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In vitro investigation on the effect of a plant preparation with antiviral activity on the functions of mice phagocyte cells

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A polyphenol extract from the aerial roots of the medicinal plant *Geranium sanguineum* L. (PC) inhibited the reproduction of influenza viruses type A and B *in vitro* and *in ovo* and protected mice from mortality in the experimental influenza infection. The *in vivo* protective effect was connected with multiple biological activities of the preparation. The present paper focuses on the *in vitro* effects of the polyphenol extract on the functions of peritoneal and alveolar macrophages and blood polymorphonuclear leucocytes (PMNs), isolated from healthy ICR mice. It was found that PC in doses of 12.5 and 25 μg ml^{-1} stimulated the phagocytic activity of peritoneal macrophages and blood PMNs. PC in the same doses did not significantly affect the phagocytic activity of alveolar macrophages, the migration of alveolar and peritoneal macrophages or the adherent activity of PMNs. Used in concentrations of 3.1–25.0 μg ml^{-1}, PC suppressed spontaneous NO production from peritoneal macrophages, while inducible NO production, provoked by LPS-, Ifn-γ and LPS + Ifn-γ inductions was not affected. The cell-toxic concentration of 100 μg ml^{-1} increased spontaneous and LPS-inducible NO production. The experimental results demonstrated a stimulating effect of PC on the phagocytic activity of murine PMNs and peritoneal macrophages as well as a beneficial effect of the preparation on spontaneous NO production.

1. Introduction

Immunomodulators of natural origin have aroused a considerable interest (for review see Bomford 1988). Plants have yielded clinically efficacious adjuvants, non-specific immunostimulants and immunosuppresants of diverse chemical structures. Some plant polysaccharides with anti-tumor activity like lentinan, derived from *Lentinus edodes* (Chihara et al. 1969) and krestin, an extract from *Coriolus versicolor* (Ohno et al. 1976) act on the macrophage level through Il-1, which may trigger T-lymphocytes. A hetero-polysaccharide, isolated from the green seaweed *Ulva lactuca,* exhibited an immunostimulating effect on the functions of macrophages and PMNs from healthy mice (Ivanova et al. 1994) and restored the suppressed functions of peritoneal macrophages, as well as the proliferating ability of spleen lymphocytes from hamsters with transplanted myeloid tumors (Toshkova et al. 1995). In this context, particular attention has been given to plant polyphenols. Ellagitannin (Feldman et al. 1999), proanthocyanidines (Lin et al. 2002) and plant derived polyphenols (Dueva and Tsorin, 1999), as well as plant extracts, containing polyphenols (Daswani and Yegnanarayan 2002; Courreges et al. 1998; Makare et al. 2001) have been reported to possess *in vitro* and *in vivo* immunomodulatory activities.

Earlier investigations proved that a semi-standardized polyphenol extract, obtained from the medicinal plant *Geranium sanguineum* L. showed a broad spectrum of antimicrobial activities. In addition to inhibiting the reproduction of influenza viruses type A and B *in vitro* and *in ovo* and protecting mice from mortality in experimental influenza infection (Serkedjieva and Manolova 1992), the preparation suppressed the growth of a series of pathogenic bacteria and fungi (Ivancheva et al. 1992).

The current study was undertaken to provide evidence for the *in vitro* effect of the plant preparation on macrophages and PMNs from healthy ICR mice.

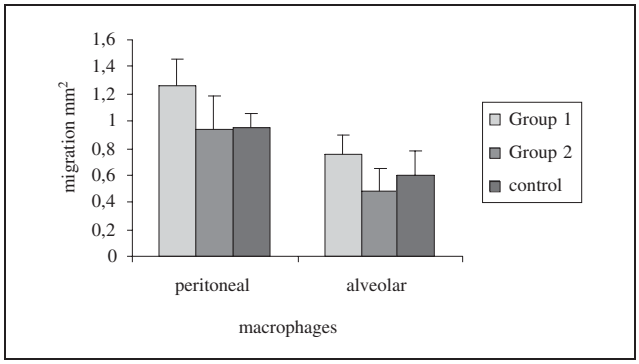

Fig. 1: Migration (mm^2) of mice peritoneal and alveolar macrophages in RPMI 1640 medium, supplemented with PC. Experimental variants: group 1 + 12.5 μg ml^{-1} PC; group 2 –+ 25.0 μg ml^{-1} PC; 3-control – absence of PC

