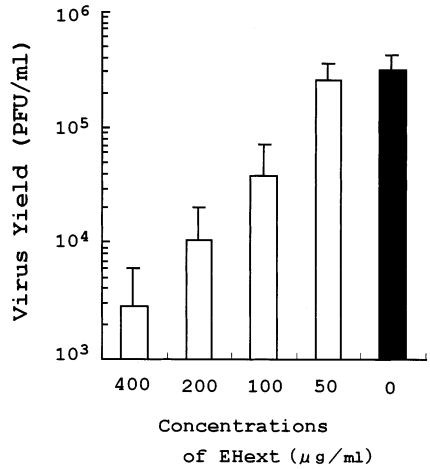

Fig. 2. Inhibitory effect of EHext on the growth of influenza A PR8 virus in MDCK cells. After infection in drug-free medium, the cells were treated with (open column) or without (closed column) various concentrations of EHext for 1 h (0–1 h p.i.) at 37°C and then further cultured in drug-free medium. After a total 10-h incubation, culture supernatants were collected to determine virus yield. Data are shown as mean (column) ± S.D. (thin bar) of triplicate cultures.

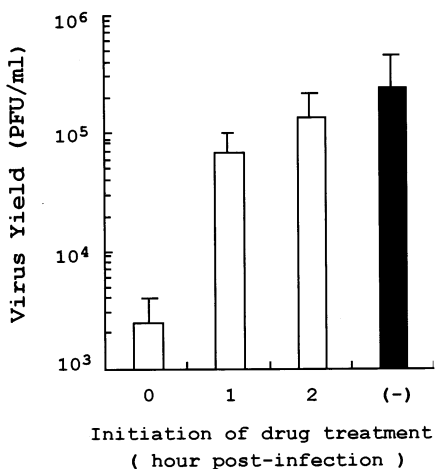

Fig. 3. Time-related effect of EHext on the growth of influenza PR8 virus in MDCK cells focusing on the initial 3 h p.i. After infection in drug-free medium, the cells received a 1-h treatment with EHext (400 μg/ml) initiated at 0, 1 or 2 h at 37°C (open column), or did not received drug treatment (closed column). After a total 10-h incubation period, culture supernatants were collected to examine virus yields. Except for the 1-h treatment, the cells were cultured in drug-free medium. Data are shown as mean (column) ± S.D. (thin bar) of triplicate cultures.

control, thus indicating that EHext exhibited no cytotoxic activity at a concentration of less than 600 μg/ml. In light of these findings, EHext was used at a concentration of less than 400 μg/ml in the experiments described below.

3.2. Effect of EHext on the growth of influenza A/PR/8/34 virus

When the cells were infected in drug-free medium and received a 1-h treatment initiated immediately after infection with various concentrations of EHext followed by further culture in drug-free medium, virus yield at 10 h p.i. decreased in a concentration-dependent manner (Fig. 2).

In addition, to clarify whether EHext affects the early stage of influenza virus infection, virus yields were determined following a 1-h treatment period initiated with 400 μg/ml of EHext at various times after infection (Fig. 3). In contrast to the virus yield of the treatment initiated immediately after infection (2.6×10^3 PFU/ml), treatment initiated at later times (1 or 2 h p.i.) did not significantly reduce virus yield as compared to the control culture (2.6×10^5 PFU/ml).

Based on the findings described above, a time-related inhibitory effect was examined with treatment started at 5, 10, 15, 20 or 30 min p.i. using EHext at a lower concentration (100 μg/ml). As shown in Fig. 4, more than 50% reduction of virus yield was obtained only when drug treatment was initiated within 5–10 min p.i. Initiation of drug treatment later than 15 min p.i. did not afford a significant inhibitory effect.

3.3. Inhibitory effect of tannin-deficient EHext on the acidification of ELS and virus growth

Taking into account the fact that tannin is one of the major components of EHext but not included in the remaining four herbs used in this study (Nishioka, 1983), we further studied the inhibitory effect of tannin-deficient EHext treated with $FeCl_3$. As shown in Fig. 5A, the inhibitory effect of EHext on acidification of ELS was completely annihilated by treatment with 4 mM of $FeCl_3$ (compare with Fig. 1E and H). Moreover, 4

mM $FeCl_3$ itself did not affect acidification of ELS (data not shown).

