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Cytotoxicity studies of membranes made with cellulose nanofibers from fique macrofibers

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ABSTRACT

We report on the fabrication of cellulose nanofibers from Fique (*Furcraea andina*), in which raw macrofibers were chemically treated and then ground. Bleaching was effective in removing lignin from the raw fibers, and this was confirmed with distinct techniques, viz. X-ray diffraction, FTIR spectroscopy, thermogravimetric analysis, and the estimation of the kappa number. From scanning electron microscopy and transmission electron microscopy images, the nanofibers were seen to form a network with 85% of nanofibers having diameters smaller than 50 nm, while their length was hundreds of nanometers. The tensile strength of membranes made with the nanofibers was 166 MPa. These membranes had no cytotoxicity in in vitro indirect tests analyzed qualitatively and quantitatively. Indeed, cell viability was higher for the nanofibers than the negative control.

Introduction

The interest in renewable resources has increased in many strategic areas, including in materials sciences where natural fibers can be employed in composites, though so far this has been mostly in handcrafts or products with low added value [1, 2]. The majority of the natural fibers contains cellulose, which is an abundant, cheap, and renewable biopolymer. With proper processing, cellulose may exhibit high mechanical strength due to its crystalline structure [3], while contributing to increase the tensile strength, Young's modulus, and impact strength of composites [4]. Applications of cellulose nanofibers are now envisaged in the automobile industry, in aircrafts, electronics, packaging, and in the biomedical industry [5–7]. Various processes involving chemical and mechanical treatments are used to obtain cellulose, depending on the source and composition [3, 8], during which other components of natural fibers are removed, especially hemicellulose, lignin, ashes, and pectins. After isolation, microstructured cellulose has to undergo another process for nanofibers to be produced,

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the most common being chemical [9, 10], enzymatic [11], and mechanical [7, 12] processes.

In this study, we used a chemical–mechanical process to obtain nanofibrillated cellulose from Fique (*Furcraea andina*), which is originally from Peru. Fique is a promising natural material because the macrofibers extracted from its leaves have high mechanical strength (Delvasto et al. 2010). Nanofibers from fique were obtained here by chemical treatments, followed by the pulping and bleaching treatment through an oxidative pathway, and being later mechanically processed. Raw macrofibers, bleached microfibers, and nanofibers were characterized with X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and thermogravimetric analysis. In addition, we show that the nanofibers may be biocompatible based on in vitro cytotoxicity tests.

Materials and methods

Fique macrofibers, originally from the Sao Paulo State, Brazil, were obtained from a genetic bank at the Rural Engineering Department of the São Paulo State University (Unesp). To obtain cellulose nanofibers, the fique macrofibers were milled and sieved through a 4-mm mesh, and then dried at 60 °C overnight. The dried macrofibers were treated with 0.05 N HCl solution for 2 h at 70 °C to remove extractives such as oil and waxes, to which a NH_4OH solution was added until reaching pH 9.5. A time lapse of 12 h was taken to extract pectic substances. The macrofibers were then washed until reaching the same pH of distilled water. The pulping process followed, with 4% NaOH solution (1:10) at 70 °C for 2 h, after which the macrofibers were washed again. In order to remove all of the non-cellulosic substances, the macrofibers were bleached using 800 mL of distilled water, 8.6 mL of acetic acid, and 9 g of $NaOCl_2$ per each 100 g of dried pulp. This reaction was kept for 4 h at approximately 70 °C. The bleached microfibers from fique were processed mechanically using the commercial grinder (Supermasscolloider MKZA 10–15 J, Masuko Sangyo Co., Japan), in a process repeated for 9, 12, or 15 times.

Membrane procedure

Membranes were prepared through filtration of 0.3 g of nanofibers suspension under vacuum using a polysulfone filter of 0.1 μm pore. The resulting membrane was covered with another polysulfone filter and then compressed at 50 psi for 30 min between paper sheets at room temperature and dried completely at 55 °C overnight.

Characterization

Mechanical strength of fique macrofibers

The mechanical strength was measured according to ASTM D3822—"Tensile Test for single Textile Fibers" using an Instron 5582 instrument and Blue Hill software. The macrofibers were segregated in single macrofibers whose diameter was measured in five different points using optical microscopy. The average diameter was used to calculate the tensile strength for ten distinct macrofibers. The gap between the grips was 2.5 cm and the speed was 1 mm/s with a load of 1000 N.

Tensile strength of nanofiber membranes

The tensile strength of ten membranes made with nanofibers was measured with an Instron Model 20 using the speed of 2.5 mm/min with load cell of 2 kN following the standard procedure ASTM-D638 [13]. The thickness of each sample was measured with a caliper rule.

