EFFECT OF IMMUNOSTIM PLUS – A STANDARDIZED FIXED COMBINATION OF SCHIZANDRA CHINENSIS WITH ELEUTHEROCOCCUS SENTICOSUS EXTRACTS ON GRANULOCYTE ACTIVITY AND TUMOUR ANGIOGENESIS IN MICE

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Abstract

The in vivo effect of fructus Schizandra and radix Eleutherococci combined extracts on various parameters of granulocyte activity in Balb/c mice were studied. The activation of respiratory burst, measured by colorimetric assay (RBA) and chemiluminescence test (CL), and activation of phagocytic activity of granulocytes (PKA test) were observed in the mice fed 300, 600 or 1200 µcg per day of the extract for 7 d. In mice receiving the extract for 3 d after the subcutaneous grafting of syngeneic L-1 sarcoma cells a diminished neovascular response, induced by transplanted cells (TIA test), was found.

Key words: mice, granulocytes, tumour angiogenesis, Schizandra chinensis, Eleutherococcus senticosus.

Schizandra chinensis (Magnoliaceae) is largely used in China owing to its adaptogenic, anti-oxidant and hepato-protective properties. It was also prescribed in cases of chronic cough and dyspnea, diarrhoea, night sweats, wasting disorders, irritability, palpitations, and insomnia (12). Decoctions of Schizandra were found to possess strong in vitro anti-bacterial activity. Data about immunotropic effects of Shizandra are lacking but anti-inflammatory and anti-tumour activity were described (1, 5-8). There are no reports on the effect of this herb on angiogenesis.

Eleutherococcus senticosus (Siberian ginseng) is a commonly used herb with adaptogenic, anti-stress, and immuno-modulatory properties (2, 4, 9, 11, 15, 16).

The aim of this study was to evaluate the combined effect of these two medicinal plant extracts on in vivo granulocyte function and tumour angiogenesis in mice.

Material and Methods

The study was performed on 8-10–week-old inbred Balb/c mice, weighing about 20 g, of both sexes, delivered from breeding colony of the Polish Academy of Sciences, and Warsaw and Mazurian (Olsztyn) Universities. Studied material was Immunostim Plus (Herbapol Lublin), 300 mg capsules, composed of dried extract of radix Eleutherococcus senticosus (155 mg), dried extract of fructus Schizandra chinensis (100 mg), and adjunctive substances (50 mg). Mice were fed Immunostim for 7 d (experiments with granulocytes) or for 3 d (tumour angiogenesis studies) in daily doses of 300, 600 or 1200 µcg.

These doses corresponded to 150, 300 and 600 mg given to a 70 kg person (applying the counter 7 for differences between mouse and human in relation of the surface to body mass). Mice received orally (feeding with use of Eppendorf pipette) Immunostim dissolved in 45 microliters of water or 10% ethyl alcohol, or 10% alcohol or water (controls).

On the day 8 mice were anaesthetized with chloral hydrate, bled from retroorbital plexus and sacrificed. Their spleen and blood were used for cellular immunity tests. Splenocytes were isolated from mice under sterile conditions by straining through a stainless
seive and centrifugation on Gradisol in order to remove erythrocytes.

**Granulocyte chemiluminescence assay.**

Briefly, the test consisted of taking 0.05 ml of heparinized venous blood and diluting it 1:4 with PBS (Biomed Lublin, Poland), supplemented with 0.1% BSA (Sigma-Aldrich, USA) and 0.1% glucose (Polfa, Poland). Next 0.05 ml of this diluted blood was added to 0.2 ml of luminol (Sigma-Aldrich, USA) solution (10⁻² M) in PBS and placed in a scintillation counter (RackBeta 1218, Sweden) in the „out of coincidence“ mode for background chemiluminescence measurement. The cells were then activated by addition of 0.02 ml solution of Zymosan (10 mg/ml) and chemiluminescence activity was measured for the next 15 min. The results were shown as cpm per 10⁶ granulocytes.

**Isolation of leukocytes.** Leukocytes were isolated from blood by centrifugation at 2000 g for 30 min at 4°C on the Gradisol G gradient (Aqua-Medica, Poland), washed three time in PBS and resuspended in RPMI 1640 medium (Sigma) supplemented with 10% of FCS (Foetal Calf Serum, Gibco-BRL) at a stock concentration of 2 x 10⁶ cells/ml of medium.

**Control of cell-mediated immunity.** The determination of metabolic activity of blood phagocytizing cells (mostly granulocytes) was based on the measurement of intracellular respiratory burst after stimulation by PMA (phorbol myristate acetate, Sigma), as described by Chung and Secombes (3) and adapted for dogs by Siwicki et al. (10) and adapted for dogs by Siwicki et al. (10) and adapted for dogs by Siwicki et al. (10) and adapted for dogs by Siwicki et al. (10). The isolated cells were resuspended in RPMI-1640 medium (Sigma) at 10⁶ cells/ml. On 96-well U-shaped microplates 100 µl of the isolated leukocytes was mixed with 100 µl of 0.2% nitro blue tetrazolium (NBT, Sigma) solution in 0.2 M phosphate buffer at pH 7.2 and added 1 µl of PMA at concentration of 1 mg/ml in ethanol. After 30 min of incubation at 37°C, the supernatant was removed from each well. The cell pellet was washed with absolute ethanol and then 3 times in 70% ethanol and dried at room temperature. The amount of extracted reduced NBT after incubation with 2M KOH and DMSO (dimethylsulfoxide, Sigma) was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech). All the samples were tested in triplicate and the mean value served as the result.

