Universidade de Aveiro Departamento de Química

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SÓNIA DOS SANTOS FERREIRA

RELAÇÃO ESTRUTURA-FUNÇÃO DE POLISSACARÍDEOS IMUNOESTIMULADORES

STRUCTURE-FUNCTION RELATIONSHIP OF IMMUNOSTIMULATORY POLYSACCHARIDES

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo Bioquímica Alimentar, realizada sob a orientação científica do Doutor Manuel António Coimbra, Professor Associado com agregação do Departamento de Química da Universidade de Aveiro e da Doutora Cláudia Pereira Passos, Bolseira de Pós-Doutoramento do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho à minha família e amigos.

"It always seems impossible until it's done."

Nelson Mandela

O júri

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palavras-chave

Café, arabinogalactanas, peso molecular, cafeína, ácidos clorogénicos, actividade immunoestimuladora, linfócitos, células dendríticas.

resumo

Os polissacarídeos do café, nomeadamente as arabinogalactanas do café instantâneo e as galactomananas da infusão de café, têm atividade imunoestimuladora *in vitro*, verificada através de uma resposta inflamatória. Os estudos anteriores mostraram que um extrato de café instantâneo com 1-5 kDa (amostra 1E), obtido por ultrafiltração, com lavagem exaustiva dos compostos de baixo peso molecular, apresentou atividade imunoestimuladora *in vitro*. No entanto, um extrato semelhante (amostra 2E), desta vez resultante de um fracionamento rudimentar, não tinha atividade. Com base na hipótese de que os compostos de baixo peso molecular podem interferir na atividade imunoestimuladora *in vitro* destes polissacarídeos, neste estudo, a amostra 2E foi purificada por cromatografia de exclusão molecular em Bio-Gel P2 (SEC-P2) e a atividade imunoestimuladora *in vitro* foi estudada em linfócitos B e T de células esplénicas de ratinhos BALB/c por expressão de um marcador precoce de ativação (CD69). Os resultados obtidos permitiram concluir que a presença de compostos de baixo peso molecular, nomeadamente ácidos clorogénicos (CGA) e cafeína, interferem com a determinação da atividade imunoestimuladora *in vitro* dos polissacarídeos do café.

Com o objetivo de saber quais as características estruturais responsáveis pela potencial atividade imunoestimuladora das arabinogalactanas do café instantâneo, a amostra 1E foi também fracionada por cromatografia de exclusão molecular em Bio-Gel P6 (1-6 kDa). Três frações foram recolhidas e liofilizadas e a sua atividade imunoestimuladora foi avaliada, permitindo observar que a atividade imunoestimuladora da amostra 1E deriva da fração com um peso molecular próximo de 5 kDa.

O tratamento da amostra 1E com uma solução de 0,1 M de NaOH diminuiu 58,2% a ativação *in vitro* de linfócitos B. Embora as análises de FTIR da amostra 1E saponificada e dialisada tenham mostrado um aumento da presença de ácidos carboxílicos quando comparado com a amostra nativa, não foram verificadas diferenças na quantidade de grupos acetilo, avaliadas por micro-extração em fase sólida da fase de vapor e análise por cromatografia em fase gasosa e detetor de ionização de chama (HS-SPME-GC-FID). A análise de GC-FID permitiu também observar uma composição de açúcares semelhante antes e após saponificação. Para testar a possibilidade de, após a saponificação, os CGA poderem ter sido libertados das estruturas das melanoidinas e ficarem adsorvidos aos polissacarídeos, mesmo após diálise exaustiva, foi realizada uma separação por SEC-P2. Uma vez que o cromatograma obtido não mostrou absorvâncias a 280 e 325 nm no volume de inclusão, foi possível deduzir que a perda de atividade imunoestimuladora não foi devida à presença de CGA adsorvidos.

A amostra 1E tinha 5,5% da proteína total. De forma a avaliar a influência da presença de proteína para a atividade imunoestimuladora, a amostra 1E foi tratada com quimotripsina. A amostra desproteinizada resultante ($1E^{dep}$) tinha uma composição de açúcares semelhante e a mesma atividade imunoestimuladora. O tratamento com uma α-L-arabinofuranosidase, que remove os resíduos de arabinose em ligação terminal, permitiu, após purificação através de SEC-P2, observar que a amostra perdeu a atividade imunoestimuladora *in vitro* de linfócitos B e T.

Para aprofundar o conhecimento sobe o modo de ativação dos linfócitos B e T pela amostra 1E, esta amostra foi testada em macrófagos (BMDM) e células dendríticas (BM-DCs) da imunidade inata, derivadas da medula óssea. Os resultados mostraram a produção de NO pelos BMDM e o aumento da expressão de marcadores de superfície de ativação, MHC-II, CD80 e CD86, pelas BM-DCs, indicando a ativação de ambos os tipos de células. Estes resultados mostram que é possível que a ativação de macrófagos e de células dendríticas possa estar envolvida na ativação dos linfócitos B e T do baço pela amostra 1E.

Os resultados obtidos também permitiram concluir que a atividade imunoestimuladora *in vitro* das frações de café instantâneo ricas em arabinogalactanas resulta de uma fração com cerca de 5 kDa. Esta atividade parece ser dependente da presença de resíduos de arabinose em ligação terminal e não da extensão da acetilação do polissacarídeo nem do conteúdo proteico presente. Foi também possível concluir que a atividade imunoestimuladora *in vitro* destas frações é influenciada negativamente pelos CGA e cafeína, caso estejam presentes. Embora estes compostos interfiram em experiências *in vitro*, não é de esperar que possam interferir *in vivo* porque durante a digestão os compostos de baixo peso molecular são absorvidos na parte superior do intestino delgado, enquanto os polissacarídeos e as melanoidinas não o são. Ao longo do trato digestivo, o efeito imunoestimulador dos polissacarídeos deve prevalecer ao interagir com as células do sistema imunitário encontradas nas placas de Peyer ou com as células dendríticas encontradas na lâmina própria do intestino delgado, antes da fermentação no cólon.

keywords

Coffee, arabinogalactans, molecular weight, caffeine, chlorogenic acids, immunostimulatory activity, B and T lymphocytes, dendritic cells.

abstract

Coffee polysaccharides, namely the arabinogalactans present in instant coffee and the galactomannans of coffee infusions have *in vitro* immunostimulatory activity, shown by an inflammatory response. Previous works showed that an instant coffee extract with 1–5 kDa (sample 1E), obtained by ultrafiltration, resultant from an exhaustive washing out of the small molecular weight compounds, presented *in vitro* immunostimulatory activity. However, another similar extract (sample 2E), this time resultant from a rudimentary fractionation, had no activity. Based on the hypothesis that small molecular weight compounds may interfere on the *in vitro* immunostimulatory activity of these polysaccharides, in this study, sample 2E was purified through Bio-Gel P2 size exclusion chromatography (SEC-P2) and the *in vitro* immunostimulatory activity in BALB/c mice spleen B and T lymphocyte cells was studied by the expression of an early activation marker (CD69). Results allowed concluding that the presence of small molecular weight compounds, namely chlorogenic acids (CGA) and caffeine, interfere with the determination of the *in vitro* immunostimulatory activity of coffee polysaccharides.

Aiming to know what could be the structural characteristics responsible for the instant coffee arabinogalactans potential immunostimulatory activity, sample 1E was also fractionated by size-exclusion chromatography using Bio-gel P6 (1-6 kDa). Three fractions were pooled and freeze-dried and their immunostimulatory activity was evaluated, allowing to observe that the immunostimulatory activity of sample 1E derived from the fraction with a molecular weight near 5 kDa.

The treatment of sample 1E with 0.1 M NaOH solution decreased by 58.2 % the *in vitro* activation of B lymphocytes. Although FTIR analyses of the saponified and dialysed sample 1E showed an increase of the presence of carboxylic acids when compared to the native sample, but no difference in the amount of acetyl groups were detected by gas chromatography of the head-space solid-phase microextraction (HS-SPME-GC-FID). Also, similar carbohydrate composition was observed by GC-FID for the sample before and after saponification. To disclose the possibility that, upon saponification, the CGA could have been released from melanoidin structures and be adsorbed to the polysaccharides, even upon exhaustive dialysis, a SEC-P2 was performed. As the chromatogram obtained did not show absorbances at 280 and 325 nm in the inclusion volume, it was possible to infer that the loss of immunostimulatory activity was not due to the presence of adsorbed CGA.

Sample 1E had 5.5% of total protein. In order to evaluate the influence of the presence of protein for the immunostimulatory activity, sample 1E was treated with chymotrypsin. The resulted deproteinised sample ($1E^{dep}$) had a similar carbohydrate composition and the same immunostimulatory activity. The treatment with an α-L-arabinofuranosidase, which should remove terminally-linked arabinose residues, allowed, after purification through SEC-P2, to observe that the sample lost the *in vitro* immunostimulatory activity to stimulate B and T lymphocytes.

In order to obtain more information on how sample 1E can activate the B and T lymphocytes, the sample 1E was tested in innate immune macrophages (BMDM) and dendritic cells (BM-DCs) derived from bone-marrow. The results showed the production of NO by BMDM and the increase of the expression of surface activation markers MHC-II, CD80, and CD86 by BM-DCs, indicating the activation of both cell types. It is possible that the activation of macrophages and dendritic cells may be involved in the activation of B and T spleen lymphocytes by sample 1E.

The results obtained allowed to conclude that the *in vitro* immunostimulatory activity of instant coffee arabinogalactan-rich fractions results from a fraction near 5 kDa. This activity seems to be dependent of the presence of arabinose terminally-linked residues but not on the acetylation of the polysaccharide neither on the protein content. Also, it was possible to conclude that the *in vitro* immunostimulatory activity of these fractions is negatively influenced by the presence of CGA and caffeine. Although these compounds interfere in *in vitro* experiments, it is not expected that they could interfere *in vivo* because during digestion the low molecular weight compounds are absorbed in the upper small intestine whereas the polysaccharides and melanoidins are not. Along the digestive tract, the immunostimulatory effect of polysaccharides should prevail when interacting with immune cells found in Peyer's patches or with dendritic cells found in the small-intestinal lamina propria, before colon fermentation.

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ABBREVIATIONS

1E	Sample rich in arabinogalactans (1-5 kDa), resultant from a exhaustive ultrafiltration of instant coffee.
2E	Sample rich in polysaccharides (1-5 kDa), resultant from a rudimentary ultrafiltration of instant coffee.
ACK	Ammonium-Chloride-Potassium
AG	Arabinogalactans
AG-I	Type I arabinogalactans
AG-II	Type II arabinogalactans
AGP	Arabinogalactan-proteins
AP-1	Activator protein-1
Ara	Arabinose
BCA	Bicinchoninic acid
BM-DCs	Bone-marrow derived DCs
BMDM	Bone-marrow derived macrophages
BRM	Biologic response modifiers
BSA	Bovine serum albumin
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CGA	Chlorogenic acids
COX-2	Cyclooxygenase-2
CR	Complement receptor
CRP	C-reactive protein
cRPMI	RPMI-1640 supplemented with penicillin (1%), and 10% of FBS
Cy	Cyclophosphamide
DCs	Dendritic cells
DS	Degree of substitution
ELSD	Evaporative light scattering detection
ERK	Extracellular signal regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FOS	Fructooligosaccharides
Fru	Frutose
FTIR	Fourier transform infrared spectroscopy
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
GC-FID	Gas chromatography with a flame ionization detector
Glc	Glucose
GlcA	Glucuronic acid
GlcNAc	N-acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor

HA	Hyaluronic acid
HBSS	Hank's balanced salt solution
HPLC	High performance liquid chromatography
HS-SPME	Headspace solid phase microextraction
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
LBP	Lypopolysaccharide-binding protein
LCCM	L929 cell-conditioned medium
LPS	Lypopolysaccharide
Man	Mannose
MAPK	Mitogen-activated protein kinase
MBL	Mannose binding lectin
MFI	Median fluoresce intensity
MO	Macrophages
MPO	Myeloperoxidase
MR	Mannose receptors
mRNA	Messenger ribonucleic acid
Mw	Molecular weight
MyD88	Myeloid differentiation protein 88
NF-κB	Nuclear factor-κB
NK	Natural killer
NO	Nitric oxide
NR	Neutral Red
PBMC	Human peripheral blood mononuclear cells
PBS	Phosphate buffered saline, 0.01 M, pH 7.4
PRRs	Pattern recognition receptors
PS	Polysaccharides
Rha	Rhamnose
ROS	Reactive oxygen species
RPMI	RPMI-1640 supplemented with penicillin (100 IU/mL), streptomycin (50 mg/L), 2-mercaptoethanol (0.05 mol/L) and 10% of FBS
RPMI-1640	Medium Roswell Park Memorial Institute
SEC-P2	Size exclusion chromatography throught Bio-gel P2
SEC-P6	Size exclusion chromatography throught Bio-gel P6
SEM	Standard error of means
SR	Scavenger receptors
TFA	Trifluoroacetic acid
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TP	Total phenolics content
TRAF-6	TNF receptor-associated factor 6
Xyl	Xylose

1. INTRODUCTION

Polysaccharides are carbohydrate polymers composed of monosaccharide units bound together by glycosidic bonds. A large number of these polymers has been reported to interact with several cells of the immune system, as well as molecules that mediate humoral immunity, showing a potential immunostimulatory activity [1]. These immunostimulatory polysaccharides are widely distributed in nature, being found in plants, fungi, bacteria, algae and animals [1,2]. In general, in studies relating immunostimulatory activity to polysaccharides there is a lack of structural characterization and, therefore, the study of structural features responsible for their activity is of a great interest for research works.

The immunostimulatory activity of polysaccharides has been related with their potential stimulation of the immune system and strengthening of the innate and adaptive immunity responses, either by exhibiting the effect themselves or by inducing effects via complex reaction cascades [1–3]. The anti-microbial [4,5], antiviral [6], antitussive [7], radioprotective [8], anti-septic shock [9], and antitumoral [10,11] immune-related properties of these polysaccharides can regard them as potential health promoting or even therapeutic agents. This wide range of bioactivities plus the wide range of sources stated above reinforces the need for studies to systematize the existing information. As a consequence, a literature review was performed to identify and systematize the information about structural features of polysaccharides with immunostimulatory potential activity. The knowledge about structure-function relationships can be crucial for future developments and identification of immunostimulatory polysaccharides.

Coffee polysaccharides, namely the galactomannans of coffee infusions [12] and the arabinogalactans (AG) present in instant coffee have *in vitro* immunostimulatory activity [7,13]. These polysaccharides isolated from coffee beverage, instant coffee, and/or spent coffee grounds have been structurally characterized, as well the structurally related *Aloe vera* galactomannans, allowing to conclude that the potential immunostimulatory activity of the galactomannans could be due to the lower branching, shorter side chains, and higher

acetylation [14]. For the AG, a relationship was found between their molecular weight (1-6 kDa) and potential immunostimulatory activity [7,13]. However, due to the complexity of the coffee bean and, on the other hand, to the structural modifications after roasting, the structural features responsible for their activity are far from being completely elucidated. With roasting, polysaccharides can undergo depolymerisation, debranching, Maillard reactions, caramelisation, isomerisation, oxidation, decarboxylation, polymerisation and melanoidins (the polymeric brown compounds) formation [15]. Moreover, without exhaustive purification steps, other compounds can remain associated to polysaccharides, namely protein, melanoidins, and/or low molecular weight material, which includes caffeine, and chlorogenic acids (CGA, the main coffee phenolic compounds). As the CGA and caffeine have been reported to present anti-inflammatory properties [16], it is important to evaluate if their presence in non-purified polysaccharide-rich extract have influence on their immunostimulatory properties.

In a previous work, it was observed that an instant coffee extract with 1–5 kDa, obtained by ultrafiltration (1E), resultant from an exhaustive washing out of the small molecular weight compounds, presented *in vitro* immunostimulatory activity by inducing the activation of B-lymphocytes [3]. However, an instant coffee extract with 1–5 kDa, resultant from a rudimentary fractionation (2E), had no *in vitro* immunostimulatory activity [17]. Based on the hypothesis that phenolic compounds and caffeine may interfere on the *in vitro* immunostimulatory activity of these polysaccharides, in this study, sample 2E was purified through Bio-gel P2 size exclusion chromatography and the *in vitro* immunostimulatory activity in BALB/c mice spleen cells was studied. Aiming to know what could be the structural characteristics responsible for the instant coffee arabinogalactans potential immunostimulatory activity, sample 1E was also fractionated by size-exclusion chromatography using Bio-gel P6 (1-6 kDa) and treated with 0.1 M NaOH solution (saponification), chymotrypsin (deproteinisation), and α-L-arabinofuranosidase. Moreover, in order to obtain more information on how sample 1E can activate the B and T lymphocytes, the sample 1E was tested in innate immune macrophages (BMDM) and dendritic cells (BM-DCs) derived from bone-marrow.

Before presenting the results obtained, a literature review focusing structure-function relationships of immunostimulatory polysaccharides is presented. This literature review is divided into four main parts. In the first part, general immunology concepts will be

described. In the second part the structural characteristics of the most studied immunostimulatory polysaccharides are presented. In the third part, it will be discussed structure-function relationships existing in the literature. In the fourth and last part, important aspects of coffee matrix in general and of coffee polysaccharides in particular are described. The experimental and detailed methodology used is presented in **chapter 2**, and results and discussion are presented in **chapter 3** subdivided according to the different studies performed. Finally, in **chapter 4** concluding remarks and perspectives for future work are presented.

1.1. IMMUNOLOGY

1.1.1. Innate and acquired immunity

The immune system comprises the body defences against foreign or potentially dangerous invaders [18]. These defences include physical barriers (skin and mucosal barriers), a number of morphologically and functionally diverse organs and tissues (**Figure 1.1**), several cells (cellular immunity), and molecules such as cytokines, chemokines, antibodies, and complement proteins (humoral immunity) [18].

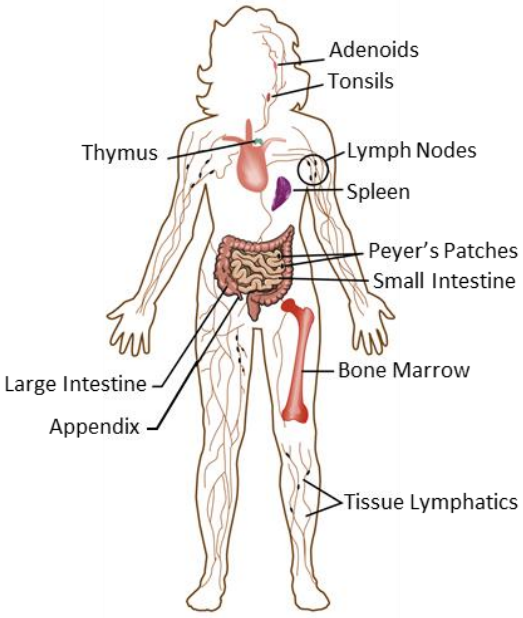

Figure 1.1. Organs and tissues of the immune system [19].

The immune system is divided into innate or nonspecific immunity and acquired or specific immunity [18]. Innate immunity is the first line of defence and it does not require a previous encounter with a microorganism or other invader to work effectively, and it has an immediate response to invaders. It involves the skin, mucosal barriers, and phagocyte white blood cells including monocytes, macrophages, neutrophils, and dendritic cells. The acquired immunity involves lymphocytes (B and T cells) and antigen-presenting cells. This immunity takes time to develop after the initial antigenic stimulus, however, thereafter, the response is quick. The activation of innate immune responses produces signals that stimulate and direct subsequent adaptive immune responses. Therefore, innate and adaptive immunity operate in cooperative and interdependent ways [18].

1.1.2. Activation of the immune system

Immunostimulatory compounds or biologic response modifiers (BRM), like polysaccharides and lipopolysaccharides (LPS), can interact direct or indirectly with cells of the immune system leading to their activation [20]. On one hand, BRM can interact with myeloid cells (monocytes, macrophages, neutrophils, and dendritic cells) or with lymphocytes, namely the Natural Killer (NK) cells, T cells, and B cells. On the other hand, BRM can interact with molecules that mediate humoral immunity, such as antibodies or proteins of the complement system. This interaction of BRM with cells and humoral immunity is mediated by receptors and binding proteins, leading to activation of certain signalling pathways that would be responsible for the expression of certain molecules and resulting responses. Consequently, the activation of the immune system by BRM will result in a better clearance of pathogens or tumour cells (**Figure 1.2**). Moreover, the activation of both humoral and cell-mediated immunity is associated to mechanisms of regulation, that together promote health [20].

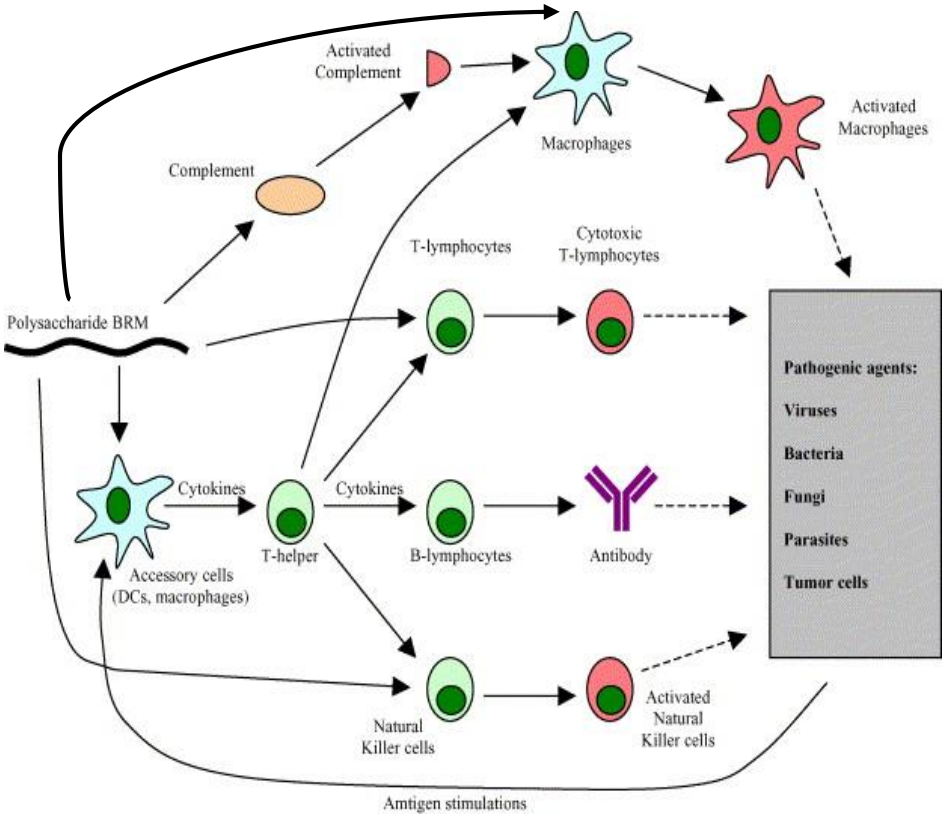

Figure 1.2. Schematic illustration of the activation of complement system and immune cells by polysaccharide BRMs. Solid arrows represent activation and dashed arrows represent suppression or destruction [20].

1.1.2.1. Immune Receptors

The biological response that BRM can elicit is determined by the cellular/molecular events triggered by its interaction with receptors of the immune system [1,20]. Interaction with different receptors will result in different responses. There are different receptors for adaptive and innate immunity (**Table 1.1**). Adaptive immunity receptors include antibodies and T-cell receptors that recognize and discriminate specific structural details of antigens [18].

The receptors of innate immunity, also called pattern recognition receptors (PRRs), recognize conserved molecular structures (pathogen-associated molecular patterns) shared by almost all microbial species but that are generally absent from the host. PRRs can occur as secreted molecules or be found on cell membrane. Furthermore, it is likely that several different receptor types cooperate with each other [1,18,20].

Table 1.1. Receptors of innate and adaptive immunity [18].

Receptor (location)	**Target** (source)
RECEPTORS OF THE ADAPTIVE IMMUNE SYSTEM	
Antibody (B-cell membrane, blood, tissue fluids)	Specific components of pathogen
T-cell receptor (T-cell membrane)	Proteins or certain lipids of pathogen
RECEPTORS OF THE INNATE IMMUNE SYSTEM	
Complement (bloodstream, tissue fluids)	Microbial cell-wall components
MBL (bloodstream, tissue fluids)	Mannose-containing microbial carbohydrates (cell walls)
CRP (bloodstream, tissue fluids)	Phosphatidylcholine (microbial membranes)
LBP (bloodstream, tissue fluids)	Bacterial LPS
TLR2 (cell membrane)	Cell-wall components of gram-positive bacteria, LPS. (β1→3)-glucans
TLR4 (cell membrane)	LPS
SR (cell membrane)	Many targets; gram-positive and gram- negative bacteria, apoptotic host cells

Abbreviations used: MBL, mannose binding lectin; CRP, C-reactive protein; LPS, lipopolysaccharides; LBP, lipopolysaccharide-binding protein; TLR2, toll-like receptors 2; TLR4, toll-like receptors 4; SR, scavenger receptors.

Soluble pattern receptors of innate immunity are present in the bloodstream and tissue fluids as soluble circulating proteins and include mannose binding lectin (MBL), C-reactive protein (CRP), lipopolysaccharide-binding protein (LBP), and proteins of alternative and classical complement pathways. The interaction of soluble receptors with BRM leads in turn to binding of the receptor:BRM complex by phagocytes, either through direct interaction with the BRM-binding receptor, or through receptors for complement, thus promoting phagocytosis and the induction of other cellular responses [21].

Scavenger receptors (SRs), the toll-like receptors (TLRs), β-glucan receptor (Dectin-1), complement receptors (CR), and mannose receptor (MR) are receptors present on cell membrane [18,20]. TLRs, in particular, are mostly found on macrophages and dendritic cells, but also are expressed on neutrophils, eosinophils, epithelial cells, and keratinocytes [22]. The TLRs are a family of ancient PRRs with identified homologues in different species, like humans and flies. Several membrane proteins belong to the TLR family, like TLR2 and TLR4 [23]. These receptors are an important link between innate and adaptive immunity [23].

