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The common lavender (*Lavandula angustifolia* Mill.) pectic polysaccharides modulate phagocytic leukocytes and intestinal Peyer's patch cells

Yordan N. Georgiev[a,b], Berit S. Paulsen[c], Hiroaki Kiyohara[d], Milan Ciz[e], Manol H. Ognyanov[a,b], Ondrej Vasicek[e,i], Frode Rise[f], Petko N. Denev[a,b], Haruki Yamada[d], Antonin Lojek[e], Vesselin Kussovski[g], Hilde Barsett[c], Albert I. Krastanov[h], Irina Z. Yanakieva[a,b], Maria G. Kratchanova[a,b,*]

[a] *Laboratory of Biologically Active Substances, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 139 Ruski Blvd., BG-4000 Plovdiv, Bulgaria*
[b] *Innovative-Technological Center Ltd., 20 Dr. G. M. Dimitrov Str., BG-4000 Plovdiv, Bulgaria*
[c] *Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, NO-0316 Oslo, Norway*
[d] *Department of Drug Discovery Science, Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, JP-108-8641 Tokyo, Japan*
[e] *Department of Free Radical Pathophysiology, Institute of Biophysics, Czech Academy of Sciences, 135 Kralovopolska, CZ-612 65 Brno, Czech Republic*
[f] *Department of Chemistry, University of Oslo, P.O. Box 1033 Blindern, NO-0315 Oslo, Norway*
[g] *The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 26, BG-1113 Sofia, Bulgaria*
[h] *Department of Biotechnology, University of Food Technologies, 26 Maritza Blvd., BG-4002 Plovdiv, Bulgaria*
[i] *International Clinical Research Center – Center of Biomolecular and Cellular Engineering, St. Anne's University Hospital Brno, 53 Pekarska, CZ-656 91 Brno, Czech Republic*

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ABSTRACT

Two pectic (chPS-L1, chPS-L2) and one polyphenolic (chPP-L) fractions were obtained from lavender flowers after boiling water extraction, exhaustive removing of alcohol-soluble molecules and SEC. chPS-L1 (52.4 kDa) contains mainly low-acetylated and high-methoxylated homogalacturonans (HG), and smaller rhamnogalacturonan (RG) I backbone fragments rich in 1,3,5-branched arabinan and arabinogalactan (AG) II side chains. chPS-L2 (21.8 kDa) contains predominantly similarly esterified HG, followed by RGI with AGII structures and RGII. The prevalence of catechin and epicatechin in chPP-L indicates that they form weak interactions with pectins. chPS-L1 and chPS-L2 enhanced β_2-integrin expression on neutrophils, inducing ROS generation and macrophage NO production. Both the effects on β_2-integrin and high complement fixation activity of chPS-L1 were proposed for its inhibitory action against PMA- and OZP-activated ROS formation. This, together with suppression of NO generation after co-stimulation with chPS-L1 and LPS, suggested anti-inflammatory activity of studied pectins. Lavender polysaccharides expressed intestinal Peyer's patch immunomodulating activity.

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Abbreviations: AGII, arabinogalactan type II; AUAC, anhydrouronic acid content; BSA, bovine serum albumin; CD18, marker for $\beta_2$integrin; CD62L, marker for L-selectin; chPP-L, polyphenolic fraction obtained after SEC from lavender PSC2; chPS-L1, first fraction of SEC purified lavender pectic polysaccharides; chPS-L2, second fraction of SEC purified lavender pectic polysaccharides; CL, chemiluminescence; CR3, omplement receptor 3; DA, degree of acetylation; DM, degree of methoxylation; DSS, sodium 4,4-dimethyl-4-silapentane-sulfonate; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; FTIR, Fourier transform infrared spectroscopy; HBSS, Hank's buffered salt solution; HG, homogalacturonan; IC_{50}, a concentration giving 50% hemolysis of erythrocytes; iNOS, inducible nitric oxide synthase; KDO, 3-deoxy-D-manno-2-octulosic acid; Lavender PSC, crude pectic polysaccharide complex from *Lavandula angustifolia*; Lavender PSC2, obtained from lavender PSC after exhaustive ethanol treatment; LDH, lactate dehydrogenase; LPS, lipopolysaccharides; NMR, Nuclear magnetic resonance spectroscopy; NO, nitric oxide; OZP, opsonized zymosan particles; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PP, Peyer's patches; RGI, rhamnogalacturonan type I; ROS, reactive oxygen species; SEC, size-exclusion chromatography; WBP, whole blood phagocytes.

* Corresponding author at: Laboratory of Biologically Active Substances, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 139 Ruski Blvd., 4000 Plovdiv, Bulgaria. Tel./fax.: +359 32 64 27 59.
E-mail address: lbas@plov.omega.bg (M.G. Kratchanova).

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1. Introduction

In Bulgaria common lavender, *Lavandula angustifolia* Miller (Lamiaceae) is cultivated predominantly in the Rose valley and since 2011 the country is the biggest lavender oil producer worldwide. Already Dioscorides (c. 40–90 AD) documented that lavender has laxative and invigorating properties and advised its tea for chest complaints (Castle & Lis-Balchin, 2002). The German Commission E has approved *L. angustifolia* tea for treatment of restlessness, insomnia and nerve disorders of the intestines. The lavender flowers express also anti-microbial, repellent, anti-inflammatory, antioxidant and diuretic activities, which are mainly attributed to the essential oils (Laekeman, 2012). However, the lavender contains also non-volatile biologically active constituents, such as triterpenoids, flavonoids and lignans (Harborne & Williams, 2002).

In a previous study, it was shown that a crude pectic polysaccharide complex (lavender PSC) from *L. angustifolia* expresses *ex vivo* immunomodulating activity against phagocytic leukocytes, T-lymphocytes and intestinal Peyer's patch cells (Georgiev, Ognyanov, Kiyohara et al., 2017). Pectic polysaccharides express different bioactivities outside the plants when they are properly extracted. Pectins contain three main building blocks – HG, RG type I and II and substituted galacturonans (Voragen, Coenen, Verhoef, & Schols, 2009). Generally, the immunomodulating activity of pectic polysaccharides is explained by the presence of immune active AG type I and II structures O-4 attached to Rha of the RGI domain (Yamada & Kiyohara, 2007; Wangensteen, Diallo, & Paulsen, 2015). Additionally, in a recent study we found that highly glucuronidated RGI isolated from *Tilia tomentosa* also exhibits immunomodulating activity (Georgiev, Paulsen et al., 2017). Immune cells express signaling receptors, such as complement receptor 3 (CR3), Fc receptor, toll-like receptors and scavenger receptors that can interact with polysaccharides to initiate up- or down-regulation of numerous immunological responses (Leung, Liu, Koon, & Fung, 2006). The immunomodulating properties of pectins are shown by exhibition of moderate complement fixation activity, activation and regulation of phagocytic leukocytes, B- and T-lymphocytes, thrombocytes, suppression of tumor cells, etc (Yamada & Kiyohara, 2007; Wangensteen et al., 2015). Because of these effects, pectins are included in *in vivo* studies and some clinical trials for their supplementary role for treatment of socially significant diseases, as reviewed by Georgiev, Ognyanov, Denev et al. (2017). Furthermore, together with smaller molecules, pectic polysaccharides are partly extracted during preparation of lavender teas or decoctions. In this way, polysaccharides are coupled with naturally occurring compounds like polyphenols, forming bioactive mixtures with combinatorial effects (Jakobek, 2015). At the same time, the increasing rate of lavender cultivation for perfume and healthcare industries expands development of different lavender-derived products and needs a proper utilization of the waste after distillation.

The aim of the current study is to characterize the chemical structure and immunomodulating properties of *L. angustifolia* pectic polysaccharides purified from the immune active crude lavender PSC obtained with boiling water (Georgiev, Ognyanov, Kiyohara et al., 2017).

2. Materials & methods

2.1. Plant material

Lavender flowers from *Lavandula angustifolia* Miller (syn. *L. vera* DC) sort "Sevtopolis" (Institute of Roses, Essential and Medical Cultures, Kazanlak, Bulgaria) were provided dried by ET "Ve Pe Pi – Vesko Pipev" (Velingrad, Bulgaria).

2.2. Isolation and purification of lavender flower polysaccharides

The crude lavender polysaccharide complex (lavender PSC) was extracted with boiling water, as previously described (Georgiev, Ognyanov, Kiyohara et al., 2017).

