# Biologically Active Constituents of a Polyphenol Extract from *Geranium sanguineum* L. with Anti-Influenza Activity

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From the aerial roots of the medicinal plant *Geranium sanguineum* L. a polyphenol-rich extract with strong anti-influenza activity has been isolated. To investigate its active fractions, the extract was partitioned by solvents with increasing polarity. The *n*-BuOH fraction contained the majority of the *in vitro* antiviral activity; the EtOAc fraction was the most effective one *in vivo*. A bioassay-directed fractionation of the *n*-BuOH and EtOAc fractions was performed to obtain information about the nature of the chemical components of the plant extract, responsible for the antiviral effect. The individual constituents were identified by spectroscopic methods and comparison with authentic samples and by HPLC. The cell-toxic and virus-inhibitory effects of the fractions and some individual polyphenol compounds, found in *Geranium sanguineum* L., were studied using the replication of representative influenza viruses in cell cultures. This study showed that the presence of a variety of biologically active compounds as well as the possible synergistic interactions between them seem to be decisive for the overall antiviral effect.

*Key words:* Bioassay-Guided Fractionation, *Geranium sanguineum*, Antiviral Activity

## Introduction

Ethnopharmacology provides scientists with an alternative approach for the discovery of antiviral agents, namely the study of medicinal plants with history of traditional use as a potential source of substances with significant pharmacological and biological activities (Vlietinck and Vanden Berghe, 1991). A large number of extracts and pure substances has been tested and a selective antiviral effect has been proved for some of them (for review see Che, 1986; Jassim and Naji, 2003). Often the virus-inhibitory effect has been attributed to the presence of polyphenol compounds (Che, 1986; Manolova and Serkedjieva, 1986).

The medicinal plant *Geranium sanguineum* L. (Geraniaceae) is wide-spread in Bulgaria. Aqueous and alcoholic extracts from its roots are used in traditional medicine to treat various infections and inflammatory conditions. The plant is widely known for the relief of pruritus, itches and skin lesions and is used for a disinfectant bath and as a poultice to the affected area (Jordanov *et al.*, 1973). A methanol extract from the aerial roots of the plant, characterized as a polyphenolic complex (PC), was shown to inhibit the reproduction of influenza A and B viruses *in vitro*; PC, administered intranasally, significantly reduced the mortality of white mice resulting from experimental influenza virus infection (Serkedjieva and Manolova, 1992).

The anti-influenza virus effect of the preparation *in vitro* was shown to be specific and selective (Serkedjieva and Hay, 1998). Expression of viral glycoproteins on the surface of virus-infected cells, virus-induced cytopathogenic effect, infectious virus yields and plaque-formation were all reduced at non-toxic concentrations of PC. The action was directed against an early stage of infection. Virus-specific protein and RNA synthesis were selectively inhibited. The selectivity of the antiviral action was confirmed by the selection of variants with reduced drug-sensitivity. High concentrations of PC ($>200$ $\mu$g/ml) showed a strong virucidal effect (Serkedjieva and Hay, 1998).

The objective of the present study was to investigate the biologically active constituents of PC, responsible for the antiviral effect, and to evaluate their relative share.

## Materials and Methods

### Chemicals

Quercetin, kaempferol, myricetin, morin, apigenin, retusin, quercetin-3-*O*-galactoside (hyperoside), (−)-catechin, (+)-catechin, (−)-epicatechin, chlorogenic, ellagic, quinic and caffeic acids were from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Rimantadine hydrochloride was from Hoffmann-La Roche (Nutley, NJ, USA). Dr. N. Mahmood and Dr. A. J. Hay (NIMR, Mill Hill, London, UK) provided some of the polyphenol compounds.

