Effect of a plant polyphenol-rich extract on the lung protease activities of influenza-virus-infected mice

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Influenza infection was induced in white mice by intranasal inoculation of the virus A/Aichi/2/68 (H3N2). The lung protease and the protease-inhibitory activities were followed for 9 days after infection. The intranasal application of a polyphenol-rich extract (PC) isolated from Geranium sanguineum L. induced a continuous rise in the anti-protease activity but did not cause substantial changes in the lung protease activity of healthy mice. Influenza virus infection triggered a slight reduction in protease activity in the lungs at 5 and 48 h post infection (p.i.) and a marked increase at 24 h and 6 day p.i.. Protease inhibition in the lungs was reduced at 24 and 48 h p.i. and an increase was observed at 5 h and 6 and 9 days p.i.. PC treatment brought both activities to normal levels. The restoration of the examined parameters was consistent with a prolongation of mean survival time and reduction of mortality rate, infectious virus titre and lung consolidation. PC reinstated superoxide production by alveolar macrophages and increased their number in virus-infected mice. The favourable effect on the protease and the protease-inhibitory activities in the lungs of influenza-virus-infected mice apparently contributes to the overall protective effect of PC in the murine experimental influenza A/Aichi infection. The antiviral effect of the individual constituents was evaluated.

Keywords: alveolar macrophages, influenza virus infection, plant polyphenols, protease, protease-inhibitory activity

Introduction

Influenza is a highly contagious, febrile, acute infection of the nose, throat, bronchial tubes and lungs caused by influenza virus. Despite the achievements in the development of new anti-influenza drugs, the need for effective therapies for this infection continues to exist. In this respect the search for viral inhibitors of plant origin is a promising approach. A large number of extracts and pure substances have been tested and antiviral effects have been proven for some of them (Che, 1991). Often the virus-inhibitory effect has been attributed to the presence of polyphenol compounds (Manolova & Serkedjieva, 1986).

Earlier research proved that a semi-standardized polyphenol-rich extract, designated as polyphenolic complex (PC), obtained from the medicinal plant Geranium sanguineum L., inhibited the reproduction of influenza viruses type A and B in vitro and in ovo and protected mice from mortality in experimental influenza virus infection (Serkedjieva & Manolova, 1992). The anti-influenza virus effect of the preparation in cell cultures was specific and selective (Serkedjieva and Hay, 1998). However the investigations showed that the in vitro virus-inhibitory activity was modest (selectivity indices: 6.1–29.1) and this was in contrast with the marked protection in vivo (indices of protection: 64–100%). Thus the therapeutic effect of PC required explanation. We presumed that it might be due to more than one biological activity, including selective antiviral effects, non-selective immunomodulating activity and some non-specific biological and pharmacological interactions known for natural polyphenols, such as protein binding, radical scavenging and antioxidant activities. Following this line of investigation, we demonstrated in model systems that the extract possessed multiple biological and pharmacological activities. In addition to the virus-inhibitory effect, PC exhibited a stimulating effect on the phagocytic activity of murine blood polymorphonuclear lymphocytes (PMNs) and peritoneal macrophages, beneficially affected the spontaneous nitric oxide (NO) production by the macrophages (Toshkova et al., 2004), had antioxidant and radical scavenging capacities (Sokmen et al., 2005), and inhibited the proteolytic activity of trypsin (Antonova-Nikolova et al., 2002).
The aim of the present work was to investigate the effect of the plant preparation on the protease and protease-inhibitory activities in the lungs of PC-treated healthy and influenza-virus-infected mice and, in this way, to provide evidence of its modulatory potential for the overall protective effect in the lethal experimental influenza A/Aichi infection.

Materials and methods

Plant material
Geranium sanguineum L. (Geraniaceae) has been introduced into the experimental field of the Institute of Botany, Bulgarian Academy of Sciences (BAS). Ground, air-dried aerial roots were defatted with petroleum ether and treated with EtOH to fully extract the polyphenol compounds. The extract was lyophilized to obtain PC and each preparation was kindly provided by Dr S Ivancheva (Institute of Botany, BAS, Sofia, Bulgaria).