Chemical analysis

The chemical composition of fique fibers was determined as to their contents of cellulose, hemicellulose, and lignin following the TAPPI standard [14]. First, the holocellulose was determined according to TAPPI T19 m-54, which consists in selective degradation of lignin, thus yielding the contents of cellulose and hemicellulose. For these procedures, fique fibers were dried, milled in 200 mesh, and then treated with a solution of sodium hypochlorite and glacial acetic acid at 75 °C under magnetic stirring for 2 h. Holocellulose was filtered and washed with hot water (at nearly 100 °C) and acetone, and dried at 60 °C in an oven until constant weight. The lignin content was calculated from the subtraction between the initial weight of fique fibers and holocellulose content. To determine the cellulose content, sodium hydroxide solution 17.5 wt% was added to holocellulose previously dried at room temperature, under magnetic stirring for 30 min to eliminate hemicellulose. Cellulose was filtered and washed until neutral pH and then dried at 60 °C in an

oven, until constant weight. Then, the hemicellulose content was determined from the subtraction between holocellulose and cellulose contents.

Microscopic study and diameter size

Scanning electron microscopy (SEM) was used to examine the microstructure of fique raw fibers and bleached fibers using a Jeol JSM-6610 LV instrument and a FEI-Quanta FEG 650 at LNANO (Brazilian Nanotechnology National Laboratory at CNPEM, Campinas, Brazil), while the nanofibers were analyzed using transmission electron microscopy (FEI-Quanta FEG 250) at the Centre For Nanostructure Imaging from Department of Chemistry at University of Toronto. For SEM analyses, untreated fibers and bleached fibers were placed on top of a carbon film on the stubs and coated with a thin layer of gold to make electrical contact. For TEM analyses, the nanofiber suspension was cast on a 400-mesh copper grid and dried. The diameter of the nanofibers was estimated using Image J program, with the size scale calibrated with the scale bar on SEM and TEM images. The diameter distribution was obtained by measuring the diameter of 50 nanofibers.

FTIR spectroscopy

FTIR spectroscopy was used to identify chemical groups of untreated fique fibers, bleached fibers, and nanofibers with a Bruker Tensor 27. The samples were dispersed in KBr and the spectra were taken with 32 scans in the range between 4000 and 400 cm^{-1}.

Thermogravimetric analysis (TGA)

The thermal stability of raw fique macrofibers, bleached microfibers, and nanofibers was investigated with a TGA Q500 V6.7 Build 203 instrument under nitrogen, with heating from 25 to 600 °C at 10 °C min^{-1}. The derivative of the mass loss curves allowed us to determine the components before and after treatment on the macrofibers.

XRD and Crystallographic Study

The cellulose nanofibers' crystallinity was determined from the diffraction patterns measured with a Philips Analytical X-ray PW 1830 diffractometer (45 kV, 100 mA) equipped with Cu Kα radiation

($\lambda = 0.1541$ nm). The angular range varied from 10 to 40° (2θ) and the step size was 0.02° (2θ). The crystallinity index of the macrofibers, microfibers, and nanofibers was calculated using an empirical method proposed by Segal et al. (1959), which measures the relative crystallinity of native cellulose, using Eq. (1) [15].

$$C.I. = \frac{I_{002} - I_{am}}{I_{002}} \times 100, \qquad (1)$$

where C.I. is the relative crystallinity index; I_{002} is the maximum diffraction intensity at the main peak around 22.5° (2θ); and I_{am} is the diffraction intensity of the amorphous material at the peak of approximately 18° (2θ), where the intensity is minimal.

Kappa number

The estimation of the kappa number serves to assess the relative bleachability or the degree of delignification of the pulp [16]. In other words, it is a measure of the bleaching treatment efficiency. The definition by TAPPI (Technical Association of the Pulp and Paper Industry) reads: "Kappa number gives essentially a straight line relationship with the total lignin content in pulps that can be estimated fairly closely by rapid, indirect methods by oxidation of the lignin (TAPPI T-236 Kappa Number of Pulp)." The procedure described in T236 cm-85 from TAPPI standard was applied on raw fique fibers before and after the bleaching process and nanofibers. According to this standard, kappa is the number of volume (mL) consumed of 20 mmol/L (0.1 N) $KMnO_4$ solution by one gram of moisture-free pulp under the conditions specified in the standard.

