Use of complementary and alternative medicine, particularly herbal remedies is known as a beneficial approach for curing the different ailments. The growing number of studies report the anti-inflammatory, antioxidant, anticancer, antidiabetic, immunomodulatory and other effects of medicinal plants, which make them as a natural, innocuous and reliable remedy for treatment of wide spectrum of human diseases, as well as attractive objects for the biotechnology [1–3].

*Portulaca oleracea* is a medicinal plant with a broad range of therapeutic activities, and one of the most commonly used medicinal plants according to the World Health Organization [4]. The most common biologic effect of *P. oleracea* and its isolated constituents is the prevention and resolution of inflammation [5, 6]. In addition, *P. oleracea* extracts have an ability to restore suppressed immune reactivity in animal models [7]. Traditional anti-inflammatory effects of this plant can be implemented through the immunosuppressive or immune-stimulant effects and there are lack of obvious and clear characteristics of its impact on different effector cells of immune system, including phagocytes which are key players of majority immune responses [8, 9].

In most cases, *P. oleracea* based phytoremedies are used in the form of herbal remedies which are prepared from only this plant. Meanwhile, herbal mixtures containing a combination of different plant species have been reported to have better biological activities than monocomponent formulations because of synergistic effects of phytochemicals from...
different medicinal plants [10, 11]. The use of such multicomponent formulations offer advantages due to the effect on different targets or improving bioavailability of crude herb extracts. There are reports showing the presence of especially prominent therapeutic effects of herb combinations that were unachievable when each plant extract was administered alone, making the study of such mixtures containing multiple plant products especially promising [12].

In this study, we aimed to explore phytochemical characteristics of water extract from polyherbal composition (WEPC) with *P. oleracea* and it’s effect on oxidative metabolism of murine peritoneal macrophages (PMs).

**Materials and Methods**

**Polyherbal composition.** A total of 8 plant species were used: *Helichrysum arenarium, Mentha piperita, Calendula officinalis, Taraxacum officinale, Polygonum aviculare, Matricaria recutita, Portulaca oleracea,* and *Hypericum perforatum* (Table 1). The material collected was identified by the standards of classical taxonomy. All plant species were collected from different regions of Azerbaijan. The flowers of species of *Asteraceae* family were collected in the region of Yardimli in June–July. The leaves of *Mentha piperita* were collected in the region of Tovuz from June until late September. The flowers of *Calendula officinalis* were collected in the region of Quba. The well-formed roots of *Taraxacum officinale* were taken up in the autumn in the region of Quba-Khachmaz. The flowers, leaves and stems of *Polygonum aviculare* were collected in Lankaran region from July until late September. The flowers of *Matricaria recutita* were used and collected in the region of Kura-Aras. Leaves and seeds of *P. oleracea* were collected in the region of Tovuz-Qazakh in July and August. The flowers and stems of *Hypericum perforatum* were collected in Lerik region in July–August.

All parts of the plants were dry out in the shade with good air ventilation, away from direct sunlight until use. To prepare herbal tea, all herbs were added in equal weight parts.

**Preparation of the aqueous extract of medicinal plants and its lyophilization.** WEPC was prepared according to [13]. 10 g of polyherbal composition were homogenized with a jar mill to a powdered state. The powder was poured with boiled distilled water in a volume of 200 ml and were infused for 30 min, after which it was filtered through a filter paper and lyophilized.

