

**A PLANT EXTRACT AMELIORATES THE DISFUNCTIONS OF ALVEOLAR MACROPHAGES IN INFLUENZA VIRUS-INFECTED MICE**

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**Summary**

A polyphenol extract from *Geranium sanguineum* L. (PC) protected mice from mortality in the experimental influenza virus infection (EIVI). The current study aimed to reveal the effect of PC on the number and functions of alveolar macrophages (aM $\phi$ ) from PC-treated healthy and influenza virus-infected mice (VIM). After infection the number of the aM $\phi$  in VIM was 2.0-2.5-fold increased with a maximum on day 6 p.i.; the migration was depressed. PC-application induced a rise of the number and migration of aM $\phi$  in the infected and healthy controls. Influenza infection impaired also the phagocytic functions of aM $\phi$ ; PC-treatment restored them and on day 9 p.i. phagocyte indices reached control values. Influenza infection provoked a significant increase of H<sub>2</sub>O<sub>2</sub>, 'O<sub>2</sub>' and NO production by aM $\phi$ , which peaked on day 9 p.i. PC-treatment decreased the release of ROS and NO; this resulted in reduced lung tissue damage.

Key words: plant extract, influenza, alveolar macrophages

Earlier research proved that a polyphenol extract from *Geranium sanguineum* L., designated as polyphenol complex (PC), inhibited the reproduction of influenza viruses type A and B *in vitro* and protected mice from mortality in the EIVI (1). We demonstrated in model systems that the extract possessed multiple pharmacological activities. In addition to the specific and selective virus-inhibitory effect (2), PC exhibited a stimulating effect on the phagocytic activity of murine PMNs and peritoneal macrophages, showed a beneficial effect on the spontaneous NO production (3), possessed antioxidant and radical scavenging capacities (4). The aim of the present work was to investigate the effect of PC on the number and functions of aM $\phi$  from PC-treated healthy and influenza VIM.

## Materials and Methods

*Plant preparation.* Ground air-dried aerial roots from *Geranium sanguineum* L. (Geraniaceae), introduced into the experimental field of the Institute of Botany, Bulgarian Academy of Sciences, were defatted with petroleum ether and treated with EtOH to fully extract the polyphenol compounds. The combined extracts were lyophilised (PC); the polyphenol content was controlled by TLC and quantitative determination of tannins, flavonoids and catechins. PC was kindly provided by Dr. S. Ivancheva.

*Mice and Experimental design.* Inbred ICR mice (16-18 g body weight) were obtained from the Animal Station, Bulgarian Academy of Sciences and were bred under conditions, accepted by the Bulgarian Veterinary Health Service. There were four experimental groups: 1, mock-infected and PBS-treated - control healthy (CH); 2, mock-infected and PC-treated (PC); 3, influenza virus-infected (VIM) and PBS-treated - virus control (VC); 4, VIM and PC-treated (VC+PC). At the end of the experiment surviving mice were sacrificed under ether anaesthesia.

*Influenza infection.* The infection was induced intranasally (i.n.) under light ether anaesthesia with A/Aichi/2/68 (H3N2) influenza strain, adapted to murine lungs. The challenge dose was 10 LD<sub>50</sub> in 0.05 ml PBS/mouse and caused lethal hemorrhagic pneumonia. Two additional groups of twelve animals each (VC and VC+PC) were observed for death daily for 14 days post infection (p.i.). Lung consolidation (score) and infectious virus titres were evaluated as described before (1).

*Alveolar macrophages.* Alveolar macrophages (aM $\emptyset$ ) were collected on days 2, 6, 9 p.i. by 5 washings of broncho-alveolar cavity with 1 ml cold Hanks' solution (pH 7.2) according to the method, described in (3).

*ROS and NO detection.* Superoxide anion (O<sub>2</sub><sup>-</sup>) was measured by the SOD-inhibitable reduction of cytochrome *c*. H<sub>2</sub>O<sub>2</sub> production was determined by phenol red oxidation. NO generation was measured by quantifying nitrite (5).

*Statistical methods.* Results are given as % of healthy controls and are analyzed by ANOVA. Student's *t*-test is used for differences in lung scores. P<0.05 is accepted for statistical significance.

## Results

The infection provoked significant changes in the number and functions of aM $\emptyset$ . Their number in VIM was 2.0-2.5-fold increased with a maximum on day 6 p.i. (Fig. 1).

The migration was markedly depressed on day 6 p.i., on day 9 it was increased 4-fold (Fig. 5). PC augmented 2.5-4-fold the number and migration of aMØ in the infected and healthy controls.

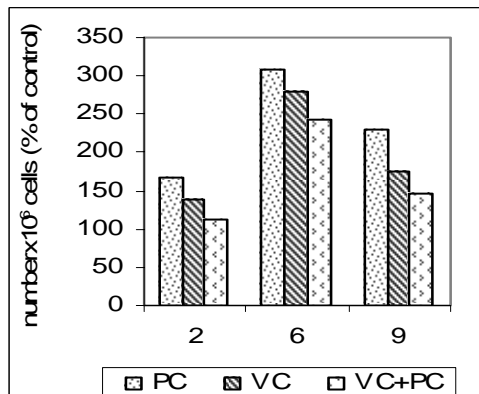


Figure 1. Number of aMØ of influenza VIM, treated with PC.

