flask) and sonicated for 15 min. Then 2 ml of this suspension were filtered through a nylon membrane filter (0.45 μm) and 1 μl (0.6 mg/ml) of this solution was injected once on the gas chromatograph (helium, 5.6, 50 kPa; total flow: 40 ml/min; temperature: 60 °C for 3 min, 6 °C/min to 220 °C, 8 °C/min to 280 °C, 280 °C for 15 min; injector temperature 250 °C; detector temperature: 300 °C, range of MS: 40–550 [m/z]).

1.3. Chromatographic purity of melatonin in tablets

The purity of melatonin was analysed by GC. Peaks in the chromatogram were compared with all libraries of spectra. MS of the following compounds were considered as reference spectra: 6-hydroxy-melatonin, 5-methoxytryptamin, N-acetylserotonin, serotonin hydrochloride, L-tryptophan, 5-methoxy-tryptophan. The same chromatographic system as described above has been used. For further details see [7].

1.4. Quantification of melatonin in tablets

The concentration of melatonin in the tablets was determined by peak integration and comparison to a reference melatonin curve, in the presence of phenacetin as the internal standard. Melatonin solutions were prepared with ethyl alcohol, sonicated and filtrated, for further details see [7]. The same chromtographic conditions described in chapter 1.2 have been used, except the range of MS: SIM (selected ion monitoring)-mode; Ions [m/z]: 108.0, 109.0, 179.0, 137.1 for phenacetin; [m/z]: 160.1, 173.1, 145.1, 232.1 for melatonin).

2. High Performance Liquid Chromatography (HPLC)

Melatonin in oral solid state formulations was identified and quantified by HPLC using a modified version [7] of a validated method described in a proposed monograph of melatonin for the United States Pharmacopeia (USP) [5].

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Immunomodulatory activity of the saponin-rich fraction from roots of *Silene vulgaris* Garcke: initial study

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Triterpenoid saponins isolated from many medicinal plants as well as mixtures of triterpenoid saponins exhibit a broad spectrum of biological and pharmacological activities, as discussed in a number of extensive reviews [e.g. 1, 2]. Various saponin-containing plants are used in traditional medicine as biomodulators of the central nervous system facilitating both physical and mental activities (*Panax ginseng*), antiphlogistics (*Aesculus hippocastanum*), diuretics (*Solidago* sp., *Herniaria* sp.), expectorants (e.g. *Hedera helix, Primula* and *Glycyrrhiza* sp.) and extracts containing standardised mixture of saponins are registered as drugs in many countries. Moreover, several new activities of saponins have been described during the last few years, such as: antitumor, cytotoxic action [1–4], antiinflammatory and immunomodulatory activities [1, 2, 5–9].

In this paper we report initial results of studies of the *in vitro* immunomodulatory activity of saponin mixtures (the S_w fraction) obtained from roots of *Silene vulgaris* Garcke (Caryophyllaceae), a perennial herb commonly found in meadows, grassy slopes and at waysides. Structures of saponins from the S_w fraction have been described in a previous paper as bidesmosides of gypsogenin and quillaic acid [15].

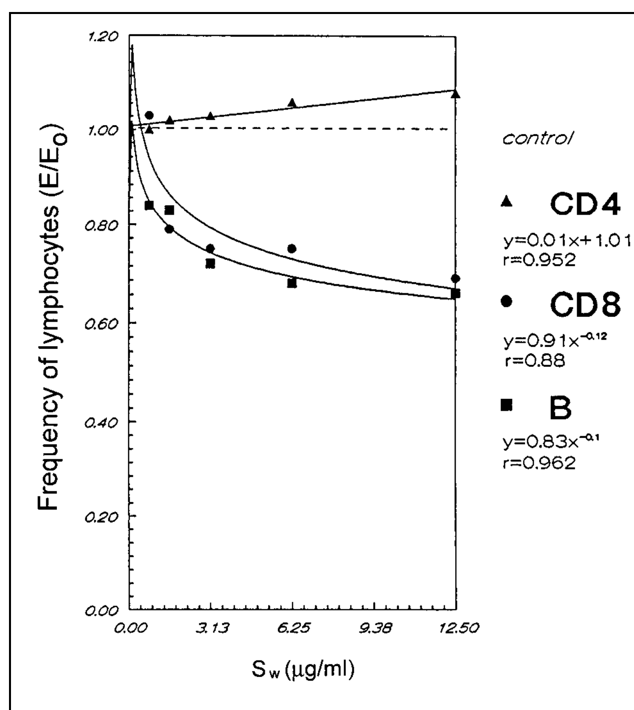

Fig.: Frequency of the main subpopulations of human lymphocytes after 72 h of culture with lectin (PHA; 10 μg/ml) and the S_w fraction. Results were compared to the relative control (without the S_w) and expressed as E/E_0 ratio. Regression equations estimate the dose-response relations

Results describing an impact of the S_w fraction on *in vitro* cultures of human blood-derived lymphocytes and granulocytes are gathered and presented in the Table.

The S_w fraction was not cytotoxic to human lymphocytes in culture up to the concentration of 25 μg/ml. However, at higher concentrations, a marked increase of dead cell numbers was noticed.

The proliferative potential of lymphocyte cultures was markedly lowered in the presence of the S_w fraction. The strongest effect was observed at the concentration of 25 μg/ml, where the PP was about four-times lower than in control cultures, whereas at lower concentrations of the S_w fraction the decrease was from 15 to 35%.

The ratio of two main subpopulations of T-lymphocytes, i.e. CD4 and CD8 cells, markedly increased at the S_w concentrations range: 3.125–12.5 μg/ml, in which CD4/CD8 ratio was by 20–50% higher than in the control culture (without the S_w). However, at the highest tested S_w concentration (25 μg/ml) the CD4/CD8 ratio decreased by about 20%.

