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Pectin-like Polysaccharide Extracted from the Leaves *Caesalpinia pulcherrima* is a Promising Antioxidant and Immunomodulator Agent

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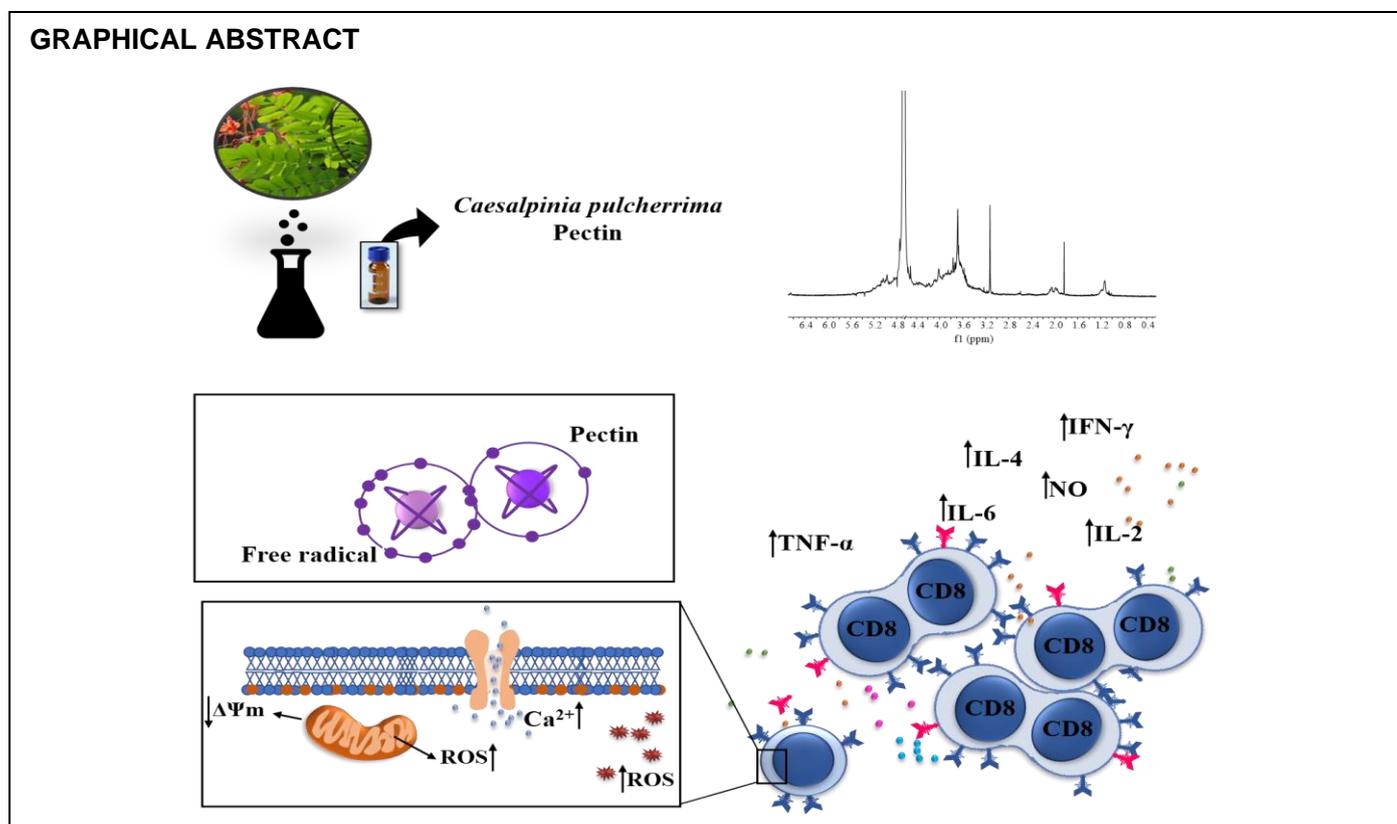*Correspondence: iranildoj@gmail.com; Tel: +558121268866; (I.J.C.F.).

HIGHLIGHTS

- The pectin-like polysaccharide was isolated from the *Caesalpinia pulcherrima* leaves.

- Showed promising results of antioxidant activity in *in vitro* assays.

- The polysaccharide is non-toxic, stimulates cell activation and cytokine production in *in vitro*.

- Promising therapeutic agent.

Abstract: Studies have shown that *Caesalpinia pulcherrima* extracts promote antioxidant, healing, immunomodulating and antiparasitic activities and its polysaccharides can be used as functional food. In this sense, this work had as objective the isolation and characterization of a polysaccharide-like pectin, extracted from the *C. pulcherrima* leaves and its possible applications as an antioxidant and immunomodulator agent. The molecule was characterized by high performance liquid chromatography, fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy. Its antioxidant potential was evaluated through the methods of phosphomolybdenum, ABTS radical scavenging [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid], DPPH (1,1-diphenyl-2-picrylhydrazyl) and nitric oxide radical. The immunostimulating effects of pectin were tested in splenocytes to evaluate its toxic, proliferative and cell activator and immunomodulatory potential. The polysaccharide obtained has structural characteristics similar to pectins. Pectin showed high *in vitro* antioxidant activity for ABTS radical scavenging, moderate activity for phosphomolybdenum and low activity for DPPH and nitric oxide. In vitro immunomodulation assays showed that pectin obtained did not promote a cytotoxic effect (viability > 90%). The increase in cytosolic ROS levels indicates a possible mechanism of cell activation without causing damage. Immunophenotyping showed that

pectin increased a subpopulation of CD8+ T lymphocytes and monocytes. In addition, it promoted a mostly pro-inflammatory response confirmed by the production of cytokines IL-2, -4, -6, IFN-γ and TNF-α. These results reinforce the ethnopharmacological use of *C. pulcherrima* leaves and expand the use of this plant for future applications as herbal medicines.

Keywords: Oxidative stress; Pro-inflammatory; Cytotoxic; Healing.

GRAPHICAL ABSTRACT

INTRODUCTION

Pectins refer to a family of oligosaccharides and polysaccharides, with common characteristics, however extremely diverse in structure [1]. The pectic skeleton is formed by α-linked galacturonic acid units (1→4), with varying degrees of esterified methyl carboxyl groups [2, 3]. This skeleton can have different side chains, formed by different neutral sugars, such as galactose, rhamnose, arabinose, fucose, among others [2, 1]. Thus, the general structure of pectin can have three different types of chains: homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII).

