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ORIGINAL ARTICLE

Electrospun PHBV nanofiber containing Tea Tree Oil: physicochemical and antimicrobial activity

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Abstract

Aiming to produce an antimicrobial dressing for wound healing applications, in this work Tea Tree oil (TTO) was incorporated into PHBV nanofibers by absorption. It was observed increase in the nanofiber diameter due to 5% TTO absorption efficiency, which also led to a 54% decrease in the contact angle. The releasing assay indicates a 6.8% oil release in the first 24 h – being probably the oil deposited at the polymer surface – followed by a minimal release at 48 h. The set of antimicrobial assays performed suggests the incorporation of TTO optimized the antimicrobial activity of the polymer for *E. coli* and *C. albicans*, while against *S. aureus* no significant difference was observed. The MTT assay showed no cytotoxicity of PHBV, but the incubation of L929 fibroblast cells with PHBV-TTO reduced cell viability. Overall, the PHBV nanofibers containing TTO present great potential as an antimicrobial dressing.

Keywords: *antimicrobial activity, nanofiber, PHBV, tea tree oil, wound healing.*

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1. Introduction

Preventing pathological infections and performing vital functions in the human body, the skin represents the interface between the internal and external environment. Being able to control the hydration state, thermoregulation, and metabolism, any skin injury must be treated to assure homeostasis maintenance[1,2]. When skin damage occurs due to physical trauma or illness, a cascade of biochemical processes takes place to restore the wounded tissue in four different stages: blood clotting, inflammation, new tissue formation, and tissue remodeling[3,4]. The human body can rapidly heal small wounds but, in case of extended damage such as burns and chronic non-healing wounds, direct exposure to the environment enhances changes of inflammation and microorganism invasion that, together with the poor blood, nutrients, and oxygenation supply, may prevent proper wound healing and management[5]. In such cases, dressings are commonly used as the first treatment to reduce damage by exposure and mechanical stress, to protect the wounded area from secondary infections, and to promote tissue regeneration by rapid epithelization[6,7].

Nanofiber-based wound dressings stand out once their structure resembles the natural skin's extracellular matrix (ECM)[8]. Due to their high surface area, porosity, and mechanical resistance, the nanofibers support cell adhesion, proliferation, infiltration, and differentiation[6,8]. Additionally, allow liquid evaporation, has oxygen permeability, absorbs exudates, and prevents wound drying; crucial properties to optimize tissue regeneration[2,6]. Polymers such as polylactic acid (PLA), Polycaprolactone (PCL), Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), chitosan and gelatin attend these properties and are widely studied for wound healing applications[9]. Besides the characteristics of the nanofiber structure itself, the material used may also play an important role in wound healing. Produced by electrospinning of a polymer solution, nanofibers of natural polymers have

been preferable over the synthetics attributable to their biocompatibility, adaptability to technological needs, and absence of residual monomers with toxicological potential[6,10].

Electrospinning is an electrohydrodynamic technique of fibers production at the micro and nano scales, in which a typical apparatus is composed of a syringe pump, a high-voltage power supply, and a grounded collector[11]. The polymer solution is loaded into a syringe coupled to a capillary metal tip and to an electrode, and then positioned into a syringe pump. The other electrode is connected to the grounded collector, enabling a potential difference between the capillary tip and the collector when the electric field is applied. As a result of this setup, the polymer droplet at the capillary tip is elongated in a straight line to the grounded collector direction while whipping motions occur together with the solvent evaporation, generating polymer fibers into the collector surface[11,12].

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) is a natural thermoplastic linear aliphatic polyester produced by bacteria, being a copolymer of hydroxybutyrate (HB) and hydroxyvalerate (HV) units[13]. PHBV is biodegradable, oxygen permeable, and has intrinsic piezoelectric properties which can improve cellular adhesion and processes such as proliferation and phenotypic maturation[7,14-16]. The PHBV degradation occurs once it is susceptible to human tissue enzymes, such as lysozyme and esterase, but also by hydrolytic degradation in a water environment at slow rates[16]. Additionally, its degradation product HV is already present in human blood and promotes fibroblast and keratinocyte proliferation[17]. It is already reported that PHBV nanofibers provide enough humidity and mechanical resistance for tissue repair[18], besides accelerated healing and minimal scarring[17], being a great material of choice for the development of soft tissue dressings.

Another crucial factor for wound healing is preventing site contamination by microorganisms. Without specific attention, these opportunistic pathogens may uncontrollably grow, promote exudate formation, lead to serious inflammations, and retard healing[19]. Thus, the development of dressing with antimicrobial activity is a constant concern of tissue engineering[10]. Considering this scenario, the antimicrobial action of nanofiber-based dressings may be achieved by the incorporation of bioactive compounds into their structure. Recently, there are increasing interest in essential oils due to their intrinsic antimicrobial and antioxidant properties, provided by their main constituents e.g., terpenes, and terpenoids[5,20]. In general, their mechanisms of action against bacteria consist in inducing cytoplasmic membrane damage by the lipid layer's hydrocarbons partition, which eventually leads to cell lysis[21]. The antioxidant properties of essential oils also have an interest in wound management once the high concentration of reactive oxygen species from inflammation at the wound site damages healthy cells and destroy proteins, and the use of these plant-based active agents prevents extensive harm to cell structures[22].

Tea tree oil (TTO) is an essential oil extracted from the *Melaleuca alternifolia* leaves and twigs, having proven efficiency among microorganisms (e.g., *E. coli*, *S. aureus*, *C. albicans*) in small quantities compared to other essential oils due to the low values of minimum inhibitory concentration[23-25]. Reports in the literature indicate an amount of 0.2 to 1.5% (v/v) of TTO is able to inhibit the growth of the before mentioned microorganisms[23-26]. TTO composition is given in the International Standard (ISO 4730-2004) and its major constituents are terpinene-4-ol (30-48%), γ-terpinene (10-28%), α-terpinene (5-13%) and 1-8 cineole (0-15%). Several studies illustrate the antimicrobial potential of TTO for a wide range of applications, e.g., active food packaging[27], dental[28], acne treatment[29] and wound healing[20,30]. Although different techniques were used to obtain the final fiber/membrane product in these studies, such as electrospinning[31], blow spinning[28], or casting technique[23], they all have a common feature: the TTO was added to the polymer solution before the fiber/membrane obtention. Similarly, Zhang et al.[31] incorporated 15% v/v of TTO into the PLA solution and proceed to the electrospinning process. The obtained nanofibers were then tested against *S. epidermidis* by the inhibition zone method, but no activity was reported due to the oil evaporation during the electrospinning technique. Indeed, essential oils in general are prone to volatilization, conversion, and degradation reactions due to their low chemical stability[32], which makes them less susceptible to maintaining their properties intact after such processes. Additionally, interactions between essential oils and polymers while in contact with the organic solvents may occur, leading to a contrary effect to expect.