1.1.2.2. Activation of signalling pathways

The interaction of BRM with receptor(s) can lead to activation of several signalling pathways, ultimately leading to induction of gene transcription. Cells of the innate immunity are the major targets of BRMs, while the direct activation of other immune cells, like NK cells and lymphocytes, can be regarded as a secondary event [20]. In macrophages, the interaction with SR and CR3 activates signalling pathways that lead to the activation of mitogen-activated protein kinase (MAPK), extracellular signal regulated kinase (ERK) and nuclear factor-κB (NF-κB). MR activation leads to activation of macrophage phagocytosis, oxidant production, endocytosis and NF-κB. TLR4 activation leads to the activation of Interleukin (IL)-1 receptor-associated kinase (IRAK) via an adaptor myeloid differentiation protein 88 (MyD88), with subsequent activation of tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF-6), MAPK (e.g. p38 and JNK) and NF-κB [1,20] (**Figure 1.3**).

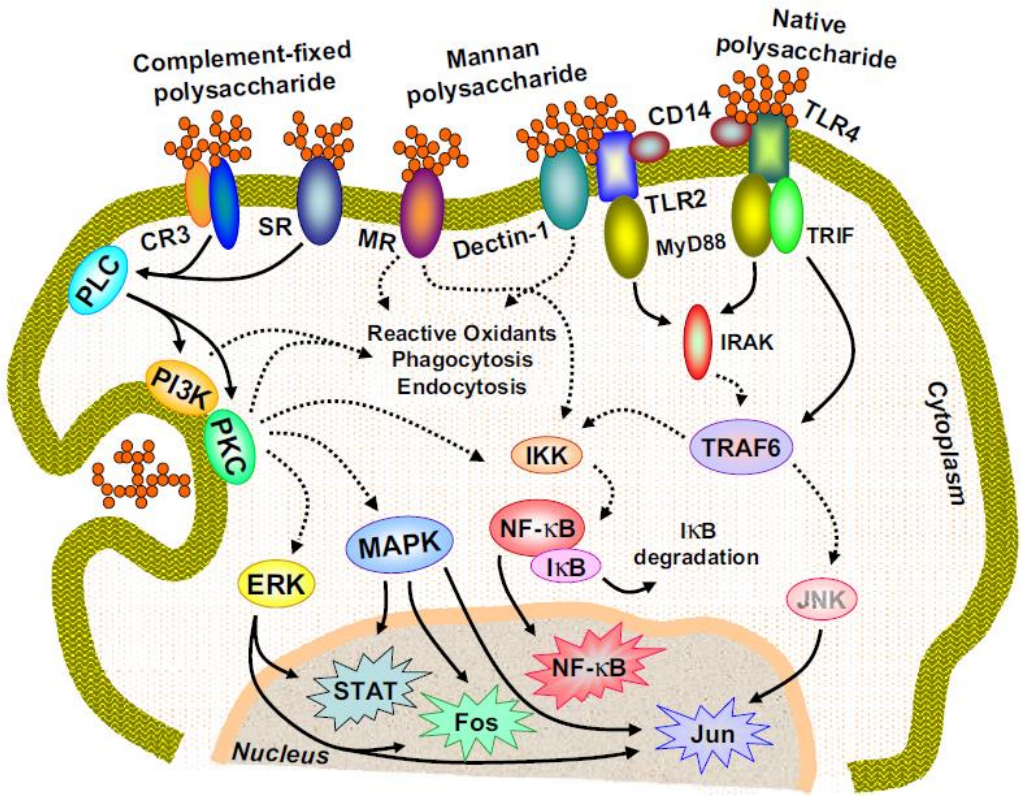

Figure 1.3. Schematic model illustrating potential signalling pathways involved in macrophage activation by polysaccharides BRMs [1].

The activation of transcription pathways induces expression of various growth factors known collectively as cytokines with pro-inflammatory activity, and inducible nitric oxide synthase (iNOS). This activation increases the reactive oxygen species (ROS) and nitric oxide (NO) production, the secretion of cytokines and chemokines, such as tumour necrosis factor-α (TNF-α), IL-1β, IL-6, IL-8, IL-12, interferon (IFN)-γ and IFN-β2, and enhances phagocytic activity. The effects of BRM can lead to further cell proliferation and differentiation [1]. Furthermore, innate immunity cells activated by BRMs increase their effector function, antigen processing capacity, and capability to modulate acquired immunity [20].

1.2. CLASSIFICATION OF IMMUNOSTIMULATORY POLYSACCHARIDES AND STRUCTURAL FEATURES

Polysaccharides are carbohydrate polymers composed of more than 10 monosaccharide units bound together by glycosidic bonds. They are classified depending on their monosaccharide composition, and they are named with the suffix "an" after the name of the residue present in higher amount. For example, a polysaccharide composed by glucose (Glc) residues is named glucan, and a polysaccharide composed by Glc but with higher amount mannose (Man) is named glucomannan. In order to know which polysaccharides have been associated to immunostimulatory activity, a search in the SCOPUS Database was performed with the topics "polysaccharides AND (immuno OR immune OR immunostimulatory OR immunomodulatory)", allowing to obtain 8370 papers, including articles and reviews. However, it was found that only 983 of these papers (12%) defined the polysaccharide studied. Glucans, mannans, pectic polysaccharides, arabinogalactans, fucoidans, galactans, hyaluronans, fructans, and xylans are the most studied polysaccharides concerning their possible immunostimulatory activity (**Figure 1.4**).

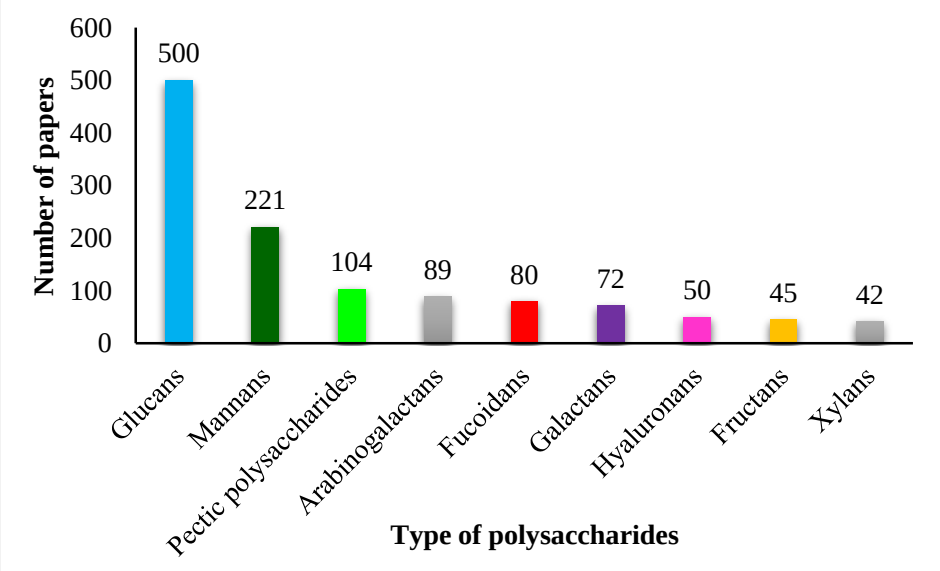

Figure 1.4. Number of papers from SCOPUS database search with the topics polysaccharides AND (immuno OR immune OR immunostimulatory OR immunomodulatory) AND type of polysaccharide. This search covers articles from 1936 until 2013.

Source-function relationships are easily established than structure-function relationships, as isolation, purification and structural characterization are usually not performed. Several immunological studies have been done with polysaccharide-rich extracts and not purified polysaccharides. This way, the presence of other compounds, like phenolic compounds or proteins [24,25], and contaminants like lipopolysaccharides (LPS) [1], could affect the measured activity. The presence of different polysaccharides in the same sample can also mask the immunostimulatory activity [26,27]. In such cases, fractionation methodologies can be important steps for purification and identification of true structure-function relationships. Moreover, the complex polysaccharides structure has made them difficult to be characterized, being necessary advanced analytical methods [28]. The chemical structures of polysaccharides, such as the monosaccharide composition, type of glycosidic linkage, and the degree of branching, may be observed by chemical analysis, chromatography and/or spectral analysis. In the next sections the main structural features of polysaccharides related with the potential immunostimulatory activity will be presented.

1.2.1. Glucans

Glucans are D-Glc*p* based polysaccharides (homoglucans) which, depending on their monosaccharides residues anomeric structure, can be α-D-glucans, β-D-glucans and mixed α,β-D-glucans [29,30]. They also present different types of glycosidic bonds originating linear or branched either (β1→4)-, (β1→3)-, and (β1→6)-glucans [27,31–39] or (α1→3)-, (α1→4)-, and (α1→6)-glucans [40–46] (**Figure 1.5. (a)-(i)**).

The complexity of glucans can further increase when there are monosaccharides present other than glucose (heteroglucans) [47–55] (**Figure 1.5. (j), (k), and (l)**), or structural differences in chain conformation, degree of branching, molecular weight or presence of functional groups [30]. All these differences result in glucans with different structural properties and therefore different possible interactions with the immune system [29,30].

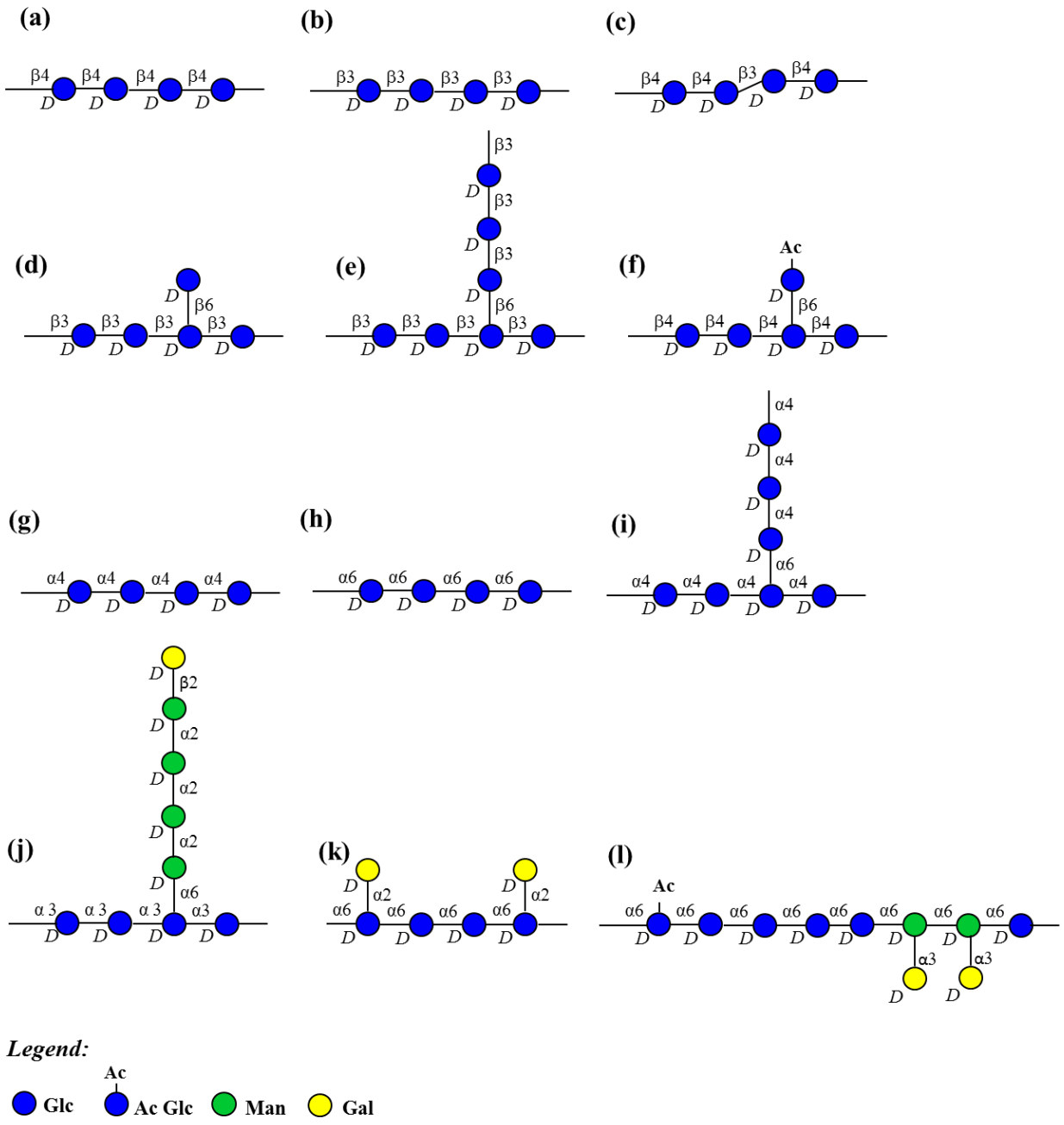

Figure 1.5. Illustration of chemical structure of several homoglucans: **(a)** cellulose, **(b)** linear (β1→3)-glucans, **(c)** mixed β-glucans from cereals, **(d)** lentinan, scleroglucan, schizophylan, laminarinan, **(e)** zymosan, **(f)** bacterial glucan, **(g)** amylose, **(h)** dextran, **(i)** amylopectin, glycogen; and **(j)(k)(l)** heteroglucans.

1.2.2. Mannans

Immunostimulatory mannans are polysaccharides with a backbone of mannopyranosyl (Man*p*) residues that can be more or less ramified with other monosaccharides. The backbone of immunostimulatory mannans mainly consists of (β1→4)-D-Man*p* [12,56–61], (β1→3)-D-Man*p* [62], (β1→2)-D-Man*p* [63], (β1→6)-D-Man*p* [64], (α1→6)-D-Man*p* [65,66] or (α1→3)-D-Manp [67–69] (**Figure 1.6**). Structural differences can also arise from the degree and sequence in which these possible backbones are substituted by various side chains containing residues of α- and β- Galactopyranosyl (Gal*p*), Man*p*, or Glc*p*, and/or functional groups, like acetyl groups [70].

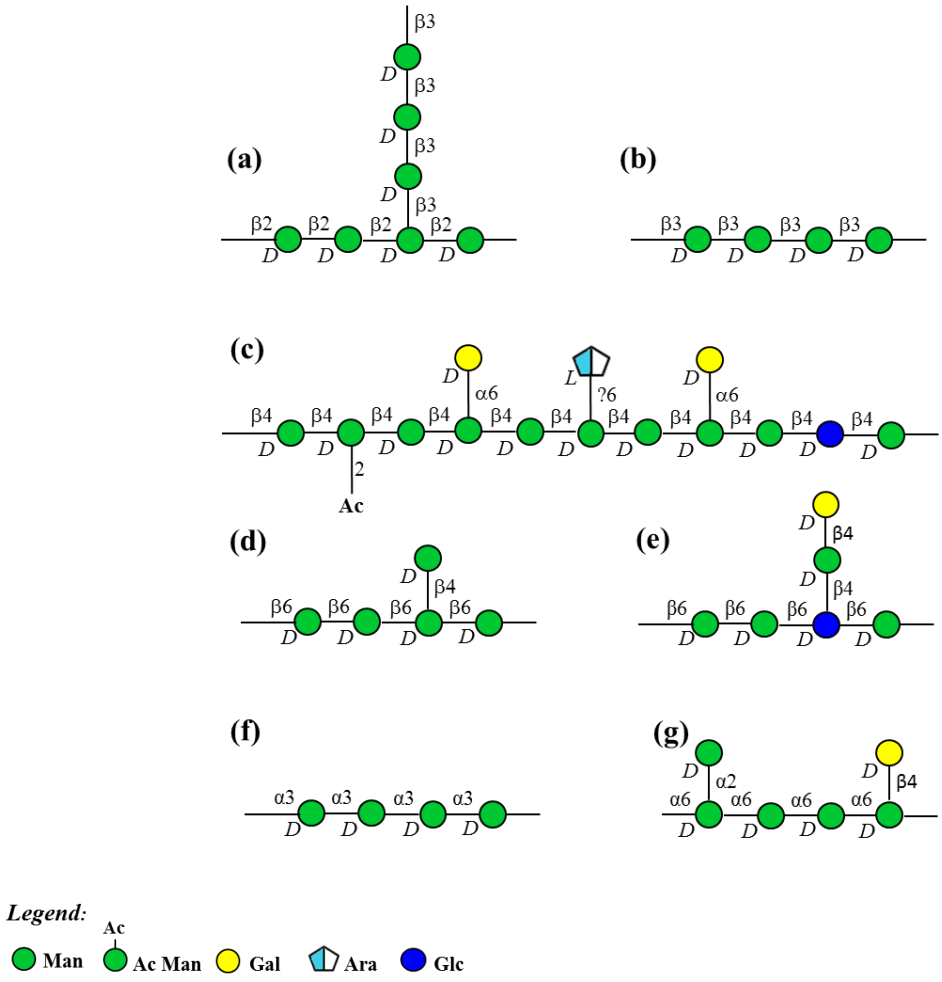

Figure 1.6. Illustration of chemical structure of possible immunostimulatory mannans: **(a)** (β1→3)-branched, β-(1→2)-D-mannan; **(b)** (β1→3)-D-mannan; **(c)** coffee galactomannan; **(d)** and **(e)** (β1→6)-D-mannans; **(f)** (α1→3)-D-mannan; **(g)** and (α1→6)-D-mannan.

1.2.3. Pectic polysaccharides

Pectic polysaccharides are complex heteropolysaccharides, which have in common a high proportion of galactopyranosyluronic acid (Gal*p*A), and can be found in plants [71]. Pectic polysaccharides include various fragments of linear and ramified regions covalently connected (**Figure 1.7**). The linear region consists of units of (α1→4)-D-Gal*p*A residues (homogalacturonan region) that can carry methyl ester groups and also be acetylated in a backbone of galacturonan [8]. A backbone of alternating (α 1→4)-D-Gal*p*A and (α 1→2)-L-Rhamnopyranosyl (L-Rha*p*) residues, ramified in the Rha by galactans [72], arabinogalactans [6,73], arabinans [74,75], of varying structure is named type I rhamnogalacturonans. Also, structures containing single xylose (Xyl) residues as pectic polysaccharide side chains has been called xylogalacturonans. Type II rhamnogalacturonans are branched structures composed of several monosaccharides, including 2-O-methylfucose, 2-O-methylxylose, and apiose, usually not observed in other polysaccharides [76–79]. Therefore, structural diversity arises from the degree of branching, degree of methyl esterification, degree of acetylation, the type of branched chains and molecular weight [80].

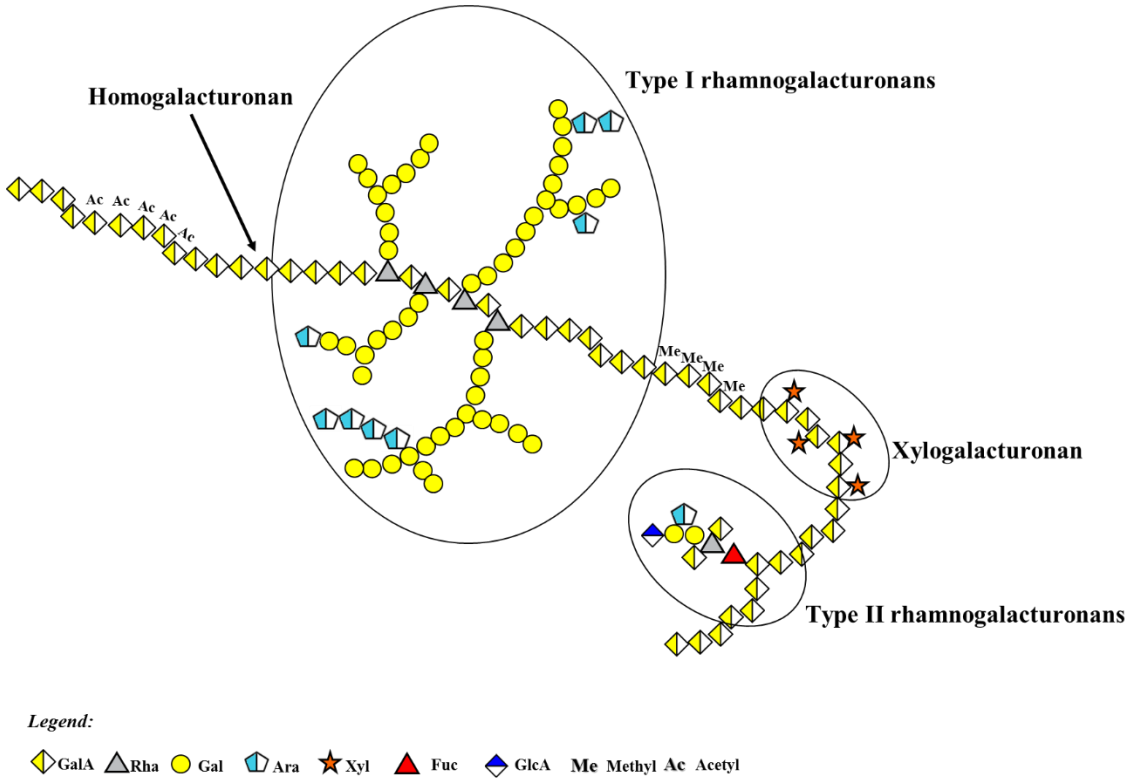

Figure 1.7. Illustration of chemical structure of the primary structure of pectic polysaccharides [adapted from 48].

1.2.4. Arabinogalactans

Arabinogalactans can be subdivided into two main structural types: type I arabinogalactans (AG-I) [6,73] and type II arabinogalactans (AG-II) [4,5,78,81–84] (**Figure 1.8**). AG-I are arabinosyl-substituted derivatives of linear (β1→4)-D-Gal*p* units. α-L-Ara*f* and β-D-Gal*p* units can be linked via position 3 along the main chain [6,73]. AG-I are found as ramified regions of rhamnogalacturonan backbones in pectic polysaccharides [73,85].

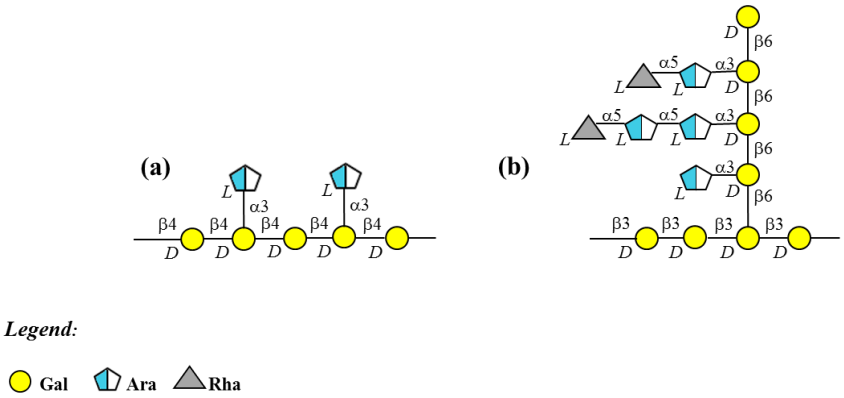

Figure 1.8. Illustration of chemical structure of **(a)** type I and **(b)** Type II arabinogalactans.

AG-II comprise highly branched polysaccharides with ramified chains of (1→3)-linked and (1→6)-linked β-D-Gal*p* units, the former predominantly in the interior and the latter in the exterior chains [4,5,78,81–84]. The arabinosyl units might be attached through different positions of the (β1→6)-D-Gal*p* side chains. AG-II may occur in a complex family of proteoglycans known as arabinogalactan-proteins (AGP) [7,86,87].

It is mainly AG-II and AGP that have been reported as immunomodulating activators. These kind of structures can be easily identified by the Yariv reagent assay [5,81,88–90].

1.2.5. Fucoidans

Fucoidans refers to sulfated fucans, that is, sulfated rich L-Fucopyranosyl (L-Fuc*p*) polysaccharides. However, like pectic polysaccharides, the chemical composition of most fucoidans is complex [91]. Nevertheless, it is generally recognized that fucoidans are heteropolysaccharides made of L-Fuc*p* (35–50%), (α1→2)-, (α1→3)- or (α1→4)-linked, that can be sulfated or acetylated at various positions (**Figure 1.9**). The other monosaccharides that can be present are Gal*p*, Man*p*, Xyl*p* and uronic acids [70]. The immunostimulatory activities of fucoidans are associated with the presence of functional groups and their major monosaccharide, fucose [70,91].

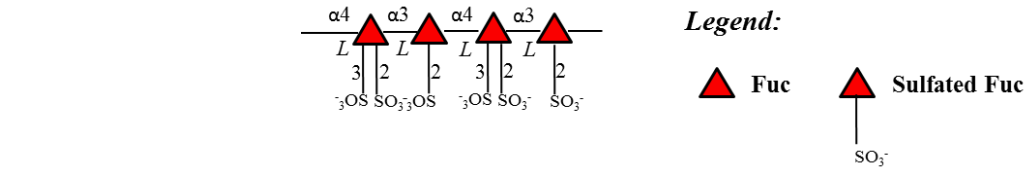

Figure 1.9. Illustration of the chemical structure of a fucoidan.

1.2.6. Galactans

Galactans are polysaccharides rich in galactose [92–96]. There are different kinds of galactans, depending in their structure. Behind the arabinogalactans already described in **1.2.4**, there are other galactans, usually sulfated, derived from marine organisms, namely carrageenan [92–94] and porphyran [95] (**Figure 1.10**), that have been studied concerning their immunostimulatory activity.

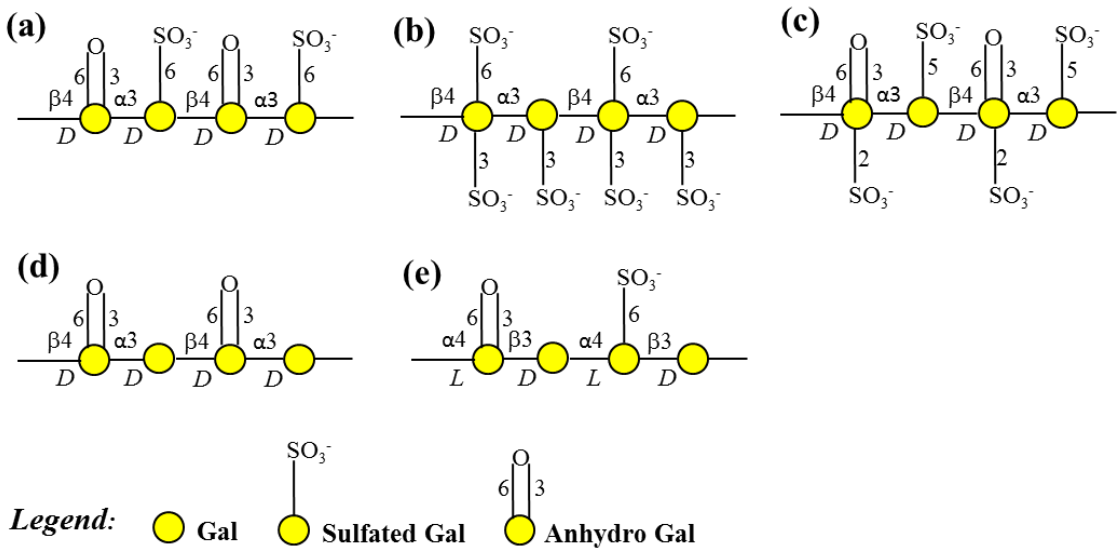

Figure 1.10. Illustration of chemical structure of some galactans: **(a)** κ-carrageenan, **(b)** λ-carrageenan, **(c)** ι-carrageenan **(d)** β-carrageenan and **(e)** porphyran.

