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Modified pectin from *Theobroma cacao* induces potent pro-inflammatory activity in murine peritoneal macrophage

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ABSTRACT

In vitro effects of acetylated pectin (OP) isolated from cacao pod husks (*Theobroma cacao* L.), its partially deacetylated and de-esterified form (MOP), and a commercial homogalacturonan (PG) were investigated on murine peritoneal macrophages. MOP stood out among the studied pectins. After 48 h of incubation, compared with the control group, it was able to promote significant macrophage morphological differentiation from resident to activated stage and also stimulated nitric oxide production, which reached a level of 85% of that of LPS stimulus. In the presence of the highest tested concentration of MOP (200 $\mu g \cdot mL^{-1}$), the levels of the cytokines TNF-α (6 h) and IL-12 and IL-10 (48 h) increased substantially in relation to untreated cells. Our results show that the partial deacetylation and de-esterification of pectin extracted from cacao pod husks (*T. cacao* L.) produced a polymer with greater ability than its native form to activate macrophages to a cytotoxic phenotype. Like this, they provide the possibility of a therapeutic application to MOP, which could lead to a decreased susceptibility to microbial infection besides antitumor activity. Additionally, the present results also corroborate with the proposition of that the chemical modifications of the biopolymers can result in an improved molecule with new possibilities of application.

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1. Introduction

Pectic substances are acidic polysaccharides present in the cell wall of higher plants, contributing to adhesion, ionic balance, and mechanical characteristics of plant tissues. With a complex chemical structure, the common feature between the pectic molecules is the predominance of galacturonic acid (GalA) units present in three main structural patterns: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II) [1,2]. HG is the linear region of the polymer, composed by D-GalA units which can be acetylated at O-2 or O-3, or methyl-esterified at carboxyl groups. High methoxyl (HM) pectins have more than 50% of carboxyl groups methyl-esterified. Otherwise, if more than 50% of carboxyl groups are not esterified, low methoxyl (LM) pectins are present. Rhamnogalacturonans encompass the ramified region of the polymer. RG-I contains D-GalA units interspersed with L-rhamnose (Rha) units, to which side chains with neutral sugars (galactans, arabinans, or arabinogalactans) are attached. RG-II is the most complex pectic polysaccharide, it is less abundant and composed of a main chain of D-GalA with four distinct side chains containing rare sugars such as apiose, 2-O-methyl-fucose, aceric acid, and others [2,3].

Commercially, pectins are obtained from citrus peel and apple pomace and are mainly used in the food industry as gelling, thickening, or stabilizing agents [2]. New sources of pectins are constantly sought and studied, with great attention targeted at byproducts from the processing of plants, such as sugar beet pulp [4], papaya peel [5], watermelon rind [6], soy hulls [7], mango peels [8], passion fruit rind [9–11], banana peels [12,13], and cacao pod husks [14–17].

However, the potential of pectins go far beyond these cited applications. Besides the benefits of its use as a natural dietary fiber, pectin has been shown to have favorable effects on human health [3,18–24]. Among the many effects exerted by pectic polysaccharides, great attention has been given to their ability to modulate the immune system. In vivo and in vitro studies suggest that pectins may contribute to the development of cellular and humoral immunity [25].

Macrophages are the main cells target of studies aiming to characterize in vitro immunomodulating activity of polysaccharides.

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These cells possess receptors able to recognize these molecules and can induce their activation [26]. Macrophages exhibit wide phenotypic diversity, which enables classifying them by various manners [27,28]. This study focuses on resident and activated macrophages, which are distinguished according to their morphological and metabolic profiles. Resident macrophages are macrophages that have not had contact with a pathogen or any stimulus; they have few cytoplasmic projections and poor spreading ability. After contact with appropriate stimulus or pathogens, these cells differentiate from resident to activated and modify their morphology exhibiting various cytoplasmic projections and increased capacity for adhering and spreading [29]. In addition to morphological alterations and increased phagocytic capacity, the macrophages in the activated stage produce and release plenty of mediators such as oxygen and nitrogen intermediates, pro- and anti-inflammatory cytokines, and so on [30].

Many researches have shown that the immunomodulating potential of pectins is directly related to specific structural features of each polymer. The presence of methyl [21,31] or acetyl groups [32,33] or ramifications in pectins, as those in the rhamnogalacturonan region [32–34], are described as playing an important role on the immunological effects of these polymers. Thus, native peculiarities of certain pectins can promote distinct effects, but also the chemical modification is a tool that can improve or abolish the immunological effects of these polymers.

Pectins extracted from cacao pod husk (*Theobroma cacao* L.) using different extractants had a high acetyl content [14–16], which confers to them a distinct pattern compared to commercial pectins. That motivates choosing a pectin sample from cacao pod husks to explore the effect of this native characteristic as well as that of this pectin submitted to a saponification method under some macrophage functions.

2. Material

2.1. Reagents

Cytochrome c from equine heart, lipopolysaccharide from *Escherichia coli* (LPS), 3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), naphthylethylene-diamine, N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid (HEPES), phorbol 12-myristate 13-acetate (PMA), polygalacturonate and sulfanilamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tissue culture materials were provided by Techno Plastic Products (Schweiz, Saxonia, Germany). Eagle medium (MEM) and gentamicin were purchased from Cultilab (Campinas, SP, Brazil). Fetal bovine serum came from Invitrogen (São Paulo, SP, Brazil). ELISA kits for mouse interleukin-10 (IL-10), interleukin-12 (IL-12), and tumor necrosis factor-alpha (TNF-α) were obtained from RayBiotech (Norcross, GA, USA). Other chemical reagents were obtained from Merck (Darmstadt, Hesse, Germany).

