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FATTY ACID COMPOSITION OF PURSLANE SEED WATER EXTRACT AND ITS EFFECT ON METABOLIC PROFILE OF MURINE PERITONEAL MACROPHAGES

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The aim of the work was to explore fatty acid composition of purslane seed water extract and its effect on the metabolic profile of murine peritoneal macrophages. Fatty acid composition was evaluated by gas chromatography–mass spectrometry. Collection of murine macrophages from the peritoneal cavity was done without preliminary sensitization. Reactive oxygen species generation was assayed by flow cytometry and nitroblue tetrazolium test. Phagocytic activity was evaluated by flow cytometry. Nitric oxide production was analyzed in cell supernatants by Griess reaction. Arginase activity was measured in cell lysates by standard colorimetric assay. Reactive oxygen species and nitric oxide production were significantly lower in murine macrophages simultaneously treated with purslane seed water extract and lipopolysaccharide in comparison to macrophages treated with lipopolysaccharide only. Also, the studied extract caused statistically significant increase in arginase activity of unsensitized peritoneal macrophages. That is consistent with the fatty acid content of this extract, since it contained comparatively higher proportion of unsaturated fatty acids exhibiting anti-inflammatory properties, than saturated fatty acids known for their pro-inflammatory effects.

Key words: purslane seed water extract, peritoneal macrophages, reactive oxygen species, phagocytosis, arginine metabolism.

Macrophages are key initiators of inflammatory processes in tissues, as well as important regulators of inflammation [1]. According to traditional views, macrophages are characterized by extreme metabolic plasticity and, depending on the nature of the external stimuli, can acquire diametrically opposite activation states: pro-inflammatory (M1) and anti-inflammatory (M2). M1 macrophages are characterized by their role in the initiation and maintenance of inflammation. Phagocytes having such metabolic profile participate in anti-infectious and antitumor immunity. Their metabolic reactions are accompanied by the synthesis and secretion of pro-inflammatory mediators: cytokines, eicosanoids, reactive oxygen and nitrogen species. M2 macrophages participate in the tissue repair and regeneration. Macrophages with such metabolic profile are involved in the resolution of inflammation and remission of inflammatory diseases [2, 3].

Portulaca oleracea, also known as purslane, is a cosmopolitan annual weed exhibiting therapeutic properties. The interest of scientists in the study of the biological effects of this medicinal plant in the last decade resulted in deciphering of some molecular mechanisms of immunomodulatory action of purslane preparations. In particular, it is known that the anti-inflammatory action of herbal preparations based on this medicinal plant is mediated by the inhibition of NF-κB and MAPK-dependent signaling pathways of myeloid cells [4, 5]. Omega-3 polyunsaturated fatty acids of *Portulaca oleracea* are

responsible for the ability of phytopreparations based on this plant to exert a potent inhibitory effect on oxidative and nitrosative stress, inhibit the synthesis of inflammatory mediators and cytotoxic function of myeloid cells, activate differentiation of regulatory cells responsible for resolution of local and systemic inflammation [6, 7]. Literature on the content and composition of fatty acids in the extract of purslane seeds are sparse and partially contradictory. In addition, phytochemical composition of medicinal plant extracts is affected by the extraction method and the origin of the plant [8].

Herbal preparations are successfully used in the complementary treatment of inflammatory diseases, especially chronic, where macrophages can both support local inflammation and activate the processes of its resolution and restoration of tissue integrity [9]. Therefore, the effect of herbal remedies on the metabolism of macrophages should be considered as one of the most important mechanisms of their anti-inflammatory action that requires careful study.

In this study, we aimed to explore fatty acid composition of purslane seed water extract (PSWE) and its effect on the metabolic profile of murine peritoneal macrophages. To evaluate the metabolic profile of macrophages, we studied their oxidative metabolism, phagocytic activity, as well as arginine metabolism, which is a commonly recognized metabolic polarization marker of these cells [10].

Materials and Methods

Plant material. Identification of *Portulaca oleracea* was performed using classical taxonomic approach. Seeds of the purslane were collected in July-August in Tovuz-Qazakh region of Azerbaijan. They were dry out with good air ventilation in the shade and kept away from direct sunlight until the use. Then, plant material was ground and weighed. Teabags containing 5 g of material were produced especially for the present study at the Nargiz Medical center.

