

NATURAL DRUGS

EFFECTS OF BILBERRY FRUIT AQUEOUS EXTRACT AND SELECTED ANTIBIOTICS ON IMMUNE RESPONSE IN MICE

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Abstract: It was demonstrated that an extract from the pharmacopoeial raw material bilberry fruit (*Fructus Myrtilli*) co-administered with selected antibiotics: cefuroxime, cefoperazone and doxycycline, positively affects the survival of mouse thymocytes in cultures with hydrocortisone (cytotoxicity test), increases the count of splenocytes forming spontaneous rosettes (E-rosette test) and increases the agglutination titre of mouse serum (active hemagglutination test). In the cytotoxicity test, the best protective effect has been obtained with the combination of doxycycline with bilberry fruit aqueous extract. The increased count of splenocytes capable of binding sheep red blood cells has been observed for cultures with cephalosporins (cefuroxime and cefoperazone). The highest agglutination titre has been demonstrated for mouse serum treated with the extract in combination with doxycycline and cefuroxime.

Keywords: bilberry fruit, immunomodulation, cefuroxime, cefoperazone, doxycycline.

Antibiotics, irrespective of their practical applications, should be regarded as inhibitors of enzymatic reactions, and as such should be defined as “inhibitors of enzymatic systems and cellular structures which determine the viability of a microorganism” (1). Given the fact that the physiological bacterial flora is a source of antigens affecting immune response, antibiotics may influence the body’s defences in many different ways (2).

Antibiotics affect various aspects of immune response, such as: phagocytosis, macrophage function and T and B cell proliferation (3, 4). The mechanisms of antibiotic action is most probably associated with the activation of cytotoxic-suppressor populations of CD4⁺ and CD8⁺ cells in the presence of monocytes (5, 6). For instance, the immunosuppressant activity of cephalosporins results from their effects on the early phases of the proliferative response in the course of both cellular and humoral immune reactions (7). Doxycycline inhibits the antibacterial activity of the serum, delayed hypersensitivity reaction (4), inhibits the formation of peroxides by leukocytes and their *in vitro* degranulation (8).

The research studies of the immunotropic activity of herbal raw materials conducted at our

Department have demonstrated that bilberry fruit extract possesses a comparable immunostimulating activity to synthetic compounds: levamisole and isoprinosine, whose mechanism of acting is the same (9).

The results of our previous studies (10, 11) allow us to believe that supportive treatment with selected herbal raw materials will be beneficial to the immune system of a patient receiving antibiotics.

Therefore, the aim of our study was to demonstrate that an extract from the pharmacopoeial raw material bilberry fruit (*Fructus Myrtilli*) co-administered with selected antibiotics positively affects the survival of mouse thymocytes in cultures with hydrocortisone (cytotoxicity test), increases the count of splenocytes forming spontaneous rosettes (E-rosette test) or increases the agglutination titre of the mouse serum (active hemagglutination test).

EXPERIMENTAL

Herbal raw materials

- *Fructus Myrtilli* (Herbapol Lublin SA, Poland)

A decoctum was prepared from the raw material in accordance with the instructions contained in

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the 6th Polish Pharmacopoeia. Determinations were performed with an undiluted extract and a 1 : 10 dilution of the extract.

Cell culture solutions

Culture medium RPMI-1640 containing Hepes 20 mM/L, physiological buffered saline, PBS, Hanks solution with the addition of 0.5% lactalbumin hydrolysate, all from Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland.

Reagents

Hydrocortisone: Corhydron (HC) 100 mg, powder and solvent, PF Jelfa, Jelenia Góra, Poland, (50 µg per 1 mL of culture), sheep red blood cells (SRBC) (Biomed, Warsaw, Poland) stabilized in Alsever's solution, Alsever's solution: glucose, sodium citrate, sodium chloride, citric acid, all from POCH, Poland, foetal calf serum (GIBCO), Tripan blue substance and Crystal violet, substance (both from Merck), Gradisol L, density 1.077 g/mL (Aqua Medica).

Antibiotics

Cephalosporins: Cefuroxime (Biofuroxim, 30.0 µg of cefuroxime per 1 mL of culture), Cefoperazone (Biocefazon, 60.0 µg of cefoperazone per 1 mL of culture) both from Biotechnology and Antibiotics Institute, Warsaw, Poland.

Doxycycline (Doxycyclinum, 4.0 µg of doxycycline per 1 mL of culture) from Polfa, Tarchomin, Poland.

Animals

The BALB/c inbred mouse strain was used in all experiments. 4-Week-old females weighing 16 g were used for the cytotoxicity test and 6- to 8-week-old females weighing about 20 g were used for the hemagglutination and rosette tests. The animal experiments were performed pursuant to the Ethics Committee decision number 18/2003 (7/NIZP/2005).