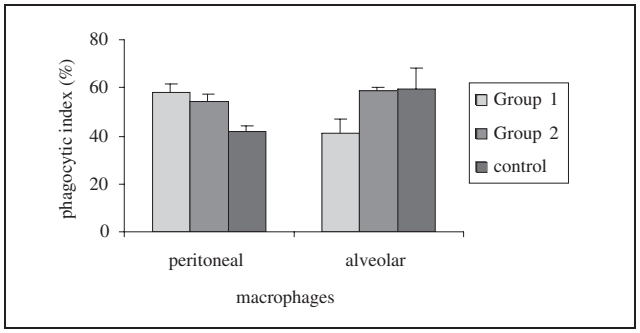

Fig. 2: Phagocytic indices (PI, %) of mice peritoneal and alveolar macrophages, cultivated in RPMI 1640 medium, supplemented with PC. Experimental variants: as in Fig. 1

2. Investigations and results

2.1. Effect of PC on the migration ability of alveolar and peritoneal macrophages

PC in doses of 12.5 and 25 μg ml^{-1} (determined previously to be optimal virus-inhibitory concentrations) did not significantly influence the migration of alveolar and peritoneal macrophages. The results are presented in Fig. 1. The lower dose (12.5 μg ml^{-1}) slightly increased migration (alveolar macrophage migration = 0.75 ± 0.15 mm^2, control – 0.60 ± 0.18 mm^2; peritoneal macrophage migration = 1.26 ± 0.2 mm^2, control – 0.95 ± 0.2 mm^2) (Fig. 1).

2.2. Effect of PC on the phagocytic indices of alveolar and peritoneal macrophages and PMNs

The results of *in vitro* determinations of the phagocyte indices of alveolar and peritoneal macrophages in the presence of 12.5 or 25 μg ml^{-1} PC are presented in Fig. 2. The extract stimulated the phagocytosis of killed *Staphylococcus aureus* Smith cells (PI = $57.7 \pm 4.2\%$ and $54.01 \pm 3.5\%$, respectively, control – $41.76 \pm 2.6\%$). The phagocytosis of alveolar macrophages was slightly suppressed by PC at a dose of 25 μg ml^{-1} (PI = $41.11 \pm 5.7\%$, control $-59.68 \pm 8.9\%$), while a dose of 12.5 μg ml^{-1} did not change the phagocytic indices.
PC, applied in doses of 12.5 and 25 μg ml^{-1} increased the phagocytic ability of blood PMNs (PI = $65.5 \pm 13.2\%$ and $61.57 \pm 11.4\%$ respectively, control $-39.83 \pm 8.9\%$) (Fig. 3).

2.3. Effect of PC on the adherence ability of blood PMNs

The adherence ability of blood PMNs was not changed after incubation for 1 h and 24 h with 12.5 and 25 μg ml^{-1} of PC. The results are presented in Fig. 4.

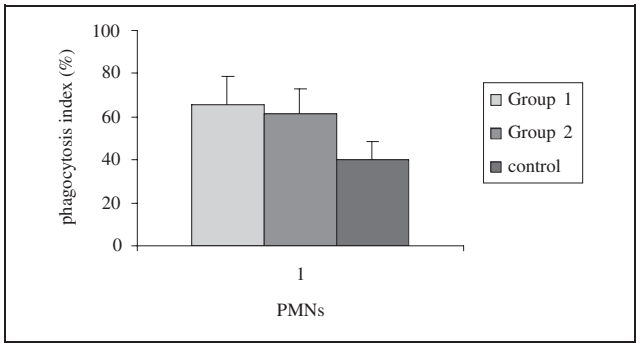

Fig. 3: Phagocytic indexes (PI, %) of mice blood PMNs cultivated in RPMI 1640 medium supplemented with PC. Experimental variants: as in Fig. 1