**Phytochemical screening.** The freshly prepared WEPC was qualitatively tested for the presence of chemical constituents. The qualitative phytochemical analysis was performed using corresponding specific color reactions with following reagents and chemicals: Alkaloids with Dragendorff’s and Mayer’s reagent, flavonoids with the use of Mg and HCI; tannins with ferric chloride and potassium dichromate solutions, coumarins with 10% aqueous NaOH solution [13], and saponins with ability to produce suds. For the analysis of the presence of triterpenes, a modified Salkowski test [14] was used. To determine the presence of polysteroids, a modified Liebermann-Burchard test [15] was

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Family</th>
<th>Morphological part of plant used in WEPC</th>
<th>% in whole mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Helichrysum arenarium</em></td>
<td><em>Asteraceae</em></td>
<td>Flowers</td>
<td>12.5%</td>
</tr>
<tr>
<td><em>Portulaca oleracea</em></td>
<td><em>Portulacaceae</em></td>
<td>Leaves and seeds</td>
<td>12.5%</td>
</tr>
<tr>
<td><em>Mentha piperita</em></td>
<td><em>Lamiaceae</em></td>
<td>Leaves</td>
<td>12.5%</td>
</tr>
<tr>
<td><em>Calendula officinalis</em></td>
<td><em>Asteraceae</em></td>
<td>Flowers</td>
<td>12.5%</td>
</tr>
<tr>
<td><em>Taraxacum officinale</em></td>
<td><em>Asteraceae</em></td>
<td>Roots</td>
<td>12.5%</td>
</tr>
<tr>
<td><em>Polygonum aviculare</em></td>
<td><em>Polygonaceae</em></td>
<td>Flowers, leaves and stems</td>
<td>12.5%</td>
</tr>
<tr>
<td><em>Matricaria recutita</em></td>
<td><em>Asteraceae</em></td>
<td>Flowers</td>
<td>12.5%</td>
</tr>
<tr>
<td><em>Hypericum perforatum</em></td>
<td><em>Hypericaceae</em></td>
<td>Flowers and stems</td>
<td>12.5%</td>
</tr>
</tbody>
</table>
Quantitative analysis of phenols in WEPC. The total amount of phenolic compounds in WEPC was determined by Siddharaju P. et. al. method [18] with minor modifications. 1 ml of 10% aqueous Folin Ciocalteu solution was added to 20 μl of the EMHT extract or standard of 10% aqueous Folin Ciocalteu solution was applied. The presence of phenolic groups was examined using modified Folin Ciocalteu test [16]. Water-soluble and water-insoluble phenolic compounds were examined as described by Cock I.E., Kukkonen L. [17]. Cardiac glycosides were detected by the modified Keller-Kiliani method [16]. Modified Kumar and Ajaiyeoba tests were used to determine the presence of anthraquinones [14, 15]. Positive reactions indicate the presence of various biologically active substances in the investigated extract.

Peritoneal macrophage isolation. PMs were isolated without preliminary stimulation as described earlier [19]. Intact C56Bl/6 mice (male, 8 weeks old, bred at animal facility of ESC “Institute of Biology and Medicine”) were sacrificed and PMs were harvested using phosphate buffered saline containing 100 U/mL of heparin. Cells were then centrifuged at 300 g for 5 min at 4 °C, washed thrice with serum-free DMEM, and resuspended in DMEM containing 10% FCS and 40 μg/mL gentamycin.

Viability assay. Cytotoxic effect of WEPC on murine peritoneal macrophages (PMs) was determined using the MTT test [20]. PMs were seeded in 96-well plates (4×10^4 at the volume of 200 μl) and treated with 1, 10, 100, and 1000 μg/mL WEPC for 24 hours at 37 °C in the presence of 5% CO₂. Ten microliters of MTT solution (5 mg/ml) (Sigma, USA) was added to each well and followed by 2 h incubation. Media was then aspirated and 100 μl of dimethyl sulfoxide (Sigma, USA) was added. The optical density was determined at 560 nm using a Multiscan RC.

Reactive oxygen generation assay. O-2 generation was assayed by the nitroblue tetrazolium (NBT) reduction method as described earlier [19]. In a 5% CO₂ atmosphere PMs (2×10^5/well) were incubated for 1 h at 37 °C in Hanks buffered saline solution containing 1 mg of NBT per ml, with or without zymosan as a stimulator of oxidative burst. The optical density at 540nm in each well was examined with a plate reader. The modulation coefficient (MC) was evaluated after the treatment of PMs with zymozan and was calculated by the formula:

$$MC = \frac{(S - B)}{B} \times 100,$$

where S — index value after treatment with zymozan; B — index value of untreated cells (baseline value).