Biological assay

The cytotoxicity assay for the nanofibers (NFs) was performed based on ISO standards (ISO 10993-5) using a Vero cell lineage (ATCC CCL-81). The cells were cultured with HAMF10 medium, 10% fetal calf serum, and 1% antibiotics at pH 7.4. The cells were maintained at 37 °C and 5% CO_2. The membranes of cellulose nanofibers were prepared using the same procedure as described above, and cut into pieces of 1 cm^2. These samples were sterilized in alcohol at 70° for 12 h and exposed to ultraviolet light for 30 min. For the cytotoxicity assay, the membranes were incubated in 5 mL culture medium for 24 h, for the nanofiber extract preparation.

Figure 1 **a** Photograph of fique plant. **b** Raw macrofibers mechanically extracted from the leaves. **c** and **d** SEM images from the raw macrofibers and bleached microfibers, respectively. **e** and **f** TEM images of cellulose nanofibers.
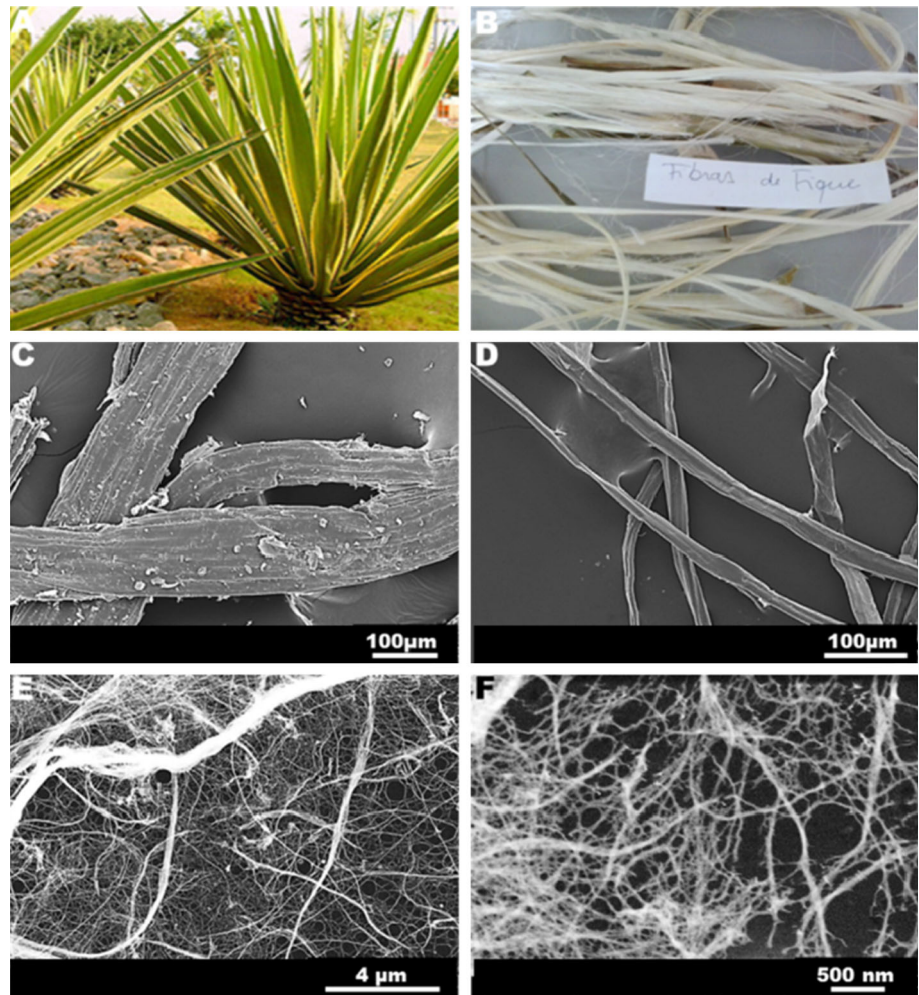
Indirect cytotoxicity–quantitative assay

The quantitative analyses of viable cells were made with MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]) assay [17, 18]. Vero cells were seeded at the density of 10^4 cells/well, in 96-well cell culture plates. The cells were incubated for 24 h until confluence. On the second day, the culture medium was replaced by the nanofiber extracts and the cells were incubated for 24 h in the presence of these extracts. The medium was removed after the incubation period, replaced by 10 μL of MTT (5 mg/mL), and prepared in phosphate buffer. The cultures were then incubated for 4 h at 37 °C. The formazan crystals were solubilized with 50 μL of sterile DMSO. After 30 min, absorbance was measured in an Elisa reader at 570 nm (Spectramax M5). Balk wells with standard culture medium were used as negative control, while the positive control had 0.25% phenol. The assay was performed in quintuplicate.

