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STRUCTURAL CHARACTERIZATION AND BIOLOGICAL ACTIVITY OF POLYSACCHARIDES FROM INFUSIONS USED IN THE POPULAR TRADITION

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CARACTERIZAÇÃO DA ESTRUTURA E ATIVIDADE BIOLÓGICA DOS POLISSACARÍDEOS PRESENTES EM INFUSÕES UTILIZADAS NA MEDICINA POPULAR

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica do Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro

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palavras-chave

Infusões, decoções, *Fraxinus angustifolia, Mentha suaveolens, Pterospartum tridentatum*, polissacarídeos pécticos, arabinogalactanas, mananas, xiloglucanas, atividade imunoestimuladora e espectrometria de massa.

resumo

O uso de plantas com fins medicinais é uma prática antiga, ainda bastante frequente em regiões de países em desenvolvimento, e que se tem vindo a disseminar em países mais industrializados. Este facto é suportado pelo elevado número de estudos etnobotânicos, onde se refere que estas plantas são frequentemente usadas na forma de decocções e infusões. Na maioria dos estudos as atividades biológicas são associadas à presença de compostos fenólicos e às suas propriedades antioxidantes, assim como aos polissacarídeos, nomeadamente devido às propriedades antitumorais.

Na região de Trás-os-Montes, algumas das infusões mais utilizadas pela medicina popular têm por base as folhas secas do freixo (*Fraxinus angustifolia*), a parte aérea seca do mondrasto (*Mentha suaveolens*) e as inflorescências secas da carqueja (*Pterospartum tridentatum*). No entanto, não existe nenhum estudo sobre os polissacarídeos presentes nas infusões das folhas secas do freixo (*Fraxinus angustifolia*), da parte aérea do mondrasto (*Mentha suaveolens*) ou das inflorescências da carqueja (*Pterospartum tridentatum*). Deste modo, com a presente dissertação pretende-se, através da caracterização estrutural dos polissacarídeos presentes nas infusões de *F. angustifolia*, *M. suaveolens* e *P. tridentatum*, avaliar a possível relação entre os polissacarídeos e a atividade imunoestimuladora que estas infusões possam apresentar.

Numa primeira fase, foram preparadas infusões tradicionais de *F. angustifolia*, tendo-se mostrado que o material solúvel das infusões continha cerca de 85% de material não retido em cartuchos C_{18} com características hidrofílicas, sendo os restantes 15% compostos por material retido de características hidrofóbicas. Mostrou-se ainda que as infusões obtidas apenas continham cerca de 2 a 4% de material de alto peso molecular (HMWM), que era composto por cerca de 30% de carboidratos. A análise de açúcares e a análise de metilação do HMWM sugeriu a presença de polissacarídeos pécticos, em conjunto com arabinogalactanas do tipo II, mananas e xiloglucanas. No entanto, a quantidade de material obtido é muito baixa para o fracionamento e a análise estrutural dos polissacarídeos presentes. A decocção de 4 h, divididas em 2 períodos de 2 h, com renovação da água, permitiu um aumento no rendimento em HMWM, relativamente às infusões tradicionais. Verificou-se ainda, que a decocção também permitiu aumentar a proporção de carboidratos no HMWM, particularmente devido ao aumento da proporção de ácido urónico presente, embora os resíduos de açúcares neutros se encontrassem em proporção semelhante. Deste modo, em todas as experiências realizadas posteriormente, o HMWM utilizado foi obtido através da decocção das folhas secas de *F. angustifolia*, da parte aérea seca de *M. suaveolens* e das inflorescências secas de *P. tridentatum*.

Após fracionamento por precipitação em etanol e cromatografia de troca aniónica dos polissacarídeos presentes no HMWM obtido através da decocção dos materiais vegetais das diferentes plantas estudadas, verificou-se a presença de elevadas proporções de polissacarídeos pécticos, contendo arabinogalactanas do tipo I, em conjunto com menores proporções de arabinogalactanas do tipo II, mananas e xiloglucanas.

A presença de polissacarídeos pécticos nos extratos de *F. angustifolia* foi também evidenciada através do tratamento enzimático com *endo*-poligalacturonase e de experiências de ESI-MS e ESI-MS/MS. A deteção de resíduos de pentose ligados a resíduos de ácido urónico também pareceu indicar a presença de domínios de xilogalacturonana nos polissacarídeos pécticos de *F. angustifolia*.

Os extratos das folhas secas de *F. angustifolia* mostraram conter arabinogalactanas do tipo II com uma maior diversidade estrutural do que as que foram detetadas nos extratos de *M. suaveolens* e *P. tridentatum*, particularmente no que diz respeito à substituição das cadeias de (1→3)-Gal*p* e à extensão das cadeias laterais compostas por resíduos de (1→5)-Ara*f*. Para além disso, verificou-se que, para todas as plantas estudadas, as arabinogalactanas do tipo II presentes nas frações obtidas durante a segunda parte do processo de extração apresentaram uma substituição das cadeias de (1→3)-Gal*p* maior do que as extraídas durante a primeira parte da extração.

Os extratos das inflorescências de *P. tridentatum* continham maiores proporções de mananas e de xiloglucanas, tendo-se detetado estruturas que apresentaram um maior grau de substituição do que as que foram detetadas em menores proporções nos extratos de *F. angustifolia* e *M. suaveolens*. Através das técnicas de ESI-MS e ESI-MS/MS, foi possível demonstrar que as mananas presentes nos extratos de *P. tridentatum* se encontravam acetiladas no *O-2* dos resíduos de manose. Verificou-se que as mananas presentes nos extratos de *P. tridentatum* se encontravam acetiladas em maior extensão do que as mananas detetadas na infusão do café, na goma de alfarroba e em outras fontes não convencionais de mananas. Para além disso, também foram detetados oligossacarídeos contendo resíduos de pentose, ligados a resíduos de hexose, sugerindo a presença de resíduos de arabinose na estrutura das mananas de *P. tridentatum*.

A atividade imunoestimuladora de três frações isoladas a partir dos extratos de *F. angustifolia*, *M. suaveolens* e *P. tridentatum* foi avaliada, tendo-se observado o aumento da produção de NO por parte de macrófagos, sem comprometer a sua viabilidade celular. As arabinogalactanas do tipo I e II parecem contribuir para a atividade imunoestimuladora observada nas frações dos extratos de *F. angustifolia* e de *M. suaveolens*

No caso da fração obtida a partir dos extratos de *P. tridentatum*, a acetilação das mananas, bem como a presença de arabinogalactanas do tipo I e II parecem ter contribuído para a atividade imunoestimuladora dos macrófagos observada. A possível presença de xiloglucanas de armazenamento, provenientes das sementes das inflorescências de *P. tridentatum*, também poderá contribuir para a atividade imunoestimuladora observada quando os macrófagos foram estimulados com maiores concentrações de extrato.

Os resultados obtidos permitem concluir que os extratos das folhas secas de *F. angustifolia*, da parte aérea de *M. suaveolens* e das inflorescências de *P. tridentatum* contêm elevadas proporções de polissacarídeos pécticos, contendo arabinogalactanas do tipo I, misturados com outros polissacarídeos, tais como arabinogalactanas do tipo II, mananas e xiloglucanas. Esta mistura de polissacarídeos mostrou contribuir para a atividade imunoestimuladora de frações isoladas a partir dos extratos das plantas estudadas. Deste modo, e como o mesmo tipo de polissacarídeos parece estar presente nas infusões e decoções, é possível que os polissacarídeos possam contribuir para as propriedades terapêuticas frequentemente atribuídas pela tradição popular às infusões destas plantas.

keywords

Infusions, decoctions, *Fraxinus angustifolia, Mentha suaveolens, Pterospartum tridentatum*, pectic polysaccharides, arabinogalactans, mannans, xyloglucans, immunostimulatory activity, and mass spectrometry.

abstract

The use of plants with medicinal purposes is an ancient practice still very common in developing regions, and is rapidly spreading in industrialized countries. This fact is evidenced by the large number of ethnobotanical studies found in the literature referring that these plants are often used as decoctions and infusions. In most studies the reported biological activities are attributed to the presence of phenolic compounds, due to their antioxidant properties, and to polysaccharides, with its anti-tumoral properties.

In "Trás-os-Montes" region, some of the most popular infusions used by the popular medicine are prepared with the dried leaves of *Fraxinus angustifolia,* the dried shoots of *Mentha suaveolens*, and the dried inflorescences of *Pterospartum tridentatum*. However, there are no studies about the polysaccharides present in these infusions. Thus, through the structural characterization of the polysaccharides present in the infusions of *F. angustifolia*, *M. suaveolens*, and *P. tridentatum*, the present PhD thesis intends to evaluate the possible relation between polysaccharides and the immunostimulatory activity that these infusions might present.

In a preliminary phase, infusions of *F. angustifolia* were prepared according to the popular tradition, and it was observed that the obtained water soluble material contained approximately 85% of material non-retained in C_{18} cartridges, with hydrophilic characteristics, with the remaining 15% comprising retained-material with hydrophobic characteristics. It was also shown that the infusions only contained between 2 and 4% of high molecular weight material (HMWM), which comprised approximately 30% of carbohydrate material. Sugar and methylation analysis of the HMWM suggested the presence of pectic polysaccharides, together with type II arabinogalactans, mannans, and xyloglucans. However, the amount of material obtained is to low for the fractionation, and structural analysis of the polysaccharides present. The 4 h decoction, divided in two periods of 2 h, with water renewal, allowed to increase the HMWM yield, relatively to the infusions traditional infusions. It was also observed that the decoction also allowed to increase the HMWM proportion of carbohydrate material, due to an increase in the proportion of uronic acid present, although the neutral sugar residues seemed to be detected in similar proportions. Therefore, in all the experiments subsequently performed, the HMWM used was obtained through the decoction of *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences.

After the fractionation, through ethanol precipitation, and anion exchange chromatography, of the polysaccharides from the HMWM obtained by the decoction of the vegetable material of the distinct studied plants, it was observed the presence of high proportions of pectic polysaccharides, containing type I arabinogalactans, together with minor proportions of type II arabinogalactans, mannans, and xyloglucans.

The presence of pectic polysaccharides in the extracts from *F. angustifolia* was also evidenced through *endo*-polygalacturonase treatment, and ESI-MS and ESI-MS/MS experiments. The detection of linked pentose and uronic acid residues, also seemed to suggest the presence of xylogalacturonan domains in the pectic polysaccharides from *F. angustifolia*.

The extracts from *F. angustifolia* dried leaves also contained type II arabinogalactans that exhibited a higher structural diversity than those detected in the M. suaveolens, and P. tridentatum extracts, particularly in the substitution degree of the galactan backbone, and in the extension of the (1→5)-Ara*f* side chains. Moreover, for all the plants studied, it was also observed that the type II arabinogalactans, extracted during the 2nd 2h of the extraction process, exhibited a substitution degree of the galactan backbone higher than those extracted during the 1st 2h.

The extracts from *P. tridentatum* dried inflorescences contained higher proportions of mannans, and also of xyloglucans, both presenting a substitution degree higher than those, which were detected in lower proportion in the extracts of *F. angustifolia* and *M. suaveolens*. Through ESI-MS and ESI-MS/MS it was possible to evidence that the mannans present in the extracts of *P. tridentatum* presented acetyl groups on the O-2 of the mannosyl residues. It was also evidenced that the *P. tridentatum* mannans were more extensively acetylated than the mannans detected in the coffee infusion, LBG, and other non-conventional mannan sources. Moreover, it was detected the presence of oligosaccharides comprising hexose residues linked to non acetylated pentose residues, suggesting the possible presence of arabinose residues in the mannans from *P. tridentatum* extracts.

The immunostimulatory activity of three fractions isolated from the extracts of *F. angustifolia*, *M. suaveolens*, and *P. tridentatum*, was tested and an increase in the NO production by macrophages, without compromising their cellular viability, was observed. The type I, and type II arabinogalactans detected in the extracts from *F. angustifolia*, and *M. suaveolens* seem to have contributed for the observed immunostimulatory activity.

For the fraction from *P. tridentatum*, the mannans acetylation, and the presence of type I, and type II arabinogalactans seemed to contribute for the macrophage immunostimulatory activity observed. The possible presence of storage xyloglucans from the inflorescences seeds, also seems to have contributed for the immunostimulatory activity registered when the macrophages were stimulated with higher extract concentrations.

The results obtained allow to conclude that the extracts of *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences contained high proportions of pectic polysaccharides, exhibiting type I arabinogalactans, together with other polysaccharides, such as type II arabinogalactans, mannans, and xyloglucans. This polysaccharide mixture seems to have contributed to the immunostimulatory activity of fractions isolated from the extracts of the studied plants. Therefore, as the same type of polysaccharides seem to be present in the decoctions and in the infusions, it seems possible that the polysaccharides might contribute for the therapeutic properties frequently associated by the popular tradition to the infusions of these plants.

Abbreviations

2-O-Me-Fuc	2-O-methyl-fucose
2-O-Me-Xyl	2-O-methyl-xylose
AG-I	Type I arabinogalactan
AG-II	Type II arabinogalactan
AGP	Arabinogalactan protein complex
AceA	Aceric acid
Api	Apiose
Ara	Arabinose
Ara*f*	Arabinosyl furanoside
Ara*p*	Arabinosyl pyranoside
CR3	Complement receptor 3
DEAE	Diethylaminoethyl
Dha	3-deoxy-D-lyxo-2-heptulosaric acid
DP	Degree of polymerization
ESI-MS	Electrospray ionization-mass spectrometry
Fuc	Fucose
Fuc*p*	Fucosyl pyranoside
Gal	Galactose
Gal*f*	Galactosyl furanoside
Gal*p*	Galactosyl pyranoside
GalA	Galacturonic acid
GC-MS	Gas chromatography-mass spectrometry
Glc	Glucose
GlcA	Glucuronic acid
HG	Homogalacturonan
HMWM	High molecular weight material
HPLC	High performance liquid chromatography
Kdo	2-keto-3-deoxy-D-manno-octulosonic acid
LBG	Locust bean gum
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccharide
Man	Mannose
MR	Mannose receptor
NO	Nitric oxide
PMB	Polymyxin B
RG-I	Type I Rhamnogalacturonan
RG-II	Type II Rhamnogalacturonan
ROS	Reactive oxygen species
Rha	Rhamnose
SR	Scavenger receptor
TLR-4	Toll-like receptor 4
XG	Xyloglucans
XGA	Xylogalacturonan
Xyl	Xylose
Xyl*p*	Xylosyl pyranoside

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CHAPTER 1

INTRODUCTION

The use of plants with medicinal purposes is a common practice in African, Asian, and Latin America countries, and is rapidly spreading in industrialized countries. The widespread use of plants with medicinal purposes can be assessed by the large number of ethnobotanical studies found in the literature that report the diversity of vegetable species used in different regions around the world, including Portugal. In most cases, the plants are used as decoctions or infusions that are ingested to achieve the desired therapeutic effect.

Although the polysaccharides represent a small fraction of the total compounds present in the decoctions or infusions, its importance has been recognized in various studies. Since the polysaccharides are extracted from the cell walls of the vegetable tissues used for the preparation of these hot water extracts, the cell wall three dimensional organization models, and its main carbohydrate components, namely pectic polysaccharides and non-starch storage polysaccharides will be described. The structural description of pectic polysaccharides distinct carbohydrate domains, and the models proposed for the description of its macromolecular structure will be addressed. Also, the structure of the main hemicelluloses will be described.

Finally, the polysaccharide interactions with the immune system, with particular focus on the structure-activity relations of pectic and mannan polysaccharides, which are frequently reported as major immunomodulatory agents, is reviewed.

1.1. The use of plants with medicinal purposes

The use of plants with medicinal purposes is an ancient practice, which is still very common on developing regions of the world, and that is rapidly increasing in industrialized countries. Consequently, a large number of ethnobotanical research studies focused on the flora of regions from diverse countries can be found in the literature: Argentina (Molares and Ladio, 2009; Hernández *et al.*, 2009), Brazil (Coelho-Ferreira, 2009), China (Au *et al*., 2008; *Meng et al.*, 2009; Zheng and Xing, 2009), Ethiopia (Giday *et al.*, 2009; Teklehaymanot, 2009), Índia (Rajakumar and Shivanna, 2009; Namsa *et al.*, 2009), Iran (Miraldi *et al.*, 2001), Italy (Palmese

et al., 2001; Idolo *et al.*, 2010), Lithuania (Petkeviciute *et al*., 2010), Pakistan (Ashraf *et al.*, 2010), South Africa (van Wyk, 2008; van Wyk *et al.*, 2008), Spain (Agelet and Vallès, 2003) and Uganda (Tabuti, 2010). Ethnobotanical studies concerning the flora of some Portuguese regions, such as "Arrábida", and "Serra de São Mamede" natural parks, "Madeira", and "Porto Santo" islands, and the "Trás-os-Montes" region, can also be found (Camejo-Rodrigues *et al.*, 2003; Novais *et al.*, 2004; Neves *et al.*, 2009; Rivera and Obón, 1995).

These studies gather and systematize a vast amount of information regarding the plants typically used by populations, such as anatomical part of the plant used, and popular application. According to these studies the medicinal plants can be used for the treatment of various illness pathologies, such as infectious and inflammatory, dermatological, respiratory, urinary, gastrointestinal, and also general health and metabolic disorders (Neves *et al.*, 2009). Depending on the intended purpose and selected plant, different anatomical parts can be used: aerial parts, flowers, fruits, leaves and roots (Novais *et al.*, 2004). Another useful information usually present in these studies is the required preparation mode, being the decoction and infusion the most frequently cited (Camejo-Rodrigues *et al.*, 2003; Novais *et al.*, 2004). The decoctions, and the infusions are water extracts prepared through the vegetable material contact with boiling water, or with water at a determined temperature, during a determined period. According to the popular tradition, the infusions are often prepared with previously boiled water.

Several physiologically active compounds can be found in medicinal plants, such as alkaloids, phenolic compounds, terpenoids, glycosides and polysaccharides (Lovkova *et al.*, 2001). However, and despite the relatively large number of ethnobotanical studies published and the even greater number of plants cited, there is a lack of consolidated and systematic scientific information focused on the chemical compounds present in the infusions of plants used with medicinal purposes, and their biological activities. In fact, with the exception of the infusion of *Camellia sinensis* leaves, commonly known as tea, only the most popular plant infusions, such as chamomile (*Matricaria recutita* L.), peppermint (*Mentha piperita* L.), rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) have their bioactivity and potential health benefits reviewed (Stagg and Millin, 1975; Wheeler

and Wheeler, 2004; McKay and Blumberg, 2006a; McKay and Blumberg, 2006b; McKay and Blumberg, 2007).

Over the years, tea has received a great deal of attention, in part due to polyphenols, and particularly flavanoids, which are frequently related to the medicinal effects of tea (Wheeler and Wheeler, 2004). Compositional data on this type of compounds could be used as the basis to a method for the differentiation of green and black tea based on the ratio between total phenolics and sum of the major catechins (Hilal and Engelhardt, 2007). In Portugal, the content of some minerals, caffeine and catechins in green tea samples commercialized were evaluated showing that green tea infusions provide significant amounts of catechins and could be an important source of some minerals, such as potassium, which was the cation present in larger amount (92 – 151 mg/L) (Reto *et al*., 2007). Aspects associated with the chemistry of tea flavanoids, such as synthesis, metabolism, antioxidant properties, relation with inflammation and cancer have also been reviewed (Wheeler and Wheeler, 2004). It is known that tea polyphenols are strong antioxidants which have demonstrated inhibitory activity against tumorigenesis in animal models. However, epidemiological studies have not provided clear conclusions concerning the protective effects of tea consumption against cancer formation in humans (Yang *et al*., 2002). The discrepancy between the results from humans and animal models could be due to the much higher doses of tea used in animals in comparison to human consumption. Another study undertaken to evaluate the role of individual flavanols in the antimutagenic potential of green tea concluded that although fractions with high flavanol content were more effective radical scavengers, the contribution of flavanols to the antimutagenic activity of green tea was limited (Bu-Abbas *et al*., 2007).

Although there is a vast amount of information associating tea health benefits with its phenolic composition and consequent antioxidant activity, only recently more attention has been dedicated to tea polysaccharides and their properties. As a fact, low molecular weight compounds have for a long time been considered as the active components of medicinal plants, but often they do not contribute to all of the therapeutic effects achieved.

Some studies have reported plant polysaccharides antioxidant and blood-glucose-reducing effects. Three polysaccharide-rich fractions presenting distinct monomeric composition, molecular weight distribution, α-glucosidase inhibitory activity, and antioxidant properties, were isolated from green tea, oolong tea, and black tea. It was observed that the black tea polysaccharide fraction composed of rhamnose (Rha), arabinose (Ara), galactose (Gal), and glucose residues (Glc), displayed the highest α-glucosidase inhibitory and antioxidant activities, which were associated with the high proportion of low molecular weight fractions detected (Chen *et al*., 2009). In 2004, a study reported that the uronic acid content of tea polysaccharide was highly correlated to the antioxidant properties exhibited (Chen *et al*., 2004). More recently, some studies reporting the antioxidant activity of polysaccharides isolated from oolong tea have been published (Chen *et al*., 2009). A tea polysaccharide complex (TPS) composed of polysaccharide and protein with the molecular weight 107 – 110 kDa, was injected in mice, originating a decrease in the levels of blood glucose and an increase of the antibody concentration, thus suggesting that the treatment of diabetes with coarse tea may be related to TPS and the content of TPS in tea (Dongfeng *et al*., 2001). An arabinogalactan-protein (AGP) isolated from tea, with a molecular weight of 100 – 120 kDa, was also reported as the major hypoglycemic factor in tea when tested on hyperglycemic diabetic mice (Zhou *et al*., 2007). The ability to inhibit pathogenic bacteria adhesion has also been reported. A green tea extract (CSI-4) composed of a major proportion of carbohydrates containing 40% uronic acids, but lacking catechins, has been reported to possess anti-adhesive activity against pathogens with no adverse effects against beneficial or commensal bacteria (Lee *et al*., 2009). A study concerning the skin protective effects of tea polysaccharides and polyphenols showed that these type of compounds had complementary advantages: while extracts rich in polysaccharides showed a better moisture absorption and retention abilities, the extracts richer in polyphenols exhibited a better skin protection against UV rays (Wei *et al*., 2009). In addition to these activities, polysaccharides are also known to exhibit immunomodulation activity (Tzianabos *et al*., 2003).

1.2. The plant cell wall

The plant cell wall, schematically represented in **Figure 1.1**, is a complex, dynamic, and highly specialized macromolecular network formed by a heterogeneous mixture of cellulose, hemicelluloses, pectic polysaccharides, and in some extent proteins and phenolic compounds (Ochoa-Villarreal *et al.,* 2012). This structure determines plant cell and organ shape, acts as a barrier against pathogens, provides signals that control growth and development, simultaneously supplying strength and flexibility that enable plants to grow and respond to a variety of environmental conditions (Freshour *et al*., 2003; Hématy *et al*., 2009).

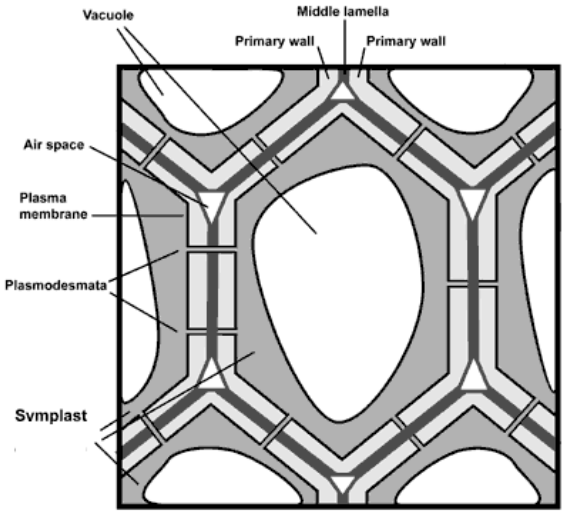

Figure 1.1- Plant cell basic structure [Adapted from Carpita and McCann, 2000].

During its growth, and according to the plant development stage and environmental conditions, the cell wall structure is selectively loosened through a controlled process of “polymer creep”, which consists on a slow, time-dependent, irreversible extension, in which the cell wall components slide within it, increasing its surface area (Cosgrove, 2005). During this process, the plant cell lays down the middle lamella and the primary wall that surrounds growing cells or cells capable of growth, typically reaching a thickness between 50 and 100 nanometers (Kaczkowski, 2003). The primary cell wall consists of a rigid skeleton of cellulose microfibrils embedded in a gel-like matrix composed of pectic polysaccharides, hemicellulose, and glycoproteins. The middle lamella is a thin adhesive layer that connects adjacent plant cells, and that mainly contains pectic polysaccharides

(Iway *et al.*, 2002), although other polysaccharides, such as some hemicelluloses, can also be found. Once cell growth has ceased, and the differentiation process begins, a secondary cell wall can be deposited between the primary cell wall and the plasma membrane. This secondary cell wall, which contains predominantly cellulose, xylan, lignin, pectic polysaccharides, and/or mannan, is more rigid and thicker than the primary cell wall, providing a greater mechanical resistance (Kaczkowsi, 2003).

1.3. Cell wall three dimensional organization models

The chemical composition of the cell wall is rather well established, however its three-dimensional organization has been a challenging matter. As a consequence, throughout the years several models have been proposed for the description of the cell wall three-dimensional complex organization. Despite their differences, all the proposed models tried to account for the mechanisms of cell growth.

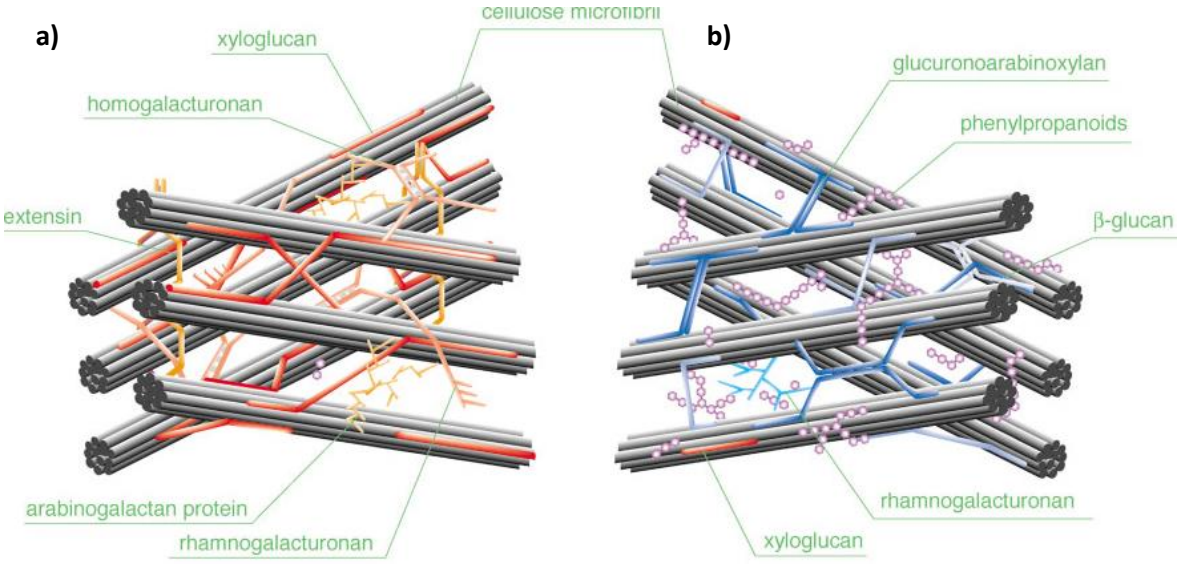

Figure 1.2– Cell wall three dimensional organization as described by the **"**Tethered network model": in **a)** type I primary cell wall (dicotyledons) and **b)** type II primary cell wall (*Poaceae* family and some other monocotyledons) [Adapted from Carpita and McCann, 2000].

The primary cell wall described in these models is classified into two major groups, according with their components chemical structure, wall architecture, and

biosynthetic processes: the type I wall, representative of all *Dicotyledonae* that have been examined in the research studies so far reported, together with some *Monocotyledonae*, and the type II wall, found in the *Poaceae*, and closely related monocot families (Carpita and Gibeau, 1993). Briefly, type I walls, shown in **Figure 1.2 a)**, contain approximately equal amounts of cellulose and cross-linking xyloglucans, with minor amounts of various polysaccharides, such as arabinoxylans, glucomannans, and galactoglucomannans. The type II walls contain cellulose microfibrils of the same structure as those of the type I wall, but the major polysaccharides that interlock the cellulose microfibrils are not xyloglucans, as happened in the type I walls, but the glucuronoarabinoxylans, as shown in shown in **Figure 1.2 b)**.

The "Albersheim model", shown in **Figure 1.3 a)**, was the first reported model that tried to describe the three-dimensional cell wall organization and was presented in 1973 (Keegstra *et al.*, 1973).

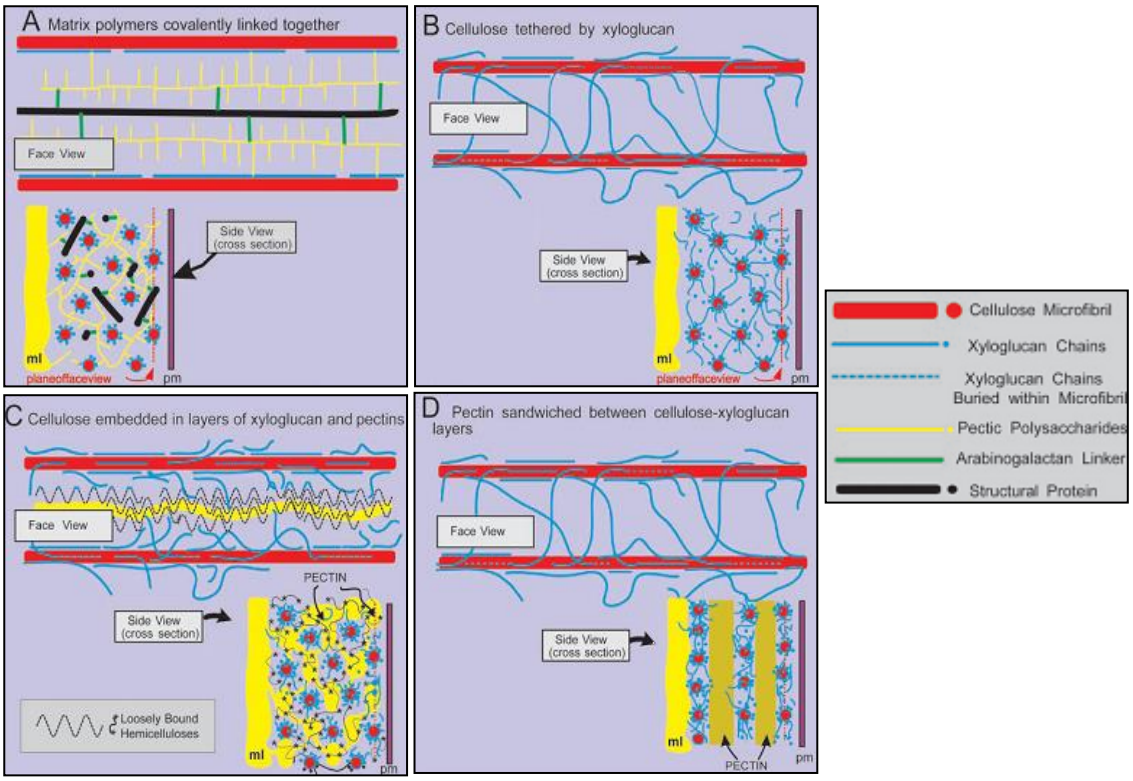

Figure 1.3- Cell wall three dimensional organization models: **a)** "Albersheim model", **b)** "tethered network model", **c)** "multicoat model" and **d)** "stratified model" [Adapted from Cosgrove, 2001].

The “Albersheim model” proposed that all the matrix components, such as xyloglucans, pectic polysaccharides, and structural proteins were assembled through covalent linkages, forming a macromolecular network. Also, it was proposed that the matrix xyloglucans are linked to the cellulose microfibrils through hydrogen bonds (Carpita and Gibeau, 1993).

However, since the existence of the pectic-xyloglucan covalent linkage, as proposed in the “Albersheim model”, could not be supported through experimental evidences, another model was then proposed. Therefore, in 1989 an alternative called the “tethered network model” suggested that the cellulose microfibrils could be tethered together by cross-linking glycans, as illustrated in **Figure 1.3 b)** (Carpita and Gibeaut, 1993; Carpita and McCann, 2000). As previously shown in **Figure 1.2**, in dicotyledons (**Figure 1.2 a)**, the cross-linking is predominantly done by xyloglucan polysaccharides whereas glucuronoarabinoxylans are involved in the cross-linking in *Poaceae* family and other monocots (**Figure 1.2 b)** (Carpita and Gibeaut, 1993; Carpita, 1996). Pectic polysaccharides, and structural proteins are regarded as independent networks that physically wrap the cellulose-xyloglucan network, although not covalently linked to it (Fry, 1989; Hayashi, 1989).

Although no definitive evidence favoring the “tethered network model” has been presented, currently the “tethered network model” is still considered the most accepted one. Nevertheless, other variations have been proposed, such as the “multicoat model”, presented in 1992, and showed in **Figure 2 c)**, which proposed that each cellulose microfibril is covered by several progressively less-tightly bound layers of polysaccharides. In this model, the cellulose microfibrils are linked together, through non-covalent interactions between the different polysaccharide layers. Later in 1997, a fourth model, illustrated in **Figure 2 d)**, consisting in a more stratified cell wall was proposed. This model suggested that the pectic polysaccharides act as spacers between the cellulose microfibrils and the xyloglucans chains.

1.4. Cell wall polysaccharides

As referred earlier, the cellulose microfibrils are the main structural element of type I primary cell walls, and are embedded in a soluble matrix, composed of two distinct groups of polysaccharides: pectic polysaccharides, as the major component of this soluble matrix, and hemicelluloses as the minor components (Cosgrove, 2005). In most plant cells, the main structural hemicellulose is xyloglucan, although hemicelluloses such as arabinoxylans can also be found in lesser amounts (Cosgrove, 2005). Additionally, hemicellulosic polysaccharides can also be found in the plant cell wall fulfilling storage functions, mainly in seeds, roots, rhizomes, tubers, bulbs, short axes, and to a lesser extent, in leaves (Meier and Reid, 1982).

1.4.1. Pectic polysaccharides

Pectic polysaccharides are a diverse group of carbohydrate polymers, which have been considered as those components of the primary cell wall extractable with hot water, dilute acid, ammonium oxalate, and other chelating agents (Northcote, 1963). Pectic polysaccharides are the most abundant class of polysaccharides present in the soluble matrix that composes the primary cell wall, and are also abundant in the middle lamella, which is present between primary cell walls, functioning as regulators of the intercellular adhesion (Willats *et al.*, 2001). Structurally, the pectic polysaccharides can be defined as a group of polysaccharides, which contain (α1→4)-linked-D-galacturonic acid as the principal constituent, and that, depending on its source, contains rhamnogalacturonan I (RG-I), and homogalacturonan (HG), with smaller amounts of xylogalacturonan (XGA), arabinan, arabinogalactans, and rhamnogalacturonan II (RG-II) (Schols and Voragen, 2002).

HG comprises approximately 65% of pectic polysaccharides, consisting of a linear homopolymer of (α1→4)-linked-D-galacturonic acid, containing between 100 and 200 galacturonic acid (GalA) residues, which can be methylesterified at the C-6 carboxylic group (O'Neill *et al*., 1990; Mohnen, 2008). In addition, the GalA

residues may be O-acetylated, predominantly at C-3, although C-2 substitution can also occur (O'Neill *et al*., 1990). GalA residues may also be substituted with residues of xylose at C-3, yielding a pectic domain known as xylogalacturonan (XGA) that appears to be quite widespread, and has been isolated from pea seed coats, apple pectic polysaccharides, watermelon fruit and carrot cells (Schols *et al*., 1995; Kikuchi et al., 1996; Mort *et al.*, 2008; Renard *et al*., 1997; Le Goff *et al*., 2001). Also, it has been reported the substitution of GalA residues with apiose (Api) at C-2 or C-3, resulting in apiogalacturonan, which has been found in the duckweeds *Lemna minor* and *Spirodela polyrrhiza* (Longland *et al*., 1989; Zhao, 2014).

RG-I is a heteropolysaccharide that accounts for 20 to 35% of pectic polysaccharides and structurally is composed by as many as 100 repeats of the disaccharide L-rhamnose-(α1→2)-D-GalA-(α1→ (O´Neill *et al.*, 1990). Between 20 and 80% of the rhamnosyl residues present in the RG-I backbone act as branching points, primarily at C-4, and, occasionally, at C-3. Whether GalA residues within RG-I can also be methylesterified as in the HGA domain is unknown. A small number of GalA residues in the RG-I backbone of sugar beet pectic polysaccharides are substituted with single glucuronic acid residues (Renard *and Jarvis*, 1999). The number and nature of the branching polymers seems to be highly dependent on the plant cell type, and its development stage, suggesting diverse functional specialization (Ridley *et al.*, 2001; Willats *et al.*, 2001). Among these branching polymers we can find polysaccharides such as arabinans and arabinogalactans. Arabinans are a group of plant polysaccharides composed of L-Ara*f*, which can be found linked to the galactan moieties in the pectic complex and can be released through enzymatic action of both *exo*- and *endo*-glycanases present in the cell walls, or by weak acid hydrolysis during the extraction process. Arabinans can be found as linear or branched polymers, being generally accepted that their core linkage are the (1→5)-linkages, and the branches occur at C-3 or C-2 (Dourado *et al*., 2004). Arabinogalactans are heteropolysaccharides found in the plant cell wall that are composed of arabinosyl and galactosyl residues. According to their structure they can be classified into two main structural types: type I arabinogalactan (AG-I), and type II arabinogalactan

(AG-II). AG-I can be present as RG-I side chains, and are composed of (β1→4)-linked D-galactan, substituted at the O-3 of some of the Gal units with single α-L-Ara*f* and/or short (α1→2)-/(α1→3)- linked L-Ara*f* residues (Carpita and Gibeaut, 1993; Yapo, 2011). AG-II is a highly branched polysaccharide with (β1→3)-, and (β1→6)-linked-D-galactosyl residues, the former predominantly in the interior and the latter in the exterior chains (Guillon and Thibault, 1989; Renard *et al*., 1991). The arabinosyl units might be attached through position 3 of the (β1→6)-linked-D-galactosyl side chains. Monosaccharides like glucuronic acid (GlcA) and its 4-O-methyl ether can also be found in AG-II (Huisman *et al*., 2001). The AG-II are mainly found associated with proteins, forming arabinogalactan proteins (AGP), which are often co-extracted with pectic polysaccharides (Vincken *et al*., 2003). It is still unclear if AG-II are part of the pectic polysaccharide complex, however some studies have reported that the pectic polysaccharides may bind to some AGP´s (Baldwin *et al*., 1993; Carpita and Gibeau, 1993; Immerzeel, *et al*., 2006). **Figure 1.4** illustrates one of the many possible structural arrangements for the AG-II isolated from the coffee hot water infusions, as described by Nunes *et al*. (2008).

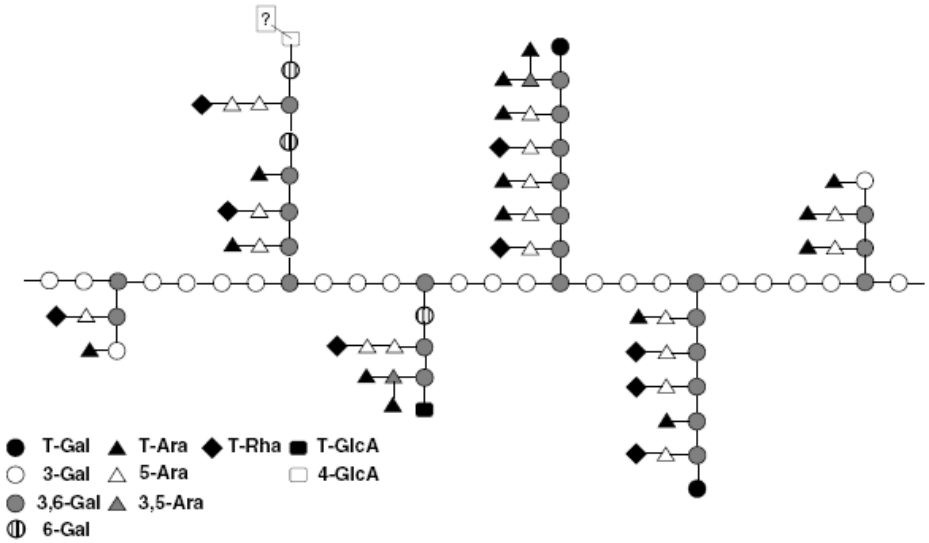

Figure 1.4- Structural arrangement proposed for the AG-II isolated from the coffee hot water infusions [From Nunes *et al*., 2008].

The presence of relatively high amounts of RG-II (20 – 150 mg/l) in wine and other fruit juices (Doco *et al*., 1997), combined with its heavy-metals binding ability (Pellerin *et al*., 1996, Pellerin and O'Neill, 1998), and immunomodulating activities (Shin *et al*., 1998) has led to a greater interest in the structure of RG-II and to the

role of this complex pectic polysaccharide in human nutrition and health. Rhamnogalacturonan-II (RG-II) is by far the most complex polysaccharide in the plant kingdom with more than 18 different types of linked sugars (Pérez *et al.*, 2003). This pectic domain, with its complex but largely conserved structure can be widely found in all primary cell walls, while considered absent from the middle lamella region (Williams *et al*., 1996; Matoh *et al*., 1997). It can be isolated by treatment of primary cell walls of higher plants with *endo*-α-(1→4)-polygalacturonase, thus releasing a low molecular weight (5–10 kDa) structurally complex pectic polysaccharide, which suggests a covalent attachment to the HG domain (Darvill *et al.*, 1978). Its largely conserved structure consists of an HG backbone of around 9 GalA residues that are (α1→4)-linked and substituted by 4 heteropolymeric side chains of known and consistent lengths (O'Neill *et al*., 1996, 2004; Vidal *et al.*, 2000), as illustrated in **Figure 1.5**.

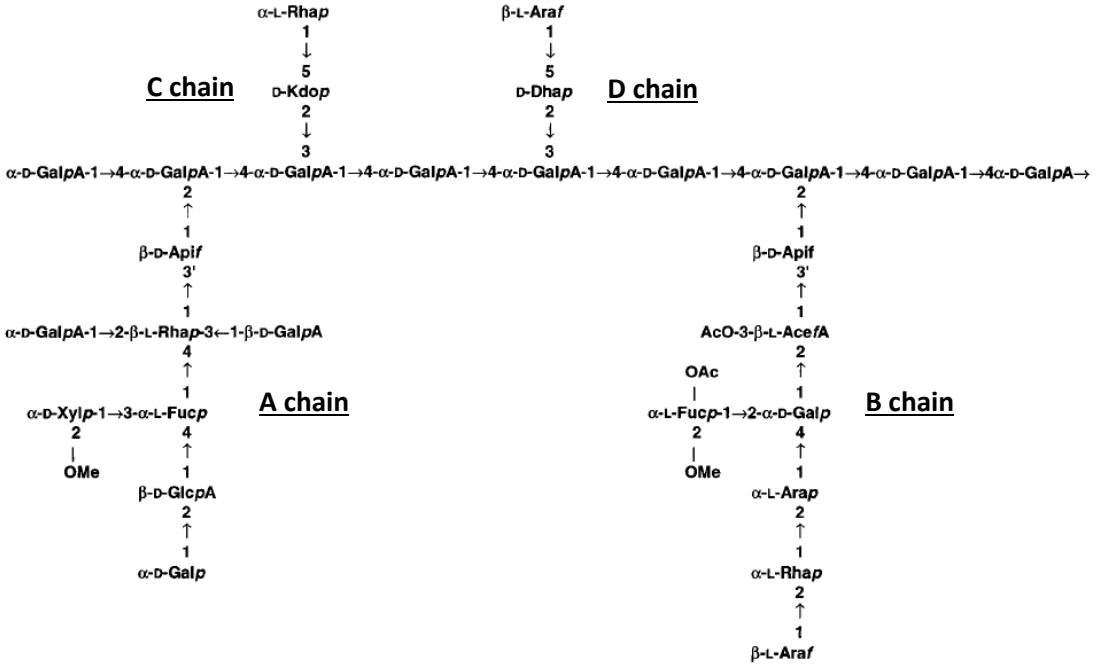

Figure 1.5- RG-II structure evidencing the 4 heteropolymeric side chains [Adapted from O´Neill *et al.*, 1996].

The RG-II side chains (A, B, C and D chains) contain eleven different sugars including Api, aceric acid (AceA), and 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) (O'Neill *et al*., 1996, 2004; Vidal *et al*., 2000). The nonasaccharide (side chain B) and the octasaccharide (side chain A) are attached to C-2 of some of the backbone GalA residues, and other two structurally different disaccharides (side

chains C and D) are attached to C-3 of the backbone (Ridley *et al.*, 2001). The backbone locations of these side chains with respect to one another have not been established with certainty.

The widespread occurrence of RG-II, its structural conservation and resistance to degradation by all the known pectic-degrading enzymes suggests a unique structural and biological role by providing stable links at key regions of HG chains within the pectic network (Vidal *et al*., 1999). In fact, RG-II presents a special structural feature that consists in its ability to dimerize by means of borate ester linkages through apiosyl residues (Kobayashi *et al*., 1996; O'Neill *et al*., 1996; Ishii *et al*., 1999). This presence of boron in RG-II dimmers seems to play an important role by satisfying the plant growth and development boron requirements besides its possible involvement in the regulation of the porosity of the pectic matrix. Fleischer *et al.* (1998, 1999) studies revealed that boron depletion of suspension-cultured *Chenopodium* album cells resulted in increased cell wall porosity while the addition of boron to these cells immediately led to the formation of RG-II dimmers with a concurrent reduction in cell wall porosity. Additionally, physiological effects of boron-deficiency have been correlated to RG-II/borate diester formation in pumpkin tissue (Ishii *et al.*, 2001). It has been reported that mutations, which caused even minor modifications to RG-II structure, also lead to reduced RG-II dimer formation, and severe growth defects, such as dwarfism, suggesting that the dimerization of RG-II in the wall was crucial for normal plant growth and development (Mohnen, 2008). Furthermore, some studies also suggested the importance of calcium for RG-II cross-links and its involvement in maintaining the structural integrity of the cell walls (Fleischer *et al*., 1999; Kobayashi *et al.,* 1999). Besides that, other studies suggested that the RG-II could be connected to proteins, since boron depletion of bean root nodules caused the loss of covalently bound hydroxyproline-rich proteins contributing to wall strength and integrity (Bonilla *et al.,* 1997).

1.4.2. Non-starch storage polysaccharides

The non-starch storage polysaccharides are a heterogenous group of partially water soluble polysaccharides (MacDougall and Selvendran, 2001), characterized by possessing a backbone of (β1→4)-linked-D-*pyranosyl* residues, and that can be found in the cell walls of plants performing a storage function (Buckeridge, 2010; Liepman *et al.,* 2007; Meier and Reid, 1982). Non-starch storage polysaccharides such as mannans, and xyloglucans are frequently reported as present in the endosperm of seeds from plants (Kai *et al*., 2010; Tester and Al-Ghazzewi, 2013). Particularly for plants from the *Fabaceae* family, in which *Pterospartum tridentatum*, one of the selected plants for this study, is included, various water soluble mannan polymers have been isolated (Olennikov and Rokhnin, 2008, 2009, 2010, 2011; Olennikov *et al.,* 2010).

Among the mannan polymers, several types of polysaccharides, such as pure mannans, glucomannans, and galactomannans can be found (Meier and Reid, 1982; Buckeridge *et al*., 2000). Mannans are formed by a backbone containing mannosyl residues linked to each other by (β1→4)-glycosidic linkages, but occasionally the backbone may also contains glucosyl residues, yielding a polymer called glucomannan. Both mannan and glucomannan backbone can be substituted with galactosyl residues through α-1,6-linkages, yielding galacto-mannans and galactoglucomannans, respectively. Results from methylation analysis confirmed the presence of galactosyl residues attached at the C6 position of mannosyl residues (Nunes and Coimbra, 2001; Cerqueira *et al.,* 2011). Isolation of these polysaccharides from hot water extracts suggested that they might be acetylated (McCleary *et al*., 1981), which has been confirmed by numerous structural studies performed on mannan polysaccharides from distinct sources, such as *Aloe vera* plant*,* green and roasted coffee, locust bean gum (LBG) from the *Fabaceae* family plant, *Ceratonia siliqua*, and other non-convencional *Fabaceae* family plants (Nunes *et al.,* 2005; Cerqueira *et al.,* 2011;. Simões *et al.,* 2010*,* 2011*,* 2012). Structural characteristics, such as substitution with galactosyl residues and/or acetylation, which will change mannans water solubility and functional features (McCleary *et al*., 1981), differ depending on the botanical

source. The ratio of mannosyl to galactosyl residues in the plants from the *Fabaceae* family may vary from fully substituted polymers (M/G = 1:1) to an average ratio of 3:1 or 4:1, according to their subfamily (Buckeridge *et al*., 2000). As an example, the galactomannans from the plants of the subfamily *Caesalpinioideae* are poorly substituted (Edwards *et al.,* 1992), which turns them into relatively less water soluble polysaccharides. Meanwhile, the subfamily *Mimosoideae* contains galactomannans that are partially branched with a M/G ratio of 2:1, whereas *Faboideae*, in which *Pterospartum tridentatum* is included, tends to have highly substituted galactomannans with a M/G near 1:1.

Furthermore, several studies using electrospray ionization mass spectrometry (ESI-MS) analysis techniques have also reported other interesting structural features of some mannan type polymers. Nunes *et al.* (2005) evidenced the presence of arabinosyl residues as structural features of mannans purified from green and roasted coffee that were previously submitted to an enzymatic hydrolysis with specific endo-β-mannanase. Using the same technique, the presence of arabinosyl residues in galactomannans extracted from non-conventional sources, such as *Gleditsia triacanthos* plant, was also reported (Cerqueira *et al.,* 2011).

Xyloglucans are another group of non-starch storage polysaccharides that can be found in the seeds cell walls of some plants from the *Fabaceae* family. Xyloglucans are characterized by possessing a (β1→4)-glucan backbone with Xyl*p*-(α1→ residues along it (Hayashi and Kaida, 2011), as shown in **Figure 1.6**.

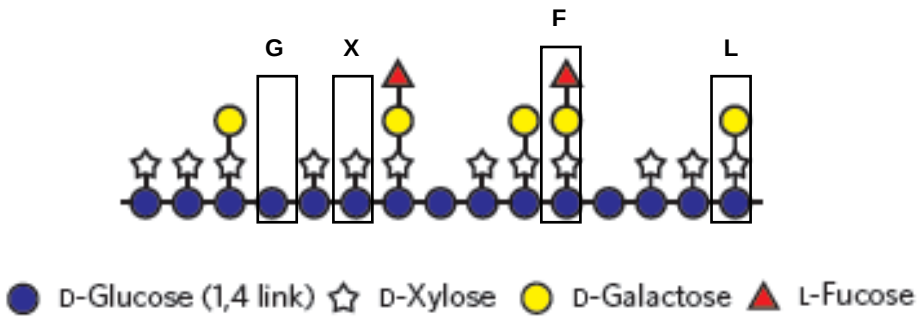

Figure 1.6- Schematic representation of a xyloglucan [Adapted from Burton *et al.*, 2010].

The number of additional galactosyl and fucosyl-galactosyl residues present along the macromolecule, as well as their distribution pattern, seems to differ according to plant species, with monocotyledons exhibiting fewer xylosyl residues than dicotyledons (Kato *et al*., 1982; Kato and Matsuda, 1985; Hayashi, 1989). Frequently, the presence of an unbranched glucose residue is denoted by the letter “G”, while a glucose residue substituted with a xylose residue is represented by the letter “X”, as shown in **Figure 1.6**. Glucose residues substituted with a galactosyl containing disaccharide, and glucose residues substituted with a fucose containing trisaccharide are denoted by the letters “L”, and “F”, respectively.

Despite the relative simplicity that the schematic xyloglucan representation presented in **Figure 1.6** might suggest, some particular structural features, such as the occurrence of some acetylated xyloglucan molecules, have been reported (Jia *et al*., 2005). Also, the presence of other substituents, such as Ara*p*-(α1→2)-Xyl*p*-(α1→ disaccharide side chains, denoted by the letter “S”, have been reported (Fry, 1988; Vincken *et al*., 1996; Hoffman *et al.,* 2005). The seed storage xyloglucans do not seem to present fucosyl residues, while structural xyloglucans present in the primary cell wall seem to exhibit this structural feature (Fry, 1988; Hayashi, 1989).

1.5. Pectic polysaccharides structure

Although the structural elements of pectic polysaccharides, illustrated in **Figure 1.7**, are rather well described, several years of research did not successfully clarified how these distinct structural entities are combined together into a single macromolecular structure. This seems to be related to the fact that the pectic polysaccharides structure seems to be dependent of several factors, such as plant species (de Vries *et al.*, 1986), plant development stage (Huisman *et al.*, 1999) and cell location (Redgwell and Selvendran, 1986).

Polysaccharide fractions enriched in RG-I, RG-II, and oligogalacturonides are generated by treating high molecular weight material (HMWM) with *endo*-polygalacturonase (O’Neill *et al.*, 1990). Therefore, it is frequently assumed that the HG, RG-I, and RG-II pectic domains present in primary walls are covalently

linked to one another, and models have been proposed for the distribution of these domains in pectic polysaccharides.

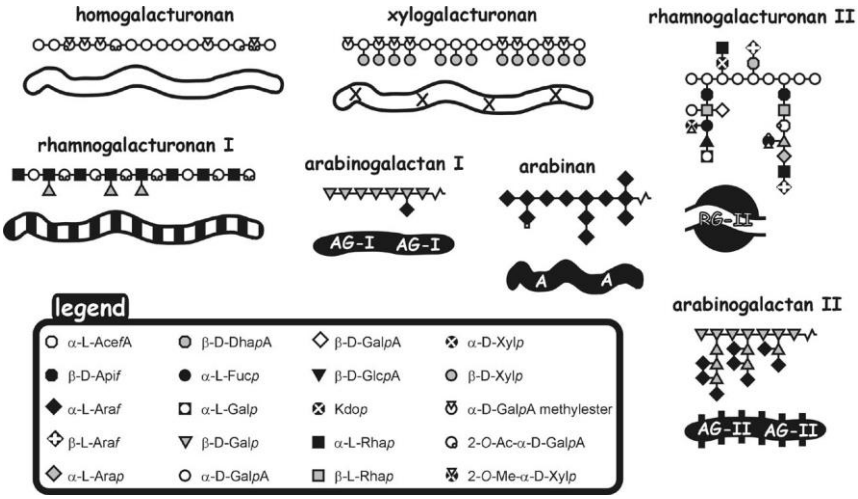

Figure 1.7- Schematic representative structures of the constituent polysaccharides of pectic polysaccharides [Adapted from Vincken *et al*., 2003].

The “rhamnogalacturonan model” was the first hypothetical model that tried to describe the pectic-complex and it was proposed based on the results from the *endo*-polygalacturonase digestion of the high molecular weight pectic polymer from sycamore primary cell walls (Talmadge *et al*., 1973). In this model, the macromolecular complex was described as being composed of a HG sequence with a degree of polymerization (DP) of approximately 8, and the alternating trisaccharide Rha-(α1→ 4)-GalA-(α1→ 2)-Rha-(α1→. The macromolecule would have a zigzag shape, due to the flexibility of (1→2)-Rha residues in the otherwise linear (1→4)-linked α-D-galacturonan chain, with the Rha residues forming a Y-shaped branch point via O-4 position substitution with linear galactan side chains.

