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Coffea arabica instant coffee—Chemical view and immunomodulating properties

Peter Capek[a,*], Ema Paulovičová[b], Mária Matulová[c], Danica Mislovičová[a], Luciano Navarini[d], Furio Suggi-Liverani[d]

[a] Institute of Chemistry, Department of Glycomaterials, Center for Glycomics, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia
[b] Institute of Chemistry, Department of Immunochemistry of Glycoconjugates, Center for Glycomics, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia
[c] Institute of Chemistry, Department of Analytical, Center for Glycomics, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia
[d] Illycaff s.p.a., Research & Innovation, via Flavia 110, I-34147 Trieste, Italy

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ABSTRACT

Results of chemical analyses and immunological studies of two *Coffea arabica* instant coffee powders obtained by freeze-dried (ICPf) and spray-dried (ICPs) procedures, and arabinogalactan-protein (AGP3) obtained from ICPf are presented. For instant coffee powders no significant differences have been found in carbohydrate (ICPf: 37%, ICPs: 38%) as well as in caffeine (ICPf: 3.0%, ICPs: 3.4%) contents. Their ^{1}H NMR spectra revealed differences in trigonelline and chlorogenic acids content and in a degree of AGP backbone substitution. Immunobiological tests of all samples (ICPf, ICPs, AGP2 and AGP3) revealed a significant immunostimulatory effect on induction of interleukin 2 and free radicals secretion by mice immunocytes. Moreover, tests revealed more pronounced effect of arabinogalactans AGP2 and AGP3 compared to instant coffee powders (ICPf and ICPs).

1. Introduction

In addition to roasted coffee beans and roasted and ground coffee, soluble or instant coffee is a form of coffee available to consumers. This coffee product is appreciated thanks to its practicity and cleaness in preparing beverages and to its long shelf life. According to the European Coffee Report (2011/12), soluble coffee exports and imports to and from non-European destinations represent currently around 41.0 and 41.2 thousand tons, respectively, per year in Europe (European Coffee Report, 2011). World soluble coffee trade has been growing very rapidly since 2000, suggesting an increase in consumption of this form of coffee, particularly in emerging markets (International Coffee Organization, 2013). Easyness in the preparation of drinks ensured by soluble coffee is counterbalanced by the poorness of aroma in the resulting beverages if compared with those prepared by fresh roasted and ground coffee. In the production of instant coffee, *Coffea canephora* (*robusta*) is preferred due to its economical attractiveness and higher extraction yield, however, *C. arabica* is also used to ameliorate the flavor and to make it more aromatic in the attempt to remedy the intrinsic lower aroma intensity of soluble coffee (Clarke & Macrae, 1987). Coffee consumption is widespread throughout the world and it is estimated that coffee brew is the second most largely consumed beverage after water. This fact, is largely due not only to pleasant organoleptic properties but also to positive physiological effects and it is also associated with social habits and country cultures.

Coffee has been subjected to extensive scientific research in order to ascertain its impact on human health (Illy & Viani, 2005). Moderate coffee consumption shows links to a positive impact being a large body of evidence in favor to benefits rather than to risks associated to this habit (Chu, 2012). Although in the past coffee has been considered just a flavorful caffeine aqueous solution nowadays, in view of observed physiological effects which cannot be related to the presence of caffeine only, the chemical complexity of this beverage is emerging. In this regards, coffee has been found to be an extremely rich dietary source of antioxidants (chlorogenic acids, hydroxycinnamic acids, caffeine and Maillard reaction products, such as melanoidins) and it represents the main contributor to the antioxidant capacity of the diet in many countries (Natella & Scaccini, 2012; Torres & Farah, 2010). In addition to antioxidant compounds, a number of studies report on coffee constituents other than caffeine, characterized by interesting biological

* Corresponding author. Tel.: +421 2 59410209; fax: +421 2 59410 222.
E-mail address: chemcape@savba.sk (P. Capek).

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activities. Such studies, however, have been aimed at investigating mostly roasted coffee, being very scarce the reports focused to soluble coffee (Alves, Mendes, Oliveira, & Casal, 2010; da Silveira, Tavares, & Gloria, 2007; Oliveira et al., 2012) and more frequent the inclusion of some instant coffee sample in investigations on coffee brews (Silva, Borges, Santos, & Alves, 2012).

Coffee brew also contains a significant amount of soluble dietary fiber (SDF), higher than other common beverages. This SDF is made up of indigestible polysaccharides (with an appreciable amount of associated polyphenolic antioxidants) (Niseteo, Komes, Belscak-Cvitanivic, Horzic, & Budec, 2012). For this reason, among the coffee bioactive compounds, attention has also been devoted to polysaccharides. As major components of the dietary fiber isolated from coffee brews, type II arabinogalactans and galactomannans have been shown to have beneficial biological activities due to rapid fermentation in the human colon and thereby contributing to the intestine health (Gniechwitz, Reichardt, Blaut, Steinhart, & Bunzel, 2007; Gniechwitz, Brueckel, et al., 2007; Reichardt, Gniechwitz, Steinhart, Bunzel, & Blaut, 2009). Beyond the prebiotic activity and dietary fiber properties of coffee polysaccharides, isolated fractions of type II arabinogalactan, arabinogalactan-proteins (AGPs) and galactomannan showed another important biological attribute: the immunostimulatory activity. In particular, type II arabinogalactan fraction isolated from green coffee beans, enhanced proliferation of splenocytes and peritoneal macrophages and activated production of T_H1-type cytokines, IL-12 and IFN-γ. The same polymer tested in vivo raised the level of IL-12 in mouse plasma (Gotoda et al., 2006) and it has been suggested that arabinogalactan from coffee beans inhibits dermatitis sensitized with TNCB by enhancing Th1 responses, and that arabinogalactan treatment could provide an effective activity for the allergic reactions (Furuya et al., 2008). These findings support previous studies on plant arabinogalactans bioactivity. In facts, several potent immunomodulatory properties of arabinogalactans were demonstrated with human and murine macrophages and enhanced liberation of both pro-inflammatory (IL-1α, IL-1β, IL-6, IL-12 and TNF-a) and anti-inflammatory (IL-10) cytokines was observed (Schepetkin & Quinn, 2006). Coffee (acetylated)-galactomannan fractions isolated from roasted and ground coffee infusions as well as coffee residue have been shown to be capable to induce the in vitro expression of the surface lymphocyte activation marker CD69 on murine B (B220) and $CD4^+$ and $CD8^+$ T lymphocytes (Simoes et al., 2009). As far as soluble coffee polysaccharides biological activity is concerned, antitussive and immunomodulating activities of instant coffee arabinogalactan-protein (AGP1), have been recently reported (Nosál'ová et al., 2011). In particular, immunological tests have shown that AGP stimulated the splenocytes' secretion of TNF-α, IFN-γ and IL-2 cytokines (Nosál'ová et al., 2011).

