

Modulatory effect of *Echinacea pallida* on cellular immunity and angiogenesis in mice

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Abstract

The aim of this work was to evaluate the *in vivo* effect of commercial preparation of *Echinacea pallida* (Lymphozil) on the *in vitro* chemokinetic activity of mouse splenic lymphocytes in tissue culture, and on their *in vivo* immunological response against foreign histocompatibility antigens in local graft-versus-host reaction (lymphocyte-induced angiogenesis, LIA). The effects of feeding mice *E. pallida* on metabolic activity of blood granulocytes (chemiluminescence test) and on the bacterial infection (*Pseudomonas aeruginosa*) were also investigated. The ability of *E. pallida* to influence neovascular response induced in mice skin by grafting of human kidney cancer cells (tumor-induced angiogenesis, TIA test) was also studied. We have found significant stimulation of LIA activity of splenic lymphocytes collected from animals fed 0.02 mg daily dose of *E. pallida*, no effect in group fed 0.2 mg and significant inhibition in group fed the highest 2 mg dose. In the case of chemokinetic activity (*in vitro* migration of cells in culture) the highest stimulatory effect was obtained when cells were obtained from animals fed 0.02 mg daily dose. Higher doses were also stimulatory but this effect was less pronounced.

In experiments with bacterial infection mice were fed for 5 days 0.08 mg daily dose of *E. pallida* and this treatment resulted in highly significant diminution of number of bacterial colonies in liver in comparison to the control.

Blood granulocytes metabolic activity was unaffected in mice fed 0.02 and 0.2 mg daily dose, and significantly lower in mice fed highest, 2 mg dose.

In experiments with kidney tumor all three doses of *E. pallida* reduced the neovascular response originated in mice skin after transplantation of cancer cells.

Key words: mice, *Echinacea pallida*, immunological angiogenesis, tumor angiogenesis, granulocytes chemiluminescence, splenocytes chemokinesis, bacterial infection.

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Introduction

Several *Echinacea* species, belonging to *Asteraceae* (*Compositae*) family, originate from North America and were traditionally used by the indigenous Indians for many illnesses. Presently, *Echinacea* preparations are widely used herbal remedies, mainly for respiratory tract infections, owing their anti-viral, anti-inflammatory and immunomodulatory properties. Apparently, out of the three most

commonly known species of *Echinacea* (*purpurea*, *angustifolia*, *pallida*), the latter one is seldom used and has not yet been thoroughly examined. However, it is not necessarily true because investigations have shown that until recent years some number of the plants cultivated in Europe under the name „*Echinacea angustifolia*” in reality had been *E. pallida* [1-3]. Moreover, there have been a few research papers published in recent years, in which the properties of all three *Echinacea* species were compared and contrasted.

The results show that *E. pallida* is a versatile species, which is at least as effective as the other two [4-7].

Most of the studies performed on the immunotropic properties of *E. pallida* were related to its effect on non-specific immunity (activation or suppression of various macrophage functions, phagocytosis of granulocytes, NK cells cytotoxicity). Only a handful of studies have investigated its adaptive immune modulation [8-11].

The aim of this work was to evaluate the effect of feeding mice for 7 days with a commercial preparation of *E. pallida* (Nutt) (Lymphozil) on some parameters of specific and non-specific cellular immunity and on the course of *Pseudomonas aeruginosa* infection in mice. The effect of this remedy on the angiogenic activity of human kidney cancer cells was also investigated.

Material and methods

The study was performed on 8- 12 weeks old inbred Balb/c and C3H female and male mice, weighing 20- 25 g, delivered from the Polish Academy of Sciences breeding colony, and on F1 hybrids C57Bl/6 × C3H (further called B6C3F1) from the breeding colony of Military Institute of Hygiene and Epidemiology. Lymphozil, pulvered hydro-alcoholic extract of *Echinacea pallida* aerial and underground parts (Cesra GmbH) was administered to mice *per os* in daily doses 0.02 mg, 0.08 mg, 0.2 mg (calculated from the doses recommended for humans, applying the counter 7 for differences between mouse and human in relation of the surface to body mass) and higher (2 mg). Mice received drug by Eppendorff pipette, in 40 µl of distilled water, for 7 days before immunological experiments, for 5 days before experiments with bacterial infection and for 3 days after tumour cells grafting. Control animals received 40 µl of distilled water.