Cytotoxicity test

The test assesses the effect of a given factor on the survival of mouse thymocytes in 18- to 20-hour cultures with hydrocortisone (12). Immature thymocytes express surface steroid receptors, making them susceptible to the lytic action of hydrocortisone, which induces the active process of autodestruction leading to apoptosis (13, 14). During the process of maturation and acquisition of immunocompetence, the cells lose these receptors and become steroid-resistant. Thymocyte cultures are a good experimen-

tal model for rapid testing of various factors with a potential to accelerate T cell maturation or, in other words, to exert thymomimetic effects similar to those of the thymic hormones.

The test was performed under sterile conditions. Four-week-old mice were euthanized with chloral hydrate. Thymuses were collected under sterile conditions and passed through a metal sieve (sieve pore diameter: approximately 300 µm, Sigma). Thymocytes obtained from the ground thymuses were washed with RPMI-1640 three times and centrifuged (at 1800 rpm for 7-8 min). After the last centrifugation, thymocytes at the concentration of 4×10^6 cells/mL were suspended in RPMI with the addition of 10% foetal calf serum. The resulting suspension of thymocytes was transferred, 1 mL each, to sterile test tubes and 10 µL of extracts from bilberry fruit and appropriate antibiotic concentrations were added. One hour later, HC was added at the dose of 50 µg/mL thymocyte culture. The resulting samples were incubated for 18-20 h at 37°C in the atmosphere of CO₂. Then the viable and dead cell counts were performed after staining with 0.04% tripan blue. The viability of thymocytes in control cultures was 80-95%. The results were converted into percentage change of the viable cell count versus control. Thymocytes alone served as controls.

Hemagglutination test

The hemagglutination test uses the reaction of specific binding of antibodies to the antigen in the form of red blood cells. Red blood cells bind with the specific antibodies to form complexes and precipitate from the reaction suspension in the form of flocculi. Six-to-eight-week-old mice were given a single intraperitoneal dose of 4×10^8 sheep red blood cells (SRBC) (15). The bilberry fruit aqueous extract was given orally and the antibiotic solutions were given subcutaneously for the next 4 days. On the fifth day following immunization, mice were euthanized with chloral hydrate and bleed. Sera were separated and spleens were collected for use in the rosette test. The collected blood was placed in the thermostat for 15 min, followed by 4°C for 30 min and the blood clots were cut off. They were then centrifuged for 10 min at 2500 rpm and serum was collected from each test tube. To activate the complement, the sera were placed in a water bath at 56°C for 30 min. 1% suspension of SRBC was added to subsequent serum dilutions prepared in microplates and the sera were incubated at 37°C for 2 h, followed by 4°C for 18-20 h.

Table 1. The effect of bilberry fruit aqueous extract on the viability of mouse thymocytes in cultures with the addition of selected antibiotics determined in the cytotoxicity test

Dilution of bilberry fruit aqueous extract	Doxycycline (control = 104.2% ± 3.24) n = 22	Cefoperazone (control = 103.9% ± 1.92) n = 22	Cefuroxime (control = 100.3% ± 2.30) n = 22
Undiluted	123.6% ± 3.11 n = 19 *	116.9% ± 3.41 n = 16	122.8% ± 3.14 n = 23 *
1 : 10 dilution	119.7% ± 3.27 n = 18 *	119.4% ± 1.84 n = 22 *	105.2% ± 3.85 n = 16

* p < 0.0001

Table 2. The effect of bilberry fruit aqueous extract and selected antibiotics on % of immune activity determined in the E-rosette test (control = 100%).

Dilution of bilberry fruit aqueous extract	Doxycycline	Cefoperazone	Cefuroxime
1 : 10 dilution	123.6 ± 17.14 n = 10	135.6 ± 8.41 n = 10 *	141.1 ± 8.63 n = 10 *

* p < 0.01

Table 3. The effect of bilberry fruit aqueous extract and selected antibiotics on % of immune activity determined in the active hemagglutination test (control = 100%).

Dilution of bilberry fruit aqueous extract	Doxycycline	Cefoperazone	Cefuroxime
1 : 10 dilution	183.8 ± 38.07 n = 10	113.7 ± 18.84 n = 10	178.6 ± 26.68 n = 9

The agglutination titre of the serum (estimated amount of antibodies in the serum) was defined as the highest serum dilution at which agglutination was still present (microscopic assessment: at least 3 agglutinates per 200× high power field).

Sera of mice given PBS served as controls.

Rosette test

It is assumed that all T cells express receptors for sheep red blood cells (16, 17). The ability of T cells to bind sheep blood cells to form spontaneous rosettes (the so-called E-rosettes) depends on the binding of glycoprotein structures present on the surface of lymphocytes with lipids present on the surface of sheep red blood cells.

