INHIBITION OF INFLUENZA VIRUS PROTEIN SYNTHESIS BY A PLANT PREPARATION FROM *GERANIUM SANGUINEUM* L.

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Summary. – A polyphenolic complex (PC) with antiviral properties has been isolated from the Bulgarian medicinal plant *Geranium sanguineum* L. A study was undertaken to investigate the effect of PC on virus-specific protein synthesis in influenza virus-infected cells. The expression of viral glycoproteins on the surface of chick embryo fibroblasts infected with virus A/FPV, strain Rostock (H7N1) was suppressed. Virus protein synthesis was selectively inhibited as shown by SDS polyacrylamide gel electrophoresis of ^{35}S-methionine-labelled proteins and proteins immunoprecipitated with monoclonal antibodies. The inhibitory effect was dose-dependent and better pronounced when PC was applied after virus infection. Two variants of influenza virus FPV/Rostock with reduced drug susceptibility were selected. PC affected to a lesser extent the synthesis of viral proteins in cells infected with the variants as compared to the sensitive parental virus.

Key words: plant polyphenolic complex; *Geranium sanguineum* L.; influenza virus; antiviral effect; protein synthesis

Introduction

The search for viral inhibitors of plant origin is a promising approach in antiviral chemotherapy as naturally occuring mixtures might be an alternative source of interesting biologically active substances. A large number of extracts and pure substances have been tested and a selective antiviral effect has been proved for some of them (for review see Van den Berghe *et al.*, 1986).

From the Bulgarian medicinal plant *Geranium sanguineum* L. PC has been isolated. It inhibited the reproduction of influenza A and B viruses *in vitro*, *in ovo* and *in vivo* (Manolova *et al.*, 1986). Phytochemical investigation revealed in PC the presence of flavonoids, catechins, gallotannins and polyphenolic acids; some of them were identified by paper and thin layer chromatography (Ivancheva *et al.*, 1987; Serkedjieva *et al.*, 1989). To investigate the active components of PC the mixture was fractionated and a butanol fraction was shown to contain the majority of the *in vitro* antiviral activity (Serkedjieva *et al.*, 1989). The virus strain-dependence of the degree of inhibition provided evidence for the selectivity of the antiviral action of PC (Manolova *et al.*, 1987). PC administered intranasally or by aerosol reduced mortality in white mice resulting from experimental influenza infection (Manolova *et al.*, 1987; Serkedjieva and Manolova, 1992). Treatment with PC in concentrations greater than 500 μg/ml completely abolished the infectious, haemagglutination and neuraminidase activities of a range of influenza viruses (Serkedjieva *et al.*, 1992).

The current study was undertaken to investigate the anti-influenza activity of PC in respect to the selectivity of the effect and specifically the synthesis of viral proteins in cells infected with PC-sensitive virus and variants with reduced susceptibility.

Materials and Methods

PC preparation. G. sanguineum L. (*Geraniaceae*), a perennial grassy plant has been introduced in the experimental field of the Institute of Botany, Bulgarian Academy of Sciences, Sofia, and a specimen has been deposited in the herbarium of the same Institute. Ground air-dried aerial roots, collected during flowering period, were defatted with petroleum ether and then treated with methanol at room temperature till the full extraction of polyphenolic substances was achieved. The extract was lyophilyzed (yield 16%) and the obtained preparation (PC) was a dark red odourless pow-

der, soluble in water. The extract was prepared and provided by Dr. S. Ivancheva from the Institute of Botany. A 10% stock solution was prepared in sterile distilled water. For the antiviral experiments further dilutions were made in cell culture medium *ex tempore*.

Rimantadine hydrochloride was from Hoffman-La Roche Inc., Nutley, NJ.

Virus. Avian influenza virus A/chicken/Germany/34/, strain Rostock (H7N1) (FPV/R), was grown in chick embryo fibroblasts (CEF). The virus was passaged in 11 day-old hen's fertile eggs and was used as allantoic fluid. In antiviral experiments the virus replication was followed in one cycle (5.5 hrs) or multiple cycles (16 hrs) of virus growth.

Cells and media. Primary CEF cultures were prepared by standard procedures. Tris-buffered Gey's medium supplemented with 5% calf serum and antibiotics served as growth medium. In antiviral experiments the medium was used without serum.

