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The effect of multi-component herbal remedy PERVIVO on cellular immunity and tumor angiogenesis in mice

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Abstract

Herbal extracts from traditional medicine can be formulated to develop novel herbal medicines as potent as synthetic drugs. We investigated properties of multicomponent digestive herbal remedy PERVIVO on innate (non-specific) immunity and adaptive (specific) cellular immunological response in various tests in mice. Previously, we reported stimulatory effect of this remedy on antibody production in mice. In the literature we have found some data about biological activity of the main active, from 27 substances of natural origin, PERVIVO components, in temporary loss of appetite, in gastrointestinal disorders as Crohn's disease and anti-inflammatory, pro-apoptotic and antitumorigenic activities. The aim of our present study was to evaluate the effect of feeding Balb/c mice with PERVIVO on the cellular immune response, and on the growth and vascularity of syngeneic tumor L-1 Sarcoma. The study was performed on 20-22 g of body mass female, F1 hybrid Balb/c × C3H mice (spleen cells recipients in GVH test), and on 8-10-weeks old inbred Balb/c mice, which received orally for 3, 7 or 14 days (40 µl/mouse/24 hours) PERVIVO, diluted in water 2, 8 and 32 times. Control mice were fed 40 µl of 10% ethyl alcohol only. Our results indicate that PERVIVO stimulates ex vivo proliferative activity of murine splenocytes, and chemokinetic activity of mouse splenic lymphocytes in tissue culture, and inhibits tumor angiogenesis. It has no effect on the number and metabolic activity of blood granulocytes, on ex vivo immunological response of lymphocytes against foreign MHC antigens in local graft-versus-host reaction (lymphocyte-induced angiogenesis, LIA), and on the tumor mass.

Key words: *herbal remedy, mice, cellular immunity, tumor angiogenesis.*

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Introduction

PERVIVO is a complex digestive herbal remedy, bitter taste traditional herbal tonic, composed of 27 substances of natural origin.

The tonic was claimed to help digestion, to be effective in the treatment of ailments of various origin, and to increase activity of immune system. However, these claims are poorly supported by scientific evidence. There are, however, some data about biological activity of the main active PERVIVO components – Herba Absinthii, Radix Zingiberis, Radix Angelicae, Radix Gentianae, and Folium Menyanthidis.

According to the European Medicinal Agency (EMEA), *Artemisia absinthium* (wormwood) is a traditional herbal

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medicinal product used in temporary loss of appetite and in mild gastrointestinal disorders. Extracts from wormwood contain a variety of phenolic and flavonoid compounds and exert antibacterial, antiplasmodial, antitrypanosomal and antihelminthic activity [1-5]. Recently, suppression of tumor-necrosis factor alpha by *A. absinthium* extracts *in vitro* was reported [6] and clinically tested in patients with Crohn's disease and IgA nephropathy [7]. Polysaccharides from *A. absinthium* stimulate NO production [8].

Recently, Craciunescu *et al.* reported a good antioxidant activity and cytoprotective effect of *A. absinthium* extract against oxidative damage in fibroblast-like cells, what provided scientific support for the traditional use of this herb in the treatment of skin disorders [9]. The effectiveness of *A. absinthium* in complex treatment of inflammatory periodontal disease was also assessed [10]. It should be remembered, however, that the intake of *Absinthii* herba preparations might influence the effect of medicinal products acting via GABA receptor (e.g. barbiturates, benzodiazepines) [1, 11].

