Food Bioprocess Technol (2016) 9:1276–1286
DOI 10.1007/s11947-016-1717-y

ORIGINAL PAPER

Characterization of Biodegradable Films Based on *Salvia hispanica* L. Protein and Mucilage

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Received: 14 September 2015 / Accepted: 11 March 2016 / Published online: 19 March 2016

Abstract Biodegradable films of chia by-products (mucilage and protein-rich fraction (PF)) incorporated with clove essential oil (CEO) were obtained and characterized. The effects of polymer concentration (PC; 1.0–3.0 %, *w*/*v*) and CEO concentration (0.1–1.0 %, *v*/*v*) were evaluated as well as the pH (7–10), using a 2^3 factorial design with four central points. The films exhibited moisture values between 11.6 and 52.1 % (d.b.), which decreased ($p<0.05$) with increasing PC and CEO. The thickness of the films increased ($p<0.05$) with increasing PC. PC and pH influenced ($p<0.05$) the lightness (*L*) and variation in color between red and green (*a*). The orientation of the color to yellow-blue hues (*b*) decreased significantly ($p<0.05$) with increasing PC. Transparency was significantly lower and higher ($p<0.05$) than PC and CEO, respectively. The film surface morphology was evaluated using atomic force miscrocope images, and thermogravimetric analysis (TGA) was performed to study the thermal stability of the films. The displacement and tensile strength were significantly lower ($p<0.05$) at higher concentrations of CEO, this variable being the only one with a significant effect. The chemical composition of the films was confirmed utilizing Fourier transform infrared (FTIR) spectroscopy. The proportion of CEO added to the films had a significant influence on antimicrobial activity, inhibiting the growth of both *Escherichia coli* and *Staphylococcus aureus*.

Keywords Biodegradable films · Physical characterization · Mechanical properties · Antibacterial activity

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Introduction

In recent years, the use of films or coatings based on biodegradable materials has been emphasized, since they help to decrease pollution problems generated by the use of containers made of synthetic polymers, which are widely used for the storage of food, beverages, and medicines, among others (Malathi et al. 2014). Thus, today, most commonly used materials for the development of films are composed of different biopolymers, such as polysaccharides, proteins and lipids, or a combination of them (Seyedi et al. 2014; Espino-Díaz et al. 2010). One of the most important functions of edible films and coatings is their ability to incorporate active ingredients, as they can serve to support additives capable of conserving and improving the quality of the product. In this sense, it is possible to improve the quality and the product service life with their application by incorporating, for example, antimicrobials, antioxidant agents, and/or texture improvers (Burt 2004; Raybaudi-Massilia et al. 2006). However, research on the use of these substances as antimicrobial agents active in the packaging materials and their effects on the properties of the film, such as its mechanical properties and barrier characteristics, are scarce. Several

studies have determined that the oils of clove, cinnamon, mustard, oregano, rosemary, and thyme possess more pronounced antimicrobial activity, since they contain a high percentage of phenolic compounds such as carvacol (a major component of oregano), thymol (from thyme), or eugenol (a major component of clove) (Deans and Ritchie 1987; Arora and Kaur 1999; Delaquis et al. 2002; Tepe et al. 2004). The action of essential oils can be improved by physical conditions such as pH, temperature, and low oxygen levels (Romero-Bastida et al. 2011). The concept of packaging with added antimicrobial materials has attracted the attention of the food industry, due in part to the increase in consumer demand for products free of preservatives with regard to fruits, vegetables, and other foods (Romero-Bastida et al. 2011).

The type of material used as a structural matrix, processing conditions and type, and/or concentration of additives will influence the film's properties such as its functional attributes, mechanical properties, optical quality, barrier function, resistance to water, and sensory attributes. Grocery films based on polysaccharides have the ability to reduce the oxygen level and increase carbon dioxide concentrations, by virtue of which they have been used to extend the life of fruit, vegetables, meat, and seafood (Vásconeza et al. 2009). However, such films are generally hydrophilic in nature and have limited water vapor barrier properties without the addition of hydrophobic substances. In this sense, the presence of lipids in the films gives them the distinction of being good barriers to moisture. Films based only on biopolymers are often fragile and difficult to manipulate mechanically. Therefore, it is necessary to employ low-molecular-weight substances, such as plasticizing agents, in their formulation, which facilitate their interaction and decrease such features (Raybaudi-Massilia et al. 2008). These substances have the ability to reduce intramolecular and intermolecular interactions of the backbone chains of biopolymers comprising edible films during their formation, causing changes in the physical and mechanical properties (Appendini and Hotchkiss 2002).

On the other hand, in recent years, consumers have shown a preference for selecting diet foods that provide components with health benefits. In this context, the chia and its by-products, such as oil (a vegetable source with a high content of α-linolenic acid, ω-3, natural antioxidants, and plant sterols), residual flour (a source of insoluble dietary fiber and protein–gluten free), and mucilage (soluble dietary fiber) can fulfill requirements for preparation of edible films. Current information regarding the functional properties of the mucilage indicates that it is a polymer with a thickening action, a high viscosity in water, and possible beneficial metabolic effects (Lin et al. 1994). Thus, intake of chia mucilage, alone or in combination with the seeds, has been proven to influence lipid metabolism by decreasing the intestinal absorption of fatty acids, cholesterol, and the drag of bile salts, increasing the loss of cholesterol through feces as well as inhibiting endogenous cholesterol synthesis and slowing down the digestion and absorption of nutrients (Hentry et al. 1990). However, few studies have been carried out on the filmogenic properties of chia mucilage. The objective of this work was to develop and characterize biodegradable films based on chia by-products (mucilage and protein fraction (PF)) with the incorporation of clove essential oil (CEO) as an antimicrobial additive.

Materials and Methods

Seeds and Chemicals

Salvia hispanica L. seeds were obtained in a local market of Merida, Yucatan, Mexico, from the February 2014 harvest. All chemicals were of reagent grade purchased from J.T. Baker (Phillipsburg, NJ) and from Sigma (Sigma Co., St. Louis, MO).

