Article

New Insights into the Biological Activities of Carboxymethylated Polysaccharides from *Lasiodiplodia Theobromae*

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Abstract: Lasiodiplodan, an exocellular *Lasiodiplodia theobromae* β-glucan exopolysaccharide (LaEPS), has attracted interest because of its antioxidant, antimicrobial, anti-inflammatory, and antiproliferative properties. LaEPS carboxymethylation enhances LaEPS water solubility and biological activities. However, carboxymethylated fractions of LaEPS (LaEPS-C): LLaEPS-C (Linear) and BLaEPS-C (Branched) were not widely studied yet. So, LaEPS-C, LLaEPS-C, and BLaEPS-C were assessed for their antioxidant, antimicrobial, antiproliferative, anticoagulant, immunomodulatory, and antiviral activities. Elementary Chemical Composition Analysis confirmed their structural characteristics by Energy Dispersive X-Ray Detector (EDS), Fourier Transform Infrared (FTIR), Nuclear Magnetic Resonance (NMR), and Scanning Electron Microscopy (SEM) techniques. The best DPPH scavenging potential was achieved for LLaEPS-C for concentrations lower than 200mg/mL. LaEPS-C showed moderate antiproliferative activity for the NCI-ADR/RES cell (GI_{50} 65.3 μg/mL), and BLaEPS-C showed weak activity for the K562 cell (GI_{50} 235 μg/mL). LLaEPS-C had a reduced Prothrombin Time (PT) and a procoagulant effect. LLaEPS-C and BLaEPS-C are inducers of pro-inflammatory activity due to their ability to induce TNF-α in human macrophages. LLaEPS-C also showed anti-hRSV, which confirmed the antiviral activity of this molecule.

Keywords: Lasiodiplodan; Anticoagulant; Immunomodulatory Activity; Antiviral activities

1. Introduction

Lasiodiplodia theobromae MMBJ is well known virulent plant pathogen in tropic and subtropic areas. This soil-borne fungus infects commodities such as mango, banana, citrus, etc., leading to significant economic loss. It affects plants in various stages, including post-harvest time. Besides its pathogenicity, the fungus was reported to produce valuable natural products [1].

Lasiodiplodans are exopolysaccharides produced by *L. theobromae*. They are glucose monomers with β-(1→3)-D-linkage or β-(1→6)-D-linkage with a triple helix conformation [2,3]. Most of the Lasiodiplodans (67%) were branched (1→3) (1→6)-β-glucans, which were insoluble in water [4]. As expected, *L. theobromae* exopolysaccharide (LaEPS) with linear (LLaEPS) and branched (BLaEPS); differ in conformation, physical properties, binding affinity to receptors, and thus biological functions[5,6].

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The (1→3)-β-D-Glucans that have β-D-glucopyranosyl units attached by (1→6) linkages enhance the immune system. This enhancement results in antitumor, antibacterial, antiviral, anticoagulatory, and wound healing activities. They also suggest that triple helical structures conformation and the presence of hydrophilic groups on the outside surface of the chain are possibly crucial for immune activity [7]. Lasiodiplodan (LaEPS) has been described as having antioxidant [8], pro-inflammatory, and antiproliferative activities [9].

Due to its triple helix structure, formed by the interaction of polyhydroxy groups in the β-D-glucan molecule, it showed reduced water solubility, which restricts its applications and inhibits its physiological function *in vivo* [10]. Carboxymethylation is recognized to be an efficient way to modify the Lasiodiplodan physiochemical properties, improving their biological activities and water solubility. These changes result from the molecular structure's alternation caused by the carboxymethyl group's conjugation [11–13].

LaEPS biological potentialities are reasonably described, but few of these mechanisms of action have yet been fully elucidated [11–13]. Also, which carboxymethylated fractions, linear or branched, have the best biological activities has not been fully established. Therefore, we seek to establish a correlation between LaEPS-C (structures, charge, and conformation) and its fractions and their biological activities: antioxidant, antimicrobial, antiproliferative, anticoagulant, antiviral, and immunomodulatory.

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2. Materials and Methods

LaEPS was carboxymethylated according to the protocol described by Wang and Zhang [15] with minor modifications. LaEPS (250 mg) was suspended in 15 mL isopropanol at room temperature and stirred for 15 min. Ten milliliters of 30% NaOH solution (w/v) were slowly added to the mixture and stirred at 50°C until the complete solubilization of LaEPS. Subsequently, 3g of chloroacetic acid (suspended in a small volume of distilled water) was slowly added while stirring. The reaction was refluxed for eight hours at 50°C. The mixture was cooled to room temperature and neutralized with glacial acetic acid. The resulting solution was dialyzed against distilled water for eight days with frequent water changes with 12-14 kDa membrane and then freeze-dried to yield LaEPS-C.

2.1. Chemical Analysis

2.1.1. Water solubility analysis

The methodology used to compare the LaEPS and LaEPS-C water solubility was adapted from Wang et al [15]. 200 mg samples of both polysaccharides were suspended in 16 mL of distilled water and stirred for 24 hours at 25°C. After centrifugation at 3000g for 15 minutes, the supernatants were collected, and the total sugar content of these samples were quantified using the phenol-sulfuric method. So, 0.5 mL of phenol solution 5% (v/v) and 2.5 ml of concentrated sulfuric acid were added to the 2 mL samples of the supernatants. After cooling to room temperature, their absorbance was read in a spectrophotometer at 490 nm. A standard curve was carried out in triplicate with 0.01 to 0.09 mg/mL glucose solution.

2.1.2 Elementary Composition Analysis by Energy Dispersive X-Ray Detector (EDS)

The concentrations of the sample's constituent elements were obtained from the Oxford X-ray Energy Dispersive Detector, model INCA-act (Cambridge, United Kingdom), coupled to Carl Zeiss benchtop scanning electron microscope, model EVO LS15 (Jena, Germany). The test used an acceleration voltage of 15.0 kV. Four iterations were completed for the LaEPS, and five for the LaEPS-C were performed following SEM analysis.

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2.1.3 Fourier Transform Infrared (FTIR) and Nuclear magnetic resonance (NMR) Spectroscopy

FTIR spectra were obtained from a Bruker FTIR spectrometer (Vertex 70, Billerica, EUA) using a KBr disc and ATR (Attenuated Total Reflection). The equipment was operated with a resolution of 4 cm^{-1}, 64 scans, and a scanning range from 4000 to 500 cm^{-1}. ^{13}C NMR spectra were obtained on a Nuclear Magnetic Resonance Imaging Spectrometer (BRUKER, model Bruker Avance III 600 MHz, 14.1T). Deuterium oxide (D_2O, 99.9%) was used as a solvent for LaEPS and LaEPS-C (10 mg/mL), which were evaluated at 313 K (40 °C). Spectra were recorded using tetramethylsilane as a standard internal reference; chemical shifts (δ) were expressed in ppm relative to the ^{13}C signals.