Chemiluminescence test

Chemiluminescence test (CL) was measured using the method of Easmon and Cole with some modifications [12, 13] at room temperature, in scintillation counter (RackBeta 1218, LKB, Sweden). Briefly: samples of 0.05 ml heparinised blood collected from Balb/c mice were diluted 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 mixed with 0.2 ml of luminol (Sigma-Aldrich, USA) solution (10^{-5} M) in PBS and placed in a scintillation counter in the "out of coincidence" mode for background chemiluminescence measurement. Then, the cells were activated by addition of 0.02 ml solution of opsonised zymosan (10 mg/ml) and chemiluminescence activity was measured for the next 15 min. Counting of leukocytes and blood smears examination were performed by routine methods and the results were shown as the maximum value of chemiluminescence (cpm) obtained for 10^3 granulocytes.

Preparation of tumour cells suspension

Material was obtained surgically from the patient with kidney clear cell carcinoma. About 5 g of tumour tissue was dispersed mechanically and subjected to enzymatic digestion by use of collagenase 0.1 mg/ml (Sigma) and DNase 0.001 mg/ml (Serva) dissolved in PBS for 45 min on a magnetic shaker in room temperature. Then, obtained suspension was filtered through a sieve, washed twice in PBS and suspended in Parker medium. Viability of tumour cells was assessed by 0.5% trypan blue exclusion method.

In vivo study of graft-versus-host reaction in mice.

Lymphozil was administered to groups of 6 C3H mice each, *per os*, in daily doses of 0.02, 0.2 or 2 mg. These doses corresponded to 10, 100 or 1000 mg given to 70 kg person. Mice received drugs by Eppendorff pipette, in 40 µl of distilled water for 7 days. Controls mice were fed 40 µl of distilled water. On the day 8 mice were sacrificed with Morbital, spleens were dissected and spleen cells suspensions prepared as described before [14]. Spleen cells suspensions were pooled within a group and grafted intradermally into F1 recipients, cells from each pool into 3-4 B6C3 F1 recipient mice. A local GVH reaction (lymphocyte-induced angiogenesis, LIA) was performed according to Sidky and Auerbach with some modifications [14]. In this test grafted C3H cells recognized foreign C57Bl/6 histocompatibility antigens and produced many immunological mediators including pro-angiogenic factors (immunological angiogenesis). The number of newly-formed blood vessels was the measure of cells reactivity. For a detail description of the test see below.

Cutaneous angiogenesis test (for immunological angiogenesis, LIA and tumor angiogenesis, TIA) [14, 15]

Multiple 0.05 ml samples of cell suspension (10^6 spleen cells from C3H mice fed for 7 days Lymphozil in LIA test, 2×10^5 tumor cells in TIA test) were injected intradermally into partly shaved, narcotised Balb/c mice in TIA test or B6C3F1 mice in LIA test (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. In TIA (tumor-induced angiogenesis) test, on the day of grafting and on the following two days recipient Balb/c mice were fed Lymphozil. In LIA test, recipient F1 hybrids were not treated. 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).

Study of spleen cells chemokinesis

Groups of 6 Balb/c mice received Lymphosil by Eppendorff pipette, in 40 µl of distilled water, in 0.02, 0.2 and 2 mg daily doses, for 7 days. Control mice were fed 40 µl of distilled water. On the day eight mice were bled in anaesthesia from retro-orbital plexus and sacrificed with Morbital. Spleens were pooled within groups and splenocytes were isolated from spleens under sterile conditions by straining through stainless sieve and cotton gauze and centrifugation on Lymphoprep in order to remove erythrocytes.

Spleen cells chemokinesis (spontaneous migration) assay *in vitro* was performed according to the Sandberg method in own modification [16]. Briefly, isolated splenocytes were resuspended in Parker culture medium with 5% inactivated FCS, at the final concentration of 30×10^6 cells/ml. Afterwards, siliconized capillary tubes were filled with cell suspension, sealed with plasticine, centrifuged (5 min 450 g) and fixed on the glass plates. Cells levels were marked. After 24 h incubation (37°C, 5% CO₂ humidified atmosphere) the distances of migration were measured in millimeters (mm) at a magnification of 6.5× and presented as migration units (1 MU = 0.18 mm).