Protein-Rich Fraction Extraction

Extraction of the protein-rich fraction (PRF) was carried out after oil extraction from milled whole seeds (Thomas-Wiley 1, model 4, Thomas Scientific, USA). Oil extraction from the milled seeds was performed with hexane to 60 °C during 80 min utilizing a Friedrich system, repeating this process four times. The remaining fraction was milled with 1-mm screen, a second oil extraction was done, and this fraction was milled with 0.5-mm screen. The protein-rich fraction was obtained by dry fractionation of the defatted flour according to Otto et al. (1997). Briefly, 500 g flour was sifted for 20 min using a Tyler 100 mesh (140-mm screen) and a Ro-Tap1 agitation system. The protein-rich fraction was passed through the mesh and stored for later use.

Obtaining the Chia Mucilage

Mucilage extraction was performed according to the method reported by Azero and Andrade (2006). Of the seeds, 4.0 kg was used in lots of 150.0 g. A suspension of whole chia seed/water in a ratio of 1:20 (*w*/*v*) was heated at 50 °C with constant agitation for 30 min. After the heating, the suspension was centrifuged (Beckman Ultracentrifuge, CA, USA) at 25, 000 rpm, 15 °C, and 10 min. The following three phases were obtained: water, seed residues, and mucilage; water was discarded, and seed residues were removed from mucilage. The process was repeated once more in order to obtain a greater amount of mucilage. Finally, the mucilage was freeze-dried at −47 °C and 13×10^{-3} mbar until use (FreeZone 4.5, Labconco, MO, USA).

Experimental Design to Prepare Films

A 2^3 factorial design with four central points was used. The variables evaluated were the polymer concentration (mucilage and protein-rich fraction of *S. hispanica* in relation 1:1), pH, and the concentration of CEO. The coded and real values of variables are presented in Table 1. Film-forming solutions were prepared by dissolving the mucilage and the protein-rich fraction in deionized water at room temperature and homogenizing for 30 min in a plate with magnetic stirring.

The pH was adjusted with 0.1 M NaOH or HCl. Afterward, glycerol was added as a plasticizing agent at a 2:1 ratio (polymer:glycerol). Once the plasticizing agent was added, the mixture was vortexed for 10 min and then subjected to heating in a water bath at 80 °C for 30 min. Then, the suspensions were left to cool down to 40 °C. The appropriate concentration of clove essential oil was then added immediately, and the suspension was sonicated for 15 min (Ultrasonic Cleaner Branson 2510, OH, USA) and poured into Petri dishes (9 × 1.5 cm). Finally, the films were dried in a convection oven (Fisher Scientific, USA) at 50 °C for 18 h and stored in a desiccator with saturated potassium carbonate solution (50 % relative humidity) until further analysis. An ANOVA test with a confidence interval of 95 % was carried out using the software Statgraphics Plus version 5.1 (Statistical Graphic Corporation, Manugistics Inc., Rockville, USA, 2005).

Characterization of the Films

Moisture Content

Moisture content was determined as the weight loss by the films when subjected to a temperature of 105 ± 2 °C for 24 h in a convection oven (Fisher Scientific, USA; AOAC 1997). The dry matter content was calculated by the difference between the moisture content and the initial weight of the film. This determination was performed in triplicate.

Thickness and Surface Density

The thickness of the films was determined using a micrometer (Mitutoyo, Japan) with a resolution of 0.001 mm. Measurements were taken at five different points on two films from each treatment (Perez-Gago and Krochta 2001). The density of the films was calculated from their weight and dimensions according to the following equation: $d = m/(A \times e)$.

where m is the dry weight of the film (g), A is the area (cm^2), e is the film thickness (cm), and d is the density of the film (g/cm^3).

Water Solubility

Solubility in water was determined according to the method described by Gontard et al. (1993). The test consists in determining the weight loss percentage of the film after being immersed in water for 24 h at room temperature. Briefly, 0.1 g of sample was taken and immersed in 30 mL of distilled water at room temperature for 24 h. Afterward, the volume of water was filtered through a Whatman no. 1 filtration paper, which was previously dried and weighed. The filter paper containing the insoluble portion of the film was then dried in a convection oven at 105 °C for 24 h. The weight of the soluble portion was determined by subtracting the weight of the insoluble matter from the initial weight of the sample.

Color Measurement

The color was determined according to the methodology described by Rhim et al. (1999). A colorimeter with a white calibration plate was used (Minolta Colorimeter CR-300,

Table 1 Experimental conditions for preparation, moisture content, thickness, solubility, and surface density of films formulated with mucilage and protein-rich fraction

Treatment	Polymer concentration (%, *w/v*)	pH	Clove essential oil concentration (%, *v/v*)	Moisture (%, d.b.)	Thickness (mm)	Solubility (%)	Surface density (g/cm^3)
T1	1 (−1)	7 (−1)	0.1 (−1)	34.89 ± 0.87	0.06 ± 0.01	70.06 ± 2.38	2.78
T2	3 (+1)	7 (−1)	0.1 (−1)	30.42 ± 0.07	0.16 ± 0.01	80.83 ± 3.37	3.72
T3	1 (−1)	10 (+1)	0.1 (−1)	45.82 ± 0.52	0.07 ± 0.01	70.23 ± 2.31	2.32
T4	3 (+1)	10 (+1)	0.1 (−1)	52.13 ± 3.94	0.19 ± 0.05	73.66 ± 0.72	2.55
T5	1 (−1)	7 (−1)	1.0 (+1)	45.86 ± 3.19	0.06 ± 0.03	75.38 ± 3.62	3.55
T6	3 (+1)	7 (−1)	1.0 (+1)	26.46 ± 0.60	0.18 ± 0.02	72.59 ± 0.62	3.58
T7	1 (−1)	10 (+1)	1.0 (+1)	37.42 ± 2.42	0.07 ± 0.03	67.21 ± 2.82	3.58
T8	3 (+1)	10 (+1)	1.0 (+1)	11.57 ± 0.69	0.18 ± 0.03	65.07 ± 4.60	3.72
T9	2 (0)	8.5 (0)	0.5 (0)	34.86 ± 2.33	0.12 ± 0.03	58.66 ± 2.86	3.04

T9 central points (4)

Minolta Camera Co., Osaka, Japan). Film fragments 3 cm in diameter were evaluated according to the Hunter scale (L^*, a^*, and b^*). This determination was performed in duplicate. Based on data obtained from L^*, a^*, and b^*, the following parameters were calculated:

$$\text{Color difference} = \Delta E = \left(\Delta L^2 + \Delta a^2 + \Delta b^2\right)^{0.5}$$

$$\text{Whiteness index} = \text{WI} : 100 - \left[(100 - L)^2 + a^2 + b^2\right]^{0.5}$$

where $\Delta L = L$ standard $- L$ sample, $\Delta a = a$ standard $- a$ sample, and $\Delta b = b$ standard $- b$ sample.