To become soluble, coffee undergoes a complex manufacture process. The beans, after selection and possible blending, are roasted, ground and subjected to successive percolation with water at temperatures up to 180 °C under pressure. The aqueous extract is then concentrated and dried. Any step in the industrial process may affect not only the sensory properties of the final product (it is well known that working conditions aimed at increasing the process yield are detrimental for the organoletic characteristics) but also the molecular integrity of the coffee polysaccharides (Redgwell, Trovato, Curti, & Fischer, 2002). In spite of the wide range of possible industrial conditions which can be adopted to manufacture soluble coffee, the final product is normally classified according to the drying process. In particular this process can be carried out according to two different technological processes: freeze- and spray-drying, and then two types of instant coffee powders are available nowadays on market: freeze- and spray-dried soluble coffees. In addition to AGP isolated from freeze dried instant coffee (Nosál'ová et al., 2011) possible immunomodulating properties of degraded products and (acetylated)-galactomannan oligosaccharides, formed during the instant coffee production process, cannot be excluded a priori. In this regard, a different behavior of instant coffee is also plausible depending on the last drying step in its manufacturing process: freeze or spray-drying, being the latter more degradative than the former.

In the present work, *C. arabica* freeze and spray-dried instant coffee powders and arabinogalactan-proteins isolated from freeze-dried instant coffee powder have been examined in term of their molecular distribution patterns, carbohydrate and protein contents, and their immunomodulating properties.

2. Materials and methods

2.1. Plant material

Coffea arabica blend for espresso coffee (100%) was dark roasted (average roasting weight loss of 19%, w/w) and industrially processed (grinding to particle size of 2.3–2.4 mm with 10% particles less than 1 mm; extracted at 150–180 °C; concentrated under vacuum at 50 °C) and freeze dried to give a freeze-dried instant coffee powder (ICPf). It was further used for isolation of arabinogalactan-protein (AGP3).

Same coffee blend but a different batch was dark roasted (average roasting weight loss of 16%, w/w) and industrially processed (grinding to particle size of 2.3–2.4 mm with 10% particles less than 1 mm; extracted at 150–180 °C; concentrated under vacuum at 50 °C) and spray dried under conventional conditions without further agglomeration step to give a spray-dried instant coffee powder (ICPs).

Caffeine contents in soluble coffee products ICPf and ICPs were determined according to ISO 20481:2008 method. Briefly: 1 g of sample is placed in a 250 mL flask, with 5 g of $MgSO_4$ and filled with Milli-Q water. After 20 min of extraction at 90 °C the flask is lead up to room temperature and after filtration an aliquot of 1 mL solution is analyzed via HPLC. A 1100 HPLC system (Agilent, Germany) was used, consisting of degasser, quaternary pump, column thermostat and diode array detector (DAD) operating at 272 nm. A XTerra MS C18 column, 5 μm 150 mm × 4.60 mm (Waters, USA) and isocratic elution (water/methanol 76/24, v/v) were used.

2.2. Isolation of arabinogalactan-protein

Arabinogalactan-protein (AGP3) isolation from the instant coffee powder was performed according the same procedure as those of AGP1 and AGP2 isolates (Capek, Matulová, Navarini, & Suggi-Liverani, 2009). The instant coffee powder (75 g) was dissolved in water (750 mL), cooled to 4 °C and formic acid (40 mL of an 85% solution) was added, and the mixture was stirred overnight in a cool room. It was centrifuged at room temperature and insoluble portion was discarded. The supernatant was treated with five volumes of 96% ethanol. The light brown precipitate was removed by centrifugation and the supernatant was discarded. The brown precipitated material was washed with ethanol and dissolved in distilled water, and freeze-dried to yield a crude polysaccharide fraction A (19.8 g).

In the second step the crude polysaccharide fraction A (19.8 g) was dissolved in 0.05 M sodium hydroxide (NaOH) solution (170 mL) and precipitated by saturated aqueous bariumhydroxide solution (220 mL). The brown precipitate formed (a crude galactomannan) was removed by centrifugation at room temperature and the supernatant was treated dropwise with 1.0 N sulfuric acid until pH of 4 was achieved. The slight precipitate formed was removed by centrifugation at room temperature and discarded. The supernatant was slowly poured into five volumes of 96% ethanol. A crude arabinogalactan precipitate was removed by

centrifugation and the supernatant was discarded. The precipitate was dissolved in distilled water (500 mL) and applied on the column (50 mm × 500 mm) of Amberlite MB 150 (H^+/OH^-), and eluted with distilled water. The water eluent containing carbohydrates was concentrated and freeze-dried to give the arabinogalactan-protein (AGP3, yield 3.6 g, i.e. 4.8%).

2.3. General methods

Solutions of carbohydrates were concentrated under diminished pressure at a bath temperature under 45 °C. Freeze-dried samples were hydrolyzed with 2 M TFA at 120 °C for 1 h and the quantitative determination of the neutral monosaccharides was performed in the form of their trifluoroacetates (Shapira, 1969) by gas chromatography on a Hewlett–Packard 5890 Series II chromatograph using a PAS-1701 column (0.32 mm × 25 m) at a temperature program of 110–125 °C (2 °C/min) to 165 °C (20 °C/min) and a flow rate of hydrogen 20 mL/min. The uronic acids content was determined with the 3-hydroxybiphenyl reagent (Blumenkrantz & Asboe-Hansen, 1973). The carbohydrates content was determined by the phenol–sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Elemental analysis was performed with EA 1108 apparatus (FISONS Instruments, East Grinstead, UK). Protein amount was calculated from the nitrogen content (%N × 6.25) and by Bradford assay (Bradford, 1976).