In general, pectins are extracted with hot dilute acid followed by recovery by alcohol precipitation. However, the use of acids can be harmful (strong acids) to the environment, in addition to increasing the cost of the process as they require additional steps to remove toxic elements [4, 5]. Thus, methodologies that use water as an extraction agent have been gaining prominence, as they do not present additional costs with sample purification, in addition to not requiring neutralization steps [4]. In aqueous extraction as well as in dilute acid, polygalacturonic acid chains, with carboxyl groups variably esterified with methanol, are soluble in water and insoluble in ethanol, which allows the recovery of the macromolecule [4,5].

The composition and structure of pectins are difficult to determine, as they may vary according to the source of pectin origin, and changes may occur during isolation, storage and processing [2, 6]. The diversity of composition and molecular arrangement of pectins enhance their biological properties. In the literature, pectins have an anti-inflammatory and analgesic action [6], gastroprotective [7], immunomodulatory [3], antioxidant activity [3,6], pro-inflammatory activity [3] and anti-tumor activity [8]. These biological activities are directly related to the chemical structure of these polysaccharides, that is, molecular weight, degree of esterification, solubility, free hydroxyl and galacturonic acid contents and different sugars present in the structure [9].

Thus, the search for polysaccharides that have effective biological properties and different plant sources are targets for obtaining these compounds has been growing. Plants in general have in their chemical

composition cellulose, polioses, lignins, extractives and ash. These constituents when isolated can have a multitude of applications.

Among the different species of plants that can be used for medicinal purposes, there is *Caesalpinia pulcherrima*, a small ornamental plant that can reach 3 to 4 meters in height in adulthood. In Brazil, the species found the necessary conditions for the development of its fruits and seeds, which are produced in large quantities throughout the year. In folk medicine it is used in the treatment of various diseases, such as fevers, infections and oral ulcers [3, 4]. In the pharmacological area, extracts obtained from flowers, fruits, seeds, bark, roots and leaves have been used in different activities, such as analgesics, anti-inflammatory [5], antioxidants, immunomodulators [6], anti-ulcer [7], and antifungal activity [10]. The lignin isolated from the leaves of this species was able to promote antioxidant, antifungal and immunostimulating activities in *in vitro* assays [10]. Mineral salts are used as a food supplement, in addition to these constituents, polysaccharides have also stood out for their beneficial effects on human and animal health. Among these we can mention the polysaccharide galactomannans formed by β-D-mannose units, which have been used as food [8, 9], in addition to promoting gastroprotective activity [11].

In this context, it becomes important to investigate the isolation, chemical characterization and evaluate the biological properties of different polysaccharides. Therefore, this work aimed to obtain and physical and chemical characterization of a polysaccharide similar to pectin, obtained from the *C. pulcherrima* leaves. Furthermore, its antioxidant, cytotoxic and immunomodulatory properties were investigated in *in vitro* assays.

MATERIAL AND METHODS

Plant material

The *C. pulcherrima* leaves were collected at the Federal University of Pernambuco - Brazil (8º 3'S, 34º 50'W). The botanical authentication was carried out at the Herbarium Geraldo Mariz of the Botany Department of the Biological Sciences Center (Federal University of Pernambuco), where the exsiccate with the registration number of 82.702 was deposited.

Analysis of the chemical composition of *Caesalpinia pulcherrima* leaves

The chemical composition (contents of extractives, ash, lignin, pectin, cellulose, and hemicellulose) of *C. pulcherrima* leaves was determined by adapting a validated methodology for analyzing the composition of sugarcane biomass proposed by Gouveia and coauthors [12]. The adaptation of the methodology was in relation to the extracting solvents used and the content of pectic polysaccharides (pectin) determined based on the methodology proposed by Habibi and coauthors [13].

Extraction of pectin from *C. pulcherrima* leaves

The dried leaves of *C. pulcherrima* (100 g) were initially ground in a knife mill (Fritsch-pulverisette 14) and sieved to obtain a material with granulation of 0.149 mm. Then, the material was subjected to two extraction steps: the first in a Soxhlet apparatus, using a toluene: ethanol (38:62, v/v) extraction system for 8 hours. This step was performed to remove organic solvent soluble extractives. The resulting solid was dried at 70°C for 48 hours. Afterwards, the solid without extractives was submitted to an aqueous extraction in Erlenmeyer flasks. This extraction was performed under the following conditions: ratio of 1:10 solid: liquid (g/L) at 60°C, 1200 rpm for 4 hours. The soluble fraction obtained was then precipitated with ethanol at a ratio of 1:2 (v/v). The solid pectin precipitate was dried at 70°C for approximately 72 hours according to the methodology of Habibi and coauthors [13]. The yield for obtaining pectin, ie precipitation in ethanol, was determined by Equation 1 proposed by Santos and coauthors [3].

$$\text{Yield (\%)} = \left(\frac{\text{Pectin weight}}{\text{dry leaves weight}}\right) \times 100\% \tag{1}$$

Determination of moisture and ash content present in pectin

The methodology used to determine the moisture and ash contents was proposed by Santos and coauthors [3]. For moisture determination, the constant weight methodology was used, where 2.0 g of pectin were placed in an oven (Tecnal, TE-393/1), at 105 ± 2ºC and weighed at regular intervals of time, until weight constant. The ash content present in the pectin (in triplicate) was also weighed approximately 2.0 g were initially calcined at 300ºC for approximately 1 hour and then at 800ºC for 2 hours. After calcination, the crucibles were cooled in a desiccator and the ash mass determined on an analytical balance (Ohaus, 214CP).

Physico-chemical characterization of pectins from *C. pulcherrima* leaves

Analysis of the composition of monosaccharides present in structure of pectin

The pectin was subjected to acid hydrolysis according to the conditions described by Lefsih and coauthors. [14] with modifications. Hydrolysis was performed at 100 ± 5°C in a thermostatic bath (Nova Instruments - NI 1246) with 2.5 mL of trifluoroacetic acid (4 M) to 25 mg of pectin for 8 hours. After removal, the samples were neutralized with 2.5 mL of ammonium hydroxide (NH_4OH - 2M), made up to 10 mL with distilled water and filtered through a microporous membrane (0.22 μm). The constituents obtained during hydrolysis were analyzed by high-performance liquid chromatography according to Gouveia and coauthors [12]. The identification of monosaccharides (glucose, arabinose, galacturonic acid, xylose and rhamnose) released during hydrolysis was performed using H_2SO_4 5mM as mobile phase (flow rate 0.6 mL/min) in Aminex HPX87H column (Bio-Rad) at 60°C. These monosaccharides were quantified on HPLC (Agilent, series 1100) using a refractive index (IR) detector. The concentration of the degradation products, furfural (for C5 monosaccharides) and 5-hydroxymethylfurfural (HMF) (for C6 monosaccharides) was determined using a reverse phase column (C-18) (Agilent Tecnologies®), with mobile phase formed by a: by a 1:8 acetonitrile-water solution containing 1% acetic acid, injection volume (5 μL), 0.5 mL/min flow and UV/Vis detector (274 nm) at 25°C. The percentage of the main pectin-bound polysaccharides, homogalacturonan (HG) and rhamnogalacturonan-I (RG-I) was given according to the following Equations 2 and 3 [13].