Considering the exposed, it is believed that dripping Tea Tree oil into the nanofiber and promoting its absorption rather than its encapsulation may be an alternative to maintain TTO properties unchanged since chemical interactions with polymers and solvents that are favored during the processing method, would be avoided in the absorption process. Moreover, to the best of our knowledge, there is no literature that had reported on the combined use of PHBV and TTO to promote antimicrobial action. Thus, this work aimed to incorporate TTO into electrospun PHBV nanofibers by absorption and evaluate its antimicrobial properties against three microorganisms (*E. coli*, *S. aureus,* and *C. albicans*), comparing the results with the action of pure oil itself.

2. Materials and Methods

2.1 Materials

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) was purchased by Nature-Plast (France), with trade name PHI002, having an HV unit concentration between 2-3% and $\bar{M}_w$ in the range of 400.000 to 500.000 g.mol^{-1}. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (Sigma-Aldrich, St. Louis/Missouri, USA) was used as an organic solvent to dissolve the PHBV, and the Tea Tree Oil (TTO) (*Melaleuca alternofilia*, PHYTOTERÁPICA, Brazil) was used as the antimicrobial agent.

2.2 Preparation of Tea Tree Oil embedded PHBV nanofibers

Using HFIP, an 8 wt.% PHBV solution was prepared under magnetic stirring. The mixture was homogenized for 24 hours at room temperature to allow its stabilization. The solution was loaded in a 5 mL syringe that has a 27 G stainless-steel

flat needle and was electrospun at a rate of 1,5 mL/h under a 14 kV electrostatic field in a proper assembly electrospinning equipment. The deposition of the nanofibers was carried out in a 3 cm diameter rotating collector at 3.000 rpm and 15 cm from the needle tip. The processes took place at 22 °C and 45% of relative humidity, and the collector was previously covered with aluminum foil to facilitate the nanofibers' handling. After 180 min, the nanofibers were collected and allowed to dry in a vacuum desiccator at room temperature for 48 hours to prevent residual solvent presence. For the development of this work, two mats were obtained by using the same conditions. Nanofibers were cut into 5 mm diameter disks and Tea Tree Oil was dripped on its surface to the saturation volume, which was 3 µL per disk. After 48 hours of absorption and drying at room temperature, the following analyses were carried out.

2.3 Morphology and mean diameter

The PHBV nanofibers were analyzed regarding their morphology before (PHBV) and after (PHBV-TTO) Tea Tree oil embedding by electron scanning microscopy (SEM, FEI Inspect S50, Hillsboro, OR, USA) of the samples coated with gold (Sputtering Quorum Q150R ES). The diameter of 100 nanofibers was collected from three different images using FIJI-Image J software, and the mean diameter and its distribution were obtained from a Gauss curve fitting in Origin ® 2018 software.

2.4 Contact angle measurement

The contact angle was measured using an optical tensiometer (TL 1000 - Invoiced freight, Theta Lite, Attension, Lichfield, Staffordshire, UK) (10 s, 50% - 30 FPS) to determine the wettability of the nanofibers. Distilled water was loaded into a glass syringe (Gastight Syringes #1001 – 1 mL) and the contact angle was recorded by the sessile drop method at room conditions. The test was carried out in quadruplicate.

2.5 Fourier-transformed infrared spectroscopy (FTIR)

The structural characterization of TTO, PHBV, and PHBV-TTO was performed to evaluate spectrum changes with the TTO embedding (n=1). FTIR was performed in a Frontier model Perkin Elmer Spectrum spectrometer from 4000 to 400 cm^{-1} in UATR mode using a diamond crystal.

2.6 Tea Tree Oil absorption efficiency

The TTO absorption efficiency by PHBV was conducted by mass change. Initially, the mass of 5 mm diameter disks of pure PHBV nanofiber (m_{PHBV}) was measured. Then, 3 µL (2.69 mg, ρ = 0.896 g/mL) of TTO was dripped into the polymer and the mass was weighed after drying for 48 hours ($m_{PHBV-TTO}$). The high-precision weighing machine used was an AS 60/220.R2 model from Radwag. The amount of oil in the nanofiber (m_{TTO}) was determined by Equation 1, while the absorption efficiency was by Equation 2. The experiment was carried out in quintuplicate.

$$m_{TTO} = m_{PHBV-TTO} - m_{PHBV} \quad (1)$$

$$\text{Absorption eficiency}(\%) = \frac{m_{TTO}}{2{,}69\ \text{mg}} \times 100\ \% \quad (2)$$

2.7 Tea Tree Oil release from PHBV-TTO

The TTO release from the PHBV-TTO nanofiber was performed by VU-Vis spectroscopy using an FS5 Edinburgh Instruments spectrometer. Firstly, a calibration curve was obtained by analysis of known concentrations of TTO in Phosphate-Buffered Saline solution (PBS) in the range of 200-400 nm and an optical path length (l) of 1 cm. The absorbance (A) of the diluted TTO solutions at the prominent peak was measured at 204 nm and the absorptivity (ε) was calculated by the Beer-Lambert Law (Equation 3), where c is the TTO concentration (mg/mL).