Chemicals
Trichloracetic acid, hydrochloric acid, bovine serum albumin (BSA), N-benzoyl-DL-arginine p-nitroanilide (BAPNA) and phorbol myristate acetate were from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany; trypsin, pepsin, proteinase K, subtilisin, chymotrypsin, cathepsin, soybean trypsin inhibitor (STI), β-casein and azocasein were from Merck, Darmshadt, Germany; cytochrome c (cyt c) and superoxide dismutase (SOD) from bovine erythrocytes were from Fluka, Diesenhofen, Germany.

Cells, media and viruses
Cell cultures from chicken embryo fibroblasts (CEF) were obtained from 11-day-old fertile hen's eggs by a standard procedure and maintained as in Serkedjieva and Hay (1998). Madin–Darby canine kidney (MDCK) cells were passaged in Dulbecco’s Eagle medium (GibcoBRL, Paisley, UK) for 3 min (interrupting sonication every 15 s). The homogenates were centrifuged (8,000 rpm, 30 min, 4°C) and the supernatants were examined for protease and protease-inhibitory activities.

Preparation and examination of lung tissue homogenates
On hour 5 and on days 1, 2, 6 and 9 post infection (p.i.) three mice of each group were anaesthetized with ether and exsanguinated by section of the subkavian arteries. Lungs were removed aseptically, washed in cold phosphate-buffered saline (PBS) and blotted dry. Tissue pieces of about 1 g were disintegrated mechanically in ice-cold PBS and subsequently by an ultrasound disintegrator (MSE, London, UK) for 3 min (interrupting sonication every 15 s). The homogenates were centrifuged (8,000 rpm, 30 min, 4°C) and the supernatants were examined for protease and protease-inhibitory activities.

Protease activity in the lungs was assessed using BAPNA as the substrate. Either milk or β-casein was used as the substrate; the inhibitory activity was determined by the reduction in millimetres of the transparent zones formed by trypsin.

Inhibitory activity towards pepsin, proteinase K, subtilisin, chymotrypsin and cathepsin. A method with azocasein as the substrate was used as described in Angelova et al. (2006).

Inhibitory activity towards trypsin.

0.5% FCS was added. The viruses were from the collection of the Institute of Microbiology, BAS, Sofia, Bulgaria.

Mice
Male and female (16–18 g), outbred ICR mice were obtained from the Experimental Animal Station, BAS, Sofia, Bulgaria. They were quarantined 24 h prior to use and maintained on standard laboratory chow and tap water ad libitum. The number of experimental animals was as small as was possible for statistical significance. The tests with animals were refined by careful planning of multifactor experiments. The animals were bred under standard conditions accepted by the Bulgarian Veterinary Health Service. Specialized personnel ensured their welfare.

Inhibitory activity towards pepsin, proteinase K, subtilisin, chymotrypsin and cathepsin. A method with azocasein as the substrate was used as described in Angelova et al. (2006).
Protease inhibitory activity in the lungs was determined as described above. The specific protease-inhibitory activity was evaluated (TIU/mg protein).

Protein determination used Bio-Rad Assay Reagent (Bio-Rad, Munich, Germany) and BSA as the standard; the protein content was expressed as mg/ml (Bradford, 1976). Absorption was measured at 595 nm.

UV-Vis spectrophotometer Shimadzu 1202 was used throughout the study.

Alveolar macrophages (aMØ)
Alveolar macrophages were collected on days 2, 6 and 9 p.i. by five washings of the broncho-alveolar cavity of at least six mice with 1 ml ice-cold Hank's buffered salt solution (HBSS) according to Holt, (1979). The phenotype of the cells was determined by light microscopy of cell populations immediately after being obtained. The cells were washed and re-suspended in complete RPMI 1640 medium.