Transparency Determination

Film transparency was evaluated according to the method described by Shiku et al. (2003), with a UV–Vis spectrophotometer (model VE-5100UV, IL, USA). Film samples were cut into rectangular shapes (2.0 × 4.0 cm) and placed in the interior of the spectrophotometer cell, and the spectra were obtained at a wavelength of 600 nm. Then, the transparency of each film was calculated according to the equation of Han and Floros (1997).

$$\text{Transparency} = A_{600}/s$$

where A = absorbance of the film at a wavelength of 600 nm and s = thickness of the film.

Morphology Characterization

Surface topography of the films was carried out utilizing a Bruker Multimode 8 atomic force microscope (AFM; Bruker, Coventry, UK) in tapping mode using Si_3N_4 tip. Scanning area was 15.0 μm square, and the micrographs were analyzed for surface roughness with the software Nanoscope 1.50.

Thermogravimetric Analysis

Thermogravimetric analysis (TGA) was performed to study the thermal stability of the films on a TGA-7 thermobalance (Perkin-Elmer, Inc., MA, USA). The sample weights examined were between 5 and 10 mg. The scans were run at 10 °C/min under nitrogen atmosphere in the temperature range from 50 to 600 °C.

Mechanical Properties

Mechanical parameters of tensile strength (TS), elongation at break (%EB), and elastic modulus (EM) were determined according to the ASTM D882 standard (ASTM 2000; Carneiro-da-Cunha et al. 2009; Osés et al. 2009). Film fragments 1.5 × 6.5 cm were obtained; previously, they had been incubated at 50 % relative humidity and 24 °C for 24 h. The mechanical parameters were determined using a universal INSTRON machine (model 4411, USA) with a cell load of 500 kgf, a distance between the jaws of 50 mm, and a test speed of 5 mm/min.

Fourier Transform Infrared Spectroscopy

The chemical composition of the films was confirmed utilizing Fourier transform infrared spectroscopy on a Nicolet 8700 spectrophotometer (Thermo Scientific Instrument, MA, USA) equipped with a Smart iTR-attenuated total reflectance (ATR) accessory. The spectra were obtained in the region 4000–600 cm^{-1} with 32 scans using a resolution of 4 cm^{-1} in the transmittance mode.

Antibacterial Activity

To evaluate the antibacterial activity of the chia films incorporated with clove essential oil, the agar diffusion technique was used based on the method of Cutter (1999). Inocule of *Staphylococcus aureus* and *Escherichia coli* were standardized at 1×10^8 cfu/mL. Petri dishes with agar soy tripticasein were inoculated with the bacterial suspensions using a cotton swab. Film sections 1 cm^2 in size were placed in the middle of the previously inoculated agar surface and incubated at 37 °C for 24 h. Antibacterial activity was determined by measuring the growth inhibition zone caused by each film. The antibacterial activity was assessed by considering the following parameters: (1) an area <10.0 mm was considered inactive, (2) areas between 10.0 and 13.0 mm were partially active, (3) areas between 14.0 and 19.0 mm were considered active, and (4) areas >19.0 mm were regarded as very active (Valgas et al. 2007).

Results and Discussion

Moisture Content, Thickness, Surface Density, and Solubility

Biodegradable films based on protein-rich fraction and mucilage from *S. hispanica* were obtained. Table 1 shows the water content, thickness, solubility, and surface density of films obtained under different conditions. The effects of the variables and their interactions on the moisture content, thickness, and solubility of films are shown in Fig. 1.1.

Film moisture content provides information about how the interaction between mucilage, PRF, and CEO could affect the water affinity of films. The results show that

the concentration of the mucilage/PRF and CEO led to a reduction in water content ($p < 0.05$). At a concentration of 3 %, the mucilage/PRF films could be forming cross-links between the mucilage and proteins; cross-linking results in a decrease in the availability of hydroxyl groups, limiting polysaccharide-water interactions by hydrogen bonding (Martins et al. 2012). From these results, it is apparent that varying the concentration of the mucilage/PRF resulted in a modification of the structure of the films, which is reflected in their moisture content. Their thickness was also significantly affected ($p < 0.05$) by the polymer concentration; it is greater film thickness by increasing this variable (Fig. 1.1). The films' thickness was greater in treatments 2, 4, 6, and 8 where higher concentrations of polymer were employed (Table 1). Surface density was significantly affected ($p < 0.05$) by the polymer concentration. However, no correlation was evident between the other properties evaluated and the surface density of the films. According to Giancone et al. (2011), changes in the surface density of the films do not change their structure. Therefore, this attribute does not determine the mechanical and functional properties of the films. On the other hand, the films exhibited high levels of solubility, varying in the range between 58.7 and 80.8 % (*w*/*v*). Their high-solubility values can be attributed to the hydrophilic nature of hydrocolloids and glycerol. This parameter is important for choosing their potential applications (Arvanitoyannis et al. 1998). Usually, when a film's solubility is high, its ability to protect a food's moisture environment and/or retard its own water loss would be decreased; however, it would be an advantage from the point of view of their biodegradability (Stuchell and Krichta 1994). Chia mucilage films exhibited higher-solubility values than that obtained for films of *Psyllium basil* and