Determination of molecular mass was performed with HPLC Shimadzu apparatus (Vienna, Austria) equipped with a differential refractometer RID-6A and a UV–vis detector SPD-10AV using the column HEMA-BIO 40 (8 mm × 250 mm) of particle size 10 μm (Tessek, Prague, Czech Republic). Phosphate buffer (0.02 M) of pH 7.2 containing NaCl (0.1 M) was used as a mobile phase at a flow rate 0.8 mL/min. A set of dextran standards (*M*w = 505, 4440, 20,500 and 62,900) was used for calibration of the column (Gearing Scientific, Polymer Lab., Hertfordshire, UK).

Fourier-transform infrared spectra (FT-IR) were obtained on a NICOLET Magna 750 spectrometer with DTGS detector and OMNIC 3.2 software, where 128 scans were recorded with 4 cm^{-1} resolution. Spectra of samples were measured in the form of KBr pellets (2 mg of a sample/200 mg of KBr).

For NMR measurements, samples were freeze-dried from 95% D_2O and dissolved in 99.98% D_2O. NMR spectra were measured at 25 °C on Varian 600 MHz UNITY INOVA 600 NB spectrometer, equipped with 5 mm multinuclear probe with inverse detection and 5 mm $^1H\{^{13}C, ^{15}N\}$PFG Triple Res IDTG600-5 with z-gradients. For both, ^1H and ^{13}C NMR spectra, chemical shifts were referenced to internal acetone (2.217 and 31.07, respectively) as a standard. ^1H ^{13}C hetero-correlated HSQC spectra were measured with optimization on one bond coupling constant of 143 Hz.

2.4. Preparation, treatment and analysis of splenic cells. ELISPOT assay

Female Balb/c mice aged 8–12 weeks were obtained from the breeding facility VELAZ, Prague (Czech Republic). In vitro experiments were conducted in compliance with GLP and OECD guidelines, according to the ethical guidelines issued by the Research Base of Slovak Medical University, Institute of Preventive and Clinical Medicine (Bratislava, Slovakia), under the approval No. Ro 2939/09-221 of State veterinary and food administration of the Slovak Republik. The animals were provided food and water ad libitum and maintained in hoods under laminar flow conditions. Spleens were removed under sterile condition and splenocytes were carefully washed out from spleens with complete RPMI-1640 (Sigma, Stockholm, Sweden) enriched with 10% fetal calf serum and 2% PenStrep (Gibco, Berlin, Germany). Cell washing was performed by centrifugation (800 × *g*/5 min, 4 °C), cell pellet was adjusted by growth medium approximately to 4×10^6 cell/mL and 100 μL aliquots were specifically stimulated with 100 μg/mL of arabinogalactans (AGP1, AGP2 and AGP3) and with ICPf (instant coffee powder – freeze dried) and ICPs (instant coffee powder – spray dried, concentrations 100 μg/mL) and plated in 24-well tissue culture plates (Nunc, Denmark). As positive control, polyclonal T-cell activator Concanavalin A (Sigma, Stockholm, Sweden) (10 μg/mL) was applied. The untreated cell suspension was used in the same protocol as baseline. Plates were incubated in humidified 37 °C CO_2 incubator for 24 h. The viability of splenocytes was determined using trypan blue dye exclusion method, before and following the specific stimulation with arabinogalactan-proteins and instant coffee powders. After then, 5×10^4 cells per well were plated for the direct production and further enumeration of interleukin 2 (IL-2) producing cells onto anti-mouse IL-2 precoated PVDF backed microplate. Following steps and procedures were according to manufacturer's recommendation (Mouse IL-2 ELISPOT, BenderMedSystems, Austria). Quantitative evaluation of spots and enumeration of IL-2 producing cells were performed via KS ELISPOT 4.10 running under AxioVision software using Imager A.1 Microscope (Zeiss, Germany).

To optimize the effective dose of immunogen, as a first step the splenocyte stimulation with several concentrations of all tested compounds has been performed. The concentration interval comprised the concentrations of AGP1, AGP2 and AGP3, and ICPf and ICPs in the range from 10 μg/mL to 1000 μg/mL (Table 3).

2.5. Determination of free radicals

Spleen cell culture supernatants following arabinogalactans (AGP1, AGP2 and AGP3) and with ICPf and ICPs treatment were assayed for total content of free radicals (Free radicals kit; Sedium R&D, Czech Republik). The assay is based on the ability of chlorophyllin to transfer electrons due to its electron-rich double-bonds structure. The quantification of free radicals was assayed using a calibration based on an Fe^{2+}/Fe^{3+}reactive shift and was expressed as mmol Fe^{2+}/L. Free radical production of unstimulated spleen cells was used to determine the baseline value.

2.6. Statistical analyses

Experimental results were calculated as means ± S.D. The normality of distribution was evaluated according to the Shapiro–Wilk's test at the 0.05 level of significance. Statistical comparisons between experimental groups were performed using one-way analysis of variance (ANOVA) and post hocBonferroni and Tukey tests. The results were significant if the difference between the analyzed groups equalled or exceeded the 95% confidence level ($P < 0.05$). Statistics were performed with ORIGIN 7.5 PRO software (OriginLab). Pearson's correlation coefficient (*R*) was used to compare the strength of the relationship between immunological parameters.

3. Results and discussion

3.1. Characterization of instant coffee powders and arabinogalactan-protein

Ripe *C. arabica* beans were industrially processed, i.e. roasted, ground, extracted, concentrated and freeze-dried to give an instant coffee powder (ICPf). Industrial process for manufacture of spray-dried instant coffee powder (ICPs) from ripe *C. arabica* beans included the same procedures excepting last one, i.e. spray drying without further agglomeration step was used to finalize an instant coffee powder (ICPs). Chemical analyses of both

Table 1
Characteristics of *Coffea arabica* freeze-dried (ICPf) and spray-dried (ICPs) instant coffee powders and arabinogalactan-proteins (AGP1, AGP2 and AGP3).

