

# The *in vivo* effect of dry hydro-alcoholic extract of *Echinacea purpurea* on angiogenic activity of human blood mononuclear cells

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## Abstract

**The aim** of this study was to evaluate the effect of dry hydro-alcoholic extract of *Echinacea purpurea* (IMMUNAL forte), or placebo, administered for seven days to 22 healthy human volunteers in daily dose of one or two tablets, on the angiogenic activity of their blood mononuclear cells (MNC), tested by mice cutaneous test (leukocyte-induced angiogenesis – LIA), and on the number of CD4+ and CD8+ T lymphocytes.

**Results:** Immunol forte administration resulted in highly statistically significant stimulation of MNC angiogenic activity in both dosage groups and significant increase of CD4/CD8 ratio in persons treated with 2 tablets daily what was connected with significant decrease of CD8+ lymphocytes in their blood. In persons treated with 1 tablet daily both CD8+ and CD4+ lymphocytes significantly increased in number. No stimulatory effect was observed after administration of placebo. All biochemical results were in normal values, and there were no differences between groups.

**Key words:** *Echinacea purpurea*, human blood mononuclear cells, angiogenesis, T lymphocytes.

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## Introduction

Herbal drugs containing *Echinacea purpurea* extracts are commonly used for treatment of upper respiratory tract infections. *Echinacea* extracts contain many compounds with immunomodulatory and anti-inflammatory activity – alkaloids, polysaccharides, polyphenols, glycoproteins, essential oils, tannins, and others. Studies in experimental animals have shown that *Echinacea* increases immune response to antigens and may enhance phagocytosis and various lymphocyte activities [1-5].

Previously, we reported stimulatory effect of *Echinacea purpurea* succus stabilized with ethanol (IMMUNAL drops, LEK, Slovenia) on various parameters of human leukocytes activity [6]. However, from experimental studies performed earlier we know that various forms of *Echinacea purpurea* preparations may vary in their immunotropic activity, and some of them were immunosuppressive in doses recom-

mended by producers. So, the aim of the present study was to evaluate the effect of other form of *Echinacea purpurea*, dry hydro-alcoholic extract (IMMUNAL forte tablets), administered for 7 days to healthy human volunteers, on the ability of their blood mononuclear leukocytes to induce neovascular cutaneous response in mice. Counting of CD4+ and CD8+ lymphocytes and some biochemical examinations were also performed.

## Material and methods

Study was performed in 22 healthy male volunteers, 20-40 years old. Informed consent was obtained from all persons. All experiments were accepted and supervised by the local Ethical Committee.

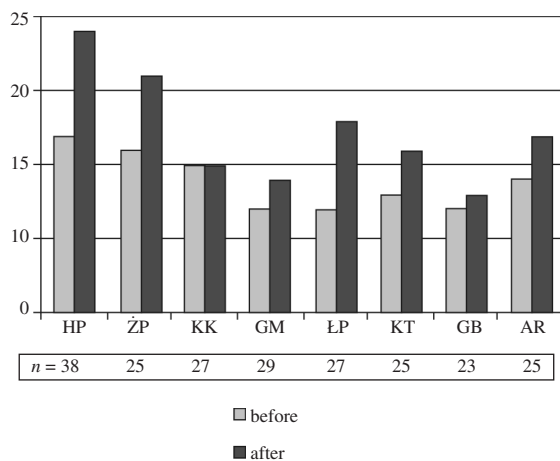
Blood from cubital vein was obtained twice, before and after 7 days treatment.

The following materials were studied:

- *Echinaceae purpureae* aerial parts dry hydro-alcoholic extract (IMMUNAL forte – tablets, LEK), 160 mg (2 tablets) daily for 7 days – 8 persons,
- IMMUNAL forte 80 mg (1 tablet) daily, for 7 days – 6 persons,
- placebo, 1 tablet daily for 7 days – 8 persons.