$$HG\ (\%) = Galacturonic\ acid\ (\%) - Rhamnose\ (\%) \tag{2}$$

$$RG\text{-}I\ (\%) = 2\ Rhamnose\ (\%) + Arabinose\ (\%) + Galactose\ (\%) \tag{3}$$

Analysis by Fourier-Transform Infrared Spectroscopy (FTIR) and determination of degree of esterification

FTIR analysis was used to identify the main functional groups present in the pectin structure. For this, 20 mg of pectin was analyzed in a spectrometer Bruker Tensor 27 (Bruker AXS, Inc., Madison, WI, EUA) using an attenuated total reflectance accessory (Platinum ATR). The spectrum was obtained in the spectral range from 4000 to 500 cm^{-1}, with a resolution of 2 cm^{-1} and 20 scans. The degree of esterification was determined according to Santos and coauthors [3]. For this, the integration of the areas of the bands of esterified carboxylic groups, attributed to the axial deformation of the carbonyl group, C=O, and non-esterified carboxylic groups, attributed to the axial deformation of the carboxylate ions, COO-, in the infrared spectrum through Equation 4:

$$DE = \left(\frac{A_{est}}{A_{nest} + A_{est}}\right) * 100\% \tag{4}$$

Where: DE is the degree of esterification (%); Aest, the peak area of the esterified carboxylic groups; Anest, the peak area of the non-esterified carboxylic groups. The values of the areas under the curve were determined using the Spectrus Processor software (ACD LABS).

Nuclear magnetic resonance spectroscopy

Nuclear Magnetic Resonance (NMR) analysis was used to determine the main hydrogens present in the structure and confirm the presence of different functional groups. Therefore, 20 mg of pectin was dissolved in D2O heated to 60°C and subjected to NMR (1H) on a Bruker Avance 300 spectrometer (Bruker AXS, Inc., Madison, WI, USA) operating at the 400 MHz frequency with a 5 mm probe.

Determination of viscosimetric molecular weight

The molecular mass of pectin was determined by viscosity using an Ostwald viscometer. The experimental determination was made by flow time. Pectin was solubilized at different concentrations (1.0 to 9.0 g/L) in water heated to 60°C. The average viscosimetric molecular mass (Mv) of pectin was calculated from the intrinsic viscosity value using Empirical Mark-Houwink-Sakurada Equation 5 [15].

$$[\eta] = K\ (Mv)^a \tag{5}$$

where: [η] is the intrinsic viscosity and K and a are constants corresponding to 1.4x10-6 and 1.34, respectively.

Determination of antioxidant activity *in vitro* promoted by pectin

DPPH radical-scavenging activity

The ability to scavenge the DPPH radical (1,1-diphenyl-2-picrylhydrazyl) was performed according to the method proposed by Santos and coauthors [3] and Cruz-Filho and coauthors [16] with modifications. 0.1mL aliquots of each dilution (concentrations ranged from 3.5 to 500 µg/mL) were added to 3.9 mL of the DPPH solution in methanol (0.06 mM). After 30 minutes, the absorbance was determined at 515 nm in a spectrophotometer (Hewlett-Packard, model 8453) and the radical scavenging capacity was calculated, according to Equation 6. The spectrophotometer blank was methanol and the standards used were ascorbic acid and butylated hydroxytoluene (BHT). The experiments were carried out in triplicate.

$$DPPH(\%)=\left\lfloor\frac{(\text{Control Absorbance - Sample Absorbance})}{\text{Control Absorbance}}\right\rfloor\times100\% \tag{6}$$

ABTS radical cation scavenging activity

The antioxidant capacity was determined by the reduction of the ABTS free radical (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) was performed according to the method proposed by Santos and coauthors [3] and Cruz-Filho and coauthors [16] with few modifications. 150 µL aliquots of each dilution (concentrations ranged from 3.5 to 500 µg/mL) were added to 2850 µL of ABTS solution (7.4 mM). After 6 minutes, a reading was taken in a spectrophotometer (Hewlett-Packard, model 8453) at 734nm. The radical scavenging capacity was calculated in relation to the control (without antioxidant), according to Equation 7. The spectrophotometer blank was methanol. The standards used were ascorbic acid and butylated hydroxytoluene (BHT). The experiments were carried out in triplicate.

$$ABTS\ (\%)=\left\lfloor\frac{(\text{Control Absorbance - Sample Absorbance})}{\text{Control Absorbance}}\right\rfloor\times100\% \tag{7}$$

Phosphomolybdenum Complex Reduction Assay (PCRA)

The determination of the antioxidant activity by the Phosphomolybdenum method was carried out according to the methodology proposed by Cruz-Filho and coauthors [16] with few modifications. Pectin was diluted in distilled water at different concentrations (3.5 to 500 µg/mL) into test tubes, 0.3 mL aliquots of each pectin concentration were added and 3.0 mL of phosphomolybdenum complex reagent solution was added. Tubes were closed and incubated at 95°C for 90 minutes. After cooling, the absorbance reading was performed in a Hewlett-Packard spectrophotometer, model 8453, at a wavelength of 695 nm. The blank consisted of 0.3 mL distilled water and 3.0 mL reagent. Assays were performed in triplicate. The antioxidant capacity was determined according to Equation 8 and the standards used were: ascorbic acid and butylated hydroxytoluene (BHT) at the same concentrations as pectin.

$$PCRA(\%)=\left\lfloor\frac{(\text{Control Absorbance - Sample Absorbance})}{\text{Control Absorbance}}\right\rfloor\times100\% \tag{8}$$