Compound	Sugar content (wt%)	Nitrogen content (wt%)	Protein content (wt%)	*Mp* (kDa)	Monosaccharide composition (wt%)						
					Rha	Fuc	Xyl	Ara	Man	Gal	Glc
ICPf	37	3.03[a]	5.9[b]	65.7; 5.4	1.0	0.1	0.6	9.7	40.2	46.7	1.7
ICPs	38	3.21[a]	6.3[b]	49.0; 3.0	1.5	0.1	0.5	14.3	33.7	48.2	1.7
AGP1[c]	84	0.25	1.6	5.4	tr.	tr.	tr.	8.2	2.7	85.0	1.4
AGP2[c]	82	0.27	1.7	5.2	tr.	tr.	tr.	8.3	2.9	85.2	1.2
AGP3	90	0.37	2.3	8.7	2.4	tr.	tr.	7.5	14.3	72.6	3.1

[a] Nitrogen content derived from protein and caffeine estimated by elemental analysis.
[b] Estimated by Bradford assay, tr. – traces.
[c] Capek et al. (2009).

commercial instant coffee powders revealed 37% and 38% of carbohydrates in ICPf and ICPs, respectively. No significant differences in carbohydrate contents in soluble coffee products were determined, although different industrial processes for their manufacture were used. Similarly, estimation of caffeine contents in both soluble coffee products did not reveal significant differences. In ICPf was determined 29.8 mg (~3.0%) and in ICPs 33.5 mg (~3.4%) of caffeine per g of soluble coffee products. Compositional analyses of ICPf and ICPs products revealed some differences in their sugar compositions (Table 1). As can be seen from Table 1, ICPf contained a higher mannose (Man) and lower arabinose (Ara) contents in comparison with that of ICPs. Besides, in ICPf product was found a higher Gal/Ara ratio (4.8:1) compared to that of ICPs (3.4:1). These findings are consistent with the different degree of roast of the two processed roasted coffees, even if both may be considered in the "dark" range of roasting degree. In facts, in view of the average water content of the processed green coffee, the organic weight loss of the roasted coffee used to produce ICPf (10%, w/w) is higher then that used to produce ICPs (8%, w/w). HPLC measurements showed similar molecular mass distribution patterns of both coffee products, although different industrial procedures for their manufacture were used (Fig. 1). From freeze-dried instant coffee powder (ICPf) the arabinogalactan-protein (AGP3) has been isolated according the Wolfrom procedure (Wolfrom & Anderson, 1967) with some modifications (Capek et al., 2009). AGP3 was recovered in 4.8% yield on a starting ICPf material. Sugar compositional analysis of AGP3 (Table 1) showed lower arabinose and galactose content, however, much higher mannose content compared to those of AGP1 and AGP2 isolates, already described (Capek et al., 2009). Besides, AGP3 showed a higher molecular mass and higher degree of polydispersity compared to those of AGP1 and AGP2 isolates (Table 1, Fig. 1).

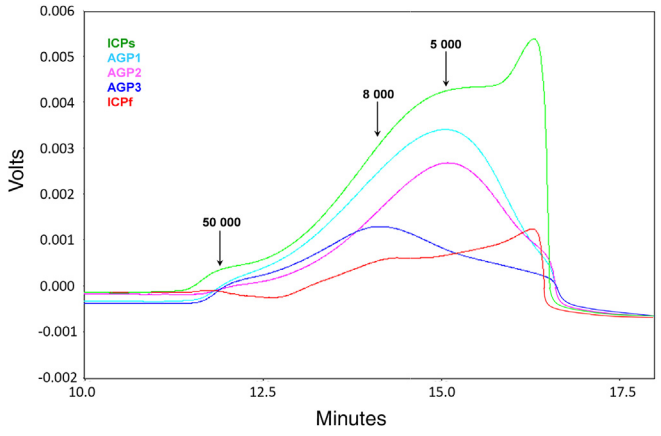

Fig. 1. HPLC molecular mass distribution patterns of *Coffea arabica* freeze-dried (ICPf) and spray-dried (ICPs) instant coffee powders, and arabinogalactan-proteins – AGP1, AGP2 and AGP3 (Capek et al., 2009).

FT-IR spectra (1900–650 cm^{-1}) of *C. arabica* freeze-dried (ICPf) and spray-dried (ICPs) instant coffee powders and arabinogalactan-proteins are displayed in Fig. 2. In both soluble coffee products ICPf and ICPs only slight differences in FT-IR patterns were observed indicating thus their structural resemblance. Bands due to carbohydrates observed at 1147, 1119, 1072 and 1036 cm^{-1} derived from two dominant polysaccharide components occurring in soluble coffee products, i.e. arabinogalactans and galactomannan (Kačuráková, Capek, Sasinkova, Wellner, & Ebringerova, 2000). In the anomeric region bands at 889 and 870 cm^{-1} are characteristic for β-type of glycosidic linkages in galactopyranosyl (AGP) and mannopyranosyl (GM) backbones, respectively. Low intensity bands at around 1769 cm^{-1} can be assigned to esters or lactones occurring in both soluble coffee products (Lyman, Benck, Dell, Merle, & Murray-Wijelath, 2003). Intensive absorption bands at 1701 and 1653 cm^{-1} were assigned to C=O stretching vibrations, and C—C and C—N stretching ones of caffeine, respectively. Moreover, several low intensive bands due to the presence of caffeine in the region of 1452–1242 cm^{-1} could be observed (Singh, Wechter, Hu, & Lafontaine, 1998). Usually in the region of 1650–1550 cm^{-1} stretching vibrations of peptide bonds ν(C=O) and bending vibrations of (N—H) groups known as Amide I and Amide II, respectively, can be observed (Arrondo, Muga, Castresana, & Goñi, 1993). Characteristic bands derived from deprotonized carboxylic groups (COO^-) of various phenolic compounds, collectively known as chlorogenic acids (esters of hydroxycinnamic acids and quinic acid) or melanoidins, can be clearly distinguishable at 1603 and 1408 cm^{-1}. The presence of phenolics can be recognized by characteristic bands appearing at 1517 cm^{-1} (C=C) and at 1242 cm^{-1} (phenyl—OH) (Gunzler & Gremlich, 2002).

FT-IR spectrum of arabinogalactan-protein (AGP3) isolated from freeze-dried instant coffee powder showed similar pattern as those of AGP1 and AGP2 already described (Capek et al., 2009), and indicated thus their structural resemblance (Fig. 2). In the spectral region of 1900–650 cm^{-1} only dominant bands at 1072 and 1036 cm^{-1} due to arabinogalactan moieties (Type II) were observed (Kačuráková et al., 2000). Besides, low intensity peaks at the wavenumber of 889 cm^{-1} indicated β-type of glycosidic linkages in galactopyranosyl backbone of coffee AGPs.

^{1}H NMR spectra of *Coffea arabica* freeze-dried (ICPf) and spray-dried (ICPs) instant coffee powders and arabinogalactan-proteins – AGP1, AGP2 and AGP3 are shown in Fig. 3.