Carrageenans are chemically characterized by repeating disaccharide units, consisting of sulfated or unsulfated D-galactose residues that are linked in alternating (β1→4)- and (*α*1→3)-bonds. There are several carrageenans, classified according to the presence of the 3,6-anhydro-bridge on the 4-linked galactose residue, and position and number of sulfate groups [92–94]. Porphyrans are characterized by a linear backbone consisting of 3-linked β-D-galactosyl units alternating with either 4-linked *α*-L-galactosyl 6-sulfate or 3,6-anhydro-*α*-L-galactosyl units [95].

1.2.7. Hyaluronans

Hyaluronan, also known as hyaluronic acid, is a major carbohydrate component of the extracellular matrix of mammalian tissue and can be found in skin, joints, eyes, and most other organs and tissues, but can also be find in other sources. It is a disaccharide repeating unit of N-acetylglucosamine (GlcNAc) and GlcA (**Figure 1.11**) and have been associated to immunostimulatory activity [9,97,98].

Figure 1.11. Illustration of chemical structure of hyaluronan.

1.2.8. Fructans

Fructans are reserve carbohydrates comprising 1–70 units of fructose, linked or not to a terminal sucrose molecule. According to the type of linkage, fructans are classified into three families, namely, inulin [(β2→1)-D-Fru*f*], levan [(β2→6)-D-Fru*f*], and mixed type [both (β2→1)- and (β2→6)-linked D-Fru*f*] [99] (**Figure 1.12**). Oligosaccharides of the fructans type act as bifidogenic agents and immune system stimulators associated with the intestinal mucosa [100].

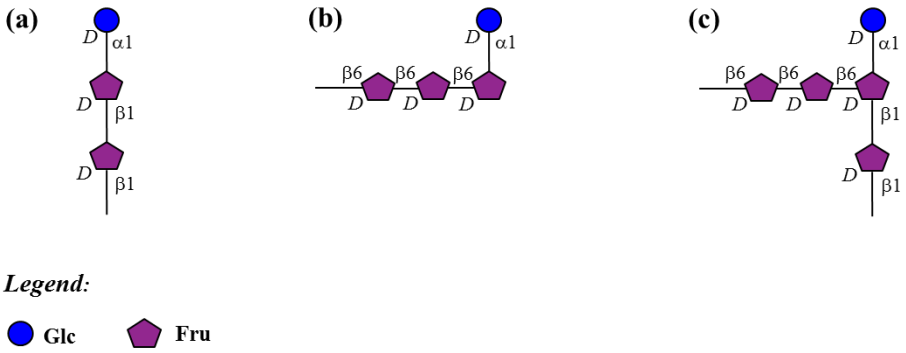

Figure 1.12. Illustration of the chemical structure of fructans: **(a)** inulin, **(b)** levan, and **(c)** mixed type.

1.2.9. Xylans

Xylans are polysaccharides present in plant cell walls and contain predominantly a backbone of (β1→4)-D-Xyl*p* residues units linked. These polysaccharides contain other sugar monomers attached to their backbone, including α-L-Ara*f* units (arabinoxylans), and α-D-Glc*p*A units (glucuronoxylans) (**Figure 1.13**), and showed immunostimulatory activity [101–105]. Their molecular weight, their degree of branching, and the presence of other compounds associated, like protein and ferulic acid, can affect the resulting immunostimulatory activity [101,104].

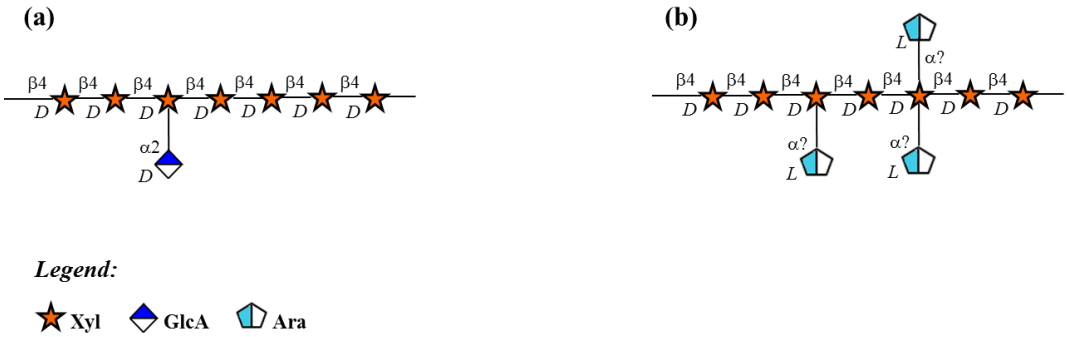

Figure 1.13. Illustration of chemical structure of **(a)** glucuronoxylans and **(b)** arabinoxylans

1.3. STRUCTURE-FUNCTION RELATIONSHIP

The previous search in the Scopus Database revealed that glucans, mannans, pectic polysaccharides, arabinogalactans, fucoidans, sulfated galactans, hyaluronans, fructans, and xylans are the most studied types of polysaccharides. In order to find structure-function relationships, a further database search was performed adding the structural feature topic to the previous ones ("polysaccharides AND (immuno OR immune OR immunostimulatory OR immunomodulatory) AND *type of polysaccharide*"). From this search it was found that in addition to the type of polysaccharide, conformation, molecular weight, presence of functional groups like acetyl and sulfate groups, charge and degree of branching are connected with the immuno topic (**Figure 1.14**). It can be noticed that some structural features have more relevance in some type of polysaccharides than in another's and that in almost all type of polysaccharides the number of papers associated to a structural feature is lower than the number of papers without assigned structural features, supporting the lack of structure-function relationships. Furthermore, the combination of several structural features may impact the resulting immunostimulatory activity in different ways.

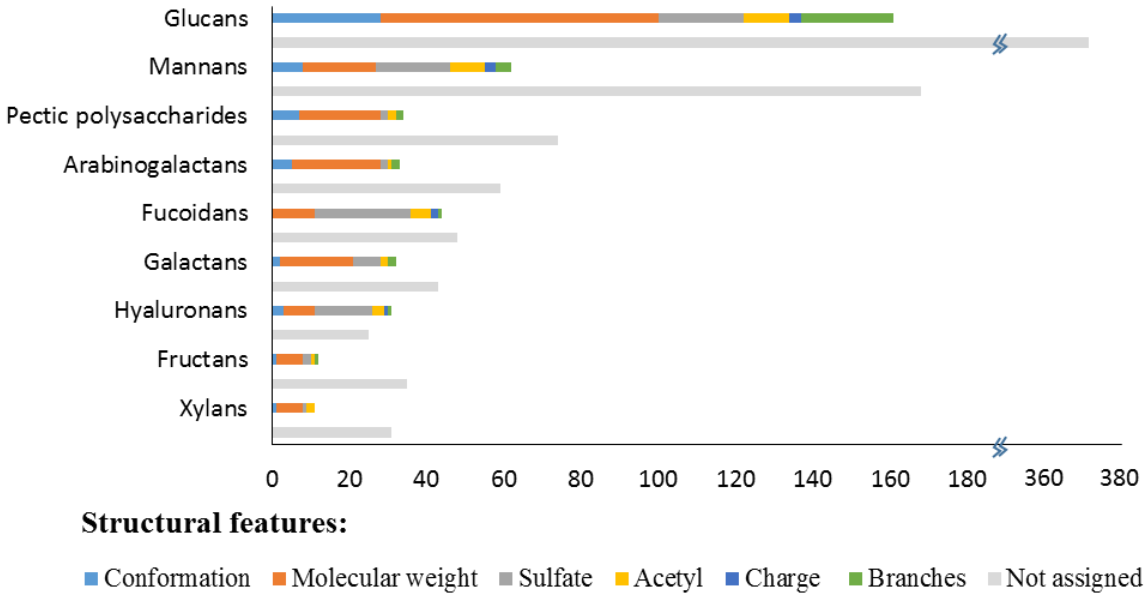

Figure 1.14. SCOPUS database search with the topics immuno AND polysaccharides AND type of polysaccharide AND structural features. This search covers the articles of years 1936 until 2013.

Based on this database search, papers that studied the conformation, molecular weight, presence of functional groups, degree of branching, and charge of polysaccharides were used to discuss the possible structure-function relationships of immunostimulatory polysaccharides, presented in the next subchapters. Moreover, the papers selected were only those that used characterised polysaccharide-rich fractions (at least giving the type of monosaccharides), studied immunostimulatory activity (*in vitro* and/or *in vivo*) and, preferably, that studied the effect of structural changes in the immunostimulatory activity potential. A summary table was constructed using the provided information, indicating the type of polysaccharide, structural features, immunostimulatory activity, as source (**Table 1.2**).

Table 1.2. Different sources and structural features of immunostimulatory polysaccharides: glucans, mannans, pectic polysaccharides, arabinogalactans, fucoidans, galactans, hyaluronans, fructans, and xylans.

Source	PS name/structure	PS features (Mw, DS, conformation)	Animal / Cell type	Immunostimulatory activity	LPS*	R
Glucans						
Aconitum carmichaeli	(α1→6)-D-glucans branched at C-3 with Glc*p*	Mw 14 kDa	BALB/c mice splenocytes	↑ mitogenic and comitogenic activity ↑ splenocyte antibody production	--	[43]
Agaricus bisporus and *Agaricus brasiliensis*	(β1→6)-D-glucans	Mw 29 and 45 kDa	THP-1 cells	↑ expression of pro-inflammatory genes	y	[27]
Armillariella tabescens	(α1→6)-D-glucan	Mw 49.5 kDa	BALB/c peritoneal MO	↑ NO, TNF-α, IL-1b and IL-6 ↑ iNOS, TNF-α, IL-1b and IL-6 mRNA	y	[42]
Chemically synthetized	Tetra- and penta-Oligo-(β1→3)-glucan	Mw 0.67-0.83 kDa; Not helical structures	BALB/c mice	↑ phagocytosis activity of peritoneal MO ↑ influx of monocytes and granulocytes into the blood ↑ influx MO into the peritoneal cavity	--	[106]
Chemically synthetized	Oligo-(β1→3)-glucan-mannose	Mw 0.83-0.99 kDa; Not helical structures	BALB/c mice	↑% of granulocytes in peripheral blood, intra-peritoneal ↓ % of lymphocytes, intra-peritoneal ↑ influx of peritoneal MO ↑ phagocytic activity of peritoneal MO ↑ IL-2 by spleen cells	--	[107]
Cordyceps sinensis (strain Cs-HK1)	Two (α1→4)-D-Glc*p*: WIPS - branched with (α1→6)-D-Glc*p* (~14%) AIPS - linear glucan.	Mw_{WIPS} 1180 kDa Mw_{AIPS} 1150 kDa Random coil structure	C57BL/6 mice inoculated with B16 cells	↑ antitumor ↑ immunostimulatory effects in splenocytes AIPS>> WIPS	--	[41]
Dendrobium huoshanense	((α1→3)-D-Gal*p*) (α1→6)-mannoglucans	Mw 22 kDa α-Glc 20% acetylated	BALB/c mice splenocytes and peritoneal MO	↑IFN-γ by splenocytes ↑TNF-α by MO	--	[50]
Dictyophora indusiata	(1→6)-branched, (β1→3)-D-glucan	Mw 480 kDa Triple-helix	Kunming (KM) mice inoculated with S180 cells	↑ Thymus and spleen indexes ↑ serum IL-2, IL-6, and TNF-α	--	[33]
Dioscorea opposita	(α1→3)-D-glucans (heteroglucan)	42 kDa	KM mice splenocytes	↑ comitogenic activity	y	[48]

Ganoderma lucidum	(β1→3)-D-glucans (highly branched)	Mw 8 kDa	CHO cells RAW264.7 cells; murine peritoneal MO; C57BL/6 and BALB/c nu/nu inoculated with Lewis lung cancer; BALB/c mice Splenocytes	↑ MAPKs- and Syk-dependent TNF-α and IL-6 Dectin-1 recognition ↑ comitogenic activity ↑ anti-tumor activity	y	[38]
	Sulfated (β1→3)-D-glucan	Mw 125 kDa; $DS_{sulfate}$ 0.94; stiff chain	BALB/c mice inoculated with S-180 solid tumors	↑ thymus and spleen index	--	[32]
	Carboxymethylated (β1→3)-D-glucan	Mw 52 kDa; $DS_{carboxymethyl}$ 1.18				
Hordeum vulgare	(β1→4)(β1→3)-D-glucans	Mw 886-1090 kDa	Human complement proteins	Activate complement system	y	[37]
Hyriopsis cumingii	Arabinoglucan (HSCP-1)	Mw_{HCPS-1} 432.2 kDa $DS_{sulfate}$ 0,3804 %	KM mice	↑ splenocyte proliferation, ↑ acid phosphatase in peritoneal MO ↑ MO phagocytosis (HCPS-3 >> HCPS-1 and HCPS-2)	--	[51–53]
	Glucan (HCPS-2)	Mw_{HCPS-2} 457.9 kDa $DS_{sulfate}$ 0.5959 %				
	Galactorhamnoglucan with fucose (HCPS-3)	Mw_{HCPS-3} 503.1 kDa $DS_{sulfate}$ 6.2938 % Not triple-helices				
Imocarpus longan	Arabinomannoglucans LPI1 and LPI2	Mw 14 kDa LPI1 - sphere-like LPI2 - single-helix	KM mice	↑ splenocyte proliferation ↑ NK cell cytotoxicity	--	[54]
Ipomoea batatas (roots)	(α1→6)-D-glucan	Mw 53.2 kDa Compact random coil	KM mice; YAC-1 cells	↑ proliferation of spleen cells ↑NK cell cytotoxicity ↑ phagocytic function of MO ↑ hemolytic activity ↑ serum IgG	--	[45]
Lentinus edodes	Arabinogalactoglucan	Mw 26 kDa Not triple-helix	RAW 264.7 cells	↑ NO, TNF-α, and IL-6 by TLR2	--	[55]
	(1→6)-branched, (β1→3)-D-glucan	Mw 1490 kDa Triple-helix	BALB/c mice inoculated with S-180 cells	↑ antitumor activity	--	[34]
Lepista sordida	(α1→6)-D-glucans (heteroglucan)	Mw 40 kDa	J774A.1 cells	↑ NO and TNF-α	y	[49].
Panax ginseng C. A. Meyer	(α1→6)-D-glucan	Mw 17 kDa	ICR mice splenocytes	↑ lymphocyte proliferation with or without LPS ↑ NO production	y	[44]
Pleurotus sajor-caju	(1→6)-branched, (β1→3)-D-glucan	Triple-helix	J774A.1 cells	↑ NO and TNF-α	y	[35]
Rhizobium sp. N613	(β1→6)- D-Glc branched, (β1→4)-D-Glc	Mw 35 kDa DS_{acetyl} 0.1	KM mices inoculated with S18, hepatoma 22, and Ehrlich ascites carcinoma	↑ spleen and thymus weight ↑ phagocytic function of MO ↑ lymphocyte proliferation ↑ serum antibody		[31]
Sclerotium rolfsii	(β1→3)-D-glucan substituted with single (β1→6)-D-Glc*p* residues at every third residue	Mw < 500 kDa or Mw> 1100 kDa Triple helix	Human monocytes	↑ TNF-α in monocytes	y	[36]
Tinospora cordifolia	(1→6)-branched, (α1→4)-D-glucan	Mw >550 kDa	Human lymphocytes; Human complement Kits	Activate NK cells, T and B cells Complement activation Th1 pathway-associated profile	y	[46]
Unknown	Carboxymethylated (α1→3)-D-glucan	Mw 80.4 kDa; DScarboxymethyl 0.28	Inbred ICR mice	↑ lymphocyte proliferation; ↑ antibody production	--	([40]

Mannans						
Aloe Vera	Acemannan	Mw 10,000 kDa, 1300 kDa, and 470 kDa. DS_{acetyl} 0.91	BALB/c mice	↑ peritoneal MO ↑ splenic T and B cell proliferation ↑ TNF-α, IL-1β, INF-γ, IL-2, and IL-6. (↑↑ for Mw 10,000 kDa)	y	[56]
	Acemannan (G2E1DS3, G2E1DS2 and G2E1DS)	$Mw_{G2E1DS3} \geq 400$ kDa; 5 kDa $\leq Mw_{G2E1DS2} \leq$ 400 kDa; $Mw_{G2E1DS1} \leq 5$ kDa	RAW 264.7 cells	↑ NO, TNF-α, IL-1β by MO (G1E2DS1 and G1E2DS3<<< G1E2DS2)	y	[57,58]
Coffea (infusion)	Galactomannans	Mw140–90 kDa DS_{acetyl} 0.08	C57BL/6 mice	↑ B lymphocyte activation	y	[12,59]
Coffea (spent coffee grounds)	Galactomannans	Mw 109 DS_{acetyl} 0.84	C57BL/6 mice	↑ B lymphocyte activation	y	[12,59]
Cordyceps militaris	Galactoglucomannan	Mw 36 kDa Random coil	RAW 264.7 cells	↑ NO, IL-1β, TNF-α	y	[64]
Haematococcus lacustris	Galactomannan	Mw 135 kDa $DS_{sulfate}$ 1.08%	RAW 264.7 cells	↑ TNF-α ↑ expression of COX-2 and iNOS	--	[108]
Hericium erinaceus (liquid culture broth)	Mannan	Mw 46 kDa Triple helix	RAW 264.7 cells	↑ NO, IL-1β and TNF-α	y	[63]
Peltigera canina	(α1→6)-mannan With (α1→2)-Man*p* and (β1→4)-Gal*p*	Mw 53 kDa	Lewis rats	↑ splenocytes proliferation ↑ IL-10 secretion ↑ TNF-α by MO	--	[65,66]
Picea abies L.	Acetylated galactoglucomannan (AcGGM) Deacetylated galactoglucomannan (GGM)	Mw_{AcGGM} 40 kDa TP_{AcGGM} 4.6 mg/100mg Mw_{GGM} 10 kDa TP_{GGM} mg/100m GGM ↓solubility	Wistar rats thymocytes	↑ proliferation of thymocytes GGM>AcGGM	--	[24]
Poria cocos (mycelia)	Heteropolysaccharide (β-D-galactofuranan, (α1→3)-D-glucan, mannan) With fucose	Mw 304 kDa and 1030 kDa; Compact random coil (close to globular shape)	HL-60 leukemia cells; Human MCF-7 cells; and Vero cells; BALB/c male mice inoculated with S180	↑antitumor activity mediated by immune system stimulation	--	[109]
Tremella aurantialba (fruit bodies)	Xylomannans: (TAPA1) TAPA1-s (sulfonated) TAPA1-ac (acetylated) TAPA1-deac (deacetylated)	Mw 1350 kDa DS_{acetyl} 0.03 $DS_{sulfate}$ 0.05 $DS_{acetylated}$ 0.23 $DS_{desacteyl}$ 0	C57BL/6 mice; lymphocytes RAW264.7 cells	↑ proliferation of spleen lymphocytes (TAPA1-s >>TAPA1) ↑ NO by MO (TAPA1-ac> TAPA1> TAPA1-deac)	--	[67–69]
Trigonella foenum-graecum L. (Fenugreek)	Galactomannans	Acetyl groups not detected	Sprague dawley rat; HB4C5 cells	↑ phagocytosis by MO ↑ proliferation of MO ↑ IgM secretion in HB4C5 cells	--	[60,61]
Polyporus albicans (Imaz.) Teng	(α1→6)-Gal*p* branched, (β1→3)-D-mannan	Mw 37 kDa	KM mice splenocytes	↑ mitogenic and comitogenic activity	y	[62]
Pectic Polysaccharides						
Avicennia marina	Branched rhamnogalacturonan type I (HAM-3-IIb-II)	DS_{acetyl} 3.1%	Mice splenocytes	↑ LPS-induced effect on B lymphocyte proliferation	--	[76]
Centella asiatica	Rhamnogalacturonan (after deacetylation and carboxyl-reduction)	Mw 77.4 kDa	Inbred ICR mice splenocytes	↑ lymphocyte proliferation	--	[77]
Monostroma angicava	Rhamnan	$DS_{sulfate}$ 21.8%	BALB/c mice	↑ spleen index, NK cytostatic activity and splenocytes activity	--	[8]
Prunus dulcis (seeds)	Arabinan-rich	Mw 762kDa	C57BL/6 mice spleen cells	↑ lymphocyte activation markers	y	[74]
Radix Astragali	Arabinan	Mw1334 kDa With O-acetyl groups Random coil	PBMC	↑ proliferation of PBMC ↑ IL-1β, TNF-α, IL-10, IL-10, GM-CSF	--	[75]

Trichilia emetic	Pectic polysaccharide with AG-II	Mw 223 kDa	Sheep erythrocytes	↑ complement fixation activity (↓ after removal of T-Ara*f*)	--	[78]
Vernonia kotschyana	Pectic polysaccharide (Vk100A2b)	Mw 1150 kDa DS_{acetyl} 7%	Sheep erythrocytes	↓ complement fixation activity	y	[82]
Arabinogalactans						
Anadenanthera colubrina	AG-II	Mw 1600 kDa	Albino Swiss mice MO; S-180 cells; albino Swiss mices inoculated with S-180 cells	↑ Phagocytosis ↑ ROS and TNF-α	y	[83]
Artemisia tripartita	AG-II	Mw 251-49 kDa N- and O-acetylated	J774.A1 cells; human and murine neutrophils	↑ ROS, NO, IL-6, IL-10, TNF-α and chemotactic protein-1.	y	[84]
Chlorella pyrenoidosa	AG	Mw 188 and 1020 kDa Not a rigid conformation	RAW264.7 cells	↑ NO	--	[86,110]
Coffea (instant coffee)	AGP	Mw 5-6 kDa	Adult male guinea pigs (strain Trik); Balb/c mice	Antitussive ↑ TNF-α, IL-2 and IFN-γ by splenocytes	--	[7]
Cordyceps militaris	AG-I	Mw 576 kDa	BALB/c mice inoculated with Influenza A virus (NWS strain, H1N1); RAW 264.7 cells	↑ survival rate of Influenza A virus infected mice ↑ TNF-α and IFN-γ in treated mice ↑ NO by iNOS in MO ↑ mRNA expression of IL-1β, IL-6, IL-10, and TNF-α by MO	y	[6]
Entada africana	AG-II	Mw 19 kDa	Sheep erythrocytes	↑ complement fixation activity (↓ after removal of T-Ara*f*)	--	[87]
Euterpe olerácea (fruit)	AG-II	Mw 4-800 kDa Presence of N- and O-acetyl groups	C57BL/6 or BALB/c mice	↑ IFN-γ by NK and γδ T cells in the lungs of C57BL/6 mice ↓ pulmonary *Francisella tularensis* and *Burkholderia pseudomallei* infections	y	[4,5]
Glinus oppositifolius	AG-I and AG-II	Mw 70 kDa DS_{acetyl} 4.3%	Sheep erythrocytes; PVG.7B strain rats lymphocytes; RNK-16 and mice MO; C3H/HeJ mice	↑ complement fixation activity B-lymphocytes proliferation ↑ IL-1β by MO ↑ mRNA for IFN-γ in NK-cells ↑ proliferation of bone marrow cells through Peyer's patch cells	y	[85,111]
Juniperus scopolorum	AG	Mw 200–680 kDa N- and O-acetylated	J774.A1 cells	↑ iNOS, NO, ROS, IL-1, IL-6, IL-12, TNF-a and IL-10	y	[90]
Lycium barbarian	AG-I	Mw 214.8 kDa	Splenocytes	↑ IgG by B-lymphocyte ↑ NF-κB and AP-1expression B-lymphocytes proliferation	--	[73]
Opilia celtidifolia	Arabinogalacturonan	Mw 1000-8400 kDa	Sheep erythrocytes; rat Wistar MO	↑ complement fixing activity ↑ NO by MO	--	[89]
Tanacetum vulgare	Acidic PS with AG-II	Presence of N/O-acetyl groups	J774.A1 cells; THP1-Blue cells; sheep erythrocytes; Human neutrophils	↑ ROS and NO by MO/monocytes ↑ TNF-α by MO ↑ NF-κB in monocytes. ↑ complement-fixing activity stimulated MPO neutrophil release	y	[81]
Vernonia kotschyana	Pectic arabinogalactan (Vk100A2a)	Mw 20 kDa DS_{acetyl} 11%	Sheep erythrocytes; C3H/HeJ mice splenocytes	↑ complement fixation activity ↑ T cell independent induction of B-cell proliferation	y	[82]
Fucoidans						
Fucus evanescens	Fucoidan (native); Hyposulfated (hypoS); Deacetylated (deAc); Hyposulfated and deacetylated (hypoSdeAc)	Mw 150 kDa and 500 kDa	Balb/c mice	↑ IL-1β, IL-6, IL-12, TNF-α by DCs and MO (Native >> hypoS> deAc>> hypoSdeAc)	y	[112]