Nitric oxide radical scavenging activity

The determination of the antioxidant activity by the nitric oxide radical scavenging method was carried out according to the methodology proposed by Cruz-Filho and caouthors [16] with few modifications. For the nitric oxide radical scavenging test, pectin was diluted in water at different concentrations (3.5 to 500 µg/mL) and submitted to nitrite analysis by the Griess colorimetric method. Nitric oxide concentration was estimated using a sodium nitrite standard curve (3.12 – 400.0 µM). Assays were analyzed on a microplate spectrophotometer (Thermo Scientific Multiskan GoW / Curvette, Waltham) at 595 nm. The antioxidant capacity was determined according to Equation 9:

$$NO(\%)=\left\lfloor\frac{(\text{Control Absorbance - Sample Absorbance})}{\text{Control Absorbance}}\right\rfloor\times100\% \tag{9}$$

Immunostimulatory activity promoted by pectin from *C. pulcherrima*

Animals, ethical considerations and splenocytes obtainment for cultures

Female BALB/c mice (5 animals aged 8 weeks) were maintained in standard laboratory conditions (20 to 22°C and 12 hours in the day and night cycle) with free access to water and standard diet in the vivarium of the Keizo Immunopathology Laboratory Asami (LIKA), Brazil. The mice were anesthetized with 2% xylazine (10 mg/kg) and 10% ketamine hydrochloride (115 mg/kg), with posterior cervical dislocation, and the spleens were removed aseptically and placed in a culture tube containing RPMI medium. 1640 (Gibco, Invitrogen, Carlsbad, CA) with fetal bovine serum [15]. Splenocytes were obtained by separation on Ficoll-Paque™ Plus (GE Healthcare Life Sciences, Sweden) with density adjusted to 1.077 g/mL according to Cruz-Filho and coauthors [16]. Cell viability was determined by the trypan blue exclusion method, considered only when viability was above 98%. All experimentals procedures were performed in accordance with the Animal Ethics Committee of the Federal University of Pernambuco (protocol number: 0048/2016).

Cell viability test in splenocytes cultures stimulated with pectin

To assess the cytotoxicity promoted by pectin, splenocyte cultures (106 cells/well) were treated with different concentrations of pectin (80; 40; 20; 10; 5.0 and 2.5 µg/mL) for 24 hours in an incubator. CO_2 cells. Then, the cells were centrifuged, stained with propidium iodide (50 µM) for 10 minutes and read in flow cytometry (FACS Calibur platform) in 10.000 events. Data were analyzed using Flowing 2.0.1® software [15].

Cell proliferation analysis

The investigation of the proliferation index of splenocytes promoted by the stimulation of pectin of C. pulcherrima was carried out by the CFSE staining method, described by Cruz-Filho and coauthors [16]. Splenocytes were treated for 24 hours with pectin at concentrations of 10; 5.0 and 2.5 µg/mL. The acquisition was performed on the FACS Calibur (BD®) platform.

Investigation of oxidative stress promoted by pectin in stimulated splenocytes

Splenocytes stimulated with 10 µg/mL pectin or 5 µg/mL Con A (positive control) were investigated for the possible mechanism of cell activation. The experiments were conducted in accordance with Cruz-Filho and coauthors [16]. Cells (106 cells/well) were incubated for 24 hours in RPMI 1640 medium at 5% CO_2. Cells only in the medium were used as a negative control. Dihydroethidium (DHE, Sigma Aldrich®) was used to determine the levels of Reactive Oxygen Species (ROS) and MitoStatus (BD Bioscience®) was used to determine the transmembrane potential. Fluorescence intensity was analyzed by flow cytometry on the FACS Calibur platform with 10.000 events collected, the results were analyzed using Flowing Software 2.5.1®.

Phenotype investigation of lymphocytes and monocytes

The lymphocyte and monocyte immunophenotyping assay were performed according to Melo and coauthors [10] and Santos and coauthors [3]. Splenocytes were also stimulated with 10 µg/mL pectin or 5 µg/mL Con A (positive control) and in medium only (negative control) for 24 hours in RPMI 1640 medium at 5% CO_2. The mouse monoclonal antibodies used were anti-CD4-FITC, anti-CD8-PE for lymphocytes, anti-CD16-PE for natural killer cells and anti-CD16/32-PercyP for monocytes (BD Bioscience®). All data were acquired in flow cytometry using the FACS Calibur platform, the results were analyzed using Flowing Software 2.5.1®.

Investigation of cytokines and nitric oxide production

Cytokines produced by pectin-stimulated cells were determined using the mouse Cytometric Array Kit (CBA) for detection of interleukins IL-2, -4, -6, -10, -17, TNF-α and IFN-γ. All data were acquired in flow cytometry using the FACS Calibur platform and the results were analyzed using Flowing Software 2.5.1® [3]. The nitric oxide released by the same cells was measured by the method of Griess [17], using a standard sodium nitrite curve (3.12 - 400 µM), as follows, ($y = 0.0025x + 0.0249 / R^2 = 0.9116$). The reading was taken on a microplate spectrophotometer (Multiskan FC, Thermo Scientific®) at 595 nm.

Statistical analysis

To test the hypothesis of normality in the variable involved in this study, the Shapiro-Wilke test was applied. The statistical difference between two groups was analyzed by Wilcoxon test and between more than three groups by one-way analysis of variance (ANOVA). They were considered significant. Differences were considered significant when $p < 0.05$. For statistical analysis, the GraphPad Prim 6.00 software was used.

RESULTS

Analysis of the chemical composition of *Caesalpinia pulcherrima* leaves

Table 1 shows the approximate results of the constituents cellulose, hemicellulose, lignin, pectin, extractives and ash present in the *C. pulcherrima* leaves.

Table 1. Chemical composition of *C. pulcherrima* leaves.

Constituents	Percent composition (%)
Cellulose	24.14 ± 1.3
Hemicellulose	15.68 ± 0.4
Lignin	21.45 ± 1.5
Pectin	11.72 ± 0.0
Extractives	15.47 ± 0.3
Ashes	10.72 ± 1.2

* SD – Standard deviation. The analysis was made in triplicate.

Physico-chemical characterization of pectin from *C. pulcherrima* leaves

The yield of obtaining pectin from *C. pulcherrima* after ethanol precipitation was 2.4 ± 0.1% (w/w of dry matter). The ash content obtained in pectin was 0.9 ± 0.0% (w/w of dry matter) and the humidity was 6.5 ± 0.0% (w/w of dry matter). Table 2 shows the composition of monosaccharides and degradation products such as Furfural and 5-hydroxymethylfurfural obtained during the acid hydrolysis of pectin. This pectin has in its polymeric or polysaccharide structure: fragments of homogalacturonan (HG) present in 45.1 ± 0.1% and rhamnogalacturonan I (RGI) 54.3 ± 0.2%. Furthermore, this pectin has in its structure low levels of galacturonic acid (<75%) molecular weight of 22.0 ± 0.5 kDa. Figure 1 shows the FTIR spectrum. The degree of esterification for the pectin under study was 30.1 ± 0.4%. Table 3 shows the band assignments for pectin extracted from *C. pulcherrima* leaves. The nuclear magnetic resonance spectrum (Figure 2, Table 4) was used to infer information about the chemical structure of pectin. The signals obtained are characteristic of a skeleton consisting of galacturonic acid monomers.