Morphological evaluation

The cells cultured in nanofibers extract were observed after 24-h incubation at a phase-contrast microscope (AxioVert A1, Zeiss) and analyzed with AxioVison SE64 (Zeiss) program. After initial observation, the extracts were removed and the adhered cells were fixed with glutaraldehyde 2.5% in phosphate buffer for 20 min, followed by washing with water and 0.15% toluidine blue staining for 30 min.

Results and discussion

Figure 1a shows a photograph of a fique plant, and raw macrofibers extracted mechanically from the leaves are shown in Fig. 1b. SEM image from the raw macrofibers is shown in Fig. 1c. The bleaching process causes the diameter of the macrofibers, of the order of 100 μm, to be reduced to 15–25 μm, and are
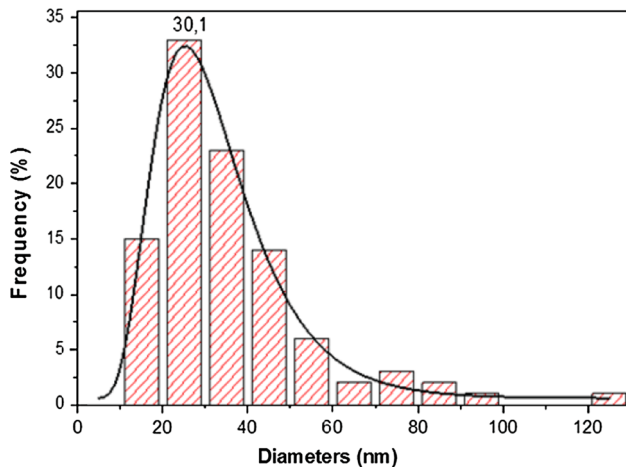
**Figure 2** Histogram with distribution of diameters for the nanofibers obtained from TEM images.

called here bleached microfibers which is shown as the SEM image in Fig. 1d. This reduction in diameter for the bleached microfiber is caused by the removal of compounds, including lignin and hemicellulose, with the release of bundles of microfibers that compose macrofibers in natural plants [19]. The concept that macrofibers in nature are composed of microfibers has been supported by different authors. For instance, the morphology of microfibrillar elements is believed to affect the macroscopic properties [20], and the elementary fibrils can be considered as individual units [21]. The presence of elementary fibrils with a hexagonal closely packed arrangement has been confirmed with small-angle X-ray scattering [22], and their diameter has been reported to vary from 10 to 50 nm [23].

The nanofibers obtained from the shearing promoted by the grinder form a network structure similar to that of bacterial cellulose where nanofibers are interconnected, as shown on TEM images of Fig. 1e, f.

The distribution of diameters of the nanofibers in Fig. 2 peaks around 30 nm. Because the nanofibers are several nanometer-to-micrometers long, they may be also referred to as microfibrillated or nanofibrillated cellulose. Similar samples of nanocellulose have been reported from raw materials such as soy hulls [24], rice husks, hemp, wood [13], and Curaua [25, 26].

The average tensile strength of a single natural macrofiber, without any treatment, was 320 ± 59 MPa, being significantly higher than the value reported in the literature for fique, which is 197 ± 65 [14] and 237 ± 51 MPa [27]. This large difference is probably due to the variability of material properties from natural sources, as they may depend on the location, climate, nutrients, period of extraction, and extraction process, in addition to the use of modifiers [23, 28]. The membranes made with nanofibers are transparent as shown in Fig. 3a. The tensile strength for the nanofibers' membranes in Fig. 3b increased with the number of grindings, where the maximum strength was 166.2 ± 10.4 MPa for nanofibers ground 15 times. The lower tensile strength for the nanofibers' membranes, compared to the macrofibers, may be associated with the removal of components that act as binders and impart high strength to the macrofibers. The strength of the fique nanofibers' membranes reported here is considerably higher than the 65 MPa for nanofibers from Curaua [25, 28] and the 102 MPa for *Aloe Vera* [29]. The Curaua nanofibers were prepared with different equipments called homogenizer but similar nanofibrillated structure, while those from *Aloe Vera* were obtained with a similar process of bleaching and grinding as for the fique nanofibers studied here. Since wood nanofibers have been reported to reach 240 MPa of maximum tensile strength [13], it may be