Superoxide anion detection
O$_2^-$ in cultures of aMØ was measured by the SOD-inhibitable reduction of cytochrome c as previously described (Dimitrova et al., 2000) with some modifications. Briefly, adherent aMØ (3x10$^5$/well) were covered with 100 μl 75 mM cytochrome c in the presence or absence of 300 U/ml SOD. The plate was incubated in a CO$_2$ incubator at 37°C for 30 min and finally cooled on ice to stop the reaction. Cyt c reduction was measured at 550 nm with ELISA reader (Packard, Pangbourne, UK).

Virology
Viral infection. The infection was induced under light ether anaesthesia by intranasal inoculation of A/Aichi/2/68 (H3N2) (A/Aichi), adapted to mice lungs with infectious titre of 10$^{6.5}$ TCID$_{50}$/ml. It was maintained by passages in mouse lungs; virus stock was kept at -80°C. The infection was induced under light ether anaesthesia by intranasal inoculation of A/Aichi infection the protease activity in the lungs of A/Aichi/2/68 (H3N2) (A/Aichi), adapted to mice lungs with infectious titre of 10$^{6.5}$ TCID$_{50}$/ml. It was maintained by passages in mouse lungs; virus stock was kept at -80°C.

To induce lethal infection, mice were challenged with 1 ml ice-cold Hank's buffered salt solution (HBSS) according to Holt, (1979). The phenotype of the cells was determined by light microscopy of cell populations immediately after being obtained. The cells were washed and re-suspended in complete RPMI 1640 medium.

Experimental design. Mice were separated into four experimental groups: Group 1: mock-infected and PBS-treated (control healthy [CH]); Group 2: mock-infected and PC-treated (PCG); Group 3: influenza-virus-infected and PBS-treated (virus control [VC]); Group 4: influenza-virus-infected and PC-treated (VC+PC). PC (10 mg/kg in 0.05 ml PBS) was inoculated by intranasal instillation 3 h before infection under light ether anaesthesia. Two groups of 12 animals each (VC and VC+PC) were observed for death daily for 14 days p.i. After the experiments surviving mice were sacrificed by cervical dislocation.

To determine infectious lung parameters three animals from each experimental group were sacrificed on days 1, 2, 6 and 9 p.i., lungs were removed aseptically and lung consolidation (score) was scored from 0 (normal) to 4 (100% consolidation). Lungs were homogenized to 10% suspensions in PBS and tenfold dilutions (0.2 ml) were assayed for infectivity in MDCK cells. Virus-induced cytopathic effect was used as a measure of viral replication and scored as described previously (Serkedjieva and Hay, 1998). Infectious virus titres were evaluated and expressed in log$_{10}$ TCID$_{50}$/ml.

Results
As a first approach we examined the protease-inhibitory effect of PC in model systems. The extract inhibited the activity of trypsin in a dose-dependent manner with 50% inhibitory concentration (IC$_{50}$)=0.48 mg/ml in the DMA and with IC$_{50}$=0.4 mg/ml in the BAPNA assay (Figure 1). STI was used as a reference.

PC also markedly reduced the protease activity of pepsin, proteinase K and catepsin in a dose-related manner, but was not inhibitory to subtilisin and chymotrypsin (data not shown). The effect of PC treatment on protease activity in the lungs is illustrated in Figure 2. During the influenza A/Aichi infection the protease activity in the lungs of virus-infected mice was slightly reduced at 5 and 48 h p.i. (75.3% and 80.5%, respectively, CH being 2,218 and 2,227 U/mg protein, respectively) and markedly increased at 24 h and 6 days p.i. (187.5 and 177.1% respectively, CH being 2,731 and 2,262 U/mg protein respectively). PC application in intact mice did not cause significant changes compared with CH. In virus-infected mice the treatment with PC lead to normalization of this activity. On day 9
Influenza virus infection triggered a decrease in the lung-protease-inhibitory activity at 24 and 48 h.p.i. (76.3% and 78.4% respectively, CH being 5,858 TIU/mg protein and 5,605 TIU/mg protein respectively) and an increase at 5, 6 and 9 days p.i. (137.7%, 126.8% and 123.5% respectively, CH being 5,229 TIU/mg protein, 5,760 TIU/mg protein and 6,045 TIU/mg protein respectively). A gradual augmentation of this activity resulting in a 1.5-fold increase was observed in the group of PC-treated healthy mice from day 1 to day 9 p.i. (116.7–153.3%). In virus-infected mice PC treatment caused a decrease in the protease-inhibitory activity on day 2 p.i. (76.3%) and an increase on days 6 and 9 p.i. (126.8% and 135.9% respectively).

