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Antibacterial activity of chitosan/collagen membranes containing red propolis extract

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In this study we developed a mucoadhesive polymeric membrane wound dressing incorporating red propolis extract (HERP). Membranes were made using a casting method employing collagen, chitosan, polyethylene glycol (15, 20, and 30v%), and hydroethanolic extract of EtOH-H_2O 70v% – 30v% (v/v) of HERP (0.5, 1.0, and 1.5%). Membranes were extensively characterized to assess the thickness, pH, morphology using Scanning Electron Microscopy (SEM), Differential Scanning Calorimetry (DSC), mechanical properties, swelling, *in vitro* mucoadhesion, cytotoxicity, and minimum inhibitory concentration (MIC). Assessment of the thickness and mechanical properties of the membranes containing HERP revealed that the most significant thickness obtained was 40.7 µm; thermal analysis suggests suggesting the hydrogen bonds between hydroxyl groups of isoflavones and the free amine present in the region of chitosan. Cell viability decreased as the amount of HERP increased. Finally, the MICs were 7.8 and 1.9 µg.mL–1 for *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853, respectively. These results were suggesting that the 0.5 % HERP membrane has the potential for future studies for wound application.

1. Introduction

Membranes have been employed to accelerate biological processes involved in wound healing (Yenilmez et al. 2015, Kamoun et al. 2017, Hissae Yassue-Cordeiro et al. 2019). With this, the method of production and the selection of polymers are crucial to obtain success with the biomaterial. Also, these polymers have to be biocompatible, biodegradable, and lacking immunogenicity (Van Onselen and Gardner 2016).

Among the natural polymers used as biomaterials, collagen, and chitosan are the most popular ones. Collagen is a natural polymer used in tissue engineering because of its low immunogenicity, biocompatibility, biodegradability, mucoadhesive properties, and capacity to enhance tissue regeneration and cell proliferation (Cheng et al. 2014). Type I collagen is the widely used in the medical and pharmaceutical industries (Guillerminet, et al. 2010). On the contrary, chitosan is a semisynthetic polymer derived from the deacetylation of chitin originating from fungi and crustaceans (Cheng et al. 2015, Barbosa et al. 2016). Chitosan is biodegradable, biocompatible, bioadhesive, and microbiostatic. Blending of polymers is used for the development of materials with properties absent in each of the polymers alone (Anjum et al. 2016). For example, hydrogen bonds formed between collagen and chitosan chains improve flexibility, swelling, and mucoadhesion of collagen/chitosan membranes (Ramasamy and Shanmugam 2015). The incorporation of antimicrobial agents in membranes has improved wound treatment to prevent infection at the site and to improve the healing process. The use of Hydroalcoholic Extract of Red Propolis (HERP) in the improvement of wound healing has also been previously reported (Souza et al. 2013). HERP is a natural product rich in phenolic compounds with antimicrobial activity against fungi, as well as Gram-negative and Gram-positive bacteria. Moreover, many studies have reported the successful use of this extract as an antimicrobial agent in the treatment of injuries (Nasiri et al. 2015).

Here we report the development of mucoadhesive collagen-chitosan blended membranes incorporating HERP for the antimicrobial activity of wounds.

2. Investigations and results

2.1. Extract yield percentage

The color of HERP was influenced by botanic origin as well as location (Borges et al. 2015; Morsy et al. 2015). The yield of dried HERP was 37.38±0.15%. The extract yield percentage showed a higher value recommended by the Brazilian Ministry of Agriculture that is at least 11% of dry extract (BRASIL 2001). Its yield is consistent with other results found in the literature, which report yields between 9.42% and 43.5% (Jug et al. 2014). According to Kubiliene et al. (2015), the yield of active biological compounds (phenols and flavonoids) is approximately ten times higher when using hydroethanolic extraction instead of aqueous extraction.

In Fig. 1 it is possible to observe peaks that elute with retention times similar to the patterns used, between 13 and 30 min, indicating more polar constituents. As can be seen, the chromatogram region between 10 and 30 min contains four peaks identified as daidzein (P1), formononetin (P2), liquiritigenin (P3) and biochanin A (P4). These components are, according to Cuestra-Rubio (2012), related to isoflavones from the UV spectrum with an absorption band of about 280 nm. In the sample used, formononetin (P2) was the substance with the highest concentration identified (48.13874

mg/g EHPV), followed by liquiritigenin (44.9398 mg/g EHPV), biochanin A (22.8276 mg/g EHPV) and daidzein (19.3521 mg/g EHPV).

According to da Silva Frozza et al. (2013), in the sample of EHPV of the state of Sergipe/Brazil, the same components were found. According to Freires et al. (2016), the EHPV from the state of Alagoas/Brazil contains several substances such as quercetin, medicarpine, ferulic acid, isoliquiritigenin. This suggests the use of the different standards in its study, allowing the identification of other substances.

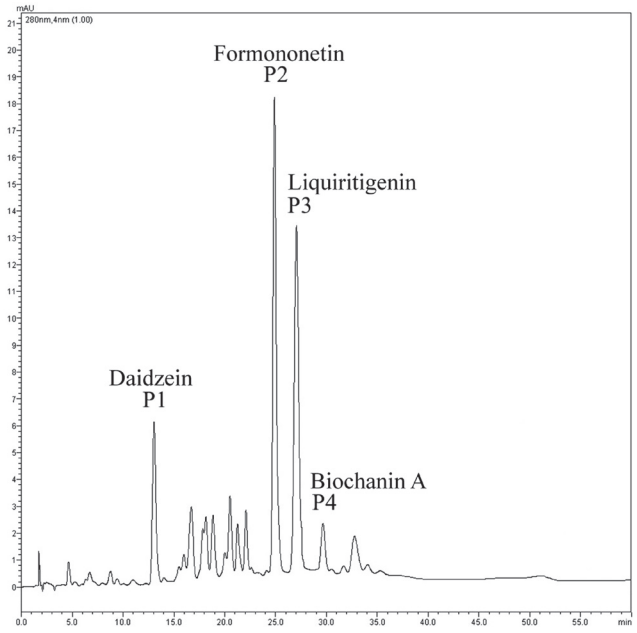

Fig. 1: sample obtained by HPLC with analytical column type C18 (150 x 4.6 mm) with an injection volume of 20 µL and with photodiode array detector set at 280 nm.

