UID/SOC/04011/2013
UID/AGR/04033/2013

ESTRUTURA E ATIVIDADE IMUNOESTIMULADORA DOS POLISSACARÍDEOS PRESENTES EM EXTRATOS AQUOSOS DE INFLORESCÊNCIAS SECAS DE P. TRIDENTATUM

STRUCTURE AND IMMUNOSTIMULATORY ACTIVITY OF POLYSACCHARIDES FROM THE AQUEOUS EXTRACTS OF P. TRIDENTATUM DRIED INFLORESCENCES

V.M.R. Martins
CIMO, Escola Superior Agrária de Bragança, Bragança, Portugal.
QOPNA, Departamento de Química, Universidade de Aveiro, Aveiro, Portugal.
E-mail: vmartins@ipb.pt

M.T. CRUZ
Centro de Neurociências e Biologia Celular de Coimbra, Coimbra, Portugal.
Faculdade de Farmácia, Universidade de Coimbra, Coimbra, Portugal.

I. FERREIRA
Centro de Neurociências e Biologia Celular de Coimbra, Coimbra, Portugal.
Faculdade de Farmácia, Universidade de Coimbra, Coimbra, Portugal.

M.A. COIMBRA
QOPNA, Departamento de Química, Universidade de Aveiro, Aveiro, Portugal.

RESUMO

As inflorescências secas da carqueja (*P. tridentatum* L.) são utilizadas na preparação de extratos aquosos que, de acordo com a tradição popular, possuem propriedades terapêuticas. Durante a preparação dos extratos ocorre a extração de compostos bioativos, tais como polissacarídeos. O presente trabalho pretende determinar o tipo de polissacarídeos presente nos extratos e avaliar o seu possível contributo para as respetivas propriedades terapêuticas. Procedeu- se à extração, caracterização estrutural e avaliação da atividade imunoestimuladora dos polissacarídeos presentes nos extratos. Foi detetada uma mistura de polissacarídeos, nomeadamente polissacarídeos pécticos, arabinogalactanas do Tipo-I e II, galactomananas e xiloglucanas, que apresenta atividade imunoestimuladora em macrófagos e que poderá contribuir para as propriedades terapêuticas atribuídas pela medicina popular a estes extratos.

ABSTRACT

The dried inflorescences of *Pterospartum tridentatum* L. are used in the preparation of water extracts that, according to the popular tradition, possess therapeutic properties. Bioactive compounds, such as polysaccharides, are extracted during the preparation of these extracts. This work intends to disclose the polysaccharides present in the extracts and also to assess their possible contribute to the therapeutic properties attributed to these extracts. The polysaccharides were extracted, structurally characterized and their immunostimulatory activity was evaluated. A mixture of polysaccharides composed of pectic polysaccharides, Type-I and Type-II arabinogalactans, galactomannans and xyloglucans was detected. This mixture exhibited a macrophage immunostimulatory activity that might contribute to the therapeutic properties that are attributed by the traditional medicine to these extracts.

1. Introduction

Pterospartum tridentatum (L.) Willk. (*Fabales* order) is a broom like Iberian shrub of the *Fabaceae* family that is very common in the Portuguese northeastern region of Trás-os-Montes, where is known as "carqueja". According to the popular tradition, the inflorescences of *P. tridentatum* inflorescences, picked during early spring and subsequently dried for the preparation of hot water extracts, provide protection against various health disorders, such as type 2 diabetes, influenza and hypertension (Carvalho, 2010; Neves *et al.*, 2009; Vitor *et al.*, 2004).

Biologically active compounds, such as polysaccharides, are extracted during the preparation of hot water extracts and can therefore contribute to the therapeutic properties attributed by the traditional medicine to the *P. tridentatum* dried inflorescences extracts. Several studies have highlighted the importance of polysaccharides as immunomodulators (Tzianabos, 2000; Paulsen and Barsett, 2005; Schepetkin and Quinn, 2006; Ramberg *et al.*, 2010; Ferreira *et al.*, 2015), however the polysaccharide components of the hot water extracts from *P. tridentatum* dried inflorescences remain unknown. Therefore, this study will evaluate the type of polysaccharides present in these hot water extracts and also assess their immunostimulatory activity and possible contribution to the therapeutic properties reported by the traditional medicine.