The level of free radicals generated *in vitro* by granulocytes during incubation with the standard granulocyte activator (PMA, 100 ng/ml) and the S_w fraction markedly decreased proportionally to the S_w concentration. At the highest S_w concentration tested (12.5 μg/ml), the diminution by 35% of free radicals generation was observed in the NBT reduction test. It should also be noticed, that the impact of the S_w on the generation of free radicals was strong and even at the lowest S_w concentration tested (0.78 μg/ml), a decrease by about 25% was estimated in relation to the control sample (without the S_w).

The S_w fraction exerted diverse influence on the main subpopulation of human lymphocytes during 72 h of *in vitro* culture.

The number of CD4 lymphocytes increased linearly in proportion to the S_w concentrations in the culture medium, whereas the number of CD8 and B cells markedly decreased in the range of the tested concentrations. The dose-effect relations were described by the regression equations given in the Fig.

In this initial study we observed that the S_w fraction exerted the immunosuppressive, potentially antiinflammatory activity on human blood cells (decrease of total lymphocyte proliferation, diminution of B cells numbers, lowering of free radicals generation by granulocytes) and, simultaneously, it had a selective immunomodulatory effect on the lymphocyte T subpopulations (an increase of CD4 cell number and a marked decrease of CD8 cells). The conclusion is valid for the lower concentrations (up to 12.5 μg/ml) of the S_w fraction. At higher concentrations (from 25 μg/ml) a marked cytostatic activity appeared, whereas at 50 μg/ml the cytotoxic action of the S_w prevailed. According to literature [1, 9], the 0.01–10 μg/ml dose range *in vitro* might be considered as representative for the saponin concentrations achievable *in vivo*, because of their low intestinal absorption level. We regard our results as an important hint for further research and clarification.

Table: *In vitro* influence of the fraction (S_w) on lymphocytes viability, proliferative potential (PP) of lymphocyte cultures, ratio of helper/suppressor cells frequencies (CD4/CD8) and on granulocyte free radicals generation (NBT reduction test)

	Lymphocytes			Granulocytes
S_w (μg/ml)	Viability[a] (% of dead cells)	PP[b]	CD4 / CD8[b]	NBT reduction[c] ($A_{515\,nm}$)
– (control)	4.26	17.80	2.80	0.216
0.78	3.85	15.84	2.73	0.160
1.56	2.15	14.24	3.59	0.158
3.125	2.25	15.13	3.84	0.155
6.25	2.45	14.95	3.98	0.153
12.50	3.44	11.57	4.42	0.149
25.00	5.33	3.74	2.18	0.142
50.00	26.15	–	–	–
100.00	41.63	–	–	–

[a] after 18 h of culture
[b] after 72 h of culture
[c] after 40 min of incubation

Experimental

1. Plant material. Extraction and separation

Roots of *Silene vulgaris* Garcke were collected in September 1995 in Trzebnica region, Poland. A voucher specimen is deposited in the Departement of Pharmacognosy, Wrocław University of Medicine. Air-dried and powdered roots (100 g) were extracted successively with dichloromethane, methanol, 50% methanol and finally with water at room temperature. Following a literature method [10] the water extract was purified by SPE method using a Bakerbond spe column with reversed phase (Octadecyl 6 ml, HC). Washing the cartridge with water and 35% methanol resulted in the removal of carbohydrates and phenolics. The fraction finally eluted with methanol contained mainly the saponin mixture [10]. The saponin-rich fraction (S_w) was dissolved with water, to make the final concentration needed, and was added in the volume of 50 μl to the cell-culture medium (2.5 ml).

2. Blood cell separation

Lymphocytes and granulocytes were isolated from heparinized venous blood obtained from two healthy volunteers. Cells were isolated by centrifugation on the discontinuous density gradient of Histopaque solutions; d = 1.077 and d = 1.119 [11]. The results of the biological activity tests presented above were the mean of two independent experiments for each blood donor.

3. Lymphocytes – cytogenetic and immunocytochemical tests

Lymphocytes'viability after 18 hrs culture in the presence of the S_w fraction (CO_2-incubator, 37 °C) was evaluated with standard staining by means of a mixture of dyes: ethidium bromide/acridine orange (1 ppm : 1 ppm) and examined under the fluorescent microscope.

Cytogenetic methods were applied to estimate mitotic indices, replication indices and proliferation potentials of lymphocytes after 72 h of culture with the standard mitogenic agent phytohemagglutinine (PHA; 10 μg/ml) and the S_w fraction (6.25–25 μg/ml) in a $C0_2$-incubator. Proliferating cells were labelled with 5-bromodeoxyuridine (30 μM), visualized and examined under a microscope following the standard techniques [12]. The proliferative potential was a product of mitotic index and replication index (PP = MI × RI). Immunocytochemical staining of cell smears prepared from lymphocyte cultures after 72 h of incubation in the presence of lectin (PHA; 10 μg/ml) and the S_w fraction was carried out following the recommended protocol with mouse monoclonal antibodies – DAKO, Denmark [13]. The frequencies of stained cell subpopulations were counted under a microscope, compared to the frequency in the control culture (without the S_w fraction), and expressed as the experimental versus the control (E/E_0) ratio.

4. Granulocytes – free radicals estimation

Free radicals generation by granulocytes *in vitro* stimulated with the standard activator – phorbol myristate acetate (PMA; 100 ng/ml) was assessed after incubation (40 min, 37 °C, shaking water-bath) with the S_w fraction by means of the quantitative reduction test of 0.4% nitroblue tetrazolium (NBT) according to the standard procedure [14]. The samples were examined spectrophotometrically ($A_{515\,nm}$), and the results were subtracted from the relative blank samples.

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