$$A = \varepsilon c l \quad (3)$$

To measure the TTO release, PHBV-TTO nanofiber was inserted in 15 mL falcon tubes filled with 3 mL of PBS solution. The tubes were maintained under agitation at 100 rpm and 37 °C in the Shaker Julabo SW 22, and after 0, 1, 3, 6, 24, and 48 hours an aliquot (1 mL) was removed from the system and analyzed by UV-Vis spectroscopy (200-400 nm). After every removal, fresh PBS was inserted into the tubes. The experiment was carried out in triplicate. The amount of TTO released from PHBV in each time point (t) was calculated using the Beer-Lambert Law, and the cumulative TTO mass released ($\Sigma\, m_{TTO\,t}$) was obtained. Regarding the release according to the absorption efficiency, the results were expressed in terms of percentage (Equation 4).

$$TTO\,released\,(\%) = \frac{\Sigma\, m_{TTO\,t}}{Absorption\,efficiency} \quad (4)$$

2.8 Antimicrobial assay

2.8.1 Microorganisms growth inhibition

The antimicrobial activity of PHBV and PHBV-TTO nanofibers was addressed by using a liquid growth inhibition assay against three strains; *E. coli* (ATCC 25922), *S. aureus* (ATCC 6537), and *C. albicans* (ATCC 10231) – which are the most common microorganisms associated with wound infection[33,34]. The pre-inoculum of the strains was prepared in MHA (Mueller Hinton Agar Medium, KASVI, K25-610034) (for bacteria) and Sabouraud Dextrose Agar (SDA, Acumedia®Lab, 7150) (for fungi) for 24 hours at 37 °C. The concentration of the inoculum was standardized at 10^8 (*E. coli*) or 10^6 (*S. aureus* and *C. albicans*) cells/mL at an absorbance of 600 nm, 630 nm, and 530 nm for *E. coli*, *S. aureus,* and *C. albicans*, respectively. The cell suspensions were adjusted to 10^3 cells/mL in Bacteriological Peptone (BP, KASVI, K25-611707) (for bacteria) or Brain Heart Infusion (BHI, KASVI, K25-610008) (for fungi). After 30 min sterilization by UV light, PHBV and PHBV-TTO were added to the 48-well plate containing 300 µL of inoculum, followed by incubation at 37 °C for 24 hours. The microorganism's growth inhibition was evaluated by measuring absorbance in the microplate reader (BioTek, Synergy H1 Hybrid Reader). The inoculum in the absence of any sample was used as a negative control of growth inhibition, while the inoculum containing 3 µL of TTO was used as a positive control. All experiments were carried out on quadruplicate in different days.

2.8.2 Nanofiber and microorganisms' morphology after liquid growth inhibition assay

The interactions of the nanofibers with the microorganisms were evaluated by SEM after the fixation and dehydration process (n=3). Firstly, samples were carefully placed into a fresh plate and the microorganisms were fixed by methanol (100%) immersion. The samples were dehydrated at different increasing concentrations of ethanol (10, 25, 50, 75, and 90% for 20 min, and 100% for 1 hour) and dried overnight at room temperature[35]. The specimens were coated with gold (Sputtering Quorum Q150R ES) and observed through an SEM (FEI Inspect S50, Hillsboro, OR, USA) in high vacuum mode at 15.0 kV electron energy and at a 24.000 x magnification.

2.8.3 Agar diffusion assay

300 µL of inoculum at 10^6 cells/mL were dispersed on the plate surface of MHA (for bacteria) or BHI (for fungi). PHBV and PHBV-TTO nanofibers were carefully placed on the plate and incubated at 37 °C for 24 hours. The diameter of the inhibition zone nanofibers was measured using a digital caliper (Mitutoyo, CD-6" CX-B, São Paulo, Brazil). Filter paper disks of 5 mm were used to separately incorporate 3 µL of TTO used as a positive control of inhibition. As a negative control and to guarantee full microorganisms' growth, plates containing only bacteria or fungi were used. The experiment was carried out in quadruplicate.

2.9 Cell viability

The cell viability assay was performed according to ISO 10993-12, being conducted by the extract method. For the obtention of extracts with a superficial area to the medium ratio of 6 mm^2/mL, 15 nanofibers disks of 5mm^2 were inserted in 1 mL of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (DMEM 10%) followed by incubation for 24 hours (37 °C, 5% CO_2) and filtration in Millipore 0.22 µm (n=3). Mouse Fibroblast Cell Line (L929) were cultured in DMEM 10%, seeded into 48-well plates (2,5 x 10^4 cells per well), and incubated (37 °C, 5% CO_2, 24 hours). The extracts of PHBV and PHBV-TTO (250 µL) were then inserted in triplicate into the 48-well plate and incubated. After 24 hours, the culture and extract medium was removed, and the well was washed with fresh Phosphate Buffered Saline (PBS). 400 µL of an MTT (3-(4,5 Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) solution (0.5 mg/mL) were added. After a 3 hours incubation period, the MTT solution was substituted with Dimethyl Sulfoxide (DMSO 20%) and solubilization of the formazan crystals was performed for 30 minutes. The absorbance of the formazan was then measured at 540 nm in a microplate reader (Synergy H1-Biotek). MEF L929 cells incubated with no sample were considered 100% of viability. The positive control consisted in 100 µL of L929 cells suspension and 150 µL of DMEM 10%, while the negative control of 250 µL DMSO 20%.

2.10 Statistical analysis

When required to discuss data, ANOVA and 2-sample t statistical test were performed at a level of significance of $p < 0.05$.

3. Results and Discussions

3.1 Morphology and diameter distribution

The morphology and diameter distribution of PHBV and PHBV-TTO nanofibers are given in Figure 1. PHBV exhibited