2. Materials and methods

2.1. Plant material, preparation of the hot water extracts (HWE) and high molecular weight material (HMWM)

The inflorescences of *P. tridentatum* were collected in early spring in the scrubland near Bragança, North-eastern Portugal. Subsequently, the collected inflorescences were dried in a dark place at room temperature, simulating general conditions of traditional folk use. A voucher specimen was deposited in the Escola Superior Agrária de Bragança Herbarium (BRESA). The hot water extracts (HWE) were prepared by the decoction with boiling water of the *P. tridentatum* dried inflorescences (0.03 g/mL), during a total of 2 h. Subsequently, the resulting 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).

2.2. 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 completely remove the ethanol, each precipitate was dissolved in distilled water, rota-evaporated and freeze-dried.

2.3. 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). The fractions of interest were pooled, dialysed and freeze-dried.

2.4. Sugar and linkage analysis

Neutral sugars were determined by gas chromatography after acid hydrolysis release and conversion to the corresponding alditol acetates, as described by Nunes and Coimbra (2002). Hexuronic acids were determined colorimetrically by a modification of the Blumenkrantz and Asboe-Hansen method (Blumenkrantz and Asboe-Hansen, 1973). The linkage analysis was performed by methylation of the polysaccharides in order to obtain partially methylated alditol acetates that were subsequently analyzed by gas chromatography-mass spectrometry (GC-MS), as described by Nunes and Coimbra (2001). Previously methylated samples were carboxyl reduced by a modification of the Lindberg and Lönngren method, as described by Coimbra *et al.* (1996).

2.5. Macrophage immunostimulatory activity

The macrophage immunostimulatory activity, expressed as nitric oxide production, was evaluated through the assessment of the nitrite accumulation in the Raw 264.7 macrophage cell line 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% (v/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.

2.6. 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 described by Mosmann (1983).

2.7. Statistical analysis

The macrophage immunostimulatory activity results 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. All statistical tests were applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA), with a significance level of $p < 0.05$.

3. Results and discussion

The hot water extracts of *P. tridentatum* dried inflorescences were dialysed in order to remove the low molecular weight compounds, therefore obtaining the high molecular weight material (HMWM), which contains the polysaccharides. The polysaccharides were subsequently fractionated according to their solubility in aqueous ethanol solutions. The mass yield, total sugars content and monomeric composition of each fraction was determined and is presented in **Table I.**

Table I- Mass yield, total sugar content and monosaccharide composition of the various fractions obtained from the ethanol precipitation of the HMWM and anion exchange chromatography of the Et_{75} fraction from the HWE of *P. tridentatum* dried inflorescences.

	Yield[a]	Total Sugar	Monosaccharide Composition (mol %)							
	(mass%)	(mass%)	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
HMWM		42.0	0.9	0.0	4.7	1.3	7.2	7.5	32.7	46.0
WI_{ppt}	16.0	26.8	2.4	0.0	2.3	0.8	1.3	4.9	73.4	15.0
Et_{50}	31.9	91.5	0.5	0.3	3.2	1.1	1.7	6.2	6.5	80.5
Et_{75}	26.5	58.2	1.0	0.5	4.4	2.4	23.6	13.6	24.2	30.3
A	73.3	85.3	0.0	0.0	3.0	2.6	26.1	11.8	15.1	41.3
B	14.5	62.1	1.9	0.2	11.1	0.6	1.4	14.6	2.3	67.7
C	5.4	31.5	3.5	0.1	13.0	1.0	2.8	15.6	11.9	52.1
D	6.8	27.7	2.3	0.0	10.0	1.3	4.5	15.3	30	36.6
SN	25.5	24.8	0.9	0.5	8.8	1.3	4.0	4.3	65.6	14.6

[a] - expressed relatively to HMWM recovered in the fractionation procedure.