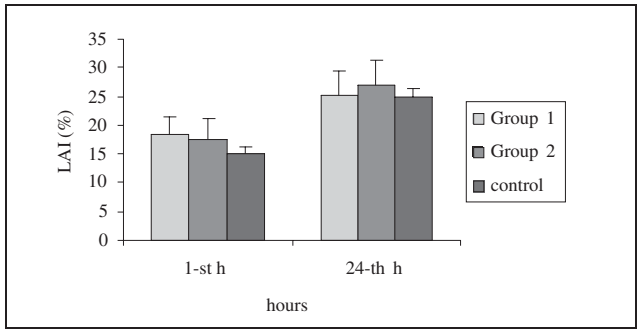

Fig. 4: Leukocyte adherence inhibition (LAI, %) of mice blood PMNs in the presence of PC. Experimental variants: as in Fig. 1

2.4. Effect of PC on the spontaneous and inducible NO production of peritoneal macrophages

Results from experiments on the NO production of peritoneal macrophages are presented in Fig. 5 and 6. PC at doses of 3.1, 6.2, 12.5 and 25 μg ml^{-1} reduced the spontaneous NO production twofold, while the cell-toxic dose of 100 μg ml^{-1} caused an increase of NO production (Fig. 5). In contrast, the inducible production of NO was not significantly influenced by PC. Only the stimulation by 100 μg ml^{-1} of PC and LPS resulted in increased macrophage NO production, while the inducible NO production remained unchanged when Ifn-γ and LPS + Ifn-γ were used as stimuli. (Fig. 6).

2.5. Virus-inhibitory effect of PC

It has been shown that in concentrations above 50 μg ml^{-1} PC causes alterations in cell monolayers and visible changes in cell morphology and viability. TC_{50} for MDCK cells was 82.0 μg ml^{-1}. PC was further tested for a specific influenza virus-inhibitory activity in multicycle experiments on A/Aichi reproduction in MDCK. PC markedly reduced the virus-induced CPE and the production of hemagglutinin as measures of viral reproduction. EC_{50} was 2.3 μg ml^{-1}, the selectivity index was 32.6. The virus-inhibitory effect was selective and dose-related and at a dose of 20.0 μg ml^{-1} the preparation practically abolished viral reproduction – infectious virus yield was reduced to 4.7 $\log_{10}$ $TCID_{50}$, virus control titre being 5.3 $\log_{10}$ $TCID_{50}$. The results are summarized in the Table.

3. Discussion

The principal objective of the study was to provide data about the *in vitro* immunostimulatory activity of a poly-

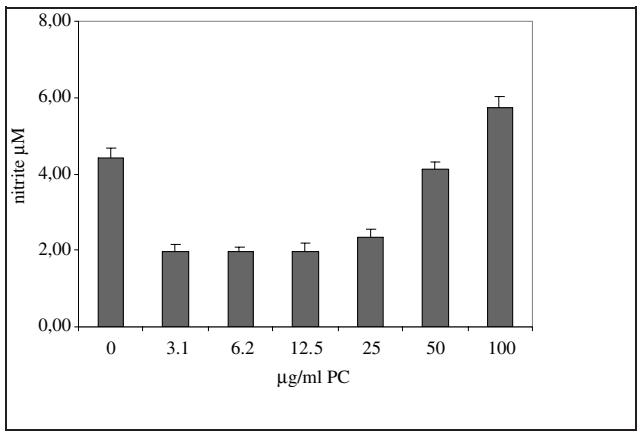

Fig. 5: Spontaneous NO production (μM) of mice peritoneal macrophages in the presence of PC