Preparation and lyophilization of PSWE. In order to prepare PSWE, 1 teabag was put into 0.2 L of water, boiled at 95 °C for half-hour without mixing. After that, teabag was squeezed out with a spoon and removed. Water extract was filtrated through a filter paper, lyophilized to obtain raw extract, which then was kept at 4 °C until use.

Evaluation of fatty acid composition by gas chromatography–mass spectrometry. Methylating mixture of methanol:toluene:sulfuric acid (44:20:2 by volume) was added to the glass vial containing sample of PSWE and heptane solution of undecanoic acid as the internal standard. The vial was heated for 4 h in a water bath, and then cooled to room temperature and centrifuged for 10 min at 5 000 r/min. Two phases appeared, where fatty acid methyl esters (FAMES) were in the upper phase. Gas chromatography–mass spectrometry (GC–MS) analysis was done using Agilent 6890N/5973 inert system (Agilent technologies, USA). Capillary column HP-88 was utilized (100 m×0.25 mm×0.25 μm; Agilent technologies, USA). Evaporator and interface temperatures were set to 250 °C and 280 °C, respectively. Temperature programming mode was used to perform separation. The initial temperature of 140 °C was kept for 5 min, then elevated to 240 °C by 4 °C/min. The final temperature was kept for 6 min. 2 μl of sample was introduced into a 1:50 flow separation mode. SCAN mode in the 38–500 m/z range was used for qualitative analysis and identification of the fatty acids. The carrier gas flow rate was set to 1.0 ml/min. The detection of fatty acid compounds of PSWE was performed by comparing their GC retention times to authentic fatty acid samples (Supelco, USA) and using the NIST 02 mass spectral database [11, 12].

Isolation of murine peritoneal macrophages (PMs) and study design. Collection of murine PMs from the peritoneal cavity was done without preliminary sensitization as previously described [13]. Male C57Bl/6 mice were housed in animal facility of ESC "Institute of Biology and Medicine". Animal protocol was approved by the ESC "Institute of Biology and Medicine" according to Animal Welfare Act guidelines. Study was performed in conformity with the standards of the Convention on Bioethics of the Council of Europe's 'Europe Convention for the Protection of Vertebrate Animals' used for experimental and other scientific purposes' (1997), the general ethical principles of animal experiments, approved by the First National Congress on Bioethics Ukraine (September 2001) and other international agreements and national legislation in this field. Intact mice were euthanized and PMs were isolated using PBS supplemented with 100 U/ml heparin. Then, cells were centrifuged at 300g for 5 min at 4 °C, and rinsed three times with serum-free DMEM. Trypan blue solution was used to quantify the number of live cells. Bacterial LPS and zymosan (Sigma, USA) were used for the pro-inflammatory macrophage activation.

Obtained PMs were treated with specified concentrations of PSWE with or without LPS (1 μg/ml) or zymosan for 18 h with subsequent analysis of reactive oxygen species (ROS) production, phagocytosis, arginase activity and NO generation. Concentration range was chosen according to the literature data on immunomodulatory properties of aqueous extracts of medicinal plants.

Measurement of intracellular ROS production by flow cytometry. 2′,7′-dichlorodihydro-fluorescein diacetate (H_2DCFDA, Invitrogen), which is a cell-permeable redox sensitive fluorescent probe, was utilized to determine the concentration of intracellular ROS, as described earlier [14]. In brief, PMs were seeded at a concentration of $2 \cdot 10^4$ cells/well into 96-well plates and incubated in PBS supplemented with 10 μM carboxy–H_2DCFDA, at 37 °C for 20 min in the dark. A short recovery time was allowed for the cellular esterases to deacetylate H_2DCFDA to 2′,7′-dichlorodihydrofluorescein (H^2DCF). When ROS are present, highly fluorescent 2′,7′-dichlorofluorescein (DCF) is formed as a result of rapid oxidation of H_2DCF. Fluorescence representing intracellular ROS concentration was determined by flow cytometry. The excitation and emission wavelengths were set at 488 nm and 525 nm, respectively. The results were expressed as mean fluorescence per cell.