2.1.4 Analysis by Scanning Electron Microscopy (SEM)

LaEPS and LaEPS-C micrographs were obtained from a Carl Zeiss benchtop Scanning Electron Microscope (model EVO LS15, Jena, Germany) equipped with secondary electron detectors (SE), high vacuum detectors (HV), variable pressure detectors (VP), backscattered electron detectors (BSE) and energy dispersive x-ray detectors (EDS). The LaEPS and LaEPS-C were previously metalized under vacuum using a Quorum gold and carbon vaporizer, model Q150R ES (Eastern Sussex, United Kingdom). Their images were performed with 200, 400, and 1500 amplitudes.

2.2 Biological assays

2.2.1 Determination of DPPH Radical Scavenging

LaEPS-C, LLaEPS-C, and BLaEPS-C radical scavenging activity were estimated with 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to Jing et al. with some modifications [16]. Thus, to an aliquot (2.0 mL) of ethanolic solution of each extract (1.56–15000 μg/mL), DPPH solution (0.5 mL, 0.03% in methanol) was added, followed by vigorous mixing, incubation for 30 min in the dark at room temperature. Absorbances were measured at 517 nm in triplicate, and Ethanol was used as a negative control. Quercetin and gallic acid were used as positive controls. The equation calculated the percentage of DPPH radical scavenging activity of the samples: % Radical Scavenging=(1-sample absorbance/control absorbance)X100.

2.2.2 Minimum Inhibitory Concentration (MIC) and In Vitro Antiproliferative Activity Assay

LaEPS, LaEPS-C, LLaEPS-C, and BLaEPS-C were tested against a yeast *Candida albicans* (ATCC10231), four gram-negative bacteria: *Escherichia coli* (ATCC 11775), *Salmonella choleraesuis* (ATCC 10708), *Pseudomonas aeruginosa* (ATCC 13388), and one gram-positive *Staphylococcus aureus* (ATCC 6538,) [17]. Chloramphenicol (0.5 mg/mL) and Nystatin (1.0 mg/mL) were used as a positive control for bacteria and yeast, respectively.

Antiproliferative activities for LaEPS, LaEPS-C, LLaEPS-C, and BLaEPS-C were assessed by the sulforhodamine B (SBR) assay [18]. In this assay, different human tumor cell lines were used: MCF-7 (breast), NCI-H460 (lung), U-251 (glioma, CNS), 786-0 (kidney), NCI-H460 (lung, non-small cell type), OVCAR-03 (ovarian), HT-29 (colon), K562 (leukemia) and PC-3 (prostate). The concentration samples required to produce total growth inhibition or cytostatic effect were determined through non-linear regression analysis using software ORIGIN 8.6® (OriginLab Corporation, Northampton, MA, USA) by using the concentration-response curve for each cell line.

2.2.3. Anticoagulant activity: Activated Partial Thromboplastin Time (APTT) test and Prothrombin Time (TP)

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The anticoagulant activity was determined using the APTT (Activated Partial Thromboplastin Time) and PT (Prothrombin Time) tests according to the commercial kit (Bios Diagnostica, Sorocaba, São Paulo, Brazil). Different concentrations of LaEPS-C, LLaEPS-C, and BLaEPS-C were incubated (1 min at 37°C) with 90 µL plasma (5-100 µg de EPS/mL de plasma). 10 µL Saline solution or heparin (2, 5, and 10 µg) were used as a negative and positive control, respectively. Rabbit cephalin was added to each sample for 3 min. Time coagulation was counted seconds after adding 0.25 M calcium chloride (APTT) or 20 µL thrombin to each sample (PT). The results were expressed as a polysaccharide(µg)/plasma (mL), considering 300 seconds as the maximum time assay.

2.2.4 Immunomodulatory Activity Assessment

The LaEPS-C potential immunomodulatory activity on cytokine secretion was evaluated in human monocyte-derived macrophage cultures. For this purpose, cells were obtained and prepared according to a previously described method [4] following procedures evaluated and approved by the local Research Ethics Committee of the Faculty of Science and Letters of Assis (approval number: CAAE 68135717.6.0000.5401). As suggested by the Committee, written informed consent was obtained from each volunteer before initiating any research procedures. Monocyte-derived macrophages were incubated with different concentrations of LaEPS-C, LLaEPS-C, and BLaEPS-C for 24 and 48h at 37ºC diluted in RPMI-medium supplemented with 10% FBS for cell viability and cytokine secretion assay. The cell viability was measured using MTT salt as previously described [19]. Quantification of cytokine production was performed by enzyme immunoassay (ELISA) using commercial kits (BD OptEIA™) following manufacturer instructions. It was assayed with TNF-α, a pro-inflammatory cytokine, and IL-10, an anti-inflammatory. Experimental data were evaluated by analysis of variance (one-way ANOVA), followed by the Bonferroni test. A significance level of $P \leq 0.05$ was considered. All assays were in triplicate.

2.2.5. Antiviral activity

Antiviral activity was evaluated by observing the cell viability of Hep-2 cells in the presence or absence of hRSV (human respiratory syncytial virus). The HEp-2 cell monolayer viability was assessed by colorimetric MTT assays [20]. HEp-2 cells (5×10^4/well) were seeded in 96-well plates and infected with hRSV previously incubated with different LaEPS solutions. Hep-2 cells with untreated RSV or Ribavirin-treated RSV were used as positive and negative infection controls. Cells were maintained in culture conditions (at 37 ºC and 5% CO_2) for three days when the antiviral activity was correlated with cellular viability (measured using salt MTT). Experimental data were evaluated by analysis of variance (one-way ANOVA), followed by the Bonferroni test. A significance level of $P \leq 0.05$ was considered. All assays were in triplicate.

3.Results and Discussion

L. theobromae cultivation yielded 5.0 g of LaEPS. From an initial mass of 3.9 g of LaEPS and by the freeze-thawing method, the process of separating constituent β-glucans has produced 1.8 g (44%) of LLaEPS and 1.9 g (56%) BLaEPS, which was a different result from achieved for Oliveira et al. (LLaEPS, 25% and BLaEPS, 75%) [4].

For the LaEPS carboxymethylation, theoretical mass yield resulted in the value of 1.36g LaEPS-C/g LaEPS. When comparing the calculated mass (0.23 g/g) and theoretical (1.36 g/g) yield values, it was observed that the carboxymethylation represented approximately 17% of the theoretical yield [12,21,22].