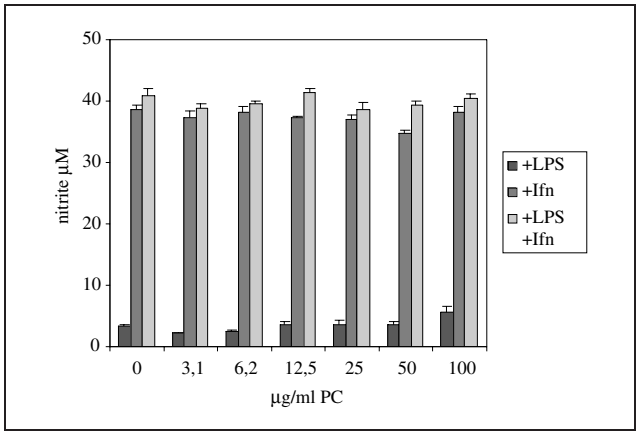

Fig. 6: Inducible NO production (μM) of mice peritoneal macrophages in the presence of different concentrations of PC (0, 3.1, 6.2, 12.5, 25.0, 50.0 and 100.0 μg ml^{-1}). NO production was inducted by: 1-LPS; 2-Ifn-γ; 3-LPS + Ifn-γ

phenol complex isolated from the Bulgarian medicinal plant *Geranium sanguineum* L. by investigation of its effect on macrophages and PMNs from healthy ICR mice. This evidence was needed to evaluate of the marked protective effect of PC in the lethal murine experimental influenza A/Aichi infection (Serkedjieva and Manolova 1992). PC administered intranasally or by aerosol reduced mortality and prolonged the survival time of mice. Investigation of the selectivity and specificity of the virus-inhibitory effect *in vitro* showed that it was rather limited (Serkedjieva and Hay 1998), in contrast with its significant protective effect *in vivo.* Thus the therapeutic effect of PC remained to be explained. We presumed that the protection might be attributed to a combination of more than one biological activity – selective antiviral action, nonselective immunomodulating activity, and some non-specific biological and pharmacological interactions which are known in natural polyphenols, such as protein binding, radical scavenging and antioxidant activities.

The immunostimulatory action of PC on peritoneal macrophage and PMN functions, established by the present investigation (Figs. 2, 3), might explain in part the marked protective effect of PC in the experimental influenza infection (Serkedjieva and Manolova 1992). Based on these results we might suggest that macrophages are the target cells for the immunostimulatory activity of the preparation. This is consistent with the findings that phagocytes, consisting mainly of macrophages and PMNs, are the crucial elements of resistance to experimental infection in mice (Ada and Jones 1986).

The effect of PC on NO production by mice macrophages was also studied. Nitric oxide is a free radical, generated from the oxidation of L-arginine by a family of constitutive or cytokine-inducible NADPH-dependent isoenzymes (Melino et al. 2000). Some of the reactive nitrogen intermediates (peroxinitrite) have strong oxidizing properties towards various cellular constituents and can cause cell death, lipid peroxidation, carcinogenesis and aging. While the secretion of high levels of NO by activated macrophages and PMNs is an important cytotoxic factor in the defense against tumor cells, fungi, protozoa, mycobacteria and virus (Croen 1993; Mackmicking et al. 1997), excessive NO production is associated with a range of diseases (Moncada et al. 1991). NO plays an important role in some viral infections as an inhibitory factor for viral replication. In viral hepatitis NO has a protective function, cooperating in viral clearance (Pinto et al. 2000). Rimelzwaan et al. (1999) showed that the replication of influenza A and B viruses in MDCK is severely impaired by the NO-donor S-nitroso-N-acetyl penicillin-amine. The data on the effect of plant extracts on NO synthesis by macrophages are controversial; Jun et al. 1998 reported suppression of NO synthesis, while Ignacio et al. 2001 and Iuvone et al. 2003 observed stimulation of induction. Our results demonstrated that the effect of PC could be either inhibitory or stimulatory, depending on the dose used (Fig. 5). In conclusion, the established *in vitro* stimulating effect of PC on the phagocytic abilities of mice peritoneal macrophages can be partly explained by the detoxifying effect of the preparation, due to a suppressive effect on spontaneous NO production by macrophages.