The second component of PERVIVO which might be responsible for its immunotropic and anti-tumor effects is *Radix Zingiberis* (ginger). Modulatory effects of ginger on *in vitro* and *in vivo* cellular and humoral immune response was observed in mice and fish [12-15]. 6-Gingerol, a natural component of ginger, has been reported to possess anti-inflammatory and antitumorigenic activities. It was shown, that this compound inhibited growth of colon cancer cells via induction of G2/M arrest [16]. The second active component of ginger, 6-Shogaol, induced apoptosis in human hepatocellular carcinoma cells [17]. Ginger treatment suppressed the proliferation and colony formation in breast cancer cell lines, and did not affect viability of nontumorigenic normal mammary epithelial cell line [18]. Active compounds of ginger (gingerols, shogaols, paradols, zingerone) are extensively studied for the antioxidant, anti-inflammatory, anti-emetic and gastroprotective properties [19]. Ginger exerted indirect inhibitory effect on the growth of influenza virus via macrophage activation leading to production of TNF-α [20]. Aqueous extract of ginger, enriched of gingerols, injected *i.p.* before airway challenge of ovalbumin – sensitized mice resulted in suppressing Th2-mediated immune responses and might thus provide a possible therapeutic application in allergic asthma [21, 22].

The next two compounds of PERVIVO, *Radix Gentianae* and *Menyanthidis folium*, was traditionally used in rheumatism, arthritis, skin diseases, and there are no available information on their immunotropic activity except one paper describing immunomodulatory effect of polysaccharides isolated from *Menyanthes trifoliata* on human blood lymphocytes and granulocytes [23].

Next compound, *Angelicae radix*, possess anti-oxidant and anti-inflammatory properties, promotes hemato- and thrombopoiesis and has inhibitory effect on platelet aggregation [24].

Previously, in pilot experiments conducted in mice grafted with syngeneic tumor, we observed some anti-angiogenic effect of PERVIVO [25]. Recently, we have reported stimulatory effect of PERVIVO on antibody production in mice [26]. The aim of the present study was to evaluate the effect of feeding Balb/c mice with PERVIVO on various parameters of specific and non-specific cellular immunity, and on the growth and vascularity of syngeneic tumor L-1 Sarcoma.

Material and methods

PERVIVO (Bittner) is a herbal drug composed of: *Absinthii herba*, *Anisi stellati fructus*, *Aurantii amari pericarpium*, *Aurantii dulcis cortex*, *Calami radix*, *Camphora racemica*, *Cardui benedicti herba*, *Caryophylli flos*, *Centauri herba*, *Cubebae fructus*, *Curacao cortex*, *Angelicae radix*, *Galangae radix*, *Gentianae radix*, *Helenii radix*, *Iridis radix*, *Ivae moschatae herba*, *Liquiritiae radix*, *Manna*, *Menyanthidis folium*, *Myristicae semen*, *Myrrha*, *Theriak*, *Verbasci flos*, *Verbasci radix*, *Zedoariae radix*, *Zingiberis radix*. These components are dissolved in 32% ethyl alcohol [25-27].

Mice

The study was performed on 20-22 g of body mass female, 8-10-weeks old inbred Balb/c mice, and F1 hybrids Balb/c × C3H mice, delivered from the Polish Academy of Sciences and from the own breeding colony.

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

Sarcoma L-1 tumor cells

L-1 Sarcoma cells from *in vitro* culture stock were delivered from Warsaw's Oncology Center collection, passaged *in vivo* and grafted subcutaneously (for evaluation of tumor growth and blood supply) or intradermally (for evaluation of angiogenic activity) to syngeneic Balb/c mice.

Treatment of mice with PERVIVO

In immunological procedures, experimental mice received orally for 7 days (40 μl/mouse/24 hours) PERVIVO diluted ×2, ×8, or ×32 in water. Control mice were fed 40 μl of 10% ethyl alcohol. On the 8th day mice were anaesthetized with chloral hydrate, bled from retro-orbital plexus and sacrificed with Morbital. Cells from their blood and spleens were used for further tests.

In the experiments with L-1 Sarcoma tumor cells, mice, after tumor cells grafting, were fed PERVIVO diluted ×2 or ×32 with water, for 3 days in cutaneous tumor-induced angiogenesis (TIA) test, or for 14 days in evaluation of the effect of PERVIVO on tumor growth and hemoglobin content.