2.2. Pectin samples

Three pectic samples named OP, MOP, and PG were employed in the present study.

OP fraction is an optimized nitric-acid-extracted pectin from cacao pod husks (*Theobroma cacao* L.). It was previously characterized by Vriesmann et al. [15], being composed of a highly methyl-esterified and acetylated homogalacturonan (degree of acetylation of 17.1% and degree of methyl-esterification of 56.6%) with rhamnogalacturonan portions containing mainly galactose-rich side chains. The fraction holds pectins with molecular mass of 1,681,000 $g{\cdot}mol^{-1}$ and 349,800 $g{\cdot}mol^{-1}$.

MOP is the OP fraction submitted to a saponification process based on Sengkhamparn et al. [35], with modifications. After solubilization of OP in distilled water, a solution of 0.1 $mol{\cdot}mL^{-1}$ NaOH in the presence of $NaBH_4$ was added to it to reach a final concentration of 0.05 $mol{\cdot}L^{-1}$ NaOH. The mixture remained under stirring at 4 °C for 16 h and was then neutralized with a solution of 0.1 $mol{\cdot}L^{-1}$ acetic acid, dialyzed against water, and freeze-dried. The resulting fraction, called MOP (Modified Optimized Pectin), was analyzed by high pressure size exclusion chromatography coupled with a differential refractometer and a multi-angle laser light scattering detector (HPSEC-MALLS-RI), nuclear magnetic resonance spectroscopy (^{13}C NMR), Fourier transform-infrared spectroscopy (FTIR), sugar composition, and acetyl content as described previously [15,36]. By both HPSEC-MALLS-RI elution profile and refractive index increment (dn/dc) value (obtained using four distinct concentrations of MOP previously solubilized in $NaNO_2$ 0.1 $mol{\cdot}L^{-1}$ containing 200 ppm NaN_3 and filtered through a 0.22 μm pore size membrane), molecular mass (Mw) was calculated employing the software Astra from the equipment.

The third polymer studied in this investigation is PG, a polygalacturonic acid with Mw of 25,000–50,000 $g{\cdot}mol^{-1}$ obtained from Sigma (product number 81325; CAS number 25990107; LOT 1294924).

The polymers PG and MOP were solubilized in ultra-pure water and OP was solubilized in phosphate buffer saline (PBS). All solutions were filtered through 0.45 μm pore size membrane, and sterilized by filtration through a sterile membrane of 0.22 μm pore size and frozen. Total carbohydrate content was determined in the samples [37] both before and after the filtration process to confirm the final concentration of the sterilized polymer.

Endotoxin was not detected in the polymers when analyzed by GC–MS according to the description [38].

2.3. Animals

Female albino Swiss mice (6–8 weeks old) were provided by the biotery of the Biological Campus of the Federal University of Paraná (UFPR). They were housed under a 12/12 h light/dark cycle and received a standard laboratory diet (Purine®) and water ad libidum. All the legal recommendations of the Brazilian legislation (law no. 6.638, 05 Nov. 1979) were respected. The animal handling procedures for scientific research were approved by the Animal Ethics Committee of UFPR.

3. Methods

3.1. Isolation of peritoneal macrophages

Peritoneal macrophages were collected from the mice by infusing their peritoneal cavity with 10 mL ice-cold sterile phosphate-buffered saline solution (HBSS). Collected cells were centrifuged and the cell pellets were resuspended in MEM medium, 5% fetal bovine serum, and 50 $\mu g{\cdot}mL^{-1}$ gentamicin or HBSS. Cell number and viability were assessed microscopically using trypan blue exclusion [39]. The number of cells was adjusted according to each experiment and incubated for 2 h at 37 °C, under 5% CO_2 in a humidified incubator. Next, non-adherent cells were removed by washing once with HBSS at 37 °C. The samples were added dissolved in the medium or HBSS and incubated for different times according to each experiment, in the same conditions.

3.2. Cytotoxicity assay

Adherent macrophages (4.10^5 cells/well) in a 96-well tissue culture plate were incubated for 48 h under the specified conditions in the absence (control) or presence of the compounds OP, MOP, and