In 1982, De Vries *et al.* (1982) proposed a series of pectic-complex models based on various apple pectic polysaccharides degradation studies, which were all characterized by an overall linear backbone comprising HG “smooth regions” alternately linked to RG-I “hairy regions”. The “hairy regions”, placed at regular intervals, and close to the ends of the macromolecular complex, were composed of neutral sugar strands with at most 5% of the total uronic acid present in the macromolecule.

Throughout the years, the model proposed by De Vries *et al.* (1982) was used as the structural model of cell wall pectic polysaccharides, irrespective of plant source (Prade *et al*., 1999; Ralet *et al.*, 2001). However, since only part of the connecting points present in the apple pectic polysaccharides were identified, and not all unknown oligomers could be identified so far, a model of pectic complex in which HG is positioned as a RG-I side chain could not be completely disregarded. Therefore, in 2003 Vincken *et al*. (2003) proposed another model, referred as the "RG-I backbone model", illustrated in **Figure 1.8 a)**.

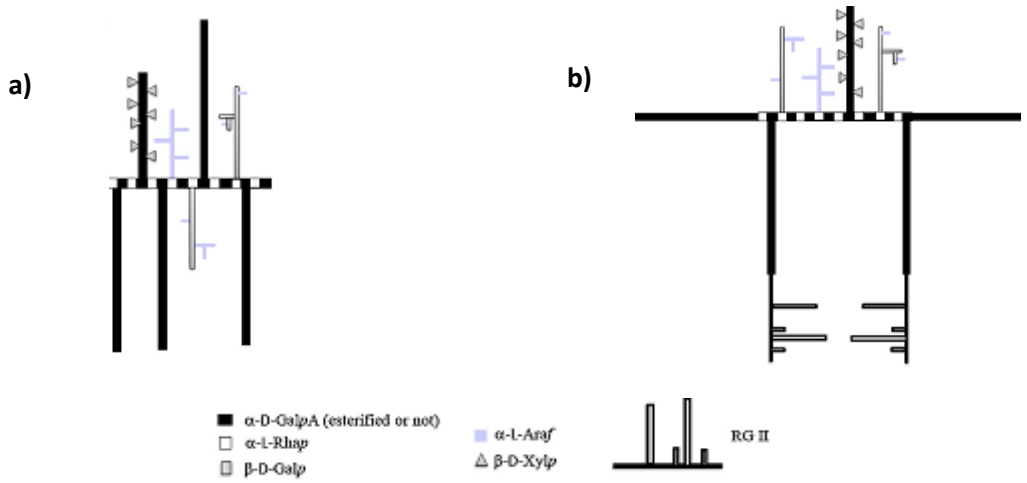

Figure 1.8- Non-traditional macromolecular models for the pectic-complex organization: **a)** the "RG-I backbone model, and **b)** the "living thing-like model" [Adapted from Yapo, 2011].

In this model the HG are arranged as side chains of the RG-I backbone, though it is not known with certainty if HG is fully a RG-I side chain and/or 'grafted' to neutral sugar side chains of RG-I. There are striking differences between the traditional alternating "smooth" and "hairy regions" models, proposed by De Vries, and the "RG-I backbone" model, such as the fact that the backbone of the pectic complex is exclusively of a RG-I type, and also that linear HG and XGA seem to be side chains of the RG-I backbone. This means that the macromolecular complex is exclusively composed of "hairy regions", which comprise strands of neutral sugars, such as arabinan, galactan, AG-I, and possibly AG-II, besides HG and XGA. However, the orientation of the HG, and XGA blocks over the RG-I backbone, the side chain kinds, and their distribution still remains unknown.

All the models described so far assumed that RG-I, and HG or XGA domains were covalently linked, however the occurrence of such a linkage was only

reported in 2007, when additional studies, using controlled acid hydrolysis of apple pectic polysaccharide modified “hairy regions”, allowed further refinement of this model (Coenen *et al.*, 2007). Despite all the updates that have been made since the first pectic polysaccharide model reported in 1973, recent structural studies of pectic polysaccharides from yellow passion fruit rind have indicated that its structural arrangement could be even more complex than that described so far, which allowed the proposal of a new model, the oddly called “living thing-like” model, which considered both the traditional, and the “RG-I backbone” models (Yapo, 2011). In this model, the backbone of the pectic-complex is alternately composed of two linear HG elements and one RG-I core, as shown in **Figure 1.8 b)**. The RG-I core is suggested to exhibit side chains composed of neutral polysaccharides, such as arabinan, galactan, and arabinogalactans, besides XGA. The two HG blocks in the main chain can take a horizontal as well as a vertical position thanks to the flexibility of the (α1→2)-linked L-Rha units to which they are attached to the RG-I core. Additionally, RG-II domains are linearly connected to these HG blocks. This hypothetical model allows to explain the excess of HG domains, evidenced in several studies reffering that more than 80% of the total uronic acid residues were usually found in the HG domains of various pectic polysaccharide sources, which suggested that these domains predominated over RG-I regions in the studied pectic polysaccharides. Furthermore, the liberation of homogenous linear HG stretches by mild acid hydrolysis, the release of XGA together with HG and RG-I oligomers by the enzymic combination of rhamnogalacturonan-hydrolase, (α1→5)-L-*endo*-arabinanase, (β1→4)-D-*endo*-galactanase, α-L-arabinofuranosidase and β-D-galactosidase, but not together with RG-I, RG-II, and oligoGalAs by homogenous *endo*-PG, and the possible dimerisation of mRGs-II can now be explained using this model.

1.6. Biological activity

Plant polysaccharides have been studied for a long time, mostly due to physical properties, such as gelling and thickening abilities, which make them widely used in the food industry (Laurent and Boulenguer, 2003). However, more

recently, diverse studies have shown that plant polysaccharides can interact with the immune system to upregulate or downregulate specific aspects of the host response, and therefore can be classified as biologic response modifiers or immunomodulators (Tzianabos, 2000). This contributes to the broad spectrum of therapeutic properties exhibited by plant polysaccharides, which combined with relatively low toxicity, and non significant side effects, turns them into perfect choices for treatments with immunomodulatory, anti-tumor, and wound-healing action (Ovodov, 1998; Sherenesheva *et al.*, 1998; Lazareva *et al.*, 2002). In fact, toxicity and significant side effects are frequently major problems associated with immunomodulatory bacterial polysaccharides and synthetic compounds (Paulsen, 2001; Ramberg *et al.*, 2010; Schepetkin and Quinn, 2006).

Despite the high complexity of the immune system, which involve many cell types with distinct but complementary roles (Janeway and Medzhitov, 2002), studies suggest that one of the mechanisms responsible for the reported plant polysaccharide therapeutic properties might involve modulation of the innate immunity and, more specifically, macrophage response which could be enhanced and/or activated leading to immunomodulation, anti-tumor activity, wound-healing and other therapeutic effects (Schepetkin and Quinn, 2006).

Macrophages represent the first line of host defense after the epithelial barrier, through the expression of a broad range of pattern recognition receptors that bind the pathogens structure, ingest bound microorganisms into vesicles, and produce reactive oxygen species (ROS), and nitrogen intermediates to destroy microorganisms (Taylor *et al*., 2005). Nitric oxide (NO) is the main reactive nitrogen intermediate, and is synthesized from L-arginine by nitric oxide synthase. NO has been shown to be the principal effector molecule produced by macrophages for cytotoxic activity, and is frequently used as a quantitative index of macrophage immunostimulation for the assessment of anti-inflammatory (Francisco *et al*., 2011), and also pro-inflammatory activities of vegetable extracts (Lee *et al.,* 2006a).

Macrophages are also involved in tissue remodeling during embryogenesis, wound repair, clearance of apoptotic cells and hematopoiesis (Lingen, 2001; Klimp *et al.*, 2002). In a review article, Schepetkin and Quinn (2006) reported several

plant polysaccharides that have been shown to increase macrophage cytotoxic activity against tumor cells and microorganisms, activate phagocytic activity, increase reactive oxygen species (ROS), and nitric oxide (NO) production besides enhancing secretion of cytokines and chemokines, such as tumor necrosis factor (TNF-α), interleukin (IL)-1 β, IL-6, IL-8, IL-12, IFN-γ and IFN-β2.

Macrophage activation by plant polysaccharides appears to be mediated primarily through the recognition of polysaccharide polymers by specific pattern recognition molecules that act as receptors (Gordon, 2002). Specifically, macrophages might bind botanical polysaccharides and/or glycoproteins via Toll-like receptor 4 (TLR4), CD14, complement receptor 3 (CR3), scavenger receptor (SR), dectin-1, and mannose receptor (MR) (Rice *et al.*, 2002; Taylor *et al*., 2002; Shao *et al.*, 2004). The activation of these receptors leads to intracellular signaling cascades, resulting in transcriptional activation and the production of pro-inflammatory cytokines, as illustrated in **Figure 1.9**.

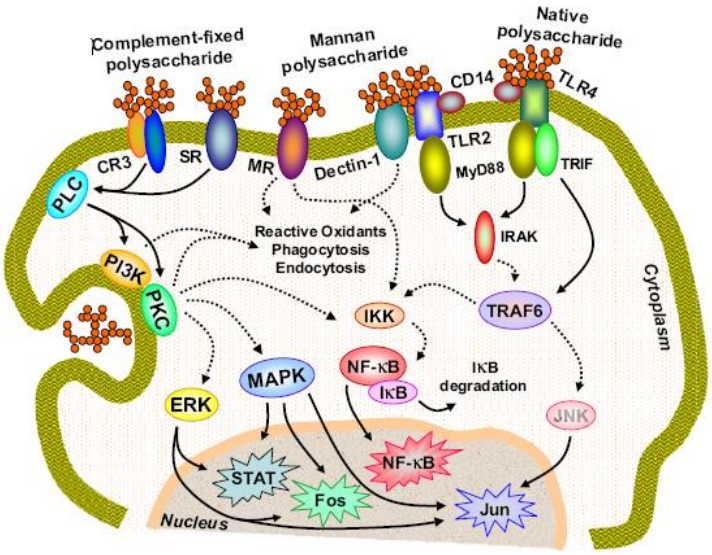

Figure 1.9- Schematic representation illustrating signaling pathways possibly involved in the interaction between plant polysaccharides and macrophage [From Schepetkin and Quinn, 2006].

Also, it has been proposed that activation of macrophages by plant polysaccharides can occur via endocytosis-dependent pathways, and it has been suggested that polysaccharides can associate with macrophage receptors and then become endocytosed (Schepetkin and Quinn, 2006).

Some studies evidenced that pectic polysaccharides isolated from the aqueous extracts of the roots, and petals of *Astralagus membranaceous*, and *Carthamus tinctorius*, respectively, were shown to activate macrophages through TLR4 (Shao *et al.,* 2004; Ando *et al.,* 2002). Angelan, a 10kDa pectic polysaccharide isolated from the aqueous extract of *Angelica gigas Nakai*, was also shown to bind to CD14 and CR3, besides TLR4 membrane receptor (Jeon *et al.,* 2000).

The different cell-surface receptors also seem to exhibit some specificity towards certain glucose-rich carbohydrate moieties, as evidenced for a polyanionic water-soluble carboxymethylated (CM) glucan that binded to macrophages via scavenger receptors (SR´s) (Dushkin *et al.,* 1996). Another study has also evidenced dectin-1 as a major β-glucan macrophage cell-surface receptor (Taylor *et al.,* 2002). For mannans, it seems that MR is the only cell-surface receptor (Harris *et al*., 1992), although Zhang and Tizard (1996) reported results that indicated that acemannan binded to four macrophage membrane proteins.

In the literature are also found several research studies that evidence the interaction of plant polysaccharides with the complement system (Nergard *et al*., 2006; Samuelsen *et al*., 2007), which is composed of over 20 serum proteins, and plays a vital role in the human immune system, such as primary defense against bacterial invasions and viral infections.

Most of the vegetable extracts assayed for the evaluation of diverse biological activities are often prepared in facilities that do not require particular equipment specifically designed to ensure aseptic conditions. Therefore, it is crucial to ensure that the observed results are not due to contamination of the assayed extracts, which could lead to misleading conclusions. Lipopolysaccharides (LPS) are cell membrane components of most Gram-negative bacteria being responsible for its organization and stability (Magalhães *et al.,* 2007), and are frequently found as a contaminant of biological preparations (Schepetkin and Quinn, 2006; Wang and Quinn, 2010). LPS is also a known immunomodulatory agent that can be recognized by immune cells, as a pathogen-associated molecule, through TLR4, and is constituted by a carbohydrate hydrophilic moiety and a hydrophobic component known as lipid A, which is responsible for its major bioactivity.

One of the methods most frequently used for the detection of LPS sample contamination involves the use of the limulus amebocyte lysate assay (LAL assay). The LAL assay is based on the clotting response of horseshoe crab (*Limulus polyphemus*) blood upon contact with LPS (Schepetkin and Quinn, 2006). However, the use of the LAL assay for the LPS sample decontamination should be considered with some caution, as various polysaccharides have been reported to give false-positive results, namely fractions containing AG-II, and β-glucans (Schepetkin and Quinn, 2005; Tanaka *et al.,* 2005). Furthermore, false-negative results have also been reported for a series of compounds, particularly calcium binding agents (Levin and Bang, 1968). Other method used for the assessment of sample contamination, consists in the sample treatment with polymyxin B (PMB), which is a peptide antibiotic that exhibits a very high binding ability for the lipid A moiety of most endotoxins (Morrison and Jacobs, 1976). This approach has been extensively used for evaluating LPS contamination in botanical polysaccharide samples (Han *et al.,* 2001, 2003; Kim *et al.,* 2007; Lee and Jeon, 2005; Lee *et al.,* 2006b).

1.7. Polysaccharides structure-activity relations

Despite the various studies that are found in the literature reporting distinct biological activities often associated with polysaccharides, and assayed by diverse methods, the information regarding relations between the structural features, and the exhibited biological activities is comparatively very sparse. Additionally, based on the literature currently available, it is easy to understand that the establishment of structure-activity relations for polysaccharides has proved a challenging task, since it is relatively common to find in the literature conflicting results concerning polysaccharides that apparently displayed similar structures. Sometimes, these conflicting results are explained because distinct biological activities are evaluated, or because different techniques are used for the evaluation of the same biological activity. However, often it is believed that these apparently conflicting results are due to structural details that could be sometimes hard to assess, such as structural spacial orientation, flexibility and steric hindrance phenomena.

The use of enzymes that are able to specifically hydrolyze certain carbohydrate structures, has proved an useful strategy for trying to unravel structure-activity relations (Tsumuraya *et al*., 1990; Schols *et al*, 1990; Kofod *et al.*, 1994; Azadi *et al*., 1995). Frequently, the research strategy adopted consisted in the *in vitro* evaluation of the biological activity, before and after the removal of certain specific regions, therefore highlighting the polysaccharide structural features that might have contributed for the reported biological activities. In order to provide an integrated view concerning structure-activity relations of polysaccharides considered major contributors for the reported biological activities, some studies considered to be the most informative are revisited. Thus, the aspects regarding structure-activity relations in pectic polysaccharides and, in mannan polysaccharides are discussed.

1.7.1. Pectic polysaccharides

1.7.1.1. RG-I domains

The pectic polysaccharides isolated from the hot water extract of the roots of *Bupleurum falcatum* have been subject of various studies regarding structure-activity relations. Two of these pectic polysaccharides, BR-2-IIb and BR-2-IIc, known for their anti-complementary (Yamada *et al*., 1989), macrophage Fc receptor up-regulating (Matsumoto *et al*., 1993), and anti-ulcer activities (Sun *et al*., 1991), were submitted to *endo*-(α1→4)-polygalacturonase digestion. Fractionation of the digestion products evidenced that both pectic polysaccharides comprised three domains with distinct molecular weight: a polygalacturonase resistant domain (PG-1), a small portion of a RG-II like domain (PG-2), and a HG domain (PG-3) (Hirano *et al.,* 1994). The PG-1 domain, considered to be responsible for the above mentioned biological activities, and that also exhibited B cell proliferation activity, was submitted to partial acid hydrolysis yielding a neutral fraction mainly composed of Ara and Gal residues, and an acidic fraction containing mainly 2-linked Rha, terminal Glc and Gal and terminal GalA, which was consistent with the presence of RG-I pectic domains (Matsumoto *et al*., 1995).

In order to further elucidate structure-activity relations for the PG-1 domain, Sakurai *et al*. (1996) prepared an “anti-bupleuran” antibody against the PG-1 domain of the pectic polysaccharide BR-2-IIc, which was used to study its distribution after oral ingestion. The results allowed to show that the antibody recognized, and attached itself to the PG-1 domain, however the experiment did not allowed to more precisely determine which structures were involved in the interaction, ie the antigenic epitopes. In order to elucidate the type of structures involved, the PG-1 domain from the BR-2-IIc pectic polysaccharide was submitted to the sequential enzymatic digestion illustrated in **Figure 1.10** (Sakurai *et al*., 1998).

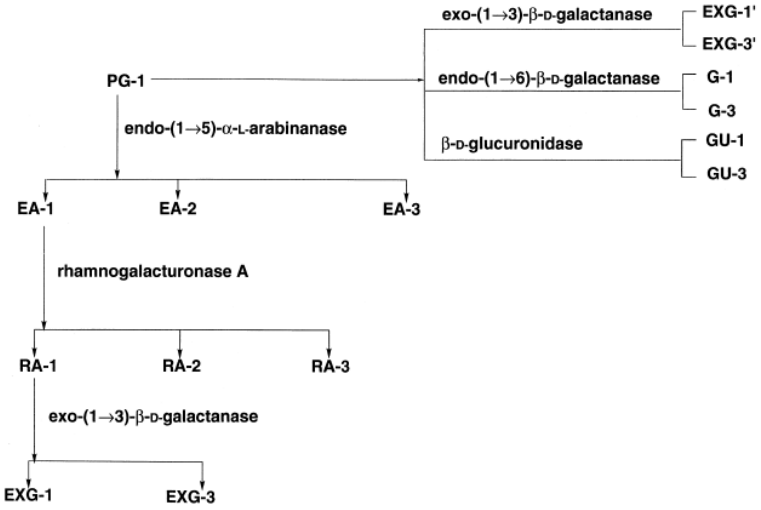

Figure 1.10– Sequential enzymatic digestion scheme of PG1 isolated from the hot water extract of the roots of *Bupleurum falcatum* [From Sakurai *et al*., 1998].

After PG-1 digestion with *endo*-(α1→5)-L-arabinase, the obtained products were separated by size exclusion chromatography, yielding a high molecular weight fraction (EA-1), an intermediate molecular weight fraction (EA-2), and a low molecular weight fraction (EA-3). The PG-1 branched (1→5)-linked arabinosyl chains removed by the digestion were recovered in EA-3, which was further fractionated, yielding a biologically inactive neutral fraction, composed mainly of Ara, and a biologically active acidic fraction, that consisted essentially of GlcA and GalA with a trace of Ara and Gal. This allowed inferring that the neutral arabinofuranosyl chains were not related to the biological activities exhibited by PG-1. Thereafter, EA-1 was digested with rhamnogalacturonase A, an enzyme that specifically cleaves the linkage of D-GalA-(α1→2)-L-Rha-(α1→ in the rhamno-

galacturonan backbone of the PG-1, leaving the Rha residue at the non-reducing end of the products. Fractionation of the digestion products originated a high molecular weight fraction (RA-1), an intermediate fraction (RA-2), and a low molecular weight fraction (RA-3). In comparison with EA-1, the RA-1 fraction, which exhibited a biological activity comparable to PG-1, mainly contained similar proportions of (1→3)-galactosyl chains, and lower proportions of (1→4)-linked GalA, and (1→2)-, and (1→2,4)-linked Rha, which were mostly recovered in RA-3 fraction that did not evidenced any biological activity. This suggested that the rhamnogalacturonan core was recovered on RA-3, and also that, by itself it was not responsible for the biological activity reported. RA-1 fraction was further digested with *exo*-(β1→3)-D-galactanase, which is known to cleave (β1→3)-D-galactan structures with or without side chains from the non-reducing terminal (Tsumuraya *et al*., 1990). The digestion products were fractionated, yielding a major high molecular weight fraction (EXG-1) and two minor intermediate and low molecular weight fractions, EXG-2 and EXG-3, respectively. Linkage and mass spectrometry analyses evidenced that EXG-3 contained mono- and di-galactosyl oligosaccharides, possessing terminal Gal, (1→6)-linked Gal*f* and Gal*p*, terminal GalA and MeGlcA4. It was also shown that the removal of the EXG-3 fraction from RA-1 by *exo*-(β1→3)-D-galactanase significantly reduced the binding ability of the antibody to the polysaccharide, suggesting that (1→6)-linked galactosyl chains that contained terminal GlcA or MeGlcA4 attached to (β1→3)-D-galactosyl chains may be involved as major antigenic epitopes in the “ramified” region (PG-1) of bupleuran BR-2-IIc. In order to confirm this assumption, PG-1 was also separately digested with *exo*-(β1→3)-D-galactanase, *endo*-(β1→6)-D-galactanase and β-D-glucuronidase, which allowed to evidence that exo-(β1→3)-D-galactanase was the most effective enzyme in the reduction of the binding ability of the antibody to the pectic polysaccharide. These results suggested that the structure illustrated in **Figure 1.11** could be involved in the expression of the reported biological activities.

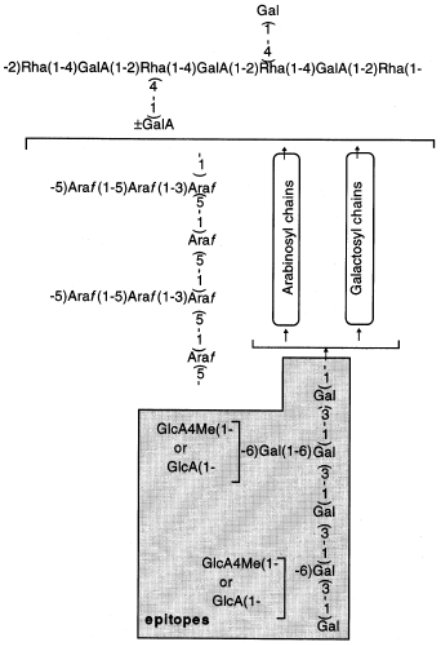

Figure 1.11- Proposed structure for the antigenic epitopes of the ramified region of bupleuran BR-2-IIC for the "anti-bupleuran" antibody [From Sakurai *et al*., 1998].

The structure shown in **Figure 1.11**, contained polygalacturonase resistant RG-I domains and, more precisely, its (1→6)-linked galactosyl chains, which exhibited terminal GlcA or MeGlcA4 attached to (β1→3)-D-galactosyl chains.

Several studies, focused on pectic polysaccharides isolated from distinct botanical sources, exhibiting other biological activities, that also supported this hypothesis have been published. As an example, we can refer the study focusing the structure-activity relations for GOA1 and GOA2, which are two pectic polysaccharides isolated from a hot water extract of the aerial parts of *Glinus oppositifolius* that exhibited complement fixing, and intestinal immune stimulating activities (Inngjerdingen *et al*., 2005). As described for *Bupleurum falcatum* PG-1 pectic polysaccharide, the studies concerning GOA1 structure-activity relation evidenced the importance of the branched moieties of the arabinogalactans, as a decrease in (1→3,6)-linked galactosyl led to a significantly lower complement fixing, and intestinal immune stimulating activities (Inngjerdingen *et al*., 2007a). Another example is the research work carried out by Inngjerdingen *et al*. (2008), that isolated two pectic polysaccharide fractions (BP 1002 and BP1002-I) from *Biophytum petersianum* that contained branched regions of arabinogalactan side

chains. However, unlike what was reported for the PG-1 pectic polysaccharide from *Bupleurum falcatum*, these pectic polysaccharides fractions did not evidenced biological activity toward B-cells proliferation, tough exhibiting activity toward macrophages and dendritic cells.

A pectic polysaccharide fraction (WGPA-2-RG) that also contained an AG with minor amounts of RG-I domains was isolated from the aqueous extracts of the roots from *Panax ginseng* (Zhang *et al.,* 2009). Partial acid hydrolysis, followed by evaluation of the *in vitro* immunomodulatory activity, showed that the AG side chains of WGPA-2-RG proved to be essential structures for stimulating NO secretion, and lymphocyte proliferation, while not appreciably affect its ability to enhance macrophage phagocytosis (Zhang *et al.,* 2012).

Despite the relevance of the research works mentioned above, some results suggested that the structure-activity relations established did not provide a full picture regarding these issues, and that a richer insight was needed. Thus, several research studies have been performed in order to provided additional information about the relevance of the arabino-3,6-galactan moieties of the RG-I domains in the expression of the pectic polysaccharides biological activity. Six biologically active pectic polysaccharide fractions from the aqueous extract of the aerial parts from *Astralagus mongholics* were obtained through anion exchange and size exclusion chromatography (Kiyohara *et al*., 2010). Linkage analyses showed that the isolated pectic polysaccharide fractions contained arabino-3,6-galactans moieties probably attached to a rhamnogalacturonan core, forming a RG-I domain, which also seemed to contribute for the expression of biological activities in *Bupleurum falcatum*, *Biophytum petersianum*, *Glinus oppositifolius*, and *Panax ginseng*, as described earlier. Enzymatic digestions of these polysaccharides evidenced that the arabino-3,6-galactan chains, which are located in the sides of the non-reducing end of these polysaccharides from *Astralagus mongholics*, strongly contributed for the activity exhibited. This evidenced the importance of the location of these arabino-3,6-galactan chains for the expression of the biological activity. This structural feature was also considered relevant by other research works, such as the one focused on the ALR-5IIa-1-1 pectic polysaccharide extracted from the hot water extracts of rhizomes from *Atractylodes lancea*. This

pectic polysaccharide exhibited intestinal immune system modulating activity, and was shown to also comprise mainly arabino-3,6- galactan domains (Yu *et al.,* 1998; Yu *et al.,* 2001a). Through glycosidase digestions, it was also suggested that the arabino-3,6-galactan moiety located at the nonreducing terminal side of ALR-5IIa-1-1 mainly contributed to expression of the activity (Yu *et al.,* 2001a). Moreover, it was also shown that the galactosyl side chains consisted of (1→6)-linked Gal*p*, but also (1→6)-linked Gal*f*, which was not found in biologically inactive larch wood arabinogalactans. Therefore, it was proposed that the galactosyl side chains consisting of (1→6)-linked Gal*p*, and (1→6)-linked Gal*f*, with a DP higher than 4, could be important for the expression of the potent intestine immune system modulating activity (Yu *et al.,* 2001a).

Some research works have also evidenced the importance of minor structural details, such as the presence of non-reducing terminal GlcA in some of the fractions isolated from the water extract of the aerial parts from *Australagus mongholics*, and from the hot water extract of the roots of *Bupleurum falcatum*. For *Australagus mongholics*, the release of GlcA by *exo*-β-D-glucuronidase digestion did not seem to have affected their activities, suggesting that GlcA in oligosaccharide side chains from neutral sugar-rich polysaccharides did not participate in the immunomodulating activity. However, another pectic polysaccharide fraction (PS14) was isolated from the same source, and submitted to *endo*-D-(α1→4)-polygalacturonase digestion. Fractionation of the digestion products by size exclusion chromatography yielded a high molecular weight fraction (PG-1), an intermediate size fraction (PG-2), and a low molecular weight fraction (PG-3). Sugar and linkage analysis, indicated that the PG-1 fractions contained RG-I domains, which comprised a rhamnogalacturonan core attached with side-chains including arabino-3,6-galactans. PG-2 fractions were considered to possess a RG-II type structure, evidenced by the presence of 2-Me-Fuc, 2-Me-Xyl, Api and AceA, characteristic residues found in RG-II. As showed in **Figure 1.12**, PG-1 from PS14 fraction exhibited a more potent activity than its precursor, while PG-2 did not show activity. Since PG-1 showed potent activity, and it was suggested that this fraction contained a RG-I domain, it was hypothesized that arabino-3,6-galactan side-chains in this RG-I fraction contributed to its activity,

similar to the six fractions from *Astralagus mongholics*, and RG-I domains from *Panax ginseng*, and *Bupleurum falcatum*, described earlier in this section.

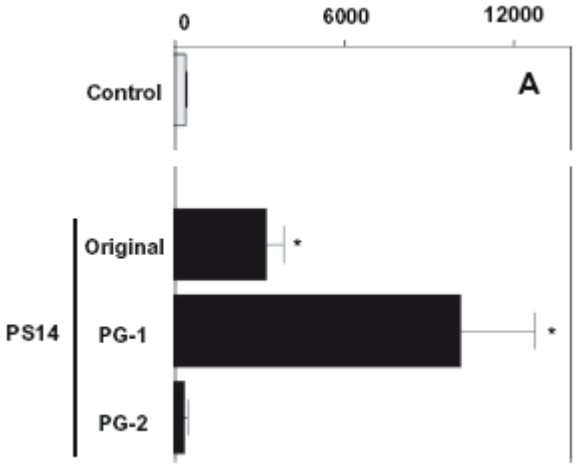

Figure 1.12- Immunomodulating activity of PG-1 and PG-2 domains from PS14 fraction isolated from the aqueous extract of the aerial parts from *Astralagus mongholics* [From Kiyohara *et al.*, 2010].

This hypothesis was confirmed by enzymatic digestion assays with *exo*-D-(β1→3)-galactanase, which cleaves (β1→3)-D-galactan structures, with or without side chains, from the non-reducing terminal (Tsumuraya *et al*., 1990), leading to a reduction of the displayed activity. As referred earlier for the six 6 fractions that were also isolated from *Australagus mongholics*, linkage analyses evidenced the presence of small proportions of GlcA located at the non-reducing end of arabino-3,6-galactan side-chains. However, in this case, upon release by *exo*-β-D-glucuronidase, a significant decrease in its activity was observed. This fact suggested that, for PS14 the sequence D-GlcA-(β1→6)-D-Gal-(β1→ of the arabino-3,6-galactan side-chains participated in the activity rather than the long D-(β1→6) galactooligosaccharide side-chains. In the case of *Bupleurum falcatum,* it was also suggested that the disaccharide, D-GlcA-(β1→6)-D-Gal-(β1→ could be involved in the expression of biological activity. Overall, these results suggested that the disaccharide D-GlcA-(β1→6)-D-Gal-(β1→ can contribute to the immunomodulating activity, when properly and sterically arranged on a specific backbone such as a D-(β1→3)-galactan attached to a rhamnogalacturonan core.

Other research work that tried to provide a deeper knowledge about the questions concerning structure-activity relations, used as starting point a water-soluble anti-complementary polysaccharide, AR-4, isolated from *Angelica acutiloba* by the addition of cetyltrimethylammonium bromide (Yamada *et al*., 1984b). Purification of AR-4 by anion-exchange chromatography, and size exclusion chromatography led to the isolation of an anti-complementary arabinogalactan (AGIIb-1) (Yamada *et al*., 1987). When submitted to mild acid hydrolysis, the AGIIb-1 fraction showed to be comprised of a rhamnogalacturonan core with at least four kinds of arabinogalactan side chains, directly attached to the Rha residues of the rhamnogalacturonan core, or through 4-linked GalA (Kiyohara and Yamada, 1989a). One of these side chains, designated N-I unit, was a typical neutral arabino-3,6-galactan, suggested to be located in the interior of one of the arabinogalactans chains (Kiyohara *et al*., 1987). N-I unit possessed the most potent anti-complementary activity, suggesting that it played an important role in the expression of the activity of AGIIb-1 among its arabinogalactan and arabinan side chains (Kiyohara *et al.*, 1989b). In order to further understand the role of the galactosyl side chains in N-I unit, in particular, and in polysaccharides containing arabinogalactans side chains, in general, the N-I unit was submitted to a series of enzymatic digestions (Kiyohara *et al*., 1997). First, the N-I unit was treated with an arabinofuranosidase, yielding a fraction (AF-N-I), which showed a potent anti-complementary activity, suggesting that trimming of Ara*f* residues from these arabinogalactan side chains did not decreased the anti-complementary activity, as earlier reported by Yamada (Yamada *et al.,* 1987). After, AF-N-I was submitted to enzymatic digestion with an *exo*-D-(β 1→3)-galactanase isolated from *Zrpex lacteus*, and the products were fractionated by size exclusion chromatography. The high molecular weight (GN-I), and intermediate molecular weight (GN-II) fractions showed a similar potent activity, but the low molecular weight fraction (GN-III) had weak activity. Linkage analysis indicated that the intermediate and low molecular weight fractions consisted mainly of 6-linked Gal residues. GN-I was further fractionated by anionic exchange chromatography, and two fractions (GN-IA and GN-IB) with similar activity were obtained. Linkage analyses showed that both GN-IA, and GN-IB consisted mainly of terminal Ara*f*, and terminal, (1→6)-

linked, and (1→3,6)-linked Gal residues. The obtained results suggested that the side chains were responsible for expression of the activity exhibited by N-I unit, and that the attachment of these side-chains to the D-(*β*1→3)-galactan backbone is necessary to the expression of a potent activity, similarly to what was also described earlier for *Astralagus mongholics* (PS1~PS12), *Atractylodes lancea* (ALR-5IIa-1-1), and *Bupleurum falcatum* (PG-1). Besides that, it also seems that chain length of the galacto-oligosaccharides might be important for expression of activity.

However, the work described above did not clarified whether the activity of AGIIb-1 isolated from *Angelica acutiloba* was mainly expressed by the action of N-I, or any of the other three types of arabinogalactans side chains. Moreover, despite the importance that the N-I unit seemed to have on the activity exhibited by AGIIb-1, the results showed that this unit was present in a relatively low proportion (Kiyohara *et al*., 1987), which suggested that other structures could also be involved in the process. In order to clarify the contribution of D-(*β*1→3)-galactan moiety, AGIIb-I was firstly digested with *exo*-α-L-arabinofuranosidase, yielding the AF-AGIIb-1 fraction, and then with *exo*-D-(β1→3)-galactanase (Kiyohara *et al*., 1997). The high molecular weight fraction obtained showed an anti-complementary activity similar to the one from AF-AGIIb-1, and comprised a rhamnogalacturonan backbone carrying D-(β1→3)-galactanase-resistant side chains, while the low molecular fraction was derived from D-(β1→3)-galactan chains in the exterior of AF-AGIIb-1 and did not show any activity. These results suggest that D-(*β*1→3)-galactans in the interior chains of AGIIb-1 mainly contributed for the expression of its activity. Linkage analysis evidenced that the high molecular weight fraction also contained D-(β1→4)-Gal residues, although *endo*-D-(β1→4)-galactanase digestion suggested that these galactan chains did not contribute to the expression of its activity, contrary to what was observed for *Vernonia kotschyana*, where it was suggested that the presence of AG-I had an important role in the expression of immunomodulating activity. Nevertheless, the enzymatic digestion only allowed the removal of about 30% of the Gal residues attached to the rhamnogalacturonan core. Thus, four fractions were obtained by submitting the high molecular weight fraction to controlled Smith degradation,

consisting in a periodate oxidation, followed by reduction, and acid hydrolysis under mild conditions. The higher molecular weight fraction (CSD-1) was mainly composed of a (1→3)-galactan, and showed a relatively potent anti-complementary activity. *Exo*-D-(β1→3)-galactanase digestion of CSD-1 gave six fractions (CSD- I-1~CSD-I-6). Linkage analyses of the fractions exhibiting potent anti-complementary activity indicated that they consisted mainly of terminal, (1→3)-, (1→6)-, and (1→3,6)-linked Gal residues, reinforcing the idea that complement activating ability of AGIIb-1 was expressed by its inner galactan chains, and also suggesting that the N-I unit was situated in a more interior position relatively to the other side-chains, which was decisive for the expression of its biological activity.

Some studies found in the literature also allowed to evaluate the importance of the arabinosyl residues present in the arabino-3,6-galactan side chains attached to the rhamnogalacturonan core. Contrary to what was observed in *Bupleurum falcatum* PG-1 (Sakurai *et al.,* 1998), and *Atractylodes lancea* ALR-5IIa-1-1 (Yu *et al.,* 1998), where the loss of arabinosyl residues did not appear to have influenced its biological activity, for *Glinus oppositifolius* GOA1 the loss of these residues led to a reduction of the exhibited biological activity. One explanation for this could be related to the distinct biological activities that were evaluated in these two studies: for PG1, the B cells proliferation activity was tested, while for GOA1, the assayed activities were complement fixing, and intestinal immune stimulating activities.

A study by Samuelsen *et al.* (1996), also reported some results that could help explain the differences regarding the importance of the arabinosyl residues, which are present in the arabino-3,6-galactan side chains, in the expression of its biological activity. A pectic polysaccharide extracted from the leaves of *Plantago major* (PMII) showed a potent anti-complementary activity, having a protective effect against *Streptococcus pneumonia* infection in mice (Hetland *et* al., 2000; Michaelsen *et al*., 2000). PMII was de-esterified with pectinesterase, and subsequently hydrolysed with pectinase, and the digestion products were further fractionated by size exclusion chromatography, originating two “hairy” regions: a high molecular weight one (PVa), showing a higher biological activity, and a lower molecular weight fraction (PVb), with lower biological activity (Samuelsen *et al*.,

1996). After removal of the arabinose residues, the activity of PVa was slightly increased, while in PVb the removal of these residues lead to a reduction in biological activity. This could be related to the fact that in PVb the arabinose residues are substituted directly on the GalA backbone, which could make them more important for the activity than the arabinose residues terminally linked on the neutral galactan side chains as in PVa. Therefore, the conflicting results described above could be explained not only by the distinct biological activities that were evaluated, but also by the location of these arabinosyl residues, depending whether they were terminally linked to the neutral galactan side chains, or present as an integrating part of the RG-I core. In order to confirm these observations reported for *Plantago major*, PMII was also submitted to weak acid hydrolysis, which resulted in a decrease of the registered anti-complementary activity, thereby reinforcing the above conclusions because the neutral side chains in the hairy regions are more easily hydrolyzed than the polygalacturonic acid backbone.

Although most of the research work found in the literature has been focused on the structural characterization of the AG-II linked to the rhamnogalacturonan core, and its implications on the reported biological activities, some of the pectic polysaccharide fractions isolated also exhibited structural features diagnostic of the presence of other polysaccharides. As an example, *endo*-(α1→4)-polygalacturonase digestion, and further fractionation by size exclusion chromatography of pectic polysaccharides isolated from the leaves of *Opilia celtidifolia* yielded four fractions that showed complement fixing, and macrophage stimulation activities (Inngjerdingen, 2008). Despite the highly complex nature of the fractions it was possible to demonstrate that the bioactive polysaccharides contained a high amount of AG-II type structure, but the presence of AG-I polysaccharides was also confirmed by the detection of (1→4)-linked Gal units. Some of the fractions also contained (1→4)- and (1→4,6)- linked Glc and terminal Xyl. These are often found as components of xyloglucans, described as having a cellulosic (1→4)- glucopyranose backbone substituted with xylopyran residues at position 6. Xyloglucan are hemicellulosic polysaccharides found in all higher plants, where they represent a quantitatively major building material of the primary cell wall (Ebringerova *et al*., 2005). In the literature we have not found reports of

xyloglucans having activity in the complement system, but other biological activities are reported for these type of polysaccharides, among those, anti-tumor activity (Mizuno *et al*., 1992), effect on lipid metabolism (Yamatoya *et al*., 1996) and promoting effects on the proliferation and differentiation of nerve PC12 cells (Ding *et al*., 2003) with the latest being the first report of exogenous plant-derived polysaccharides with biological activity on nerve cell. Despite the similar structural features identified in these fractions and in others also reported (Inngjerdingen *et al*., 2005; Nergard *et al.,* 2005), the polysaccharide fractions isolated showed the ability to fix complement and activate macrophages to release nitric oxide, while polysaccharides isolated from *Glinus oppositifolius* and *Vernonia kotschyana* have shown no ability to release nitric oxide from macrophages, while they had activity in the complement system.

1.7.1.2. RG-II domains

Besides some distinct structures present in the RG-I pectic domains, such as the AG-I, and AG-II, which were reviewed above, the literature also provides some information regarding the importance of other pectic domains, such as RG-II domains in some of the reported biological activities. However, the information about the structure-activity relations for RG-II seems to be less abundant, and detailed than the one that was found in the literature for RG-I.

Fractionation of two pectic polysaccharides fraction (ALR-a, and ALR-b), isolated from a hot water extract of rhizomes from *Atractylodes lancea*, yielded RG-II domains with intestinal immune system modulating activity (Yu *et al*., 1998; Yu *et al.,* 2001b). A RG-II complex pectic polysaccharide (GL-RI) has also been isolated from the leaves of *Panax ginseng*, and shown to be a macrophage Fc receptor expression-enhancing polysaccharide (Shin *et al*., 1997). The intestinal immune system modulating activity of RG-II from ALR-b (“PG2 from ALR-b”) was compared with RG-II from other medicinal plants, such as *Bupleurum falcatum* (“RG-II from bupleuran 2IIb”), and *Panax ginseng* (“GL-RI”), as showed in **Figure 1.13**, evidencing that the tested RG-II´s exhibited different degrees of biological activity. The RG-II´s from *Panax ginseng* and *Atractylodes lancea* exhibited a

potent activity, while the RG-II domains isolated from *Bupleurum falcatum* did not seem to express the biological activity.

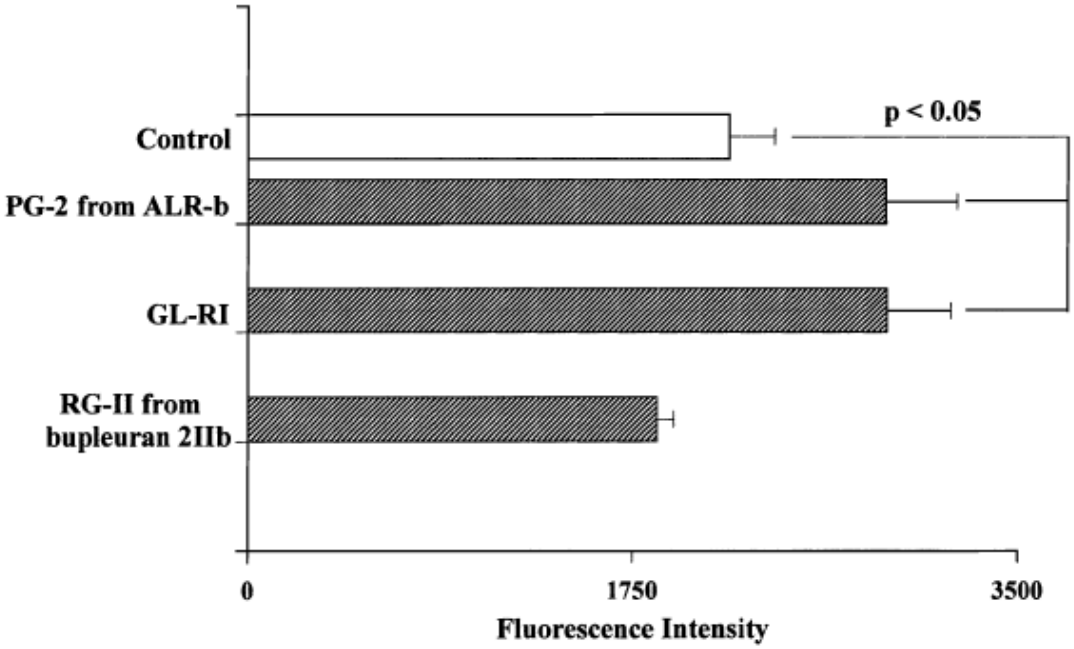

Figure 1.13- Comparison of intestinal immune system modulating activity of RG-II from *Bupleurum falcatum* (RG-II from bupleuran 2IIb), *Panax ginseng* (GL-RI) and *Atractylodes lancea* PG-2 (PG-2 from ALR-b) [From Yu *et al*., 2001b].

Other studies have shown that RG-II domains isolated from polysaccharide fractions of *Angelica acutiloba*, *Astralagus mongholics*, *Biophytum petersianum*, and *Glinus oppositifolius*, also did not seem to express the biological activity. These results suggest that there must be some fine structural details responsible for the observed differences. It is known that RG-II can associate through borate ion interaction into the dimeric form. High performance liquid chromatography (HPLC) analysis permitted the detection of two chromatographic peaks in the *Atractylodes lancea* fraction that contained the RG-II domain (PG-2), suggesting the presence of RG-II in both the monomeric and dimeric forms. This observation is in agreement with the reduction in the estimated molecular weight of *Panax ginseng* fraction GL-4IIb2, which comprised a RG-II domain, after acidic treatment (Shin *et al.,* 1997). However, it was not clear that dimer formation affected the intestinal immune system modulating activity of the RG-II´s tested.

1.7.2. Mannans

Mannan type polysaccharides can be found in various plants where they perform storage and/or structural functions, as referred in **Section 1.4.2**. The plant of *Aloe vera* is one of the most popular mannan sources, due to its widely reported medical applications, particularly in the dermatology field where it can be used for the treatment of radiation-caused skin conditions. Additionally, a relatively large collection of literature documenting medical applications in digestive problems, and in gynaecological conditions can also be found (Grindlay and Reynolds, 1986).

Polysaccharides comprise more than 60% of the *Aloe vera* dry weight (McAnalley, 1993) and through sequential extraction it was possible to evidence the presence of two main types of mannose-containing polymers: a storage polysaccharide, detected in the filet and gel fractions, and a structural polysaccharide, present in the skin tissue (Femenia *et al.,* 1999). The storage polysaccharide is one of the most studied bioactive polymers, commonly known as acemannan and commercially available as Carrysin™. Acemannan has been reported as the main active substance of *Aloe vera* (McAnalley, 1993), and is widely known as an immunomodulatory polysaccharide (Manna and McAnalley, 1993).

The *Aloe vera* acemannans are composed by a backbone that comprises acetylated (β1→4)-mannose residues, with (α1→6)-galactose residues as single side chains (Reynolds, 1985). The acetylation occurs at the C-2, C-3 and C-6 of mannose residues with an acetyl/mannose ratio of approximately 1:1, and the mannan backbone also contains interspersed (β1→4)-linked glucose residues (Manna and McAnalley, 1993; Talmadge *et al*., 2004).

The wide range of reported medical applications suggested the possible involvement of macrophage activation as one of the mechanisms responsible for the biological activities usually associated with acemannans (Zhang and Tizard, 1996). In fact, several studies evidenced that acemannan activates macrophages in diverse ways, such as to produce inflammatory cytokines (Zhang and Tizard, 1996), to increase NO production (Karaca *et al*., 1995; Ramamoorthy *et al.*, 1996;

Djeraba and Quere, 2000), and to upregulate the phagocytic and candidicidal activities (Stuart *et al*., 1997).

Some studies performed in order to evaluate the effects of acemannan on NO production by the mouse macrophage cell line RAW 264.7 have reported that a minimum amount of NO was released when the cells were exposed to acemannan alone (Ramamoorthy *et al.*, 1996; Zhang and Tizard, 1996). However, a dose dependent increase in NO production was registered in macrophage cells treated with acemannan and IFN-γ, suggesting that more macrophages were activated, or that a higher level of macrophage activation was achieved (Zhang and Tizard, 1996). On the other hand, it has been reported that cultures of normal chicken spleen cells, and HDI 1 line cells produced NO in response to acemannan alone (Karaca *et al*., 1995)

In the literature, a large number of studies have reported mannans as macrophage activators, however only a minor fraction of these studies tried to establish a relation between the activation attributed to mannans, and its structural features. Karaca *et al*. (1995) showed that acemannan induced, in a dose-dependent manner, NO production in cells of a chicken macrophage cell line, while the non-acetylated yeast mannan did not. This observation was consistent with the results from another study, which suggested that acetylated mannose-specific receptors could have been involved in the observed macrophage activation (Zhu *et al*., 1993). Previous *in vivo* and *in vitro* studies have shown that mannans actually bind to macrophages via mannose-specific protein receptors being then internalized (Aderem and Underhill, 1999; Tietze *et al.,* 1982). Following this internalization acemannan is able to activate macrophages *in vitro* by increasing the release of cytokines such as IL-1, IL-6 and TNF-α (Zhang and Tizard, 1996). Im *et al*. (2005) obtained a modified *Aloe* polysaccharide through partial cellulase digestion, followed by an 80% ethanol extraction, and protein removal with DEAE-Sephacel column chromatography. The protein-free modified *Aloe* polysaccharide was further fractionated and three fractions with distinct molecular weight were obtained. It was observed that the intermediate molecular weight polysaccharides (5 – 400 kDa) exhibited the most potent macrophage-activation activity as determined by nitric oxide release of mouse macrophage cell

line RAW 264.7 (Im *et al*., 2005). Therefore, it seems that structural features such as acetylation patterns, and molecular weight may have an important role in the macrophage activation associated with mannans.

Simões *et al*. (2009) evaluated the *in vitro* immunomodulatory activity of different mannans towards murine lymphocytes. It was reported that the mannans from the coffee infusions, and chemically acetylated mannans from the spent coffee grounds had an activity similar to those from *Aloe vera*, and that LBG mannans did not exhibit any stimulatory activity. However, despite having a comparable molecular weight (90 – 110 kDa), and similar glycosidic-linkage composition, the chemically acetylated mannans from the spent coffee grounds showed a slightly lower potency than the ones from the coffee infusions. This could be justified be the distinct acetylation patterns of these polymers, with the mannans from spent coffee grounds preferentially acetylated in the side chain residues, whereas those from the coffee infusions only exhibited acetyl groups directly linked to the backbone residues (Simões *et al.,* 2010). The same research group also investigated the acetylation pattern of the mannans from *Aloe vera*, reporting that these mannans contained a rather unusual high, and also non-homogenous acetylation content, with an average of 2.08 acetyl groups per sugar residue (Simões *et al.,* 2012). These values were much higher than those observed for the mannans of coffee infusions and spent coffee grounds, 0.08 and 0.84 – 0.94, respectively (Simões *et al.,* 2009). It was also reported the presence of acetylated arabinose side chains, a structural feature that had only been observed in the chemically acetylated mannans with immunostimulatory activity prepared from the spent coffee grounds (Simões *et al.,* 2012). On the other hand, mannans from LBG, which showed no immunostimulatory activity, exhibited an acetylation content below the detection limit of the method used. Also, it was shown that for *Aloe vera* the ratio of total Man/T-Man, which provides information regarding the length of the polymers, and the ratio of total Man/(1→4,6)-Man, which accounts for the degree of branching, was comparable or slightly inferior to those exhibited by mannans from coffee infusions and from chemically acetylated spent coffee grounds but much smaller than the observed for LBG (Simões *et al.,* 2012). This seems to suggest that lower branching, shorter chains, and higher

acetylation contents may have contributed for the immunostimulatory activity exhibited by mannans.

1.8 Aim of the study

The thesis expressed in this dissertation is that the infusions of *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences contain polysaccharides that exhibit immunostimulatory activity, and therefore might contribute for the therapeutic properties frequently attributed by the popular tradition to their ingestion. This dissertation should also contribute to the valorization of these natural resources.

In order to support the thesis expressed above, a series of preliminary experiments were performed on the infusions of *F. angustifolia* dried leaves, prepared according to the popular tradition. These traditional infusions were prepared by contacting the dried leaves with previously boiled water, during 5 min. The yield of water soluble material was determined, together with the carbohydrate material content and its monomeric composition. Also, the content of total phenolic compounds was determined, together with the antioxidant activity, through the DPPH-scavenging activity, which is frequently used as a first assessment of the biological activity. The type of polysaccharides present in the infusions of *F. angustifolia* dried leaves was determined through methylation of the high molecular weight material (HMWM), which was fractionated using several techniques in order to separate the polysaccharides detected in the infusions. As the carbohydrate material content of the HMWM from the *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences traditional infusions was low, extensive hot water extractions were prepared, and the method considered to be the most adequate in order to achieve the aims of this dissertation was used in the preparation of the HMWM used in subsequent experiments. These results are presented, and discussed in Chapter 3.

The polysaccharides present in the HMWM obtained wit the most adequate method were fractionated by ethanol precipitation, and anion exchange chromatography. Sugar, and glycosidic linkage analysis were performed in order

to determine the quantity, and the type of polysaccharides present in the various hot water extracts, and also to evaluate its structural features. The HMWM was also submitted to *endo*-polygalacturonase treatments, and the digestion products were fractionated by size-exclusion chromatography. The material recovered in the higher molecular fraction was submitted to sugar, and glycosidic linkage analysis, in order to confirm the structural features previously determined for the polysaccharides present in the HMWM. In order to reveal other structural details, ESI-MS, and ESI-MS/MS experiments were also performed on the digestion products resulting from the *endo*-polygalacturonase, and *endo*-mannase treatment of fractions isolated from the *F. angustifolia*, and *P. tridentatum* extracts, respectively. These results are shown, and discussed throughout Chapters 4 to 6.

The anti-, and pro-inflammatory activities of selected fractions from the hot water extracts of *Fraxinus angustifolia* dried leaves, *Mentha suaveolens* dried shoots, and *Pterospartum tridentatum* dried inflorescences, were evaluated. The immunostimulatory activity observed, expressed through an increase in the macrophage NO production, was related with the polysaccharide composition of the hot water extracts. These results are revealed in Chapter 7.

In Chapter 8, the conclusions that allow to support the previously presented thesis are presented.

CHAPTER 2

EXPERIMENTAL SECTION

2.1. Vegetable material

The vegetable species selected for the preparation of the various hot water extracts were “freixo” (*Fraxinus angustifolia*), “mondrasto” (*Mentha suaveolens*), and “carqueja” (*Pterospartum tridentatum*). These vegetable species were selected based on an ethnobotanical study about “Parque Natural do Montesinho” (Carvalho, 2005), a region located in Bragança district, at the northeast of Portugal, as shown in **Figure 2.1**.

Figure 2.1- Portugal map with the location of the “Parque Natural do Montesinho” at the Bragança district.

F. angustifolia, M. suaveolens, and *P. tridentatum* were selected among the various vegetable species, which are used for medicinal purposes, and that were mentioned in the referred study. The choice was based on certain criteria, such as:

- frequency of citation by the population surveyed;
- number of medicinal applications reported;
- regularity of distribution;
- quantity available;
- species survival issues.

Most of the plants used for medicinal purposes, and that are referred in the various ethnobotanical studies found in the literature, can be used as aqueous

infusions or decoctions, prepared by contacting the vegetable material with previously boiled water, or with boiling water, for a determined period. Also according to these studies, the aqueous infusions can be prepared from distinct parts of the vegetable material, which needs to be collected at adequate times of the year. Therefore, the leaves of *F. angustifolia*, and the shoots of *M. suaveolens* were harvested during early summer in 2007, and the inflorescences of *P. tridentatum* were collected during the early spring of 2008. The leaves from *F. angustifolia,* and the *M. suaveolens* shoots were harvested at a location known as "Quinta do Poulão", property of the "Escola Superior Agrária de Bragança", and the inflorescences of *P. tridentatum* were collect at a location in "Parque Natural do Montesinho". Subsequently, the collected vegetable material was dried in a dark and cool place, according to the popular tradition.

F. angustifolia, illustrated in **Figure 2.2 a)**, is a tree that belongs to the *Oleaceae* family, from the *Lamiales* order, and its dried leaves are used in the prevention of high blood pressure, rheumatism and high levels of uric acid (Carvalho, 2005).

Figure 2.2- Vegetable species selected for the study: **a)** *F. angustifolia*, **b)** *M. suaveolens*, and **c)** *P. tridentatum*.

M. suaveolens, shown in **Figure 2.2 b)**, is a member of the *Lamiaceae* family, from the *Lamiales* order, whose dried shoots infusions exhibit beneficial effects for stomach, diarrhea, cold, and are also reported as anti-haemorrhagic, and anti-cholesterolemic (Carvalho, 2005). *P. tridentatum* is a small shrub, illustrated in **Figure 2.2 c)** that belongs to the *Fabaceae* family, from the *Fabales* order. According to the popular tradition, its dried inflorescence infusions protect against cold, headache, stomachache, throat irritation, diabetes, high blood pressure, urinary tract diseases, and heart problems (Carvalho, 2005).

