O-ATRP synthesized poly(β-pinene) blended with chitosan for antimicrobial and antioxidant bio-based films production

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ABSTRACT

Antioxidant and antimicrobial activities are important characteristics of active film packaging designed to extend food preservation. In this study, functional bio-based films were produced using different concentrations of antioxidant poly(β-pinene) bio-oligomer synthesized via organocatalyzed atom transfer radical polymerization (O-ATRP) and blended with chitosan of different molecular weights. The structural, mechanical, thermal, solubility, antioxidant, and antimicrobial properties of the films were investigated. The poly(β-pinene)-chitosan blends presented significant pores and irregularities with the increase of poly(β-pinene) concentration over 30%. Chitosan molecular weight did not show any important influence in the physical properties of the blends. Poly(β-pinene) load decreased the materials' tensile strength and melting temperature, exhibiting a plasticizing effect on chitosan chains. The antioxidant and antimicrobial activities of the films were improved by poly(β-pinene) incorporation and mainly depended on its concentration. Therefore, the incorporation of poly(β-pinene) in chitosan films can be an alternative for active packaging production.

1. Introduction

The continuous increase of plastics production and its inadequate disposal have created an extensive problem of environmental pollution. This mentioned issue is detrimental to natural ecosystems and threatens the sustainability of human life on the planet. The production of biocompatible and biodegradable films from bio-based polymers is a promising alternative to replacing petroleum-based plastics in many applications, reducing the impacts caused by synthetic films, mitigating carbon footprint and greenhouse gas emissions [1–3].

Among the bio-based renewable polymers, chitosan stands out for the formulation of edible films due to its abundance and physicochemical characteristics. Chitosan is an abundant polycationic polysaccharide obtained from the deacetylation of chitin, which is mainly found in crustacean exoskeleton. It consists of two sub-units, D-glucosamine and N-acetyl-D-glucosamine, linked linearly by 1,4-glycosidic bonds. Besides, chitosan possesses diverse characteristics of interest, such as biocompatibility, biodegradability, non-toxicity, antioxidant and antimicrobial activities, and even anticancer properties [4–7].

Chitosan presents good film-forming properties. The films produced using this polysaccharide have been experimentally tested with success in food packaging, offering protection against contamination and microbial spoilage [8–10]. Furthermore, chitosan can be produced with different molar masses. Molecular size is a fundamental property for polymers, which directly impacts their performance and applications [11]. This availability of chitosan of different molecular weights can be explored to assess their effect on the production of films for packaging.

Different strategies have been reported to improve chitosan films properties, including fillers incorporation, ultrasound and enzyme treatments, cross-linking, surface coating, and blending with other materials [12–17]. Regarding the blending strategy, chitosan blended films present improved performance compared with that obtained for each constituent of the blend separately. This strategy also represents economic advantages over the synthesis of new polymers due to its execution simplicity and considering the possibility to produce tailored materials for specific applications [8].

Blends are constituted of two or more polymers incorporated to each other by a physical method. Depending on the composition and

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characteristics of the blend, the addition of a compatibilizer agent can also be required [18]. Many natural and synthetic polymeric materials have been applied in blends with chitosan, such as gelatin, starch, polyethylene, poly (vinyl alcohol), poly (lactic acid), and poly-caprolactone [19–23]. Kan and collaborators reported the incorporation of gelatin and Chinese hawthorn fruit extract to chitosan to produce antioxidant films [19]. Huang et al. demonstrated that blending poly (vinyl alcohol) and attapulgite with chitosan can enhance the mechanical properties and water resistance of the polysaccharide matrix [23].

Additionally, the application of essential oil constituents in chitosan-based films has been used to enhance their antimicrobial and antioxidant activities, producing efficient nontoxic natural food preservatives. However, due to their low water solubility and high volatility, the essential oils' content that can be added in the chitosan films is low. Therefore, its presence in the composition of films over long periods can be substantially reduced [24–26].

Essential oils also have very characteristic strong smells, intensely affecting the attributes of the films. Furthermore, the microstructure of chitosan/essential oils composite films is exceedingly heterogeneous. The hydrophobic character of the oil disrupts the continuous phase of the chitosan matrix, which leads to inferior thermal and mechanical properties of the composite films [24,27].

Recently, our research group reported the production of poly (β-pinene) oligomers through the controlled polymerization of β-pinene (O-ATRP), a renewable monomer widely distributed in nature and produced by plants as an essential oil constituent. The oligomers synthesized exhibited antioxidant activity more than twice that observed for β-pinene, which represents a very significant advance since essential oil constituents are already known for their very markedly antioxidant activities [28]. Therefore, the incorporation of poly(β-pinene) oligomer in chitosan matrix might imply in the production of efficient active bio-based films.

β-pinene can also undergo free radical or cationic polymerization. However, there are many challenges to these techniques, such as extreme reaction conditions and poor quality of the resulting polymer [29]. Hence, β-pinene polymerization via O-ATRP can constitute an important strategy to introduce new chemical groups to poly(β-pinene), broadening its applications and functionalities. Additionally, the employment of a light-mediated polymerization system has the advantage of using mild reaction conditions, making the process greener.

In the present work, functional bio-based films were produced using different concentrations of poly(β-pinene) oligomers blended with chitosan of different molecular weights. The films were comprehensively investigated and the influence of poly(β-pinene) oligomer concentration on the films' structural, mechanical, thermal, solubility, antioxidant, and antimicrobial properties were assessed. Distinctively, this study represents the first report on the properties of blend films obtained from poly (β-pinene) oligomer incorporation in chitosan matrix, which can be an easy alternative to circumvent the mentioned drawbacks regarding the use of essential oils.