Galactans						
Chondrus ocellatus	Galactan (λ-carrageenans)	Mw 9.3-650 kDa Sulfate 21.8-30.5 %	ICR mice inoculated with S180 and H22 cells; YAC-1 cells	↑ NK cells activity ↑ lymphocyte proliferation	--	[92]
Chlorella pyrenoidosa	(β1→3)-D-galactans	Acetylated	RAW 264.7 cells	↑ NO	--	[96]
Gigartinaceae and *Tichocarpaceae*	Galactans (κ-,β-,ι-,λ-carrageenans)	Mw 200-500 kDa Sulfate 20-28%	ICR mice human blood cells; BALB/C mice peritoneal fluid	↑MO-phosphatase activity ↑TNF-α, IL-6 ↑lysosomal activity of MO ↑ROS (λ-carregannan)	--	[93]
Porphyra vietnamensis	Porphyran (sulfated galactan)	$DS_{sulfate}$ 1.15 DS_{methyl} 0.62	Wistar albino rats and albino mice; Sheep erythrocytes	↑ weight of the thymus, spleen and lymphoid organ cellularity ↑ phagocytic activity ↑ neutrophil adhesion ↑ alkaline phosphatase activity ↓ Cy-induced myellosuppression	--	[95]
Solieria chordalis	Galactans (carrageenans)	Mw< 20 kDa $DS_{sulfate}$ 33.54±0.3	Daudi (Human Burkitt's lymphoma); PBMC	↑ phagocytosis, ↑ cytotoxicity by NK-cells, and antibody-dependent cell cytotoxicity ↑ lymphocyte proliferation.	--	[94]
Hyaluronans						
Streptococcus equi subsp. zooepidemicus	HA (CP-3)	Mw 1338.0 kDa	KM mice	↑ splenocyte proliferation ↑ increase the activity of acid phosphatase in peritoneal MO	--	[97]
Unknown	Hyaluronans	Mw 1050, 145, and 45.2 kDa	KM mice	↑ splenocyte proliferation ↑ indices of spleen and thymus ↑ activity of lysozyme in serum ($Mw_{145\ and\ 45.2} > Mw_{1050}$)	--	[98]
Fructans						
Allium sativum (Aged extract)	Two fructans (HF and LF)	Mw_{HF} >3.5 kDa; Mw_{LF} <3 kDa	BALB/c mice and CFT Wistar rats	↑ mitogenic activity ↑ intra-peritoneal MO activity ↑ phagocytosis of MO	--	[113]
Asparagus racemosus Linn.	(β2→1)-D-fructo-oligosaccharides	Mw 1.1-1.2 kDa	PBMC	↑ NK cell activity	--	[114]
Bacillus subtilis (fermentation of soybeans)	(β2→1)-D-Fru branched, (β2→6)-D-fructan	--	J744.1 RAW264.7 C3H/HeN and C3H/HeJ	↑ IL-12 and TNF-a	y	[115]
Platycodon grandiflorum	(β2→1)-D-fructans	--	BDF1 mice	↑ IgM ↑ B cells proliferation ↑ iNOS mRNA and NO in MO	y	[116]
Ophiopogon japonicus	Fructans	Mw 14 kDa Globular to helical fibrous shape at increasing concentrations	Balb/c mice	↑ lymphocytes proliferation	--	[117]
Xylans						
Several sources	Glucuronoxylans Aarabinoxylans	Mw 21.5-990 kDa	Wistar rats thymocytes	↑ mitogenic and comitogenic activity	y	[104][105]
Triticum spp. (bran)	Arabinoxylans (AXa and AXe)	Mw_{AXa} 351,7 kDa Mw_{AXe} 32,52 kDa AXe had ferulic acid	BALB/c mice	↑ MO phagocytosis ↑ lymphocyte proliferation ↑ hypersensitivity reaction	y	[101]

*LPS contamination evaluation or decontamination: y, evaluated; --, not evaluated. Abbreviations: PS, polysaccharides; Mw, molecular weight; DS, degree of substitution; LPS, lipopolysaccharide; R, references; MO, macrophages; TNF-α, tumor necrosis factor α; IL, interleukin; iNOS, inducible nitric oxide synthase; mRNA, messenger ribonucleic acid; IFN, interferon; KM, Kunming; MAPK, mitogen-activated protein kinase; NK, natural killer; IgG, immunoglobulin G; TLR2, toll-like receptor 2; COX-2, cyclooxygenase-2; TP, total phenolics content; IgM, immunoglobulin M; PBMC, human peripheral blood mononuclear cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; AG-I, type I arabinogalactan; AG-II, type II arabinogalactan; AG, arabinogalactan; AGP, Arabinogalactan-protein: ROS, reactive oxygen species; NF-κB, nuclear factor-κB; AP-1, activator protein-1; MPO, myeloperoxidase; HA, hyaluronic acid; DCs, dendritic cells; Cy, cyclophosphamide.

1.3.1. Conformation

The conformation of polysaccharides has been related to their immunostimulatory activity. Polysaccharides may exhibit different conformations in solution, such as helical chains, including single- and triple-helix, and random coil chains, that can be more or less stiff (rigid) or flexible chains [28]. Analysis of the conformation is a difficult task not only because polysaccharides are complex structures but also because they often have high molecular weights, and tend to form aggregates in solution that can mask the behaviour of individual macromolecules [75]. Conformation can influence the direct contact between the polysaccharides and the cells or others components of the immune system and, therefore, the resulting immunostimulatory activity [118].

Glucans

Some paradoxical data appeared about the importance of triple-helix conformation tightness for the immunostimulatory activity of β-glucans. First studies indicated that triple-helix conformation conferred higher immunostimulatory activity to (β1→3)-D-glucans with side chains of (β1→6)-D-Glc [35,36]. The importance of triple-helix was also shown when the destruction of this structure lead to the reduction of activity [34]. Furthermore, it has been evidenced that triple-helix with a rigid conformation in solution had the highest activity [118,119]. However, contrasting results showed that a less tight triple-helix, obtained after a denaturation and renaturation process, had the highest activity [33,120]. Other studies show that single-helix glucans had also immunostimulatory activity, suggesting that the immunostimulatory activity of (βl→3)-D-glucans may be depend on the existence of an helical conformation [20,34,121].

In contrast with homoglucans, heteroglucans without helical conformations showed also immunostimulatory activity [47,55]. This suggests that the presence of other monosaccharides surpasses the requirement of helical conformations for the exhibition of immunostimulatory activity.

Mannans

Mannans with random coil conformation have higher immunostimulatory activity [64,109]. Furthermore, the compactness and the globular shape of this random coil conformation has been also associated with the immunostimulatory activity [109]. Nevertheless, a less active mannan was described with a triple-helix conformation [63].

Arabinogalactans

Immunostimulatory pectic polysaccharides with arabinogalactan structures exhibited random coil conformations [75,86]. In this kind of polysaccharides their activity was associated to a flexible chain conformation and not rigid conformations.

Fructans

The importance of helical conformation in immunostimulatory activity was also showed for fructans [117]. This conformation was evidenced at increasing concentrations and the immunostimulatory activity was also concentration dependent.

1.3.2. Molecular Weight

A large range of molecular weights, from low (1,1 kDa) [114] to high molecular weights (10,000 kDa) [56], have been attributed to immunostimulatory polysaccharides. This large range hampered the establishment of molecular weight-function relationships. Therefore, most of the studies stated the molecular weight as an important structural feature in a perspective source-function relationship [78,81,86,87,98] (**Table 1.2**).

Glucans

A large range of molecular weight glucans were tested, from 7.70 to 28,300 kDa, showing that (β1→3)-D-glucans, with side chains of (β1→6)-D-Glc, with molecular weight around 1,020-1,490 kDa had the highest immunostimulatory activity [34,118]. However, it was suggested, by the study of oligosaccharides of (β1→3)-D-Glc*p*, that high molecular weight was not necessary to obtain immunostimulating effects [106,107]. Such results suggest that the molecular is not an exclusive property, but is intrinsically related to other structural features, e.g. conformation [118].

Mannans

The first studies suggested that high immunostimulatory activity of (β1→4)-D-mannans was associated to the high molecular weight (10,000 kDa) [56,122]. However, further fractionation of these structures, gave smaller polysaccharides (5 to 400 kDa) with stronger immunomodulatory activities [12,57–59]. Furthermore, also (β1→3)-D-mannans, (α1→6)-D-mannans and (α1→3)-D-mannans were active in the same molecular weight range as (β1→4)-D-mannans [24,62,63,108].

Hyaluronans

Hyaluronans with molecular weight of 1,050 and 1,338 kDa showed immunostimulatory activity [9,97]. However, a size-effect study showed that after hydrolysis, the resulting hyaluronans with 45.2 and 145 kDa exhibited much stronger immunostimulatory activity [9,97].

Galactans

Low molecular weight (<20 kDa) fractions of carrageenans are associated to higher immunostimulating properties [92,94]. Moreover, the low molecular weight is also associated to lower viscosity, which facilitates the immunostimulatory assays [92].

Fructans

Immunostimulatory properties of fructans have been linked to the molecular weight. Studies have shown that fructooligosaccharides (FOS) and fructans with less than 13 kDa showed immunostimulatory activity [113–117,123].

1.3.3. Functional groups

The presence of functional groups, like acetyl and/or sulfate groups, have been attributed to immunostimulatory polysaccharides [12,91]. It is known that they affect the polysaccharide solubility and conformation [118,119], however it is still unclear how they influence the triggered immunostimulatory response.

Glucans

Most of glucans isolated from natural sources do not have functional groups, but there are a few exceptions. From these, acetylated mixed (β1→6)-(β1→4)-linked glucans and (α1→6)-glucans with Gal and/or Man residues presented high immunostimulatory activity [31,50]. On the other hand, several studies have chemically functionalized glucans to improve their solubility, resulting in soluble glucans with higher immunostimulatory activity [32,40]. Furthermore, it was reported that also glucans conformation was modified after functionalization, leading to stiffener chains [32,40]. It is important to notice that this conformation, however, had lower contribution to the immunostimulatory activity than the triple-helix conformation of non-sulfated (β1→3)-D-glucans with side chains of (β1→6)-D-Glc [118,119].

Pectic polysaccharides

Acetyl groups were identified in immunostimulatory pectic polysaccharides [76,77,82]. The pattern of acetylation, in particular the degree of acetylation and the localization of acetyl groups, are also important features with impact in the resulting activity [77]. A higher degree of acetylation, due to acetyl groups localized in the backbone, may be associated to lower immunostimulatory activity, as shown by an increase of activity after a deacetylation procedure [77].

Arabinogalactans

Acetyl groups were identified in immunostimulatory AG-II structures [81,84,90] and mixed type I and type II structures [75]. Additionally, sulfated pectic polysaccharides also showed immunostimulatory activity, as shown by several sulfated AG-II, with at least 3.4% of sulfate groups [84].

Mannans

A relationship of acetyl groups and the immunostimulatory function has been identified for naturally and chemically acetylated (β1→4)-mannans [12,24,56,58]. The importance of acetyl groups was reinforced as non-acetylated (β1→4)-mannans did not show immunostimulatory activity [12]. On the other hand, the position of the acetyl group seems not to be an essential feature in (β1→4)-mannans, since both, more acetylated in their backbone, and more acetylated side chains, showed similar immunostimulatory activity [12,14,59]. The importance of acetyl groups were also described for (α1→3)-mannans, where the pattern of acetylation, in particular the degree of acetylation and the localization of acetyl groups, are also important features with impact in the resulting activity [69]. While the deacetylation of these mannans gave markedly lower effects in the immune system, on the other hand, further chemically acetylation increased their immunostimulatory effect [69], evidencing that higher degree of acetylation is associated to higher immunostimulatory activity. Furthermore, the localization of the acetyl groups in the peripheral residues of side chains contributes positively to the immunostimulatory activity [69].

Beyond the importance of acetyl groups for immunostimulatory activity, the presence of sulfate groups was also described in immunostimulatory mannans [108]. Additionally, chemically sulfated mannans were markedly more stimulatory than the original ones [68].

Hyaluronans

Hyaluronans are natural acetylated polysaccharides. They have in their disaccharide repeating unit an N-acetyl group that may contribute to their activity [9,97,98].

Fucoidans

The naturally higher content of sulfate groups in fucoidans is associated with a higher immunostimulatory activity [51,91,124]. Moreover, removal of almost every sulfate groups lead to a markedly reduced activity [112].

The presence of acetyl groups also seems to be an important structural feature because, after a deacetylation treatment, the immunostimulatory activity of fucoidans decreased [112]. Furthermore, the simultaneous presence of acetyl and sulfate groups was crucial for fucoidans activity, since a prepared deacetylated and hyposulfated fucoidan lost almost all activity [112].

Galactans

Sulfated galactans from algae have shown immunostimulating effects [92–95]. However, in some cases, they trigger an uncontrolled pro-inflammatory response with associated harmful effects, after long term exposition [125,126].

Additionally, the presence of acetyl groups in algae mannogalactans was also associated to immunostimulatory activities [96].

1.3.4. Degree of branching

The degree of branching of polysaccharides is a structural feature associated with the presence of linked monosaccharide residues to their backbone. Immunostimulatory polysaccharides may have a linear backbone without branches (linear polysaccharides) or have more or less complex branches linked to their backbone. Depending on this structural feature, solubility and other structural features will be affected, namely conformation and molecular weight [118].

Glucans

High degree of branching in β-glucans is positively associated to immunostimulatory activity [33,35,38,118,120]. Highly branched (β1→3)-D-glucans, with side chains of (β1→6)-D-Glc, with an average of a side chain branch on every third glucose residue unit along the backbone, had higher immunostimulatory activity when comparing with less branched or linear (β1→3)-D-glucans [33,35,118,120]. However, it must be taken into account that to the higher branched (β1→3)-D-glucans were associated tighter triple-helix conformations, already described as important structural features for immunostimulatory activity [118]. Therefore, the effect of the degree of branching might not be directly related to the immunostimulatory activity, but be an important structural feature for (β1→3)-D-glucans conformation.

Moreover, both linear and/or branched non-starch (α1→6)-glucans and (α1→4)-glucans have shown immunostimulatory activity [42–46], suggesting that other structural characteristics may be involved, like conformation and molecular weight. However, when comparing two (α1→4)-glucans with similar conformations and molecular weight, linear (α1→4)-glucans showed higher immunostimulatory activity than (α1→4)-glucans with branches of short chains of (α1→6)-D-Glc*p* [41], indicating that the lower degree of branching was the possible structural feature responsible for the higher activity.

The immunostimulatory activity of heteroglucans is positively related with the degree of branching, either in α-heteroglucans or β-heteroglucans with galactose and/or mannose residues. The loss of the activity was observed after hydrolysis of these branching residues [47–49].

Pectic polysaccharides

The structure-function studies of pectic polysaccharides suggests that their linear and branched regions have different effects in immunostimulatory activity by decreasing or increasing it, respectively [80] (**Figure 1.15**). Therefore the ratio of these regions in pectic polysaccharides will influence their activity.

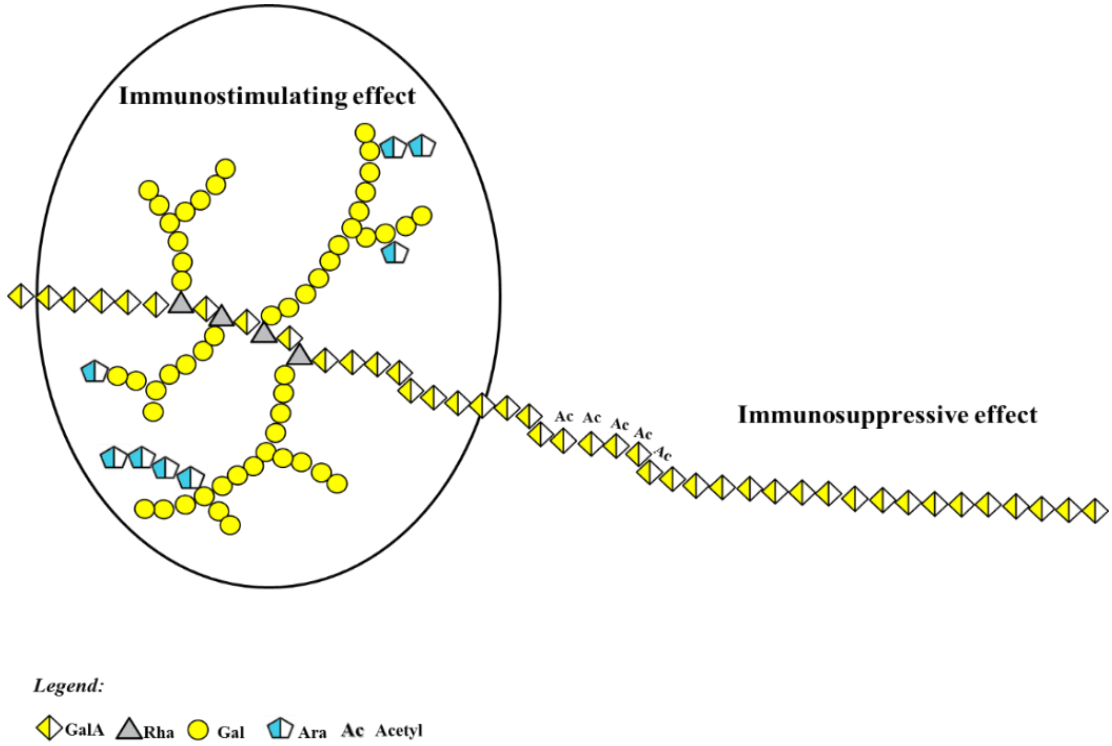

Figure 1.15. Diagram illustrating the immunomodulatory properties of pectic polysaccharides

Removal of the linear regions by enzymatic treatments with endo-polygalacturonase resulted in higher immunostimulatory activity [89]. Furthermore, the isolated branched regions, characterized as galactans, arabinans, and arabinogalactans, are responsible for the resulting immunostimulatory activity [80,101–105,127].

The own branching pattern of branched regions is also crucial for the resulting activity, as was demonstrated by the loss of activity after an enzymatic treatment with *exo*-α-L-arabinofuranosidase and *exo*-β-D-galactosidase, where the enzyme resistant part of the polysaccharide exhibited a diminished immunostimulatory activity [82]. Moreover, the pattern of Ara*f* residues in pectic polysaccharides was associated to the immunostimulatory activity, not only because arabinan-rich pectic polysaccharides showed high immunostimulatory activity [74,76] but also because after the removal of Ara*f* residues, by weak acid hydrolysis treatment, immunostimulatory activity decreased [77,78,85,87,128].

Mannans

The degree of branching of (β1→4)-mannans with (1→6)-D-Gal*p* units contributed differently to immunostimulatory activity [56–58,61], which can be related to different molecular weights, early described as an important structural feature. In one hand, it was shown that when associating a higher degree of branching and a higher molecular weight (10 MDa), mannans showed higher activity [56]. One the other hand, the association of a lower degree of branching and a lower molecular weight (5 to 400 KDa) was also associated to higher immunostimulatory activity [57,58].

Moreover, similar degrees of branching were found in structurally different immunostimulatory mannans, a (β1→3)-linked to (β1→2)-mannan [63] and a mixed linked mannan with a backbone of (β1→2)-D-Man*p* and (β1→6)-D-Man*p* residues, with branches at O-6 of (1→4)-D-Gal*p* units [64], resulting in different immunostimulatory activity. The first described was less active, suggesting that the kind of branches are important and, therefore, branches of Gal*p* units might result in higher immunostimulatory activity [63,64].

Summarily, the presence of branches of D-Gal*p* residues might be an important structural feature in mannans, in the form of galactomannans [56–58,61,64–66]. However, knowing that other structural features, like molecular weight, could affect the effect of these branching units, more research is needed to know how the degree of branching may affect the activity.

1.3.5. Charge

Although several neutral polysaccharides, such as glucans and mannans, present immunostimulatory activity, charged polysaccharides were also linked to immunostimulatory activities. However, charges from functional groups, like sulfate, phosphate, amino, carboxymethyl groups, or carboxylic groups of uronic acids showed a different impact in the polysaccharide immunostimulatory activity. It was already discussed in **section 1.3.3** that polysaccharides with sulfate groups have enhanced activity [51,68,84,91,108,112,118,124]. In contrast, a high percentage of carboxylic groups in pectic polysaccharides provides an immunosuppressive activity [80]. Moreover, polysaccharides with both positively and negatively charged moieties, termed zwitterionic polysaccharides, enhance the immune system [9].

Glucans

The presence of charges from sulfate and carboxymethyl groups in linear (β1→3)-glucans have been related to a stiffness conformation when comparing with neutral glucans [32]. This effect may be explained by the impact of these groups in intramolecular and intermolecular hydrogen bonding, strengthening the effect of electrostatic repulsion and enabling the adoption of a certain structure [40]. As conclusion, the presence of charges can be an important structural feature for β-glucans because it is related to conformation, an important feature to immunostimulatory activity already described.

Pectic polysaccharides

A high content of galacturonic acid (that can be negatively charged) associated to high content of linear regions in pectic polysaccharides is related to immunosuppressive activity [80]. This effect can be overtaken by methyl esterification, carboxyl-reduction, or removal of the linear regions by enzymatic treatments with *endo*-polygalacturonase [77,80,89]. Therefore, the presence of charges from carboxyl groups is negatively correlated with immunostimulatory activities in pectic polysaccharides.

1.4. COFFEE POLYSACCHARIDES

Galactomannans and arabinogalactans, in addition to cellulose, represent almost half of green coffee beans compounds [129]. For the preparation of coffee beverage, coffee beans are roasted, a process that contributes to coffee aroma and under which the arabinogalactans and galactomannans undergo several structural modifications, increasing their extractability to the coffee beverage [130]. Beyond polysaccharides, melanoidins, lipids, protein, minerals, chlorogenic acids, caffeine, other nitrogenous compounds, and volatiles are part of the complex matrix of roasted coffee [131].

Galactomannans from coffee are composed by a linear (β1→4)-D-Man*p* residues backbone substituted at O-6 with single residues of α-D-Gal*p* residues [59]. They also contain single arabinose residues as side chains and (β1→4)-Glc*p* residues interspersed in the main backbone [132]. These galactomannans are acetylated polysaccharides [133], as acetyl groups have been observed at the O-2 or O-3 of mannose residues [132].

Coffee type II arabinogalactans are polysaccharides usually covalently linked to proteins, giving a positive Yariv test [129] . They have a main backbone of (β1→3)-D-Gal residues, with some substitutions at the O-6 position with short chains of (β1→6)-D-galactose residues [134]. The galactose residues of these (β1→6)-D-Gal side chains can be substituted at the O-3 position with single α-arabinose residues, (1→5)-linked arabinose disaccharides [129], rhamnoarabinose disaccharides or rhamnoarabinoarabinose trisaccharides [135]. Terminally linked to these (β1→6)-D-galactose side chains can be GlcA residues [129]. Therefore, arabinogalactans are heterogeneous both with regard to the degree of branching and the degree of polymerisation of their side chains.

With roasting, polysaccharides can undergo depolymerisation, debranching, Maillard reactions, caramelisation, isomerization, oxidation, decarboxylation, transglycosylation, and melanoidins formation [15,129,130,136–144]. These structural modifications increase their complexity and difficult polysaccharides structural characterization.

Several compounds from coffee have biological activities, namely caffeine, chlorogenic acids (CGA), and melanoidins present anti-inflammatory properties [16]. On other way, a special attention has been given to coffee polysaccharides, and potential activities have been demonstrated too, such as lowering colon cancer risk [145,146], contributing to diary dietary fibre intake [147,148], having prebiotic effect [149], and having immunostimulatory activity shown by an inflammatory response [7,12,13].

The study of the structural features responsible for immunostimulatory potential activity have been hampered by the complex structural features, but also to the difficult separation from the complex matrix. Nevertheless, some structural features, relating, essentially, molecular weight and functional groups, have been identified in coffee galactomannans and arabinogalactans with potential immunostimulatory activity.

Immunostimulatory galactomannans from coffee beverage and chemically acetylated ones from spent coffee grounds have a comparable molecular weight (90–110 kDa), and similar glycosidic-linkage composition. However, they have different acetylation patterns, as galactomannans from spent coffee grounds were preferentially acetylated in the side chain residues whereas the galactomannans recovered from coffee infusions only had acetyl groups directly linked to the backbone residues [59], these polysaccharides present comparable immunostimulatory properties [12].

Arabinogalactans from instant coffee have potential immunostimulatory activity. Two distinct immunostimulatory assays conducted in BALB/c spleen cells revealed that a purified arabinogalactan and a fraction rich in arabinogalactans, with molecular weights of 5-6 kDa and 1-5 kDa, respectively, have immunostimulatory activity [7,13]. As arabinogalactans are polysaccharide-protein complexes, the importance of the protein content for the immunostimulatory activity was not assessed, remaining the question if the activity is caused by the polysaccharide or not. In both studies with arabinogalactans from instant coffee, traces of galactomannans were present, in one case they were considered as contaminants of the purified arabinogalactans [7], in the other case mannose residues accounted for 10.5 mol% [13]. In contrast with galactomannans from coffee beverage, where the presence of acetyl groups was associated to potential immunostimulatory activity [12,14,59], it was not evaluated the presence of an acetylation pattern in these arabinogalactans.

As already described for arabinogalactans from different sources, the immunostimulatory potential of arabinogalactans from coffee may also be associated to the pattern of Ara. The importance of these residues was assessed for other arabinogalactans after removal of Ara residues enzymatically or by weak acid hydrolysis treatment [77,78,82,85,87,128].

1.4.1. Aims

In a previous work, it was observed that an instant coffee extract with 1–5 kDa, obtained by ultrafiltration (1E), resultant from an exhaustive washing out of the small molecular weight compounds, presented *in vitro* immunostimulatory activity by inducing the activation of B-lymphocytes [13]. However, an instant coffee extract with 1–5 kDa, resultant from a rudimentary fractionation (2E), had no *in vitro* immunostimulatory activity [17]. Based on the hypothesis that phenolic compounds and caffeine may interfere on the *in vitro* immunostimulatory activity of these polysaccharides, in this study, sample 2E was purified through Bio-Gel P2 size exclusion chromatography and the *in vitro* immunostimulatory activity in BALB/c mice spleen cells was studied.

Furthermore, structural changes were studied in sample 1E after 1) fractionation on Bio-gel P6 (1-6 kDa); 2) treatment with 0.1 M NaOH solution (saponification); 3) deproteinisation with chymotripsin; and 4) α-L-arabinofuranosidase treatment, to evaluate their impact in the *in vitro* immunostimulatory activity in BALB/c mice spleen cells, in order to find structure-function relationships.

Samples were also studied for their *in vitro* immunostimulatory activity in innate immune cell cultures derived from bone marrow, namely macrophages (BMDM) and dendritic cells (BM-DCs).