Figure 3 a The nanofiber membrane; **b** the average of maximum tensile strength measured for ten membranes prepared with nanofibers ground 9, 12 and 15 times.
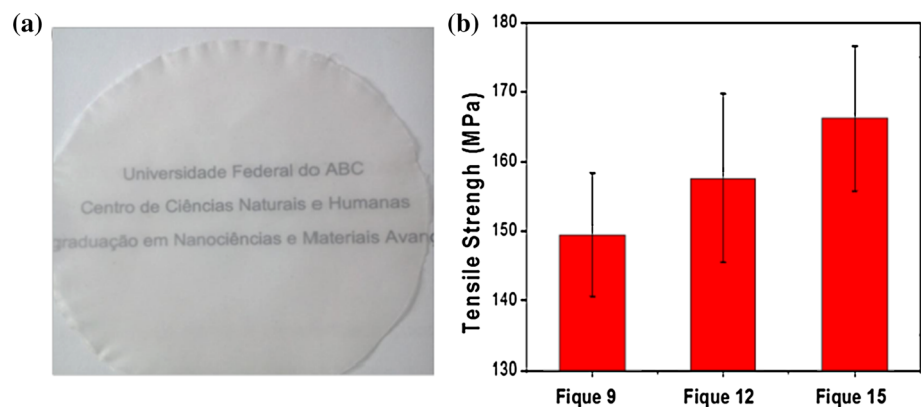
possible to optimize the process of preparation of fique nanofiber membranes to achieve even higher strengths.

The XRD patterns of raw macrofibers, bleached microfibers, and nanofibers are shown in Fig. 4. The crystallinity of cellulose was recognized early in X-ray diffraction studies, and the XRD patterns from native cellulose were assigned as Cellulose I [30]. Treating this native cellulose affects the XRD pattern; for example, regenerated and mercerized celluloses yield patterns referred to as Cellulose II [3]. The main peaks in all samples in Fig. 4 are typical of Cellulose I [30]: a narrow peak at $2\theta = 22°$ and a smaller peak at $2\theta = 16°$ associated with crystalline planes of [101] and [002] [13, 29], though there are small differences in width and intensity. The crystallinity index (CI) estimated from the XRD patterns was 77.3% for the bleached microfibers, 73.5% for the nanofibers, and 62.5% for the raw macrofibers. The highest CI for the bleached microfibers is due to a higher content of cellulose, since amorphous components, such as pectin, lignin, and hemicellulose, are eliminated from the raw macrofibers during bleaching. Furthermore, the alkali treatment was effective in removing most lignin content but did not convert Cellulose I into II, since no peak appears between 18° and 22° in the bleached microfibers and nanofibers [31]. The nanofibers had slightly lower CI owing to breaking of cellulose chains during the grinding process to reach the nanoscale. Indeed, a larger number of passes
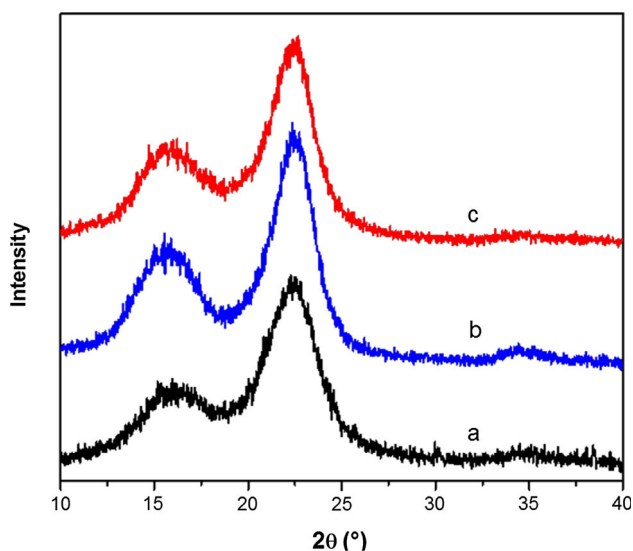
through the grinder has been reported to decrease the degree of crystallinity [12].

The FTIR spectra in Fig. 5 were used to identify the functional groups and understand changes induced by reaching the nanoscale in the fique macrofibers. All of the fibers displayed a band at 2900 cm^{-1} assigned to stretching vibrations of C–H groups from cellulose and a broad band between 3300 and 3500 cm^{-1} (not shown in the figure) due to water molecules absorbed on the cellulose structure. The band at 890 cm^{-1} assigned to stretching of aliphatic groups on the carbohydrate chain has increased intensity in the bleached microfibers and nanofibers because of the removal of contaminant species [24, 32]. The bands at 1737 and at 1251 cm^{-1} appear mostly on the spectrum for the raw fique macrofibers, as they are assigned, respectively, to stretching of carbonyl groups (C=O) from lignin and hemicellulose and O-acetyl esters from hemicellulose, extractives, pectin, and lignin [29, 33, 34]. These results confirm that the bleaching process was efficient in removing lignin and hemicellulose; in particular, the spectrum for the nanofibers is similar to that of cellulose, with high-intensity bands at 890, 1060, and 1370 cm^{-1}.