The LaEPS-C had 3.6 times more soluble sugar (0.68 mg/mL H_2O) than LaEPS (0.2 mg/mL H_2O). Interactions of hydroxyl groups in LaEPS decrease after carboxymethylation,

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producing the molecule more hydrophilic [5,6]. So, the LaEPS carboxymethylation could be associated with changes in the solubility in water.

3.1 Chemical Analysis

From LaEPS-C and LaEPS Elementary Composition Analysis by Energy Dispersive X-Ray Detector (EDS) was observed an increase in oxygen (43.95% to 45.08%) and carbon decrease (55.59% to 52.71%) proportions. These results were related to substituting a hydroxyl (OH) of the glucose ring with a carboxymethyl radical (-CH_2COOH). The percentage of silica (0.24%) resulted from the fungus culture. Na (1.12%) remaining in the LaEPS-C sample is a residue of the carboxymethylation reaction. Also, from fungus culture, LaEPS and LaEPS-C detected magnesium (0.13 and 0.23%) and calcium (0.11 and 0.43%), respectively. The Au percentages in the LaEPS and LaEPS-C (0.22%, 0.19%) correspond to the insertion of the element in the LaEPS metallization procedure under vacuum, so the analysis by Electron Microscopy of Scan could be performed should be disregarded.

3.1.1. Analysis by Fourier Transform Infrared (FTIR) and Nuclear magnetic resonance (NMR) Spectroscopy

For evaluation of the success of LaEPS carboxymethylation, FT-IR and NMR spectroscopic analyses were done. LaEPS and LaEPS-C FTIR spectrum analysis of the absorption bands assigned revealed one typical polymeric structure of the carbohydrate. In the LaEPS-C spectra, two new intermediate absorption bands were observed, in 1604 cm^{-1} and 1421 cm^{-1}, respectively, from stretching the asymmetric and symmetrical COO^-. These bands indicate the occurrence of carboxymethylation of the polysaccharide. A weak absorption band at 1720 cm^{-1} belonging to carboxyl groups suggests that the LaEPS-C sample exists predominantly in the form of a salt [12]. The strong absorption band in the region between 3310 cm^{-1} and 3425 cm^{-1} for both spectra is attributed to OH stretching vibrations. The peak at 2920 cm^{-1} was attributed to the CH stretching of the methylene groups (CH_2). The peak at 1648 cm^{-1} found in the LaEPS spectrum was attributed to the glucose ring. At 1604 cm^{-1}, a higher peak in the LaEPS-C sample can be attributed to the glucose ring associated with the absorption band corresponding to COO^- [23].

The results above, plus Nuclear Magnetic Resonance (NMR), also confirm LaEPS carboxymethylation. The characteristic signals of LAEPS-C were confirmed through chemical shifts of ^{13}C NMR and comparison with literature data [5,6]. Among them, δ 178.1 (carbonyl of the carbomethoxy group) and δ 70.0 were attributed to methylene of the carbomethoxy group. Shift signals for C3 (76.3 to 85.1 ppm) and C4 (71.0 to 79.6 ppm) suggest that this carboxymethylation may have occurred at carbons 3 and 4. The peaks at 102.8 were attributed to glucopyranose units (C-1), 73.3 (C-2), 60.9 (C-5), and 71.2 (C-6) [23,24].

3.1.2. Analysis by Scanning Electron Microscopy (SEM)

As can be seen in Figures 1A, 1C and 1E, LaEPS exhibited structures with different sizes and shapes, similar to leaves. As expected, carboxymethylation promoted morphological changes in the biopolymer and increased porosity [25]. Analyzing one unmodified polysaccharide composed of glucose and rhamnose, an equally structured surface in the form of leaves is formed [16]. LaEPS-C presented a more rigid structure, and their irregular fragments were arranged in the form of cracked plates as described by Kagimura et al. (Figure 1B, 1D, and 1F) [5]. These results suggest that carboxymethylation can produce different surface morphologies in polysaccharides.

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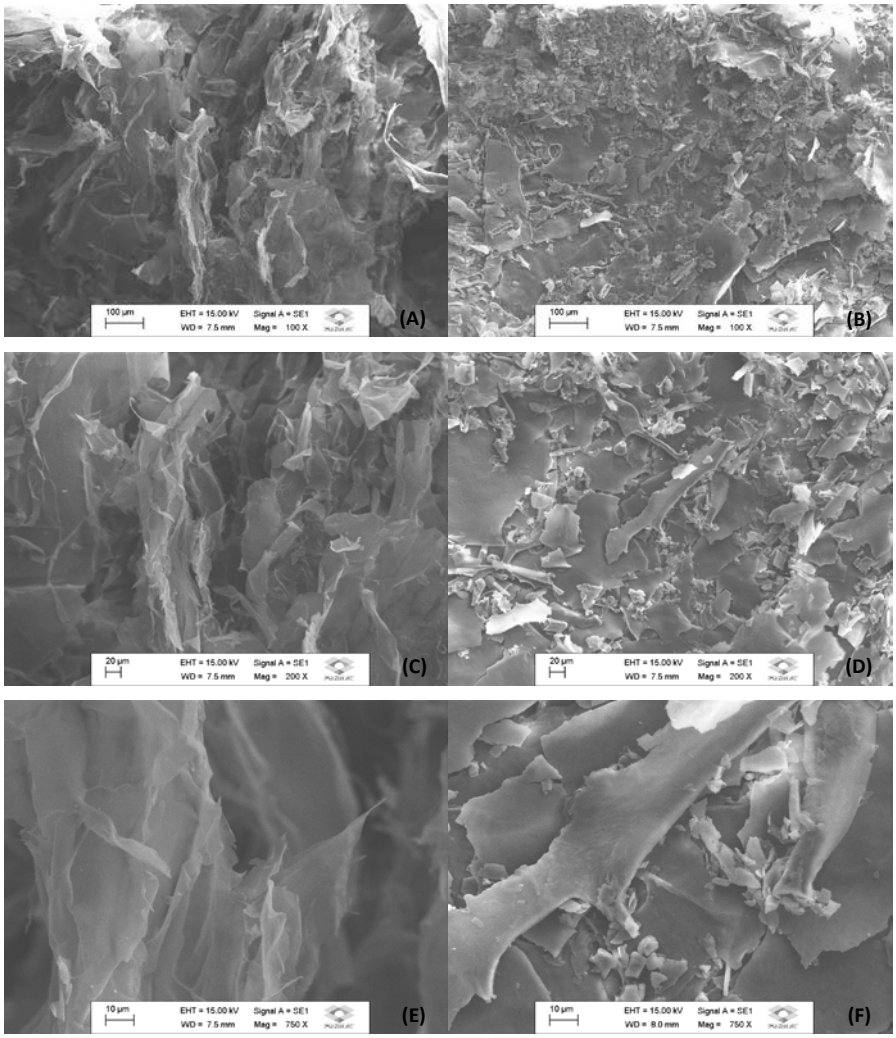

Figure 1. Micrograph of unmodified and carboxymethylated LaEPS obtained by scanning electron microscopy. (A) LaEPS at 200X magnification, (B) LAEPS-C at 200X magnification, (C) LaEPS at 400X magnification, (D) LaEPS-C at 400X magnification, (E) LaEPS at Magnification at 1500X, (F) LaEPS-C at 1500X magnification.