Evaluation of intracellular ROS production by nitroblue tetrazolium test. PMs ($2 \cdot 10^5$/well) were incubated in HBSS supplemented with 1 mg/ml of NBT (Sigma-Aldrich) at 37 °C and 5% CO_2 for 1 h. Then, 2M KOH and 50% DMSO were added to stop the reaction. The absorbance of the formazan in each well was measured at 630 nm with a plate reader.

The flow cytometry phagocytosis assay was carried out as described previously [15]. Briefly, 5 μl of the heat-inactivated fluorescein isothiocyanate-labeled *Staphylococcus aureus* Cowan I cell suspension (collected by employees of Department of Microbiology and Immunology at Taras Shevchenko National University of Kyiv) at the concentration of $1 \cdot 10^7$ cells/ml were added to PMs. The samples were incubated for 30 min at 37 °C. Then, cold stop solution (PBS with 0.02% EDTA and 0.04% paraformaldehyde) and lysis buffer were added to arrest phagocytosis and lyse erythrocytes, respectively. The results were analyzed using FACSCalibur flow cytometer and CellQuest software (Becton–Dickinson, USA). Macrophages were identified through forward and side scatter gating. The results were presented as phagocytosis index (PI) that represents the mean fluorescence per one phagocytic cell (quantity of engulfed bacteria per one cell), and the percentage of cells emitting fluorescence after a defined culture period (percentage of cells performing phagocytosis).

Evaluation of NO release. Concentration of NO^{2-}, the main stable product of nitric oxide oxidation, was measured with the Griess method to evaluate nitrite levels in PM culture supernatants as described earlier [14]. In brief, equal volumes of the PM culture supernatants and Griess reagent were mixed and incubated for 10 min at room temperature. The absorbance of the formed chromophore was measured on a microplate reader at a wavelength of 550 nm. Sodium nitrite was used as a standard to measure nitrite content. Each value was divided by the number of viable PMs and presented as nitrite level for 10^6 cells. Mean and SD values were calculated with normalized quantities.

Arginase activity detection. Standard colorimetric assay with minor modifications was used to evaluate arginase activity in cell lysates [16]. 100 μl of lysis buffer (pH 7.5) was added to each PMs sample. The mixture was heated at 56 °C for 7 min to activate phagocytic arginase. Then, 100 μl of L-arginine (0.5 M; pH 9.7) was added. After that, the mixture was incubated at 37 °C for 2 h, and 800 μl of stop solution (H_2SO_4:H_3PO_4:H_2O = 1:3:7 by volume) was added to stop L-arginine hydrolysis. For colorimetric detection of urea, 40 μl of 9% α-isonitrosopropiophenone solution in ethanol was added, and the mixture was kept for half-hour at 95 °C and then for half-hour at 4 °C. Plate reader was used to estimate urea levels at a wavelength of 540 nm. Each value was divided by the number of viable cells and presented as urea concentration/h for 10^6 cells. Mean and SD values were calculated with normalized quantities.

Statistical analysis. Each sample was assayed in triplicate. Experimental results are presented as mean ± SD. Statistical significance of the data was determined by the one-way ANOVA and Student's *t*-test. The values of $P < 0.05$ were considered as significant.

Results and Discussion

Purslane seed water extract contains fatty acids with anti-inflammatory properties. The fatty acid quantitative composition of PSWE is shown in Table. As one can see from the table,

the concentration of α-linolenic (9Z,12Z,15Z-octadecatrienoic) omega-3 acid is the highest among other fatty acids in the studied extract. It is known that omega-3 polyunsaturated fatty acids have high anti-inflammatory potential due to both competition for the synthesis of proinflammatory, omega-6-derived mediators and their enzymatic conversion into specialized proresolving mediators, which stimulate resolution of inflammation [17]. Also, PSWE has high content of linoleic (9Z,12Z-octadecadienoic) omega-6 acid and oleic (9Z-octadecenoic) omega-9 acid. The influence of omega-6 acids on inflammation is complex: they can be converted into both pro-inflammatory eicosanoids and lipid mediators involved in inflammation resolution. There is still a controversy about whether their effects are pro- or anti-inflammatory [18]. Oleic acid is reported to have anti-inflammatory properties [19]. Relative proportion of saturated fatty acids, characterized by their ability to induce pro-inflammatory response [20, 21], such as palmitic (hexadecanoic) and stearic (octadecanoic) acids, is low.