The Et_{50} and Et_{75} fractions were the richest in carbohydrate material (91.5 and 58.2%, respectively) and 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 the poorer in carbohydrate material, each one comprising approximately 25% of sugar material. These results show that 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.

The polysaccharides recovered in the Et_{50} and Et_{75} fractions exhibited a monomeric composition rich in UA, suggesting the presence of pectic polysaccharides in both fractions. The proportion of UA residues detected in the Et_{50} fraction was higher than the detected in the Et_{75} fraction, suggesting that the polysaccharides that precipitated in 50% ethanol aqueous solutions were more charged than those that precipitated in 75% ethanol aqueous solutions. It is also possible that the polysaccharides that precipitated in 75% ethanol aqueous solutions might exhibit lower molecular weight, 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 hot water extracts. Particularly for the Et_{75} fraction, it was also possible to detect significant proportions of neutral sugar residues, such as mannose (23.6%), glucose (24.2%) and galactose (13.6%), suggesting that besides the pectic polysaccharides, significant amounts of other types of polysaccharides might have precipitated in 75% ethanol aqueous solutions.

In order to try to separate the polysaccharides that seemed to be present in the Et_{75} fraction based on their charge, an additional fractionation by anion exchange chromatography on DEAE-Sepharose FF was performed. The fractionation procedure yielded one not retained neutral fraction (A), eluted with buffer, and three retained acidic fractions (B, C and D), eluted with buffer containing 0.125, 0.250, and 1.0 M NaCl, respectively. The mass yield, total sugars content and monomeric composition of each fraction was determined and is presented in **Table I**. The neutral Et_{75}A fraction was the most abundant, comprising 73.3% of the HMWM recovered, and the richest in carbohydrate material,

containing 85.3% of carbohydrate material. The carbohydrate material recovered in the $Et_{75}A$ fraction contained 41.3% of UA residues together with lower proportions of Man (26.1%), Glc (15.1%) and Gal (11.8%) residues. The less abundant $Et_{75}B$, $Et_{75}C$ and $Et_{75}D$ acidic fractions, which comprised 14.5, 5.4 and 6.8% of the HMWM recovered, respectively, also exhibited carbohydrate material contents lower than the neutral $Et_{75}A$ fraction: 62.1% for the $Et_{75}B$ fraction, 31.5% for the $Et_{75}C$ fraction and 27.7% for the $Et_{75}D$ fraction. The carbohydrate material recovered in the acidic $Et_{75}B$, $Et_{75}C$ and $Et_{75}D$ fractions exhibited a similar monomeric composition, comprising significant proportions of UA residues mixed with lower proportions of Ara and Gal residues.

The previous results evidenced that most of the polysaccharides that precipitated in 75% ethanol aqueous solutions were recovered in the neutral fraction. Moreover, considering that the $Et_{75}A$ fraction was the most abundant and also the richest in carbohydrate material, it can be inferred that the majority of the Gal, Glc, Man and UA residues, which were the most abundant in the Et_{75} fraction, were recovered in the neutral fraction. Therefore, in order to determine what type of carbohydrate material, besides the pectic polysaccharides, were precipitated in the 75% ethanol aqueous solutions, the material recovered in the $Et_{75}A$ fraction was methylated and the results for the neutral sugars are shown in **Table II**.

Table II- Deduced linkages from the methylation analysis of the neutral $Et_{75}A$ fraction obtained from the HWE of *P. tridentatum* dried inflorescences.