2. MATERIAL AND METHODS

2.1. SAMPLES

Instant coffee extracts with 1–5 kDa available in our laboratory and previously studied by Passos *et al.* [13] (sample 1E) and Cepeda [17] (sample 2E) were used. Sample 1E, obtained by ultrafiltration, resultant from an exhaustive washing out of the small molecular weight compounds, presented *in vitro* immunostimulatory activity. However, another similar extract (sample 2E), was resultant from a rudimentary fractionation, and had no activity.

2.2. SIZE EXCLUSION CHROMATOGRAPHY OF INSTANT COFFEE FRACTIONS

Size exclusion chromatography using Bio-gel P2 (SEC-P2) was performed on a XK 1.6/40 column with a flow rate of 0.3 mL/min. The samples were dissolved in 1 mL of distilled water, centrifuged and loaded on the column previously equilibrated with water. For samples 2E and 1Es 50 mg were used, and for sample $1E^{Arase}$ 5.7 mg were used. Exclusion and inclusion volumes were estimated with Blue Dextran (2,000 kDa) and Glc (180 Da), respectively. Fractions of 1 mL were collected and monitored with evaporative light scattering detection (ELSD). ELSD was performed by setting the temperature to 57 ºC, the pressure to 1.9 bar, and introducing 70 µL of each fraction interspersed with water using a flow of 4 mL/min. The absorbance at 280, 325 and 405 nm of 1:20 dilution of each fraction were measured using a quartz cuvette in a double beam ultraviolet–visible (UV/Vis) spectrophotometer (Lambda 35, Perkin-Elmer, USA). The appropriate fractions were pooled and freeze-dried.

Size exclusion chromatography using Bio-gel P6 was performed on a XK 2.6/70 column using the conditions described for SEC-P2, except the sample amount (10.10 mg of Sample 1E), fractions of 2 mL were collected until a total volume of 160 mL, and the remaining volume was collected on a single container (160-350 mL). The collected fractions were monitored with ELSD and at 280, 325 and 405 nm, as already described, and were also assayed for sugars by the phenol-sulfuric acid method (absorption at 490 nm) (section **2.11.5. Phenol-sulfuric acid method**).

2.3. FOURIER INFRARED SPECTROSCOPY

Fourier transform infrared (FTIR) spectra were recorded on a PerkinElmer Spectrum BX FTIR spectrometer, using a horizontal one single reflection ATR Golden Gate (Specac, Germany). Between determinations, the crystal was carefully cleaned with water. The spectra were registered between 4000 and 600 cm^{-1}, collected at a resolution of 8 cm^{-1}, with 64 scans co-added before Fourier transformation. All spectra are the average of two independent measurements after baseline-correction and smooth correction; moreover background spectrum was subtracted to aid clarity.

2.4. CHLOROGENIC ACIDS (CGA) AND CAFFEINE QUANTIFICATION

The methodology used for quantification of chlorogenic acids (CGA) and caffeine was adopted from Nunes *et al.* [138]. Aliquots of 10 g/L were prepared with ultrapure water and filtered (0.20 μm). Samples were characterized for their total free CGA and caffeine content through reversed-phase high performance liquid chromatography (HPLC), using a Gilson solvent delivery system equipped with a UV–Vis-156 Gilson detector. Separation was performed by gradient elution on a Spherisorb S10 ODS2, PS (10 μm particle size; 200 mm; 4.6 mm). Eluent A was a 5% formic acid aqueous solution, and eluent B was methanol. The eluent program was as follows: 0−5 min, 5% eluent B; 5−45 min, 40% B; 45−65 min, 70% B; 65−75 min, 5% B. The sample volume injected was 20 μL, the flow rate was 0.8 mL/min and the column temperature was maintained at 30 ºC during the run. The eluent was continuously monitored from 240 to 600 nm with a UV/Vis – 156 Gilson detector. Quantification of total CGA was performed using a standard curve made with 5-cafeoylquinic acid (0.10-0.58 g/L) and expressed as 5-cafeoylquinic acid equivalents. Caffeine was quantified by using a calibration curve made with pure caffeine (0.02-0.92 g/L).

2.5. SAPONIFICATION OF INSTANT COFFEE FRACTION

Sample 1E was subjected to a saponification procedure by dissolution of 50 mg in 5 mL of 0.1 M NaOH. This solution was stirred for 24 h at room temperature, neutralized with glacial acetic acid, and dialysed for 6 h using a membrane cut-off of 1,000 Da (Spectrum, Breda, The Netherlands), with water changes until reaching the distilled water conductivity. Then, the retentate was freeze-dried to yield sample 1Es.

2.6. DETERMINATION OF THE DEGREE OF ACETYLATION

The degree of acetylation (DA) of samples 1E and 1Es was determined by quantification of the acetic acid released by saponification of acetyl groups, acidification of solution, headspace solid phase microextraction (HS-SPME) and analysis by gas chromatography with a flame ionization detector (GC-FID), in accordance to the method developed by Nunes *et al.* [150]. The samples (1-5 mg) were dispersed in water (2.4 mL) in vials with 10 mL capacity and the saponification of the polysaccharides occurred by the addition of 0.8 mL of 2 M NaOH, with a reaction time of 1 h at room temperature. The reaction was finished and the solution was acidified (pH 2) by the addition of 0.95 mL of 2 M HCl. The vials (10 mL) containing 4.15 mL of sample suspension (sample dispersed in 2.4 mL of water, 0.8 mL of 2 M NaOH and 0.95 mL of 2 M HCl or standard solutions (4 mL of standard solution, and 0.15 mL of 2 M HCl) were thermostatised at 40 ºC in a water bath, with continuous stirring. After 15 min, the SPME fibre coated with 50/30 μm divinylbenzene/carboxen on polydimethylsiloxane (DVB/carboxen/PDM) was manually inserted through the Teflon septum into the headspace of the vial and exposed at 40 ºC during 30 min. The SPME coating fibre containing the headspace volatile compounds was introduced into the GC injection port at 250 ºC and kept for 3 min for the desorption. A Hewlett-Packard 5890 series II gas chromatographer (Hewlett-Packard, Wilmington, USA), equipped with a split/splitless injector and a flame ionization detector (FID) was used. The desorbed compounds were separated in a 30 m length DB-Wax column (J&W) with 0.53 mm i.d. and 1.0 μm film thickness and hydrogen as carrier gas was used at 6 mL/min linear velocity. An oven temperature programme was done between 50 and 220 ºC with three rates, 5 ºC min^{-1} until 65 ºC, 20 ºC min^{-1} until 185 ºC, and 35 ºC min^{-1} until 220 ºC, and held 1 min at 220 ºC. The detector temperature was at 250 ºC.

2.7. DEPROTEINISATION PROCEDURE

Sample 1E, presenting an immunostimulatory potential activity, contained 5.5% of total protein [13]. In order to ascertain the importance of the protein fraction sample 1E immunostimulatory activity, it was submitted to an enzymatic deproteinisation procedure using α-chymotrypsin from bovine pancreas (EC 3.4.21.1, Sigma, St. Louis, USA). The sample obtained after the saponification procedure (sample 1Es) was subjected to the same procedure.

Samples (50 mg) were deproteinized with 20 U of α-chymotrypsin during 24 h at 25 ºC with continuous stirring in a 100 mM Tris-HCl buffer, pH 7.8, and 10 mM $CaCl_2$. Enzymatic digestion was terminated by adjusting the pH to 2.0 by the addition of 2 M HCl during 15 min and following neutralization with 2 M NaOH. Samples were dialysed using a membrane cut-off of 1,000 Da (Spectrum, Breda, The Netherlands). The filtrates from the first hour of dialysis were recovered for future analysis (samples $1E^{dialysis}$ and $1Es^{dialysis}$). Dialysis was maintained for two days with renewal of filtrate water until reach distilled water conductivity. Retentates were freeze-dried yielding $1E^{dep}$ and $1Es^{dep}$.

2.8. PROTEIN TOTAL CONTENT

Protein content was calculated by the bicinchoninic acid (BCA) assay and from the nitrogen content (% N×6.25) [7].

The BCA Working Reagent (2 mL) (Sigma, St. Louis, USA) was added to 0.1 mL of blank (water), bovine serum albumin (BSA) protein standards, and samples diluted in water in test tubes. Following vortex the test tubes were let to incubate at 37 ºC for 30 min. Test tubes were let to cool to room temperature and then the absorbance was measured at 562 nm. The protein concentration was determined by comparison of the absorbance of the samples to the standard curve prepared using the BSA protein standards (0.2-1.0 g/L).

The elemental analysis for nitrogen content (%N), was performed using Truspec 630-200-200 elemental analyser.

2.9. α-L-ARABINOFURANOSIDASE TREATMENT

In order to remove the terminally-linked arabinose from arabinogalactans, an enzymatic assay with high purity α-L-arabinofuranosidase was performed [59]. Sample 1E (15 mg) was hydrolysed with 1 U of *Clostridium thermocellum* arabinofuranosidase 51A (EC 3.2.1.55, nzytech), purified from a recombinant *Escherichia coli* strain, during 48 h at 37 ºC with continuous gently stirring in a 100 mM Na-acetate buffer, pH 5.5, containing 0.02% sodium azide. It was freeze-dried (sample $1E^{Arase}$) and purified through SEC-P2.

2.10. YARIV ASSAY FOR ARABINOGALACTAN PROTEINS

Arabinogalactans-proteins were identified by the Yariv assay [7, 8]. A solution containing 1% w/v agar-agar (V. Reis, Lisboa), 0.15 M NaCl, 0.02% w/v NaN_3 and 0.002% (w/v) of Yariv phenyl glycoside (1,3,5-tri[4-β-D-glucopyranosyl-oxyphenylazo] 2,4,6-trihydroxybenzene, Biosupplies, Victoria, Australia) was prepared and heated to boiling. Petri dishes were covered with a layer of approximately 3 mm thick of the prepared solution. Samples (20 μL) were poured in wells of 5 mm width made on the gel formed. The Petri dishes were sealed with Parafilm and left in the dark at room temperature for 2 days, to allow the colored halo to develop (positive result).

Samples were dissolved in water (2 mg/mL) and were pipetted into wells. Gum arabic (Biosupplies, Victoria, Australia) (2 mg/mL), and a galactomannan (from Locust Bean Gum) (2 mg/mL) were used as positive and negative test polysaccharides, respectively. Water was used as blank.

2.11. SUGAR ANALYSIS

The neutral sugars were determined after acid hydrolysis, derivatisation to alditol acetates and analysis by GC-FID [10, 11]. The total sugars content was determined by the sum of the amount of the individual sugars, taking into account that the hydrolysis of a glycosidic linkage results in an addition of a water molecule into the sugar structure. All determinations were performed in duplicate.

2.11.1. Acid hydrolysis

To carry out hydrolysis, about 1-2 mg of each sample was weighted in 10 mL tubes, and 200 µL of 72% H_2SO_4 were added. After incubation for 3 h at room temperature with occasional stirring, 1.0 mL of distilled water was added and incubated for another 1 h at 120 ºC. The tubes were cooled down in a cold water bath.

2.11.2. Reduction and acetylation

After adding 200 µL of internal standard (2-deoxy-glucose 1 g/L), 0.5 mL of sample was transferred to other tube and neutralized with 200 µL 25% NH_3. The reduction was performed adding 100 µL of 15% (m/v) $NaBH_4$ in 3 M NH_3 to samples and incubating for 1 h at 30°C. After cooling down the tubes in a cold water bath and adding 2x50 µL of acetic acid, 300 µL of sample were transferred to *sovirel* tubes. The acetylation was performed by adding 450 µL of 1-methylimidazole and 3 mL of acetic anhydride. After mixing on vortex, samples were incubated for 30 min at 30 °C. The resulting alditol acetates were extracted to an organic phase by adding 3 mL of distilled water and 2.5 mL of dichloromethane, followed by vigorous stirring, and separation by centrifugation (30 s, 3000 rpm). Afterwards, the aqueous phase was removed by suction with vacuum. Addition of distilled water and dichloromethane, stirring, centrifuging and removing the aqueous phase was repeated. Later the organic phase was washed twice with 3 mL of distilled water, mixed, and centrifuged, after which the aqueous phase was completely removed. The organic phase was transferred to specific *speedvac* tubes and the dichloromethane was evaporated. Afterwards, 1 mL of anhydrous acetone was added and evaporated, twice.

2.11.3. GC-FID analysis

The alditol acetates were dissolved in 50 µL of anhydrous acetone and analysed by GC-FID. The GC was equipped with a 30 m column DB-225 (J&W Scientific, Fol-som, CA, USA) with i.d. and film thickness of 0.25 mm and 0.15 µm, respectively. The oven temperature program used was: initial temperature 200 ºC, a rise in temperature at a rate of 40 ºC/min until 220 ºC, standing for 7 min, followed by a rate of 20 ºC/min until 230 ºC and maintain this temperature 1 min. The injector and detector temperatures were, respectively, 220 and 230 ºC. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min.

2.11.4. Method for small amount samples

To analyse smaller amounts of sample which have been previously solubilized in water or in phosphate buffer saline (PBS), 0.01 M, pH 7.4 (dry powder packet, SigmaAldrich, St. Louis) , 20-50 μL of sample were transferred to *speedvac* tubes and evaporated. To carry out hydrolysis, 1 mL of 2 M trifluoroacetic acid (TFA) was added and incubated for 1 h at 120 ºC. Afterwards the TFA was evaporated.

After adding 20-50 μL of internal standard (2-deoxy-glucose 0.1-1 g/L), the reduction was performed adding 200 μL of 15 % (m/v) $NaBH_4$ in 3 M NH_3 to hydrolysed samples and incubating for 1 h at 30°C. After cooling down the tubes in a cold water bath and adding 2x50 μL of acetic acid, the acetylation was performed by adding 450 μL of 1-methylimidazole and 3 mL of acetic anhydride. After mixing on vortex, samples were incubated for 30 min at 30°C. The resulting alditol acetates were extracted as previously described, dissolved in 10-20 μL of anhydrous acetone, and analysed by GC-FID.

2.11.5. Phenol-sulfuric acid method

Fractions of SEC-P6 were assayed for total sugars by the phenol-sulfuric acid method, measuring the absorbance at 490 nm [152]. Briefly, to 80 μL of samples, galactose standards (0.01-0.8 mg/mL) or blank solution (water), 150 μL of phenol (5 %) and 1 mL of concentrated sulfuric acid were added to the test tubes. Test tubes were manually shaken and kept in a water bath for 5 min at 100 ºC. After cooling to room temperature in a water bath, the absorbance of each test tube was measured at 490 nm. The total sugar content was determined from a regression analysis using serial dilutions of the standard solution.

2.12. IMMUNOSTIMULATORY ACTIVITY ASSAYS

Samples *in vitro* immunostimulatory activity was tested with several cellular cultures, namely BALB/c mice spleen mononuclear cells, bone-marrow derived dendritic cells (BM-DCs), and bone-marrow derived macrophages (BMDM).

2.12.1. Mice

Six- to eight-week old BALB/c mice were purchased from Charles River laboratories and maintained at the animal facilities of Instituto de Ciências Biomédicas de Abel Salazar (ICBAS) until the time of experiment. All procedures were performed in order to minimize mice suffering, according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92).

2.12.2. *In vitro* splenic mononuclear cell cultures

The spleen from BALB/c mice was aseptically removed and the spleen cells were obtained by gently teasing the organ in RPMI-1640 medium (Sigma, St. Louis, USA) supplemented with penicillin (100 IU/mL), streptomycin (50 mg/L), 2-mercaptoethanol (0.05 mol/L) and 10% of fetal bovine serum (FBS, Sigma, St. Louis, USA) (RPMI). Spleen cell suspensions were distributed on 96-well plates ($0.5\text{-}1\times10^6$ cells/well) and stimulated as followed described for cytotoxicity with Neutral Red uptake, lymphocyte proliferation and lymphocyte stimulation assays.

2.12.3. Neutral Red uptake assay for the estimation of cell viability/cytotoxicity

The assessment of *in vitro* toxic effects due to treatment with the immunostimulatory sample 1E was performed by the uptake of Neutral Red (NR) dye (PMID: 18600217). BALB/c mice spleen cell cultures were stimulated with RPMI (negative control), 1 mg/L of LPS from *Salmonella abortus equi* (Sigma, St. Louis, USA) (positive control), and with 25-75 mg/L of sample 1E, and cultured for 20 h at 37 ºC, in 95% humidified atmosphere containing 5% CO_2. Afterwards, stimulated cells were washed in PBS and then incubated with NR in RPMI (40 μg/mL) for 3 h at 37 ºC, in 95% humidified atmosphere containing 5% CO_2. At the end of the incubation period, the medium was removed and cells were washed three times in PBS. NR accumulated within cells was extracted with a mixture of 1% (v/v) acetic acid and 50% (v/v) ethanol (extracting solution). The cultures were allowed to stand for 10 min at room temperature, and 30 min with gentle stirring in an orbital shaker

to enhance mixing of the solubilized dye. The absorbance of each well was measured at 540 nm in a microtiter plate reader.

Data were analysed with GraphPad Prism 5.01 software (OriginLab Corporation, Northampton, MA). The significance of the difference was evaluated with one-way ANOVA, followed by Dunnett's test to statistically identify differences between the cells treated with negative control and samples.

2.12.4. Evaluation of the in vitro lymphocyte proliferation effect by flow cytometry analysis

In order to measure proliferation in the presence of the immunostimulatory sample 1E, the BALB/c mice spleen cells were labelled with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE). The cells were washed and re-suspended at 2 x 10^6 cells/mL in Hank's balanced salt solution 10x (HBSS, Sigma, St. Louis, USA) diluted 1:10 in $NaHCO_3$ and labelled with a final concentration of 5 μM CFSE. Cells were vortexed and incubated at room temperature for approximately 10 min. Excess CFSE was quenched by addition of 10% FBS and of 3 volumes of ice-cold RPMI. After 5 min incubation on ice, the cells were pelleted by centrifugation and re-suspended in fresh media. To remove any unlabelled CFSE, the cells were pelleted and washed twice in RPMI. Before a final wash, the cells were incubated at 37 °C for 10 min. Following the final wash, the cells were re-suspended in RPMI and the effectiveness of CFSE labelling confirmed by using an EPICS XL flow cytometer and the EXPO32ADC software (Beckman Coulter) with 488 nm excitation and appropriate fluorescein emission filters.

CFSE-labelled spleen cells were cultured as described above for NR uptake assay, with controls and sample 1E. Cells were incubated for 2 days at 37 ºC, in 95% humidified atmosphere containing 5% CO_2. For flow cytometry analysis, the cells were washed and re-suspended in PBS supplemented with 10 mmol/L of sodium azide and 1% BSA (FACS buffer).

2.12.5. Evaluation of the *in vitro* lymphocyte stimulating effect by flow cytometry analysis

Spleen BALB/c mice cells suspensions (0.5-1×10^6 cells/well) were stimulated with RPMI (negative control), 1 mg/L of LPS (positive control), and with 20-150 mg/L of samples, and cultured for 6.5 h or 16 h at 37 ºC (two different experiments), in 95% humidified atmosphere containing 5% CO_2. After incubation, the cells supernatant was removed by centrifugation at 500 g for 5 min (~ 1500 rpm, 7 min) and the spleen cells were re-suspended in 25 µL of monoclonal antibodies 1:100 in FACS buffer. After incubation for 30 min at 4 ºC in the dark, cells were washed by centrifugation with 150 µL of FACS buffer to remove unlabelled antibodies. Ammonium-Chloride-Potassium (ACK) lysing buffer (40 µL) was used for lysing erythrocytes from incubated spleen cells before cytometry analysis. After 2/3 min incubation with ACK lysing buffer diluted in 1:10 in water, cells were washed and re-suspended in 150 µL FACS buffer. Cells suspension was collected in FACS tubes in a final volume of 200-300 µL.

The following monoclonal antibodies specific for: CD19 (PE; clone 6D5; Biolegend), CD3 (FITC; clone 145-2C11; BD Bioscience) and early activation marker CD69 (PE; clone H1.2F3; Biolegend) were used for immunofluorescence cytometric analysis in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter).

In each assay, propidium iodide was added to stimulated spleen mononuclear cell before flow cytometry analysis. After flow cytometry analysis, unviable cells were counted and excluded in the respective dot plots (**Figure 2.1**) for further evaluation of expression of CD69.

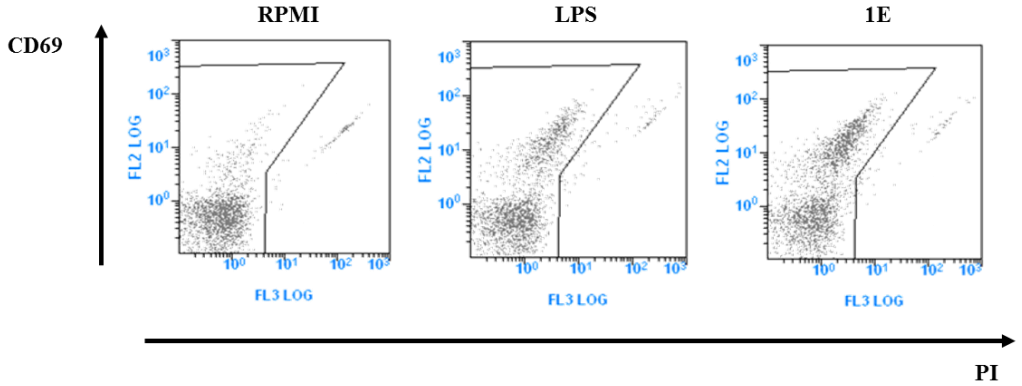

Figure 2.1. Dot plots of CD69 expression and propidium iodide incorporation by cells stimulated with RPMI, LPS and 1E (75 µg/mL). Only selected cells will be further analysed.

2.12.6. Generation of bone-marrow-derived macrophages (BMDM)

Bone marrow cells from femora and tibiae (5×10^6) were seeded *per* well in 6-well plates in complete RPMI (cRPMI; 10% FBS, 1% penicillin/streptomycin, Sigma, St. Louis, USA) supplemented with 10% L929 cell-conditioned medium (LCCM) at 37 ºC in a 5% CO_2 atmosphere. Four days post culture the cell media was replaced and at day 6 cells were collected and plated in 96 well plates in cRPMI at a density of 2×10^5 cells *per* well. At day 7 BMDM were stimulated. This method allows for the differentiation of a homogeneous primary culture of macrophages that retain the characteristic morphological, physiological, and surface markers.

2.12.7. Measurement of nitrite production by Griess reagent

At day 7 of culture, BMDMs were incubated at 37 ºC in an atmosphere of 5% CO_2 with complete RPMI (negative control), LPS (positive control), and samples 1E, 2E, and 2E-$P2^{F1}$. After 24 or 48 h incubation, the culture supernatant was removed and assayed for NO production, using a colorimetric reaction with the Griess reagent [153]. In brief, supernatants were mixed with equal volumes of 1% (w/v) sulphanilamide containing 5% (w/v) phosphoric acid and then of 0.1% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride. After 5-10 min at room temperature, the absorbance was measured at 570 nm in the microtiter plate reader Multiskan Ex spectrophotometer (Thermo Electron Corporation, Corston, UK) using the Ascent software (Thermo Electron Corporation). Culture medium was used as blank and nitrite concentration was determined from a regression analysis using serial dilutions of sodium nitrite as standard (2-50 μM).

2.12.8. Generation of bone-marrow-dendritic cells (BM-DCs)

Briefly, 5×10^6 bone marrow cells (from femora and tibiae of BALB/c mice) were seeded *per* well in 6-well plates in complete RPMI (cRPMI; 10% FBS, 1% penicillin/streptomycin, Sigma, St. Louis, USA) supplemented with 10% of J558 – cell conditioned medium as a source of granulocyte/macrophage colony-stimulating factor (GM-CSF) and incubated at 37 °C in 5% CO_2. On days 2 and 4, the medium was renewed. On day 6, non-adherent cells were collected and re-suspended in fresh medium, without GM-CSF, and 2×10^5 cells/well were cultured overnight in a 96 well plate, in a total medium volume of 100 μL *per* well. On day 7, cultures were stimulated in a volume up to 200 μL *per* well. Approximately 60 - 70% were positive for $CD11c^+$ cell surface expression.

2.12.9. Flow cytometric analysis of BM-DCs

BM-DCs (2×10^5) were stimulated with cRPMI (negative control), LPS (positive control), and samples 1E, 2E and 2E-$P2^{F1}$ for 6 and 14 h. After stimulation, BM-DCs were incubated with anti-FcγR mAb solution for 15 min on ice and then incubated with surface markers for 30 min at 4 ºC in the dark. Cells were then washed with FACS buffer. The surface markers used were the anti-mouse antibodies: CD11c (FITC; clone HL3; BD Pharmingen); CD80 (PE; clone 16-10A1; BD Pharmingen); CD86 (PE-Cy7; clone GL1; BD Pharmingen); and MHC Class II (I-A/I-E) (PerCP; clone M5/114.15.2; Biolegend). Samples were run on the EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter, Miami, FL). The collected data files (100 000 events *per* sample) were converted for analysis with the CELLQUEST software, v3.2.1f1 by using FACS CONVERT, v1.0 (both from Becton Dickinson, San Jose, CA). Data were analysed using Cell Quest software, v3.2.1f1 (Becton Dickinson).

3. RESULTS AND DISCUSSION

3.1. PURIFICATION OF INSTANT COFFEE FRACTION

In a previous work, it was observed that an instant coffee extract with 1–5 kDa, obtained by ultrafiltration (1E), resultant from an exhaustive washing out of the small molecular weight compounds, presented *in vitro* immunostimulatory activity by inducing the activation of B-lymphocytes [13]. However, an instant coffee extract with 1–5 kDa, resultant from a rudimentary fractionation (2E), had no *in vitro* immunostimulatory activity [17]. **Table 3.1** shows the sugar composition, protein, total chlorogenic acids (CGA), caffeine and melanoidin content of samples 1E and 2E. The high proportions of galactose (Gal) and arabinose (Ara) show that both fractions are composed by arabinogalactans; these fractions contain also mannose (Man), namely in 2E, which is a characteristic sugar component of galactomannans. The estimation of total sugars shows that 1E is richer in carbohydrates than 2E. In fact, sample 2E, although obtained from the same pore size as sample 1E, shows a much higher amount of CGA and caffeine, probably due to the less exhaustive washing performed for this sample. In addition, these fractions were brown, which is diagnostic of the presence of the high molecular weight compounds known as melanoidins.