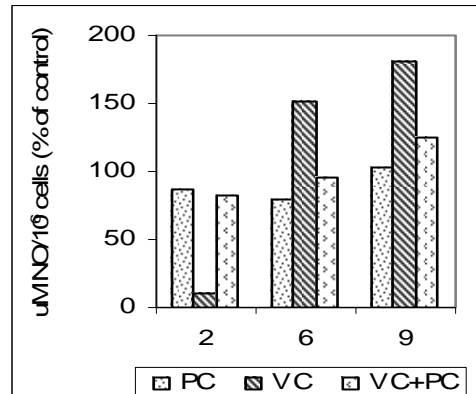


Figure 2. NO production in aMØ of influenza VIM, treated with PC VIM.

In the infected and healthy controls PC induced a continuous 2.5-4-fold rise of the number and migration of aMØ, the maximum being on day 6 p.i. (Fig. 1, 5). EIVI impaired also the phagocyte functions of aMØ; PC-treatment restored them and on day 9 p.i. phagocyte indices reached control values (Fig. 6). In healthy mice the phagocyte abilities of aMØ were enhanced markedly after the application of PC. Interestingly, *in vitro* PC (25 µg/ml) did not affect the phagocyte activity and the migration of aMØ (Fig. 7).

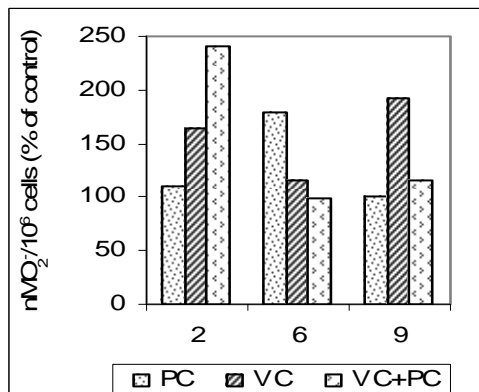


Fig. 3. O<sub>2</sub><sup>-</sup> production in aMØ of influenza VIM, treated with PC

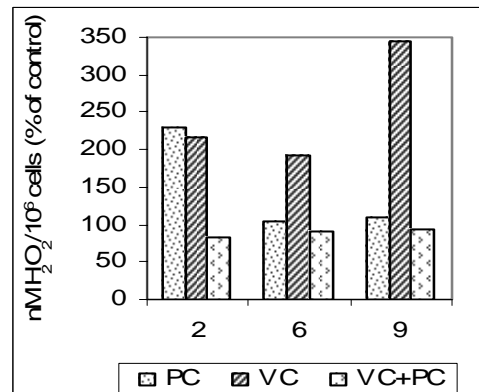


Fig. 4. H<sub>2</sub>O<sub>2</sub> production in aMØ of influenza VIM, treated with PC

The infection caused also a marked increase of  $H_2O_2$ ,  $O_2^-$  and NO production, which peaked on day 9 p.i. (Figs. 2, 3, 4). PC-treatment decreased the release of ROS and NO; this resulted in reduced lung tissue damage (Fig. 8). The restoration of the compromised functions of aMØ in VIM was consistent with a prolongation of the mean survival time and reduction of the mortality rate and the infectious virus titer (results not shown). PC-treatment of CH resulted in 1.5-fold increase in  $O_2^-$  production by aMØ on day 6 p.i. and significant induction of  $H_2O_2$  release on day 2 p.i. (Fig. 2).

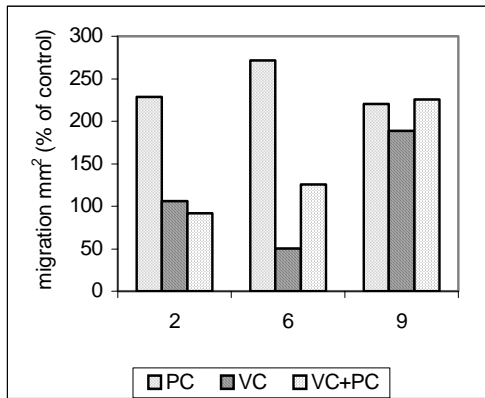


Fig. 5. Migration of aMØ of influenza VIM, treated with PC.