In the Table we summarize the results from the present research. PC, used in non-toxic concentrations, was virus-inhibitory, stimulated phagocytosis of peritoneal macrophages and PMNs and suppressed spontaneous NO production. In a cell-toxic dose of 100 μg ml^{-1} the extract increased both the spontaneous and inducible NO production. *In vivo* experiments are in progress to investigate the interactions between the various biological activities of the plant preparation and to evaluate their role in the protective effect of PC in the experimental murine influenza infection.

The variety of biological activities of the plant extract is related to the presence of large quantities of polyphenol compounds. Phytochemical analysis of PC showed that it contained tannins (34%) flavonoids (0.17%), catechins and proanthocyanidines (2 mg kg^{-1}) (Ivancheva et al. 1992).

Identification of the individual compounds showed that flavonoids – aglycones and glycosides (quercetin, quercetin 3-O-galactoside, morin, myricetin, kaempferol, rhamnasin, retusin, apigenin), phenolic acids (caffeic, ellagic, quinic), gallotannins, catechins and maltol were present (Ivancheva et al. 1992, 1996). It has been demonstrated that the virus-inhibitory effect of the extract could not be attributed to one or a few separate constituents. The presence of a diversity of biologically active compounds, as well as the possible synergistic action between them, seemed to be quite significant for the total antiviral effect.

In conclusion, the results of the experiments demonstrate the *in vitro* stimulatory effect of PC on the phagocytic abilities of mice peritoneal macrophages and PMNs, as

Table 1: *In vitro* effects of PC on the functions of mice phagocyte cells and on the reproduction of influenza A/Aichi virus

PC (μg ml^{-1})	ICR mice peritoneal macrophages				MDCK	A/Aichi Viral inhibition
	Phagocytosis	Migration	NO production	Inducible NO production	Cytotoxicity	
12.5	stimulation	no effect	suppression	no effect	no effect	effective
25.0	stimulation	no effect	suppression	no effect	no effect	effective
100.0	n.d.	n.d.	increase	increase	toxic	n.d.

n.d. not determined

well as the beneficial action of the preparation on spontaneous NO production by peritoneal macrophages. These *in vitro* properties might contribute to the overall protective effect of the plant preparation in lethal murine experimental influenza A/Aichi infection.

4. Experimental

4.1. Medicinal plant and extraction

The medicinal plant *Geranium sanguineum* L. (Geraniaceae) has been introduced into the experimental plots of the Institute of Botany, Bulgarian Academy of Science, Sofia and a voucher specimen has been deposited in the Herbarium of the Institute (SOM-5/86). A methanol extract from the aerial roots was obtained as described earlier (Ivancheva et al. 1992) and characterized as the polyphenol complex (PC). In summary – ground air-dried aerial roots, collected during the flowering period, were defatted with petroleum ether and treated with methanol to fully extract the polyphenol components. The extract was lyophilized (yield 16%). The polyphenol content of the preparation was monitored by thin layer chromatography and by quantitative determination of tannins, flavonoids and catechins (Ivancheva et al. 1996).

4.2. Animals

Male and female inbred ICR mice, body weight 18–20 g, were obtained from the Experimental Animal Station of the Bulgarian Academy of Sciences in Slivnitza. The animals were bred in the animal house of the Institute of Microbiology, Bulgarian Academy of Sciences under conditions accepted by the Bulgarian Veterinary Health Control Service. The animals were killed under ether anaesthesia.

4.3. Peritoneal macrophages

The peritoneal macrophages were collected from the peritoneal cavities by 3 washings of the peritoneal cavity with 5 ml cold Hanks solution. The cells were washed by centrifugation at 1200 rpm at 0 °C and resuspended in RPMI 1640 (Fluca) medium, supplemented by 10% fetal calf serum, 100 IU penicillin, 100 μg ml^{-1} streptomycin, 2 mM L-glutamine and 2 mM HEPES and adjusted to different concentrations for the various tests described below.

