**In vivo** effect of two complex herbal remedies Echinasal and Bioaron C on antibody production and immunological angiogenesis in mice

EWA SKOPIŃSKA-RÓŻEWSKA1,2, ALEKSANDER WASIUTYŃSKI1, PIOTR SKOPIŃSKI1, DOROTA SIWICKA4, ROBERT ZDANOWSKI5, PAWEŁ BODERÁ1

1Department of Pathology, Center for Biostructure Research, Medical University of Warsaw, Poland
2Department of Microwave Safety, Military Institute of Hygiene and Epidemiology, Warsaw, Poland
3Department of Histology and Embryology, Center for Biostructure Research, Medical University of Warsaw, Poland
4Department of Clinical Immunology, Faculty of Medicine, Medical University in Lublin, Poland
5Department of Pharmacology and Toxicology, Military Institute of Hygiene and Epidemiology, Warsaw, Poland

**Abstract**

The in vivo effect of two composed herbal remedies Echinasal and Bioaron C on specific cellular and humoral immunity in mice was studied. Stimulatory effect on the local graft-versus-host reaction (immunological angiogenesis) was presented by feeding donor mice for 7 days with both remedies. Angiogenic activity of maternal donor spleen lymphocytes was more efficiently stimulated by Bioaron C than by Echinasal. Stimulatory effect on anti-SRBC antibody production was presented by feeding mice for 7 days before immunization with 40 or 80 µl daily dose of Echinasal (p < 0.01 and p < 0.001, respectively), and 30 µl daily dose of Bioaron C (p < 0.01). Higher (90 µl) daily dose of Bioaron C presented stimulation on the border of statistical significance (p < 0.1).

**Key words:** Echinasal, Bioaron C, humoral immunity, immunological angiogenesis, mice.

(Centr Eur J Immunol 2011; 36 (3): 139-144)

**Introduction**

Previously, we reported the in vivo modulatory effects of various substances of natural origin on the ability of parental mice splenic lymphocytes to induce local graft-versus-host reaction in F1 recipients (lymphocyte-induced angiogenesis, LIA test) and on the antibody production in mice [1-9]. In this paper we describe the in vivo effect of two commercial herbal drugs, Echinasal and Bioaron C, on above parameters of cellular and humoral immunity. Bioaron C syrup is a complex remedy, composed of bioystomin (aloë extract), succus aroniae and vitamin C, used for the treatment of upper respiratory tract infections in children. This remedy contains water extract of aloë leaf (Aloe arborescens Mill.), chokeberry fruit juice (Aronia melanocarpa Elliot) and vitamin C [10, 11].

Echinasal syrup, used for the treatment of respiratory tract infections with accompanying cough, is an extractum compositum ex: Plantaginis lanceolatae folio, Grindeliae herba, Rosae fructus, Thymi herbae, and Echinaceae purpureae herbae succus.

**Material and methods**

Preparats: Bioaron C (Phytopharm)
Preparats: Echinasa (Herbapol Wroclaw)

**Mice:** The study was performed on 7-9 weeks old inbred female Balb/c mice and on F1 hybrids Balb/c × C3H, 20-25 g of body mass, delivered from the Polish Academy of Sciences and from the own breeding colony.

**Study of antibody production:** Mice were fed remedy or water (controls) for 7 days, before intraperitoneal
injection of 0.2 ml 10% sheep red blood cells (SRBC) suspension. Animals received daily 40 and 80 µl of Echinusal or 30 and 90 µl of Bioaron C (feeding with use of Eppendorf pipette).

These doses corresponded to 20 and 40 ml of Echinusal or 15 and 45 ml of Bioaron C given to 70 kg person (applying the counter 7 for the differences between mouse and human in relation of the surface to body mass). Each experimental or control group consisted of ten animals.

Mice were bled in anaesthesia from retroorbital plexus 7 days after immunization.