2.2. Membranes

All membranes produced with a blend of chitosan and collagen had similar macroscopic characteristics. Also, the incorporation of PEG in different concentrations as a plasticizer was investigated and chosen according to homogeneity, flexibility, continuity, and handling. The membrane with 15% PEG showed the best macroscopic characteristics highlighting the flexibility.

2.3. Scanning electron microscopy (SEM)

Surface morphology of the membranes was evaluated using SEM micrographs of the surface and the cross-section, as illustrated in Fig. 2. Membranes had homogeneous microstructures with no irregularities such as air bubbles; they were uniform, compact, and without cavities, cracks, or pores.

Fig. 2 (a), (b), and (c) show an increase in surface roughness with the addition of HERP. Surface roughness increased as the amount of HERP increased. Fig. 2 (d), (e), and (f) depict the freeze-drying of these membranes, which leads to a homogenous structure morphology. Similar results were observed for all samples, which formed densely packaged membranes. These results agree with those of Bodini et al. (2013). They also observed the formation of an irregular surface when an ethanolic extract of propolis was incorporated into the hydroxypropyl methylcellulose membranes.

2.4. Differential scanning calorimetric (DSC)

Figure 3 shows the DSC curves for all chitosan/collagen membrane samples. The heating curves had two peaks. The first peak (T_1, endothermic) was observed between 30 and 130 ºC, which can be attributed to the evaporation of water bound to the chitosan chain by different physical bonds (hydrogen bonds, electrostatic interactions). The second peak (T_2, exothermic) was observed at a temperature above 250 ºC owing to the degradation of polymers. Similar results were reported by Ostrowska-Czubenko et al. (2009) explaining that water binds the chitosan chain *via* hydroxyl groups

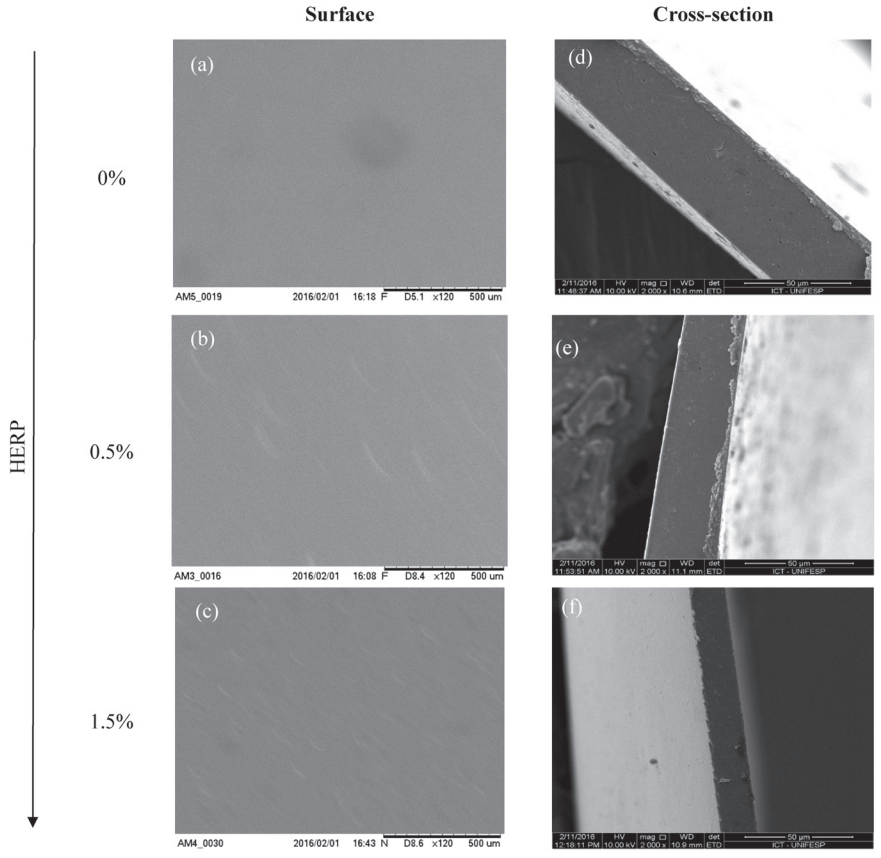

Fig. 2: SEM of membrane surfaces/sections of membrane fracture.

