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Antimicrobial activity and cellulose acetate membrane characterization with tangerine peel extract (Citrus reticulata) for bio packing

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ABSTRACT

Biopacking material was obtained by incorporating tangerine peel extract (Citrus reticulata) in cellulose acetate (CA) film. The acetate film with tangerine extract was shown to have a homogeneous material characteristic by FTIR and DSC analysis, being reinforced in MEV and MFA, and showed absence of pores in the film with extract. These characteristics justify the reduced water absorption and release of the CA membrane extract. Low water absorption is important for the film to act as a barrier with external environment and the release of the extract was sufficient to prevent the growth of the strains investigated on the sample surface. The introduction of the extract also reduced the tensile strength and deformation of the film. This study showed the good potential of biomass for active bio packing that can gradually replace non-renewable packaging and take advantage of agricultural waste.

Keywords: Bio packing; tangerine peel; Citrus reticulata; antimicrobian activity

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1 INTRODUCTION

The use of renewable materials for industrial applications is increasing due to the demand for alternatives for non-renewable and environmentally friendly supplies. The use of biomass in the development of packaging technologies, known as bio packages, is especially attractive due to the abundance of raw material origin, for being renewable, due to mechanical properties and with nano-dimensions that open a wide range of possible properties to be exploited (Dannenberg *et al*. 2017; Brinchi *et al*., 2013; Gontard; Guilbert, 1994).

The Citrus genre is one of the most important in the Rutaceae family because of its fruits, which are estimated primarily for food (Waheed *et al*. 2009; Rafiq *et al*. 2018). The Rutaceae family is comprised of about 150 genres, 1600 species, distributed in tropical and subtropical regions around the world, being more abundant in Tropical America, South Africa and Australia.

In Brazil, the family is represented by approximately 29 genera and 182 species, with some of medicinal, ecological and economic importance (Melo; Zickel, 2004).

According to the United States Department of Agriculture (USDA), Brazil was the largest producer in the 2018/2019 harvest, and accounts for more than three quarters of global orange juice exports. Global tangerine production for 2018/2019 was a record of 32.0 million, with larger harvests in China and the European Union (CEPEA, 2017; USDA, 2019).

Worldwide, citrus plants such as orange, lemon and tangerine are widely consumed by the population, being fruits rich in vitamin C, dietary fibers, carotenoids and flavonoids, thus having a high antioxidant potential (Rafiq *et al*. 2018; Duzzioni *et al*. 2010). The medicinal and nutritional properties of tangerine (*Citrus reticulata*) have been known since antiquity, generating several studies on the use of its resources (Rafiq *et al*. 2018; Al-Rejaie *et al*. 2013; Wilcox *et al*. 1999).

The peel of *Citrus reticulata* has also been studied in some animal models, showing antiangiogenic activity by increasing the expression of vascular endothelial growth factor, inhibiting the formation of blood vessels and increasing bone

density (Adelina *et al*. 2008; Chrisnanto *et al*., 2008). The peel had a higher content of flavonoids such as hesperidin, narirutin, nobiletine and tangeretine (Nogata *et al*. 2006; Bermejo *et al*. 2011) and greater amount of nutrients compared to other Citrus, including calcium, magnesium, carotenoids, dietary fibers and total polyphenols (Rincón *et al*. 2005). Despite these findings, no study has evaluated the release of active ingredients of cellulose acetate membrane with extract produced from *Citrus reticulata* tangerine peels for antibacterial action.

According to the above, the peel of the mandarin (*Citrus reticulata*) has different biological activities, and is a biomass still underutilized, being a plant grown in different parts of the world. On the daily use of this citrus, the fruit is used by discarding its peel, which in turn contains high amounts of compounds beneficial to health.

Fruit peels when thought of as residues can represent loss of biomass and nutrients. On an industrial scale, they can also increase the polluting potential of soil and water bodies when associated with inadequate disposal, still leading to the spread of rodents and other vectors that may cause public health problems. On the other hand, costs associated with the treatment, transport and final disposal of waste generated have a direct effect on the price of the final product (Egea *et al*., 2005; Blades *et al*., 2017). Special attention has been focused on minimizing or reusing waste from agribusiness (biomass), and establishing new uses for agricultural products.