4.4. Alveolar macrophages

Alveolar macrophages were collected by 5 washings of the trachealbronchoalveolar cavity with 1 ml cold Hanks' solution (pH 7.2) by the method of Holt et al. (1979). The cells were washed and resuspended in culture medium as described for peritoneal macrophages.

4.5. Polymorphous nuclear leukocytes (PMNs)

Blood samples were obtained from the left neck vessels (A. carotis and V. ingularis), collected in glass tubes and immediately diluted 1 : 3 with 2% sodium citrate solution. PMNs were separated from other blood cells by gradient centrifugation on Hisopaque (Sigma, Grunwald, Germany) according to Boyoum (1968). Briefly, citrate blood samples were over-layered subsequently with two gradients (Histopaque 1107 and Histopaque 1119) with a ratio of gradients to blood of 1 : 3 and centrifuged at 300 × g for 30 min at room temperature. The PMNs from the inter-phase between the two layers were collected and washed with Hanks' balanced salt solution (HBSS) and contaminating erythrocytes were destroyed by short lysis in distilled water.

4.6. Phagocyte index

Phagocyte indices of peritoneal and alveolar macrophages were determined according to the method of Osada et al. (1982). Briefly, macrophages at a concentration of 1×10^7 cells ml^{-1} in RPMI 1640 medium without supplements were allowed to adhere to 3 glass lamellae (1 cm^2 in size) for 2 h at 37 °C in a humidified 5% CO_2 atmosphere. After washing with phosphate-buffered saline (PBS), fresh complete RPMI medium and a suspension of killed *Staphylococcus aureus* Smith cells were added to the macrophage monolayers (the ratio of macrophages to bacteria was 1 : 20). The cells were allowed to interact for 1 h under the same conditions, and were then washed and stained by the Papenheim procedure. The phagocyte index (PI) was determined as the number of macrophages participating in phagocytosis per 100 counted macrophage cells. PI values for macrophages in PC-containing medium were estimated and compared to controls.

4.7. Migration of peritoneal and alveolar macrophages

Migration of peritoneal and alveolar macrophages in complete 1640 RPMI medium was examined according to Leu et al. 1972. Briefly, macrophages at a concentration of 6×10^7 ml^{-1} were introduced in silicon-coated hematocrit glass capillaries (75/1 mm for laboratory use), with one end stopped with bees wax. The capillaries were centrifuged at 500 rpm for 5 min at 4 °C, then cut at the visible level of cell pools and put in Petri dishes, containing supplemented RPMI 1640 (Fluca) medium. After 24 h incubation in a CO_2-incubator the migration zones were estimated using enlargement by a projection apparatus and a planimeter. Migration of macrophages in PC-containing media was estimated and compared to controls.

4.8. NO production

NO production was determined by quantifying nitrite, a metabolic product of NO, in macrophage supernatants with a micro-assay (Ding et al. 1988). Briefly, the peritoneal macrophage suspension was inoculated in 96-well plastic plates (100 μl/well) and incubated for 2 h at 37 °C in a CO_2-incubator. The plate was washed three times with Hanks' solution to eliminate of non-adherent cells. Fresh complete medium was added to each well. To investigate the influence of PC on NO production, macrophages were cultivated in the presence of increasing concentrations of PC. The spontaneous and inducible NO production was evaluated using *E. coli* lipopolysaccharide (LPS), Ifn-γ and LPS+Ifn-γ as stimuli. The quantity of the NO produced was estimated using Greiss reagent (1% sulfanilamide/0.1% naphtylethylene-diamine dihydrochloride/2.5% H_2PO_4). 100 μl of Greiss reagent was added to 100 μl of the respective supernatants and incubated for 10 min at room temperature. Optical density at 570 nm was determined by ELISA-spectrophotometer and nitrite concentrations were calculated by comparison with a $NaNO_3$ standard curve.