The antibody level was evaluated with haemagglutination assay in heat inactivated (56°C, 30 min) sera. After performing a series of sera dilutions, 0.5% SRBC were added and the mixture was incubated for 60 min at room temperature, then centrifuged (10’, 150 g) and shaken. The hemagglutination titer was evaluated in a light microscope – as the last dilution in which at least 3 cell conglomerates were present in at least 3 consecutive fields at objective magnification 20×.

**Study of immunological angiogenesis**

Drugs were administered to the groups of 6 Balb/c mice each, per os, in daily doses of 40 and 80 µl (Echinusal) or 30 and 90 µl (Bioaron C). Mice received drugs by Eppendorf pipette, for 7 days. Controls mice were fed 80 µl of distilled water.

On the 8th day mice were sacrificed with Morbital, spleens were dissected and spleen cells suspensions prepared. Spleen cells suspensions were pooled within a group and grafted intradermally into F1 recipients, cells from each pool into 3-4 F1 recipient mice. A local GVH reaction (lymphocyte-induced angiogenesis – LIA) was performed according to Sidky and Auerbach with some modifications [3, 5]. In this test grafted Balb/c cells recognize foreign C3H histocompatibility antigens and produce many immunological mediators including pro-angiogenic factors (immunological angiogenesis). The number of newly-formed blood vessels is the measure of cells reactivity.

Multiple 0.05 ml samples, containing 10⁶ spleen cells each, from Balb/c mice fed remedies or water were injected intradermally into partly shaved, narcotised F1 mice (3-4 mice per group, 4-6 injections per mouse). In order to facilitate the localisation of injection sites later on, the suspension was coloured 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 6×, 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, differ from the background vasculature in their tortuosity and divercations. All experiments were performed in anaesthesia (3.6% chloral hydrate, 0.1 ml per 10 g of body mass).

For all experiments animals were handled according to the Polish law on the protection of animals and NIH standards. Experiments were approved by the Local Ethical Committee.

**Statistical analysis**

Statistical evaluation of the results was done by one-way analysis of variance ANOVA (GraphPad Prism software) and the significance of differences between the groups was verified by Newman-Keuls Multiple Comparison Test (immunological angiogenesis) and Tukey’s Multiple Comparison Test (antibody production).

**Results**

According to one way analysis of variance (ANOVA) the P value is < 0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance. Both remedies have stimulated humoral and cellular immunity. Stimulatory effect on anti-SRBC antibody production was similar for both tested remedies (Fig. 1 and Table 1), except for the higher dose of Bioaron C, statistically non-different from the control (difference on the border of statistical significance).

Angiogenic activity of maternal donor spleen lymphocytes was more efficiently stimulated by Bioaron C than by Echinusal (Fig. 2 and Table 2).

**Discussion**

In this paper we present the evidence of stimulatory activity of complex herbal remedies Echinusal and Bioaron C on cellular and humoral immunity in mice. Both remedies stimulated the ability of maternal mice splenic lymphocytes to induce local graft-versus-host reaction in F1 recipients (immunological angiogenesis, LIA test). In
Invivoeffectoftwocomplexherbalremedies Echinasaland Bioaron Con at antibody production and immunological angiogenesis in mice

thisteststimulatoryeffectof Bioaron C was more pronounced than the effect of Echinasal.

In the case of the effect on anti-SRBC humoral immunity, Echinasal and Bioaron C exerted stimulatory effects, except in the experiments where mice were fed with 90 µl of Bioaron C daily dose (difference on the border of statistical significance).