Statistical analysis. Each sample was assayed for generation in triplicate, and results are presented as mean ± SE. The statistical significance of the experimental results was determined by Student’s t test. For all analyses, P < 0.05 was accepted as a significant probability level.

Results and Discussion

Investigated polyherbal composition includes medicinal plants with proven immunomodulatory properties. In particular, Calendula officinalis was found to inhibit bacterial LPS-induced pro-inflammatory phagocyte activity [21]. Hypericum perforatum is a medicinal plant with anti-inflammatory and immunostimulating properties. Aghili et al. (2014) demonstrated that H. perforatum affects leukocyte, monocyte and neutrophil migration [22]. Taraxacum officinale (dandelion herb) is a well-known medicinal plant with numerous therapeutic properties including immunomodulation. Lee et al. (2012) reported that dandelion extract potentiates effect of interferon-gamma and increase nitric oxide production by peritoneal macrophages [23]. Chamomille (Matricaria recutita) activates immunoregulatory cells and
increases sensitivity of effector cells to helper signals [24]. The Polygonum genus includes species containing diverse pharmacologically active constituents with various properties [25]. Immunomodulatory potential of P. aviculare, as well as of other plants from this genus, hasn’t been extensively studied. However, George et al. (2014) showed that aqueous extract of P. minus caused significant increase in phagocytic activity of blood-derived phagocytes in vivo [26]. Peppermint is popular herb in traditional medicine that also has immunomodulatory effects [27, 28]. Cosentino et al. (2009) demonstrated the ability of peppermint oils to increase stimulated oxidative burst of peripheral blood monocytes and to decrease IL-4 production [29].

The abovementioned plants produce huge amount of different secondary metabolites playing protective role against infection or other harmful stimuli. A large number of these plant-derived secondary metabolites, also known as phytochemicals, possess immunomodulatory activity [1]. Among others, phenolic compounds are one of the most abundant phytochemicals. They can regulate immune system by targeting various receptors expressed on the surface of lymphoid cells and activating signaling pathways to initiate immune responses [30].

WEPC phytochemical characteristics. The results of qualitative WEPC phytochemical screening are given in Table 2.

Qualitative phytochemical analysis demonstrated the presence of water-soluble and insoluble phenols, tannins, saponins, flavonoids, cardiac glycosides and coumarins. Quantitative phytochemical analysis showed that studied WEPC contained 165 μg of phenolic compounds expressed in terms of gallic acid per 1 g of dry matter. Considering exceptional macrophage functional plasticity [31], these cells represent perfect targets for immunoregulatory impact of mentioned phytochemicals. Tannins and saponins are known to increase phagocytic activity [32, 33]. Saponins have the ability to stimulate cell mediated immune system as well as to enhance antibody production [34]. Flavonoids and other phenolic compounds are able to stimulate cellular and humoral immune response, lymphocyte proliferation, interferon secretion and phagocytic activity [2, 35–38].

Ye et al. (2004) showed enhancement of mitogen induced proliferation of murine splenocytes cultured in vitro in presence of three cardiac glycosides [39]. Coumarins obtained from vegetables and spices increased activation and IFN-γ secretion of lymphocytes [40].

Major constituents of P. oleracea are flavonoids, alkaloids, coumarins, monoterpene glycoside and fatty acids including Omega-3 [4]. Combination of P. oleracea with other medicinal plants allows to supplement this herbal remedy with other potent phytochemicals which can promote its immunomodulatory effect.