Cytotoxicity. The effect of PC on CEF growth was determined in 96-well microtiter plates by tetrazolium dye MTT-based spectrophotometric method – MTT assay (Pauwels *et al.*, 1988). The maximal tolerated concentration (MTC) of drug was defined as that causing no detectable drop of cell growth, i.e. no drop of (A_{540}-A_{690}) virus control.

ELISA. The expression of viral haemagglutinin (HA), neuraminidase (NA) and nucleoprotein (NP) on the surface of infected cells was assayed by ELISA as described previously (Belshe *et al.*, 1988). Before assay of NP, cells were permeabilized with 1% Triton X-100. Monoclonal antibodies (MoAbs) to H7, N1 and NP (Rostock) were kindly provided by Dr. A. Douglas from the World Centre of Influenza, Mill Hill, London, UK. The minimal inhibitory concentration (MIC) of drug was the lowest one causing at least 50% reduction in A_{450} of cells inoculated with 10 – 100 $TCID_{50}$ of the virus. Rimantadine hydrochloride (0.1 – 1 mg/ml) was used as a positive control in all antiviral experiments.

Total viral protein synthesis. Single-cycle growth experiments were performed in 24-well plates to determine the effect of PC on total viral protein synthesis in cells infected with influenza virus FPV/R or FPV/R-r. The procedure was described by Hayden *et al.* (1990). The cell monolayers were labelled with 10 μCi of ^{35}S-methionine per well for 1 hr (4.5 – 5.5 hrs p.i.), lyzed and analyzed by SDS-PAGE.

Viral HA, NA and NP synthesis in infected cells was determined by immunoprecipitation of cell lysates with MoAbs followed by SDS-PAGE of the immune complexes. The procedure was described by Sugrue and Hay (1991).

Selection of PC-resistant variants of FPV/R virus was done by standard procedures (Hay *et al.*, 1985).

Results and Discussion

Studies on the cytotoxicity of PC revealed that PC in concentrations below 50 μg/ml did not reduce the $A_{540,690}$ values of CEF in MTT assay as compared to controls, i.e. did not reduce their growth, and the MTC was thus estimated at 50 μg/ml.

The inhibitory effect of PC on the expression of viral HA, NA and NP on the surface of FPV/R virus-infected CEF was observed by ELISA (Fig. 1). In some experiments low virus doses were used and ELISA was performed after multiple cycles of viral reproduction. In one-cycle growth experiments high virus doses (10^7 $TCID_{50}$) were used.

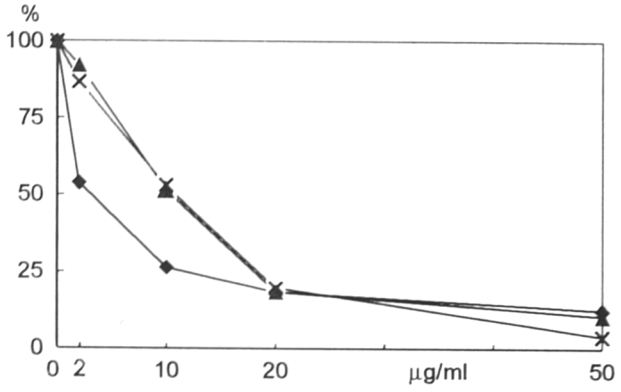

Fig. 1
Effect of PC on expression of viral proteins on surface of CEF infected with influenza virus FPV/R
Abscissa: PC concentration; ordinate: percentage of A_{450} of control
◆ HA, × NA, ▲ NP.

The inhibitory effect of PC depended on the concentration of the substance (Fig. 1) and on the virus inoculum (results not shown). Most sensitive to the action of PC was the expression of HA (MIC 2 μg/ml).