### Plant material and extraction

*Geranium sanguineum* L. was collected in June–August in the stage of flowering in the Lulin Mountain. The plant was identified by Assoc. Prof. S. Ivancheva from the Institute of Botany, Bulgarian Academy of Sciences, Sofia, Bulgaria. A specimen was deposited in the Herbarium of the same institute (SOM-5/96). Air-dried aerial roots (500 g) were extracted with ethanol ($3 \times 600$ ml) for 24 h at room temperature. The EtOH extracts were combined and lyophilized (yield 16.7%). The total EtOH extract was further extracted by $(C_2H_5)_2O$, $CHCl_3$, $CH_3CHCl_2$, EtOAc, *n*-BuOH and $H_2O$. The fractions were purified by column chromatography. Qualitative determination of polyphenols was according to Markham (1982). Quantitative determination of tannins was according to European Pharmacopoeia (2003), of flavonoids was according to Pharmacopoeia Helvetica (2003), of catechins and proanthocyanidins was according to Ivancheva and Borzeix (1999).

### Column chromatography (CC)

Sorbents: Sephadex LH-20 – eluent system: MeOH; silica gel – eluent system: EtOAc/MeOH in different ratios; Polyamide S – eluent system: MeOH/$H_2O$ 70:30.

### Thin layer chromatography (TLC)

Sorbents: Polygram silica gel UV$_{254}$ (Merck) – eluent system: EtOAc/HCOOH/$CH_3$COOH/$H_2O$ 100:11:11:27; Kieselgel 60 F$_{254}$ (Merck) – eluent systems: EtOAc/HCOOH/$CH_3$COOH 100:11:11; $CHCl_3$/MeOH 50:50, 75:25, 85:15; Polyamide 11F$_{254}$ (Merck) – eluent systems: EtOAc/HCOOH/ $H_2O$ 10:2:3; $CHCl_3$/MeOH 8:1; Kieselgel 60

(without a fluorescent indicator) – eluent system: $CHCl_3$/MeOH 10:50, 85:15. Spray reagents: NTS/ PEG (1% solution of diphenylborate aminoethanol in MeOH) for flavonoids; 0.5% Echtblausalz in MeOH for tannins. The individual compounds were identified by comparison with authentic samples on different sorbents and by diverse eluent systems and observed at UV$_{364}$ before and after spraying with NTS for flavonoids and Echtblausalz for catechins. Authentic samples were provided by Prof. E. Wollenweber (Institute of Botany, Darmstadt, Germany).

### High performance liquid chromatography (HPLC)

Standards and chemicals

HPLC-grade acetonitrile, HPLC-grade MeOH, formic acid and *ortho*-phosphoric acid (analytical grade) were purchased from Merck (Germany). All other chemicals and solvents were high-analytical grade. Bi-distilled water was prepared in the laboratory from de-ionized water. The reference standards of the flavone aglycones were supplied by Roth (Germany).

Sample preparation

0.2 g dried extracts from a plant sample were dissolved in 20 ml MeOH, treated on a ultrasonic bath for 30 min, dissolved in 20 ml 1.5 M HCl, hydrolyzed on a water bath under reflux for 20 min at 100 °C, cooled at room temperature and diluted 1/50 with MeOH.

Chromatographic equipment and conditions

The chromatographic analysis was performed on a Shimadzu 4A (Japan) chromatographic system, which includes a tertiary pump, a Rheodyne injector with 100 $\mu$l sample loop and a UV-Vis detector. Knauer Chromatography software and workstation were used for controlling the system and collecting the data. The separation was performed using an Atlima, C18, Rocket (57 mm × 7 mm) 3 $\mu$m column (Alltech Inc., Lancashire, UK) maintained at room temperature.

Procedure

A gradient elution (Fig. 1) was carried out using the following solvent systems: mobile phase A, bi-distilled water/methanol/formic acid (74.7:25:0.3; v/v/v); mobile phase B, acetonitrile/formic acid (99.7:0.3; v/v). The linear gradient elution system