Deduced Linkage	Molar %
1,2,4-Rha*p*	0.2
Total	***0.2[a](0.0)[b]***
T-Fuc*p*	0.2
Total	***0.2(0.0)***
T-Ara*f*	1.5
1,2-Ara*f*	0.3
1,3-Ara*f*	0.5
1,5-Ara*f*	1.6
1,3,5-Ara*f*	0.2
Total	***4.1(5.1)***
T-Xyl*p*	2.4
1,2-Xyl*p*	1.0
1,4-Xyl*p*	1.1
1,2,4-Xylp	0.5
Total	***5.0(4.6)***
T-Man*p*	1.5
1,4-Man*p*	27.7
1,2,4-Man*p*	1.9
1,3,4-Man*p*	3.5
1,4,6-Man*p*	9.5
Total	***44.1(44.5)***
T-Gal*p*	9.5
1,2-Gal*p*	0.2
1,3-Gal*p*	0.7
1,4-Gal*p*	5.0
1,6-Gal*p*	2.9
1,3,6-Gal*p*	1.4
Total	***19.7(20.1)***
T-Glc*p*	1.3
1,3-Glc*p*	0.4
1,4-Glc*p*	18.1
1,6-Glc*p*	0.3
1,2,4-Glc*p*	1.8
1,3,4-Glc*p*	0.6
1,4,6-Glc*p*	3.4
Total	***25.9(25.7)***

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

Galactosyl residues in various linkages, namely (1→3)-, and (1→3,6)-Gal*p*, were detected in the $Et_{75}A$ fraction, evidencing the presence of type II arabinogalactans (AG-II). Terminal-, and (1→6)-

Gal*p* residues, frequently reported as AG-II components were also detected. The detection of arabinosyl residues, preferentially present as terminally- and (1→5)-linked, all in furanosidic form, also suggests the presence of AG-II (Fischer *et al.*, 2001). Other residues, that also have been reported in fractions containing AG-II, were also detected, reinforcing the presence of these structures, namely (1→2)-, (1→3)-, and (1→3,5)-Ara*f* (Nergard *et al.*, 2005; Togola *et al.*, 2008; Grønhaug *et al.*, 2010). The detection of (1→4)-Gal*p* residues suggests the presence of type I arabinogalactan (AG-I) structures, which have been reported in the water extracts of diverse plants with therapeutic properties (Samuelsen *et al.*, 1996; Inngjerdingen *et al.*, 2007, 2008). The AG-I structures are usually reported as pectic polysaccharides side chains, while AG-II may occur as side chains of pectic polysaccharides or in a complex family of proteoglycans known as arabinogalactan-proteins (Ferreira *et al.*, 2015).

The detection of relevant proportions of (1→4)-Glc*p*, together with the non-detection of starch by the iodine assay, suggests that at least some of the (1→4)-Glc*p* residues that were detected are present as xyloglucan components. The presence of these polysaccharides has been reported in the water extracts of various *Fabaceae* vegetable species (Rosário *et al.*, 2011). Also, the presence of mannosyl residues, mainly detected as (1→4)-Man*p* and (1→4,6)-Man*p* residues, evidences the presence of mannans. The detection of similar proportions of (1→4,6)-Man*p* and T-Gal*p* residues is compatible with the presence of galactomannans. Galactomannans are polysaccharides with a backbone composed by (1→4)-Man*p* residues that are substituted at *O*-6 by single Gal*p* residues (Nunes and Coimbra, 2001). The galactomannans exhibited a substitution degree, which can be evaluated through the value of the (1→4,6)-/(1→4)-Man*p* ratio, of 0.34. This value is 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). Mannans with lower substitution degree values have been 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 sugar and linkage analysis of $Et_{75}A$ fraction allowed to detect the presence of pectic polysaccharides mixed with AG-I and AG-II structures, galactomannans and xyloglucans in the HWE of *P. tridentatum* dried inflorescences. The effect of the fraction $Et_{75}A$ isolated from the HWE of *P. tridentatum* dried inflorescences on RAW 264.7 macrophage cellular viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the obtained results are shown in **Figure 1**. The results showed that for fraction concentrations comprised between 5 and 200 μg/mL, the macrophage cellular viability was similar and always higher than 75%, as happened for the control and LPS-induced macrophages. This evidences that over the entire concentration range tested, the $Et_{75}A$ fraction had no cytotoxic effects on the macrophages.