Complementary experiments investigating the effect of PC treatment on the number of aMØ are presented in Figure 4. The number of the aMØ in virus-infected mice was augmented 2.0–2.5-fold with a maximum on day 6 p.i. (280%, CH being 2.4×10⁶ cells). In the group of PC-treated virus-infected mice a twofold increase in the number of aMØ on day 9 was found (190%, CH being 3.2×10⁶ cells). PC treatment most noticeably increased the number of aMØ in the healthy animals (160–260%).

The experimental data on the effect of PC treatment on superoxide production by aMØ are shown in Figure 5. PC treatment of healthy mice resulted in enhancement of the superoxide production by aMØ on days 6 and 9 p.i. (230% and 120% respectively, CH being 14.1 nM O₂⁻/10⁶ cells and 12.1 nM O₂⁻/10⁶ cells respectively). The application of PC caused a decrease in the influenza virus-induced O₂⁻.
Influenza virus strains have HAs with monobasic cleavage site and are usually cleaved only in a restricted number of cell types, so these viruses cause local infection. In general these glycoproteins are activated by secreted proteases such as serum plasmin, kallikrein, urokinase, thrombin, acrosin, trypsin Clara and mini-plasmin in rat lungs, and mast cell tryptase and trypase TC30 in porcine lungs (Steinhauer, 1999 and references cited therein). Cleavage activation of influenza monobasic HAs by host proteases is generally thought to occur extracellularly on the surface and/or in the lumen of the respiratory tract.

The induction of influenza virus infectivity by host cell proteases is strictly regulated by inhibitors of the serine proteases such as human mucus protease inhibitor (MPI) in the upper respiratory tract (Kido et al., 1999) and pulmonary surfactant (Kido et al., 1993) in the lower respiratory tract. MPI accounts for 70–90% of the protease-inhibitory capacity of normal bronchial secretions.

The results reported here show that the influenza A/Heinjy infection in mice induces changes in the lung protease and protease-inhibitory activities. In the first hours after viral inoculation the infection caused marked increase in the protease-inhibitory activity; the equilibrium between the measured activities was disturbed and as a result the protease activity levels decreased. Furthermore, the protease-inhibitory activity decreased on days 1 and 2 p.i., and increased on days 6 and 9 p.i. (Figure 3). The changes in the protease activity followed another pattern: the protease levels on days 1 and 6 p.i. were particularly

Discussion

It has been postulated that the pneumotropism of influenza viruses is determined by the presence of specific trypsin-like protease(s) in the respiratory tract, which cleave precursors of the envelope glycoprotein of the progeny viruses (Kido et al., 1996). The main influenza virus glycoprotein, haemagglutinin, is synthesized as a single polypeptide chain (HA0), but upon maturation it is modified post-translationally by host cell proteases, giving rise to covalently linked HA1 and HA2 subunits and allowing fusion of viral and host cell membranes (Kido et al., 1996; Steinhauer, 1999). The mammalian and the non-pathogenic avian

**Figure 4. Number of alveolar macrophages of PC-treated influenza-virus-infected mice**

The procedure is as described in Materials and methods section. Lavages from six mice were pooled for every sample and three to five measurements were made for every sample. *P<0.05. †P<0.01. ‡P<0.001. PCG, mock-infected and phenolic complex (PC)-treated mice; VC, virus-infected and phosphate-buffered-saline-treated mice; VC+PC, virus-infected and PC-treated mice.

