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Characterization and Biological Activity of Native and Sulfated Noni (*Morinda citrifolia* Linn.) Pectin

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The aim of this work was to extract and characterize pectin from lyophilized noni pulp (*Morinda citrifolia* Linn.) at three different pHs using different extractor agents and to evaluate anti-viral activity. The extraction of high yield (acid extraction) was subjected to sulfation procedure. Fourier transform infrared spectroscopy and proton nuclear magnetic resonance analysis were used to calculate the degree of methylation of pectin from acid extraction and its sulfated derivative. The data obtained for the degree of methylation of noni pectin and sulfated pectin were 30.3 and 30.7, respectively. Pectin was subjected to acute oral toxicity testing in mice. The results showed that pectin had a pro-inflammatory effect, and sulfated pectin had an anti-inflammatory effect. Both pectins were also evaluated for herpes simplex virus activity *in vitro*, and although they successfully inhibited virus replication, the sulfated pectins were cytotoxic.

Keywords: noni pectin, cytotoxicity, HSV

Introduction

Many plants are known to have biological potential,[1] among which can be promising for antibacterial,[2,3] anticancer,[4,5] and in the replication of human immunodeficiency virus (HIV)[6] infection process. Among these plants the *Morinda citrifolia* Linn., known as noni, can be highlighted.

Preliminary phytochemical investigation on leaf of *Morinda citrifolia* Linn. indicated the presence of steroids, terpenoids, flavonoids, glycosides, phenolic compounds, α-amino acids, reducing sugars, carbohydrates, saponins, tannins and alkaloids.[7] These extracts are used for synthesis of metal nanoparticles with important anti-cancer activity.[8] Studies[9] have shown that herbal treatment has positive effects in the prevention of various diseases, with similar action to conventional drugs. However, a deeper investigation is necessary to identify the real effects in the elimination of several diseases.[10-12] In this context, research on *Morinda citrifolia* in countries where the planting and consumption of noni are directed to its bioactive properties has received increasing interest. Among the activities can be mentioned the antibacterial, antioxidant, antiviral, antifungal activity, as well as its effect on the immune system, among others.[13-15]

There is little research on noni pectin and all its biological properties. Pectin from other sources have been

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studied to find the biological properties observed when anti-metastasis was verified in the treatment of prostate cancer in rats, and its use in the prevention of metastasis in different organs.[16] However, studies have shown that polysaccharides extracted from *Morinda citrifolia* Linn. showed anti-inflammatory potential[17] and protective effect against intestinal disease.[18]

Another way to use the pectin from noni is by modifying its chemical composition by sulfation to improve its bioactivity. A study carried out by other authors[19] showed that a sulfated polysaccharide has a good effect on blood clotting, being anti-thrombotic. The substitution of carboxylic groups of the pectin by esters of sulfate increases the pharmaceutical potential compared with the non-sulfated pectin. Recent studies confirm the improvement of sulfated pectin[20] through blood anti-clotting behavior. The same property was found by other authors[21] associated with the effect of fibrinogen inhibition. The sulfated polysaccharides have demonstrated antiviral activities, especially against herpes simplex virus (HSV).[22]

In this work, noni pectin and its sulfated derivative were chemically characterized and then submitted to *in vitro* tests to evaluate their toxicity and antiviral activity. In addition, both anti-inflammatory activity and toxicity were also investigated through *in vivo* assays, where it was possible to identify the action of the tested substances. Finally, the *in vitro* anti-herpes simplex virus activity of pectin was evaluated.

Experimental

Materials

The citric pectin (PC) was obtained from Sigma-Aldrich (St. Louis, United States). All other reagents were analytical grade. The dry pulp of *Morinda citrifolia* Linn. was obtained from the dehydration of the ripe fruit, collected in Fortaleza-CE, northeastern of Brazil (3°49'52.0"S and 38°33'03.2"W). The fruit dehydration process occurred by lyophilization using an Alpha 1-2 LDplus® lyophilizer (Analítica, São Paulo, Brazil) operating at −55 ºC and 0.021 mbar. The use of this species was registered in the Brazilian National System of Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) by registration number A53C3CE.

Polysaccharide extraction

The extraction was adapted from literature.[23] Three portions of the dried noni pulp (20.0 g) were dispersed in 900 mL of the extracting agent, 0.25% ammonium oxalate solution ($(NH_4)_2C_2O_4$ at pH 3.0) (Dinâmica, São Paulo, Brazil), distilled water (pH 7.0) and a sodium hydroxide solution (NaOH, pH 10.0) (Dinâmica, São Paulo, Brazil). The suspension was stirred for 1 h at 80 °C, then the extract was filtered and centrifuged at 800 rpm for 10 min. After concentration by evaporation under reduced pressure, the pH was adjusted to 7.0 (NaOH solution in the acid extraction and oxalic acid in the basic extraction). The polysaccharide was separated by precipitation after the addition of ethanol (Dinâmica, São Paulo, Brazil) (1:3 v/v). After lyophilization the isolated material was denominated PDN-a, PDN-n and PDN-b to pectin extracted from pH 3, pH 7 and pH 10, respectively.