Lavender PSC was dissolved in water (10 mg/mL) and precipitated five times (each for 24 h) with three volumes of 95% ethanol. This step was for partly removing of ethanol-soluble molecules prior to size exclusion chromatography (SEC). After each run, the polysaccharide-containing precipitates were obtained by centrifugation at 4000 rpm for 30 min at 20 °C. Then, the recovered polysaccharide-containing fraction (lavender PSC2) was dissolved in water, centrifuged and the supernatant was applied to a Sephacryl® S-200 Superfine (GE Healthcare Life Sciences) column (100 × 1.5 cm) coupled with a FPLC BioLogic® LP system (BioRad). The sample was eluted with 0.2 M NaCl at 0.2 mL/min flow rate and 5 mL fractions were collected. Two pectic fractions chPS-L1 and chPS-L2, and one polyphenolic fraction chPP-L were obtained. The carbohydrate peaks were visualized by the *m*-hydroxydiphenyl and phenol-sulfuric acid methods, and polyphenols by the Folin-Ciocalteu reagent, and UV–Vis fingerprinting (200–800 nm). The chPP-L fraction was concentrated under vacuum to 50 mL and loaded on an Amberlite® XAD7HP (Sigma-Aldrich) column (12 × 3 cm), followed by column elution with 200 mL water and the polyphenols were eluted with 150 mL 95% ethanol. The polyphenolic fraction was evaporated to dryness under vacuum, dissolved in water and freeze-dried. chPS-L1 and chPS-L2 were concentrated under vacuum, dialyzed against water (MWCO 12–14 kDa, 72 h), filtered through 0.2 μm cellulose nitrate filters (Sartorius) and freeze-dried. The three samples were stored in a desiccator in dark.

2.3. General methods

Anhydrouronic acid content (AUAC) was determined by the *m*-hydroxydiphenyl method in Skalar San++ autoanalyzer (Analytical BV, Breda, The Netherlands), using galacturonic acid as a standard (Blumenkrantz & Asboe-Hansen, 1973). Total amount of sugars was quantified by the phenol-sulfuric acid method, using 1:1 mixture of glucose and galacturonic acid as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Degree of methoxylation (DM) was determined by alcohol oxidase (*P. pastoris*, Sigma-Aldrich) and Purpald® (Sigma-Aldrich) (Anthon & Barrett, 2008). Degree of acetylation (DA) was measured according to McComb and McCready (1957), using β-D-glucose pentaacetate as a standard. The relative molecular weight was determined by HPSEC-RID (Waters) with Shodex standard P-82 kit (pullulan standards, Showa DENKO, Tokyo, Japan) (Georgiev, Ognyanov, Yanakieva, Kussovski, & Kratchanova, 2012). Total phenolics (gallic acid standard) and protein content (BSA standard) were analyzed according to Singleton and Rossi (1965) and Bradford (1976), respectively.

2.4. Monosaccharide composition and glycosidic linkage determination

Polysaccharide samples (1 mg) with added 100 μg mannitol as an internal standard were subjected to methanolysis with anhydrous 3 M HCl in methanol for 24 h at 80 °C. The formed methyl glycosides of different monosaccharides were converted into their corresponding TMS derivatives and analyzed by GC-FID on a Focus GC system (Thermo Scientific, Milan, Italy), according to Chambers & Clamp (1971) and Barsett, Paulsen, & Habte (1992). The presence of KDO (3-deoxy-D-manno-2-octulosic acid) was detected by the thiobarbituric acid assay (York, Darvill, McNeil, & Albersheim, 1985).

The glycosidic linkage composition of chPS-L1 (2 mg) was examined after methylation. Prior to methylation uronic acids in the sample were reduced with $NaBD_4$ to 6,6′-dideuterio-sugars that could be differentiated from the neutral sugars (Kim & Carpita, 1992). The procedure was completed by methylation, acid hydrolysis and preparation of alditol acetates, partly as described by Ciucanu & Kerek (1984). The hydrolysis was performed with 2.5 M TFA for 2 h at 100 °C. The partly methylated alditol acetates were analyzed by a GC-EI/MS-QP2010 system (Shimadzu, Kyoto, Japan), coupled with a Restek Rxi-5MS column (30 m, 0.25 mm i.d., 0.25 μm film) in a Scan mode (40–450 ions), as described by Ho, Zou, Aslaksen, Wangensteen, and Barsett (2016). The detection and quantification of the relative amounts of each glycosidic linkage type was based on the chromatographic behavior (RT), ionization patterns and results from the monosaccharide composition.

The presence of AGII was detected by precipitation with the Yariv *ß*-glucosyl reagent (Van Holst & Clarke, 1985).

2.5. Contamination with lipopolysaccharides (LPS)

Possible LPS contamination was checked by the detection of 3-hydroxy fatty acids as their specific chemical markers in the form of acetylated fatty acid methyl esters on the GC-EI/MS-QP2010 system (Shimadzu, Kyoto, Japan), coupled with a Restek Rxi-5MS column (30 m, 0.25 mm i.d., 0.25 μm film) in a SIM mode (257 ion intensity for calculation), as described by de Santana-Filho et al. (2012) and Ho et al. (2016).

2.6. Fourier transform infrared spectroscopy (FTIR)

Infrared (IR) spectra of chPS-L1 (2 mg) 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).

2.7. Nuclear magnetic resonance (NMR) spectroscopy

chPS-L1 (12 mg) was dissolved in D_2O, freeze-dried and redissolved in 0.7 mL D_2O. 0.5 mL of chPS-L1 was investigated by the following NMR experiments. 1D ^1H, ^{13}C, DEPT 135 and 2D $^1H/^{13}C$ HSQC, $^1H/^1H$ COSY, $^1H/^1H$ TOCSY, $^1H/^1H$ ROESY, $^1H/^1H$ NOESY, $^{13}C/^1H$ HMBC experiments were performed on a Bruker Avance III HD Ascend 800 MHz spectrometer operating at 800.03 MHz (Bruker, Fällanden, Switzerland) with the Bruker TopSpin™ 3.5 software at a temperature of 60 °C. The chemical shifts were referenced to the internal standard DSS (sodium 4,4-dimethyl-4-silapentane-sulfonate).

2.8. Immunomodulatory effect

2.8.1. Ethics

Blood sampling procedure was in accordance with the Helsinki Declaration of 1975, as revised in 1983. 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.

2.8.2. 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). 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*.

2.8.3. Complement fixation assay

The inhibition of complement hemolysis (complement fixation) of target antibody sensitized sheep red blood cells (classical pathway) or rabbit erythrocytes (alternative pathway) by normal porcine serum pre-treated with chPS-L1, chPS-L2 or chPP-L was studied. The analyses were performed according to Klerx, Beukelman, Dijk, and Willers (1983) and Klerx et al. (1985), as described by Georgiev et al. (2012). The complement fixation activity induced by the test samples was calculated on the basis of a colorimetric measurement of hemoglobin released from lysed cells after incubation with PS-treated normal porcine serum by the formula: $[A_{control} - A_{test}]/A_{control} \times 100\%$. From these data a dose-response curve was constructed and the concentration of test sample giving 50% inhibition of hemolysis (IC_{50}) was calculated (Fig. S2). The samples were analyzed in triplicates.

2.8.4. Reactive oxygen species (ROS) production from human whole blood phagocytes (WBP) and isolated neutrophils

Heparinized (50 IU/mL) blood samples were obtained from the cubital vein of healthy human volunteers after overnight fasting. The isolation of human neutrophils from venous blood was accomplished according to the procedure of Nauseef (2014). The kinetics of ROS production by WBP and isolated neutrophils 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 (Vasicek, Lojek, Jancinova, Nosal, & Ciz, 2014). Briefly: 25 μL of ten times diluted blood with Hank's buffered salt solution (HBSS) or 0.2×10^6 cells/well of isolated neutrophils in phosphate-buffered saline (PBS) 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 chPS-L1, chPS-L2 or chPP-L (50 and 100 μg/mL) for spontaneous (non-stimulated with known activators) CL. Furthermore, 12.5 μL or 5 μL of ten times diluted blood (or 0.2×10^6 cells/well) mixed with 25 μL luminol were used for incubation of each fraction 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 percentage of the control (mean ± SEM) from five independent experiments.