2. Materials and methods

2.1. Chemicals

β-Pinene (Sigma Aldrich, 97% purity) was used as received. Benzophenone (Sigma Aldrich) was used a photocatalyst, 1-bromoethyl benzene (Sigma Aldrich) as the initiator, and N, N, N', N'', N''-pentamethyldiethylenetriamine (PMDETA) (Sigma Aldrich) as an electron donor amine for poly(β-pinene) synthesis via O-ATRP. Acetone (Synth, 99.8%) was used for poly(β-pinene) washing and cleaning process. Chitosan of high (CH − Mw ~ 600,000 g mol^{-1}) and medium (CM − Mw ~ 400,000 g mol^{-1}) molecular weights were obtained from Fluka BioChemika. Sodium dodecyl sulfate (Dinâmica) and ethanol (Synth, 99.5%) were used as surfactant and compatibilizer, respectively, for poly(β-pinene) and chitosan in the blends produced. Acetic acid (Synth,

99.7%) was used to produce the chitosan solutions.

2.2. β-Pinene polymerization and molecular weight determination

Poly(β-pinene) oligomer (Fig. 1a) was produced via O-ATRP in bulk reaction. The molar ratio of 200:1:25:5 was used as the reaction condition for monomer (β-pinene), initiator (1-bromoethyl benzene), catalyst (benzophenone), and electron donor amine (PMDETA), respectively [28]. As illustrated in Fig. 1a, the polymerization was processed in vials placed under a luminous plastic shell (Beaufly SK-818) equipped with 4 UV lamps with a wavelength of 250 nm and irradiation intensity of approximately 3800 lx, for a 6-h period. The reactions occurred at 40 °C, yielding poly(β-pinene) oligomers of 863.81 g mol^{-1} molecular weight (M_w) and dispersity (Đ) of 1.2. The monomer conversion (15.10%) was determined gravimetrically in three replicates. Poly(β-pinene) oligomer characterization is reported by Rodrigues et al. [28]. More detailed reaction steps and the oligomer molecular weight determination are presented in the Supplementary material.

2.3. Poly(β-pinene)-chitosan blends formulation

The poly(β-pinene)-chitosan blends were prepared with chitosan of high (C_H) and medium (C_M) molecular weights. Poly(β-pinene) (P) was added in the concentrations of 10, 30 and 50% *w/w* (P10:C90, P30:C70 and P50:C50, respectively). Films prepared using solely chitosan of high (C_H100) and medium (C_M100) molecular weights were used as control.

To prepare the film solutions, poly(β-pinene) (P) 1% (*w/v*) was dissolved in ethanol with the addition of 2.5% (of the polymer amount) of sodium dodecyl sulfate. Each chitosan, high and medium molecular weights (C_H and C_M, respectively) at 1% (w/v), was dissolved in a 2% (*v/v*) acetic acid solution. Glycerol 0.5% (*w/w* in respect to chitosan) was used as plasticizer. The film forming solutions were kept at 25 °C for 2 h under vigorous stirring.

The poly(β-pinene)-chitosan blend solutions were obtained by mixing the film forming solutions at different poly(β-pinene):chitosan ratios; 10:90%, 30:70% and 50:50% (P10:C90, P30:C70 and P50:C50, namely). The resulting mixtures were stirred during 30 min at 25 °C. Furthermore, in all formulations, 30 mL of each solution was cast onto a plate (9 cm × 9 cm) and oven dried (Venticell - LSIS-B2V) at 50 °C for 24 h. The dried films were manually peeled off and submitted to further analysis.
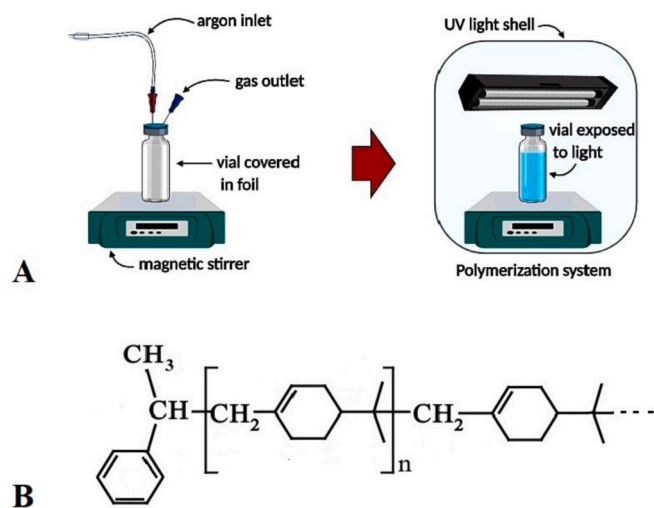

Fig. 1. (A) β-Pinene O-ATRP preparation scheme; from atmosphere purge to the polymerization system configuration and (B) poly(β-pinene) oligomer structure.

2.4. Films characterization

2.4.1. Scanning electron microscopy (SEM)

Microstructure of surface and cryo-fractured cross-section of the films was visualized using a scanning electron microscope with X-ray dispersive energy detector (LEO Electron Microscopy/Oxford) at an accelerating voltage of 15.0 kV. The film samples were cryo-fractured by immersion in liquid nitrogen. Prior to visualization, film samples were mounted on brass stub and sputtered with gold to make the sample conductive. Cryo-fractured Samples were photographed with an angle of 90°. Magnification was of 5000 times.