Mitogen-induced (PHA) splenocytes proliferation assay performed according to [28]

Briefly, spleen cells cultures were incubated in microplates (culture medium RPMI-1640 with L-glutamine, 10% FBS and antibiotics) without or with mitogen PHA at different concentrations: 0.5, 1.0 and 2.0 ml/ml. After 48 hours of incubation 10 ml of tritiated thymidine (^{3}HTdR 0.2 mCi) was added. After further 24 hours cells were harvested and incorporation of tritiated thymidine was counted using β-scintillation counter (RackBeta 1218, LKB Wallac). The arithmetical mean of quadruplicate count was calculated and expressed as counts per minute (CPM).

Measurement of metabolic activity of mice blood granulocytes

A modified granulocyte chemiluminescence (CL) test [29], previously described by Easmon *et al.* [30] was performed on blood samples. Chemiluminescence test is used to estimation of bactericidal activity of stimulated phagocytes by measurement of their light energy.

Briefly, the test consisted of taking 0.05 ml of blood from retroorbital plexus (with 50 units of heparin/ml) with a 0.2 ml of phosphate buffered saline (PBS), supplemented with 0.1% bovine serum albumin and 0.1% glucose. Next, 0.05 ml of this diluted blood was added to 0.2 ml of luminol (10^{-5} M) solution and placed in a scintillation counter. Chemiluminescence spontaneous activity was measured for the next few minutes in room temperature. Next, 0.2 ml opsonized zymosan solution in PBS (1 mg/ml) was added to cell suspension and CL stimulated activity was measured for 33 minutes (99 counts). In addition total number of leukocytes and granulocytes percentage were counted. The results of CL_{max} were expressed as cpm per 10^3 granulocytes.

Graft-versus-host reaction

Local reaction GvH (graft-versus-host reaction, lymphocyte-induced angiogenesis – LIA) was performed according to Sidky and Auerbach [31] with some own modifications [32].

Briefly, Balb/c mice were fed for 7 days with PERVIVO preparations, as described above, next spleens were dissected and after isolation spleen cells were grafted intradermally (3-6× of 500 thousands of cells in 0.05 ml of Parker medium per mouse) into shaved with a razor blade flanks of (Balb/c × C3H) F1 mice. Cell suspensions were supplemented with 50 μl/ml of 0.01% trypan blue in order to facilitate recognition of injection sites later on. Grafted cells recognized C3H antigens and produced many immunological mediators including proangiogenic agents (immunological angiogenesis). In this case number of newly formed blood vessels identified and counted 3 days later in dissection microscope on the inner skin, was the measure of cells reactivity.

Spleen cells chemokinesis assay (spontaneous migration)

Under sterile conditions splenocytes were isolated from mice by straining through surgical cotton gauze and centrifugation on Histopaque-1077 to remove the erythrocytes.

Test was performed according to Sandberg method in own modification [33]. 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 hours incubation (37°C, 5% CO_2 humidified atmosphere) the distances of migration were measured in millimeters (mm) at a magnification of 6.5×, and presented in migration units (1 MU = 0.18 mm).

Evaluation of sarcoma L-1 growth and angiogenic activity was performed according to [34, 35]

1. Preparation of tumor cells after *in vivo* passage

Briefly, sarcoma L-1 cells from *in vitro* stock were grafted (10^6/0.1 ml) subcutaneously into subscapular region of Balb/c mice. After 14 days the tumors were excised, cut to smaller pieces, rubbed through sieve and suspended in 5 ml of PBS. The suspension was left for 10 min at room temperature.

After sedimentation the supernatant was collected and centrifuged for 10 min at 1500 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 4×10^6/ml or 10^7/ml.

2. Cutaneous angiogenesis assay (tumor-induced angiogenesis – TIA test)

Multiple 0.05 ml samples of 200 thousand of cells were injected intradermally into partly shaved, narcotised Balb/c mice (at least 2-4 mice per group). In order to facilitate the localization of cell injection sites, the suspension was colored with 0.1% of trypan blue. Mice were fed with PERVIVO for 3 days. 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 are thin and (or) 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).