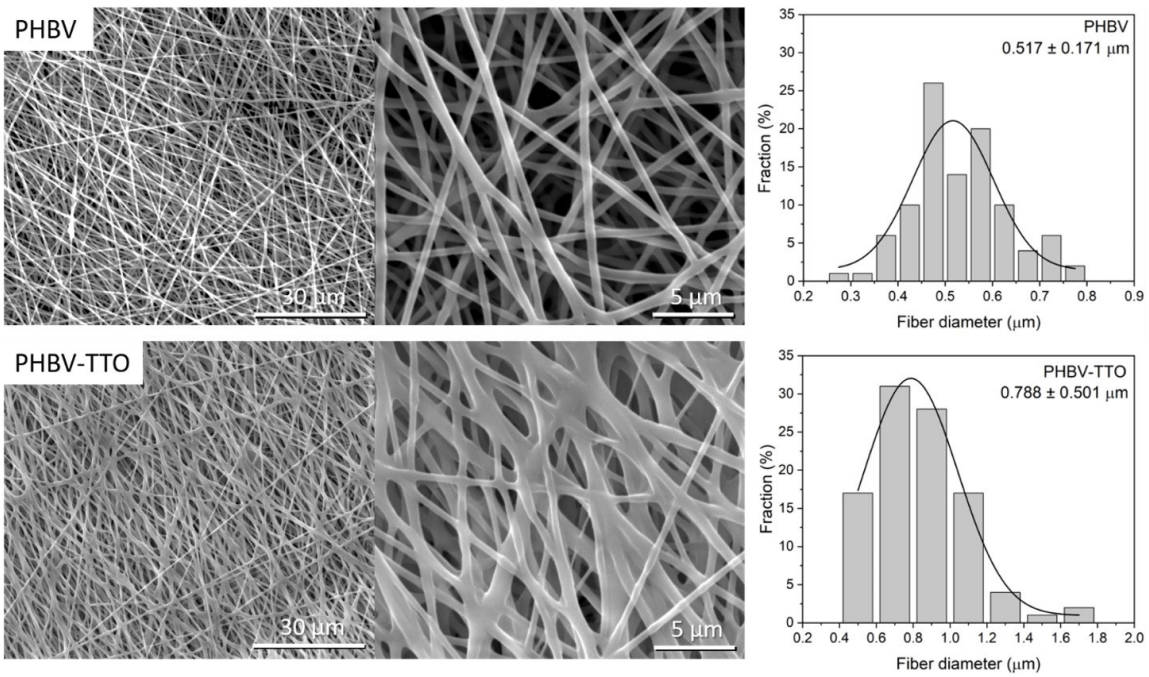

Figure 1. SEM images and fiber diameter distribution of PHBV and PHBV-TTO nanofibers.

randomly oriented, homogeneous-sized, and bead-free nanofibers with a mean diameter of 0.517 ± 0.171 μm. The morphology of PHBV-TTO, however, revealed a different structure as the result of the evident essential oil absorption by the polymer and its spreading in the PHBV surface, which reflected in diameter increase to 0.788 ± 0.501 μm. When the essential oil is added to the polymer solution before the electrospinning, the outcome means diameter usually decreases due to the plasticizer effect typically induced[36,37]. Once the essential oil was added after the electrospinning process, TTO was absorbed and covered the nanofibers, resulting in the increased mean diameter fiber.

3.2 Contact angle

To study the nanofibers' wettability the contact angle was measured. As can be observed in Figure 2, the contact angle of PHBV (90.2 ± 6.2°) is significantly higher than PHBV-TTO nanofibers (48.6 ± 2.4°); with the addition of the essential oil, the contact angle decreased by approximately 54%. Once the PHBV contact angle is higher than 65°, its surface is considered to be hydrophobic[23], while PHBV-TTO exhibited a hydrophilic character. This last result is against the expected, once the lipid nature of essential oils results in water droplets repulsion. Considering that rough surfaces tend to have a higher contact angle than smooth surfaces due to cavities and larger surface area, and that the TTO caused the flattering of the PHBV nanofiber surface due to its absorption and spreading, the result may be explained. Indeed, Figueroa-Lopez et al. (2019) also reported a decrease in contact angle with the addition of essential oils (oregano essential oil, rosemary extract, and green tea extract) in PHBV electrospun nanofibers and attributed this effect to the decrease in the surface tensions caused by the presence of oily molecules on the nanofiber surface[38]. Similarly, Unalan et al. (2019) reported a decrease in contact angle with the incorporation of clove essential oil in a nanofiber blend composed of poly(ε-caprolactone) and gelatin[39].

3.3 Fourier transformed infrared spectroscopy (FTIR)

Figure 3 shows the FTIR spectra of PHBV and PHBV-TTO nanofibers, where Figure 3a indicates the full range spectra, while Figure 3b shows the range between

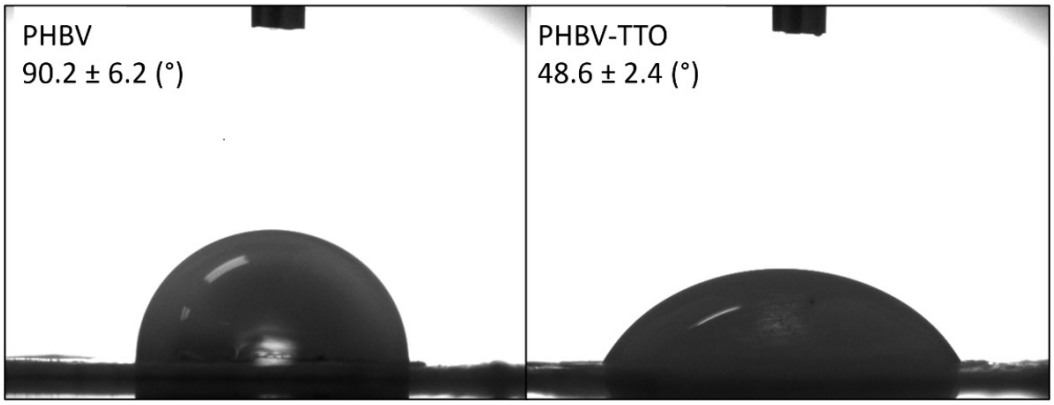

Figure 2. Water contact angle of the PHBV and PHBV-TTO nanofibers.