The effectiveness of the bleaching treatment was tested by calculating the kappa number, Fig 6, whose value is an indirect indication of the lignin content TAPPI. Since the higher the kappa number the higher the lignin content is, the values of 27.0 ± 2.6 and 2.7 ± 2.6 for raw macrofibers and nanofibers, respectively, indicate a decrease of approximately 90% of lignin content upon bleaching. Kappa number
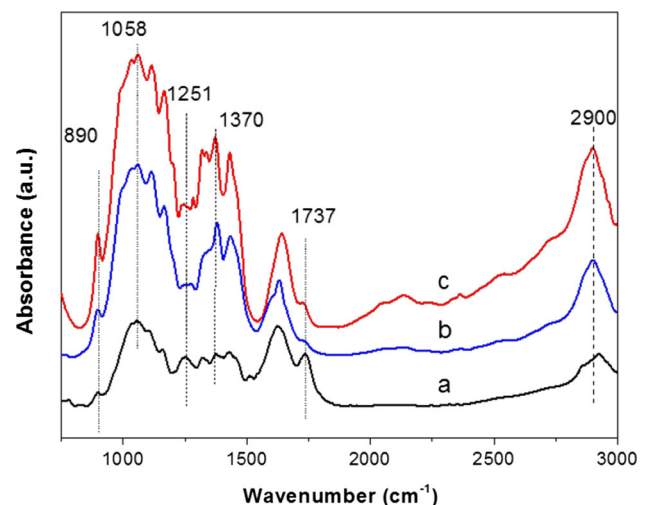

Figure 4 XRD patterns for raw fique macrofibers (*a*), bleached microfibers (*b*), and nanofibers grinded 15 times (*c*).

Figure 5 FTIR spectra taken with raw fique macrofibers (*a*), bleached microfibers (*b*), and nanofibers (*c*).

Figure 6 Kappa number for the raw macrofibers and nanofibers.

Figure 7 TG and DTG curves of raw macrofibers, bleached microfibers, and nanofibers.
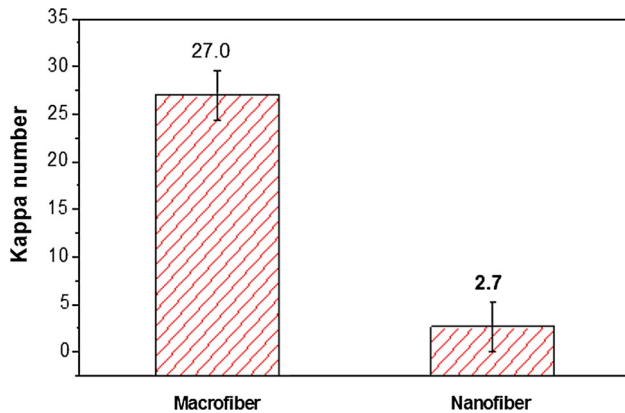
for the bleached microfibers was the same as for nanofibers, which shows that the mechanical process of grinding does not remove lignin from the matrix, as expected from the literature [24].

The thermogravimetric (TG) and differential thermogravimetric (DTG) curves shown in Fig. 7 allow us to investigate degradation and identify the material components based on the literature [35]. The TG curve for the bleached microfibers is shifted to higher temperatures, compared to the curve for the raw macrofibers, because bleaching turns the microfibers more resistant to degradation owing to the higher relative contents of the highly crystalline cellulose. The difference is better visualized in the DTG curves, where a shift in the peak for maximum degradation is observed from 364 °C for raw fibers to 380 °C for the bleached fibers. This peak is ascribed to degradation of cellulose [36]. Also to be noted are the additional features in the DTG curve for the raw macrofibers, with a shoulder at 301 °C owing to degradation of hemicellulose and a small peak at 497 °C owing to lignin degradation [37]. As for the nanofibers, Fig. 7 shows that maximum degradation occurs at a lower temperature (341 °C), probably due to the shearing process that favors heat permeation in the nanoscale. Furthermore, the DTG curve is broader for the nanofibers because of the higher dispersion in dimensions for the nanofibrillated material.