The fatty acid composition of purslane seeds water extract in our experiments almost completely coincide with the results from other research groups. The most complete analysis of the fatty acid composition of purslane seeds collected in Iran is presented in the article of Delfan-Hosseini et al. [22]. According to these authors, the composition of purslane seeds revealed the same list of monounsaturated and polyunsaturated fatty acids, which was registered as a result of our research. Quantitative characteristics of the total content of saturated, unsaturated, polyunsaturated and monounsaturated fatty acids also almost completely coincide. The only difference is the presence in the fatty acid profile of purslane seeds isolated in Iran, a small amount of palmitoleic acid — a monounsaturated fatty acid, which is part of most vegetable oils. We suggest that this difference may be due to the territorial conditions of plant growth.

Purslane seed water extract abrogates macrophage oxidative burst caused by PRR-agonists. ROS generation is one of the key macrophage metabolic reactions. ROS have been demonstrated to get involved in the functional and phenotypic regulation of tissue macrophages. ROS can control the proliferation, cell death, phagocytic ability and motility of macrophages. In addition, ROS play a complicated role in regulating macrophage polarization: can promote both M1 and M2 macrophage metabolic profile [23–25].

Treatment of non-sensitized macrophages with PSWE caused a slight increase in their oxidative metabolism, regardless of the concentration used (Fig. 1). Macrophage treatment with LPS resulted in a strong 2.4-fold increase in ROS synthesis. Co-treatment of macrophages with PSWE at low concentrations and LPS did not affect the stimulatory effect of the latter. The use of PSWE at higher concentrations was associated with a slight inhibition of ROS synthesis induced by LPS. High content of alpha-linoleic acid in PSWE can be the reason of such effect, since it is known that PUFAs of both omega-3 and omega-6 groups at high concentrations can inhibit ROS and RNS formation by stimulated macrophages [26].

Treatment with zymosan was also associated with the increase of ROS production by macrophages. PSWE inhibited the oxidative metabolism of macrophages induced by this fungal glucan without dose dependence of the effect (Fig. 1, *F*).

Fatty acid composition of PSWE

Retention time	Fatty acid methyl ester	PSWE (mg/g biomass)
20.9729	Hexadecanoic acid (C16:0)	16.27
24.2692	Octadecanoic acid (C18:0)	7.24
25.1624	9Z-Octadecenoic acid (9-C18:1)	16.65
25.29	11Z-Octadecenoic acid (11-C18:1)	1.51
26.4916	9Z,12Z-Octadecadienoic acid (9,12-C18:2)	28.60
27.3263	Eicosanoic acid (C20:0)	0.98
27.9962	9Z,12Z,15Z-Octadecatrienoic acid (9,12,15-C18:3)	38.25