Chemical modification by sulfation (PDN-s)

The following experimental procedure was adapted from O'Neill,[24] in which 300.1 mg of PDN-a were added in a mixture of solvents composed by pyridine (Sigma-Aldrich, St. Louis, United States) and *N*,*N*-dimethylformamide (50:10 v/v) (Sigma-Aldrich, St. Louis, United States), under stirring at 25 °C for 12 h. After this procedure, the mixture was placed in a close container at 4 °C where 4.0 mL of chlorosulfonic acid (HSO_3Cl) (Sigma-Aldrich, St. Louis, United States) were slowly added, under stirring in an ice bath. After the acid was added, the solution was neutralized with a saturated solution of sodium hydrogen carbonate ($NaHCO_3$) (Dinâmica, São Paulo, Brazil). The sulfated pectin (PDN-s) was dialyzed for 120 h and lyophilized.

Polysaccharides characterization

The infrared spectra for the commercial pectin (PC), the pectin from the acid extraction (PDN-a) and the sulfated pectin (PDN-s) were recorded with a Shimadzu IR spectrophotometer (Model IRTracer-100) (Kyoto, Japan) by scanning over a range wavelength from 4000 to 400 cm^{-1} using KBr pellet. The proton nuclear magnetic resonance (^1H NMR) was obtained in a Bruker Avance-DPX 500 (Kyoto, Japan), with the pectin samples (PDN-a and PDN-s) dissolved in deuterium oxide (D_2O) (Sigma-Aldrich, St. Louis, United States) in a concentration of 20 mg mL^{-1}. Samples were left in D_2O for 24 h before analysis. The degree of methylation (DM) of the pectin was obtained by the ratio between the areas under the ^1H NMR peaks of methyl-ester group (I_{COOCH3}) and the total amount of proton H-3 (I_{H-3}), according to equation 1.[25] Due to overlapping signals, it was not possible to precisely calculate the value of I_{COOCH3}, but it was possible to estimate the sum $I_{COOCH3} + I_{H-2}$ (I_{H-2} is the integral of the ^1H NMR signal which matches to the total amount of sugar proton H-2).

$$DM = \frac{I_{COOCH_3} + I_{H-2}}{I_{H-3}} \quad (1)$$

The PDN-a and PDN-s samples were analyzed to find the C, H, S and N contents in a microanalyzer Carlo ERBA EA 1108 (Milan, Italy). The degree of sulfation (DS) was calculated according to the equation 2.

$$DS = [162 \times (\%S/32)]/\{100 - [(80/32) \times)\%S)]\} \quad (2)$$

Atomic force microscopy (AFM) analyzes were performed using a Multimode 8 Microscope (Bruker, CA) (Massachusetts, United States) in Quantitative Nanomechanics mode, using probes model Scanasyst Air (Bruker Probes) with a nominal spring constant of 0.4 N m^{-1}. For each probe, the real spring constant was calibrated by the thermal noise method. Approximately 10 µL of PDN-a and PDN-s solutions were placed on the surface of previously cleaved mica and allowed to dry until a film was formed. Three-dimensional topographic and mechanical maps (256 × 256 samples *per* line) were obtained for each sample.

In vivo assay

Animals and housing conditions

Twenty conventional female mice of Swiss strain, three weeks old, were obtained from the Central Animal Facility of the Federal University of Ceará (Fortaleza, Brazil). The animals were kept in the Biology Department at 23.0 ± 2.0 °C, of 12 h of light/12 h dark photoperiod and controlled humidity between 45-55%. The mice were kept in adequate numbers in polypropylene cages with pine shavings as substrate and water. They were fed (Biobase, Bio-Tec, São Paulo, Brazil) *ad libitum* until they reached an approximate weight of 20 g. All protocols with animals adopted in this work were approved by the Ethics Committee in Animal Research of the Federal University of Ceará under the protocol number 34/09, which follows Law No. 11,794 of October 2008[26] on the use of animals in scientific research in Brazil.

Acute oral toxicity assay

An acute oral toxicity study was performed following the general principles of guideline of the Organization for Economic Cooperation and Development (OECD).[27] Some modifications were made to the method, among them, the inclusion of a control group (vehicle), and the performance of biochemical analyzes of blood, hematology, histopathology, and relative wet weights of the organs in all animals at the end of the test. These modifications may increase our certainty about potential deleterious effects. Female mice (n = 5 *per* group), 5 weeks old and weighing 18-22 g were used. The PDN-a was resuspended in distilled water and administered orally at doses of 5, 50 and 300 mg kg^{-1} of body weight, totaling 3 test groups. Higher doses were not tested because they exceeded the solubility of the sample in water. A control group received only distilled water. All mice were observed within the first hours after PDN-a administration and then twice daily for 14 days. The possible intervention of PDN-a in the natural behavior of mice was observed, as well as evidence of toxicity from the verification of the following symptoms or characteristics: piloerection, anesthesia, motor activity, vocal tremor, touch response, balance, contortions, tremors, ptosis and deformed or dark stools. Body weight (g) of all mice was recorded on days 0, 4, 7, 10 and 14. On day 14, the animals were lightly sedated with halothane (Zeneca, São Paulo, Brazil) and exsanguinated via the retro-orbital sinus to determine hematological and serum biochemical parameters. The animals were sacrificed by cervical dislocation and dissected for observation (monitored by a pathologist) of the anatomical and morphological condition of 16 organs. Then, carefully weighed to obtain the relative wet weight.

Hematology and serum biochemistry

At the end of the test, a part of the blood was collected and kept in heparinized tubes for the determination of hematological parameters. Another part was collected to obtain serum for determination of biochemical parameters. For hematological analysis, an aliquot of 15 µL of blood from each animal was applied to a veterinary hematology analyzer (Sysmex, model 100iV Poch, Diff, Kobe, Japan). The analyzed parameters were number of white blood cells (WBC), number of red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), red cell distribution width measured as coefficient of variation (RDW-CV) and red cell distribution width measured as standard deviation (RDW-SD).