3. Subcutaneous tumor growth assay

Suspensions of sarcoma cells were grafted (2 millions of cells) subcutaneously into mice. On the day of cells grafting and on the following 13 days mice were fed PERVIVO, or ethyl alcohol as a control. After 14 days mice were sacrificed and tumors were removed, weighed and measured

with electronic caliper (The Fowler Ultra-Cal Mark III caliper).

4. Estimation of Hb concentration in tumors

It was done according to the method described [36]. Briefly, tumors were homogenized in PBS using an ultrasonic sonificator (Virsonic, USA), then centrifuged for 20 min at 4000 × g. 20 µl of the supernatant was added to 5 ml of Drabkin reagent. The absorbance was read in a spectrophotometric reader Elx800 (Biotek Instruments, USA) at 570 nm. The reader for the Hb measurement was calibrated with haemoglobin standard solutions (Sigma). The results were shown as µg Hb in 1 mg of tumor mass.

Statistical methods

Statistical analysis of the results was performed with one – way and two-way ANOVA with Bonferroni post-test, Student *t* test and Mann-Whitney test.

Results

The effect of PERVIVO on the proliferative activity of mouse spleen cells, stimulated with different doses of PHA, was evaluated in the first stage.

In this assessment the greatest stimulatory activity, regardless of PHA dose, was observed for the concentration of PERVIVO, which was diluted twice (Table 1, Figs. 1 and 2).

In the next stage, the effect of PERVIVO was evaluated by the number and activity of the CL of granulocytes. In the studies which were conducted, irrespective of applied dilution, PERVIVO had no effect on the number and phagocytic capacity of granulocytes, assessed by the CL assay (Table 2).

In the following stage of this study the *in vivo* effect of PERVIVO 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 (LIA) was evaluated.

We have not found significant stimulation of LIA activity of splenic lymphocytes collected from animals which were fed with PERVIVO in all doses applied (Table 3).

In the case of chemokinetic activity of splenocytes *ex vivo* (*in vitro* migration of cells in culture) we observed significant stimulation (Fig. 3) after all doses applied.

The study also evaluated the PERVIVO effect on the process of neoangiogenesis regarding the transplanted tumor of Sarcoma L-1.

In its assessment PERVIVO, in a double dilution, exerted an inhibitory effect on the early neoangiogenesis (TIA test) in relation to the control group. The same effect was not observed concerning 32-folds dilution (Table 4).

Another study confirmed these results, which examined the process of neoangiogenesis by measuring hemoglobin

Table 1. Statistical analysis of the results presented on Fig. 1

Two-way ANOVA				
Source of variation	**% of total variation**	***P* value**		
Interaction	5.45	0.0081		
Treatment	10.91	< 0.0001		
Concentration	63.86	< 0.0001		
Bonferroni multiple comparisons			**Number of comparisons: 12**	
Control vs. Pervivo dil. × 2				
Concentration	**Difference**	***t***	***P* value**	**Summary**
PHA 0	19.00	1.461	> 0.05	NS
PHA 0.5	76.00	6.051	< 0.0001	****
Control vs. Pervivo dil. × 8				
Concentration	**Difference**	***t***	***P* value**	**Summary**
PHA 0	12.00	0.8846	> 0.05	NS
PHA 0.5	49.00	3.612	< 0.01	**
Control vs. Pervivo dil. × 32				
Concentration	**Difference**	***t***	***P* value**	**Summary**
PHA 0	–14.00	1.077	> 0.05	NS
PHA 0.5	49.00	3.612	< 0.01	**