Structural features of AGP1 and AGP2 isolates have been compared in our previous study (Capek et al., 2009;Capek, Matulová, Navarini, & Suggi-Liverani, 2010). In the ^{1}H NMR spectrum of AGP3 characteristic H1 anomeric signals of sugar residues could be identified: internal 1,3-linked βGal of the backbone at δ 4.69; terminal βGal linked to O_3 of the neighboring Gal unit at δ 4.62; reducing end 3-linked βGal at δ 4.63; internal 1,6-linked βGal at δ 4.45 and of terminal βGal residue at δ 4.43. Signals due to 1,3,6-linked βGal residues were found to be downfield shifted to δ 4.48. Two H1 signals due to α-arabinofuranosyl (α-Ara*f*) residues have been

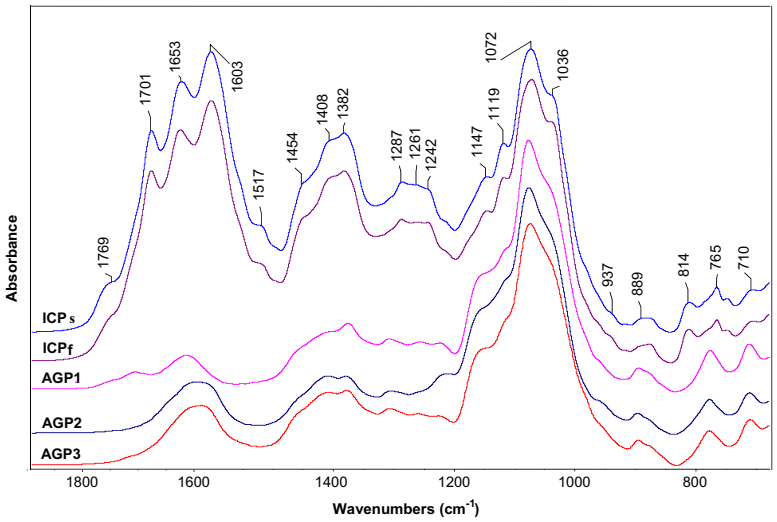

Fig. 2. FT-IR spectra of *Coffea arabica* freeze-dried (ICPf) and spray-dried (ICPs) instant coffee powders, and arabinogalactan-proteins – AGP1, AGP2 and AGP3 (Capek et al., 2009).

identified: at δ 5.24 due to a terminal Ara*f* unit linked to O_3 of βGal and at δ 5.09 which could be assigned to 1,5-linked αAra*f* linked to C5 of neighboring Ara*f* (Redgwell & Fischer, 2006) or αAra*f* units linked to O6 of βGal of the side chain (Delgado, Vignoli, Siika-aho, & Franco, 2008). The latter type of linkage was confirmed by analysis of oligomers issued from an enzymatic hydrolysis of the AGP polymer (Matulová, Capek, Kaneko, Navarini, & Liverani, 2011). Intensity of H1 anomeric signals in ^{1}H NMR spectra of AGP3 isolate (Fig. 2) varied from those of AGP1 and AGP2, suggesting thus differences in βGal substitution and Araf content (Table 2).

Intensities of H1 anomeric signals in ^{1}H NMR spectra reflect different type of linkages of sugars in tested samples. Relative intensities of βGal H1 signals in AGP1-3 revealed differences in the degree of substitution of oligosaccharides as well as arabinose content. The highest intensity of 1,3-βGalint signal was found in AGP1. This fact indicated that oligosaccharides present in this isolate have had the lowest degree of the substitution. The intensities of βGal signals present in side chains (1,6-βGal and branched 1,3,6-βGal) in AGP1 were the lowest, they were higher in AGP2 and the highest in AGP3. This observation was in accordance with an increasing intensity of αAraf residue signals observed at δ 5.24 and 5.09 ppm. Moreover, the presence of branched 1,3,6-βGal units signal (δ 4.50 ppm) with increasing intensity from AGP1 to AGP2 and AGP3 confirmed that in AGP3 oligosaccharides with the highest degree of substitution are present. In arabinogalactans the backbone could be substituted mostly by βGal of 1,6-linked βGal side chains, while side chains βGal units are substituted by both, Araf and βGal (Redgwell & Fischer, 2006). Intensity of αAraf and 1,3,6-βGal signals suggest that in AGP3 oligosaccharides with shorter backbone are highly substituted by longer sidechains. The degree of substitution deceased in AGP2 and was the lowest in AGP1.

^{1}H NMR spectra of instant coffee powders ICPf and ICPs have been more complex. Signals due to anomeric protons of arabinogalactan moiety have been partially overlapped by other signals (Fig. 3). However, the intensity of the 1,3-linked βGal signal in the ICPs spectrum suggest their slightly higher content in the AGP backbone in comparison with ICPf. Overlapped signals due to substituted 1,3,6-βGal units are large in both spectra, ICPf and ICPs giving thus an evidence about a high degree of AGP substitution. In both spectra also signals due to trigonelline (T), caffeine (C), chlorogenic acids (ChA), mannan composed of 1,4-β-Man residues (M), N-methyl-pyridinium (Me), choline (Ch) (Wei et al., 2012) could be identified. ^{1}H NMR spectrum of ICPs showed higher content of trigonelline and chlorogenic acids.

3.2. Immunobiological effects of instant coffee powders and their arabinogalactan-proteins

It is evident that coffee is nowadays one of the most popular beverages due to its typical aroma and stimulating effect on organism. Coffee became a part of our food chain due to its every day consumption. Results of chemical and spectroscopic analyses on soluble coffee products – ICPf and ICPs, and ICPf-derived arabinogalactan-proteins – AGP1, AGP2 and AGP3 revealed small differences in their monosaccharide composition, molecular mass, and in FT-IR and NMR spectral patterns. These slight structural differences could have some impact on their immunomodulating activities. It has been found that *C. arabica* AGP1 affected some mediators of immunocompetent cells of immune system as tumor necrosis factor α (TNF-α), interleukin 2 (IL-2) and interferon γ (IFN-γ) cytokines (Nosál'ová et al., 2011). To get more information about possible immunobiological effects of soluble coffee products and their arabinogalactan-protein components, IL-2 induction in mice splenocytes was followed up using previously optimized activation protocol for detection of polysaccharide specific IL-2 secreting T-cells (Nosál'ová et al., 2011; Paulovicova et al., 2013). The applied concentration of 100 μg/mL of AGP2, AGP3, ICPs and ICPf formulations in indirect ELISPOT assay with 24 h pre-incubation activation

Table 2
Content of βGal and Ara*f* in different linkage types in AGP1-3 isolates based on anomeric signal intensities in ^{1}H NMR spectra.