2.4.2. Fourier transform infrared spectroscopy (FTIR)

The materials functional groups were evaluated by FTIR spectroscopy (PerkinElmer Spectrum 100) with 4 cm^{-1} resolution and between the wavenumbers range from 4000 cm^{-1} to 675 cm^{-1}, using a single-bounce attenuated total reflection accessory with a germanium crystal.

2.4.3. Deacetylation degree (DD)

For both chitosan types studied, high and medium molecular weights, the amino groups percentage was determined by FTIR spectroscopy (PerkinElmer Spectrum 100), according to the method reported by Martínez-Camacho et al. [17]. The DD percentage was calculated according to the following equation:

$$DD(\%) = 100 - \left[\left(\frac{A_{1655}}{A_{3450}} \right) \times \frac{100}{1.33} \right] \tag{1}$$

In which A_{1655} and A_{3450} are the absorbances noted at 1655 cm^{-1} (amide I band) and 3450 cm^{-1} (hydroxyl band), respectively.

2.4.4. Thermogravimetric (TGA) and differential scanning calorimetry (DSC) analysis

Thermogravimetry (Perkin Elmer Model Pyris 1TGA) was executed to determine the samples initial degradation temperature (T$_{onset}$) and their maximum decomposition temperature (T$_{max}$). Six milligrams of each sample were placed in a platinum tray (cross-sectional area of 2.47 10^{-5} m^2) and heated at a rate of 10 °C min^{-1} from 25 °C to 600 °C under a nitrogen flow rate of 25 mL min^{-1}. DSC was performed using a Mettler-Toledo differential scanning calorimeter (DSC1, Greifensee) under a N$_2$ atmosphere in a 50 mL min^{-1} flow. The samples (3 mg) were heated from −40 to 250 °C at a rate of 10 °C min^{-1}.

2.4.5. Thickness and mechanical properties

The samples were conditioned for 48 h at 25 °C and 50% relative humidity prior to the mechanical properties' tests. The determinations were made with 15 mm wide specimens, cut with high-precision equipment to avoid burrs (RDS-100-C, Chem-Instruments, OH, USA). All tests were performed with five repetitions. The tensile properties were determined according to ASTM D882-18 [61], using a universal testing machine (Instron, 5966-E2, Norwood, USA) operating with 0.1 kN load cell. The test speed was 12 mm min^{-1} and the distance between test devices in the machine direction was 50 mm. The films thickness was measured using a micrometer (Mitutoyo, MDC-SX, Kawasaki, Japan) [62]. Ten random locations around each film sample were used for thickness determination.

2.4.6. Water moisture content (MC) and water solubility (WS)

For moisture content measurements, the prepared composite films were cut into squares of 2 × 2 cm. The samples were weighed and the mass loss was determined after drying for 24 h at 105 °C [30]. MC was calculated using Eq. (2):

$$MC (\%) = \frac{W_0 - W_1}{W_0} \times 100 \tag{2}$$

where W_0 represents the initial weight of the film and W_1 represents the

film weight after drying. Measurements were performed in triplicate for each sample.

WS was calculated using dried films (105 °C for 24 h) cut into squares (2 × 2 cm). The referred samples were put into distilled water at 25 °C for 24 h. Subsequently, the immersed films were dried overnight at 105 °C to constant weight. The samples WS was calculated using the Eq. (3). W_2 is the weight of the immersed film after drying.

$$WS (\%) = \frac{W_1 - W_2}{W_1} \times 100 \tag{3}$$

2.4.7. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The free radical scavenging activity of poly(β-pinene)-chitosan films was measured using the DPPH method [31]. The films electron donation ability was evaluated using an ethanol solution containing 0.1 mM of DPPH. First, 15 mg from each film was dissolved in 1 mL of DPPH solution, then the mixture was incubated for 30 min in a dark room. The absorbance decrease was monitored at a wavelength of 517 nm using a UV–Vis spectrophotometer (Kasuaki IL-592-LC-BI). The free radical scavenging activity was calculated according to Eq. (4):

$$DPPH \text{ scavenging activity } (\%) = \frac{Abs_{DPPH} - Abs_{SAMPLE}}{Abs_{DPPH}} \times 100 \tag{4}$$

where Abs$_{DPPH}$ represents the absorbance of the DPPH solution, and Abs$_{SAMPLE}$ corresponds to the absorbance of the sample.

2.4.8. Antimicrobial activity

Three bacterial strains (*Escherichia coli* ATCC 11229, *Staphylococcus aureus* ATCC 23235, and *Salmonella enterica* serotype Typhi ATCC 14028) and three filamentous fungi (*Aspergillus niger* ATCC 1004, *Colletotrichum gloesporioides* ISO024, and *Fusarium oxysporum* ATCC 48112) were obtained from the culture collection of the Microbiology Laboratory of the Human at the Federal University of Paraíba (Bananeiras – Paraíba, Brazil). All bacteria were cultured on Mueller-Hinton agar (MH) at 37 °C, and the filamentous fungi were cultured on potato dextrose agar (PDA) at 25 °C.

The antimicrobial activities of poly(β-pinene) oligomer and poly(β-pinene)-chitosan blend films were evaluated by the disc diffusion method on agar [32]. Aliquots of 100 μL of microbial suspensions at 10^8 CFU mL^{-1} were inoculated onto the surface of the plates containing MH agar for the bacteria and PDA for the fungal strains.

For the antimicrobial activity of the oligomer, three paper discs with a diameter of 6 mm were first impregnated with 10 μL of the referred material and placed on plates containing the culture medium inoculated with the investigated microorganisms.