Fig. 1. RP-HPLC, standard solution analysis, detection at 270 nm. The linear gradient elution system was realized as follows: 100% mobile phase A (water/methanol/formic acid, 74.7:25:0.3, v/v/v) for 5 min, to 100% mobile phase B (acetonitrile/formic acid, 99.7:0.3, v/v) after additional 10 min, standing at 100% B for 5 min and returning to 100% A after other 5 min. 1, (+)-Catechin; 2, chlorogenic acid; 3, caffeic acid; 4, (−)-epicatechin; 5, rutin; 6, hyperoside.
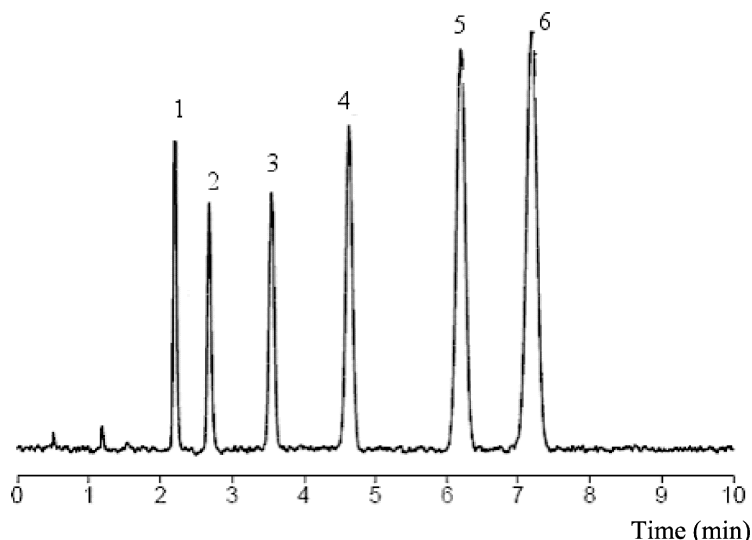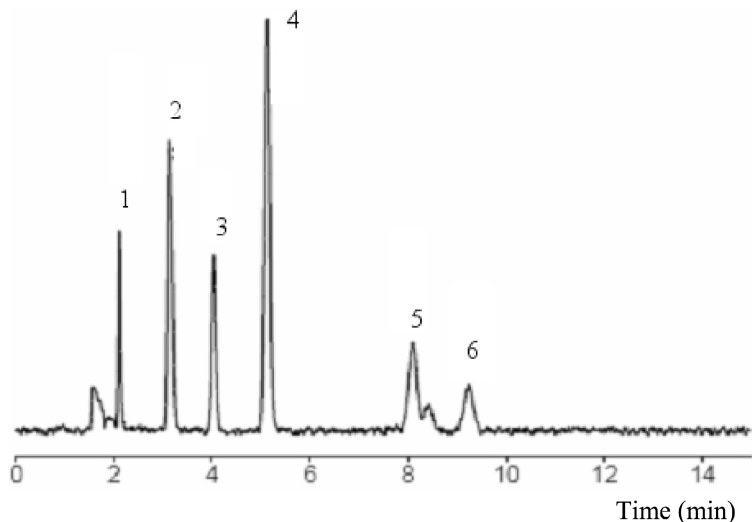
Fig. 2. RP-HPLC isocratic separation: standard solution analysis, detection at 254 nm. Mobil phase: water/acetonitrile (65:35, v/v, adjusted to pH 2.3 by *ortho*-phosphoric acid), flow rate 1.0 ml/min. 1, Caffeic acid; 2, myricetin; 3, morin; 4, quercetin; 5, apigenin; 6, kaempferol.

was: 100% mobile phase A, for 5 min, to 100% mobile phase B after additional 10 min, standing at 100% B for 5 min and returning to 100% A after other 5 min. The flow rate was 1.0 ml/min and the quantification of catechins was performed at 270 nm. 20 µl of each sample were injected, after filtration through a 0.45 µm (Millipore Ireland B.V., Tullagreen, Carrigtwohill, County Cork, Ireland) filter disk.

The mobile phase for isocratic HPLC separation (Fig. 2) containing a volume ratio water/acetonitrile 65:35 (adjusted to pH 2.3 by *ortho*-phosphoric acid) was filtered through a 0.45 µm filter

and de-gassed before use in a ultrasonic bath. The flow rate was 1.0 ml/min. The chromatograms were recorded at 254 nm according to the specific UV absorption of the assayed compounds.