When virus yields were compared for the infected cells that had received a 1-h treatment with tannin-deficient EHext treated with $FeCl_3$ in the concentration range of 0.5–4 mM (Fig. 5B), virus yields recovered amounted to 8, 37, 62 and 80% of the control at $FeCl_3$ concentrations of 0.5, 1.0, 2.0 and 4.0 mM, respectively. These data suggest that tannin is one of the antivirally active components in EHext.

3.4. Reversibility of inhibitory effect of EHext on acidification of ELS and virus growth

When the cells were initially treated with EHext at 37°C for 1 h and then further cultured in drug-free medium, acidified ELS reappeared to a comparable level as in the untreated cells at 4 h after removal of the drug (Fig. 6A; compare with Fig. 1H). Based on this finding, the culture period of infected cells, which received the 1-h treatment

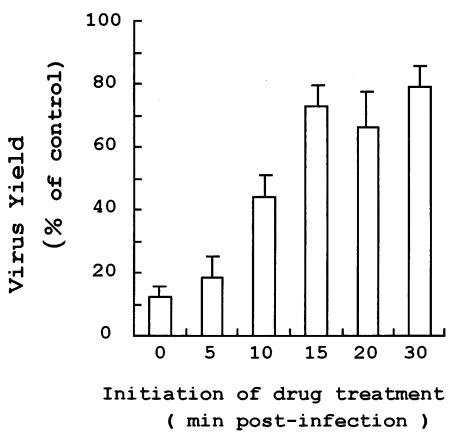

Fig. 4. Time-related effect of EHext on the growth of influenza PR8 virus in MDCK cells focusing on the initial 30 min p.i. After infection in drug-free medium, the cells received EHext (100 μg/ml) treatment initiated at 0, 5, 10, 15, 20 or 30 min p.i., lasting until 3 h p.i. at 37°C. After a total 10-h incubation, culture supernatants were collected and examined for virus yield. Except for the drug treatment period ending at 3 h p.i., the cells were cultured in drug-free medium. Data are expressed as percent of the control culture yielding 1.8×10^5 PFU/ml which was obtained in drug-free medium throughout the whole experiment. Representative data from three experiments are presented (mean ± S.D.).