Blood samples for serum biochemistry analyses were kept during 12 h at 4 ºC until complete coagulation. Then, the coagulated blood was centrifuged at 600 rpm for 10 min, and the serum was collected. The following biochemistry parameters were measured: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, creatinine, urea, total protein, albumin, and total cholesterol. All analyses of blood serum were performed using specific kits (Labtest, Minas Gerais, Brazil) for each parameter, according to the instructions of the manufacturer.

Relative organ weights

At the end of the acute toxicity experiment, the animals were carefully dissected. The organs/tissues were checked for appearance (color, presence of stains and/or anatomical irregularities) by a histopathologist, and then weighed. The organs/tissues dissected were brain, thymus, heart, lungs, liver, spleen, pancreas, stomach, small intestine (duodenum, jejunum, and ileum), large intestine, kidneys, bladder, oviduct + uterus and ovaries.

Statistical analyzes of chemical characterization and biological evaluations

Statistical comparisons were designed to determine whether differences in response variables described above between groups were attributable to the PDN-a test compared to the control group. The homogeneity of variance was performed by simple analysis of variance (one-way ANOVA) with the software GraphPad Prism 6.0 (San Diego, CA, USA).[28] Human leucocyte-rich blood from healthy adults was obtained from HEMOCE (blood bank), Fortaleza, Brazil. The use of human samples in this study was approved by the Federal University of Ceará under protocol No. 34/09. In the present study, the cells suspension contained 80-90% neutrophils with a viability of 90 ± 2.0% established by the exclusion with Trypan blue. The neutrophils (2.5×10^6 cells mL^1) were incubated at 37 °C, for 15 min, with the Hanks (the medium, non-treated cells), water (vehicle, negative control), the Triton X-100 (0.2%, positive control), pectin (1, 6.25, 12.5, 25, 50 and 100 μg mL^{-1}) or sulfated pectin (1, 6.25, 12.5, 25 and 50 μg mL^{-1}). Analyzes were performed in triplicate and in separate tubes. After the incubation, the tubes were placed in a centrifuge at 4 °C for 10 min. The supernatants were separated and kept at low temperature for measurement using a spectrophotometer with a wavelength of 340 nm. The liquiform lactate dehydrogenase (LDH) kit (substrate pre-incubated at 37 ºC for 3 min) is responsible for stimulating the activity of LDH. Thus, it is possible to verify the decrease in absorbance in the times of 1 and 3 min after the insertion of the substrate. This is due to the oxidation of nicotinamide adenine dinucleotide (NADH). The decrease occurs because this enzyme is released when lysis or cellular necrosis happen. The LDH activity, according to the kit manual, can be calculated using equation 3.

$$A = \left[\frac{(A_1 - A_2)}{2}\right] \times 1746.03 \quad (3)$$

where: A: LDH enzymatic activity ($U\ L^{-1}$); A_1: absorbance at the time of 1 min; A_2: absorbance at the time of 3 min; 1746.03: current factor calculated by the manufacturer.

The neutrophils (5×10^6 cells mL^{-1}) were suspended in Hanks and incubated for 15 min at 37 °C with water (vehicle, positive control), pectin (1, 6.25, 12.5, 25, 50 and 100 μg mL^{-1}), sulfated pectin (1, 6.25, 12.5, 25 and 50 μg mL^{-1}) or indomethacin (100 μmol L^{-1}, standard drug) (Sigma-Aldrich, St. Louis, United States). Human neutrophils were stimulated by the addition of phorbol myristate acetate (PMA, 0.1 μmol L^{-1}) (Sigma-Aldrich, St. Louis, United States) for 15 min at 37 °C. After incubation, the suspension was centrifuged at 4 ºC for 10 min and the supernatant was separated. Aliquots of the supernatants were added to phosphate-buffered saline (PBS) (100 μL) (Sigma-Aldrich, St. Louis, United States), phosphate buffer (50 μL, pH 7.0) (Sigma-Aldrich, St. Louis, United States) and H_2O_2 (0.012%) (Synth, São Paulo, Brazil). After 5 min at 37 °C, TMB (3,3',5,5'-tetramethylbenzidine, 1.5 mmol L^{-1}, 20 μL) (Sigma-Aldrich, St. Louis, United States) was added, and the reaction was stopped with 30 μL of sulfuric acid (4 mol L^{-1}) (Dinâmica, São Paulo, Brazil). The results are expressed as percentage of release of myeloperoxidase (MPO) by stimulated human neutrophils.

Antiviral activity

Cells and virus

HEp-2 cells (human larynx epithelial cells carcinoma, ATCC CCL-23) were grown in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum (Invitro-gen/Gibco, Waltham, USA), 100 IU mL^{-1} penicillin (Novafarma Ind. Farm., Fortaleza, Brazil), 100 μg mL^{-1} streptomycin (Gibco BRL, Waltham, USA) and 2.5 μg mL^{-1} of amphotericin B (Meizler Biopharma S/A, São Paulo, Brazil). The clinical isolate HSV-1 was provided by the Virology Department (IMPPG/UFRJ, Brazil).

Cytotoxicity assay

The cytotoxicity of the PDN-a and PDN-s were evaluated by MTT (dimethyl-thiazolyl-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, United States) in HEp-2 cells. The 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis as the concentration of the substance capable of reducing the optical density of MTT product by 50% in comparison to control.