3.2 Biological assays

3.2.1 Determination of DPPH Radical Scavenging

LaEPS is a suitable candidate for antioxidant compounds since they are often non-toxic, compatible, and thermally stable [26]. LaEPS is correlated to antioxidant activity, which is affected by molecular structure and water solubility. Lasiodiplodans of branched structure (BLaEPS), i.e., with (1→3;1→6)-β-D-glucan, showed a high antioxidant effect, but linear Lasiodiplodan (LLaEPS), on the other hand, showed minimal antioxidant activity[8].

LaEPS-C has enhanced antioxidant activity compared to LaEPS due to the increase in water solubility, which results from the structural changes with a decreasing hydrogen bonding and apparent low viscosity [23]. These structural modifications from LaEPS, with electron-donating or electron-withdrawing functional groups, improve their antioxidant activity. The carboxymethyl group increases the electronic cloud along the LaEPS structure, creating the hydroxyl groups more "active". As a result, the potential for hydrogen donation and polysaccharide solubility is improved [6,27].

From Figure 2, it could also be observed that scavenging potential is greater for LLaEPS-C for concentrations lower than 200mg/mL. Since LLaEPS-C has a low steric hindrance, the interactions among the hydroxyl groups of LLaEPS-C with the DPPH radical are more viable than the hydroxyl groups of BLaEPS or LaEPS-C. Between 250 and 2000

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mg/mL LaEPS-C has a better antiradical effect than BLaEPS-C and LLaEPS-C. In addition, above 5000mg/mL, LaEPS-C and BLaEPS steric effects seemed less significant because these glucans have a lower viscosity than LLaEPS-C. So, the radical-polysaccharide interactions are less effective.

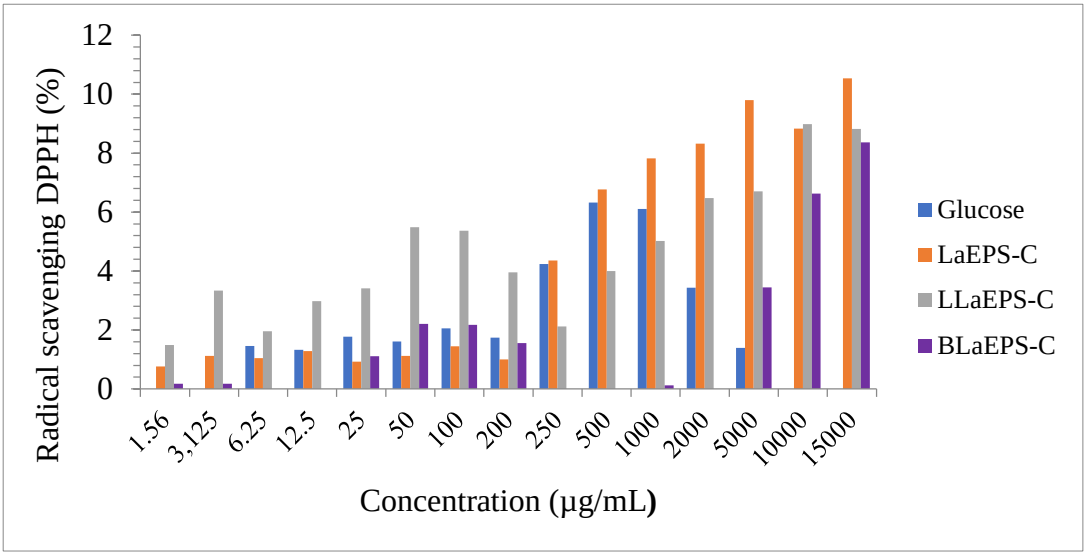

Figure 2. DPPH radical scavenging for LaEPS-C, LLaEPS-C, and BLaEPS-C compared to glucose.

Although the antioxidant effects of polysaccharides are negligible when compared to the standard phenolic compounds such as gallic acid and quercetin, they have, in general, a more significant antioxidant potential than non-polymeric carbohydrates [28](KOZARSKI et al., 2012). Even though phenolics compounds, which are recognized as antioxidants, they have an absence of certain qualities, for example, a molecular size that is suitable for intestinal epithelium absorption, quantity limitations, ability to cross the brain barrier as a drug molecule, etc [26].

3.2.2 Evaluation of antimicrobial and antiproliferative activities

Some natural polysaccharides are reported to have antibacterial (bacteriostatic) activity after carboxymethylation. This effect of carboxymethyl groups occurs due to its increased water-solubility, strength chain stiffness, and changing conformation. LaEPS has described the fungistatic effect against *Candida albicans* and *C. tropicalis* at 0.15 and 0.26 mg/mL concentrations, respectively. LaEPS revealed a bacteriostatic effect against *Listeria monocytogenes* (gram-positive, MIC of 0.15 mg/mL) and a bactericidal effect against *Escherichia coli* (gram-negative, MBC of 0.05 mg/mL) [27]. Carboxymethylated β-glucans (CMGs) of barley bran against *Staphylococcus aureus* presented a MIC value of 20 mg/mL [24]. So, it was expected that LaEPS-C and their fractions might yield candidate compounds for developing new antimicrobial drugs.

For one potential antibiotic application, it showed the following bactericidal classification: weak (MIC≥1.6 mg mL^{-1}), moderate (0.6 mg mL^{-1}≤MIC≤1.5 mg mL^{-1}), or strong (MIC≤0.5 mg mL^{-1}) [29]. From this kind of analysis, LaEPS and LaEPS-C, as well as their fractions LLaEPS-C and BLaEPS-C, did not show bactericidal activity (MIC values ≥1.0 mg/mL) for none of the tested strains.