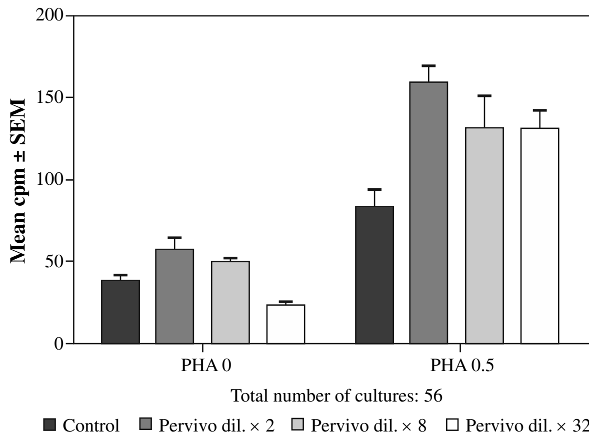

Fig. 1. Proliferation activity of mice spleen cells after 7-days feeding with PERVIVO (low concentration of PHA, 3H thymidine incorporation test)

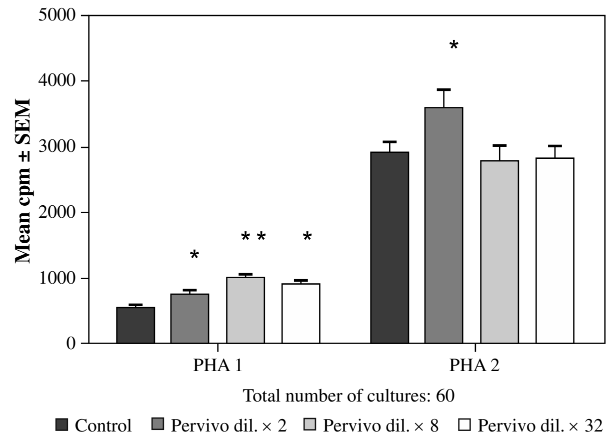

Fig. 2. Proliferation activity of mice spleen cells after 7-days feeding with PERVIVO (intermediate [1 µg/ml] and high [2 µg/ml] concentration of PHA, 3H thymidine incorporation test); $*p < 0.05$, $**p < 0.01$

Table 2. No effect of PERVIVO administration on the number and chemiluminescent activity of mice blood granulocytes

PERVIVO dilution	Mean number of leukocytes ± SEM	Mean number of granulocytes ± SEM	Mean chemiluminescent activity cpm ± SEM	Statistical significance of differences in comparison to the control
× 2 (10 mice)	1870 ±172	250 ±36	33284 ±3087	NS
× 8 (10 mice)	1725 ±120	238 ±25	31219 ±5047	NS
× 32 (10 mice)	2355 ±263	302 ±27	25317 ±3204	NS
Control (10 mice)	2035 ±281	286 ±32	30568 ±6157	–

Table 3. No effect of PERVIVO on the graft-versus-host activity of spleen cells (LIA test)
Donor mice: Balb/c females; recipients: F1 (Balb/c × C3H) females

Exp. group	Number of tests	Mean number of blood vessels ± SEM	Statistical significance of differences
Control	25	13.2 ±0.24	–
PERVIVO dil. 32 ×	25	12.2 ±0.41	NS
PERVIVO dil. 8 ×	23	13.3 ±0.33	NS
PERVIVO dil. 2 ×	20	12.4 ±0.33	NS

concentration in the tumor mass 14 days after tumor cells grafting (Fig. 4). The analysis of the group receiving formulation PERVIVO revealed, that hemoglobin concentration was significantly lower in relation to the values observed in the control group.

In order to complete the analysis, in the next stage of the study, the influence of 14-day supply PERVIVO on the tumor mass was evaluated.