2.8.5. Determination of the cell surface expression of adhesion molecules

The measurements were performed according to the method described by Gallova, Kubala, Ciz, and Lojek (2004). Briefly, isolated human neutrophils (1×10^5 cells) were incubated in plastic tubes (Falcon, USA) with chPS-L1 or chPS-L2 (100 μg/mL) for 1 h and with the activators fMLP (N-formyl-met-leu-phe, 0.1 μM) and PMA (0.01 μg/mL) for 10 min. After incubation anti-CD18 (anti-β_2-integrin) and anti-CD62L (anti-L-selectin) monoclonal antibodies (5 μL of each) were added. The samples were incubated at 4 °C for 15 min, subsequently centrifuged, re-suspended in PBS and analyzed by a flow cytometer FACSVerse™ (Becton Dickinson, USA). The median fluorescence intensity was determined and corrected for unspecific staining by subtracting the fluorescence of cells stained with the control antibody (isotype control). The data are expressed as $CD62L^-/CD18^+$ population (mean% of total leukocytes ± SEM) from five independent experiments.

2.8.6. Nitric oxide (NO) production from murine macrophages

Murine macrophage cell line RAW 264.7 (ATCC, USA) was incubated in 12-well flat bottom culture plates at 1×10^5 cells/well with chPS-L1 or chPS-L2 (50 and 100 μg/mL) alone in DMEM-high Glc supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (500 μL final volume) (HyClone™, GE Healthcare Life Sciences) for 24 and 48 h at 37 °C and 5% CO_2. The cells were also cultured by combination of polysaccharides with 10 ng/mL LPS (*E. coli*/026:B6, Sigma-Aldrich). The generation of NO was determined indirectly as the accumulation of nitrites in the cell culture supernatants. At the end of the incubation period the culture media were collected and centrifuged at 16 000 × *g*, 4 °C for 10 min. Then, 150 μL of supernatants were mixed with an equal volume of Griess reagent (Sigma-Aldrich) in a 96-well plate and the mixture was incubated at room temperature for 30 min in dark. The absorbance was measured at 546 nm and $NaNO_2$ (0–52 μM) was used as a standard (Lojek et al., 2011). The results are expressed as mean ± SEM from three independent experiments.

2.8.7. Western blot analysis of inducible nitric oxide synthase (iNOS)

RAW 264.7 cells, which were obtained as described in **2.8.6.**, were lysed in the lysis buffer (1% SDS, 0.1 M Tris pH 7.4, 10% glycerol, 0.001 M sodium *o*-vanadate, 0.001 M PMSF). The protein concentrations were determined by using the BCA™ protein assay (Pierce, USA), with BSA as a standard. Equal amounts of protein (18 μg) were then subjected to SDS-PAGE, using 10% separating gel. The expression of iNOS protein was quantified by a Western blot analysis (Pekarova et al., 2011). Anti-iNOS/NOS Type II mouse monoclonal antibody (Cell signaling Technology, 1:5000) and ECL™ anti-rabbit IgG horseradish peroxidase linked whole antibody (from goat; Cell Signaling Technology, 1:2000) were used. The immunoreactive bands were detected using an ECL™ detection kit (Pierce) and exposed to a radiographic film (AGFA). The relative protein levels were quantified by scanning densitometry, using the ImageJ™ programme, and the individual band density value was expressed in arbitrary units. The results are shown as mean ± SEM from three independent experiments.

2.8.8. Cytotoxicity assay

Cytotoxicity was estimated by lactate dehydrogenase (LDH) activity in the cell culture supernatants of RAW 264.7 cells, as obtained in **2.8.6.**, using the LDH Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, Switzerland). The results are expressed as mean ± SEM from three independent experiments (Fig. S4).

2.8.9. Immunomodulating activity against murine Peyer's patch (PP) cells

Immunomodulating activity was measured by the enhanced production of bone marrow cell-proliferative cytokines from PP cells of pathogen-free C3H/HeJ mice ($Tlr4^{Lps-d}$), according to Hong, Matsumoto, Kiyohara, and Yamada (1998). Suspensions (1.2×10^6 cells/mL, 180 μL) of PP cells (5–9 patches obtained per mouse) from the small intestine of C3H/HeJ mice were cultured with H_2O (negative control), AMOL-1 (pectic polysaccharide from *Astragalus mongholics* as a positive control, Kiyohara et al., 2010) or 100 μg/mL of the pectic fractions (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 (Hong et al., 1998). The results are shown as mean ± SEM.

2.9. Statistical analysis

The 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. Purification and structural characterization of lavender pectic polysaccharides

3.1.1. Purification and chemical composition of lavender fractions

After the ethanol pretreatment of lavender PSC, the obtained lavender PSC2 having 41% AUAC and low protein content (2.9%) was subjected to SEC (Fig. 1(A)). The presence of non-carbohydrate compounds with diene and aromatic structure (e.g. polyphenols, fatty acids, proteins) in all fractionation tubes was estimated by reading of the absorbance at 254/280 nm. This in addition to the UV–Vis fingerprinting of selected tubes helped for determination of two polysaccharide and one polyphenolic fractions. As can be seen from Fig. 1(B) the three lavender fractions are distinguished by the intensity of two domains at 201–202 nm and around 280 nm. The first domain originates from different π-bonds, such as C=O of carboxyl group in pectin (202 nm) and other compounds and the second has λ_{max}=276 nm typical for catechin and tannins.

The total yield of the two polysaccharide fractions in dried lavender flowers is 1.6% (w/w). The monosaccharide composition of these fractions shows that they contain mainly pectic polymers with the highest content of GalA, followed by Gal, Ara and Rha (Table 1). This result is in agreement with the relatively high AUAC between 41 and 45%. chPS-L1 and chPS-L2 give a cross-reaction with the *ß*-glucosyl Yariv reagent, which indicates that both fractions contain 3,6-linked galactans or AGII. The reaction ring for chPS-L1 is more intensive, thus it has higher AGII content than chPS-L2 (gels not shown). Furthermore, the presence of small amounts of GlcA, Xyl and Fuc, which are specific for some pectic side chains are detected. The presence of the rare monosaccharides 2-OMeXyl and 2-OMeFuc in chPS-L2 indicated that it contains RGII, which was further confirmed by the presence of KDO. Additionally, negligible contamination with other polysaccharides containing Man and Glc is also found. Both polysaccharides are classified as high methoxylated pectins with DA <20%. As was expected chPS-L2 (2.18×10^4 Da) has lower molecular weight (relative to pullulan) than chPS-L1 (5.24×10^4 Da) and higher total polyphenol content (see Fig. 1(A) & (B)).

Direct SEC fractionation of lavender PSC2 was chosen because of the interest for identification of some polyphenols retained associated to pectins after an exhaustive extraction of ethanol-soluble molecules from lavender PSC. The yield of chPP-L is 9.2% from lavender PSC2. Interestingly, 10 phenolic acids and 8 flavonoids were identified in minor amounts in chPP-L (Table S1). Ferulic acid, protocatechuic acid, quercetin, catechin and epicatechin are presented in the highest concentrations. For comparison, in the alcohol-soluble fraction obtained from lavender PSC, only the catechin (385.4 μg/100 mg) and epicatechin (497.7 μg/100 mg) contents are much lower than in chPP-L based on the identical polyphenols detected in the two samples. Both flavonoids are ethanol-soluble, thus weak interactions with pectic polysaccharides might occur and they could be broken during the saline SEC fractionation.