In an additional experiment it was established that PC treatment of virus-infected mice led to significant reduction of mortality rates (IP=77.8%; Table 1) and marked prolongation of MST (+5.2 days). Lung infectious virus titres (log_{10} TCID_{50}/ml=2.2–3.2; Figure 6), lung weights and lung indices (Table 1) were reduced; lung lesions as shown by macroscopic and microscopic examination were markedly alleviated. The infectivity in the lung tissue of PC-treated virus-infected mice could be enhanced by stimulation with trypsin before titration (Figure 6).

**Figure 5. Production of O_2 in alveolar macrophages from PC-treated influenza-virus-infected mice**

The procedure is as described in Materials and methods section. Lavages from six mice were pooled for every sample and three to five measurements were made for every sample. *P<0.05. †P<0.01. PCG, mock-infected and phenolic complex (PC)-treated mice; VC, virus-infected and phosphate-buffered-saline-treated mice; VC+PC, virus-infected and PC-treated mice.
The infectivity in the lung tissue from PC-treated virus-infected mice could be enhanced by in vitro stimulation with trypsin before the infectivity assay (Figure 6). This shows that part of the virus produced in the presence of PC was non-infectious, in contrast with the results obtained from virus-infected mice. In the latter case virus titres could not be increased by trypsin stimulation.

It should be noted that PC demonstrated a distinct inhibition of trypsin protease activity in two complementary in vitro assays with IC\textsubscript{50}=0.48 mg/ml in the DMA and 0.4 mg/ml in the BAPNA assay (Figure 1). EC\textsubscript{50}s in diverse virus inhibitory assays have been found to vary from 2.1 to 10 μg/ml (Serkedjieva and Hay, 1998). Thus the antiviral effect in cell cultures could not be accounted for by protease inhibition as IC\textsubscript{50}s largely exceeded the EC\textsubscript{50}s. Moreover, the investigations of the mode of anti-influenza virus activity of PC in cell cultures showed that PC was most effective when applied 1–3 h after virus infection: presumably it affected the early synthetic stages of viral replication (Serkedjieva and Hay, 1998). These results support our proposition that the in vitro virus-inhibitory effect of PC was specific, whereas the in vivo protective effect was due to a combination of activities of the extract.

In the lungs, the resident alveolar macrophages and monocytes act as antigen-presenting cells and produce cytokines and reactive oxygen species (ROS), which limit the spread of the virus (Arora and Houde, 1992). However, the excessive production of ROS has the potential to injure uninfected cells and to induce local inflammation and oxidative stress in the infected lungs. Oxidative stress could contribute to proteolytic lung tissue injury by inactivation of the protease inhibitors (Oda et al., 1989). In view of these findings we followed the effect of PC on the number of aMØ and the level of O\textsubscript{2} release by aMØ. It has been reported that the application of PC in virus-infected mice induces a continuous rise in the number and function of peritoneal macrophages and stimulates the phagocytic activities of blood PMNs (Ivanova et al., 2005).

Influenza infection induced a 2–2.5-fold increase in the number of aMØ with a maximum on day 6 (Figure 4) and about 1.8-fold increase in O\textsubscript{2} production (Figure 5). PC treatment restored the number of aMØ although an increase was found at the end of observation. A substantial increase in O\textsubscript{2} production was detected on day 2 p.i. in PC-treated virus-infected mice (Figure 5). This could explain, in part, the reduced level of lung anti-protease activity (Figure 2) and a subsequent rise in lung protease activity.

increased, corresponding to the periods of active viral replication in the lungs. Late in infection, on day 9, the protease activity returned to normal levels (Figure 2).

Yang et al. (2002) observed a continuous rise of both activities in the bronchoalveolar lavage fluid of virus-infected mice until day 6 p.i. Although our results contrast these data, they are in agreement with the changes in both lung activities, described by Divocha et al. (1990).

The application of the polyphenol-rich extract from Geranium sanguineum 3 h before viral infection restored the virus-induced alterations in the lung protease activities. An unexpected finding of our studies was the PC-induced steady augmentation of the protease-inhibitory activity in healthy mice; the lung protease activity was not affected. It could be assumed that in the course of the infection PC exerts its inhibitory effect on the virus protein proteolysis, and hence the activation of viral particles, indirectly through an increase in the protease-inhibitory activity.