2.2. Hot water extracts

2.2.1. Traditional infusions

The infusions prepared according to the popular tradition, consisted in contacting the dried vegetable material with previously boiled water during 5 min. For the preparation of the infusions, dried leaves of *F. angustifolia*, dried shoots of *M. suaveolens*, and dried inflorescences from *P. tridentatum* were used. For *F. angustifolia*, proportions of vegetable material to water volume of 0.01, 0.05, 0.10, and 0.14 g/mL were used, while for *M. suaveolens*, and *P. tridentatum* 0.10, and 0.07 g/mL were used, respectively. The resulting liquid was filtered through a glass fiber filter (Whatman GF/C), and concentrated. The concentrated extracts were submitted to solid phase extraction and/or dialysis. The solid phase extraction was performed on previously activated C_{18} cartridges, eluted with water to obtain the not retained material, and subsequently eluted with acidic methanol for recovering the retained material. The dialysis (cut-off 12-14 kDa) was performed to obtain the high molecular weight material (HMWM) and also to eliminate salts.

2.2.2. Extensive hot water extracts

The extensive hot water extracts were prepared by the infusion of the dried vegetable material with water at 50ºC, or by the decoction of the dried vegetable material with boiling water, during a total of 4 h, divided in two equal parts of 2 h. For the preparation of the extensive hot water extracts of *F. angustifolia*, and *M. suaveolens* a proportion of vegetable material to water volume of 0.05 g/mL was used, while for *P. tridentatum* the extracts were prepared with a proportion of 0.03 g/mL. After the 1st 2h, the liquid was filtered through a glass fiber filter (Whatman GF/C), concentrated, dialysed (cut-off 12-14 kDa) and freeze-dried to obtain the high molecular weight material (HMWM) from the 1st 2h. The vegetable residue was further mixed with renewed distilled water at 50 ºC or with boiling distilled water (0.07 g/mL for *F. angustifolia* and *M. suaveolens*, and 0.03 g/mL for *P. tridentatum*) during additional 2 h. The liquid was filtered through a glass fiber filter

(Whatman GF/C), concentrated, dialysed (cut-off 12-14 kDa) and freeze-dried, to obtain the HMWM from the 2nd 2h.

2.3. Ethanol precipitation

The HMWM was dissolved in distilled water (10.0 mg/mL), the solution was stirred for 1 hour at room temperature and centrifuged at 24400 g for 20 minutes at 4 °C. The cold water insoluble residue obtained (**WI_{ppt}**) was suspended in distilled water, frozen, and freeze-dried. Absolute ethanol was added (50% ethanol, assuming additive volumes) and left for 1 hour at 4 °C. This solution was then centrifuged, and the precipitate obtained (**Et_{50}**) was removed by centrifugation. Absolute ethanol was added to the supernatant (75% ethanol, assuming additive volumes), and the resulting solution was left for 1 hour at 4 °C, and centrifuged. The precipitate obtained (**Et_{75}**) was removed from the supernatant solution (**SN**). In order to remove the ethanol completely each precipitate was dissolved in distilled water, and rota-evaporated. All fractions were freeze-dried.

2.4. Column chromatography separations

2.4.1. DEAE-Sepharose FF anion exchange chromatography

Anion exchange chromatography on DEAE-Sepharose FF (Pharmacia), was performed on a 100 × 1.6 cm column (XK 100/16, Pharmacia), at a flow rate of 0.5 mL/min. The samples were suspended in 50 mM potassium phosphate buffer pH 6.5 (1.0 mg/mL). The samples were sequentially eluted in the same phosphate buffer, and buffer with 0.125, 0.250, 0.500, and 1.000 M NaCl. Fractions (3.0 mL) were collected and assayed for sugars, according to a modification of the Dubois method (Coimbra *et al.*, 1996), as described in **Section 2.5**. The fractions of interest were pooled, dialysed and freeze-dried.

2.4.2. Bio gel P30 size-exclusion chromatograpy

Gel filtration chromatography on Bio-Gel P30 (Pharmacia), was performed on a 100 × 1.6 cm column (XK 100/16, Pharmacia), at a flow rate of 0.5 mL/min. Blue dextran, and glucose solutions were separately eluted in order to determine the columns exclusion, and inclusion volumes, respectively. The samples were eluted with 50 mM acetate buffer, pH 5.0. Fractions (3.0 mL) were collected and assayed for sugars, according to a modification of the Dubois method (Coimbra *et al.*, 1996), as described in **Section 2.5**. The fractions of interest were pooled, dialysed and freeze-dried.

2.4.3. Bio-Gel P2 and P6 gel-exclusion chromatograpy

Gel filtration chromatography on Bio-Gel P2 or P6 (Pharmacia), was performed on a 100 × 1.6 cm column (XK 100/16, Pharmacia), at a flow rate of 0.2 mL/min. The column was previously calibrated with DP4 (stachyose), DP2 (cellobiose), and monosaccharide (glucose), using a flow of 0.2 mL/min. The samples were eluted with distilled water. Fractions (1.0 mL) were collected and assayed for sugars, according to a modification of the Dubois method (Coimbra *et al.*, 1996), as described in **Section 2.5**. Fractions containing oligosaccharides were evaporated until all the eluent was removed. No lyophilization was performed since it has been shown that it promotes O-acetyl migration on galactomannans (Nunes *et al*., 2005).

2.5. Determination of total sugars

To test tubes, previously washed with acid, a sample aliquot (80 µL), concentrated H_2SO_4 (1.0 mL), and phenol solution (160 µL, 5% m/v) was added. Then, the mixture was shaken vigorously, and heated in boiling distilled water for 5 min. After cooling, the absorbance at 490 nm was registered.

2.6. Determination of total phenolic compounds

The extracts were dissolved in a 0.5 % (v/v) acetic acid solution, and 0.5 mL of this extract solution was mixed with 250 μL of the Folin–Ciocalteu reagent. After homogeneously mixing, 1.0 mL of sodium carbonate solution (200 g/L) and 3.25 mL of distilled water were added. The tubes were vortexed, and allowed to stand for 10 min. at 70ºC and subsequently for 30 min. at room temperature for colour development. Absorbance was then measured at 700 nm. Gallic acid was used to calculate the standard curve, and the results were expressed as gallic acid equivalents (GAE) per g of dry weight. The results are expressed as mean of samples prepared in triplicate.

2.7. DPPH radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity was monitored according to the method reported by Hatano *et al.* (Hatano *et al.*, 1988). Various concentrations of sample extracts (0.3 mL) were mixed with 2.7 ml of a methanolic solution containing DPPH radicals (6×10^{-5} mol/L). After vigorous shaking, the mixture was left to stand for 60 min. in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation:

$$\%RSA = \left[\left(A_{DPPH} - A_S\right) / A_{DPPH}\right] \times 100,$$

where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radical-scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration, and the results are expressed as mean of samples prepared in triplicate.

2.8. Fructose determination

Fructose was determined using the improved resorcinol reagent, as described by Yaphe and Arsenault (1965). The resorcinol reagent (5 mL) was added to capped Soviril tubes containing the sample (1 mL), and previoulsly cooled in an ice bath. The mixture was stirred and left to stand for 5 min. The Soviril tubes were put in a water bath at 25 ºC, during 4 min., and subsequently heated at 80 ºC, during 10 min. After cooling in an ice bath, the absorbance at 555 nm was registered. Fructose was used as standard, and the results are expressed as mean of samples prepared in triplicate.

2.9. Sugar analysis

Neutral sugars were determined by gas chromatography (GC), after acid hydrolysis release, and conversion to the corresponding alditol acetates. The polysaccharides were treated with 72% (w/w) H_2SO_4 during 3 h at room temperature with occasional stirring followed by hydrolysis for 2.5 h with 1 M sulfuric acid at 100ºC. The monosaccharides were reduced with $NaBH_4$ (15% w/v in NH_3 3 M) during 1 h at 30ºC, and subsequently acetylated with acetic anhydride (3 mL) in the presence of 1-methylimidazole (450 μL) during 30 min. at 30ºC. The alditol acetate derivatives were then separated with dichloromethane and analyzed by GC with a flame ionization detector (FID), and equipped with a 30 m column DB-225, with an internal diameter and film thickness of 0.25 mm and 0.15 μm, respectively. The oven temperature program used was: an initial temperature of 200ºC, a rise in temperature at a rate of 40ºC/min. up to 220ºC, standing for 7 min., followed by a rate of 20ºC/min. up to 230ºC, and maintained at this temperature for 1 min.. The injector and detector temperatures were, respectively, 220 and 230ºC. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min. (Nunes and Coimbra, 2001). The hydrolysis of all samples was done in duplicate.

Uronic acids (UA) were determined colorimetrically, according to a modification (Coimbra *et al*., 1996) of the Blumenkrantz and Asboe-Hansen (1973) method. Briefly, the samples were prepared by pre-hydrolysis in 72% (w/w) H_2SO_4 (200 μL) for 3 h at room temperature, followed by an 1 h hydrolysis in 1 M H_2SO_4 at 100ºC. In triplicate, an aliquot of the hydrolyzed material, previously diluted with distilled

water, was transferred to ice cooled test tubes and mixed with 50 mM boric acid in concentrated H_2SO_4 (3.0 mL). After stirring, the test tubes were heated in boiling water during 10 min. Upon cooling, 3-phenylphenol (100 μL, 0.15% in 0.5% NaOH,) were added to two out of the three test tubes, which were then left for 0.5 hour in the dark. After that, the absorbance at 520 nm was registered, and the uronic acid content was estimated using a D-galacturonic acid calibration curve. The results are expressed as mean of samples prepared in duplicate.

2.10. Linkage analysis

Linkage analysis was carried out by methylation, using a modification of the method described by Ciucanu and Kerek (1984). The sample was dissolved in anhydrous dimethylsulfoxide (DMSO) (1 mL), then powdered NaOH (40 mg) was added, and the samples were methylated with CH_3I (3 × 80 μL) during 20 min. The samples were dissolved in $CHCl_3$/MeOH (1:1 v/v) (3 mL) and dialysed against an acidified ethanol:distilled water mixture (pH 3.0, 1:1 v/v) (cut-off 12-14 kDa) at 4ºC with 2 renewals. At the end of the dialysis procedure, the samples were evaporated to dryness and remethylated to ensure complete methylation of the polysaccharides (Nunes and Coimbra, 2001). A portion of the methylated material (1/3 of the total volume) was hydrolyzed with TFA 2 M at 121ºC for 1 h and the remaining volume was carboxyl-reduced as described in **Section 2.11**. The hydrolyzed methylated material was then reduced with $NaBD_4$ and acetylated as previously described for neutral sugar analysis in **Section 2.9**. The partially methylated alditol acetates (PMAA) were separated and analyzed by gas chromatography–mass spectrometry (GC–MS). The GC was equipped with a 30 m length DB-1 capillary column, with an internal diameter and film thickness of 0.25 mm and 0.15 μm, respectively. The samples were injected in *splitless* mode (time of *splitless* 5 min.) using the following temperature program: The oven temperature program used was: an initial temperature of 45ºC, standing for 5 min., with a linear increase of 10ºC/min. up 140ºC, and standing for 5 min., a rise in temperature at a rate of 0.5ºC/min. up to 170ºC, and standing for 1 min. at this temperature, followed by a rise in temperature at a rate of 15ºC/min. up to 280ºC,

with further 5 min. at 280ºC. The injector and detector temperatures were, respectively, 220 and 230ºC. The helium carrier gas had a flow rate of 1.7 mL/min. and a column head pressure of 2.8 psi. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range m/z 40–500 in a 1 s cycle in a full scan mode acquisition.

2.11. Carboxyl-reduction of the methylated alditol acetates

Previously methylated samples, according to the method described in **Section 2.10**, were carboxyl reduced by a modification of the Lindberg and Lönngren method (1978), as described by Coimbra *et al*. (1996). A portion of the previously vacuum dried methylated samples (2/3 of the total volume) were dried under vacuum in the presence of P_2O_5. Under argon atmosphere, $LiAlD_4$ (20 mg) and tetrahydrofuran dried with molecular sieves 3A (1.0 mL) were added to each sample. Then, the tube was sealed and left to react at 65°C during 4 h. The excess of $LiAlD_4$ was destroyed, first with 2 to 3 drops of ethanol, and then with 2 to 3 drops of distilled water. The solution was neutralized with H_3PO_3 2M, and a $CHCl_3$/MeOH solution (2.0 mL, 2:1 v/v) was added. The precipitated was removed by centrifugation, and washed twice with 2 mL of the same $CHCl_3$/MeOH solution. The supernatant was vacuum dried, and transferred to a soviril tube for hydrolysis, reduction, and acetylation, as described in **Section 2.9**. The carboxyl reduced partially methylated alditol acetates were separated and analyzed by GC–MS as described in **Section 2.10**.

2.12. Determination of the degree of methyl esterification

The samples were dissolved in a glass vial with distilled water (2.2 mL), and saponified with 2 M NaOH (800 μL), with a reaction time of 1 h at 25ºC, as described by Waldron and Selvendran (1990). The saponification reaction was finished with 2 M HCl (800 μL). As internal standard, 1-propanol (200 μL, 2.5 g/L) was also added to the vial after saponification. The sample suspension was filtered through a 20.0 μm nylon membrane filter. The filtrate was injected (500 μL)

in a GC-FID equipment with a 30 m length DB-Wax column, with an internal diameter, and film thickness of 0.53 mm, and 1.0 mm, respectively. The oven temperature program used was: an initial temperature of 50ºC, a rise in temperature at a rate of 5ºC/min. until 65ºC, another rise in temperature at 20ºC/min. until 185ºC, and a final rise at 35ºC/min. until 220ºC, and held 1 min. at 220ºC. The injector, and the detector temperatures were set at 250ºC. The flow rate of the carrier gas (H_2) was set at 6 mL/min. A calibration curve was constructed for methanol final concentrations from 40 to 400 mg/L. Estimated concentrations were made by peak area comparisons with the area of the known concentration of 1-propanol. The results are expressed as mean of samples prepared in duplicate, and each run in duplicate.

2.13. Enzymatic treatments

2.13.1. *Endo*-D-(α1→4)-polygalacturonase digestion

The HMWM was dissolved in 0.1 M NaOH for de-esterification (10.0 mg/mL) and left for 24 h at ambient temperature. After neutralization with acetic acid, the de-esterified sample in 50 mM acetate buffer (5.0 mg/mL), pH 5.5, was treated with *endo*-α-D-(1→4)-polygalacturonase (0.3 U) for 72 h at 40ºC, with continuous stirring. The reaction was terminated by heating at 100ºC. The digested material was fractionated according to the procedure described in **Section 2.4.2**.

2.13.2. *Endo*-D-(β1→4)-mannanase digestion

The HMWM was dissolved in a 100 mM Na-acetate buffer (3.0 mg/mL), pH 5.5, and treated with *endo*-β-(1→4)-D-mannanase (1.0 U), for 48 h at 37ºC, with continuous stirring. The digested material was freeze-dried, and subsequently dissolved in distilled water for fractionation according to the procedure described in **Section 2.4.3**.

2.14. Electrospray ionization mass spectrometry (ESI-MS)

The fractions obtained after the enzymatic treatments described in **Sections 2.13.1** or **2.13.2**, and fractionated according to the procedure presented in **Section 2.4.3**, were dissolved in Mili-Q water (200 μL), and further diluted in methanol/water/formic acid (50:49.9:0.1, v/v/v). Positive ion ESI-MS and MS/MS spectra were acquired using a LXQ linear ion trap mass spectrometer (Finningan). Typical ESI conditions were as follows: nitrogen sheath gas 30 psi, spray voltage 5 kV, heated capillary temperature 275ºC, capillary voltage 1 V, and tube lens voltage 40 V. The flow rate was set to 8 L/min., and the voltage applied was 5 kV. Nitrogen was used as nebulizing and drying gas. Full scan mass spectra ranging from m/z 100 to 1500 were acquired in the positive mode. In the MS/MS experiments, collision energy varied between 15 and 25 of normalized collision. Data acquisition was carried out with Xcalibur data system.

2.15. Immunostimulatory activity

2.15.1. Cell culture

Raw 264.7, a mouse leukaemic monocyte macrophage cell line from American Type Culture Collection, was cultured in Iscove's Modified Dulbecco's Eagle Medium supplemented with 10% noninactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37ºC in a humidified atmosphere of 95% air, and 5% CO_2. Along the experiments, cells were monitored by microscope observation, in order to detect any morphological change.

2.15.2. Determination of cell viability

Assessment of metabolically active cells was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction colorimetric assay as reported by Mosmann (Mosmann, 1983). Raw 264.7 cells (6×10^5 cells/well) were plated and allowed to stabilize for 12 h. Following this period, cells were either maintained in culture medium (control) or pre-incubated with the

extracts for 1 h, and later activated with 1μg/ml LPS for 24 hours. After the treatments, a MTT solution (5 mg/ml in phosphate buffered saline) was added and cells incubated at 37ºC for 15 min., in a humidified atmosphere of 95% air, and 5% CO_2. Supernatants were then removed, and dark blue crystals of formazan solubilized with acidic isopropanol (0.04M HCl in isopropanol). Quantification of formazan was performed using an SLT ELISA automatic microplate reader at 570 nm, with a reference wavelength of 620 nm.

2.15.3. Measurement of nitrite production

The production of nitric oxide (NO) was measured by the accumulation of nitrite in the culture supernatants, using a colorimetric reaction with the Griess reagent (Green *et al*., 1982). Briefly, 170 μL of culture supernatants were diluted with equal volumes of the Griess reagent [0.1% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H_3PO_4], and maintained during 30 min. in the dark. The absorbance at 550 nm was measured using an SLT ELISA automatic microplate reader. Culture medium was used as blank and nitrite concentration was determined from a regression analysis using serial dilutions of sodium nitrite as standard.

2.15.4. Statistical analysis

The results presented in **Chapter 7** are expressed as the mean±standard deviation from three independent experiments. When comparing the effect of different treatments to non-stimulated (Ctrl.) or to LPS-stimulated cells (LPS) one-way ANOVA followed by Dunnett's test was used. Comparison between assays using distinct extract concentrations was performed by one-way ANOVA followed by Tukey's HSD test. Statistical analysis of the effect of PMB on macrophage NO production and cellular viability was performed between two groups (with and without pre-incubation with PMB), and analysed using two-sided unpaired t-test. All statistical tests were applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA), with a significance level of $p < 0.05$.

CHAPTER 3

PRELIMINARY EXPERIMENTS

In this chapter, traditional infusions of *Fraxinus angustifolia* dried leaves were prepared according to the popular tradition, using various proportions of vegetable material to water volume. These infusions were divided in two portions of equal volume, and one portion was submitted to solid phase extraction, while the other one was dialysed. For the various extracts obtained, the mass yield, carbohydrate content, and its monomeric composition were determined, together with the content of total phenolic compounds, and its DPPH scavenging activity.

In order to evaluate the type of polysaccharides present in the infusions, the polymeric material present in the high molecular weight extract (HMWM) was methylated. The polymeric material was also submitted to a series of fractionation procedures, and the mass yield, carbohydrate content, and the monomeric composition of the various fractions were determined. The efficiency of the fractionation techniques, determined by the sugar enrichment of the fractions, and also by its total mass recovery, was also evaluated.

Alternative extensive hot water extraction methods were also tested, namely the infusion with distilled water at 50ºC, and the decoction with boiling distilled water, during a total period of 4h, divided in two periods of 2h each. The HMWM yield, carbohydrate content, and its monomeric composition, together with the content of total phenolic compounds were determined. Also, the *in vitro* antioxidant activity was evaluated using the DPPH radical-scavenging activity.

3.1. Dialysis and solid phase extraction of *Fraxinus angustifolia* infusions

The leaves of *Fraxinus angustifolia*, used for the preparation of the infusions, are highly abundant during the early summer, which is the adequate season for the gathering of this vegetable material, according to the popular tradition. Thus, large amounts of *F. angustifolia* leaves were collected and subsequently dried, in order to perform a series of preliminary experiments that would enable to evaluate the total material yield, carbohydrate material, and total phenolic compounds contents. The *in vitro* antioxidant activity, which is frequently used as a first indicator of the potential biological activity of vegetable extracts, was also evaluated using the DPPH radical-scavenging activity.

The infusions of *F. angustifolia* were prepared according to the popular tradition by the infusion of the dried leaves in previously boiled distilled water (0.01 g/mL), during 5 min. Subsequently, the total volume of the infusion was equally divided in two portions. One portion was submitted to solid phase extraction, yielding two extracts: SP_{nret}, and SP_{ret}, which comprised the not retained hydrophilic, and retained hydrophobic material, respectively. The second portion of the infusion was dialysed (cut-off 12-14 kDa), yielding the high molecular weight (HMWM) extract, containing the polymeric material.

The infusions were prepared using proportions of vegetable material to water volume comparable to those frequently used in the preparation of infusions (0.01 g/mL), and also proportions 5 times higher (0.05 g/mL). In both cases, it was observed that the amount of polymeric material obtained in the HMWM extracts was not enough to carry out the determinations that had been defined: 1.3 and 5.8 mg, for the infusions prepared with proportions of vegetable material to water volume of 0.01 g/mL, and 0.05 g/mL, respectively. Therefore, higher proportions were used for the preparation of the infusions: 0.10 and 0.14 g/mL.

The material yield, carbohydrate content and its monomeric composition, total phenolic compounds content, as well as the DPPH radical scavenging activity, expressed as the EC_{50} value, of the various extracts were determined and are shown in **Table 3.1**.

Table 3.1- Mass yield, total sugar content and monosaccharide composition, total phenolic compounds content, and DPPH scavenging activity, expressed by the EC_{50} value, of the extracts from *Fraxinus angustifolia* dried leaves.

	Yield[a] (mass %)	Total Sugars (mass%)	Monosaccharide Composition (mol %)								Total Phenolics (mass%)	EC_{50} (mg/mL)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA		
0.10 mg/mL												
SP_{nret}	6.9	34.9	0.6	0.5	0.9	0.6	47	4.9	39.6	5.9	1.46	1.23
SP_{ret}	1.2	30.4	11.8	0.4	0.5	1.8	1.0	1.1	76.2	7.4	17.07	0.14
HMWM	0.16	31.2	7.7	1.1	14.7	2.4	5.5	17.9	29.4	21.3	7.84	0.34
0.14 mg/mL												
SP_{nret}	8.2	37.4	0.6	0.5	0.8	0.9	50.1	4.9	32.8	9.4	1.82	1.00
SP_{ret}	1.5	32.6	12.8	0.5	0.6	1.6	1.2	1.2	69.4	12.8	16.17	0.13
HMWM	0.19	29.4	8.2	1.0	15.2	2.9	6.1	17.7	30.1	18.9	8.52	0.38

[a]- expressed relatively to the vegetable material dry weight

The extracts SP_{nret}, which comprised the hydrophilic material of the infusion, exhibited the highest total material yields, while the HMWM ones, which contained the infusions polymeric material, displayed the lowest. The extracts SP_{nret} presented a total material yield that was approximately 6 and 43 times higher than those of the SP_{ret} and HMWM extracts, respectively. It was observed that the total carbohydrate material content of the various extracts was similar, while the total phenolic compounds content was distinct. The SP_{ret} extracts presented the highest total phenolic compounds content, followed by the HMWM, and the SP_{nret} extracts, which presented the lowest content. The antioxidant activity of the three types of extracts, evaluated by the DPPH scavenging activity, and expressed as EC_{50} values, was also distinct. The SP_{nret} extracts exhibited the highest EC_{50} values, while the HMWM, and SP_{ret} extracts showed lowest EC_{50} values, evidencing the higher antioxidant activity of the HMWM and SP_{ret} extracts. The registered antioxidant activity seems to be directly related to the phenolic compounds content of the extracts, as phenolic compounds are widely known for their biological and pharmacological properties, particularly for the antioxidant activity (Shahidi and Wanasundara, 1992).

Considering that the SP_{nret}, and SP_{ret} extracts combined contained the totality of the water soluble material present in the infusions, it is possible to observe that the HMWM extracts only accounted for 2% of that material. This evidenced that the infusions of *F. angustifolia* dried leaves mostly contained low molecular weight compounds, which were preferentially recovered in the SP_{nret} extracts.

The various extracts that were obtained from the infusions of *F. angustifolia* dried leaves presented a distinct monomeric carbohydrate composition, as evidenced in **Table 3.1**. High contents of mannitol, expressed as Man (47-51%), and glucitol, expressed as Glc (32-40%), were quantified in the SP_{nret} extracts. The occurrence of readily water soluble sugar alcohols, such as glucitol, and mannitol, in the leaves from plants is widespread (Beck and Hopf, 1990). Therefore, it seems possible that the infusions of *F. angustifolia* dried leaves contained glucitol, and mannitol, which were recovered in the SP_{nret} extracts. Additionaly, the high content of mannitol in these C_{18} non-retained extracts may also be explained by the presence of fructose residues, as the determination of the monomeric

composition of the carbohydrate material present in the various extracts involves, after the hydrolysis step, the reduction of the anomeric carbon. If fructose residues are present, the reduction step will yield 57% of glucitol, and 43% of mannitol, which are subsequently detected in the sugar analysis as their alditol acetate derivatives, attributed to Man and Glc, respectively. The fructose content of SP_{nret} extracts was determined by the resorcinol method (Yaphe and Arsenault, 1965), allowing the detection of 123.2, and 114.7 mg fructose/g vegetable material for the infusions prepared with proportions of vegetable material to water volume of 0.10 and 0.14 mg/mL, respectively. This suggests that the *F. angustifolia* dried leaves infusions also contain fructose residues, possibly sucrose, although it cannot be excluded the presence of fructooligosaccharides (FOS), as reported for the roots and leaves of various plants, including members of the *Oleacea* family, in which *F. angustifolia* is included (Oliveira *et al*., 2011).

The carbohydrate material from the SP_{ret} extracts was particularly rich in Glc (69-77%), besides exhibiting relevant proportions of Rha (11-13%). These extracts also presented the highest proportions of total phenolic compounds. Phenolic compounds can be found as aglycone moieties of glycosides, which often comprise glucosyl, and rhamnosyl residues as its glycones (Amaral *et al*., 2005; Awaad, 2006). This suggests that the infusions of *F. angustifolia* dried leaves contained hydrophobic glycosides.

The HMWM extracts showed a diverse monomeric composition rich in Glc (29 – 31%), Gal (18%), and UA residues (18 – 22%), together with Ara (14 – 16%), and Rha (7 – 9%), which are diagnostic of the presence of pectic polysaccharides, widely reported as present in the infusions of various plants used with medicinal purposes, and known for their diverse biological activities.

The results obtained allowed to observe that, to have enough material for analysis, it is necessary to have a proportion of vegetable material to water volume higher than the ones frequently used to prepare an infusion. It was observed that when distinct proportions of vegetable material to water volume were used, the carbohydrate material and total phenolic compounds contents were comparable, similarly to what was observed for the monomeric composition, and DPPH scavenging activity of the various extracts. Thus, as a compromise, the

methylation analysis, and further fractionations performed used a vegetable material to water volume proportion of 0.14 g/mL.

The polymeric material present in the HMWM extracts was methylated and the deduced linkages are presented in **Table 3.2**. An agreement between the molar fractions from sugar (alditol acetates) and linkage analysis (partially methylated alditol acetates) was registered.

Table 3.2- Deduced linkages from the methylation analysis of the HMWM extract from the infusions of *Fraxinus angustifolia* dried leaves.

Linkage	Molar %	Linkage	Molar %
T-Rha*p*	6.9	2-Man*p*	0.8
2-Rha*p*	1.1	4-Man*p*	5.2
Total	**8.0[a] (10.1)[b]**	4,6-Man*p*	1.0
		Total	**7.0 (7.5)**
T-Fuc*p*	1.2		
Total	**1.2 (1.2)**	T-Gal*p*	6.3
		3-Gal*p*	1.8
T-Ara*f*	13.8	6-Gal*p*	8.9
2-Ara*f*	1.0	3,6-Gal*p*	5.6
3-Ara*f*	3.4	**Total**	**22.6 (21.8)**
5-Ara*f*	5.0		
Total	**23.2 (18.7)**	T-Glc*p*	13.8
		4-Glc*p*	10.1
T-Xyl*p*	3.9	4,6-Glc*p*	7.1
2-Xyl*p*	0.5	**Total**	**31.0 (37.1)**
4-Xyl*p*	0.9		
2,4-Xyl*p*	1.7		
Total	**7.0 (3.5)**		

molar % obtained through: [a] linkage analysis, and [b] sugar analysis

The results evidenced the presence of (1→3)-, (1→6)-, and (1→3,6)-linked galactosyl residues, in 1:5:3 proportion, besides a high proportion of terminally-linked Ara residues, which together with the presence of uronic acid residues detected in the sugar analysis, could be diagnostic of the presence of type II arabinogalactans (AG-II), and pectic polysaccharides. Also, the presence of (1→4)-, and (1→4,6)-linked glucosyl residues allowed to infer the possible presence of xyloglucans. This seems to be supported by the detection of other glycosidic residues often found as xyloglucan side chains, such as terminally-,

and (1→2)-linked xylosyl residues, besides terminally-linked Gal and Fuc residues. The presence of (1→4)-linked mannosyl residues might suggest the presence of mannans in lower proportion.

The results obtained so far evidenced that the infusions from *Fraxinus angustifolia* dried leaves contained water soluble material, comprising 85% hydrophilic material. This material presented approximately 10% of fructose residues. The infusions from *F. angustifolia* dried leaves also contained water soluble polymeric material, which comprised approximately 2% of the total water soluble material, and that contained nearly 30% of carbohydrate material, mainly containing pectic polysaccharides, AG-II, and possibly xyloglucans. The DPPH scavenging activity of the various extracts obtained from the infusions from *F. angustifolia* dried leaves seemed to be directly related to the total phenolic compounds content, with the hydrophobic material, which contained the highest proportions of these compounds, also exhibiting the highest DPPH scavenging activity.

3.2. Fractionation experiments of the HMWM extracts from *Fraxinus angustifolia* dried leaves infusions

The preliminary experiments previously performed, and described in **Section 3.1**, showed that the polymeric material recovered in the HMWM extracts obtained from the infusions of *F. angustifolia* dried leaves was composed of a mixture of polysaccharides, including pectic polysaccharides, AG-II, and possibly xyloglucans. However, the carbohydrate material content of the HMWM extracts was only estimated as approximately 30%. It is well reported that some types of polysaccharides require more aggressive hydrolysis conditions for the breakage of the glycosidic linkages, and subsequent release of monosaccharides that, after alkaline reduction and acetylation, will provide the alditol acetates that are detected and quantified by GC-FID. It has been also reported that the presence of inorganic material might compromise the acidic hydrolysis. Thus, in order to evaluate the influence of the hydrolysis conditions on the estimated carbohydrate content, the HMWM from the infusions of *F. angustifolia* dried leaves was also

submitted to hydrolysis with 2 M H_2SO_4, at 100ºC during 2.5 h, and 2 M TFA, for 1.0 h at 121ºC. Also, a treatment with 1-methylimidazole, which acts as a chelating agent, was performed prior to the HMWM hydrolysis, in order to minimize the effect of the possible presence of salts. The obtained results were compared with the previous ones obtained using 1 M H_2SO_4 hydrolysis, at 100ºC for 2.5 h, and are presented in **Table 3.3**.

Table 3.3- Total sugar content and monosaccharide composition of the HMWM from *F. angustifolia* infusions, submitted to hydrolysis with 2 M H_2SO_4, 1 M H_2SO_4, 2 M TFA ($HWMW_{H2SO4-2M}$, $HWMW_{H2SO4-1M}$, and $HMWM_{TFA-2M}$, respectively), and also treated with a chelating agent ($HMWM_{chel}$) prior to hydrolysis with 1 M H_2SO_4.

Fraction	Total Sugars (mass %)	Monosaccharide Compostion, (mol %)							
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
$HMWM_{H2SO4-1M}$	29.4	8.2	1.0	15.2	2.9	6.1	17.7	30.1	18.9
$HMWM_{H2SO4-2M}$	25.2	0.0	0.0	16.0	3.0	9.2	15.8	25.4	30.6
$HMWM_{TFA-2M}$	24.6	5.3	0.6	18.9	3.2	6.3	19.8	31.6	14.3
$HMWM_{chel}$	30.5	8.2	1.2	15.7	2.6	4.1	17.6	26.6	24.1

Table 3.3 shows that the hydrolysis step performed in distinct conditions did not affect the carbohydrate material content of the HMWM. Also, the treatment of the HMWM with 1-methylimidazole, which acts as a chelating agent, prior to the hydrolysis step, allowed to obtain an estimated carbohydrate material content similar to those obtained without this pre-treatment. It was also observed that the HMWM monomeric compositions determined using the hydrolysis with 1 M H_2SO_4, and 2 M TFA were similar. The determination of the HMWM monomeric composition using 2 M H_2SO_4 for the hydrolysis step, did not allow to detect rhamnosyl, and fucosyl residues, possibly promoting their degradation. This may explain the excess of uronic acid residues relatively to the monomeric composition determined with the use of a mild hydrolysis step performed with 1 M H_2SO_4. Therefore, all the subsequent sugar analyses were performed using 1 M H_2SO_4 hydrolysis, at 100ºC for 2.5 h, which were the initial conditions.

Since the glycosidic content of the HMWM extract is relatively low, several fractionation experiments (DEAE-Sepharose FF anion exchange chromatography, C_{18} cartridges solid phase extraction, and ethanol precipitation) were performed in order to obtain fractions enriched in sugar material that would allow further structural characterization, and biological activity evaluation studies.

3.2.1. Anion exchange chromatography

The material present in the HMWM extracts was redissolved in distilled water (1.0 mg/mL), and submitted to anion exchange chromatography on DEAE-Sepharose FF. The obtained chromatographic elution profile is shown in **Figure 3.1**, exhibiting several chromatographic bands that evidenced the presence of both phenolic compounds (Abs 280 nm), and polysaccharides (Abs 490 nm) in the polymeric material.

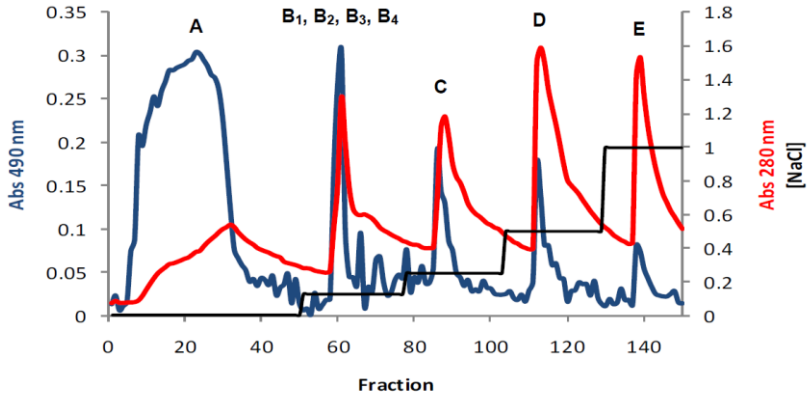

Figure 3.1 - DEAE-Sepharose FF anion exchange chromatographic profiles for the HMWM obtained from the infusions of *F. angustifolia* dried leaves.

One not retained neutral fraction (A), eluted with buffer, four retained acidic fractions eluted with buffer containing 0.125 M NaCl (B_1, B_2, B_3, and B_4), and three retained acidic fractions eluted with buffer containing 0.250, 0.500, and 1.0 M NaCl (C, D, and E, respectively), were obtained. The presence of these chromatographic bands evidenced the heterogeneity of the polymeric material contained in the infusions of *F. angustifolia* dried leaves.

The mass yield, total carbohydrate material content, and the monomeric composition of the polysaccharides present in the various fractions were determined, and are shown in **Table 3.4**.

The neutral (A), and one of the less acidic fractions (B_1) were the richest in carbohydrate material, with 60.2 and 37.2%, respectively. These fractions, which comprised 17.0 and 20.9% of the recovered polymeric material, respectively, contained polysaccharides with a monomeric composition rich in arabinosyl and galactosyl residues, suggesting the presence of arabinogalactans in both fractions.

Table 3.4- Mass yield, total sugar content, and monosaccharide composition of the various fractions obtained by anion exchange chromatography on DEAE-Sepharose FF of the HMWM extracts from the infusions of *Fraxinus angustifolia* dried leaves.

	Yield[a] (mass%)	Total Sugar (mass%)	Monosaccharide Composition (mol %)							
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
A	17.0	60.2	3.2	0.5	26.0	4.0	6.3	29.9	20.4	9.8
B_1	20.9	37.2	6.1	0.6	19.6	3.1	2.4	22.0	8.9	37.3
B_2	7.6	11.2	7.8	0.4	7.8	2.3	3.6	9.1	45.3	23.8
B_3	6.9	12.7	9.5	0.5	7.8	1.8	3.9	9.0	34.9	32.7
B_4	9.8	10.2	9.4	0.6	7.6	2.3	3.9	9.0	42.4	24.7
C	10.1	32.5	4.6	0.7	7.5	3.2	4.0	11.6	17.8	50.5
D	17.3	14.9	10.0	0.7	8.4	1.5	4.3	10.2	41.4	23.4
E	10.4	16.4	9.6	0.4	5.4	1.4	3.1	6.6	42.3	31.2

[a]- expressed relatively to the HMWM recovered

The polysaccharides present in the A fraction also exhibited glucosyl residues. Since no starch was detected by the iodine assay, the presence of other glucose-rich polysaccharides, such as xyloglucans seems possible. Besides the arabinosyl and galactosyl residues, the monomeric composition of the B_1 fraction polysaccharides was also rich in uronic acid residues, contrary to the polysaccharides from the A fraction, which contained the lowest proportion of uronic acid residues. The presence of higher proportions of uronic acid residues in the composition of the polysaccharides from the B_1 fraction suggested the presence of higher proportions of pectic polysaccharides in the retained fraction, which could have contributed for their retention and subsequent elution with a solution with higher ionic strength.

Similarly to the B_1 fraction, the fractions B_2, B_3, and B_4 were also eluted with buffer containing 0.125 M NaCl, but contained lower proportions of carbohydrate material: 11.2, 12.7, and 10.2%, respectively. The B_2, B_3, and B_4 fractions were also less abundant than the B_1: 7.6, 6.9, and 9.8% of the recovered polymeric material, respectively. The C fraction contained 32.5% of carbohydrate material, which is the third highest value, and comprised 10.1% of the recovered polymeric material. The D and E fractions contained 14.9 and 16.4 % of carbohydrate material, and accounted for 17.3 and 10.4% of the recovered polymeric material. The polysaccharides recovered in the B_2, B_3, B_4, C, D, and E fractions presented a

monomeric composition that mainly comprised uronic acid, which suggested the presence of pectic polysaccharides, and glucosyl residues. In comparison with the A fraction, all of the remaining fractions exhibited a darker coloration. The chromatographic elution profile also showed higher absorbance values at 280 nm, for the bands that corresponded to the acidic fractions, suggesting the presence of higher proportions of phenolic compounds in these fractions. Thus, it is possible that the glucosyl residues are present as the glycone moiety of glycosides.

The multiplication of each fraction mass yield by the respective carbohydrate material content allows the calculation of the proportion of carbohydrate material recovered in the various collected fractions. Through this, it is evidenced that the polysaccharides present in the infusions of *Fraxinus angustifolia* dried leaves were mainly recovered in the neutral A and in the less acidic B_1 fractions: 36.2, and 27.5%, respectively, which seem to mostly contain arabinogalactans, pectic and also glucose-rich polysaccharides, possibly xyloglucans.

3.2.2. Solid phase extraction and anion exchange chromatography

The elution of the HMWM on DEAE-Sepharose FF without any type of pre-treatment left some colored compounds retained in the column. This material, which possibly contained phenolic compounds, could not be removed even using the cleaning solutions recommended by the manufacturer. The retention of these compounds may explain the low total material recovery of 10.2% that was registered. As the recovery was low, another HMWM fractionation approach was tested by the use of solid phase extraction on C_{18} cartridges, eluted with distilled water and then with acidic methanol, yielding a not retained and a retained fraction, respectively. The not retained, and the retained fractions collected were submitted to DEAE-Sepharose FF anion exchange chromatography, and the chromatographic profiles obtained are shown in **Figure 3.2**. The chromatographic elution profile of the not retained fraction (**Figure 3.2 a)**) showed a neutral fraction (A_{nret}), eluted with buffer, one acidic fraction eluted with buffer containing 0.125 M NaCl (B_{nret}), and two retained acidic fractions eluted with buffer containing 0.250, and 0.500 NaCl (C_{nret}, and D_{nret}, respectively). When the retained material was

eluted (**Figure 3.2 b)**), one neutral fraction (A_{ret}), eluted with buffer, two acidic fractions eluted with buffer containing 0.125 M NaCl (B_{1ret}, and B_{2ret}), and three retained acidic fractions eluted with buffer containing 0.250, 0.500, and 1.000 NaCl (C_{ret}, D_{ret}, and E_{ret} respectively).

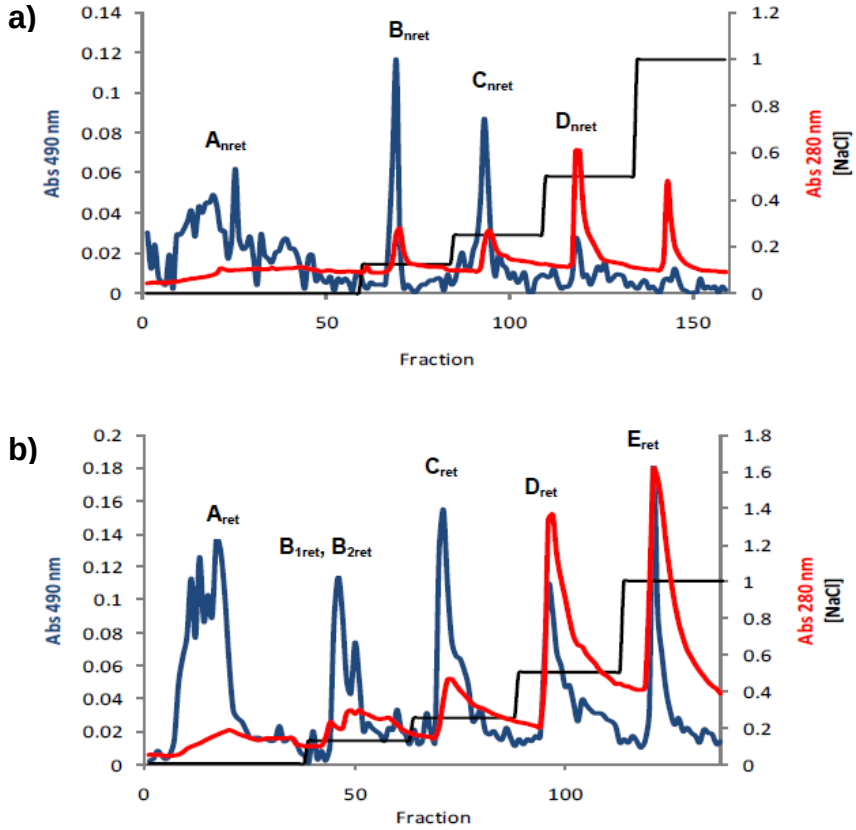

Figure 3.2- DEAE-Sepharose FF anion exchange chromatographic elution profiles for the **a)** not retained and **b)** retained material obtained from the solid phase extraction of the HMWM from the infusions of *F. angustifolia* dried leaves.

The chromatographic elution profile of the material retained in the solid phase extraction C_{18}, and subsequently eluted with acidic methanol, presented higher values for the absorbance registered at 280 nm, suggested the presence of higher proportion of total phenolic compounds. The mass yield, carbohydrate material content, and monomeric composition of the various fractions was determined, and is presented in **Table 3.5**.

Table 3.5 shows that for the fractionation of the not retained polymeric material, the A_{nret} and B_{nret} fractions were the most abundant, and also the richest in sugars, comprising 35.4 and 30.8% of the recovered material, containing 64.6 and 55.5% of carbohydrate material, respectively.

Table 3.5- Mass yield, total sugar content and monosaccharide composition of the various fractions obtained by solid phase extraction followed by anion exchange chromatography on DEAE-Sepharose FF of the HMWM extracts from the infusions of *F. angustifolia* dried leaves.

	Yield[a] (mass%)	Total Sugar (mass%)	Monosaccharide Composition (mol %)							
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
			Not retained polymeric material							
A_{nret}	35.4	64.6	1.1	0.0	4.7	1.6	2.7	14.1	12.9	63.0
B_{nret}	30.8	55.5	5.1	0.4	9.3	2.5	1.6	16.1	9.6	55.4
C_{nret}	26.2	49.4	0.4	0.7	3.2	4.9	1.3	6.0	4.9	78.7
D_{nret}	7.6	38.4	9.9	0.0	2.1	7.2	11.6	4.1	50.6	14.5
			Retained polymeric material							
A_{ret}	26.5	34.6	3.0	0.8	29.5	4.9	6.2	26.2	15.5	13.9
B_{1ret}	23.4	39.2	6.5	0.0	17.2	1.9	3.3	18.1	11.9	41.2
B_{2ret}	4.6	25.1	5.6	0.0	6.7	0.0	0.0	10.0	26.1	51.6
C_{ret}	17.4	31.2	6.2	0.0	10.9	4.3	2.7	12.1	20.1	43.7
D_{ret}	14.5	20.6	10.7	0.0	8.6	1.8	3.5	9.1	45.1	21.2
E_{ret}	13.6	13.6	16.7	0.0	5.0	0.0	2.8	4.9	50.7	19.9

[a]- expressed relatively to the HMWM recovered

It was also possible to observe that the A_{nret} and B_{nret} fractions exhibited higher carbohydrate material contents than the HMWM extracts that were fractionated, evidencing a sugar enrichment in these fractions. These fractions also exhibited higher proportions of carbohydrate material than the most sugar rich fractions that resulted from the fractionation of the retained material (the A_{ret}, and B_{1ret} fractions that contained 34.6 and 39.2% of carbohydrate material, respectively). The A_{ret} and B_{1ret} fractions were the most abundant from the fractionation of the retained material, accounting for 26.5 and 23.4% of the recovered material.

Calculating the proportion of carbohydrate material recovered in each fraction that resulted from the fractionation of the not retained and retained materials, it was possible to observe that the A_{nret} and B_{nret} fractions together accounted for 71.6% of the recovered carbohydrate material, and that the A_{ret} and B_{1ret} fractions comprised 61.6% of the recovered carbohydrate material, respectively.

The polysaccharides present in the A_{nret} and B_{nret} fractions showed a similar monomeric composition, rich in uronic acid residues, suggesting that the polysaccharides from the neutral fraction should contain methylesterified uronic

acid residues or that some sterical phenomena should have occurred due to the occurrence of branching neutral polysaccharides. The polysaccharides of the A_{ret} and B_{1ret} fractions also presented a similar monomeric composition that mainly comprised arabinosyl, galactosyl, glucosyl, and uronic acid residues. The material present in the A_{ret} and B_{1ret} fractions were darker than the A_{nret} and B_{nret} fractions. This fact may indicate the presence of higher proportions of phenolic compounds in the fractions from the retained material, which is in agreement with the higher absorbance values at 280 nm that were registered for the fractioning of the retained material, as shown in **Figure 3.2**. Thus, it is possible that some of the arabinosyl, galactosyl, and glucosyl residues appear as glycone moieties of glycosides present in the fractions from the retained material.

The fractionation of the polymeric material contained in the HMWM extracts from the infusions of *F. angustifolia* dried leaves by the use of solid phase extraction followed by anion exchange chromatography on DEAE-Sepharose FF allowed to obtain fractions enriched in carbohydrate material, which resulted from the fractionation of the not retained material of the infusions. Nevertheless, the total material recovery of the fractionation procedure was lower than 10.0%.

3.2.3. Sequential ethanol precipitation, solid phase extraction and anion exchange chromatography

Ethanol precipitation of the polymeric material from *F. angustifolia* HMWM extracts was performed and a total material recovery of 91.2% was obtained. After the fractionation process, the HMWM collected in each fraction was immediately weighed after freeze-drying. It was possible to observe that the sum of the HMWM mass collected in each fraction was lower than the mass of the HMWM submitted to the ethanol fractionation procedure. This could be explained by the hygroscopicity of carbohydrates, such as pectins, which were reported to contain amounts of water that might reach to 48.3% (Copikova *et al*., 2006). Therefore, although stored in a desiccator with phosphorous pentoxide as desiccating agent, it seems plausible that the HMWM weighed at the beginning of the ethanol fractionation contained some amount of water.

The mass yield, carbohydrate and total phenolic compound contents, and also the monomeric composition of the polysaccharides recovered in the various fractions were determined, and are shown in **Table 3.6**.

Table 3.6- Mass yield, total sugar content and monosaccharide composition, and total phenolic compounds content of the various fractions obtained by sequential fractionation by ethanol precipitation, solid phase extraction, and anion exchange chromatography on DEAE-Sepharose FF of the HMWM extracts from the infusions of *F. angustifolia* dried leaves.

	Yield[a] (mass%)	Total Sugar (mass%)	Monosaccharide Composition (mol %)								Total Phenolics (mass %)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
WI_{ppt}	20.3	10.2	8.7	0.7	5.7	0.0	7.0	21.7	29.3	26.9	nd
Et_{50}	17.9	21.2	9.1	0.6	11.3	3.8	4.0	18.2	29.4	23.6	8.9
SP_{nret}	41.1	29.2	6.5	0.8	13.5	4.1	3.8	27.1	9.1	35.2	5.6
SP_{ret}	58.9	11.2	17.9	0.5	6.3	2.2	3.9	6.4	48.9	13.9	7.1
Et_{75}	21.1	31.8	6.8	0.5	19.9	3.5	4.4	28.0	21.1	15.8	7.7
SP_{nret}	40.7	38.0	5.7	0.5	17.2	2.1	4.5	35.7	8.4	25.9	4.9
A	29.0	51.1	2.9	0.4	17.0	0.9	5.0	49.9	4.5	19.3	nd
B	28.1	54.0	5.5	0.4	23.8	1.9	1.8	38.4	3.5	24.8	nd
C	29.5	37.6	8.4	0.9	14.0	3.2	3.5	22.8	6.7	40.4	nd
D	8.0	22.1	5.5	0.0	12.4	2.5	6.5	24.9	18.3	29.9	nd
E	5.4	11.3	8.1	0.0	9.6	0.0	0.0	18.2	29.0	35.1	nd
SP_{ret}	59.3	14.2	14.4	0.5	10.0	2.7	4.1	11.2	41.7	15.3	9.1
SN	40.7	27.0	9.4	1.6	14.5	1.7	7.2	10.4	44.7	10.5	11.9
SP_{nret}	26.9	25.9	6.3	6.3	7.8	4.1	8.0	17.6	29.0	20.9	6.7
SP_{ret}	73.1	22.8	13.0	0.0	11.6	0.6	5.4	5.7	53.0	10.8	11.3

[a]- expressed relatively to the HMWM recovered; nd- not determined

The results show that approximately 40% of the polymeric material contained in the HMWM extracts remained soluble in 75% ethanol aqueous solutions. For the remaining material, it was observed that similar proportions were insoluble in distilled water and precipitated in 50% and 75% ethanol aqueous solutions: 20.3, 17.9, and 21.1%, respectively. The Et_{50}, Et_{75}, and SN fractions were the sugar richest, with 21.2, 31.8, and 27.0% of carbohydrate material, while the WI_{ppt} fraction only contained 10.2% of carbohydrate material. Nevertheless, no sugar enrichment was observed relatively to the carbohydrate content of the HMWM that

was fractionated, which presented approximately 30% of carbohydrate material. The total phenolic compounds content of the Et_{50} and Et_{75} fractions was similar, while the SN fractions showed a slightly higher proportion of total phenolic compounds.

The calculation of the proportion of carbohydrate material recovered in each fraction evidenced that 46.7% of the recovered carbohydrate material remained soluble in 75% ethanol aqueous solutions, while 28.4, and 16.1% precipitated in 75%, and 50% ethanol solutions, respectively.

The polysaccharides from the Et_{50}, Et_{75}, and SN fractions presented a similar monomeric composition that contained mostly arabinosyl, galactosyl, glucosyl, and uronic acid residues, however present in distinct proportions. The SN fraction contained higher proportions of glucosyl residues, concomitant with lower proportions of galactosyl, and uronic acid residues, in comparison with the polysaccharides from the Et_{50}, and Et_{75} fractions. It was observed that the polysaccharides that precipitated in solutions with lower proportion of ethanol contained higher proportions of uronic acid residues. This had been also observed during the fractionation of olive extracts rich in pectic substances with increasing ethanol concentrations (Silva, 1993).

The Et_{50}, Et_{75}, and SN fractions, because they presented the highest proportions of carbohydrate material, were further submitted to C_{18} solid phase extraction, and total mass recoveries of 81.7, 73.4, and 90.9% were obtained, respectively. Although no visible compound retention was detected during the cartridge elution, a possible compound retention in the cartridge packing due to hydrophobic interactions may have occurred. However, it is also possible that the hygroscopicity of the materials fractionated can explain the discrepancy of the mass added to the column and the mass recovered.

The mass yield, carbohydrate and total phenolic compound contents, and also the monomeric composition of the polysaccharides recovered in the various fractions were determined, and are shown in **Table 3.6**. For the solid phase extraction of the Et_{50} and Et_{75} fractions, approximately 40% of the material was recovered as SP_{nret}. Considering the carbohydrate content of the various fractions and its relative abundance, it was also possible to observe that for these fractions

almost 65% of the carbohydrate material was recovered as SP_{nret}, evidencing the more pronounced hydrophilic nature of the polysaccharides present in these fractions. According to the results of the monomeric composition, it was possible to observe that the not retained polysaccharides that resulted from the solid phase extraction of the Et_{50} and Et_{75} fractions exhibited a similar composition that mostly comprised arabinosyl, galactosyl, and uronic acid residues.

For the SN fraction, nearly 75% of the material was recovered as SP_{ret} material that accounted for approximately 70% of the recovered carbohydrate material, evidencing the more pronounced hydrophobic nature of the compounds, including the carbohydrate material, present in these fractions. The carbohydrate material was composed of high proportion of glucosyl residues. The SP_{ret} material obtained from the solid phase extraction of the SN fraction presented the highest proportion of total phenolic compounds, therefore it seems plausible that part of the glucosyl residues detected might be linked to the phenolic compounds. It was also observed that the polysaccharides recovered in the SP_{ret} material from the solid phase extraction of the Et_{50} and Et_{75} fractions exhibited a similar composition.

The SP_{nret} material from the solid phase extraction of the Et_{75} fraction presented the highest proportion of carbohydrate material, and was further submitted to anion exchange chromatography on DEAE-Sepharose FF. The mass yield, carbohydrate material content, and also the monomeric composition of the polysaccharides recovered in the various fractions were determined, and are shown in **Table 3.6**.

The neutral (A), and the less acidic (B) fractions were the sugar richest, containing 51.1 and 54.0% of carbohydrate material, respectively. These fractions, which were also abundant, represented a sugar enrichment relatively to the SP_{nret} material from the solid phase extraction of the Et_{75} fraction, which contained 38.0% of carbohydrate material. These fractions together accounted for almost 70% of the carbohydrate material recovered, which exhibited a similar monomeric composition that comprised high proportions of galactosyl residues, and also arabinosyl, and uronic acid residues, suggesting the presence of the arabinogalactans, and pectic polysaccharides that according to the sugar and

methylation analysis (**Tables 3.1** and **3.2**) seemed to be present in the HMWM extracts from the infusions of *F. angustifolia* dried leaves.

The anion exchange chromatography fractionation procedure presented a total material recovery of 48.5%, suggesting a possible interaction between the gel and the sample compounds, although no visible interaction was observed. This recovery value was much lower than the recoveries registered for the ethanol precipitation (91.2%), and for the solid phase extraction of the Et_{50}, Et_{75}, and SN fractions (81.7, 73.4, 90.9%, respectively). Thus, it is possible that the poor carbohydrate content of the HMWM could have contributed for the lower recoveries obtained for the anion exchange chromatography fractionation.