Likewise, polysaccharides have been attracting attention because of their activity in different types of cancer. They can act as adjuvant medicines combined with chemotherapy/radiotherapy to deal with various cancers [30]. LaEPS had already been shown in

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MCF-7 cells (IC_{50} 100 mg/mL) in a time and dose-dependent manner. It was also demonstrated that LaEPS could decrease cell proliferation by inducing cell cycle arrest, apoptosis, and oxidative stress [31]. In this manuscript, LaEPS-C showed a moderate Growing Inhibition Activity for the NCI-ADR/RES cell (GI_{50} 65.3 μg/mL), and BLaEPS-C showed a weak activity for the K562 cell (GI_{50} of 235 μg/mL). All the others were inactive for the tested strains, including MCF-7 cells. Therefore, these results lead us to infer that LaEPS carboxymethylation changed tumor-cell interaction because of the structural changes with new conformational arrangements.

3.2.3 Anticoagulant activity: Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT) bioassays

Activated partial thromboplastin time (APTT) and Prothrombin time (PT) are valuable tools for diagnosing and monitoring coagulation disorders. The APTT measures the coagulation activity of the intrinsic pathway (coagulation factors II, V, VIII, IX, X, XI, and XII) and common pathways. PT is used to evaluate the extrinsic and common pathways of coagulation, which would detect deficiencies of factors II, V, VII, and X and low fibrinogen concentrations. PT range for healthy donors is between 11 and 13.5, and the time range of APTT is between 25 and 32 s [32,33].

From the genus Botryosphaeria, sulfated β-glucans exert anticoagulant activity because of their negative charge. They were able to prolong the APPT in a concentration-dependent manner, and they showed values >100 seconds for 20 μg/mL [34].

Despite the good activity presented by sulfated β-glucans, the conventional sulfation method has high costs and generates toxic waste due to the use of chlorosulfonic acid and organic solvents, such as pyridine [11]. On the other side, carboxymethylation possesses advantages, such as low-cost reagents, safe and low-toxic reaction products [23]. So, carboxymethylation should also be an alternative to sulfation.

The increase in the density of negative charges also happens in LaEPS-C and promotes electronegativity similar to that found in heparin [35]. But, compared with sulfated β-glucans, carboxymethylated molecules are at a disadvantage in this regard. For instance, the antiplatelet activities of carboxymethylated β-glucans from *Saccharomyces cerevisiae* were only detected for concentrations above 100 μg/mL and antithrombotic activities above 300 μg/mL[36].

There was no significant difference between positive and negative controls for APTT and LaEPS-C and fractions. So, the inhibition of the clotting may be due to an interaction with different coagulation factors of the common pathway.

Prothrombin, also known as clotting factor II, is a protein produced by the liver and, when activated, promotes the conversion of fibrinogen into fibrin, which, together with platelets, forms a layer that prevents bleeding. Thus, prothrombin is an essential factor for blood clotting to take place. So, Prothrombin Time (PT) is the time of blood clotting begins.

Unlike sulfated polysaccharides reported above, the carboxymethylated b-glucans showed activity for PT. LaEPS-C revealed a significant reduction in PT when compared to the negative control and heparin ($p>0,05$) in a dose-dependent fashion from 50 μg/mL (Table 1). BLaEPS-C showed the same effect only at 50 μg/mL (12.28 ± 1,07s), and LLaEPS-C from 5 μg/mL to 25 μg/mL (11.85 ± 0.87s and 14.26 ± 0.10s). The anticoagulant effect of heparin is not mediated by modulation of the extrinsic system, but LaEPS-C and fractions seem to be an inhibitor of this pathway [34]. In LLaEPS-C, the low steric effects plus the carboxymethyl charge seemed to be key factors in reducing Prothrombin Time (PT) and a procoagulant effect. Above 50 μg/mL LLaEPS, its viscosity grows, increasing Prothrombin Time (PT) and reducing the procoagulant effect. Also, at this point, only the charge effect appears to be significant for PT reduction.

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Table 1. Assessment of anticoagulant activity by APTT and PT.

Sample	Concentration (µg/mL)	APTT (s)	PT (s)
Negative Control*	-	38.59 ± 0.60 a	15.04 ± 0.49 a
Heparin	10	34.31 ± 3.38 a	14.05 ± 1.30 a
	25	130.36 ± 4.85 bc	20.69 ± 0.77 bc
	50	>300 bcd	24.76 ± 0.98 bcd
	100	>300 bcde	29.68 ± 0.94 bcde
LaEPS-C	5	36.87 ± 2.79 a	14.90 ± 0.64 a
	10	36.24 ± 0.74 a	14.65 ± 0.90 a
	25	35.59 ± 1.50 a	12.95 ± 0.17 a
	50	31.29 ± 0.23 a	11.73 ± 0.24 b
	100	31.75 ± 0.86 a	10.73 ± 0.63 b
BLaEPS-C	5	38.91 ± 0.45 a	15,73 ± 0,26 a
	10	41.13 ± 1,38 a	14.98 ± 0,85 a
	25	32.29 ± 4,98 a	14.56 ± 1,14 a
	50	32.32 ± 0,65 a	12,28 ± 1,07 b
	100	33.55 ± 4.02 a	13.38 ± 1.86 a
LLaEPS-C	5	44.21 ± 1.92 a	12.63 ± 0.28 b
	10	37.56 ± 1.35 a	11.85 ± 0.87 b
	25	34.69 ± 1.61 a	14.26 ± 0.10 b
	50	35.31 ± 1.82 a	12.51 ± 0.66 a
	100	32.76 ± 0.34 a	13.43 ± 1.24 a

*Blood plasma with saline only (no LaEPS or heparin added)** Clotting time value followed by the same letters do not differ statistically from each other ($p > 0.05$). The reference values for normality are 30-45 seconds and 10-14 seconds for the APTT and PT, respectively.

3.2.4. Immunomodulatory Activity Assessment

Macrophages are essential immune innate cells able to eliminate different microorganisms or tumor cells by cytokines secretion. Here, it was evaluated whether different fractions from LaEPS-C could stimulate macrophages to induce pro- or anti-inflammatory cytokines without cytotoxicity.

The macrophage cell viability culture showed different patterns according to the polysaccharide type (LaEPS-C, LLaEPS-C, or BLaEPS-C), dose, and incubation time. For LaEPS-C, the highest significative toxicity (lowest viability, $p>0.05$) were found at 100 µg/mL for 24h (61.19 ± 46.97%) and 48h (52.84 ± 24.06 %) of incubation time. For LLaEPS-C, there was no significant toxicity found. BLaEPS-C, at 48h, from 25-100µg/mL, showed significant toxicity ($p >0.05$) (Figure 3, Table 2).

