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Acidic polysaccharide complexes from purslane, silver linden and lavender stimulate Peyer's patch immune cells through innate and adaptive mechanisms

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ABSTRACT

Three polysaccharide complexes (PSCs) were isolated from the aerial parts of common purslane (*Portulaca oleracea* L.), and the flowers of common lavender (*Lavandula angustifolia* Mill.) and silver linden (*Tilia tomentosa* Moench) by boiling water extraction and ethanol precipitation. The chemical composition and immunomodulating effects of isolated PSCs were characterized. The chemical characterization revealed that the three samples contain mainly pectic polysaccharides. They exhibited *ex vivo* intestinal immunomodulating activity through the murine Peyer's patch-mediated bone marrow cell proliferation test at 100 μg/ml concentration. At the same time, they stimulated *ex vivo* human blood T-cell populations ($CD4^+/CD25^+$ and $CD8^+/CD25^+$), phagocytic leukocytes ($CD14^+$ and $CD64^+$ cells) and induced IL-6 production from human white blood cells and Peyer's patch cells. The herbal PSCs stimulated *ex vivo* ROS production from whole blood phagocytes and showed unspecific *in vitro* anti-proliferative activity against normal and A549, HeLa and LS180 tumor cells. This is the first report on immunomodulating studies of linden flower pectins and chemical and biological activity characterization of lavender polysaccharides. Our study demonstrates that similarly to purslane, lavender and silver linden herbal materials contain immunomodulating polysaccharides that could be useful for support of compromised immune system.
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1. Introduction

Herbal medicine is a symbol of human knowledge, because it has been accompanying every nation through its development and people will always prepare herbal infusions, decoctions and extracts [1]. The thorough research in the last 30 years in the Chinese and especially the Japanese Kampo medicine, traditional European, African and Indian Ayurvedic medicines has shown that herbal pectic polysaccharides contribute to the biological activity of widely used herbs [2–5]. Already in 1988, polysaccharide hydrocolloids were included in the German Rote Liste® as antitussive, antipyretic, wound healing, nutraceutical, laxative, immune stimulating and gastric agents [6]. Furthermore, examples of commercial products containing immunomodulating polysaccharides are modified citrus pectin, Bazoton®, *Viscum album* extract, *Astragalus* polysac-

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charide extract and Kampo medicine formulations Juzen-taiho-to and Hochuekkito [7–10].

Generally, pectic polysaccharides contain three main building blocks – unbranched homogalacturonan (HG), ramified rhamnogalacturonan (RG) I and II [11]. The unbranched HG is composed of an α-(1 → 4)-linked poly-D-Gal*p*A chain, which COOH groups are methyl-esterified to different extent and some OH groups are acetylated at C-2 and/or C-3. The ramified RGI has a backbone structure of the linear repeating fragment [→2)-α-L-*R*hap-(1 → 4)-α-D-Gal*p*A-(1 →]$_n$. The main fragment carries side chains of α-(1 → 5)-L-arabinans, β-(1 → 4)-D-galactans, arabinogalactan (AG) type I and II at the OH group of C-4 of the L-Rha residues. The AGI is composed of a linear β-D-(1 → 4)-linked galactan, which is branched at O-3 mainly with α-L-(1 → 5)-linked Ara*f* residues. The AG II is composed of a β-D-(1 → 3)-linked galactan backbone with side chains at O-6 of β-(1 → 6)-linked galactans. 1,3- and 1,6-galactans are substituted mainly with α-L-(1 → 3)-linked Ara*f* residues. The glycosidic coupling of L-Ara in the AG structures is highly diverse (1,2; 1,3; 1,5) [2,4,11].

The most important immune active pectic side chains are AGs [2,4]. The immunomodulating effects of herbal pectins in the gastrointestinal tract expand a lot the classical concept for dietary fibers and human health. The pectic polysaccharides from herbal infusions, traditional foods and drinks pass easily through the gastrointestinal tract, where they can interact with mucosal immune system. Stimulation of the mucosal immune system through immunocompetent cells in Peyer's patches by polysaccharides has shown perspective application for supplementary treatment of infectious diseases [10,12–14]. The Peyer's patches contain B-, T-cells and phagocytic leukocytes, such as macrophages, dendritic cells and neutrophils, which can be affected by the pectic polysaccharides [2,3,15]. The pectins activate oxidative burst and phagocytosis of neutrophils and macrophages, thus influencing the innate immune response [16]. Furthermore, the activated macrophages and dendritic cells could stimulate adaptive immune cells [17,18]. Direct or indirect immunomodulating effect of polysaccharides on T-cell subpopulations can be used for prevention and treatment of tumor and autoimmune diseases [19,20].

Silver linden (*Tilia tomentosa*) and common lavender (*Lavandula angustifolia*), together with the edible weed common purslane (*Portulaca oleracea*) are traditionally used in the herbal medicine [21]. Bulgaria is a major exporter of wild and cultivated aromatic medicinal plants and their derived products, which are raw materials for the pharmaceutical, cosmetic and nutraceutical industries. It is the biggest lavender oil producer in the world since 2011 and leading country in the export of Tiliae flos. Interestingly, *T. tomentosa* forms coenoses only in the Balkans and although it is widely used in the gemmotherapy, it is still poorly studied [22]. On the other hand, the medicinal use of lavender is focused on its essential oil. The third herb, purslane is a very popular leaf vegetable in the Indian and Mediterranean cuisine, which is named the "global panacea" by WHO [23]. The immunomodulating activity of the three herbs is related mainly to the lavender essential oil, linden tiliroside, scopoletin and lignans, and only for purslane to polysaccharides [24,25,23]. The purslane contains acidic pectic polysaccharides and neutral AGs, arabinoglucomannans and starch [26–28]. It is shown that purslane polysaccharides have potential to be used in the treatment of tumor diseases because they exhibit immune-enhancing activities, but studies on their intestinal immunomodulating activity are missing [19,29,30]. Kram & Franz [31] reported that small-leaved linden *Tilia cordata* flower mucilage is rich in pectin, as later Schmidgall et al. [32] showed that its raw polysaccharide complex expresses moderate bioadhesion to epithelial tissue *ex vivo*. To our knowledge investigations on the chemical composition and biological activity of silver linden and lavender pectic polysaccharides are absent.

The aim of the current study was to isolate and characterize the chemical composition and immunomodulating effects of polysaccharide complexes from *L. angustifolia* and *T. tomentosa*, and to compare their effects with the known bioactive polysaccharide complex from *P. oleracea*.

2. Materials & methods

2.1. Plant material

Common purslane aerial parts were collected from natural habitats in September 2013 in Plovdiv region, washed, cut into small pieces, freeze-dried and stored in a desiccator until use. The plant material was identified as *Portulaca oleracea* L. (Portulacaceae) by Assoc. Prof. Dr. Ivanka Dimitrova-Dyulgerova (Plovdiv University Paisii Hilendarski). Lavender flowers *Lavandula angustifolia* Miller (syn. *L. vera*) sort "Sevtopolis" (Institute of Roses, Essential and Medical Cultures, Kazanlak, Bulgaria) were provided dried by ET "Ve Pe Pi – Vesko Pipev" (Velingrad, Bulgaria). Linden flowers were collected in June 2013 in Plovdiv, air-dried for two weeks and stored in a desiccator until use. The plant material was identified as silver linden, *Tilia tomentosa* Moench (Tiliaceae), by Dr. Evgeny Tsavkov (University of Forestry, Sofia). A voucher specimen with given number SOA061426 was deposited in the Herbarium of the Agricultural University of Plovdiv.