3.3. Influence of temperature and time of extraction on the mass yield and composition of the HMWM extracted

3.3.1. Fraxinus angustifolia dried leaves

As referred in **Section 3.1**, the HMWM extracts obtained from the infusions of *F. angustifolia* dried leaves, prepared according to the popular tradition, exhibited a carbohydrate material content of approximately 30.0% (**Table 3.1**). This poor carbohydrate material content could have contributed for the low recovery of the anion exchange chromatography technique used during the fractionation of the HMWM from the infusions of *F. angustifolia* dried leaves, as referred in **Section 3.2**. It were also observed very low mass yields for the HMWM extracts obtained from the infusions of *F. angustifolia* prepared according to the popular tradition, which consisted in the infusion of the dried leaves in previously boiled distilled water during 5 min. Therefore, two new methodologies for the preparation of the infusions from *F. angustifolia* dried leaves were tested. Both methodologies consisted in the extensive water extraction of the *F. angustifolia* dried leaves during a total of 4 h, divided in two parts with equal duration. One of the methodologies consisted in an infusion of the vegetable material with distilled water at 50ºC (INF_{50}), while in the other methodology a decoction consisting in the use of boiling distilled water (INF_{100}) during the extraction was performed. The extracts obtained using the distinct methodologies were concentrated, and

dialysed, in order to remove the salts, and obtain the high molecular weight material fraction (HMWM).

The mass yield, carbohydrate material and total phenolic compounds contents, of the HMWM obtained from the infusion at 50ºC, and from the decoction of *F. angustifolia* dried leave were determined. Sugar analysis was performed for the evaluation of the monomeric composition of the carbohydrate material present in the various HMWM. The results, which are shown in **Table 3.7**, were compared with those of the HMWM that was obtained from the infusion of *F. angustifolia* dried leaves in previously boiled distilled water during 5 minutes. These results are also presented in **Table 3.7**, and represented as INF.

Table 3.7- Mass yield, carbohydrate material and total phenolic compound contents, and monosaccharide composition of the various extracts from the hot water extraction of *F. angustifolia* dried leaves.

	Yield[a]	Total Sugar	Monosaccharide Composition (mol %)								Total Phenolics
	(mass%)	(mass%)	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	(mass %)
INF	0.2	29.4	8.2 (10.1)[b]	1.0 (1.2)	15.2 (18.7)	2.9 (3.6)	6.1 (7.5)	17.7 (21.8)	30.1 (37.1)	18.9	0.085
INF_{50-1}	1.2	25.4	22.1	0.0	5.3	0.7	2.6	6.8	48.3	14.2	16.0
INF_{50-2}	0.5	30.0	20.4	0.0	5.0	1.2	2.6	6.7	49.1	15.1	12.8
DEC_{100-1}	2.2	57.0	4.2 (12.8)[b]	0.0 (0.0)	7.6 (23.2)	1.6 (4.9)	2.7 (8.3)	5.3 (16.2)	11.2 (34.3)	67.3	4.2
DEC_{100-2}	1.6	76.1	2.5	0.0	11.6	1.8	1.5	5.5	5.3	71.7	3.7

[a]- expressed relatively to the vegetable material dry weight; [b]- molar % calculated without the UA residues contribution

According to the results presented in **Table 3.7,** it is evident that the longer extraction times used in the methodologies INF_{50} and DEC_{100}, allowed to obtain HMWM yields higher than those obtained with the methodology INF, where an infusion time of 5 min was used. From the methodologies that used an extraction time of 4 h, DEC_{100} showed a higher HMWM yield, which is explained by the use of an extraction temperature of 100 ºC, while in INF_{50} a temperature of 50ºC was used. It was also observed that the use of boiling distilled water allowed to obtain a HMWM yield in the 2nd 2h of the extraction of 1.6%, which was still superior than those obtained when using previously boiled distilled water during 5 min. or distilled water at 50ºC during a total of 4 h. Besides displaying the highest HMWM yields, for both parts of the extraction, DEC_{100} also contained the HMWM with the

highest carbohydrate material contents: 57.0 and 76.1%, for the 1st and 2nd 2 h of the hot water extraction, while the INF, and the INF_{50} extracts showed a similar carbohydrate material content. The HMWM from the INF_{50} extracts, contained carbohydrate material that comprised mainly rhamnosyl, glucosyl, and uronic acid residues. The INF_{50-}, and INF_{50-2} extracts contained the highest proportion of total phenolic compounds. Since rhamnosyl, and glucosyl residues are frequently reported as glycones of glycosides, it seems possible that some of these sugar residues are present linked to phenolic compounds. The monomeric composition of the carbohydrate material contained in the HMWM from the DEC_{100} extracts, evidenced the presence of high proportion of uronic acid residues. When the molar composition of the DEC_{100-1} extract was calculated without the contribution of the uronic acid residues (values presented in brackets), and compared with the monomeric composition of the INF extract, calculated in the same manner, it was possible to observe that these extracts presented a similar monosaccharide composition. This evidenced that, despite the higher proportion of uronic acid residues exhibited by the DEC_{100-1} extract, the neutral sugar residues are present in a proportion similar to the neutral sugar proportion registerd for the INF extract. This suggests that the boiling water extracts of *F. angustifolia* dried leaves, obtained during the 1st 2h, contain proportions of pectic polysaccharides higher than the infusions prepared according to the popular tradition, but the polysaccharides composed of neutral sugars are present in similar proportions.

3.3.2. Mentha suaveolens dried shoots and *Pterospartum tridentatum* dried inflorescences

The results reported in **Section 3.3.1** evidenced that for *F. angustifolia* dried leaves, longer extraction times, combined with the use of higher water temperatures, allowed the preparation of extracts that contained higher HMWM yields, and also higher carbohydrate material contents. The preparation of the infusions of *M. suaveolens*, and *P. tridentatum* involved the use of distinct vegetable materials, which might behaved differently in comparison with the *F. angustifolia* dried leaves, thus the three methodologies referred in **Section 3.3.1**

were also evaluated for the dried shoots and dried inflorescences from *M. suaveolens*, and *P. tridentatum*, respectively.

The mass yield, carbohydrate material and total phenolic compound contents, together with the monomeric composition of the HMWM obtained using the various methodologies for the extraction of *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences, were determined, and are shown in **Table 3.8**.

Table 3.8- Mass yield, carbohydrate material and total phenolic compound contents, and monosaccharide composition of the various extracts from the hot water extraction of *M. suaveolens* dried shoots and *P. tridentatum* dried inflorescences.

	Yield[a] (mass%)	Total Sugar (mass%)	Monosaccharide Composition (mol %)								Total Phenolics (mass %)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
M. suaveolens											
INF	0.5	12.2	4.2 (7.2)[b]	0.0 (0.0)	11.7 (20.1)	2.7 (4.6)	4.7 (8.1)	11.0 (18.9)	23.9 (41.1)	41.8	25.5
INF_{50-1}	1.3	27.1	3.4	0.4	10.3	0.9	1.4	16.1	9.1	58.4	22.2
INF_{50-2}	0.6	28.5	1.6	0.2	4.6	1.0	2.0	27.4	14.8	48.4	21.6
DEC_{100-1}	2.5	46.4	1.8 (6.2)	0.0 (0.0)	5.3 (18.3)	0.9 (3.1)	3.0 (10.4)	8.6 (29.8)	9.4 (32.5)	71.1	14.5
DEC_{100-2}	1.7	69.7	1.6	0.0	6.8	0.8	1.7	8.1	4.8	76.4	10.2
P. tridentatum											
INF	0.7	36.1	1.2 (2.1)[b]	0.0 (0.0)	4.2 (7.3)	1.6 (2.8)	4.9 (8.5)	6.4 (11.1)	39.1 (68.0)	42.5	21.3
INF_{50-1}	0.9	36.0	1.4	0.3	8.2	1.8	9.4	13.0	35.6	30.3	23.9
INF_{50-2}	0.7	28.1	1.1	0.2	6.7	1.8	9.4	9.1	46.0	25.7	26.2
DEC_{100-1}	3.4	44.1	0.9 (1.7)	0.0 (0.0)	4.7 (8.7)	1.3 (2.4)	7.2 (13.3)	7.5 (13.9)	32.7 (60.6)	46.0	19.4
DEC_{100-2}	1.5	68.8	1.0	0.0	10.8	1.0	4.0	8.6	11.4	63.2	11.2

[a]- expressed relatively to the vegetable material dry weight; [b]- molar % calculated without the UA residues contribution

Distinct extracts were obtained through the infusion with previously boiled distilled water during 5 min (INF), the infusion with distilled water at 50ºC during a total of 4 h (INF_{50}), and the decoction with boiling distilled water during a total of 4 h (DEC_{100}). As evidenced for the *F. angustifolia* dried leaves (**Table 3.7**), the results showed that also for *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences, the use of longer extraction times in INF_{50}, and DEC_{100} methodologies allowed to obtain higher HMWM yields than the obtained with a shorter extraction time in the INF methodology. This effect is most noticeable for

the DEC_{100-1} extracts, which were obtained in the 1st 2h, particularly those prepared with the dried inflorescenses of *P. tridentatum*. Although the HMWM yields for the DEC_{100-1} extracts were higher than those of the DEC_{100-2} extracts, the amount of material extracted with boiling distilled water in the 2nd 2h was still relevant. This suggests that even though the extraction rate diminished throughout the boiling water extraction process, according to the usual kinetics of the extraction processes, still a large amount of material was extracted when the vegetable material was once again suspended in boiling distilled water for an additional 2h. This allowed extracting more 68% of the material extracted in the first 2h for *M. suaveolens* dried shoots, and more 44% for *P. tridentatum* dried inflorescences.

It was also observed that the various HMWM extracts obtained with DEC_{100} methodology contained the highest carbohydrate material contents: 46.4%, in the 1st 2h, and 69.7%, in the 2nd 2h, for *M. suaveolens*, and 44.1%, in the 1st 2h, and 68.8%, in the 2nd 2h, for *P. tridentatum*. The HMWM from the 2nd 2h of the boiling water extraction contained higher proportions of carbohydrate material than the ones from the 1st 2h, evidencing a higher polysaccharide extractability during the 2nd 2h of the extraction process. For the phenolic compounds, the oposite behaviour was registered, with the highest proportions of phenolic compounds being extracted in the 1st part of the extraction process.

For *M. suaveolens* dried shoots, it was observed that the HMWM from the INF, INF_{50}, and DEC_{100} extracts presented carbohydrate material with a monomeric composition rich in uronic acid residues. Besides the high proportions of uronic acid residues, the HMWM from the INF extracts contained relevant proportions of arabinosyl, galactosyl, and glucosyl residues, while the HMWM from the INF_{50} extracts presented galactosyl residues. For *P. tridentatum* dried inflorescences, the HMWM from the INF, INF_{50}, and DEC_{100} extracts contained carbohydrate material rich in uronic acid residues, together with relevant proportions of glucosyl residues.

The monomeric composition of the carbohydrate material contained in the extracts from *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences suggests the presence of high proportions of pectic polysaccharides that seem to

be mixed with other polysaccharides, similarly to what was observed for the extracts from *F. angustifolia* dried leaves. Also, it was observed that, as evidenced for *F. angustifolia*, the proportion of neutral sugar residues, estimated without the contribution of the uronic acid residues, was similar in the INF, and $DEC_{100\text{-}1}$ extracts from both *M. suaveolens*, and *P. tridentatum*. This suggests the presence of similar proportions of neutral sugar rich polysaccharides in the *M. suaveolens*, and *P. tridentatum* boiling water extracts, obtained during the 1st 2h, and in the infusions prepared according to the popular tradition, despite the higher proportion of pectic polysaccharides present in the boiling water extracts.

3.4. Concluding remarks

The infusions of *P. tridentatum* dried leaves prepared according to the popular tradition, and using proportions of vegetable material to water volume similar to those frequently used yielded very low amounts of HMWM. The use of higher proportions allowed to increase the HMWM yields, however only approximately 30% of carbohydrate material, mainly containing pectic polysaccharides, AG-II, and possibly xyloglucans, was obtained. The HMWM also contained approximately 8% of total phenolic compounds, which seemed to have contributed for the DPPH-scavenging activity of the polymeric material. It was also observed that the fractionation of the HMWM using anion exchange chromatography, alone or as part of a sequential procedure, yielded low total material recoveries.

For all the vegetable materials, the hot water extracts prepared by decoction using boiling distilled water during a total of 4 h, divided in two periods of 2 h, originated higher HMWM yields, in comparison with the extracts prepared with lower water temperature, or prepared with lower infusion times. It was also observed that the HMWM from the extracts prepared with boiling distilled water during 4 h, contained higher proportions of carbohydrate material, relatively to the other methodologies used. Thus, the methodology that consists in the extensive extraction of the distinct vegetable materials with boiling distilled water, during a total of 4 h, seems to be the more adequate, since it provides higher yields of

HMWM that contains high proportions of polysaccharides, which are the main subject of this PhD thesis.

The monomeric composition of the HMWM obtained by the three methodologies used in the preparation of the extracts suggested the presence of the same type of polysaccharides, particularly pectic polysaccharides, possibly mixed with other polysaccharides. Despite the higher proportion of uronic acid residues detected in the boiling water extracts, the neutral monosaccharide residues seem to be present in proportions similar to the registered for the infusions prepared according to the popular tradition. Therefore, although the goal of this PhD thesis was to structurally characterize the polysaccharides present in the infusions prepared according to the popular tradition, which consisted in the infusion of the vegetable material in previously boiled distilled water during 5 min, the decoction of the vegetable material during a total of 4 h, will also provide the same type of polysaccharides. Moreover, the decoction of the distinct vegetable materials will provide the sufficient amounts of HMWM with an adequate carbohydrate content, which will allow to structurally characterize the polysaccharides present, and also to evaluate their biological activity.

CHAPTER 4

STRUCTURAL FEATURES OF *FRAXINUS ANGUSTIFOLIA* POLYSACCHARIDES EXTRACTED WITH HOT WATER

In this chapter the structural features of the polysaccharides from the extensive hot water extraction of *Fraxinus angustifolia* dried leaves during a total period of 4 h (2 h + 2 h) are described. The extracted material was dialysed and the high molecular weight material (HMWM) was fractionated according to its solubility in aqueous ethanol solutions. The carbohydrate and phenolic compounds content of the various fractions obtained were determined, as well as the antioxidant activity, expressed as the DPPH radical scavenging activity. The carbohydrate composition of the polysaccharides present in the various fractions obtained was evaluated. The fractions precipitated with 50 and 75% of aqueous ethanol solutions were further fractionated by anion exchange chromatography on DEAE-Sepharose FF. The carbohydrate composition, the type of glycosidic linkages and the degree of methyl esterification of the polysaccharides present in the various fractions obtained were evaluated. Selected fractions, rich in pectic polysaccharides, were also digested with *endo*-polygalacturonase, fractionated by size exclusion chromatography on Bio Gel and analysed by ESI-MS.

4.1. Isolation and fractionation of the polysaccharides from the HMWM

4.1.1. Ethanol fractionation

The HMWM obtained from the decoctions of *F. angustifolia* dried leaves was suspended in distilled water and the material insoluble in cold water was separated, yielding the WI_{ppt} fraction. The remaining HMWM was fractionated by ethanol precipitation, yielding three fractions: Et_{50}, HMWM insoluble in 50% aqueous ethanol solution; Et_{75}, HMWM insoluble in 75% aqueous ethanol solution; and SN, HMWM soluble in 75% aqueous ethanol solution. The ethanol fractionation procedure was performed in duplicate and HMWM mass recoveries of 88.9 ± 5.1% and 88.2 ± 4.9% for the 1^{st} and the 2^{nd} 2 h of the hot water extraction process were registered, evidencing the hygroscopicity of the carbohydrate material present in the HMWM.

The mass yield, total carbohydrate and total phenolic compound contents, and the EC_{50} values, which expressed the DPPH scavenging activity of the various

fractions, together with the composition of the polysaccharides recovered in the various fractions obtained by ethanol precipitation are shown in **Table 4.1**.

Table 4.1- Mass yield, total sugar content and monosaccharide composition, and DPPH scavenging activity, expressed by the EC_{50} value, of the Et_{50} and Et_{75} fractions obtained by anion exchange chromatography of the HMWM from the decoctions of *F. angustifolia* dried leaves.

	Yield[a] (mass %)	Total Sugars (mass %)	Monosaccharide Composition (mol %)							Total Phenolics (mass %)	EC_{50} (mg/mL)
			Rha	Ara	Xyl	Man	Gal	Glc	UA		
1st 2h											
Starting HMWM		57.0	4.2	7.6	1.6	2.7	5.3	11.2	67.3	4.2	nd
WI_{ppt}	10.4	3.3	17.5	4.9	0.0	0.0	5.6	39.3	32.8	nd	nd
Et_{50}	45.8	81.0	2.1	3.5	1.6	0.5	3.2	3.0	86.3	1.6	1.69
Et_{75}	15.6	55.8	3.4	11.2	2.6	2.6	12.8	9.8	57.8	4.3	0.52
SN	28.2	33.0	8.4	17.0	1.2	11.0	5.3	33.9	23.3	14.2	0.10
2nd 2h											
Starting HMWM		76.1	2.5	11.6	1.8	1.5	5.5	5.3	71.7	3.7	nd
WI_{ppt}	9.2	16.2	10.6	8.5	0.0	0.0	10.5	35.7	34.8	nd	nd
Et_{50}	49.8	90.1	1.6	4.3	1.5	0.2	2.9	1.1	88.5	1.4	> 2.5
Et_{75}	18.8	84.7	2.5	10.6	2.6	1.1	9.5	4.1	69.8	2.5	1.50
SN	22.2	38.8	5.3	35.7	1.2	8.0	4.5	22.3	23.1	10.7	0.19

[a] - expressed relatively to the HMWM recovered; [nd] - not determined

The Et_{50} fractions were the most abundant and sugar rich fractions, comprising 45.8, in the 1st 2h, and 49.8%, in the 2nd 2h, of the HMWM recovered, with 81.0 and 90.1% of carbohydrate material, respectively. These fractions were the poorest in total phenolic compounds with antioxidant activity, determined by Folin-Ciocalteau method, and therefore also exhibited the highest EC_{50} values, evidencing its lower antioxidant activity. The Et_{75} fractions comprised 15.6, in the 1st 2h, and 18.8%, in the 2nd 2h, of the HMWM recovered, with 55.8 and 84.7% of carbohydrate material, respectively. These fractions were slightly richer in total phenolic compounds with antioxidant activity than the Et_{50} fractions, and therefore also exhibited lower EC_{50} values, evidencing its higher antioxidant activity. The SN fractions accounted for 28.2, in the 1st 2h, and 22.2%, in the 2nd 2h, of the HMWM recovered, with 33.0 and 38.8% of carbohydrate material, respectively. These fractions were the richest in total phenolic compounds with antioxidant activity, exhibiting the lowest EC_{50} values, which evidenced its higher antioxidant activity.

The WI_{ppt} fractions, which only accounted for 10.4, in the 1st 2h, and 9.2%, in the 2nd 2h, of the HMWM recovered, were also the poorest in carbohydrate material: 3.3, in the 1st 2h, and 16.2%, in the 2nd 2h.

The total phenolic compounds were determined by the Folin-Ciocalteau method, and were expressed as gallic acid equivalents. This could have resulted in an underestimation of the total phenolic compounds content, because of the high antioxidant activity of gallic acid, which has been reported to be much higher than those of chlorogenic acid, rutin, Trolox, and vitamin C (Kim *et al*., 2002). This seems to be supported by the importance of the gallic acid moiety in the antioxidant activity of flavonoids (Rice-Evans *et al*., 1997).

The Folin-Ciocalteau assay has been used for many years as a measure of the total phenolic content of natural products, but the method principle is based on an oxidation/reduction reaction and, thus it can be also considered as another method for the determination of antioxidant activity (Prior *et al*., 2005). Since it was observed that the fractions that contained the highest proportions of total phenolic compounds, determined by the Folin-Ciocalteau method, also showed the highest antioxidant activities, evaluated by the DPPH scavenging activity, it seems plausible to attribute the antioxidant activity registered to the total phenolic compounds detected in the Folin-Ciocalteau assay.

Through the multiplication of the mass yield of each fraction by the respective proportion of total sugars, it is possible to calculate the amount of carbohydrate material present in each fraction. This way, it was possible to observe that 66.9, in the 1st 2h, and 63.3%, in the 2nd 2h, of the polysaccharides present in the HMWM, recovered in the ethanol fractionation procedure, precipitated in 50% ethanol aqueous solutions, while only 15.7, in the 1st 2h, and 22.5, in the 2nd 2h, precipitated in 75% ethanol aqueous solutions. This suggests that the decoctions of *F. angustifolia* dried leaves might contain higher proportions of charged polysaccharides, that tend to precipitate in 50% ethanol aqueous solutions, and lower proportions of less charged polysaccharides and/or lower molecular weight polysaccharide structures that will precipitate in 75% ethanol aqueous solutions.

The HMWM from the 1st and 2nd 2 h of the hot water extraction process submitted to ethanol fractionation contained approximately 57 and 76% of

carbohydrate material, respectively. Et_{50} fractions from the 1st and 2nd 2 h of the hot water extraction process contained 81.0 and 90.1% of carbohydrate material, respectively, evidencing the carbohydrate enrichment of the Et_{50} fractions obtained through ethanol precipitation. For the Et_{75} fractions, only a slight enrichment was observed for the 2nd 2h of the hot water extraction process. The Et_{75} fractions from the 1st and 2nd 2 h of the hot water extraction process contained 55.8 and 84.7 % of carbohydrate material, respectively.

The Et_{50} and Et_{75} fractions, that contained the highest amounts of carbohydrate material, presented a monomeric composition rich in UA, suggesting the presence of pectic polysaccharides. It was observed that the Et_{50} fractions contained polysaccharides that comprised higher proportions of uronic acid residues in its composition, relatively to those recovered in the Et_{75} fractions. During the fractionation of olive extracts rich in pectic substances with increasing ethanol concentrations, it was also observed that the polysaccharides that precipitated in lower ethanol concentrations exhibited higher proportions of uronic acid residues (Silva, 1993). This seems to support the assumption made earlier, which suggested that the polysaccharides insoluble in 50% ethanol aqueous solutions should be more charged those insoluble in 75% ethanol aqueous solutions.

Besides the high proportions of UA residues, the Et_{75} fractions also displayed Ara and Gal, indicating the possible presence of arabinogalactans, together with Glc residues. The iodine assay performed on these fractions turned out negative, suggesting the absence of starch polymers. Therefore the presence of xyloglucans is more likely, since it is a major hemicellulose present in the primary cell walls of dicotyledons, and has been reported in several studies, although these compounds are also known to give positive results in the iodine assay (Bauer *et al.,* 1973). However, some authors have also reported xyloglucans that did not possess the ability to form coloured complexes with iodine (Aspinall *et al.,* 1969). According to Gould *et al.* (1971), the ability of xyloglucans from white mustard seeds to form complexes with iodine involved the interaction of iodine molecules within the interstices between aggregated xyloglucan chains.

4.1.2. Anion exchange chromatography

The fractions Et_{50} and Et_{75}, because were those with the highest proportions of carbohydrate material, were further fractionated by anion exchange chromatography on DEAE-Sepharose FF, in order to try to separate the polysaccharides according to their charge. For both Et_{50} and Et_{75} fractions, one not retained neutral fraction (A), eluted with buffer, and two retained acidic fractions (C and E), eluted with buffer containing 0.250 and 1.0 M NaCl, respectively, were obtained. The mass yield, total sugars as well as their monomeric composition and methyl esterification degree, are shown in **Table 4.2**.

Table 4.2- Mass yield, total sugar content and monosaccharide composition, and methyl esterification degree, shown by the methanol released from polysaccharides, of the Et_{50} and Et_{75} fractions obtained by anion exchange chromatography of the HMWM from the decoctions of *F. angustifolia* dried leaves.

	Yield[a] (mass %)	Total Sugar (mass %)	Methanol (mmol/mol UA)	Monosaccharide Composition (mol %)							
				Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
Et_{50} *1st 2h*											
A	22.3	83.9	631.2	0.5	0.0	3.9	3.5	1.3	4.3	4.8	81.7
C	59.4	86.9	320.4	1.8	0.1	5.1	1.9	0.0	4.2	0.4	86.4
E	18.3	22.1	nd	7.7	0.4	8.5	1.8	0.7	10.4	8.2	62.4
Et_{75} *1st 2h*											
A	54.5	85.6	509.9	0.9	0.1	7.8	3.9	3.7	10.8	5.7	67.1
C	30.5	55.8	334.0	5.7	0.3	17.5	1.6	0.3	13.4	2.8	58.2
E	15.0	13.9	nd	17.9	0.3	5.4	0.6	1.3	5.8	43.7	25.0
Et_{50} *2nd 2h*											
A	21.6	87.1	430.7	0.4	0.0	5.5	3.2	0.8	3.8	2.8	83.4
C	65.1	94.2	320.0	2.1	0.2	6.6	2.0	0.0	4.5	0.4	84.3
E	13.3	13.3	nd	7.1	0.1	7.5	0.6	0.2	11.0	10.6	62.9
Et_{75} *2nd 2h*											
A	53.3	82.5	569.7	0.7	0.1	7.3	4.3	2.8	8.9	3.6	72.3
C	41.7	71.0	343.0	4.1	0.2	18.6	2.0	0.1	10.4	0.9	63.6
E	5.0	13.7	nd	14.3	0.2	9.0	1.0	1.6	10.6	39.9	23.5

[a] - expressed relatively to the HMWM recovered; [nd] - not determined

For the Et_{50} fractionation, total mass recoveries of 81.4 and 76.3% were registered for the 1st and 2nd 2 h of the hot water extraction process, respectively, while for Et_{75} fractionation, mass recoveries of 66.4 and 77.5% were observed for the 1st and 2nd 2 h of the hot water extraction process, respectively. These mass recovery values suggest that some interaction between the HMWM present in the Et_{50} and Et_{75} fractions and the stationary phase may have occurred. During the chromatographic separation it was observed that the top of the column became darker, evidencing an interaction between these coloured compounds and the DEAE-Sepharose FF gel. At the end of the chromatographic separation, the gel was washed with 2.0 M NaCl, and 1.0 M NaOH solutions in a reversed flow direction, according to the manufacturer instructions, for the removal of the bound compounds. During the cleaning procedure it was possible to observe the removal of the coloured compounds, and after this cleaning procedure, the darker colour at the top of the column disappeared, suggesting the removal of the bound compounds. The lower recovery value was registered for the fractionation of the Et_{75} fraction obtained in the 1st 2h of the hot water extraction process, which also exhibited the higher proportion of total phenolic compounds, among the fractions submitted to the anion exchange chromatography process (**Table 4.2**). This suggests that the lower recovery exhibited by the Et_{75} fraction, from the 1st 2h of the hot water extraction process, could be related to the retention of phenolic compounds by the DEAE-Sepharose FF gel, which could only by eluted with solutions with higher ionic strength than the ones used during the normal elution process.

For all the fractionations performed, most of the HMWM was collected in the neutral (A), and the less acidic (C) fractions, that together contained more than 80% of the HMWM recovered, and also showed the highest proportions of total sugars. The most acidic (E) fractions were the less abundant, and also the poorest in carbohydrate material.

For Et_{50} fractionation, the most abundant were the $\underline{Et_{50}C}$ fractions that contained 59.4, in the 1st 2h, and 65.1%, in the 2nd 2h, of the HMWM recovered, respectively, while for the Et_{75} fractionation, the most abundant were the $\underline{Et_{75}A}$ fractions that presented nearly 55% of the HMWM recovered, for both parts of the

hot water extraction process. This evidenced that the HMWM insoluble in 50% ethanol aqueous solutions that was present in the decoctions from *F. angustifolia* dried leaves, contained a higher proportion of HMWM composed of polymers with structural characteristics that enable them to be retained, and later eluted with 0.250 M NaCl from the stationary phase, while the Et_{75} fractions comprised polymers that were not retained by the DEAE-Sepharose FF stationary phase. These differences in the elution of the HMWM insoluble in 50 and 75% ethanol aqueous solutions might be related to the distinct content of uronic acid residues that was reported for these fractions, but also with the methyl esterification degree of these uronic acid residues.

The $\underline{Et_{50}A}$ and $\underline{Et_{50}C}$ fractions showed a similar monomeric composition, which was also similar to the monomeric composition of the Et_{50} fractions, which comprised very high proportions of UA. It was observed that the uronic acid residues present in the neutral fraction were methyl esterified in a greater extent than those from the acidic fraction: 63.1 and 32.0%, for the $\underline{Et_{50}A}$ and $\underline{Et_{50}C}$ fractions of the 1st 2h, respectively; 43.1 and 32.0%, for the $\underline{Et_{50}A}$ and $\underline{Et_{50}C}$ fractions of the 2nd 2h, respectively. This suggests that the fractionation process was achieved based on the methyl esterification degree of the pectic polysaccharides present in the Et_{50} fraction, rather than on the respective uronic acid content. This type of behaviour was also observed during the fractionation by anion exchange chromatography on DEAE-Trisacryl of extracts enriched in pectic polysaccharides from the olive pulp cell walls (Silva, 1993). This also evidenced that the majority of the polysaccharides present in the decoctions of *F. angustifolia* that precipitated in 50% ethanol aqueous solutions are composed of high proportions of non methyl esterified uronic acid residues, which renders them more insoluble in 50% ethanol aqueous solutions and also allows them to be preferentially retained by the gel, and later eluted with 0.250 M NaCl from the stationary phase.

The $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ fractions exhibited a monomeric composition that mainly comprised UA residues, although present in a lower proportion when compared with the correspondent Et_{50} fractions, together with small proportions of Ara and Gal. Although the proportion of UA residues recovered in a fraction is usually

increased when eluting solutions presenting an increasing ionic strength, it was observed that the neutral fractions contained proportions of UA residues higher than the acidic ones. Similarly to what was observed for the fractionation of $\underline{Et_{50}}$, the UA residues of the neutral fractions exhibited a methyl esterification degree higher than the ones from the acidic fractions: 51.0 and 33.4%, for the $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ fractions of the 1st 2h, respectively; 57.0 and 34.3%, for the $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ fractions of the 2nd 2h, respectively. This might have contributed for the lack of interaction with the stationary phase of the fractions with higher proportions of UA residues. This also evidenced that the majority of the polysaccharides present in the decoctions of *F. angustifolia* that precipitated in 75% ethanol aqueous solutions are composed of high proportions of methyl esterified uronic acid residues, which renders them more insoluble in 75% ethanol aqueous solutions, and also allows them preferentially not to be retained by the gel.

In comparison with the Et_{75} fractions, the $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ fractions seemed to contain polysaccharides with similar proportions of Gal residues. It was also possible to observe that in comparison with the Et_{75} fractions, the $\underline{Et_{75}C}$ fractions contained polysaccharides with higher proportions of arabinosyl residues, while the $\underline{Et_{75}A}$ fractions comprised polysaccharides with lower proportions of arabinosyl residues in their composition. It is possible that these arabinosyl residues are present as arabinogalactans side chains. Furthermore, it was registered that the rhamnosyl residues that were detected in minute proportions in the Et_{75} fractions, were mainly recovered in the $\underline{Et_{75}C}$ fractions, suggesting its presence as components of RG-I moieties of the pectic polysaccharides.

4.1.3. Glycosidic linkage analysis

In order to elucidate the type of polysaccharides present in the decoctions from *F. angustifolia* dried leaves, the fractions that showed the highest amounts of carbohydrate material were methylated and analysed as partially methylated alditol acetates. A carboxyl reduction of the methylated polysaccharides was also performed for the detection of the uronic acid residues and assessment of the respective types of linkages.

The monomeric composition of the $\underline{Et_{50}A}$, $\underline{Et_{50}C}$, $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ fractions from the 1st 2h of the hot water extraction was similar to the correspondent obtained in the 2nd 2h. Therefore, assuming that the same types of polysaccharides are being extracted in both parts of the extraction process, we performed the linkage analysis on the fractions obtained in the 1st 2h of the hot water extraction process. For all the fractions, the carboxyl reduction of the previously methylated polysaccharides allowed the detection of the 1,4,5,6-tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-hexitol residue with a mass spectra with a fragmentation pattern that exhibited some ions with +2 m/z units, indicating the presence of (1→4)-HexA. As the pectic polysaccharides seem to be the major type of polymer present in the decoctions from *F. angustifolia* dried leaves it is plausible to attribute the (1→4)-HexA residues to the presence of (1→4)-GalA, which is one of the main components of pectic polysaccharides. The presence of *t*-GalA and *t*-GlcA as pectic polysaccharide components has been also reported (Matsumoto *et al*., 1995; Kiyohara *et al*., 2010), however they are found in much lower proportions, in comparison with the (1→4)-GalA residues, which, together with the very low recovery of the uronic acid residues (<5%), could have contributed for its non detection. Due to the high proportion of UA residues present in these fractions, and as only (1→4)-GalA residues were detected, we opted for presenting the linkage analysis referring only to the neutral sugar residues, contributing for a better understanding of the possible distinct structural features of the polysaccharides present in the isolated fractions, as shown in **Table 4.3**.

Good agreement between the molar fractions obtained from sugar (alditol acetates) and linkage (partially methylated alditol acetates) analysis was observed. Also a good agreement between the proportion of terminal and ramified residues was registered for each fraction: 20.1 and 25.0%, 22.6 and 25.8%, 27.6 and 25.8%, and 26.1 and 23.2%, for the terminal and ramified residues of $\underline{Et_{50}A}$, $\underline{Et_{50}C}$, $\underline{Et_{75}A}$, and $\underline{Et_{75}C}$, respectively.

The fractions $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ presented higher proportions of total galactosyl residues (32.5 and 36.8%, respectively) than the correspondent $\underline{Et_{50}A}$ and $\underline{Et_{50}C}$ fractions (24.0 and 30.0%, respectively).

Table 4.3- Deduced linkages from the methylation analysis of the neutral (A) and acidic (C) fractions from the Et_{50} and Et_{75} fractions of the 1st 2h of the decoctions from *F. angustifolia* dried leaves.

Linkage	Fraction			
	Et_{50} A	Et_{50} C	Et_{75} A	Et_{75} C
t*-Rha*p	0.4	2.3	0.6	2.0
1,2-Rha*p*	0.0	2.9	0.0	1.5
1,3-Rha*p*	0.0	1.3	0.0	0.0
1,2,4-Rha*p*	0.0	5.2	0.0	3.2
1,3,4-Rha*p*	0.0	2.2	0.0	0.0
Total	***0.4[a] (2.7)[b]***	***13.9(13.2)***	***0.6(2.7)***	***6.7(13.6)***
t-Fuc*p*	0.4	2.7	0.4	1.2
Total	***0.4(0.0)***	***2.7(0.7)***	***0.4(0.3)***	***1.2(0.7)***
t*-Ara*f	7.6	5.3	15.0	11.5
t*-Ara*p	0.0	1.4	2.0	2.0
1,2-Ara*f*	0.8	0.7	0.9	0.9
1,3-Ara*f*	2.5	2.1	3.3	4.5
1,5-Ara*f*	9.0	20.6	4.9	17.9
1,3,5-Ara*f*	2.5	5.6	0.6	2.4
Total	***22.4(21.3)***	***35.7(37.5)***	***26.7(23.7)***	***39.2(41.9)***
t-Xyl*p*	3.9	2.0	1.9	1.4
1,2-Xyl*p*	3.4	0.3	1.9	0.0
1,4-Xyl*p*	1.0	2.0	3.9	1.5
1,2,3-Xylp	0.0	1.2	0.0	0.0
1,2,4-Xylp	3.7	4.2	2.0	3.4
Total	***12.0(19.1)***	***9.7(14.0)***	***9.7(11.9)***	***6.3(3.8)***
t*-Man*p	0.9	0.0	0.7	0.3
1,4-Man*p*	9.2	0.0	8.7	0.0
1,2,4-Man*p*	1.2	0.0	0.0	0.0
1,4,6-Man*p*	0.9	0.0	2.2	0.0
Total	***12.2(7.1)***	***0.0(0.0)***	***11.6(11.2)***	***0.3(0.7)***
t*-Gal*p	5.1	8.3	5.1	5.9
1,2-Gal*p*	0.7	2.5	0.5	0.0
1,3-Gal*p*	2.5	3.5	3.0	8.1
1,4-Gal*p*	0.0	8.2	0.0	1.8
1,6-Gal*p*	9.6	3.2	7.9	7.7
1,3,6-Gal*p*	6.1	4.3	16.0	13.3
Total	***24.0(23.5)***	***30.0(30.9)***	***32.5(32.8)***	***36.8(32.1)***
t*-Glc*p	1.8	0.6	1.9	1.8
1,3-Glc*p*	1.5	0.4	2.3	0.6
1,4-Glc*p*	14.5	3.9	9.1	5.1
1,6-Glc*p*	0.2	0.0	0.2	1.1
1,2,4-Glc*p*	0.3	1.8	0.0	0.0
1,4,6-Glc*p*	10.3	1.3	5.0	0.9
Total	***28.6(26.2)***	***8.0(2.9)***	***18.5(17.3)***	***9.5(6.7)***

molar % obtained through: [a] linkage analysis, and [b] sugar analysis

The detection of galactosyl residues in various linkages, namely (1→3)-, and (1→3,6)-Gal*p,* for all the fractions analysed is diagnostic of the presence of the AG-II. Terminal-, and (1→6)-Gal*p* residues, frequently reported as AG-II components, were also detected. These residues were present in higher proportion in the Et_{75} fractions, evidencing the presence of higher proportions of AG-II in these fractions, as suggested earlier by the higher proportion of galactosyl residues in the monomeric composition of these fractions, in comparison with those from the Et_{50} fractions, as shown in **Table 4.2**.

The structural features of the AG-II extracted from coffee are well established, and have been reported in various studies (Fischer *et al.,* 2001; Nunes and Coimbra, 2001; Redgwell *et al.,* 2002; Oosterveld *et al.,* 2003; Nunes *et al*., 2008). Therefore the structural features of the AG-II from the decoctions of *F. angustifolia* dried leaves will be compared with those of the AG-II from coffee throughout this section.

The fractions $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ presented a substitution degree, expressed by the value of the ratio (1→3,6)-Gal*p*/(1→3)-Gal*p*, of 5.3 and 1.6, respectively. The substitution degree of the correspondent $\underline{Et_{50}A}$ and $\underline{Et_{50}C}$ fractions was lower (2.4 and 1.2, respectively), suggesting that the AG-II present in the Et_{75} fractions had a more substituted galactosyl backbone than those present in the correspondent Et_{50} fractions. It was also observed that the neutral fractions possessed a more substituted galactosyl backbone in comparison with the acidic ones.

Several studies reported the extraction of AG-II from coffee with distinct substitution degrees of the galactosyl backbone. AG-II isolated from the water insoluble residue of green coffee beans were reported as possessing a substitution degree of 0.33, evidencing a lower substitution degree in comparison with the AG-II from the decoctions of *F. angustifolia* dried leaves (Wolfrom and Patin, 1964; Bradbury and Halliday, 1990). These results seem to be in agreement with some studies that have shown that the AG-II present in more easily extracted water soluble fractions exhibited higher substitution degree values than those present in water insoluble residues (Oosterveld *et al.,* 2003).

Redgwell *et al.* (2002), using a combination of chemical extraction and enzymatic hydrolysis, extracted several AG-II structures from the green coffee

beans, which presented substitution degree values that ranged between 0.76 and 0.54. Nunes and Coimbra (2001) reported the presence of AG-II in the HMWM from the hot water extracts of Brazil and Costa Rica *arabica* green coffees that showed a substitution degree value of 1.0. Also, a rich AG-II fraction insoluble in 75% ethanol aqueous solutions, and that presented a substitution degree value of 1.3 was isolated from the HMWM of the hot water extracts of Brazil *arabica* green coffees (Nunes *et al.,* 2008).

All the fractions isolated from the decoctions of *F. angustifolia* dried leaves presented a substitution degree value higher than the range of values (0.76 – 0.54) reported for the AG-II fractions from the green coffee beans isolated by Redgwell *et al.* (2002), suggesting the presence of more substituted arabinogalactan structures in the hot water extracts from *F. angustifolia.* The degree of substitution values presented by the $\underline{Et_{50}C}$, and $\underline{Et_{75}C}$ fractions (1.2, and 1.6, respectively) seem to be in agreement with the substitution degree value of 1.3 reported for the AG-II fraction isolated from the HMWM of the hot water extracts of Brazil *arabica* green coffees (Nunes *et al.,* 2008). The higher degree of substitution values presented by the $\underline{Et_{50}A}$, and $\underline{Et_{75}A}$ fractions (2.4, and 5.3, respectively) evidenced the presence of AG-II with a more substituted galactosyl backbone. These results reflect that the AG-II present in the decoctions from *F. angustifolia* dried leaves seem to possess highly substituted galactosyl chains.

The AG-II isolated from coffee contain proportions of (1→3)-Gal*p* residues that usually exceeds the ones of (1→6)-Gal*p* (Fischer *et al.,* 2001; Nunes and Coimbra, 2001; Oosterveld *et al.,* 2003; Gniechwitz *et al.,* 2007), similarly to what was reported for the AG-II present in the hot water extracts from the leaves of *Opilia celtidifolia* (Togola *et al.,* 2008). For the AG-II isolated from the decoctions of *F. angustifolia* dried leaves, the contrary was observed, similarly to what was reported for the AG-II isolated from the hot water extracts of other plants (Duan *et al.,* 2003; Nergard *et al.,* 2005; Inngjerdingen *et al.,* 2007b).

Arabinosyl residues, preferentially present as terminally- and (1→5)-linked, all in furanosidic form, were detected, which also suggests the presence of AG-II (Fischer *et al.,* 2001). Other residues, that also have been reported in fractions containing AG-II have also been reported, were also detected, namely T-Ara*p*,

(1→2)-, (1→3)-, and (1→3,5)-Ara*f* (Nergard *et al.,* 2005; Togola *et al.,* 2008; Grønhaug *et al.,* 2010). The $\underline{Et_{50}A}$ and $\underline{Et_{50}C}$ fractions presented (1→5)-Ara*f*/T-Ara*f* ratio values higher than the correspondent $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ fractions (1.2 and 3.9 *vs* 0.33 and 1.6, respectively), suggesting that in the Et_{50} fractions the (1→5)-linked arabinosyl residues probably can be arranged as more extended AG-II side chains, and also that in the $\underline{Et_{75}A}$ fraction most of these side chains might be composed of a single arabinosyl residue (Fischer *et al.,* 2001). The difference in the (1→5)-Ara*f*/T-Ara*f* ratio values observed for the $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ fractions supports the hypothesis earlier expressed, suggesting that the arabinosyl residues content of these fractions could be related to the presence of arabinosyl side chains, which seem to be more extended in the $\underline{Et_{75}C}$ fraction.

Usually, the (1→5)-linked arabinosyl are side chains linked at *O*-3 of the (1→6)-linked galactosyl residues, which are arranged as side chains of the (1→3)-linked galactan backbone (Nunes *et al*., 2008). The presence of (1→3,5)-Ara*f* in all the fractions also seems to suggest that the (1→5)-linked arabinosyl side chains can themselves be substituted, possibly with single arabinosyl residues, as proposed by Nunes *et al.,* (2008). For the $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ fractions, it was also observed that the ratio (1→3,5)-/(1→5)-Ara was lower than those of the correspondent Et_{50} fractions (0.12 and 0.13 *vs* 0.28 and 0.27), possibly evidencing the presence of less substituted arabinosyl side chains. The values of 0.12 and 0.13, registered for the $\underline{Et_{75}A}$ and $\underline{Et_{75}C}$ fractions, respectively, were in agreement with the value of 0.11 shown by a AG-II fraction isolated from the Brazil A*rabica* green coffee (Nunes *et al.,* 2008).

The AG-II are frequently described as being mainly associated with proteins, forming the AGP´s structures (Fry, 1988; Vincken *et al.,* 2003), which are widely distributed in plants, and are mainly located at the plasma membrane, and in the cell wall (Serpe and Nothnagel, 1999). Fry (1988) also supports that most of the AG-II structures reported in the literature were in fact AGP´s in which the protein moiety has been overlooked. Nunes *et al.* (2008) reported the T-Rha*p* residues as structural features of the side chains from AGP´s extracted from green coffee (Nunes *et al.,* 2008). Since rhamnosyl residues terminally linked were detected in all the fractions, it is possible that at least some of the AG-II from the decoctions of

F. angustifolia dried leaves could be present as AGP´s. The green coffee AGP´s reported by Nunes *et al.* (2008) and the AG-II present in the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions were obtained using similar experimental procedures: recovery in not retained fractions, which resulted from the fractionation by anion exchange chromatography of hot water soluble HMWM, previously fractionated by ethanol precipitation. Therefore, it is possible that the AG-II present in the neutral fractions from the decoctions of *F. angustifolia* dried leaves are also present as AGP´s. Altogether, this seems to support the presence of AGP´s in the neutral fractions, without totally excluding the possible presence of AGP´s also in the $\underline{Et_{50}C}$ and $\underline{Et_{75}C}$ fractions, which also contained T-Rha*p* residues.

Besides terminally linked rhamnosyl residues, other rhamnosyl residues were detected in the acidic $\underline{Et_{50}C}$ and $\underline{Et_{75}C}$ fractions, such as (1→2)-, and (1→2,4)-Rha*p* residues. The presence of these residues, together with the presence of (1→ 4)-GalA residues, suggested the presence of RG-I moieties that seemed to be absent in the neutral $\underline{Et_{50}A}$, and $\underline{Et_{75}A}$ fractions. The ratio of (1→2,4)-/(1→2)-Rha indicated that approximately 64 and 68% of the rhamnosyl residues from the $\underline{Et_{50}C}$ and $\underline{Et_{75}C}$ fractions were substituted, respectively. These values were in accordance with those reported by Albersheim *et al.* (1996), which referred that the proportion of substituted rhamnosyl residues generally varied from approximately 20 to 80%, depending on the source of the polysaccharide and isolation method.

The acidic fractions, particularly $\underline{Et_{50}C}$, also exhibited galactosyl residues (1→4)-linked, which are diagnostic for the presence of type I arabinogalactans (AG-I). The (1→4)-Gal*p* residues compose the backbone of AG-I, which can be substituted at *O*-3 with arabinosyl residues (Ridley *et al.,* 2001; Vincken *et al.,* 2003). Since no (1→3,4)-linked galactosyl residues were detected, these AG-I seem to be mainly non substituted structures, similarly to what was reported for the AG-I from the hot water extracts of *Cochlospermum tinctorium* and *Vernonia kotschyana* (Nergard *et al., 2005,* 2006) and contrasting with the highly unusual structural features of the AG-I extracted from the fruits of *Lycium barbarium*, that possessed a backbone of (1→4)-Gal*p* residues, which are all branched at position 3 with chains of different compositions (Peng *et al*., 2002). Nevertheless, as

(1→3)-Gal*p* were detected it can not be excluded their possible presence as substituents, linked at *O*-3 of the (1→4)-Gal*p* residues located at the non-reducing end.

The (1→2,4)-Rha*p* residues are frequently reported as the main binding site for various types of side chains often found attached to the RG-I moiety of pectic polysaccharides, namely arabinogalactans. The linkage between the AG-I reducing end and the (1→2,4)-Rha*p* residues of RG-I moiety from pectic polysaccharides seems consensual, and has been reported by several authors (Yamada and Kiyohara, 1999; Nergard *et al.,* 2005). Therefore, it is possible that the AG-I, that co-eluted in the acidic $\underline{Et_{50}C}$ and $\underline{Et_{75}C}$ fractions with the (1→2)- and (1→2,4)-linked rhamnosyl residues, are covalently linked to RG-I elements.

Also, relevant proportions of (1→4)-, besides (1→4,6)-linked glucosyl residues, were observed in the neutral $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, while the acidic $\underline{Et_{50}C}$ and $\underline{Et_{75}C}$ fractions presented minor proportions. As no starch was detected by the iodine assay, it was assumed that at least some of these glucosyl residues should be part of other polysaccharide structures. Xyloglucans contain a (1→4)-Glc*p* backbone often substituted at *O*-6 with single xylosyl residues, and are also a major hemicellulose present in the primary cell walls of dicotyledons (Carpita and Gibeaut, 1993), therefore it seems plausible that the (1→4)-, and (1→4,6)-linked glucosyl residues present in the decoctions of *F. angustifolia* dried leaves originated from xyloglucans.

It was also observed that the xyloglucans were detected in the neutral fractions at higher proportions than those registered for the acidic ones. The neutral $\underline{Et_{50}A}$, and $\underline{Et_{75}A}$ fractions presented higher (1→4,6)-/(1→4)-Glc*p* ratio values than the correspondent acidic $\underline{Et_{50}C}$, and $\underline{Et_{75}C}$ fractions (0.71 and 0.55 *vs* 0.33 and 0.18), evidencing the presence of more substituted xyloglucan structures in the neutral fractions. The neutral fractions, and particularly the $\underline{Et_{50}A}$, also contained higher proportions of (1→4,6)-Glc*p* residues in comparison with the proportion of T-Xyl*p* residues (10.3% *vs* 3.9% and 5.0% *vs* 1.9%, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively), suggesting that besides single xylosyl residues, these xyloglucans should also contain in its structure other terminally linked residues.

The (1→4)-Glc*p* backbone of xyloglucans can be substituted at *O*-6 with single xylosyl residues, but the Gal*p*-(β1→2)-Xyl*p*-(α1→ disaccharide and/or the Fuc*p*-(α1→2)-Gal*p*-(β1→2)-Xyl*p*-(α1→ trisaccharide have also been reported as xyloglucan side chains (Fry, 1988; Hoffman *et al.,* 2005). Additionally, Vincken *et al.* (1996) reported that the potato xyloglucans also possessed the disaccharide Ara*p*-(α1→2)-Xyl*p*-(α1→ as side chain. Therefore, besides T-Xyl*p*, some of the terminally linked arabinosyl, fucosyl and galactosyl residues detected in neutral fractions could also be present in the xyloglucan structure. Based on the sum of the proportions of (1→3,5)-Ara*f* and (1→3,6)-Gal*p* residues of $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, that are present in the AG-II structure, it can be inferred that 8.6% and 16.6%, respectively, of the T-Ara*f* and T-Gal*p* residues, that can be present in both AG-II and xyloglucans structures, should be present as AG-II components. Therefore, the remaining proportions of these terminally linked residues (4.1% and 3.5%, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively), together with other detected terminally linked residues that have also been reported as present in xyloglucans structures, such as T-Fuc*p* (0.4% and 0.4%, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively) and T-Xyl*p* (3.9% and 1.9%, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively), could be present as components of the xyloglucan structures present in the neutral fractions. The sum of the molar proportions of these terminally linked residues (8.4% and 5.8%, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively) seems to be reasonably compatible with the 10.3% and 5.0% of (1→4,6)-Glc*p* residues detected in the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively. It was previously reported that xyloglucans produced by *Olea europaea*, from the *Oleaceae* family like *F. angustifolia*, contained arabinosyl and galactosyl residues (Hoffman *et al.,* 2005). These results, together with the presence of galactosyl and xylosyl residues, both (1→2)-linked*,* reinforce the possible presence of xyloglucan structures in the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, bearing not only single xylosyl side chains, but also the Ara*p*-(α1→2)-Xyl*p*-(α1→ and Gal*p*-(β1→2)-Xyl*p*-(α1→ disaccharides, and the Fuc*p*-(α1→2)-Gal*p*-(β1→2)-Xyl*p*-(α1→ trisaccharide.

The presence of mannosyl (1→4)-linked residues in the neutral fractions $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$, also suggested the presence of mannans, which are another hemicellulosic polysaccharide composed of a (1→4)-Man*p* backbone that can be

substituted at *O*-6 with galactosyl, and/or glucosyl residues (Meier and Reid, 1982). The mannans present in the $\underline{Et_{50}A}$, and $\underline{Et_{75}\ A}$ fractions exhibited a ratio (1→4,6)-/(1→4)-Man*p* ratio values of 0.10 and 0.25, respectively, suggesting that approximately 10, and 20% of the mannosyl residues were substituted at *O*-6, respectively.

It was also possible to infer the presence of xylans in very in small proportions. This hemicellulosic polysaccharide was mainly diagnosed through the detection of terminally-, (1→4)- and (1→2,4)-linked xylosyl residues in all the fractions. The xylans are hemicellulosic polysaccharides composed of a (1→4)-Xyl*p* backbone that can be substituted at *O*-2 and/or *O*-3 with single arabinosyl and/or glucuronic acid residues (Fry, 1988). The presence of minute proportions of (1→2)-Ara*f* also seems to support the presence of xylans, as the side chains may also contain these arabinosyl residues besides the T-Ara*f* residues (Fry, 1988).

4.2. *Endo*-polygalacturonase treatments

The fractions obtained from the decoctions of *F. angustifolia* dried leaves contained high proportions of uronic acid residues that suggested the presence of pectic polysaccharides. Particularly for the neutral fractions obtained from the anion exchange chromatography of the Et_{75} fractions, it was observed that besides the presence of high proportions of pectic polysaccharides, these fractions also contained other polysaccharides in lower amounts, such as arabinogalactans, xyloglucans, mannans, and xylans.

The treatment of pectic polysaccharides with *endo*-polygalacturonase, and subsequent fractionation of the digestion products by size exclusion chromatography has been used for a long time as a tool for the structural analysis of polysaccharides (Talmadge *et al*., 1973). The *endo*-polygalacturonase enzyme cleaves glycosidic linkages between de-esterified (1→4)-Gal*p*A residues present in the homogalacturonan domains of pectic polysaccharides, generating diverse digestion products, among them oligogalacturonides with DP´s ranging from 1 to 5 (O´Neill and York, 2003).

Thus, to further elucidate the structural features of the polysaccharides present in the $\underline{Et_{75}A}$ fractions, an *endo*-polygacturonase treatment was performed in the material from both parts of the hot water extraction process. The material present in the $\underline{Et_{75}A}$ fractions was previously de-esterified with NaOH solutions, in order to provide the substrate for the enzyme, and subsequently submitted to an enzymatic digestion, followed by size exclusion chromatography on Bio Gel P30, which presents a typical fractionation limit ranging from 2.5 to 40 kDa. The chromatographic elution profiles of the fractions, with and without *endo*-polygalacturonase treatment, were obtained, and are presented in **Figure 4.1**.

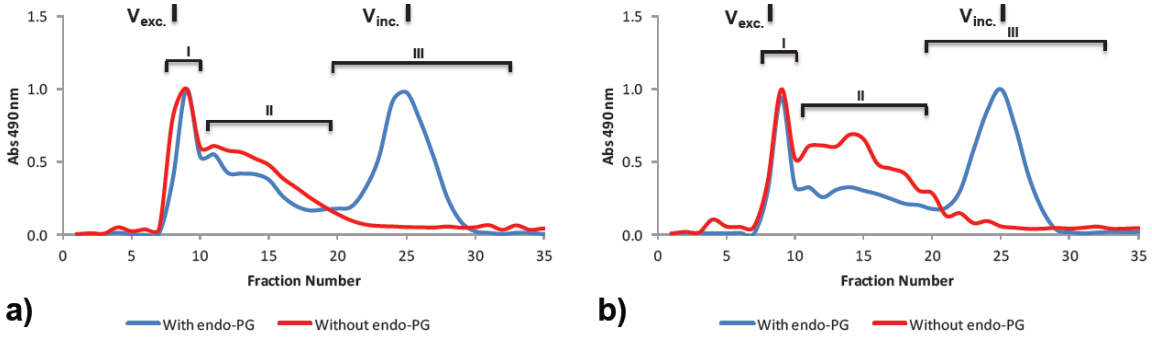

Figure 4.1- Elution profiles from the size exclusion chromatography on Bio Gel P30 of the $\underline{Et_{75}A}$ fractions, with and without the *endo*-polygalacturonase treatment, obtained from the **a)** 1st 2h, and **b)** 2nd 2h of the hot water extraction of *F. angustifolia* dried leaves.