Study of bacterial infection [17]

Animals

Studies were performed on B6C3F1 hybrid mice, males, at the age of 10-12 weeks, delivered from own breeding colony.

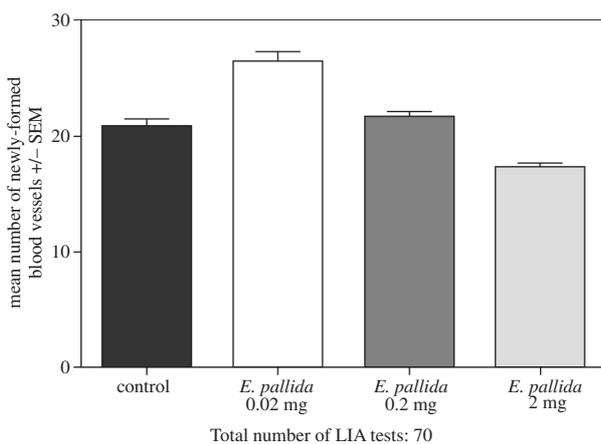


Fig. 1. The effect of feeding mice *Echinacea pallida* on the activity of their splenic lymphocytes in local Graft – versus Host reaction (LIA intra-dermal test)

Bacterial infection

Groups of 8 mice were fed Lymphosil 0.08 mg daily, in distilled water, or distilled water only, by Eppendorff pipette, for 5 days. On the day six mice were infected intraperitoneally (i.p.) with *Pseudomonas aeruginosa* strain ATCC (27853). Four hours after administration of 0.1 ml of bacteria suspension (3×10^7 CFU) the mice were anaesthetized with barbiturates and killed by spinal dislocation after which the livers were isolated. The livers were homogenized and the number of viable bacteria were estimated by plating after 24 hours growth on Cetrymide agar (Merck). The results are presented as the mean number of colony forming units (CFU)+/- SEM per 1 g of liver tissue $\times 10^3$.

For all experiments animals were handled according to the Polish law on the protection of animals and NIH standards. All experiments were accepted by the local Ethical Committee.

Statistical evaluation of the results was performed by one-way ANOVA and the significance of differences between the groups was verified with a Tukey's Multiple Comparison PostTest (GraphPadPrism software package).

Results

The results of immunological experiments are presented graphically on the Fig. 1-3.

We have found highly significant stimulation of LIA activity of splenic lymphocytes collected from animals fed 0.02 mg daily dose of *E. pallida* ($p < 0.001$), no effect in group fed 0.2 mg and significant inhibition ($p < 0.01$) in group fed the highest 2 mg dose (Fig. 1). In the case of chemokinetic activity (in vitro migration of cells in culture) the highest stimulatory effect was obtained when cells was obtained from animals fed 0.02 mg daily dose ($p < 0.001$).

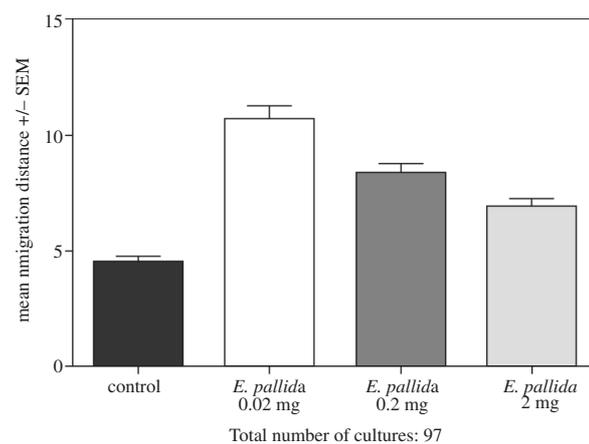


Fig. 2. The effect of feeding mice *Echinacea pallida* on the *in vitro* migratory activity of their splenic lymphocytes in tissue culture