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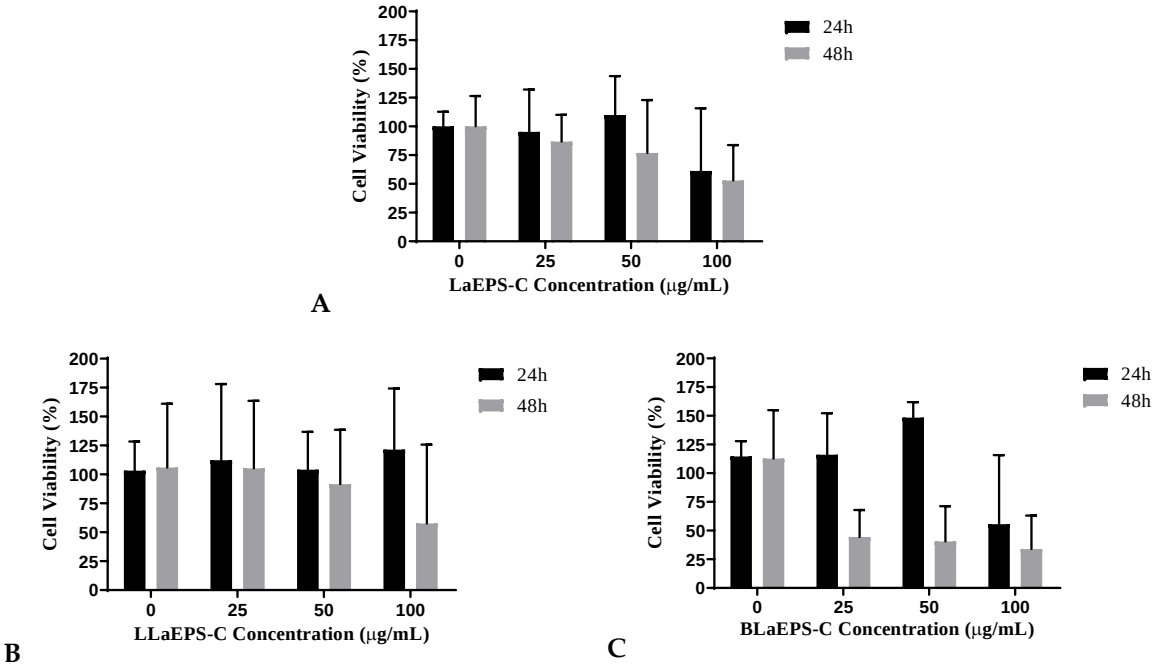

Figure 3. Cell viability analysis by MTT at four different concentrations and two incubation periods. The concentration of 0 µg/mL corresponds to the cell control, and the bars' values correspond to the means. (A) LaEPS-C. (B) LLaEPS-C. (C) BLaEPS-C.

Table 2. Cell viability values of LaEPS-C and their derivatives.

	24h	48h
LaEPS-C (µg/mL)		
0	100.00 ± 9.78a*	100.00 ± 21.87a
25	95.23 ± 28.73a	86.82 ± 18.90ab
50	109. 89 ± 28.85a	76.93 ± 41.69ab
100	61.19 ± 46.97b	52.84 ± 24.06b
LLaEPS-C (µg/mL)		
0	103.33 ± 20.85 a	105.98 ± 39.13a
25	112.33 ± 45.70 a	105.22 ± 48.87a
50	104.06 ± 25.78 a	79.13 ± 31.13a
100	121.40 ± 39.26 a	57.61 ± 46.96a
BLaEPS-C (µg/mL)		
0	114.57 ± 8.44a	112.74 ± 33.68a
25	116.08 ± 31.66a	44.355 ± 20.16b
50	148.49 ± 11.31a	40.645 ± 25.55b
100	55.65 ± 51.13a	33.871 ± 25.16b

* Means, with standard deviation, followed by at least one equal letter, indicate non-significant differences ($p>0.05$).

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The standard curves used to calculate the cytokine concentration (pg/mL) were the following: y = 180.35x – 8.0576 for TNF-α and y = 156.44x +6.4008 for IL-10. The quantification of the production of TNF-α and IL-10 by LaEPS-C, LLaEPS-C, and BLaEPS-C are represented in Table 3 and Figure 4.