3.1.2. Glycosidic linkage determination

The quantitative glycosidic linkage analysis of chPS-L1 indicated that it contains mainly 1,4-linked Gal*p*A (47.3 mol%) with considerably lower amounts of 1,2- and 1,2,4-linked Rha*p*. It is

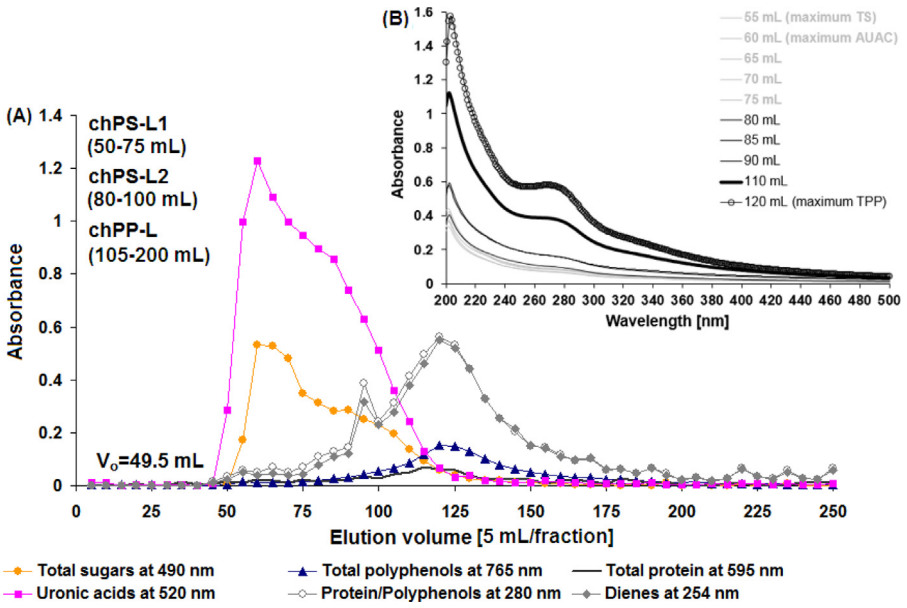


Fig. 1. Purification and fractionation of lavender pectic polysaccharides. (A) Elution profile of lavender PSC2 on Sephacryl S-200. (B) UV–Vis fingerprinting analysis of obtained pectic (chPS-L1 and L2) and polyphenolic (chPP-L) fractions. TS (total sugars), TPP (total polyphenols).

rich in AGII structures (26.7 mol%), shown by 1,3,6-linked Gal*p* (6.9 mol%), T-Ara*f* (5.6 mol%) and 1,5-linked arabinan (3.3 mol%) (Table 2). chPS-L1 is a pectic type polymer composed mainly of HG with a smaller RGI backbone fragment, containing 1,3,5-branched arabinan and highly branched AGII side chains. The molar ratio 1,2:1,2,4-linked Rha*p* in chPS-L1 is 41:59, indicating a high degree of branching on the Rha*p* present in RGI. The 3,6-linked Gal and 1,5-arabinans in the Rha side chains are 25.8 and 12.4 mol% calculated from the total galactan and arabinan structures. The predominance of 3,6-linked Gal is in a good agreement with the Yariv's test results. chPS-L1 is also characterized with a minor amount of 1,3,4-linked Gal*p*A, which probably indicates the presence of some substituted galacturonan, such as RGII or others. The other uronic acid in the fraction – GlcA is T-, 1,2- and 1,2,4-linked most probably in AG side chains (Yamada & Kiyohara, 2007). Additionally, the methylation analysis showed that Glc in chPS-L1 is T- and 1,4-linked probably in an independent glucan polymer, such as starch. Concluding, HG (GalA – Rha) and RGI (2Rha + Ara + Gal) content in chPS-L1 are roughly estimated to be 47.6 mol% and 39.1 mol%, respectively (Table 1) (Denman & Morris, 2015).

3.1.3. FTIR analysis

The IR spectrum of chPS-L1 (Fig. S1) contains the typical bands for polysaccharides and pectins, as follows: 3408, 2935, 1743, 1616, 1331, 1146 and 1101 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 ionized (free) carboxyl group, C—H in the ring, C—O—C of the glycosidic bond (or within the ring) and C—C or C—O in the ring, respectively (Synytsya, Čopíková, Matějka, & Machovič, 2003). According to the formula: $[A_{1743}/(A_{1743} + A_{1616})] \times 100$, where A is the area of the corresponding signal, the DM of chPS-L1 is 61.4%. This result is in agreement with the already determined DM of 63.4% (Table 1). Additionally, the signal at 1373 cm^{-1} is attributed to $\delta_s(CH_3)$ of methoxyl ester and two overlapped bands at 1242–1259 cm^{-1} originate from ν(C—O—C) of acetyl esters (Synytsya, Čopíková, Matějka et al., 2003). The signals at 1146, 1101 and 1018 cm^{-1}, as well as the peaks at 833 (C1-OH, α-anomer), 764, 638 and 536 cm^{-1} confirm the predominance of the HG structure in the studied polysaccharide (Kačuráková, Capek, Sasinková, Wellner, Ebringerová, 2000). The presence of arabinogalactorhamnoglycan structure or the hairy region in chPS-L1 is demonstrated by the characteristic signals at 1078, 1050 and 914 cm^{-1} (Kačuráková et al., 2000).

3.1.4. NMR structural studies

In Table 3 are presented ^{13}C and ^{1}H assignments for the identified structural fragments of chPS-L1 found from specific correlations in the HSQC spectrum, as well as from the COSY, TOCSY, ROESY and HMBC spectra. The NMR result interpretation was done with the help of literature data and in accordance with the monosaccharide and glycosidic linkage compositions (Shakhmatov, Atukmaev, & Makarova, 2016). The absolute configurations were assumed to be the same as already found for pectins (McNeil, Darvill, Albersheim, 1980). The detailed NMR study confirmed that chPS-L1 is a pectic polysaccharide with a predominant HG fragment with existence of methoxyl and acetyl esters, followed by a RGI branched with arabinans and AGII.

The presence of a HG fragment, revealing the 1,4-linked α-D-polygalacturonic acid in chPS-L1 is shown by the correlations H1/H4 of α-D-Gal*p*A-6-OMe units at 4.95/4.45 ppm (**A**) and H1/H4 of α-D-Gal*p*A units at 5.10 (5.05)/4.45 ppm (**B**) in the ROESY spectrum (Fig. 2). This was also proved by through bond correlations in the COSY spectrum. The C/H correlations at 55.5/3.81 ppm for methyl carbon and proton of CH_3O group, at 22.8/2.08 ppm and 22.5/2.17 ppm for methyl carbon and proton of CH_3CO group in the HSQC spectrum show the presence of methoxyl and acetyl esters in chPS-L1. The intensive through space correlations between the CO signal at 173.4 ppm and the $\underline{CH_3}O$ group at 3.81 ppm, as well as the cross-peaks at 173.4/5.05 ppm and 173.4/5.10 ppm in the HMBC spectrum indicate that the methoxyl groups are located at C6 of α-D-Gal*p*A units in the HG. The calculated DM by NMR is 62%, using the introduced by Synytsya, Čopíková, and Brus (2003) formula:

$$DM = A_{\underline{COOCH_3}}/A_{C\text{-}6\,tot.} \times 100\,(\%) \quad (1)$$

where A is the peak area and $A_{C\text{-}6\ tot.}$ includes the combined area of the C6 peak regions of GalA. The ratio between the integral intensities of the proton signals of CH_3CO- at 2.08 and 2.17 ppm is 69:31. According to the literature data the peak at 2.08 ppm is attributed to O-3 acetylation and this at 2.17 ppm to O-2 acetylation of GalA in the HG (Perrone et al., 2002). The CO signal of the acetyl group attached at O-3 of GalA was determined with the help of a