Plaque reduction assay (PRA)

The antiviral activity of PDN-a and PDN-s was evaluated by plaque reduction assay according to Melo *et al.*[29] Briefly, cell cultures were infected with HSV-1 and simultaneously treated with varying concentrations of the compounds (100, 50, 25 and 12.5 μg mL^{-1}). The percent of viral inhibition (VI) was calculated as previously described.[30]

The 50% inhibitory concentration (IC_{50}) determined by regression analysis is the concentration capable of reducing virus activity in 50% and the ratio CC_{50}/IC_{50} defined as the selectivity index (SI). The activity was performed under the protocols of the time-of-addition, the inhibition of adsorption and virucidal assay. Time-of-addition assay: varying concentrations of the compounds were added to cell cultures before (1 h), during (time 0 h) and after (+1 h and +2 h) infection.[31] Inhibition of adsorption assay: cell cultures were subjected to various concentrations of compounds simultaneously with viral infection. Before the procedure, the cells were incubated at 4 ºC for 1 h and after that, they were incubated under the same conditions (4 ºC for 1 h).[32] Virucidal assay: virus dilutions were incubated with varying concentrations of the substances before infection.[33] Sodium acyclovir (ACV) (Novafarma Ind. Farm., Brazil) was used as positive control.

For statistical analysis ANOVA followed by Tukey's test (BioEstat 5.0 for Windows XP)[34] were used and $p < 0.05$ was considered significant.

Results and Discussion

Polysaccharide characterization

Several factors can be applied for the optimization in the process of obtaining pectin and may cause changes on the yield and DM, such as effect of temperature, extraction time, pH, and solvent to sample ratio.[35,36] It was observed that extractions obtained at neutral and basic pH presented low yields. Some authors[35] reported that the extraction conditions have effects not only on the extraction itself, but also on the chemical structure and yield of the extracted material. Reduction of the initial extraction pH allows better yields, and their extreme decrease may be disadvantageous since they can accelerate polymer degradation and pectin esterification. An average yield of 19.39% was obtained for the acid-pH sample by means of the extraction and purification procedures.

Through the extraction and purification procedures performed, the average yields described on "Polysaccharide extraction" sub-section were obtained. Our results are remarkably similar to those from some authors[17] (see Table 1).

Table 1. The percentage of yield from different pH values during extraction

Pectin	Mass / g	Yield / %
PDN-a	3.8789	19.39
PDN-n	2.9681	14.84
PDN-b	2.4582	12.29

The FTIR results for the samples PC, PDN-a and PDN-s are shown in Figure S1 (Supplementary Information section), where the bands at 3454 cm^{-1} correspond to the stretching mode vibration of the hydroxyl groups. The band at 2942 cm^{-1} corresponds to the stretching of the C–H bond and the band at 1744 cm^{-1} corresponds to the stretching modes of the esterified carboxyl groups. The other two bands at 1635 and 1419 cm^{-1} correspond to the vibrations of the O=C–O bond and the other two bands at 1100 and 956 cm^{-1} correspond to the digital impression, specific to polysaccharides.[17]

The part of the FTIR spectra from 1500 to 1800 cm^{-1} is of special interest due to the esterification degree evaluation, once it has the absorption bands associated with the carboxylic acid and its esters in the pectin molecule. The presence of sulfonate groups in the PDN-s structure was also confirmed by FTIR spectroscopy. Absorption bands at 827 and 1266 cm^{-1} were found to be very similar to those related in the literature. These values are in accordance with those found by Bae *et al*.[37] They found values of 810 cm^{-1} for vibrations characteristic of the C–O–S bond and 1250 cm^{1} for S=O bonds, thus showing the sulfation of the PDN-a sample. The results on the esterification degree by the integration method are 30.71 by ^{1}H NMR (DM), degree of esterification (DE) (30.28) by FTIR for PDN-a and of 34.15 for PC. There is a correlation between values found for the PC and the PDN-a. Both presented DE lower than 50%, characterized as low DE. The pectin extracted from apple bagasse using the method of citric acid 5%, time of extraction of 30 to 80 min and temperature from 50 to 100 ºC shows low DE (14.3 to 29.4%)[38] and pectin extracted from lemon, in HCl solution pH 1.5 at 85 ºC for one hour shows the esterification degree of 49%.[23] As the biological activities of pectin are related to its structural characteristics, chemical modification suggests an improvement in its properties.

The values observed in Table 2 show that for the PDN-s sample the amount of carbon, nitrogen and hydrogen is reduced. This may be associated with the sulfation that produces the polymer chain degradation.[16] The sulfur content for the samples PDN-s confirms the sulfation, presenting, according to equation 4, the value for DS of 0.65.

$$DS = \frac{1.62 \times S\%}{32 - (1.02 \times S\%)} \quad (4)$$

where S is the content of sulfur.[39,40]

From ^{1}H NMR analysis, the DM of PDN-a was calculated (Figure 1b). The signals from protons H-1 and H-5 adjacent to the ester groups can be seen in the graph of Figure 1b with the shifts at 5.5-5.4 ppm. And the signal

Table 2. Elemental analysis for pectin (PDN-a) and sulfated pectin (PDN-s)

Sample	Carbon / %	Sulfur / %	Nitrogen / %	Hydrogen / %
PDN-a	30.11	Trace	6.35	5.50
PDN-s	9.46	9.72	0.66	2.40

of H-5 next to the carboxylate groups (I_{COO^-}) used to be around 4.8 ppm. These signals (H-1 and H-5) should appear around 5.2 and 4.9 ppm, respectively.