Table 2. Composition of monosaccharides, and degradation products obtained during acid hydrolysis of pectin.

Monosaccharide identified in pectin	(% ± SD)
Galacturonic acid	47.75 ± 0.3
Glucose	34.89 ± 0.1
Xylose	6.47 ± 0.2
Rhamnose	2.62 ± 0.2
Arabinose	1.35 ± 0.1
Degradation product during hydrolysis (%)	
5-hydroxymethylfurfural	6.10 ± 0.3
Furfural	0.50 ± 0.0

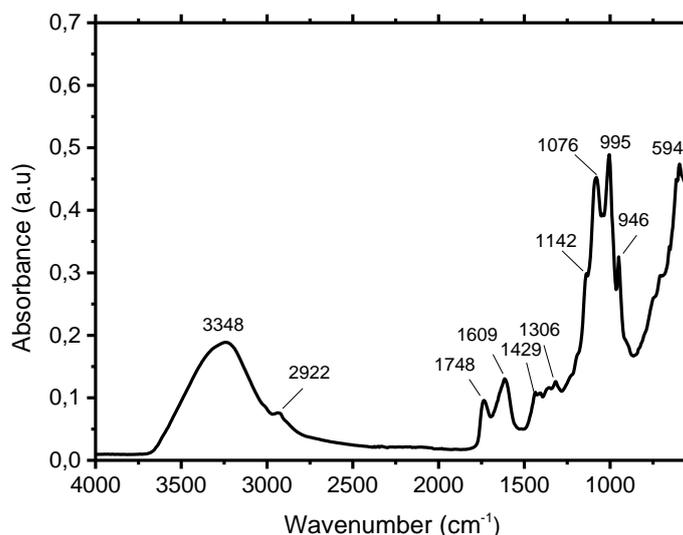* SD – Standard deviation. The analysis was made in triplicate.

Figure 1. FTIR spectrum of the pectin extracted from the *C. pulcherrima* leaves.

Table 3. Assignment of absorption bands in the FT-IR spectra of the *C. pulcherrima* pectin.

Band position (cm^{-1})	Assignment
3420-3300	O-H stretching
2938-2920	$-CH$, $-CH_2$ and $-CH_3$, methyl esters of galacturonic acid
1720-1675	C = O of esterified carboxylic groups ($-COOCH_3$)
1610-1595	free carboxylic groups ($-COOH$)
1430-1422	C–H asymmetric deformation in $-OCH_3$
1306	C-O stretch
1143-1000	(C–O–C) glycosidic bond vibration
1047-900	Deformation vibrations of C-O bands in primary alcohols
1000-600	β-glycosidic and α-glycosidic

References: Santos et al. [3]; Venzon et al. [4]; Sharma et al. [19]; Gnanasambandam and Proctor [25]; Černá et al. [26]; Oberemko et al. [29]
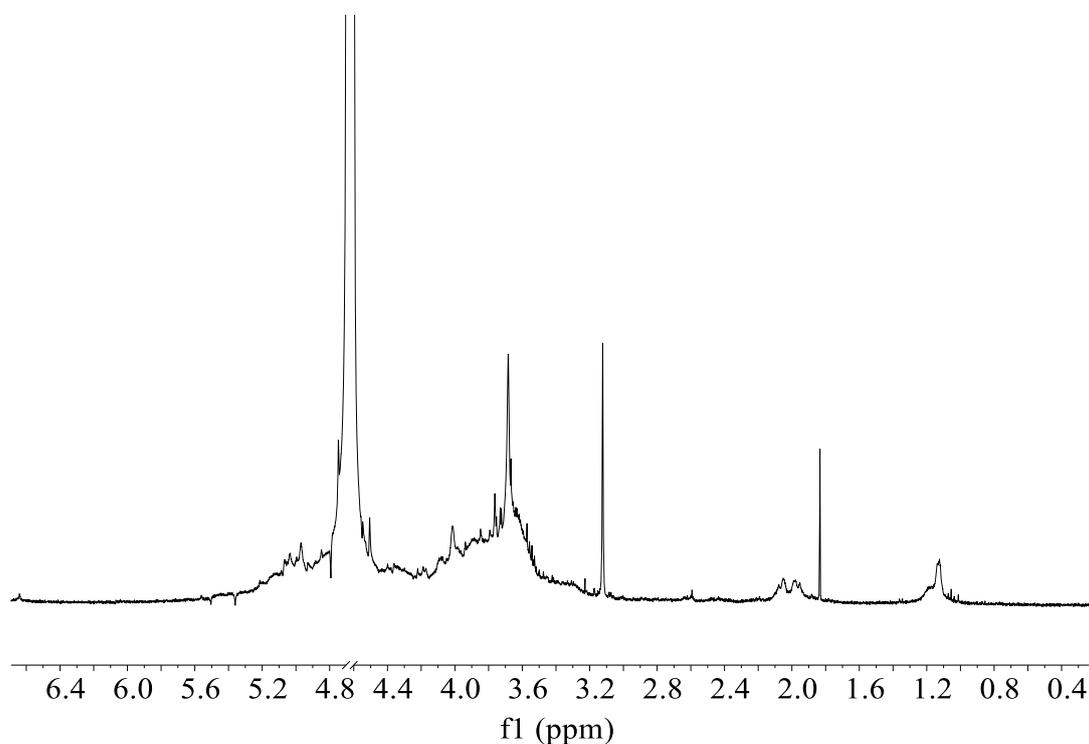

Figure 2. ^{1}H NMR spectrum of the pectin extracted from the *C. pulcherrima* leaves.

Table 4. Signal assignments in the ¹H NMR spectrum of *Caesalpinia pulcherrima* pectin.

Displacement (ppm)	Type of proton
~1.5	Methyl groups
2.12	Acetyl Groups of 2-O- and 3-O-Galacturonic Acids
3.73	H-2 of esterified galacturonic acids methyl groups
3.81	Methyl groups attached to carboxylic groups
3.97	H-3 of esterified galacturonic acids methyl groups
4.30	Deuterated water solvent

References: Marcon et al. [27]; Taboada et al. [28].

