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

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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.*

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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 biostymin (aloe extract), succus aroniae and vitamin C, used for the treatment of upper respiratory tract infections in children. This remedy contains water extract of aloe 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: Echinasal (Herbapol Wrocław)

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

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injection of 0.2 ml 10% sheep red blood cells (SRBC) suspension. Animals received daily 40 and 80 µl of Echinasal or 30 and 90 µl of Bioaron C (feeding with use of Eppendorf pipette).

These doses corresponded to 20 and 40 ml of Echinasal 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.

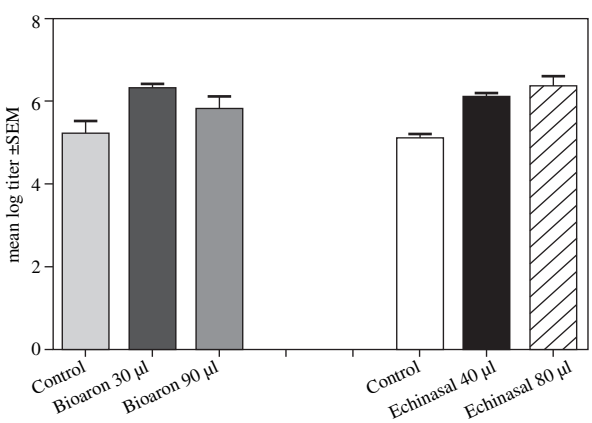
Mice were bled in anaesthesia from retroorbital plexus 7 days after immunization.

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 (Echinasal) or 30 and 90 µl (Bioaron C). Mice received drugs by Eppendorff 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.



Mice were fed Bioaron C or Echinasal for 7 days before intraperitoneal SRBC injection

Fig. 1. The *in vivo* effect of herbal remedies Bioaron C and Echinasal on the antibody production in mice

Multiple 0.05 ml samples, containing 10^6 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 divarications. 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 standarts. 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 Echinasal (Fig. 2 and Table 2).

Discussion

In this paper we present the evidence of stimulatory activity of complex herbal remedies Echinasal 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

Table 1. Statistical analysis of the effects on antibody production

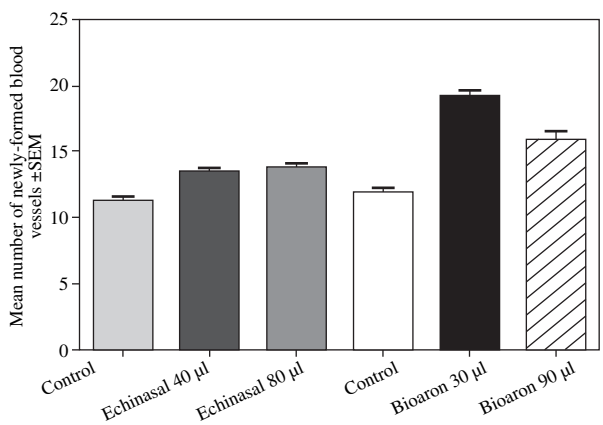
One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (*P* < 0.05)	Yes			
Number of groups	6			
F	7.937			
R square	0.4104			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	16.79			
P value	0.0049			
P value summary	**			
Do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

NS – not significant

this test stimulatory effect of 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,



Donor mice 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

Table 2. Statistical analysis of the effects on immunological angiogenesis

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? ($P < 0.05$)	Yes			
Number of groups	6			
F	74.57			
R square	0.7489			
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	SS	df	MS	
Treatment (between columns)	762.4	5	152.5	
Residual (within columns)	255.6	125	2.045	
Total	1018	130		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? $P < 0.05$?	Summary
Control vs. Bioaron 30 µl	−7.970	24.63	Yes	***
Control vs. Bioaron 90 µl	−4.700	15.12	Yes	***
Control vs. Echinasal 80 µl	−2.500	9.332	Yes	***
Control vs. Echinasal 40 µl	−2.200	7.907	Yes	***
Control vs. Control	−0.7000	2.252	No	NS
Echinasal 40 µl vs. Bioaron 30 µl	−5.770	17.47	Yes	***
Echinasal 40 µl vs. Bioaron 90 µl	−2.500	7.865	Yes	***
Echinasal 40 µl vs. Echinasal 80 µl	−0.3000	1.087	No	NS
Echinasal 80 µl vs. Bioaron 30 µl	−5.470	17.01	Yes	***
Echinasal 80 µl vs. Bioaron 90 µl	−2.200	7.123	Yes	***
Bioaron 90 µl vs. Bioaron 30 µl	−3.270	9.129	Yes	***

NS – not significant

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* leafs 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

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, PGE2, TNF-α) and metalloproteinases 1, 3, 7, 8, 9, and 13 [23]. Studies on neutrophils revealed quercetin-3-methylether 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 VEGF-induced migration in human umbilical vein endothelial cells [30].