Isolate	Content of sugar (mol%)				
	Ara*f* (δ 5.09, 5.24)	1,3-βGal$_{int}$ (δ 4.69)	βGal$_{red,\ term}$ (δ 4.63)	1,3,6-βGal (δ 4.48)	1,6-βGal (δ 4.45–4.43)
AGP1	9	46	13	12	20
AGP2	9	37	13	19	22
AGP3	12	27	12	21	28

Red, inter and term – reducing, internal and terminal βGal units, respectively.

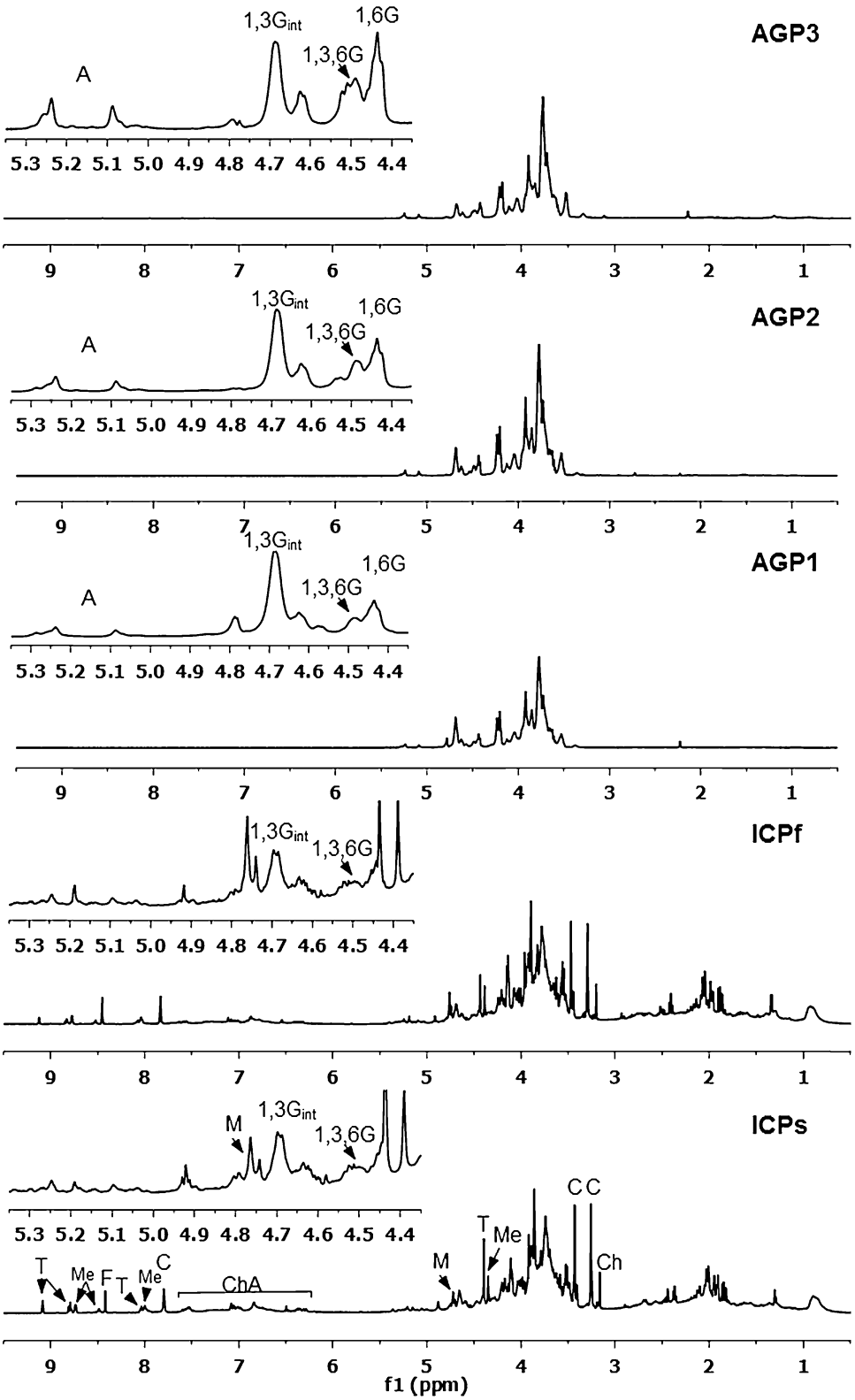

Fig. 3. ^{1}H NMR spectra of ICPf and ICPs and AGP1, AGP2 and AGP3 isolates. T – trigonelline, C – caffeine, ChA – chlorogenic acids, M – mannan (1,4-β-Man), Me – N-methylpyridinium, Ch – choline, 1,3G – 1,3-linked βGal internal, terminal and reducing units, 1,3,6G – 1,3,6-linked βGal and 1,6G – 1,6-linked βGal, F – formic acid.

step, was selected, based on the results reached with concentration interval from 10 to 1000 μg/mL (Table 3).

Evaluation of instant coffee powders (ICPf and ICPs) and arabinogalactan-proteins (AGP2 and AGP3) induced IL-2 secretion following 24 h treatment of splenocytes revealed the maximal stimulated increase with AGP2 (3.05 times) ($P < 0.001$) and AGP 3 (2.59 times) ($P < 0.01$) in comparison with untreated splenocytes (Fig. 4). Previously published results on IL-2 induced secretion following AGP1 treatment revealed 5.75 times increase compared with unstimulated splenocytes (Nosál'ová et al., 2011). Both coffee

Table 3
Concentration-dependent release of IL-2 by splenocytes arabinogalactan-proteins' (AGP2 and AGP3) and freeze/spray-dried instant coffee powders' (ICPf/ICPs) activated. Determination of frequency of IL-2 secreting cells (spot forming cells, SFC) among Balb/c mice splenocytes treated with 10, 100 and 1000 μg/mL of AGP2, AGP3, ICPf and ICPs. The results of two independent parallel measurements are expressed as average ± S.D.