For the antimicrobial activity determination of the composite films, 1 × 1 cm of each film (previously disinfected under UV light for 30 min) was applied to the agar plates and allowed to stand on them for 30 min at room temperature to favor the bioactive compounds diffusion from the films to the media.

The plates were incubated at 37 °C during 24 h for the bacteria and at 25 °C during 120 h for the fungi. After the incubation period, the diameter of the growth-inhibition zones was measured using a digital caliper.

2.4.9. X-ray diffraction (XRD)

The *X-ray diffraction analysis* is described in the Supplementary material.

2.4.10. Statistical analysis

Data treatment was performed using the tools available in the software Statistica8.0 (Statsoft Inc., Tulsa). Statistical significance was calculated by Tukey's test ($p < 0.05$).

3. Results and discussion

The chitosan-based films appeared transparent, with color gradually turning to yellow with the increase of poly(β-pinene) oligomer load. No characteristic smell was detected. All films prepared were submitted to thermal, chemical, physical, and biological characterizations to evaluate their properties and characteristics that would endorse their application.

3.1. Scanning electron microscopy (SEM)

Scanning electron microscopy was conducted to visualize the top and cross-section topography of different chitosan films and presented in Fig. 2. It is possible to observe the microstructural changes in the samples of the materials due to poly(β-pinene) addition at different concentrations. There were no microstructural differences between chitosan films of medium and high molecular weight for the same poly(β-pinene) concentration.

Surface and cross-section micrographs of chitosan control films, C_H100 and C_M100, displayed a smooth and flat morphology. However, the poly(β-pinene)-chitosan blends presented numerous pores most likely due to proper coalescence of droplets of the nonpolar oligomer molecules in the process of film formation due to the volatilization of solvent [24]. These irregularities on the blends were notably perceived with the increase of poly(β-pinene) concentration. With further addition of the oligomer, many droplets were embedded in the composite films, disrupting the continuity of the chitosan matrix, as can be noted in Fig. 2.

Yaoyao Xu et al. [24] reported the production of chitosan-based films with much larger pores and irregularities with the addition of clove essential oil Pickering emulsion in chitosan blends in a concentration as low as 0.6% (w/w), the films were prepared with zein colloid particles as stabilizer. Akhter et al. [27] found that the surface roughness of chitosan composite films increased significantly due to the incorporation of only 0.5% of mint and rosemary essential oils.

3.2. Fourier transform infrared spectroscopy (FTIR) and deacetylation degree (DD)

Chitosan control films C_H100 and C_M100 deacetylation degree (DD) were $83.62 \pm 0.83\%$ and $82.51 \pm 0.20\%$, respectively. The DD corresponds to the number of acetyl groups removed from chitin, releasing the amino groups from the *N*-acetylglucosamine monomers that constitute the chitin chain. A DD ranging from 70 to 85% indicates a medium deacetylation degree of chitosan, which may be partly dissolved in water. Also, a higher DD is associated with superior biological properties [33,34].

FTIR spectra of chitosan-based films are given in Fig. 3. The broad peak between 3200 and 3570 cm^{-1} (Fig. 3) is attributed to stretching of OH bonds in the chitosan molecule [17]. The sharp peak observed in the range of 1424–1504 cm^{-1} is associated to the absorption bands of the amide groups (–CO–NH– peptide bonds). The wavenumbers of 1640, 1590, and 1370 cm^{-1} are characteristics for the amide I (C=O), amide II (NH$_2$) and amide III (C—N) groups [35]. As the poly(β-pinene) oligomers concentration was increased in the film blends, characteristic peaks for this material appeared clearer in the FTIR spectra. The sharp peak noted at 700 cm^{-1} in poly(β-pinene)-chitosan blends, for example, is related to the presence of a benzyl group fragment coming from the initiator used on the poly(β-pinene) photopolymerization [28]. The geminal methyl group bands of poly(β-pinene) appear at the wavenumbers of 1317 and 1281 cm^{-1} [36].

3.3. Thermogravimetric (TGA) and differential scanning calorimetry (DSC)

Poly(β-pinene)-chitosan films thermogravimetric characterization is displayed in Fig. 4. The results show the mass loss and maximum degradation temperatures of the samples during thermal decomposition. The materials produced from chitosan of high molecular weight displayed slightly higher degradation temperatures than those based on medium molecular weight chitosan. The degradation of all samples tested occurred in three different thermal events, which is typical for chitosan-based films [30,37].

The first thermal event (70–80 °C) is associated with the evaporation of water in the films [38]. The second event, observed around 160–190 °C, corresponds to the decomposition of glycerol [30]. Finally, the third thermal event noted over 280–290 °C is related to chitosan and poly(β-pinene) degradation in the film [28,30]. There were no important changes in chitosan films thermal behavior due to poly(β-pinene) addition. The thermal degradation of this polyterpene oligomer occurs around 251.52 °C [28], which may explain this phenomenon. Furthermore, weight loss of poly(β-pinene)-chitosan films are less pronounced than that of chitosan control films up until 250–300 °C, indicating that the addition of poly(β-pinene) contributes to the integrity of the films under heat exposure. However, in temperatures superior to 300 °C, the poly(β-pinene)-chitosan blends displayed higher rates of weight loss than those observed for chitosan control films due to fast degradation of poly(β-pinene).