The displacement observed is caused by the conditions of storage and conservation of the sample prepared before analysis. Five signals can be seen in the ^{1}H NMR spectra (Figure 1b) and are attributed to protons in the methoxy groups of esterified pectin (H-2) at 3.78 ppm. The signal at 3.93 ppm was assigned to H-3. The H-4 appears as two signals at 4.29 and 4.45 ppm. The protons adjacent to the ester groups (H-5) had shifted to 5.1, and H-1 appears at 5.26 and 5.33 ppm.

Atomic force microscopy (AFM)

The AFM can be used to determine the ultrastructure and aggregation pattern of pectin molecules due to its ability to obtain images in nanometric and subnanometric resolution. Figure 2 shows 2D topographic images of the PDN-a and PDN-s samples (Figures 2a and 2b) and their respective three-dimensional topographies (Figures 2c and 2d).

The topography maps of the PDN-a sample reveal a reticulated structure, formed by fibers with approximately 50 nm in diameter. The PDN-s sample does not present the reticulated pattern, suggesting a globular arrangement with ultra-structures of a few nanometers in height (as observed in the z scale bar of Figure 2b). AFM images suggest that the sulfation procedure of pectins extracted from noni pulp results in a depolymerization process, associated with increased solubilization of the PDN-s sample. The depolymerization process is associated with the arrangement of the PDN film. From the z scale bar observed in the PDN-a sample (Figures 2a, 2c) there is a difference in the z axis that can reach 102 nm, while for the PDN-s sample (Figures 2b, 2d), the difference in height between the higher and lower structure on image does not exceed 11.1 nm. The increase in the solubilization and depolymerization is generally correlated with the decrease of the strength on the tissue.

Figure 3 shows AFM data that reveals changes in the ultrastructural arrangement of the noni pectin after the sulfation process. In Figure 3a it is possible to observe twisted chains with a diameter of approximately 50 nm, as shown in the cross section of Figure 3e (corresponding to the region marked in Figure 3a with the dotted line). Figure 3c shows the adhesion force map of the region corresponding to the image shown in Figure 3a. The adhesion maps are excellent indicative of changes in the properties of a sample, not only revealing materials with different composition (different contrasts in the adhesion map), as changes in a material after a process (as in the case of noni, after sulfation). In the PDN-a sample, the adhesion force map (Figure 3c) has maximum adhesion force with the AFM probe of up to 3 nN. On the chains (zoom in the region of Figure 3c corresponding to the green dotted square), it is possible to observe different adhesion contrasts, with a globular structure (Figure 3g, blue arrows).

Figure 3b of the PDN-s sample reveals structures of a smaller diameter than those observed in the non-sulfated sample. The arrangement of these structures suggests molecular groupings, as shown in Figure 3g, corresponding to the region bounded by the green dotted square in Figure 3b. The adhesion map for the corresponding region in Figure 3b shows that the adhesion forces are lower for the sulfated sample (with maximum values of approximately

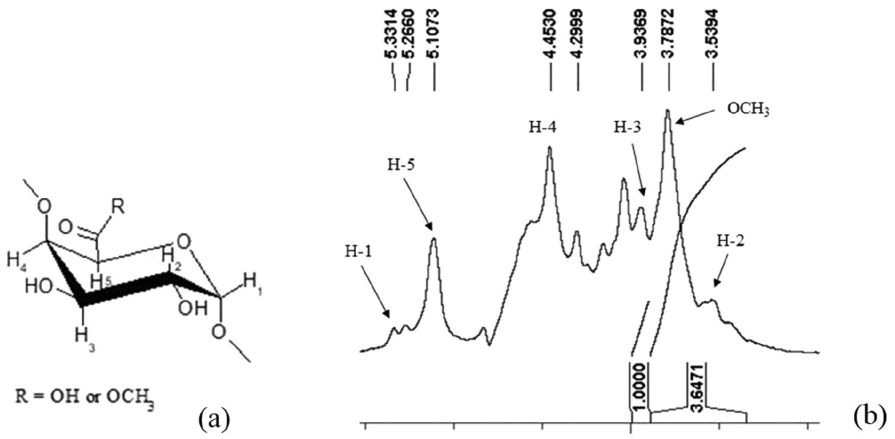

Figure 1. Chemical structure of the monomer unit of pectin (a) and expansion of a region of the ^{1}H NMR spectrum (300 MHz, D_2O) of PDN-a sample showing the peak integrals used for DM estimation (b).