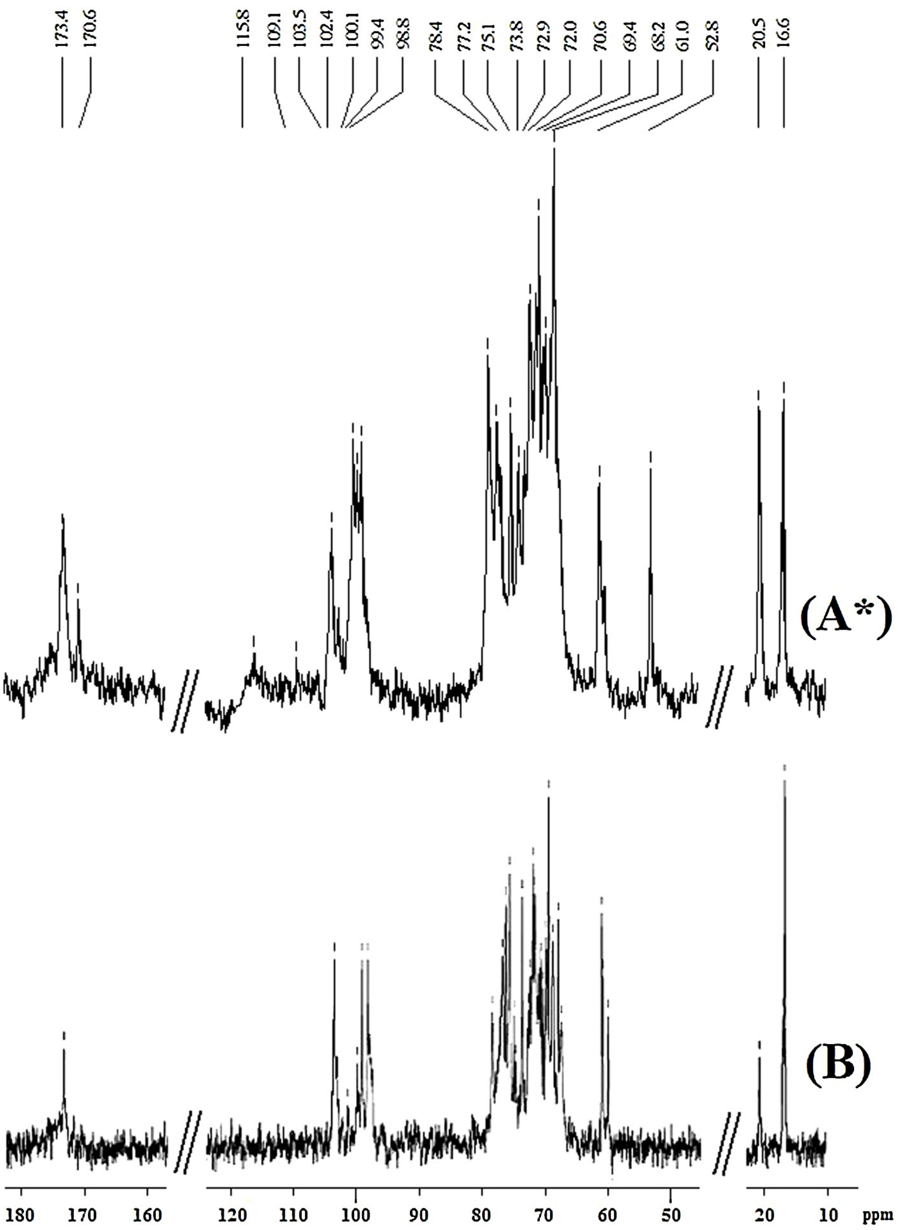

Fig. 1. Comparative ^{13}C NMR spectra of initial OP fraction from cacao pod husks (A*) and its modified form MOP (B). Solvent D_2O at 70 °C. * Reprinted from Vriesmann, Teófilo and Petkowicz [15], p. 1235, with permission from Elsevier.

PG at various concentrations (25, 50, 100, 200 and 400 µg·mL^{-1}). Cytotoxicity was evaluated using the MTT method as described by Mosmann [40].

3.3. Assay of cell activation by morphology analysis

Adherent macrophages (4.10^5 cells/well) on glass coverslips contained in a 24-well tissue culture plate were incubated in the standard medium in the presence or absence of OP, MOP, and PG (100 and 200 µg·mL^{-1}). After 48 h at 37 °C under 5% CO_2, the glass coverslips were washed twice with HBSS pH 7.4, fixated for 5 min in Bouin's fixative, stained with hematoxylin-eosin for 1 min and with eosin for 30 s and dehydrated in acetone. The slides were then mounted with Entellan and examined microscopically [41]. The number of activated and resident macrophages was counted in all groups.

3.4. Phagocytic activity assay

Phagocytic activity was assayed according to standard protocols using yeasts as the phagocytizing particles. In short, a macrophage monolayer (4.10^5 cells/well), adhered to a glass coverslip and contained in a tissue culture plate (24 wells), was incubated with the standard medium in the absence (control) or presence of OP, MOP, and PG at 100 and 200 µg·mL^{-1} concentrations. After 48 h at 37 °C under 5% CO_2, the cells were washed twice with the MEM and the yeast (ratio of macrophages to yeast, 1:10) was added, followed by incubation under the same conditions for up to 1 h. After each incubation time, non-phagocytic yeasts were removed by rinsing with HBSS. The coverslips were then fixated with Bouin's fixative, stained with Giemsa for 1 h, and dehydrated in acetone and xylol [41]. The slides were mounted with Entellan. To obtain the ratio of phagocytic yeasts/macrophage, the slides were examined microscopically.

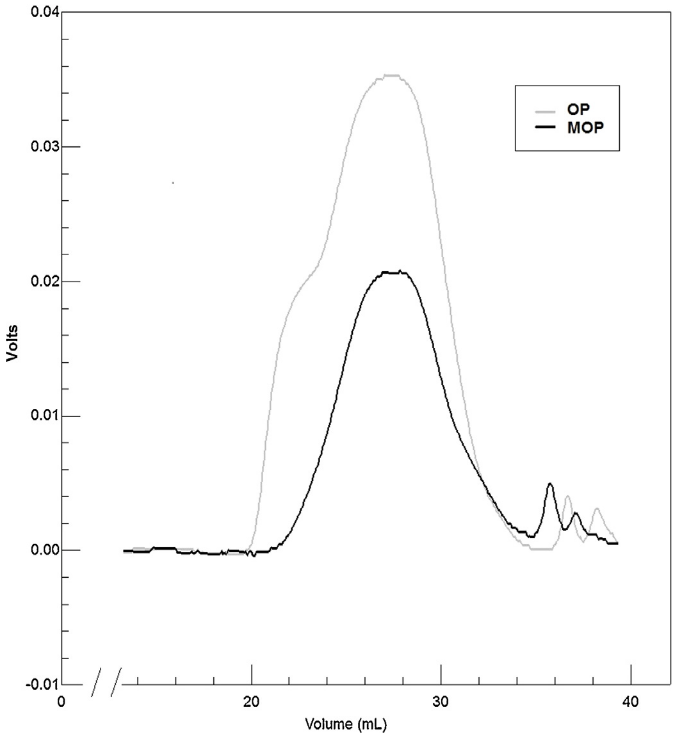

Fig. 2. Elution profile obtained by high-pressure size exclusion chromatography (HPSEC): comparative detection of differential refractometer (RI) of the OP fraction from cacao pod husks and its modified form—MOP fraction.