Differential scanning calorimetric analysis of the chitosan-based films was conducted to investigate the interactions between the polymers used in the blends. Fig. 5 presents the melting enthalpy (ΔH_m), as well as the melting (T_m) temperatures of all film samples tested. The ΔH_m values were higher for high molecular weight chitosan composites
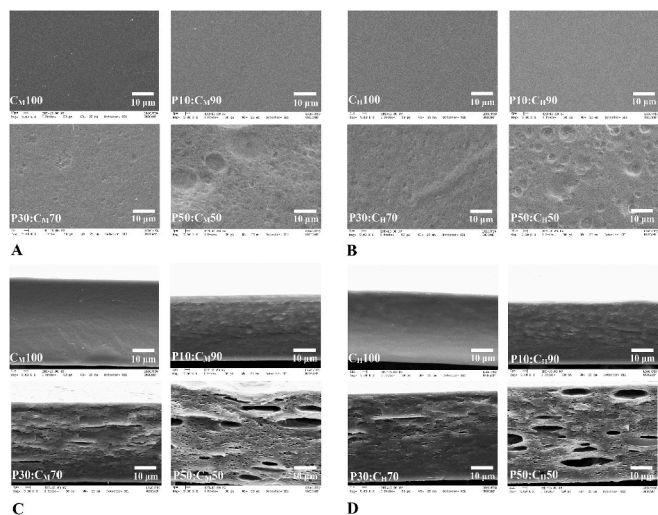

Fig. 2. Scanning electron micrography of poly(β-pinene)-chitosan with chitosan of medium (A, C) and high (B, D) molecular weights and different poly(β-pinene) percentages in top (A, B) and cross-section (C, D) views (5000 times magnification).

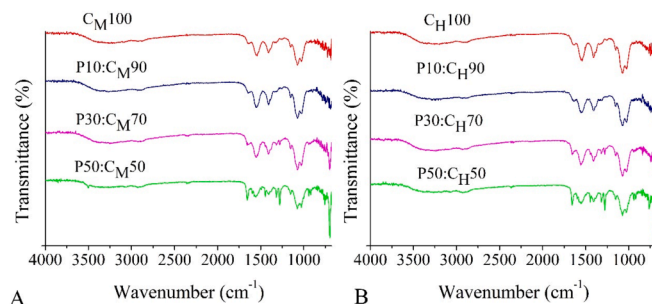**Fig. 3.** FTIR spectra of poly(β-pinene)-chitosan blend films with chitosan of medium (A) and high (B) molecular weights and different poly(β-pinene) percentages.

Sample	Degradation peaks (°C)		
C_M100	76.38	160.81	281.86
P10:C_M90	77.72	165.16	283.20
P30:C_M70	77.96	171.01	283.19
P50:C_M50	73.73	172.32	283.35

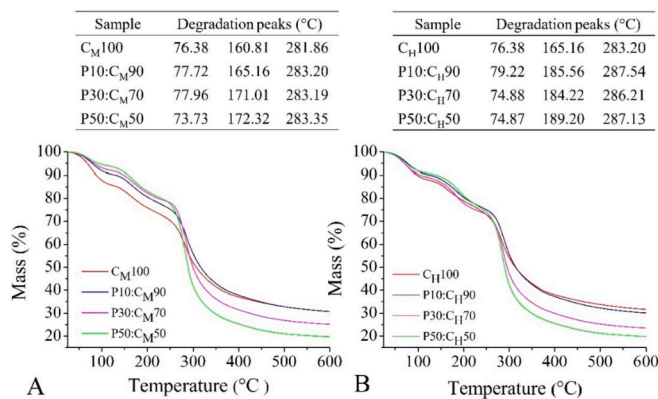| Sample	Degradation peaks (°C)		
C_H100	76.38	165.16	283.20
P10:C_H90	79.22	185.56	287.54
P30:C_H70	74.88	184.22	286.21
P50:C_H50	74.87	189.20	287.13

Fig. 4. TGA curves of poly(β-pinene)-chitosan blend films with chitosan of medium (A) and high (B) molecular weights and different poly(β-pinene) percentages.

Sample	T_m (°C)	ΔH_m (J g^{-1})
C_M100	98.90	309.69
P10:C_M90	93.09	282.97
P30:C_M70	92.06	258.04
P50:C_M50	85.04	205.66

Sample	T_m (°C)	ΔH_m (J g^{-1})
C_H100	98.42	344.60
P10:C_H90	95.51	334.53
P30:C_H70	93.99	243.18
P50:C_H50	86.21	217.34

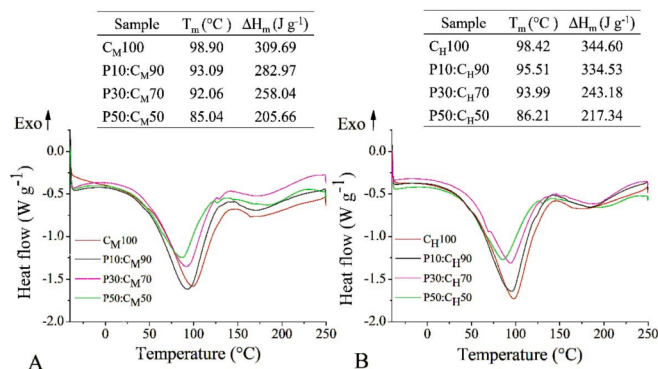**Fig. 5.** DSC curves of poly(β-pinene)-chitosan blend films with chitosan of (A) medium and (B) high molecular weights and different poly(β-pinene) percentages.

than those observed for chitosan blends of medium molecular weight since more energy is needed to melt a polymer with higher molecular weight, to a certain extent [39]. ΔH_m decreased with the increase of poly (β-pinene) concentration for both high and medium molecular weight chitosans used. This parameter decrease indicates a decrease in the crystallinity of the films [40]. The poly(β-pinene) presence in the composites may have contributed to a disarrange of chitosan chains, disturbing the interactions between them and, therefore, decreasing the material crystalline character. X-ray diffraction analysis, presented in the Supplementary material, confirmed the reduction in the crystalline organization of the films produced due to the poly(β-pinene) load on the blends.