### Table 1. Mortality and lung parameters of mice infected with influenza A/Aichi virus and treated with PC

<table>
<thead>
<tr>
<th>Group</th>
<th>Day p.i.</th>
<th>Survival, n/total</th>
<th>Lung parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC</td>
<td>1</td>
<td>20/20</td>
<td>0.30 ±0.06 0.0 ±0.0 1.5</td>
</tr>
<tr>
<td>VC+PC*</td>
<td>1</td>
<td>20/20</td>
<td>0.30 ±0.02 0.0 ±0.0 1.5</td>
</tr>
<tr>
<td>VC</td>
<td>2</td>
<td>20/20</td>
<td>0.32 ±0.03 1.0 ±0.2 1.5</td>
</tr>
<tr>
<td>VC+PC*</td>
<td>2</td>
<td>10/10</td>
<td>0.31 ±0.01 0.5 ±0.1 1.5</td>
</tr>
<tr>
<td>VC</td>
<td>6</td>
<td>16/20</td>
<td>0.4 ±0.03 3.0 ±0.4 2.2</td>
</tr>
<tr>
<td>VC+PC*</td>
<td>6</td>
<td>10/10</td>
<td>0.31 ±0.01 1.5 ±0.3 1.8</td>
</tr>
<tr>
<td>VC*</td>
<td>9</td>
<td>7/20</td>
<td>0.38 ±0.04 2.7 ±0.5 2.1</td>
</tr>
<tr>
<td>VC+PC*</td>
<td>9</td>
<td>8/10</td>
<td>0.30 ±0.01 1.5 ±0.4 1.5</td>
</tr>
</tbody>
</table>

*Treated with the polyphenolic complex (PC) 10 mg/kg. †P<0.01. ‡P<0.001. p.i., post infection; VC, virus control; VC+PC, virus-infected and PC-treated mice.

The procedure is as described in Materials and methods section. Three lung homogenates were pooled for every sample; three measurements were made for every sample. *Lung viruses stimulated in vitro with trypsin before the assay. TCID\textsubscript{50}, 50% tissue culture infectious dose; VC, virus-infected and phosphate-buffered saline-treated mice; VC+PC, virus-infected and PC-treated mice.
(Figure 3). These events corresponded to the start of viral replication in the lungs of PC-treated virus-infected mice (Figure 6). The superoxide release in virus-infected mice on days 6 and 9 p.i. was not influenced significantly by PC. A possible explanation for the increase in O$_2^-$ production on day 2 could be a synergistic oxidative burst as a result of the combined effect of the virus infection and the application of the plant preparation. ROS are known to have a dual role during influenza infection. They can induce apoptosis in virus-infected cells, thus contributing to the decrease in the total viral load. In this way they offer the first line of defence against infection, preceding the alternative defence mechanisms of the organism (Arora and Houde, 1992). Although additional experimental data are needed to draw a conclusion about the effect of PC on oxidative-stress response, it could be speculated that the effect on ROS production in the first stages of infection is due to an alternative mode of action of the plant extract, in addition to its selective virus-inhibitory activity.

Because it is acknowledged that the virulence of a particular influenza virus strain depends on the ability of its haemagglutinin to be cleaved by cellular proteases (Steinhauer, 1999), it seems reasonable that the prevention of this cleavage would result in inhibition of subsequent rounds of viral replication and spread in the respiratory tract. In influenza viruses with monobasic HA, exogenous inhibitors of serine proteases, including e-aminocaproic acid (Lozitsky et al., 1997), aprotonin (Ovcharenko & Zhirnov, 1994) and ambroxol (Yang et al., 2002) have been shown to reduce HA cleavage and virus activation in cultured cells, in chick embryos and in the lungs of infected mice.

Plant polyphenols, particularly flavonoids, are known for their strong tryptase-inhibitory ability (Malix et al., 2004). Moreover Rajbhandari et al. (2001) and Wegner et al. (2006) demonstrated that the anti-influenza virus effect of plant polyphenols was often associated with strong protease inhibition.