Both remedies are composed from many substances of natural origin. Some of them are known as immunomodulators (Echinacea purpurea, Plantago lanceolata, Aloe arborescens). There is strong evidence that Echinacea enhances both cellular immunity and antibody production [12-16]. Plantago extracts, however, and isolated from them compounds (for example plantagoside) have been described as suppressing antibody response to SRBC and, in higher doses, lymphocyte proliferation. This may explain, why in Echinasal remedy stimulatory effect of Echinacea was less pronounced than in other Echinacea containing remedies. However, Plantago extracts possess strong anti-viral, cytotoxic and anti-inflammatory activity [17-20]. Ursolic acid,

\[
\begin{array}{c|c|c|c}
\text{Table 1. Statistical analysis of the effects on antibody production} \\
\hline
\text{One-way analysis of variance} & & \\
\hline
P \text{ value} & <0.0001 & \\
\hline
P \text{ value summary} & *** & \\
\hline
\text{Are means signif. different? (P < 0.05)} & Yes & \\
\hline
\text{Number of groups} & 6 & \\
\hline
F & 7.937 & \\
\hline
R \text{ square} & 0.4104 & \\
\hline
\text{Bartlett’s test for equal variances} & & \\
\hline
\text{Bartlett’s statistic (corrected)} & 16.79 & \\
\hline
P \text{ value} & 0.0049 & \\
\hline
P \text{ value summary} & ** & \\
\hline
\text{Do the variances differ signif. (P < 0.05)} & Yes & \\
\hline
\text{ANOVA Table} & SS & df & MS \\
\hline
\text{Treatment (between columns)} & 16.71 & 5 & 3.342 \\
\hline
\text{Residual (within columns)} & 24.00 & 57 & 0.4211 \\
\hline
\text{Total} & 40.71 & 62 & \\
\hline
\text{Tukey’s Multiple Comparison Test} & Mean Diff. & q & \text{Significant? P < 0.05?} & Summary \\
\hline
\text{Control vs. Bioaron 30 µl} & -1.100 & 5.872 & Yes & ** \\
\hline
\text{Control vs. Bioaron 90 µl} & -0.6000 & 2.965 & No & NS \\
\hline
\text{Control vs. Control} & 0.1000 & 0.5090 & No & NS \\
\hline
\text{Control vs. Echinasal 40 µl} & -0.9000 & 4.581 & Yes & ** \\
\hline
\text{Control vs. Echinasal 80 µl} & -1.200 & 6.108 & Yes & *** \\
\hline
\end{array}
\]

NS – not significant

Donormice were fed Echinasal or Bioaron C for 7 days before grafting their splenic cells to recipient’s skin.

Fig. 2. The in vivo effect of two herbal remedies on the angiogenic activity of splenic lymphocytes
a triterpenoid compound found in *Plantago* extracts inhibited tumor-associated capillary formation [21]. However, in hind limb ischemia model in mice, ursolic acid enhanced collateral blood flow recovery through induction of neovascularization [22] what corresponds to our present results.

Extracts of *Echinacea* and *Plantago* are used not only in human medicine. They are also useful in veterinary medicine, for example in the treatment of Kennel cough (a highly contagious respiratory infection affecting dogs and cats). This infection is caused by a combination of bacterial and viral agents (*Bordetella bronchiseptica*, *Canine Parainfluenza*, less commonly *Adenovirus* types 1 and 2 and *Mycoplasma*).

*Grindelia*, expectorant herb with bronchospasmolytic activity was used by the Indian natives from California before the conquest of the country by white men. Hispanics used *Grindelia* species in a similar way to the Native American use — that is, primarily for asthma, neuralgia, bladder infections. *Grindelia* leaves and flowering tops was introduced into the general practice in 1875 for external (rashes, burns and insect bites) and internal (spasmodic respiratory conditions such as asthma and bronchitis) use. In 1880, it was introduced into the U.S. Pharmacopoeia. Anti-inflammatory activity of

**Table 2. Statistical analysis of the effects on immunological angiogenesis**

<table>
<thead>
<tr>
<th>One-way analysis of variance</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P value</strong></td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td><strong>P value summary</strong></td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Are means signif. different?</td>
<td>(P &lt; 0.05)</td>
<td>Yes</td>
</tr>
<tr>
<td>Number of groups</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>74.57</td>
<td></td>
</tr>
<tr>
<td>R square</td>
<td>0.7489</td>
<td></td>
</tr>
</tbody>
</table>