Mononuclear cells (MNC) were isolated from heparinised blood on Lymphoprep gradient, washed thrice with phosphate buffered saline (PBS), and resuspended in Parker medium. Cutaneous angiogenesis assay (LIA test) was performed according to Majewski *et al.* [7] with some modifications. Briefly, MNC were injected intradermally into 6-7 weeks old female inbred Balb/c mice (multiple 0.05 ml samples of  $5 \times 10^5$  cells each, at least 2-4 mice per group). Before performing injections the mice were anaesthetized with 3.6% chloralhydrate (0.1 ml per 10 g of body mass) and both flanks of each mouse were finely shaved with razor blade. On each flank 2-3 injections were localized. In order to facilitate the localization of cell injection sites later on, the suspension was colored with 0.1% of trypan blue. After 72 hours 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. Identification was based on the fact that new blood vessels, directed to the point of cells injection are thin and/or differ from the background vasculature in their tortuosity and divarications.

Analysis of T lymphocytes was done by monoclonal antibody staining of specific markers CD4 and CD8, using DAKO APAP KIT System 40, USA, in Lymphoprep-isolated blood mononuclear cell suspensions, according to



**Fig. 1.** Angiogenic activity of human blood mononuclear cells (MNC) before and after 7-days treatment with 2 IMMUNAL tablets, presented as mean number of newly-formed blood vessels, n = number of LIA tests

producer instruction. Triplicate samples were established from each material.

Biochemical studies of volunteers. The following laboratory studies were performed in both placebo and subject groups: number of leukocytes and their subpopulations, prothrombin time, plasma level of fibrinogen, kaolin-kephalinic time, serum concentration of IgG, IgA, IgM, and C3 and C4 fractions of complement.

All samples were analysed using autoanalysers: haematological SYSMEX SF-3000; biochemical HITACHI 911 and BCT (for coagulation system).

Statistical analysis of the results was performed using unpaired t test and a one-way ANOVA. The significance of differences between the groups was verified with a Bonferroni Multiple Comparison post test (GraphPad InStat3).

## Results

The results of angiogenic activity of MNC and CD4/CD8 ratio are presented in Fig. 1 and on the table 1 and table 2. IMMUNAL forte administration resulted in highly statistically significant stimulation of MNC angiogenic activity in both dosage groups (the two-tailed p-value is 0.0001, considered extremely significant). Variation among column means is significantly greater than expected by chance. We also found significant increase of CD4/CD8 ratio in persons treated with 2 tablets daily what was connected with significant decrease of CD8+ lymphocytes in their blood. In persons treated with 1 tablet daily both CD8+ and CD4+ lymphocytes significantly increased in number. No stimulatory effect was observed after administration of placebo.

All hematological and biochemical results were in normal values, and there were no differences between groups.

**Table 1.** The effect of IMMUNAL tablets or placebo administration to human volunteers for 7 days on angiogenic activity of their blood mononuclear cells

|  | N            | Stimulation index ± SE |
|--|--------------|------------------------|
| IMMUNAL 2 tablets (8 persons)                        | 219          | 1.24 ±0.03             |
| IMMUNAL 1 tablet (6 persons)                         | 172          | 1.15 ±0.02             |
| Placebo (8 persons)                                  | 238          | 1.04 ±0.01             |
| Statistical significance of differences from placebo | $p < 0.0001$ |                        |

The results are presented as mean stimulation indices (value after treatment/value before treatment); n = total number of LIA tests

Unpaired t test (GraphPad InStat3)

The two-tailed p-value is 0.0001, considered extremely significant.