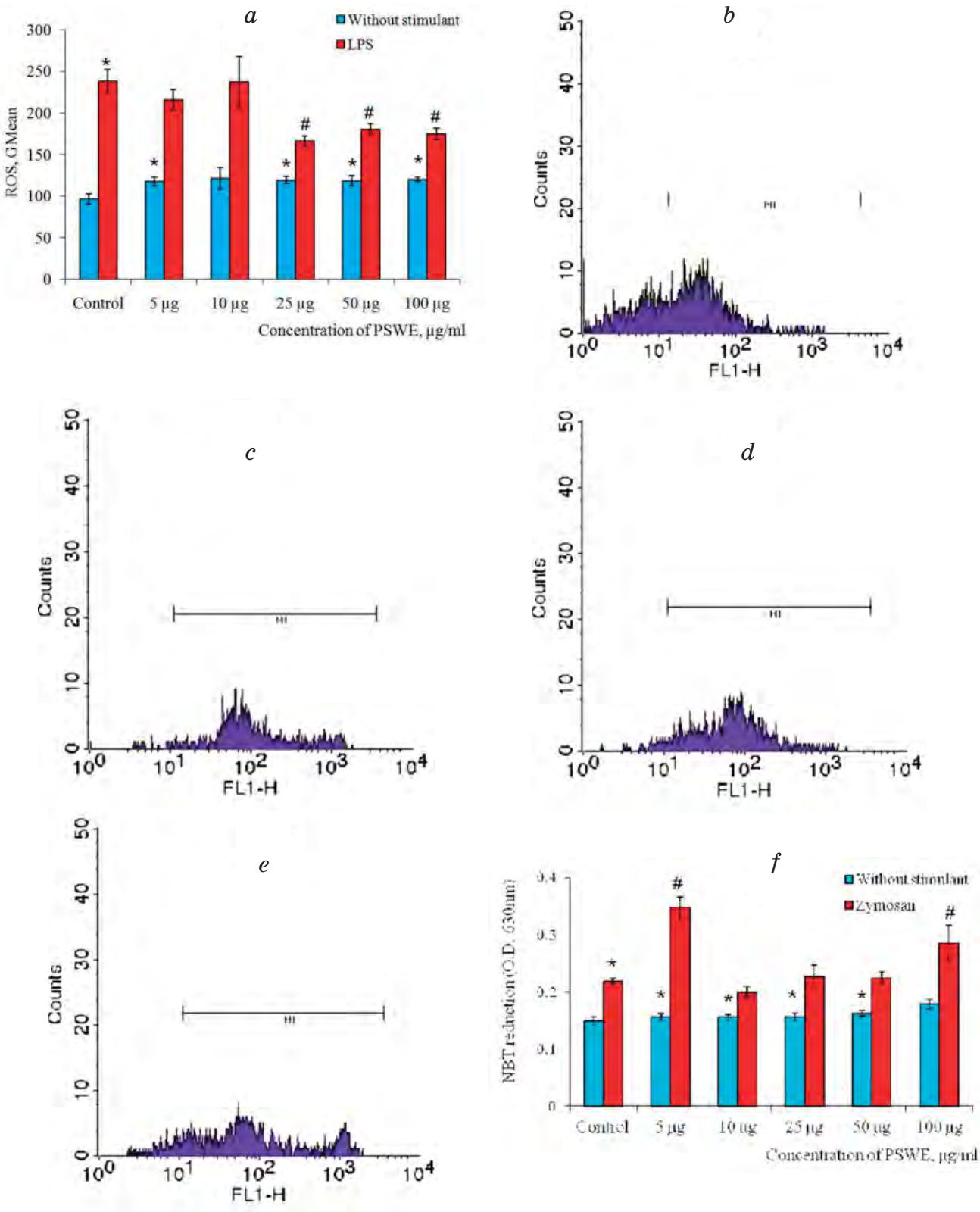

Fig. 1. **Influence of PSWE on the oxidative metabolism of macrophages:**
a — effect of PSWE on the oxidative metabolism of peritoneal macrophages measured by flow cytometry;
b–e — flow cytometry histograms showing ROS production by untreated (*b*) peritoneal macrophages and peritoneal macrophages treated with LPS (*c*), PSWE (*d*); and both LPS and PSWE (*e*); *f* — effect of PSWE on the oxidative metabolism of peritoneal macrophages measured by NBT test
* — $P \leq 0.05$ were considered significant compared to control without stimulant;
# — $P \leq 0.05$ were considered significant compared to control treated with bacterial lipopolysaccharide/zymosan.

Both stimulatory agents, which were used in our experiments, belong to ligands of macrophage pattern recognition receptors (PRRs): LPS — for CD14 and TLR4, and zymosan — for Dectin 1 and TLR2 [27, 28]. Alpha-linoleic acid is able to inhibit the translocation of the major transcription factor involved in the synthesis of pro-inflammatory mediators, NF-kB, and is capable to reduce the expression level of some pattern recognition receptors [29]. Therefore, inhibition of ROS generation by PSWE can be stipulated by both down-regulation of PRRs and suppression of NADPH-activity.

Purslane seed water extract slightly increase phagocytic activity of peritoneal macrophages. Phagocytic activity is an essential macrophage feature. Phagocytosis is significant for maintaining tissue homeostasis, controlling inflammation and the adaptive immune response. Increased phagocytic activity is characteristic for scavenging function of M2-macrophages [30]. PSWE had no significant effect on the phagocytic activity of macrophages (Fig. 2). There was only a slight increase in the proportion of phagocytic cells in the treated samples and a tendency to increase in their engulfing activity.