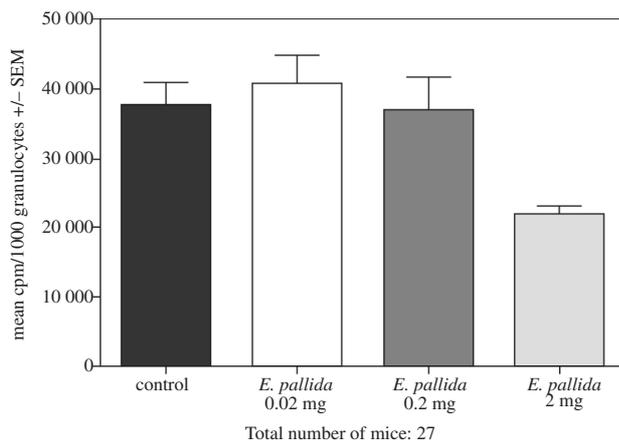


Fig. 3. The effect of feeding mice *Echinacea pallida* on metabolic activity of their blood granulocytes (CL)

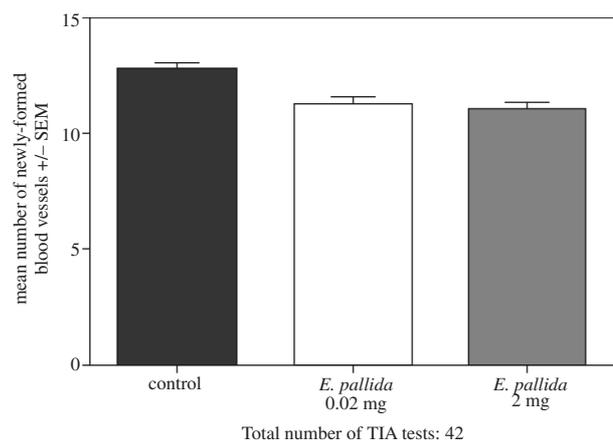


Fig. 4. The effect of feeding mice *Echinacea pallida* for 3 days after intra-dermal grafting of human kidney cancer cells on neovascular response in local TIA test

Higher doses were also stimulatory ($p < 0.01$), but this effect was less pronounced (Fig. 2).

Blood granulocytes metabolic activity (Fig. 3) was unaffected in mice fed 0.02 and 0.2 mg daily dose, and significantly lower in mice fed highest, 2 mg dose ($p < 0.05$).

In experiments with bacterial infection mice were fed for 5 days 0.08 mg daily dose of *E. pallida* and this treatment resulted in highly significant diminution of number of bacterial colonies in liver in comparison to the control (4.1 ± 1.3 in comparison to 17.2 ± 4.1 CFU/g $\times 10^3$), $p < 0.01$.

In experiments with kidney tumor (Fig. 4) both low and high doses of *E. pallida* significantly diminished neovascular response originated in mice skin after transplantation of cancer cells ($p < 0.01$).

Discussion

In this paper we present for the first time the *in vivo* effect of *Echinacea pallida* herb extract on such parameters of cellular immunity as graft-versus-host reaction, spleen cells chemokinesis and blood granulocytes' chemiluminescence in mice. We obtained evidence, in agreement with our previous findings with other *Echinacea* species, that the effect may be stimulatory or inhibitory, depending on the dose of remedy and studied parameter [18]. Inhibition of granulocytes metabolic activity (CL test) is probably linked to the presence of anti-oxidants. Lowering of chemokinetic activity and ability to react in GVH test may be coupled with stimulation of some suppressory mechanisms by yet unidentified compounds present in *Echinacea pallida* herb.

The roots of *E. pallida* contain caffeoyl derivative echinacoside, which is also present in underground parts of

E. angustifolia, but is absent in *E. purpurea*. Caffeic acid derivatives are effective antioxidants in free radical generation systems and have an antihyaluronidase activity, which is beneficial for scarless wound repair. In rats, excision wounds treated topically with echinacoside, showed reduced inflammatory response and higher hyaluronan content [19-21]. The anti-inflammatory activity of *Echinacea* might be due to multiple active metabolites, which work together to switch macrophage activation from classical activation towards alternative activation. Echinacoside has both antibacterial and antiviral effects *in vitro* [22]. Both pressed juice and hydro-alcoholic extracts of *Echinacea pallida* exhibited inhibitory activity against herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) [23]. Cytotoxic activity of acetylenic compounds isolated from *Echinacea pallida* roots to cancer cell lines was also described [24, 25]. In this paper we describe for the first time anti-angiogenic effect of *E. pallida* in TIA test, where human tumor cells isolated from kidney cancer release angiogenic growth factors and induce neovascular response in mice skin. Previously, we described such inhibitory effect for other natural remedies and isolated from them compounds in TIA tests performed on various tumors of human origin as well as murine tumors syngeneic to the recipient mice [13, 26-30]. We suppose that *E. pallida* in lower doses may be effective immunostimulator without danger of tumor angiogenesis stimulation.