In this analysis, no significant difference between the mass of the tumor was observed in the control group

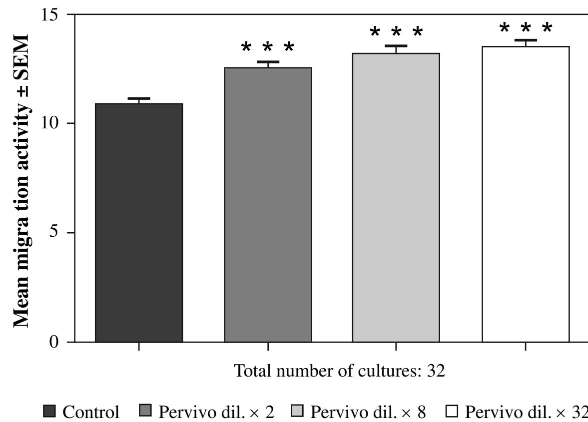

Fig. 3. Stimulatory effect of feeding mice with PERVIVO on the *ex vivo* chemokinetic activity of their splenocytes; $p < 0.001$

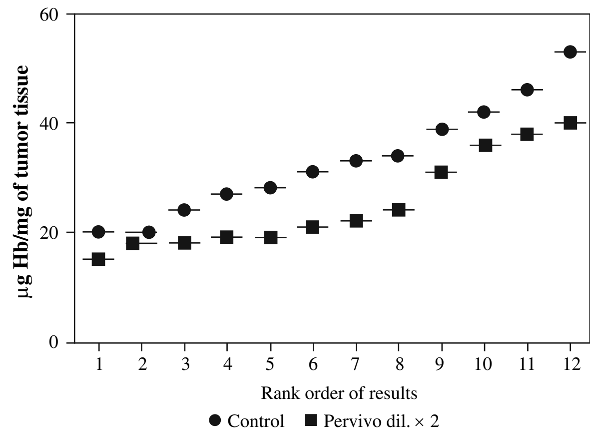

Fig. 4. Inhibitory effect of PERVIVO dil. × 2 on L-1 Sarcoma tumors hemoglobin content. Median values: control: 32, Pervivo: 21.5; Mann-Whitney U: 37, $p = 0.0462$

Table 4. The effect of PERVIVO on skin cutaneous angiogenesis induced in mice by grafting of Sarcoma L-1 tumor cells

Passage *in vivo*	Control (L-1 Sarcoma alone)		*PERVIVO* dil. × 32		*PERVIVO* dil. × 2	
	Number of tests	Mean number of blood vessels ± SEM	Number of tests	Mean number of blood vessels ± SEM	Number of tests	Mean number of blood vessels ± SEM
2nd	22	18 ±0.67	17	17.6 ±1.17 NS	21	15.3 ±1.11 $P < 0.05$

Table 5. Statistical analysis of the results presented on Fig. 3 (chemokinetic activity of splenocytes)

One-way analysis of variance
P value < 0.0001
P value summary ****
Are means signif. different? ($P < 0.05$) Yes

Bonferroni's Multiple Comparison Test	Mean Diff.	*t*	Significant?	*P* < 0.05?
Control vs. Pervivo dil. × 2	–1.630	4.433	Yes	****
Control vs. Pervivo dil. × 8	–2.320	6.310	Yes	****
Control vs. Pervivo dil. × 32	–2.650	9.040	Yes	****

(439 ±70 mg, $n = 12$) and the group treated with the formulation PERVIVO (339 ±40 mg, $n = 12$).

Summary of results

PERVIVO affects:
- *ex vivo* proliferative activity of murine splenocytes (stimulation),
- *ex vivo* chemokinetic activity of mouse splenic lymphocytes in tissue culture (stimulation),
- tumor angiogenesis (inhibition),
- tumor hemoglobin content (inhibition).

It has no effects on:
- the number and metabolic activity of granulocytes,
- *ex vivo* immunological response of lymphocytes against foreign histocompatibility antigens in local graft-versus-host reaction (LIA),
- tumor mass.

Discussion

The role of the immune system cells in the development and destruction of abnormal tissues, or those changed by inflammation or cancer, in the light of numerous studies, is still open to discussion.