Identification of the flavanols and phenolic acids was carried out by comparing the retention times and the UV absorbance of the unknown peaks with those of the standards. A standard mixture containing (+)-catechin (0.14 mg/ml), (−)-epicatechin (0.38 mg/ml), chlorogenic acid (0.6 mg/ml), caffeic acid (0.14 mg/ml), rutin (0.8 mg/ml), hyperoside (0.8 mg/ml), myricetin (0.9 mg/ml), morin (0.8 mg/ml), apigenin (0.7 mg/ml) and

kaempferol (0.8 mg/ml) in bi-distilled water/formic acid 99.7:0.3 (v/v) was prepared and analyzed. Calibration curves were prepared, using standard solutions, containing between 25% and 125% from the standard mixtures. Each solution was prepared twice and analyzed by HPLC. All sample preparations from dried extracts were prepared in duplicate and analyzed in triplicate for a total of six replicates per sample.

### Virology

#### Cells, media and viruses

Cell cultures from chicken embryo fibroblasts (CEF) were obtained from 11-day-old fertile hens' 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, Pasley, Scotland, UK), supplemented with 5% fetal calf serum (FCS) (BioWhittaker Europe, Verviers, Belgium) and antibiotics; cell cultures were cultivated at 37 °C in the presence of 5% $CO_2$ till the formation of confluent monolayers. In the antiviral experiments 0.5% FCS was added. MDCK cells were provided by Mrs. I. Roeva, Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria. The avian influenza viruses A/Germany/34, strain Rostock (H7N1) (A/Rostock) and A/Germany/27, strain Weybridge (H7N7) (A/Weybridge), grown in CEF and the human influenza virus A/Aichi/2/68 (H3N2) (A/Aichi), adapted to MDCK and cultivated in the presence of 2 μg/ml trypsin (Sigma) were used as test viruses. A/Aichi adapted to mice lungs (A/Aichi-ad) was used for the animal experiments in the dose 5 $LD_{50}$. The virus stocks were stored at −80 °C. The virus infectious titres were in the range $10^{6.3}$–$10^{7.5}$ $TCID_{50}$ (50% tissue culture infectious doses)/0.2 ml and infection was induced with 100 $TCID_{50}$/0.2 ml. The viruses were from the collection of the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria.

#### Mice

Male and female (16–18 g), inbred ICR mice were obtained from the Experimental Animal Station, Bulgarian Academy of Sciences, Sofia, Bulgaria. They were quarantined 24 h prior to use and maintained on standard laboratory chow and tap water *ad libitum* for the duration of the studies. After the end of the experiments surviving mice were sacrificed by cervical dislocation.

#### Ethical aspects

Experiments with animals are necessary in investigations concerning the treatment of experimental influenza infection. The number of experimental animals was reduced as much as possible, depending on statistical significance. The animals were bred under standard conditions, accepted by the Bulgarian Veterinary Health Service. Specialized personnel took care of their welfare. We have adhered most strictly to all national and international ethical provisions applicable to Bulgaria where the investigations with experimental animals were carried out.

#### Cellular toxicity

The cell-toxic effect was examined following the cytopathogenic effect (CPE) of the preparations as described by Serkedjieva and Hay (1998). The dose causing visible changes in cell morphology or sheet density in 50% of intact cells was evaluated from graphic plots (50% toxic concentration, $TC_{50}$).

#### Cytopathogenic effect (CPE) reduction assay

CPE reduction assay was as described before (Serkedjieva and Hay, 1998). The antiviral effect was studied in multicycle experiments of viral growth. The virus-induced CPE was used as a measure of viral replication. The substances were inoculated simultaneously with viral infection. The dose reducing CPE by 50% with respect to virus control was estimated (50% effective concentration, $EC_{50}$). The selectivity index (SI) was found from the ratio $TC_{50}/EC_{50}$. A selectivity index > 4 was considered to indicate a significant selective antiviral effect. For comparative reasons the effects of caffeic acid and respective solvents were tested. All antiviral experiments were carried out in parallel with the selective anti-influenza drug rimantadine hydrochloride.