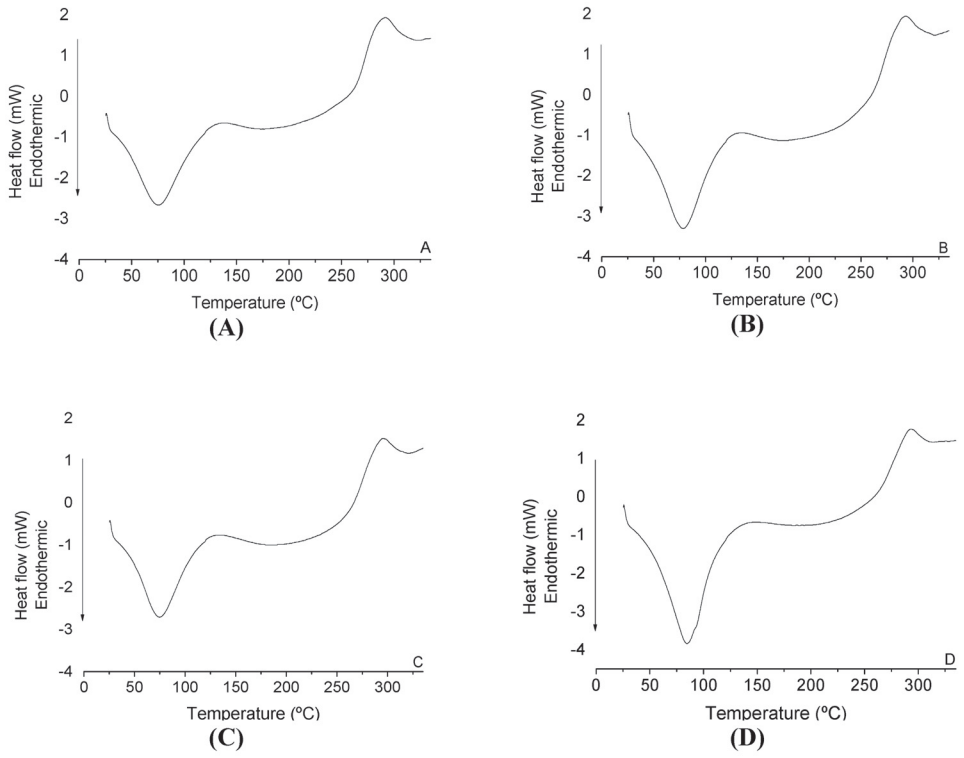

Fig. 3: DSC thermogram of chitosan/collagen membranes HERP-free (a) membrane chitosan/collagen with HERP 0.5 %, (b) membrane chitosan/collagen with HERP 1.0 % (c) membrane chitosan/collagen with HERP 1.5 % (d).

and the free amine present in the amorphous region of chitosan. Elevation of the endothermic peak temperature from 75 to 78.3 °C and 84.4 °C in membranes with 0%, 0.5%, or 1.5% HERP, respectively, may be related to the interaction between polymer and propolis. According to chromatography, the HERP shows the main components of the isoflavones as daidzein, formononetin, liquiritigenin and biochanin. These components show one or more hydroxyls attached to one or more aromatic rings suggesting the hydrogen bonds between hydroxyl groups of isoflavones and the free amine present in the region of chitosan (Panos et al. 2008; Hsiao et al. 2017).

The antioxidants from interactions with the amide groups of chitosan (Benbettaïeb, et al. 2015), and thus the main interaction between water and chitosan is through the hydroxyl groups, which form stronger hydrogen bonds with water than does the amine group; therefore, higher temperatures are required to remove water (Neto et al. 2005; Ghosh et al. 2010; Somers et al. 2015). Furthermore, loss of water is more difficult because water molecules become trapped in a cross-linking net. The increase of crosslinks promoted by HERP is demonstrated by an elevation in the degradation temperature from 290 to 294 °C.

According to a study by Ghosh et al. (2010), the most important functional groups that bind water to the chitosan chain are the hydroxyl groups and the free amine present in the amorphous region of chitosan. The addition of HERP promoted an elevation in the temperature required for the onset of water evaporation, which can be attributed to the hydrophobic molecules of HERP. Such molecules would enhance membrane hydrophobicity and thus hamper the evaporation of water. Therefore, based on the studies mentioned above, the elevation in endothermic peak temperature may be related to the cross-linking of chitosan promoted by propolis. Some compounds like flavonoids (e.g., quercetin) and phenolic acid (e.g., ferulic acid) were identified and quantified in red propolis (Park et al. 2004; Silva et al. 2008). Such compounds deserve attention because they have already been incorporated into biopolymer films or even used as cross-linking agents. With this, the thermal behavior of the membrane by adding the EHPV increases the stability of the composite discreetly.

The results of the TGA that was performed to evaluate the thermal stability of membranes are shown in Fig. 4. Weight loss occurred in three stages for the HERP-free chitosan/collagen membrane. The first and second stages represent the loss of water, but the second stage represents the structural water linked stronger than the first (Fernandes et al. 2011). The last stage represents the decomposition (thermal and oxidative) of both biopolymers. Collagen combustion occurs at about 311 ºC, which is almost the same as the temperature of chitosan decomposition (León-Mancilla et al. 2016). It was observed that the incorporation of HERP in the membrane tended to shift the last thermal event to a slightly higher temperature, and such a shift was attributed to an increase in thermal stability, which supports the cross-linking action of HERP as observed in the DSC results.

2.5. Mechanical properties of membranes

The measurement of mechanical properties is of paramount importance because natural polymers have limitations on these

Table 1: Mechanical properties of the membranes

Concentration of HERP (%)	**Elongation at Break (%)**	**Young's modulus MY (MPa)**	**Tensile Strength (MPa)**
Membrane free HERP	46.429 ± 1.403 [a]	230.240 ± 65.508 [a]	97.561 ± 22.660 [a]
M + HERP 0.5%	10.000 ± 0.208 [d]	584.225 ± 84.873 [a]	59.455 ± 15.751 [b]
M + HERP 1.0%	14.857 ± 0.121 [c]	632.920 ± 139.382 [a]	89.463 ± 14.789 [a]
M + HERP 1.5%	20.571 ± 0.282 [b]	531.317 ± 41.663 [a]	103.012 ± 19.021 [a]

M: membrane; HERP: hydroethanolic extract red propolis. The mean presented, followed by the same letter in the columns, belongs to the same group according to Tukey's post-test ([a]$p < 0.05$).