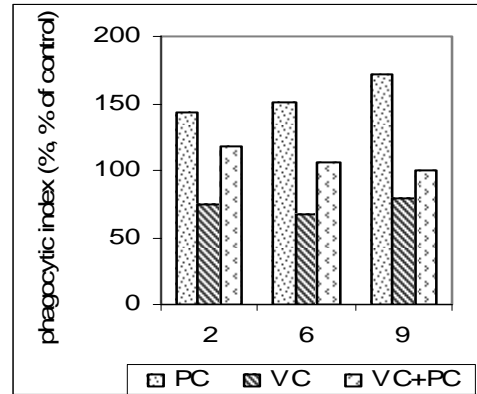


Fig. 6. Phagocytosis of aMØ of influenza VIM, treated with PC

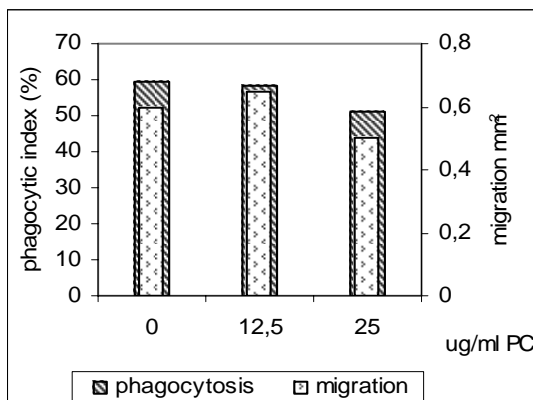


Fig. 7. Migration and phagocytosis of aMØ in vitro

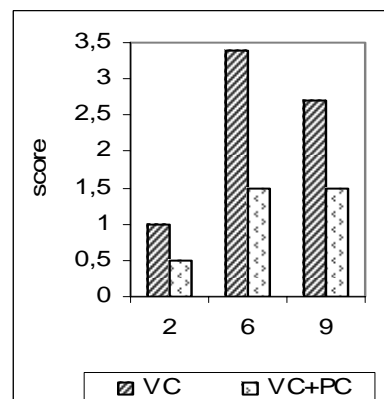


Fig. 8. Lung score of influenza VIM, treated with PC

### Discussion

Macrophages are part of the innate immune system, which is stimulated by foreign agents to produce cytokines and reactive oxygen and nitrogen species. As activation of the immune system is a possible therapeutic approach we investigated the effect of a plant polyphenol extract on the number and functions of aM $\phi$  from PC-treated healthy and influenza VIM and in this way to provide evidence for the implication of its immunomodulatory potential for the overall protective effect in the lethal experimental influenza virus infection.

The results from the present experiments provide evidence that PC ameliorated the dysfunctions of phagocytes in A/Aichi virus-infected mice. Its *in vitro* enhancing effect on pM $\phi$  and PMNs functions and the beneficial action on the spontaneous NO production has been reported previously (3); PC stimulated immune reactivity and caused a rise of antigen-binding cells; it has been found also that the i.p. application of PC induced low titers of serum interferon in intact mice (6).

Based on the results, obtained *in vitro* and *in vivo*, we suggest that M $\phi$  are the target cells for the immunoenhancing activity of the extract. The favorable immunopotentiating capacity of PC in VIM was in concert with the alleviation of disease symptoms: the severity of the lung lesions was notably decreased; mortality and infectious virus load in the lungs were also markedly reduced. We suggest that the immunorestoring effect of the extract contributes substantially to its overall protective effect in the EIVI. This is consistent also with the findings that phagocytes are a crucial element of resistance to influenza virus infection in mice (7). Moreover Watanabe *et al.*, 2005 proved that phagocytosis of virus-infected cells helped suppress the progress of influenza in mice (8).

The presented results show also that PC modulated the excessive production of O $_2^-$ , H $_2$ O $_2$  and NO during the experimental influenza virus infection. 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 decrease in ROS and RNS production is an alternative mode of action of the plant extract in addition to its specific virus-inhibitory activity.

Phytochemical analysis of PC showed that it contained tannins (34%) flavonoids (0.17%), catechins and proanthocyanidines (2 mg/kg) (9). The identification of individual compounds showed that flavonoids - aglycones and glycosides (quercetin, quercetin 3-O-galactoside, morin, myricetin, kaempferol, rhamnasin, retusin, apigenin), phenolic acids (caffeic, ellagic, quinic and chlorogenic), gallotannins, catechins and maltol were present (9, 10). It has been demonstrated that the virus-inhibitory effect of the extract could not be attributed to one or few separate ingredients.

The presence of a diversity of biologically active compounds, as well as the possible synergistic action between them seemed to be more significant for the total antiviral effect.

Immunomodulators of natural origin have raised a considerable interest (for review see 11). Plants have yielded clinically efficacious adjuvants, non-specific immunostimulants and immunosuppressants of diverse chemical structures. In this context, particular attention has been given to plant polyphenols. Ellagitannin (12), proanthocyanidines (13), plant derived polyphenols (14) as well as plant extracts, containing polyphenols (15, 16, 17) have been reported to possess *in vitro* and *in vivo* immunomodulatory activities.

Based on our results we could suggest that the polyphenol-rich extract from *Geranium sanguineum* is a promising candidate as an immune modifier, useful for the treatment of influenza-virus infection. Further studies are warranted to elucidate the primary biological effects of its immunopotentiating activity.

### Acknowledgments

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