#### Protective effect *in vivo*

The protective effect was examined in white mice, infected nasally with 10 $LD_{50}$ of A/Aichi-ad as described by Serkedjieva and Ivanova (1997). PC and its fractions were inoculated under light ether anesthesia by nasal instillation 3 h before infection, at the dose 10 mg/kg, in the volume of 0.05 ml PBS. Mice were observed for death daily for 14 d after viral challenge. The protection was estimated by the reduction of the rate of mortality and prolongation of mean survival time (MST).

The index of protection (PI) was determined from the equation $(PR - 1)/PR \times 100$, where PR (ratio of protection) is $M_{control}/M_{experiment}$ and M is the mortality.

Statistical methods

Student's *t*-test was used for the statistical analyses of the results. All experiments were done in triplicate at least.

## Results and Discussion

To investigate the biologically active constituents of PC, responsible for its antiviral effect, and to evaluate their relative share, an EtOH extract was prepared from the aerial roots of the medicinal plant *Geranium sanguineum* L. as described in Materials and Methods. This was the extraction of choice, as the MeOH extract could not be used in animal experiments. The preparation contained 16.15% tannins, 0.126% flavonoids and 2.12 mg/kg catechins and proanthocyanidines. 12% free sugar moieties and a small amount of aminoacids were found, no proteins and saponins were detected. Total soluble phenolic constituents of the extract, measured by Folin-Ciocalteu reagent, were found

as 34.60% (w/w). The total extract was fractionated by organic solvents with increasing polarity – $(C_2H_5)_2O$, $CHCl_3$, $CH_3CHCl_2$, EtOAc, *n*-BuOH, $H_2O$. The fractions were purified by CC on silica gel and re-chromatographed on Polyamide S.

The phytochemical characteristics and antiviral effects of the obtained fractions are presented in Table I. Only the *n*-BuOH fraction showed a significant antiviral effect.

The EtOAc and *n*-BuOH fractions were further separated by CC on Sephadex LH 20 (Table II) and subjected to phytochemical analysis to identify the biologically active constituents.

The *n*-BuOH fraction contained the majority of the antiviral activity in cell cultures (Table I), its virus-inhibitory effect was comparable to the effect of the total extract, SI 24.2% and 32.6%, respectively. Conversely it was not effective *in vivo* (Table II). Eleven subfractions were obtained (*n*-BuOH-1 – *n*-BuOH-11). Four of the subfractions (*n*-BuOH-1, -5, -6, -7) exhibited significant antiviral activity; two of them (*n*-BuOH-5, -6) surpassed the overall effect of the total EtOH extract (SI > 50%). They were studied by TLC, carried out on Polygram silica gel 60, and identification of indi-

Table I. Polyphenol content and anti-influenza virus effect of the total EtOH extract from *Geranium sanguineum* L. and its fractions.

| Plant preparation | Polyphenol content and constituents | Anti-influenza A/Aichi virus effect | |
| --- | --- | --- | --- |
| | | in MDCK cells SI[a] (%) | in mice PI[b] (%) |
| Total EtOH extract | tannins – 16.15% flavonoids – 0.126% catechins and proanthocyanidines – 2.12 mg/kg | 32.6 | 71.4 |
| $(C_2H_5)_2O$ | 4 flavonoids, 1 gallotannin, 2 catechins, 1 polyphenolic acid | no effect[c] | no effect[d] |
| $CHCl_3$ | 1 flavonoid, 1 catechin, condensed tannins | no effect[c] | no effect[d] |
| $CH_3CHCl_2$ | 3 flavonoids, 2 catechins | no effect[c] | no effect[d] |
| EtOAc | 6 flavonoids, hyperoside, 4 gallotannins, 1 polyphenolic acid | no effect[c] | 67.4 |
| *n*-BuOH | 6 flavonoids, 2 catechins, epicatechin, 3 polyphenolic acids, gallotannins, ellagitannin, condensed tannins | 24.2 | no effect[d] |
| $H_2O$ | condensed tannins | no effect[c] | no effect[d] |
| Caffeic acid | | 12.4 | +[e] |