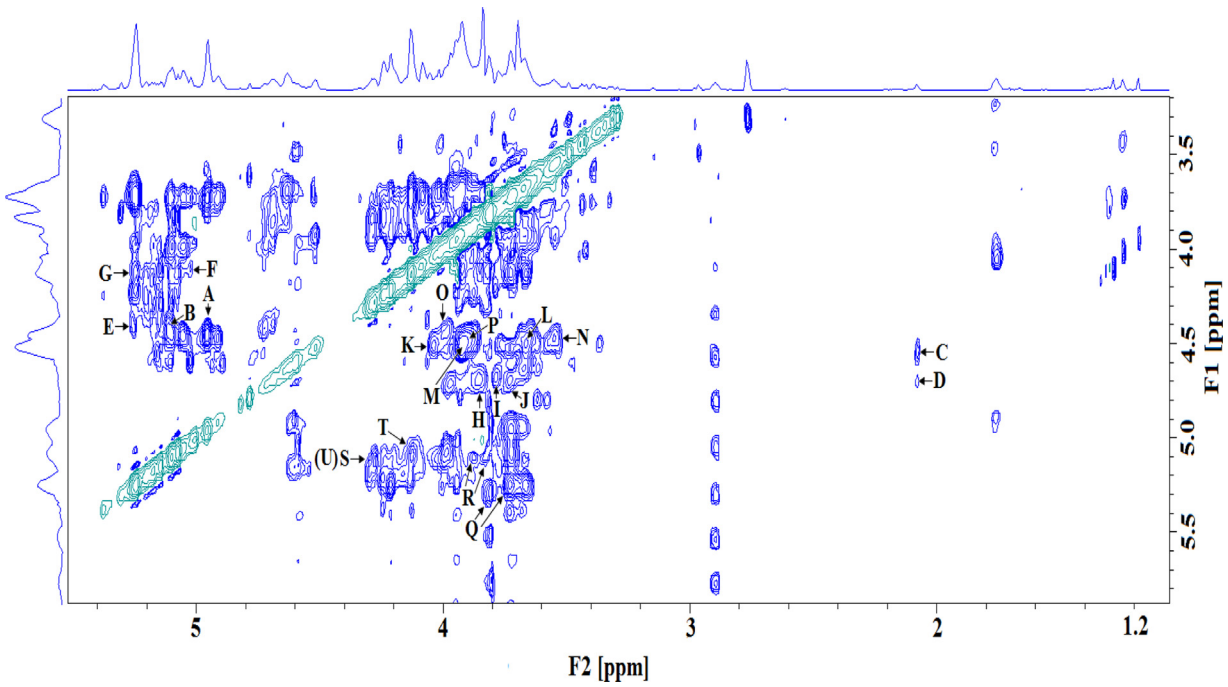


Fig. 2. $^{1}H/^{1}H$ ROESY spectrum of chPS-L1 pectic fraction. Each letter (see Section 3.1.4.) corresponds to important correlations in the HG fragment (**A**–**D**); RGI backbone (**E**–**G**); 1,3-Gal chain (**H**–**J**); 1,6-Gal (**N**–**P**); 1,3,6-Gal (**K**–**M**); 1,5-Ara (**Q**–**R**); 1,3,5-Ara (**S**–**U**).

Table 1
Yields and chemical characterization of lavender pectic fractions obtained after SEC of lavender PSC2.

	chPS-L1	chPS-L2
Yield [% of lavender PSC]	26.9	19.6
Yield [% of lavender PSC2]	35.2	25.6
Yield [% of dried flowers]	0.9	0.7
Total sugars [%]	73.7	58.5
AUAC [%]	40.8	44.9
DM[a] [mol%] (Methoxyl content [%])	63.4 (4.7)	61.0 (5.0)
DA[a] [mol%] (Acetyl content [%])	16.8 (1.7)	15.3 (1.7)
Protein content [%]	1.1	4.2
Total polyphenols [%]	0.7	2.1
LPS [%]	n.f.	n.f.
Monosaccharide composition [w/w%]		
Ara	10.1[b] (12.2)[c]	4.6 (5.7)
Rha	5.6 (6.2)	3.7 (4.2)
Fuc	0.7 (0.8)	0.9 (1.0)
Xyl	2.8 (3.4)	2.1 (2.6)
Man	1.7 (1.7)	0.5 (0.5)
Gal	14.3 (14.5)	6.5 (6.7)
Glc	4.7 (4.7)	3.7 (3.8)
GlcA	2.9 (2.8)	1.5 (1.4)
GalA	57.3 (53.8)	76.6 (73.9)
2-OMeXyl	n.f.	+
2-OMeFuc	n.f.	+
Kdo	n.d.	++
AGII	++	+
Relative molecular weight [Da]	5.24×10^4	2.18×10^4

n.d. – not determined. n.f. – not found.
[a] Calculated on AUAC basis, moles of methoxyl or acetyl groups per 100 mol of GalA.
[b] ww%, calculated on total carbohydrate content basis.
[c] mol% of total carbohydrate content.

Table 2
Glycosidic linkage composition of chPS-L1 fraction.

Glycosidic linkages	chPS-L1
T-Ara*f*	4.6[a] (5.6)[b]
1,2-Ara*f*	trace
1,3-Ara*f*	1.4 (1.7)
1,5-Ara*f*	2.8 (3.3)
1,3,5-Ara*f*	1.3 (1.5)
T-Rha*p*	1.4 (1.6)
1,2-Rha*p*	1.7 (1.8)
1,4-Rha*p*	0.1 (0.2)
1,2,4-Rha*p*	2.4 (2.6)
T-Gal*p*	3.3 (3.4)
1,3- Gal*p*	2.0 (2.1)
1,6-Gal*p*	2.1 (2.2)
1,3,6-Gal*p*	6.8 (6.9)
T-Glc*p*	1.9 (1.9)
1,4-Glc*p*	2.8 (2.8)
T-Glc*p*A	0.7 (0.6)
1,4-Glc*p*A	1.7 (1.7)
1,2,4-Glc*p*A	0.5 (0.5)
T-Gal*p*A	4.7 (4.5)
1,4-Gal*p*A	50.4 (47.3)
1,3,4-Gal*p*A	2.2 (2.0)

[a] w/w%, calculated on total carbohydrate content basis.
[b] mol% of total carbohydrate content.

correlation at 176.2/2.08 ppm in the HMBC spectrum. The proton sequence within α-D-Gal*p*A-3-OAc unit was determined according to Perrone et al. (2002) by the following correlations: 2.08/4.52 ppm (**C**) and 2.08/4.72 ppm (**D**) in the ROESY spectrum, revealing interactions between the $\underline{CH_3}CO$- with H4 and H5 of GalA, respectively; 176.2/4.09 ppm and 176.2/4.52 ppm in the HMBC spectrum, showing through space interaction between the $CH_3\underline{C}O$- with H2 and H4 of GalA; finding of the adjacent protons in the COSY spectrum and checking the correlations in the TOCSY spectrum. The gross calculation of DA of chPS-L1 by NMR is 15.4%, using the formula (Synytsya, Čopíková, Matějka et al., 2003):

$$DA = A_{O\underline{C}OCH_3} / A_{C\text{-}6\,tot.} \times 100\,(\%) \quad (2)$$

Table 3
^{13}C and ^{1}H NMR chemical shift assignment (δ in ppm) of chPS-L1 pectic fraction, referenced to DSS.

Residue	C-1	C-2	C-3	C-4	C-5	C-6	$\underline{CH_3}CO$[b]
	H-1	H-2	H-3	H-4	H-5:5′	H-6:6′	
→4)-α-Gal*p*A-6-OMe-(1→	102.7	70.6	71.4	81.5	73.2	173.4[a]	
	4.95	3.72	4.00	4.45	5.10; 5.05		
→4)-α-Gal*p*A-3-OAc-(1→	n.d.	71.6	73.5	79.3	74.0	177.2	22.8
	5.31	4.09	5.14	4.52	4.72		2.08
→4)-α-Gal*p*A-(1→	102.0	70.6	71.4	81.5	74.0	177.2	
	5.10	3.75	4.00	4.45	4.72		
→4)-α-Gal*p*A-(1→2	100.3	71.4	n.d.	81.5	74.4	177.4	
	5.02	3.93	4.13	4.45	4.69		
→2)-α-Rha*p*-(1→	101.7	79.3	71.9	74.7	71.9	19.2	
	5.25	4.13	4.00	3.44	3.72	1.25	
→2,4)-α-Rha*p*-(1→	101.7	79.3	72.6	82.8	72.1	19.4	
	5.25	4.13	4.09	3.67	3.82	1.30	
α-Ara*f*-(1→	111.8	84.0	79.4	86.7	64.1	–	
	5.25	4.21	3.98	4.09	3.72; 3.81		
→3,5)-α-Ara*f*-(1→	110.2	n.d.	85.1	83.6	69.6	–	
	5.10	4.28	4.13	4.29	3.81; 3.93		
→5)-α-Ara*f*-(1→	110.2	84.0	79.4	85.1	68.4	–	
	5.11	4.13	4.03	4.21	3.81; 3.87		
→3)-β-Gal*p*-(1→	106.1	72.9	84.8	71.1	n.d.	63.9	
	4.69	3.78	3.87	4.24	3.73	3.81	
→6)-β-Gal*p*-(1→	106.1	74.7	75.5	71.3	n.d.	63.9	
	4.45	3.54	3.67	3.98		3.93	
→3,6)-β-Gal*p*-(1→	106.1	73.2	83.6	71.4	76.4	72.6	
	4.51	3.67	4.03	4.13	3.93	3.93; 4.03	

[a] -O$\underline{CH_3}$ δ at 55.5/3.81 ppm.
[b] $CH_3\underline{CO}$- δ at 176.2 ppm.

where A_{OCOCH_3} presents the area of the peak at 176.2 ppm, as found in the HMBC spectrum. This result is in agreement with the colorimetric determination of DA (16.8%).