The T_m values of the chitosan control films (C_M100 and C_H100) are similar to those typically reported for chitosan pure films [41,42]. Due to crystallinity reduction with the presence of poly(β-pinene), T_m values also decreased with the increment of the oligomer in the chitosan matrix. Differences in chitosan molecular weight did not interfere in the films T_m. Glass transition temperatures (T_g) were not identified in the DSC curves, several studies have demonstrated that determination of chitosan T_g is difficult [43,44]. However, decrease in T_m and crystallinity values with the addition of poly(β-pinene) are a direct indicative of T_g reduction, revealing that this oligomer causes a plasticizing-like effect on the polymer matrix, increasing the mobility of the composite macromolecular chains compared to the chitosan-based controls. Plasticizers can increase the free volume between polymer chains upon their incorporation into the matrix, allowing the chain segments to move and rotate more easily increasing movement of polymer chains with respect to each other and decreasing the polymer T_g and melt viscosity [45].

3.4. Thickness and mechanical properties

Adequate mechanical properties are of paramount importance for films applications in packaging since they must be able to withstand external stress and maintain their integrity during usage. Therefore, thickness, tensile strength (TS), and elastic modulus (E_M) of poly (β-pinene)-chitosan blend films are presented in Table 1.

The films thickness varied from about 80 to 50 μm, values corresponding to C_M100 and P50:C_H50, respectively. This parameter presented a noticeable decrease due to higher poly(β-pinene) incorporation and consequent lower chitosan percentage in the composites. Nonetheless, the thickness of the films produced in the present study is within the range (124 to 44.68 μm) commonly reported for chitosan-based films [35,41].

TS of chitosan control films did not reveal any statistical difference as a result of molecular weight changes (M_w of 600,000 and 400,000 g mol^{-1}), agreeing with the results reported by Yixin Xu et al. [30]. However, the E_M was 23% higher for the control film produced with high molecular weight chitosan since higher molecular weights often lead to better polymer mechanical properties. Similar values of TS and E_M for pure chitosan films of medium molecular weight were described by Di Filippo et al. [37] and Kaya et al. [38].

Overall, the inclusion of poly(β-pinene) oligomer in the chitosan-based films importantly reduces the TS and E_M of the composites produced, especially for the film blends composed of 50% w/w poly (β-pinene) (P50:C_H50), which is consistent with the general expectation for a plasticized polymer. These results confirm that the oligomer addition has a similar effect to that obtained by the introduction of a plasticizer into the composition of chitosan films [46], which was also noted in the thermal characterization.

In a study of functional properties of chitosan films modified by snail mucus extract, Di Filippo et al. [37] reported a reduction in TS from 32 to 7 MPa and in E_M from 1200 to 170 MPa with the incorporation of 30% of mucus in their films. Hight TS and E_M reduction were also reported by Kaya et al. [38] in chitosan films incorporated with stem, leaf, and seed extracts of *Pistacia terebinthus* in a concentration of 33% (w/w). Akhter et al. [27] published TS values of 11.7 and 7.7 MPa, and E_M values of 0.8 and 1.5 MPa for chitosan-pectin-starch films with and without mint essential oil (0.5%) incorporation, respectively. These results illustrate the important mechanical properties obtained in the present work, even in high concentrations of poly(β-pinene) addition.

3.5. Moisture content and water solubility

The moisture content (MC) and water solubility (WS) values of the poly(β-pinene)-chitosan blend films produced in this study are listed in Table 2. The results show that chitosan molecular weight does not significantly affect the MC of the composite films. Furthermore, poly (β-pinene) addition does not represent a critical factor in this parameter.

Table 1
Thickness and mechanical properties of poly(β-pinene)-chitosan blend films produced with chitosan of medium and high molecular weights and different poly(β-pinene) percentages.

Sample	Thickness (μm)	Tensile strength (MPa)	Elastic modulus (MPa)
C_M100	80.4 ± 9.2[a]	28.6 ± 0.8[a]	1634.4 ± 46.7[a]
P10:C_M90	57.8 ± 8.4[def]	22.2 ± 3.9[bcd]	2161.2 ± 172.4[b]
P30:C_M70	62.8 ± 3.9[cde]	16.8 ± 3.5[b]	1146.5 ± 215.6[c]
P50:C_M50	67.8 ± 9.5[bc]	3.2 ± 0.7[e]	352.6 ± 95.4[d]
C_H100	77.1 ± 7.2[ab]	26.7 ± 4.2[ad]	2014.7 ± 301.7[b]
P10:C_H90	67.1 ± 9.2[cd]	23.0 ± 0.6[acd]	1470.7 ± 38.4[ac]
P30:C_H70	56.3 ± 5.2[ef]	19.7 ± 1.0b[c]	1206.7 ± 86.6[c]
P50:C_H50	49.6 ± 5.1[f]	4.1 ± 1.0[e]	498.9 ± 73.9[d]

Averages followed by different letters, in the upper right, show differences between the films in the same column block, determined by Tukey's test ($p < 0.05$).

Table 2
Moisture content and water solubility of poly(β-pinene)-chitosan blend films produced with chitosan of medium and high molecular weights and different poly(β-pinene) percentages.