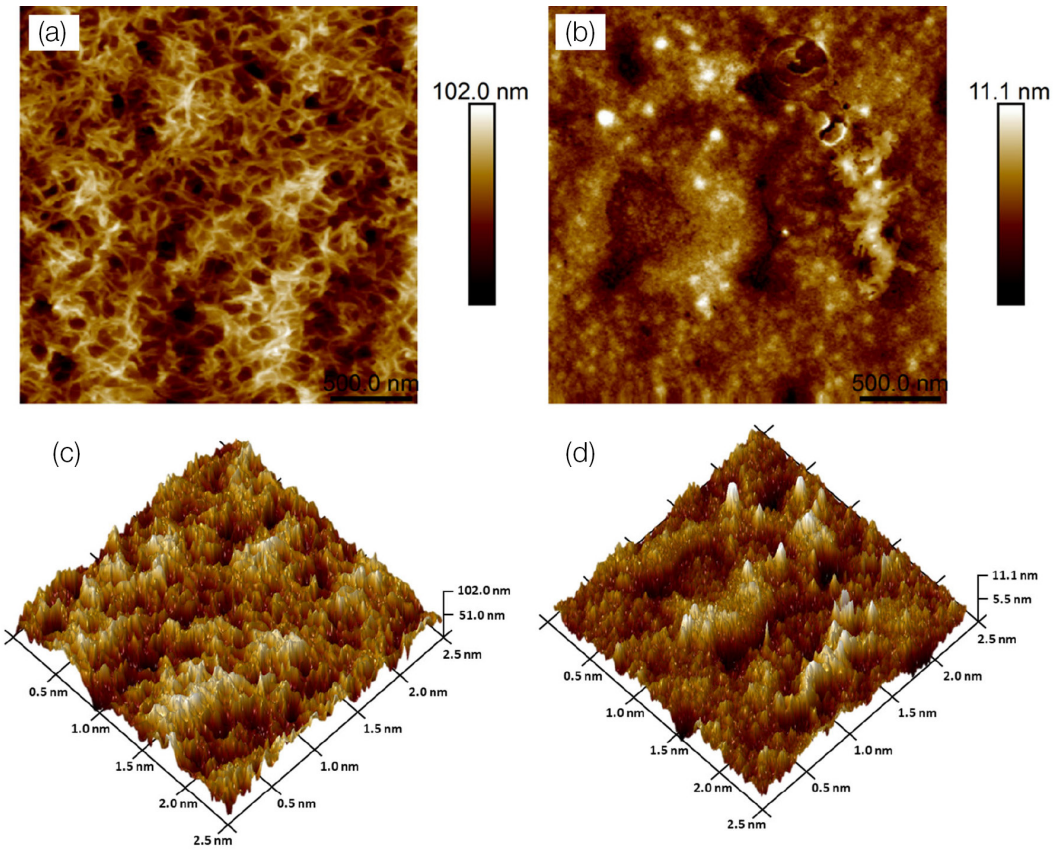

Figure 2. Topographic AFM maps (2 µm scan size) of the PDN-a (a) and PDN-s (b) samples and their respective three-dimensional maps (c, d). The scale bars represent the heights (maximum values) of the structures observed in the images.

350 pN), suggesting that the properties of this sample are different from the non-sulfated sample.

In vivo assay

The acute oral administration of PDN-a in single doses of 5, 50 and 300 mg kg^{-1} resulted in no mortality or any apparent symptoms of systemic toxicity or behavioral changes in mice. In addition, mice in the test groups showed no significant ($p > 0.05$) changes in the body weight gain when compared to the control group (water) over 14 days (Figure S2, Supplementary Information section). Any significant reduction in body weight of the animals in the test groups could be indicative of toxic and/or antinutritional effects inherent to the samples tested.[41] Based on the mortality data, it is possible to affirm preliminarily that the median lethal dose (LD_{50}) of the PDN-a is > 300 mg kg^{-1} being therefore classified in the categories of lower acute oral toxicity hazard (category 4 or higher) according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS).[42]

The results of hematological and biochemical parameters measured, at the end of the experiment (14th day), are listed in Tables S1 and S2 (Supplementary Information section), respectively. For the hematological parameters, just the platelet counting had a significant ($p < 0.05$) increase in the group administered with the PDN-a, at 50 mg kg^{-1}. However, this result seems not to be significant since in the highest dose tested (300 mg kg^{-1}) the platelets number did not change ($p > 0.05$).

Regarding the data on serum biochemical parameters (Table S2), differences ($p < 0.05$) in ALT, AST, creatinine and urea between the PDN-a and control group were observed. Nevertheless, these differences were not dose-dependent related to any of the parameters analyzed. For albumin, total protein, and cholesterol, no changes related to the control group (water) were detected in the test groups.

The sample did not affect the relative organs weight of mice, except of the thymus and pancreas (Table S3, Supplementary Information section). For the thymus, it was not possible to establish a crescent correlation between the sample tested and increase/decrease of this organ. As for the relative wet weight of pancreas, it is difficult to correlate any toxic effect to PDN-a since the larger concentrations tested (50 and 300 mg kg^{-1}) did not present adverse effects. Changes in organ weights (increase or decrease), especially in organs involved in detoxification processes, may represent inherent toxic effects of the administered