The following important through space cross-peaks are also found in the ROESY spectrum (Fig. 2): H1 of 1,2(,4)-α-L-Rha*p* and H4 of 1,4-α-D-Gal*p*A residues at 5.25/4.45 ppm (**E**); H2 of α-L-Rha*p* and H1 of α-D-GalpA residues at 4.13/5.02 ppm (**F**); H1 of α-L-Rha*p* with H3 of α-D-Gal*p*A units at 5.25/4.13 ppm (**G**). These correlations confirm the presence of the RGI backbone structure [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→]$_n$ in chPS-L1. The ratio between the integral intensities of H6 of 1,2:1,2,4-α-L-Rha*p* gives 45:55, showing the prevalence of O-4 substituted Rha in agreement with the glycosidic linkage results.

The anomeric proton at 4.69 ppm shows through bond correlation peaks between H1/H3 (4.69/3.87 ppm) (**H**), H1/H2 (4.69/3.78 ppm) (**I**) and H1/H5 (4.69/3.73 ppm) (**J**) within β-D-Gal*p* residues in the ROESY spectrum confirming the presence of [→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→]$_n$ fragment (Makarova, Patova, Shakhmatov, Kuznetsov, & Ovodov, 2013). Additionally, the through space interaction between C1 and H6 of β-D-Gal*p* units at 106.1/3.93 ppm in the HMBC spectrum demonstrates 1,6-glycosylation in the galactan chains. The presence of 3,6-galactan structure was elucidated by the COSY correlations between the adjacent protons and the following interactions in the ROESY spectrum: H1/H3 (4.51/4.03 ppm) (**K**), H1/H2 (4.51/3.67 ppm) (**L**) and H1/H6 (4.51/3.93 ppm) (**M**) of the β-D-Gal*p* units. Similarly, the found adjacent proton correlations for 1,6-β-D-Gal*p* units in the COSY spectrum and H1/H2 (4.45/3.54 ppm) (**N**), H1/H4 (4.45/3.98 ppm) (**O**) and H1/H6 (4.45/3.93 ppm) (**P**) of the same units in the ROESY spectrum demonstrate the presence of [→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→]$_n$ fragment (Shakhmatov, Toukach, Kuznetsov, & Makarova, 2014). Generally, the 3,6-galactans in pectins are linked to O-4 of α-L-1,2,4-Rha*p* through the 1,3-galactan backbone (Voragen et al., 2009). The ROESY spectrum also shows the presence of [→5)-α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→]$_n$ structure by the cross-peaks 5.25/3.81;3.72 ppm (**Q**) for H1/H5 of the terminal Ara*f* residues and 5.10/3.87;3.81 ppm (**R**) for 1,5-Ara*f* residues (Shakhmatov et al., 2014). Furthermore, the correlations H1/H2 (5.10/4.28 ppm) (**S**); H1/H3 (5.10/4.13 ppm) (**T**) and H1/H4 (5.10/4.29 ppm) (**U**) for 1,3,5-substituted Ara*f* units are found in the ROESY spectrum in addition to the respective COSY correlations (Makarova et al., 2013). This confirms the presence of 1,3,5-branching of the Ara*f* units most probably presenting the 1,3,5-arabinans attached to O-4 of α-L-1,2,4-Rha*p* units (Voragen et al., 2009). The presence of terminal α-L-Ara*f* units linked to O-6 and/or O-3 of β-D-Gal*p* residues within the 3,6-galactans is also suggested because of the positive reaction of chPS-L1 with the β-glucosyl Yariv reagent.

3.2. Immunomodulatory effect

chPS-L1 and chPS-L2 were tested for presence of LPS, which are considered to be contaminants that might influence the biological activity of studied compounds. It appears that there is no LPS contamination in the two fractions because the LPS marker 3-hydroxy tetradecanoic acid (3-OH-C14:0) was not detected (de Santana-Filho et al., 2012).

3.2.1. Complement fixation activity

The IC_{50} values of the pectic fractions chPS-L1 and chPS-L2 via the classical pathway were 94.0 and 456.3 μg/mL, revealing the higher activity of the first fraction (Fig. S2). The IC_{50} values of chPS-L1 and chPS-L2 through the alternative pathway were 38.9 and 689 μg/mL, as LPS were not detected in both fractions. It was important to be tested the complement fixation activity of the polyphenolic fraction chPP-L, because it practically accompanies polysaccharides in tea and water extracts. The IC_{50} values of chPP-L were 86.9 and 539.4 μg/mL via the classical and alternative pathways, respectively. In this case polyphenols expressed a similar effect to the most active chPS-L1, which means that they could also contribute to the observed polysaccharide activity in a proper combined formulation.

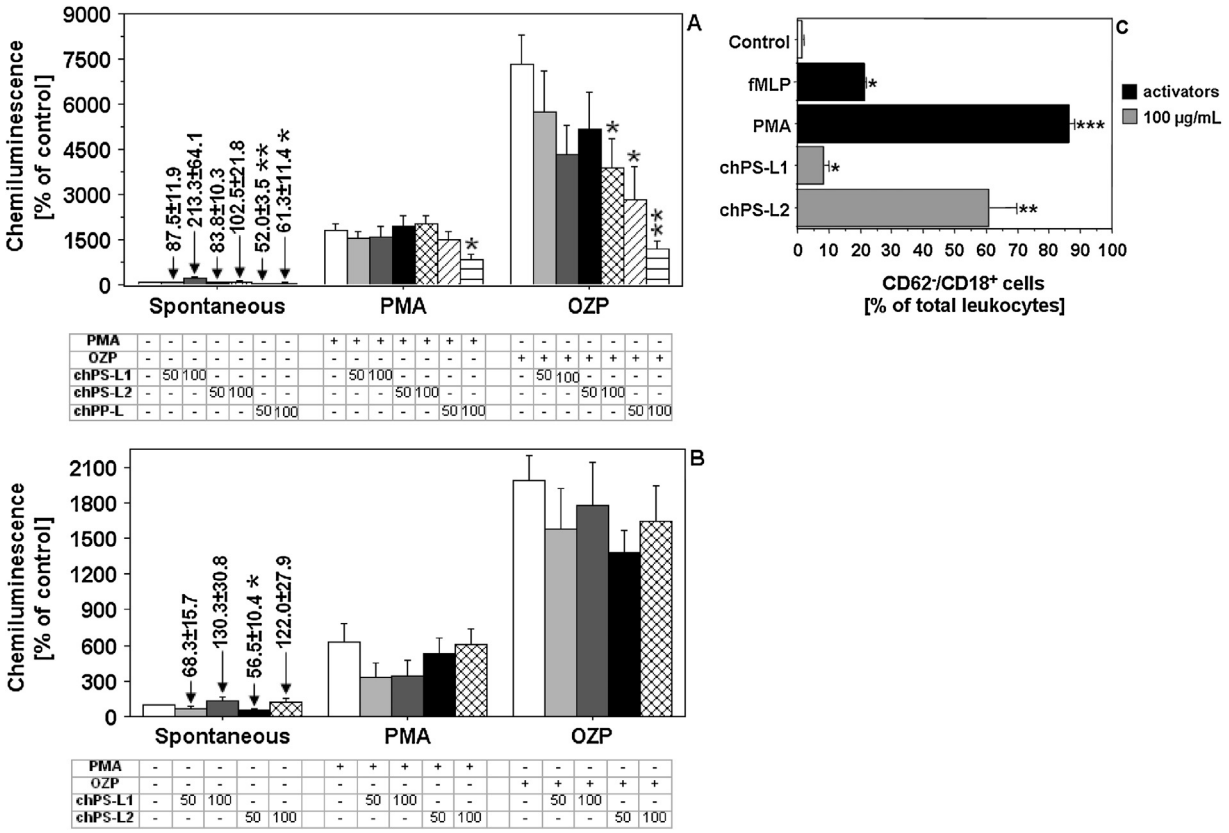


Fig. 3. Effect of lavender pectic (chPS-L1 and chPS-L2) and polyphenolic (chPP-L, only in A) fractions on **A**) ROS production from non-stimulated (spontaneous), PMA- and OZP-activated human whole blood phagocytes. **B**) ROS production from isolated neutrophils. **C**) Proportion of $CD62L^{-}/CD18^{+}$ population of total human neutrophils treated with lavender pectins. The asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) vs. control. 50 (50 μg/mL) and 100 (100 μg/mL).