In particular biopackages have a passive action in relation to food, merely acting as a barrier between it and the external environment. However, the incorporation of active substances can promote desirable interactions with food, such as antimicrobial activity (Hafsa *et al*., 2016; Calo *et al*., 2015).

Active packaging, increases microbiological safety (Rizzolo *et al*., 2016), and allows producers to reduce the use of synthetic additives added directly to food (Moradi *et al*., 2016). As a result, with the aim of exploring all the resources available by a plant and reducing the amount of waste generated by the agro-industry, cellulose acetate membranes were prepared with and without *Citrus reticulata* peel

extract in order to assess mechanical properties, absorption of water, extract release and antimicrobial activity, and these too were characterized by infrared and differential scanning calorimetry.

2 MATERIALS AND METHODS

The cellulose acetate reagent (AC Mn-900 $gmol^{-1}$) was obtained from Sigma-Aldrich Chemicals Co. (St. Louis, USA) and the acetone from Vetec SA (Rio de Janeiro, Brazil), the reagents were used as purchased. The peel was removed from ripe fruits harvested in June 2015, in the city of Araranguá-SC. The tangerine species was botanically identified as being from the Rutaceae Family, common name Ponkan, and scientific name *Citrus reticulata*. An exsiccata of the same was deposited in the Herbarium Laelia purpurata under Voucher number: SRS 0050047.

To prepare the tangerine extract, the peels were collected the day after the 10 kg tangerine harvest. The peels were dried at 40 °C in an oven for 24 h (Figure 1A). The dry peels were grinded and classified by granulometry using 400 mm sieves. The bark powder (Figure 1B) was stored in a dry and sealed plastic bag. Figure 1 shows the dry peels (A), powder of the peels (B) and (C) the hydroalcoholic extract of the tangerine peels.

Figure 1 - (A) Tangerine peel, (B) powder after drying and grinding and (C) the hydroalcoholic extract of the tangerine peels.

A fraction of 100 g of tangerine peel powder was added in 1000 mL of absolute ethanol, subjected to constant stirring (about 120 rpm) for 24 h. The resulting suspension was vacuum filtered and the ethanol removed in the rotary evaporator at

a maximum temperature of 40 °C. The concentrated extract was stored in a closed container, at room temperature (Figure 1C).

2.1 Preparation of membranes and microstructure

In a beaker, 1 g of cellulose acetate was added in 10 ml of acetone stirring until complete solubility at 25 °C. The making of membranes with extract included the addition of 100 mg of the tangerine extract. The solution was poured into a petri dish and the solvent evaporated at room temperature (25 °C) until the membrane formed (Meier al., 2004). The membrane was weighed to constant weight, then the final thickness of the membranes was checked, being 0.17±0.005 mm for membranes with or without tangerine extract.

The micrographs of the membranes were obtained by scanning electron microscopy (SEM), using a JEOL JSM-6390LV device, with a voltage of 15 kV, and the samples were fractured in liquid nitrogen. The three-dimensional images were obtained by atomic force microscopy (AFM), using an Atomic Force Microscopy (AFM) equipment, Agilent Technologies 5500 equipment. Samples cut into 2 cm x 2 cm squares and kept under pressure with clips, the measurements were performed at room temperature, in non-contact mode. High-resolution probes SSS-NCL (Nanosensors, force constant = 48 N/m, resance frequency = 154 kHz). Images captured by PicoView 1.14.4 software and analyzed by PicoImage 5.1 (both from Molecular Imaging Corporation).

2.2 Differential scanning calorimetry

The DSC curves of tangerine extract, PCLT and PCLT/extract were obtained using a differential scanning calorimeter (DSC 50, Shimadzu) by heating the samples from 25 °C to 200 °C at a rate of 10 °Cmin^{-1}, after a first run from 20 to 120 °C to destroy the thermal history. The average sample size was 10 mg and the nitrogen flow rate was 50 cm^{3}min^{-1}. Standard calibration (156.6 °C) and zinc (419.5 °C) were used for calibration.