The size exclusion elution profile of the untreated fractions showed two distinct chromatographic bands, corresponding to a high molecular weight fraction (I), eluting near the exclusion volume, and that should contain material with an estimated molecular weight higher than 40 kDa, and one intermediate molecular weight fraction (II), containing material with an estimated molecular weight comprised between 40 and 2.5 kDa. Besides the high, and intermediate molecular weight fractions, the elution profile of the treated fractions also presented a low molecular weight fraction (III), which evidenced the *endo*-polygalacturonase action, and the presence of 1,4-linked α-galacturonic acid residues, which are frequently found as components of the homogalacturonan domains of pectic polysaccharides.

After the *endo*-polygalacturonase treatment of the $\underline{Et_{75}A}$ fractions, and subsequent size exclusion chromatography on Bio Gel P30 of the digestion products, the fractions of interest were pooled, dialysed using a tubing with a

molecular weight cut-off of 1.0 kDa, and freeze-dried. The use of a tubing with a molecular weight cut-off of 1.0 kDa allowed to eliminate salts, and also to reduce losses of the digestion products. The mass yield, carbohydrate material content, and monomeric composition of the fractions obtained are presented in **Table 4.4**.

Table 4.4- Mass yield, total sugar content, and monosaccharide composition of the fractions obtained through *endo*-polygalacturonase digestion of the $\underline{Et_{75}A}$ fractions, isolated from the decoctions of *F. angustifolia* dried leaves.

	Yield[a] (mass %)	Total Sugars (mass %)	Monosaccharide Composition (mol%)							
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
			1st 2h							
	$Et_{75}A$	85.6	0.9	0.1	7.8	3.9	3.7	10.8	5.7	67.1
$Et_{75}A$-I	31.3	85.0	1.8	0.2	35.0	4.0	3.4	37.1	10.5	8.1
$Et_{75}A$-II	35.6	78.4	1.8	0.3	12.6	8.6	12.5	19.6	18.6	26.0
$Et_{75}A$-III	33.1	95.6	0.4	0.0	0.8	2.8	0.6	1.2	1.8	92.4
			2nd 2h							
	$Et_{75}A$	82.5	0.7	0.1	7.3	4.3	2.8	8.9	3.6	72.3
$Et_{75}A$-I	23.2	78.6	1.7	0.1	37.0	4.5	2.7	37.7	4.9	11.5
$Et_{75}A$-II	25.0	83.6	1.0	0.3	13.6	11.5	10.9	17.7	13.7	30.3
$Et_{75}A$-III	51.8	97.5	0.2	0.0	0.5	2.6	0.3	0.9	1.2	94.3

[a] - expressed relatively to the HMWM recovered

The enzymatic digestion of the $\underline{Et_{75}A}$ fraction, isolated from the 1st 2h of *F. angustifolia* dried leaves hot water extraction, yielded similar proportions of $\underline{Et_{75}A\text{-I}}$, $\underline{Et_{75}A\text{-II}}$, and $\underline{Et_{75}A\text{-III}}$ fractions, which contained high proportions of carbohydrate material: 85.0, 78.4, and 95.6%, respectively. For the 2nd 2h of the hot water extraction, the $\underline{Et_{75}A\text{-I}}$ and $\underline{Et_{75}A\text{-II}}$ fractions were also recovered in similar proportions (≈ 25%), which were lower in comparison with those from the 1st 2h. The $\underline{Et_{75}A\text{-III}}$ fraction comprised twice more material, evidencing that, for the 1st 2h of the hot water extraction process, approximately the same proportions of carbohydrate material were recovered in the different fractions (30.9, 32.4, and 36.7%, for the $\underline{Et_{75}A\text{-I}}$, $\underline{Et_{75}A\text{-II}}$, and $\underline{Et_{75}A\text{-III}}$ fractions, respectively), while for the 2nd 2h, most of the carbohydrate material was recovered in the low molecular weight fraction (20.3, 23.3, and 56.3%, for the $\underline{Et_{75}A\text{-I}}$, $\underline{Et_{75}A\text{-II}}$, and $\underline{Et_{75}A\text{-III}}$ fractions, respectively). This suggests the presence of higher proportions of *endo*-polygalacturonase resistant carbohydrate material, with a molecular weight higher

than 40 kDa, in the $\underline{Et_{75}A}$ fractions isolated from the 1st 2h of the hot water extraction of *F. angustifolia* dried leaves, while the carbohydrate material from the 2nd 2h seemed more susceptible to the action of the enzyme.

It was possible to observe that for all the fractions resulting from the *endo*-polygalacturonase treatment, the carbohydrate material from both 1st and 2nd 2h of the hot water extraction process, exhibited a similar monomeric composition. This evidenced that the carbohydrate material extracted during both parts of the hot water extraction contained the same monosaccharide proportions.

The proportion of uronic acid present in the $\underline{Et_{75}A\text{-I}}$ and $\underline{Et_{75}A\text{-II}}$ fractions was lower than the observed for the untreated $\underline{Et_{75}A}$ fractions, while for the $\underline{Et_{75}A\text{-III}}$ fractions the carbohydrate material was almost exclusively composed of uronic acid residues (>90%), which resulted from the *endo*-polygalacturonase enzymatic cleavage of the (1→4)-Gal*p*A residues from the pectic polysaccharides present in the $\underline{Et_{75}A}$ fractions. The polysaccharides present in the $\underline{Et_{75}A\text{-I}}$ fractions were mostly composed of arabinosyl and galactosyl residues, present in similar proportions (approximately 35% of each residue). Lower proportions of glucosyl and uronic acid residues were also detected. The polysaccharides of the $\underline{Et_{75}A\text{-II}}$ fractions were composed of uronic acid residues (26.0 and 30.3%, for the 1st and 2nd 2h, respectively), together with lower proportions of various sugar residues, such as arabinosyl (12.6 and 13.6%, for the 1st and 2nd 2h, respectively), galactosyl (19.6 and 17.7%, for the 1st and 2nd 2h, respectively), glucosyl (18.6 and 13.7%, for the 1st and 2nd 2h, respectively), mannosyl (12.5 and 10.9%, for the 1st and 2nd 2h, respectively) and xylosyl (8.6 and 11.5%, for the 1st and 2nd 2h, respectively) residues.

The monomeric composition of the $\underline{Et_{75}A\text{-I}}$ and $\underline{Et_{75}A\text{-II}}$ fractions, together with the sugar and linkage analysis of the $\underline{Et_{75}A}$ fraction from the 1st 2h (**Tables 4.2**, and **4.3**), suggests that the AG-II previously detected in the $\underline{Et_{75}A}$ fractions were mostly recovered in the high molecular weight fractions, while the mannans, xylans, and xyloglucans, which were also detected in the $\underline{Et_{75}A}$ fraction in lower proportion than the AG-II, seemed to be mainly recovered in the intermediate molecular weight fractions.

The polysaccharides present in the higher molecular weight fraction, isolated from the decoctions from *F. angustifolia* dried leaves, were methylated and analysed as partially methylated alditol acetates. As these fractions exhibited low proportions of UA resides, no carboxyl reduction of the previously methylated polysaccharides was performed. The results presented in **Table 4.5** show a good agreement between the molar fractions obtained from sugar (alditol acetates) and linkage (partially methylated alditol acetates) analysis. The proportion of terminal and ramified residues was registered for each fraction: 32.9 and 28.5%, and 28.7% and 37.0%, for $\underline{Et_{75}A\text{-}I}$ fractions from the 1st and 2nd 2h of the hot water extraction process, respectively.

The AG-II contained in the $\underline{Et_{75}A\text{-}I}$ fractions presented a substitution degree, expressed by the value of the ratio (1→3,6)-Gal*p*/(1→3)-Gal*p*, of 5.3 and 7.2, for the 1st and 2nd 2h of the hot water extraction process. Another structural feature of AG-II that is usually assessed is the extension of its (1→5)-Ara*f* residues side chains, evaluated through the (1→5)-Ara*f*/T-Ara*f* ratio. The $\underline{Et_{75}A\text{-}I}$ fractions exhibited (1→5)-Ara*f*/T-Ara*f* ratio values of 0.31 and 0.33, for the 1st and 2nd 2h of the hot water extraction process, respectively. The substitution degree of the (1→5)-Ara*f* residues side chains was also evaluated and expressed as the (1→3,5)-Ara*f*/(1→5)-Ara*f* ratio value: 0.13 and 0.16, for the 1st and 2nd 2h of the hot water extraction process, respectively. These results suggested that the AG-II, insoluble in 75% ethanol aqueous solutions, which were extracted in the 1st 2h, possess a less substituted (1→3)-Gal*p* residues backbone, in comparison with the ones extracted during the 2nd 2h of the hot water extraction of *F. angustifolia* dried leaves. Moreover, the AG-II extracted in the 1st and 2nd 2h showed similar extent and degree of substitution of the (1→5)-Ara*f* residues side chains.

When comparing the structural features of the AG-II present in the $\underline{Et_{75}A\text{-}I}$ fraction, which resulted from the enzymatic digestion of the $\underline{Et_{75}A}$ fraction from the 1st 2h, with those of the AG-II present in the $\underline{Et_{75}A}$ fraction, it is possible to notice that the substitution degree of the (1→3)-Gal*p* residues backbone, and the extent and substitution degree of the (1→5)-Ara*f* residues side chains are very similar.

Table 4.5- Deduced linkages from the methylation analysis of the $\underline{Et_{75}A\text{-}I}$ fractions obtained from the *endo*-polygalacturonase treatment of the $\underline{Et_{75}A}$ fractions isolated from the decoctions of *F. angustifolia* dried leaves.

Deduced Linkage	Fraction	
	$\underline{Et_{75}A\text{-}I}$ (1st 2h)	$\underline{Et_{75}A\text{-}I}$ (2nd 2h)
t*-Rha*p	0.5	0.2
1,2-Rha*p*		
1,4-Rha*p*	0.6	0.4
1,2,4-Rha*p*		0.2
Total	***1.1[a](2.0)[b]***	***0.8(1.9)***
t-Fuc*p*	0.0	0.0
Total	***0.0(0.2)***	***0.0(0.1)***
t*-Ara*f	26.0	23.4
t*-Ara*p	1.4	
1,2-Ara*f*	2.0	1.0
1,3-Ara*f*	4.4	1.8
1,5-Ara*f*	8.0	7.7
1,3,5-Ara*f*	1.0	1.2
Total	***42.8(38.1)***	***35.1(41.8)***
t-Xyl*p*	1.4	0.8
1,2-Xyl*p*	1.4	0.5
1,4-Xyl*p*	1.9	1.0
1,2,4-Xylp	2.2	2.6
Total	***6.9(4.4)***	***4.9(5.1)***
t*-Man*p	0.2	0.2
1,4-Man*p*	1.4	3.3
1,6-Man*p*	0.3	0.3
1,4,6-Man*p*	0.0	0.2
Total	***1.9(3.7)***	***4.0(3.1)***
t*-Gal*p	3.0	3.6
1,2-Gal*p*		
1,3-Gal*p*	4.3	4.3
1,4-Gal*p*		
1,6-Gal*p*	7.5	9.0
1,3,6-Gal*p*	22.6	30.9
Total	***37.4(40.4)***	***47.8(42.6)***
t*-Glc*p	0.4	0.5
1,3-Glc*p*	1.4	1.3
1,4-Glc*p*	5.4	3.6
1,6-Glc*p*		0.1
1,3,4-Glc*p*		0.2
1,4,6-Glc*p*	2.7	1.7
Total	***9.9(11.4)***	***7.4(5.5)***

molar % obtained through: [a] linkage analysis, and [b] sugar analysis

This strengthens the presence of AG-II in the decoctions of *F. angustifolia* dried leaves that exhibit the structural features that had been referred in **Section 4.1.3**, but also shows that some of these AG-II have a molecular weight higher than 40 kDa, which is the Bio Gel P30 exclusion limit.

The presence of (1→4)-Glc*p* and (1→ 4,6)-Glc*p* residues in the $\underline{Et_{75}A}$ fractions isolated from the hot water extraction of *F. angustifolia* dried leaves was attributed to the possible presence of xyloglucans, that also seem to be present in the $\underline{Et_{75}A\text{-I}}$ fractions due to the presence of the same type of residues. The xyloglucans present in the $\underline{Et_{75}A\text{-I}}$ fractions presented (1→ 4,6)-Glc*p*/(1→4)-Glc*p* values of 0.50 and 0.47, for the 1st and 2nd 2h of the hot water extraction process, respectively. These values suggest the presence of similarly substituted xyloglucans in the 1st and 2nd 2h of the *F. angustifolia* decoctions.

The value of 0.50 registered for the $\underline{Et_{75}A\text{-I}}$ fraction obtained in the 1st 2h of the hot water extraction process, was similar to the value of 0.55 that had been reported for the correspondent untreated $\underline{Et_{75}A}$ fraction, suggesting the presence of similarly substituted xyloglucans in both fractions. These results allow to confirm the xyloglucan structural features that had been reported in **Section 4.1.3** (**Table 4.3)**, and also showed that some of these xyloglucans possess an estimated molecular weight higher than 40 kDa, which is the columns exclusion volume.

The proportion of (1→4,6)-Glc*p* residues detected seemed compatible with the proportion of T-Xyl*p* residues, indicating that the side chains of the xyloglucans recovered in the $\underline{Et_{75}A\text{-I}}$ fractions could be composed of single xylosyl residues. However, the detection of (1→2)-Xyl*p* residues does not allow to exclude the presence of side chains composed of Ara*p*-(α1→2)-Xyl*p*-(α1→ and Gal*p*-(β1→2)-Xyl*p*-(α1→ disaccharides, which had been proposed to be present as side chains of the xyloglucans detected in the untreated $\underline{Et_{75}A}$ fraction, as referred in **Section 4.1.3** (**Table 4.3**).

The T-Fuc and (1→2)-Gal*p* residues were not detected in the xyloglucans from the treated $\underline{Et_{75}A\text{-I}}$ fraction, as opposed to what was observed for the xyloglucans present in the untreated $\underline{Et_{75}A}$ fraction, as mentioned in **Section 4.1.3**. This suggests that the xyloglucans recovered in the $\underline{Et_{75}A\text{-I}}$ high molecular weight fraction, resulting from the *endo*-polygalacturonase treatment of $\underline{Et_{75}A}$ fraction, do

not seem to present the Fuc*p*-(α1→2)-Gal*p*-(β1→2)-Xyl*p*-(α1→ trisaccharide side chains. The presence of glucosyl, and xylosyl residues also in the intermediate molecular weight fraction $\underline{Et_{75}A\text{-}II}$, suggests that the untreated $\underline{Et_{75}A}$ fraction could contain xyloglucans with distinct molecular weight that could not be fractionated by its charge, but were fractionated according to their molecular weight.

4.3. Electrospray ionization mass spectrometry (ESI-MS) experiments

Mass spectrometry (MS) has been successfully used for the study of the structural details of polysaccharides, namely using soft ionization methods such as electrospray (ESI). ESI-MS gives information about the molecular weight of oligosaccharides, even when present in mixtures, and, in combination with the sugar residues composition, allows to infer the structural details of oligo and polysaccharides (Zaia, 2004). Tandem mass spectrometry (MS/MS) has proved to be a valuable tool in the structural characterization of carbohydrates, allowing to obtain detailed information about their structure (Asam and Glish, 1997; Zhou *et al*., 1990). This approach presents advantages over other commonly used methods due to its feasibility even on complex and in trace amount of samples.

The $\underline{Et_{75}A}$ fraction from the 1st 2h of the hot water extraction of *F. angustifolia* dried leaves was submitted to an *endo*-polygalacturonase treatment. The *endo*-polygalacturonase treatment comprised a previous de-esterification with NaOH, and subsequent enzymatic digestion with *endo*-polygacturonase. Following the enzymatic treatment, the products of the *endo*-polygalacturonase treatment were concentrated, and loaded on a column containing Bio Gel P2, which presents a typical fractionation range comprised between 1.8 and 0.1 kDa. The column was previously equilibrated with distilled water, calibrated with blue dextran and glucose. The obtained chromatographic elution profile is shown in **Figure 4.2 a)**, evidencing that all the carbohydrate material eluted in a single band near the columns exclusion volume.

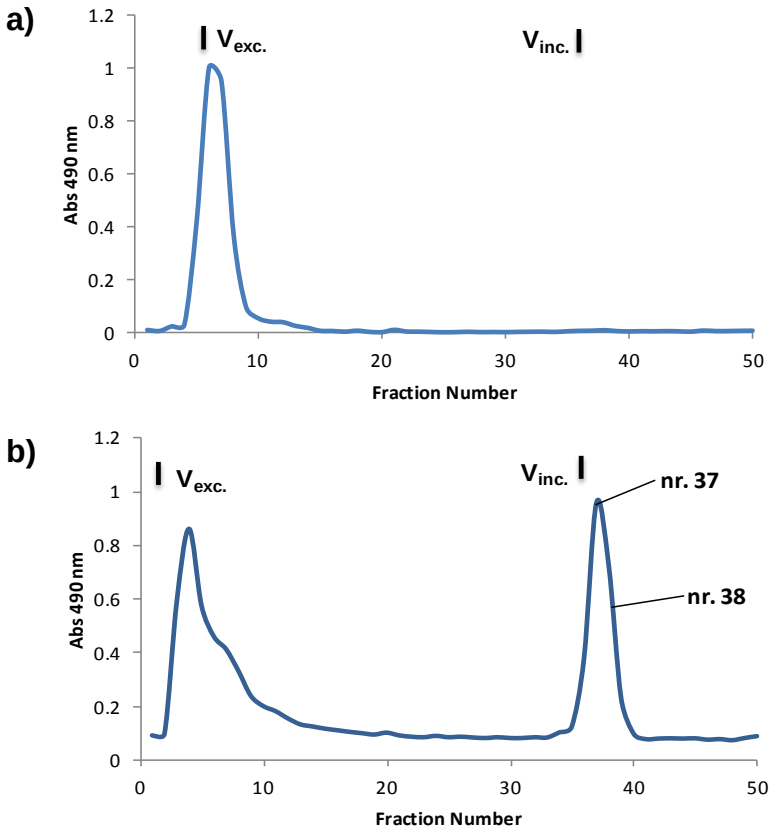

Figure 4.2- Size exclusion chromatography on **a)** Bio Gel P2, and **b)** Bio Gel P6 of the oligosaccharides obtained after *endo*-polygalacturonase treatment of the $\underline{Et_{75}A}$ fraction, obtained from the 1st 2h of the hot water extraction of *F. angustifolia* dried leaves.

This could indicate that the *endo*-polygalacturonase treatment did not work, or that all the products, which resulted from the *endo*-polygalacturonase treatment, had an estimated molecular weight higher than 1.8 kDa, which is the columns exclusion volume. Thus, in order to clarify what had happened, the material that eluted in the referred band was pooled, freeze-dried, re-dissolved in distilled water, and loaded on a column containing Bio Gel P6 that has a wider typical fractionation range, comprised between 6.0 and 1.0 kDa. The Bio Gel P6 was also previously equilibrated with distilled water, calibrated with blue dextran and glucose. The chromatographic elution profile obtained, which is shown in **Figure 4.2 b)**, presents one band that eluted near the columns exclusion volume, suggesting the presence of material with an estimated molecular weight higher than 6.0 kDa, which in accordance with the observed when the material was fractionated on Bio Gel P2. Also, one band eluting near the columns inclusion

volume was observed, which indicates the presence of material that should present an estimated molecular weight lower than 1.0 kDa, which seems to conflict with the observed in the Bio Gel P2 fractionation, where it was inferred that all the material that eluted near the columns exclusion volume should present an estimated molecular weight higher than 1.8 kDa. This suggests that, besides molecular weight, some other factor should have contributed for the interaction between the analytes, and the Bio Gel P2 and P6. These results could be explained by the presence of anionic groups, which are known to elute earlier than the corresponding neutral oligosaccharides, due to electrostatic interactions, which are stronger when using water as the eluent (Churms, 2002), as occurred in this case, in order to enable the MS analysis of the fractions of interest.

The fractions of the chromatographic band that eluted near the column inclusion volume, and that contains the oligosaccharides resulting from the *endo*-polygalacturonase treatment, can provide information regarding the structural features of the pectic polysaccharides present in the decoctions of *F. angustifolia* dried leaves. Thus, selected fractions from the chromatographic band that eluted near the column inclusion volume were analysed by ESI-MS and ESI-MS/MS. The ESI-MS spectra of the fractions number 37, and 38 are shown in **Figure 4.3**.

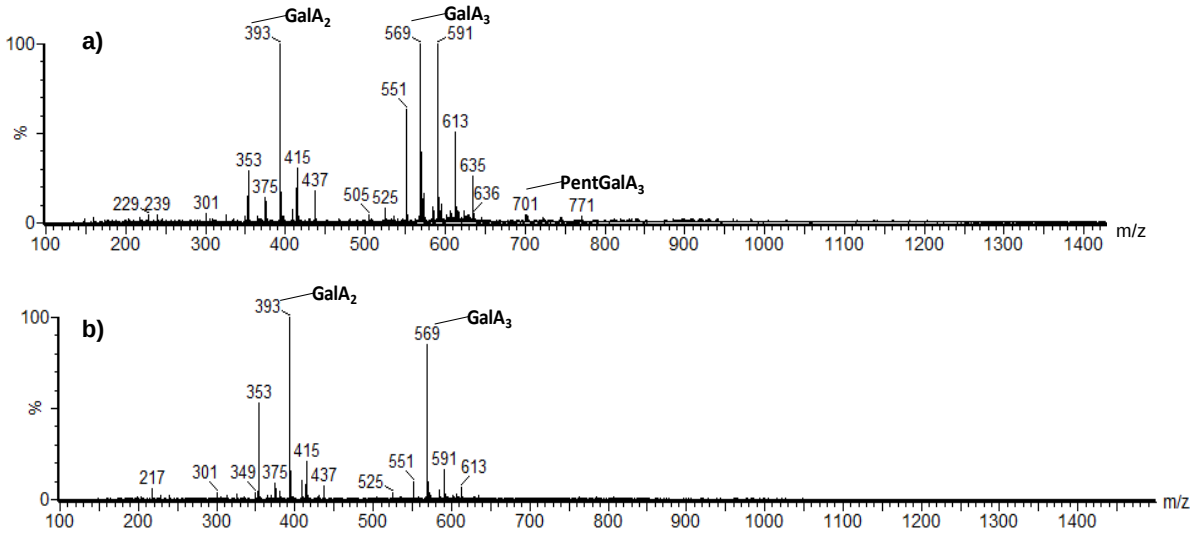

Figure 4.3- ESI-MS spectra of the fractions **a)** number 37, and **b)** number 38 from the $\underline{Et_{75}A}$ fraction, obtained from the 1st 2h of the hot water extraction of *F. angustifolia* dried leaves, and submitted to *endo*-polygalacturonase treatment.

The MS spectra exhibited ion fragments that have been previously identified as sodium adduct ions ($[M+Na]^+$), typical of oligogalacturonides ionization by ESI-MS (Nunes *et al*., 2012). Based on the MS spectra of both fractions, it can be observed the presence of $[M+Na]^+$ ions that mainly corresponded to oligosaccharides constituted only by galacturonic acid residues ($GalA_n$, n = 2 – 3), at *m/z* 393, and 569. The MS spectrum of the fraction number 37 also presented a less abundant $[M+Na]^+$ ion at *m/z* 701, which suggested the presence of an oligosaccharide composed by three galacturonic acid residues, and an additional pentose residue.

To confirm these assignments, ESI-MS/MS was performed for the ion fragments at *m/z* 569, and 701, and the respective spectra are presented in **Figure 4.4**. The ESI-MS/MS spectrum of the ion at *m/z* 569, presented in **Figure 4.4 a)**, showed product ions at *m/z* 393 and 217.

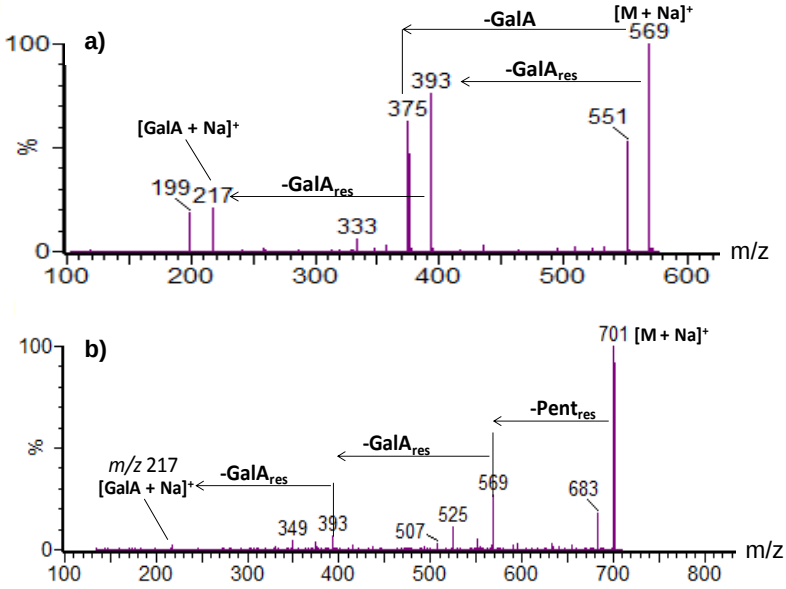

Figure 4.4- ESI-MS/MS spectra of the $[M+Na]^+$ ions at **a)** *m/z* 569 and **b)** *m/z* 701 of the $\underline{Et_{75}A}$ fraction, obtained from the 1st 2h of the hot water extraction of *F. angustifolia* dried leaves, and submitted to *endo*-polygalacturonase treatment.

These product ions are attributed to $[GalA_2+Na]^+$, and $[GalA+Na]^+$, respectively, resultant from the loss of 176 and 352 Da, correspondent to the loss of $GalA_{res}$ and $GalA_{2res}$. Product ions at *m/z* 551, 375, and 199, due to the loss of 18, 194, and 370 Da, were also detected and attributed to the loss of H_2O, GalA, and $GalA_2$. These results confirm the assignment of the fragment ion detected in the

MS spectrum at *m/z* 569 to the $[GalA_3+Na]^+$ oligosaccharide, reinforcing the presence of (α1→4)-linked galacturonic acid residues.

The ESI-MS/MS spectrum of the ion at *m/z* 701, presented in **Figure 4.4 b)**, showed a product ion at *m/z* 683, due to the loss of 18 Da, which was attributed to the loss of H_2O. The ESI-MS/MS spectrum of the ion at *m/z* 701 also showed a product ion at *m/z* 569. This product ion is due to the loss of 132 Da, attributed to the loss of a pentose residue, which confirms the presence of the pentose residue in the ion at *m/z* 701. Also ions at *m/z* 525, 393, 349, and 217, attributed to $[GalA_2Pent+Na]^+$, $[GalA_2+Na]^+$, $[GalAPent+Na]^+$, and $[GalA+Na]^+$, respectively, were detected. These product ions are due to the loss of 176, 308, 352, and 484 Da, correspondent to the loss of $GalA_{res}$, $GalAPent_{res}$, $GalA_{2res}$, and $GalA_2Pent_{res}$. This fragmentation pattern confirms the presence of a pentose residue attached to galacturonic acid residues in the oligosaccharides that were released by the *endo*-polygalacturonase treatment of the $\underline{Et_{75}A}$ fraction. The methylation analysis of the $\underline{Et_{75}A}$ fraction from the 1st 2h of the decoctions of *F. angustifolia* dried leaves, revealed the presence of T-Xyl*p* residues (**Table 4.3**). Although the methylation analysis also evidenced the presence of T-Ara*f* residues, as far as we know, there seems to be no reports of arabinosyl residues directly attached to the galacturonic acid residues of pectic polysaccharides. Thus, it seems plausible to consider that the pentose residue is due to xylosyl, and that this oligosaccharide might be present as a structural component of the xylogalacturonan regions of the pectic polysaccharides. The occurrence of oligosaccharides containing pentose residues, attributed to the presence of xylose, was reported for the pectic polysaccharides from plum and pear cell walls, and commercial pectic polysaccharides obtained from citrus (Nunes *et al*., 2012).

4.4. Concluding remarks

The decoctions of *F. angustifolia* dried leaves contains high proportions of pectic polysaccharides, together with type I, and AG-II, mannans, and also xyloglucans, all present in lower proportions. The pectic polysaccharides, AG-II, and xyloglucans were detected in all fractions, while the AG-I, and mannans were only detected in the acidic, and neutral fractions, respectively. It seems plausible that the decoctions of *F. angustifolia* dried leaves might contain AG-II present as AGP´s, and also that the pectic polysaccharides possess xylogalacturonan domains.

CHAPTER 5

STRUCTURAL FEATURES OF *MENTHA SUAVEOLENS* POLYSACCHARIDES EXTRACTED WITH HOT WATER

In this chapter, the structural features of the polysaccharides from the extensive hot water extraction of *Mentha suaveolens* dried shoots during a total period of 4 h (2 h + 2 h) are described. The extracted material was dialysed and the high molecular weight material (HMWM) was fractionated according to its solubility in aqueous ethanol solutions. The content of carbohydrates and phenolic compounds of the various fractions were determined, as well as the antioxidant activity, expressed as the DPPH scavenging activity. The carbohydrate composition of the polysaccharides present in the various fractions obtained was determined. The fractions precipitated with 50 and 75% of aqueous ethanol solutions were further fractionated by anion exchange chromatography on DEAE-Sepharose FF. The carbohydrate composition, the type of glycosidic linkages and the degree of methyl esterification of the polysaccharides present in the various fractions obtained were evaluated. Selected fractions, rich in pectic polysaccharides, were also digested with *endo*-polygalacturonase, fractionated by size exclusion chromatography on Bio Gel.

5.1. Isolation and fractionation of the polysaccharides from the HMWM

5.1.1. Ethanol fractionation

The HMWM obtained from the infusions of *M. suaveolens* dried shoots was suspended in water (10.0 mg/mL) and the material insoluble in water at ambient temperature was separated, yielding the WI_{ppt} fraction. The remaining HMWM was fractionated by ethanol precipitation, yielding three fractions: Et_{50}, HMWM insoluble in 50% ethanol solution; Et_{75}, HMWM insoluble in 75% ethanol solution; and SN, HMWM soluble in 75% ethanol solution. HMWM mass recoveries of 91.1 ± 0.2% and 92.9 ± 1.0% for the 1^{st} and the 2^{nd} 2 h of the infusion process were registered. These values are comparable to the HMWM mass recoveries registered for the decoctions of *Fraxinus angustifolia* dried leaves, evidencing the hygroscopicity of the compounds present in the HMWM, namely the polysaccharides.

The mass yield, total carbohydrate and total phenolic compound contents, and the EC_{50} values, which expressed the DPPH scavenging activity of the various

fractions, together with the composition of the polysaccharides recovered in the various fractions obtained by ethanol precipitation are shown in **Table 5.1**.

Table 5.1- Mass yield, total sugar content and monosaccharide composition, and DPPH scavenging activity, expressed by the EC_{50} value, of the Et_{50} and Et_{75} fractions obtained by anion exchange chromatography of the HMWM from the decoctions of *Mentha suaveolens* dried shoots.

	Yield[a]	Total Sugars	Monosaccharide Composition (mol %)								Total Phenolics	EC_{50}
	(mass %)	(mass %)	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	(mass %)	(mg/mL)
1st 2h												
Starting HMWM		46.0	1.8	0.0	5.3	0.9	3.0	8.6	9.4	71.1	14.5	nd
WI_{ppt}	15.1	12.9	1.4	0.0	6.4	3.2	2.3	7.2	31.8	47.6	nd	nd
Et_{50}	38.5	72.0	1.6	0.8	4.0	0.5	0.7	4.0	2.0	86.3	4.3	0.43
Et_{75}	18.8	63.5	1.7	0.8	6.3	1.3	4.0	10.0	6.5	69.3	10.9	0.29
SN	27.6	26.4	3.1	1.0	4.9	1.5	8.3	16.7	23.4	41.0	34.2	0.05
2nd 2h												
Starting HMWM		70.0	1.6	0.0	6.8	0.8	1.7	8.1	4.8	76.4	10.2	nd
WI_{ppt}	11.4	18.2	1.6	0.0	7.2	1.0	1.6	10.5	33.0	45.2	nd	nd
Et_{50}	54.3	91.4	1.1	0.3	3.8	0.4	0.2	3.8	0.9	89.4	2.2	1.02
Et_{75}	12.8	64.2	1.6	0.4	8.2	1.2	3.2	11.3	4.6	69.3	6.6	0.32
SN	21.5	29.6	2.1	0.6	9.5	1.7	7.5	22.0	19.4	37.2	25.8	0.06

[a] - expressed relatively to the HMWM recovered; [nd] - not determined

The data presented in the **Table 5.1** under the designation of "Starting HMWM" is relative to the HMWM that was submitted to the ethanol fractionation procedure. This data has been presented and discussed in Chapter 3, however it is shown here as a reference for comparisons with the fractions that were obtained from HMWM ethanol precipitation. The starting HMWM from the 1st and 2nd 2 h of the hot water extraction process contained 46 and 70% of carbohydrate material, respectively. Et_{50} fractions from the 1st and 2nd 2 h of the hot water extraction process contained 72.0 and 91.4% of carbohydrate material, respectively, evidencing the carbohydrate enrichment of the Et_{50} fractions obtained through ethanol precipitation. The Et_{75} fractions from the 1st and 2nd 2 h of the hot water extraction process contained 63.5 and 64.2 % of carbohydrate material, respectively, showing that only for the 1st 2h of the hot water extraction process carbohydrate enrichment was registered. In the ethanol precipitation of the HMWM from the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried

shoots, it was observed that the Et_{50} fractions, obtained in both parts of the hot water extraction, presented a carbohydrate content higher than the ones from the respective starting HMWM. Relatively to the Et_{75} fractions, for *F. angustifolia* the carbohydrate material enrichment was registered in the fraction from the 2nd 2h, while for *M. suaveolens* it was observed in the fraction from the 1st 2h of the hot water extraction.

Besides being the most sugar richest, the Et_{50} fractions from the decoctions of *M. suaveolens* dried shoots were also the most abundant ones, comprising 38.5 and 54.3%, in the 1st and 2nd 2h, respectively. These fractions were the poorest in total phenolic compounds with antioxidant activity, determined by Folin-Ciocalteau method, and therefore also exhibited the highest EC_{50} values, evidencing its lower antioxidant activity. The Et_{75} fractions were poorer in carbohydrate material and also less abundant than the Et_{50} fractions, comprising 18.8 and 12.8%, in the 1st and 2nd 2h, respectively. These fractions were richer in total phenolic compounds with antioxidant activity than the Et_{50} fractions, and therefore also exhibited lower EC_{50} values, evidencing its higher antioxidant activity. The SN fractions accounted for 27.6, in the 1st 2h, and 21.5%, in the 2nd 2h, of the HMWM recovered, with 26.4 and 29.6% of carbohydrate material, respectively. These fractions were the richest in total phenolic compounds with antioxidant activity, exhibiting the lowest EC_{50} values, which evidenced its higher antioxidant activity. The WI_{ppt} fractions, which only accounted for 15.1, in the 1st 2h, and 11.4%, in the 2nd 2h, of the HMWM recovered, were also the poorest in carbohydrate material: 12.9, in the 1st 2h, and 18.2%, in the 2nd 2h.

Through the multiplication of the mass yield of each fraction by the respective proportion of total sugars, it is possible to calculate the proportion of carbohydrate material present in each fraction. Thus, it was observed that 56.7, in the 1st 2h, and 74.9%, in the 2nd 2h, of the polysaccharides present in the HMWM precipitated in 50% ethanol aqueous solutions, while only 24.4 and 12.4% precipitated in 75% ethanol aqueous solutions, in the 1st and 2nd 2h, respectively. These results evidenced that the decoctions of *M. suaveolens* dried shoots contained higher proportions of HMWM that precipitated in 50% ethanol aqueous

solutions, similarly to what had been observed for the decoctions of *F. angustifolia* dried leaves.

The Et_{50} and Et_{75} fractions of *M. suaveolens* dried shoots decoctions presented a monomeric composition rich in UA, suggesting the presence of pectic polysaccharides. The Et_{75} fractions also displayed Ara and Gal, concomitant with a lower proportion of uronic acid residues, indicating the possible presence of arabinogalactans. It was also possible to observe the presence of glucosyl residues, which based on the results from *F. angustifolia* may indicate the presence of xyloglucans. It was observed that the fractions precipitated with lower concentration of ethanol contained polysaccharides with higher proportions of uronic acid residues, similarly to what had been observed for the decoctions of *F. angustifolia* dried leaves. This suggests that the ethanol precipitation is fractioning the polysaccharides according to their proportion of uronic acid residues.

5.1.2. Anion exchange chromatography

The fractions Et_{50} and Et_{75}, which were those with the highest proportions of carbohydrate material, were further fractionated by anion exchange chromatography on DEAE-Sepharose FF, in order to separate the polysaccharides present in these fractions based on their charge. For both Et_{50} and Et_{75} fractions, one not retained neutral fraction (A), eluted with buffer, and three retained acidic fractions (B, C and E), eluted with buffer containing 0.125, 0.250, and 1.0 M NaCl, respectively, were obtained. Comparing to the anion exchange chromatography of the *F. angustifolia* recovered fractions, an extra fraction was obtained, with 0.125 M NaCl.

The mass yield, total sugars as well as their monomeric composition and methyl esterification degree, are shown in **Table 5.2**. For the Et_{50} fractionation, total mass recoveries of 91.0 and 96.4% were registered for the 1^{st} and 2^{nd} 2 h of the hot water extraction process, respectively, while for Et_{75} fractionation, mass recoveries of 85.1 and 83.4% were observed for the 1^{st} and 2^{nd} 2 h of the hot water extraction process, respectively. Similarly to was referred for the anion exchange chromatography of the Et_{50} and Et_{75} fractions, isolated from the HMWM

of *F. angustifolia* dried leaves decoctions, these recovery values suggested a possible interaction between some compounds present in these fractions and the DEAE-Sepharose FF gel. This hypothesis was supported by the appearance of a darker colour at the top of the column, that could only be removed after washing the gel with 2.0 M NaCl and 1.0 M NaCl solutions in a reversed flow direction, similarly to what had been observed for the fractionation of *F. angustifolia* Et_{50} and Et_{75} fractions.

Table 5.2- Mass yield, total sugar content and monosaccharide composition, and methyl esterification degree, shown by the methanol released from polysaccharides, of the Et_{50} and Et_{75} fractions obtained by anion exchange chromatography of the HMWM from the decoctions of *M. suaveolens* dried shoots.

	Yield[a] (mass %)	Total Sugar (mass %)	Methanol (mmol/mol UA)	Monosaccharide Composition (mol %)							
				Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
Et_{50} *1st 2h*											
A	20.8	55.1	511.2	0.4	0.7	2.9	1.1	3.2	4.8	6.2	80.7
B	44.7	96.5	403.6	0.9	0.4	2.2	0.2	0.1	1.8	0.5	94.0
C	20.5	59.4	279.5	2.5	1.1	5.3	0.5	0.2	4.4	0.9	85.2
E	14.0	86.6	nd	2.9	0.0	8.8	1.2	2.0	12.7	5.7	66.7
Et_{75} *1st 2h*											
A	31.9	75.2	446.7	0.5	0.0	4.7	2.1	6.7	9.6	9.8	66.6
B	35.2	94.6	283.1	1.8	0.2	5.6	0.4	0.3	5.4	0.8	85.5
C	16.7	65.4	192.4	4.7	0.4	10.4	0.7	0.5	7.4	1.0	74.9
E	16.2	12.9	nd	3.8	0.0	26.1	1.8	0.0	3.9	26.2	38.3
Et_{50} *2nd 2h*											
A	13.4	45.6	491.4	0.5	0.0	3.9	1.7	2.1	5.2	4.4	82.2
B	46.5	99.5	418.7	1.1	0.1	3.3	0.3	0.1	2.4	0.4	92.2
C	26.4	81.1	203.3	3.0	0.2	6.7	0.3	0.1	4.8	0.2	84.7
E	13.7	16.8	nd	4.3	0.0	32	0.9	0.7	14.3	2.9	45.0
Et_{75} *2nd 2h*											
A	41.3	97.5	423.5	0.7	0.2	7.6	2.8	10.9	14.5	12.7	50.7
B	32.3	75.8	312.5	3.4	0.4	14.2	0.4	0.3	13.7	0.8	66.8
C	12.9	50.0	205.4	6.7	0.3	25.7	0.5	0.7	19.1	1.5	45.5
E	13.6	13.8	nd	3.3	0.0	26.8	1.3	0.0	2.8	31.2	34.7

[a] - expressed relatively to the HMWM recovered; [nd] - not determined

It was observed a higher recovery for the anion exchange chromatography of the Et_{50} fractions, which contained lower proportions of total phenolic compounds

with antioxidant activity, in comparison with the Et_{75} ones. This behaviour, which had also been observed for the fractionation of the Et_{50} and Et_{75} fractions from *F. angustifolia* dried leaves decoctions, suggests the possible involvement of the phenolic compounds in this interaction with the DEAE-Sepharose FF gel.

For Et_{50} fractionation, the most abundant were the $\underline{Et_{50}B}$ fractions that contained 44.7%, in the 1st 2h, and 46.5%, in the 2nd 2h, of the HMWM recovered, both comprising more than 95% of polysaccharides. For the Et_{75} fractionation, the most abundant were the $\underline{Et_{75}A}$ and $\underline{Et_{75}B}$ fractions that represented 31.9 and 35.2%, in the 1st 2h, and 41.3 and 32.3%, in the 2nd 2h, of the HMWM recovered, respectively. The $\underline{Et_{75}A}$ and $\underline{Et_{75}B}$ fractions also exhibited high proportions of carbohydrate material: 75.2 and 94.6%, in the 1st 2h, and 97.5 and 75.8%, in the 2nd 2h, respectively. The HMWM recovered in the most acidic (E) fractions was the less abundant and also the poorest in carbohydrate material. These results evidenced that the carbohydrate material that precipitated in 50% ethanol aqueous solutions comprised higher proportions of polysaccharides that were retained by the DEAE-Sepharose FF gel and subsequently eluted with low ionic strength solutions. It was also evidenced that the carbohydrate material that precipitated in 75% ethanol aqueous solutions was composed of similar proportions of polysaccharides that were not retained by the gel and also polysaccharides that were retained by the gel and subsequently eluted with low ionic strength solutions. These results are similar to those observed for the DEAE-Sepharose FF chromatography of the fractions from *F. angustifolia* dried leaves decoctions.

The $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$ fractions exhibited a monomeric composition very rich in uronic acid residues, which was similar to that presented by the Et_{50} fractions that were submitted to the fractionation by anion exchange chromatography on DEAE-Sepharose FF. It was observed that the neutral $\underline{Et_{50}A}$ fractions contained lower proportions of uronic acid residues, while the $\underline{Et_{50}B}$ presented the highest proportions. It was also observed that the uronic acid residues of the polysaccharides present in the neutral fractions were methyl esterified in a greater extent than those from the polysaccharides of acidic B and C fractions, with the polysaccharides of the C fractions exhibiting the lower degree of

methyl esterification (51.1, 40.4, and 28.0%, in the 1st 2h; 49.1, 41.9, and 20.3%, in the 2nd 2h, for the $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$ fractions, respectively).

The polysaccharides from the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ fractions presented a monomeric composition that mainly comprised uronic acid residues, however in lower proportions than the correspondent $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$ fractions, which render them more soluble in ethanol aqueous solutions. This difference between the uronic acid content of the fractions referred above was more evident for the 2nd 2h of the hot water extraction process. The uronic acid residues content increased in the following order: $\underline{Et_{75}A}$ < $\underline{Et_{75}C}$ < $\underline{Et_{75}B}$, while the methyl esterification degree of the uronic acid residues increased in the following sequence: $\underline{Et_{75}C}$ < $\underline{Et_{75}B}$ < $\underline{Et_{75}A}$, similarly to what had been observed for the fractions that resulted from the anion exchange chromatography of the $\underline{Et_{50}}$ fractions. The methyl esterification degree of the polysaccharides present in the various fractions analysed suggests that the fractionation was accomplished based on the methyl esterification degree of the uronic acid residues of the pectic polysaccharides present in both Et_{50} and Et_{75} fractions of the decoctions from *M. suaveolens* dried shoots, similarly to what had been observed for the decoctions of *F. angustifolia* dried leaves.

Comparing the monomeric composition of the polysaccharides present in the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ fractions with the monomeric composition of the Et_{75} fractions that were fractionated by anion exchange chromatography on DEAE-Sepharose FF, it was possible to observe some differences in the proportion of neutral sugars residues. The neutral $\underline{Et_{75}A}$ fractions comprised higher proportions of glucosyl and mannosyl residues, while the less acidic $\underline{Et_{75}B}$ fractions contained lower proportions of these residues. The more acidic $\underline{Et_{75}C}$ fractions also contained lower proportions of glucosyl and mannosyl residues, concomitant with higher proportions of arabinosyl and rhamnosyl residues. These differences were more pronounced for the 2nd 2h of the hot water extraction process. For the 2nd 2h of the hot water extraction process, the more acidic $\underline{Et_{75}C}$ fraction also contained higher proportions of galactosyl residues in comparison with the fractionated Et_{75} fraction.

5.1.3. Glycosidic linkage analysis

In order to elucidate the type of polysaccharides present in the decoctions from *M. suaveolens* dried shoots, the fractions that showed the highest amounts of carbohydrate material were methylated and analysed as partially methylated alditol acetates. A carboxyl reduction of the methylated polysaccharides was also performed for the detection of the uronic acid residues and assessment of the respective types of linkage.

It was observed that for the 1st and 2nd 2h of the hot water extraction the monomeric composition of the $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$ was similar. For the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ it was observed a higher variability between the monomeric composition of the fractions from the 1st and 2nd 2h of the hot water extraction process. The fractions from the 1st 2h exhibited higher proportions of uronic acid residues, concomitant with lower proportions of arabinosyl and galactosyl residues, in comparison with those from the 2nd 2h. Therefore, the linkage analysis was performed for the $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$ fractions obtained in the 1st 2h, whereas for the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ fractions the linkage analysis was performed for both decoctions, and the results are shown in **Table 5.3**.

For all the fractions, the carboxyl reduction of the previously methylated polysaccharides allowed the detection of 1,4,5,6-tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-hexitol residue with a mass spectra with a fragmentation pattern that exhibited some ions with +2 m/z units, indicating the presence of (1 → 4)-HexA. As the pectic polysaccharides seem to be the major type of polymer present in the decoctions from *M. suaveolens* dried shoots it is plausible to consider that the presence of the (1→4)-HexA residues to the presence of (1 → 4)-GalA, which is one of the main components of pectic polysaccharides. The presence of *t*-GalA and *t*-GlcA as pectic polysaccharide components has been also reported, however they are found in much lower proportions, in comparison with the (1 → 4)-GalA residues.

Table 5.3- Deduced linkages from the methylation analysis of the neutral (A) and acidic (B and C) fractions from the Et_{50} and Et_{75} fractions of the 1st 2h of the decoctions from *Mentha suaveolens* dried shoots.

Linkage	Fraction					
	Et_{50}A	Et_{50}B	Et_{50}C	Et_{75}A	Et_{75}B	Et_{75}C
t*-Rha*p	0.7	3.4	2.7	0.3	1.9	2.4
1,2-Rha*p*	0.5	2.2	6.1	0.4	2.6	4.8
1,3-Rha*p*			1.2		1.2	0.8
1,2,4-Rha*p*	0.2	2.9	6.2		2.7	4.3
1,3,4-Rha*p*			1.9		4.1	1.9
Total	***1.4[a] (2.1)[b]***	***8.5(15.0)***	***18.1(16.9)***	***0.7(1.5)***	***12.5(12.4)***	***14.2(18.7)***
t-Fuc*p*	0.4	2.7	2.7	0.2	2.5	1.7
Total	***0.4(3.6)***	***2.7(6.7)***	***2.7(7.4)***	***0.2(0.0)***	***2.5(1.4)***	***1.7(1.6)***
t*-Ara*f	7.3	10.0	10.5	2.9	6.6	11.1
t*-Ara*p	1.3	3.3	2.3	0.6	3.8	2.2
1,2-Ara*f*	0.8	0.8	0.8	0.6	0.7	1.3
1,3-Ara*f*	2.9	2.5	2.6	0.9	2.3	3.8
1,4-Ara*p*					9.0	4.3
1,5-Ara*f*	3.7	8.5	11.5	1.8	5.9	11.4
1,3,5-Ara*f*	1.5	4.4	6.3	1.0	3.0	6.6
Total	***17.5(15.0)***	***29.5(36.7)***	***34.0(35.8)***	***7.8(14.1)***	***31.3(38.6)***	***40.7(41.4)***
t-Xyl*p*	2.7	2.6	1.7	1.1	1.4	2.9
1,2-Xyl*p*	2.7			1.4		
1,4-Xyl*p*	3.0	6.0	3.7	1.4	1.9	3.3
1,2,4-Xylp	0.7	0.9	0.7	0.9	1.2	1.5
Total	***9.1(5.7)***	***9.5(3.3)***	***6.1(3.4)***	***4.8(6.3)***	***4.5(2.8)***	***7.7(2.8)***
t*-Man*p	1.3	0.6	0.3	1.2	0.3	0.3
1,4-Man*p*	9.9	0.0	0.0	18.5	0.0	0.0
1,2,4-Man*p*				0.9		
1,4,6-Man*p*	2.0			4.6		
Total	***13.2(16.6)***	***0.6(1.7)***	***0.3(1.4)***	***25.2(20.1)***	***0.3(2.1)***	***0.3(2.0)***
t*-Gal*p	5.6	6.2	9.0	8.2	9.1	7.5
1,2-Gal*p*						
1,3-Gal*p*	1.7	3.4	4.6	1.8	3.9	3.0
1,4-Gal*p*		5.6	4.4		3.3	5.2
1,6-Gal*p*	12.6	8.6	7.0	16.5	8.6	4.9
1,3,6-Gal*p*	7.5	14.3	5.9	6.0	13.7	5.3
Total	***27.4(24.9)***	***38.1(30.0)***	***30.9(29.7)***	***32.5(28.7)***	***38.6(37.2)***	***25.9(29.5)***
t*-Glc*p	2.6	1.8	2.1	1.8	0.7	1.4
1,3-Glc*p*	2.7	1.2	0.9	2.5	1.1	1.1
1,4-Glc*p*	20.5	2.3	1.3	20.1	3.9	4.8
1,6-Glc*p*	0.4	0.9		0.4		
1,2,4-Glc*p*		2.6	2.5		2.2	1.3
1,3,4-Glc*p*					1.4	
1,4,6-Glc*p*	4.8	2.3	1.1	4.0	1.0	0.9
Total	***31.0(32.1)***	***11.1(8.3)***	***7.9(6.1)***	***28.8(29.3)***	***10.3(5.5)***	***9.5(4.0)***

molar % obtained through: [a] linkage analysis, and [b] sugar analysis

This, combined with the very low recovery of the uronic acid residues (<5%), could have contributed for the non detection of these residues. Due to the high proportion of UA residues present in these fractions, and as only (1 → 4)-GalA residues were detected, we opted for presenting the linkage analysis referring only to the neutral sugar residues, contributing for a better understanding of the possible distinct structural features of the polysaccharides present in the isolated fractions.

Good agreement between the molar fractions obtained from sugar (alditol acetates) and linkage (partially methylated alditol acetates) analysis was observed. Relatively good agreement between the proportion of terminal and ramified residues was registered for each fraction: 21.9 and 16.7%, 30.6 and 27.4%, 31.3 and 24.6%, for the terminal and ramified residues of $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$, respectively. For the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ fractions obtained during the 1st 2 h, 16.3 and 17.4%, 26.3 and 29.3%, 29.5 and 21.8% were registered for the terminal and ramified residues, respectively. The $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ fractions obtained during the 2nd 2 h, exhibited 23.9 and 15.1%, 30.2 and 28.8%, and 30.8 and 28.4% of terminal and ramified residues, respectively.

It was possible to observe that the polysaccharides that precipitated in 50 and 75% of ethanol aqueous solutions that were recovered in the neutral fractions, exhibited lower proportions of terminal and branched residues, suggesting the presence of less ramified carbohydrate material in the neutral fractions, in comparison with the polysaccharides recovered in the acidic fractions.

For all the fractions analysed, it was possible to detect galactosyl residues in linkages that indicated the presence of AG-II, namely terminal-, (1→3)-, (1→6)-, and (1→3,6)-Gal*p* residues. The polysaccharides present in the fractions $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$ presented a substitution degree, expressed by the value of the ratio (1→3,6)-Gal*p*/(1→ 3)-Gal*p*, of 4.4, 4.2, and 1.3, respectively, suggesting the presence of AG-II with a more substituted backbone in the neutral $\underline{Et_{50}A}$ and less acidic $\underline{Et_{50}B}$ fractions. The correspondent $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ fractions contained AG-II with substitution degrees of 3.3, 3.5, and 1.8, respectively, also suggesting the presence of more substituted (1→3)-Gal*p* backbone in the $\underline{Et_{75}A}$ and $\underline{Et_{75}B}$ fractions, similarly to what was observed in the $\underline{Et_{50}}$ fractions.

Also arabinosyl residues usually found in AG-II, such as T-Ara*f*, T-Ara*p*, (1→2)-, (1→3)-, (1→5)-, and (1 →3,5)-Ara*f* residues, were detected. The neutral $\underline{Et_{50}A}$ fraction contained AG-II that presented (1→5)-Ara*f*/T-Ara*f* ratio values lower than the acidic fractions $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$ (0.51, 0.85, and 1.10, for the $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$, respectively), suggesting the presence of more extended (1 → 5)-linked arabinosyl residues side chains in the acidic fractions. The same tendency was also registered for the fractions that resulted from the anion exchange chromatography of the HMWM insoluble in 75% ethanol aqueous solutions: 0.62, 0.89, and 1.0, for the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$, respectively. These (1→5)-Ara*f*/T-Ara*f* ratio values also suggest that the AG-II present in the $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$ fractions exhibit (1→5)-linked arabinosyl residues side chains with a similar extent comparatively to the correspondent $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ fractions.

The (1→5)-linked arabinosyl residues side chains can be substituted at O-3, as evidenced by the presence of (1→3,5)-Ara*f* residues in all the fractions analysed, and particularly in the acidic fractions. The substitution degree of the (1→ 5)-linked arabinosyl residues side chains of the neutral fractions was similar, as expressed by the (1→3,5)-Ara*f*/(1→5)-Ara*f* ratio values: 0.41 and 0.33, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$, respectively. These values were only slightly lower than the substitution degree of the acidic fractions: 0.52 and 0.55, for the $\underline{Et_{50}B}$ and $\underline{Et_{50}C}$ fractions, respectively; 0.51 and 0.58 for the $\underline{Et_{75}B}$ and $\underline{Et_{75}C}$ fractions, respectively. This suggests the presence of AG-II with similarly substituted (1→5)-linked arabinosyl residues side chains.

The linkage analysis of the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$ and $\underline{Et_{75}C}$ fractions from the 2nd 2h of the hot water extraction process was also performed, since these fractions showed monomeric compositions that were distinct from those of the correspondent $\underline{Et_{75}A}$, $\underline{Et_{75}B}$ and $\underline{Et_{75}C}$ fractions from the 1st 2h of the hot water extraction process. As referred earlier the fractions from the 1st 2h presented a monomeric composition richer in uronic acid residues and concomitantly poorer in arabinosyl and galactosyl residues, in comparison with the fractions obtained in the 2nd 2h of the hot water extraction process. The results of the linkage analysis of the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$ and $\underline{Et_{75}C}$ fractions from the 2nd 2h of the hot water extraction of *M. suaveolens*

dried shoots are presented in **Table 5.4**. Relatively good agreement between the proportion of terminal and ramified residues was registered for each fraction: 24.1 and 16.6%, 30.2 and 28.8%, 30.8 and 28.4%, for the terminal and ramified residues of the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ fractions obtained during the 2nd 2h, respectively.

For the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$ and $\underline{Et_{75}C}$ fractions from the 2nd 2h of the hot water extraction process, arabinosyl and galactosyl residues diagnostic of the presence of AG-II were also detected. The AG-II showed galactan backbone substitution degrees of 5.8, 4.3, and 2.5 for the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$ and $\underline{Et_{75}C}$ fractions, respectively.