3.5. Superoxide anion assay

Adherent macrophages were incubated with cytochrome c at 80 μmol·L^{-1} in HBSS in the absence or presence of the compounds (100 and 200 μg·mL^{-1}). PMA (1 μg·mL^{-1}) was used as positive control. The absorbance at 550 nm was measured after 2 h and the superoxide anion concentration was calculated by dividing the difference in the absorbance of the samples by the extinction molar coefficient $C_{oxid.red.\Delta e} = 2.1.10^4\ M^{-1}\ cm^{-1}$ for reduced cytochrome c [42]. The results are expressed as μmol·L^{-1} of reduced cytochrome c per 4.10^5 cells.

3.6. Nitrite assay

Adherent macrophages (4.10^5 cells/well) in a 96-well tissue culture plate were incubated with polymers (100 and 200 μg·mL^{-1}) and LPS (100 ng·mL^{-1}) as positive control. After 48 h, the supernatants (100 μL) were mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min [43]. The absorbance was measured at 550 nm and the nitrite concentration was calculated from a $NaNO_2$ standard curve. The results are expressed as μmol·L^{-1}/4.10^5 cells.

3.7. Interleukins IL-10, IL-12, and TNF-α assay

Adherent macrophages (4.10^5 cells/well) were incubated in a 96-well tissue culture plate with standard medium in the absence (control) or presence of OP, MOP, and PG (100 and 200 μg·mL^{-1}). LPS (100 ng·mL^{-1}) was used as positive control. After incubation for 6 h for TNF-α and 48 h for IL-10 and IL-12, the supernatant was collected and kept at −80 °C until assayed. Interleukin concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol.

Table 1
Composition of the chemically modified pectin (MOP) from the optimized pectin (OP[a]) extracted from cacao pod husks (*Theobroma cacao* L.).

Composition	MOP	OP[a,b]
Monosaccharides[c]		
Rha	18.0 ± 0.1	10.0 ± 1.0
Ara	2.5 ± 0.4	2.7 ± 1.8
Xyl	0.8 ± 0.1	0.7.±0.5
Man	tr	1.0 ± 0.5
Gal	19.7 ± 0.5	16.8 ± 0.9
Glc	3.0 ± 0.1	2.8 ± 0.6
Uronic acid[d]	56.0 ± 0.2	66.0 ± 2.2
DE[e]	20.8 ± 1.1	56.6 ± 1.4
DA[f]	9.4 ± 0.1	17.1 ± 0.5

tr: Trace.

[a] Pectin previously characterized by Vriesmann, Teófilo, & Petkowicz [15].

[b] Special credit from Vriesmann, Teófilo and Petkowicz [15], p. 1234, with permission from Elsevier.

[c] Determined by GLC, similarly to Vriesmann, Teófilo & Petkowicz [15], mean of triplicate ± SD.

[d] Determined by colorimetric method, similarly to Vriesmann, Teófilo & Petkowicz [15], mean of triplicate ± SD.

[e] Degree of esterification, determined by FT-IR, as described by Vriesmann and Petkowicz [36], mean of duplicate ± SD.

[f] Degree of acetylation, determined by colorimetric method, similarly to Vriesmann and Petkowicz [36], mean of triplicate ± SD.

3.8. Statistical analysis

The data of the present study were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. $p < 0.05$ was considered statically significant. Results are expressed as means ± standard deviation (SD) or means ± standard error of means (SEM).

4. Results

4.1. Characterization of the saponified pectin – MOP

After the saponification process of OP, an MOP sample was obtained at a yield of 88.15% in relation to the initial fraction. Its ^{13}C NMR analysis is showed in Fig. 1B, evidencing similar structural patterns with OP pectin (Fig. 1A) [15]. De-esterification of GalA units in MOP was evidenced by the absence of signals at 170.5 ppm and 52.8 ppm, assigned, respectively, to C-6 from esterified GalA units and $-OCH_3$ groups in OP fraction. The signal at 20.5 ppm from methyl carbons of acetyl groups remained, although with less intensity than in the native OP fraction [15]. The fraction was chemically characterized, as shown in Table 1.

After the alkaline treatment, comparing OP initial fraction with MOP sample, the degree of acetylation (DA) decreased from 17.1% to 9.4% of GalA, whereas the degree of methyl-esterification (DE) decreased from 56.6% to 20.8%, showing that the saponification more easily removed the methyl groups than the acetyl groups. Furthermore, the content of uronic acid decreased at the same rate as the content of rhamnose and galactose increased together.

As expected, the composition data together with those of ^{13}C NMR confirmed the presence of homogalacturonan and rhamnogalacturonan-I with side chains mainly of galactans in the modified pectin (MOP), similarly to native pectin (OP) from cacao pod husks.