[a]  SI, selectivity index = $TC_{50}/EC_{50}$, where $TC_{50}$ is the 50% toxic concentration to MDCK cells and $EC_{50}$ is the 50% effective virus-inhibitory concentration.
[b]  PI, protective index = $(PR - 1)/PR \times 100$, where PR (ratio of protection) is $M_{control}/M_{experiment}$ and M is the mortality.
[c]  Concentration $\leq TC_{50}$.
[d]  Dose 10 mg/kg.
[e]  Pollikoff *et al.* (1965).

Table II. Polyphenol compounds in the subfractions of the EtOAc and *n*-BuOH fractions and their inhibitory effect on the replication of the influenza viruses A/Rostock and A/Weybridge (*) in CEF.

| Preparation[a] | Main polyphenol constituents | Antiviral effect | | |
|---|---|---|---|---|
| | | $TC_{50}$[b] [$\mu$g/ml] | $EC_{50}$[c] [$\mu$g/ml] | SI[d] (%) |
| EtOAc | | | | |
| -I-2 | (+)-catechin, (−)-catechin, 3 flavonoids[e] | 25.0 | <3.1 | > 8 |
| -I-4 | quercetin, maltol, 1 flavonoid[e] | >200 | 12.5 | >20 |
| -II-3 | hyperoside, apigenin | >100 | 9.0 | >10 |
| -II-4 | hyperoside, caffeic acid | > 50 | 5.0 | >10 |
| -II-5 | myricetin | > 50 | 2.5 | >20 |
| -III-5 | apigenin, quercetin, 1 flavonoid[e] | >100 | 5.0 | >20 |
| *n*-BuOH | | | | |
| -1 | gallotannin[e] | 200.0 | 24.0 | 8.3 |
| | | | 31.2* | 6.6 |
| -5 | caffeic acid, gallotannin[e] | 80.0 | 5.0 | 16.0 |
| | | | 1.6* | 50.0 |
| -6 | (+)-catechin, (-)-epicatechin | 80.0 | 1.6 | 50.0 |
| | | | <1.6* | >50.0 |
| -7 | ellagitannin[e] | 120.0 | 12.0 | 10.0 |
| | | | 8.0* | 15.0 |
| Rimantadine | | > 32 | 0.2 | >160 |

[a] Only subfractions with significant antiviral effect (SI > 4).
[b] $TC_{50}$, 50% toxic concentration to CEF cells.
[c] $EC_{50}$, 50% effective virus-inhibitory concentration.
[d] SI, selectivity index = $TC_{50}/EC_{50}$.
[e] Non-identified.

vidual constituents was done with authentic samples. There were found quercetin, myricetin, morin, kaempferol, retusin, rhamnetin, (+)-catechin, (−)-epicatechin, ellagic, caffeic and chlorogenic acids. The most effective subfractions (*n*-BuOH-5, -6) contained caffeic acid and gallotannins and (+)-catechin and (−)-epicatechin, respectively. It should be noted that though lacking antiviral activity, some of the ineffective fractions contained the same substances (results not shown).
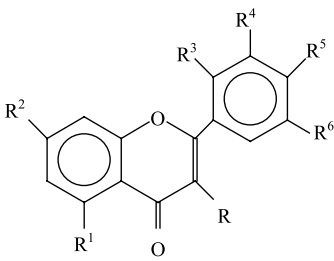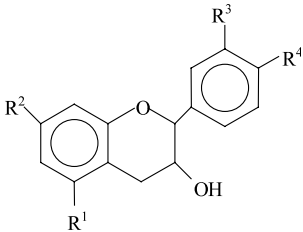
The EtOAc fraction was the most effective fraction *in vivo*, although it was not active in cell cultures. It inhibited influenza virus pathology comparably to the total extract, PI 67.4% and 71.4%, respectively. Eight major subfractions were obtained (EtOAc-I-1 – EtOAc-I-8). I-1 was re-chromatographed on silica gel and 12 more subfractions were obtained (EtOAc-II-1 – EtOAc-II-12). II-6 was re-chromatographed on Polyamid S and yielded 6 more subfractions (EtOAc-III-1 – EtOAc-III-6). Three subfractions have been obtained previously (EtOAc-1 – EtOAc-3). As mentioned before, the EtOAc fraction protected mice in experimental influenza infection but was not effective *in vitro* (Table II). However 6 of all 29 sub-