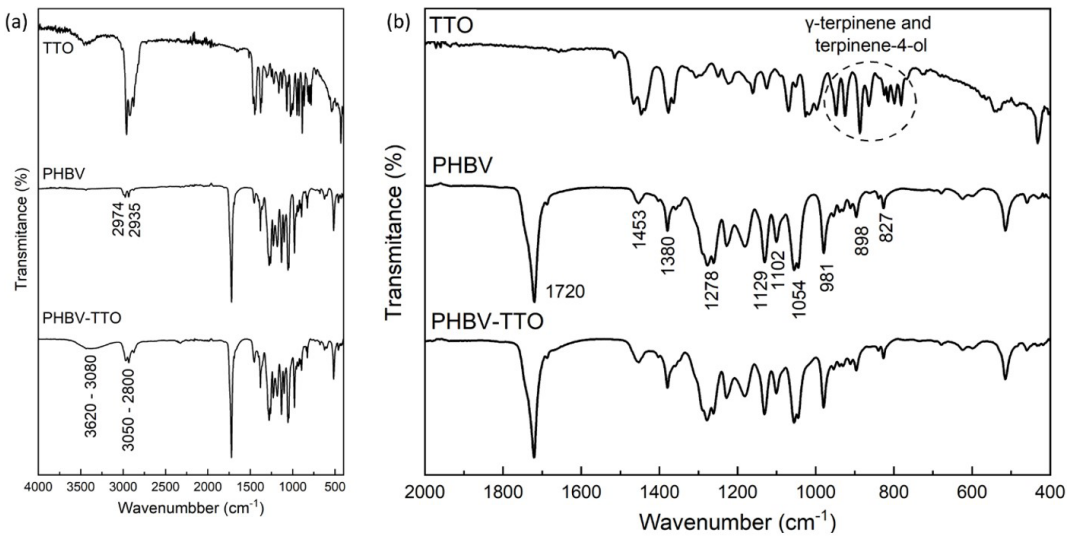

Figure 3. FTIR spectra of pure TTO, PHBV and PHBV-TTO. (a) 4000-400 cm^{-1} and (b) 2000-400 cm^{-1}.

2000 to 400 cm^{-1} to evidence PHBV bands. Pure TTO is reported to present infrared active bands at 780, 815, and 830 cm^{-1} from γ-terpinene and 799, 864, and 889 cm^{-1} from terpinene-4-ol[40]. These bands are readily observed in the IR spectra (dashed ellipse). Furthermore, the broad between 3620 and 3080 cm^{-1} is attributed to the O-H stretching vibration of terpene alcohols[41], while the bands in the 2900 cm-1 region correspond to its C-H stretching vibration[42].

PHBV spectra revealed the polymer characteristic bands. The stretching vibration of the C-H groups is observed in 2974 and 2935 cm^{-1}, while its bending mode is shown in 1453 and 1380 cm^{-1}[43]. The band at 1720 cm^{-1} is attributed to C=O stretching vibration, and the presence of an interval (1792-1700 cm^{-1}) rather than a peak indicates the coexistence of an amorphous phase[7,13]. The anti-symmetrical stress vibration of the C-O-C bond is indicated by the bands at 1129, 1102, and 1054 cm^{-1}, while this same group's symmetrical stretching is observed in 981, 898, and 827 cm^{-1}[7,43].

The main bands characteristic of γ-terpinene and terpinene-4-ol were not found in the PHBV-TTO obtained spectra, being probably hidden by PHBV which happens to have absorption bands in very close wavenumbers than TTO. Besides the presence of a large band positioned between 3620 and 3080 cm^{-1} (Figure 3a) and an intensity increase in the 3050-2800 cm^{-1} region, PHBV-TTO spectra reveal no significant change compared with the PHBV – although these suggest the TTO is effectively present in the polymer. Also, once no significant changes were observed comparing both spectra, it is safe to state no chemical reactions occurred between the polymer and the essential oil, and a physical interaction rather than a chemical took place.

3.4 Tea Tree Oil absorption efficiency

The absorption efficiency performed by mass change indicated a 5.08 ± 0.51% of TTO remaining in the nanofiber after the incorporation and the 48 hours of the drying process (134 μg or 0.145 μL). The volatile compounds of low molecular weight that comprehend the TTO composition are now absent in PHBV-TTO, while compounds of higher molecular weight and less volatile remain.

3.5 Tea Tree Oil release

The representative UV-vis spectra of the aliquots are shown in Figure 4a, while the essential oil cumulative release from PHBV-TTO nanofibers is observed in Figure 4b. As demonstrated by Figure 4a, the highest UV-vis absorption band appears at 204 nm and this wavelength was chosen for cumulative release studies. This absorption band is in agreement with other references[41]. The cumulative release of Tea Tree Oil from PHBV-TTO is shown in Figure 4b, where a progressive, slow, and controlled release is observed in the first 24 hours, followed by a minimal release at 48 hours. The results suggest the release of TTO deposited in the surface of the nanofiber, while in the next hours the release of the oil that was effectively absorbed and entrapped by the polymer. There is no statistical difference in the release measurements at 24 and 48 hours.

3.6 Antimicrobial assay

3.6.1 Microorganisms growth inhibition

The antimicrobial efficacy of PHBV and PHBV-TTO nanofiber compared with pure TTO (control +) was first investigated by liquid growth inhibition assay against *E. coli* (gram-negative bacteria), *S. aureus* (gram-positive bacteria), and *C. albicans* (fungi), as shown in Figure 5. Pure TTO was highly efficient against the three microorganisms, PHBV nanofiber exhibited great activity against the bacteria only, and PHBV-TTO revealed a similar behavior to pure oil; indicating the amount of oil released from the nanofiber was enough to promote antimicrobial activity.

As previously stated, the antimicrobial action of Tea Tree oil is attributable to its hydrophobicity, in which its lipids alter cell membrane balance turning it more permeable with inevitable cell wall rupture[25]. The TTO compounds play an important role in antimicrobial activity, majorly attributable to terpenes. Although 1,8-cineole is not as stronger against bacteria as terpinene-4-oil, it is agreed it collaborates with the cytoplasmic membrane permeabilization, facilitating the terpinene-4-ol action to promote damage and cell lysis[23,44]. According to the seller's specifications, the TTO