The (1→5)-Ara*f*/T-Ara*f* ratio values suggested the presence of AG-II with more extended (1→5)-linked arabinosyl residues side chains in the acidic fractions (0.53, 0.82, and 0.95 for the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$ and $\underline{Et_{75}C}$ fractions, respectively), which also seemed to be more substituted than the neutral fraction, as evidenced by the higher (1→3,5)-Ara*f*/(1→5)-Ara*f* ratio values (0.29, 0.46, and 0.62 for the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$ and $\underline{Et_{75}C}$ fractions, respectively).

These results suggested that for the AG-II that precipitated in 75% ethanol aqueous solutions, those extracted during the 2nd 2h exhibited a more substituted (1→3)-Gal*p* backbone than the correspondent AG-II from the 1st 2h of the hot water extraction process.

In the decoctions of *F. angustifolia* dried leaves, it was possible to observe that the AG-II with a more substituted (1→3)-Gal*p* backbone were recovered in the neutral fractions. For the decoctions of *M. suaveolens* dried shoots, AG-II with a more substituted (1→3)-Gal*p* backbone were recovered in the neutral and also in the less acidic fractions. In both situations, the the AG-II with the less substituted (1→3)-Gal*p* backbone were recovered in the more acidic fractions.

Rhamnosyl residues terminally-linked were detected in all the fraction analysed, particularly in the acidic ones, thus it is possible that the AG-II present in the decoctions of *M. suaveolens* dried shoots might be present as AGP´s, since T-Rha*p* have been reported as structural features of coffee AG-II (Nunes *et al*., 2008).

Table 5.4- Deduced linkages from the methylation analysis of the neutral (A) and acidic (C) fractions from the Et_{75} fractions of the 2nd 2h of the decoctions from *Mentha suaveolens* dried shoots.

Linkage	Fraction		
	Et_{75}A	Et_{75}B	Et_{75}C
t*-Rha*p	0.3	1.5	1.1
1,2-Rha*p*	0.3	2.7	3.8
1,3-Rha*p*			
1,2,4-Rha*p*		2.2	5.2
1,3,4-Rha*p*		0.8	1.0
Total	***0.6[a](1.4)[b]***	***7.2(10.2)***	***11.1(12.3)***
t-Fuc*p*	0.2	0.9	0.8
Total	***0.4(0.4)***	***0.9(1.2)***	***0.8(0.6)***
t*-Ara*f	10.7	15.2	16.7
t*-Ara*p	1.4	3.9	3.5
1,2-Ara*f*	0.7	0.8	0.9
1,3-Ara*f*	1.9	2.0	4.3
1,5-Ara*f*	4.1	12.5	13.0
1,3,5-Ara*f*	0.4	5.7	10.5
Total	***19.2(15.4)***	***40.1 (42.8)***	***48.9(47.2)***
t-Xyl*p*	1.3	1.2	1.0
1,2-Xyl*p*	1.0	0.0	0.0
1,4-Xyl*p*	4.7	1.6	1.5
1,2,4-Xylp	1.0	1.2	1.8
Total	***8.0 (5.7)***	***4.0(1.2)***	***4.3(0.9)***
t*-Man*p	0.8		0.2
1,4-Man*p*	15.9	1.1	0.0
1,2,4-Man*p*			
1,4,6-Man*p*	3.7		0.1
Total	***20.4(22.1)***	***1.1(0.9)***	***0.3(1.3)***
t*-Gal*p	7.6	6.9	6.5
1,2-Gal*p*			
1,3-Gal*p*	1.5	4.3	3.5
1,4-Gal*p*			4.2
1,6-Gal*p*	11.2	11.7	6.3
1,3,6-Gal*p*	8.7	18.6	8.8
Total	***29.0(29.4)***	***41.5(41.3)***	***29.3(35.0)***
t*-Glc*p	1.6	0.6	1.0
1,3-Glc*p*	3.1	0.5	1.1
1,4-Glc*p*	14.7	3.8	2.1
1,6-Glc*p*	0.2		0.1
1,2,4-Glc*p*			0.6
1,4,6-Glc*p*	2.8	0.3	0.4
Total	***22.4(25.8)***	***5.2(2.4)***	***5.3(2.8)***

molar % obtained through: [a] linkage analysis, and [b] sugar analysis

The coffee AGP´s that were reported to present T-Rha*p* residues as a structural feature were also obtained through anion exchange chromatography of HMWM precipitated in ethanol aqueous solutions, similarly to what was done in the present study.

Besides terminally linked rhamnosyl residues, other rhamnosyl residues were detected mainly in the acidic fractions, such as (1→2)- and (1→2,4)-Rha*p* residues. The presence of these rhamnosyl residues, along with the presence of (1→4)-GalA residues, suggested the presence of RG-I moieties that seemed to be present in lower proportion in the neutral fractions, similarly to what had been observed for the decoctions of *F. angustifolia* dried leaves. For the HMWM insoluble in 50% ethanol aqueous solutions, the ratio (1→2,4)-/(1→2)-Rha*p* values indicated that approximately 59% and 50% of the rhamnosyl residues of the $\underline{Et_{50}B}$ and $\underline{Et_{50}C}$ fractions were substituted, respectively. For the HMWM insoluble in 75% ethanol aqueous solutions, values of 51% and 47% were registered in the 1st 2h of the hot water extraction, evidencing the presence of RG-I moieties insoluble in 50% ethanol aqueous solutions with a higher substitution degree than those insoluble in 75% ethanol aqueous solutions. For the 2nd 2h, values of approximately 45% and 58% were observed for the $\underline{Et_{75}B}$ and $\underline{Et_{75}C}$ fractions, respectively. These values were in accordance with those reported by Albersheim *et al.* (1996), which referred that the proportion of substituted rhamnosyl residues generally varied from approximately 20 to 80%, depending on the source of the polysaccharide and isolation method.

The (1→2,4)-Rha*p* residues are frequently reported as the main binding site for various types of side chains often found attached to the RG-I moiety of pectic polysaccharides, namely AG-I (Yamada and Kiyohara, 1999; Nergard *et al.,* 2005). In the acidic fractions, which displayed the highest proportions of (1→2,4)-Rha*p* residues, it was also possible to observe the presence of (1→4)-Gal*p* residues which are diagnostic for AG-I structures. Therefore, it is possible that the AG-I, that co-eluted in the acidic fractions with the (1→2)- and (1→2,4)-linked rhamnosyl residues, are covalently linked to the RG-I domains. The AG-I present in the decoctions of *M. suaveolens* dried shoots seemed to be mostly linear structures, as no (1→3,4)-Gal*p* residues were detected. The presence of linear AG-I

structures in the acidic fractions had also been observed for the decoctions of *F. angustifolia* dried leaves.

Relevant proportions of glucosyl (1→4)-, besides (1→4,6)-linked residues, were observed in the neutral $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, while the acidic fractions presented minor proportions. Similarly to what had happened for the decoctions of *F. angustifolia* dried leaves, no starch was detected by the iodine assay, and thus it was assumed that at least some of these glucosyl residues should be part of xyloglucans, which is a major hemicellulose in the primary cell walls of dicotyledons (Carpita and Gibeaut, 1993).

When comparing the xyloglucans present in the neutral $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, from the 1st 2h, it was possible to observe that they showed a similar substitution degree, as evidenced by the (1→4,6)-/(1→4)-Glc*p* ratio values of 0.23 and 0.20. Also, the xyloglucans detected in the $\underline{Et_{75}A}$ fraction, from the 2nd 2h, presented a (1→4,6)-/(1→4)-Glc*p* value of 0.19, suggesting the presence of similarly substituted xyloglucans in the $\underline{Et_{75}A}$ fractions from both parts of the hot water extraction process. It was also possible to observe that the xyloglucans recovered in the neutral fractions seemed to be less substituted than the correspondent acidic ones: 0.23, 1.00, and 0.85, for the $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{50}C}$, respectively. For the xyloglucans insoluble in 75% ethanol aqueous solutions, this tendency was not evident, as suggested by the similar (1→4,6)-/(1→4)-Glc*p* ratio values: 0.20, 0.26, and 0.19, for the $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ fractions, respectively. Furthermore, for the 2nd 2h of the hot water extraction process, it was possible to observe that the less substituted xyloglucans insoluble in 75% ethanol aqueous solutions were recovered in the neutral and less acidic fractions: 0.19, 0.08, and 0.33, $\underline{Et_{75}A}$, $\underline{Et_{75}B}$, and $\underline{Et_{75}C}$ fractions, respectively.

For the neutral $\underline{Et_{50}A}$, and $\underline{Et_{75}A}$ fractions, from the 1st 2h, the proportion of (1→4,6)-Glc*p* residues detected were slightly higher than those of T-Xyl*p* residues, which combined with the detection of (1→2)-Xyl*p* residues, might suggest that the Gal*p*-(β1→2)-Xyl*p*-(α1→ and Ara*p*-(α1→2)-Xyl*p*-(α1→ disaccharides could be present as xyloglucan side chains, together with single xylosyl residues. Thus, besides T-Xyl*p*, some of the terminally linked arabinosyl and galactosyl residues detected in neutral fractions could also be present in the xyloglucan structure.

Based on the sum of the proportions of (1→3,5)-Ara*f* and (1→3,6)-Gal*p* residues of $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, that are present in the AG-II structure, it can be inferred that 9.0% and 7.0%, respectively, of the T-Ara*f* and T-Gal*p* residues, that can be present in both AG-II and xyloglucan structures, should be present as AG-II components. Therefore, some of the remaining proportions of these terminally linked residues (3.9, and 4.1%, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively), together with T-Fuc (0.4, and 0.2%, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively), and T-Xyl*p* (2.7, and 1.1%, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively), could be present as components of the xyloglucan structures present in the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions. The sum of the molar proportions of these terminally linked residues (7.0, and 5.4%, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively) seems to be reasonably compatible with the 4.8, and 4.0% of (1→4,6)-Glc*p* residues detected in the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively.

For the $\underline{Et_{75}A}$ fraction from the 2nd 2h, the proportion of (1→4,6)-Glc*p* residues detected seemed to be compatible with the proportions of T-Xyl*p* residues, although the presence of (1→2)-Xyl*p* residues, does not allow to completely exclude the possible presence of Gal*p*-(β1→2)-Xyl*p*-(α1→, and Ara*p*-(α1→2)-Xyl*p*-(α1→ disaccharides as side chains.

The presence of relevant proportions of mannosyl (1→4)-linked residues in the neutral fractions $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$, also suggests the presence of mannans, which are another hemicellulosic polysaccharide that had also been reported in the neutral fractions isolated from the decoctions of *F. angustifolia* dried leaves. The neutral $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions from the 1st 2h of the hot water extraction process, presented values of 0.20 and 0.25 for the (1→4,6)-/(1→4)-Man*p* ratio, respectively, which were also similar to the ratio value of 0.23 exhibited by the mannans insoluble in 75% ethanol aqueous solutions extracted in the 2nd 2h. The mannans insoluble in 50% ethanol aqueous solutions present in the decoctions of *M. suaveolens* dried leaves seemed to be more substituted than the correspondent mannans extracted from the *F. angustifolia* decoctions. The mannans insoluble in 75% ethanol aqueous solutions present in the dried leaves *F. angustifolia* and dried shoots *M. suaveolens* decoctions exhibited similar substitution degrees.

The presence of xylans in very in small proportions was also possible to infer, due to the detection of terminally-, (1→4)- and (1→2, 4)-linked xylosil residues in all the fractions, similarly to what had been observed for the decoctions of *F. angustifolia* dried leaves. The presence of minute proportions of (1→2)-Ara*f* also seems to support the presence of xylans, as the side chains may also contain these arabinosyl residues besides the T-Ara*f* residues (Fry, 1988).

5.2. *Endo*-polygalacturonase treatments

The fractions that were isolated from the decoctions of *M. suaveolens* dried shoots contained high proportions of pectic polysaccharides, similarly to what was observed for the decoctions from *F. angustifolia* dried leaves. Particularly for the neutral fractions obtained from the anion exchange chromatography of the Et_{75} fractions, it was also possible to observe the presence of other polysaccharides, such as arabinogalactans, mannans and xyloglucans, besides the possible presence of xylans in minute proportions. Thus, for a better structural characterization of these carbohydrate polymers, the $\underline{Et_{75}A}$ fractions, from both parts of the hot water extraction process, were de-esterified with NaOH, in order to ensure that the enzyme acted adequately, and subsequently submitted to an enzymatic digestion with *endo*-polygacturonase, followed by size exclusion chromatography on Bio Gel P30. According to the manufacturer, the typical fractionation range of Bio Gel P30 is comprised between 2.5 and 40 kDa. The chromatographic elution profiles of the fractions with and without *endo*-polygalacturonase treatment were obtained and are presented in **Figure 5.1**.

It is possible to observe that the elution profiles before and after the *endo*-polygalacturonase digestion, for both 1st and 2nd 2h of the hot water extraction process, are distinct. After the *endo*-polygalacturonase digestion, the elution of the $\underline{Et_{75}A}$ fractions yielded a chromatographic band near the column inclusion volume, which was absent in the elution profile of the undigested samples, evidencing the enzymes action.

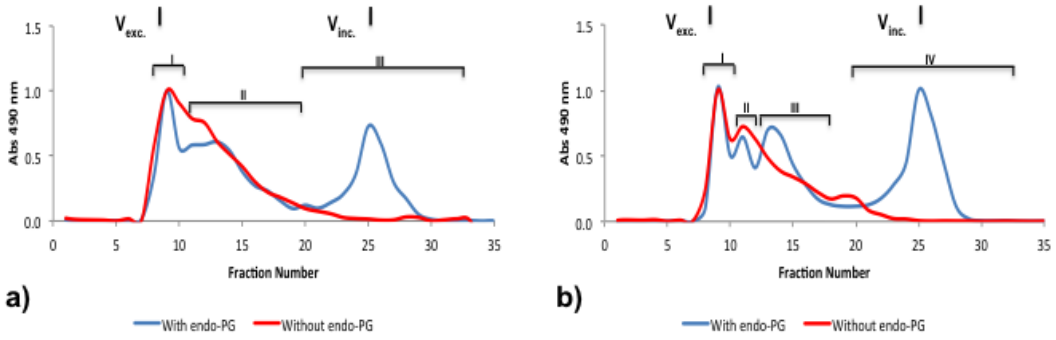

Figure 5.1- Elution profiles from the size exclusion chromatography on Bio Gel P30 of the $\underline{Et_{75}A}$ fractions, with and without the *endo*-polygalacturonase treatment, obtained from the **a)** 1st 2h and **b)** 2nd 2h of the hot water extraction of *Mentha suaveolens* dried shoots.

The fractionation of the digestion products allowed obtaining three and four distinct molecular weight fractions, for the 1st and 2nd 2h of the decoctions from *M. suaveolens* dried shoots, respectively. For the 1st 2h of the hot water extraction process, a high molecular weight fraction (I), one intermediate molecular weight fraction (II), and a low molecular weight fraction (III) were obtained. For the 2nd 2h of the hot water extraction process, besides the presence of one high molecular weight fraction (I) and one low molecular weight fraction (IV), two intermediate molecular weight fractions were recovered (II and III). The chromatographic elution profile for the 1st 2h of the hot water extraction process was similar to the ones obtained for *F. angustifolia*. For the 2nd 2h of the hot water extraction of *M. suaveolens* dried shoots, two intermediate molecular weight fractions were obtained, while for *F. angustifolia* only one intermediate molecular weight fraction was recovered.

The high molecular weight fractions that eluted near the exclusion volume might comprise *endo*-polygalacturonase-resistant carbohydrate material with a molecular weight higher than 40 kDa, which is the upper limit of gel fractionation range. The *endo*-polygalacturonase-resistant carbohydrate material present in this fraction might have resulted of the *endo*-polygalacturonase action or could comprise polysaccharides that were not digested by the enzyme. The intermediate molecular weight fractions are reported to exhibit a diverse monomeric composition, evidencing the presence of various structural elements (Inngerdingen *et al*., 2007, 2008). In this case, the elution profiles of the $\underline{Et_{75}A}$ fractions presented in **Figure 5.1** showed that the intermediate molecular weight fractions might

contain portions of pectic polysaccharides that were digested by *endo*-polygalacturonase and present a molecular weight comprised between 40 and 2.5 kDa, which is the typical fractionation range of Bio Gel P30, and also polysaccharides that were not digested. The low molecular weight fractions should contain galacturonic acid residues released by the action of *endo*-polygalacturonase on the pectic polysaccharides structure.

After the de-esterification, enzymatic digestion and size exclusion chromatography on Bio Gel P30 of the $\underline{Et_{75}A}$ fractions digestion products, the fractions of interest were pooled, dialysed with a tubing of a molecular weight cutoff of 1.0 kDa, for the elimination of salts, and also allowing to reduce the losses of digestion products, and freeze-dried. The mass yield, carbohydrate material content, and monomeric composition of the fractions obtained are presented in **Table 5.4**.

Table 5.4- Yield, carbohydrate material content, and monosaccharide composition of the fractions obtained through *endo*-polygalacturonase digestion of the $\underline{Et_{75}A}$ fractions, isolated from the decoctions of *Mentha suaveolens* dried shoots.

	Yield[a] (mass %)	Total Sugars (mass %)	Monosaccharide Composition (mol%)							
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
			1st 2h							
	$Et_{75}A$	75.2	0.5	0	4.7	2.1	6.7	9.6	9.8	66.6
$Et_{75}A$-I	28.5	69.6	1.5	0.3	20.0	4.4	6.8	25.9	15.0	26.1
$Et_{75}A$-II	41.2	75.2	0.9	0.4	5.1	4.8	26.9	16.8	29.9	15.1
$Et_{75}A$-III	30.3	94.2	0	0	0.2	0.4	0.9	2.2	1.8	94.5
			2nd 2h							
	$Et_{75}A$	97.5	0.7	0.2	7.6	2.8	10.9	14.5	12.7	50.7
$Et_{75}A$-I	24.5	90.1	1.8	0.3	30.4	5.6	4.9	36.9	10.7	9.4
$Et_{75}A$-II	11.8	88.5	0.7	0.1	7.1	4.7	30.2	23.7	26.6	6.9
$Et_{75}A$-III	22.6	81.4	0.9	0.4	4.2	5.0	29.6	19.7	30.3	9.9
$Et_{75}A$-IV	41.1	98.4	0.0	0.0	0.3	0.5	1.2	3.4	1.5	93.1

[a] - expressed relatively to the HMWM recovered

The enzymatic digestion of the $\underline{Et_{75}A}$ fraction isolated from the 1st 2h of *M. suaveolens* dried shoots hot water extraction yielded similar proportions of $\underline{Et_{75}A\text{-I}}$ and $\underline{Et_{75}A\text{-III}}$ fractions (28.5 and 30.3%, respectively) that together comprised approximately 60% of the recovered material. These values are comparable with those registered for the correspondent fractions from *F. angustifolia* decoctions

(31.3 and 33.1%, for the Et_{75}A-I and Et_{75}A-III, respectively). The remaining 41.2% were recovered in the Et_{75}A-II fraction, which was slightly more abundant than the correspondent fraction from *F. angustifolia* decoctions (35.6%). The Et_{75}A-I fraction contained 69.6% of carbohydrate material, a value lower than the registered for the same fraction obtained from the *F. angustifolia* decoctions. The Et_{75}A-II and Et_{75}A-III fractions exhibited higher proportions of carbohydrate material (75.2 and 94.2%, respectively), which were similar to those observed for the Et_{75}A-II and Et_{75}A-III fractions from the decoctions of *F. angustifolia* dried leaves.

By multiplying the mass yield of each fraction by the respective proportion of total sugars, the proportion of carbohydrate material present in each fraction can be determined. The results evidenced that the majority of the polysaccharides present in the Et_{75}A fractions, isolated from the decoctions of *M. suaveolens* dried shoots, and recovered from the size exclusion chromatography on Bio Gel P30, were recovered in the Et_{75}A-II and Et_{75}A-III fractions (39.0 and 36.0%, respectively). The Et_{75}A-I fraction, which contained the polysaccharides resistant to the *endo*-polygalacturonase action, accounted for 25.0% of the carbohydrate material recovered. This value was slightly lower than the one registered for the Et_{75}A-I fraction isolated from *F. angustifolia* decoctions, which evidenced that 30.9% of carbohydrate material was recovered in this fraction.

For the 2nd 2h of the hot water extraction of *M. suaveolens* dried shoots, the most abundant was the Et_{75}A-IV fraction that accounted for 41.1% of the recovered material. The two intermediate molecular weight fractions combined accounted for 34.4%, while for the Et_{75}A-I fraction 24.5% of the recovered material was collected. These fractions contained high proportions of carbohydrate material: 90.1, 88.5, 81.4, and 98.4, for the Et_{75}A-I, Et_{75}A-II, Et_{75}A-III, and Et_{75}A-IV fractions, respectively. These results evidenced that 24.2% of the carbohydrate material was collected in the Et_{75}A-I fraction, while 44.3% was comprised in the Et_{75}A-IV fraction.

For the decoctions of *M. suaveolens* dried shoots, the proportion of carbohydrate material present in the low molecular weight fraction that resulted from the enzymatic digestion of the Et_{75}A fraction from the 1st 2h was lower than

the registered for the 2nd 2h: 36.0 *vs* 44.3%, for the 1st and 2nd 2h, respectively. This is similar to what had been observed for the decoctions of *F. angustifolia* dried leaves, which presented an even higher proportion of carbohydrate material present in the low molecular weight fraction from the 2nd 2h: 56.3%. This seems to suggest the presence of higher proportions of carbohydrate material susceptible to the *endo*-polygalacturonase action in the 2nd 2h of the hot water extraction process, particularly for the decoctions of *F. angustifolia* dried leaves.

For the 1st 2h of *M. suaveolens* dried shoots hot water extraction it was possible to observe that the polysaccharides from the $\underline{Et_{75}A\text{-}I}$ fraction were mainly composed of arabinosyl (20.0%), galactosyl (25.9%), and uronic acid residues (26.1%), besides relevant proportions of glucosyl residues (15.0%). The fraction $\underline{Et_{75}A\text{-}II}$ contained polysaccharides that mainly comprised glucosyl (29.9%) and mannosyl residues (26.9%), together with galactosyl (16.8%) and uronic acid residues (15.1%). The $\underline{Et_{75}A\text{-}III}$ fraction contained polysaccharides composed of 94.5% of uronic residues.

For the fractions obtained from the 2nd 2h of the *M. suaveolens* decoctions, it was possible to observe that the polysaccharides present in the $\underline{Et_{75}A\text{-}I}$ fraction were mainly composed of arabinosyl (30.4%) and galactosyl residues (36.9%), present in higher proportion in comparison with the correspondent $\underline{Et_{75}A\text{-}I}$ fraction isolated from the 1st 2h of the decoctions. The two intermediate molecular weight fractions ($\underline{Et_{75}A\text{-}II}$ and $\underline{Et_{75}A\text{-}III}$) contained polysaccharides with identical monomeric composition, mainly composed of glucosyl (26.6 and 30.3%, for the $\underline{Et_{75}A\text{-}II}$ and $\underline{Et_{75}A\text{-}III}$ fractions, respectively), mannosyl (30.2 and 29.6%, for the $\underline{Et_{75}A\text{-}II}$ and $\underline{Et_{75}A\text{-}III}$ fractions, respectively), and galactosyl residues (23.7 and 19.7%, for the $\underline{Et_{75}A\text{-}II}$ and $\underline{Et_{75}A\text{-}III}$ fractions, respectively), similarly to what had been observed for the $\underline{Et_{75}A\text{-}II}$ fraction from the 1st 2h of the hot water extraction process. It was also observed that the high and the two intermediate molecular weight fractions from the 2nd 2h contained lower proportions of uronic acid residues than the correspondent fractions from the 1st 2h of the *M. suaveolens* dried shoots hot water extraction process. This seems to suggest the presence of *endo*-polygalacturonase resistant polysaccharide structures in the fractions from the 1st 2h of the decoctions of *M. suaveolens* dried shoots. The $\underline{Et_{75}A\text{-}IV}$ fraction

contained the carbohydrate material that resulted from the *endo*-polygalacturonase, which was composed of very high proportions of uronic acid residues (93.1%), similarly to the $\underline{Et_{75}A\text{-III}}$ fraction from the 1st 2h of the hot water extraction process.

The monomeric composition and linkage analysis performed for the $\underline{Et_{75}A}$ fractions that was described in **Section 5.1**, suggested the presence of a high proportions of pectic polysaccharides, mixed with lower proportions of arabinogalactans and minute proportions of mannans and xyloglucans. The monomeric composition of the high and intermediate molecular weight fractions ($\underline{Et_{75}A\text{-I}}$ and $\underline{Et_{75}A\text{-II}}$, for the 1st 2h; $\underline{Et_{75}A\text{-I}}$, $\underline{Et_{75}A\text{-II}}$, and $\underline{Et_{75}A\text{-III}}$, for the 2nd 2h), which were obtained from $\underline{Et_{75}A}$ fractions through *endo*-polygalacturonase digestion, suggests the presence of the same type of polysaccharides, with the arabinogalactans present in higher proportion in the high molecular weight fractions, while the intermediate molecular weight ones seemed to contain higher proportions of mannans and xyloglucans. The monomeric composition of the $\underline{Et_{75}A\text{-III}}$ fraction revealed the presence of carbohydrate material mainly composed of uronic acid residues, which resulted from the *endo*-polygalacturonase action on the pectic polysaccharides.

The carbohydrate material contained in the $\underline{Et_{75}A\text{-I}}$ fractions isolated from the decoctions from *M. suaveolens* dried shoots was methylated and analysed as partially methylated alditol acetates. The results are showed on **Table 5.5**. As these fractions exhibited low proportions of UA resides, no carboxyl reduction of the previously methylated polysaccharides was performed.

Good agreement between the molar fractions obtained from sugar (alditol acetates) and linkage (partially methylated alditol acetates) analysis was observed. The proportion of terminal and ramified residues was registered for each fraction: 20.0 and 22.9%, and 22.4% and 28.1%, for $\underline{Et_{75}A\text{-I}}$ fractions from the 1st and 2nd 2h of the hot water extraction process, respectively.

The AG-II detected in the $\underline{Et_{75}A\text{-I}}$ fractions from the 1st and 2nd 2h of the hot water extraction process, only differed in the substitution degree of the galactan backbone, which was higher for the 2nd 2h: 3.4, and 5.8, for the 1st and 2nd 2h, respectively.

Table 5.5- Deduced linkages from the methylation analysis of the $\underline{Et_{75}A\text{-}I}$ fractions obtained from the *endo*-polygalacturonase treatment of the $\underline{Et_{75}A}$ fractions isolated from the decoctions of *Mentha suaveolens* dried shoots.

Deduced Linkage	Fraction	
	$\underline{Et_{75}A\text{-}I}$ (1st 2h)	$\underline{Et_{75}A\text{-}I}$ (2nd 2h)
t*-Rha*p	0.6	0.3
1,2-Rha*p*	0.8	0.6
1,4-Rha*p*	1.2	0.6
1,2,4-Rha*p*		
Total	***2.6[a](2.0)[b]***	***1.5(2.0)***
t-Fuc*p*	0.1	0.2
Total	***0.1(0.4)***	***0.2(0.3)***
t*-Ara*f	11.0	15.6
t*-Ara*p	1.1	0.9
1,2-Ara*f*	1.5	1.4
1,3-Ara*f*	3.3	3.1
1,5-Ara*f*	6.0	7.0
1,3,5-Ara*f*	2.0	1.1
Total	***24.9(27.1)***	***29.1(33.6)***
t-Xyl*p*	1.5	0.7
1,2-Xyl*p*		
1,4-Xyl*p*	0.5	5.2
1,2,4-Xylp	1.6	2.0
Total	***3.6(6.0)***	***7.9(6.2)***
t*-Man*p	0.5	0.3
1,4-Man*p*	6.8	4.3
1,6-Man*p*	0.8	0.5
1,4,6-Man*p*	1.4	0.7
Total	***9.5(9.2)***	***5.8(5.4)***
t*-Gal*p	3.9	3.3
1,2-Gal*p*		
1,3-Gal*p*	4.4	4.0
1,4-Gal*p*		
1,6-Gal*p*	17.0	12.7
1,3,6-Gal*p*	15.0	23.2
Total	***40.3(35.0)***	***43.2(40.7)***
t*-Glc*p	1.3	1.1
1,3-Glc*p*	3.7	4.0
1,4-Glc*p*	10.8	5.9
1,6-Glc*p*	0.3	0.2
1,4,6-Glc*p*	2.9	1.1
Total	***19.0(20.3)***	***12.3(11.8)***

molar % obtained through: [a] linkage analysis, and [b] sugar analysis

The extension (0.55 and 0.48, for the 1st and 2nd 2h, respectively) and substitution degree (0.33 and 0.30, for the 1st and 2nd 2h, respectively) of the (1→5)-Ara*f* residues side chains was similar. The structural features of the AG-II present in the $\underline{Et_{75}A\text{-}I}$ fractions, which resulted from the enzymatic digestion of the $\underline{Et_{75}A}$ fractions, were similar to those of the AG-II present in the correspondent untreated $\underline{Et_{75}A}$ fractions. This strengthens the presence of AG-II in the decoctions of *M. suaveolens*, presenting the structural features that had been referred in **Section 5.1**, and also suggest that these polysaccharides present an estimated molecular weight higher than 40 kDa.

The presence of (1→4)-Glc*p* and (1→4,6)-Glc*p* residues in the $\underline{Et_{75}A}$ fractions isolated from the hot water extraction of *M. suaveolens* dried shoots was attributed to the possible presence of xyloglucans, that also seemed to be present in the $\underline{Et_{75}A\text{-}I}$ fractions. The xyloglucans detected in the $\underline{Et_{75}A\text{-}I}$ fractions presented (1→4,6)-Glc*p*/(1→4)-Glc*p* ratio values of 0.27 and 0.19, for the 1st and 2nd 2h, suggesting the presence of similarly substituted xyloglucans in both parts of the hot water extraction process. These values were also similar to those of the correspondent untreated $\underline{Et_{75}A}$ fractions (0.20 and 0.15, for the 1st and 2nd 2h, respectively).

The $\underline{Et_{75}A\text{-}I}$ fractions contained very low proportions of t-Fuc, and did not seem to present (1→2)-Gal*p*, similarly to what had been reported for the correspondent untreated $\underline{Et_{75}A}$ fractions, suggesting the absence of the Fuc*p*-(α1→2)-Gal*p*-(β1→2)-Xyl*p*-(α1→ trisaccharide as side chain of the xyloglucans detected in both treated and untreated fractions. Also, the treated $\underline{Et_{75}A\text{-}I}$ fractions did not contained (1→2)-Xyl*p* residues, suggesting that the xyloglucans present in the treated fractions did not contained the Gal*p*-(β1→2)-Xyl*p*-(α1→, Ara*p*-(α1→2)-Xyl*p*-(α1→ disaccharides as side chains, unlike what had been observed for the untreated fractions. The detection of glucosyl and xylosyl residues in the intermediate molecular weight $\underline{Et_{75}A\text{-}II}$ fractions suggests the presence of xyloglucans also in these intermediate molecular weight fractions. Thus, it seems possible that the xyloglucans that presented the Gal*p*-(β1→2)-Xyl*p*-(α1→, Ara*p*-(α1→2)-Xyl*p*-(α1→ disaccharides as side chains exhibit are being recovered in the intermediate molecular weight fractions.

5.3. Concluding remarks

The results showed that the decoctions from *M. suaveolens* dried shoots contain high proportions of pectic polysaccharides, with AG-I side chains, together with AG-II, mannans, and xyloglucans. These polysaccharides were detected in the decoctions from both parts of the extraction process, and it was showed that the mannans and xyloglucans, from the 1st, and 2nd 2h presented similar structural features, while the AG-II from the 1st 2h seemed to possess a less branched galactan backbone, in comparison with those extracted during the 2nd 2h.

CHAPTER 6

STRUCTURAL FEATURES OF *PTEROSPARTUM TRIDENTATUM* POLYSACCHARIDES EXTRACTED WITH HOT WATER

In this chapter, the structural features of the polysaccharides from the extensive hot water extraction of *Pterospartum tridentatum* dried inflorescences during a total period of 4 h (2 h + 2 h) are described. The extracted material was dialysed and the high molecular weight material (HMWM) was fractionated according to its solubility in aqueous ethanol solutions. The content and composition of the carbohydrate material, together with the phenolic compounds content of the various fractions were determined, as well as its antioxidant activity, expressed as the DPPH scavenging activity. The fractions precipitated with 50 and 75% of aqueous ethanol solutions were further fractionated by anion exchange chromatography on DEAE-Sepharose FF. The carbohydrate composition, the type of glycosidic linkages and the degree of methyl esterification of the polysaccharides present in the various fractions obtained were evaluated. Various fractions rich in pectic polysaccharides were further submitted to *endo*-polygalacturonase treatment, the resulting digestion products were fractionated by size exclusion chromatography on Bio Gel P30, and the carbohydrate material composition of the higher molecular weight fraction, together with the type of glycosidic linkages were assessed. Also, the fraction that contained the highest proportion of mannans was digested with *endo*-mannanase, the resulting digestion products were fractionated on Bio Gel P2, and selected fractions were analysed by ESI-MS and ESI-MS/MS in order to reveal the mannans structural details. The structural features of the mannans were compared with the structural features of mannans that are already well studied, such as the chemically acetylated mannans from spent coffee ground, and also the naturally acetylated mannans extracted from *Aloe vera*, coffee infusions, and LBG.

6.1. Isolation and fractionation of the polysaccharides from the HMWM

6.1.1. Ethanol precipitation

The HMWM obtained from the decoctions of *P. tridentatum* dried inflorescences was suspended in water at room temperature (10.0 mg/mL), and the water insoluble material was separated, yielding the WI_{ppt} fraction. The

remaining HMWM was fractionated by ethanol precipitation, yielding three fractions: Et_{50}, HMWM insoluble in 50% ethanol solution; Et_{75}, HMWM insoluble in 75% ethanol solution; and SN, HMWM soluble in 75% ethanol solution.

Mass recoveries of 86.5 ± 4.8%, for the 1st 2h, and 91.0 ± 2.1%, for the 2nd 2h, were registered for the ethanol fractionation procedure, which was performed in duplicate. The mass recoveries registered are comparable to those obtained for the ethanol fractionation of the HMWM from the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots, evidencing the hygroscopic character of the polysaccharides present in the HMWM.

The mass yield, total carbohydrate and total phenolic compound contents, and the EC_{50} values, which expressed the DPPH scavenging activity of the various fractions, together with the composition of the polysaccharides recovered in the various fractions are shown in **Table 6.1**.

Table 6.1- Mass yield, total sugar content and monosaccharide composition, total phenolics content, and DPPH scavenging activity, expressed by the EC_{50} value, of the Et_{50} and Et_{75} fractions obtained by ethanol precipitation of the HMWM from the decoctions of *Pterospartum tridentatum* dried inflorescences.

	Yield[a] (mass%)	Total Sugar (mass%)	Monosaccharide Composition (mol %)								Total Phenolics (mass %)	EC_{50} (mg/mL)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA		
1st 2 h												
Starting HMWM		42.0	0.9	0.0	4.7	1.3	7.2	7.5	32.7	46.0	19.4	nd
WI_{ppt}	16.0	26.8	2.4	0.0	2.3	0.8	1.3	4.9	73.4	15.0	nd	nd
Et_{50}	31.9	91.5	0.5	0.3	3.2	1.1	1.7	6.2	6.5	80.5	9.1	0.19
Et_{75}	26.5	58.2	1.0	0.5	4.4	2.4	23.6	13.6	24.2	30.3	13.9	0.15
SN	25.5	24.8	0.9	0.5	8.8	1.3	4.0	4.3	65.6	14.6	35.1	0.05
2nd 2 h												
Starting HMWM		69.0	1.0	0.0	10.8	1.0	4.0	8.6	11.4	63.2	11.2	nd
WI_{ppt}	14.1	37.9	4.1	0.0	6.1	0.9	1.1	13.1	41.1	33.7	nd	nd
Et_{50}	21.4	96.6	0.9	0.4	7.0	1.0	2.0	8.7	4.0	76.0	3.3	0.57
Et_{75}	27.5	90.0	1.0	0.4	8.2	1.4	7.1	11.1	6.8	64.0	4.6	0.32
SN	36.9	37.6	1.2	0.6	39.2	1.5	4.8	5.0	32.9	14.8	26.2	0.07

[a] - expressed relatively to the HMWM recovered; [nd] - not determined

Besides the data regarding the various fractions that were obtained by the ethanol fractionation, it is also presented information relative to the "Starting HMWM", which is the HMWM submitted to the fractionation process. The "Starting

HMWM" contained 42 and 69% of carbohydrate material, for the 1st and 2nd 2h of the hot water extraction process, respectively. These values were lower than those of the Et_{50} and Et_{75} fractions: 91.5 and 96.6% for the Et_{50} fractions of the 1st and 2nd 2h, respectively; 58.2 and 90.0% for the Et_{75} fractions of the 1st and 2nd 2h, respectively. These results evidence carbohydrate enrichment for the Et_{50} and Et_{75} fractions from both parts of the hot water extraction process. For the Et_{50} fractions from the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots, it was also registered a carbohydrate enrichment for both parts of the hot water extraction process. Relatively to the Et_{75} fractions, for *F. angustifolia* and *M. suaveolens*, the carbohydrate enrichment was observed only for the 2nd and 1st 2h, respectively.

For the 1st 2h of the hot water extraction process, the Et_{50} and Et_{75} fractions, besides being the richest in carbohydrate material, were also the most abundant (31.9 and 26.5%, respectively). The SN and WI_{ppt} fractions were the least abundant (25.5 and 16.0%, respectively), and also the poorer in carbohydrate material, each one comprising approximately 25% of sugar material.

For the 2nd 2h of the hot water extraction process, the most abundant were the SN and Et_{75} fractions (36.9 and 27.5%, respectively). The SN fraction contained 37.6% of carbohydrate material, which is a lower proportion than the one of the Et_{75} fraction. The Et_{50} fraction, which was the richest in carbohydrate material, only accounted for 21.4% of the HMWM recovered in the ethanol precipitation procedure. The WI_{ppt} fraction was the least abundant and presented a carbohydrate material content similar to the exhibited by the SN fraction.

It was observed that for *P. tridentatum*, the relative abundance of the various fractions was distinct for the 1st and 2nd 2h of the dried inflorescences hot water extraction process. For the 1st 2h of the hot water extraction process, the most abundant was the Et_{50} fraction, followed closely by the Et_{75} and SN, which presented a similar abundance, and the WI_{ppt}. For the 2nd 2h, the SN fraction was the most abundant, followed by the Et_{75}, Et_{50}, and WI_{ppt} fractions. In the ethanol fractionation of the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots, the relative abundance of the various fractions was the same in both parts of the hot water extraction process: the Et_{50} fraction was the most abundant,

followed by the SN, Et_{75}, and WI_{ppt} fractions. The Et_{50} fractions comprised between 38.5 and 54.3% of the HMWM recovered, which are values higher than the registered for the Et_{50} fractions from *P. tridentatum*. These results evidence that the decoctions from *P. tridentatum* dried inflorescences, in comparison with those from *F. angustifolia* dried leaves and *M. suaveolens* dried shoots, contained lower proportions of HMWM insoluble in 50% ethanol aqueous solutions.

By calculating the proportion of carbohydrate material present in each fraction, it was observed that for the 1st 2h of the hot water extraction of *P. tridentaum* dried inflorescences, approximately 53% of the polysaccharides present in the recovered HMWM, precipitated in 50% ethanol aqueous solutions, while only 28% precipitated in 75% ethanol aqueous solutions. For the 2nd 2h, approximately the same amounts of recovered polysaccharides precipitated in 50% and 75% ethanol aqueous solutions (32 and 38%, respectively). This pattern was distinct from the observed for the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots, where most of the polysaccharides precipitated in 50% ethanol aqueous solutions in both parts of the hot water extraction process. These results evidenced that the higher proportion of HMWM that precipitated in 75% ethanol aqueous solutions during the 2nd 2h was due to the higher proportion of polysaccharides that precipitated in that conditions.

It was observed that, for both parts of the hot water extraction process, the fractions Et_{50}, Et_{75} and SN contained higher proportions of phenolic compounds, accompanied by a higher DPPH scavenging activity, expressed by lower EC_{50} values, than the correspondent fractions from the decoctions of *F. angustifolia* dried leaves. Relatively to the fractions from the decoctions of *M. suaveolens* dried shoots, it was observed that the fractions from *P. tridentatum* dried inflorescences exhibited comparable proportions of phenolic compounds and also DPPH scavenging activity.

The Et_{50} fractions exhibited a monomeric composition richer in UA than the Et_{75} fractions, particularly for the 1st 2h, suggesting that the polysaccharides that precipitated in 50% ethanol aqueous solutions were more charged than those that precipitated in 75% ethanol aqueous solutions, similarly to what had been described for the decoctions of *F. angustifolia* dried leaves, and *M. suaveolens*

dried shoots. It is also possible that the polysaccharides that precipitated in 75% ethanol aqueous solutions might exhibit lower molecular, which would render them more soluble in ethanol aqueous solutions. The presence of lower molecular weight polysaccharides could result from β-elimination reactions of pectic polysaccharides. These reactions have been reported to occur in neutral or alkaline solutions, being favoured in methyl esterified pectic polysaccharides (Albersheim *et al*., 1960). However, Keijbets and Pilnik (1974) have found that β-elimination could also occur in slightly acidic pectin solutions (pH 6.1) boiled for 30 minutes, which are conditions similar to those used in the present study for the preparation of the various hot water extracts.

The Et_{75} fractions exhibited a carbohydrate material monomeric composition that was distinct for the 1st and 2nd 2h of the hot water extraction. In the 1st 2h of the hot water extraction process, the carbohydrate material contained similar proportions of Man, Glc and UA residues, together with Gal residues present in minor amounts. For the 2nd 2h of the hot water extraction process, the carbohydrate material presented high proportions of UA residues, together with minor proportions of Ara, Man, Gal, and Glc residues.

The monomeric composition of the Et_{50} and Et_{75} fractions suggested the presence of pectic polysaccharides, in distinct proportions, together with other polysaccharides, as described for the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots. However, the carbohydrate material present in the Et_{75} fraction from the 1st 2h of the hot water extraction of *P. tridentatum* dried inflorescences, contained the lowest proportion of UA residues, concomitant with the presence of the highest proportions of Man and Glc residues registered until now for the various decoctions prepared. This suggested that the decoctions of *P. tridentatum* dried inflorescences obtained during the 1st 2h might contain higher proportions of mannans, and xyloglucans than those of *F. angustifolia* and *M. suaveolens*.

6.1.2. Anion exchange chromatography

The fractions Et_{50} and Et_{75}, because were those with the highest proportions of carbohydrate material, were further fractionated by anion exchange chromatography on DEAE-Sepharose FF, in order to try to separate the polysaccharides that seemed to be present in these fractions based on their charge. Similarly to what had been observed for *M. suaveolens* decoctions, both Et_{50} and Et_{75} fractions yielded one not retained neutral fraction (A), eluted with buffer, and three retained acidic fractions (B, C and E), eluted with buffer containing 0.125, 0.250, and 1.0 M NaCl, respectively. The mass yield, total carbohydrate material content and monomeric composition, and the sugars methanol content are shown in **Table 6.2**.

For the Et_{50} fractionation, total mass recoveries of 73.1 and 97.2% were registered for the 1st and 2nd 2 h of the infusion process, respectively, while for Et_{75} fractionation, mass recoveries of 73.2 and 82.4% were observed for the 1st and 2nd 2 h of the infusion process, respectively. The higher recoveries registered for the fractions obtained in the 2nd 2h of the hot water extraction, together with the lower content of total phenolic compounds presented by these fractions relatively to the correspondent from the 1st 2h, suggest the occurrence of interactions between these compounds and the DEAE-Sepharose FF gel. This assumption was supported by the development of a dark coloration at the top of the column, similarly to what had been observed for the fractionation of *F. angustifolia* and *M. suaveolens* Et_{50} and Et_{75} fractions. The dark coloration could only be removed after washing the gel with 2.0 M NaCl and 1.0 M NaCl solutions in a reversed flow direction.

For Et_{50} fractionation, the most abundant were the $\underline{Et_{50}A}$ and $\underline{Et_{50}B}$ fractions that comprised 51.8 and 32.4%, in the 1st 2h, and 44.9 and 36.2%, in the 2nd 2h, respectively, of the HMWM recovered. These fractions were also the richest in carbohydrate material: 87.1 and 89.7%, in the 1st 2h, and 88.3 and 93.1%, in the 2nd 2h, respectively. For the Et_{75} fractionation, the most abundant and sugar rich were the $\underline{Et_{75}A}$ fractions, comprising 73.3, in the 1st 2h, and 60.3%, in the 2nd 2h, of the HMWM recovered, with 85.3 and 97.8% of carbohydrate material, respectively.

These results evidenced that most of the polysaccharides present in the decoctions of *P. tridentatum* dried inflorescences were recovered in the neutral $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, and also in the less acidic $\underline{Et_{50}B}$ and $\underline{Et_{75}B}$ fractions.

Table 6.2- Mass yield, total sugar content and monosaccharide composition, and methyl esterification degree, shown by the methanol released from polysaccharides, of the Et_{50} and Et_{75} fractions obtained by anion exchange chromatography of the HMWM from the decoctions of *Pterospartum tridentatum* dried inflorescences.

	Yield[a] (mass %)	Total Sugar (mass %)	Methanol (mmol/mol UA)	Monosaccharide Composition (mol %)							
				Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
				Et_{50} *1st 2h*							
A	51.8	87.1	759.0	0.3	0.2	3.7	2.3	5.0	8.5	6.2	73.8
B	32.4	89.7	545.8	0.7	0.0	3.5	0.4	0.1	4.4	0.8	90.1
C	9.4	35.9	372.3	2.0	0.1	7.5	0.9	0.4	10.9	5.8	72.5
E	6.4	16.9	nd	2.5	0.0	9.4	1.9	1.4	19.0	23.4	42.4
				Et_{75} *1st 2h*							
A	73.3	85.3	418.1	0.0	0.0	3.0	2.6	26.1	11.8	15.1	41.3
B	14.5	62.1	541.1	1.9	0.2	11.1	0.6	1.4	14.6	2.3	67.7
C	5.4	31.5	358.4	3.5	0.1	13.0	1.0	2.8	15.6	11.9	52.1
E	6.8	27.7	nd	2.3	0.0	10.0	1.3	4.5	15.3	30	36.6
				Et_{50} *2nd 2h*							
A	44.9	88.3	645.0	0.3	0.3	7.6	2.4	5.6	11.3	5.9	66.6
B	36.2	93.1	483.1	0.8	0.1	4.2	0.4	0.0	3.5	0.9	90.1
C	12.5	31.2	341.7	2.2	0.1	13.8	0.4	0.2	10.7	2.5	70.0
E	6.4	18.6	nd	4.0	0.2	21.1	1.2	0.6	20.5	7.8	44.8
				Et_{75} *2nd 2h*							
A	60.3	97.8	423.5	0.1	0.0	2.9	1.3	9.1	7.7	5.4	73.5
B	26.1	98.8	384.2	1.5	0.1	11.8	0.5	0.2	9.3	0.5	76
C	6.2	33.5	227.6	4.1	0.0	24.2	0.8	0.9	19.5	3.6	46.9
E	7.4	21.8	nd	4.5	0.0	26.1	1.2	0.0	2.4	27.5	38.4

[a] - expressed relatively to the HMWM recovered; [nd] - not determined

The acidic fractions C from the decoctions of *P. tridentatum* dried inflorescences were less abundant and also contained lower proportions of carbohydrate material than the correspondent fractions from the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots. It was also observed that the neutral $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions from *P. tridentatum* decoctions were

more abundant than those from the decoctions of *F. angustifolia* and *M. suaveolens.* This evidenced that the decoctions of *P. tridentatum* dried inflorescences contained higher proportions of neutral and less acidic polysaccharides than the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots.

For the various fractions resulting from the anion exchange chromatography of the $\underline{Et_{50}}$ and $\underline{Et_{75}}$ fractions, and for both parts of the hot water extraction, it was observed that the uronic acid residues present in the neutral fractions were methyl esterified in greater extent than those from the acid B and C fractions, with the uronic acid residues of the C fractions exhibiting the lower degree of methyl esterification. Based on these results, it seems possible that the fractionation of the polysaccharides present in the Et_{50} and Et_{75} fractions was accomplished based on the methyl esterification degree of the uronic acid residues of the pectic polysaccharides, similarly to what had been registered for the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots.

The polysaccharides present in the $\underline{Et_{50}A}$ and $\underline{Et_{50}B}$ fractions exhibited a similar monomeric composition, which comprised high proportions of UA residues, similarly to the Et_{50} fractions. Despite the similar monomeric composition of the polysaccharides present in these fractions, it was possible to observe slight differences regarding the proportions of some neutral sugar residues. The polysaccharides from the $\underline{Et_{50}A}$ fractions contained higher proportions of galactosyl, glucosyl, and mannosyl residues than the $\underline{Et_{50}B}$ acidic fraction.

For the polysaccharides recovered in the fractions that resulted from the anion exchange chromatography of the Et_{75} fractions, it was also possible to observe evident differences in its monomeric composition, besides the distinct proportions of uronic acid residues. For both parts of the hot water extraction process, it was possible to observe that the $\underline{Et_{75}A}$ fraction contained polysaccharides with higher proportions of mannosyl residues than the $\underline{Et_{75}B}$ fraction, together with lower proportions of arabinosyl residues.

The anion exchange chromatography of the HMWM that precipitated in 50% ethanol aqueous solutions allowed the recovery of the majority of the polysaccharides that mostly comprised galactosyl, glucosyl, and mannosyl

residues in the neutral $\underline{Et_{50}A}$ fractions. For the fractionation of the HMWM that precipitated in 75% ethanol aqueous solutions it was observed that the majority of the mannosyl containing polysaccharides were recovered in the neutral $\underline{Et_{75}A}$ fraction, while those that contained arabinosyl residues were mainly recovered in the less acidic $\underline{Et_{75}B}$ fraction.

6.1.3. Glycosidic linkage analysis

In order to elucidate the type of polysaccharides present in the decoctions from *P. tridentatum* dried inflorescences, the fractions that showed the highest amounts of carbohydrate material were methylated and analysed as partially methylated alditol acetates. A carboxyl reduction of the methylated polysaccharides was also performed for the detection of the uronic acid residues and assessment of the respective types of linkage.

It was observed that the monomeric composition of the $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{75}B}$ fractions was similar for both parts of the hot water extraction. For the $\underline{Et_{75}A}$, it was observed a higher variability in the monomeric composition of the fractions from the 1st and 2nd 2h of the hot water extraction process. Thus, for the $\underline{Et_{75}A}$ fraction the linkage analysis was performed on the material from both parts of the hot water extraction process, while for the $\underline{Et_{50}A}$, $\underline{Et_{50}B}$, and $\underline{Et_{75}B}$ fractions the material from the 1st 2h was analysed, and the results are shown in **Table 6.3**. For all the fractions, the carboxyl reduction of the previously methylated polysaccharides allowed the detection of 1,4,5,6-tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-hexitol residue with a mass spectra with a fragmentation pattern that exhibited some ions with +2 *m/z* units, indicating the presence of (1 → 4)-HexA. As the pectic polysaccharides seem to be the major type of polymer of the decoctions from *P. tridentatum* dried inflorescences it is plausible to attribute the detection of the (1 → 4)-HexA residues to the presence of (1 → 4)-GalA, which is one of the main components of pectic polysaccharides.

Table 6.3- Deduced linkages from the methylation analysis of the neutral (A) and acidic (B and C) fractions from the Et_{50} and Et_{75} fractions of the 1st 2h of the decoctions from *P. tridentatum* dried inflorescences.

Linkage	Fraction				
	1st 2h				2nd 2h
	Et_{50}A	Et_{50}B	Et_{75}A	Et_{75}B	Et_{75}A
t*-Rha*p		2.1		1.4	
1,2-Rha*p*		2.9		1.3	
1,3-Rha*p*				0.5	
1,2,4-Rha*p*	0.5	2.8	0.2	1.7	0.3
1,3,4-Rha*p*				0.9	
Total	***0.5[a](1.1)[b]***	***7.8(7.1)***	***0.0(0.0)***	***5.8(5.9)***	***0.0(0.4)***
t-Fuc*p*	0.9	1.7	0.2	1.1	0.2
Total	***0.9(0.8)***	***1.7(0.0)***	***0.2(0.0)***	***1.1(0.6)***	***0.2(0.0)***
t*-Ara*f	5.3	11.6	1.5	9.4	3.8
t*-Ara*p		2.9		1.9	
1,2-Ara*f*		0.9	0.3	0.7	0.1
1,3-Ara*f*	0.7	1.2	0.5	1.6	0.3
1,5-Ara*f*	8.5	11.7	1.6	15.9	4.0
1,2,4-Ara*p*		4.0		2.0	
1,3,5-Ara*f*	0.7	1.5	0.2	1.6	0.5
Total	***15.2(14.1)***	***34.0(35.4)***	***4.9(5.1)***	***33.5(34.6)***	***8.7(10.9)***
t-Xyl*p*	4.0	1.6	2.4	0.8	2.4
1,2-Xyl*p*	1.4	0.5	1.0		0.9
1,4-Xyl*p*	1.7	1.2	1.1	0.8	1.0
1,2,4-Xylp	2.1	2.5	0.5	3.7	1.0
Total	***9.2(8.8)***	***5.8(4.0)***	***5.0(4.6)***	***5.3(1.9)***	***5.3(4.9)***
t*-Man*p	0.8		1.5	0.4	1.7
1,4-Man*p*	12.0	0.5	27.7	2.4	23.9
1,2,4-Man*p*			1.9	0.3	1.2
1,3,4-Man*p*			3.5	0.3	
1,4,6-Man*p*	4.3		9.5	0.5	7.9
Total	***17.1(19.1)***	***0.5(1.0)***	***44.1(44.5)***	***3.9(4.4)***	***34.7(34.3)***
t*-Gal*p	8.7	5.2	9.5	5.8	9.9
1,2-Gal*p*	1.1		0.2		0.2
1,3-Gal*p*	0.95	1.3	0.7	3.0	0.4
1,4-Gal*p*	9.0	10.8	5.0		11.9
1,6-Gal*p*	12.9	19.7	2.9	20.9	2.7
1,3,6-Gal*p*	1.5	4.7	1.4	12.0	1.3
Total	***34.2(32.4)***	***41.7(44.4)***	***19.7(20.1)***	***41.7(45.5)***	***26.4(29.1)***
t*-Glc*p	1.5	2.3	1.3	2.5	1.2
1,3-Glc*p*			0.4	0.5	0.7
1,4-Glc*p*	8.6	6.2	18.1	4.0	19.0
1,6-Glc*p*			0.3	0.2	
1,2,4-Glc*p*			1.8	0.9	0.3
1,3,4-Glc*p*			0.6		
1,4,6-Glc*p*	12.8		3.4	0.6	3.2
Total	***22.9(23.7)***	***8.5(8.1)***	***25.9(25.7)***	***8.7(7.2)***	***24.4(20.4)***

molar % obtained through: [a] linkage analysis, and [b] sugar analysis

The presence of *t*-GalA and *t*-GlcA as pectic polysaccharide components has been also reported, however they are found in much lower proportions, in comparison with the (1→4)-GalA residues, which, together with the very low recovery of the uronic acid residues (<5%), could have contributed for the non detection of detect these residues. Due to the high proportion of UA residues of the polysaccharides from these fractions, and as only (1→4)-GalA residues were detected, we present the linkage analysis results relative to the neutral sugar residues, looking for a clearer understanding of the distinctive structural features of the polysaccharides present in the analysed fractions.