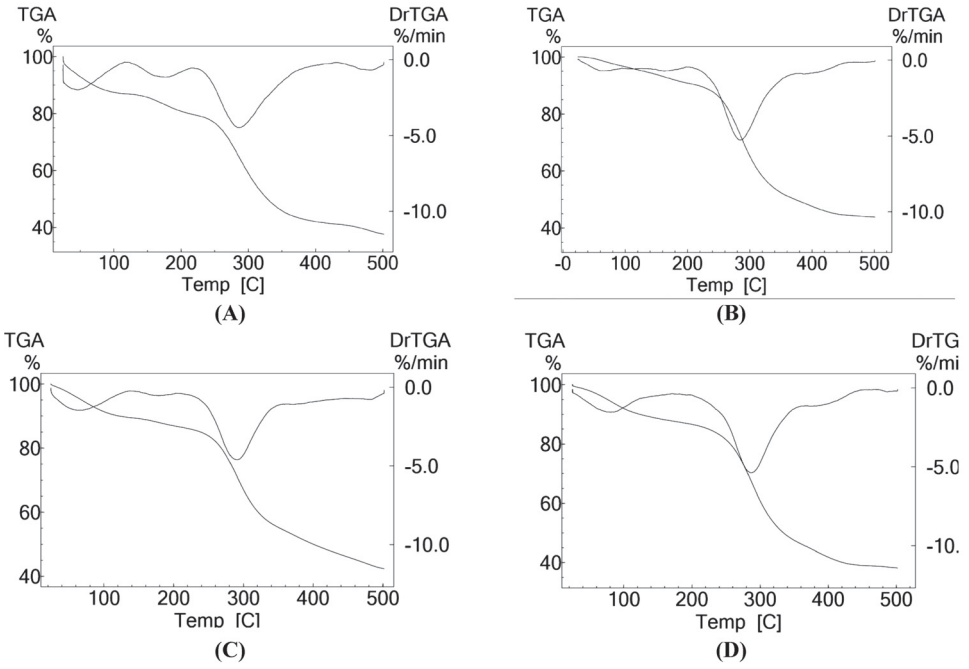

Fig. 4: Thermogravimetric analysis (TGA) of chitosan/collagen membranes HERP-free (a) membrane chitosan/collagen with HERP 0.5 %, (b) membrane chitosan/collagen with HERP 1.0 % (c) membrane chitosan/collagen with HERP 1.5 % (d).

characteristics such as low tensile strength, elongation at break, and flexibility. Also, mechanical properties of the membrane are essential for coating and protecting the wound, and they also need to allow flexibility and maintain integrity to facilitate application and manipulation. In the present study, the mechanical properties of the membranes were evaluated as given in Table 1.

The membrane-free HERP, membrane chitosan/collagen HERP-free, had the highest percentage of elongation and the least stiffness, with values of 46.42% and 230.240 MPa, respectively. The membrane with 0.5% HERP had the lowest rate of elongation (10%) and the most moderate tensile strength (59.455 MPa). The membrane with 1.5% HERP had the highest elongation (20.571%) and the highest tensile strength (103.012 MPa).

Young's Modulus was not affected by HERP incorporation in different quantities. Young's modulus is an indication of the hardness (stiffness) of the material when it is subjected to external tensile stress, being the maximum stress that the biomaterial supports without undergoing permanent deformation (Dall'Antonia et al. 2009). Among the various characteristics of a dressing, maintaining its integrity during use is paramount, so the evaluation of this property is significantly crucial to aid in the storage, handling and application of the device.

Tensile strength was significantly different from the incorporation of the HERP 0.5%, in this case indicating that low quantity of HERP favours structure rigidity (Pastor, Sánchez-González et al. 2010). Regarding the evaluation of the elongation at Break of the membranes, there was a difference among the samples, assuming that HERP concentration alters this parameter.

2.6. Mucoadhesion

In mucoadhesion testing, contact time and force required to remove each system from the mucin disk were evaluated (Fig. 5). The test was performed in triplicate per sample (n = 3). The force (N) for separating the membrane without HERP and the membrane with 0.5% HERP were similar, lasting 2 s and reaching a force of 3 N to complete separation. We also observed that increasing the amount of HERP in the membrane increased contact time. The strength necessary for separation was near 0.4 N for the membrane with 1.5% HERP, and 0.7 N for the membrane with 1.0% HERP. Therefore, this result suggests a weaker interaction of the membrane with the mucin as some HERP increases.

Andrews et al. (2015) tested two different concentrations of poly(vinyl alcohol), poly(acrylic acid), and poly(methyl vinyl ether-co-maleic anhydride) at three different molecular weights and obtained 1.23 N or less for all the different formulations. Guler et al. (2015) tested different amounts of montmorillonite incorporated into a hydrogel for application to the vaginal mucosa and found that the higher the percentage of the montmorillonite the better the mucoadhesion properties owing to the better interaction between the hydrogel and the mucus. The best result was 0.36 N. In both studies; it was possible to observe a force comparable to that obtained in membrane formulations with HERP at 1.0 and 1.5%.

2.7. Cell viability (MTT)

Cell viability of the membranes was determined using an indirect method. Cell viability results indicated that membranes without HERP had slightly toxic effects. This can be attributed to chitosan's biological property of stimulating cell proliferation. In chitosan membranes containing HERP, we observed a more significant toxic effect. In the membrane containing 1.5% HERP, 5.5% of the cells remained viable; in the membrane with 1.0% HERP, 42.8% of the cells were viable; and in the membrane with 0.5% HERP, we obtained a cell viability value of 54.7%.

Figure 6 shows that membrane cytotoxicity increases with increasing concentrations of HERP. The membrane with 0.5% HERP had the highest cell viability, exceeding 50%, compared to

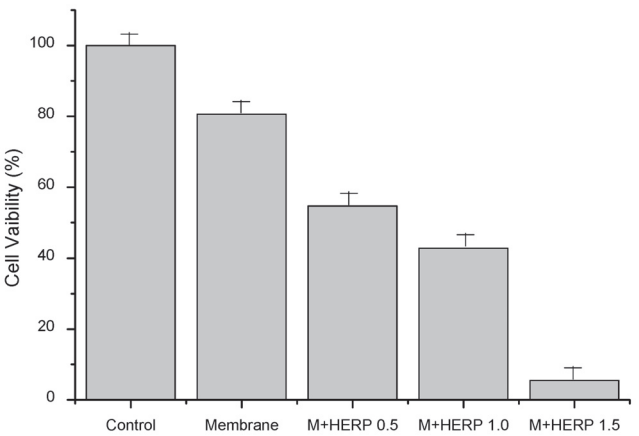

Fig. 6: Cell viability of fibroblasts cells treated with chitosan/collagen membranes HERP-free, membrane chitosan/collagen with HERP 0.5%, membrane chitosan/collagen with HERP 1.0%, membrane chitosan/collagen with HERP 1.5% (d).