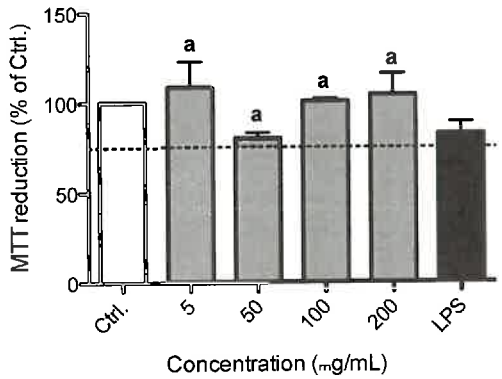

Figure 1- Influence of the $Et_{75}A$ fraction isolated from the *P. tridentatum* HWE on the macrophage cellular viability. LPS-induced (LPS) and non-stimulated (Ctrl.) macrophages were used as positive and negative controls, respectively. (* compared to Ctrl.; # compared to LPS; different letters denote significant differences between assays using distinct extract concentrations; $p<0.05$).

The immunostimulatory activity of the mixture of polysaccharides detected in the $Et_{75}A$ fraction was evaluated through the assessment of the accumulation of nitrite, which is the stable end product of NO, in the RAW 264.7 macrophage cells supernatant and the results are shown in **Figure 2**.

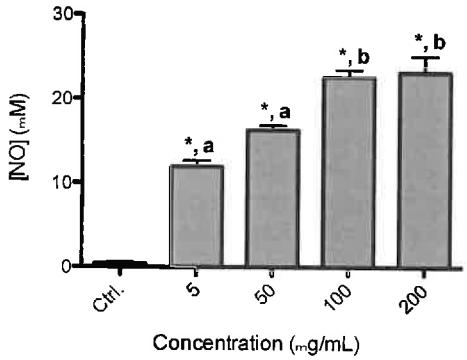

Figure 2- Influence of the $Et_{75}A$ fraction isolated from the *P. tridentatum* HWE on macrophage NO production. Non-stimulated (Ctrl.) macrophages were used as negative control. (* compared to Ctrl.; different letters denote significant differences between assays using distinct extract concentrations; $p<0.05$).

When the macrophages were stimulated with concentrations of the $Et_{75}A$ fraction that ranged from 5 to 200 μg/mL, an increase in the macrophage NO production, relatively to the control, was registered, evidencing the macrophage immunostimulatory activity of the $Et_{75}A$ fraction. In order to clarify the contribution of the various polysaccharides detected in the $Et_{75}A$ fraction to the macrophage immunostimulatory activity, the $Et_{75}A$ fraction was submitted to various treatments and the NO production of the resulting fractions was evaluated. The $Et_{75}A$ fraction was submitted to a saponification treatment, which promotes de-acetylation, and the results for the macrophage NO production of the resulting $Et_{75}A$-sap are shown in **Figure 3**.

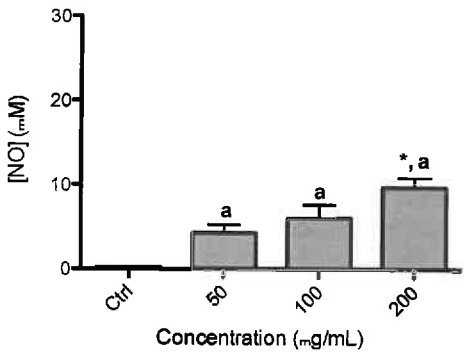

Figure 3- Influence of the $Et_{75}A$-sap resulting from the saponification treatment of the $Et_{75}A$ fraction isolated from the *P. tridentatum* HWE on macrophage NO production. Non-stimulated (Ctrl.) macrophages were used as negative control. (* compared to Ctrl.; different letters denote significant differences between assays using distinct extract concentrations; $p<0.05$).