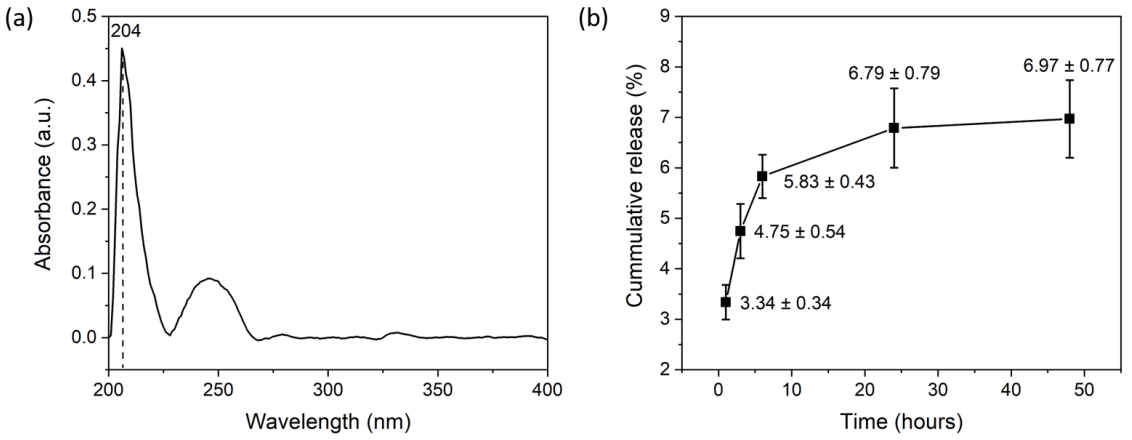

Figure 4. (a) Representative UV-vis spectra from the aliquots and (b) cumulative release of TTO from PHBV-TTO nanofibers (1, 3, 6, 24, and 48 hours).

used has 42.3% of terpinene-4-oil, 19.4% of γ-terpineno, 8.5% of α-terpineno, 2.9% of 1,8-cineole, and a few other compounds in smaller content.

Silveira et al.[23] incorporated 1.5% of TTO into the polymeric solution to produce cassava starch cellulose nanofiber-based films and evaluated its efficacy against the same three microorganisms in this study. While a growth inhibition of 68 and 64% were evidenced for *S. aureus* and *C. albicans*, no activity was performed against *E. coli* when a TTO containing 60.42% terpinene-4-oil was used. Thus, not only the influence of the content of the compound was illustrated, but also the incorporation methodology. The stronger activity of the Tea Tree Oil together with the PHBV and the incorporation methodology reflected in the excellent inhibition growth observed in the liquid growth inhibition assay.

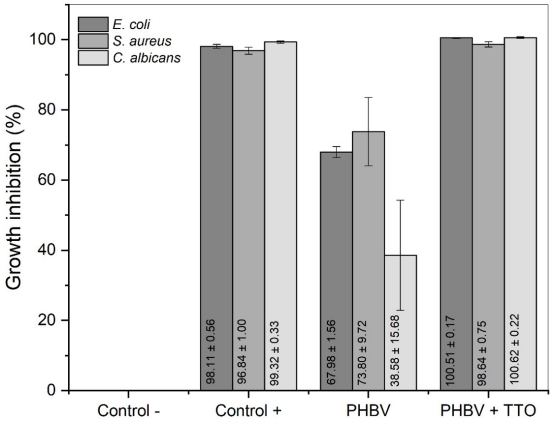

Figure 5. Growth inhibition of PHBV and PHBV-TTO against three strains.

3.6.2 Nanofiber and microorganisms' morphology after liquid growth inhibition assay

After the growth inhibition assay, the microorganisms were fixed to the nanofiber's surfaces and observed by SEM, as in Figure 6. The microorganisms present their typical morphology; *E. coli* rods, *S. aureus* spheres and *C. albicans* pseudohyphae. Disregarding *E. coli* in the PHBV, there was general difficulty in finding the microorganisms on the samples, especially for PHBV-TTO. It is believed that due to the initial burst release of the essential oil in PHBV-TTO, differently from the PHBV nanofiber, the *E. coli* was rapidly inhibited and given no time for adhesion, as well as *S. aureus* and *C. albicans*.

3.6.3 Agar diffusion assay

To further investigate the interactions of PHBV and PHBV-TTO nanofibers with the microorganisms, the agar diffusion assay was performed. The zone of inhibition generated is observed in Figure 7, and Table 1 summarizes the measured halo diameters. PHBV nanofiber presents limited activity compared with the growth inhibition assay; at higher concentrations of microorganisms, it firstly showed some zone of inhibition but few colonies are visible in the clear area after 24 hours for both bacteria, and no zone at all was observed for fungi. Thus, the antimicrobial activity of PHBV itself is limited according to the microorganism's strain and concentration. When TTO is added (PHBV-TTO),

Table 1. Zone of inhibition measurements (mm).

Strain	PHBV	PHBV-TTO	TTO
E. coli	7.45 ± 0.42	13.84 ± 1.33	21.63 ± 1.60
S. aureus	13.83 ± 1.59 *	13.68 ± 1.83 *	15.41 ± 1.52
C. albicans	0	10.13 ± 0.44	18.22 ± 1.53

*No significant difference (p>0.05 in 2-sample t).

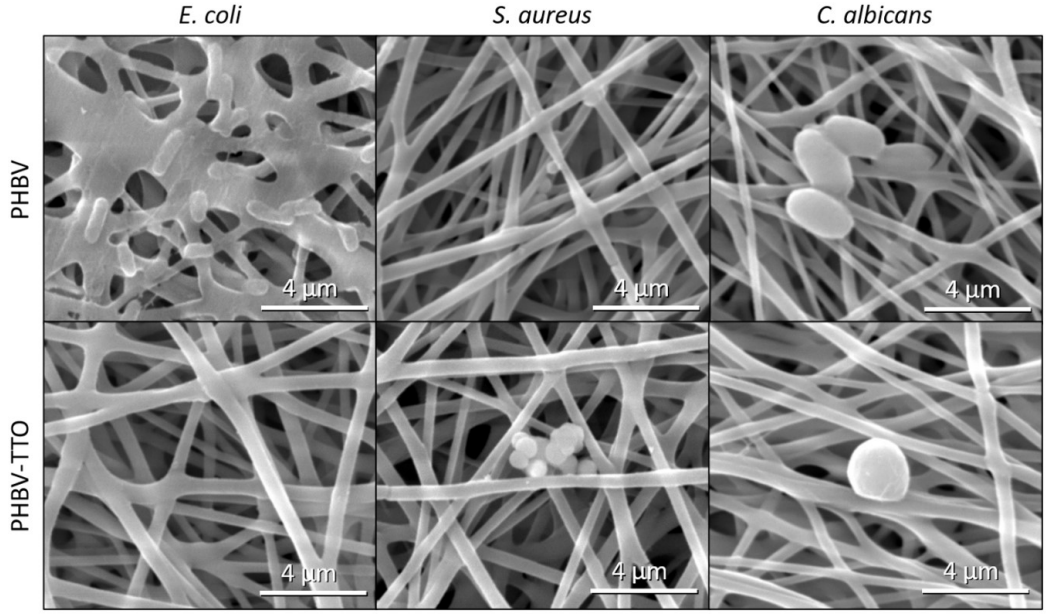

Figure 6. Microorganisms' morphology at PHBV and PHBV-TTO nanofibers after growth inhibition assay.