4.9. Leukocyte adherence inhibition (LAI) technique

LAI assay was performed by the tube method, described by Grosser and Thomson (1975). Briefly, 0.1 ml of 0.5×10^6 PMNs and 0.1 ml of PC in the corresponding concentrations, both made in complete RPMI 1640 medium, were mixed in glass tubes (16 × 150 mm). The tubes were placed horizontally, so that the contents covered $^3/_4$ of the lower areas of the tubes. After incubation for 1 h and 24 h at 37 °C in CO_2-incubator, the tubes were placed vertically and shaken. The number of non-adherent cells was counted in a Burker's chamber. The LAI index was calculated according to the formula:

$$\text{LAI index} = \frac{\text{Experimental cell adherence} - \text{control cell adherence}}{\text{Control cell adherence}} \times 100$$

4.10. Virology

4.10.1. Cells and media

Madin-Darby canine kidney (MDCK) cells were suspended in Dulbecco's Eagle medium (GibcoBRL, Scotland, UK), supplemented with 5% fetal calf serum (FCS) (BioWhittaker Europe, Germany) and antibiotics (100 IU ml^{-1} benzyl penicillin and 100 μg ml^{-1} streptomycin). MDCK cells were cultivated at 37 °C in the presence of 5% CO_2 until the formation of confluent monolayers. In the antiviral experiments 0.5% FCS was added. A/Aichi was cultivated in the presence of 2 μg ml^{-1} trypsin (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). MDCK cells were kindly provided by Mrs. I. Roeva, Institute of Microbiology, Bulgarian Academy of Sciences, Sofia.

4.10.2. Virus

Human influenza virus – A/Aichi/2/68 (H3N2) (A/Aichi) was adapted to MDCK cells; the virus stock was stored at −70 °C. The virus infectious titre was 10^6 $TCID_{50}$ ml^{-1} (50% tissue culture infectious doses ml^{-1}). The virus was from the collection of the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia.

4.10.3. Cellular toxicity

The cell-toxic effect of PC was examined as described earlier (Serkedjieva and Hay 1998). Briefly, confluent MDCK cell monolayers in 96-well plastic plates were overlaid with two-fold dilutions of PC in growth medium and were observed microscopically for changes in cell morphology and viability at 24, 48 and 72 h of incubation. The cytopathic effect (CPE) was scored under an inverted microscope as described before. The dose causing visible changes in cell morphology or viability in 50% of intact cells was evaluated from graphic plots (50% toxic concentration, TC_{50}).

4.10.4. Cytopathogenic effect (CPE) reduction assay

The antiviral effect of PC was examined as described earlier (Serkedjieva and Hay 1998). Briefly, quadruplicate confluent monolayers in 96-well plates were overlaid with 2× PC-containing medium (0.1 ml) and an equal volume of virus suspension (100 $TCID_{10}$ 0.1 ml^{-1}). The virus-induced CPE was scored after 72 h as described before. Virus-induced CPE and the pro-

duction of HA were used as measure of viral growth. The dose reducing CPE by 50% with respect to virus control was estimated (50% effective concentration, EC_{50}). Reduction of CPE > 50% was considered to indicate a significant virus-inhibitory effect. In all the experiments non-drug treated, mock-infected cells were used as cell controls and non-drug treated, virus-infected cells as virus controls. The selective anti-influenza drug rimantadine hydrochloride (2 μg ml^{-1}) was used as a positive control. The selectivity index (SI) was determined by the ratio TC_{50}/EC_{50}.

4.10.5. 50% End point titration technique (EPTT)

EPTT was performed according to Vanden Berghe et al. (1986). The antiviral activity was determined by the difference between the virus titres of control and treated viruses ($\delta \log_{10} TCID_{50}$/ml). The significance of the difference was estimated by Student's *t* test.

4.11. Statistical methods

Student's *t*-test was used for the statistical analyses of the results. All experiments were done in triplicate. Results are given as arithmetic mean values ±SD. ** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

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