Analysis of MOP fraction by HPSEC-MALLS/RI (Fig. 2) shows the presence of polymers eluting at higher volume than those of OP fraction, evidencing the presence of pectins with lower molecular mass (Mw 251,900 g·mol^{-1}; dn/dc 0.104) than the native pectin. Additionally, the higher molecular mass portion of the OP fraction which eluted at 22–24 mL disappeared after the chemical treatment. These data together with monosaccharide composition indicate that some degradation might have happened in MOP in relation to the OP fraction. Pectin is susceptible to degradation in alkaline and neutral conditions. During the saponification process, along with the de-esterification, some β-elimination of GalA can happen due to the alkaline environment of the reaction media [44].

4.2. Morphological analysis and phagocytic activity of macrophages treated with pectins

In order to determine the non-toxic concentration of the pectins, adherent macrophages were incubated with OP, MOP, and PG at different concentrations as indicated in Fig. 3. After 48 h, the cell viability was analyzed as described. OP polymer did not show any cytotoxic effects under the evaluated concentrations and conditions; however MOP and PG polymers, both at 400 $\mu g{\cdot}mL^{-1}$, decreased cell viability by ~80% in respect to untreated macrophages. Thus, in the subsequent experiments, polymers were employed at concentrations of 100 and

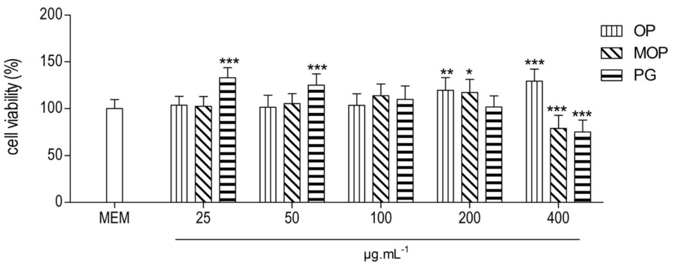

Fig. 3. Effects of OP, MOP and PG on the viability of peritoneal macrophages. Murine peritoneal macrophages were exposed for 48 h to the pectins at the indicated concentrations. Cell viability was determined by MTT assay. Culture medium was used as negative control, corresponding to 100% viability. The results are expressed as mean ± SD (n = 3, each experiment in quadruplicate). Significant difference from the negative control (medium) group and samples by Tukey's test, *p < 0.05, **p < 0.01 and ***p < 0.001.

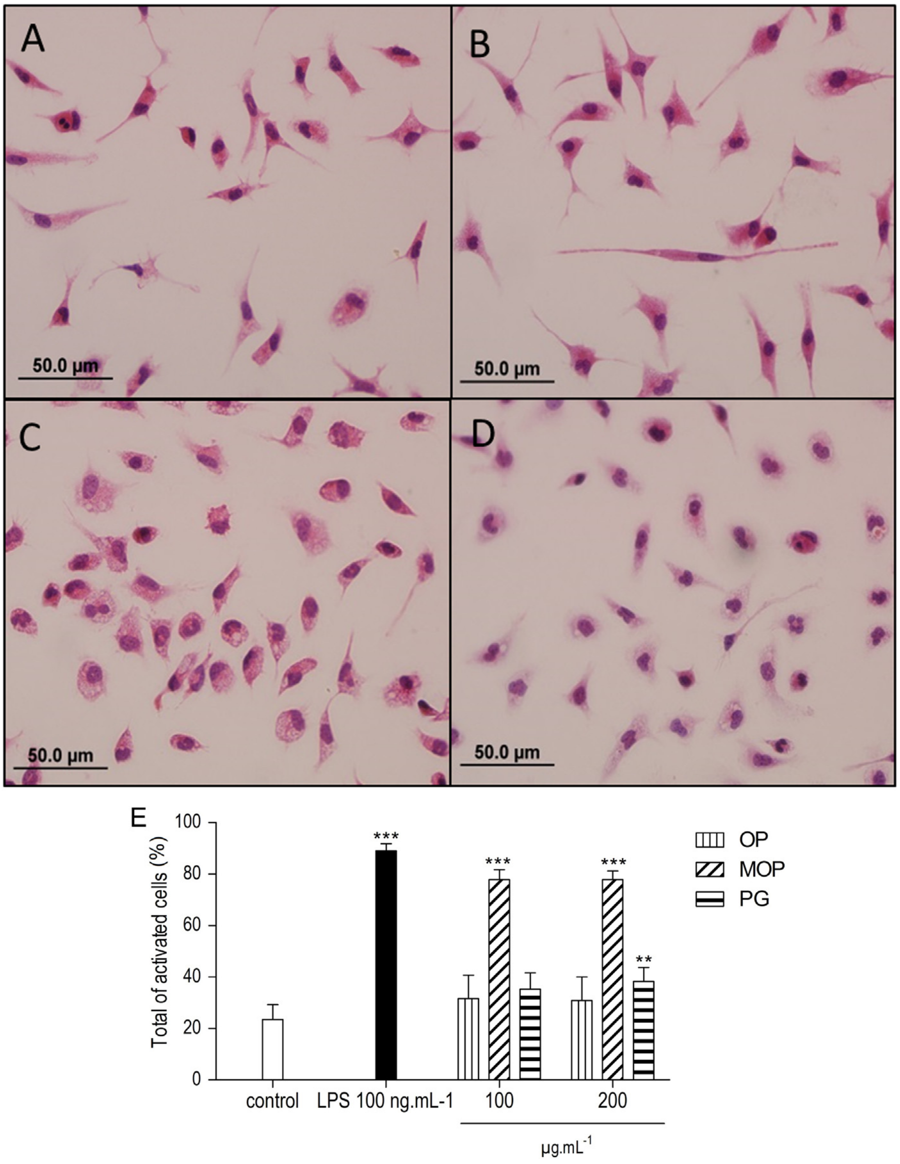

Fig. 4. Representative micrographs of mice peritoneal macrophages incubated with OP, MOP, and PG at concentrations of 200 $\mu g{\cdot}mL^{-1}$. After 48 h of treatment with the polymers at 37 °C under 5% CO_2, the cells were processed for light microscopy by staining with hematoxylin-eosin. A) Control, B) OP, C) MOP, and D) PG. E) Percentage of activated macrophages in relation to total counted macrophages. Results are expressed as mean ± DP for three independent experiments in duplicate. Significant difference from the control (medium) group and samples by Tukey's test, ** p < 0.01 and *** p < 0.001.