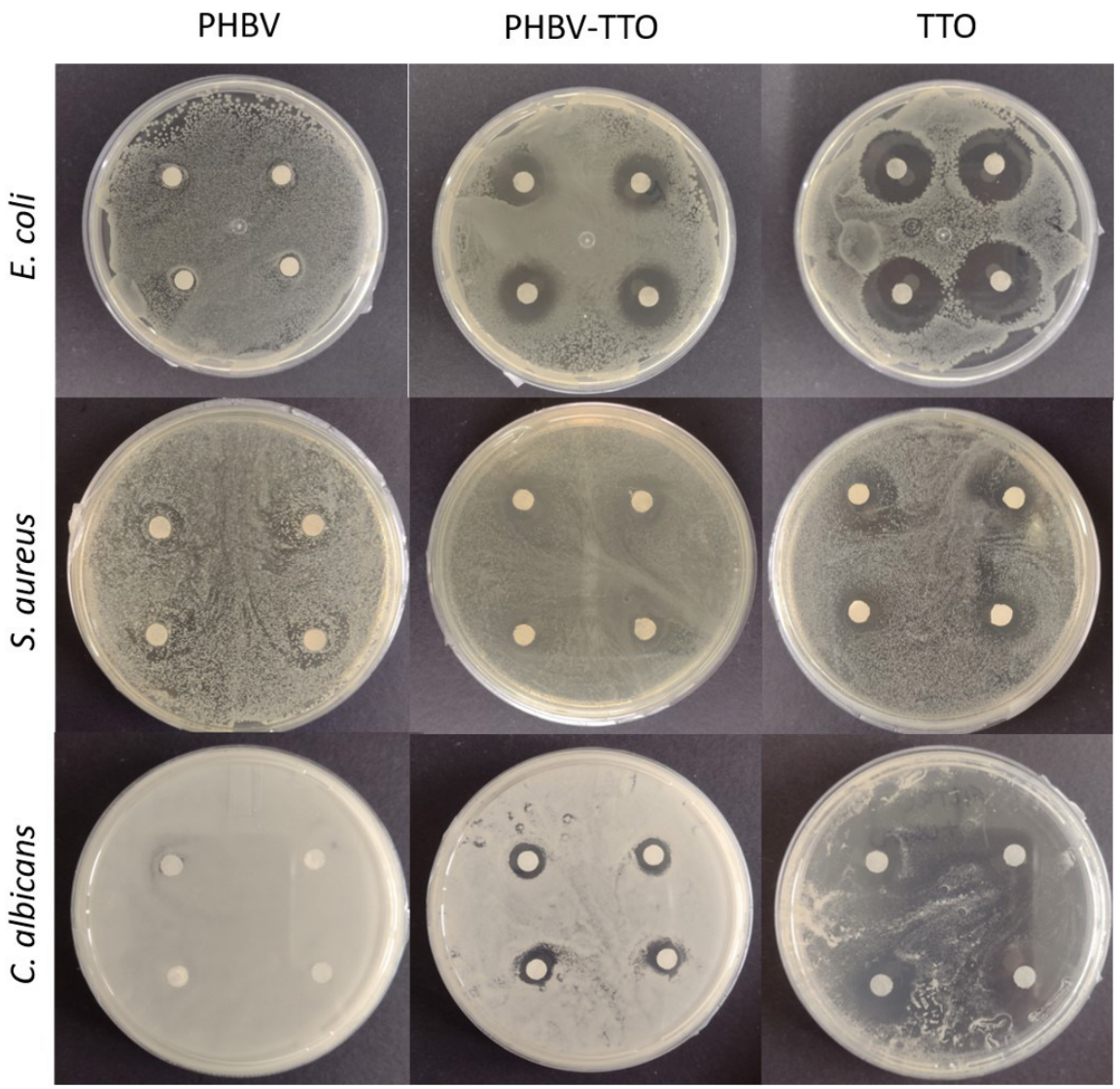

Figure 7. Images of agar plates showing the PHBV, PHBV-TTO nanofibers and controls on *E. coli*, *S. aureus* and *C. albicans*.

however, an increase in the activity of the membrane for *E. coli* (from 7.45 ± 0.42 mm to 13.84 ± 1.33 mm) (p<0.05 in 2-sample t) and *C. albicans* (from 0 mm to 10.13 ± 0.44 mm) (p<0.05 in 2-sample t) is observed and, although no significant difference in the zone of inhibition was noticed against *S. aureus* (p>0.05 in 2-sample t), no colonies were found in the clear area. The pure oil exhibited a higher zone of inhibition for all strains compared to the nanofibers (PHBV and PHBV-TTO), being effective against *E. coli*, then *C. albicans* and *S. aureus*, respectively – a reflection of the concentration used aiming to guarantee its efficacy as a negative control.

Few studies agree TTO is more active against gram-positive bacteria than gram-negative once the latter has an extra protective liposaccharide membrane[5], but here the opposite was found – probably due to the content of the active compound in the Tea Tree Oil composition. In PHBV-TTO, the essential oil amount released by the polymer was demonstrated to be enough to promote antimicrobial action against *E. coli* and *C. albicans*, but a higher content is necessary to extend the activity to *S. aureus*.