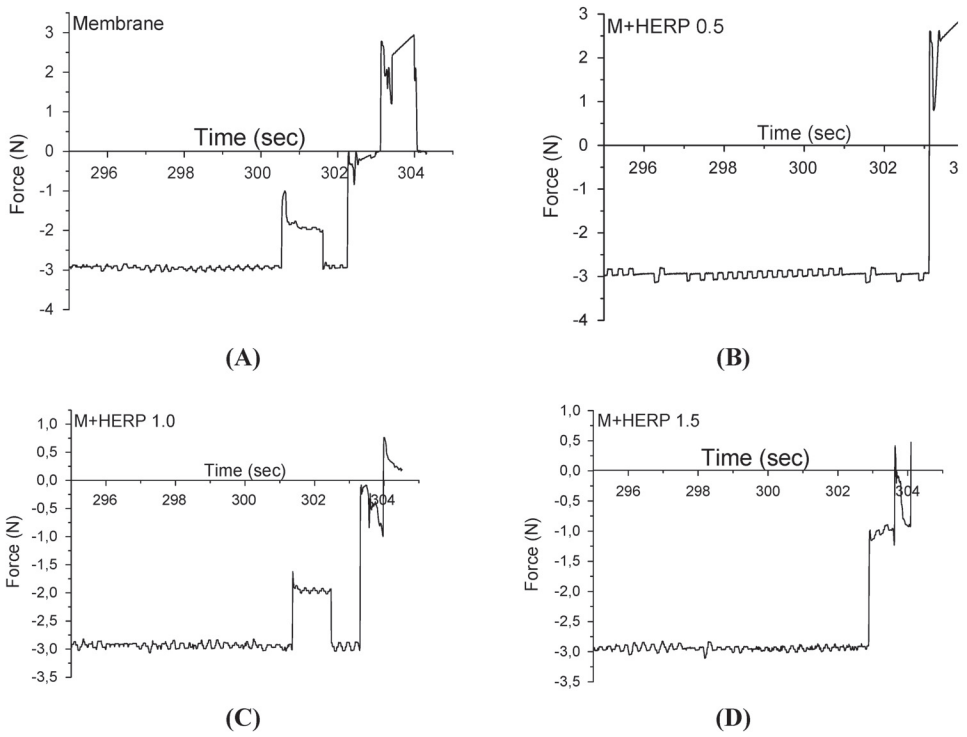

Fig. 5: Mucoadhesion testing of chitosan/collagen membranes HERP-free (a) membrane chitosan/collagen with HERP 0.5 %, (b) membrane chitosan/collagen with HERP 1.0 % (c) membrane chitosan/collagen with HERP 1.5 % (d).

the other membranes with HERP. The cell viability of membranes with EHPV was lower than 70%, indicating that these samples were cytotoxic for human keratinocytes. Oliveira et al. (2015) and de Funari et al. (2007) obtained a similar result about the toxicity of fibroblasts. However, wound healing treatment with propolis showed accelerated tissue repair, reduced inflammation and stimulation the reepithelialization, which could improve healing.

Mendonca et al. (2015), reported that a concentration of 50 µg/mL of HERP resulted in high cytotoxicity in SF-295, OVCAR-8, and HCT 116 cells, with approximately 10%, 20%, and 30% cell viability, respectively. Begnini et al. (2014) used Brazilian red propolis at concentrations of 25, 50, and 100 µg to test for cell viability using the CHO-K1 cell line (regular) and the 5637 cell line (carcinoma). Lower concentration did not have a significant effect on cell viability in both cell lines, whereas higher levels resulted in 51% cell viability in the CHO-K1 and 8% in the 5637 lines.

2.8. Minimum inhibitory concentration (MIC) test

The MIC values for chitosan, collagen, chitosan:collagen, chitosan:collagen with HERP (0.5%, 1.0%, and 1.5%), and HERP (0.5%, 1.0%, and 1.5%) from *Pseudomonas aeruginosa* and *Staphylococcus aureus* are reported in Table 2.

The MIC values of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were similar for chitosan (39.1 $\mu g.mL^{-1}$), proving the antimicrobial action already described in the literature. Adding HERP to chitosan: collagen significantly reduced *Pseudomonas aeruginosa* (1.9 $\mu g.mL^{-1}$) and *Staphylococcus aureus* (7.8 $\mu g.mL^{-1}$) MICs. However, increasing the HERP concentration did not result in a further increase in the antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These concentrations are similar to literature values from Freires et al. (2016), who reported MIC values between 3.8 and 100 $\mu g.mL^{-1}$ for *Staphylococcus aureus*.

The antimicrobial action of HERP is not entirely understood owing to its complex composition (Gebara et al. 2002). The antimicrobial activity of chitosan is also unclear, but studies suggest that chitosan can increase the permeability of cell membranes and thereby disrupt the bacterial cell membrane (Shanmugam et al. 2016), which would explain the better results observed in samples with chitosan.

2.9. Conclusion

Membranes with HERP had adequate properties, which rendered them suitable for use as a dressing for wound healing. Besides, the use of 15% PEG had the best macroscopic characteristics highlighting the flexibility and the addition of HERP improved mechanical properties and thermal stability. Results indicate that the membrane with 0.5 % HERP has potential and should be further studied.

3. Experimental

3.1. Material

Chitosan (82.83±3.63% deacetylation degree and 296.6-kDa molar weight) was purchased from Polymar (Fortaleza, Brazil). Hydrolyzed collagen was acquired from

Table 2: Minimum Inhibitory Concentration (MIC) of the chitosan (CS), collagen (C), chitosan: collagen (CS:C), chitosan:collagen with HERP at concentrations of 0.5% (CS:C HERP 1), 1.0% (CS:C HERP 2), and 1.5% (CS:C HERP 3), and HERP at concentrations of 0.5% (HERP 1), 1.0% (HERP 2), and 1.5% (HERP 3)

Bacteria	CS	CS:C	CS:C HERP 1	CS:C HERP 2	CS:C HERP 3	HERP 1	HERP 2	HERP 3
Pseudomonas aeruginosa (ATCC 27853)	39.1	39.1	1.9	1.9	1.9	7.8	39.1	7.8
Staphylococcus aureus (ATCC 25923)	39.1	15.6	7.8	7.8	15.6	31.2	7.8	31.2

CS: chitosan; HERP: hydroethanolic extract red propolis. Results are expressed as (µg/mL) the mean ± standard deviation (n = 4).