<table>
<thead>
<tr>
<th>Qualitative test</th>
<th>Positive reaction</th>
<th>Studied WEPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test for tannins</td>
<td>Dark blue color</td>
<td>+</td>
</tr>
<tr>
<td>Test for saponins</td>
<td>Persistent foam</td>
<td>+</td>
</tr>
<tr>
<td>Test for flavonoids</td>
<td>Yellow color with precipitate</td>
<td>+</td>
</tr>
<tr>
<td>Test for polysteroids</td>
<td>Reddish-brown color</td>
<td>−</td>
</tr>
<tr>
<td>Test for triterpenes</td>
<td>Red-violet color</td>
<td>−</td>
</tr>
<tr>
<td>Test for phenols</td>
<td>Blue-gray color</td>
<td>+</td>
</tr>
<tr>
<td>Test for water-soluble phenolic compounds</td>
<td>Red color</td>
<td>+</td>
</tr>
<tr>
<td>Test for water-insoluble phenolic compounds</td>
<td>Change in color of the mixture</td>
<td>+</td>
</tr>
<tr>
<td>Test for cardiac glycosides</td>
<td>Pink color</td>
<td>+</td>
</tr>
<tr>
<td>Test for anthraquinones</td>
<td>Bright pink color</td>
<td>−</td>
</tr>
<tr>
<td>Test for coumarins</td>
<td>Yellow color</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: “−” — absence; “+” presence of the phytochemical.

Table 2. Phytochemical characteristics of WEPC
The effect of WEPC on macrophage viability. Despite the great therapeutic potential of phytochemicals, they can exert adverse toxic effects resulting in the development of different pathologic conditions such as liver failure, contact dermatitis, hemolytic anemia, cancer and so on [41, 42]. Therefore, it was necessary to investigate potential cytotoxic activity of studied WEPC. MTT assay was used to assess toxic effect of WEPC on peritoneal macrophages in vitro. Cells were treated with increasing concentrations of studied aqueous phytoextract. As it can be seen from Fig. 1, there weren’t any significant changes in the number of viable cells in samples of murine peritoneal macrophages that had been exposed to WEPC over a range of concentrations from 1 μg/ml to 1000 mg/ml. These results indicate safety of the studied plant mixture.

The effect of WEPC on macrophage oxidative metabolism. Reactive oxygen species (ROS) production is an important mechanism used by phagocytic cells to defend host from pathogens. It is known that in addition to toxic effect on bacterial cells ROS also activate other antimicrobial defenses due to their role as secondary messengers of cellular signaling. ROS affect migration of phagocytes, regulation of their differentiation and phagocytic activity [43, 44]. In addition, ROS are involved in tissue regeneration and wound healing [45]. We used NBT test to evaluate the influence of WEPC on the ability of murine PMs to produce ROS. WEPC at all concentrations caused statistically significant dose-dependent increase in spontaneous ROS production by murine peritoneal macrophages (Fig. 2, A). Particularly, WEPC at the concentration of 100 μg/ml increased baseline oxidative burst by 2 times compared to control.

Treatment PMs with zymosan allowed us to evaluate the effect of WEPC on reactivity reserve (the remaining capacity of a cell to fulfill given metabolic activity under stress) of phagocyte oxidative metabolism, that was characterized by MC (see Materials and Methods). Treatment with WEPC PMs which were previously treated with zymosan resulted in the decrease of reactivity reserve of this phagocyte function (Fig. 2, B). However, WEPC at the concentration of 50 μg/ml didn’t affect reactivity reserve of macrophage oxidative metabolism. Data concerning ROS-
stimulating properties of phytochemicals are controversial and scarce. Cosentino et al. (2009) demonstrated increase in stimulated oxidative burst of polymorphonuclear cells stimulated with essential oils of *Mentha piperita* [29]. Conversely, Lee et al. described ROS-scavenging (antioxidant) properties of phytorextracts [46]. The effects of different herbal extracts on ROS equilibrium may depend on their composition, determined by presence of different species of plants, their organs used in preparation of the extract, as well as the nature of solvent (water, methanol etc.).

In conclusion, studied WEPC didn’t exhibit any leukotoxic effects and dose-dependently stimulated oxidative burst of murine peritoneal macrophages. Further research is needed to elucidate mechanisms of action of multiple compounds that have been detected in studied WEPC, as well as to explore immunomodulatory activity of this extract in vivo.