fractions obtained from this fraction exhibited a significant antiviral effect in cell cultures (EtOAc-I-2, -4; EtOAc-II-3, -4, -5; EtOAc-III-5). This observation suggested that the presence of one or few virus-inhibitory constituents not necessarily resulted in the manifestation of antiviral activity for the whole preparation.

The identification of individual compounds was performed by TLC and comparison with authentic samples. Quercetin, apigenin, myricetin, kaempferol, morin, quercetin-3-*O*-galactoside, (+)-catechin, (−)-catechin, quinic and caffeic acids and maltol were identified. Myricetin, apigenin and (+)-catechin were found for the first time in *G. sanguineum* L.

In order to establish the relative share of the individual components, 15 pure substances, analogues to the compounds identified in PC, were tested for anti-influenza virus activity. The results are presented in Table III. Not surprisingly, almost all tested substances (with the exception of morin, chlorogenic and quinic acids) significantly inhibited the viral growth. None of the compounds identified in PC showed a virus-inhibitory effect as high as that of the total extract. The decreasing

Table III. Antiviral effect of polyphenol compounds, identified in PC on the replication of influenza virus A/Rostock in CEF.

| Substance | Chemical structure | | | | | | | $TC_{50}{}^a$ [$\mu$g/ml] | $EC_{50}{}^b$ [$\mu$g/ml] | $SI^c$ (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Total EtOH extract | | | | | | | | 100.0 | 3.1 | 32.0 |



| | R | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | $R^6$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Quercetin | OH | OH | OH | H | OH | OH | H | 60.0 | 12.2 | 4.9 |
| Kaempferol | OH | OH | OH | H | H | OH | H | 60.0 | 12.2 | 4.9 |
| Myricetin | OH | OH | OH | H | OH | OH | OH | 100.0 | 18.8 | 5.3 |
| Morin | OH | OH | OH | OH | H | OH | H | 100.0 | $>TC_{50}$ | |
| Apigenin | H | OH | OH | H | H | OH | H | 60.0 | 4.1 | 14.6 |
| Retusin | $OCH_3$ | OH | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | H | 100.0 | 24.7 | 4.1 |
| Quercetin-3-*O*-galactoside | galac-tose | OH | OH | H | OH | OH | H | 100.0 | 14.7 | 6.8 |



| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | | | | $TC_{50}$ | $EC_{50}$ | SI |
|---|---|---|---|---|---|---|---|---|---|---|
| (+)-Catechin | OH | OH | OH | OH | | | | 100.0 | 8.3 | 2.0 |
| (−)-Catechin | OH | OH | OH | OH | | | | 120.0 | 8.5 | 14.1 |
| (−)-Epicatechin | OH | OH | OH | OH | | | | 120.0 | 12.0 | 10.0 |
| Chlorogenic acid | 1,3,4,5-tetrahydroxycyclohexane carboxylic acid 3-(3,4-dihydroxycinnamate) | | | | | | | 100.0 | 10.8 | 9.3 |
| Quinic acid | 1,3,4,5-tetrahydroxycyclohexane carboxylic acid | | | | | | | 20.0 | $= TC_{50}$ | |
| Ellagic acid | 4,4′,5,5′,6,6′-hexahydroxydiphenic acid 2,6,2′,6′-dilactone | | | | | | | 60.0 | $>TC_{50}$ | |
| Caffeic acid | 3,4-dihydroxycinnamic acid | | | | | | | 150.0 | 12.5 | 12.0 |
| Rimantadine | | | | | | | | $>32$ | 0.2 | $>160$ |

a, b, c As in Table II.

order of activity for the polyphenolic acids was: chlorogenic = caffeic > ellagic = quinic (not active). Apigenin was the most effective flavonoid. The order of decreasing activity for the flavonoids was: apigenin > quercetin = kaempferol = myricetin = retusin = hyperoside (borderline) > morin (not active).