Physicochemical analysis of PC revealed that the extract contained tannins (34%), flavonoids (0.17%), catechins and proanthocyanidines (2 mg/kg). The identification of individual compounds by chromatographic methods showed that flavonoids (aglycones and glycosides: quercetin, quercetin 3-O-galactoside, morin, myricetin, kaempferol, rhamnatin, retusin and apigenin), phenolic acids (caffeic, ellagic, quinic and chlorogenic), gallo-tannins and catechins were present (Pantev et al., 2006). The results from previous and present investigations (Table 2) suggest that the presence of a variety of biologically active compounds, as well as the possible synergistic interactions between the constituents, is decisive for the overall virus-inhibitory effect.

On the basis of our results, obtained in vitro and in vivo, we suggest that PC restores the balance between the protease and the protease-inhibitory activities. This favourable capacity of the plant extract corresponded in virus-infected mice with the reduction of mortality (Table 1) and reduction of infectious virus load in the lungs (Figure 6). The severity of the macroscopic lung lesions was also markedly decreased. Lung scores and lung indices were ameliorated and brought to normal levels (Table 1). PC treatment of virus-infected mice produced a noticeable reduction of mortality rates (IP=75.1%) and marked prolongation of MST (+4.1 days). We feel that the restoring effect of the extract on the lung proteolytic balance contributes substantially to its overall protective effect in the murine experimental influenza virus infection.

It is possible that other treatment schedules for administering the extract would have produced different results; however, we previously established that the intranasal inoculation of PC 3 h before viral challenge yielded the best protection from mortality (Ivanova et al., 2005). Compared with other routes of application it could be the best application for the treatment of viral respiratory infections, because of an increased amount of the drug reaching the viral-targeted tissues.

In conclusion, the presented results demonstrate that the polyphenol-rich extract from *Geranium sanguineum* interfered with influenza virus infection by regulation of the host lung protease activities in addition to its selective virus-inhibitory activity. Further studies are warranted to elucidate the primary biological effects of this restoring effect.

### Table 2. Antiviral effect of polyphenol compounds identified in polyphenolic complex on the replication of influenza virus A/Weybridge in CEF

<table>
<thead>
<tr>
<th>Substance</th>
<th>TC$_{50}$ μg/ml</th>
<th>EC$_{50}$ μg/ml</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total EtOH extract</td>
<td>100.0 ±11.4</td>
<td>3.1 ±0.3</td>
<td>32.0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>60.0 ±3.2</td>
<td>3.0 ±0.0</td>
<td>20</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>60.0 ±2.2</td>
<td>20.0 ±3.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Myricetin</td>
<td>100.0 ±6.7</td>
<td>20.0 ±2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Morin</td>
<td>100.0 ±4.5</td>
<td>&gt;TC$_{50}$</td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>60.0 ±4.2</td>
<td>4.8 ±1.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Quercetin-3-O-galactoside</td>
<td>100.0 ±8.5</td>
<td>12.5 ±3.1</td>
<td>8.0</td>
</tr>
<tr>
<td>(+)-catechin</td>
<td>100.0 ±7.8</td>
<td>10.0 ±2.5</td>
<td>10.0</td>
</tr>
<tr>
<td>(-)-catechin</td>
<td>120.0 ±8.0</td>
<td>8.5 ±0.9</td>
<td>14.1</td>
</tr>
<tr>
<td>(-)-epicatechin</td>
<td>&gt;120.0</td>
<td>10.0 ±2.1</td>
<td>&gt;12.0</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>&gt;150.0</td>
<td>10.0 ±1.8</td>
<td>&gt;15.0</td>
</tr>
<tr>
<td>Rimantadine</td>
<td>&gt; 32</td>
<td>±0.05</td>
<td>&gt;160</td>
</tr>
<tr>
<td>hydrochloride</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CEF, chicken embryo fibroblast; EC$_{50}$ 50% effective virus-inhibitory concentration; SI, selectivity index; TC$_{50}$ 50% toxic concentration.
Acknowledgments

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References


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