## Discussion

The modified allogeneic and xenogenic leukocyte-induced angiogenesis tests were introduced by Kaminski *et al.* [8] and used up today in the experimental and clinical studies for evaluation of total reactivity of cells isolated from lymphatic tissues or blood mononuclear leukocytes [9]. Lymphocytes and monocytes, grafted into mice skin, released various cytokines and growth factors important for the development of neovascular response. It was demonstrated in rabbits with experimental hyperlipidemia that their blood lymphocytes express enhanced angiogenic activity, and significant increase of FcGR positive cells was detected [10, 11]. In rats, similar effect was obtained by physical training [12]. *In vivo* exposure of mice to essential oil of *Melaleuca alternifolia* (tea tree) or feeding them *Echinacea purpurea*, *Rhodiola rosea*, *Rhodiola kirilowii*, *Rhodiola quadrifida* or *Panax ginseng* extracts increased angiogenic capability of their spleen cells. Administration of *Centella asiatica*, however, decreased spleen cells angiogenic activity [13-16].

It was shown in further studies in man, that the highest angiogenic activity was expressed by T cells forming rosettes with SRBC and bearing both the receptor for the Fc portion of IgG and a CD4 surface antigen [17, 18]. More detailed studies discovered two subpopulations of highly angiogenic human T lymphocytes: first, CD4+ FcG+ with CD2 receptors sensitive to theophylline, and second, CD4+ FcG- with CD2 receptors resistant to theophylline [19, 20]. High angiogenic activity of activated macrophages was also described [21].

In our present study CD4/CD8 lymphocytes ratio increased significantly in volunteers treated with 2 tablets of Immunal for seven days, and accordingly, MNCs from this group of people exerted the highest angiogenic activity.

Angiogenic activity of human blood mononuclear leukocytes decreased in elderly, in comparison with young adults, but no difference depending on sex was observed [22]. Abnormalities were observed in angiogenic activity of MNCs collected from patients with systemic scleroderma [23, 24]. Lowered angiogenic activity of blood MNCs was also observed in patients with coronary heart disease and some patients with oral candidosis [25].

A lot of studies was performed by our scientific team for evaluation the effect of various natural and synthetic substances on human blood MNCs angiogenic capability. In those studies MNCs were isolated from human blood, grafted intradermally to mice, and tested substances were introduced to recipients orally or subcutaneously, for following three days.

In this test system, several natural drugs and herbal extracts behaved as stimulators of immunological angiogenesis: among them Tołpa peat preparation (TPP), stimulating angiogenic activity of T cells and monocytes, water extracts of *Hypericum perforatum* L and *Melissa*

**Table 2.** The effect of IMMUNAL tablets or placebo administration to human volunteers for 7 days on CD4+/CD8+ T lymphocytes ratio in their blood

|  | N  | CD4/CD8 ratio ± SE                       | Statistical significance of differences from "before treatment" |
|--|----|--|---|
| Before treatment (22 persons)                        | 66 | 1.66 ±0.04                               |   |
| IMMUNAL 2 tablets (8 persons)                        | 24 | 2.08 ±0.09                               | p < 0.001   |
| IMMUNAL 1 tablet (6 persons)                         | 18 | 1.71 ±0.04                               | Non significant   |
| Placebo (8 persons)                                  | 24 | 1.37 ±0.05                               | p < 0.05  |
| Statistical significance of differences from placebo |    | 1 tablet p < 0.05<br>2 tablets p < 0.001 |   |

*officinalis* L; ether fraction of poplar leaves water extract and isolated from this fraction phenolic acids: caffeic, gallic, salicylic, ferulic and gentisic. Chlorogenic acid behaved as immunomodulator, it increased angiogenic activity of MNCs collected from healthy donors, and decreased abnormally high angiogenic activity of MNCs collected from the blood of rheumatoid arthritis patients. The same was observed for TPP [26-29].

*Echinacea purpurea* increased angiogenic activity of MNCs collected from the blood of patients with oral candidosis and from the blood of healthy donors [30].

## Conclusion

Stimulation by *Echinacea purpurea* extracts of angiogenic factors release by mononuclear leukocytes opens new fields for application of this drug (ischemic diseases, prolonged repair and healing processes, chronic infections and ulcerations).

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