The *n*-BuOH and the EtOH fractions were investigated also by HPLC. (+)-Catechin, (−)-epicatechin and caffeic acid were identified in the *n*-BuOH fraction; myricetin, morin, quercetin, api-

genin, kaempferol and caffeic acid were identified in the EtOAc fraction.

From the data in Tables II and III follows, that pure flavonoids [myricetin, (+)-catechin, (−)-catechin and (−)-epicatechin] were less active than the corresponding fractions containing them. This observation suggested that these fractions contained additional compounds with higher specific activity that escaped the analytical detection.

The principal objective of the present investigation was to provide data about the individual

chemical components of the polyphenol extract from *Geranium sanguineum* L. responsible for its antiviral effect. This was necessary for the elucidation of the active principle(-s) of the significant anti-influenza virus activity *in vitro* (Serkedjieva and Hay, 1998; Serkedjieva and Manolova, 1992) and the marked protective effect in the murine model of experimental influenza virus infection (Serkedjieva and Manolova, 1992). Altogether 6 fractions and 44 subfractions were screened for antiviral activity. The fractionation of the total EtOH extract did not lead to the isolation of fractions with increased virus-inhibitory effect. Only the *n*-BuOH fraction showed an effect, comparable to that of the total extract. It should be noted that in some cases the activity was considerably reduced or disappeared with fractionation as observed also by Houghton (1996). No clear relationship could be shown between the polyphenolic composition and the antiviral actvity of the fractions. Further fractionation achieved two subfractions (*n*-BuOH-5, -6), which surpassed the effect of the total extract (Table II).

Most of the biologically active compounds identified in the preparation – flavonoids, catechins, gallotannins, polyphenolic acids – belong to chemical groups, known as inhibitors of viral growth. Influenza virus, though, has not been investigated intensively in that order. Apigenin, quercetin and their glycosides, found in *V. thapsiphorme* (Skwarek, 1979), quercetin and catechins from *H. perforatum* (Derebenzeva *et al.*, 1972), kaempferol, quercetin and myricetin discovered in *E. hirsutum* (Ivancheva *et al.*, 1992) were shown to inhibit influenza virus replication *in vitro*. Data on the pro-

tective effect of polyphenols in experimental influenza infection are limited. Caffeic acid (Pollikoff *et al.*, 1965), the flavonoid gossipol from *G. hirsutum* (Vickanova *et al.*, 1970), the flavonoid F36 from *S. baicaliensis* (Nagai *et al.*, 1992), a flavonoid from Euphorbiaceae (Sidwell *et al.*, 1994) and rutin and a rutin-quercetin mixture from propolis (Esanu *et al.*, 1981) improved various influenza infection parameters.

We have examined previously the antiviral effects and the polyphenol content of a variety of extracts from *Geranium sanguineum* L.: diverse extracts from the aerial roots, extracts from different parts of the same plant, extracts prepared from plant material, collected over a period of 10 years in different growth stages as well as extracts from other species of Geraniaceae (Serkedjieva *et al.*, 1998). No direct relationship could be established between the antiviral activity and the polyphenol content of the investigated extracts.

In conclusion the results from the present study and from the previous extensive experiments imply that the antiviral effect of PC could not be accounted for simply in terms of polyphenol content, neither could be attributed to one or few separate ingredients. The presence of a variety of biologically active compounds as well as the possible synergistic interactions between the constituents seem to be more significant for the overall virus-inhibitory effect.

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