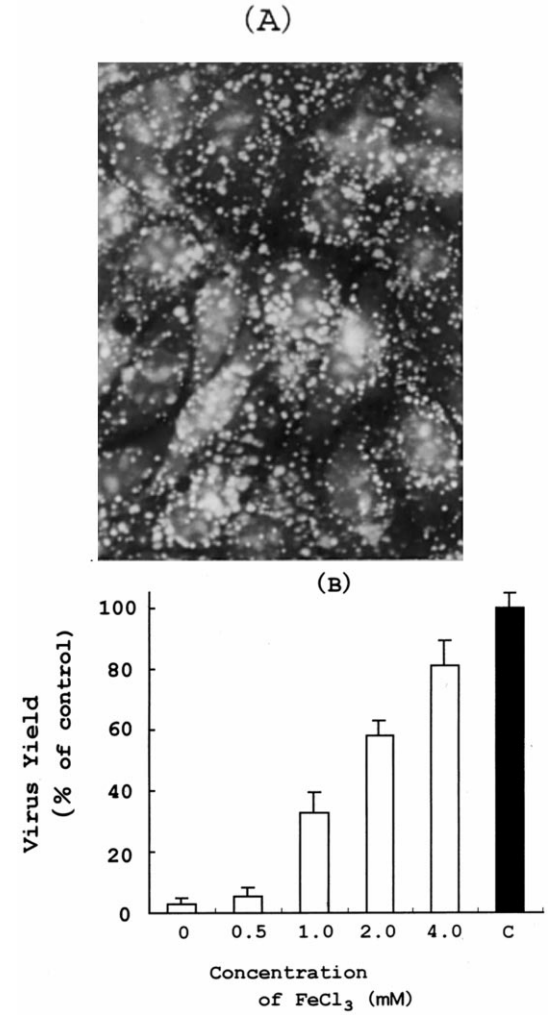

Fig. 5. Effect of $FeCl_3$-treated EHext on acidification of ELS and the growth of influenza PR8 virus. (A) MDCK cells received a 1-h treatment with 400 μg/ml EHext which was previously reacted with 4 mM $FeCl_3$ and then processed for vital fluorescence staining. (B) After infection in drug-free medium, the cells received a 1-h treatment (0–1 h p.i.) with EHext (400 μg/ml), which was previously reacted with various concentrations of $FeCl_3$ (0–4 mM) and then cultured in a drug-free medium at 37°C. After a total 10-h incubation, culture supernatants were collected to examine virus yields. Data are expressed as percent of the control culture (C) yielding 9.5×10^5 PFU/ml which was processed in drug-free medium throughout the whole experiment (mean ± S.D., $n = 3$).

initiated immediately after infection, was prolonged to as long as 15 h at the maximum. As shown in Fig. 6B, virus yields were recovered in proportion to the length of the culture period, that is, this value (less than 5% of the control at 11 h p.i.) was increased to 29 and 96% of the control at 14 and 15 h p.i., respectively. These data suggest that the inhibitory effect on virus growth is reversible in strict association with reappearance of acidified ELS. These data also suggest that the inhibition of virus growth is not due to the cytotoxic activity of the extract.

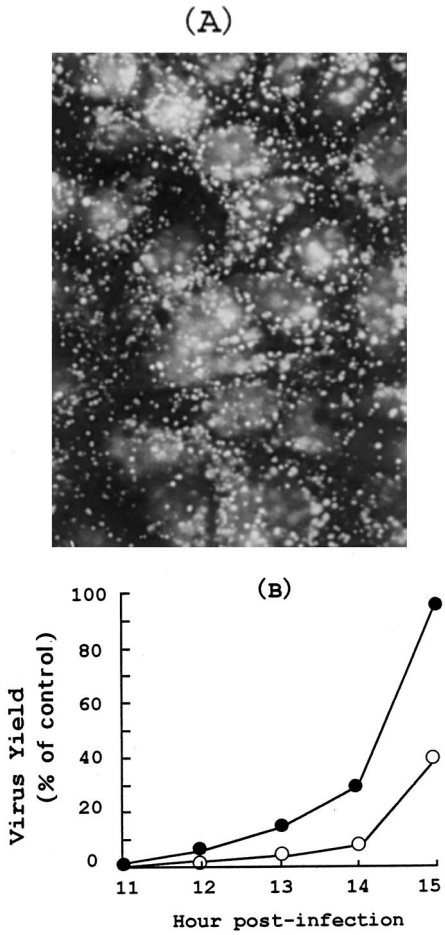

Fig. 6. Reversibility of inhibitory effect of EHext on acidification of ELS and virus growth. (A) MDCK cells were initially treated with 400 μg/ml EHext at 37°C for 1 h and then further cultured for 4 h in drug-free medium. Thereafter the cells were processed for vital fluorescence staining. (B) Infected cells received a 1-h treatment (0–1 h p.i.) with 400 μg/ml (open circle) or 200 μg/ml (closed circle) of EHext at 37°C and were then washed twice with serum-free medium to remove the drug, followed by incubation in drug-free medium up to 15 h p.i. At the indicated times p.i., culture supernatants were collected to examine virus yields. Data are expressed as percent of control cultures yielding $4.6–4.9 \times 10^5$ PFU/ml at each experimental point.

4. Discussion

It has been shown that acidic condition in ELS is essential for the uncoating process of influenza virus infection by triggering the viral envelope fusion activity (for a review, see White et al., 1983). Thus, drugs affecting acidification of ELS might be expected to exhibit an inhibitory effect on influenza virus growth. Indeed, chloroquine, an endosomotropic weak base, and Bafilomycin A1 inhibit influenza virus growth by raising the pH within ELS (Shibata et al., 1983; Ochiai et al., 1995). In addition to these agents, this study has clearly demonstrated that of the five herbal extracts EHext has the potential to inhibit the growth of influenza A PR8 virus, at least in part, through affecting the acidity of ELS.