Table 3.1. Chemical characterization and *in vitro* B lymphocyte stimulatory effect of 1-5 kDa instant coffee fractions (samples 1E and 2E).

Samples	Sugar content (%)	Total protein (%)	Caffeine (mg/100g)	Total CGA (mg/100g)	Unknown material[a] (%)	K_{mix}[b]			MBI[c]	R[d]
						280nm	325nm	405nm		
1E	43.8	5.5	2.6	4.3	50.7	4.45	2.91	0.94	1.85	[13]
2E	36	6.3	3779	3722[c]	58.0	6.11	4.31	0.35	0.61	[17]

[a] Non-carbohydrate and non-proteic material (usually referred as unknown material); [b] Expressed in $mL.mg^{-1}.cm^{-1}$; [c] Determined by the ratio of $K_{mix,405nm}$ and unknown material; [d] References

Based on the hypothesis that phenolic compounds and caffeine may interfere on the *in vitro* immunostimulatory activity of these polysaccharides, in this study, sample 2E was purified through Bio-Gel P2 size exclusion chromatography (SEC-P2) (**Figure 3.1.a**) and the *in vitro* immunostimulatory activity in BALB/c mice spleen B and T lymphocyte cells was studied by the expression of an early activation marker (CD69).

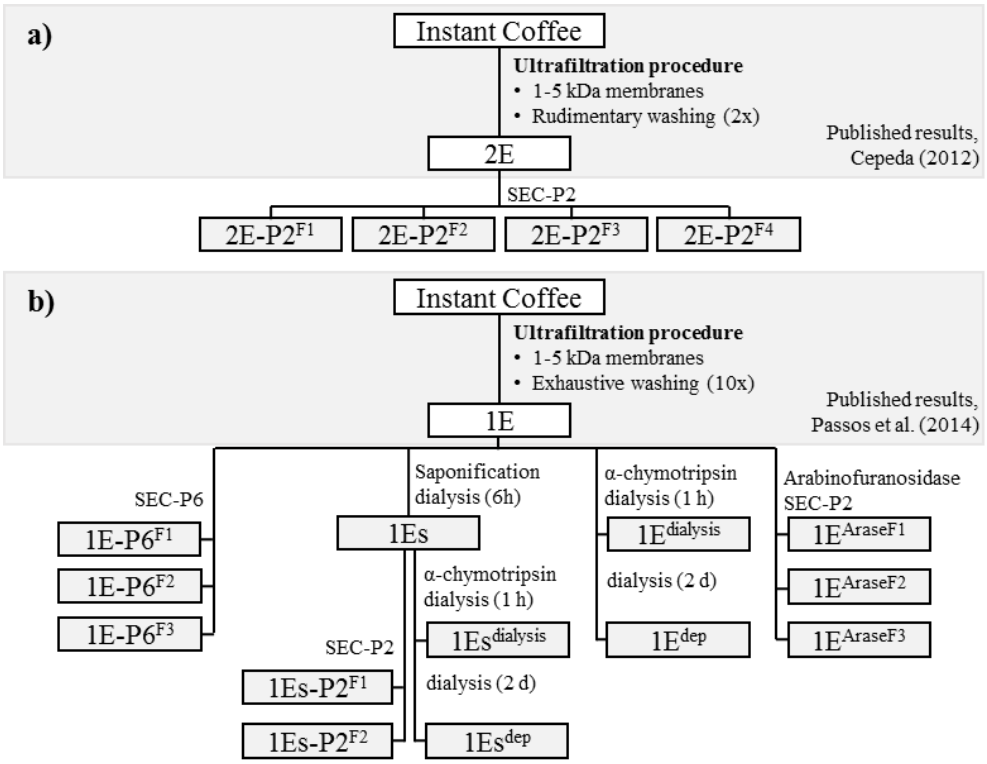

Figure 3.1. Schematic representation of treatments performed to **a)** sample 1E and **b)** sample 2E.

3.1.1. Characterization of samples obtained by SEC-P2

Sample 2E was purified by solubilisation of 50 mg in 1 mL of water and passage through SEC-P2. The eluent chosen for the SEC-P2 was water, in order to facilitate the preparation of samples for the immunostimulatory assays. After passage of the sample, the gel became slightly yellow, demonstrating that some brown compounds were retained on the column. These compounds were released after washing the column with 0.15 M NaCl. There is awareness that not using an eluent with ionic strength of at least 20 mM would not eliminate the effect of small amounts of negatively charged groups on the gel (supplier instructions) and therefore SEC could not be interpreted solely as molecular weight information, however some insights were done into molecular weight and diversity of obtained fractions.

Eluted fractions from SEC-P2 were analysed with evaporative light scattering detection. The light scattering profile of sample 2E (**Figure 3.2**) shows, in addition to the high molecular weight material eluted in the void volume (12 mL), some material in all inclusion volumes (from 12 to 52 mL). Since sample E2 had compounds that entered in Bio-gel P2 pores (1,800 Da to 100 Da) and were eluted in inclusion volumes, it can be concluded that it had compounds with lower molecular weight than 1,000 Da, confirming the non-removal of all lower molecular weight compounds with the rudimentary ultrafiltration procedure carried on, by which sample 2E was obtained.

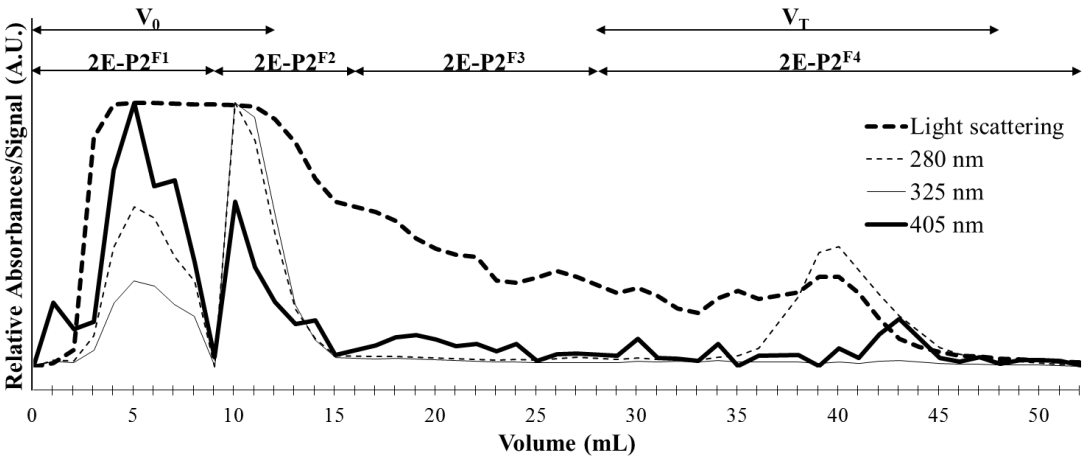

Figure 3.2. Size-exclusion chromatography profiles of sample 2E using light scattering and spectrometric detection at 405, 325 and 280 nm. Void volumes (V_0), elution volume of monomers (V_T), and fractions of sample 2E are indicated (2E-P2^{F1}, 2E-P2^{F2}, 2E-P2^{F3}, and 2E-P2^{F4}).

The eluted samples were also analysed for their absorbances at 280, 325, and 405 nm (**Figure 3.2**). The absorption maximum at 280 nm can be explained by the presence of the aromatic rings of proteins, caffeine, chlorogenic acids, and caffeic acid. The absorption maximum at 325 nm can be explained by the presence of chlorogenic acid and caffeic acid. The absorption at 405 nm can be explained by the presence of melanoidins, since this is a wavelength often chosen to measure the intensity of the brown colour and where only melanoidins absorb [154]. Moreover, as melanoidins result from structural changes that components of coffee, namely polysaccharides, proteins, and chlorogenic acids, undergo during the roasting process, it is generally accepted that melanoidins contain conjugated systems which result in light absorption throughout the whole spectrum [155], including the maximum wavelengths at 280 and 325 nm of aromatic rings and CGA, respectively.

The SEC-P2 profiles of sample 2E obtained by the measure of absorbances at 405 nm, 325 nm, and 280 nm show the presence of two melanoidin populations with different molecular weights (2E-P2^{F1}, and 2E-P2^{F2}, **Figure 3.2**). 2E-P2^{F1} was eluted in the void volume, and therefore has a molecular weight between 1.8 kDa and 5 kDa, 2E-P2^{F2} was mainly eluted in the inclusion volume and therefore has a molecular weight near 1.8 kDa. Another fraction that can be distinguished by the absorbance profiles is fraction 2E-P2^{F4}, containing low molecular weight material that absorbed only at 280 nm. Since this fraction did not absorb at the wavelength of CGA (325 nm), and that sample 2E contained high amounts of free caffeine, this absorption peak at 280 nm must be attributed to the elution of caffeine.

The fractions were pooled and analysed, allowing to determine that 55.4 % of sample 2E was recovered in the void volume fraction (2E-P2^{F1}) and 13 % in the inclusion volume fractions (**Table 3.2**). The recovery of only 68,4 % of material after SEC-P2 of sample 2E may be associated to the retention of material on Bio-gel P2.

The fractions pooled from SEC-P2 of sample 2E were characterized for their sugar content. Sample 2E-P2^{F1} presented an intense brown colour, as also shown by the profile of the absorbance at 405 nm. It was composed mainly by Gal (82 mol%), Ara (8 mol%) and Man (6 mol%) (**Table 3.2**), suggesting an enrichment in arabinogalactans, when compared with the sugars content of sample 2E (8.3 mol% of Ara, 42.1 mol% of Man, and 44.3 mol% of Gal). The sugar composition of 2E-P2^{F2} was similar to 2E-P2^{F1} and fractions 2E-P2^{F3} and 2E-P2^{F4} were rich in Man (51 and 41 mol%, respectively). As Man was eluted in the lower molecular weight fractions, this residue is part of oligosaccharides [degree of polymerization lower than 10 (<1.8 kDa)] that were not separated in the ultrafiltration procedure. However, as 2E-P2^{F3} and 2E-P2^{F4} represent only 9 % of sample 2E, the Man recovered in these low molecular weight fractions does not reach the Man present in sample 2E. As hypothesis, Man residues might be incorporated in melanoidins, that remained retained to the Bio-gel P2 of column and contributed to the yellow colour of gel seen after passage of sample 2E, as it was identified in the literature a type of coffee melanoidins where low molecular weight brown compounds were covalently linked to galactomannans [139].

Table 3.2. Yield and sugar composition of fractions obtained after size-exclusion chromatography on Bio-gel P2 of sample 2E.

Samples	Yield (% m/m)[a]	Sugar Composition (% mol)					% Total Sugars	Total CGA (mg/100g)	Caffeine (mg/100g)
		Rha	Ara	Man	Gal	Glc			
1E		1.4±0.1	6.7±0.1	10.5±0.1	75.0±0.8	6.3±0.6	43.8[b]	4.3[b]	2.6[b]
2E		1.1±0.0	8.3±0.1	42.1±0.1	44.3±0.8	3.9±0.7	36.0[e]	3779	3722
2E-P2^{F1}	55.4	1.8±0.2	8.2±0.4	6.1±0.2	82.0±0.2	1.9±0.1	37.2±6.6	40	nd
2E-P2^{F2}	7.0	nd	7.7±0.4	8.9±0.1	82.1±0.4	1.3±0.1	50[f]	4276	nd
2E-P2^{F3}	3.8	nd	9.9±2.1	51.2±1.7	35.8±0.3	3.1±0.1	60.9±9.4	--	--
2E-P2^{F4}	2.2	nd	20.7±0.3	40.6±1.3	34.6±1.7	4.1±3.4	6.4±0.6	--	--

[a]Relative yield; [b]Passos et al 2014; [c]Not detected; [d]Not determined; [e]Cepeda 2012; and [f] Determined after filtration with a 0,20 μm filter.

Sample 2E and fractions were analysed by HPLC to quantify free CGA and caffeine. Only high molecular weight ones (2E-P2^{F1} and 2E-P2^{F2}) were analysed since fractions 2E-P2^{F3} and 2E-P2^{F4} gave low yields and there was not enough sample to perform the analysis. Sample 2E contained 3.8 g/100g of total free CGA. Fractionation allowed to separate the free CGA from the high molecular weight material, as 2E-P2^{F1} had only 1% of 2E total CGA and 12% of 2E total CGA was recovered in the inclusion material (2E-P2^{F2}) (**Table 3.2**). Therefore, free CGA also contributed to the absorbances at 280 and 325 nm seen in chromatograms of fraction 2E-P2^{F2} (**Figure 3.2**).

The caffeine that remained in sample 2E after the rudimentary fractionation by ultrafiltration accounted for 3.7 g/100g (**Table 3.2**). SEC-P2 fractionation allowed to separate the free caffeine from the high molecular weight material since it was not detected in 2E-P2^{F1}, neither in 2E-P2^{F2}. Caffeine must been recovered in 2E-P2^{F4}, as shown by the chromatogram of absorbances at 280 and 325 nm where it was only observed absorption at 280 nm (**Figure 3.2**). Since caffeine may have been recovered in sample 2E-P2^{F4} and the sample had only vestigial total sugar content (6 %), it must be rich in caffeine.

It was already described in the literature that phenolic compounds are retained by polysaccharides [156]. With the fractionation of sample 2E by SEC-P2, it is shown that CGA and caffeine were retained by the polymeric material of sample 2E. Moreover, it is also shown that CGA were strongly retained by the polymeric material than caffeine, as CGA were still eluted in 2E-P2^{F2} (with a molecular weight near 1,800 Da) in contrast with caffeine that was eluted in the monomers elution volumes (2E-P2^{F4}).

In order to study the structural differences between samples 1E, 2E, and 2E-P2^{F1}, Fourier transform infrared spectroscopy (FTIR) spectra were obtained. In the spectra of these samples (**Figure 3.3**), characteristic bands in the region 3400-2800 cm^{-1} and 1200-900 cm^{-1} due to polysaccharide moiety were observed. Characteristic bands of protein, caffeine and CGA were also observed in the wavenumbers of 1700-1150 cm^{-1}.

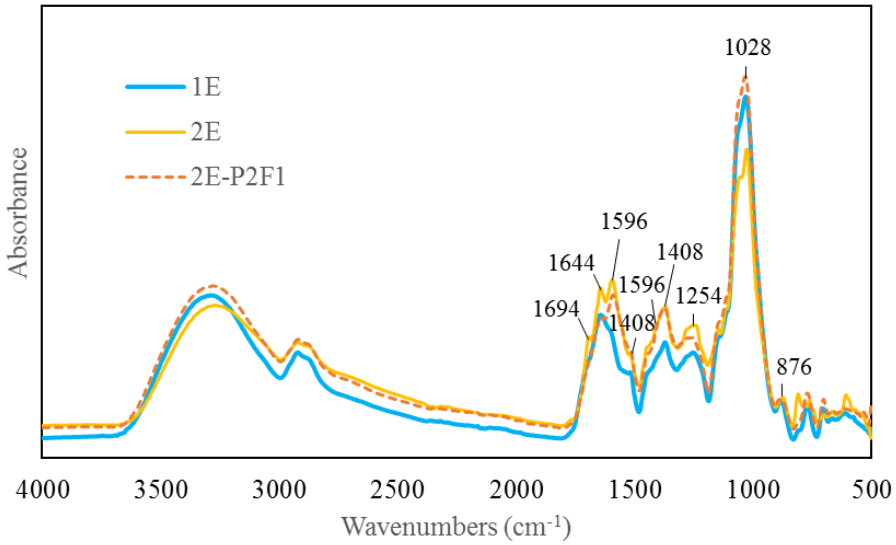

Figure 3.3. FTIR spectra of samples 1E, 2E, and 2E-P2^{F1} acquired by ATR sampling technique (shown after baseline correction and smooth correction; background spectrum subtracted to aid clarity).

The fingerprint region, in which characteristic bands due to polysaccharide appear, showed a strong band around 1028 cm^{-1} for samples 1E, E2, and 2E-P2^{F1}, which are characteristic for a galactopyranose backbone in Type II arabinogalactan [157–159]. Besides, in the anomeric region, absorption bands around 876 cm^{-1} were observed, indicated β-nature of glycosidic linkages [160].

In the region of 1650-1550 cm^{-1} stretching vibrations of peptide bonds (C═O) and bending vibrations of (N─H) groups known as Amide I and Amide II, respectively, can contribute to the intensities observed [157]. In the literature, caffeine is responsible for two large bands in the region 1550-1750 cm^{-1}, assigned to C═O stretching vibrations, and C─C and C─N stretching ones [161]. CGA has major bands in the region 1150-1300 cm^{-1} derived from the deprotonated carboxylic groups (COO^{-}) of these compounds [161]. Since melanoidins have incorporated all these functional groups, they are also responsible for the absorption bands at these wavelengths.

In spite of similar spectral pattern of FTIR spectra of samples 1E and 2E, some differences were observed in band intensities between the wavenumbers of 1700 and 1000 cm^{-1} caused by different protein, caffeine, CGA, and polysaccharide content (**Table 3.1**). In accordance with increasing content of protein, CGA and caffeine from 1E to 2E, increasing of band intensities in 1700-1550 cm^{-1} region was observed. In the 2E spectra, lower intensities of bands in the carbohydrate fingerprint regions (1200–900 cm^{-1}) indicated lower carbohydrate contents, in agreement with the lower sugar content (**Table 3.2**).

Samples 1E and 2E-$P2^{F1}$ showed similar spectral patterns, in accordance with comparable sugars composition, and lacking of CGA and caffeine (**Table 3.2**). Moreover, both spectra show similarities with those of arabinogalactan-protein (AGP) isolated from instant coffee [157–159]. The immunostimulatory activity of coffee samples were evaluated.

3.1.2. Immunostimulatory activity of purified instant coffee fractions

The *in vitro* immunostimulatory activity in BALB/c mice spleen B and T lymphocyte cells of samples 1E, 2E, 2E-$P2^{F1}$, and 2E-$P2^{F2}$ was studied by the expression of an early activation marker (CD69). Sample 1E was also evaluated by Neutral Red assay for cell viability and by CFSE assay for proliferation potential. Moreover all samples were tested for their cytotoxicity adding propidium iodide before flow cytometric analysis.

3.1.2.1. Cell viability/cytotoxicity

To evaluate the existence of possible cytotoxicity of tested compounds, two assays were performed: a) the Neutral Red (NR) uptake assay and b) the addition of propidium iodide before flow cytometric analysis.

With NR uptake assay, only viable cells uptake the dye NR by active transport and incorporate it into lysosomes. Therefore, a decrease of incorporation of NR, measured by the 540 nm absorbance, means a decrease in the number of viable cells. The viability of cells was studied in comparison with cells cultured with medium alone. In **Figure 3.4.a** the % viability of cells cultured with sample 1E was not significantly different from cells cultured with RPMI, the negative control (100 % viability).

In the propidium iodide assay, it crosses the membrane and stains intracellular components if the cell membrane has been compromised. Therefore, healthy cells will not

be stained with propidium iodide. Results of labelling with propidium iodide show that 1E is not cytotoxic for spleen cells (**Figure 3.4.b**), confirming the results from the NR assay.

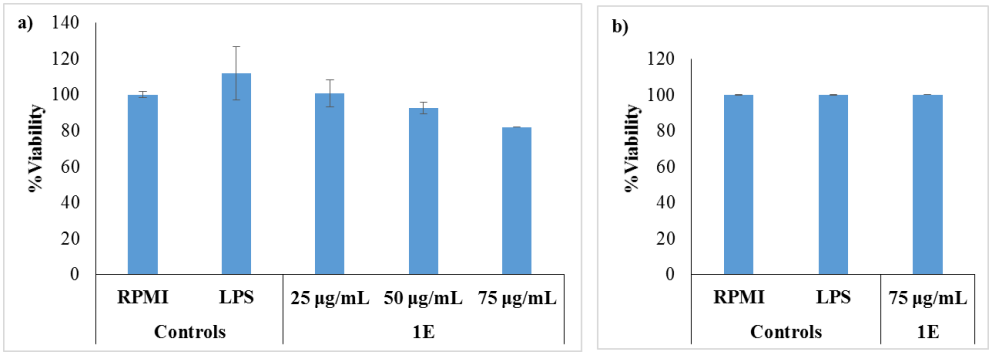

Figure 3.4. Viability of spleen mononuclear cell cultures after stimulation with negative (RPMI) and positive (LPS) controls, and sample 1E (25, 50, and 75 µg/mL) by: **a)** Neutral red (NR) assay; **b)** Propidium iodide incorporation. The viability of tested samples considers RPMI as 100% viable. The results are expressed as mean ± SEM - standard error of means - of duplicates. Results are not significantly different from RPMI ($p>0.001$).

3.1.2.2. Proliferation assays

Sample 1E was studied for the potential of induction of proliferation of mononuclear spleen cells. Results of stimulation of cells with 1E (not shown) were not different from negative control (RPMI) in contrast with the proliferation induced by positive control (LPS). The different response of 1E and LPS also indicates that 1E stimulation of B lymphocytes is not a consequence of LPS contamination.

3.1.2.3. Evaluation of B-lymphocytes activation

Figure 3.5 shows representative examples of dot plots from flow cytometric analysis of surface CD69 expression on the surface of B cells ($CD19^+$) stimulated with the coffee samples 1E, 2E, 2E-$P2^{F1}$, as well as the negative (RPMI) and positive (LPS) controls. In the dot plots, each dot represents a cell. Vertical axis represents the fluorescence intensity of anti-CD69 and horizontal axis represents that of anti-CD19. Gates were set as shown to delineate activated cells (expression of CD69, an early activation marker, $CD69^+$ cells) and B lymphocytes (expression of CD19, $CD19^+$ cells), therefore we can measure the number of $CD69^+$ B lymphocytes (1^{st} quadrant). Furthermore, the percentage of activation (% of activation) can be calculated by the ratio of activated B lymphocytes (1^{st} quadrant) with the total number of B lymphocytes (sum of 1^{st} and 4^{th} quadrants).

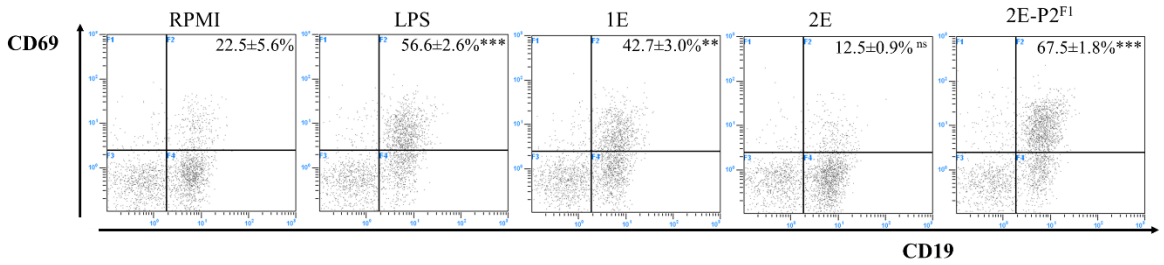

Figure 3.5. Dot plots showing CD69 expression on the surface of B lymphocytes ($CD19^{+}$) in BALB/c mice spleen mononuclear cell cultures stimulated for 6.5 h with RPMI, 1 µg/mL of LPS, with 75 µg/mL of samples 1E, and 2E, and with 50 µg/mL of sample 2E-$P2^{F1}$. Numbers inside dot plots indicate the mean % of activation ± SEM. The significance of the results, as compared with control RPMI, is also indicated (**, $p < 0.01$; ***, $p < 0.001$; ns, $p > 0.05$)

The results of the evaluation of B-lymphocytes activation are also represented in a bar chart of mean % of activation (**Figure 3.6**), confirming the immunostimulatory potential of sample 1E (42.7% of activation) [13] and the absence of activity of sample 2E (12.5% of activation, not statistically different from the negative control) [17]. In contrast with sample 2E, sample 2E-$P2^{F1}$ activated 67.5% of the B lymphocytes, comparable with the activation of sample 1E and the positive control. The potential immunostimulatory activity of 2E-$P2^{F1}$ shows that sample 2E had compounds with immunostimulatory potential. However, the presence of CGA and caffeine, compounds with described anti-inflammatory activity [16], are possibly negatively influencing the *in vitro* immunostimulatory activity.

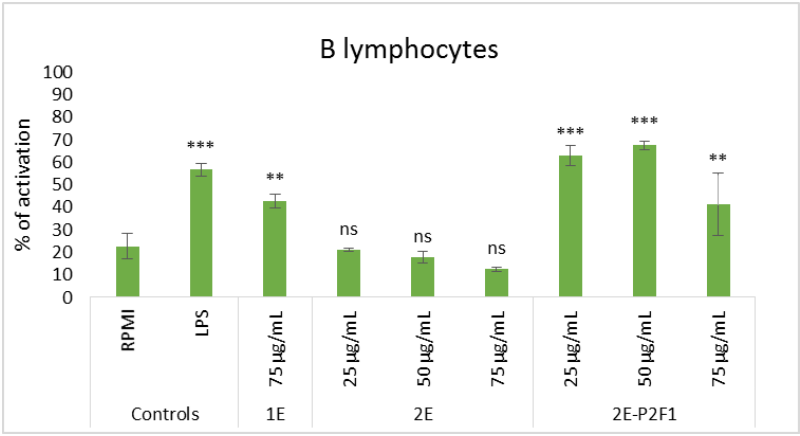

Figure 3.6. Immunostimulatory effect after 6.5 h of stimulation with samples 1E, 2E, and 2E-$P2^{F1}$ (25-75 µg/mL) expressed as % of activation of $CD69^{+}$ B lymphocytes. The significance of the results, as compared with control RPMI, is also indicated (ns, not significant; ***, $p < 0.001$).

3.1.2.4. Evaluation of T-lymphocytes activation

Figure 3.7 shows representative examples of dot plots from flow cytometric analysis of surface CD69 expression on the surface of T lymphocytes ($CD3^+$) stimulated with the coffee samples 1E, 2E, 2E-$P2^{F1}$, as well as the negative (RPMI) and positive (LPS) controls. These dot plots are interpreted as already described for $CD19^+$ cells. Therefore, the T cells % of activation can be calculated by the ratio of activated cells in the 1st quadrant ($CD3^+$ $CD69^+$) with all T lymphocytes from the 1st and 4th quadrants ($CD3^+$).