There is an evidence of the ability of alpha-linoleic acid to exert a pronounced stimulating effect on the endocytic activity of phagocytes [29]. It is probable that the concentration of this omega-3 acid in the tested PSWE is not sufficient to produce this biological effect. Also, it is known that palmitic acid, which is also present in the studied extract (16.27 mg/g of PSWE biomass) (Table), may suppress phagocytosis in macrophages by inducing COX-2 and increasing the downstream production of prostaglandins [31]. Thus, the effect of alpha-linoleic acid on phagocytosis may be counteracted by other compounds of the extract.

Purslane seed water extract shifts macrophage arginine metabolism to M2 metabolic profile. Key facets of macrophage biology are substantially driven by the phenotype of arginine metabolism. In M1 macrophages, iNOS pathway predominates: nitric oxide synthase metabolizes arginine to nitric oxide (NO) and citrulline. Arginase pathway dominates in M2 macrophages: arginase hydrolyzes arginine to ornithine and urea. For this reason, arginine metabolism is the most validated metabolic marker for distinguishing M1 and M2 macrophage activation profile [32, 33]. PSWE did not have a significant effect on NO production by non-sensitized macrophages regardless of the concentration used, but inhibited LPS-stimulated synthesis of this proinflammatory mediator (Fig. 3). Also, dose dependence of this effect was observed. PSWE enhances arginase activity of unsensitized peritoneal macrophages (Fig. 4). Clear dose dependence of the stimulatory effect of PSWE was absent; when using PSWE at high concentration (100 μg/ml) the difference between the arginase activity of treated and untreated cells was statistically insignificant. Given that PSWE did not influence the synthesis of NO by unsensitized phagocytes, but caused moderate increase in arginase activity, the modulating effect of this extract on the metabolic profile of macrophages can be considered as moderately anti-inflammatory.

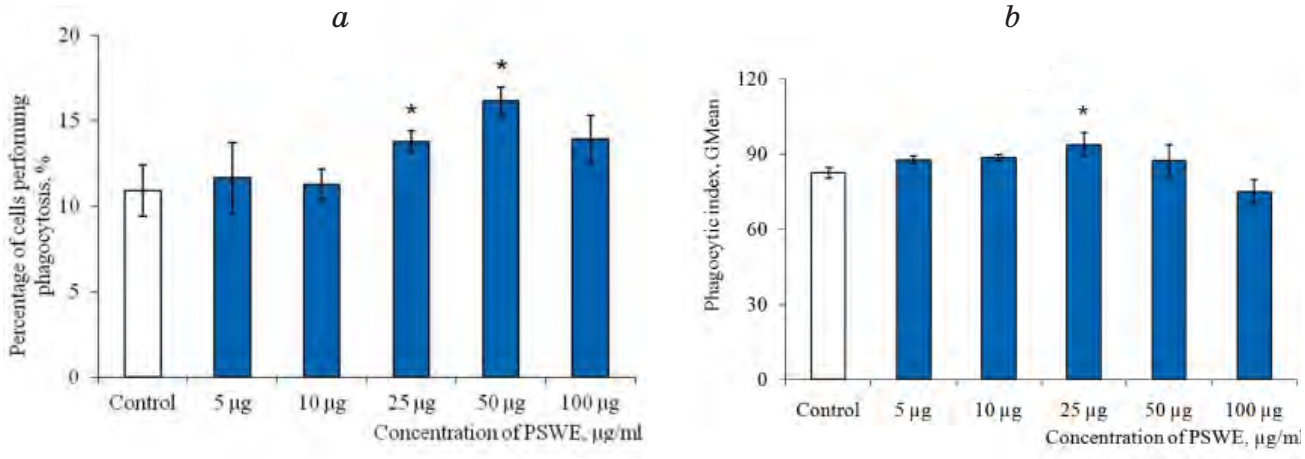

Fig. 2. **Influence of PSWE on phagocytic activity of macrophages:**
a — number of cells performing phagocytosis; *b* — fluorescence intensity (phagocytic index)
* — $P \leq 0.05$ were considered significant compared to control