Nutryervas (São Paulo, Brazil), and all other reagents were bought by Sigma-Aldrich (São Paulo, Brazil). Red propolis was donated by Uniprópolis (São Paulo, Brazil), and Millipore® (home supplied) filtered double distilled water was used. All other reagents were bought by Sigma.

3.2. Extract preparation and characterization

HERP was prepared according to the protocol by Cavalcanti et al. (2011). Briefly, propolis (1.0% w/w) was dissolved in an ethanol solution and ultrasound-treated for 1 h at 25 °C (Ultra Cleaner 1400A, Unique/Brazil). Subsequently, the extract was centrifuged (Hettich centrifuge – Routine 380R/Germany) at 1,800 ´ g for 15 min. The supernatant was evaporated. The extraction yield was a measure of the solvent's efficiency in extracting specific components, and it was defined as the mass of extract recovered from the initial mass of the whole material, presented as a percentage (%). The chromatogram of the EHPV sample was done using HPLC. Analyses were performed on a Shimadzu system consisting of a degasser (DGU-20A3), an automatic sampler (SIL-20A), two pumps (LC-20AD), and a photodiode arrangement detector (SPDM20Avp-DAD) together with a CBM20A interface. Chromatographic separation was performed using an analytical column type C18, 150 x 4.6 mm (5 µ particle size). The flow rate was 1.0 mL/min and the injection volume was 20 µL. The column was eluted with a linear gradient using methanol (B), water Milli-Q: 1% acetic acid (v / v) (A) as the mobile phase. The elution started with 40% B for 10 min. 45-50% B, between 10-15 min. 50-55% B, between 15-20 min. 55% B for 20-35 min. 55-65% B, 35-40 min. 65-75% B, 40-45 min. 75-85% B, between 45-50 min. 85-40% of B between 50 – 60 min were returning to initial conditions. A photodiode array detector was set at 280 nm for the acquisition of the chromatograms.

3.3. Production of membranes

Membranes were prepared using the casting technique published by Barbosa et al. (2016). Briefly, chitosan (1.0%) and collagen (0.1%) were dissolved in an aqueous acetic acid solution (0.5 M), separately, using a magnetic stirrer (Kasvi, K40-182OH, Brazil). Three chitosan/collagen ratios (1:3, 1:1, and 3:1) were evaluated. Then, PEG-400 concentrations (15, 20, and 30% mg/mL), and three HERP concentrations (0.5, 1.0, and 1.5 mg/mL) were incorporated into the membrane. Solutions were stirred for 12 h (Kasvi, K40-182OH, Brazil) and then poured into polyethylene Petri dishes (0.21 g/cm^2). All dishes were dried in a forced-air circulation oven (Tecnal, ET-394/3, Brazil) at 40 °C for 24 h.

3.4. Scanning electron microscopy (SEM)

Morphologies of the membranes were examined using SEM (Shimadzu SSX-550, Japan) at 10 kV and 100 pcA. Membranes were cryofractured in liquid nitrogen to obtain cross-sectional images. Cryofracture cross-sections and surfaces of gold-sputtered films were analyzed.

3.5. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)

DSC analyses were performed using a DSC-60 thermal analysis system (Shimadzu, Japan). Samples weighing approximately 10 mg were weighed into an aluminum pan, sealed, and heated in a DSC furnace at temperatures ranging from 25 to 320 °C and a heating rate of 5 °C min $^{-1}$ in an inert atmosphere (N_2) (45 mL/min). TGA curves were determined using a thermoanalytical balance (DTG-60H, Shimadzu, Japan). Membranes were carefully weighed (~1.50 mg) in a platinum pan and heated to temperatures ranging from 25 to 500 ºC (10°C/min) under a dynamic nitrogen atmosphere (100 mL/min).

3.6. Mechanical properties of membranes

The elongation at break, tensile strength, and Young's modulus was measured using a texture analyzer (TA.XT2, United Kingdom) equipped with a 5 kg load cell. Membranes were cut into 8 cm^2 (2 x 4 cm) sections. Specimens were tested to breaking at a cross-head speed of 2 mm·s^{-1} and a displacement of 10 mm. Analyses were performed in triplicate.

3.7. Mucoadhesion testing

Mucoadhesion testing was conducted using a texture analyzer (TA.XT2, United Kingdom) as described by Woertz et al. (2013). Mucin disks were manufactured in a hydraulic press, and they were hydrated with phosphate buffer 0.1 M (pH 7.4) for 30 min. The compression mode was set at a rate of 0.5 mm·s^{-1} under a force of 2 mN. After 3, 5, and 15 min of contact, the specimen was moved in the opposite direction (2.0 mm·s^{-1}). The maximum force required to separate the sample from the mucin disks was assessed in sextuplicate.

3.8. Cell viability (MTT assay)

The cell viability assay was performed using L929 mouse fibroblasts. The methodology followed was based on the ISO 10993-5 (2009) protocol (ISO 2009). L929 cells were seeded into 96-well plates (0.1 x 10^6 cells/well) and cultured in Dulbecco's Modified Eagle Medium (DMEM). Initially, the membranes samples were cut in 6 cm^2 under sterile conditions and then placed in falcon tubes with 1 mL of DMEM culture medium for 24 h. Cells were subjected to different concentrations of membranes with HERP for 24 h at 37 °C and 5% CO_2. Cells were exposed to different membranes containing 0.5 mg/mL, 1.0 mg/mL, and 1.5 mg/mL of HERP. Cell viability was assessed colorimetrically using methyl thiazolyl tetrazolium (MTT). Optical density was measured at a wavelength of 570 nm using an automated plate reader (DTX880 Multimode Detector, Beckan Coulter Inc., Packard, ON, Canada). Tests were performed in quadruplicate and then normalized according to Eq. (1).

$$\% \text{ Cell viability} = \frac{\text{Abs (treated cells)} - \text{Abs (white)}}{\text{Abs (positive control)} - \text{Abs (white)}} \times 100$$

where Abs (treated cells) is the absorbance in the presence of the tested membrane, Abs (positive control) is the absorbance of the cells without treatment, and Abs negative control is the absorbance with the membrane without HERP.