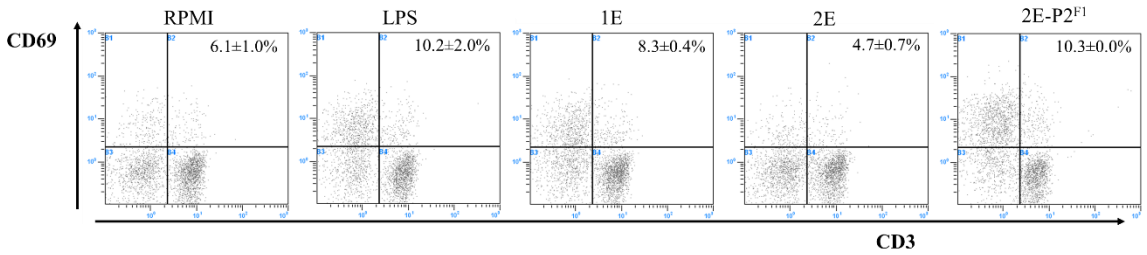

Figure 3.7. Dot plots showing CD69 expression on the surface of T lymphocytes ($CD3^+$) in BALB/c mice spleen mononuclear cell cultures stimulated for 6.5 h with RPMI, 1 µg/mL of LPS, with 75 µg/mL of samples 1E, and 2E, and with 50 µg/mL of sample 2E-$P2^{F1}$. Numbers inside dot plots indicate the mean % of activation ± SEM. Results were not significantly different of the results from RPMI results.

The expression of CD69 by T lymphocytes is lower than that expressed by B lymphocytes, which is in accordance with literature [12,74], showing lower % of activation of $CD3^+$ cells by CD69 expression after stimulation with LPS (10.2%) (**Figure 3.7** and **Figure 3.8)**. Data were not significantly different from the negative control. However, a similar tendency with B lymphocytes activation can be seen. Sample 1E activated 8.3 % of T lymphocytes, sample 2E activated only 4.7 %, lower than the % of activation of negative control. In contrast with sample 2E, sample 2E-$P2^{F1}$ activated 10.3% of the T lymphocytes, comparable with the activation of sample 1E and the positive control. The potential immunostimulatory activity of sample 2E-$P2^{F1}$ to activate T lymphocytes confirms the results of B lymphocytes activation, showing that the presence of CGA and caffeine in the sample 2E influenced negatively the *in vitro* immunostimulatory activity.

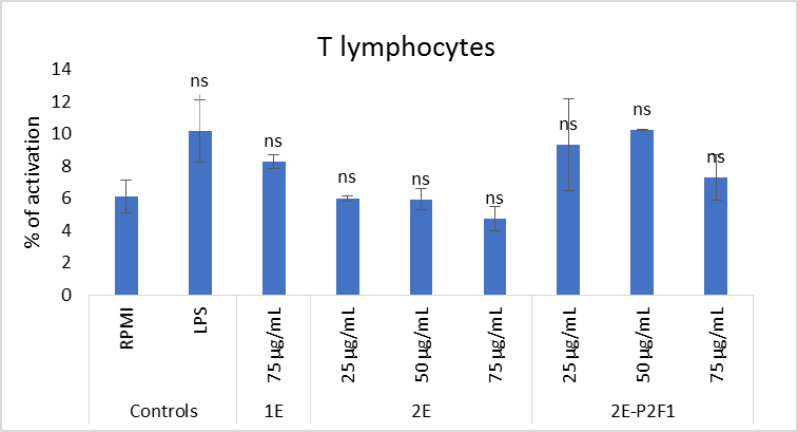

Figure 3.8. Immunostimulatory effect after 6.5 h of stimulation with samples 1E, 2E, and 2E-P2^{F1} (25-75 µg/mL) expressed as % of activation of CD69^{+} T lymphocytes. The significance of the results, as compared with control RPMI, is also indicated (ns, not significant).

3.2. FRACTIONATION ON BIO-GEL P6

Aiming to know what could be the structural characteristics responsible for the instant coffee arabinogalactans potential immunostimulatory activity, sample 1E (1-5 kDa) was submitted to some procedures, as schematised in **Figure 3.1.b**. From fractionation by size-exclusion chromatography using Bio-gel P6 (SEC-P6, 1-6 kDa)) three fractions were pooled and freeze-dried (1E-P6^{F1}, 1E-P6^{F2}, and 1E-P6^{F3}) and their immunostimulatory activity was evaluated.

3.2.1. Characterization of samples obtained by SEC-P6

The SEC-P6 light scattering profile of sample 1E shows a broad band along all the eluted fractions analysed (**Figure 3.9**). This shows that compounds present in sample 1E have a heterogeneity of molecular weight covering all the molecular weight range of Bio-gel P6 (1-6 kDa). The eluted samples were also analysed for their absorbances at 405 nm, 325 nm and 280 nm to evaluate the presence of melanoidins, as discussed in the **section 3.1.1**. Moreover, the sugar content of each fraction was evaluated by absorbances at 490 nm after phenol-sulfuric acid assay. These profiles show that sample 1E comprises two partially overlapping bands, the first related to the presence of carbohydrates with maximum at ~102 mL and another of brown compounds with maximum value eluted at ~124 mL (**Figure 3.9**). Observing the profiles of SEC-P6 in **Figure 3.9**, three fractions of interest were pooled, one with the material of higher molecular weight (first 78 mL eluted volumes, 1E-P6^{F1}) (~5 kDa), another with the intermediate molecular weight material with the peak of sugar content (80 mL till 108 mL eluted volumes, 1E-P6^{F2}) and another with the peak of brown compounds and all smaller molecular weight compounds (110 mL till 160 mL eluted volumes plus 160 till 350 mL not illustrated in the SEC-P6 profiles, 1E-P6^{F3}).

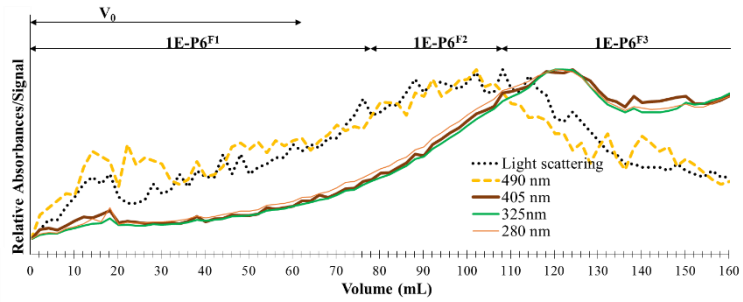

Figure 3.9. Size-exclusion chromatography profiles of sample 1E using light scattering, direct spectrometric detection at 280, 325, and 405 nm, and spectrometric detection after phenol sulfuric acid assay at 490 nm. Void volumes (V_0), fractions of sample 1E (1E-P6^{F1}, 1E-P6^{F2}, and 1E-P6^{F3}).

The SEC-P6 profile given by the absorbance at 405 nm allows to infer that fraction 1E-P6^{F3} was the fraction with higher contribution of melanoidins, followed by 1E-P6^{F2} and 1E-P6^{F1}. 51% of material was recovered in the two high molecular weight fractions, 1E-P6^{F1} and 1E-P6^{F2} after SEC-P6, and fraction 1E-P6^{F3} yielded the remaining 49% (**Table 3.3**). Sugar analyses showed that fraction 1E-P6^{F2} was the fraction richer in carbohydrates (53.5 %). This was in accordance with SEC-P6 profiles, where fraction 1E-P6^{F2} contained the peak of carbohydrates. 1E-P6^{F1} and 1E-P6^{F3} had 42.0 and 26.6 % of total sugars content. However, considering the yields, the sugars recovered in all fractions comprised 30-35 % of total sugars recovered.

1E-P6^{F1} eluted near the void volume, with a molecular weight near 5 kDa, contained 42.0 % of total sugars, and was composed mainly by Gal (80 mol%), Ara (11 mol%), and Man (4 mol%) (**Table 3.3**), therefore it was rich in arabinogalactans. Similarly to 1E-P6^{F1}, 1E-P6^{F2} was rich in Gal (85 mol%), and also contained Ara (10 mol%), and Man (4 mol%). 1E-P6^{F3} contained higher Man content, than the other fractions (19 mol%). Therefore, their sugar composition was similar to sample 1E, although 1E-P6^{F1} and 1E-P6^{F2} were enriched in Gal, and 1E-P6^{F3} was enriched in Man. These results and the SEC-P6 profiles (**Figure 3.9**) show that 1E-P6^{F1} was rich in arabinogalactans and with a lower contribution of melanoidins; 1E-P6^{F2} was rich in arabinogalactans, but had a higher contribution of melanoidins; and 1E-P6^{F3} was composed by all small molecular weight material, melanoidins, arabinogalactans and oligosaccharides.

Table 3.3. Yield and sugar composition of fractions obtained after size-exclusion chromatography on Bio-gel P6 of sample 1E

Samples	Yield (%)[a]	Sugar Composition (%mol)					% Total Sugars	% Recovered sugars
		Rha	Ara	Man	Gal	Glc		
1E		1.4±0.1	6.7±0.1	10.5±0.1	75.0±0.8	6.3±0.6	43.8[b]	
1E-P6^{F1}	30.6	nd[c]	11.1±0.7	4.4±0.5	80.4±0.5	4.1±0.3	42.0±1.0	35
1E-P6^{F2}	20.7	nd	9.8±0.1	3.9±0.5	84.8±0.4	1.5±0.7	53.5±7.7	30
1E-P6^{F3}	48.8	nd	9.6±0.4	18.7±1.3	67.6±0.6	4.2±0.3	26.6±3.3	35

[a] Relative yield; [b] Passos *et al.* [13]; [c] not detected

3.2.2. Immunostimulatory activity of samples obtained after SEC-P6

Similarly to sample 1E, all fractions obtained after SEC-P6 did not show cytotoxicity (data not shown). Moreover, *in vitro* stimulation of B and T lymphocytes effect of 1E-P6^{F1}, 1E-P6^{F2} and 1E-P6^{F3} (20-150 μg/mL) were evaluated.

Figure 3.10 (primary axis) shows that only 1E-P6^{F1} and 1E-P6^{F3} stimulated B lymphocytes, in contrast with 1E-P6^{F2} that did not activate B lymphocytes even when using 150 μg/mL of sugar concentration. Although 1E-P6^{F1} showed lower activity than sample 1E, as it did not reach the same % of activation after stimulation with 150 μg/mL, the activation of B lymphocytes by 1E-P6^{F1} indicates that the compounds with a molecular weight near 5 kDa are responsible for 1E activity. Fraction 1E-P6^{F3} also activated B lymphocytes indicating that the activity of 1E results from synergy between compounds with different molecular weights. The material recovered in this fraction may have a lower contribution to 1E activity as it was necessary to stimulate with 60 μg/mL to reach the stimulation of 50 μg/mL of 1E-P6^{F1} (~59 % of activation).

In concordance with B lymphocytes activation results, significant activation of T cells was observed for 1E-P6^{F1} with only 50 μg/mL, and 1E-P6^{F3} with 60 μg/mL (**Figure 3.10**, secondary axis). Moreover, 1E-P6^{F2} % of activation of T lymphocytes was not significantly different from RPMI. As fraction 1E-P6^{F1} is rich in arabinogalactans, these results indicate that arabinogalactans with ~5 kDa contributed to the immunostimulatory activity of sample 1E. This is in accordance with the literature, where arabinogalactans purified from instant coffee and with a molecular weight of 5-6 kDa were described with immunostimulatory activity, showed by a pro-inflammatory response [7].

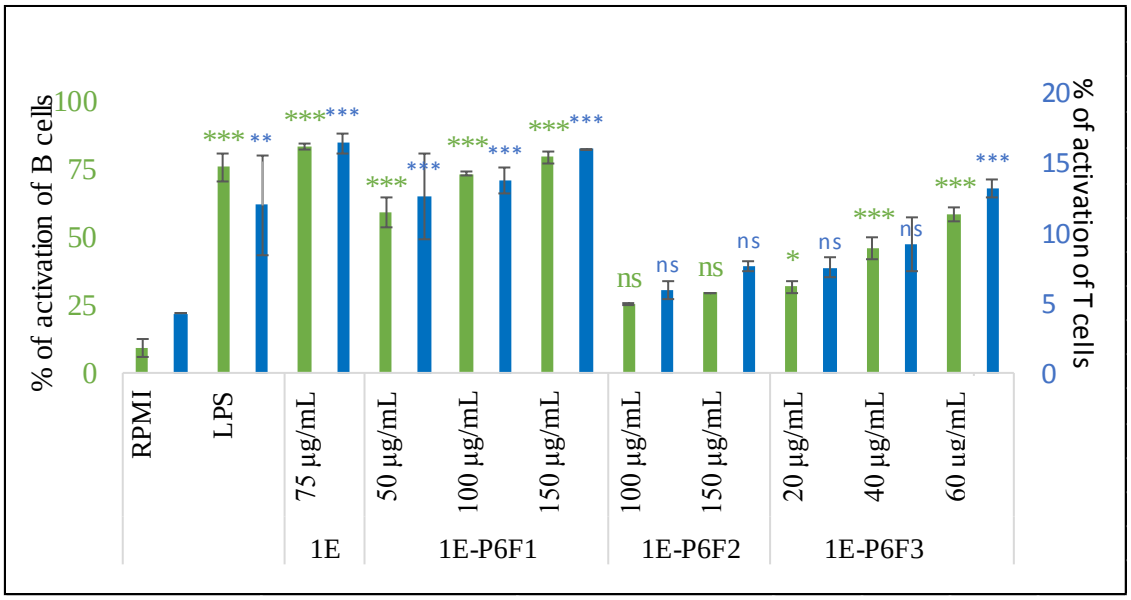

Figure 3.10. Immunostimulatory effect after 16h of stimulation with samples obtained by fractionation on Bio-gel P6 of 1E, namely 1E-P6^{F1}, 1E-P6^{F2} and 1E-P6^{F3} (20-150 µg/mL), expressed as % of activation of CD69^{+} B (green bars) and T lymphocytes (blue bars). The significance of the results, as compared with control RPMI, is also indicated (ns, not significant; *, $p<0.05$; **, $p < 0.01$; ***, $p <0.001$).

3.3. SAPONIFICATION OF INSTANT COFFEE FRACTION

The presence of acetyl groups has been associated to the potential immunostimulatory activity of galactomannans [12,14,59]. However, it was not yet evaluated if the presence of an acetylation pattern could also be associated with the immunostimulatory activity of arabinogalactans, although these polysaccharides, when active, can also contain acetyl groups [81,84,90]. Therefore, sample 1E was treated with 0.1 M NaOH solution for saponification, dialysed for 6 h, and the immunostimulatory activity of the resulting sample (1Es) was evaluated.

3.3.1. Characterization of samples obtained after saponification

The sample obtained after saponification and dialysis of sample 1E yielded 66% of mass weight and showed a similar monosaccharide composition when compared to the native (**Table 3.4**). Therefore, the saponification procedure did not affect the sugar composition.

Table 3.4. Yield and sugar composition of fractions obtained after size-exclusion chromatography on Bio-gel P2 of sample 1Es.

Samples	Yield (%)	Sugar Composition (%mol)					% Total Sugars	DA[a]
		Rha	Ara	Man	Gal	Glc		
1E		1.4±0.1	6.7±0.1	10.5±0.1	75.0±0.8	6.3±0.6	43.8[b]	nd[c]
1Es	65.8	1.8±0.0	7.9±0.1	9.1±0.0	71.8±0.4	9.4±0.3	45.7±5.1	nd
1Es-P2^{F1}	59.1	2.2±0.2	9.5±0.2	5.1±0.6	80.5±0.2	2.6±0.8	30.5±1.2	--[d]

[a] Degree of acetylation; [b] Passos *et al.* [13]; [c] Not detected; [d] Not determined.

For the determination of the degree of acetylation of sample 1E before and after saponification (1Es), the acetic acid released by saponification of acetyl groups, acidification of solution, headspace solid phase microextraction (HS-SPME) and analysis by gas chromatography with flame ionization detector (GC-FID) was measured. However, although this method has been implemented as routine analyses in the laboratory, it was not possible to detect acetic acid in these samples.

In order to have an alternative study to the alterations of the saponification, a FTIR spectrum was obtained for 1Es. The FTIR spectrum of 1Es is very similar to 1E in the carbohydrate fingerprint, confirming the carbohydrate analysis (**Figure 3.11**). Differences were found in the region of 1574 cm^{-1} (COO^- of CGA, [157]) and 1380 cm^{-1} (C=O symmetric stretching of COO- group). The higher contribution of this functional group in 1Es, indicates that with the saponification some deesterification may have occurred when preparing 1Es.

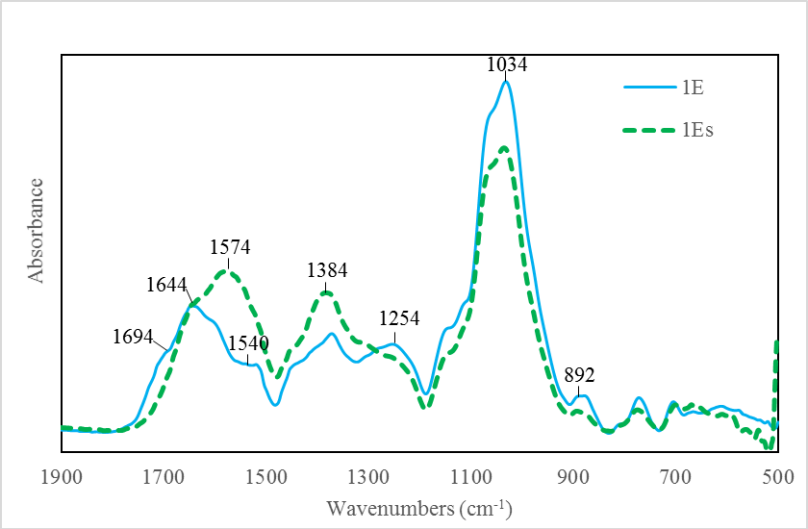

Figure 3.11. FTIR spectra of fingerprint regions of 1E and 1Es acquired by ATR sampling technique (shown after baseline correction and smooth correction; background spectrum subtracted to aid clarity)

To disclose the possibility that, upon saponification, the CGA could have been released from melanoidin structures and be adsorbed to the polysaccharides, even upon exhaustive dialysis, a SEC-P2 was performed. Eluted fractions were analysed with evaporative light scattering detection (ELSD) and absorbances at 405, 325 and 280 nm (**Figure 3.12**). According SEC-P2 profiles, two fractions were obtained, namely 1Es-$P2^{F1}$, from the void volume, with melanoidins, and 1Es-$P2^{F2}$, from the inclusion volume, that did not absorbed at any of the wavelengths measured. If deesterification of CGA linked to melanoidins occurred in a great extension, free CGA would be detected in the inclusion volume. However as it did not happen, CGA were thus inferred to be covalently linked to the melanoidins by a linkage resistant to the alkali conditions, but not by an ester linkage. This information is in accordance with Bekedam *et al.* [155] that suggested that CGA are most probably incorporated to melanoidins via the caffeic acid moiety through non-ester linkages.

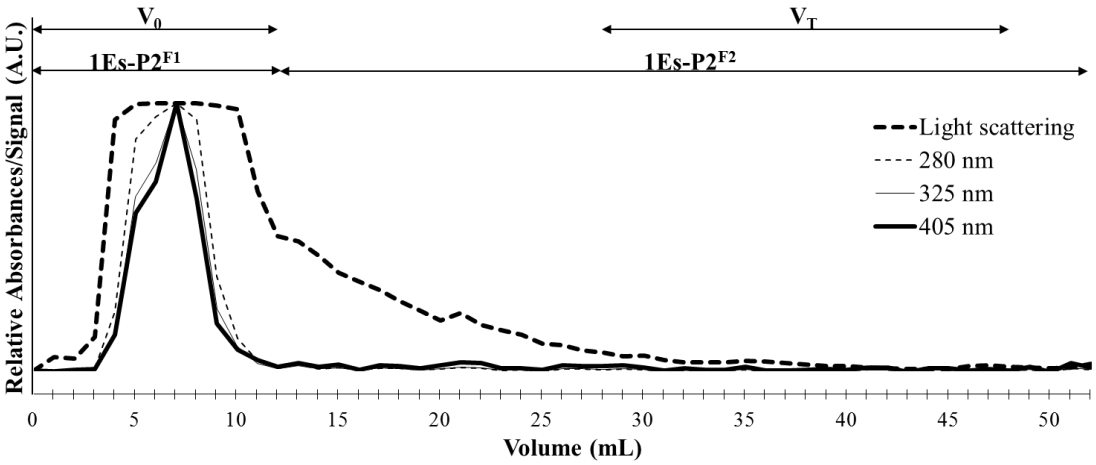

Figure 3.12. Size-exclusion chromatography profiles of sample 1Es using light scattering and spectrometric detection at 280, 325, and 405 nm. Void volumes (V_0), fractions of sample 1Es (1Es-$P2^{F1}$, and 1Es-$P2^{F2}$).

3.3.2. Immunostimulatory activity of samples obtained after saponification

The saponification procedure decreased from 58.2% to 34.9% the immunostimulatory activity of sample 1E using the same concentration of total carbohydrates (75 µg/mL) (**Figure 3.13**). Therefore, saponification must have removed some structural characteristic crucial for activation of B lymphocytes.

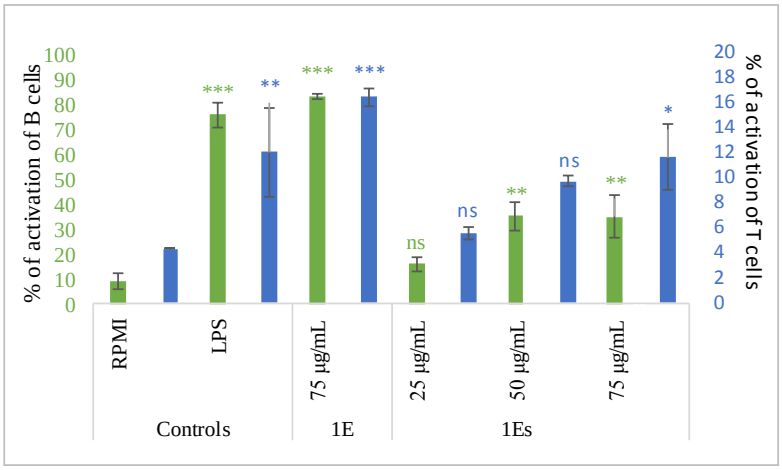

Figure 3.13. Immunostimulatory effect of sample obtained by saponification of 1E (1Es, 25-75 µg/mL) expressed as % of activation of $CD69^+$ B and T lymphocytes. The significance of the results, as compared with control RPMI, is also indicated (ns, not significant; *, $p<0.05$; **, $p < 0.01$; ***, $p <0.001$).

As other acetylated polysaccharides from coffee [7,13,157], sample 1E may have an acetylation pattern that was removed with saponification, but that cannot be quantified with HS-SPME-GC-FID. With the possible existence of an acetylation pattern and the fact that it is not detectable, indicates that it is not necessary a higher amount of acetyl groups.

3.4. DEPROTEINISATION OF INSTANT COFFEE FRACTION

In order to disclose the possibility of the origin of the immunostimulatory activity arise from protein, sample 1E, which contained 5.5% of protein, was submitted to a deproteinisation procedure to eliminate or, at least, change the protein structure associated to polysaccharides.

3.4.1. Characterization of samples obtained after deproteinisation

The deproteinisation treatment with chymotrypsin followed by dialysis of samples 1E and 1Es yielded two deproteinized samples (retentate), $1E^{dep}$(63.8 %) and $1Es^{dep}$(60.2 %), respectively. The solutions (concentrated to 15 mL) of the 1st h of dialyses ($1E^{dialysis}$ and $1Es^{dialysis}$) presented a yellow colour, meaning that some coloured low molecular weight material was washed out through the dialysis membranes (cut off of 1 kDa). However, differences of protein content between initial and deproteinized samples were not detectable by BCA assay and also by elemental analysis (**Table 3.5**). Moreover, as these two methods have inferences from phenolic substances in the case of BCA reagent [162], and by non-amino-acid-nitrogen-containing compounds in the case of the determination of total nitrogen content by elemental analysis, the achievement of other conclusions is prevented.

Table 3.5. Yield and protein content according elemental analysis (%N×6.25) and BCA assay before and after deproteinisation procedure.

Samples	Yield (%)	Protein (%)	
		%Nx6.25	BCA assay
1E		17.6	38.7±0.0
$1E^{dep}$	63.8	18.5	35.8±0.0
$1E^{dialyses}$			1.5±0.0
1Es		20.0	35.4±1.2
$1Es^{dep}$	60.2	22.5	51.0±2.9
$1Es^{dialyses}$			1.2±0.1

The total sugar content and monosaccharides composition was analysed for initial and deproteinized samples (**Table 3.6**). $1E^{dep}$ had a similar sugar content when comparing with sample 1E, showing that with deproteinisation sugars were not affected. However, as sample $1Es^{dep}$ had low solubility, only soluble material was analysed ($1Es^{dep}_{soluble}$). Sample $1Es^{dep}_{soluble}$ had lower total sugar content than 1Es, showing that some modification occurred with the deproteinisation procedure, namely sugars solubility.

Table 3.6. Yield and sugar composition of samples obtained after deproteinization of samples 1E and 1Es

Samples	Sugar composition (% mol)					Total sugars
	Rha	Ara	Man	Gal	Glc	%
1E	1.4±0.1	6.7±0.1	10.5±0.1	75.0±0.8	6.3±0.6	43.8[a]
$1E^{dep}$	1.7±0.1	7.5±0.1	7.3±0.1	77.7±0.4	5.7±0.4	42.5±4.3
1Es	1.8±0.0	7.9±0.1	9.1±0.0	71.8±0.4	9.4±0.3	45.7±5.1
$1Es^{dep}_{soluble}$	nd[b]	11.8±0.1	7.4±0.3	73.3±0.6	5.7±0.3	32.9±1.8

[a] Passos *et al.* [13]; [b] not detected

FTIR spectra were also obtained for deproteinized samples and compared with initial samples. The fingerprint region of FTIR spectra of deproteinized samples are displayed in **Figure 3.14**, where it can be seen no differences between 1E and $1E^{dep}$. On the other hand, 1Es and $1Es^{dep}$ displayed differences in the region of characteristic bands of protein, caffeine, and CGA (1700-1150 cm^{-1}). The spectrum of 1Es had two peaks with in the 1574 and 1384 cm^{-1} wavenumbers that were not present in $1Es^{dep}$ spectrum. It is hypothesized that the deproteinisation was higher when a previous saponification was performed because saponification enhanced the availability of amino acids for the protease action.