Biological assay

Indirect cytotoxicity–quantitative assay

One important issue in the use of nanomaterials is related to the possible cytotoxicity, both during their production or upon their use. The first test of
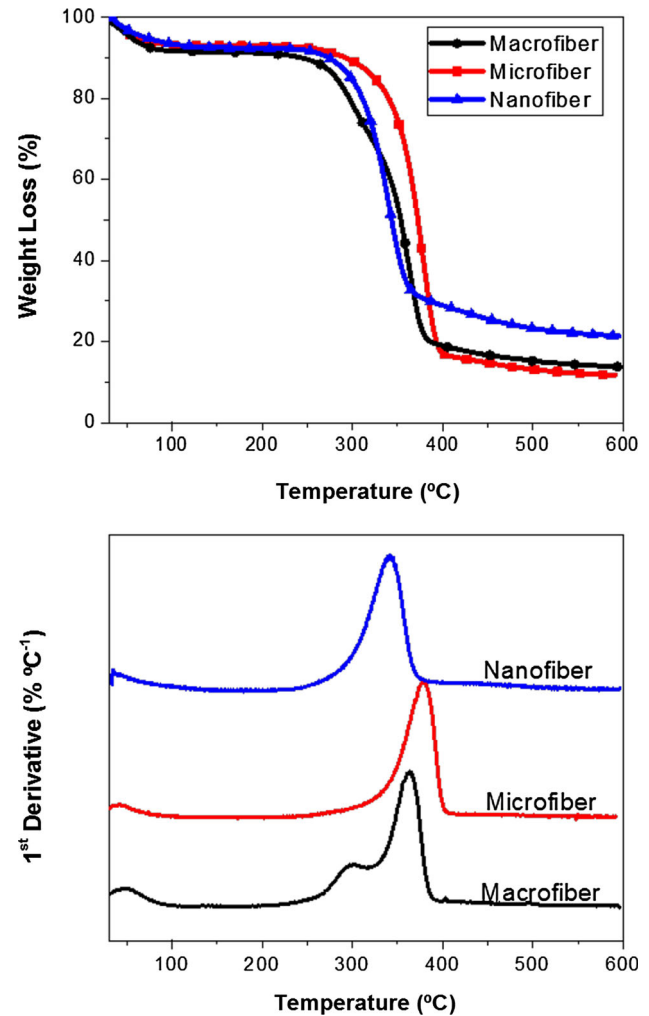
biocompatibility can be performed with in vitro cytotoxicity studies [38–40], as is the case of the mitochondrial activity to quantify viable cells in the MTT assay [41, 42]. Figure 8 shows the Vero cell viability in an indirect cytotoxicity assay, using negative control as the pattern of 100% viable cells. The measurements showed similar pattern of cells cultured in the presence of nanofiber extracts compared to the non-cytotoxicity control, where the viable cells were around 109 ± 25 %. The statistical analyses (One-way ANOVA) showed significant difference ($p < 0.05$) between negative and positive controls, and no significant difference was observed between negative control and the nanofibers. Therefore, the membranes made with nanofibers were considered not cytotoxic in this assay.
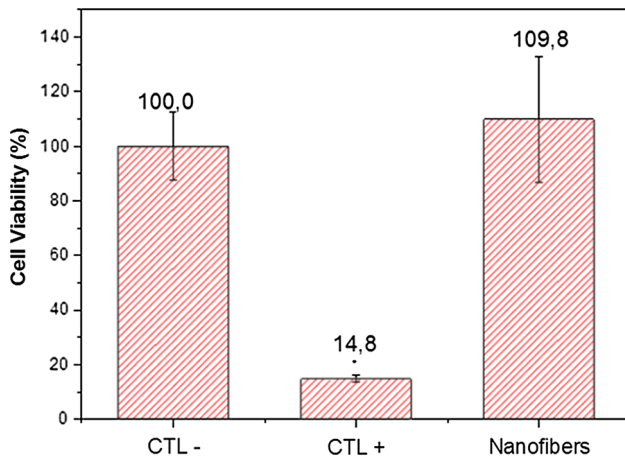
Figure 8 Cell viability with MTT assay and absorbance measurements. Negative control (CTL−) was considered as 100% viable cells. Positive control (CTL+) was obtained with 0.25% phenol. The cells cultured in the presence of cellulose nanofibers extract had 109.8% of viable cells.

The morphological evaluation of cytotoxicity is illustrated in Fig. 9, where the negative control (CTL−) with non-cytotoxic behavior shows a confluent monolayer of fibroblast cells. The positive, cytotoxic control (CTL+) was characterized by round, non-adhered cells in the presence of debris. The cells cultured in the presence of nanofibers extract showed a morphological pattern similar to the negative control cells, forming a confluent monolayer of spread cells. Also, the morphology and basic cell functions appear not to be affected by contact with the extract medium, since the morphology displays features of fibroblast cells. These morphological results corroborate the cellular activity (MTT) evidenced through the cell viability test, confirming that the nanofibers produced did not exhibit any cytotoxic behavior. Cellulose nanofibers from the wood of *Pinus radiate*, *Eucalyptus nitens*, and *Eucalyptus globulus* were not