3.9. Minimum inhibitory concentration (MIC)

For determining the MIC, *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) strains were used. Colonies were harvested and resuspended to 1.5 × 10^8 CFU.mL^{-1} (turbidity equivalent to 0.5 McFarland standard scale). Samples were diluted in dimethyl sulfoxide at concentrations from 1.0 until 0.0019531 mg.mL^{-1}. The negative control was 0.1 mL of Mueller Hinton Broth, and the positive control was the bacterial solution diluted in Mueller Hinton Broth to obtain 1 × 10^5 CFU.mL^{-1}. Plates were incubated at 37 °C for 20 h.

3.10. Statistical analysis

Statistical analyses were performed using the R software. The data presented correspond to the mean±standard deviation of three independent experiments performed in duplicate and evaluated by one-way analysis of variance (ANOVA) with Tukey post-test. $^a p < 0.05$ compared to the negative control.

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Conflict of Interest: The authors report no conflicts of interest.

References

Andrews GP, Laverty TP, Jones DS (2015) Rheological analysis of polymer interactions and ageing of poly(methylvinylether co maleic anhydride)/poly(vinyl alcohol) binary networks and their effects on mucoadhesion. J Pharm Sci 104: 4329–4338.

Anjum S, Arora A, Alam M,. Gupta B (2016) Development of antimicrobial and scar preventive chitosan hydrogel wound dressings. Int J Pharm 508: 92–101.

Barbosa GP, Debone HS, Severino P, Souto EB, da Silva CF (2016) Design and characterization of chitosan/zeolite composite films—Effect of zeolite type and zeolite dose on the film properties. Mat Sci Engin C 60: 246–254.

Barbosa GP, Debone HS, Severino P, Souto EB, da Silva CF (2016). Design and characterization of chitosan/zeolite composite films—Effect of zeolite type and zeolite dose on the film properties. Mater Sci Eng C Mater Biol Appl 60: 246–254.

Begnini KR, Moura de Leon PM, Thurow H, Schultze E, Campos VF, Martins Rodrigues F, Borsuk S, Dellagostin OA, Savegnago L, Roesch-Ely M, Moura S, Padilha FF, Collares T, Pêgas Henriques JA, Seixas FK (2014) Brazilian red propolis induces apoptosis-like cell death and decreases migration potential in bladder cancer cells. Evid-Based Complem Alt Med 2014: 639856.

Benbettaïeb N, Karbowiak T, Brachais CH, Debeaufort F (2015) Coupling tyrosol, quercetin or ferulic acid and electron beam irradiation to cross-link chitosan–gelatin films: a structure–function approach. Eur Polym J 67: 113–127.

Bodini R, Sobral P, Favaro-Trindade C, Carvalho R (2013) Properties of gelatin-based films with added ethanol–propolis extract. LWT-Food Sci Technol 51: 104–110.

Borges AF, Silva C, Coelho JF, Simões S (2015) Oral films: current status and future perspectives: I—galenical development and quality attributes. J Control Release 206: 1–19.

BRASIL (2001) BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa nº 3, de 19 de janeiro de 2001.

Cavalcante DRR, d. Oliveira PS, Góis SM, Soares AF, Cardoso JC, Padilha FF, d. Albuquerque Júnior RLC (2011) Effect of green propolis on oral epithelial dysplasia in rats. Braz J Otorhinolaryngol 77: 278–284.

Cheng SY, Wang BJ, Weng YM (2015) Antioxidant and antimicrobial edible zein/chitosan composite films fabricated by incorporation of phenolic compounds and dicarboxylic acids. LWT-Food Sci Technol 63: 115–121.

Cheng Y, Lu J, Liu S, Zhao P, Lu G, Chen J (2014) The preparation, characterization and evaluation of regenerated cellulose/collagen composite hydrogel films. Carbohydrate Polym 107: 57–64.

Cuesta-Rubio O, Piccinelli AL, Rastrelli L (2012) Tropical propolis: recent advances in chemical components and botanical origin. Medicinal plants: Biodiversity and drugs. 1st ed. New York: CRC Press, Taylor & Francis Group, pp. 209-240.

da Silva Frozza CO, Garcia CSC, Gambato G, de Souza MDO, Salvador M, Moura S, Padilha FF, Seixas FK, Collares T, Borsuk S (2013) Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. Food Chem Toxicolog 52: 137-142.

Dall'Antonia AC, Martins MA, Moreno R, Mattoso LH, Gonçalves PS, Job AEJP (2009) Caracterização mecânica e térmica da borracha natural formulada e vulcanizada dos clones: GT 1, IAN 873, PB 235 e RRIM 600. 63-71.

de Funari CS, de Oliveira Ferro V, Mathor MB (2007) Analysis of propolis from Baccharis dracunculifolia DC.(Compositae) and its effects on mouse fibroblasts. J Ethnopharmacol 111: 206–212.

de Mendonça ICG, de Moraes Porto ICC, do Nascimento TG, de Souza NS, dos Santos Oliveira JM, dos Santos Arruda RE, Mousinho KC, dos Santos AF, Basílio-