2.2. Isolation of the herbal PSCs

Preparation of the herbal PSCs was performed partly as the Japanese Kampo medicine decoctions are prepared [33]. Thirty grams of each milled herbal material were mixed with 600 ml water and boiled until the mixture volume was reduced to half. The extracts were filtered through a nylon cloth and the plant residue was rinsed with 150 ml water and re-boiled with 600 ml water again, following the same procedure. The collected extracts and washes were centrifuged (4000 rpm, 20 min), filtered through a Büchner funnel and concentrated four times in volume under vacuum at 50 °C. The plant extracts were centrifuged again and coagulated with 4 vol. 95% ethanol at 4 °C over night. The obtained precipitates were collected by a Büchner funnel separation for linden and purslane PSCs (linden PSC and purslane PSC) or centrifugation (4000 rpm, 30 min, 4 °C) for lavender PSC. Then, the three crude polysaccharides were re-dissolved in water, dialyzed for 72 h against water at 4 °C (VISKING®, SERVA Electrophoresis, MWCO 12–14 kDa), centrifuged, freeze-dried and stored in a desiccator in dark until use.

2.3. General methods

The polyuronic acid content (PUC) in the plant materials was determined titrimetrically, according to Owens et al. [34]. The anhydrouronic acid content (AUAC) and simultaneous determination of GalA and GlcA (in the absence of 4-*O*-methyl-GlcA) in the herbal PSCs were measured colorimetrically by the *m*-hydroxydiphenyl (GalA as a standard) and 3,5-dimethylphenol (GalA and D-glucuronolactone as standards) methods [35,36]. Degree of methoxylation (DM) was analyzed with alcohol oxidase (*P. pastoris*, Sigma-Aldrich) and Purpald® (Sigma-Aldrich) [37]. Degree of acetylation (DA) was assayed by the method of McComb & McCready [38], using β-D-glucose pentaacetate as a standard. Molecular weight was determined by HPSEC-RID (Waters) with Shodex standard P-82 kit (pullulan standards, Showa DENKO, Japan) [39]. Protein content (BSA standard), total phenolics (gallic acid standard) and presence of total saponins were analyzed according to Bradford [40], Singleton & Rossi [41] and Pasaribu et al.

[42], respectively. Only distilled water was used for all experiments. All analyses were performed in duplicates (n = 6).

2.4. Monosaccharide composition

Polysaccharide samples (10 mg) with added *myo*-inositol as an internal standard (1 mg) were pre-hydrolyzed with 72% (w/w) H_2SO_4 for 1 h at 30 °C. Then, the samples were diluted with water to 1 M H_2SO_4 and further hydrolyzed for 3 h at 100 °C. The released monosaccharides were converted into their alditol acetates, according to Selvendran et al. [43]. The formed alditol acetates were analyzed by GC/MS on a HP 6890 Series Plus gas chromatograph coupled with a HP 5973 mass selective detector (Hewlett–Packard, Palo Alto, USA), on a Supelco SP-2380 column (30 m × 0.25 mm ID × 0.25 μm). The monosaccharide composition (including GalA and GlcA) of the three samples (1 mg) containing 100 μg *myo*-inositol was also determined by HPAEC-PAD after methanolysis with anhydrous 2 M HCl in methanol for 16 h at 80 °C, followed by hydrolysis with 2 M TFA for 1 h at 121 °C [44]. All samples were analyzed in duplicates.

The presence of arabinogalactan type II (AGII) was detected by precipitation with the *ß*-glucosyl Yariv reagent [45].

2.5. Fourier transform infrared spectroscopy (FTIR)

The lavender and linden PSCs were dissolved in water (10 mg/ml) and coagulated several times with 95% ethanol with final alcohol concentration of 70%. After each run the polysaccharides were separated by centrifugation at 4000 rpm for 30 min at 20 °C, dissolved in water and freeze-dried. FTIR spectra of lavender and linden PSCs (2 mg) after an exhaustive removing of alcohol-soluble molecules were obtained on a Nicolet™ Avatar (Thermo Fisher Scientific, Waltham, USA) spectrometer in a KBr tablet. The spectra were recorded in the range 4000 and 400 cm^{-1} and analyzed by the SpectraGryph software (Friedrich Menges).

2.6. Immunomodulatory effect

2.6.1. Ethics

Blood sampling procedure was in accordance with the Helsinki Declaration of 1975, as revised in 1983. All participants signed a written informed consent prior to initiation of the study. The animal experiments were approved by the Animal Research Committee of the Kitasato University, and performed in accordance with the Guidelines for Care and Use of Laboratory Animals at the Kitasato University and the National Research Council Guide for the Care and Use of Laboratory Animals in Japan.

2.6.2. Animal procedure and care

Female C3H/HeJ mice (6–8 weeks old) were purchased from Japan SLC (Shizuoka, Japan), and male ICR mice (4 weeks old) were from Charles River (Tokyo, Japan). The mice were housed in plastic cages in an air-conditioned room at 23 ± 2 °C with a relative humidity of 55 ± 10% under a 12 h-light-dark cycle, and fed a standard laboratory diet with water given *ad libitum*.

2.6.3. Flow cytometric analyses

Peripheral venous blood was collected from healthy volunteers (n = 3; gender = 2 men and 1 women, age range 25–39 years) using Venoject® tubes (Terumo Corporation, Japan). The blood samples were centrifuged at 1500*g* for 15 min. Then, the serum was removed and the red blood cells were lysed using 0.84% NH_4Cl buffer. The samples were washed twice with phosphate-buffered saline (PBS) buffer. The isolated white blood cells were pooled, centrifuged at 1000 rpm for 10 min and the cell pellet was re-suspended in a complete DMEM culture medium (Gibco, Invitrogen, USA) supplemented with 10% fetal calf serum and antibiotics. The cell suspension was seeded in four 25 cm^2 culture flasks (TPP, Switzerland), as one flask served as a control (cells cultured in a standard growth medium), the cells in the other three culture vessels were treated with 100 μg/ml purslane, lavender and linden PSCs, respectively for 48 h in a humidified incubator at 37 °C and 5% CO_2 content. At the end of the incubation period, the control and the PSC-treated cells were harvested and centrifuged at 1000 rpm for 10 min. The cell pellets were re-suspended in FACS buffer (PBS containing 5% fetal calf serum and 0.05% NaN_3). Then, the cells were stained with fluorochrome labeled antibodies specific for CD3, CD4, CD8, CD14, CD20, CD64 markers. The cell samples were incubated with antibody mixes for 10 min at room temperature in dark. After that, the cells were washed twice with the FACS buffer and acquired on a FC500 flow cytometer (Beckman Coulter, USA). The levels of different leukocytes were compared between the control and PSC-treated cells.