2.3 Infrared spectroscopy

FTIR spectra were performed on a Bruker infrared spectrometer, Alpha model, using direct analysis of the thin film of tangerine extract, PCLT and PCLT/extract on ZnSe cell. The scan occurred within 400–4000 cm^{-1} wavelength range.

2.4 Mechanic Properties

The mechanical tests were performed on EMIC® equipment of a universal testing machine using two claws (one stationary and the other mobile) operating at a speed of 25 $mmmin^{-1}$ and equipped with a load cell with a capacity of 100N. Strips of membrane film (50 mm long, 10 mm wide and thickness range from 0.100 to 0.120 mm) were used to determine final strength and elongation at break. The eight specimens of each sample were conditioned at 25 °C, the graphs were generated from the average of the data collected.

2.5 Water Absorption

Six samples from each membrane remained in the oven at 50 °C for 24 hours. Then, the samples were immersed in a static distilled water bath at a temperature of 25 °C for 24 h. After that, they were dried with paper towels and weighed. Water absorption was calculated using the equation: Water absorption (%) = (Wet x 100)/Dry (D 570-98).

2.6 Zeta Potential

Zeta potential measurements were performed with the SurPASS ™ 3 instrument using the adjustable opening cell. For each measurement, a pair of dry membranes was attached to the sample holders (with a 20 mm x 10 mm cross section) using face adhesive tape. The sample holders were inserted into the adjustable opening cell in such a way that the membrane surfaces were facing each other. A range of approx. 100 µm was adjusted between the sample surfaces. A 0.001 $molL^{-1}$ aqueous KCl solution was used for the analysis of the zeta potential. The pH was adjusted automatically by the integrated titration unit using 0.05 $molL^{-1}$ of HCl and 0.05 mol L^{-1} of NaOH, respectively.

2.7 Extract release

Each membrane was cut into pieces and shaken in a Becker containing a solution of 5 ml of ethanol, to 995 ml of distilled water. The tests were performed in triplicate, and the aliquots were removed every 15 min. Since a calibration curve was drawn for the tangerine extract solution using the same solvents. The absorbance readings were performed at a wavelength of 211 nm on the UV-Vis U-2001 spectrophotometer (Hitachi).