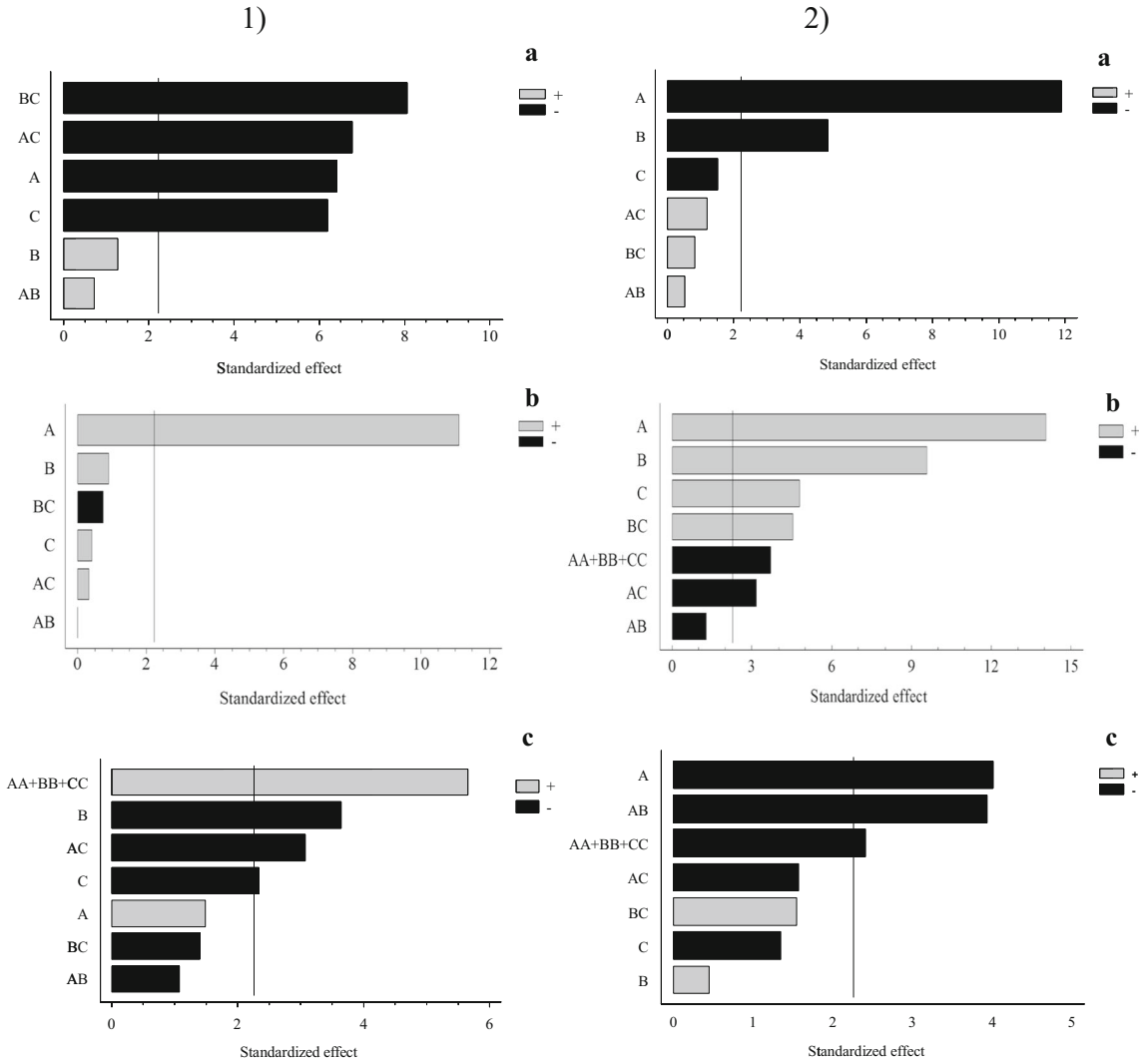

Fig. 1 (1) Pareto chart of the moisture content (**a**), thickness (**b**), and solubility (**c**). (2) Pareto chart of the *L* (**a**), *a* (**b**), and *b* (**c**).The *black line* indicates the critical level above which the variables presented a significant effect ($p < 0.05$). (**A**) Polymer concentration, (**B**) pH, and (**C**) concentration of clove essential oil

Lepidium perfoliatum mucilages (Reza et al. 2012; Khazaei et al. 2014; Seyedi et al. 2014).

Color Measurement and Transparency

Color parameters (L, a, b, WI, and ΔE) and transparency are presented in Table 2. The color is an important factor in a film's characterization since it determines its acceptance by consumers (Mali et al. 2004). The luminosity (L) and color variation between red and green (a) were significantly affected ($p < 0.05$) by polymer concentration and pH, decreasing and increasing these parameters, respectively, serving to increase these variables (Fig. 1.2a, b). Moreover, the CEO significantly affected a value. The orientation toward yellow and blue tones (b) decreased significantly ($p < 0.05$) when the concentration of polymers was increased.

Color changes due to incorporating different concentration of CEO can be more fully described using other color functions such as WI, which indicates the degree of whiteness, and ΔE, which indicates the degree of total color difference from the standard color palate. The addition of CEO resulted in an increase ($p < 0.05$) in ΔE but decreased ($p < 0.05$) WI. Transparency is a valuable property in films since they are part of the packaging system and therefore interfere with consumers' choice. With respect to transparency, it was significantly lower ($p < 0.05$) at higher concentrations of polymers and significantly higher at higher concentrations of CEO (Fig. 2.1).

The addition of essential oils to edible films may change the native color of the films. The degree of change is concentration dependent. Du et al. (2009) reported that darker films were produced by the addition of cinnamon, allspice, and clove bud oils into film-forming solutions.

Morphology Characterization

AFM imaging provides structural information for a sample in its more natural state, without dehydration or coating. An AFM image of the film surface is created by monitoring motion of the probe tip across surface. Therefore, the film surface morphology was evaluated using AFM images (Fig. 2.2). In general, non-homogeneous, non-compact, and rough film networks were observed; this behavior could be the result to incorporate hydrophobic substances (clove oil) into hydrophilic matrices.

Thermogravimetric Analysis

TGA thermograms of films made with mucilage and protein-rich fraction of *S. hispanica* L. (in the temperature range of 50 to 600 °C under nitrogen) are shown in Fig. 3.

The initial weight loss of all samples at approximately 60 °C is due to the evaporation of water. The weight loss in the second range (250–350 °C) corresponds to a complex process including the dehydration of the monosaccharide rings and depolymerization (Mathew and Dufresne 2002). The TGA curves showed that all films are stable up to 250 °C with a maximum rate of decomposition occurring at about 340 °C.