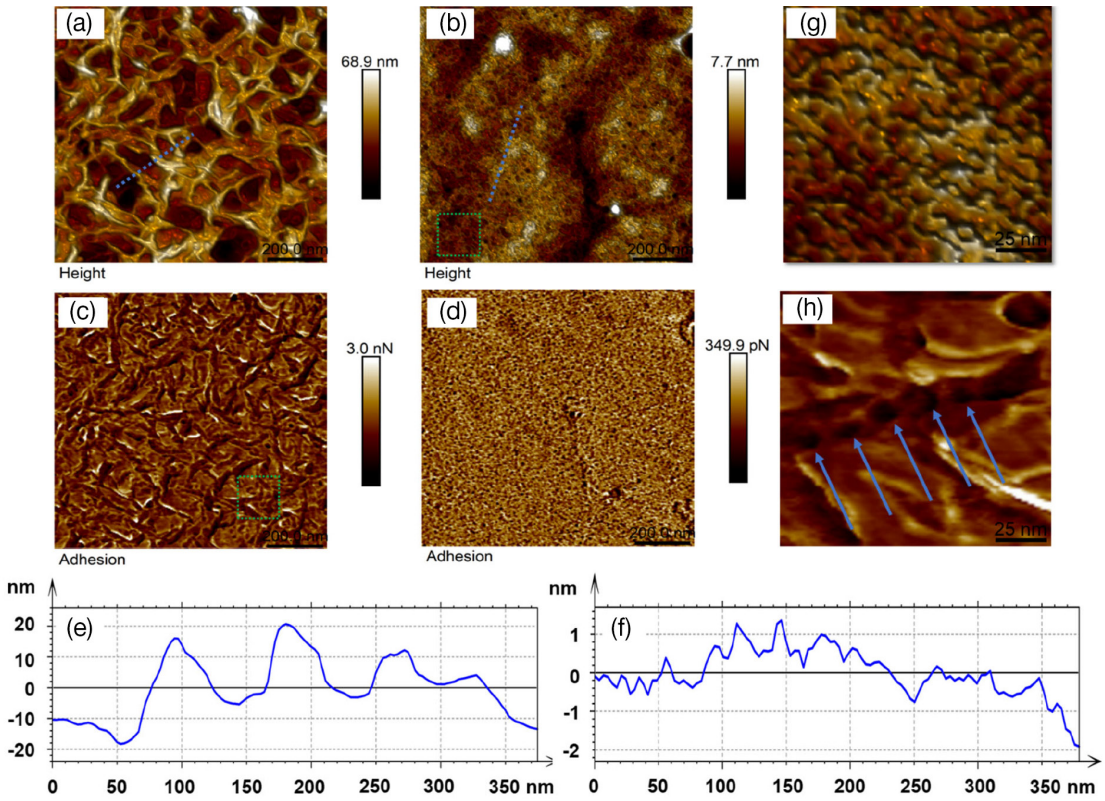

Figure 3. Topographic images of the PDN-a (a) and PDN-s (b) samples. The scan has 1 × 1 μm. Adhesion maps (c and d) corresponding to the scan regions shown in (a) and (b). (e) Cross section of the blue dotted line in (a). (f) Cross section of the blue dotted line in image (b). (g) Image corresponding to the region of the green dotted square observed in image (b). (h) Adhesion map corresponding to the detail shown in (c) de-limited by the green dotted square. The blue arrows show globular structures that make up the chains that form the network observed in the PDN-a sample.

substances when compared to control samples.[41] Regarding the punctual changes observed in some parameters discussed above, they could not be consistently assigned to the tested sample. Such variations presented within specific groups are commonly identified in mammalian toxicity studies, especially when specific strains are used.[43]

Biological evaluation on human neutrophil

LDH activity

As shown in Figure S3 (Supplementary Information section), the pectin and sulfated pectin at the concentrations investigated did not interfere significantly in the LDH activity released by human neutrophils when compared to the control group. The positive control, 0.2% Triton X-100 (cytotoxic standard) increases the LDH activity.

The enzyme LDH occurs in the cytoplasm, being used as a marker to assess possible damage in the plasma membrane. So, when its activity is significantly increased in the extracellular environment it means that the plasma membrane suffers an injury.[44] Therefore, based on the obtained results, both pectin and sulfated pectin seems not to be toxic to human neutrophil plasma membranes.

Degranulation assay

Pectin was able to induce release of the MPO enzyme (1 and 6.25 μg mL^{-1}) exhibiting a pro-inflammatory effect. On the other hand, the sulfated pectin at higher concentration (25 and 50 μg mL^{-1}) showed anti-inflammatory effect reducing significantly the MPO release induced by PMA ($p < 0.05$) (Figure 4). Neutrophils are the most abundant leukocytes in the human body and constitute the first line of defense of innate immunity against invading pathogens, but it also contributes to the adaptive immune responses.[45] The neutrophil azurophilic granules is the major source of MPO which has a key role in the phagocytosis producing a microbicide agent (hypochlorous acid-HOCl), using H_2O_2 as substrate. The phagocytosis is one of the major antimicrobial mechanisms of defense of the human organism, acting at the early period of infection, when the humoral response and T-cell-mediated immunity are beginning.

A previous study[46] showed that neutrophils activated by the supernatants of human corneal cells treated with virus components inhibited the virus growth through the release of various mediators, including MPO, H_2O_2, nitric oxide and chemokines, such as monokine induced by interferons and interferon-inducible protein 10 (IP-10). These data demonstrate that neutrophil acts not only as phagocytic cell

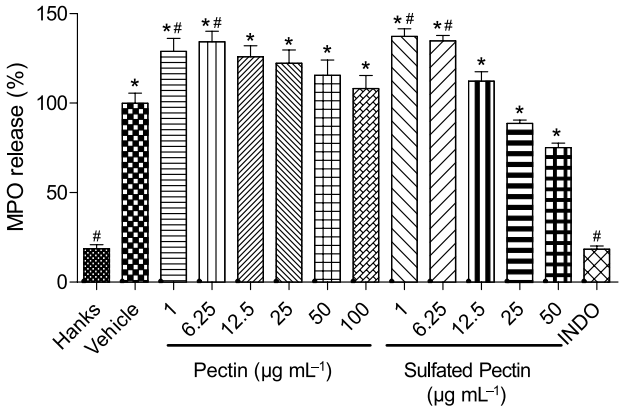

Figure 4. Effects of pectin and sulfated pectin on the release of the myeloperoxidase enzyme in human neutrophils stimulated by PMA (0.1 µM). Hanks: non-treated cells, vehicle: water (control group), INDO (indomethacin, 100 µM). **vs*. Hanks (untreated cells) #*vs*. vehicle (positive control). Results represent means ± equine protozoal myeloencephalitis. ($p < 0.05$; ANOVA and Tukey's post hoc test).