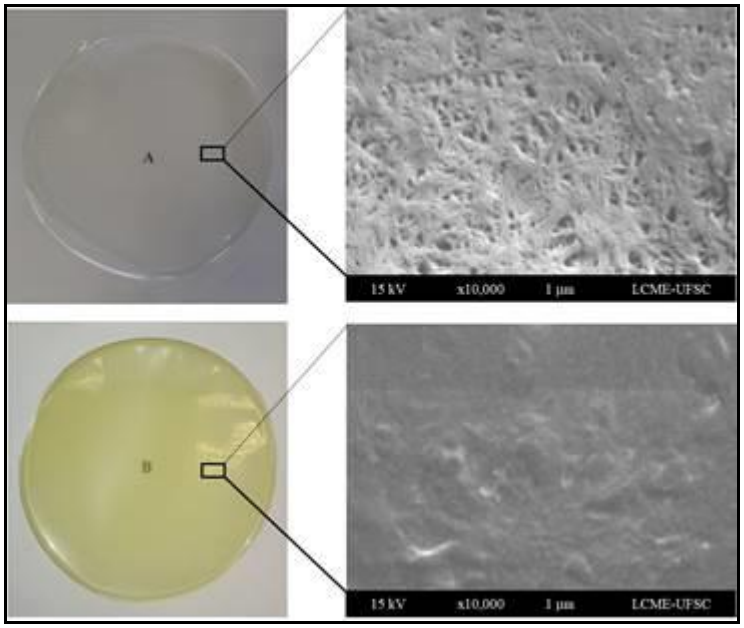
2.8 Antimicrobian Activity

Antimicrobial activity was tested using the agar diffusion sensitivity method. Gram positive (Staphylococcus aureus ATCC 25923) and Gram negative (Pseudomonas aeruginosa ATCC 0027) strains inoculated in Petri dishes containing Müeller Hinton agar were used. The concentration of microorganisms was standardized through the turbidity of the suspensions in a UV-Vis spectrophotometer LGS 53 (Bel Engineering) at 625 nm with absorbance between 0.08 to 0.13 corresponding to 1 x 108 to 2 x 108 UFC.mL^{-1}. First, 100 µL of the microbial suspension was spreaded using a swab over the surface of the culture medium. Then, the membranes were cut in circles and were deposited in the culture medium containing the microorganisms. In addition, 50 mL of the extract was also analyzed. The plates were incubated in an oven at 35±2 °C for 24 h.

3 RESULTS AND DISCUSSION

Figure 2 shows the prepared membranes and corresponding scanning electron microscopy with a magnification of 10,000 times, Figure 2A membrane without adding the extract and Figure 2B with added mandarin extract (*Citrus reticulata*).

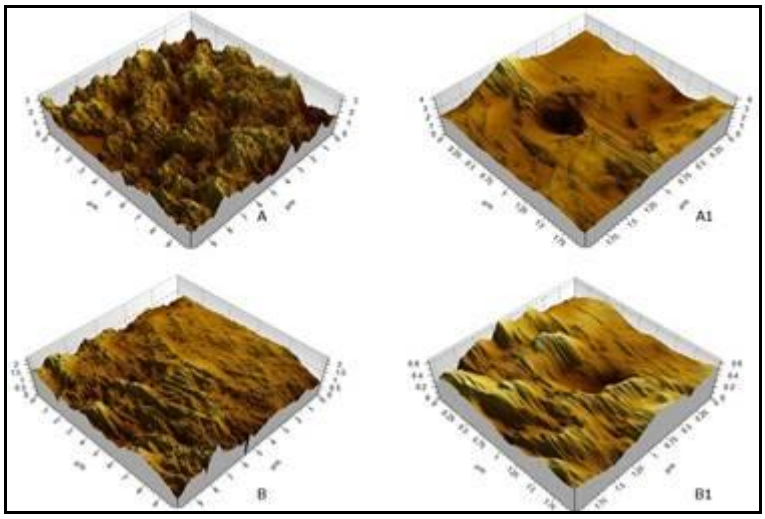
Figure 2 - Cellulose acetate membranes without extract (A) and with tangerine extract (B), and respective SEM with 10,000 times magnification



The membranes obtained from cellulose acetate with tangerine extract were translucent (Figure 2B), being indicative of a homogeneous mixture between the polymer and the extract and also a characteristic of the amorphous microstructure of cellulose acetate similar to that seen for pure polymer (Figure 2A) (Meier al., 2004; Kanis *et al*., 2014).

The SEM images showed that the membranes have a different surface, that is, the addition of the extract modified the morphology of the membrane. However, the two membranes are regular and dense, and the membrane without extract is woven while the membrane with extract showed smooth morphology in the SEM, without the presence of pores or wefts. In the three-dimensional images in Figure 3, made by AFM, it is confirmed that the two materials do not have pores.

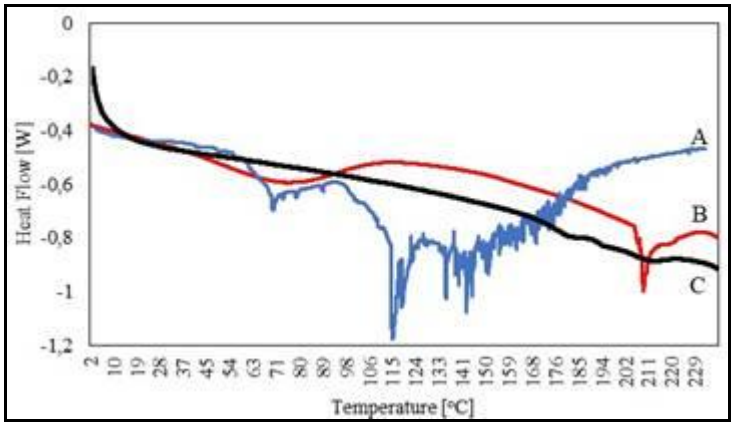
Figure 3 - AFM of cellulose acetate membranes with an area of 100μm² (A), 4μm² (A1) and an extract membrane with cellulose acetate with an area of 100μm² (B) and 4μm² (B1)