Júnior ID, Parolia A (2015) Brazilian red propolis: phytochemical screening, antioxidant activity and effect against cancer cells. BMC Complem Altern Med 15: 1.
Fernandes LL, Resende CX, Tavares DS, Soares GA, Castro LO, Granjeiro JM (2011) Cytocompatibility of chitosan and collagen-chitosan scaffolds for tissue engineering. Polimeros 21: 1–6.
Freires IA, de Alencar SM, Rosalen PL (2016) A pharmacological perspective on the use of Brazilian Red Propolis and its isolated compounds against human diseases. Eur J Med Chem 110: 267–279.
Gebara EC, Lima LA, Mayer M (2002) Propolis antimicrobial activity against periodontopathic bacteria. Brazil J Microbiol 33: 365369.
Ghosh A, Ali MA, Walls R (2010) Modification of microstructural morphology and physical performance of chitosan films. Int J Biol Macromol 46: 179–186.
Guillerminet F, Beaupied H, Fabien-Soulé V, Tomé D, Benhamou CL, Roux C, Blais A (2010) Hydrolyzed collagen improves bone metabolism and biomechanical parameters in ovariectomized mice: an in vitro and in vivo study. Bone 46: 827834.
Güler MA, Gök MK, Figen AK, Özgümüş S (2015) Swelling, mechanical and mucoadhesion properties of Mt/starch-g-PMAA nanocomposite hydrogels. Appl Clay Sci 112: 44-52.
Hissae Yassue-Cordeiro P, Zandonai CH, Pereira Genesi B, Santos Lopes P, Sanchez-Lopez E, Garcia ML, Camargo Fernandes-Machado NR, Severino P, Souto EB, Ferreira da Silva CJP (2019) Development of chitosan/silver sulfadiazine/zeolite composite films for wound dressing. Pharmaceutics 11: 535.
Hsiao YH, Hsia SY, Chan YC, Hsieh JF (2017) Complex coacervation of soy proteins, isoflavones and chitosan. Molecules 22: pii: E1022.
ISO E (2009) 10993-5. Biological evaluation of medical devices-Part 5: Tests for in vitro cytotoxicity. Geneva: International Organization for Standardization.
Jug M, Končić MZ, Kosalec I (2014) Modulation of antioxidant, chelating and antimicrobial activity of poplar chemo-type propolis by extraction procures. LWT-Food Sci Technol 57: 530-537.
Kamoun EA, Kenawy ERS, Chen X (2017) A review on polymeric hydrogel membranes for wound dressing applications: PVA-based hydrogel dressings. J Adv Res 8: 217–233.
Kubiliene L, Laugaliene V, Pavilonis A, Maruska A, Majiene D, Barcauskaite K, Kubilius R, Kasparaviciene G, Savickas A (2015) Alternative preparation of propolis extracts: comparison of their composition and biological activities. BMC Complem Altern Med 15: 156.
León-Mancilla B, Araiza-Téllez M, Flores-Flores J, Piña-Barba M (2016) Physico-chemical characterization of collagen scaffolds for tissue engineering. J Appl Res Technol 14: 77–85.
Morsy A, Soltan Y, Sallam S, Kreuzer M, Alencar S, Abdalla A (2015) Comparison of the in vitro efficiency of supplementary bee propolis extracts of different origin in enhancing the ruminal degradability of organic matter and mitigating the formation of methane. Animal Feed Sci Technol 199: 51-60.
Nasiri E, Hosseinimehr SJ, Azadbakht M, Akbari J, Enayati-fard R, Azizi S (2015) Effect of Malva sylvestris cream on burn injury and wounds in rats. Avicenna J Phytomed 5: 341–354.
Neto CDT, Giacometti JA, Job AE, Ferreira FC, Fonseca JLC, Pereira MR (2005). Thermal analysis of chitosan based networks. Carbohydrate Polymers 62: 97–103.
Oliveira RN, McGuinness GB, Rouze R, Quilty B, Cahill P, Soares GD, Thiré RMSM (2015) PVA hydrogels loaded with a Brazilian propolis for burn wound healing applications. J Appl Polym Sci 132: 42129.
Ostrowska-Czubenko J, Gierszewska-Drużyńska M (2009) Effect of ionic crosslinking on the water state in hydrogel chitosan membranes. Carbohydr Polym 77: 590–598.
Panos I, Acosta N, Heras A (2008) New drug delivery systems based on chitosan. Curr Drug Discov Technol 5: 333–341.
Park YK, Paredes-Guzman JF, Aguiar CL, Alencar SM, Fujiwara FY (2004) Chemical constituents in Baccharis dracunculifolia as the main botanical origin of southeastern Brazilian propolis. J Agricult Food Chem 52: 1100–1103.
Pastor C, Sánchez-González L, Cháfer M, Chiralt A, González-Martínez C (2010) Physical and antifungal properties of hydroxypropylmethylcellulose based films containing propolis as affected by moisture content. Carbohydr Polym 82: 1174–1183.
Ramasamy P, Shanmugam A (2015) Characterization and wound healing property of collagen–chitosan film from Sepia kobiensis (Hoyle, 1885). Int J Biol Macromol 74: 93–102.
Shanmugam A, Kathiresan K, Nayak L (2016) Preparation, characterization and antibacterial activity of chitosan and phosphorylated chitosan from cuttlebone of Sepia kobiensis (Hoyle, 1885). Biotechnol Rep 9: 25–30.
Silva BB, Rosalen PL, Cury JA, Ikegaki M, Souza VC, Esteves A, Alencar SM (2008) Chemical composition and botanical origin of red propolis, a new type of Brazilian propolis. Evid-Based Complem Alt Med 5: 313–316.
Somers P, Roosens A, De Somer F, Cornelissen M, Van Nooten G (2015) Non-cytotoxic crosslinkers for heart valve tissue engineering. J Heart Valve Dis 24: 92–100.
Souza ICL, do Nascimento MF, de Souza Neta RG, Dos Santos JC, Costa LP, Cardoso JC, Cavalcanti de Albuquerque-Junior (2013) Effect of the maltodextrin-induced chemical reticulation on the physical properties and healing potential of collagen-based membranes containing Brazilian red propolis extract. Int J Med Med Sci 5: 514–524.
Van Onselen J, Gardner S (2016) Wound dressings—classification and selection: an introduction to Tissue Viability Focus 2016. Dermatol Nurs 15: 10–12.
Woertz C, Preis M, Breitkreutz J, Kleinebudde P (2013) Assessment of test methods evaluating mucoadhesive polymers and dosage forms: an overview. Eur J Pharm Biopharm 85: 843–853.
Yenilmez E, Başaran E, Arslan R, Berkman MS, Güven UM, Bayçu C, Yazan Y (2015) Chitosan gel formulations containing egg yolk oil and epidermal growth factor for dermal burn treatment. Pharmazie 70: 67–73.