All the test procedures were performed while samples and media were kept on ice and centrifugation was performed in a centrifuge fitted with a cooling system.

The mice used for the hemagglutination test were also the source of splenocytes. Splenocytes obtained from the spleens were homogenized, placed on Gradisol L (density 1.077 g/mL) and centrifuged for 15 min at 3000 rpm. Lymphocytes obtained from the phase boundary were washed twice with Hanks solution, centrifuged again and suspended in the medium achieving the final cell count of 2×10^6 per 1 mL of culture. Next, 1% SRBC suspension in Hanks solution was added to the splenocyte culture. Following 15 min of incubation at 37°C, the cultures were placed at 4°C for 20 h. After staining with 0.1% crystal violet, the percentage of splenocytes around which rosettes had formed was counted under the microscope. A rosette was defined as a splenocyte closely surrounded by at least three sheep red blood cells. Splenocytes originated from animals immunised with 0.2 mL of 10% SRBC in PBS suspension.

Cultures of splenocytes collected from mice given PBS served as controls.

We used the Medistat software to analyze all our results (18) and calculated standard error (SE) and statistical significance for two matched samples.

RESULTS

To find out if bilberry fruit extract (undiluted and diluted at 1:10) co-administered with antibiotics has protective effects on the immune system, three tests: cytotoxicity test, E-rosette test and active hemagglutination test were used. These tests have allowed to evaluate both cellular and humoral immune response.

The following doses of antibiotics per 1 mL of culture were used in the experiments: 30.0 µg of cefuroxime (Biofuroxim), 60.0 µg of cefoperazone (Biocefazon) and 4.0 µg of doxycycline (Doxycyclinum). Selection of antibiotic concentrations was based on bibliographical data regarding therapeutic concentrations in human serum (19).

Bilberry fruit aqueous extract was added to thymocyte cultures or administered to animals both undiluted and diluted at 1:10.

The calculation of the viability of mouse thymocytes in the cytotoxicity test was corrected for the cytotoxic effects of antibiotics added to the cultures compared to controls (culture without the addition of hydrocortisone).

The results summarized in Table 1 suggest that adding bilberry fruit aqueous extract to thymocyte cultures containing selected antibiotics (cefuroxime, cefoperazone and doxycycline) significantly increases the viability of mouse thymocytes, although this is not always in proportion to the dilution of the extract.

The significantly high viability of mouse thymocytes in cultures with doxycycline to which bilberry fruit extract is given is achieved irrespective of the dilution of extract.

In the case of cephalosporins, the results are not so straightforward. An addition of bilberry fruit extract diluted at 1 : 10 to the culture with cefoperazone markedly increases the viability of thymocytes, similarly to the addition of an undiluted aqueous extract to the culture with cefuroxime. However, when both cefuroxime plus the extract at 1 : 10 dilution and cefoperazone plus undiluted extract were added to the culture, no significant differences in the viability of mouse thymocytes were observed.

The active hemagglutination and E-rosette tests were performed for 1 : 10 dilutions of bilberry fruit aqueous extract to reduce the number of animals required to perform the experiment.

The results presented in Table 2 demonstrate the statistically significant effect of the extract reflected by the increased count of splenocytes capable of binding sheep red blood cells (formation of rosettes) in cultures with the addition of cephalosporins. The addition of bilberry fruit aqueous extract to the culture with doxycycline increased the number of rosettes, although the increase did not reach statistical significance. It is very likely that using an undiluted extract would have allowed to demonstrate a statistically significant effect, which would only confirm our findings.

The results concerning the effect of bilberry fruit extract regarding the increased agglutination titre with sheep red blood cells are summarized in Table 3. They demonstrate a considerable increase of the agglutination titre of mouse serum in the case of combined administration of bilberry fruit with doxycycline and cefuroxime. When bilberry fruit aqueous extract was co-administered with cefoperazone no effect was observed.

CONCLUSIONS

The results of the cytotoxicity, active hemagglutination and E-rosette tests have demonstrated stimulation of the immune system in mice when antibiotics were co-administered with an extract from the herbal raw material bilberry fruit (*Fructus Myrtilli*).

Bilberry fruit extract improved the viability of the highest percentage of mouse thymocytes when doxycycline was added to the culture.

Increased counts of splenocytes capable of binding sheep red blood cells were achieved in cultures with cephalosporins (cefuroxime and cefoperazone) to which bilberry fruit aqueous extract was added.

The highest agglutination titre was demonstrated in mouse serum after the addition of bilberry fruit extract and doxycycline plus cefuroxime.

The results concerning the antibiotics used demonstrated a variable effect on the immune system of mice, while bilberry fruit extract always exerted an immunostimulating action.

It seems justified to use the traditionally employed herbal raw material as a supportive measure to improve the immune status of patients undergoing antibiotic therapy.

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