The topography of the cellulose acetate membrane with extract (Figure 3B, 3B1) is more pronounced than that of the membrane without extract (Figure 3A, 3A1), both are rough, but closed and with low pore incidence. There are two surfaces with different roughness, probably due to the tangerine extract having a variety of low molecular weight substances, which interact specifically with cellulose acetate polymer filling the free spaces in the woven microstructure, resulting in a different topography.

To evaluate the thermal stability of the tangerine extract (*Citrus reticulata*), cellulose acetate membrane and cellulose acetate membrane with extract, differential scanning calorimetry analysis was performed. Figure 4 shows that the DSC curves obtained from pure components, cellulose acetate and tangerine extract, and from the cellulose acetate membrane with tangerine extract.

Figure 4 - DSC curve of extract (A), cellulose acetate (B) and extract with cellulose acetate (C)



The DSC curve of the tangerine extract (Figure 4, curve A) exhibits three thermal events. The first between 64 and 91 °C showing an endothermic peak at 72 °C, probably referring to the loss of water in the sample and evaporation of low molecular weight organic compounds. The second region with an endothermic peak was between 117 and 121 °C related to the evaporation of other organic compounds present in the extract. And the third region was between 135 and 185 °C indicating the degradation of the organic compounds present in the extract (Kanis *et al*., 2014; Vieira *et al*., 2012).

Figure 4 (curve B) shows the cellulose acetate thermogram, which revealed two endothermic transitions, the first being a wide region with a peak at 78 °C, typical of water interaction with the polymer. The second transition, an acute endothermic peak in the range of 206 and 223 °C, which corresponded to the melting temperature of the crystalline region (Taniguchi; Horigome, 1975; Barud *et al*., 2008).

The cellulose extract/acetate DSC curve (Figure 4C) showed two broad endothermic peaks, little pronounced, with values around 185 and 211 °C. Therefore, it is possible that an interaction between the mandarin extract (*Citrus reticulata*) and cellulose acetate has occurred, since the disappearance of the melting peak of a pure substance in a mixture or the reduction of the melting temperature suggests an interaction or the dissolution of the substance in the medium (Costa *et al*., 2013; Fernandes *et al*. 2013). Intramolecular polymer-extract interactions can restrict the

mobility of the polymeric chain and prevent nucleation, preventing micro-crystallization.

FTIR analyzes were performed to detect the presence of polymer-extract interaction. The FTIR spectra of the components, tangerine extract and cellulose acetate, and of the cellulose acetate membrane with tangerine extract are shown in Figure 5.

Figure 5 - FTIR spectrum of the mandarin extract (A), cellulose acetate (B) and the cellulose acetate membrane with extract (C).