Figure 9 Vero cells, morphological cytotoxicity assay. The live cell culture is shown on the *left column* and the toluidine blue-stained cells are shown on the *right column*. The positive control (CTL+) shows fewer rounded cells. The negative control (CTL−) shows elongated cell morphology in a monolayer. Cells cultured in the presence of NF show a morphological pattern similar to the negative control. This morphological observation confirms the quantitative assay for the non-cytotoxic effects of the nanofibers.
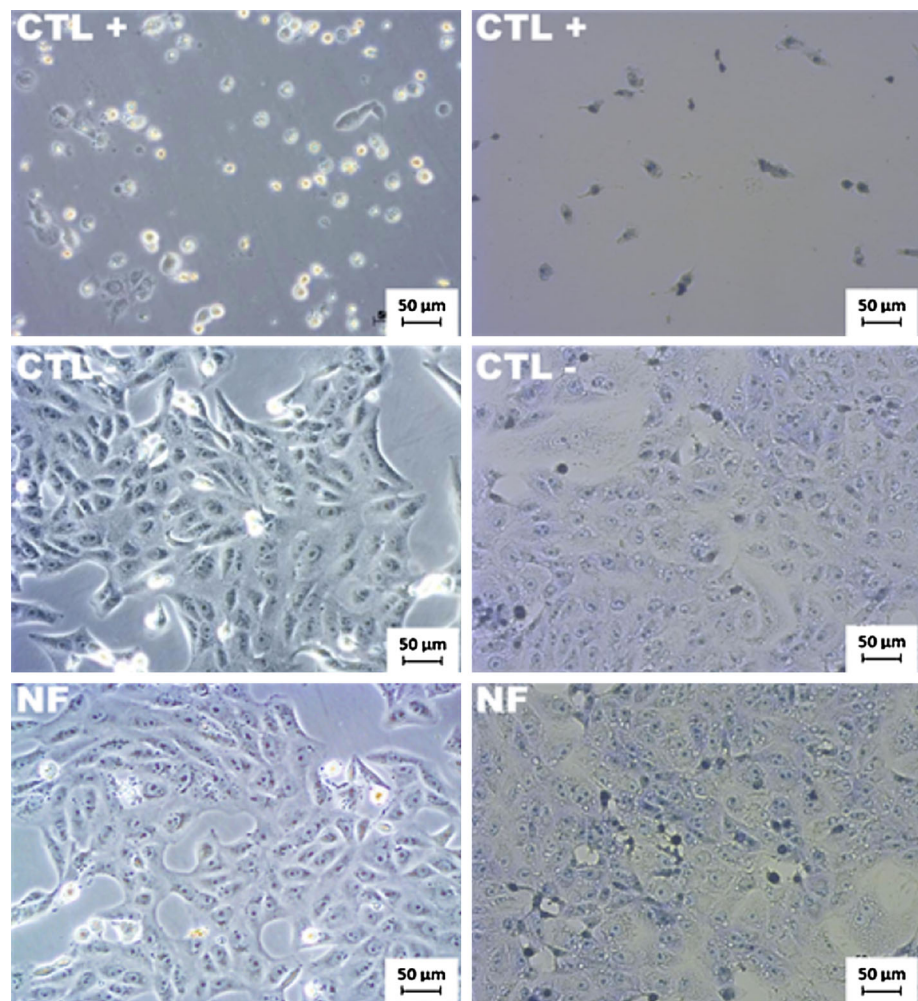
cytotoxic in direct and indirect contact assays [43]. Similar results were shown with cellulose nanofibers obtained from birch cellulose from kraft process, whereas microcrystalline cellulose did not show any effect on inflammatory system [44]. Cellulose nanowhiskers from bacteria were tested for cytotoxicity and no morphological alteration or genotoxicity was observed [45]. Since the cytotoxicity evaluation is the first test for any biocompatible material [46], the results presented here point to the nanofibers from fique crop as being promising materials.

Conclusions

Nanofibers from fique raw macrofibers could be obtained from bleached microfibers using grinding process, and the membranes made with the nanofibers were transparent. Bleaching caused the microfibers to resist to higher temperatures owing to the larger relative contents of crystalline cellulose in the bleached microfibers. The tensile strength of the nanofiber membranes increased with the number of grindings, though one may need to consider the increased cost with more energy being necessary with repeated grindings. From XRD patterns, we could infer that the macrofibers adopted a conformation typical of Cellulose I. The nanofibers seem to be biocompatible, judging by the cytotoxicity studies, since the mitochondrial activity was similar to the negative control, while the morphology was typical of the lineage used, with the cells expanding continuously.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

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