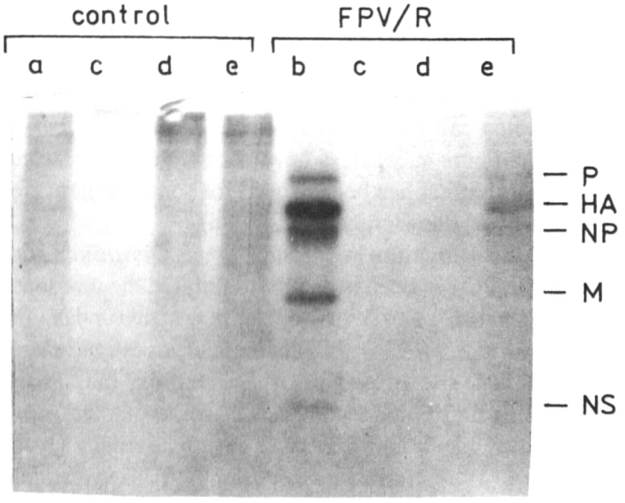

Fig. 2
Inhibition by PC of synthesis of ^{35}S-methionine-labelled proteins in CEF infected with influenza virus FPV/R
P – proteins of the polymerase complex; M – matrix protein; NS – non-structural protein; HA, NP – standard abbreviations. Lanes: a,b – PC absent, c – 100 μg/ml PC, d – 50 μg/ml PC, e – 20 μg/ml PC.

The effect of PC on virus-specific protein synthesis was studied also by labelling of CEF with ^{35}S-methionine and SDS-PAGE of cell lysates in one-cycle virus growth experiments (Fig. 2). The inhibitory effect of PC was dose-dependent (Fig. 3) and was more pronounced when PC was applied after viral infection (Fig. 4). The presence of PC in the culture medium during the whole infectious process was necessary for the full expression of the antiviral effect. In higher concentrations PC inhibited the infectious virus growth also when applied before or simultaneously with virus. These results showed that the inhibition of virus-specific protein synthesis was of limited specificity.

The effect of PC on the synthesis of HA, NA and NP was determined by immunoprecipitation with MoAbs and by SDS-PAGE of the immune complexes. The synthesis of all three proteins as well as total protein synthesis (Fig. 5) was inhibited by doses of PC above 10 μg/ml. The HA synthesis was most sensitive to the inhibitory effect of PC.

After 11 passages in CEF in the presence of inhibitory concentrations of PC and by isolation of resistant plaques in three plaque inhibition experiments two variants of FPV/R less sensitive to PC were selected. It was observed that the inhibition by PC of the expression of HA in CEF with FPV/R-r variants was lower in comparison with the parental virus (Table 1). The same was observed for the expression of NA and NP (data not shown). To a lesser extent was affected also the total viral protein synthesis in CEF infected with resistant variant FPV/R-r compared to the parental FPV/R (Fig. 6), as well as the HA, NA and NP synthesis (results not shown).

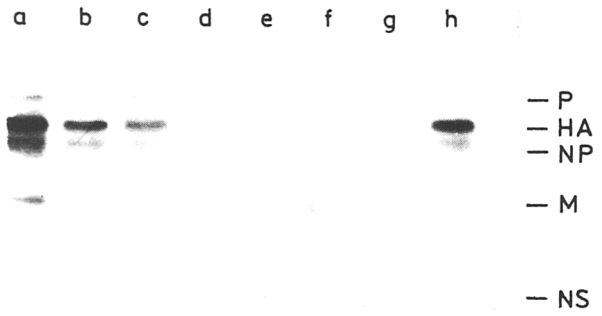

Fig. 3
Dose-dependence of the inhibition of virus protein synthesis by PC
Lanes: a – PC absent, b,c,d,e,f,g,h – PC 100, 80, 40, 20, 10, 5, 2.5 μg/ml. For the rest of legend see Fig. 2.

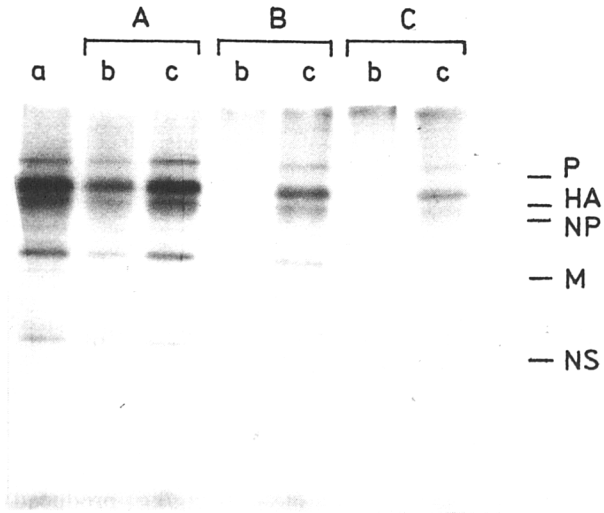

Fig. 4
Dependence of the inhibitory effect of PC on virus protein synthesis on time of addition
A – PC added 1 hr prior to infection; B – PC added simultaneously with virus; C – PC added 1 hr after virus. Lanes: a – PC absent, b – 50 μg/ml PC, c – 20 μg/ml PC. For the rest of legend see Fig. 2.