Antioxidant activity of *Caesalpinia pulcherrima* pectin

Antioxidant activity was determined through different *in vitro* assays (DPPH, ABTS, NO and PCRA). The standards used for the assay were: ascorbic acid and BHT. Figure 3 shows the curves for each assay of antioxidant activity, indicating the increase in antioxidant activity with increasing concentration of pectin. Table 5 presents the percentage values for each of the assays at the concentration of 500 µg/mL of pectin and the IC_{50} values (Inhibitory concentration or minimum antioxidant equivalent to scavenge 50% of the initial or complex radicals). The pectin in our study showed high scavenging activity for the ABTS radical, moderate activity for the phosphomolybdenum assay and low antioxidant activity for scavenging the nitric oxide and DPPH radical.
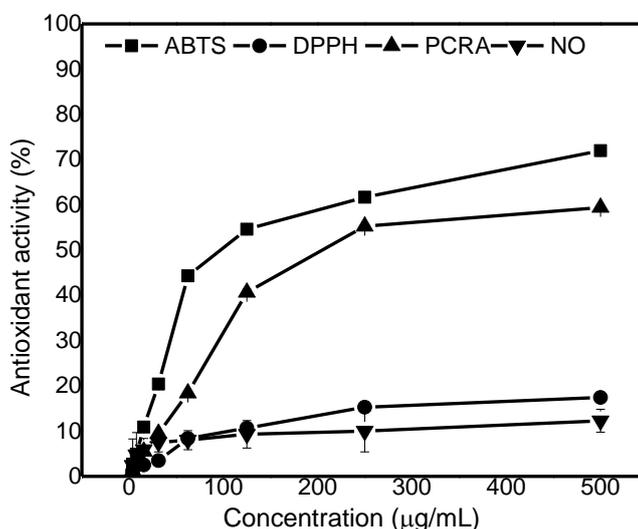

Figure 3. Antioxidant activity curve for the different *in vitro* assays: percentage of antioxidant activity vs pectin concentration.

Table 5. Antioxidant activity promoted by pectin from *C. pulcherrima* leaves.

Sample	ABTS		PCRA		NO	DPPH
	IC₅₀ (µg/mL)	% (0.5 mg/mL)	IC₅₀ (µg/mL)	% (0.5 mg/mL)	% (0.5 mg/mL)	% (0.5 mg/mL)
Pectin	112.05 ± 1.90	72.53 ± 1.60	248.97 ± 0.20	54.86 ± 0.39	11.9 ± 0.30	15.0 ± 0.30
Ascorbic acid	90.93 ± 1.30	90.47 ± 0.34	*Nd*	100	*Nd*	90.04 ± 0.16
BHT	7.67 ± 1.20	95.55 ± 0.07		98.9 ± 0.20	*Nd*	93.13 ± 0.58

PCRA: Phosphomolybdenum complex reduction assay; NO: nitric oxide; Nd: not determined.

Immunostimulatory activity promoted by *C. pulcherrima* pectin

Splenocytes were incubated for 24 hours with different concentrations (80 to 2.5 µg/mL) of pectin, in order to evaluate the cytotoxic effect promoted by this polysaccharide. The results showed that the pectin in this study was not able to cause significant death in animal cells (showing viability greater than 90%) and can be safely used at all concentrations evaluated in this study (Figure 4A). After verifying the high cell viability promoted by pectin, the cell proliferation assay was performed using the fluorescent dye CFSE. For this, concentrations of 10, 5.0 and 2.5 µg/mL were used (concentrations described in the literature capable of promoting cell proliferation *in vitro*). The results showed that pectin was not able to induce cell proliferation under the conditions studied (Figure 4B). In tests for the evaluation of oxidative stress parameters it was found that pectin promoted a significant increase in cytosolic ROS levels (Figure 4C) without promoting significant cell damage (result that can be observed in the evaluation of cell viability). This increase in ROS

levels without promoting cell death indicates a possible cell activation mechanism. Furthermore, it did not promote changes in the membrane potential (Figure 4D) of the cells when compared to the control. These results were compared with the lectin Concanavalin A (ConA), a macromolecule already used as an immunostimulator. The results showed that ConA was able to significantly increase the levels of cytosolic ROS and, in addition to promoting changes in membrane potential, essential events for the activation of immune cells.

Figure 4. Effects of *Caesalpinia pulcherrima* leaf pectin on the viability and proliferation of Splenocyte cells in 24 hours of incubation. A – Cytotoxic effect of pectin in human Splenocytes. B – Proliferation induction by pectin in splenocytes. C – cytosolic reactive oxygen species (ROS) levels, D – membrane mitochondrial potential (ΔΨm). In negative control, cells were incubated only in culture medium while in positive control cells were treated with concanavalin A (5 µg/mL). The bars represent the average of the experiment performed in quintuplicate.

The quantitative evaluation of proliferation by the CFSE method showed that *C. pulcherrima* pectin did not induce cell proliferation at the concentrations tested, however, the cell phenotyping assay showed that pectin influenced cell populations (lymphocytes and monocytes) when compared to the experimental control (only culture medium and cells) and Concanavalin A (ConA). Immunophenotyping assays showed that *C. pulcherrima* pectin significantly influenced the increase in the number of CD8+ T lymphocytes (Figure 5A), when compared to negative (cell culture in medium only) and positive controls (cells treated with ConA). ConA, in turn, influenced the increase in the population of TCD4+ Lymphocytes (Figure 5A). However, it was observed that both *C. pulcherrima* pectin and ConA were not able to promote a significant difference in CD16+ cell populations when compared to the control (only culture medium and cells). In addition, an increase in the population of monocytes (Figure 5B) treated with *C. pulcherrima* pectin was observed when 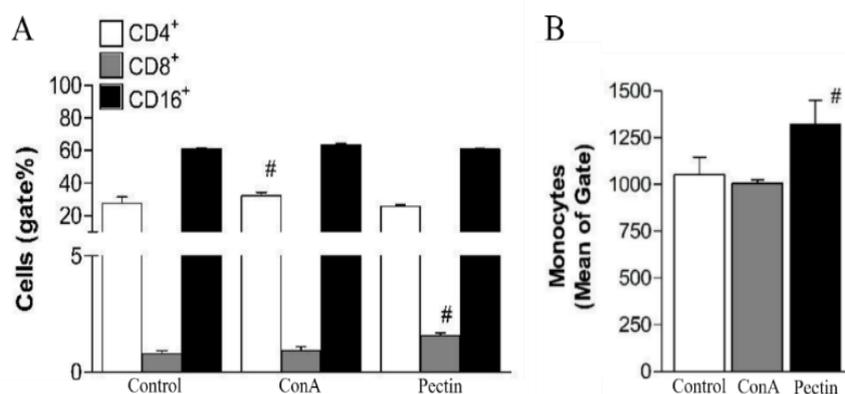compared to ConA and the negative control. CD16+ cell populations when compared to the control (only culture medium and cells). In addition, an increase in the population of monocytes (Figure 5B) treated with *C. pulcherrima* pectin was observed when compared to ConA and the negative control.