Good agreement between the molar fractions obtained from sugar (alditol acetates) and linkage (partially methylated alditol acetates) analysis was observed. Relatively good agreement between the proportion of terminal and ramified residues was registered for most of the analysed fractions: 21.2 and 21.9%, 27.4 and 15.5%, for the terminal and ramified residues of $\underline{Et_{50}A}$ and $\underline{Et_{50}B}$, respectively. For the $\underline{Et_{75}A}$ and $\underline{Et_{75}B}$ fractions obtained during the 1st 2 h, 16.4 and 23.0%, 23.3 and 24.5%, were registered for the terminal and ramified residues, respectively. The $\underline{Et_{75}A}$ fraction obtained during the 2nd 2h, exhibited 19.2 and 15.7% of terminal and ramified residues, respectively.

Similarly to what had been observed for the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots, arabinosyl and galactosyl residues diagnostic of the presence of AG-II were detected in all the fractions analysed. For the 1st 2h of the hot water extraction, the AG-II recovered in the neutral $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions exhibited lower (1→3,6)-Gal*p*/(1→3)-Gal*p* values than those of the acidic $\underline{Et_{50}B}$ and $\underline{Et_{75}B}$ fractions: 1.6 and 2.0, for the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, respectively; 3.6 and 4.0 for the $\underline{Et_{50}B}$ and $\underline{Et_{75}B}$ fractions, respectively. It was also observed that the AG-II that precipitated in 50% ethanol aqueous solutions also presented a galactan backbone less branched than the correspondent arabinogalactans that precipitated in 75% ethanol aqueous solutions.

The AG-II, recovered in the neutral $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, and acidic $\underline{Et_{50}B}$ and $\underline{Et_{75}B}$ fractions, presented (1→5)-Ara*f*/T-Ara*f* values of 1.6, 1.1, 1.0, 1.7, respectively, which did not allowed to detect a defined tendency relatively to the extension of the (1→5)-Ara*f* residues side chains. The AG-II present in the various

fractions exhibited similar (1→3,5)-/(1→5)-Ara*f* values that ranged from 0.08 to 0.13.

The AG-II present in the $\underline{Et_{75}A}$ fraction from the 2nd 2h of the decoctions of *P. tridentatum* dried inflorescences presented (1→3,6)-Gal*p*/(1→3)-Gal*p*, (1→5)-Ara*f*/T-Ara*f*, and (1→3,5)-Ara*f*/(1→5)-Ara*f* values of 3.13, 1.05, and 0.13, respectively. These results evidenced that the AG-II present in the $\underline{Et_{75}A}$ fractions from both parts of the hot water extraction process possess similar extension and substitution degree and (1→ 5)-Ara*f* residues side chains, only differing in the substitution degree of the galactan backbone, which was higher for the arabinogalactans from the 2nd 2h. The acidic $\underline{Et_{50}B}$ and $\underline{Et_{75}B}$ fractions exhibited T-Rha*p* residues, suggesting that the AG-II detected in these fractions might be present as AGP´s.

Most of the fractions analysed also presented (1→4)-Gal*p* residues that are diagnostic for the presence of AG-I structures. AG-I have also been detected in the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots. In the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots, the AG-I were recovered in the acidic fractions, while for *P. tridentatum* these polysaccharides were also detected in the neutral fractions, which presented low proportions of (1→2,4)-Rha*p* residues that are frequently reported as the main binding site for AG-I often found attached to the RG-I moiety of pectic polysaccharides.

For all the analysed fractions, relevant proportions of (1→4)-Glc*p* were detected. Similarly to what had been observed for the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots, no starch was detected by the iodine assay. Thus, it seems plausible that the (1→4)-Glc*p* residues that were detected in the neutral fractions are present as xyloglucan components. Particularly for the $\underline{Et_{50}A}$ fraction, relevant proportions of (1→4,6)-Glc*p* residues were detected, which suggest the presence of highly substituted xyloglucans, evidenced by the (1→4,6)-/(1→4)-Glc*p* value of 1.5, which was higher than the values of 0.19 and 0.17 registered for the $\underline{Et_{75}A}$ fractions from both parts of the hot water extraction. It was also observed that the proportion of (1→4,6)-Glc*p* residues of the $\underline{Et_{50}A}$ fraction clearly exceeded the proportion of T-Xyl*p* residues, suggesting that these

xyloglucans must possess other side chains, besides those composed of single xylosyl residues. The detection of T-Fuc*p*, (1→2)-Gal*p*, and (1→2)-Xyl*p* residues, which suggest the presence of other side chains, namely Gal*p*-(β1→2)-Xyl*p*-(α1→, Ara*p*-(α1→2)-Xyl*p*-(α1→ disaccharides, and Fuc*p*-(α1→2)-Gal*p*-(β1→2)-Xyl*p*-(α1→ trisaccharide, seem to reinforce this assumption. For the $\underline{Et_{50}A}$ fraction, the proportions of (1→3,5)-Ara*f* and (1→3,6)-Gal*p* residues accounted for 2.2%, while the T-Ara*f* and T-Gal*p* residues added to 14.0%. Thus, only 2.2% of these terminally linked residues should be present as AG-II structural components. The remaining 11.8% of T-Ara*f* and T-Gal*p* residues, together with 0.9 and 4.0% of T-Fuc*p* and T-Xyl*p* residues might be present in the neutral $\underline{Et_{50}A}$ fraction as part of the xyloglucan side chains. The total value of 16.7% for these terminally linked residues seems compatible with the 12.8% of (1→4,6)-Glc*p* residues detected in this fraction, which also strengthens the presence of Gal*p*-(β1→2)-Xyl*p*-(α1→, Ara*p*-(α1→2)-Xyl*p*-(α1→ disaccharides, and Fuc*p*-(α1→2)-Gal*p*-(β1→2)-Xyl*p*-(α1→ trisaccharide, besides single xylosyl residues, as xyloglucan side chains. The detection of T-Fuc*p* residues suggests that a portion of the xyloglucans might perform a structural role, as storage xyloglucans do not seem to contain these residues (Fry, 1988; Hayashi, 1989).

The lower (1→4,6)-/(1→4)-Glc*p* values presented by the xyloglucans detected in the $\underline{Et_{75}A}$ fractions, from the 1st and 2nd 2h of the hot water extraction process, evidenced their less substituted character in comparison with the xyloglucans detected in the $\underline{Et_{50}A}$ fraction. The proportion of (1→4,6)-Glc*p* residues detected in the $\underline{Et_{75}A}$ fractions seemed in agreement with the proportion of T-Xyl*p* residues, suggesting that the detected xyloglucans possessed few side chains, which might be composed of single xylosyl residues. However, the detection of (1→2)-Xyl*p* residues, and also (1→2)-Gal*p* residues in minor proportions, does not allow to exclude the possible presence of Gal*p*-(β1→2)- Xyl*p*-(α1→, Ara*p*-(α1→2)-Xyl*p*-(α1→ disaccharides, and Fuc*p*-(α1→2)-Gal*p*-(β1→2)-Xyl*p*-(α1→ trisaccharide as side chains.

The neutral $\underline{Et_{75}A}$ fractions from the 1st 2h of the hot water extraction process exhibited a monomeric composition rich in mannosyl residues, mainly detected as (1→4)-Man*p* and (1→4,6)-Man*p* residues, evidencing the presence of mannans.

The neutral $\underline{Et_{75}A}$ fraction from the 2nd 2h of the hot water extraction process contained mannosyl residues, although in lower proportion, which were also mostly present as (1→4)-Man*p* and (1→4,6)-Man*p* residues. The (1→4,6)-/(1→4)-Man*p* values of 0.34 and 0.33, for the 1st and 2nd 2h of the hot water extraction, respectively, suggested the presence of mannans with a similar substitution degree. These values were higher than those exhibited by mannans extracted from the coffee infusion, spent coffee grounds, and *Aloe vera* (Nunes *et al*., 2006; Simões *et al*., 2010, 2012), which presented values of 0.04, 0.09, and 0.09, respectively. The mannans from the neutral $\underline{Et_{75}A}$ fractions exhibited (1→4,6)-/(1→4)-Man*p* values similar to those of mannans extracted from non-conventional sources, such as *Gleditsia triacanthos*, which presented a ratio of 0.32 (Cerqueira *et al*., 2011).

6.2. *Endo*-polygalacturonase treatments

The fractions that were isolated from the decoctions of *P. tridentatum* dried inflorescences contained high proportions of pectic polysaccharides, similarly to what had been observed for *F. angustifolia* dried leaves and *M. suaveolens* dried shoots decoctions. Also, for the neutral $\underline{Et_{75}A}$ fraction, obtained from the 1st 2h of the hot water extraction, it was observed the presence of the highest proportions of mannans, mixed with minor proportions of other polysaccharides, such as arabinogalactans and xyloglucans. Furthermore, it was observed that the neutral $\underline{Et_{50}A}$ fractions from both parts of the hot water extraction presented higher proportions of neutral sugars than the correspondent fractions isolated from the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots. Therefore, for a better structural characterization of the carbohydrate polymers that were detected together with the pectic polysaccharides, the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, from both parts of the hot water extraction, were submitted to an enzymatic digestion with *endo*-polygacturonase.

After the de-esterification and enzymatic digestion of the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions, the digestion products were fractionated by size exclusion chromatography on Bio Gel P30. The $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ samples without the *endo*-

polygalacturonase treatment were also fractionated on Bio Gel P30, the chromatographic profiles registered, and compared with those from the samples submitted to the enzymatic treatment, as shown in **Figure 6.1**.

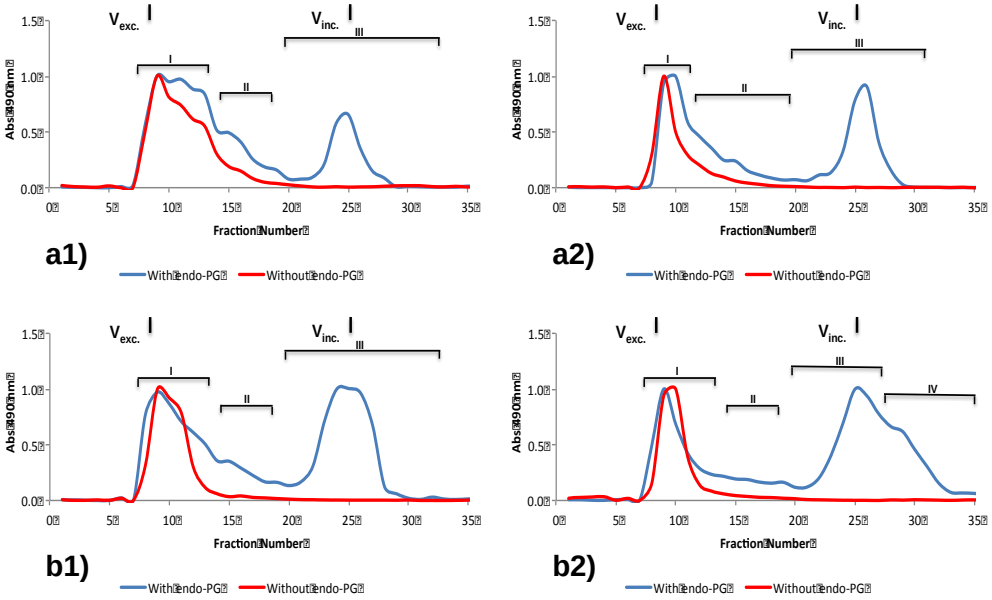

Figure 6.1- Elution profiles from the size exclusion chromatography on Bio Gel P30 of the **a)** $\underline{Et_{75}A}$ and **b)** $\underline{Et_{50}A}$ fractions, with and without the *endo*-polygalacturonase treatment, obtained from the **a1)**, **b1)** 1st 2h and **a2)**, **b2)** 2nd 2h of the hot water extraction of *P. tridentatum* dried inflorescences.

The chromatographic elution profiles of the fractions with and without *endo*-polygalacturonase treatment are similar to those from *F. angustifolia* and *M. suaveolens*, evidencing the *endo*-polygalacturonase action, through the presence of an additional band, eluting near the column inclusion volume, in the chromatogram resulting from the elution of the digestion products.

The mass yield, carbohydrate material content, and monomeric composition of the fractions obtained after the enzymatic digestion of the $\underline{Et_{75}A}$ fractions were determined and are presented in **Table 6.4**. The enzymatic digestion of the $\underline{Et_{75}A}$ fraction isolated from the 1st 2h of *P. tridentatum* dried inflorescences hot water extraction yielded higher proportions (67.2%) of the $\underline{Et_{75}A\text{-I}}$ fraction, which contained 86.7% of carbohydrate material. The $\underline{Et_{75}A\text{-II}}$ and $\underline{Et_{75}A\text{-III}}$ fractions were recovered in similar abundance (14.7 and 18.1%, respectively), and contained 74.1 and 97.4% of carbohydrate material, respectively.

Table 6.4- Yield, carbohydrate material content, and monosaccharide composition of the fractions obtained through *endo*-polygalacturonase digestion of the $\underline{Et_{75}A}$ fractions, isolated from the decoctions of *Pterospartum tridentatum* dried inflorescences.

	Yield[a] (mass %)	Total Sugars (mass %)	Monosaccharide Composition (mol%)							
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
			1st 2h							
	$Et_{75}A$	85.3	0.0	0.0	3.0	2.6	26.1	11.8	15.1	41.3
$Et_{75}A$-I	67.2	86.7	0.2	0.2	4.3	4.3	46.9	16.4	25.6	2.2
$Et_{75}A$-II	14.7	74.1	1.1	0.2	5.6	3.4	32.4	25.3	21.0	10.9
$Et_{75}A$-III	18.1	97.4	0.0	0.0	0.1	0.2	1.1	0.9	1.2	96.5
			2nd 2h							
	$Et_{75}A$	97.8	0.1	0.0	2.9	1.3	9.1	7.7	5.4	73.5
$Et_{75}A$-I	35.8	90.9	0.3	0.3	12.6	4.5	35.9	20.8	20.5	5.3
$Et_{75}A$-II	22.2	82.1	0.8	0.3	7.6	3.9	29.3	26.7	19.4	12.0
$Et_{75}A$-III	42.0	98.6	0.0	0.1	0.4	0.5	0.9	1.4	0.9	95.9

[a] - expressed relatively to the HMWM recovered

These results evidenced that the majority of the carbohydrate material that resulted from the enzymatic digestion was present in the $\underline{Et_{75}A\text{-I}}$ fraction (67.1%). For *F. angustifolia* and *M. suaveolens* it was observed that the $\underline{Et_{75}A\text{-I}}$ fractions only accounted for 20.3 to 30.9% of the carbohydrate material. This should be due to the lowest proportion of uronic acid residues that the polysaccharides present in the $\underline{Et_{75}A}$ fractions from the decoctions of *P. tridentatum* dried inflorescences contained.

For the 2nd 2h of *P. tridentatum* dried inflorescences hot water extraction it was possible to observe that the majority of the material was recovered in the $\underline{Et_{75}A\text{-III}}$ fraction (42.0%), while the remaining was recovered in the $\underline{Et_{75}A\text{-I,}}$ and $\underline{Et_{75}A\text{-II}}$ fractions (35.8 and 22.2%, respectively). These fractions contained high proportions of carbohydrate material: 90.9, 82.1, and 98.6% for the $\underline{Et_{75}A\text{-I}}$, $\underline{Et_{75}A\text{-II}}$, and $\underline{Et_{75}A\text{-III}}$ fractions, respectively. These results evidenced that most of the carbohydrate material was recovered in the $\underline{Et_{75}A\text{-III}}$ fraction (44.9%), suggesting the presence of higher proportions of carbohydrate material susceptible to the *endo*-polygalacturonase action in the $\underline{Et_{75}A}$ fractions isolated from the 2nd 2h of the hot water extraction of *P. tridentatum* dried inflorescences, similarly to what had been observed for *F. angustifolia* and *M. suaveolens* decoctions.

For both parts of *P. tridentatum* dried inflorescences decoctions, it was possible to observe that the monomeric composition of the polysaccharides

present in the $\underline{Et_{75}A\text{-}I}$ and $\underline{Et_{75}A\text{-}II}$ fractions was rich in mannosyl residues, together with galactosyl and glucosyl residues. The $\underline{Et_{75}A\text{-}III}$ fractions contained carbohydrate material almost exclusively composed of uronic acid residues, which were released during the *endo*-polygalacturonase action on the pectic polysaccharides present in the $\underline{Et_{75}A}$ fractions.

For the 1st 2h of the hot water extraction process, the chromatographic elution profile of the $\underline{Et_{50}A}$ fraction submitted to enzymatic treatment showed three distinct molecular weight fractions: $\underline{Et_{50}A\text{-}I}$, $\underline{Et_{50}A\text{-}II}$, and $\underline{Et_{50}A\text{-}III}$, as shown in **Figure 6.1 b1)**. The mass yield, carbohydrate material content, and monomeric composition of the fractions obtained after the enzymatic digestion of the $\underline{Et_{50}A}$ fractions, from both parts of the hot water extraction process, were determined and are presented in **Table 6.5**.

Table 6.5- Yield, carbohydrate material content, and monosaccharide composition of the fractions obtained through *endo*-polygalacturonase digestion of the $\underline{Et_{50}A}$ fractions, isolated from the decoctions of *Pterospartum tridentatum* dried inflorescences.

	Yield[a] (mass %)	Total Sugars (mass %)	Monosaccharide Composition (mol%)							
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
			1st 2h							
	$Et_{50}A$	87.1	0.3	0.2	3.7	2.3	5.0	8.5	6.2	73.8
$Et_{50}A$-I	40.6	73.0	1.2	1.1	17.8	8.6	16.4	26.2	24.2	4.6
$Et_{50}A$-III	59.4	99.1	0.1	0.0	0.3	0.4	0.4	0.9	1.1	96.8
			2nd 2h							
	$Et_{50}A$	88.3	0.3	0.3	7.6	2.4	5.6	11.3	5.9	66.6
$Et_{50}A$-I	29.8	75.8	0.7	0.7	21.9	5.8	14.9	29.8	17.5	8.8
$Et_{50}A$-II	5.5	65.4	1.1	0.5	11.5	1.9	6.0	30.8	6.4	41.8
$Et_{50}A$-III	55.0	99.5	0.1	0.0	0.3	0.3	0.2	1.0	1.7	96.4
$Et_{50}A$-IV	9.7	97.4	0.0	0.0	0.2	0.3	0.3	0.7	1.4	97.1

[a] - expressed relatively to the HMWM recovered

It was observed that approximately 60% of the HMWM was recovered in the low molecular weight $\underline{Et_{50}A\text{-}III}$ fraction, while the high molecular weight $\underline{Et_{50}A\text{-}I}$ fraction comprised the remaining 40%. After dialysis and freeze-drying, no amount of HMWM was recovered in the $\underline{Et_{50}A\text{-}II}$ fraction. The $\underline{Et_{50}A\text{-}I}$ fraction contained 73% of carbohydrate material, which mainly comprised arabinosyl, galactosyl, glucosyl, and mannosyl residues. The $\underline{Et_{50}A\text{-}III}$ fraction contained 99.1% of carbohydrate

material, almost exclusively composed of uronic acid residues that resulted from the *endo*-polygalacturonase digestion.

For the 2nd 2h of the hot water extraction process, the chromatographic elution profile of the $\underline{Et_{50}A}$ fraction submitted to enzymatic treatment showed four distinct molecular weight fractions. The high molecular weight $\underline{Et_{50}A\text{-}I}$ fraction comprised approximately 30% of the HMWM recovered, and contained 75.8% of carbohydrate material, mainly comprising arabinosyl, galactosyl, glucosyl, and mannosyl residues, similarly to the $\underline{Et_{50}A\text{-}I}$ fraction from the 1st 2h. The intermediate molecular weight $\underline{Et_{50}A\text{-}II}$ fraction only accounted for 5.5% of the HMWM recovered, and exhibited the lowest proportion of carbohydrate material, which was mainly composed of uronic acid residues, together with arabinosyl and galactosyl residues. The $\underline{Et_{50}A\text{-}IIII}$ and $\underline{Et_{50}A\text{-}IV}$ fractions combined, accounted for approximately 65% of the HMWM recovered and contained high proportions of carbohydrate material almost exclusively composed of uronic acid residues.

The previous results showed that, for both parts of the hot water extraction of *P. tridentatum* dried inflorescences, the majority of the carbohydrate material recovered from the $\underline{Et_{50}A}$ enzymatic treatment was contained in the low molecular weight fractions: 66.5 and 71%, for the 1st and 2nd 2h, respectively. These values, which are higher than those registered for the enzymatic treatment of the $\underline{Et_{75}A}$ fractions from the decoctions of *P. tridentatum* dried inflorescences, but also from *F. angustifolia* dried leaves and *M. suaveolens* dried shoots, can be attributed to the higher proportion of uronic acid residues that compose the polysaccharides that precipitated in 50% ethanol aqueous solutions.

The carbohydrate material contained in the $\underline{Et_{50}A\text{-}I}$ and $\underline{Et_{75}A\text{-}I}$ fractions, isolated from both parts of the hot water extraction of *P. tridentatum* dried leaves, was methylated, and analysed as partially methylated alditol acetates. The results are shown in **Table 6.6**.

As these fractions exhibited low proportions of UA resides, no carboxyl reduction of the previously methylated polysaccharides was performed. Good agreement between the molar fractions obtained from sugar (alditol acetates) and linkage (partially methylated alditol acetates) analysis was observed.

Table 6.6- Deduced linkages from the methylation analysis of the Et_{50}A-I and Et_{75}A-I fractions isolated from both parts of the decoctions of *Pterospartum tridentatum* dried inflorescences.

Linkage	Fraction			
	1st 2h		2nd 2h	
	Et_{50}A-I	Et_{75}A-I	Et_{50}A-I	Et_{75}A-I
t*-Rha*p	0.2			
1,2-Rha*p*				
1,3-Rha*p*				
1,2,4-Rha*p*	0.3		0.3	
1,3,4-Rha*p*				
Total	***0.5[a](1.3)[b]***	***0.0(0.2)***	***0.3(0.8)***	***0.0(0.3)***
t-Fuc*p*	1.0	0.2	0.8	0.2
Total	***1.0(1.2)***	***0.2(0.2)***	***0.8(0.8)***	***0.2(0.3)***
t*-Ara*f	5.9	1.3	6.3	4.9
t*-Ara*p		0.8		
1,2-Ara*f*	0.2	0.3	0.3	0.2
1,3-Ara*f*	0.5	0.5	0.4	1.9
1,5-Ara*f*	8.3	1.5	9.0	4.8
1,3,5-Ara*f*	0.7	0.2	1.3	0.6
Total	***15.6(18.7)***	***5.9(4.4)***	***17.3(24.0)***	***12.4(13.3)***
t-Xyl*p*	5.0	1.9	4.1	3.0
1,2-Xyl*p*	1.8	0.5	1.9	0.7
1,4-Xyl*p*	1.0	1.5	1.3	1.7
1,2,4-Xylp	3.1	0.5	4.7	1.8
Total	***10.9(9.0)***	***4.4(4.4)***	***9.7(6.4)***	***7.2(4.8)***
t*-Man*p	0.7	1.4	0.6	0.9
1,4-Man*p*	12.8	32.2	12.4	25.1
1,2,4-Man*p*	0.8	1.8	0.9	1.5
1,4,6-Man*p*	4.3	10.6	4.3	7.6
Total	***17.3(17.2)***	***46.0(48.0)***	***18.2(16.3)***	***35.1(37.7)***
t*-Gal*p	7.1	8.8	6.0	7.4
1,2-Gal*p*	1.1	0.2	1.2	0.3
1,3-Gal*p*	1.1	0.6	0.85	1.1
1,4-Gal*p*	13.8		17.0	9.0
1,6-Gal*p*	5.5	2.6	5.0	3.0
1,3,6-Gal*p*	1.8	1.2	2.2	3.6
Total	***30.4(27.4)***	***13.4(16.7)***	***32.3(32.6)***	***24.4(22.0)***
t*-Glc*p	3.8	1.2	0.6	0.8
1,3-Glc*p*	0.2		0.4	0.4
1,4-Glc*p*	9.3	22.9	8.4	15.7
1,6-Glc*p*		0.4	0.1	0.2
1,2,4-Glc*p*	0.1	1.8	0.3	0.2
1,4,6-Glc*p*	13.9	3.8	11.6	3.4
Total	***24.3(25.4)***	***30.1(26.2)***	***21.4(19.2)***	***20.7(21.6)***

molar % obtained through: [a] linkage analysis, and [b] sugar analysis

A good agreement between the proportion of terminal and ramified residues was also registered for each fraction: 20.7 and 24.7%, and 15.6% and 19.9%, for the Et_{50}A-I and Et_{75}A-I fractions from the 1st 2h of the hot water extraction process, respectively. The Et_{50}A-I and Et_{75}A-I fractions from the 2nd 2h of the hot water extraction process, exhibited 18.4 and 23.3%, and 17.2 and 18.7% of terminal and ramified residues, respectively.

The Et_{50}A-I fraction obtained in the 1st 2h of the hot water extraction of *P. tridentatum* dried inflorescences contained AG-II that presented (1→3,6)-Gal*p*/(1→3)-Gal*p*, (1→5)-Ara*f*/T-Ara*f*, and (1→3,5)-Ara*f*/(1→5)-Ara*f* values of 1.6, 1.4, and 0.08, respectively. The AG-II that were detected in the correspondent untreated Et_{50}A fraction exhibited (1→3,6)-Gal*p*/(1→3)-Gal*p*, (1 → 5)-Ara*f*/T-Ara*f*, and (1 → 3,5)-Ara*f*/(1 → 5)-Ara*f* values of 1.6, 1.6, and 0.08, respectively. For the Et_{75}A-I fraction, from the 1st 2h, it was observed that the AG-II presented (1→3,6)-Gal*p*/(1→3)-Gal*p*, (1→5)-Ara*f*/T-Ara*f*, and (1→3,5)-Ara*f*/(1→5)-Ara*f* values of 2.0, 1.2, and 0.13, which are similar to those of the correspondent untreated Et_{75}A fraction. This was also observed for the Et_{75}A-I fractions from the 2nd 2h and the correspondent untreated Et_{75}A fraction.

The mannans present in the Et_{50}A-I and Et_{75}A-I fractions, from the 1st 2h, showed a substitution degree, expressed by the values of the (1→4,6)-Man*p*/(1→4)-Man*p*, of 0.34, and 0.33, which are similar to the values of 0.36, and 0.34 registered for the correspondent untreated Et_{50}A and Et_{75}A fractions. The mannans detected in the Et_{75}A-I fractions, from the 2nd 2h, showed a substitution degree of 0.30, which was similar to the value of 0.33 registered for the mannans detected in the correspondent Et_{75}A fraction.

Relatively to the structural features of the xyloglucans recovered in the Et_{50}A-I fraction, from the 1st 2h of the hot water extraction process, it was possible to observe a substitution degree, expressed by the (1→4,6)-Glc*p*/(1→4)-Glc*p* value, of 1.49, equal to the value registered for the correspondent untreated Et_{50}A fraction. The xyloglucans detected in the Et_{75}A-I fraction, from the 1st 2h of the hot water extraction process, presented a substitution degree of 0.17, similar to the value of 0.19 registered for the xyloglucans of the correspondent untreated Et_{75}A fraction. For the 2nd 2h, it was also observed that the xyloglucans detected in the

$\underline{Et_{75}A\text{-}I}$ fraction exhibited a substitution degree similar to the observed for the untreated $\underline{Et_{75}A}$ fraction: 0.22 and 0.17, for the $\underline{Et_{75}A\text{-}I}$ and $\underline{Et_{75}A}$ fractions, respectively. For both parts of the hot water extraction, the $\underline{Et_{50}A\text{-}I}$ fractions contained proportions of (1→4,6)-Glc*p* residues that clearly exceeded the proportion of T-Xyl*p* residues, as observed for the untreated $\underline{Et_{50}A}$ fraction obtained in the 1st 2h (**Table 6.3**). This suggests that these xyloglucans must possess other side chains, besides those composed of single xylosyl residues. The detection of T-Fuc*p*, (1→2)-Gal*p*, and (1→2)-Xyl*p* residues, which suggest the presence of other side chains, namely Gal*p*-(β1→2)-Xyl*p*-(α1→, Ara*p*-(α1→2)-Xyl*p*-(α1→ disaccharides, and Fuc*p*-(α1→2)-Gal*p*-(β1→2)-Xyl*p*-(α1→ trisaccharide, seem to reinforce this assumption. For the $\underline{Et_{50}A\text{-}I}$ fraction obtained from the 1st 2h of the hot water extraction, the proportions of (1→3,5)-Ara*f* and (1→3,6)-Gal*p* residues accounted for 2.5%, while the T-Ara*f* and T-Gal*p* residues added to 13.0%. Thus, only 2.5% of these terminally linked residues should be present as AG-II structural components, and the remaining 10.5% of T-Ara*f* and T-Gal*p* residues, together with 1.0 and 5.0% of T-Fuc*p* and T-Xyl*p* residues, might be present as part of the xyloglucan side chains. The total value of 16.5% for these terminally linked residues seems compatible with the 13.9% of (1→4,6)-Glc*p* residues detected in this fraction. The same type of agreement was observed for the $\underline{Et_{50}A\text{-}I}$ fraction obtained from the 2nd 2h of the hot water extraction. These results evidenced that the xyloglucans recovered in the $\underline{Et_{50}A\text{-}I}$ fraction from the 1st 2h of the hot water extraction seem to present structural features similar to the xyloglucans detected in the untreated $\underline{Et_{50}A}$ fraction, suggesting that some of these xyloglucans might have an estimated molecular weight higher than 40 kDa. Since glucosyl, and xylosyl residues were also detected in the intermediate molecular weight fraction $\underline{Et_{50}A\text{-}II}$, it seems possible that the untreated $\underline{Et_{50}A}$ fraction also contained xyoglucans with distinct molecular weight.

It was observed that the (1→4)-linked galactosyl residues detected in the untreated fractions, and that suggested the presence of AG-I, were not detected in the correspondent fractions submitted to the *endo*-polygalacturonase treatment. This may be attributed to the action of the *endo*-polygalacturonase, which may release fragments from the pectic polysaccharides structure with lower molecular

weight, and bearing AG-I side chains. The intermediate molecular weight $\underline{Et_{50}A\text{-}II}$, and $\underline{Et_{75}A\text{-}II}$ fractions also contained arabinosyl and galactosyl residues, which are indicative for the presence of arabinogalactans. Thus, it is possible that some of these residues may be present as components of the AG-I, previously detected in the untreated fractions.

The results referred above show that the high molecular weight fractions that resulted from the *endo*-polygalacturonase treatment, contained AG-II, mannans, and xyloglucans that exhibit structural features similar to those reported for the correspondent untreated fractions (**Section 6.1**). This allows to confirm the structural features of the AG-II, mannans, and also of the xyloglucans detected in the decoctions from *P. tridentatum* dried inflorescences, and also show that some of these polysaccharides possess a molecular weight higher than 40 kDa.

6.3. Electrospray ionization mass spectrometry (*ESI-MS*) experiments

The decoctions of *P. tridentatum* dried inflorescences contained higher proportions of mannans than the decoctions from both *F. angustifolia* dried leaves and *M. suaveolens* dried shoots. In order to know more about the structural features of the mannans from the decoctions of *P. tridentatum* dried inflorescences, the $\underline{Et_{75}A}$ fraction isolated from the 1st 2h of the hot water extraction process, which contained the highest proportion of mannans among the various fractions isolated, was submitted to an enzymatic treatment with *endo*-β-(1→4)-D-mannanase. According to the known enzymatic mechanism of *endo*-β-(1→4)-D-mannanase, the selective degradation procedure cleaves the mannan backbone between adjacent (β1→4)-linked mannose residues yielding mannan oligosaccharides with distinct degree of polymerization (DP). The digestion products were fractionated by size exclusion chromatography on a column containing Bio Gel P2, which has a typical fractionation range between 1.8 and 0.1 kDa, and the chromatographic elution profile is shown in **Figure 6.2 a)**. The column was previously equilibrated with water, and calibrated with blue dextran, stachyose (DP_4), cellobiose (DP_2), and glucose.

The chromatographic elution profile on Bio Gel P30 of the $\underline{Et_{75}A}$ fraction without enzymatic digestion is also presented in **Figure 6.2 b)**. The

chromatographic elution profile of the mannanase treated material, when compared to the untreated sample, exhibited one big hump, which spreaded across the chromatogram, and that should correspond to oligosaccharides resulting from the enzyme action.

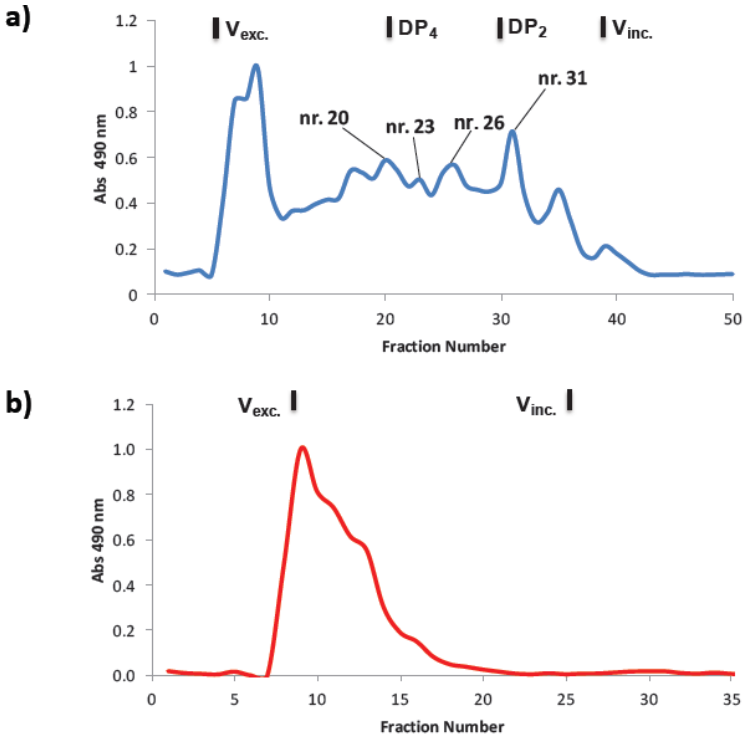

Figure 6.2- Size exclusion chromatography **a)** of the digestion products resulting from the *endo*-β-(1→4)-D-mannanase digestion of the $\underline{Et_{75}A}$ fraction on Bio Gel P2, and **b)** of the $\underline{Et_{75}A}$ fraction without enzymatic on Bio Gel P30, both obtained from the 1st 2h of the hot water extraction of *P. tridentatum* dried inflorescences.

These results allow to infer the presence of (β1→4)-D-mannose residues. The resultant oligosaccharides should have a molecular weight within 1.8 and 0.1 kDa, with distinct DP.

The chromatographic elution profile exhibited one chromatographic band that eluted near the column exclusion volume that should comprise material with a molecular weight higher than 1.8 kDa, which is the column exclusion volume. This is in accordance with the results of methylation analysis and the polygalacturonase treatment performed, which showed the presence of other types of polysaccharides besides the mannans. It is also possible that the material that eluted near the gel exclusion volume could also comprise mannan moieties with structural features that hindered the enzyme action, such as high acetylation

and/or branching degree, which are known to influence the range of oligosaccharides released after enzymatic hydrolysis (Daas *et al*., 2000; Simões *et al*., 2010, 2011, 2012). The low molecular weight oligosaccharides that resulted from the *endo*-β-(1→4)-D-mannanase digestion should also retain structural details of the polysaccharide backbone (Dhawan *et al*., 2007; Moreira *et al*., 2008). SInce mass spectrometry has proved to be a valuable tool for the assessment of polysaccharides structural features, particularly when using soft ionization methods, such as electrospray (ESI), the fractions number 20, 23, 26, and 31, marked in **Figure 6.2 a)**, were analysed by ESI-MS, and the respective spectra are shown in **Figure 6.3**.

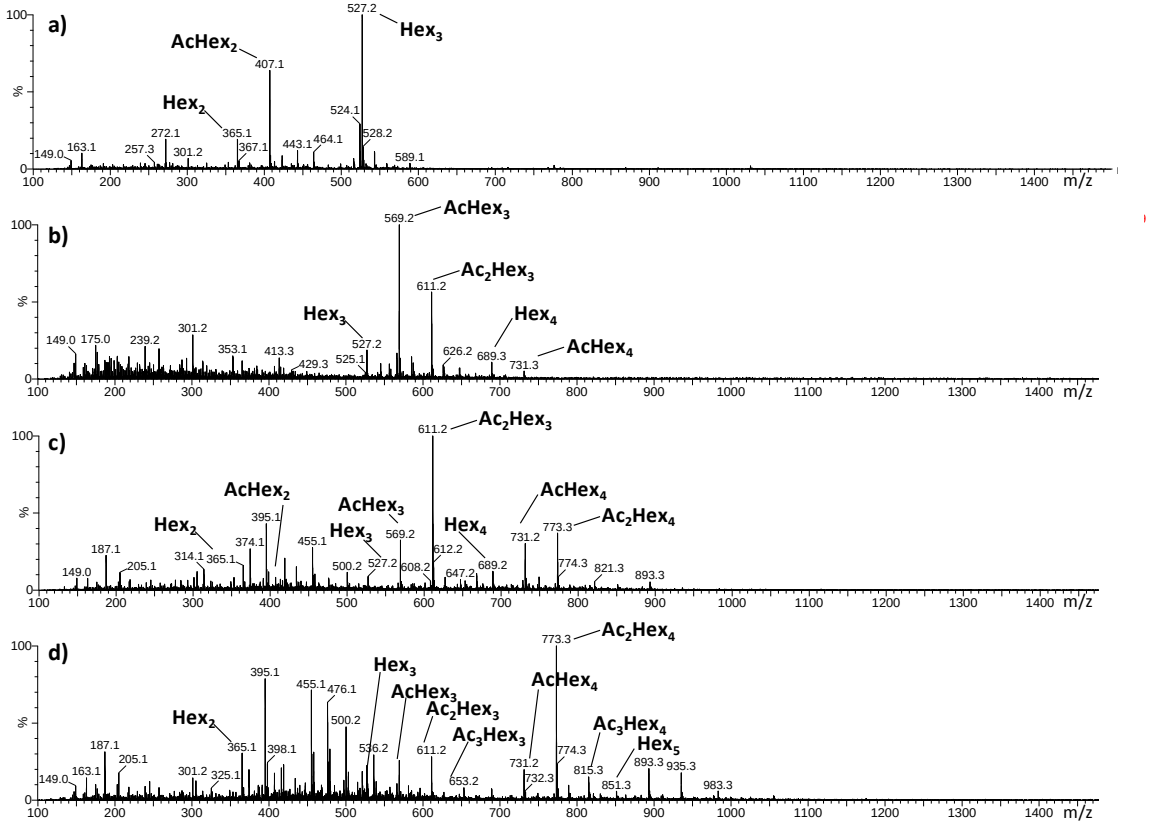

Figure 6.3- ESI-MS spectra of the $\underline{Et_{75}A}$ fraction oligosaccharides, from the *P*. *tridentatum* 1st 2h hot water extract, obtained after size-exclusion chromatography on Bio-Gel P2 with the identification of the most probable composition of the $[M+Na]^+$ ions: **a)** nr. 31, **b)** nr. 26, **c)** nr. 23, and **d)** nr. 20 fractions.

The ions observed in the MS spectra have been previously identified as sodium adduct ions ($[M+Na]^+$), typical of ESI-MS from mannooligosaccharides ionization (Cerqueira *et al*., 2011; Simões *et al*., 2010, 2012). Based on the sugar analysis of the $\underline{Et_{75}A}$ fraction (**Table 6.2**), and on the known structure of mannans from other

sources, it is possible that the mannans from the decoctions of *P. tridentatum* dried inflorescences contain hexose residues besides mannose, namely glucose and galactose (Meier and Reid, 1982; Buckeridge *et al*., 2000). Since mannose, galactose, and glucose residues possess the same mass/charge ratio, it is not possible to distinguish them by MS, thus, these hexose residues are represented generically by “Hex”.

Figures 6.3 a), **b)**, **c)**, and **d)**, evidenced the presence of fragment ions in the mass range of *m/z* 350 to 900, where it is possible to observe $[M+Na]^+$ ions of oligosaccharides that can correspond to Hex_{2-5} series. It was also possible to detect $[M+Na]^+$ ions of oligosaccharides that can correspond to acetylated hexose series, namely $Ac_{1-3}Hex_3$ and $Ac_{1-3}Hex_4$. In order to confirm the presence of acetylated hexose oligosaccharides, ESI-MS/MS was performed for the ion fragments at the correspondent *m/z* values. The ESI-MS/MS spectra of the acetylated oligosaccharides Ac_2Hex_3 (*m/z* 611), and Ac_3Hex_4 (*m/z* 815) are presented in **Figure 6.4 a)**, and **b)**, respectively.

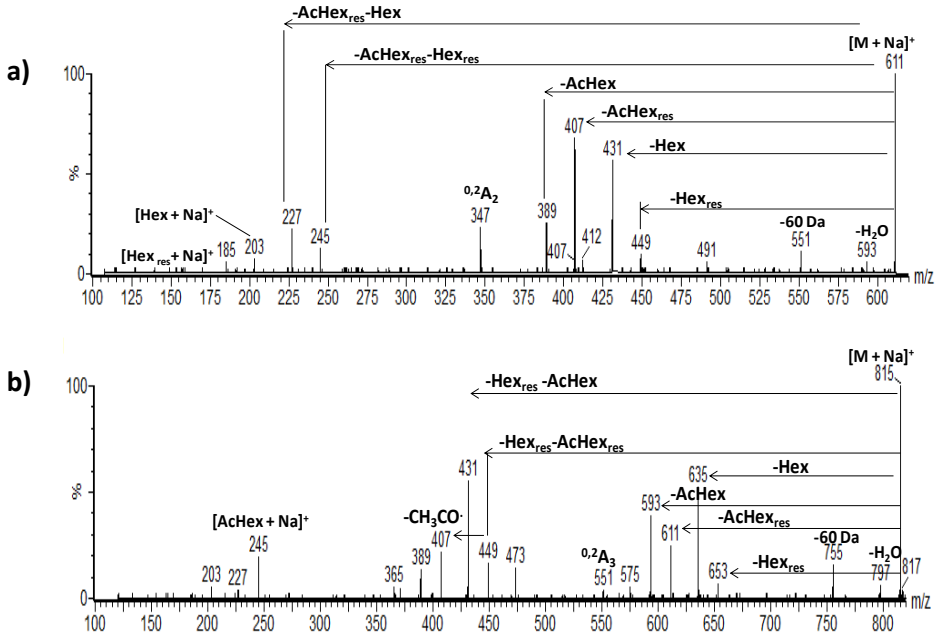

Figure 6.4- ESI-MS/MS spectra of the $[M+Na]^+$ adducts of **a)** Ac_2Hex_3 (*m/z* 611), and **b)** Ac_3Hex_4 (*m/z* 815), obtained from the decoctions of *P. tridentatum* dried inflorescences.

The ESI-MS/MS spectrum of the ion at *m/z* 611 presented ions at *m/z* 449 and 431, identified as $[Ac_2Hex_2+Na]^+$ and $[Ac_2Hex_{2res}+Na]^+$, due to the loss of a hexose residue (-162 Da), and a hexose (-180 Da), respectively. Ions at *m/z* 407 and 389 were also identified and attributed to the presence of $[AcHex_2+Na]^+$ and

$[AcHex_{2res}+Na]^+$, due to the loss of an acetylated hexose residue (-204 Da), and an acetylated hexose (- 222 Da), respectively. Ions at *m/z* 245 and 227 were also identified and attributed to the presence of $[AcHex+Na]^+$, and $[AcHex_{res}+Na]^+$, respectively. These ions were due to the loss of a hexose residue combined with the loss of an acetylated hexose residue, and to the loss of a hexose plus an acetylated hexose residue. These results showed that the trisaccharide contains nonacetylated and acetylated hexose residues. The two acetyl groups present seem to occur in different hexose residues, as the loss of a hexose bearing two acetyl groups has not been observed. It was also detected the presence of an ion at *m/z* 347, due to the combined loss of 162 Da plus the loss of 102 Da, and correspondent to an $^{0,2}A_2$ cross-ring fragment, showing that the acetyl group should be placed at *O*-2 position of the hexose residue (Nunes *et al*., 2005). These results evidenced the presence of acetyl groups in the structure of oligosaccharides composed by three hexose residues and that resulted from the *endo*-β-(1→4)-D-mannanase digestion of the $\underline{Et_{75}A}$ fraction. This was also observed by the analysis of the ESI-MS/MS spectrum of $[Ac_3Hex_4+Na]^+$ (*m/z* 815) (**Figure 6.4b**), which exhibited fragment ions at *m/z* 653 and 635, attributed to the presence of $[Ac_3Hex_3+Na]^+$ and $[Ac_3Hex_{3res}+Na]^+$, due to the loss of a hexose residue (-162 Da), and a hexose (-180 Da), respectively. Also, the fragment ions at *m/z* 611 and 593 were identified as $[Ac_2Hex_3+Na]^+$ and $[Ac_2Hex_{3res}+Na]^+$, due to the loss of an acetylated hexose residue (-204 Da), and an acetylated hexose (-222 Da). The presence of ions at *m/z* 449 and 431 correspondent to $[Ac_2Hex_2+Na]^+$, and $[Ac_2Hex_{2res}+Na]^+$, respectively, attributed to the loss of a hexose residue combined with the loss of an acetylated hexose residue and to the loss of a hexose plus an acetylated hexose residue, was also observed. The presence of ions at *m/z* 407 and 389, was attributed to the presence of $[AcHex_2+Na]^+$ and $[AcHex_{2res}+Na]^+$, due to the loss of two acetylated hexose residues, and to the loss of a acetylated hexose plus an acetylated hexose residue. The presence of a fragment ion at *m/z* 551 corresponding to the combined loss of a hexose residue (-162 Da) plus the loss of 102 Da due to the $^{0,2}A_3$ cross-ring fragment containing the acetyl group at *O*-2 position was also observed.

For the mannans from the decoctions of *P. tridentatum* dried inflorescences, it was possible to detect triacetylated oligosaccharides. This number of detected acetyl groups is higher than the registered for coffee infusion (Nunes *et al*., 2005; Simões *et al*., 2010), LBG (Simões *et al*., 2011), and also non-conventional sources of mannans (Cerqueira *et al*., 2011). For the coffee infusion mannans diacetylated oligosaccharides were detected, while for the mannans from LBG, and from non-conventional sources only monoacetylated oligosaccharides were identified. The oligosaccharides resulting from the *endo*-β-(1→4)-D-mannanase digestion of *P. tridentatum* mannans contained a lower number of acetyl groups than those from *Aloe vera*, which exhibited oligosaccharides presenting up to eight acetyl groups (Simões *et al*., 2012).

The distinct acetylation patterns seemed to contribute for the different characteristics of chromatographic elution profiles obtained using mannans enzymatic digestions performed in the same conditions as the used for this study. The chromatographic elution profiles obtained through *endo*-β-(1→4)-D-mannanase digestion of mannans presenting high number of acetyl groups, such as those from *Aloe vera* and *P. tridentatum*, suggested the presence of oligosaccharides with DP´s higher than 4, while for the coffee infusion and LBG low acetylated mannans, the most abundant fractions had a DP value of 2 (Simões *et al*., 2010), and a DP value comprised between 2 and 4 (Simões *et al*., 2011), respectively.

Besides the most abundant fragment ions highlighted in **Figure 6.3**, it was also possible to identify lower abundant $[M+Na]^+$ fragment ions with *m/z* values that evidenced the occurrence of acetylated oligosaccharides with hexoses and a pentose residue, such as $AcPentHex_{2-4}$. The presence of such oligosaccharides seems plausible, since the occurrence of mannan oligosaccharides containing pentose residues, identified as arabinose, has also been observed in *Aloe vera* (Simões *et al*., 2012), coffee infusion (Nunes *et al*., 2005), LBG (Simões *et al*., 2011), and spent coffee ground (Simões *et al*., 2010). To confirm the presence of these oligosaccharides, ESI-MS/MS was performed for the ion fragments at the correspondent *m/z* values, and the spectrum for the acetylated oligosaccharide $AcPentHex_4$ (*m/z* 863) is presented in **Figure 6.5**.

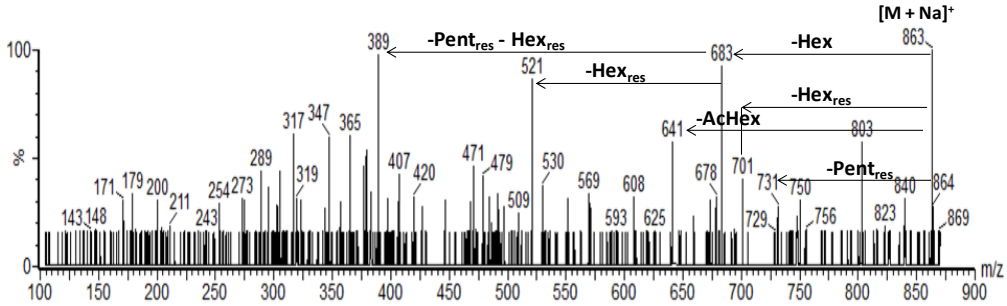

Figure 6.5- ESI-MS/MS spectra of the $[M+Na]^+$ adducts of $PentAcHex_4$ (*m/z* 863), obtained from the decoctions of *P. tridentatum* dried inflorescences.

The ESI-MS/MS spectrum of $[AcPentHex_4+Na]^+$ (*m/z* 863), shown in **Figure 6.5**, exhibited a fragment ion at *m/z* 731 attributed to the presence of $[AcHex_{4res}+Na]^+$, due to the loss of a pentose residue (-132 Da). It was also observed a fragment ion at *m/z* 389, resulting from the combined loss of a hexose, a hexose residue plus a pentose residue, and attributed to the presence of $[AcHex_{res}+Na]^+$. These results evidenced the presence of pentose in the oligosaccharides that resulted from the *endo*-β-(1→4)-D-mannanase digestion of the $\underline{Et_{75}A}$ fraction. Moreover, these results also evidenced that the acetyl group was not linked to the pentose, but to the hexose units, which is reinforced by the presence of the fragment ion at *m/z* 641, attributed to the presence of $[PentHex_{3res}+Na]^+$, due to the loss of an acetylated hexose (-222 Da) from the $[M+Na]^+$ adduct of $PentAcHex_4$ (*m/z* 863). The presence of acetyl groups linked to the hexose units was also observed in mannans from coffee infusion (Simões *et al*., 2010), and LBG (Simões *et al*., 2011), while for the *Aloe vera* mannans also acetylated pentose units were detected (Simões *et al*., 2012), besides acetylated hexoses.

The ESI-MS analysis allowed to identify the various oligosaccharide series represented in **Figure 6.3** based on its molecular weight. The presented assignments were further confirmed by ESI-MS/MS. All the identified $[M+Na]^+$ ions of the digestion products of $\underline{Et_{75}A}$ fraction and the correspondent confirmed assignments are presented on **Table 6.7**.

Table 6.7- Oligosaccharides identified in ESI-MS spectra of the digestion products of $\underline{Et_{75}A}$ fraction, and further confirmed by ESI-MS/MS, with the identification of the $[M+Na]^+$ ions *m/z* value, and its composition.

		Number of acetyl groups			
		0	**1**	**2**	**3**
	n	***m/z***			
Hex$_n$	**2**	365			
	3	527	569	611	653
	4	689	731	773	815
	5	851	893	935	977
	6		1055		
PentHex$_n$	**2**		539		
	3		701		
	4		863		

6.4. Concluding remarks

The results obtained for the decoctions of *P. tridentatum* dried inflorescences showed the presence of high proportions of pectic polysaccharides, together with lower proportions of both AG-I and AG-II, similarly to what was observed for the decoctions of *F. angustifolia* dried leaves and *M. suaveolens* dried shoots. The decoctions of *P. tridentatum* also presented mannans and xyloglucans in higher proportions than the registered for the other two hot water extracts. It seems possible that these polysaccharides might have been extracted from the seeds, which are present in *P. tridentatum* inflorescences, possibly performing storage functions (Buckeridge *et al*., 2000; Meier and Reid, 1982). In fact, methylation analysis allowed to observe the presence of terminally-linked galactose residues and (1→4,6)-linked mannose residues, characteristic of galactomannans, as observed for other plant seeds. The mannans of *P. tridentatum* exhibited an acetylation extent that was higher than the registered for the galactomannans extracted from coffee infusion, LBG, and also non-conventional galactomannan sources, but lower than the exhibited by the *Aloe vera* galactomannans.

CHAPTER 7

BIOLOGICAL ACTIVITY

In this chapter, three fractions isolated from the distinct decoctions were selected, and its macrophage cytotoxic effect, anti-, and pro-inflammatory activities, together with the possible LPS contamination, were evaluated. The possible contribution of the various polysaccharide structures present in the assayed fractions to the observed biological activity is also discussed. The selected fractions comprised two pectic polysaccharide-rich fractions, from the decoctions of *F. angustifolia* dried leaves, and *M. suaveolens* dried shoots, and one pectic polysaccharide-rich fraction, isolated from the decoctions of *P. tridentatum* dried inflorescences, that also contained mannans, and xyloglucans in considerable proportions.

7.1. Macrophage cellular viability

The influence of the fractions $\underline{Et_{50}C}$, $\underline{Et_{50}B}$, and $\underline{Et_{75}A}$, isolated from the decoctions of *F. angustifolia*, *M. suaveolens*, and *P. tridentatum*, respectively, on RAW 264.7 macrophage cellular viability was tested. The cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and the results are shown in **Figure 7.1**.

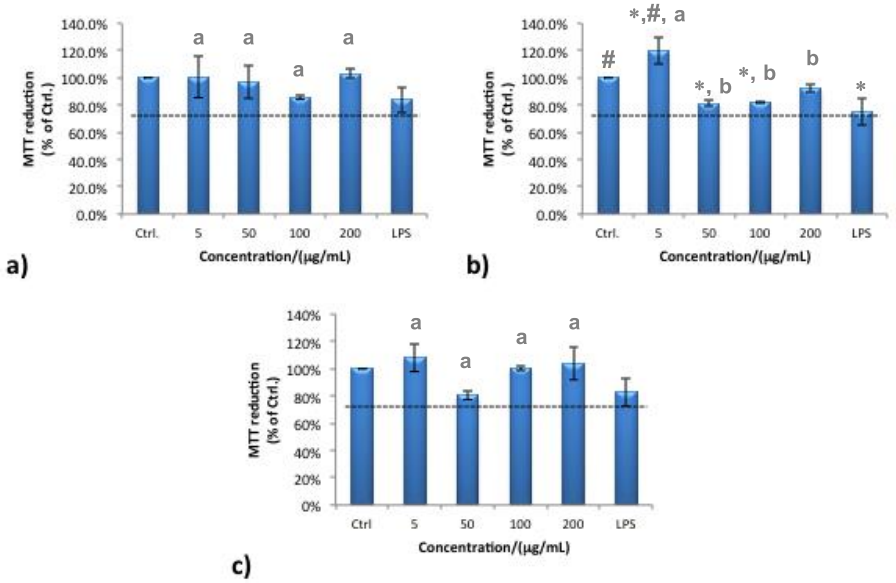

Figure 7.1- Influence of **a)** $\underline{Et_{50}C}$, from *F. angustifolia*, **b)** $\underline{Et_{50}B}$, from *M. suaveolens*, and **c)** $\underline{Et_{75}A}$, from *P. tridentatum*, fractions on macrophage cell viability. LPS-induced macrophages (LPS), and non-stimulated macrophages (Ctrl) were used as positive, and negative controls, respectively. (* compared to Ctrl.; # compared to LPS; different letters denote significant differences between assays using distinct extract concentrations; $p < 0.05$).