The results showed an increase in NO production relatively to the control for the different extract concentrations assayed, evidencing that the $Et_{75}A$-sap fraction possessed a macrophage immunostimulatory activity. However, the observed macrophage NO production was lower than the registered for the unsaponified $Et_{75}A$ fraction. Through mass spectrometry analysis (data not shown) it was possible to evidence that the galactomannans detected in the HWE of *P. tridentatum* dried inflorescences were acetylated. The acetylation has been reported as an important structural feature in the expression of mannans immunostimulatory activity (Karaca *et al.*, 1995; Simões *et al.*, 2009, 2012). Thus, it seems plausible to considerer that the acetylation contributed to the macrophage

immunostimulatory activity of the galactomannans from *P. tridentatum* dried inflorescences decoctions.

However, the saponified Et_{75}A-sap fraction still exhibited significant immunostimulatory activity, suggesting that the presence of the pectic polysaccharides, along with both AG-I and AG-II, and xyloglucans may have contributed for the remaining immunostimulatory activity registered. In fact, the contribution of AG-I (Inngjerdingen, 2008) and AG-II (Sakurai *et al.*, 1998) structures to the macrophage immunostimulatory activity of plant HWE has been reported. Also, studies have evidenced the importance of storage xyloglucans, isolated from some *Fabaceae*, which is also the botanical family of *P. tridentatum,* as macrophages activators (Rosário *et al.*, 2008, 2011). The Et_{75}A fraction was also submitted to a saponification treatment followed by an *endo*-polygalacturonase digestion and the resulting digestion products were fractionated in Bio-Gel P30. A high molecular weight (Et_{75}A-I) and a low molecular weight (and Et_{75}A-II) fraction were isolated and their macrophage immunostimulatory activity was evaluated and is presented in **Figure 4**.

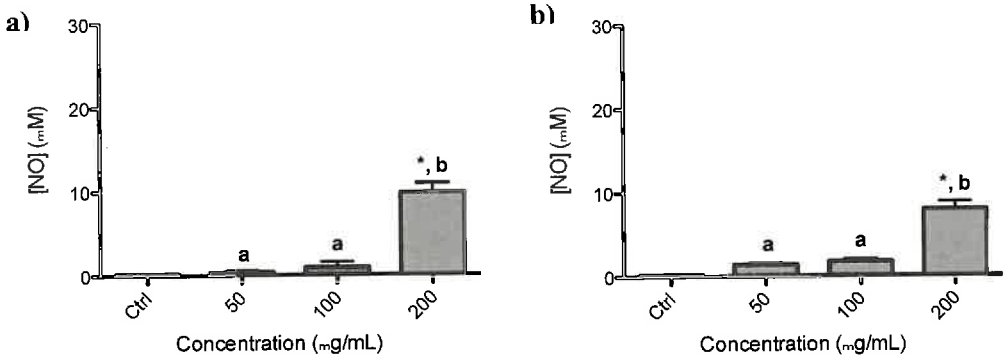

Figure 4- Influence of the **a)** Et_{75}A-I and **b)** Et75A-II fractions isolated from the *P. tridentatum* HWE on macrophage NO production. Non-stimulated (Ctrl.) macrophages were used as negative control. (* compared to Ctrl.; different letters denote significant differences between assays using distinct extract concentrations; $p<0.05$).

For both Et_{75}A-I and Et_{75}A-II fractions, it was observed that when the macrophages were stimulated with 50 and 100 μg/mL, the macrophage immunostimulatory activity 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. However, when 200 μg/mL of both Et_{75}A-I and Et_{75}A-II fractions were used for macrophage stimulation, an immunostimulatory activity higher than the observed for the lower concentrations and control was registered. This suggests that the polysaccharides present in the Et_{75}A-I and Et_{75}A-II fractions require adequate concentrations for the expression of the macrophage immunostimulatory activity.

Sugar and linkage analysis showed that the Et_{75}A-I fraction did not contained residues diagnostic for the presence of pectic polysaccharides comprising AG-I structures, as shown in **Table III**. Since residues diagnostic for the presence of pectic polysaccharides comprising AG-I structures were detected in the Et_{75}A fraction, as shown previously in **Table II,** this seems to reinforce the possible contribution of AG-I structures to the observed macrophage immunostimulatory activity.