2.6.4. Reactive oxygen species (ROS) production from human whole blood phagocytes (WBP)

Heparinized (50 IU/ml) blood samples were obtained from the cubital vein of healthy human volunteers after overnight fasting. The kinetics of ROS production by WBP was analyzed by luminol-enhanced chemiluminescence (CL) for a period of 60 min at 37 °C, using 96-well white flat bottom culture plates on luminometer (Orion II Berthold Detection Systems GmbH, Germany). The principle of the method was previously described [46]. Briefly: 25 μl of ten times diluted blood with Hank's buffered salt solution (HBSS) were mixed with 25 μl 1 mM luminol (10 mM stock solution in 0.2 M borate buffer) HBSS solution and incubated with HBSS solutions of the three PSCs (10 and 100 μg/ml) for spontaneous (without activators) CL. Furthermore, 12.5 μl or 5 μl of ten times diluted blood mixed with 25 μl luminol were used for incubation of the samples with 25 μl (0.5 μg/ml) phorbol myristate acetate (PMA-activated, Sigma-Aldrich) or 25 μl (62.5 μg/ml) opsonized zymosan particles (OZP-activated). The final volume for spontaneous, PMA- and OZP-activated CL tests was 250 μl. The recorded values included the intensity of CL emitted during the studied time interval (integral of CL). The results are expressed as mean percentage of the control ± SEM from six independent experiments.

2.6.5. Ex vivo *immunomodulating activity against murine Peyer's patch (PP) cells*

Immunomodulating activity was measured by the enhanced production of bone marrow cell-proliferative cytokines from the PP cells of pathogen-free C3H/HeJ female mice ($Tlr4^{Lps-d}$), according to Hong et al. [47]. Suspensions (1.2×10^6 cells/ml, 180 μl) of PP cells (5–9 patches obtained per mouse) from the small intestine of the C3H/HeJ mice were cultured with H_2O (negative control), AMOL-1 (pectic polysaccharide from *Astragalus mongholics* as a positive control, Kiyohara et al. [48]) or 100 μg/ml of the three PSCs (20 μl) in a 96-well flat bottom culture plate (FALCON 3072) for 6 days at 37 °C in a humidified atmosphere of 5% CO_2–95% air. The resulting culture supernatants (50 μl) were further cultured with a bone marrow cell suspension (5.0×10^5 cells/ml) from male ICR mice for 6 days. The numbers of proliferated bone marrow cells were measured by Alamar Blue™, as described previously [47]. IL-6 production from the murine PP cells stimulated by the tested samples was measured in the cell culture supernatants by ELISA, according to the company manual (BD Pharmingen) and as described by Kiyohara et al. [48]. The results are expressed as mean ± SEM.

2.6.6. In vitro *tumor cell growth inhibition*

The growth inhibitory effect of the three PSCs was studied against FL cell line (ATCC CCL 62, NBIMCC 94) derived from human

amniotic cells, normal human fibroblasts (isolated from testicular tissue; cultures obtained following the 5th passage) and different human tumor cell lines: A549 (ATCC CCL 185), LS180 (ATCC CL-187) and HeLa (ATCC CCL-2). All cell lines were expanded in 75 cm^2 culture flasks (TPP, Switzerland) in a DMEM growth medium, supplemented with 10% fetal calf serum and antibiotics. The cells were trypsinized and adjusted to a concentration of 2×10^5 cells/ml and then seeded on 96-well plates (TPP, Switzerland) with a working volume of 200 μl/well and cultured for 24 h at 37 °C, 5% CO_2 and high humidity. After that, the growth medium was replaced with a fresh medium containing 2, 10, 50, 100 or 200 μg/ml polysaccharide samples and the cells were cultured for 24, 48 and 72 h. At the end of each test-period, MTT (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide) (Sigma–Aldrich) solution in a final concentration of 0.5 mg/ml was added to all wells. The cells were incubated for 4 h in a humidified incubator at 37 °C and 5% CO_2 content. Eventually, the MTT-containing medium was removed; 100 μl DMSO were pipetted to each test well and incubated for 15 min at room temperature on a shaker, in order to dissolve the formazan crystals accumulated in the cells. The absorbance was measured at 570 nm using a Synergy-2 spectrophotometer (BioTek, USA). The percent inhibition of cell growth was calculated using the mean absorbance units from each test sample and the mean data from the control cells incubated in the absence of polysaccharides. All samples were assayed in triplicates and the results are expressed as $IC_{50} \pm$ SEM (48 h).

2.7. Statistical analysis

Statistical significance was analyzed by the Student's *t*-test, using the Statview software (SAS Institute, Inc.). The mean results ± SEM were compared to those from the control. Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Isolation and chemical characterization

In Table 1 are presented results from the chemical characterization of the three crude PSCs. The polyuronide content in the initial herbal materials ranges between 14.1–22.2%, which indicates the presence of pectic type polysaccharides in all of them. The differences between the polyuronides in the initial materials and the residues after extraction reveal that about 77%, 57% and 71% of the detected polyuronides in the used parts of purslane, lavender and linden are recovered by the double extraction with boiling water. The corresponding PSCs were obtained with yields of 8.6%, 3.2% and 6.1%, respectively. The three PSCs contain >55% total sugars. The AUAC in the samples is in the range 37.0–45.9%. The most of the AUAC in the purslane and lavender PSCs is represented by GalA according to the 3,5-dimethylphenol method (Table 1). Interestingly for the linden PSC we found 33.6% GalA and 14.3% GlcA by the 3,5-dimethylphenol, which in total is in agreement with the AUAC result (45.9%). The three PSCs contain methoxyl and acetyl esters in different extents, which are normally located in the GalA units in the pectins and rarely found in the neutral sugars [49]. The linden PSC has the lowest methoxyl (2.5%) and the highest acetyl content (3.3%) in comparison with the other two samples. The lavender and purslane PSCs have also relatively high acetyl contents. The AUAC and methoxyl-ester content of the linden PSC are in a good agreement with the data obtained by Yakovlev [50], however he did not mention the presence of acetyl groups. According to the HPSEC-RID analysis, linden PSC is characterized by two main populations with molecular weights 1.23×10^6 and 4.72×10^4 g/mol. The other two samples are characterized by predominant populations with molecular weights in the range 1.5×10^4–5.3×10^4 g/mol.

Table 1
Chemical characterization of the three herbal PSCs. The results are presented as mean (w/w% DS).