Mechanical Properties

The properties desired in a food packaging material depend on its application. In general, a food packaging may be an undeformable material to provide structural integrity or reinforce food structure or a deformable film for other applications (Mali et al. 2004). Tensile strength is one of the most common indicators of the mechanical properties of an edible film. It expresses the maximum stress developed in a film specimen during tensile testing (Gennadios et al. 1994). The values of tensile strength (kgf) obtained for the different films are shown in Table 3. Tensile strength was significantly affected ($p < 0.05$) only by the concentration of CEO, this parameter being diminished as the concentration of CEO was increased (Fig. 4a). This behavior was similar that observed by Pranoto et al. (2005) and Jouki et al. (2014) in alginate-based edible film incorporated with garlic oil and in forming quice seed

Table 2 Color settings including L, a, b, color difference (ΔE), whiteness index (WI), and transparency (T) of films formulated with mucilage and protein-rich fraction

Treatment	L	a	b	ΔE	WI	T
T1	66.55 ± 1.28	4.42 ± 0.31	33.67 ± 1.50	43.58 ± 1.99	52.33 ± 1.98	8.39 ± 0.16
T2	48.30 ± 1.15	13.94 ± 0.55	40.41 ± 2.39	62.80 ± 2.61	31.91 ± 1.02	3.66 ± 0.04
T3	60.53 ± 1.30	7.81 ± 0.33	42.67 ± 1.97	54.75 ± 2.37	41.35 ± 2.35	7.31 ± 0.38
T4	35.57 ± 1.13	15.62 ± 1.08	27.72 ± 1.00	63.42 ± 3.20	28.09 ± 1.60	5.30 ± 0.25
T5	64.91 ± 0.33	6.34 ± 0.02	35.66 ± 0.18	46.41 ± 0.35	49.57 ± 0.36	8.59 ± 0.44
T6	42.18 ± 0.37	12.27 ± 0.09	28.67 ± 1.58	60.70 ± 0.45	34.28 ± 0.39	4.97 ± 0.24
T7	53.21 ± 0.16	13.89 ± 0.02	41.44 ± 0.41	59.91 ± 0.37	35.97 ± 0.37	9.45 ± 0.48
T8	41.43 ± 2.07	20.35 ± 0.89	27.62 ± 1.60	65.91 ± 3.29	35.81 ± 1.80	5.78 ± 0.23
T9	47.62 ± 0.00	14.44 ± 0.44	39.56 ± 1.22	64.10 ± 2.71	30.52 ± 3.89	5.41 ± 0.07

T9 central points (4)

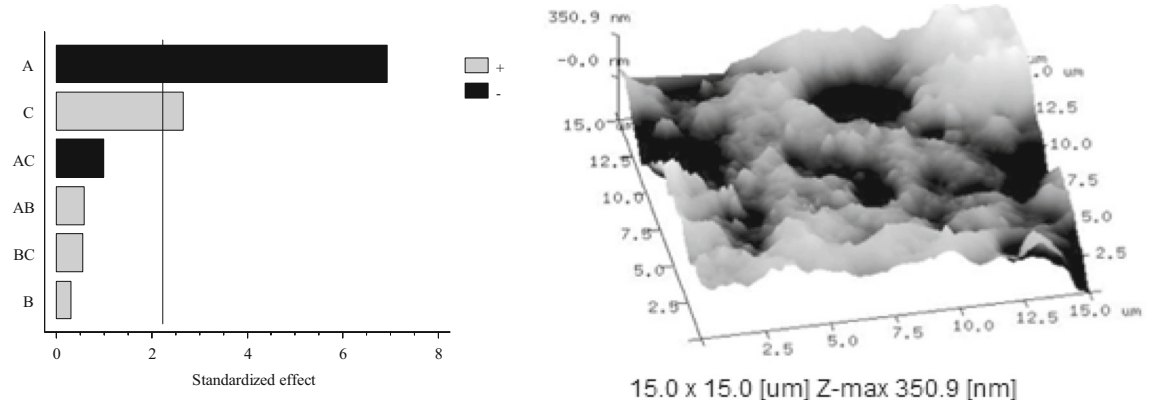

Fig. 2 (1) Pareto chart of the transparency of the films. The *black line* indicates the critical level above which the variables presented a significant effect ($p < 0.05$). (**A**) polymer concentration, (**B**) pH, and (**C**) concentration of clove essential oil. (2) 3D image obtained from AFM of edible films. This figure clearly showed surface morphology of biodegradable film fabricated. It is observed that the nodules are not merged and have some peaks. T8 was chosen as more representative

mucilage films incorporated with oregano essential oil, respectively.

Elongation at break (%) is a measure of film stretchability prior to breakage. High tensile strengths are generally necessary for edible films in order to withstand the normal stress encountered during their application, subsequent shipping, and food handling. However, the flexibility of edible films, i.e., elongation at break, should be adjusted according to the intended application of an edible film. The values of elongation at break (%) obtained for the different films are shown in Table 3. Elongation at break was significantly affected ($p < 0.05$) only by the concentration of CEO, this parameter decreasing as the concentration of CEO was increased (Fig. 4b). Films made with the highest concentration of CEO had less resistance and were less extensible than those with smaller amounts of CEO. The higher the elastic modulus (kgf/