3.2.2. *Effects on phagocytic leukocytes*

3.2.2.1. *Human whole blood and isolated neutrophils.*

The effect of lavender fractions on WBP and isolated neutrophils was evaluated *ex vivo* by the change of CL emitted by the cells in response to ROS formation. From both studied concentrations of the three samples, only chPS-L1 enhanced the spontaneous ROS generation from WBP at the higher dose of 100 μg/mL (Fig. 3A). Its effect was negligible in comparison with the independent PMA and OZP treatments alone. Under the co-stimulation with PMA, chPP-L expressed an inhibitory activity against the ROS production in comparison with the PMA control. This inhibition was observed also in the absence of activator and it could be explained by the ROS-scavenging properties of the polyphenols. Interestingly, under the co-stimulation with OZP, the three lavender samples showed an inhibitory activity against the ROS formation, as it was statistically significant for chPS-L2 and chPP-L at 100 μg/mL.

At the higher concentration, chPS-L1 and chPS-L2 slightly increased the CL response of isolated human neutrophils, but again their effect was much lower in comparison with the effect of PMA and OZP activators (Fig. 3B). Because the activity of chPP-L on generation of free radicals was predictable, it was not investigated further. Both pectic fractions did not increase the CL response of PMA-activated cells and chPS-L1 showed an evident inhibitory activity. The observed inhibitory effect of chPS-L2 on the OZP-treated WBP was also seen with isolated neutrophils, but it was not so clearly expressed like in the blood environment. Furthermore, both pectic fractions exhibited a statistically significant induction of the neutrophil activity, which was accompanied with an increased expression of CD18 and a decreased expression of CD62L (Figs. 3 C & S3). Interestingly, the effect of chPS-L2 was much higher than those of fMLP. It was proposed that both polysaccharide fractions could interact with CD18. Our findings suggest that lavender pectins are active ingredients in the crude lavender PSC, affecting ROS production of human WBP and neutrophils.

3.2.2.2. *Murine macrophages.*

The effect of the lavender pectins on macrophages was estimated *in vitro* by measurement the production of NO from treated cells. The pectin behavior in a simulated macrophage infection by a co-stimulation with LPS was also studied. Firstly, both polysaccharide fractions increased in a similar manner the expression of iNOS after 24 h of incubation (Fig. 4A). The enzyme expression in the growth medium control was negligible, which showed that the cells were not artificially activated by the plate surface. As was proposed, under the co-stimulation with LPS the induction was higher than this for pectin-treated cells. Contrary to chPS-L1, chPS-L2 did not increase the iNOS levels of the independent polysaccharide and combined with LPS treatments at the higher concentration of 100 μg/mL. After 48 h of incubation, the effects on the iNOS expression of both fractions alone or in the combinations with LPS were comparable (Fig. 4A). chPS-L2 again did not increase its induction ability at 100 μg/mL, as the iNOS level was the highest at 50 μg/mL with LPS. The enzyme expression with this fraction at the two border points – 50 μg/mL alone and under the co-stimulation with LPS at 100 μg/mL was almost identical.

There was no a significant difference between the activity of the two lavender polysaccharides, concerning stimulation of NO production after 24 or 48 h. However, chPS-L2 showed a negligible prevalence after 24 h (Fig. 4B). Furthermore, the enhancement of NO production induced by chPS-L1 or chPS-L2 at 100 μg/mL after 48 h of culturing were 9 or 6 fold (on mean value basis) higher than the corresponding NO release after 24 h. These results demon-

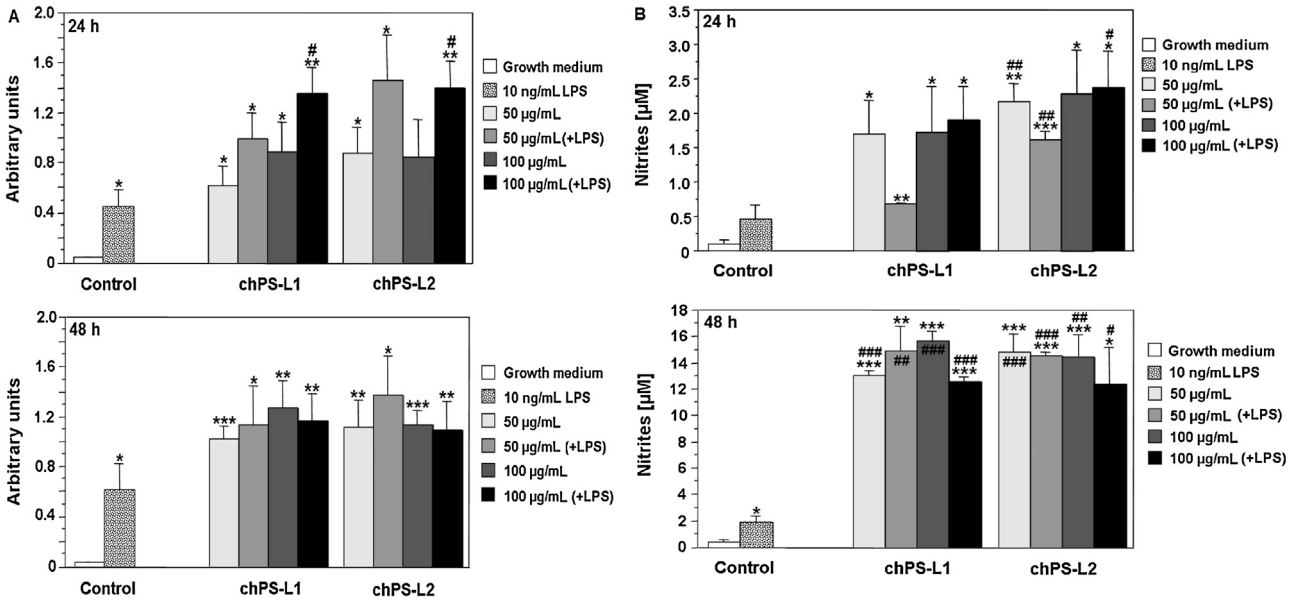


Fig. 4. Effect of lavender pectic fractions on A) iNOS protein expression in murine macrophages Raw 264.7 cells cultured with samples alone or co-stimulated with LPS. B) NO production from murine macrophages cultured with samples alone or co-stimulated with LPS. LPS from *E. coli* were used as a positive control. The asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) vs. control – untreated cells (growth medium) and (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$) vs. LPS.

strate that lavender pectins similarly to other already investigated polysaccharides could unlock macrophage activation. Additionally, lavender pectins were not toxic for the macrophages (Fig. S4). Under the conditions of co-stimulation with LPS and both fractions, the NO production increased at the higher concentration of pectins used after 24 h. After 48 h, the macrophage stimulation with chPS-L1 did not lead to the same effect, as at 100 μg/mL the NO level was decreased in the presence of LPS and elevated in the absence of LPS. Generally, it was observed that combining 100 μg/mL lavender pectins with 10 ng/mL LPS did not lead to a synergistic elevation of the iNOS expression and NO production after 48 h. Interestingly, the results from the LDH test for co-stimulation of macrophages with 100 μg/mL pectins and 10 ng/mL LPS after 48 h gave absorbance values close to that from the control cells (Fig. S4).

3.2.3. *Intestinal immunomodulating activity*

Normally, polysaccharides are administered orally (e.g. tea, folk remedies and food) and they can interact with the intestinal immune system through different immunocompetent cells localized in the PP, including the phagocytes. We investigated the intestinal immunomodulating activity of the lavender polysaccharides by the PP-mediated bone marrow cell proliferation test (Fig. 5). It was found that both lavender fractions express intestinal immunomodulating activity ($p < 0.01$) comparable to that of the positive control AMOL-1. This result confirmed that lavender pectins are involved in the exhibited PP stimulating activity of the crude lavender PSC (Georgiev, Ognyanov, Kiyohara et al., 2017).

4. Discussion

The lavender pectic polysaccharides are chemically characterized with predominant HG fragments that are acetylated (<20%) and highly methoxylated (>60%), followed by smaller RGI domains, which are extremely branched with arabinans and AGII structures. chPS-L1 has a higher Mw and AGII content than chPS-L2, which could explain its higher complement fixation and intestinal immunomodulating activities (Yamada & Kiyohara, 1999; 2007). chPS-L2 is immune active on PP cells and phagocytes and it has a lower Mw than chPS-L1 and contains monosaccharides characteristic for RGII. It exhibited very potent stimulating activity against CD18 expression (Figs. 3 C & S3).