	Purslane PSC	Lavender PSC	Linden PSC
Polyuronides[a], initial herb [%]	14.1	15.5	22.2
Polyuronides[a], residue after extraction [%]	3.3	6.6	6.5
Yield [%]	8.6	3.2	6.1
Total sugars[b] [%]	82.5	64.9	56.0
AUAC [%]	43.2	37.0	45.9
GalA[c]	40.1	35.8	33.6
DM[d] [mol%]	68.2	70.8	40.5
(Methoxyl content [%])	(5.0)	(4.6)	(2.5)
DA[d] [mol%]	16.1	25.7	40.4
(Acetyl content [%])	(1.6)	(2.3)	(3.3)
Molecular weight distribution [g/mol]	5.3×10^4 (49.0)	1.6×10^6 (2.5)	1.23×10^6 (67.5)
	1.7×10^4 (51.0)	1.5×10^4 (97.5)	4.72×10^4 (32.5)
Monosaccharide composition [%]			
Ara	9.5[e] (11.0)[f]	5.7 (5.4)	4.4 (4.8)
Rha	3.8 (1.1)	2.5 (0.8)	11.5 (1.2)
Fuc	trace	0.2 (0.1)	trace
Xyl	1.4 (1.0)	1.6 (1.0)	1.2 (0.8)
Man	0.7 (0.5)	2.3 (1.0)	(0.1)
Gal	11.3 (10.8)	6.3 (5.2)	3.3 (2.8)
Glc	13.2 (10.1)	5.1 (3.1)	1.0 (0.5)
GalA	39.1	35.8	24.0
GlcA	3.5	5.4	10.6
Protein content [%]	3.3	2.2[g]	2.2
Total phenolics [%]	0.8	11.0	2.7
Presence of saponins	+	+	+

[a] Determined titrimetrically according to Owens et al. [34].
[b] Sum of the monosacharides determined by the HPAEC-PAD analysis.
[c] Determined on the basis of the differentiation between GalA and GlcA with the 3,5-dimethylphenol method.
[d] Calculated on the GalA basis, moles of methoxyl or acetyl groups per 100 mol of GalA.
[e] Monosaccharide composition determined by HPAEC-PAD analysis.
[f] Neutral monosaccharide composition determined as alditol-acetates.
[g] Determined after an exhaustive treatment with 95% ethanol.

Because of the proposed complex polysaccharide composition of the samples, two different techniques for determination of the monosaccharides were used. When we compared the results from both monosaccharide composition analyses, we found that the neutral monosaccharides and especially Rha are detected in lower amounts after H_2SO_4 hydrolysis, as was already described [31,44] (Table 1). For the comments presented below and for estimation of the total amount of sugars are used the data from the HPAEC-PAD analysis. The monosaccharide composition confirmed that the three samples contain pectic polysaccharides with predominant GalA and the neutral monosaccharides Ara, Gal and Rha. They also have Glc, Xyl, Man and Fuc, as the latter is related to RGII structures [11]. The purslane PSC is characterized by the highest GalA content within the three PSCs, which indicates that the HG fragments in the purslane pectins are in considerable amounts. It has also the highest Glc content and relatively comparable amounts of Gal (11.3%) and Ara (9.5%). The sum GalA + GlcA in this sample is 42.6%, which is in agreement with the determined AUAC (43.2%). The ratio Gal/Ara in the complex is 1.2 and it is comparable to the ratio of 1.1 found by Amin & El-Deeb [26] in mucilage B from the leaves of *P. oleracea*. It was found that *P. oleracea* contains an AG polysaccharide with a low GlcA content that was elucidated to be AGII [27,28]. The high Glc content in the PSC could be attributed to the presence of different glucans, as previously found [27,28].

Similarly, the lavender PSC is characterized by high Gal (6.3%), Ara (5.7%) and Glc (5.1%) contents. To best of our knowledge, there are not studies on the polysaccharides from lavender described in the literature so far. The considerable amount of GalA and the low Rha content indicate that the HG segments are predominantly presented in the lavender polysaccharides. The ratio Gal/Ara in the lavender PSC is 1.1, suggesting the presence of AG structures similarly to the purslane PSC. Furthermore, the detected Fuc is indicative for presence of RGII structures. Interestingly, it has relatively high Man (2.3%) and Glc (5.1%) contents. Both purslane and lavender PSCs contain low amounts of GlcA, which is in agreement with other studies on herbal polysaccharides [13,48]. The results for GalA of both PSCs are in a good agreement with the colorimetric data (Table 1).

The linden PSC is characterized by high Rha and GlcA contents, as the latter finding is confirmed by the colorimetric quantification of GalA and GlcA. The high Rha content, in addition to the high GalA amount suggest the existence of a RGI fragment in a representable proportion in the linden PSC. The GlcA is present in a surprisingly high content, comparable to the amount of Rha. We calculated that GlcA is 30%-31% from uronides in the linden PSC on the basis of the colorimetric and chromatographic determination of both uronic acids. Similarly, Kram & Franz [31] and Yakovlev [50] found high Rha content in the crude linden polysaccharide, and the first group mentioned the simultaneous presence of GalA and GlcA, but they did not provide any quantitative data for both uronic acids. The linden PSC had the lowest Gal content, but we found by the Yariv's test that the sample contains the immune active AGII structures (data not shown).

The purslane and linden PSCs are also characterized by low protein and polyphenol contents. The lavender PSC has a high polyphenol content (11%), which interferes with the quantification of total proteins, as was observed by Kiyohara et al. [9]. After an exhaustive removing of the alcohol-soluble molecules from the lavender PSC, it was determined that it contains 2.2% proteins by the Bradford method. The presence of saponins was evaluated only qualitatively by the vanillin-sulfuric acid method because of a possible Rha interference. The qualitative and quantitative analyses of the non-carbohydrate constituents were necessary for a better explanation of the observed immunomodulatory effects.

3.2. FTIR spectroscopy

After an exhaustive removing of alcohol-soluble molecules from the lavender and linden PSCs, the recovered PSCs were analyzed by FTIR spectroscopy (Fig. 1). Both spectra contain the typical bands for polysaccharides and pectins: 3446, 2920 (2929), 1751, 1734, 1635 and 1420, 1146 (1151) cm^{-1}, arising from O-H of hydroxyl groups, C-H of CH_2, C=O of methoxyl ester group ($COOCH_3$), C=O of acetyl ester group ($OCOCH_3$), C=O of ionized (free) carboxyl group and C-O-C of the glycosidic bond (or within the ring), respectively [51]. For the linden PSC the signal at 1751 cm^{-1} is negligible and the peak at 1734 cm^{-1} is with equal intensity to the signal at 1716 cm^{-1}. The latter is proposed to arise only from ν(C=O) of the acetyl group [51]. The absorption band at 1255 or 1244 cm^{-1} is from ν(C-O-C) of the acetyl group [51]. The absence of a characteristic peak at 1751 cm^{-1}, the higher intensities of the two signals at 1255 and 1716 cm^{-1} in the spectrum of linden PSC than in the lavender PSC spectrum confirm its lower DM and higher DA. Furthermore, the signal at 1373 (1375) cm^{-1} originates from $\delta_s(CH_3)$ of ester groups. It is clearly seen that the intensity of the band at 1635 cm^{-1} for the linden PSC is higher than this in the spectrum of lavender PSC, which is related to the higher AUAC and free COOH derived from the GalA and GlcA in the first sample. The region of 1200–1000 cm^{-1} is the so called "fingerprinting region" for each pectin and it contains mainly the following skeletal ring vibrations: C-OH, C-C and C-O-C glycosidic bond vibrations [52]. We found five signals in this region for the lavender PSC – 1146, 1101 (νC-O ring), 1076, 1051 and 1016 (νC-OH, C-C) cm^{-1} and three signals for the linden PSC – 1151, 1070 and 1041 cm^{-1}. The bands at 1101 and 1019 cm^{-1} are the strongest for the lavender PSC, indicating the predominance of the HG structure in it. Conversely, for the linden PSC the signals at 1070 and 1041 cm^{-1} show that it contains a considerable amount of the RGI structure [53]. In both spectra are found bands in the anomeric region (C1-H, ring) at 822 or 833 cm^{-1} and at 908 (893) cm^{-1} for $\gamma(C\text{-}OH)_{ring}$ characteristic for α- and β-linked aldopyranoses [51]. In the region 1600–1500 cm^{-1} are expected signals derived from different functional groups, such as COOH, NH_2 and aromatic ring vibrations.