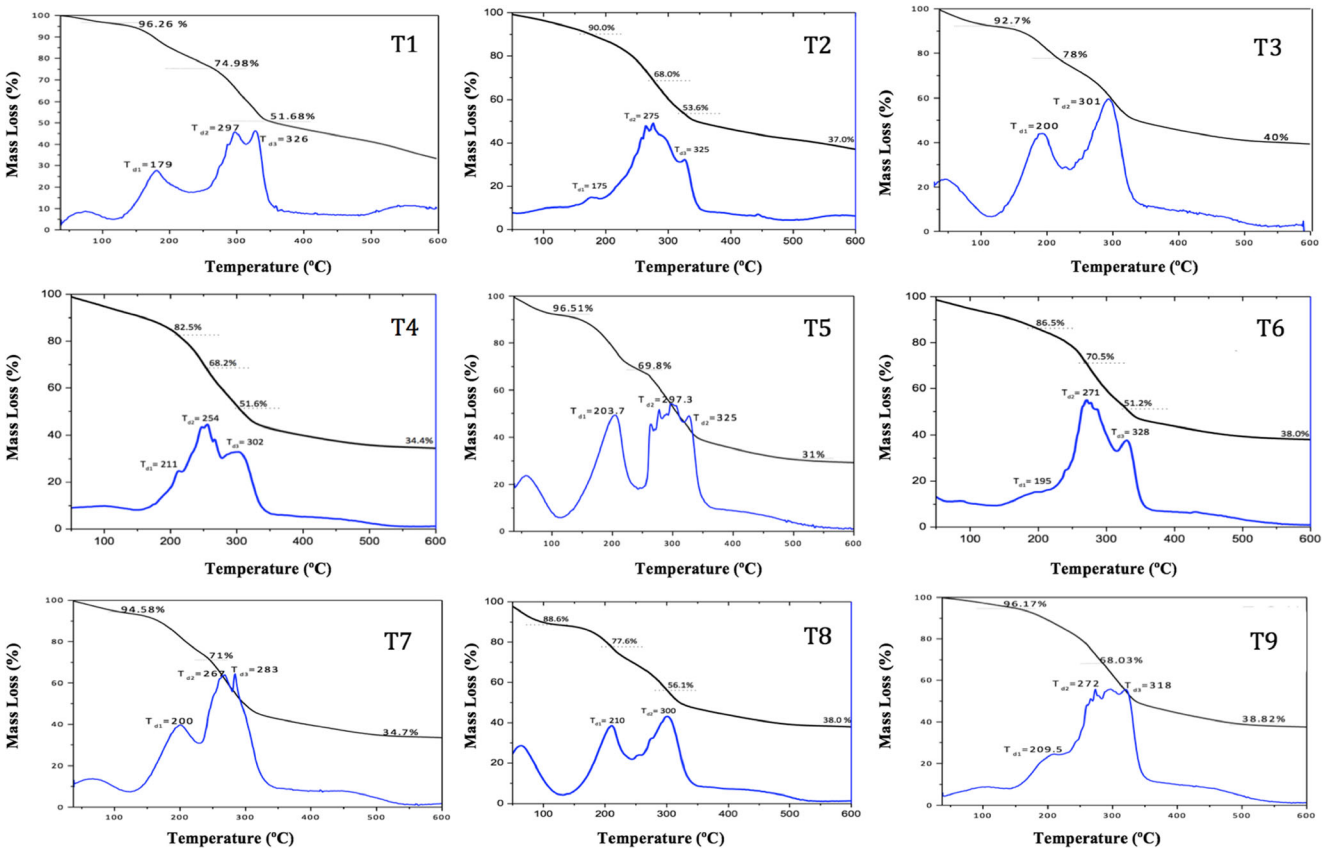

Fig. 3 TGA thermograms of films made with mucilage and protein-rich fraction of *Salvia hispanica* L. (T1–T8 = 2^3 factorial design treatments and T9 = central points). *Black graphs* indicate mass loss (%), and *blue graphs* indicate transition temperatures (°C)

Table 3 Tensile strength (TS), elongation at break (EB), and elastic module (EM) of films formulated with mucilage, protein-rich fraction, and clove essential oil

Treatment	TS (kgf)	EB (%)	EM (kgf/mm)
T1	2.26 ± 0.40	6.12 ± 0.30	0.54 ± 0.16
T2	2.77 ± 0.73	3.22 ± 1.57	2.49 ± 0.42
T3	1.52 ± 0.58	4.37 ± 0.83	0.92 ± 0.47
T4	2.25 ± 0.34	4.72 ± 1.31	1.19 ± 0.88
T5	0.64 ± 0.37	3.25 ± 2.30	0.48 ± 0.03
T6	1.99 ± 1.89	2.78 ± 0.40	1.95 ± 0.28
T7	0.97 ± 1.09	3.22 ± 2.51	0.48 ± 0.13
T8	0.47 ± 0.22	1.51 ± 0.26	6.02 ± 0.96
T9	2.54 ± 0.49	4.57 ± 0.67	1.80 ± 0.27

T9 central points (4)

mm) of a material is, the stiffer or, in other words, less flexible the material is. The values of elastic modulus (kgf/mm) obtained for the different films are shown in Table 4. In this study, reducing CEO and PRF increased the film's flexibility. The use of both plasticizer and CEO reduces the intermolecular forces between mucilage and protein, and increases the mobility of mucilage chains, thus enhancing the flexibility of the films. Concentration of glycerol was fixed, so tensile strength, elongation at break, and elastic modulus were significantly affected only by the concentration of CEO; these parameters were being diminished, as the concentration of CEO was increased. When the amount of CEO is above 1 % (*w*/*v*), only part of the CEO locates in the interphase area, and the excess is dispersed in the mucilage/protein matrix. This affects its homogeneity and consequently reduces the tensile strength of the films.

Also, films with lower moisture content were more elastic. According to Diab et al. (2001), biopolymers are extremely brittle and fragile at low moisture levels. Their structure collapses very rapidly and they offer no resistance to an applied load. Failure propagates very quickly in such a structure and causes its fragmentation and disintegration. At moderate moisture content levels, the partially plasticized biopolymer matrices become more cohesive and hence tougher; i.e., the materials deform rather than disintegrate. As a result, structural elements that would have been completely destroyed and eliminated by brittle failure at low levels of moisture content can remain intact or only partially degraded and continue to offer resistance to an applied force.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) analyses were used to evaluate chemical interactions between the polymeric film and the bioactive agents incorporated in it. Table 4 represents the FTIR profile obtained for mucilage and PRF films