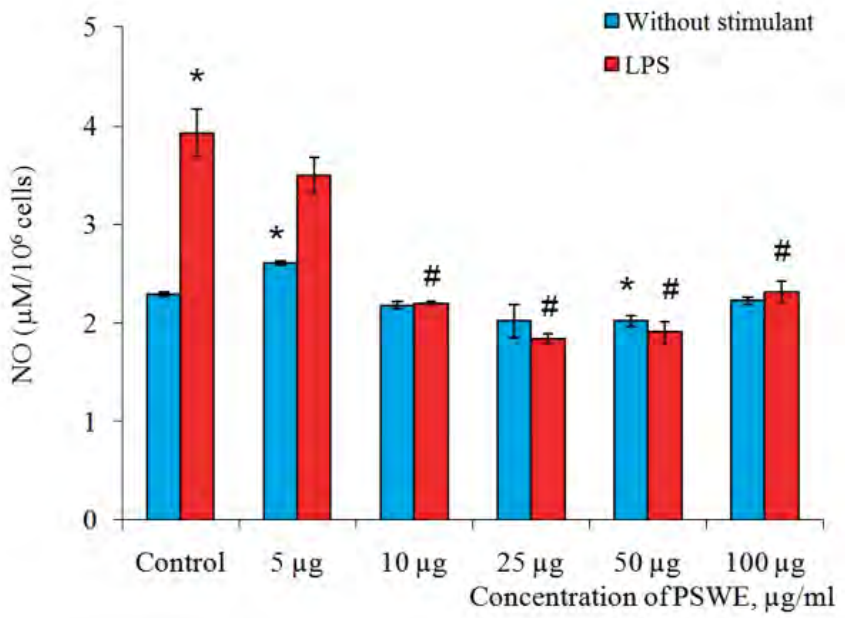

Fig. 3. **Effect of PSWE on NO production by peritoneal macrophages**
Hereinafter: * — $P \leq 0.05$ were considered significant compared to control without stimulant;
— $P \leq 0.05$ were considered significant compared to control treated with bacterial lipopolysaccharide

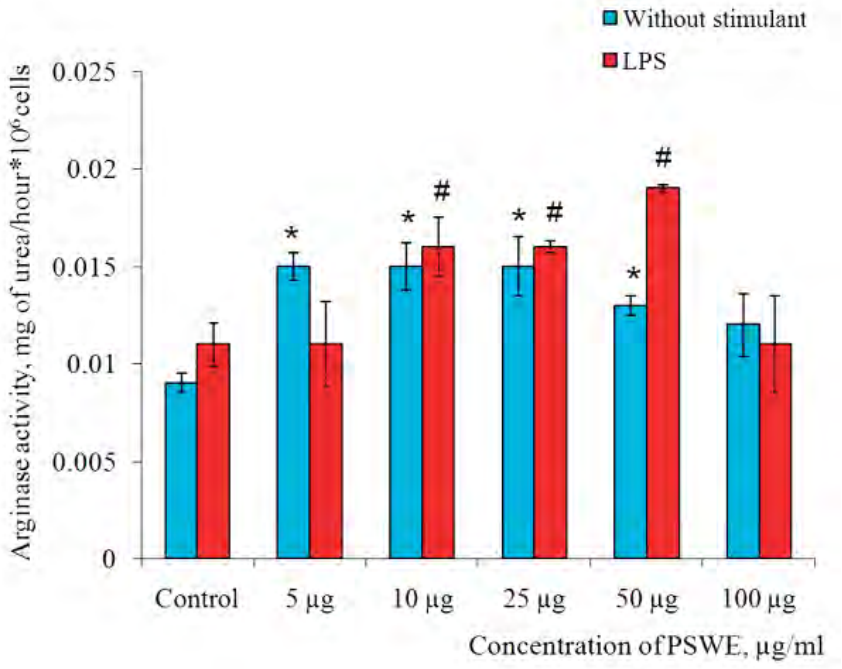

Fig. 4. **Effect of PSWE on arginine metabolism of peritoneal macrophages**

The arginase activity of macrophages co-treated with LPS and PSWE was higher than those in cell samples treated with LPS only. Therefore, it can be assumed that this phenomenon is due to the simultaneous increase in the activity of Arg-I, caused by the action of omega-3 acids, and increase in the activity of Arg-II, caused by the use of a high dose of endotoxin. Reduced LPS-stimulated NO generation at the same time with increased arginase activity can be regarded as markers of the anti-inflammatory modulation of macrophage arginine metabolism by studied PSWE.