Previously, we have estimated *in vivo* the effect of Biostimine (main active compound of Bioaron C) on some parameters of humoral and cellular immunity in mice [31]. Biostimine is a water-soluble extract of the leaves of triennial plants *Aloe arborescens* Mill., which is planted in green-houses of the Herbal Industry Phytopharm Klęka.

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

1. Skopińska-Różewska E, Różycka B, Białas-Chromiec B, et al. (1999): Immunostimulatory effect of essentials oils. Protetyka Stomatologiczna 49: 15-18.
2. Skopińska-Różewska E, Niemirowska-Mikulska H, Zwolska Z, et al. (2001): Immunotropic activity of essentials oils. Terapia 9: 47-49.
3. Skopińska-Różewska E, Furmanowa M, Guzewska J, et al. (2002): The effect of *Centella asiatica*, *Echinacea purpurea* and *Melaleuca alternifolia* on cellular immunity in mice. Centr Eur J Immunol 27: 142-148.
4. Skopińska-Różewska E, Gibka J, Gliński M, et al. (2006): Immunotropic effects of undecan-2 one in mice. Centr Eur J Immunol 31: 57-62.
5. Siwicki AK, Skopińska-Różewska E, Hartwich M, et al. (2007): The influence of *Rhodiola rosea* extracts on non-specific and specific cellular immunity in pigs, rats and mice. Centr Eur J Immunol 32: 84-91.
6. Gibka J, Majda T, Tichek A, et al. (2008): Study of the effect of 3-undecanone and 3-undecanol on cellular and humoral immunity in mice. J Essential Oils Res 20: 282-286.
7. Gibka J, Skopińska-Różewska E, Siwicki AK, et al. (2008): Stimulation of humoral immunity in mice by undecan-2-one, undecan 2-ol and their derivatives. Centr Eur J Immunol 33: 47-49.
8. Skopińska-Różewska E, Siwicki K, Sommer E (2009): Stimulation of humoral immunity in mice by some commercial fragrances. Centr Eur J Immunol 34: 232-234.
9. Skopińska-Różewska E, Sommer E (2010): Stimulation of humoral immunity in mice by complex herbal remedy PERVIVO. Centr Eur J Immunol 34: 146-149.
10. Demkow U, Skopińska-Różewska E: Wpływ preparatu Bioaron C na odporność. In: Rola immunomodulatorów pochodzenia naturalnego. Skopińska-Różewska E, Siwicki AK (eds). Wyd. Medyk, Warszawa 2003; 51-56.
11. Horoszkiewicz-Hassan M, Beuscher N, Lehnfeld R, et al. (2005): The tolerance and efficacy of Bioaron C syrup in the treatment of upper respiratory tract infections in children. Herba Polonica 5: 45-54.
12. Skopińska-Różewska E, Sokolnicka I, Radomska-Leśniewska D, et al. (2003): The *in vivo* effect of *Echinacea purpurea* succus on various functions of human blond leukocytes. Centr Eur J Immunol 28: 126-130.
13. Bałan BJ, Nartowska J, Skopińska-Różewska E, et al.: Wpływ wyciągów *Echinacea purpurea* na reakcje odpornościowe oraz procesy angiogenezy. In: Endogenne i egzogenne modulatory odporności i angiogenezy. Skopińska-Różewska E, Siwicki AK (eds). SPW EDYCJA, Olsztyn 2007; 27-60.
14. Skopińska-Różewska E, Sommer E, Bałan BJ, et al. (2010): The *in vivo* effect of dry hydro-alcoholic extract of *Echinacea purpurea* on angiogenic activity of human blood mononuclear cells. Centr Eur J Immunol 35: 223-226.
15. Skopińska-Różewska E, Wasiutyński A, Sommer E, et al. (2011): Modulatory effect of *Echinacea pallida* on cellular immunity and angiogenesis in mice. Centr Eur J Immunol 36: 18-23.
16. Skopińska-Różewska E, Sokolnicka I, Siwicki AK, et al. (2011): Dose-dependent *in vivo* effect of *Rhodiola* and *Echinacea* on the mitogen-induced lymphocyte proliferation in mice. Pol J Vet Sci 14: 265-272.
17. Yamada H, Nagai T, Takemoto N, et al. (1989): Plantagoside, a novel alpha-mannosidase inhibitor isolated from the seeds of Plantago asiatica, suppresses immune response. Biochem Biophys Res Commun 165: 1292-1298.
18. Rezaeipoor R, Saeidnia S, Kamalinejad M (2000): The effect of Plantago ovata on humoral immune responses in experimental animals. J Ethnopharmacol 72: 283-286.
19. Chiang LC, Ng LT, Chiang W, et al. (2003): Immunomodulatory activities of flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds of *Plantago* species. Planta Med 69: 600-604.
20. Chiang LC, Chiang W, Chang MY, Lin CC (2003): *In vitro* cytotoxic, antiviral and immunomodulatory effects of *Plantago major* and *Plantago asiatica*. Am J Chin Med 31: 225-234.
21. Kanjoormana M, Kuttan G (2010): Antiangiogenic activity of ursolic acid. Integr Cancer Ther 9: 224-235.
22. Lee AW, Chen TL, Shih CM, et al. (2010): Ursolic acid induces allograft inflammatory factor-1 expression via a nitric oxide – related mechanism and increases neovascularization. J Agric Food Chem 58: 12941-12949.
23. La VD, Lazzarin F, Ricci D, et al (2010): Active principles of *Grindelia robusta* exert antiinflammatory properties in a macrophage model. Phytother Res 24: 1687-1692.