3.3. Immunomodulating effects

3.3.1. Effect on human white blood cells

The effect of the three PSCs on various human white blood cells was studied at a concentration of 100 μg/ml by a flow cytometric immunophenotyping analysis (Fig. 2). The purslane PSC stimulated $CD4^+$ cells, whereas the lavender PSC stimulated $CD8^+$ cells. The three samples did not express stimulating activity on $CD20^+$ cells, representing B-cell population. They activated to different extent $CD25^+$ cells, which in addition to the B-cells are also related to T-cells. The linden and lavender PSCs even showed inhibitory activity on $CD20^+$ B-cells, which could be partly associated with the non-carbohydrate constituents. The purslane and lavender PSCs exhibited activity on $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T-cells, and the stimulatory effect of the linden PSC was weak. In addition to the effect on the T-cell subpopulations the herbal polysaccharides can activate phagocytes and thus again exhibit immunostimulating activity. The three PSCs showed activity on $CD14^+$ cells or monocytes. The linden and lavender PSCs had activity on $CD64^+$ cells, representing mainly macrophages. Conversely, the effect of purslane PSC on the $CD14^+$ cells was not observed on the $CD64^+$ cells, which could be difficultly explained. We also found that all samples increased IL-6 production from white blood cells, which was in agreement with the stimulation of monocytes and T-cells (data not included).

3.3.2. Effect on ROS formation by human WBP

The three herbal PSCs stimulated to different extent the oxidative burst in WBP at 10 and 100 μg/ml, which was expressed by the increase in spontaneous CL response, as a measure of ROS generation (Fig. 3). The highest activity at 100 μg/ml was observed for the purslane PSC and then for the linden PSC. Both samples have some important differences in the monosaccharide composition in respect to Gal, Ara, Glc, GalA, Rha and GlcA contents and in their molecular weights. The purslane PSC is characterized with higher Gal, Ara and Glc, which are indicators for presence of immune active AGs and glucans. On the other hand, the lavender PSC expressed a higher stimulating activity at 10 μg/ml than at 100 μg/ml, which was expected because of the mimic antioxidant effect from polyphenols in it. As can be seen from Fig. 3 the control values for PMA- and OZP-activated CL were much higher than those for the spontaneous CL response of the polysaccharide-treated cells. Interestingly, the three PSCs did not increase CL response of the PMA-activated phagocytes. The lavender PSC decreased the PMA-activated CL at 100 μg/ml, expressing a suppressive or protective-like effect on the activated cells. This mode of action was related to polyphenols, as reviewed by Lojek et al. [16]. Similarly, the lavender PSC induced a decrease of the OZP-stimulated CL at 100 μg/ml. Contrary, purslane and linden PSCs increased the CL response in the OZP-treated cells at both studied concentra-

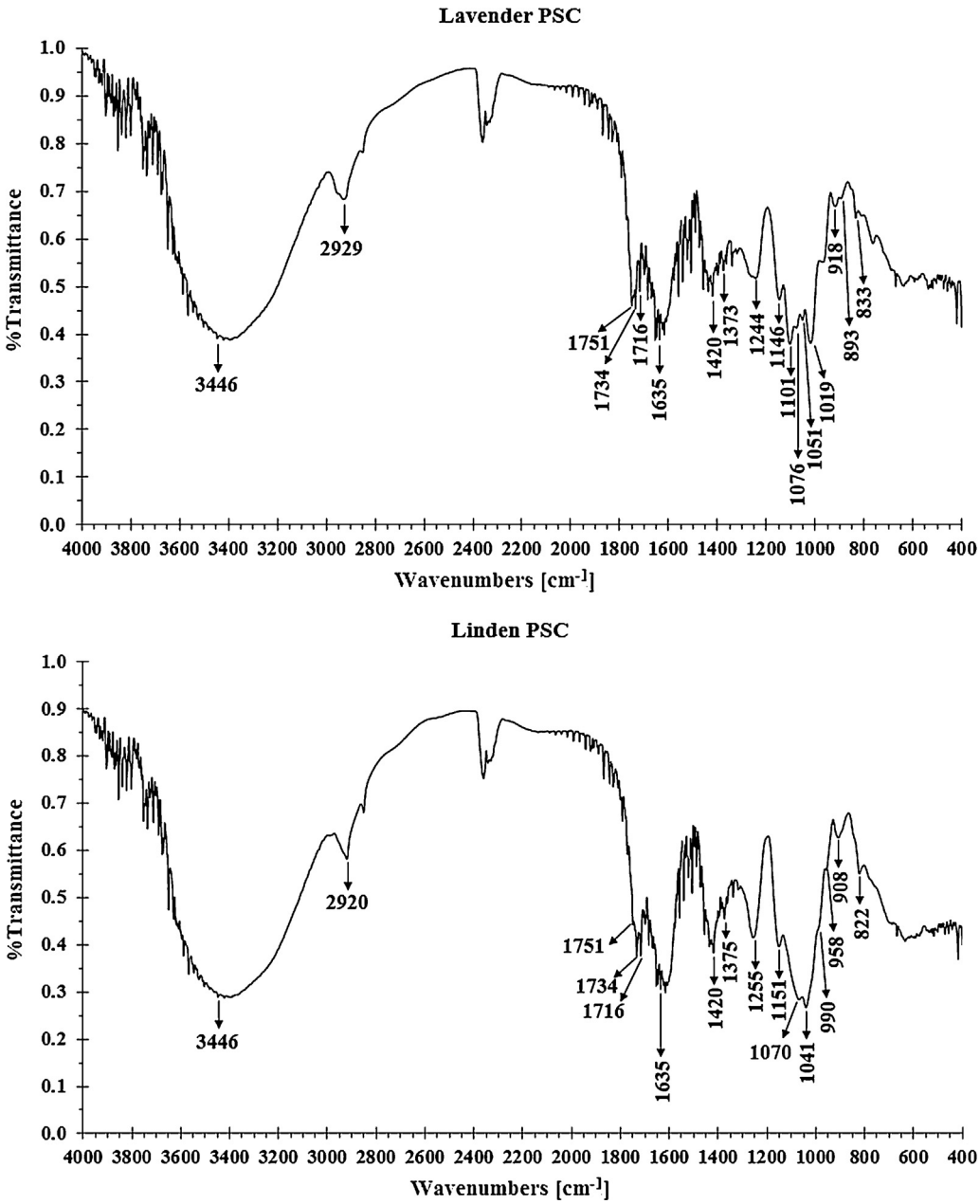


Fig. 1. FTIR spectra of selected herbal PSCs.

tions, which was probably in agreement with the spontaneous ROS generation.