In conclusion, PSWE exhibited anti-inflammatory effect on the metabolism of murine peritoneal macrophages. PSWE action was manifested by decrease of ROS and NO production in macrophages stimulated by LPS, and moderate increase in arginase activity of unsensitized PMs. It is consistent with the fatty acid content of this extract, since it contains comparatively higher proportion of unsaturated fatty acids exhibiting anti-inflammatory properties, than saturated fatty acids known for their pro-inflammatory effects. The results obtained give grounds to recommend the inclusion of purslane seeds in the composition of complex herbal remedies with anti-inflammatory action.

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No potential conflict of interest was reported by the authors.

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ЖИРНОКИСЛОТНИЙ СКЛАД ВОДНОГО ЕКСТРАКТУ НАСІННЯ ПОРТУЛАКУ ГОРОДНЬОГО ТА ЙОГО ВПЛИВ НА МЕТАБОЛІЧНИЙ ПРОФІЛЬ ПЕРИТОНЕАЛЬНИХ МАКРОФАГІВ МИШЕЙ

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Метою роботи було дослідити жирнокислотний склад водного екстракту насіння портулаку городнього та його вплив на метаболічний профіль перитонеальних макрофагів мишей. Жирнокислотний склад оцінювали методом газової хроматографії/мас-спектрометрії. Макрофаги отримували з перитонеальної порожнини мишей без попередньої сенситизації. Продукцію активних форм оксигену вимірювали з використанням протокової цитометрії та тесту із нітросинім тетразолієм. Фагоцитарну активність оцінювали методом протокової цитометрії. Продукцію оксиду нітрогену аналізували в супернатантах клітин за допомогою реакції Гріса. Активність аргінази встановлювали в клітинних лізатах стандартним колориметричним методом. У макрофагів, що їх культивували в середовищі з додаванням водного екстракту насіння портулаку городнього та ліпополісахариду, продукція активних форм оксигену й нітрогену була достовірно нижчою порівняно з макрофагами, які культивували у присутності лише ліпополісахариду. Також досліджуваний екстракт спричинював статистично вірогідне зростання активності аргінази несенситизованих перитонеальних макрофагів мишей. Це узгоджується з жирнокислотним складом екстракту, оскільки він містить більшу частку ненасичених жирних кислот, що мають протизапальні властивості, порівняно з відомими насиченими жирними кислотами з протизапальною активністю.

Ключові слова: водний екстракт насіння портулаку городнього, перитонеальні макрофаги, активні форми оксигену, фагоцитоз, метаболізм аргініну.

ЖИРНОКИСЛОТНЫЙ СОСТАВ ВОДНОГО ЭКСТРАКТА СЕМЯН ПОРТУЛАКА ОГОРОДНОГО И ЕГО ВЛИЯНИЕ НА МЕТАБОЛИЧЕСКИЙ ПРОФИЛЬ ПЕРИТОНЕАЛЬНЫХ МАКРОФАГОВ МЫШЕЙ

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Целью работы было изучить жирнокислотный состав экстракта семян портулака огородного и его влияние на метаболический профиль перитонеальных макрофагов мышей. Жирнокислотный состав оценивали методом газовой хроматографии/масс-спектрометрии. Макрофаги получали из перитонеальной полости мышей без предварительной сенситизации. Продукцию активных форм кислорода измеряли с использованием проточной цитометрии и теста с нитросиним тетразолием. Фагоцитарную активность оценивали методом проточной цитометрии. Продукцию оксида азота анализировали в супернатантах с помощью реакции Гриса. Активность аргиназы измеряли в клеточных лизатах стандартным колориметрическим методом. У макрофагов, которые культивировались в среде с добавлением водного экстракта семян портулака огородного и липополисахарида, продукция активных форм кислорода и азота была достоверно ниже по сравнению с макрофагами, которые культивировались в присутствии только липополисахарида. Также исследуемый экстракт вызывал статистически значимое повышение активности аргиназы несенсибилизированных перитонеальных макрофагов мышей. Это согласуется с жирнокислотным составом экстракта, поскольку он содержит большую долю ненасыщенных жирных кислот, обладающих противовоспалительными свойствами, по сравнению с насыщенными жирными кислотами, известными своей провоспалительной активностью.

Ключевые слова: водный экстракт семян портулака огородного, перитонеальные макрофаги, активные формы кислорода, фагоцитоз, метаболизм аргинина.