Table 1. Inhibition by PC of HA expression in CEF infected with FPV/R and FPV/R-r viruses

PC (μg/ml)	HA expression (A_{450}) % of control	
	FPV/R virus	FPV/R-r virus
10	38.4	100
25	8.2	58.6

One-cycle virus growth experiments.

The principal objective of the study was to characterize the anti-influenza activity of the plant PC isolated from the Bulgarian medicinal plant *Geranium sanguineum* L. in respect to virus infection and specifically the effect of PC on the synthesis of viral proteins. In previous studies (Manolova *et al.*, 1988) it was shown that the inhibitory effect was strain-dependent and indicated its selectivity.

By the use of various strains of human, avian and equine influenza viruses it was further confirmed that the inhibitory effect was strain-dependent that was consistent with its apparent selectivity (selectivity index 10-250). The decrease in the production of infectious virus was shown in drug-susceptibility, virus yield and plaque assays (data not shown).

The present studies concerning the effect of PC on the synthesis of specific viral proteins showed its limited specificity. These studies were interesting with regard to the pronounced therapeutic effect of PC found in experimental influenza in-

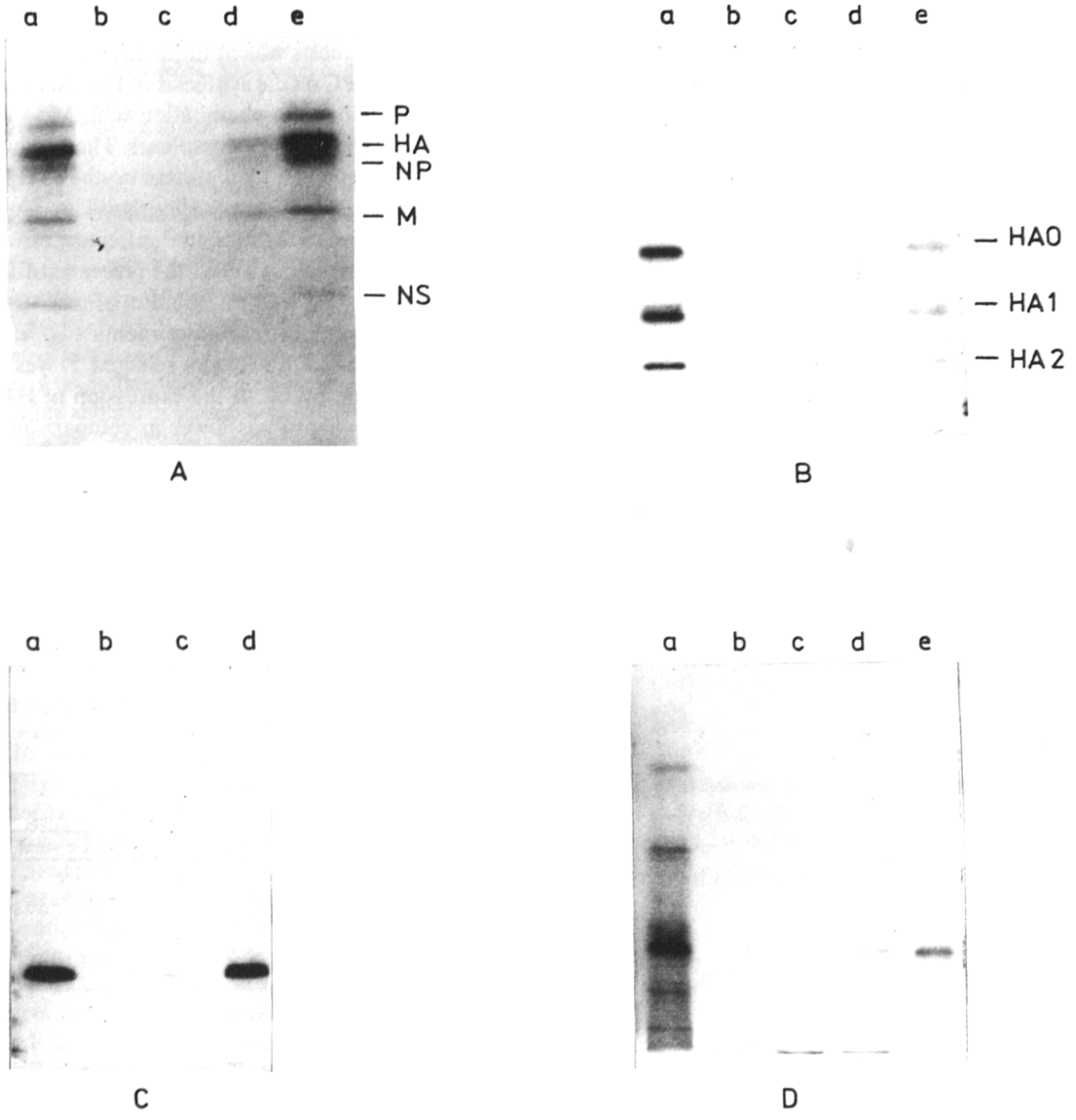

Fig. 5
Inhibition of viral HA, NA and NP synthesis in virus-infected CEF by PC
Immune complexes precipitated with specific MoAbs were analyzed by SDS-PAGE. A – total viral proteins; B – HA; C – NA; D – NP. Lanes: a – PC absent, b, c, d, e – 50, 25, 12.5, 6.25 μg/ml PC. HAO – uncleaved HA. For the rest of legend see Fig. 2.