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ФІТОХІМІЧНИЙ СКРИІНІГ ЗБОРУ ЛІКАРСЬКИХ РОСЛИН НА ОСНОВІ Portulaca oleracea ТА ЙОГО ВПЛИВ НА ОКСИДАТИВНИЙ МЕТАБОЛІЗМ МАКРОФАГІВ

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Метою роботи було дослідити фітохімічні характеристики водного екстракту збору лікарських рослин, до складу якого входить Portulaca oleracea та його вплив на оксидативний метаболізм перитонеальних макрофагів мишей. Якісний фітохімічний аналіз проводили колориметричним методом, кількісний аналіз фенолів здійснювали в тесті з використанням галової кислоти як стандарта. Перитонеальні макрофаги мишей виділяли без попередньої сенситизації. Лейкотоксичний ефект водного екстракту збору лікарських рослин оцінювали в МТТ-тесті. Генерацію реактивних форм кисню досліджували в тесті з нітросинім тетразолієм. Фітохімічний аналіз виявив у складі водного екстракту збору лікарських рослин присутність водорастворимих та водонерастворимих фенолов, танів, сапонінів, флавоноїдів, серцевих глікозидів і кумаринів. Водний екстракт досліджуваного збору лікарських трав у діапазоні концентрацій 1–1000 мкг/мл (ліофілізата в дистилірованій воді) не чинив токсичного ефекту на перитонеальні макрофаги, спричинював статистично достовірне дозозалежне посилення їх оксидативного метаболізму. Отже, результати цієї роботи дозволяють зробити висновок про модуляторне дію досліджуваного екстракту збору лікарських трав на оксидативний метаболізм перитонеальних макрофагів, що має важливе значення для створення нових методів відновлення оксидативного стресу.

Ключові слова: водний екстракт збору лікарських рослин, Portulaca oleracea, перитонеальні макрофаги, реактивні форми кисню.

ФИТОХИМИЧЕСКИЙ СКРИНИНГ СБОРА ЛЕКАРСТВЕННЫХ РАСТЕНИЙ НА ОСНОВЕ Portulaca oleracea И ЕГО ВЛИЯНИЕ НА ОКСИДАТИВНЫЙ МЕТАБОЛИЗМ МАКРОФАГОВ

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Целью работы было исследовать фитохимические характеристики водного экстракта сбора лекарственных растений, в состав которого входит Portulaca oleracea и его влияние на оксидативный метаболизм перитонеальных макрофагов мышей. Качественный фитохимический анализ проводили колориметрическим методом, количественный анализ фенолов выполняли в тесте с использованием галовой кислоты в качестве стандарта. Перитонеальные макрофаги мышей выделяли без предварительной сенситизации. Лейкотоксический эффект водного экстракта сбора лекарственных растений оценивали в МТТ-тесте. Генерацію реактивных форм кислорода исследовали в тесте с нитросиним тетразолием. Фитохимический анализ выявил в составе водного экстракта сбора лекарственных растений присутствие водорослевых и воденосщих фенолов, таннов, сапонинов, флавоноидов, сердечных гликозидов и кумаринов. Водный экстракт исследуемого сбора лекарственных трав в диапазоне концентраций 1–1000 мкг/мл (лиофилизата в дистилированной воде) не оказывал токсического эффекта на перитонеальные макрофаги мышей, вызывал статистически достоверное дозозависимое усиление их оксидативного метаболизма. Отсутствие токсического действия и способность усиливать оксидативный метаболизм перитонеальных макрофагов позволяют предположить модуляторные свойства исследуемого водного экстракта сбора лекарственных растений относительно эффекторов врожденного иммунитета.

Ключевые слова: водный экстракт сбора лекарственных растений, Portulaca oleracea, перitoneальные макрофаги, реактивные формы кислорода.