Table III- Total sugar content, and polysaccharide composition of $Et_{75}A$ fraction and of the $Et_{75}A$-sap, $Et_{75}A$-I and $Et_{75}A$-II that resulted from the saponification and saponification followed by an *endo*-polygalacturonase treatment of the $Et_{75}A$ fraction from the HWE of *P. tridentatum* dried inflorescences.

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

the presence of the various polysaccharides was: [a] determined by sugar and 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 $Et_{75}A$-II fraction was mostly composed of oligogalacturonides resulting from the *endo*-polygalacturonase action, suggesting that, in adequate concentrations, the galacturonic acid rich moieties of pectic polysaccharides might also be involved in the expression of the observed macrophage immunostimulatory activity.

4. Conclusions

The HWE of *P. tridentatum* dried inflorescences contained a mixture of polysaccharides with distinct solubility in ethanol aqueous solutions. This mixture was mainly composed of pectic polysaccharides insoluble in 50 and 75% ethanol aqueous solutions that comprised AG-I and possibly also AG-II structures. It was also evidenced that the presence of galactomannans and xyloglucans, mostly insoluble in 75% ethanol aqueous solutions.

The mixture of polysaccharides exhibited immunostimulatory activity, without compromising the macrophages cellular viability. The acetylation of the galactomannans, together with the AG-I of pectic polysaccharides seemed to have contributed to the immunostimulatory activity observed. Moreover, the results also suggested that the AG-II and xyloglucans, when present in adequate concentrations, could contribute to the immunostimulatory activity. Therefore, it seems plausible that the polysaccharides might contribute to the therapeutic properties frequently associated by the traditional medicine to the HWE of *P. tridentatum* dried inflorescences.

5. References

Albersheim, P.; Neukom, H.; Deuel, H. Splitting of pectin chain molecules in neutral solutions. *Archives of Biochemistry and Biophysics*, v. 90, p. 46-51, 1960.

Blumenkrantz, N.; Asboe-Hansen, G. New Method for Quantitative Determination of Uronic Acids. *Analytical Biochemistry*, v. 54, p. 484-489, 1973.

Carvalho, A. M. Plantas y sabiduría popular del Parque Natural de Montesinho. Un estudio etnobotánico en Portugal. *Biblioteca de Ciencias*, v. 35, p. 496, 2010.

Cerqueira, M. A. et al. Structural and Thermal Characterization of Galactomannans from Non-Conventional Sources. *Carbohydrate Polymers*, v. 83, p. 179-185, 2011.

Coimbra, M.A. et al. Isolation and Analysis of Cell Wall Polymers from Olive Pulp. In: H.F. Linskens and J.F. Jackson (Orgs.) *Modern Methods of Plant Analysis, vol. 17*, Springer-Verlag, 1996. p. 19-44.

Ferreira, S.S. et al. Structure-Function Relationships of Immunostimulatory Polysaccharides: A Review. *Carbohydrate Polymers*, v. 132, p. 378-396, 2015.

Fischer, M., S. et al. Polysaccharides of Green Arabica and Robusta Coffee Beans. *Carbohydrate Research*, v. 330, p. 93-101, 2001.

Green, L. C. et al. Analysis of Nitrate, Nitrite, and [^{15}N] Nitrate in Biological Fluids. *Analytical Biochemistry*, v. 126, p. 131-138, 1982.

Grønhaug, T. E. et al. Bioactive Arabinogalactans from the Leaves of *Opilia celtidifolia* Endl. Ex Walp. (*Opiliaceae*). *Glycobiology*, v. 20, p. 1654-1664, 2010.

Inngjerdingen, K. T. et al. Immunological and Structural Properties of a Pectic Polymer from *Glinus oppositifolius*. *Glycobiology*, v. 17, p. 1299-1310, 2007.