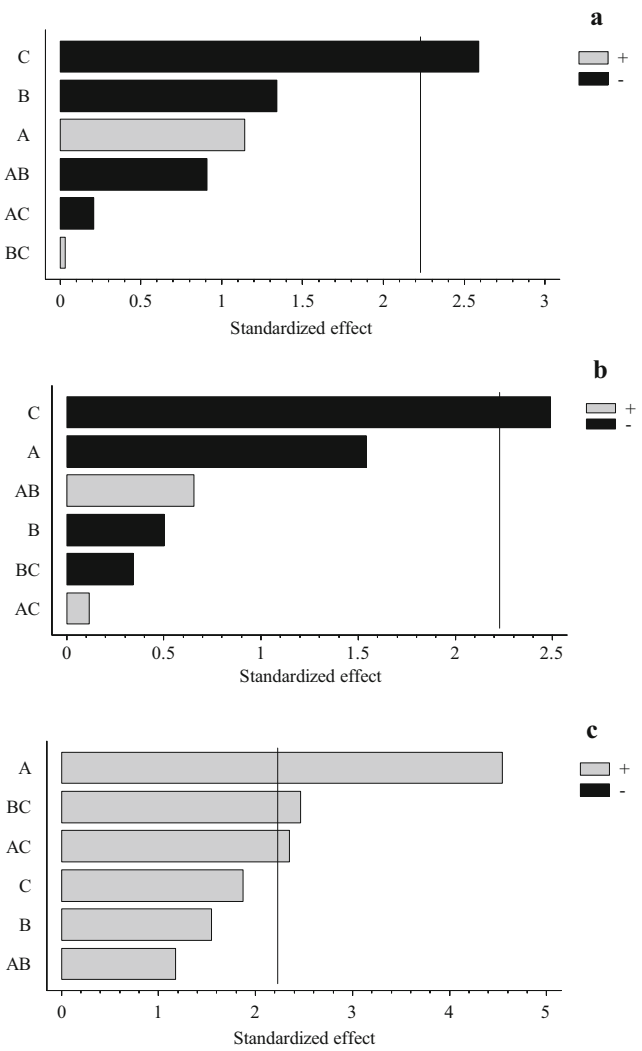

Fig. 4 Pareto chart of the tensile strength (**a**), elongation at break (**b**), and elastic module (**c**).The *black line* indicates the critical level above which the variables presented a significant effect ($p < 0.05$). (**A**) polymer concentration, (**B**) pH, and (**C**) concentration of clove essential oil

incorporated with CEO. Spectra show similar patterns with absorption peaks between 3400 and 840 cm^{-1}. Peaks between 3400 and 1020 cm^{-1} correspond to –OH stretching of hydroxyl group, −C-H stretching vibration, (C = O) bonded to aromatic group, $-COO^{-}$ stretching (ion), and C-O as ester group in a cycle. These groups were reported by Muñoz-Hernández (2012) for FTIR analyses of mucilage form *S. hispanica* seeds.

The absorption bands for mucilage-PRF-based composite films showed a peak between 1690 and 1600 cm^{-1}, which corresponds to C = O stretching amide I, a representative group of proteins; the amide I band is the most useful peak for infrared analysis of secondary protein structures (Nur-Hanani et al. 2013). Finally, a peak at 960 and 840 cm^{-1} corresponds to trans-CH out-of-plane ring deformation, for eugenol derivatives in clove essential oils like isoeugenol, methyl eugenol, and acetyl eugenol (Wang and Sung 2011).

Table 4 FTIR absorption bands of films formulated with mucilage, protein-rich fraction, and clove essential oil

Component	Functional group class	Band position (cm^{-1})	Wave number films (cm^{-1})
Mucilage	–OH stretching of hydroxyl group	3200–3400	3280–3330
	–C-H stretching vibration	2926 (±10)	2923–2937
	(C = O) bonded to aromatic group	1600–1700	1513–1598
	$–COO^-$ stretching (ion)	1400–1550	1407–1429
	C-O as ester group in a cycle	1230–1270, 1020–1075	1234–1268, 1029–1035
Protein-rich fraction	C = O stretching amide I	1600–1690	1660–1625
Clove essential oil	Trans-CH out-of-plane ring deformation	960, 804	914–921, 836–850

Antibacterial Activity

Plant-derived essential oils, the odorous, volatile products of an aromatic plant's secondary metabolism, normally formed in special cells or groups of cells, are well-known antimicrobial agents that could be used to control food spoilage and foodborne pathogenic bacteria. The antimicrobial activity of plant essential oils is assigned to a number of small terpenoid and phenolic compounds, which in pure form also exhibit antibacterial or antifungal activity (Burt 2004). The antibacterial activity of the film formulations against two pathogenic bacteria was expressed in terms of zone inhibition. Treatments with a low concentration of CEO (0.1 %) and central treatments (0.5 %) failed to produce a zone of inhibition. Among the films studied, treatments with a high concentration of CEO (1.0 %) exhibited a prominent inhibitory effect on both pathogenic bacteria (Fig. 5.1).

The concentration of CEO as the only variable had a significant effect ($p < 0.05$) on this property, with a higher