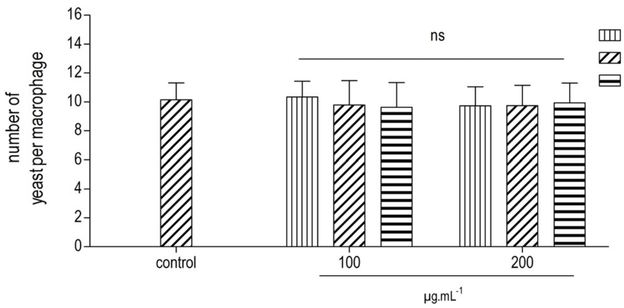

Fig. 5. Effect of pectins on phagocytic activity of peritoneal macrophages. Adherent macrophages were incubated in the absence (control) or presence of OP, MOP, and PG (100 and 200 μg·mL^{-1}) for 48 h as described in the Methods section. The phagocytic activity was obtained from the ratio of phagocytized yeasts to macrophage, which contained 500 cells per group. The results are expressed as mean ± SD for three independent experiments in duplicate. Significant difference from the control (medium) group and samples by Tukey's test ns = $p < 0.05$.

200 μg·mL^{-1}. An apparent increase in the cell viability observed in the concentration up to 200 μg·mL^{-1} could be due the activation of macrophages, since when these cells are activated, an increasing in their metabolism may occur, which could be evidenced by MTT assay [45].

Regarding macrophage modulation, initially the effects of OP, MOP, and PG on macrophages morphology were investigated. Fig. 4 shows representative images of the distribution of resident and activated macrophages in control and in the presence of polymers (Fig. 4 A–D). In the control group and macrophages incubated with OP polymer, approximately 20% of macrophages were in activated state. However, the treatment with MOP and PG (Fig. 4C and D) showed significant alterations in macrophage morphology, such as greater ruffling of the plasma membrane and presence of vacuoles, when compared with the control group. In the presence of MOP at 100 and 200 μg·mL^{-1}, about 80% of macrophages in activated stage were observed, whereas for PG polymer, alteration in morphology was observed in ~40% of cells only at 200 μg.mL^{-1} (Fig. 4E).

In the present study, the phagocytic activity was determined from the ratio of phagocytized yeasts/macrophage treated and untreated with pectins. OP, MOP, and PG did not produce a significant increase in the phagocytic activity of murine peritoneal macrophages compared with the control group (Fig. 5).

4.3. Effect of pectin polymers on superoxide anion and nitric oxide production by macrophages

The effect of OP, MOP, and PG on respiratory burst in macrophages was measured through superoxide anion production. Under the conditions tested, only PG polymer at 200 μg·mL^{-1} stimulated superoxide anion production, with a ~60% increase compared to the control group (Fig. 6A). Cacao pod husk pectins did not show any increase in stimulus compared to the control.

Regarding NO production, macrophages incubated with 100 and 200 μg·mL^{-1} of MOP for 48 h increased the NO level by ~250% compared to control (medium alone). But OP and PG polymers did not stimulate NO production in the tested conditions (Fig. 6B).

4.4. Effect of pectins on the production of IL-10, IL-12, and TNF-α interleukins by macrophages

The ability of structure-related pectins to induce the production and secretion of cytokines IL-10, IL-12, and TNF-α by macrophages treated with the polysaccharides at 100 and 200 μg·mL^{-1} was investigated. Compared with the medium alone, IL-10, IL-12, and TNF-α productions were significantly increased by LPS stimulation. IL-10 production was increased by OP and PG polymers only at 200 μg·mL^{-1} treatment compared with the control group, with increases of 10 and 12%, respectively. Stimulation of IL-10 production by MOP was observed at both concentrations (100 and 200 μg·mL^{-1}), with increases by 140 and 165%, respectively, when compared with the control (Fig. 7A).