fection in mice (Manolova *et al.*, 1986; 1987). PC administered intranasally (1 – 3 mg/kg) 6 hrs before infection reuced the mortality rate (protection index 33 – 67%) and prolonged the survival time (1 – 3 days). When PC was administered by aerosol in a dose of 20 mg/ml 2, 24, 48 and 78 hrs after virus infection the protection index was 64% (Serkedjieva and Manolova, 1992). Administration of PC in combination with rimantadine hydrochloride (1 – 10 mg/kg) produced a synergistic therapeutic effect and the protection index reached 77.8% (Gegova *et al.*, 1993).

It has been shown also that PC exhibited a stimulating effect on cell-type immune response and induced low production of interferon after intraperitoneal application (Manolova *et al.*, 1989).

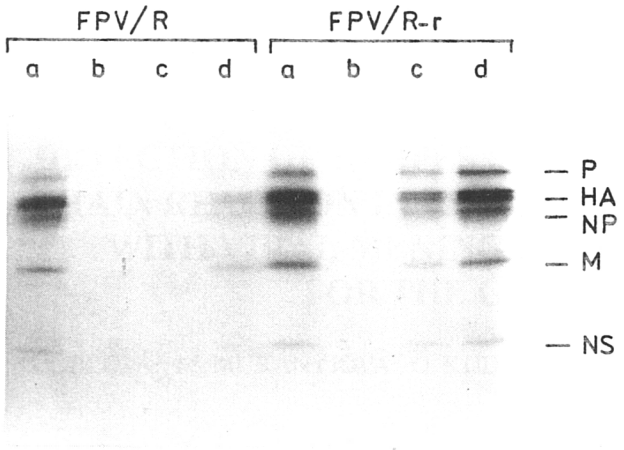

Fig. 6
Inhibition of total virus protein synthesis in CEF infected with FPV/R and FPV/R-r viruses
Lanes: a – PC absent, b, c, d – 100, 50, 20 μg/ml PC. For the rest of legend see Fig. 2.

The therapeutic effect of PC on the course of experimental influenza infection *in vivo* might be attributed to the combination of expression of more than one biological activities of the plant product – selective antiviral action, immunostimulating effect, some important non-specific biological and pharmacological interactions, well known for plant polyphenols, such as protein binding (Okuda *et al.*, 1992), antioxidant activity (Costantino *et al.*, 1992) etc.

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