Bartlett’s test for equal variances

| Bartlett’s statistic (corrected) | 9.710 |
| **P value**                      | 0.0839 |
| **P value summary**              | NS     |

Do the variances differ signif. (P < 0.05) No

ANOVA Table

<table>
<thead>
<tr>
<th>Treatment (between columns)</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>762.4</td>
<td>5</td>
<td>152.5</td>
</tr>
</tbody>
</table>

Residual (within columns)

|                      | 255.6| 125 | 2.045|

Total

|                      | 1018 | 130 |

Newman-Keuls Multiple Comparison Test

<table>
<thead>
<tr>
<th>Control vs. Bioaron 30 µl</th>
<th>Mean Diff.</th>
<th>q</th>
<th>Significant? P &lt; 0.05?</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs. Bioaron 90 µl</td>
<td>−7.970</td>
<td>24.63</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Control vs. Echinusal 80 µl</td>
<td>−4.700</td>
<td>15.12</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Control vs. Echinusal 40 µl</td>
<td>−2.500</td>
<td>9.332</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Control vs. Control</td>
<td>−0.700</td>
<td>2.252</td>
<td>No</td>
<td>NS</td>
</tr>
<tr>
<td>Echinusal 40 µl vs. Bioaron 30 µl</td>
<td>−5.770</td>
<td>17.47</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Echinusal 40 µl vs. Echinusal 80 µl</td>
<td>−2.500</td>
<td>7.865</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Echinusal 40 µl vs. Bioaron 90 µl</td>
<td>−0.3000</td>
<td>1.087</td>
<td>No</td>
<td>NS</td>
</tr>
<tr>
<td>Echinusal 80 µl vs. Bioaron 30 µl</td>
<td>−5.470</td>
<td>17.01</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Echinusal 80 µl vs. Bioaron 90 µl</td>
<td>−2.200</td>
<td>7.123</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Bioaron 90 µl vs. Bioaron 30 µl</td>
<td>−3.270</td>
<td>9.129</td>
<td>Yes</td>
<td>***</td>
</tr>
</tbody>
</table>

NS – not significant
Grindelia extracts was investigated in vitro. In macrophage model, Grindelia exerted anti-inflammatory effect through its capacity to reduce the accumulation of inflammatory mediators (IL-6, RANTES, MCP-1, FGE2, TNF-α) and metalloproteinases 1, 3, 7, 8, 9, and 13 [23]. Studies on neutrophils revealed quercetin-3-methyl ether as the most active compound of Grindelia robusta acetonic extract in inhibition of neutrophil elastase, what also contributed to the anti-inflammatory activity of the drug [24]. Successful treatment of poison oak dermatitis with Grindelia extract was described [25]. Thyme (Thymus vulgaris L.) and its major components thymol and carvacrol display antimicrobial activity, antioxidant properties against aflatoxin-induced oxidative stress in rats, anti-fungal activity against Aspergillus flavus and Aspergillus ochraceus, and inhibitory effect on human oral cavity squamous cell carcinoma [26-29].

When thyme plants were exposed to highly vascular mint plants, inducible vascular factor arose which prevented properties against aflatoxin-induced oxidative stress in rats, anti-fungal activity against Aspergillus flavus and Aspergillus ochraceus, and inhibitory effect on human oral cavity squamous cell carcinoma [26-29].

Biostimine exerted high stimulatory effect on migration activity of mouse splenocytes, chemiluminescence activity of blood granulocytes, anti-SRBC antibody production, and angiogenic activity of mononuclear leukocytes isolated from the blood of healthy human volunteers and patients with oral infection, in mouse cutaneous LIA test. Similarly as in the present study, in higher doses stimulatory effect disappeared. It was also demonstrated by other authors, that Aloe vera gel possess pro-angiogenic activity [32, 33].

References


27. El-Nekeety AA, Mohamed SR, Hathout AS, et al. (2011): Antioxidant properties of *Thymus vulgaris* oil against aflatoxin-induced oxidative stress in male rats. Toxicon 57: 984-991.