Compounds	Concentration (μg/mL)	Number of SFC (average ± S.D.)
AGP2	10	104.3 ± 9.2
	10^2	135.2 ± 15.4
	10^3	125.7 ± 21.8
AGP3	10	53.6 ± 14.6
	10^2	113.0 ± 18.3
	10^3	75.2 ± 8.1
ICPf	10	83.7 ± 11.4
	10^2	93.0 ± 14.0
	10^3	90.5 ± 5.8
ICPs	10	86.1 ± 9.4
	10^2	96.5 ± 10.7
	10^3	90.3 ± 15.5

powders (ICPf and ICPs) were significantly less immunologically active than AGP-proteins ($P < 0.01$), the IL-2 producing cell count being 2.13 times with ICPf and 2.22 times with ICPs in comparison with unstimulated splenocytes (Fig. 4). Evidently, following 24 h-activation period, the frequencies of IL-2 secreting cells induced by AGP2, AGP3, ICPs, and ICPf reflected the specific response, since the frequency of spot forming cells (SFC) detected in presence of AGP 2, AGP3, ICPf and ICPs is at least two times frequency of SFC detected in the absence of antigen (Kalyuzhny, 2005).

AGP1 and AGP2 showed very similar sugar composition while AGP3 slightly differed from them in higher mannose content and higher molecular mass (Table 1). The observed difference of immunobiological efficacy of both coffee powders with those of AGP1 (Nosál'ová et al., 2011), AGP2 and AGP3 is reasonable with respect to total amount of arabinogalactan-proteins. Total amount of carbohydrates in ICPf represented 37% and in ICPs 38% and about one third of that amount corresponded to AGPs (Capek et al., 2009). Slightly increased amount of interleukin-2 (IL-2) producing cells (1.04 times) with ICPs in comparison with ICPf suggests the structure-immunomodulation interactivities due to different composition of coffee powders (Table 1).

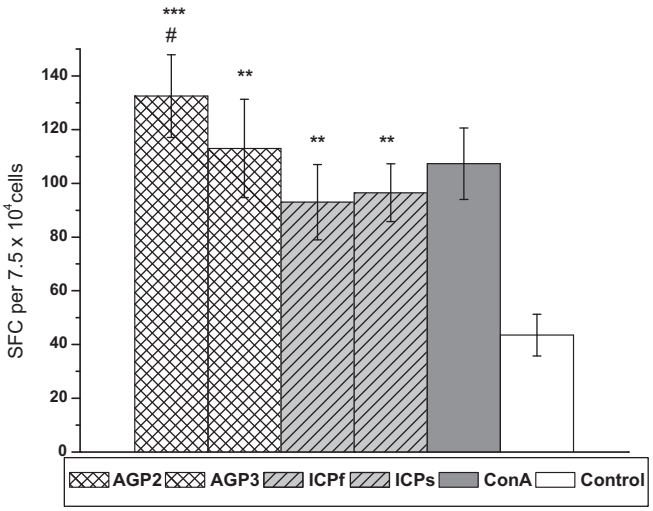

Fig. 4. Enumeration of IL-2 secreting cells (spot forming cells, SFC) among Balb/c mice splenocytes treated with 100 μg/mL of arabinogalactan-proteins (AGP2 and AGP3), instant coffee powders – freeze dried (ICPf) and spray dried (ICPs), and Concanavalin A (Con A). Experimental data groups were compared with untreated cells, i.e. baseline IL-2 cell secretion. The results of two independent parallel measurements are expressed as mean ± S.D. Levels of significance were expressed as follows: $^{***}P < 0{,}001$, $^{**}P < 0.01$, $^{*}P < 0.05$. Experimental data sets comparison with ConA levels: $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$. Differences were considered significant where $P < 0.05$.

Interleukin-2, is a T_H1-cell-derived lymphoid cell growth factor which is produced in a response to antigenic or mitogenic stimuli and exerts its biological activity to promote the continuous proliferation and maturation of additional T-cells. Additionally, IL-2 has been found to be important in the regulation of growth and differentiation of B-cells, natural killer cells, glioma cells, and cells of the monocyte lineage after specifically interacting with its receptors. Generally, IL-2 has pivotal role in the generation and regulation of the immune responses (Gómez, García-Domingo, Martínez, & Rebollo, 1997; Smith, 1988). IL-2 has multiple, sometimes opposing functions during an inflammatory response. IL-2 contributes to both the induction and the termination of inflammatory immune responses (Hoyer, Dooms, Barron, & Abbas, 2008). The participation of IL-12p40 cytokine and $T_H1$cytokine IFN-γ in cell response to coffee bean AGPs in mice splenocytes and bone marrow-derived dendritic cells was revealed (Gotoda et al., 2006). IL-12p40 builds heterodimers with IL-12p35 (as IL-12 p70) and induces the polarization toward T_H1-cells, as IL-12p70 is an essential inducer of the differentiation of T cells toward a T_H1 phenotype. Moreover, they also observed the proliferation of macrophage and splenocyte in the presence of AGP from coffee beans. (Gotoda et al., 2006).

The effect of AGP from coffee beans was investigated on allergic dermatitis-like skin in mice, suggesting that AGP inhibits dermatitis in mice sensitized with 2, 4, 6-trinitrochlorobenzene by enhancing T_H1 responses. Thus, AGP treatment could provide an effective activity for the allergic reactions (Furuya et al., 2008). The 4.06 times increase of IFN-γ following the AGP1 treatment (100 μg/mL) vs non-stimulated control has been observed in previous our study with AGP1 (Nosál'ová et al., 2011). Evidently, based on the induced secretion of IFN-γ by AGP1-activated splenocytes, the coffee AGP1 reflected the pro-$T_H1$character. Our experiments on liberation of IL-2, accompanied by direct counting of splenocytes producing IL-2 cytokine from mice immunocytes exposed to AGP2, AGP3, ICPf and ICPs also supported proposed T_H1 polarization by arabinogalactan-proteins from coffee beans. Observed lower counts of immunocytes secreting IL-2 after ICPf and ICPs treatment compared to those of AGP1 (Nosál'ová et al., 2011), AGP2, and AGP3 suggested the role of next immunomodulatory components in instant coffee possibly with blocking/suppressive effect on overall IL-2 secretion, e.g. caffeine. Caffeine has been reported to reduce IL-2 secretion. The significantly increasing IL-2 suppression following in vitro coffee treatment in accordance with increasing coffee concentration was observed (He, Noda, & Sugiyama, 2001; Ohta, Lukashev, Jackson, Fredholm, & Sitkovsky, 2007). Thus, the caffeine in coffee might contribute to the suppression of splenocyte proliferation through a decrease in IL-2 secretion (Goto, Takano-Ishikawa, & Shinmoto, 2011). Moreover, coffee directly applied to naïve mouse splenocytes induced T_H1 polarization based on secretion of antigen-specific IFNγ and IL-2 levels over the secretion of pro-T_H2 IL4 and IL-5 cytokines (Goto et al., 2011).