3.3.3. *Intestinal immunomodulating activity*

In the current study the three PSCs exhibited *ex vivo* intestinal immunomodulating activity through the PP-mediated bone marrow cell proliferation test and increased IL-6 production from the PP cells in comparison with the positive control AMOL-1 (Fig. 4). The PP cells were isolated from the LPS low responder ($Tlr4^{Lps-d}$) C3H/HeJ mice, thus it was considered that the observed activity was not a result of possible LPS contamination. The bone-marrow cell proliferation was measured to estimate the relative production of hemopoietic growth factors from the polysaccharide-stimulated PP cells. The highest stimulating activity was expressed by the purslane PSC, followed by the linden and lavender PSCs, as in the same order was IL-6 production. The purslane PSC even induced a higher IL-6 production than the positive control. Therefore, the IL-6 production from the white blood cells induced by the three herbal PSCs was also confirmed by the animal immune cells. The control of IL-6 production in the PP cells is important because this cytokine can induce IgA antibody production through the B-cell proliferation in the patches.

3.3.4. *Anti-tumor activity* in vitro

In Table 2 are presented IC_{50} values of the three PSC samples against the growth of different human tumor cell lines after 48 h of incubation. Normal human fibroblasts (NF) and amniotic cells (FL) were used as controls. The linden PSC was the most active sample, expressing inhibitory activities against A549, HeLa and LS180 cells with IC_{50} values after 48 h of incubation as follows: 92.6 ± 2.7 μg/ml, 96.7 ± 1.6 μg/ml and 185.9 ± 6.3 μg/ml, respectively. The IC_{50} values for A549 and HeLa cell lines after 48 h of culturing with the lavender PSC were 162 ± 2.6 μg/ml and 170 ± 2.5 μg/ml, and for the purslane PSC were 169.9 ± 9.2 μg/ml

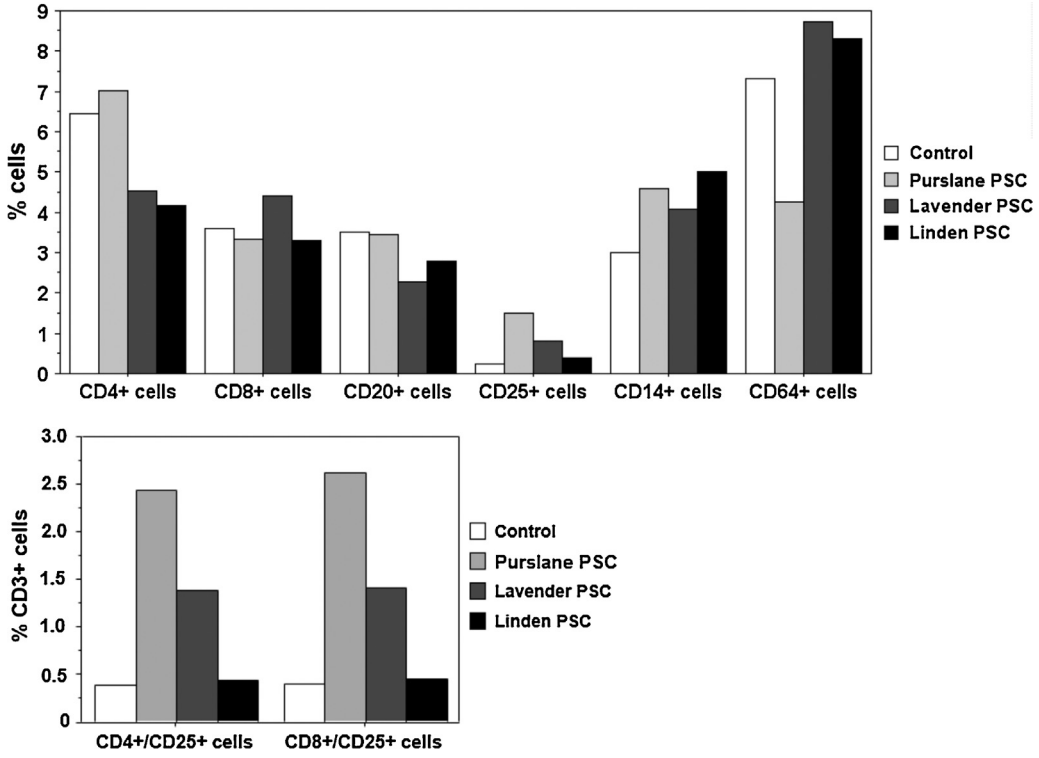


Fig. 2. Flow cytometric analysis for immunophenotyping of human white blood cells treated with 100 μg/ml purslane, lavender and linden PSCs.

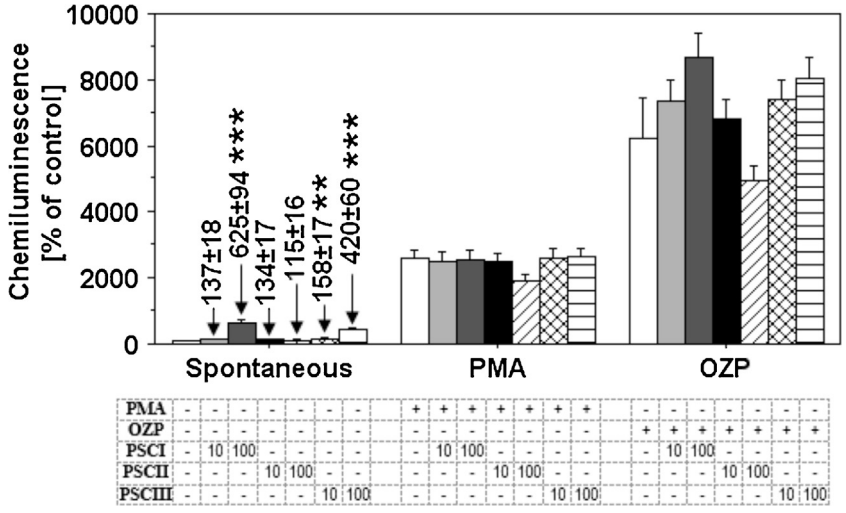


Fig. 3. Effect of purslane (PSCI), lavender (PSCII) and linden PSCs (PSCIII) on ROS production from non-stimulated (spontaneous), PMA- and OZP-activated whole blood phagocytes. 10 (10 μg/ml) and 100 (100 μg/ml). The asterisks indicate statistical difference (**p < 0.01, ***p < 0.001) vs. control.