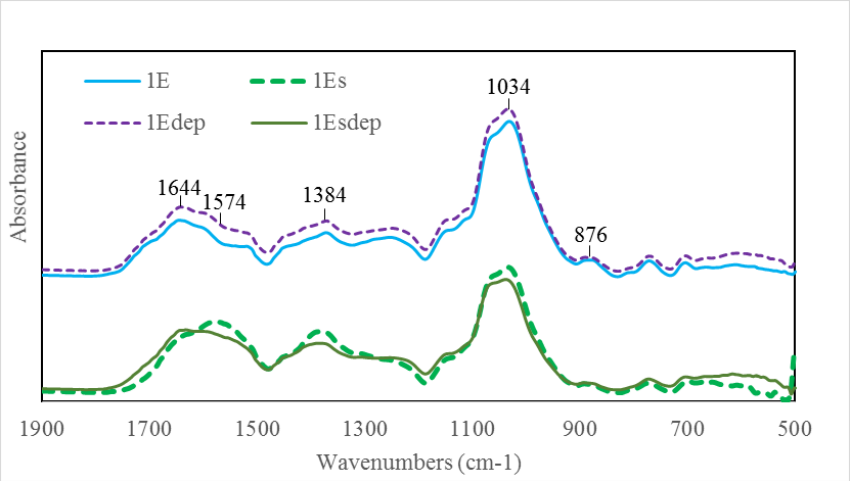

Figure 3.14. FTIR spectra of fingerprint regions of 1E and 1Es before and after deproteinisation treatment, acquired by ATR sampling technique (shown after baseline correction and smooth correction; background spectrum subtracted to aid clarity).

3.4.2. Immunostimulatory activity of samples obtained after deproteinisation

The results of B and T lymphocytes stimulation of samples obtained after deproteinisation are displayed in **Figure 3.15**. From the results of B lymphocytes activation, the deproteinisation procedure did not change the immunostimulatory activity of the resulting sample (83.2%) in comparison with 1E (83.4%). Moreover, deproteinisation of 1Es also did not change significantly the immunostimulatory activity. Therefore we can conclude that the protein content is not important for the activation of B lymphocytes.

Sample $1E^{dep}$ had some significantly activation of T cells in comparison with RPMI, although less than sample 1E. T cells % of activation was affected after deproteinisation in contrast with B lymphocytes % of activation.

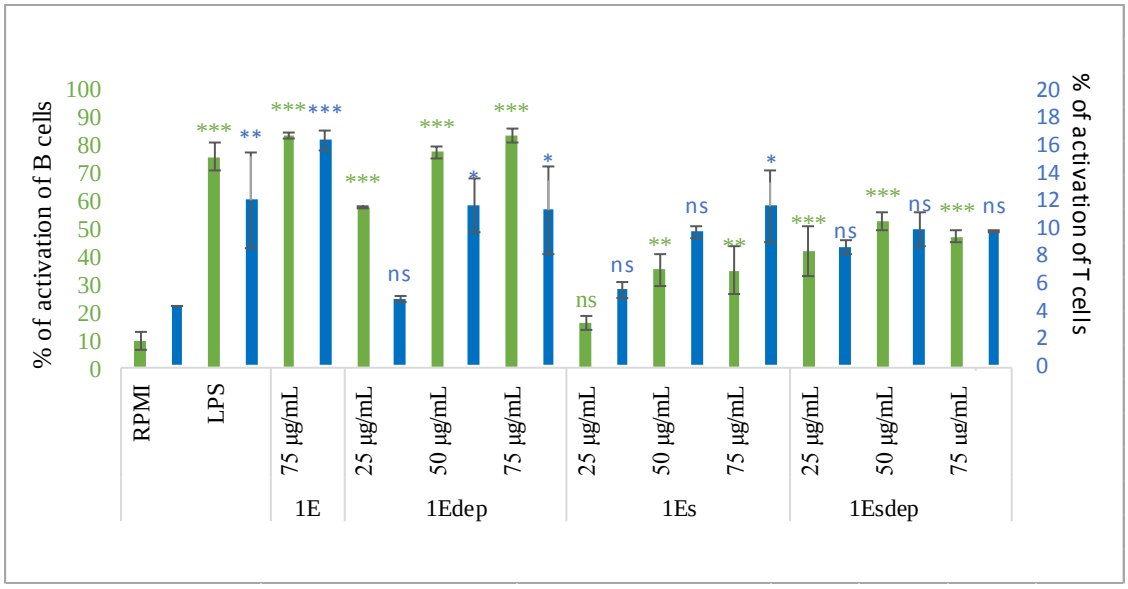

Figure 3.15. Immunostimulatory effect of samples obtained by deproteinisation of 1E and 1Es (25-75 µg/mL) expressed as % of activation of $CD69^+$ B (green bars) and T lymphocytes (blue bars). The significance of the results, as compared with control RPMI, is also indicated (ns, not significant; *, $p<0.05$; **, $p < 0.01$; ***, $p <0.001$).

3.5. α-L-ARABINOFURANOSIDASE TREATMENT AND FRACTIONATION

Arabinose (Ara) residues have been described as important for the immunostimulatory potential of arabinogalactans from different sources [77,78,82,85,87,128]. Therefore, sample 1E with 3.0 % of terminal Ara (T-Ara) was submitted to an enzymatic hydrolysis with an α-L-arabinofuranosidase to evaluate the effect of the resulting sample in *in vitro* immunostimulatory potential.

3.5.1. Characterization of samples obtained by α-L-arabinofuranosidase treatment

After α-L-arabinofuranosidase treatment, $1E^{Arase}$ was submitted to a fractionation step to remove Ara residues through Bio-Gel P2 size-exclusion chromatography (SEC-P2). ELSD profile shows that $1E^{Arase}$ had two populations of brown compounds, as showed by the absorbance profiles at 405, 325 and 280 nm ($1E^{AraseF1}$ and $1E^{AraseF2}$, 1.8 to 5 kDa, **Figure 3.16**). A fraction from inclusion volume was also pooled (1,800-180 Da, $1E^{AraseF3}$), noting this last fraction includes elution volume of monomers.

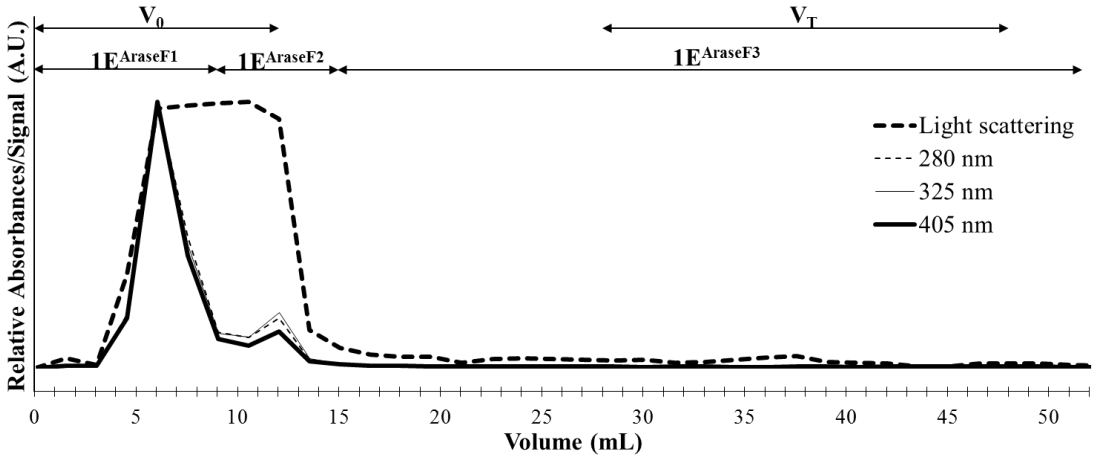

Figure 3.16. Size-exclusion chromatography profiles of sample $1E^{Arase}$ using light scattering and spectrometric detection at 280, 325, and 405 nm. Void volumes (V_0), fractions of sample $1E^{Arase}$ ($1E^{AraseF1}$, $1E^{AraseF2}$, and $1E^{AraseF3}$).

As low amounts of material were recovered in each fraction after passage of $1E^{Arase}$ on SEC-P2, it was not possible to determine the yield of material after lyophilisation, but just the percentage of sugar recovered in each fraction (yield, **Table 3.7**). 64.4% of sugars were recovered in the $1E^{AraseF1}$ and 25.8% in $1E^{AraseF2}$, summing up to 90.2 % of the sugars recovered in the void volume. The inclusion volume, $1E^{AraseF3}$, contained 9.8% of total sugars recovered.

Sample $1E^{AraseF1}$ was rich in Gal (77.1 mol%), followed by Ara (12.6 mol%), and Man (6.3 mol%). Comparing this sample with 1E, $1E^{AraseF1}$ had higher Gal and Ara contents and less Man (**Table 3.7**), therefore it was richer in arabinogalactans. Sample $1E^{AraseF2}$ was also rich in arabinogalactans than sample 1E, but it has less Ara than sample $1E^{AraseF1}$. The higher percentage of Ara in sample $1E^{AraseF3}$ (17.4 %) indicates that α-L-arabinofuranosidase released Ara from arabinogalactans.

Table 3.7. Yield and sugar composition of samples obtained after α-L-arabinofuranosidase treatment and fractionation on Bio-gel P2 of sample 1E

Samples	Sugar composition (% mol)					Yield
	Rha	Ara	Man	Gal	Glc	(%)
1E	1.4±0.1[a]	6.7±0.1	10.5±0.1	75.0±0.8	6.3±0.6	
$1E^{AraseF1}$	nd[b]	12.6±1.5	6.3±0.4	77.1±0.8	4.1±1.9	64.67
$1E^{AraseF2}$	nd	6.6±0.1	6.6±0.4	83.5±0.1	3.4±0.2	25.69
$1E^{AraseF3}$	nd	17.4±0.6	33.8±3.0	32.6±0.1	16.2±2.4	9.64

[a] Passos *et al.* [13]; [b] not detected

3.5.2. Immunostimulatory activity of samples obtained after α-L-arabinofuranosidase

After α-L-arabinofuranosidase treatment and fractionation of sample 1E, sample $1E^{AraseF1}$ was assayed for its immunostimulatory potential of B and T lymphocytes. Results are displayed in **Figure 3.17**, where it can be seen that for $1E^{AraseF1}$ values of % of activation of B and T lymphocytes are not significantly different from RPMI. Therefore, after α-L-arabinofuranosidase treatment of 1E B and T lymphocytes were not significantly activated. This indicates that as other arabinogalactans described with immunostimulatory activity, T-

Ara residues are important for 1E activity. These residues are side chains of arabinogalactans and as degree of branching of polysaccharides affects their conformation [163], arabinose residues potentially participate in the conformation of the polysaccharide and their removal could affect the final conformation.

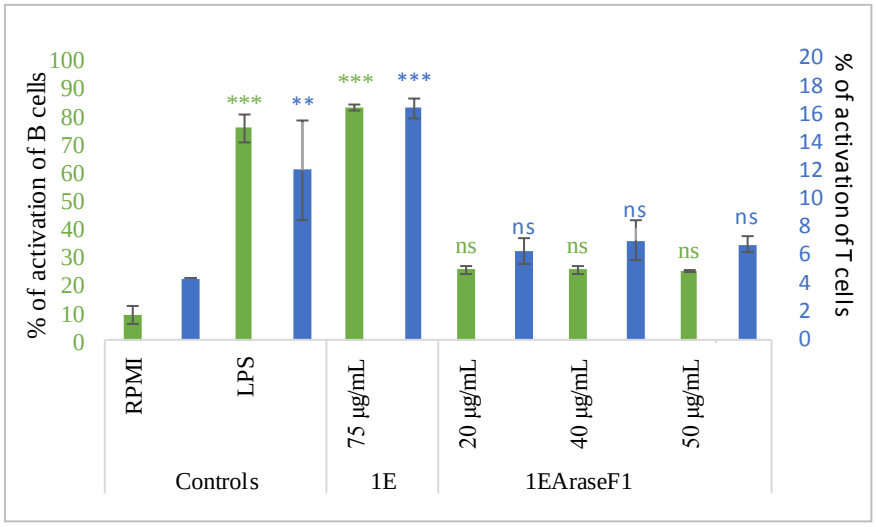

Figure 3.17. Immunostimulatory effect of sample $1E^{AraseF1}$ obtained after α-L-arabinofuranosidase treatment and fractionation of sample 1E (20-50 µg/mL) expressed as % of activation of $CD69^{+}$ B lymphocytes (green bars) and T lymphocytes (blue bars). The significance of the results, as compared with control RPMI, is also indicated (ns, not significant; *, $p<0.05$; **, $p < 0.01$; ***, $p <0.001$).

3.6. YARIV ASSAY

Several arabinogalactan-proteins (AGP) with a positive reaction with Yariv phenylglycosides showed immunostimulatory activity [5,81,88–90]. Yariv reagents are widely used for the detection, quantification, fractionation, and staining of arabinogalactan-proteins (AGP). However, other arabinogalactans without a positive reaction with Yariv showed potent immunostimulatory activity, showing that AGP do not appear to be the main structures responsible for immune system activation [81,88]. Nevertheless, AGP structures are important structural features and therefore fractionated arabinogalactans from instant coffee (samples 1E and 2E) and arabinogalactans resulting from fractionation, deproteinisation, saponification and α-L-arabinofuranosidase treatment were tested for their reactivity with Yariv reagent.

Results from Yariv assay showed that all tested samples did not react with the Yariv reagent (**Figure 3.18**).

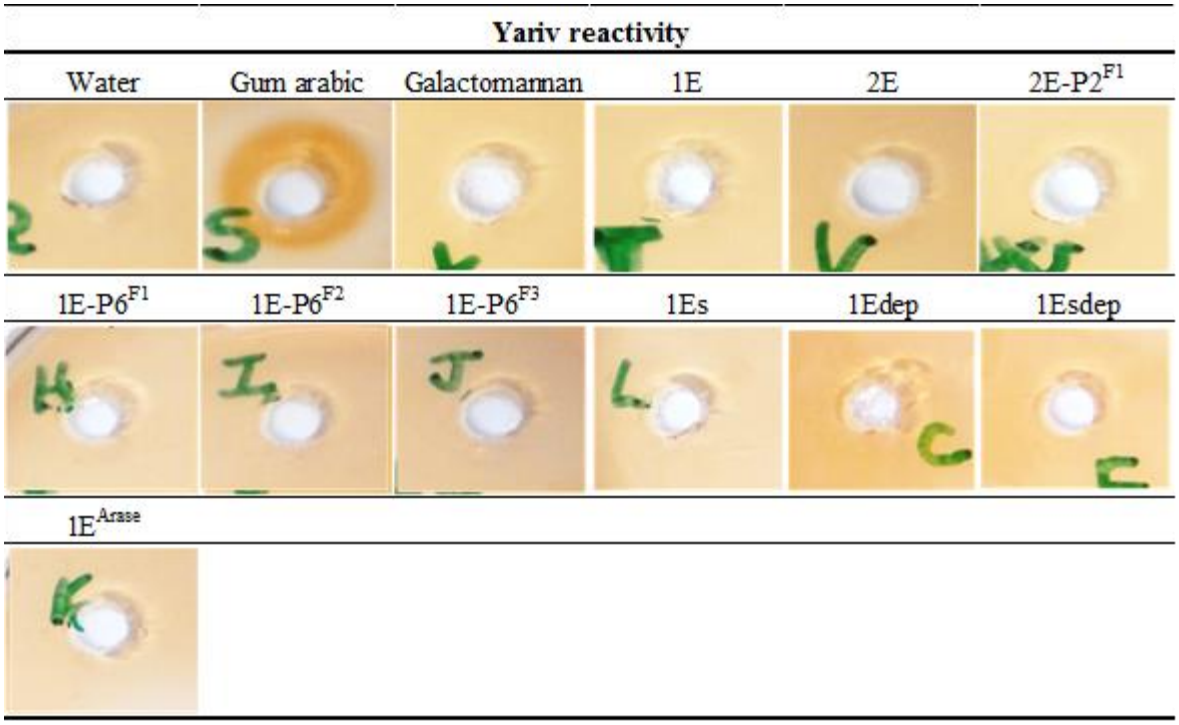

Figure 3.18. Yariv-gel diffusion assay reactivity results of controls (water, gum arabic, and galactomannan) and samples.

The target structure in AGP to which Yariv phenylglycosides bind has not been fully determined. Kitazawa *et al.* [164] found, after combining data from the base hydrolysis of the peptide backbone, and from the analysis of the carbohydrate components with different residues number and glycosidic linkages, that the β1,3-galactan chains longer than five residues are a target structure for Yariv reagent. More than seven residues are needed for cross linking and further precipitation in radial gel-diffusion assays like the ones performed in this study. Moreover they found that neither α-L-Ara*f* residues nor β1,6-galactan side chains are involved in Yariv reactivity.

It is known that AGP (molecular weight near 500 kDa) from green coffee beans react with Yariv reagent [135,165], however roast and further treatments to obtain instant coffee change AGP structures, namely by depolymerisation, debranching, Maillard reactions, caramelisation, isomerization, oxidation, decarboxylation, transglycosylation, and melanoidins formation [15,129,130,136–144]. Moreover, sample 1E with 1-5 kDa has arabinogalactans with a maximum molecular weight near 5 kDa, and glycosidic linkage composition analysis [13] showed that it has 19 mol% of terminal linked Gal and 14 mol% of 3,6-linked Gal. Therefore, these arabinogalactans with 5 kDa (~28 residues) have ~6 3-linked Gal and ~4 are 3,6-linked Gal residues, comprising short chains that do not react with Yariv in accordance with Kitazawa *et al.* [164]. These arabinogalactans possibly have some structural features without Yariv reactivity that are responsible for the immunostimulatory activity shown in sample 1E.

3.7. EVALUATION OF INNATE IMMUNE CELLS ACTIVATION

3.7.1. Evaluation of macrophages activation

Several polysaccharides modulate innate immunity, more specifically, macrophage immune responses [1]. In this study, to evaluate the pro-inflammatory potential by production of NO by macrophages, bone-marrow derived macrophages (MBDM) were prepared and stimulated with samples 1E, 2E, and 2E-P2F1, and controls (cRPMI and LPS) for 24 and 48 h. **Figure 3.19** show a clear stimulation of BMDM by samples 1E and 2E-$P2^{F1}$ (significantly different from the negative control, cRPMI), in contrast with sample 2E. After 24 h, the stimulation of BMDC by samples 1E and 2E-$P2^{F1}$ was similar to LPS. After 48 h, the stimulation by these samples was significantly higher than LPS. These results show that samples1E and 2E-$P2^{F1}$ have a pro-inflammatory potential by activation of macrophages.

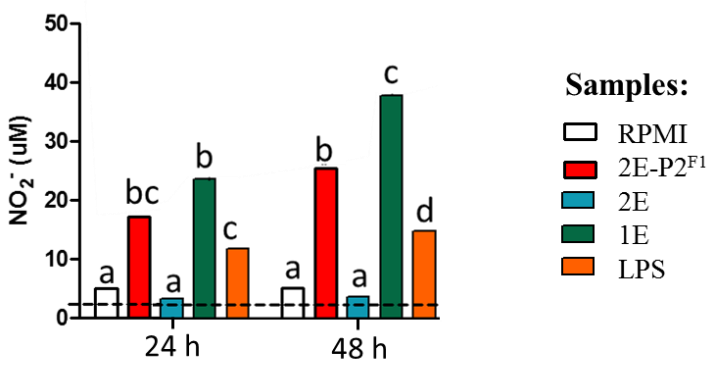

Figure 3.19. Levels of NO_2^- production by BMDM with 75 μg/mL of samples 1E, 2E, and 2E-$P2^{F1}$, after 24 and 48 h compared with non-stimulated cells (RPMI) and positive control (LPS). Means with different letters are significantly different ($p<0.05$).

3.7.2. Evaluation of dendritic cells activation

Dendritic cells (DCs) are the most potent antigen presenting cells of the immune system that induce and modulate immune responses [166]. To study the potential immunostimulatory effect of coffee polysaccharides in DCs, bone-marrow derived DCs (BM-DCs) were prepared and stimulated with samples 1E, 2E, and 2E-$P2^{F1}$ for 6 and 14 h to evaluate the surface expression of MHC-II, CD80, and CD86 in comparison with non-stimulated cells (RPMI) and cells stimulated with LPS (positive control).

Figure 3.20 shows a significant increased expression of MHC-II, CD80, and CD86 after 6 h of stimulation with samples 1E and 2E-P2^{F1} comparing with non-stimulated cells. Sample 2E did not increase the expression of these activation markers, even after 14 h of stimulation. These results of BM-DCs stimulation showed the same tendency as previously observed for the activation of B lymphocytes (1E and 2E-P2^{F1}), where CGA and caffeine are not present.

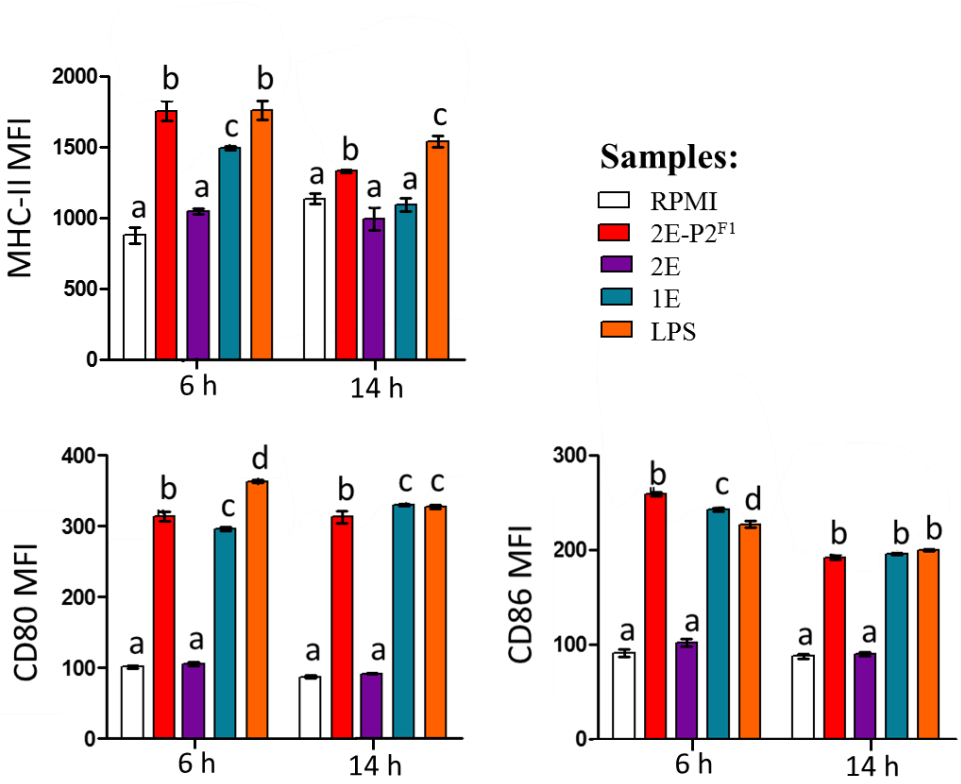

Figure 3.20. Median fluoresce intensity (MFI) of activation markers (MHCII, CD80, CD86) of BM-DCs stimulated with 75 µg/mL of samples, after 6 h and 14 h, compared with non-stimulated cells (RPMI) and positive control (LPS). The results are expressed as mean ± SEM of triplicates. Means with different letters are significantly different ($p<0.05$).

4. CONCLUDING REMARKS AND PERSPECTIVES FOR FUTURE WORK

The study of instant coffee extracts rich in arabinogalactans, using BALB/c mice spleen B and T lymphocyte cells, allowed to conclude that the presence of small molecular weight compounds, namely chlorogenic acids (CGA) and caffeine, interfere with the determination of the *in vitro* immunostimulatory activity of coffee polysaccharides. This result highlight the importance of purification procedures before the study of polysaccharides immunostimulatory activity.

The study of structure-function relationships of instant coffee polysaccharides showed that the *in vitro* immunostimulatory activity of arabinogalactan-rich fractions is dependent of the molecular weight of the compounds, having higher activity the fraction near 5 kDa. This activity seems to be dependent of the presence of terminally-linked arabinose residues, but not on the acetylation of the polysaccharide neither on the presence of protein. However, these results show that the *in vitro* immunostimulatory activity of instant coffee fractions are related to the arabinogalactans, it will be necessary to purify these polysaccharides from other structures, namely melanoidins, to establish with more certainty polysaccharide structure-immunostimulatory activity relationships.

The fraction presenting BALB/c mice spleen B and T lymphocyte stimulatory activity stimulates also innate immune cells, shown by the NO produced from bone-marrow derived macrophages (BMDM) and which increased expression of surface activation markers MHC-II, CD80, and CD86 by dendritic cells derived from bone-marrow (BM-DCs). It is possible that the activation of macrophages and dendritic cells may be involved in the activation of B and T spleen lymphocytes by these polysaccharides. To address this possibility it will be necessary to test these compounds in purified cell cultures of B and T lymphocytes. On the other hand, it will be important to study the type of immune responses induced by instant coffee samples, by evaluation of cytokines production. For example, by evaluation of IL-10, IFN-γ and IL-12, it can be disclosed if the activation of immune system

is associated to T helper type 1 (TH1) responses (increase of IFN-γ and IL-12) versus TH2 responses (increase of IL-10) [167].

Although the interference of CGA and caffeine was observed in the *in vitro* experiments, it is not expected that it could occur *in vivo*. During digestion, CGA and caffeine are absorbed in the upper small intestine whereas the polysaccharides and melanoidins are not. Along the digestive tract, the immunostimulatory effect of polysaccharides *in vivo* should prevail when interacting with immune cells found in Peyer's patches, localized in the lowest portion of the small intestine, the ileum, before colon fermentation.

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