but also as a source of lymphocyte-recruiting chemokines which contribute to the adaptive immune response for viral infections, including the herpes simplex virus type 1. In the present study, the addition of sulfated pectin or non-sulfated pectin in neutrophils suspension treated with PMA induced a similar response pattern. They significantly increased the activation status of cells induced by PMA at lowest concentrations (pro-inflammatory response), while in the highest concentration it was observed a tendency to reduce the cell activation (non-sulfated pectin) or inhibit it significantly (anti-inflammatory response) as observed by the addition of sulfated pectin. Studies[47,48] have shown the biological potential of polysaccharides such as anti-viral, anti-tumor, anti-inflammatory and as stimulating of the immune response. Some studies[49] highlight the potential immunostimulatory effect of pectin on immune cells. Microparticle systems containing pectin and propolis extract were able to increase the generation of HOCl in human neutrophils.[50] In other reports, pectin isolated from *Bergenia crassifolia* or *Lavandula angustifolia* increased the phagocytic activity of cells increasing the production of reactive oxygen species and nitrite production in murine macrophages.[51] A previous study[52] developed by our group determined the anti-inflammatory, hypoglycemic and hypotriglyceridemic effects of pectin from *Passiflora edulis*. There is evidence that sulfated polysaccharides may purpose new or stronger biological activities when related to non-sulfated. Wang *et al*.[53] demonstrated that sulfation of rice bran polysaccharide improves its antitumor activity, while Li *et al*.[54] showed that sulfated polysaccharide from *Enteromorpha prolifera* presented higher free radical scavenging activity than non-sulfated polysaccharides determined through superoxide anion, hydroxyl, and 2,2-diphenyl-1-picrylhydrazyl assays. Thus, our results suggest that the sulfation of pectin allowed it to show anti-inflammatory activity.

Antiviral activity

The CC_{50} for PDN-a and PDN-s were 605 and 44 µg mL^{-1}, respectively. Both compounds showed IC_{50} approximately 46 µg mL^{-1}, and, therefore, PDN-s was excluded (CC_{50} almost equal to IC_{50}). Alternatively, the PDN-a demonstrated significant antiviral activity when used concomitantly with viral infection, reaching 100% of viral inhibition at 100 µg mL^{-1} ($p < 0.05$). This activity was maintained when the compound was added 1 and 2 h after infection ($p < 0.05$). No inhibition was observed when cells were treated before infection or in virucidal and inhibition of adsorption assays. These data are summarized in Table S4 (Supplementary Information section). The pectin (PDN-a) demonstrated anti-HSV activity when added during and after the infection, even at low concentrations (Figure S4, Supplementary Information section). The antiviral properties of polysaccharides mainly involve blocking virus attachment and penetration due to the interaction of their negatively charged polyanions with positively charged domains of viral glycoprotein spikes. Despite this being one of the main targets of antiviral drugs, sterically suppressing the binding of the virus to the host cell, our results demonstrated the action of PDN-a in later stages of viral adsorption. Similar results were suggested by other natural or chemically modified polysaccharides, possibly associated with immunomodulatory properties and/or changes in intracellular or transmembrane signaling pathways, blocking viral replication.[55-59]

Conclusions

Extraction with oxalate of ammonia seems appropriate for the recovery of noni pectin relative to income, on certain conditions (pH 3.0 at 80 °C for 60 min) compared to other pH values (7 and 10). Both FTIR and ^{1}H NMR spectroscopy showed very efficient characterization of noni pectin and sulfated pectin, enabling the determination of the degree of methylation (30.3 and 30.7, respectively). The elemental analysis confirmed the procedure of sulfation of the sample, since the AFM analysis confirmed the structural differences between both pectins studied (PDN-a and PDN-s). The pectin (PDN-a) did not cause deaths or apparent symptoms of toxicity in mice, even after oral administration of the highest dose tested (300 mg kg^{-1}). Thus, the LD_{50} of PDN-a > 300 mg kg^{-1} in mice. Regarding the cytotoxicity assessment, pectins (PDN-a and PDN-s) did not affect the viability of neutrophils measured by LDH activity. Pectin (PDN-a) shows a pro-inflammatory effect, while the

sulfated pectin (PDN-s) shows an anti-inflammatory effect. The pectin (PDN-a) demonstrated anti-HSV activity when added during the infection and post-infection treatment was also effective even at lower concentrations. The sulfated pectin (PDN-s) was excluded for its toxicity. Therefore, a potential antiviral activity is foreseen for PDN-a and further studies must be carried out to get a better insight on how pectin acts in the replication of HSV-1. By extension, other enveloped viruses could be elicited with no drug with significant clinical effect yet.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Nágila M. P. S. Ricardo, Denilton G. Santos, Arcelina P. Cunha and Ana C. B. Ribeiro were responsible for conceptualization; Davi F. Farias, Ana F. F. U. Carvalho, João A. C. Sousa for methodology; Denilton G. Santos and Z. Z. for validation; Luciana M. R. Alencar for formal analysis; Luzia K. A. M. Leal, Nayara Lopes, Rosa E. C. Linhares, Carlos Nozawa for investigation; Nágila M. P. S. Ricardo for resources; Maria Elenir N. P. Ribeiro, X. X. for resources; Débora H. A. Brito, Nágila M. P. S. Ricardo for writing-original draft preparation; Nágila M. P. S. Ricardo, Débora H. A. Brito for writing - review and editing; Antonia F. J. Uchoa, Maria E. N. P. Ribeiro for visualization; Nágila M. P. S. Ricardo for supervision; Nágila M. P. S. Ricardo for project administration; Nágila M. P. S. Ricardo for funding acquisition. All authors have read and agreed to the published version of the manuscript.

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