Interestingly, chPS-L1 expressed a high complement fixation activity through both tested pathways, but it was more potent through the alternative pathway. Therefore, it was suggested that the complement activation by chPS-L1 was not only antibody-dependant, but it could be a result of a direct polysaccharide (AGII) interaction with C3 component (C3b), initiating the alternative pathway (Michaelsen, Gilje, Samuelsen, Høgåsen, & Paulsen, 2000; Kiyohara, Matsumoto, Nagai, Kim, & Yamada, 2006). In fact, Wang et al. (2016) found that pectic fraction Fs-8-ba2 from *Forsythia suspensa* selectively interacts with C1q, C1r, C1s, C2, C3 and C9, but not with C4 and C5 complement proteins.

It was supposed that the high complement fixation activity of chPS-L1 would reflect on the phagocyte immune response, because the fraction could activate antibody-initiated and direct C3-initiated complement system (Leung et al., 2006). chPS-L1 and chPS-L2 induced CD18 expression on isolated neutrophils (Fig. 3C). Thus, the inhibitory effect of lavender pectins on the OZP-activated ROS production from WBP (Fig. 3A) could be due to their direct interaction with CD18 and the complement fixation activity developed in the tested blood environment. The CD18 is a constructive unit of CR3, CR4 and LFA-1 receptor expressed on leukocytes. CR3 is expressed on phagocytes and involved in the phagocytosis via iC3b-bound antigens. Zymosan is a complement activator via the alternative pathway by binding to (i)C3b and it can interact with CR3 on neutrophils inducing phagocytosis and superoxide generation (Fearon & Austen, 1977; Ross & Větvička, 1993). OZP (62.5 μg/mL) and the pectins (50 and 100 μg/mL) could compete for the neutrophil receptor recognition. The inhibitory effect of lavender pectins on the OZP-activated ROS generation from isolated neutrophils was less pronounced probably because of the lack of blood environment containing complement components and immunoglobulins (Fig. 3B). Pectins (100 μg/mL) induced higher CL responses with WBP than with isolated neutrophils. Conversely, chPS-L1 expressed a better inhibitory effect against the PMA-activated ROS generation with isolated neutrophils. PMA induced the CD18 expression, as it can bind to CR3, initiating phagocytosis and oxidative burst (Ross & Větvička, 1993). Thus, chPS-L1 could compete with PMA for a direct CD18 binding.

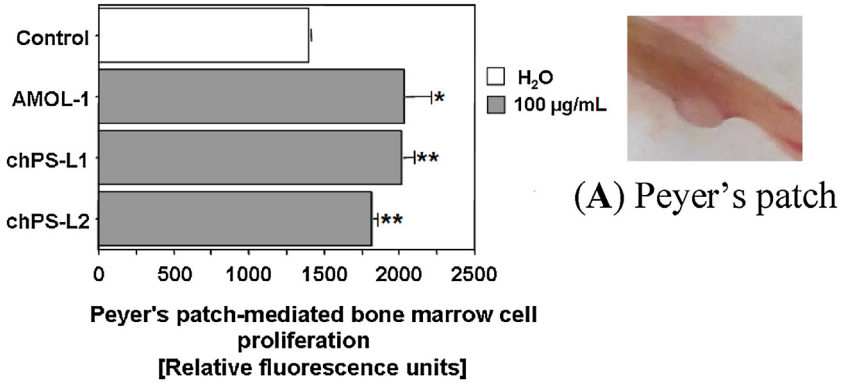


Fig. 5. Enhancement of production of bone marrow cell-proliferating cytokines from Peyer's patch cells cultured in presence or absence of lavender pectic fractions. The asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$) vs. water control. AMOL-1 is an active pectic polysaccharide from *A. mongholics* used as a positive control. (**A**) is a picture of a Peyer's patch.

The elevation of iNOS levels and NO production induced by the lavender pectins confirmed that they could stimulate the macrophage activation. Therefore, the lavender polysaccharides are at least partly responsible for the observed enhancement of $CD14^+$ monocytes and $CD64^+$ cell population (phagocytes) provoked by the lavender PSC (Georgiev, Ognyanov, Kiyohara et al., 2017). CD64 is a marker for Fc-γ receptor 1 expressed on macrophages, which can be activated by the pectic ramified RGI regions, which are also present in the studied pectins (Matsumoto et al., 1993). In a previous study it was found that *T. tomentosa* pectins exhibit suppression-like effect on LPS-induced iNOS expression and NO production from macrophages (Georgiev, Paulsen et al., 2017). chPS-L1 (100 µg/mL) showed a clear reduction of the NO generation under the co-stimulation with LPS in comparison with its separate action after 48 h. According to Chen et al. (2006), the observed phenomenon could be a result from a pectin-LPS interaction or a competition for the receptor binding (TLR4), but not a pectin inhibition of the LPS-signaling protein activities in the macrophages. Gallet et al. (2009) found that pectic hairy regions inhibiting the LPS-induced NO production in J774.2 macrophages reduced the TNF-α production stimulated by LPS. Therefore, chPS-L1 may exhibit anti-inflammatory effects in the simulated microbial infection, regulating the synthesis of inflammatory molecules. This regulation could result in a reduction of the NO generation, which was initially induced by chPS-L1 alone.

Pectins are little absorbed by the intestinal epithelium, but they may interact with it and most importantly with the mucosal immune system through immunocompetent cells in the PP. Functionally modulated immunocompetent cells by polysaccharides are proposed to result in modulations of several mucosal and systemic immune systems (Yamada & Kiyohara, 2007). Undoubtedly, the complement fixation activity and the stimulatory effect on the neutrophils and macrophages of lavender pectins contributed to the PP-mediated bone-marrow cell proliferation. However, lavender PSC stimulated not only phagocytes, but also $CD4^+/CD25^+$ and $CD8^+/CD25^+$ T cells that constitute about 10% and 5% of PP cells, as 5–30% of $CD4^+$ T-cells in the mouse bone marrow are $CD4^+/CD25^+$ Treg (Georgiev, Ognyanov, Kiyohara et al., 2017; Jung, Hugot, & Barreau, 2010; Zhao et al., 2012). Therefore, in the intestinal immunomodulating activity of lavender pectins might be involved also T-cells. And as well, the CD18 expression, which was found to be induced by the lavender pectins is important for establishment of the intestinal mucosal T-cell compartment (Huleatt & Lefrançois, 1996).

Investigation of the type of attached polyphenols to pectin enables a better understanding of the chemistry of the polyphenol-polysaccharide complexes and the polysaccharide affinity to different bioactive polyphenols. In a previous study, it was found that pectic polysaccharides in rosehip are at least partly involved in the observed synergism in ORAC antioxidant activity after combining rosehip with other herbal extracts (Kratchanova, Denev, & Kratchanov, 2014). The physico-chemically attached polyphenols to pectin have valuable physiological activities in the human gastrointestinal tract (Jakobek, 2015). From a technological point of view, the observed affinity of catechin and epichatechin to the studied polysaccharides reveals the pectin potential for reduction of catechin astringency in drinks. Interestingly, Slavov, Kiyohara, and Yamada, (2013) showed that distilled rose petals from *Rosa damascena* contain pectins with intestinal immunomodulating activity. Méndez-Tovar, Herrero, Pérez-Magariño, Pereira, and Asensio-S.-Manzanera (2015) reported that a distilled lavender waste could serve for recovery of polyphenolic antioxidants. Therefore, the lavender by-product could be subjected to a further extraction of bioactive non-volatile compounds for production of infusion formulations and nutraceuticals with immunomodulating and antioxidant activities.

5. Conclusion

The detailed chemical analysis revealed that chPS-L1 and chPS-L2 are acetylated and highly methoxylated pectic type polymers with a predominant HG fragment, followed by a RGI with AGII structural motifs. Our findings with chPS-L1 and chPS-L2 confirmed that lavender pectins are at least partly responsible for the observed *ex vivo* and *in vitro* effects for lavender PSC. The lavender pectins activated innate and adaptive immune response through the complement system, WBP, neutrophils, macrophages and PP immunocompetent cells. Therefore, they could be considered as immunomodulating compounds in the lavender flowers. The observed inhibitory effects of chPS-L1 against the PMA- and OZP-activated ROS production, and the suppression of NO generation under the co-stimulation between chPS-L1 and LPS suggested anti-inflammatory activity of the studied pectins. chPP-L showed a high complement fixation activity via the classical pathway and was effective against the PMA and OZP inflammatory activation of WBP. The proper formulation or individual use of lavender polysaccharides and polyphenols could be of benefit in the supplementary treatment of a compromised immune system and control of inflammation. Furthermore, different immunomodulating and antioxidant nutraceutical formulations could be developed on the basis of the polysaccharide and polyphenol interaction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2017.07.011.

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