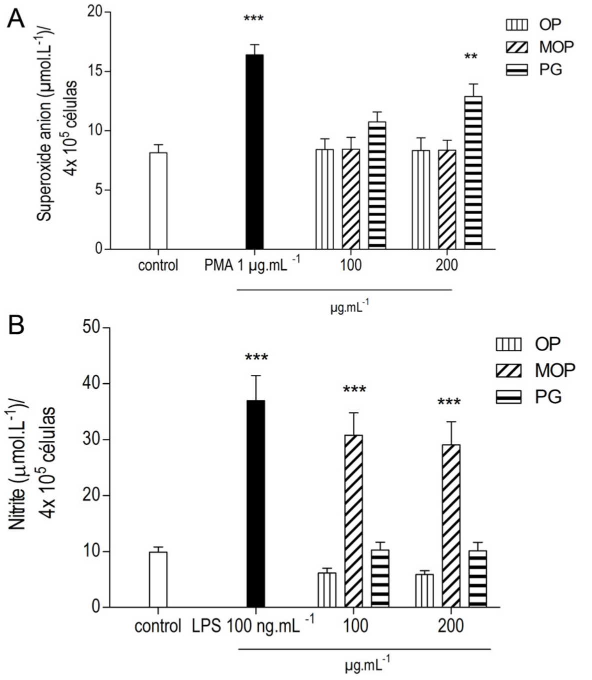

Fig. 6. Effect of OP, MOP, and PG on superoxide anion and nitric oxide production by macrophages. (A) Adherent macrophages were incubated for 2 h in a mixture consisting of HBSS containing ferricytochrome c (80 μmol·L^{-1}) in the absence or presence of PMA (1 μg·mL^{-1}) or the polysaccharides OP, MOP, and PG (100 and 200 μg·mL^{-1}). (B) Adherent macrophages were incubated for 48 h with MEM in the absence or presence of LPS (100 ng·mL^{-1}) or the polysaccharides OP, MOP, and PG. The nitrite concentration was measured as described in the Methods section. The results are expressed as mean ± SE from four independent experiments in triplicate. Significant difference from the control (medium) group and samples by Tukey's test ** $p < 0.01$ and *** $p < 0.001$.

Fig. 7B shows the results of IL-12 production. OP did not interfere in the secretion of IL-12. MOP promoted a 70% increase in IL-12 production at the lowest concentration (100 μg·mL^{-1}), reaching 95% at 200 μg·mL^{-1}, compared to the untreated cells. At both concentrations (100 and 200 μg·mL^{-1}), PG promoted an increase by 30% in IL-12 production versus the control group.

TNF-α production following the macrophage treatment by pectins is shown in Fig. 7C. OP and PG did not promote an enhancement of TNF-α production in relation to the control. However, for MOP, the effect occurred at both tested concentrations, 100 and 200 μg·mL^{-1}, and the enhancement of TNF-α production ranged from 60% to 87%, respectively, in relation to the control.

LPS contamination was not present in the polymers, confirmed by GC–MS [38], thus, it was not responsible for the observed immunomodulatory activity of the polymers.

5. Discussion

The results of the present study showed a varied profile regarding macrophage morphology in the presence of pectins. A great increase of approximately 80% of macrophages with the activated-stage morphology were observed when these cells were incubated with MOP at 100 and 200 μg·mL^{-1}, while its native acetylated pectin (OP) did not promote any change. The linear polymer PG (200 μg·mL^{-1}) also increased the number macrophages with morphology characteristics of activated, but it was twofold less potent

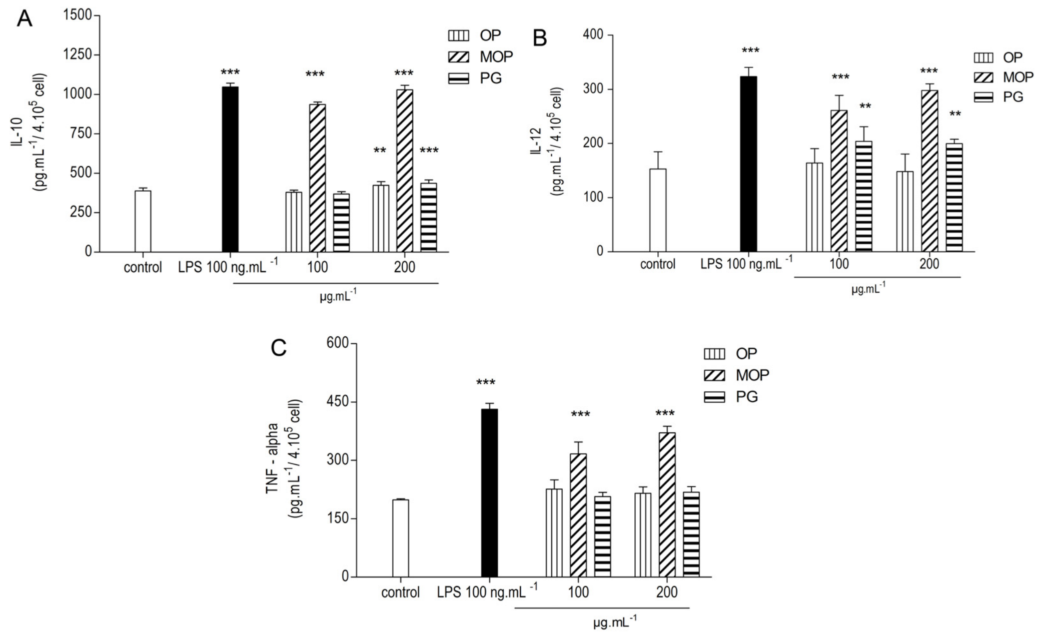

Fig. 7. IL-10, IL-12, and TNF-α production by peritoneal macrophages in the presence of pectins.
The adherent macrophages were incubated with MEM in the absence or presence of LPS (100 ng·mL^{-1}) or the polysaccharides OP, MOP, and PG (100 and 200 μg·mL^{-1}). The cytokines were measured by ELISA in the culture supernatants collected after 48 h for quantification of IL-10 (A) and IL-12 (B) and after 6 h for TNF-α (C). The results are expressed as mean ± SD for five independent experiments in duplicate. Significant difference from the control (medium) group and samples by Tukey's test * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

than MOP. Such information corroborates the proposition that the effects of pectins on macrophage modulation are directly correlated with structural aspects of the polymers.

Interestingly, other studies also showed activation of macrophages by polysaccharides through morphological evaluation similar of the present work [41,46,47]. However, none of them correlated the effect with a particular structural feature.

One of the most important characteristics of macrophages is their high phagocytic capacity, which is important to maintain homeostasis and innate immunity [30]. Pectins evaluated in the present study did not induce any stimulus in yeast phagocytosis by macrophages. These results are different from literature data for some structure-related polysaccharides [41,48,49].