24. Krenn L, Wollenweber E, Steyrleuthner K, et al. (2009): Contribution of methylated exudates flavonoids to the anti-inflammatory activity of *Grindelia robusta*. Fitoterapia 80: 267-269.
25. Canavan D, Yarnell E (2005): Successful treatment of poison oak dermatitis treated with *Grindelia* spp. (Gumweed). J Altern Complement Med 11: 709-710.
26. Voidarou C, Alexopoulos A, Plessas S (2011): Antibacterial activity of different honeys against pathogenic bacteria. Anaerobe Apr 16.
27. El-Nekeety AA, Mohamed SR, Hathout AS, et al. (2011): Antioxidant properties of *Thymus vulgaris* oil against aflatoxin-induce oxidative stress in male rats. Toxicon 57: 984-991.
28. Centeno S, Calvo MA, Adelantado C, Figueroa S (2010): Antifungal activity of extracts of *Rosmarinus officinalis* and *Thymus vulgaris* against *Aspergillus flavus* and *A. ochraceus*. Pak J Biol Sci 13: 452-455.
29. Sertel S, Eichhorn T, Plinkert PK, Efferth T (2011): Cytotoxicity of *Thymus vulgaris* essential oil towards human oral cavity squamous cell carcinoma. Anticancer Res 31: 81-87.
30. Krill D, Madden J, Huncik K, Moeller PD (2010): Induced thyme product prevents VEGF-induced migration in human umbilical vein endothelial cells. Biochem Biophys Res Commun 403: 275-281.
31. Białas-Chromiec B, Skopińska-Różewska E, Strzelecka H, et al. (2000): The immunomodulatory effect of Biostimine – water soluble extract of the leaves of triennial plants *Aloe arborescens* Mill. Onkol Pol 3: 85-89.
32. Lee MJ, Lee OH, Yoon SH, et al. (1998): *In vitro* angiogenic activity of *Aloe vera* gel on calf pulmonary artery endothelial (CPAE) cells. Arch Pharm Res 21: 260-265.
33. Choi S, Kim KW, Choi JS, et al. (2002): Angiogenic activity of beta-sitosterol in the ischaemia/reperfusion-damaged brain of Mongolian gerbil. Planta Med 68: 330-335.