Inngjerdingen, M. et al. Pectic Polysaccharides from *Biophytum petersianum* Klotzsch, and Their Activation of Macrophages and Dendritic Cells. *Glycobiology*, v. 18, p. 1074-1084, 2008.

Karaca, K.; Sharma, J.M.; Nordgren, R. Nitric Oxide Production by Chicken Macrophages Activated by Acemannan, a Complex Carbohydrate Extracted from *Aloe Vera*. *International Journal of Immunopharmacology*, v. 17, p. 183-188, 1995.

Keijbets, M. J. H.; Pilnik, W. β- Elimination of Pectin in the Presence of Anions and Cations. *Carbohydrate Research*, v. 33, p. 359-362, 1974.

Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods*, v. 65, p. 55-63, 1983

Nergard, C. S., et al. Structural and Immunological Studies of a Pectin and a Pectic Arabinogalactan from *Vernonia kotschyana* Sch Bip. Ex Walp. (*Asteraceae*). *Carbohydrate Research*, v. 340, p. 115-130, 2005.

Neves, J. M. et al. Ethnopharmacological Notes About Ancient Uses of Medicinal Plants in Trás-os-Montes (Northern of Portugal). *Journal of Ethnopharmacology*, v. 124, p. 270-283, 2009

Ramberg, J. E.; Nelson, E. D.; Sinnott, R.A. Immunomodulatory Dietary Polysaccharides: A Systematic Review of the Literature. *Nutrition Journal*, v. 9, p. 1-60, 2010.

Rosário, M. M. T. et al. Effect of Storage Xyloglucans on Peritoneal Macrophages. *Phytochemistry*, v. 69, p. 464-472, 2008.

Rosário, M. M. T. et al. Storage Xyloglucans: Potent Macrophages Activators. *Chemico-Biological Interactions*, v. 189, p. 127-133, 2011.

Samuelsen, A. B. et al. Characterization of a Biologically Active Pectin from *Plantago major* L. *Carbohydrate Polymers*, v. 30, p. 37-44, 1996.

Sakurai, M. H. et al. Characterization of Antigenic Epitopes in Anti-Ulcer Pectic Polysaccharides from *Bupleurum falcatum* L. Using Several Carbohydrases. *Carbohydrate Research*, v. 311, p. 219-229, 1998.

Schepetkin, I. A.; Quinn, M.T. Botanical Polysaccharides: Macrophage Immunomodulation and Therapeutic Potential. *International Immunopharmacology*, v. 6, p. 317-333, 2006.

Simões, J. et al. Immunostimulatory Properties of Coffee Mannans. *Molecular Nutrition and Food Research*, v. 53, p. 1036-1043, 2009.

Simões, J. et al. Structural Features of Partially Acetylated Coffee Galactomannans Presenting Immunostimulatory Activity. *Carbohydrate Polymers*, v. 79, p. 397-402, 2010.

Simões, J. et al. Mass Spectrometry Characterization of an *Aloe vera* Mannan Presenting Immunostimulatory Activity. *Carbohydrate Polymers*, v. 90, p. 229-236, 2012.

Paulsen, B.S.; Barsett, H. Bioactive Pectic Polysaccharides. *Advances in Polymer Science*, v. 186, p. 69-101, 2005.

Togola, A. et al. Polysaccharides with Complement Fixing and Macrophage Stimulation Activity from Opilia Celtidifolia, Isolation and Partial Characterisation. *Journal of Ethnopharmacology*, v. 115, p. 423-431, 2008.

Tzianabos, A. O. Polysaccharide Immunomodulators as Therapeutic Agents: Structural Aspects and Biologic Function. *Clinical Microbiology Reviews*, v. 13, p. 523-533, 2000.

Vitor, R.F. et al. Flavonoids of an Extract of *Pterospartum tridentatum* Showing Endothelial Protection Against Oxidative Injury. *Journal of Ethnopharmacology*, v. 93, p. 363–370, 2004.