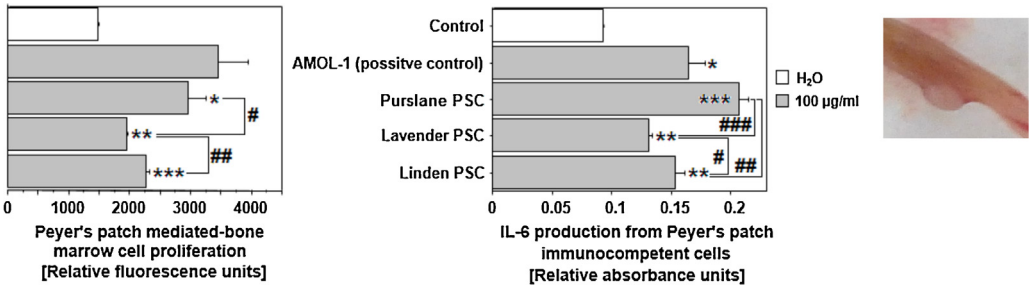


Fig. 4. Enhancement of production of bone marrow cell-proliferating cytokines from Peyer's patch cells cultured in presence or absence of purslane, lavender and linden PSCs. (A). Enhancement of production of IL-6 from Peyer's patch immunocompetent cells cultured in presence or absence of polysaccharides (100 μg/ml) (B). The symbols indicate statistical difference (*p < 0.05, **p < 0.01, ***p < 0.001) vs. water control or among samples (#p < 0.05, ##p < 0.01, ###p < 0.001). AMOL-1 is an active pectic polysaccharide isolated from *A. mongholics*, containing AGII.

Table 2
IC_{50} values (± SEM) of the three herbal PSCs on the growth of different cell lines after 48 h of treatment.

Polysaccharide complex	Cell line	IC_{50} [μg/ml]
Purslane PSC	NF[a]	>200
	FL[b]	173.9 ± 1.6
	A549[c]	169.9 ± 9.2
	HeLa[d]	195.4 ± 0.9
	LS180[e]	>200
Lavender PSC	NF	>200
	FL	183.7 ± 5.4
	A549	162.0 ± 2.6
	HeLa	170 ± 2.5
	LS180	>200
Linden PSC	NF	>200
	FL	167.5 ± 17.2
	A549	92.6 ± 2.7
	HeLa	96.7 ± 1.6
	LS180	185.9 ± 6.3

[a] **NF** (normal fibroblasts).
[b] **FL** (normal amniotic cells).
[c] **A549** (lung adenocarcinoma).
[d] **HeLa** (cervical adenocarcinoma).
[e] **LS180** (colon adenocarciona cells).

and 195.4 ± 0.9 μg/ml. The three PSCs were the most effective against the lung adenocarcinoma A549 cells. It was found that only the linden PSC expressed anti-proliferative activity against the growth of colon adenocarcinoma LS180 cells with IC_{50} value under 200 μg/ml. However, the results from the control treatment of NF and FL cells with the three PSCs showed that control cells were inhibited in a similar manner like abnormal cells. This means that the three samples exhibited non-specific inhibitory activity on the basis of the chosen control cell lines.

4. Discussion

Only on the basis of the monosaccharide composition data and the ester content (Table 1) could be suggested that the purslane and lavender PSCs contain methoxylated and acetylated pectic polysaccharides with predominant HG fragments, followed by AG structures most probably attached in the RGI fragments. The chemical features of the purslane PSC, such as the highest Gal, Ara and Glc contents among the three PSCs must be important for the potent activity of this sample in most of the observed effects. Similarly, it is proposed that the linden PSC contains high molecular weight pectins with considerable DA and low DM and with high RGI and GlcA contents, which could also influence the observed activities. From the three studied PSCs only polysaccharides of the purslane are investigated previously for expression of *in vitro* and *in vivo* immunomodulating activity [19,29]. Our findings on the stimulation of human white blood cells (Fig. 2) showed that the purslane PSC elevated the $CD4^+$ and $CD25^+$ cells, $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T-cells, and $CD14^+$ monocytes. The lavender PSC stimulated the $CD8^+$ and $CD25^+$ cells, T-cells and phagocytes. The linden PSC expressed activity mainly against the $CD14^+$ and $CD64^+$ cells. CD14 and CD64 proteins related to innate immune response are expressed on phagocytes, whereas CD4 and CD8 are related mainly to T-cells and adaptive immunity. CD64 or FcγRI receptor is expressed on monocytes, macrophages, neutrophils and other granulocytes and it interacts with antibody-coated antigens (IgG-Ag), thus triggering cell activation, such as phagocytosis and antibody-dependent cell-mediated cytotoxicity [54]. Its increased CD64 expression in treated with the studied herbal PSCs blood cells could mean Ig interaction with polysaccharides or other molecules in them [55]. When herbal pectins and probably their AG side chains interact with neutrophils through different receptors, they serve as antigens and similarly to macrophages activate phagocytosis with production of ROS [2]. The neutrophils comprise between 50 and 70% of all leukocytes in the blood and they are considered as main contributor to the total luminol CL [16]. Furthermore, the neutrophils exhibit more rapid rates of phagocytosis and a more intense oxidative response than the macrophages. Only the lavender PSC showed inhibitory effects on the PMA- and OZP-activated ROS generation (Fig. 3). Unlike the lavender, purslane and linden PSCs have low phenolic content and did not show any prominent antioxidant activity by the ORAC method (data not included). Similar effects to these of the lavender PSC on the spontaneous, PMA- and OZP-activated WBP were observed by Nikolova et al. [56] for leek pectic polysaccharides, which also showed antioxidant activity through the ORAC method. PMA is a potent inflammatory activator of protein kinase C, which could induce NADPH-oxidase activity and further the neutrophil degranulation and death [57]. OZP are opsonized zymosan particles that are coated with immunoglobulins and some complement factors which could be recognized through Fc and CR3 on neutrophils or other phagocytic leukocytes [58]. The stimulation of WBP by the three studied PSCs was in agreement with the elevated levels of $CD14^+$ and $CD64^+$ cells (Fig. 2). Therefore, the studied herbal PSCs could activate phagocytic leukocytes, which are involved in the first innate mechanism for immune defense against different pathogens and in inflammation processes. Interestingly, polysaccharides can affect positively the immune potentiation and negatively the inflammation [3]. This dual mode of action makes them perspective candidates for development of polysaccharide-derived healthcare products.

It was proposed that the studied herbal PSCs stimulated Tregs cells, represented most probably by the $CD4^+/CD25^+$ T-cells. The $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T-cell subpopulations are responsible for regulation of the immune response in autoimmune, tumor diseases and immune homeostasis. The stimulatory effect of herbal polysaccharides on T-cells could be useful in the supplementary therapy of infectious and tumor diseases [59]. For example, Shen et al. [19] demonstrated that polysaccharide POP from *P. oleracea* exhibited a potent anti-tumor activity *in vivo* and increased the animal's immune responses, including increase in the number of white blood cells and $CD4^+$ T-cells, as well as the ratio of $CD4^+/CD8^+$ cells. Similarly, Shu et al. [60] showed that polysaccharide PSSC from *Salvia chinensis* expressed an anti-tumor immunostimulatory activity *in vivo* and elevated $CD4^+$ T-cells in both spleen and lymph nodes and the cytotoxic activity of NK cells and $CD8^+$ T-cells.