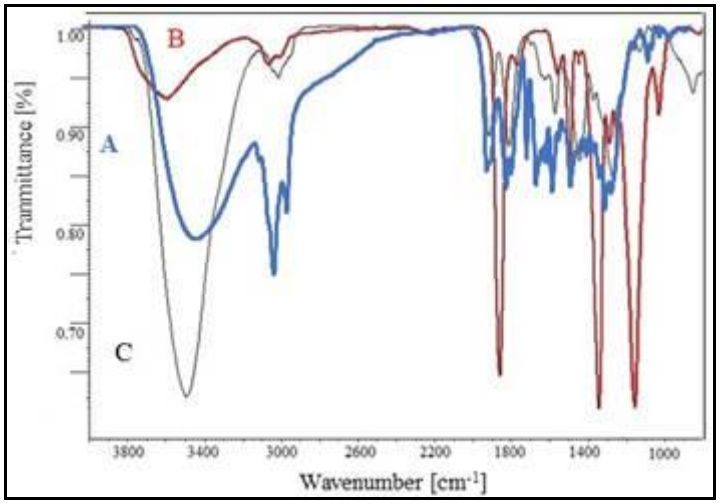


Figure 5 (spectrum A) shows the FTIR spectrum of the extract obtained from tangerine peel showed a broad band at 3480 cm^{-1} related to the hydroxyls hydrogen bond. The 2960 cm^{-1} and 2850 cm^{-1} bands (CH stretch of the CH_2 and CH_3 groups), 1750 cm^{-1} and 1740 cm^{-1} intense band of the carbonyl group deformation vibration. The absorbance values at the wavelengths 1625 cm^{-1}, 1580 cm^{-1}, 1500 cm^{-1} vibrations of aromatic nuclei, and the 1250 cm^{-1} and 1050 cm^{-1} vibrations of the C-O stretch (ether and esters).

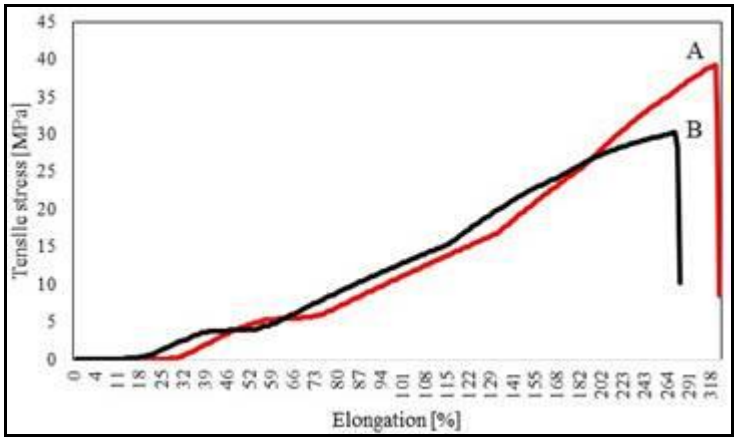
The cellulose acetate spectrum (Figure 5, spectrum B) showed a broad band at 3477 cm^{-1} related to the intramolecular hydrogen bonds of the hydroxyl groups (OH stretch). The bands 2943 cm^{-1} and 2868 cm^{-1} (CH stretch of the CH_2 and CH_3 groups), 1733 cm^{-1} intense band of the deformation vibration of the carbonyl group of the esters (C=O stretch of the ester). The bands 1215 cm^{-1} and 1028 cm^{-1} correspond to the C-O stretch (ester and ether). Both cellulose acetate and mandarin extract, due to

the presence of groups with CO and OH bonds, can form hydrogen bonds (Meier *et al*., 2004; Barud *et al*., 2008; Schmidt; Soldi, 2006).

The cellulose acetate membrane with tangerine extract (10: 1), in Figure 5 (spectrum C), presented a 3447 cm^{-1} band characteristic of hydroxyls with hydrogen bonding of the components. Due to the formation of a hydrogen bond between cellulose acetate and extract, it provided a shift in the wavelength value, from 3477 cm^{-1} of cellulose acetate to 3447 cm^{-1} in the membrane with extract and cellulose acetate. This and the transparency of the cellulose acetate membrane and tangerine extract are indicative of miscibility. These bands can also be attributed to the water contained in the extract, as the drying temperature of the shells and evaporation of the solvent was 40 °C. The bands at 2926 cm^{-1} (aliphatic C-H), 1750 cm^{-1} intense band of the carbonyl group deformation vibration. 1633 cm^{-1} aromatic vibration, which must be from the flavonoids present in the tangerine extract. Thus, it is suggested that polymer and extract are miscible, since the FTIR spectrum of cellulose acetate with extract showed considerable differences when compared to the pure components, changes in the band intensity of the OH group were observed.