Free radicals liberation (Fig. 5) after co-cultivation of splenocytes with AGP1 was significantly higher ($P < 0.001$), than after co-cultivation of splenocytes with AGP2 and AGP3 ($P < 0.01$ and $P < 0.05$, respectively). Free radicals maximal stimulated increase (3.6 and 1.4 times) was revealed with AGP1 treated immunocytes compared to untreated cells and Concanavalin A treated. AGP2 and AGP3 treatment induced free radicals liberation to lesser degree compared with untreated cells, i.e. 2.8 and 2.5 times, respectively, than observed with the AGP1 treatment. Splenocytes' treatment with ICPf and ICPs resulted in decreased production ($P < 0.05$) of free radicals in comparison with those of AGP1-3. ICPs induced slightly more (1.04 times) free radicals release from splenocytes compared

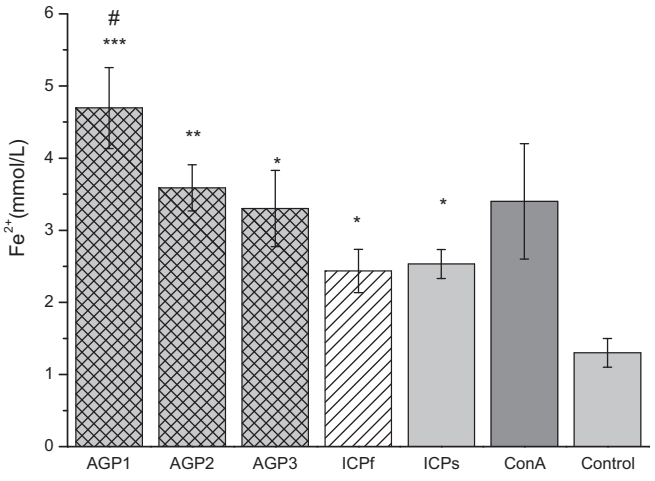

Fig. 5. Splenocyte release of free radicals induced by 100 μg/mL of arabinogalactan-proteins (AGP1, AGP2 and AGP3), ICPf (freeze dried instant coffee powder) and ICPs (spray dried instant coffee powder) and Concanavalin A (Con A) treatment. Experimental data groups were compared with untreated cells, i.e. baseline free radical production. The results of two independent parallel measurements are expressed as mean ± S.D. Levels of significance were expressed as follows: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. Experimental data sets comparison with ConA levels: $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$. Differences were considered significant where $P < 0.05$.

to ICPf. This event could be explained by decreased immunobiological potency of ICPf, presumably because of lower AGP content in instant coffee comparing with pure AGP1-3. To compare the strength of the relationship between immunological parameters following splenocytes' activation by co-cultivation with AGP1, AGP2, AGP3, ICPf and ICPs Pearson's correlation coefficient was used. Statistically significant correlation ($R = 0.94152$, $P = 0.032$) revealed the tight relationship and interreactivity between free radicals liberation and IL-2 secretion following mice splenocytes activation.

Free radicals are produced in mammalian cells in a response to the activation of diverse cell surface receptors (Rhee, Chang, Bae, Lee, & Kang, 2003). Free radicals participate in host defence biological activities. The generation of free radicals plays important physiological roles in anti-microbial defence as well as in pro-inflammatory cell-signaling (Forman & Torres, 2001; Droge, 2002). It has been proposed that redox signaling plays an important role in the regulation of inflammatory responses. Activation of the NADPH oxidase pathway initiates an intracellular reactive oxygen species signaling pathway that amplifies the production of pro-inflammatory cytokines (Forman & Torres, 2002). Arabinogalactans extracted from higher plants had potent immunomodulatory properties for human and murine immunocytes, as demonstrated by the induction of iNOS expression and NO production, priming of free radicals and ROS production (Schepetkin & Quinn, 2006).

The close correlation between immunobiological effectiveness of AGP1 (Nosál'ová et al., 2011), AGP2, AGP3, ICPf and ICPs treatment of immunocytes and carbohydrate composition was supported by ^{1}H NMR spectra. It was shown that AGP1 induced the highest levels of IL-2 (Nosál'ová et al., 2011) and free radicals. The ^{1}H NMR spectrum of AGP1 has shown the highest content of non-substituted internal 1,3-linked βGal units of the polymer backbone. ^{1}H NMR spectra revealed that ICPs, which showed enhanced immunobiological activities in comparison with ICPf, contained more internal 1,3-linked βGal residues. Obtained results suggest that the immune responsiveness of immunocytes might depend on the content of internal 1,3-linked βGal in AGP1, AGP2, AGP3, ICPf and ICPs. Similar effect on the immunological activity of fungal mannans and glucans was associated to the localization and frequency of β-1,3-linked mannose units (Iorio et al., 2008). Moreover, the diagnostic significance of β-1,3-linked mannosyl units as *S. cerevisiae* and *C. albicans* epitopes in Crohn disease has been previously stressed (Standaert-Vitse et al., 2006).

4. Conclusion

Coffea arabica is one of the most spread species used for cultivation of coffee beans and manufacture of coffee products. Chemical analysis of its freeze and spray-dried coffee instant products did not show significant differences in their chemical composition. Slight differences have been found by ^{1}H NMR spectra, which reside mainly in the AGP backbone substitution, in trigonelline and chlorogenic acids content. Chemical and spectroscopic analysis of AGP3 isolate revealed some differences in the backbone, side chains substitution, sugar composition and molecular mass in comparison with those of AGP1 and AGP2 (Capek et al., 2009). Immunobiological studies on instant coffee products (ICPs and ICPf) and AGP2 and AGP3 isolates have shown that they possess immunomodulatory properties, i.e. they effectively stimulated immunocytes, enhanced beneficial pro-T_H1 cell immune responses and ROS liberation, engaged in cell proliferation and host anti-microbe immune responses. Results of immunological tests have shown more pronounced effect of arabinogalactan-proteins in comparison with those of instant coffee products. They suggest that arabinogalactan-proteins are one of the main contributors of immunological activity of instant coffee powders. Obtained results contribute to broadening of knowledge concerning immunobiological effect of soluble coffee products and their AGP components.

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