Herbal polysaccharides are mainly administrated in the human organism by herbal infusions, foods or other formulations. They can express intestinal immunomodulating activity through interaction with immunocompetent cells in the PPs localized in the small intestine [2]. Although, polysaccharides are little absorbed by intestinal epithelial cells, their communication with the epithelium could also lead to immunomodulation of the underlying immunocompetent cells [10]. The AGII, together with the AGI structures in pectins are important for expression of the intestinal immunomodulating activity through the PPs [2]. It was found by the Yariv's test that the linden PSC contains AGII. Similarly, literature data indicate that the purslane pectins also contain AGII [28]. Therefore, the purslane PSC logically showed the most potent activity because it had the highest amount of Gal, which was assumed to be mostly linked in the AGII and AGI structures. It also contained a considerable amount of Glc, derived probably from starch that activates PP cells as well [61]. Our results were in a good agreement with those obtained for a lignin-carbohydrate complex from the Kampo extract preparation TJ48 (Juzen-Taiho-To), BP-II pectic fraction from *Biophytum petersianum* and RP-1 pectic fraction from *Rosa damascena* [9,13,62].

Murine PPs contain about 60% B-cells ($B220^+$), 25% T-cells ($CD3^+$), 10% dendritic cells ($CD11c^+$), and less than 5% macrophages ($F4/80^+$) or polymorphonuclear neutrophils ($Ly\text{-}6G^+$). Among the

T-cells 45% are $CD4^+$, 35% are $CD8^+$ and 20% are $CD4^-/CD8^-$ T-cells. Among the $CD4^+$ T-cells, 85% are memory T-cells ($CD25^-CD45RB^{lo}$), 10% are Naïve ($CD25^-CD45RB^{hi}$) and 5% are regulatory T-cells ($CD25^+CD45RB^{lo}$). Furthermore, bone marrow contains T-cells, B-cells, neutrophils, dendritic cells and monocytes, as it has 8%–20% lymphocytes with T/B-cell ratio of 5:1. The mouse bone marrow contains 1–5% $CD3^+$, 1.5% $CD4^+$, 2.0%–2.5% $CD8^+$ T-cells. Importantly, $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T-cells are found to be about 10% and 5% in the human PPs and 5–30% of the $CD4^+$ T-cells in the mouse bone marrow are $CD4^+/CD25^+$ Tregs cells [63,64]. Therefore, on the basis of our results with human white blood cells (Fig. 2) and WBP (Fig. 3), it could be proposed that the studied polysaccharides expressed activity against the PP mediated-bone marrow cell proliferation through the phagocytic leukocytes and T-lymphocytes. Additionally, the three samples induced IL-6 production from PP cells and human white blood cells, which is produced by T-cells and macrophages [2]. Administration of herbal polysaccharides active against the intestinal immune system could support the compromised immune health. For example, Hou et al. [59] found that treatment (i.p.) of mice suffering from polymicrobial sepsis with 100 and 200 mg/kg *Astragalus* polysaccharide APS increased the percentage of activated Th cells in the PPs.

When it was found that the herbal PSCs expressed immunostimulating activity against innate and adaptive human immune cells and intestinal murine PP cells, it was also important to be tested their effect on abnormal cells. This was necessary in respect to the perspective application of the herbal PSCs and related products in the prophylaxis and prevention of infectious, autoimmune and tumor diseases. Only the anti-tumor activity of purslane polysaccharides was investigated, so far [29,30]. Zhao et al. [30] obtained IC_{50} = 489 μg/ml after 48 h treatment of HeLa cells with polysaccharide fraction POL-P3b isolated from *P. oleracea*, which was 2.5 fold higher than those we obtained for the purslane PSC (Table 2). The purslane PSC expressed a higher *in vitro* activity, but this could be related primarily to the presence of a mixture of purslane neutral and pectic polysaccharides, as well as to the supplementary effect of smaller molecules, which were expected to be <18% (Table 1). Our results with the A549 cells for the purslane and lavender PSCs were in agreement with those obtained for crude polysaccharides extracted from the fruits of *Cyphomandra betacea*, which showed IC_{50} = 170 μg/ml after 48 h with A549 cells [65]. Even though, the combinatorial effects of different compounds in herbal extracts are rather complex, it cannot be excluded that the polysaccharides and some of the non-carbohydrate constituents act in a synergistic manner. For example, it was found that supplementation of breast cancer patients undergoing postoperative radiation therapy with aronia juice enriched with apple pectin led to an immune system stimulation expressed by a significant increase of the $CD4^+$ and $CD8^+$ T-cells levels [66]. Although, studies of the polysaccharide effect on LS180 cells are still scarce, the anti-tumor activity of the herbal polysaccharides against gastrointestinal tumors expands their immunomodulating potential and deserves more studies for their protective and suppressive effects. However, our results showed that the studied herbal PSCs expressed non-specific *in vitro* anti-proliferative activity against normal and tumor cells. This effect needs to be further studied with the purified polysaccharide fractions, otherwise it seems rather unspecific and not promising.

Further immunomodulating characterization of the purslane PSC is necessary because of the increasing popularity of purslane as a food and its use in the functional and dietary nutrition [23]. The immunomodulating studies on the lavender and linden PSCs are important primarily due to the traditional use of these herbs and their water extracts in the folk medicine and cosmetics. The increasing lavender oil production implies better utilization of the bioactive compounds, including the pectins in the distilled residue into secondary value-added products, such as water extracts for nutraceuticals and cosmetics. We found that the polyuronides, representing pectins in industrially distilled lavender flowers are 18.9% with a very close value of 19.1% for the stalks, thus the obtained by-product could be a source of bioactive polysaccharides in addition to smaller molecules.

For further explanation of the biological results it was necessary to differentiate between the herbal polysaccharides and the other constituents in the samples. This was done for linden and lavender PSCs in subsequent studies, confirming the polysaccharide role in the observed immunomodulating activities and will be published.

5. Conclusion

We demonstrated that similarly to the edible herb purslane, the other two widely used medicinal plants – common lavender and silver linden contain also immune active acidic PSCs rich in pectic polysaccharides. Interestingly, GlcA is about 31% of the uronides (HPAEC-PAD analysis basis) in the linden PSC, which is also characterized by high Rha content in addition to high GalA amount, suggesting the existence of a considerable amount of a RGI fragment in this sample. The three PSCs expressed *ex vivo* stimulating activity against different blood T-cell populations and phagocytic leukocytes. These herbal PSCs also stimulated *ex vivo* ROS production from WBP and inhibited non-specifically the growth of different tumor cell lines *in vitro*. Furthermore, they showed *ex vivo* intestinal immunomodulating activity against PP cells at 100 μg/ml. It was proposed that the studied herbal PSCs can modulate the PP immunocompetent cells through the $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T-cells and phagocytes, because they are also found in the patches and in the bone marrow. The purslane PSC expressed the highest stimulating activity against normal immune cells and the linden PSC showed the highest inhibitory activity against tumor cells with an IC_{50} value of 93 μg/ml for A549 cells. Our results suggest that similarly to the purslane polysaccharides, these isolated from lavender and silver linden have also perspectives to be studied their supplementary role in treatment of health conditions characterized with compromised immune system.

Acknowledgements

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