In experiment with bacterial infection we obtained beneficial effect of *E. pallida*, probably connected with the activation of peritoneal macrophages and, possibly, with direct antibacterial effect of some *Echinacea* compounds present in mouse tissues after 5-days feeding. This finding might have practical implications, because in people immunocompromised due to immunosuppressive treatment, cancer therapy, severe burns or other reasons, opportunistic

infections are often seen. *Pseudomonas aeruginosa* is one of the most frequently observed pathogens [31-34].

Conclusion

Lymphosil may be recommended as immunostimulatory drug in daily doses from 10 (preferably) to 40 mg. Higher doses may be less effective, ineffective, or even inhibitory in respect to some immune reactions.

References

1. Bauer, H. Echinacea. In: Bauer and Lawson (Editors) Phytomedicines of Europe. Oxford University Press, London 1998.
2. Bauer R, Wagner H (1991): *Echinacea* species as potential immunostimulatory drugs. *Econom Med Plant Res* 5: 253-261.
3. Hostettmann K (2003): History of a plant: the example of *Echinacea*. *Forsch Komplementarmed Klass Naturheilkd* 10 (suppl 1): 9-12.
4. Sloley BD, Urichuk LJ, Tywin C, et al. (2001): Comparison of chemical components and antioxidants capacity of different *Echinacea* species. *J Pharm Pharmacol* 53: 849-857.
5. Speroni E, Govoni P, Guizzardi S, et al. (2002): Anti-inflammatory and cicatrizing activity of *Echinacea pallida* Nutt. root extract. *J Ethnopharmacol* 79: 265-272.
6. Brauning B, Knick E (1993): Therapeutical experiences with *Echinacea pallida* for influenza-like infections. *Naturheilpraxis* 1: 72-75.
7. Dorn M, Knick E, Lewith G (1997): Placebo controlled double blind study of *Echinaceae pallidae* radix in upper respiratory tract infections. *Complement Ther Med* 5: 40-42.
8. Egger M, Pellett P, Nicki K, et al. (2008): Synthesis and cannabinoid receptor activity of ketoalkenes from *Echinacea pallida* and non-natural analogues. *Chemistry* 14: 10978-10984.
9. LaLone CA, Rizshsky L, Hammer KD, et al. (2009): Endogenous levels of Echinacea alkylamides and ketones are important contributors to the inhibition of PGE2 and nitric oxide production in cultured macrophages. *J Agric Food Chem* 57: 8820-8830.
10. Zhai Z, Liu Y, Wu L, et al. (2007): Enhancement of innate and adaptive immune functions by multiple *Echinacea* species. *J Med Food* 10: 423-434.
11. Block KI, Mead MN (2003): Immune system effects of *Echinacea*, *Ginseng*, and *Astragalus*: a review. *Integr Cancer Ther* 2: 247-267.
12. Easmon CSF, Cole PJ, Williams AJ, Hastings M (1980): The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescence. *Immunology* 41: 67-74.
13. Skopińska-Różewska E, Krotkiewski M, Sommer E, et al. (1999): Inhibitory effect of shark liver oil on cutaneous angiogenesis induced in Balb/c mice by syngeneic sarcoma L-1, human urinary bladder and human kidney tumor cells. *Oncology Rep* 6: 1341-1344.
14. Skopińska-Różewska E, Wójcik R, Siwicki AK, et al. (2008): The effect of *Rhodiola quadrifida* extracts on cellular immunity in mice and rats. *Pol J Vet Sci* 11: 105-111.
15. Skopińska-Różewska E, Hartwich M, Siwicki AK, et al. (2008): The influence of *Rhodiola rosea* extracts and rosavin on cutaneous angiogenesis induced in mice after grafting of syngeneic tumor cells. *Cent Eur J Immunol* 33: 102-107.
16. Skopińska-Różewska E, Bychawska M, Białas-Chromiec B, Sommer E (2009): The *in vivo* effect of *Rhodiola rosea* and *Rhodiola quadrifida* hydro-alcoholic extracts on chemokinetic activity of spleen lymphocytes in mice. *Centr Eur J Immunol* 34: 42-45.