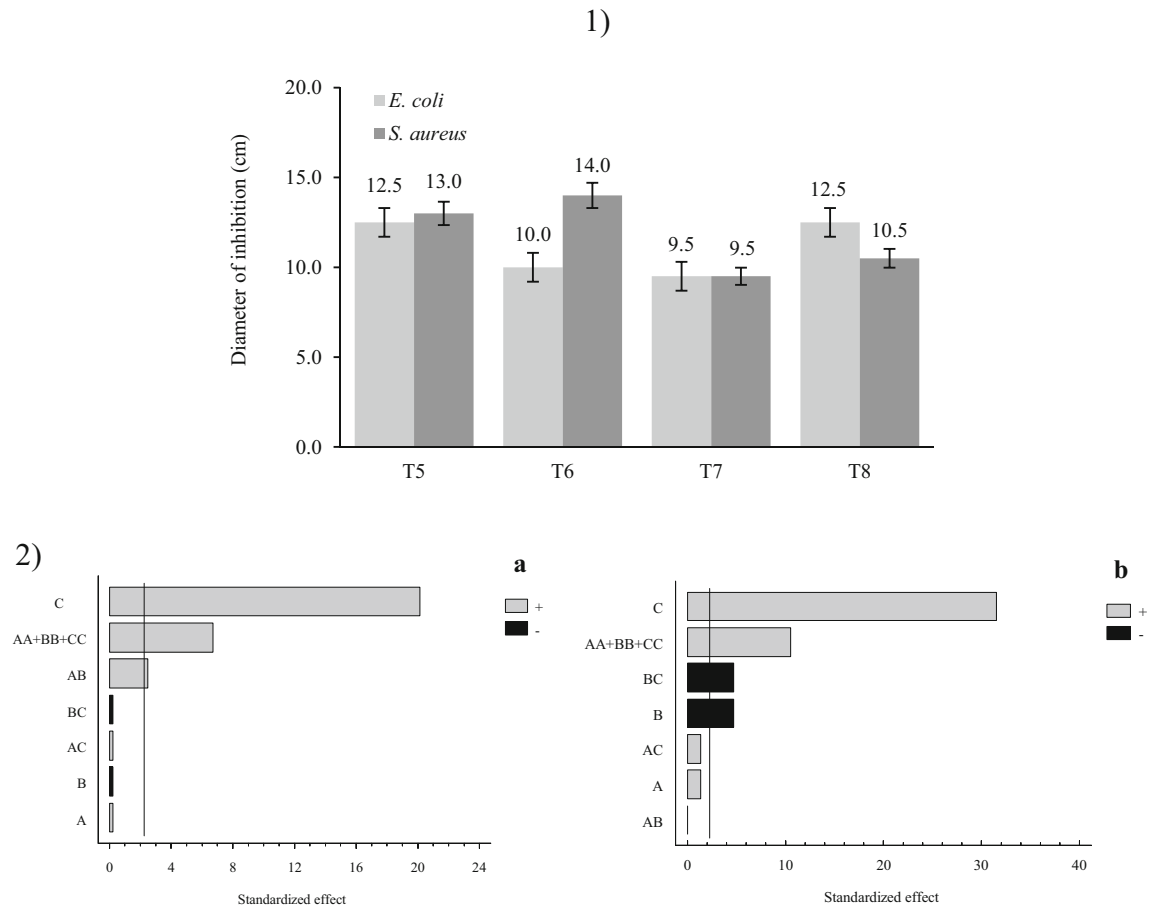

Fig. 5 (1) Diameter of inhibition of films made with mucilage and protein-rich fraction of *Salvia hispanica* L. (2) Pareto chart of the capacity growth inhibition of *E. coli* (**a**) and growth inhibition of *S. aureus* (**b**). The *black line* indicates the critical level above which the variables presented a significant effect ($p < 0.05$). (**A**) Polymer concentration, (**B**) pH, and (**C**) concentration of clove essential oil

percentage of bacterial inhibition at a higher concentration of CEO (Fig. 5.2). In order for a film to exhibit inhibitory activity against bacterial growth, it is necessary that the antimicrobial agent be spread out over the agar. The films obtained with a low concentration of CEO did not diffuse their active agents sufficiently; therefore, they did not inhibit the growth of bacteria. Clove oil contains eugenol, carvacrol, and thymol, phenolic compounds with proven antimicrobial activity; such activity is dependent on the concentration (Nonsee et al. 2011). This explains the behavior observed in the present study, where a higher content of CEO (3 %, *w*/*v*) increased the inhibitory effect on bacterial growth. The mechanism of antimicrobial activity of essential oils is related with the attack on the phospholipid present in cell membranes, which causes increased permeability and leakage of cytoplasm, or in their interaction with enzymes located on the cell wall. Thus, the resistance of Gram-negative bacteria to the essential oils likely lies in the protective role of their cell wall lipopolysaccharides or outer membrane proteins. Considering the proposed parameters for Valgas et al. (2007), the antimicrobial activity of the films obtained with treatment 7 can be considered as inactive against both bacteria. In the case of treatments 5 and 8, the films were partially active against both bacteria. The film obtained with treatment 6 was partially active against *E. coli* and active against *S. aureus*. Results obtained showed that antibacterial activity of films tasted in this study is dependent of polymers concentration (3 %, *w*/*v*), pH (7), and content of CEO (1 %, *w*/*v*). Among the two tested pathogens, *E. coli* was the most resistant. The lower antimicrobial activity against *E. coli* can be attributed to the fact that Gram-negative bacteria are in general more resistant due to the external lipopolysaccharide wall surrounding the peptidoglycan cell wall. The direct application of antimicrobial substances in foods can have limited effects, especially if applied to the surface of the food (Quintavalla and Vicini 2002). Instead, the use of films containing antimicrobial agents is more efficient for the slow release of the bioactive compounds from the film to the surface of the product, in addition to the more prolonged effect.

Conclusions

In this study, the capacity of *S. hispanica* mucilage and protein-rich fraction to form films was analyzed, along with the addition of clove essential oil as an antimicrobial agent. The films corresponding to treatments with a lower concentration of polymer (1 %) were more transparent, thin with a yellowish hue, and softer to the touch than films made with the highest concentration of polymer (3 %, *w*/*v*). The latter presented a dark brown hue, were thicker, and were quite brittle. The best features were obtained in the film corresponding to the central point (2 % polymer and pH 8.5), exhibiting a yellowish color while maintaining transparency, flexibility, an appropriate thickness, and without invasive to clove scent. In general, non-homogeneous, non-compact, and rough film networks were observed in AFM images. The TGA curves showed that all films are stable up to 250 °C with a maximum rate of decomposition occurring at about 340 °C. FTIR analyses showed the chemical interactions between the polymeric film and the bioactive agents incorporated in it. Films obtained with higher content of CEO (treatments 5, 6, 7, and 8) showed antibacterial active against both pathogens. These results indicate that CEO is an effective antimicrobial on some important pathogenic bacteria and can be added to packaging materials made with mucilage and protein from *S. hispanica* due to their absorbance on various surfaces. Therefore, it is necessary to continue with this research in order to define the best conditions to obtain films with better properties (resistance, flexibility, proper permeability, thickness, biological activity, morphological characteristics, and thermal gravimetric analysis), in such a way that they can be used for coating food such as breads, fruits, vegetables, meats, and cheese, among others.

Acknowledgments This research forms part of Project 189741 "Aspectos tecnológico-funcionales de subproductos de chía (*Salvia hispánica* L.) aplicables al desarrollo de alimentos," financed by the Consejo Nacional de Ciencia y Tecnología (CONACYT), Universidad Autónoma de Yucatán–Facultad de Ingeniería Química (UADY), México and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 1735), Universidad Nacional de La Plata (UNLP, 11/X610), and Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Argentina.

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