**Angiogenesis induced in the skin after grafting of L-1 sarcoma syngeneic cells.** Cutanous angiogenesis assay was performed according to Sidky and Auerbach with own modifications (14). Sarcoma cells were delivered from the Warsaw Oncology Center Bank and then passaged through several generations of Balb/c mice. Briefly, sarcoma cells were grafted (10⁶/0.1 ml) intradermally into subscapular region. After 14 days the tumours were excised, cut to smaller pieces, rubbed through sieve and suspended in 5 ml of PBS. The suspension was left for 15 min at room temperature. After sedimentation, the supernatant was collected and centrifuged for 10 min at 1400 rpm. Obtained sarcoma cells were washed once with PBS for 10 min, then centrifuged at 1500 rpm, and resuspended in Parker medium in concentration of 4x10⁶/ml. Multiple 0.05 ml samples of cells were injected intradermally into partly shaved, narcotised Balb/c mice. In order to facilitate the localisation of cell injection sites, the suspension was coloured with 0.1% of trypan blue. On the day of cell grafting and on the following two days mice were fed Immunostim or water. After 72 h mice were sacrificed with lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope on the inner skin surface, at magnification of 6x, in 1/3 central area of microscopic field. The identification was based on the fact that new blood vessels, directed to the point of cells injection, differ from the background vasculature in their tortuosity and diversications. All the experiments were performed in anaesthesia (3.6% chloral hydrate, 0.1 ml per 10 g of body mass).

Experiments were approved and supervised by Local Ethical Committee. Statistical analysis was performed by Mann-Whitney and Student tests.

**Results**

The results are presented in Tables 1 and 2 (granulocyte activity tests) and on Fig. 1 (tumour angiogenesis). As can be seen from the tables and figure Immunostim Plus highly significantly increased leukocyte activity evaluated by all the methods applied, without influencing leukocyte count in blood. On the other hand, Immunostim Plus administrated after tumour cell grafting highly significantly diminished neovascular reaction induced in mice by syngeneic tumour cells.

**Discussion**

Our experiments revealed, for the first time, that combination of *Schizandra chinensis* and *Eleutherococcus senticosus* extracts behaves as strong immunostimulator of non-specific cellular defense, dependent on the first line cells, granulocytes. Wagner et al. (17) showed stimulation of granulocytes and carbon-clearance tests by polysaccharide fractions of *Eleutherococcus senticosus*. 
Table 1
The *in vivo* effect of Immunostim Plus on the metabolic (RBA test) and phagocytic (PKA test) activity of blood and spleen leukocytes (x OD 620 nm ± SE)

<table>
<thead>
<tr>
<th>Dose of Immunostim (mcg/day)</th>
<th>RBA spleen</th>
<th>RBA blood</th>
<th>PKA spleen</th>
<th>PKA blood</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (n=5) (control)</td>
<td>0.163 ± 0.007</td>
<td>0.209 ± 0.004</td>
<td>0.214 ± 0.002</td>
<td>0.215 ± 0.005</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>600 (n=5)</td>
<td>0.234 ± 0.005</td>
<td>0.283 ± 0.003</td>
<td>0.257 ± 0.005</td>
<td>0.284 ± 0.003</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>1200 (n=5)</td>
<td>0.243 ± 0.001</td>
<td>0.290 ± 0.002</td>
<td>0.290 ± 0.004</td>
<td>0.298 ± 0.001</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Significance P < 0.001

Table 2
Effect of Immunostim Plus on chemiluminescent activity and number of blood leukocytes

<table>
<thead>
<tr>
<th>Dose of Immunostim (mcg/day)</th>
<th>Leukocytes /mm³ x ± SE</th>
<th>Significance</th>
<th>cpm/1000 granulocytes x ± SE</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (n=10) (control)</td>
<td>6390 ± 344</td>
<td>-</td>
<td>4722 ± 521</td>
<td>-</td>
</tr>
<tr>
<td>300 (n=10)</td>
<td>6195 ± 302</td>
<td>n.s.</td>
<td>7549 ± 996</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>600 (n=10)</td>
<td>5765 ± 225</td>
<td>n.s.</td>
<td>9514 ± 112</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

n.t. – not significant

**Fig. 1.** Effect of Immunostim Plus on neovascular reaction induced by Sarcoma L-1 tumour cells in syngeneic mice skin.
Wildfeuer and Mayerhofer (18) showed that *Eleutherococcus senticosus* increased in vitro phagocytosis of *Candida albicans* by granulocytes and monocytes from healthy donors. However, there are no papers about influence of *Schizandra* on granulocyte functions. In our present study combination of *Eleutherococcus* with *Schizandra* exerted strong anti-angiogenic activity in cutaneous angiogenesis test induced by syngeneic tumour cells in Balb/c mice. In available literature there are no communications concerning the effect of *Eleutherococcus* or *Schizandra* on angiogenesis. In our earlier study we evaluated the influence of *Eleutherococcus senticosus* on cellular lymphocyte-dependent and humoral immune response (9). We have shown that this plant had immunomodulatory properties, and had no influence on the angiogenic activity of human renal carcinoma cells. So, we might suppose that angio-inhibitory effect of Immunostim Plus is mainly connected with *Schizandra* extract.

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**References**


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