17. Bany J, Skopińska-Różewska E, Chorostowska-Wynimko J, et al. (2004): The effect of complex herbal remedy on the angiogenic activity of L-1 sarcoma cells, L-1 sarcoma tumor growth and on the bacterial infection in mice. *Centr Eur J Immunol* 29: 29-34.
18. Skopińska-Różewska E, Sokolnicka I, Siwicki AK, et al. (2011): *In vivo* stimulatory or suppressive effects, depending on the dose of herbal remedy, on lymphocyte mitogen-induced proliferation in mice. *Pol J Vet Sci* in press.
19. Goldhaber-Fiebert S, Kemper KJ (1999): *Echinacea* (*E. angustifolia*, *E. pallida* and *E. purpurea*). Longwood Herbal Task Force, August 20, <http://www.mcp.edu/herbal/default.h>
20. Bauer R, Khan IA, Wagner K (1988): TLC and HPLC analysis of *Echinacea pallida* and *Echinacea angustifolia* roots. *Planta Medica* 54: 426-430.
21. Barnes J, Anderson LA, Gibbons S, Phillipson JD (2005): Review *Echinacea species* [*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench]: a review of their chemistry, pharmacology and clinical properties. *J Pharm Pharmacol* 57: 929-954.
22. Bauer R, Woelkart K. *Echinacea*. In: Coates P (Editor) *Encyclopedia of Dietary Supplements*. Dekker, New York 2005; 177-187.
23. Schneider S, Reichling J, Stintzing FC, et al. (2010): Antiherpetic properties of hydro-alcoholic extracts and pressed juice from *Echinacea pallida*. *Planta Medica* 76: 265-272.
24. Morandi S, Pellati F, Ori C, et al. (2008): Isolation, structure elucidation and total synthesis of a cytotoxic dienone from *Echinacea pallida*. *Org Biomol Chem* 6: 4333-4339.
25. Chicca A, Pellati F, Adinolfi B, et al. (2008): Cytotoxic activity of polyacetylenes and polyenes isolated from roots of *Echinacea pallida*. *Br J Pharmacol* 153: 879-885.
26. Skopińska-Różewska E, Pastewka K, Wasiutyński A, et al. (2010): the influence of herbal remedies on cutaneous angiogenesis induced in mice after grafting of human kidney cancer tissue. *Centr Eur J Immunol* 35: 199-203.
27. Rogala E, Skopińska-Różewska E, Wasiutyński A, et al. (2008): *Echinacea purpurea* diminishes neovascular reaction induced in mice skin by human cancer cells and stimulates non-specific cellular immunity in humans. *Centr Eur J Immunol* 33: 127-130.
28. Skopińska-Różewska E, Janik P, Przybyszewska M, et al. (1998): Inhibitory effect of theobromine on induction of angiogenesis and VEGF mRNA expression in v-raf transfectants of human urothelial cells HCV-29. *Int J Mol Med* 2: 649-652.
29. Skopińska-Różewska E, Sommer E, Demkow U, et al. (1997): Screening of angiogenesis inhibitors by modified tumor-induced angiogenesis (TIA) test in lung cancer. *Ann Acad Med Bialostocensis* 42: 287-296.
30. Wasiutyński A, Bałan BJ, Skopińska-Różewska E, et al. (2009): The effect of *Echinacea purpurea* on the morphology, angiogenic activity and VEGF concentration of murine L-1 sarcoma tumors. *Centr Eur J Immunol* 34: 38-41.
31. Spencer RC (1996): Predominant pathogens found in the European prevalence of infection in intensive care study. *Eur J Clin Microbiol Infect Dis* 15: 281-285.

32. Pruitt BA, McManus AT, Kim SH, et al. (1997): Burn wound infections: current status. *World J Surg* 22: 135-145.
33. Griffith SJ, Nathan RK, Selender RK, et al. (1989): The epidemiology of *Pseudomonas aeruginosa* in oncology patients in a general hospital. *J Inf Dis* 160: 103-106.
34. Korvick JA, Marsh JW, Starlz TE, et al. (1991): *Pseudomonas aeruginosa* bacteriemia in patients undergoing liver transplantation: an emerging problem. *Surgery* 109: 62-68.