The application of biomass in bio packages also depends on mechanical properties, as the material must withstand transport, handling and storage. Figure 6 shows the stress-strain curves obtained from mechanical tensile strength analysis of membranes at room temperature.

Figure 6 - Elastic stress versus stretching for cellulose acetate (A) and extract with cellulose acetate (B).

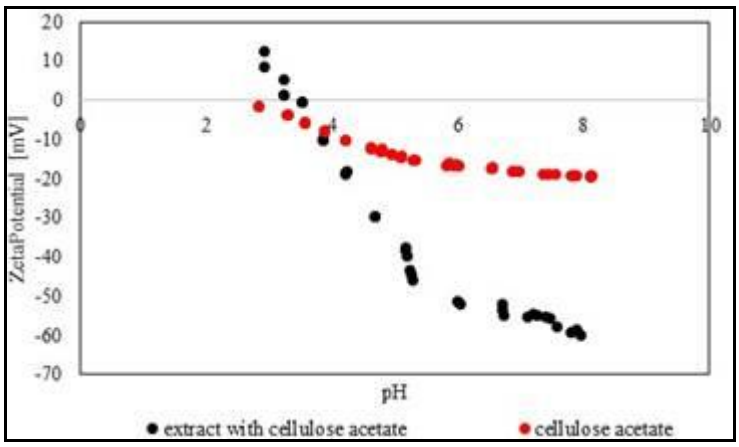


The results revealed that the introduction of tangerine extract in the cellulose acetate membrane reduces the elongation at break by about 40% (Figure 6), with an elongation of 327.5% for the cellulose acetate membrane while for the film with extract was 288.4%. It was also observed a reduction of 24% in the maximum stress supported by the cellulose acetate film with tangerine extract, being 29.69 MPa while the film without extract showed a mechanical resistance of 39.30 MPa (Figure 6). The results were similar for the elasticity modules, with 9.78 MPa for the extract film and 9.17 MPa for pure cellulose acetate film. The extract probably has a variety of low molecular weight substances, which interact with cellulose acetate, reducing intramolecular bonds (polymer-polymer), thereby reducing the elongation capacity and the mechanical resistance of the membrane prepared with extract.

The cellulose acetate membrane with tangerine extract showed water absorption slightly higher than the membrane without extract, with an average of 10.52±0.01% of water absorption for membranes without extract and 12.23±0.01% absorption for membranes with extract when in contact with water for 24 h. The greater water absorption capacity of the membrane with tangerine extract is attributed to the possibility of the components of formation of hydrogen bonds with water. For applications such as bio packages, low water absorption is desired so that the film remains intact to act as a barrier between the packaged product (food or not) and the external environment (Hafsa *et al*., 2016; Calo *et al*., 2015).

Results on the evolution of the zeta potential of the membranes, as a function of the pH of the solution used in the analysis, can be seen in Figure 7.

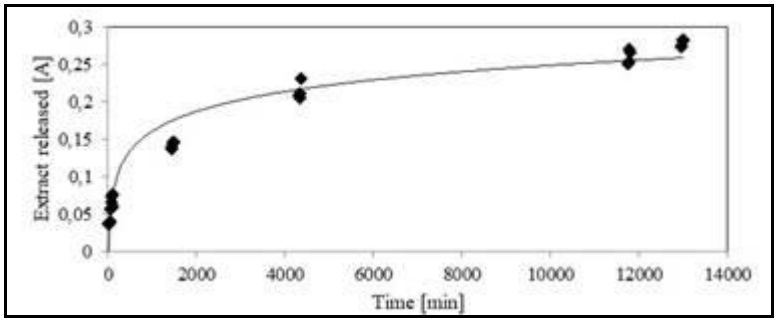
Figure 7 - Zeta potential of cellulose acetate membranes, depending on the pH of the aqueous KCl solution at 0.001 mol/l



In Figure 7, the result shows that the zeta potential measured on the surfaces, depending on the pH of the solution, is different for the studied membranes. The cellulose acetate membrane showed a potential variation between 0 to -20mV, while the membrane containing the tangerine extract showed a potential variation between 10 to -60mV, when the pH of the solution varies from 2.7 to 8. It can also be seen that on the cellulose acetate membrane, an isoelectric point (IEP) of 2.7 was found, while the IEP of the cellulose acetate membrane with extract was 3.4. Therefore, an effect of the plant extract on the zeta potential of the membrane surface is observed, indicating that the presence of the plant extract makes the material more stable and increases the hydrophobicity of the membrane.