Figure 5. Immunophenotyping of T lymphocytes and monocytes in cultures treated with *Caesalpinia pulcherrima* pectin (10 µg/mL). In negative control, cells were incubated only in culture medium while in positive control cells were treated with concanavalin A (5 µg/mL). Vertical bars represent the average of one experiment performed in quintuplicate.

The evaluation of cytokine production showed that pectin-treated cells significantly produced all investigated cytokines: IL-2 (1500 ± 10.9 pg/mL) (Figure 6A); IL-4 (3500 ± 50.8 pg/mL) (Figure 6B); TNF-α (3445 ± 9.8 pg/mL) (Figure 6C); IL-6 (800 ± 4.9 pg/mL) (Figure 6D); IFN- γ (750 ± 14.8 pg/mL) (Figure 6E) compared to the negative control (Figure 6A-E). In addition, it stimulated a significant increase in the release of nitric oxide (34.1± 1.8 µM) when compared to the control (culture medium and cells), however, they expressed the cytokines in smaller amounts when compared to ConA (Figure 6F).
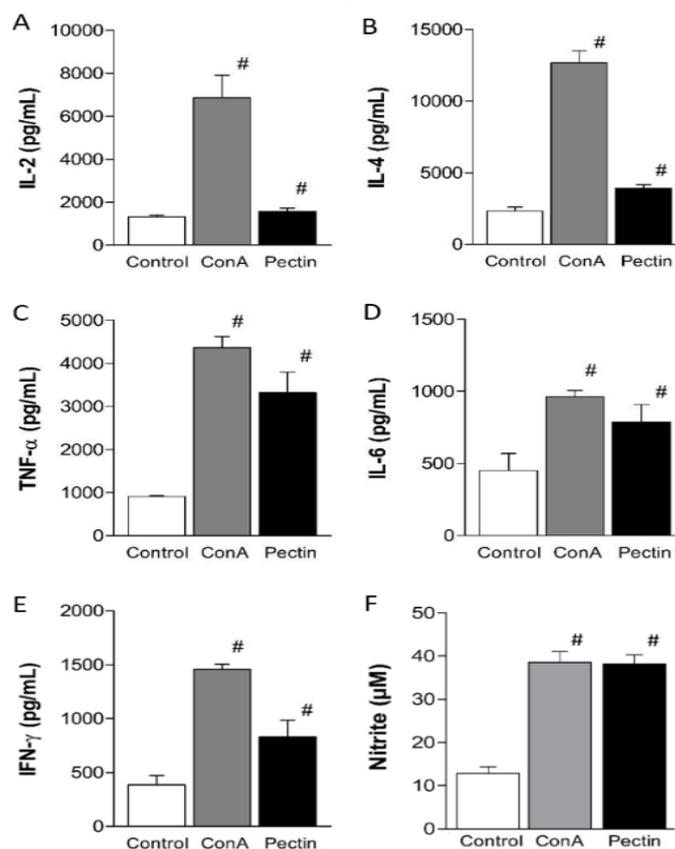

Figure 6. Cytokine (A-E) and nitric oxide (F) splenocytes cultures treated with *Caesalpinia pulcherrima* pectin for 24 hours. In negative control, cells were incubated only in culture medium while in positive control cells were treated with concanavalin A (5 µg/mL). Vertical bars represent the average of the experiment performed in quintuple.

DISCUSSION

The yield for obtaining pectins, moisture content and total ash vary according to the method of extraction, quantification and plant source. The literature presents different results for these parameters. Hosseini, Parastouei and khodaiyan [17] optimizing the extraction process of a pectin obtained from sour cherry pomace obtained yields ranging from 2.0 to 11.80% according to the process conditions. Regarding ash and

moisture contents, the authors obtained values of 3.73% and 8.32%, respectively. Wathoni and coauthors [18] characterizing the pectin of Indonesian mangosteen obtained yield of 1.16 ± 0.17% and the moisture and ash contents of pectin were 9.85 ± 0.12% and 3.91 ± 0.17%. Santos and coauthors [3] characterizing a pectin-like polysaccharide extracted from *Conocarpus erectus* leaves obtained extraction yield of 3.3 ± 0.2% moisture and ash contents were 81.4 ± 0.3% and total ash of 14.7 ± 0.5%. Liu and coauthors [20] extracting pectin from mulberry bark without epidermis and with epidermis obtained extraction yields of 11.88 ± 0.38% and 11.76 ± 0.98%, moisture contents 6.46 ± 0.35% and 6.60 ± 0.21% and ash contents of 1.39 ± 0.06 and 2.08 ± 0.08 respectively.

Regarding the monosaccharide composition, *C. pulcherrima* pectin presented in its structure glucose, xylose, rhamnose, arabinose and galacturonic acid characteristics of the composition of pectins. Other authors who characterize different pectins also found these monosaccharides in the structure in different amounts. Lefsih and coauthors [14] characterizing pectins from the cladodes of *Opuntia ficus indica* obtained: galactose (31.85%), glucose (25.1%), Galacturonic acid (23.2%), arabinose (18.8%) and xylose (1.1%). Al-Amoudi and coauthors [21], characterizing pectins obtained from Mespilus germanica fruits, obtained: galacturonic acid (71.4 ± 4.85%), arabinose (3.28 ± 0.07%), rhamnose (1.06 ± 0.095%), galactose (7.92 ± 0.16%), glucose (3.72 ± 0.07%). Santos and coauthors [3] characterizing a pectin obtained from *Conocarpus erectus* leaves obtained: galacturonic acid (35.98 ± 0.03%), glucose (3.93 ± 0.01%), xylose (14.45 ± 0.06%), rhamnose (9.86 ± 0.1%) and arabinose (16.17 ± 0.5%). The authors did not obtain 100% monosaccharide mass balance, this was expected due to the formation of degradation products obtained during acid hydrolysis [22].