Sample	Moisture content (%)	Water solubility (%)
C_M100	17.54 ± 0.29^{ab}	7.77 ± 0.20^{abc}
$P10:C_M90$	16.59 ± 0.68^{bc}	12.16 ± 1.37^{de}
$P30:C_M70$	17.20 ± 0.27^{abc}	9.84 ± 0.39^{bcd}
$P50:C_M50$	16.87 ± 0.60^{abc}	13.90 ± 0.27^{e}
C_H100	18.86 ± 0.74^{a}	7.06 ± 0.27^{ab}
$P10:C_H90$	17.19 ± 0.74^{abc}	4.98 ± 0.55^{a}
$P30:C_H70$	15.33 ± 0.19^{c}	11.41 ± 0.43^{cde}
$P50:C_H50$	16.44 ± 0.44^{bc}	22.8 ± 1.73^{f}

Averages followed by different letters, in the upper right, show differences between the films in the same column block, determined by Tukey's test ($p < 0.05$).

However, it is possible to note a slight trend of film MC decrease as the polyterpene oligomer concentration is raised.

Average MC values were 17.05 ± 0.52 and $16.95 \pm 1.2\%$ for the films prepared with chitosan of medium and high molecular weights, respectively. Similar behavior was found by Sedlaříková et al. (2019) in their work on the physical properties of chitosan films incorporated with different concentrations of thyme (*Thymus vulgaris*) essential oil (0.5 to 2% *w/w*).

The WS is a direct indication of the polymer films' resistance to water and humidity, which is a fundamental feature for their application in packaging and food preservation. Poly(β-pinene) incorporation in the chitosan matrix caused a general trend of WS increase due to the oligomer percentual increment in the film blend (Table 2). However, the WS values did not exceed 23% in the conditions tested.

This increase can be related to the plasticizing effect noted for poly(β-pinene), leading to an expansion in the free volume between chitosan chains, promoting a greater exposure of their hydrophilic groups. In turn, this effect favors the diffusion of water molecules into the film matrix and, consequently, increases their solubility [46].

Sanyang et al. [47] revealed that the increase in the content of different plasticizers in biodegradable films based on sugar palm (*Arenga pinnata*) starch intensifies the WS of the materials. The same behavior was reported by Boesel [46] in his study on the effect of plasticizers on the barrier and mechanical properties of biomimetic composites of chitosan and clay.

Nonetheless, compared with the composite films of chitosan and ascorbate produced by Tan et al. [48] and the thermoprocessed starch-polyester bilayer films added with gellan or xanthan gum studied by Hernández-García et al. [49], the poly(β-pinene)-chitosan blend films reported in the present work exhibit a lower water solubility in 24 h.

3.6. DPPH free radical scavenging activity

Chitosan films can be used as active packaging to prevent the oxidation of food and commodities, and poly(β-pinene) oligomer has demonstrated higher antioxidant activity than its composing monomer, β-pinene [4,28]. The antioxidant activity of the chitosan-based films

Table 3
DPPH free radical scavenging activity of poly(β-pinene) and poly(β-pinene)-chitosan blend films produced with chitosan of medium and high molecular weights and different percentages of the oligomer.

Sample	DPPH (%)	Sample	DPPH (%)
C_M100	19.80 ± 0.16^{a}	C_H100	20.81 ± 0.07^{a}
$P10:C_M90$	25.81 ± 0.10^{b}	$P10:C_H90$	24.46 ± 0.26^{f}
$P30:C_M70$	33.80 ± 0.26^{c}	$P30:C_H70$	28.84 ± 0.29^{g}
$P50:C_M50$	48.91 ± 0.10^{d}	$P50:C_H50$	$49,29 \pm 0.51^{d}$
Poly(β-pinene)	44.44 ± 0.37^{e}	–	–

Averages followed by different letters, in the upper right, show differences between the films in both column blocks, determined by Tukey's test ($p < 0.05$).

produced in this study was determined by the DPPH method and is displayed in Table 3. The DPPH free radical scavenging activity of poly(β-pinene) is also shown.

Reported values of pure chitosan films antioxidant activity (DPPH) commonly ranges from 12 to 38% [44,50]. In the present work, there were no significant differences in the DPPH free radical scavenging activity of the chitosan control films produced with different molar weights, which was around 20%. Chitosan antioxidant response is mainly attributed to the ability of its residual free amino groups to react with free radicals yielding ammonium groups and stable macromolecular radicals [50].

Poly(β-pinene) oligomer addition in the composite films greatly extended the antioxidant activity displayed by the chitosan controls. This effect was notably superior in high oligomer concentrations (30 and 50%). The combined effect of poly(β-pinene) and chitosan in the P50:C_M50 and P50:C_H50 films generated materials with higher antioxidant activity than those observed for the individual components of the blend. This result suggests a synergic effect of both materials when incorporated at a 50–50% weight ratio. This synergism is most likely due to the simple summation of the individual effects of radical scavenging exhibited by poly(β-pinene) and chitosan.

Kaya et al. [38] reported DPPH free radical scavenging activity ranging from 93.98 to 95.91% for chitosan films incorporated with stem, leaf, and seed extracts of *Pistacia terebinthus*. Liu et al. also reported similar radical scavenging activity for chitosan films incorporated with 1.0–2.0% protocatechuic acid [51]. Still, the results shown in the current study were superior to those found in chitosan films blended with sea urchin spine powder, cellulose-curcumin-chitosan blends, and chitosan-kefiran composites [30,35,52].

3.7. Antimicrobial activity

Poly(β-pinene) oligomer and poly(β-pinene)-chitosan composite films antimicrobial activity were investigated against different microorganisms (Table 4). This is the first report on the antimicrobial activities of poly(β-pinene) oligomer blended with chitosan. The microbial cultures used in this study included bacterial strains and filamentous fungi selected due to their pathogenic and/or food-decomposing capabilities.