The results showed that the cellular viability of macrophages stimulated with distinct concentrations of $Et_{50}C$, and $Et_{75}A$ fractions was similar to the observed for the control, where non-stimulated macrophages were used. Significant differences relatively to the control assay were registered when the macrophages were stimulated with 5, 50, and 100 μg/mL of $Et_{50}B$. When compared with the control assay, the use of 5 μg/mL of $Et_{50}B$ seemed to increase cellular viability, while macrophage stimulation with 50, and 100 μg/mL of $Et_{50}B$ decreased it. However, it was observed that the macrophage cellular viability was always higher than 75%, as happened for LPS-induced macrophages. This value is considered the reference for the assessment of possible cytotoxic effects. Thus, these results evidenced that over the entire concentration range tested (5-200 μg/mL), the $Et_{50}C$, $Et_{50}B$, and $Et_{75}A$ fractions had no cytotoxic effects on the macrophages.

7.2. Macrophage nitric oxide (NO) production

The influence of $Et_{50}C$, $Et_{50}B$, and $Et_{75}A$ on macrophage NO production was assessed by measuring the accumulation of nitrite, which is the stable end product of NO, in the RAW 264.7 macrophage cells supernatant. Both pro-, and anti-inflammatory activities (blues, and red bar charts, respectively) were evaluated, and the results are presented in **Figure 7.2**. The pro-inflammatory activity is evidenced when macrophages stimulated with a determined concentration of the assayed fraction, exhibit an increase in NO production, relatively to the control. When LPS-induced macrophages, stimulated with a determined concentration of the assayed fraction, exhibit a decrease in the NO production, relatively to the LPS-induced macrophages, an anti-inflammatory effect is observed.

Upon stimulating the macrophages with $Et_{50}C$ (**Figure 7.2 a1)**), $Et_{50}B$ (**Figure 7.2 b1)**), and $Et_{75}A$ (**Figure 7.2 c1)**) fractions (5, 50, 100 and 200 μg/mL), an increase in the macrophage NO production was observed relatively to the control. For the $Et_{50}C$ fraction from *F. angustifolia* a similarly potent macrophage NO production was observed for extract concentrations of 5.0 and 50 μg/mL. When stimulated with 100 and 200 μg/mL the macrophage NO production increased

relatively to the situation where an extract concentration of 5.0 μg/mL was used. For *M. suaveolens* $\underline{Et_{50}B}$ fraction, the stimulation of macrophages with a concentration of 5.0 μg/mL caused an increase in the NO production relatively to the control, however this increase was lower than the registered for the *F. angustifolia* fraction, when the macrophages were stimulated with the same concentration.

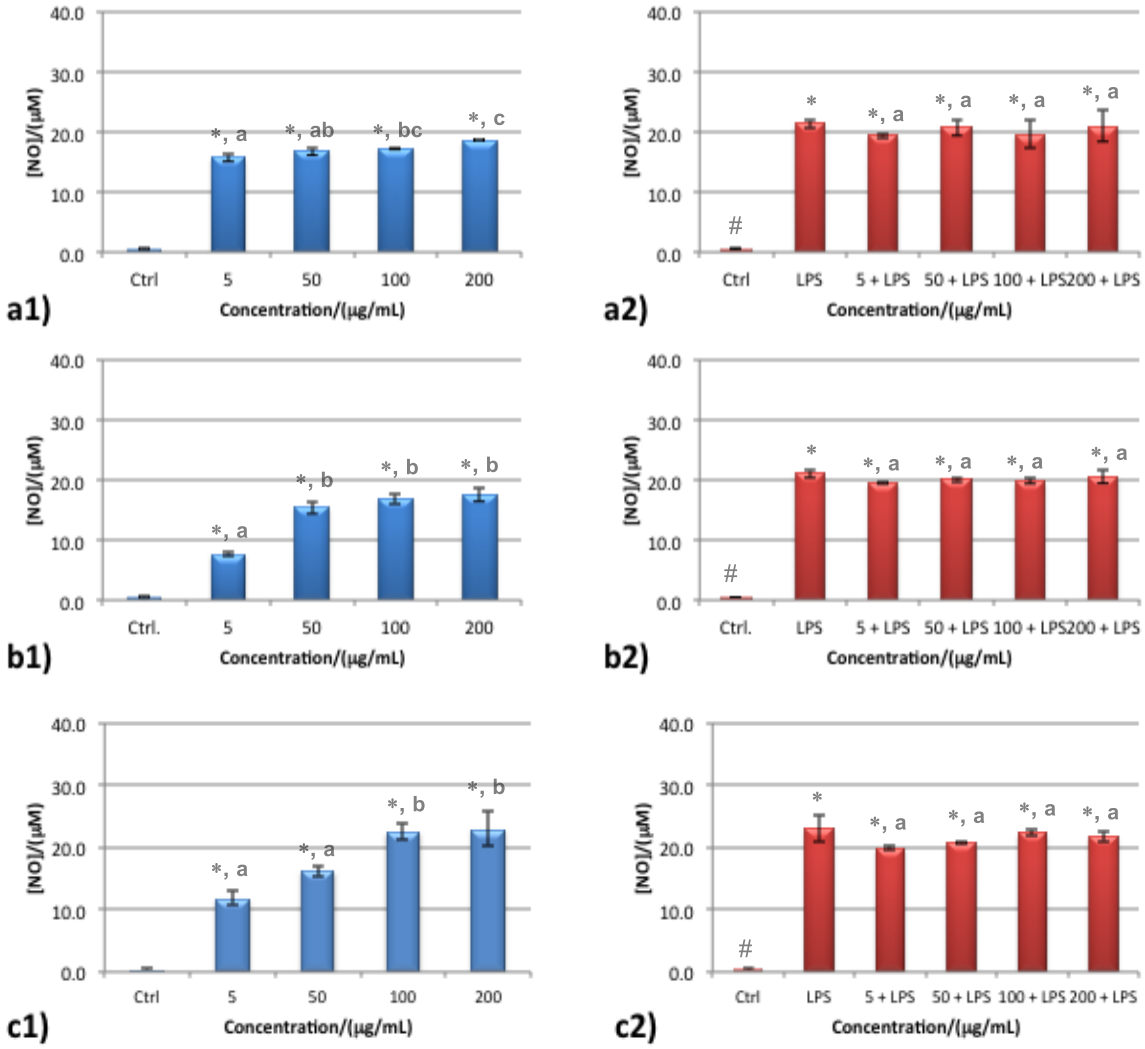

Figure 7.2- Influence of $\underline{Et_{50}C}$, $\underline{Et_{50}B}$, and $\underline{Et_{75}A}$ on macrophage NO production: **a1)**, **b1)**, and **c1)** pro-inflammatory activity; **a2)**, **b2)**, and **c2)** anti-inflammatory activity. LPS-induced macrophages (LPS), and non-stimulated macrophages (Ctrl) were used as positive, and negative controls, respectively. (* compared to Ctrl.; # compared to LPS; different letters denote significant differences between assays using distinct extract concentrations; $p < 0.05$).

When stimulated with a $\underline{Et_{50}B}$ concentration ten times higher, the NO production doubled, relatively to the lower concentration tested. The macrophage stimulation

with $Et_{50}B$ concentrations of 100, and 200 μg/mL did not seem to cause a further increase in the NO production. For the $Et_{75}A$ fraction from *P. tridentatum*, a potent and similar effect was observed stimulating the macrophages with 5 and 50 μg/mL. Upon stimulation with 100 and 200 μg/mL, the macrophage NO production did not seem to be affected, relatively to the lowest concentrations assayed. The results suggested that for the range of concentrations assayed, the various fractions exhibited a macrophage immunostimulory effect, expressed by the increase in the macrophage NO production. Also, it was observed that for the lowest concentration assayed, the $Et_{50}C$ fraction, from the decoctions of *F. angustifolia*, showed the most potent macrophage NO production.

When the macrophages were pre-incubated with $Et_{50}C$ (**Figure 7.2 a2)**), $Et_{50}B$ (**Figure 7.2 b2)**), and $Et_{75}A$ (**Figure 7.2 c2)**) fractions (5, 50, 100 and 200 μg/mL), and then treated with LPS (1 μg/mL), no change in the macrophage NO production was observed, relatively to the LPS-induced macrophages. Also, the macrophage NO production was similar over the entire range of extract concentrations assayed. This suggests that, for the range of concentrations tested, the various fractions did not present anti-inflammatory activity, which would be expressed by a decrease in the macrophage NO production, relatively to the LPS-induced macrophages. Also, as no increase in the macrophage NO production, relatively to the LPS-induced macrophages, was registered, it can be inferred that no synergistic effect between LPS, and the various assayed fractions occurred.

7.3. Influence of polymyxin B on macrophage NO production

The results referred in **Sections 7.1** and **7.2** suggested that, for the range of concentrations tested, the various fractions assayed presented a macrophage immunostimulatory activity, expressed by the NO production, without compromising the macrophage cellular viability.

Endotoxin (LPS) is a contaminant that can be found in biological preparations, and that is known for its macrophage immunostimulatory activity. The HMWM from the various hot water extracts prepared was fractionated in a biochemistry laboratory that was not specifically equipped for the prevention of bacterial

contamination. Therefore, to exclude the possibility of contamination with LPS, the fractions (200 μg/mL) were pre-incubated with polymyxin B (PMB) for 1h, and subsequently used for stimulating macrophages, alone or mixed with LPS. The macrophage NO production was evaluated, and the results are shown in **Figure 7.3**.

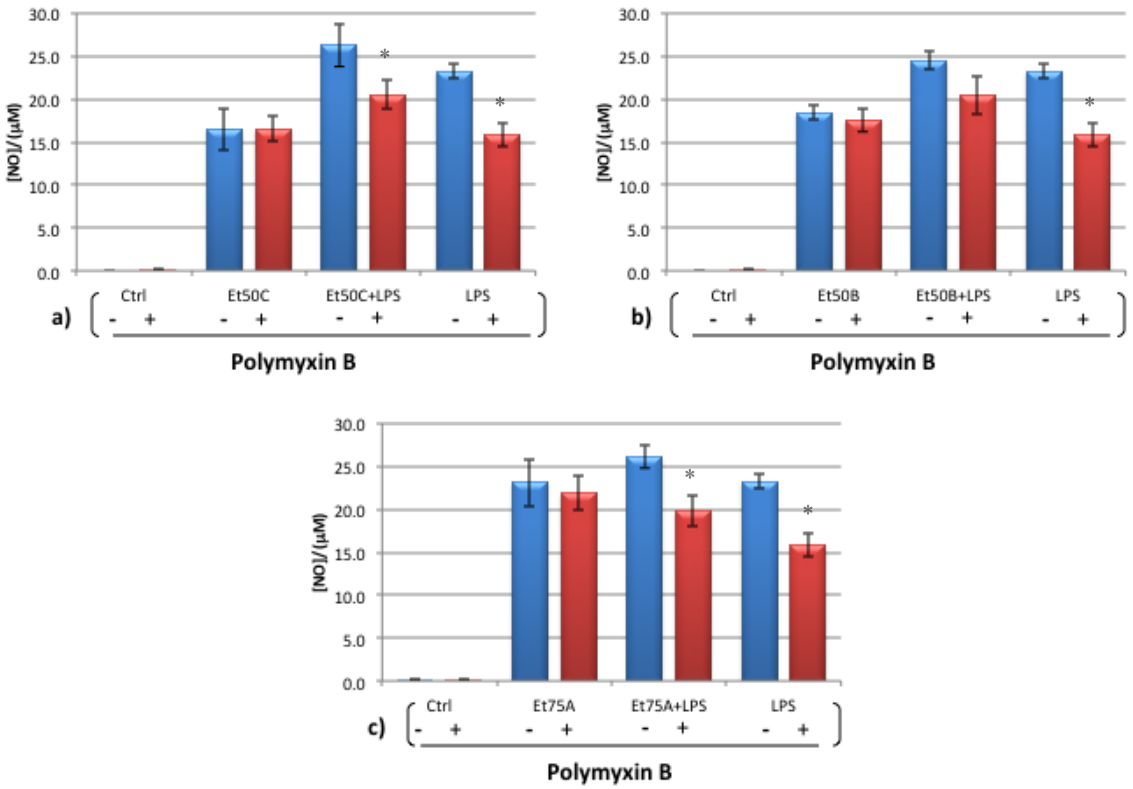

Figure 7.3- Effect of PMB on $\underline{Et_{50}C}$-, $\underline{Et_{50}B}$- and $\underline{Et_{75}A}$-stimulated (200 μg/mL), or LPS-induced (1 μg/mL) NO production. The fractions, and LPS were pre-incubated with PMB (1000 U/mL) for 1h, before macrophage stimulation. (* compared to assay without pre-incubation with PMB; $p < 0.05$).

The results showed that the pre-incubation with PMB did not seem to affect NO production by macrophage stimulated by any of the three fractions assayed, while reducing it in LPS-induced macrophages. It was also observed a reduction in the NO production of co-stimulated macrophages with LPS, and $\underline{Et_{50}C}$ or $\underline{Et_{75}A}$. It is known that PMB inhibits the LPS-induced activation of B cells, and macrophages by binding the lipid A moiety of LPS (Morrisson and Jacobs, 1976), and therefore the NO generation decrease registered in LPS-induced macrophages, and co-stimulated macrophages with LPS and $\underline{Et_{50}C}$ or $\underline{Et_{75}A}$ was according to the literature.

Since the macrophage NO generation induced by $\underline{Et_{50}C}$, $\underline{Et_{50}B}$, and $\underline{Et_{75}A}$ was not affected by the PMB treatment, it seems that the fractions assayed were not contaminated by LPS. Therefore, these results allowed to evidence that the increase in the NO macrophage production that was reported in **Section 7.2** can be attributed to the macrophage immunostimulatory activity of the compounds present in the $\underline{Et_{50}C}$, $\underline{Et_{50}B}$ or $\underline{Et_{75}A}$ fractions.

7.4. Influence of polymyxin B on macrophage cellular viability

In order to confirm that the results observed in **Section 7.3** were not influenced by changes in the macrophages cellular viability, the possible influence of PMB on the cellular viability of macrophages alone, and macrophages stimulated with $\underline{Et_{50}C}$, $\underline{Et_{50}B}$ or $\underline{Et_{75}A}$, alone or mixed with LPS, and LPS-induced was evaluated by the MTT assay and the results are shown in **Figure 7.4**.

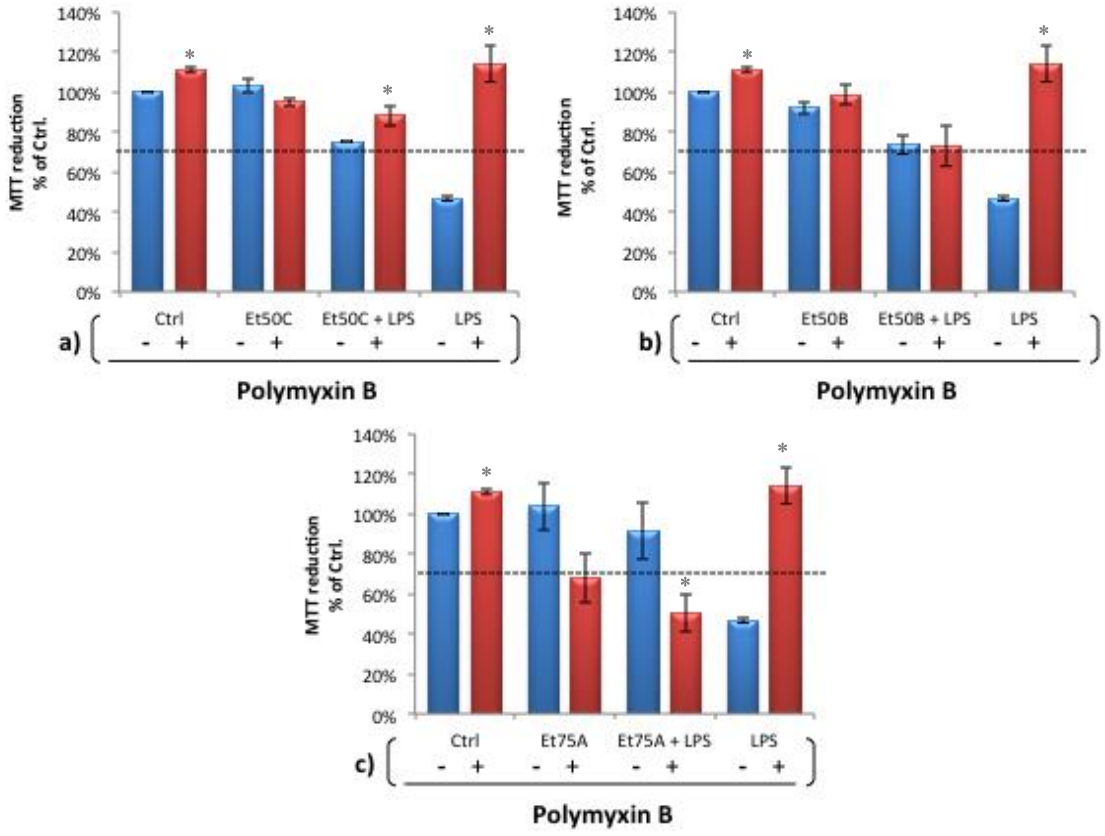

Figure 7.4- PMB influence on cell viability of $\underline{Et_{50}C}$-, $\underline{Et_{50}B}$-, and $\underline{Et_{75}A}$-stimulated (200 μg/mL), or LPS-induced (1 μg/mL) macrophage cell viability. The fractions, and LPS were pre-incubated with PMB (1000 U/mL) for 1h, before macrophage stimulation. (* compared to assay without pre-incubation with PMB; $p < 0.05$).

The control experiments showed that for the macrophages alone, the cellular viability was slightly increased by the pre-incubation with PMB, while the pre-incubation with PMB highly increased the cellular viability of LPS-induced macrophages. This was probably due to the high affinity of PMB to the lipid A moiety of LPS (Morrisson and Jacobs, 1976) that will decrease the amount of LPS available for binding to the membrane receptors, and immunostimulate macrophages. The cellular viability of the macrophages stimulated with $\underline{Et_{50}C}$, $\underline{Et_{50}B}$, and $\underline{Et_{75}A}$ alone was not affected by the pre-incubation with PMB.

For macrophages stimulated with $\underline{Et_{50}C}$, and $\underline{Et_{75}A}$, mixed with LPS, an increase and a decrease in the cellular viability was observed, respectively.

The $\underline{Et_{75}A}$ fraction from *P. tridentatum* contained the highest proportion of mannans, amongst all the fractions isolated from the distinct decoctions studied, as referred in **Section 6.1.3**. Previous *in vivo* and *in vitro* studies have shown that mannans are capable to bind to macrophages through mannose-specific protein receptors, and are subsequently internalized (Aderem and Underhill, 1999; Tietze *et al.,* 1982). Therefore, the possible influence of a mannan-PMB complex on the macrophage cellular viability should be considered.

The results obtained reinforced that the similar macrophage NO production, with or without the PMB pre-incubation, that was exhibited by the macrophages stimulated with various concentrations of the distinct fractions was not due to the possible influence of PMB in the macrophage cellular viability. This also allows to confirm that the macrophage immunostimulatory activity observed for the $\underline{Et_{50}C}$ from *F. angustifolia*, $\underline{Et_{50}B}$ from *M. suaveolens*, and $\underline{Et_{75}C}$ from *P. tridentatum* decoctions is related to the presence of biologically active compounds in these fractions.

7.5. Polysaccharide contribution for the immunostimulatory activity

As referred in the previous section, the results obtained evidenced that the macrophage immunostimulatory activity of the assayed fractions could be attributed to the presence of biologically active compounds. The total sugar content, and the polysaccharide composition of the assayed $\underline{Et_{50}C}$, $\underline{Et_{50}B}$ and $\underline{Et_{75}A}$ fractions, is shown in **Table 7.1**. The presence of a determined polysaccharide structure is denoted by the "+" symbol, while its absence is represented by the "-" signal.

Table 7.1- Total sugar content, and polysaccharide composition of $\underline{Et_{50}C}$, $\underline{Et_{50}B}$ and $\underline{Et_{75}A}$ fractions, from the decoctions of *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences, respectively, and in the $\underline{Et_{75}A\text{-sap}}$, $\underline{Et_{75}A\text{-I}}$, and $\underline{Et_{75}A\text{-III}}$ fractions, resulting from the NaOH treatment, and *endo*-polygalacturonase treatment, respectively, of the $\underline{Et_{75}A}$ fraction.

	Total Sugar (mass %)	Pectic Polysaccharides		AG-II	Acetylated Mannans	Xyloglucans
		Galacturonans	AG-I			
$Et_{50}C$[a]	86.9	+	+	+	–	–
$Et_{50}B$[a]	96.5	+	+	+	–	–
$Et_{75}A$[a]	85.3	+	+	+	+	+
$Et_{75}A$-sap[b]	85.3	+	+	+	–	+
$Et_{75}A$-I[a]	86.7	–	-	+	–	+
$Et_{75}A$-III[c]	97.4	+	-	–	–	–

the presence of the various polysaccharides was: [a] determined by glycosidic linkage analysis; [b] inferred based on the NaOH treatment performed on $Et_{75}A$ fraction; [c] inferred based on the *endo*-polygalacturonase treatment performed on $Et_{75}A$ fraction, and also on the sugar analysis of the $Et_{75}A$-III fraction.

The $\underline{Et_{50}C}$, $\underline{Et_{50}B}$ and $\underline{Et_{75}A}$ fractions contained high proportions of carbohydrate material, and exhibited macrophage immunostimulatory activity, expressed by a pro-inflammatory activity. As evidenced in **Section 1.7**, several studies found in the literature evidenced the immunostimulatory activity of polysaccharides, suggesting that the polysaccharides present in these fractions should have contributed to the observed macrophage NO production.

The monomeric composition, and glycosidic linkage analysis of the polysaccharides recovered in the $\underline{Et_{50}C}$ fraction from *F. angustifolia* (**Tables 4.2**, and **4.3**), and in the $\underline{Et_{50}B}$ fraction from *M. suaveolens* (**Tables 5.2**, and **5.3**), evidenced the presence of high proportions of pectic polysaccharides, particularly

galacturonans, mixed with minor proportions of AG-I, and AG-II, as shown in **Table 7.1**. Various studies have reported the immunostimulatory activity of pectic polysaccharide fractions, as evidenced in **Section 1.7**. Most of these studies attributed the observed biological activities to AG-II structures present in the pectic polysaccharide fractions. Thus, it seems possible that the macrophage immunostimulatory activity exhibited by the $\underline{Et_{50}C}$, and $\underline{Et_{50}B}$ fractions was due to the presence of the AG-II. Although less frequently than the observed for AG-II, some studies found in the literature have also reported the presence of AG-I in fractions of bioactive pectic polysaccharides. Therefore, the possible contribution of these polymers for the macrophage immunostimulatory activity of these fractions should be considered.

The monomeric composition of the polysaccharides present in the $\underline{Et_{75}A}$ fraction from *P. tridentatum*, together with its glycosidic linkage analysis (**Tables 6.2**, and **6.3**, respectively), evidenced the presence of a mixture containing pectic polysaccharides, mannans, and xyloglucans, together with AG-I, and also AG-II in minor proportions, as shown in **Table 7.1**. Besides the contribution of pectic polysaccharide AG-I structures, and of AG-II often found in rich pectic polysaccharide fractions, several studies have also evidenced the biological activity of mannans, and xyloglucans.

In order to try to clarify the contribution of the various polysaccharides in the expression of the macrophage immunostimulatory activity, the $\underline{Et_{75}A}$ fraction was submitted to various treatments, and the NO production was evaluated. The $\underline{Et_{75}A}$ fraction was de-esterified, through treatment with NaOH solution, yielding the $\underline{Et_{75}A\text{-sap}}$ fraction. The $\underline{Et_{75}A}$ fraction was also submitted to an *endo*-polygalacturonase treatment, as described in **Section 6.2**, and the digestion products were fractionated by size exclusion chromatography on Bio Gel P30, as also described in **Section 6.2**. The macrophage immunostimulatory activity of the high, and low molecular weight fractions obtained ($\underline{Et_{75}A\text{-I}}$, and $\underline{Et_{75}A\text{-III}}$, respectively) was evaluated, and is shown in **Figure 7.5**.

From **Figure 7.5 a1)**, it was observed that stimulating macrophages with 50 μg/mL of $\underline{Et_{75}A\text{-sap}}$ fraction did not increased in a statistically significant way the macrophage NO production relatively to the control. Macrophage stimulation with

100 and 200 μg/mL led to a similar increase in the macrophage NO production relatively to the control and to the lowest concentration tested.

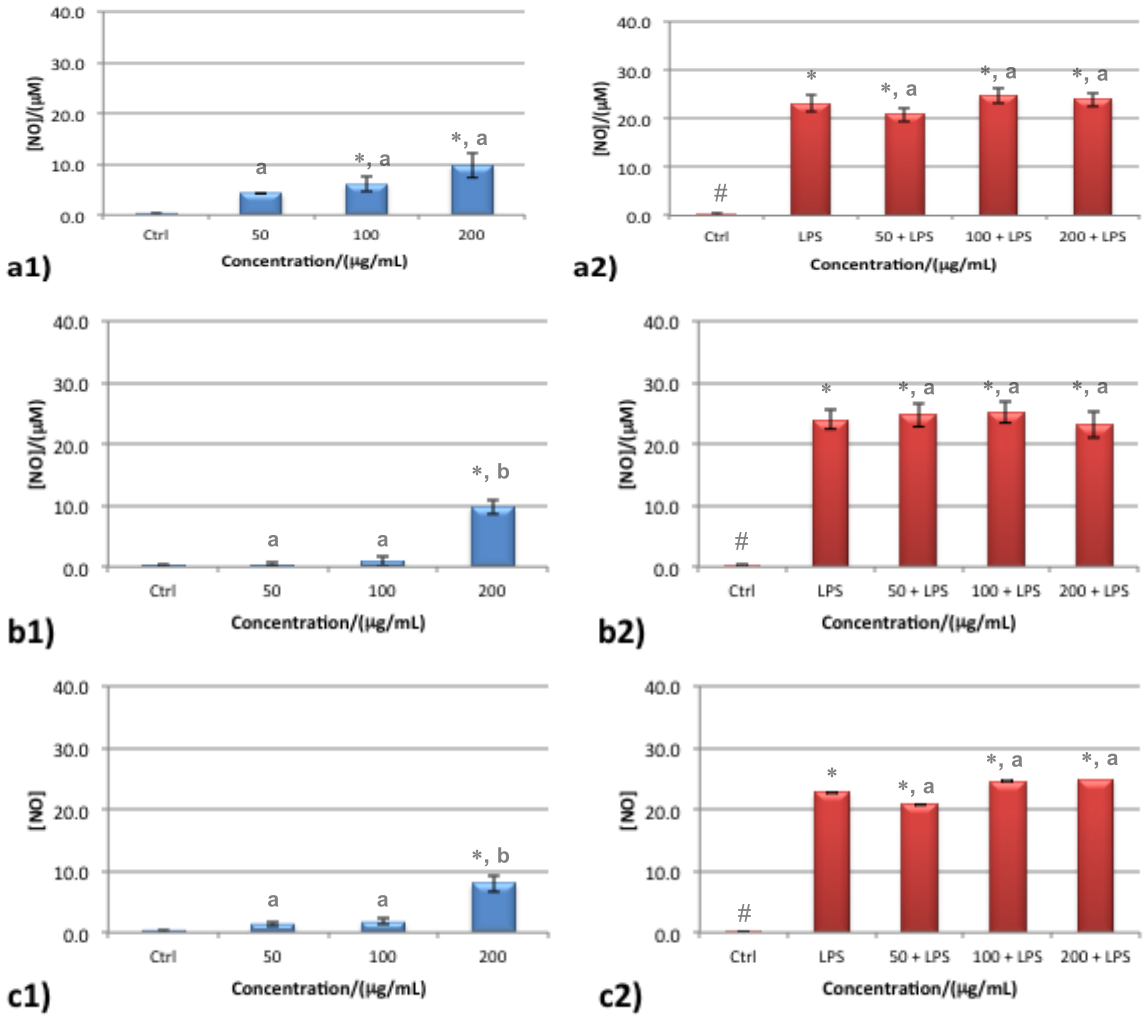

Figure 7.5- Influence of $\underline{Et_{75}A\text{-sap}}$, $\underline{Et_{75}A\text{-I}}$, and $\underline{Et_{75}AIII}$ on macrophage NO production: **a1)**, **b1)** and **c1)** pro-inflammatory activity; **a2)**, **b2)** and **c2)** anti-inflammatory activity. LPS-induced macrophages (LPS), and non-stimulated macrophages (Ctrl) were used as positive, and negative controls, respectively. (* compared to Ctrl.; # compared to LPS; different letters denote significant differences between assays using distinct extract concentrations; $p < 0.05$).

This increase in the macrophages NO production suggests a macrophage immunostimulatory effect. However, compared to the observed for $\underline{Et_{75}A}$ fraction (**Figure 7.2 c1)**), the reported immunostimulatory activity was less potent, as evidenced by the reduction in the macrophages NO production of approximately 50, 45, and 35%, registered for 50, 100 and 200 μg/mL concentrations, respectively. These results suggested that the NaOH treatment of the $\underline{Et_{75}A}$ fraction, which yielded the de-acetylated $\underline{Et_{75}A\text{-sap}}$ fraction, contributed for the

decrease observed in the macrophage immunostimulatory activity of the resulting fraction. As previously referred, the $Et_{75}A$ fraction comprised mannans, and through the use of ESI-MS and ESI-MS/MS experiments, it was evidenced that these mannans were acetylated (**Section 6.3**). Therefore, it seems possible that the acetylation of the polysaccharides, and particularly from the mannans present in the $Et_{75}A$ fraction, could have contributed for its macrophage immunostimulatory activity, and that their de-acetylation could have contributed for the less potent macrophage immunostimulatory activity of the correspondent de-acetylated $Et_{75}A$-sap fraction. The importance of the mannans acetylation suggested by these results, seems to be supported by some studies found in the literature, as referred in **Section 1.7.2**.

The $Et_{75}A$-sap fraction still evidenced immunostimulatory activity (**Figure 7.5 a1)**, suggesting that the other polysaccharides present in $Et_{75}A$ fraction could have contributed for the remaining macrophage NO production observed. Therefore, the $Et_{75}A$ fraction was submitted to an *endo*-polygalacturonase treatment, which consisted in a previous treatment with NaOH solution, followed by the *endo*-polygalacturonase digestion. The macrophage immunostimulatory activity observed when the macrophages were stimulated with 50, and 100 μg/mL of $Et_{75}A$-I fraction, was similar to the registered for the non-stimulated macrophages, and negligible when compared to the registered for macrophages stimulated with the same concentrations of $Et_{75}A$ fraction. This evidenced the influence of the *endo*-polygalacturonase treatment of $Et_{75}A$ fraction on its immunostimulatory activity. The high molecular weight fraction ($Et_{75}A$-I) that resulted from the *endo*-polygalacturose treatment performed was shown to mainly contain mannans and xyloglucans, together with small proportions of AG-II, as shown in **Table 7.1**. Previously, it has been shown that the NaOH treatment, which yielded the $Et_{75}A$-sap fraction, decreased the immunostimulatory activity of the $Et_{75}A$ fraction, possibly through mannan de-acetylation. The *endo*-polygalacturonase digestion subsequently performed yielded the $Et_{75}A$-I fraction that did not contain the AG-I, which had been detected in the $Et_{75}A$ fraction, suggesting the possible contribution of these structures to the immunostimulatory ativity exhibited by $Et_{75}A$ fraction.

Also, it was observed that when the macrophages were stimulated with 200 μg/mL of the Et_{75}A-I fraction, an immunostimulatory activity higher than the exhibited for the lowest concentrations, and control was registered. This immunostimulatory activity was similar to the registered for the macrophage stimulation with Et_{75}A-sap fraction at the same concentration level. This could be related to the presence of AG-II in the Et_{75}A-I fraction, however for the fractions Et_{50}C, and Et_{50}B from *F. angustifolia*, and *M. suaveolens*, respectively, the AG-II detected seemed to express their immunostimulatory activity at lower concentrations. This could be related with differences in the structural features of the AG-II isolated from the various decoctions.

The Et_{75}A fraction contained xyloglucans that were also detected in the Et_{75}A-I fraction. Some studies have evidenced the importance of storage xyloglucans, isolated from the seeds of vegetable species from the *Fabaceae* family, as macrophages activators (Rosário *et al*., 2008, 2011). Thus, since *P. tridentatum* belongs to the same family, and also as the inflorescences used for the preparation of the decoctions contain the seeds, the possible contribution of xyloglucans for the macrophage immunostimulatory activity registered for Et_{75}A fraction should be considered. Also, it seems possible that these xyloglucans need to be present in higher concentrations for the expression of their macrophage immunostimulatory activity.

The macrophage stimulation with 50, and 100 μg/mL of Et_{75}A-III, caused an immunostimulatory activity that was comparable to the observed for the non-stimulated macrophages, and that was negligible to the registered for macrophages stimulated with the same concentrations of Et_{75}A fraction. When 200 μg/mL of Et_{75}A-III were used, an immunostimulatory activity that was approximately 50% lower than the registered for the same concentration of Et_{75}A fraction was observed. The Et_{75}A-III fraction, which is the low molecular weight fraction resulting from the *endo*-polygalacturonase treatment of the Et_{75}A fraction, was mostly composed of oligogalacturonides that resulted from the enzymes action. This suggest that the presence of these oligogalacturonides in adequate concentrations, should have contributed for the registered activity. Thus, it seems

possible that the homogalacturonan moiety of pectic polysaccharides could have contributed for the immunostimulatory activity of $\underline{Et_{75}A}$ fraction.

7.6. Concluding remarks

The results obtained evidenced the immunostimulatory activity of the $\underline{Et_{50}C}$, $\underline{Et_{50}B}$, and $\underline{Et_{75}A}$ fractions isolated from the decoctions of *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences, respectively, without compromising the macrophage cellular viability. It was also showed that the observed immunostimulatory activity was not related to some bacterial contamination, but attributed to the presence of bioactive polysaccharides. The immunostimulattory activity exherted by the rich-polysaccharide fractions seemed to be related to the presence of AG-I, and AG-II in all the fractions assayed, and also to the presence of acetylated mannans for the $\underline{Et_{75}A}$ fraction from *P. tridentatum*. Also, the presence of xyloglucans, and homogalacturonan domains in adequate concentrations seemed to have contributed to the macrophage immunostimulatory activity of the fraction from *P. tridentatum*.

CHAPTER 8

CONCLUSIONS

The infusions of *Fraxinus angustifolia* dried leaves prepared according to the popular tradition, and using distinct proportions of vegetable material to water volume, yielded approximately between 0.45 and 12.9 mg of water soluble material per mL of infusion, correspondent to approximately 45 to 97 mg of water soluble material per g of dried leaves. The water soluble material comprised approximately 85% of hydrophilic material, which contained approximately 35% of carbohydrate material, and 1.5% of total phenolic compounds. It was also detected between 11.5 and 12.3% of fructose in the hydrophilic material, suggesting the possible presence of sucrose, although it cannot be excluded the presence of fructooligosaccharides (FOS). The remaining 15% of the water soluble material was composed by material with hydrophobic characteristics, which contained proportions of carbohydrate material similar to those of the hydrophilic material, mainly comprised of glucosyl residues, and a proportion of total phenolic compounds approximately ten times higher than the detected in the hydrophilic material. The high proportions of glucosyl residues, together with the also high proportions of total phenolic compounds, suggest the presence of glycosides in the water soluble material that showed hydrophobic characteristics. DPPH scavenging activity of the hydrophilic and hydrophobic materials was related with the presence of total phenolic compounds, with the hydrophobic material exhibiting an activity that was approximately ten times higher than the observed for the hydrophilic material.

It was also observed that the water soluble material of the *F. angustifolia* infusions, only contained between 2 and 4% of HMWM. The HMWM contained approximately 30% of carbohydrate material, with a monomeric composition rich in uronic acid residues, which suggested the presence of pectic polysaccharides. Also, through linkage analysis, it was possible to detect sugar residues diagnostic for the presence of AG-II, mannans, and also xyloglucans, all present in lower proportions. The HMWM also contained approximately 8% of total phenolic compounds, which was a higher content than the one of the hydrophilic material, and lower than the observed for the hydrophobic material. The HMWM showed a DPPH scavenging activity approximately three times higher than the one

registered for the hydrophilic material, and three times lower than the one of the hydrophobic material.

The fractionation of the HMWM through the use of anion exchange chromatography alone, and by the sequential use of solid phase extraction, followed by anion exchange chromatography, and ethanol precipitation, followed by solid phase extraction and further anion exchange chromatography, allowed to obtain neutral fractions that contained approximately twice more carbohydrate material than the starting one. For the solid phase extraction and ethanol precipitation, mass recoveries higher than 70%, and of approximately 90%, respectively, were observed. For the anion exchange chromatography technique, the highest mass recovery value registered was approximately 50%. This evidenced that the infusions of *F. angustifolia*, which were prepared by contacting the dried leaves during five minutes with previously boiled water, contained high proportions of HMWM that strongly interacted with the DEAE-Sepharose FF gel.

The use of extensive extraction with water at 50ºC, and with boiling water, allowed to increase approximately eight, and twenty times, the *F. angustifolia* HMWM yield, in comparison with the infusions prepared according to the popular tradition. For *M. suaveolens*, four- and eight-fold increases in the HMWM yields were registered, when extensive extractions with water at 50ºC, and with boiling water were performed, respectively. Extensive extractions using water at 50ºC, and boiling water permitted to achieve HMWM yields that were two and seven times higher, respectively, than the registered for the infusions of *P. tridentatum* prepared according to the popular tradition. These results evidenced that the use of extensive hot water extractions permitted to achieve higher HMWM yields, particularly in the case of *F. angustifolia* dried leaves.

The use of an extensive extraction with water at 50ºC, only allowed to obtain HMWM with proportions of carbohydrates that were higher than the HMWM from the infusions prepared according to the popular tradition, for the *M. suaveolens* dried shoots. The use of extensive extractions with boiling water, permitted to achieve HMWM with higher proportions of carbohydrate material for *F. angustifolia* dried leaves, *M. suaveolens* dried shots, and also *P. tridentatum* dried inflorescences. The increase in the HMWM carbohydrate content was mainly

attributed to the presence of higher proportions of uronic acid residues in the extracts prepared with boiling water, while the remaining monosaccharide residues continued to be present in proportions similar to the observed for the infusions prepared according to the popular tradition.

The fractionation by ethanol precipitation of the HWMW obtained through the extensive extraction with boiling water of *F. angustifolia* dried leaves, and *M. suaveolens* dried shots, yielded mostly material that precipitated in 50% ethanol aqueous solutions. For *P. tridentatum* this trend was less evident, and inclusively, for the 2nd 2h of the hot water extraction, only approximately 20% of the recovered HMWM precipitated in 50% ethanol aqueous solutions, while approximately 25% of the material precipitated in 75% ethanol aqueous solutions, and 35% remained soluble. The material that precipitated in 50 and 75% ethanol aqueous solutions was rich in polysaccharides that contained high proportions of uronic acid residues. The polysaccharides that precipitated in 50% ethanol aqueous solutions contained higher proportions of uronic acid residues than those that precipitated in75%, evidencing that the fractionation by ethanol precipitation was based on the uronic acid content of the polysaccharides.

When fractionated by anion exchange chromatography, most of the *F. angustifolia*, and *M. suaveolens* polysaccharides that precipitated in 50% ethanol aqueous solutions, were recovered in the acidic fractions, while the polysaccharides that precipitated in 75% were more equitatively recovered in the neutral and acidic fractions. For *P. tridentatum,* most of the polysaccharides that precipitated in 50 and 75% ethanol aqueous solutions were mainly recovered in the neutral fractions. This allows to conclude that the decoctions from *P. tridentatum* dried inflorescences contained higher proportions of neutral polysaccharides, in comparison with those from *F. angustifolia* and *M. suaveolens.* It was observed that the polysaccharides recovered in the neutral fractions presented a higher proportion of methylesterified uronic acid residues, in comparison with those recovered in the acidic fractions, suggesting that the anion exchange chromatography fractionation was achieved based on the methyl esterification degree of the uronic acid residues

The fractionation of the HMWM by the use of ethanol precipitation followed by anion exchange chromatography on DEAE-Sepharose FF, allowed to separate the polysaccharides present in the decoctions from *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences according to their uronic acid content, and proportion of methylesterified uronic acid residues. The use of this fractionation procedure also permitted to obtain fractions that were richer in carbohydrate material than the correspondent starting HMWM.

The methylation of the HMWM from the *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences decoctions evidenced the presence of high proportions of pectic polysaccharides that were present in the various fractions resulting from the ethanol precipitation, and subsequent anion exchange chromatography. Also, the presence of lower proportions of type AG-I, AG-II, mannans, and xyloglucans, was detected.

The mass of uronic acid residues, and the mass of the main polysaccharides present in the 1st 2h of the various decoctions was estimated, and is shown in **Table 8.1**. Also, the structural features of the main polysaccharides, previously referred in **Sections 4.1.3**, **5.1.3**, and **6.1.3**, were compiled, and are also shown in **Table 8.1**. The mass of each polysaccharide was estimated based on the molar fractions of the main glycosidic residues present in the different polysaccharides. The (1→4)-Gal*p* residues were considered for the calculation of the AG-I estimated mass, while for AG-II, T-Gal*p*, (1→3)-, (1→6)-, and (1→3,6)-Gal*p* residues, together with T-Ara*f*, (1→5)-, and (1→3,5)-Ara*f* residues were considered. The mannans mass was estimated based on (1→4)-, (1→4,6)-Man*p*, and T-Gal*p* residues, and the xyloglucans mass was calculated based on the T-Xyl*p*, together with (1→4)-, (1→4,6)-Glc*p*, and also (1→2)-Gal*p*, and (1→2)-Xyl*p* residues. The mass yield, and carbohydrate content of the main fractions obtained through anion exchange chromatography, combined with the mass yield of the main fractions from the ethanol fractionation procedure, and the HMWM mass yield for the 1st 2h of the various decoctions were also used for the calculation.

The results evidence that the decoctions of *P. tridentatum* dried inflorescences contained approximately twice more polysaccharides than those from *F. angustifolia* dried leaves, and *M. suaveolens* dried shoots, which exhibited similar

estimated amounts of polysaccharides. When compared with the decoction from *F. angustifolia* dried leaves, and *M. suaveolens* dried shoots, the decoctions from *P.tridentatum* dried inflorescences contained higher amounts of all the main polysaccharides. Besides galacturonans, the mannans, and xyloglucans were the polysaccharides that mostly contributed to the mass differences registered. It is possible that the mannans, and xyloglucans detected in the decoctions of *P. tridentatum* was extracted from the seeds of the inflorescences, where they performed storage functions.

The AG-I from the decoctions of *F. angustifolia*, and *M. suaveolens* were detected in the acidic fractions, while for *P. tridentatum*, the AG-I were mostly detected in the neutral fractions. The AG-II from the decoctions of the various vegetable materials were detected in both neutral and acidic fractions, while the mannans, and xyloglucans were mostly detected in the neutral fractions.

The decoctions of *F. angustifolia* dried leaves, obtained during the 1st 2h, contained AG-II with a substitution degree of the galactan backbone, expressed by the (1→3,6)-Gal*p*/(1→3)-Gal*p* value, comprised between 1.2 and 5.3, as shown in **Table 8.1**. For the 1st 2h of the hot water extraction, the AG-II of *M. suaveolens* showed a substitution degree of the galactan backbone that ranged between 1.3 and 4.4, while for *P. tridentatum* the values were comprised between 1.6 and 4.0. The AG-II isolated from the decoctions of *F. angustifolia* dried leaves, which exhibited the most and the less substituted galactan backbone, presented the lowest and the highest value of (1→5)-Ara*f*/T-Ara*f* (0.33, and 3.9, respectively). This suggests that the decoctions from *F. angustifolia* contain AG-II with the wider range of galactan backbone substitution, and also extension of the (1→5)-linked arabinosyl side chains. Relatively to the substitution degree of the (1→5)-linked arabinosyl side chains, it was observed that the AG-II detected in the decoctions of *F. angustifolia* contained more substituted arabinosyl side chains than those detected in the extracts of *P. tridentatum*, and less substituted than those of *M. suaveolens* extracts.

The decoctions from *P. tridentatum* dried inflorescences contained mannans in higher proportions than the hot water extracts from the other plants. The majority of the mannans from *P. tridentatum* precipitated in 75% ethanol aqueous

solutions, and were mostly recovered in the neutral fraction from the HMWM extracted during the 1st 2h, exhibiting a (1→4,6)-/(1→4)-Man*p* value of 0.34. Through the use of ESI-MS and ESI-MS/MS experiments, it was possible to show that these mannans, which could have been extracted from the seeds contained in the *P. tridentatum* inflorescences, are more acetylated than other mannans, such as the galactomannans extracted from the coffee infusion, locust bean gum, and also from some non-conventional sources, and are less acetylated than the galactomannans from *Aloe vera*. The acetylation was determined to occur at the *O*-2 of the hexose residues. It was also possible to evidence the presence of oligosaccharides comprising hexose residues linked to a pentose residue, similarly to what was observed in the mannans from *Aloe vera*, coffee infusion, locust bean gum and spent coffee ground. In these mannans, the pentose residue was identified as arabinose, which was also detected in the fractions that contained the *P. tridentatum* mannans.

Although present in lower proportion, the HMWM extracted during the 2nd 2h, also contained mannans that precipitated in 75% ethanol aqueous solutions, and that were recovered in the neutral fraction. These mannans exhibited a substitution degree of 0.33, similar to the observed for the mannans extracted during the 1st 2h. Although present in lower proportions than the registered for the extracts of *P. tridentatum*, the decoctions of *F. angustifolia*, and *M. suaveolens* also contained mannans, that were mostly recovered in the neutral fractions of the HMWM that precipitated in 50 and 75% ethanol aqueous solutions, and that presented (1→4,6)-/(1→4)-Man*p* values that ranged between 0.10 and 0.25. This suggests the presence of more branched mannans in the *P. tridentatum* decoctions.

Table 8.1- Content, and structural features of the polysaccharides present in the major fractions isolated from the decoctions of *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *Pterospartum tridentatum* dried inflorescences.

	Estimated Total Content (mg/100 mL)	Uronic Acid Content (mg/100 mL)	AG-I Content (mg/100 mL)	AG-II				Mannans		Xyloglucans					
				Content (mg/100 mL)	GSD[a]	AE[b]	ASD[c]	Content (mg/100 mL)	MSD[d]	Content (mg/100 mL)	XSD[e]	X[f]	L	S	F
***Fraxinus angustifolia* decoctions (1st 2h)**															
$Et_{50}A$	43.0	7.9	--	0.55	2.4	1.2	0.28	0.16	0.10	0.05	0.71	√	√	√	√
$Et_{50}C$		23.0	0.27	1.23	1.2	3.9	0.27	--	nd	0.06	0.33	√	?	?	?
$Et_{75}A$		5.6	--	1.21	5.3	0.33	0.12	0.32	0.25	0.04	0.55	√	√	√	√
$Et_{75}C$		1.8	0.02	0.71	1.6	1.6	0.13	--	nd	0.01	0.18	√	--	--	--
		38.30	**0.29**	**3.70**				**0.49**		**0.16**					
***Mentha suaveolens* decoctions (1st 2h)**															
$Et_{50}A$	46.2	4.6	--	0.33	4.4	0.51	0.41	0.14	0.20	0.25	0.23	√	√	√	--
$Et_{50}B$		19.7	0.06	0.54	4.2	0.85	0.52	--	nd	0.05	1.0	√	--	--	--
$Et_{50}C$		5.1	0.04	0.27	1.3	1.1	0.55	--	nd	0.02	0.85	√	--	--	--
$Et_{75}A$		3.9	--	0.57	3.3	0.62	0.33	0.50	0.25	0.40	0.20	√	√	√	--
$Et_{75}B$		6.8	0.04	0.56	3.5	0.89	0.51	--	nd	0.07	0.26	√	--	--	--
$Et_{75}C$		2.0	0.03	0.20	1.8	1.0	0.58	--	nd	0.04	0.19	√	--	--	--
		42.10	**0.17**	**2.46**				**0.64**		**0.83**					
***Pterospartum tridentatum* decoctions (1st 2h)**															
$Et_{50}A$	91.2	24.9	0.73	2.0	1.6	1.6	0.08	1.7	0.36	2.2	1.5	√	√	√	√
$Et_{50}B$		19.2	0.21	0.80	3.6	1.0	0.13	0.01	nd	0.15	nd	√	?	?	--
$Et_{75}A$		16.5	1.1	1.70	2.0	1.1	0.13	10.0	0.34	5.1	0.19	√	√	√	√
$Et_{75}B$		3.9	--	1.05	4.0	1.7	0.10	0.05	nd	0.09	0.15	√	--	--	--
		64.50	**2.04**	**5.55**				**11.85**		**7.44**					

[a,d,e]- galactan, mannan, and xyloglucan backbone substitution degree; [b]- arabinosyl side chains extension; [c]- arabinosyl side chains substitution degree; [f]- X, L, S, and F, denote the presence of single xylosyl, Gal*p*-(β1→2)-Xyl*p*-(α1→, and Ara*p*-(α1→2)-Xyl*p*-(α1→ disaccharides, and Fuc*p*-(α1→2)-Gal*p*-(β1→2)-Xyl*p*-(α1→ trisaccharide, respectively, as possible xyloglucan side chains; ? (1→4,6)-Glc*p* does not exceed T-Xyl*p*, however diagnostic residues were detected.

The decoctions of *P. tridentatum* dried inflorescences also contained higher proportions of xyloglucans than the decoctions of *F. angustifolia* dried leaves, and *M. suaveolens* dried shoots. Most of the xyloglucans detected in the decoctions from *P. tridentatum* dried inflorescences were recovered in the neutral fractions, particularly in those obtained from the HMWM that precipitated in 75% ethanol aqueous solutions, similarly to what was observed for the decoctions of *M. suaveolens* dried shoots. Most of the xyloglucans detected in the decoctions from *P. tridentatum*, which were recovered in the $\underline{Et_{75}A}$ fraction, exhibited a substitution degree of 0.19 that was similarly to the substitution degree of the xyloglucans detected in the $\underline{Et_{50}A}$ and $\underline{Et_{75}A}$ fractions isolated from *M. suaveolens* decoctions. The xyloglucans detected in the $\underline{Et_{50}A}$ fraction from *P. tridentatum* decoctions presented a substitution degree value of 1.5, suggesting the presence of highly substituted polymers. This contrasts with the xyloglucans detected in minor proportions in the decoctions from *F. angustifolia* dried leaves which presented substitution degree values comprised between 0.18 and 0.71.

For all the decoctions, it was also possible to observe that the AG-II extracted during the 2nd 2h, presented structural features that only differed from those of the AG-II of the 1st 2h, in the galactan backbone substitution degree. The AG-II extracted during the 2nd 2h, exhibited a more branched galactan backbone than the AG-II present in the extracts from the 1st 2h. The mannans and xyloglucans extracted during the 1st and 2nd 2h showed similar substitution degree.

The *endo*-polygalacturonase treatment combined with the size exclusion fractionation of the digestion products permitted to confirm the AG-II, mannan, and xyloglucan structural features that had been previously determined for the polysaccharides present in the untreated fractions. It was also evidenced that the AG-II, mannans, and xyloglucans detected in the higher molecular weight fraction that resulted from the size exclusion of the *endo*-polygalacturonase treatment, had an estimated molecular weight higher than 40 kDa. It was also suggested the presence of AG-II, mannan, and xyloglucan with an estimated molecular weight lower than 40 kDa.

The effect of the *F. angustifolia* $\underline{Et_{50}C}$, *M. suaveolens* $\underline{Et_{50}B}$, and *P. tridentatum* $\underline{Et_{75}A}$ fractions on the cellular viability of macrophages was tested, and the results

allow to conclude that, for concentrations comprised between 5 and 200 μg/mL, no reduction on cell viability was registered. It was also observed that for the same range of concentrations, these fractions exhibited an immunostimulatory effect, expressed by the macrophage NO production, while no reduction on the NO production by LPS-stimulated macrophages was observed, evidencing the pro-inflammatory activity, and absence of anti-inflammatory activity of the assayed fractions. The polysaccharides from fractions $\underline{Et_{50}C}$ and $\underline{Et_{50}B}$ contained high proportions of uronic acid (86.4 and 94.0%, respectively), and also exhibited residues that permitted to infer the presence of AG-II, which are often found in fractions containing pectic polysaccharides, and are frequently considered to be the main contributors for the immunostimulatory effects reported. Thus, it is possible to conclude that the AG-II structures detected in the $\underline{Et_{50}C}$ and $\underline{Et_{50}B}$ fractions contributed for the immunostimulatory effect observed. It was possible to detect glycosidic residues that confirmed the presence of AG-I structures, particularly in the $\underline{Et_{50}C}$ fraction. The presence of AG-I in fractions of bioactive pectic polysaccharides has also been reported, although less frequently than the observed for AG-II structures. Thus, the contribution of the AG-I structures for the macrophage immunostimulatory activity exhibited by the $\underline{Et_{50}C}$ and $\underline{Et_{50}B}$ fractions should be considered.

The $\underline{Et_{75}A}$ fraction from *P. tridentatum* contained high proportions of pectic polysaccharides, comprising AG-I I, AG-II, mannans, and also xyloglucans. The treatment of the $\underline{Et_{75}A}$ fraction with NaOH solution, led to a decrease of approximately 50% in the macrophage immunostimulatory activity. This permitted to conclude that the acetylation of the polysaccharides present in the $\underline{Et_{75}A}$ fraction, and particularly of the mannans, should have contributed for the observed macrophage immunostimulatory activity. The remaining macrophage immunostimulatory activity, should be attributed to the presence of the pectic polysaccharides, with its AG-I side chains, and also to the presence of AG-II. The contribution of these polysaccharides was demonstrated by the residual activity of the $\underline{Et_{75}A}$ submitted to a treatment with NaOH solution. The results from the *endo*-polygalacturonase treatment of $\underline{Et_{75}A}$ fraction, also suggested the possible contribution of storage xyloglucans, and homogalacturonan moiety of pectic

polysaccharides, when present at adequate concentrations, for the observed immunostimulatory activity.

The results obtained during this work allow us to conclude that the extensive hot water extracts of *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences contained a mixture of various polysaccharides composed of high proportions of pectic polysaccharides, which are easily extracted by hot water, and also lower proportions of AG-II, mannans, and xyloglucans. The decoctions of *P. tridentatum* dried inflorescences proved to be particularly rich in mannans. Also, it is possible to conclude that these hot water extracts contain bioactive polysaccharides, which proved to possess macrophage immunostimulatory activity. Since the HMWM from the decoctions of *F. angustifolia* dried leaves contained the same type of polysaccharides as the HMWM from the infusions prepared according to the popular tradition, it seems plausible to conclude that the same happened to the other plants used in this study. Thus, it seems possible that the infusions of *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences also contained macrophage immunostimulatory polysaccharides that may contribute for the therapeutic properties that are frequently associated to the ingestion of these infusions by the popular tradition.

FUTURE PERSPECTIVES

The preparation of decoctions from *F. angustifolia* dried leaves, *M. suaveolens* dried shoots, and *P. tridentatum* dried inflorescences allowed to obtain higher amounts of carbohydrate material, in comparison with the obtained with traditional infusions. Despite the higher proportion of uronic acid residues detected in the decoctions, this carbohydrate material contained the same type of polysaccharides that seemed to be present in the traditional infusions. Therefore, the same methodology could be used in order to obtain enough carbohydrate material for future experiments. The glycosidic linkage analysis, and the biological activity assays evidenced the presence of a mixture of polysaccharides that exhibited macrophage immunostimulatory activity, expressed by an increase in the NO

production. Thus, it should be interesting to have a better understanding about the contribution of each type of polysaccharide to the observed immunostimulatory activity. This could be done performing selective enzymatic treatments on selected fractions isolated from these plants hot water extracts, followed by diverse biological activity assays, and re-evaluation of the structural features of the purified polysaccharides.

As the infusions contain lower amounts of the polysaccharides detected in the decoctions, the possible effects frequently attributed to their ingestion should be related to cumulative effects, which could be evaluated through animal, and human clinical trials. Also, the development of a functional food containing an adequate mixture of these polysaccharides could be considered.

CHAPTER 9

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