For EHext to exhibit an inhibitory effect on virus growth, a 1-h treatment period initiated as early as 5–10 min p.i. was required. Time-related drug effect of EHext was almost equal to those of Bafilomycin A1 and chloroquine (Shibata et al., 1983; Ochiai et al., 1995), confirming that these drugs affect the early phase of influenza virus infection. On the other hand, acidified ELS reappeared after removal of EHext with a lag time of $\sim$4 h which was 2 h longer than that of Bafilomycin A1 (Ochiai et al., 1995). In correlation with the reappearance of acidified ELS, virus growth also recovered in the cell cultures incubated for a prolonged time in drug-free medium after a 1-h treatment with EHext. These findings show that the drug effect is reversible and unfused virus particles are protected from acidophilic hydrolytic enzymes in ELS under drug treatment, in accordance with previous data (Yoshimori et al., 1991). Both Bafilomycin A1 (Yoshimori et al., 1991) and EHext require a 1-h treatment period for the induction of disappearance of acidic ELS, in contrast to chloroquine which raises pH within

ELS within 5 min (Shibata et al., 1983). Thus, the mode of inhibitory effect of EHext is similar to Bafilomycin A1 rather than chloroquine. To obtain a better insight into the inhibitory mechanism of EHext, further studies are needed to clarify whether EHext actually inhibits the V-H^+ pump.

As for active components in EHext responsible for these phenomena, tannin is strongly suggested to be one of the candidate components based on the complete or partial reversal of the inhibitory effect after $FeCl_3$ treatment of the extract. It has been reported that EHext contains many kinds of condensed tannins as one of its major components (Nishioka, 1983). Condensed tannin is composed of flavan units, mostly (+)-catechin, (−)-epicathechin or their analogs, which form polymers with each other via carbon–carbon bonds (Nishioka, 1983). In this connection, we have recently found that virus yield (influenza A PR8) decreases following 1-h treatment (during 0 and 1 h p.i.) with purified (+)-catechin in a concentration-dependent manner (2–10 mM), accompanying the disappearance of acidified ELS (data not shown).

It is well known that these tannins are contained in green tea, black tea, wine and many herbs (for a review, see Okuda, 1995). Several tannin-containing compounds have been reported to exert various biological activities such as antibacterial (Toda et al., 1990) and antiviral effects (Hatano et al., 1988; Fukuchi et al., 1989; Nakashima et al., 1992). Nakayama et al. (1993) have reported that tea-condensed tannins, (−)-epigallocatechin gallate and teaflavin diagallate, inhibit the growth of influenza A and B viruses due to prevention of hemagglutin (HA) activity essential for virus adsorption. Indeed, we observed that 200 μg/ml EHext reduces HA activity of PR8 virus from 512 to 16 HA titers when virus was incubated with the extract for 1 h at 37°C (data not shown). Thus, EHext could inhibit influenza virus growth not only by affecting the uncoating process as shown in this study but also by preventing virus adsorption.

In a mouse infection model with mouse-adapted PR8 virus, Nagai et al. (1996) have shown that virus yields in the broncho-alveolar cavity are reduced by treatment with Sho-seiryu-to which incidentally contains *Ephedrae herba*. The paper of Nagai et al. made no reference to the contribution of *E. herba* to the inhibitory activity. It may be speculated that EHext could exert an inhibitory effect on influenza virus growth even in vivo, if the compound could reach the infected sites at effective concentrations.

In summary, it has been demonstrated that EHext inhibits the growth of influenza A PR8 virus in strict relation with the disappearance of acidified ELS. The fact that these inhibitory effects were completely or partially reversed by $FeCl_3$, a tannin-reactive agent, strongly suggests that tannin is one of the active components in the extract. Although the cytotoxic:therapeutic ratio (900 vs. 400 μg/ml) was quite small, EHext may be considered as a novel inhibitor of influenza A virus growth.

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