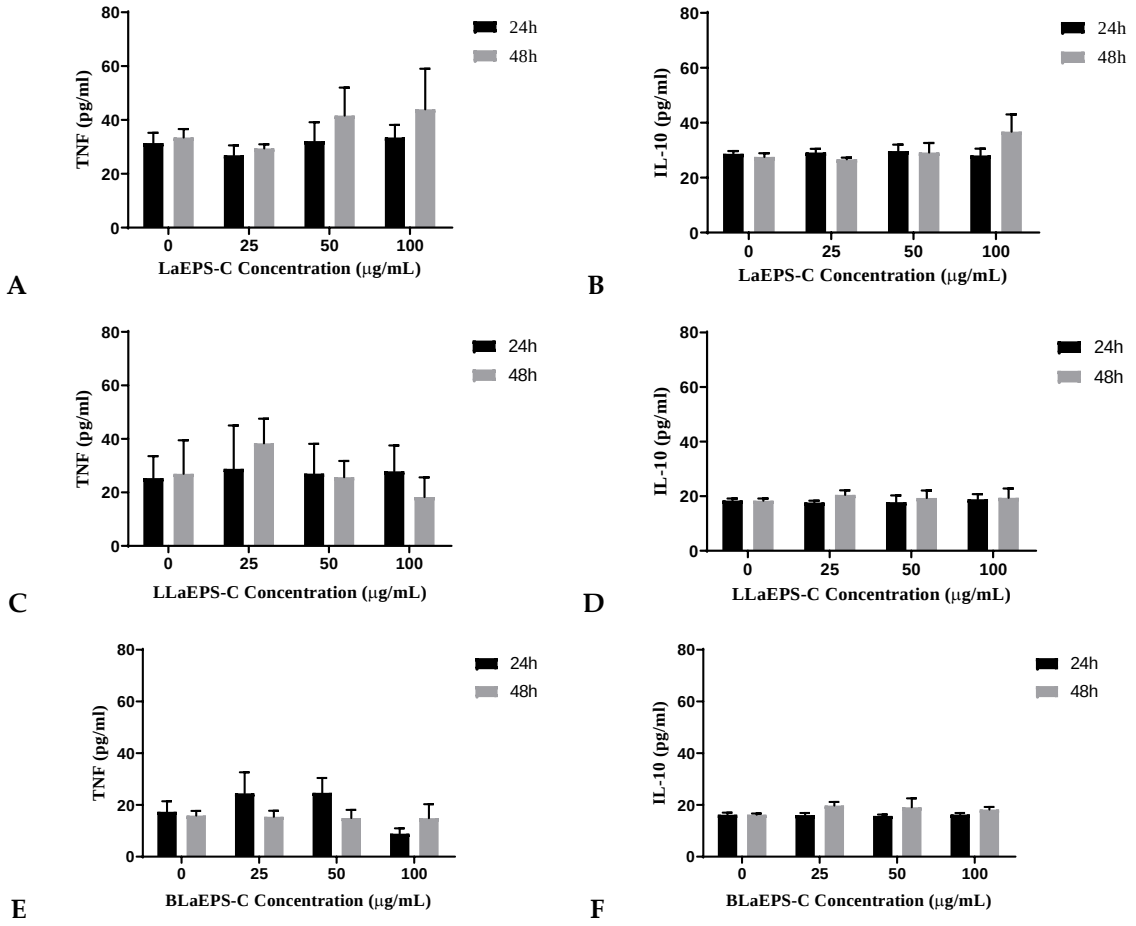

Figure 4. Analysis of the quantification of TNF-α and IL-10 cytokines produced by the culture of human macrophages in pg/mL. (A) Production of TNF-α by LaEPS-C. (B) Production of IL-10 by LaEPS-C. (C) Production of TNF-α by LLaEPS-C. (D) Production of IL-10 by LLaEPS-C. (E) Production of TNF-α by BLaEPS-C. (F) Production of IL-10 by BLaEPS-C.

Table 3. Mean values quantification for TNF-α IL-10 and IL-10, with standard deviation, for the different values of glucan concentration and incubation time.

	TNF-α		IL-10	
	24h	**48h**	**24h**	**48h**
LaEPS-C (µg/mL)				
0	31.44 ± 2.70a*	33.51 ± 1.99a	28.68 ± 0.72a	27.58 ± 1.03a
25	26.89 ± 2.55a	29.46 ± 1.26a	29.15 ± 0.93a	26.68 ± 0.52a
50	32.20 ± 5.58a	41.611 ± 7.78a	29.68 ± 1.73a	29.16 ± 2.40a
100	33.51 ± 3.47a	43.97 ± 10.07a	28.09 ± 2.24a	36.75 ± 5.53b
LLaEPS-C (µg/mL)				

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0	25.36 ± 4.91a	26.98 ± 7.91a	18.45 ± 0.42a	18.39 ± 0.63a
25	28.80 ± 12.06a	38.40 ± 7.42a	17.706 ± 0.50a	20.47 ± 1.34a
50	27.00 ± 6.70a	25.69 ± 5.21a	17.810 ± 1.86a	19.38 ± 2.09a
100	27.89 ± 7.22a	18.27 ± 5.80ab	18.895 ± 1.43a	19.41 ± 2.56a
BLaEPS-C (µg/mL)				
0	17.35 ± 2.24a	15.88 ± 0.96a	16.26 ± 0.626a	16.26 ± 0.36a
25	24.50 ± 6.15b	15.46 ± 1.60a	16.12 ± 0.633a	19.77 ± 1.22b
50	24.69 ± 3.55b	14.94 ± 2.21a	15.79 ± 0.438a	19.12 ± 2.29c
100	8.88 ± 1.74c	14.90 ± 3.93a	16.29 ± 0.438a	18.22 ± 0.87c

* Means followed by at least one equal letter indicate non-significant differences (p>0.05).

For the macrophage culture stimulated with LaEPS-C, for cytokine TNF-α there was no significant difference (p> 0.05) for all concentrations and both times (Table 3, Figure 5A). For IL-10, there was an observed significant difference (p> 0.05) for 100 µg/mL and 48h (36.75 ± 5.53) (Table 3, Figure 4B). Therefore, LAEPS-C showed higher production of IL-10, that is, an anti-inflammatory character. LLaEPS-C showed no significant difference in TNF-α production at 24 hours. For 48h, in 100 µg/mL, there was a significant decrease of TNF-α (18.27 ± 5.80, p> 0.05) (Table 3, Figure 4C). There was no significant difference in IL-10 production for all concentrations and incubation times tested (Table 3, Figure 4D). At 24 h, TNF-α biogenesis was stimulated by BLaEPS-C for 25 and 50 µg/mL (24.50 ± 6.15 and 24.69 ± 3.55, respectively), while for 100 µg/mL it was observed one opposite effect. At 48 hours, there is no significant difference (p> 0.05) (Table 3, Figure 4E). For IL-10 synthesis, there was a significant difference (p> 0.05) at 24h (Table 3, Figure 5F). BLaEPS ensured the production of an anti-inflammatory effect from 25 µg/mL and 50 µg/mL, but there is no significative difference between 50 µg/mL and 100 µg/mL. The dataset of cell viability and cytokine production indicates that, in the absence of cellular cytotoxicity, LLaEPS-C and BLaEPS-C are inducers of a pro-inflammatory pattern due to their ability to induce TNF-α in human macrophages.

The pro-inflammatory character of β-glucans that induce TNF-α secretion by human macrophages was demonstrated without cytotoxicity [4,37]. The literature describes that the interaction of linear β-glucans β-(1→3) and/or β-(1→6) with the transmembrane protein Dectin-1 on the surface of macrophages induces the secretion of TNF-α [38,39]. Other cellular receptors could be involved in the immunomodulatory activities of β-(1→3)(1→6) branched β-glucans, including the induction of TNF-α by macrophages. It is suggested carboxymethylation preserved the pro-inflammatory activity of β-glucans obtained from *L. theobromae*.

3.2.5. Antiviral activity

The human respiratory syncytial virus (hRSV) is an infectious agent in infants and young children with no vaccine or treatment drugs. Anionic polysaccharides have attracted attention over the past decades for their inhibitory activity against viruses, such as hRSV [40]. They inhibit the first step of infection, where the glycoprotein on the viral envelope utilizes its positive charges to interact with negative charges of heparan sulfate (HS), one of the host cell surface receptors. By inhibiting this step, polysaccharides mimic HS, thus blocking the virus from entering the host cell [41,42].

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The cytotoxic activity analysis determined that the CC50 (Compound Concentration can kill 50% of the cells in the culture). LaEPS, LaEPS-C, and fractions showed cytotoxic activity for Hep-2 cells. LaEPS were the least (14.2 mg/ml) cytotoxic polysaccharide, followed by LaEPS-C (6.8 mg/ml), LLaEPS-C (1.4 mg/ml), and BLaEPS-C, the most cytotoxic b-glucan (0.8 mg/ml) (Figure 5).