Development of new diagnostic methods, allowing the more precise definition of the nature of cancer entails progress in the development of therapeutic approaches, where the most sophisticated, targeted way to treat cancer is the therapeutic use of monoclonal antibodies.

The documented history of cancer treatment began in 1777, when the Duke of Ken's surgeon injected the cancerous tissue as an first attempt to produce a vaccine against cancer.

Despite a sequence of failures, the idea of manufacturing specific cancer vaccines has not been abandoned up to date.

Vaccines based on heat shock proteins, peptides, viruses, DNA or vaccines based on genetically modified dendritic cells or tumor cells have been tested. So far, the results of the evaluation of the therapeutic effects of these vaccines are rather disappointing.

The first report concerning successful immunotherapy after vaccination with killed bacteria containing endotoxin from nine *streptococcus* and *Serratia marcescens* was published in 1891 by a doctor from Sloan Kettering Cancer Institute in New York, William Coley.

A long-term remission was observed in soft tissue sarcomas in 10% of patients. The vaccine composition, cited above, has the adopted name of the vaccine creator – Coley. Coley's vaccine gave rise to the development of today's powerful industry, based on immunotherapy, using non-specific immunostimulants and immunomodulators, both plant and bacterial.

Despite numerous studies, supporting the therapeutic efficacy of adjuvant therapy in the treatment of cancer, non-specific immunostimulants and immunomodulators have not found a permanent position in routine clinical practice. However, in the case of certain cancers, such as lung cancer (adenocarcinoma) the suspension, based on the killed *Mycobacterium vaccae* was proved to be more effective in comparison to chemotherapy [37, 38]. Recent positive results of clinical trials in castration-resistant prostate cancer and metastatic melanoma with novel immunoactive drugs (anti-cancer vaccine Sipuleucel-T and a monoclonal antibody ipilimumab) raised hope, that immunotherapy will play important role in the cancer treatment in the future [39].

In view of the above mentioned data, the aim of this study was an evaluation of the impact of a complex herbal preparation PERVIVO on selected parameters, characterizing the non-specific immune response in mice and within the specific context of a possible usefulness in supporting the preparation of anticancer therapy. In this analysis, the non-specific response was characterized by assessment of the number of cells and phagocytic capacity of granulocytes, based on the chemiluminescence test. Specific response was assessed by capacity of proliferative activity of mouse spleen cells on PHA.

Complement work, which was subject of this evaluation, examined the impact of PERVIVO on tumor tissue environment through the assessment of the impact on neoangiogenesis process and tumor mass.

PERVIVO impact on some aspects which characterize the non-specific response was not observed in this study (except splenocytes locomotory activity, which was increased) but PERVIVO 2-fold dilution increased significantly the size of the proliferative capacity of splenocytes stimulated with PHA, with no observed dose dependent PHA effect.

Similar effects of PERVIVO impact on some aspects of a specific answer, was presented in the study of Skopińska-Różewska and Sommer [26].

In an experimental animal model, authors of the study observed a statistically significant effect of PERVIVO on the synthesis capacity of antibodies in mice. In summary, this study postulated a possible therapeutic efficacy of the adjuvant treatment of PERVIVO of diseases with a deficiency of immunoglobulins.

Effective anticancer therapy, beyond the effector cells' control, involved in the anticancer response, should not stimulate the growth of tumor tissue, neither indirectly, through its influence on the neoangiogenesis process, nor directly by providing stimuli to the cancer cells.

In its assessment, which was the subject of the present study, the utilized preparation PERVIVO, had no stimulatory effect on either of the processess: neoangiogenesis which was assessed by hemoglobin concentration in tumor tissue, or TIA test, and on the tumor mass. In fact, PERVIVO treatment of L-1 sarcoma recipients diminished early (TIA test) and late (hemoglobin content) tumors vascularity.

In conclusion, in view of the results stated above, it seems that PERVIVO formulation may be found as the safe adjuvant therapy of such conditions as immunodeficiencies, including cancer.

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