Table 4
Antimicrobial activity of poly(β-pinene) and poly(β-pinene)-chitosan blend films produced with chitosan of medium and high molecular weights and different percentages of the oligomer.

Sample	Bacteria zone of inhibition (mm)		
	E. coli	*S. typhi*	*S. aureus*
Oligomer	9.13 ± 0.75	6.83 ± 0.08	13.30 ± 0.31
C_M100	n.d.	n.d.	n.d.
$P10:C_M90$	n.d.	n.d.	n.d.
$P30:C_M70$	n.d.	n.d.	11.41 ± 0.80
$P50:C_M50$	n.d.	n.d.	13.20 ± 1.15
C_H100	n.d.	n.d.	n.d.
$P10:C_H90$	n.d.	n.d.	n.d.
$P30:C_H70$	n.d.	n.d.	10.62 ± 0.43
$P50:C_H50$	n.d.	n.d.	12.15 ± 0.11

Sample	Fungi zone of inhibition (mm)		
	A. niger	*C. gloesporioides*	*F. oxysporum*
Oligomer	16.60 ± 0.77	19.04 ± 0.40	n.d.
C_M100	n.d.	n.d.	n.d.
$P10:C_M90$	n.d.	n.d.	n.d.
$P30:C_M70$	18.15 ± 1.14	16.38 ± 0.47	n.d.
$P50:C_M50$	18.55 ± 1.03	17.89 ± 1.47	15.59 ± 0.30
C_H100	n.d.	n.d.	n.d.
$P10:C_H90$	n.d.	n.d.	n.d.
$P30:C_H70$	15.38 ± 0.33	n.d.	n.d.
$P50:C_H50$	16.61 ± 0.83	16.52 ± 0.81	18.45 ± 1.43

Poly(β-pinene) exhibited antimicrobial activity against all microorganisms tested, except for *F. oxysporum*. The oligomer inhibition zone varied from 6.83 to 19.04 mm. Oppositely, chitosan control films did not present any antimicrobial activity against the microorganism analyzed, which is consistent with literature reports [37,53].

Additionally, the chitosan minimum inhibitory concentration varies widely depending on the microorganism strain and it is unable to diffuse through the surrounding agar media in a film form, making unfeasible the microorganism growth inhibition [54,55].

The films produced from the poly(β-pinene)-chitosan blend revealed antimicrobial activity against *S. aureus*, *A. niger*, *C. gloesporioides*, and *F. oxysporum*. However, the inhibition zone varied largely (6.83 to 18.55 mm) considering the film oligomer content. Films produced with poly(β-pinene) concentrations up to 30% were not efficient to inhibit the strains analyzed in this work.

Interestingly, neither poly(β-pinene) or chitosan presented antimicrobial activity against *F. oxysporum* individually. Nonetheless, the P50:C_M50 and P50:C_H50 formulation displayed inhibitions of 15.59 and 18.45 mm against the referred fungi. This data indicates a synergic inhibition effect of poly(β-pinene) and chitosan against *F. oxysporum* when incorporated at a 50–50% weight ratio that must be studied in more depth for more precise conclusions.

Essential oils and their constituents are known to possess significant antimicrobial activity. Their hydrophobic nature allows them to move across the cell membranes of microorganisms and disrupt these structures, making them more permeable [56]. Additionally, the potential of β-pinene to inhibit phospholipase and esterase activities was also reported in the literature [57].

Considering β-pinene structural modifications in the polymerization process, the antimicrobial activity demonstrated by the poly(β-pinene) oligomer is likely associated with the presence of delocalized electrons, its hydrophobic nature, and its relatively small molecular weight, which may have made possible its diffusion through the cell walls of the microorganisms tested [58].

The presence of bromine at the oligomers chain end coming from the initiator used in the polymerization is another factor likely contributing to poly(β-pinene) antimicrobial activity. Halogenation is a strategy used to confer antimicrobial characteristics to a chemical compound [59,60]. Bouthenet and her collaborators reported an antimicrobial activity in resorcinol dimers after the bromination of this material. The brominated dimers showed inhibition of the glyoxalate pathway enzyme. The importance of the halogen substitution in achieving high inhibitory values for isocitrate lyase was also demonstrated [60].

4. Conclusions

Functional films were unprecedented and successfully produced using different concentrations of poly(β-pinene) oligomer blended with chitosan of high and medium molecular weights. The morphology of the blends was considerably altered by poly(β-pinene) concentration on the polysaccharide matrix in high oligomer concentrations, which negatively influenced the materials' mechanical properties. Chitosan molecular weight did not show appreciable influence on the physical properties of the blends produced. Poly(β-pinene) promoted a plasticizing effect on chitosan macromolecular chains increasing the films' water solubility and affecting their thermal behavior. T_m values were decreased due to poly(β-pinene) load. However, the material degradation temperature was not altered. The antioxidant and antimicrobial activities of the films were importantly improved by poly(β-pinene) incorporation, and these mentioned characteristics were mainly dependent on the oligomer concentration. Although higher concentrations of oligomer provided the most pronounced functional effects, it is noted that the P30:C_M70 formulation displayed the best balance comparing antimicrobial and antioxidant activities with mechanical properties performance. This oligomer concentration (30%) provided biopolymer films with satisfactory tensile strength (>16 MPa)

simultaneously with inhibitory activity against bacteria and fungi, in addition to antioxidant activity approximately 70% higher than that of pure chitosan films.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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