The literature search revealed that some carboxymethylated polysaccharides enhance the antitumor activity of polysaccharides. Thus, their carboxymethylated derivatives can successfully suppress tumor cells' growth. Specifically, the effects of carboxymethylation on the β-glucan cytotoxic activity could be attributed to the hidrossolubility and structural changes in molecular conformation [10,24,43].

Next, each LaEPS-C fraction had its anti-hRSV activity tested. For this, were assayed concentrations equal to or smaller than CC_{50} values. The better anti-hRSV results obtained from each tested LaEPS fraction are shown in Figure 5. Ribavirin, a known anti-hRSV drug, was used as negative infection control. LaEPS (7 mg/ml), LaEPS-C (3.4 mg/ml) and BLaEPS-C (0.4 mg/ml) show discrete anti-hRSV activity that was not statistically significant. LLaEPS-C (0.7 mg/ml) was the only fraction that showed anti-hRSV activity statistically significant, demonstrating that the carboxymethylation improved the antiviral activity from LLaEPS.

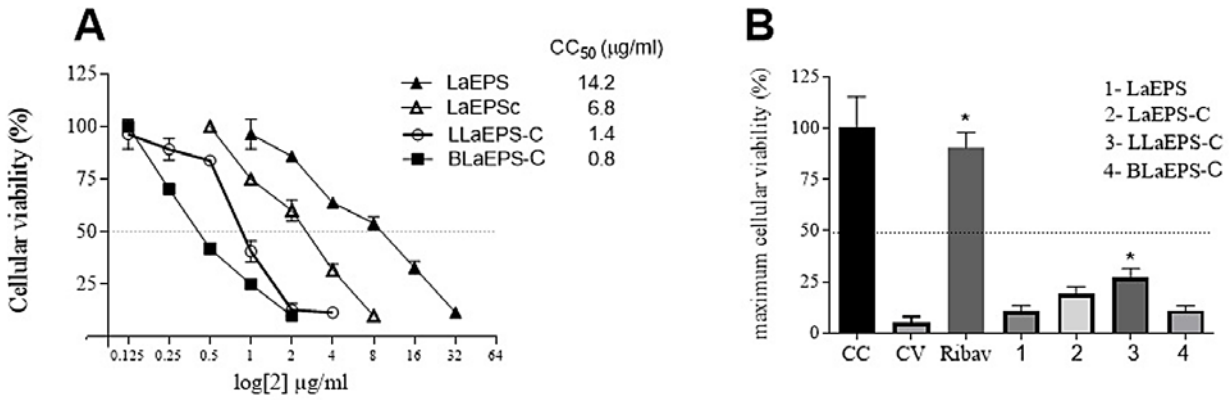

Figure 5. Determination of CC_{50} and anti-hRSV activity. (A) Different fractions from EPS were incubated in different concentrations with Hep-2 cells. The cellular viability was evaluated by MTT, and CC_{50} was calculated by linear regression. (B) Anti-hRSV from different EPS fractions was tested using a lower CC_{50} concentration than that obtained in Figure 5A. Just EPS concentration that resulted the maximum cellular viability is shown in the graphic: LaEPS (7 µg/ml), LaEPS-C (3.4 µg/ml), LLaEPS-C (0.7 µg/ml) and BLaEPS-C (0.4 µg/ml). Cells incubated with culture medium (CC), only virus (CV) or antiviral drug Ribabirin (Ribav) were used as control.

Möller et al. (2012) reported carboxymethyl groups on hyaluronan derivatives did not contribute to antiviral activity against HSV-1 [44]. Lopes et al. 2021 compared the antiviral activity between sulfated and carboxymethylated polysaccharide derivatives [42] (LOPES et al., 2021a). They suggest introducing carboxymethyl groups may change the triple-helix conformation of polysaccharides [2]. Consequently, the hydrophobic interactions responsible for antiviral activity could be damaged [45]. Finally, the negative charges introduced by the carboxymethylation of LaEPS-C might not be sufficient to confer more extensive hRSV binding.

4. Conclusions

LaEPS carboxymethylation provided products with enhanced water solubility and new or improved bioactivities. Comparing LAEPS-C and fractions, LLaEPS-C provided the best antiradical activity at lower concentrations. So, interactions among the hydroxyl

groups of LLaEPS-C with the DPPH radical indicated the best viability, because of its lower sterical effect. Still, structural changes and new conformational arrangements could be responsible for the antiproliferative activity of LaEPS-C (NCI-ADR/RES) and the BLaEPS-C (K562). Also, the LLaEPS-C reduced steric effects seemed to be a critical factor in the lack of antiproliferative activity for tested lineages.

Charge plus steric effects LaEPS-C and fractions promote a reduction in PT and a procoagulant effect. Again, LLaEPS-C, with reduced steric effect compared to LaEPS-C and BLaEPS-C, showed better PT activity, especially at low concentrations. As LLaEPS-C concentration increases, the interaction between LLaEPS-C-fibrinogen decreases because of the increase in viscosity. LaEPS-C and BLaEPS-C, with higher sterical effects, do not suffer the effects of increased viscosity and become more effective for higher concentrations. Therefore, the steric effect seems to be more significant for PT assay.

LLaEPS-C and BLaEPS-C are inducers of pro-inflammatory activity due to their ability to induce TNF-α in human macrophages. LLaEPS-C also showed anti-hRSV, revealing that the carboxymethylation improved the antiviral activity of this molecule. The negative charges introduced by carboxymethylation seemed insufficient to confer more extensive hRSV binding, since the hydrophobic interactions responsible for antiviral activity could be broken. Therefore, it can be inferred that the carboxymethylation of LaEPS resulted in more active polysaccharides. LLaEPS stands out mainly since the effect of charge, conformation, and low steric effect resulted in better activity in most of the biological assays tested in this manuscript. Further assessment of the LaEPS-C structure-activity relationship studies is required to detect new and desirable bioactivities from these polysaccharides.

Author Contributions: C.S., K.A.T., and V.M.G.N., conceived and designed the experiments. M.C.P., N.G. A, B.R.P.L, A.L.T.G.R., and C.D, performed the experiments. M.C.T.D., K.A.T, and C.S. analyze the data. CS and K.A.T. wrote the paper.

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Institutional Review Board Statement and Informed Consent Statement: The LaEPS-C potential immunomodulatory activity on cytokine secretion was evaluated in human monocyte-derived macrophage cultures. For this purpose, cells were obtained and prepared according to the procedures evaluated and approved by the local Research Ethics Committee of the Faculty of Science and Letters of Assis (approval number: CAAE 68135717.6.0000.5401). As suggested by the Committee, written informed consent was obtained from each volunteer before initiating any research procedures.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are not available from the authors.

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