The respiratory burst is another important early pathway involved in the microbicidal effects by macrophages. It can be triggered concomitantly or not with phagocytosis and ROS are its main products, which are toxic to microbes [30,50]. In the present study, only PG (200 μg·mL^{-1}) promoted an effect on respiratory burst when incubated for 2 h, increasing by 60% the superoxide anion level compared to the control.

Schepetkin et al. [51] proposed that the ability to activate ROS production seemed to be directly proportional to the increase in molecular weight of the polymer. In the present work only the pectin with the lowest molecular mass (PG) showed an effect on superoxide production, showing that other factors, besides molecular mass, are involved in this process. In fact, there is a complexity in evaluating ROS production by phagocytic cells, because it depends not only on the class of polymers, but also on their structural composition and the type of cells, as showed by Xie et al. [52].

The nitric oxide (NO) pathway has been also widely investigated for the action of polysaccharide biological response modifiers, since its activation in macrophages trigged microbicidal and antitumor effects [53]. Concerning to the present study, OP and PG polymers did not stimulate NO production in murine peritoneal macrophages, but MOP fraction increased NO level by approximately 2.5-fold compared to control. In addition, considering the LPS stimulus at 100%, MOP reached 85%, characterizing this pectin as a potent activator of the NO pathway. However, other distinct polysaccharides also exhibited NO production [54,55].

Besides the NO pathway, the effects of plant polysaccharides on cytokine production by macrophages can indicate their potential application as immunomodulators, whether for anti- or pro-inflammatory purposes. It has been described that IL-10, IL-12, and TNF-α are released in a coordinated manner from activated macrophages. The release of TNF-α plays an important role in inflammation during the acute phase, promotes inhibition of tumor cell division and macrophage-mediated tumor cytotoxicity mechanisms, and this cytokine may induce IL-12 production. After the production of IL-12 by macrophages, this cytokine plays a role in stimulating the production of TNF-α and IFN-γ in T and NK cells, which leads to tumoricidal action. IL-10 has been reported to inhibit cytokine synthesis and deactivate macrophages, thereby preventing or reducing potential detrimental effects from excessive macrophage activation during inflammation [56].

Of the cytokine secretions evaluated in the present study, MOP was the most potent activator among the studied pectins, since it promoted an expressive increase in anti-inflammatory (IL-10) and pro-inflammatory (TNF-α and IL-12) cytokines. MOP exhibited a similar profile to LPS with respect to macrophage modulation when compared to the untreated macrophages. Additionally, IL-10 level was increased at approximately sixteen-fold by MOP compared to OP and PG. Regarding to the releasing of IL-12 and TNF-α, MOP increased at approximately twofold the level of these cytokines compared to control, while OP did not increase the IL-12. Both polymers, OP and PG, did not alter the TNF-α production.

This pattern is not similar for all polysaccharides, a predominantly non-methoxylated homogalacturonan (85% GalA) from rhizomes of *Acorus calamus* did not influence murine macrophage IL-10 production at 20 μg·mL^{-1}, but increased IL-12 production sixfold. For human mononuclear cells stimulated with LPS, the same pectin stimulated TNF-α production by 1.7 times, not changing the production of IL-10, both in relation to LPS individually [54].

In the present study, we evaluated several effects of OP and MOP from cacao pod husks compared to a commercial homogalacturonan pectin (PG) in macrophages and we observed that several aspects related to pectin structure could be responsible for the observed differences and these polymers. Monosaccharide composition varied between the samples as well as the content of methyl and acetyl groups, besides the molecular mass. While PG is the lowest polymer and is composed by de-esterified homogalacturonans, the pectic fractions from cacao pod husks (OP and MOP) are more complex polymers, composed by homogalacturonans with insertions of rhamnogalacturonans carrying mainly galactans as side chains. Furthermore, both OP and MOP have hydrophobic portions due the presence of methyl and acetyl groups, both at lower proportion in MOP. The fractions are partially methyl-esterified, but in distinct amount and manner, leading to different charge distribution along the molecules. All of these structural features probably lead to distinct conformation and exposure of the molecules in solution, and, thus, to peculiar interaction with macrophages to modulate them.

6. Conclusions

Optimized pectin (OP) extracted from cacao pod husk (*T. cacao* L.) and its partially deacetylated and de-esterified form (MOP) and a commercial homogalacturonan pectin (PG) are able to modulate some macrophage functions. Alkaline treatment during the saponification of acetylated pectin from cacao pod husks (OP) generated chemical modifications in MOP which promoted distinct and increased forms of biological activities in relation to OP initial fraction.

MOP showed to be the most potent since it increased a number of activated peritoneal macrophages and triggered the secretion of pro- (NO, TNF-α, and IL-12) and anti- (IL-10) inflammatory mediators by these cells. OP showed a slightly anti-inflammatory profile with reduced IL-10 production. PG showed an increase in the number of activated macrophages, amount of superoxide anion, and IL-12 level, which suggests that the pro-inflammatory properties are predominant.

Taken together, the results of the present research show that the partial deacetylation and de-esterification of pectin extracted from cacao pod husk (*T. cacao* L.) produced a polymer with greater ability than its native form to activate macrophages to a cytotoxic phenotype. Like this, they provide the possibility of a therapeutic application to MOP, which could lead to a decreased susceptibility to microbial infection besides antitumor activity. Additionally, the present results also corroborate with the proposition of that the chemical modifications of the biopolymers can result in an improved product with new possibilities of application.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2016.08.015.

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