3.7 Cell viability assay

The cytotoxicity of the PHBV and PHBV-TTO nanofibers was evaluated by the MTT *in vitro* assay with L929 cells, as observed in Figure 8. Compared with L929 (positive control) cell viability, the one obtained for PHBV of 86.4% was considered non-cytotoxic while for PHBV-TTO of 33.5% is considered highly cytotoxic (cell viability < 70%) (ISO 10-993-5) reaching values close to the negative control DMSO 20% of 31.09%. Considering the essential oil release pattern (Figure 4), it is believed the initial burst release of TTO caused a negative impact on cell metabolism leading to its low viability. A considerable number of studies regarding the influence of TTO in the metabolism of different cell lines discuss cytotoxicity as a

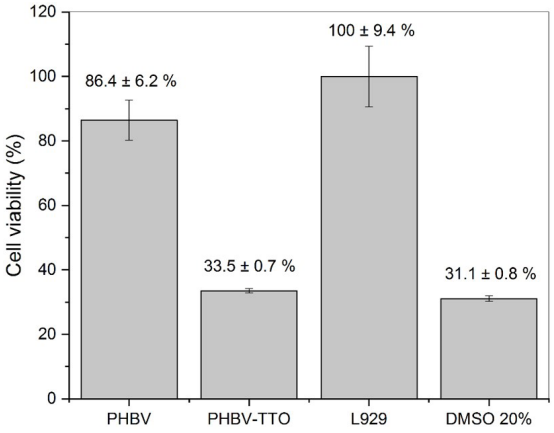

Figure 8. Cell viability percentage of PHBV and PHBV-TTO nanofibers after 24 hours incubation.

limiting factor in wound healing management, being closely related to its concentration, time of exposure, and main constituents' content[45-47]. It is known that terpinene-4-ol is the main component responsible for the *in vitro* cell growth inhibition[45,48], while concentrations up to 1% (v/v)[46] and 10-1000 µg/mL[49] are reported to also perform this effect in a membrane-associated reaction similar to the already described antimicrobial mechanism[48]. Assmann et al.[47] performed an MTT assay on HFF-1 (human fibroblasts) cell line and found a high cytotoxic effect after a 24 hours exposure to 10-1000 µg/mL TTO concentrations, but after 72 hours at 300 µg/mL, however, cell proliferation was induced. In future investigations, the exposure time of PHBV-TTO to cells should be addressed to verify the occurrence of the same phenomena for L929 cells. As demonstrated, many factors should be considered when assessing the *in vitro* cytotoxicity of TTO, while *in vivo* the outcome may be quite different. The use of the essential oil in a dressing mode composed of pads and gauzes applied in *S. aureus* infected wounds in ten volunteers in a clinical study, however, demonstrated accelerated healing of abscessed wounds[30]. As noticed, there are great divergences and controversies related to the use of TTO in wound healing; while there is great concordance with respect to its excellent antimicrobial activity, the cytotoxicity of Tea Tree Oil is still a concern. Considering a hypothetical application of the PHBV-TTO nanofiber in an infected wound, it is believed the progressive release of the essential oil within the first 24 hours followed by minimal release from that time will inhibit further microorganisms' growth while bringing no harm to healthy cells. Thus, after 24 hours, the PHBV-TTO nanofiber will behave just as pure PHBV, allowing cell proliferation and remodeling in extended periods of time with no further cytotoxicity.

4. Conclusions

In this work, a different method of essential oil incorporation on nanofibers for wound healing application was performed; rather than encapsulation, the oil was embedded by absorption on the polymer matrix. Nanofiber's micrographs, contact angle measurements, and FTIR spectra indicate the presence of the essential oil in the PHBV-TTO nanofiber. The absorption efficiency was proven to be around 5%, which corresponds to the less volatile compounds remaining after the drying period of 48 hours. The UV-vis analysis indicated a progressive TTO release from PHBV-TTO nanofiber until reaching 6.8% in the first 24 hours, corresponding to the oil deposited at the surface of the mat, followed by a minimal release at 48 hours as TTO was trapped inside the polymer. The growth inhibition and agar diffusion assays demonstrated the antimicrobial efficacy of not only the PHBV-TTO nanofiber, but also pure PHBV nanofiber (especially against the bacteria) – although no significative differences were shown for *S. aureus* between PHBV and PHBV-TTO nanofibers. The MTT assay performed in the nanofibers indicated the absence of cytotoxicity for PHBV alone, while when associated with the essential oil in PHBV-TTO, its release resulted in low cell viability. As a suggestion of application in wound healing and considering the set of characterizations performed, PHBV-TTO nanofiber may be used in an urgent action to manage wound infection, while no change of dressing will be necessary to promote tissue repair after 24 hours owing to the oil minimal release after this period. Overall, the results of this work suggest the absorption of Tea Tree oil by the PHBV generated a nanofiber with great potential to be used as an antimicrobial dressing.

5. Author's Contribution

- **Conceptualization** – Eliandra de Sousa Trichês.
- **Data curation** – Verônica Ribeiro dos Santos.
- **Formal analysis** – Verônica Ribeiro dos Santos.
- **Funding acquisition –** Verônica Ribeiro dos Santos; Samara Domingues Vera; Eliandra de Sousa Trichês.
- **Investigation** – Verônica Ribeiro dos Santos; Samara Domingues Vera; Gabrielle Lupeti de Cena; Adrielle de Paula Silva.
- **Methodology** – Verônica Ribeiro dos Santos; Samara Domingues Vera; Gabrielle Lupeti de Cena; Adrielle de Paula Silva.
- **Project administration** – Eliandra de Sousa Trichês.
- **Resources** – Dayane Batista Tada; Kátia da Conceição; Ana Paula Lemes; Alexandre Luiz Souto Borges; Eliandra de Sousa Trichês.
- **Software** – NA.
- **Supervision** – Dayane Batista Tada; Kátia da Conceição; Ana Paula Lemes; Alexandre Luiz Souto Borges; Eliandra de Sousa Trichês.
- **Validation** – Verônica Ribeiro dos Santos.
- **Visualization** – Verônica Ribeiro dos Santos; Eliandra de Sousa Trichês.
- **Writing – original draft** – Verônica Ribeiro dos Santos.
- **Writing – review & editing** – Dayane Batista Tada; Kátia da Conceição; Ana Paula Lemes; Alexandre Luiz Souto Borges; Eliandra de Sousa Trichês.

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