The average of the absorbances detected from the cellulose acetate film samples with tangerine extract was performed over a period of 10 days. The behavior of the cellulose acetate membrane extract release behavior can be observed in Figure 8, in the abscissas is the time variation in minutes and on the ordinate axis, the absorbance measured.

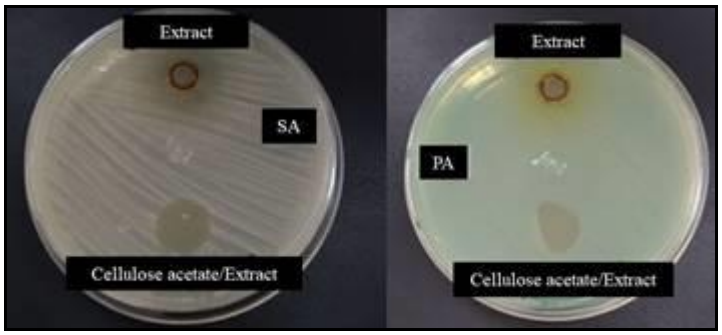
Figure 8 - Release curve of the mandarin extract from the cellulose acetate membrane with extract in a solution of 5 mL of ethanol and 995 mL of water



As shown in Figure 8, the release test shows that the release rate of tangerine extract from the cellulose acetate film was intense in the initial phase, probably due to the solubilization of the film surface extract. After that, the extract present inside the film was retained, leading to a decrease in the release rate, and release of the membrane extract was limited to 0.011% in 8 h. The release of the extract was possibly reduced due to the structural characteristic of the cellulose acetate film, as observed in the SEM, it did not present pores, making it difficult for the solvent to access the extract, thus with greater resistance to mass transfer from the matrix to the solvent, in due to less solvent access to the extract (Azuma *et al*., 2013).

Samples of tangerine extract and cellulose acetate film with extract were tested for antimicrobial activity, the tests are in Figure 9.

Figure 9 - Antimicrobial activity of the mandarin extract and the cellulose acetate membrane with extract using the agar diffusion sensitivity method. On the left with Gram positive strains (SA, Staphylococcus aureus ATCC 25923) and on the right with Gram negative strains (PA, Pseudomonas aeruginosa ATCC 0027)



Although the extract showed antimicrobial activity with an inhibition zone of 1.8±0.1 cm for Staphylococcus aureus and 0.9±0.2 cm for Pseudomonas aeruginosa, the membrane did not present antimicrobial activity against the tested

microorganisms, but there was also no growth of strains on the samples surface. This result suggests a possible application in bio packing technology, for example for medicines and food, maintaining the quality and shelf life.

4 CONCLUSION

Bio packing material was obtained by incorporating tangerine peel extract into cellulose acetate film, which showed to be a homogeneous material characteristic by FTIR, DSC, SEM and MFA analysis and does not exhibit pores. These characteristics justify the reduced water absorption and release of the CA membrane extract.

Low water absorption is important for the film to act as a barrier with external medium and the release of the extract was sufficient to prevent growth on the sample surface of the strains investigated. The presence of the extract reduced the tensile strength and the deformation of the film. Although the extract showed antimicrobial activity for the microorganisms Staphylococcus aureus and Pseudomonas aeruginosa, the membrane did not show antimicrobial activity, but there was also no microbiological growth on the material surface. In this way, a method for reusing agricultural waste was presented, with the use of citrus residues presenting the potential of bio packing technology, increasing profitability, exploring more effectively the planting of this fruit and minimizing environmental stress.

For future research, aiming at applications in packaging technology, studies on high temperature volatility and flavor for food applications should be considered.

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