Molecular weight was obtained through the viscosity of pectin using the empirical equation of Mark-Houwink-Sakurada. The pectin in this work had a molecular weight of 22 kDa. This value was similar (22 kDa) to that obtained by Cantu-Jungles and coauthors [7], studying pectins from *Euterpe oleraceae*. It was lower than those obtained by Santos and coauthors [3] that obtain 24.0 ± 0.2 kDa for pectin-like polysaccharide extracted from *Conocarpus erectus* leaves. According to Nascimento and Melo [23] natural pectin masses range from 20 to 360 kDa while commercial samples range from 35 to 120 kDa.

The FTIR results are in agreement with the studies of Venzon and coauthors [4]; Sharma and coauthors [19]; Begum and coauthors [24] and Oberemko and coauthors [29], where the O-H, methyl and carboxylic groups were highlighted. In addition to being underweight, she had a low degree of esterification (< 50%). Low-grade pectins, when in contact with solutions with a pH between 2.5 to 6.5 are able to form gel in the presence of divalent ions, such as calcium [3]. Other authors also obtained a low degree of esterification, Santos and coauthors [3] for pectin from *Conocarpus erectus* leaves (37.5 ± 0.3%) and Bayar and coauthors [30], pectin from the cladodes of *Opuntia ficus indica* (41.42%). This variation may be related to the extraction method, where polygalacturonic acid chains with carboxyl groups variably esterified with methanol are soluble in water, promoting a decrease in the degree of esterification [4,5]. Nuclear magnetic resonance (^{1}H NMR) allowed the identification of methoxy groups and different fragments of galacturonic acid, H-2, H-3. These signs corroborate those obtained by Vriesmann and coauthors [31], Makarova and coauthors [32], Godoi and coauthors [33] and Dias and coauthors [34] featuring different pectins.

The antioxidant and immunomodulatory activities promoted by pectins are directly related to their chemical structure [35]. This is molecular weight, composition of monosaccharides, glycosidic bonds, branches, configuration of the polysaccharide ring, content of free hydroxyl groups, among others [36]. Pectins with higher levels of free hydroxyl groups can promote an increase in antioxidant activity when viscosity is not very high [35]. This fact was confirmed by different works found in the literature, among which the ones carried out by Santos and coauthors [3]. These authors verified that pectin isolated from *Conocarpus erectus* leaves presented promising results of antioxidant activity for the ABTS and DPPH assays. Wathoni and coauthors [18] characterizing Indonesian mangosteen pectin found that pectin had moderate antioxidant activity for the d DPPH assay and for Ro and coauthors [37] characterizing pectins obtained from apple also obtaining promising results of antioxidant activity for the DPPH assay. An advantage of using pectin as antioxidants is the fact that these polysaccharides do not have toxic effects on animal cells [3, 19, 38].

Cytotoxicity assays performed in splenic cells of Balb/c mice showed that pectin from *C. pulcherrima* leaves did not present toxic effects at the concentrations studied. Similar results were observed by Lefsih and coauthors [14], studying the effect of water-soluble pectins of *Opuntia ficus-indica* cladodes by microwave. They found that the pectin fractions obtained exerted cytotoxic activity selectively affecting LAN5 cancer cells, without any effect on normal NIH 3T3 cells. Santos and coauthors [3] verified that pectin obtained from *Conocarpus erectus* leaves also did not promote cytotoxic effects on peripheral blood mononuclear cells. Chen and coauthors [39], verified that polysaccharides isolated from *Abelmoschus*

esculentus L. do not present cytotoxic effects on RAW 264.7 cells at a concentration of 200 µg/mL. Pectin was not able to induce cell proliferation. This fact was also noted by Busato and coauthors [40] which verified in pectin obtained from *Brassica oleracea* did not show cytotoxic effect. Furthermore, it was not able to promote cell proliferation in in vitro assays, at concentrations of 250 and 500µg/mL when compared to the control (ConA). In addition to cytotoxicity and proliferation assays, assays were performed for the determination of reactive oxygen species (ROS) and membrane potential assessment.

Intracellular ROS is an important indicator of immune function activation, as indicated by the immunostimulator ConA used as a positive control in this study. Just like our results, Rocca and coauthors [41], evaluating the effect of pectin-coated nanotubes obtained from apple on RAW 264.7 macrophage cells, they observed a significant increase ($p < 0.05$) in ROS production, without causing cell death. Yang and coauthors [8] demonstrated in their studies that the polysaccharide isolated from the herbal tea *Gynostemma pentaphyllum* in the range of 100 - 400 µg/mL when in contact with macrophages significantly stimulates intracellular ROS increase in a dose-dependent manner ($p < 0.05$), when compared to control. Despite not showing a significant difference in the membrane potential of immune cells, the increase in cytosolic ROS promoted by *C. pulcherrima* pectin induced a mechanism of proliferation and activation of lymphocytes and monocytes. These cell activation results are reinforced by the increased number of CD8+ T lymphocytes and monocytes subpopulation, production of cytokines and nitric oxide, showing that pectin has the ability to activate immune cells to develop an immune response [3].

Regarding the cytokines produced, even pectin is able to stimulate the production of all cytokines analyzed in our study. Thus, it is possible to observe the predominant pro-inflammatory response profile, through the higher levels of cytokines characteristic of the pro-inflammatory response (IL-2, and TNF-α and IFN-γ), when compared to the level of the anti-inflammatory profile cytokine (IL-4). Similar answer was obtained by Santos and coauthors [3] for pectin from *Conocarpus erectus* leaves, where, despite stimulating the high production of all cytokines under study, the pro-inflammatory response was more prevalent when analyzing the relationship of antagonist response cytokines IFN-γ: IL-4 (3:1) and TNF-α: IL 10 (3:1).Yin and coauthors [42] evaluating a water-soluble polysaccharide from the leaves of *Plantago asia* found an increase in the production of pro-inflammatory cytokines. IL-6, a pleiotropic cytokine, is possibly responsible for the induction of proliferation and activation of the CD8+ T cell subpopulation [43]. The production of nitric oxide by cells incubated with pectin was also greater than that measured in the control. Cytokines with a pro-inflammatory profile together with nitric oxide produced at significant levels may be related to a pro-healing action profile [44]. Corroborating our results, the pectin from *Plantago* major leaves has pro-healing properties, in addition to having a mitogenic effect on macrophages and stimulating the production of TNF-α and IL-6 [45].

CONCLUSION

The *C. pulcherrima* pectin promotes antioxidant and immunomodulatory activity, suggesting that the molecule in question may be a compound capable of stimulating immune cells to perform an effector response with a mostly pro-inflammatory profile without causing cell damage, in addition to having the ability to eliminate free radicals, emphasizing its potential for preventing cell damage, and can be considered as a targeted candidate for cell repair.

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