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Research Article

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Functional properties and in vitro antioxidant and antibacterial effectiveness of pigskin gelatin films incorporated with hydrolysable chestnut tannin

Cristina Peña-Rodriguez[1], Josefa F Martucci[2], Laura M Neira[2], Aitor Arbelaiz[1], Arantxa Eceiza[1] and Roxana A Ruseckaite[2]

Abstract

The impact of the incorporation of 10% w/w of hydrolyzable chestnut tannin into pigskin gelatin (G) films plasticized with glycerol (Gly) on the physicochemical properties as well as the in vitro antioxidant and antibacterial effectiveness against food-borne pathogens such as *Escherichia coli* and *Streptococcus aureus* was investigated. A higher tendency to both redness (a*) and yellowness (b*) coloration characterized gelatin films incorporated with chestnut tannin. The reduced lightness (L) and transparency of gelatin–chestnut tannin films plasticized with 30% w/w Gly might be associated with certain degree of phase separation which provoked the migration of the plasticizer to the film surface. The incorporation of chestnut tannin and glycerol affected the chemical structure of the resultant films due to the establishment of hydrogen interactions between components as revealed by Fourier transform infrared spectroscopy. These interactions reduced gelatin crystallinity and seemed to be involved in the substantial decrease of the water uptake of films with tannin, irrespective of the glycerol level. Such interactions had minor effect on tensile properties being similar to those of the control films (without chestnut tannin) at the same glycerol level. Films modified with 10% w/w chestnut tannin showed significant ($P < 0.05$) 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, ca. from 0 ± 0.033 to 87.1 ± 0.002% for chestnut tannin-free and chestnut tannin-containing gelatin films. The limited inhibitory activity of films incorporated with 10% w/w chestnut tannin against the selected bacteria evidenced by disk diffusion method probably resulted from the interactions within the film restricting the diffusion of the active agent into the agar medium. The more modest protective effect observed against a Gram-positive bacterium (*S. aureus*) was also discussed.

Keywords

Gelatin, chestnut tannin, physicochemical properties, antioxidant, antibacterial

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In memoriam of Prof. Iñaki Mondragon

INTRODUCTION

Quite recently the interest in the potential uses of films from biodegradable polymers particularly polysaccharides and protein-based materials has increased, mainly because these biopolymers can decompose more readily in the environment than

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their synthetic polymeric counterparts (Kuorwel et al., 2011). The combination of biodegradable polymers with active agents, such as antimicrobial and antioxidants, is emerging as a promising active packaging system, with the advantage of being environmentally benign (Tharanathan, 2003).

Gelatin (G) is a water-soluble protein derived from animal sources, obtained from the hydrolysis of skin, bone-collagen, or connective tissues of mammals and fish (Arvanitoyannis, 2002; Martucci and Ruseckaite, 2009; Vanin et al., 2005). The acceptance of gelatin as a "Generally Recognized as Safe" (GRAS) substance by the U.S. Food and Drug Administration (FDA), along with its excellent film forming ability through different processing technologies has turned it into an attractive alternative for the design and development of functional films with high potential applications in the food sector. Gelatin films exhibit good oxygen barrier properties at low or intermediate relative humidity (RH) and satisfactory mechanical properties making them suitable for use as coating or food packaging materials (Andreuccetti et al., 2009; Arvanitoyannis, 2002; Martucci et al., 2012; Martucci and Ruseckaite, 2009, 2010; Vanin et al., 2005). However, under high RH these desired properties deteriorate dramatically owing to the fact that gelatin contains large amounts of hydrophilic groups which readily might interact with water (Arvanitoyannis, 2002). Additionally, proliferation of food-borne pathogens is favored in such wet environments, thus compromising the potential application of gelatin films as food packaging material (Bigi et al., 2001; Martucci et al., 2012; Martucci and Ruseckaite, 2009; Vanin et al., 2005).

Several attempts have been undertaken to modulate gelatin film properties to meet comparable performance to those of the conventional synthetic plastics used in food packaging. A generally accepted way to enhance moisture repellency is by cross-linking through physical, enzymatic, and chemical routes (Arvanitoyannis, 2002). Gelatin features several side-chain groups (i.e. NH_2, COOH, OH) capable of reacting with a variety of chemical reagents such as dialdehydes which have been successfully used to stabilize gelatin films (Bigi et al., 2001; De Carvalho and Grosso, 2004; Farris et al., 2010; Martucci et al., 2012). However, the suspected toxicity of such aldehydes has somehow put into question their application in food contact materials (Bigi et al., 2001). This issue has motivated in search of natural compounds as alternative cross-linking agents, including genipin (Bigi et al., 2002), oxidized starch (Martucci and Ruseckaite, 2009, 2010), and lignin (Núñez-Flores et al., 2013). Natural tannins comprise a wide range of oligomeric and polymeric phenols; condensed tannins (i.e. proanthocyanidins) have flavanol units in their structure, while hydrolysable ones are composed of one molecule of sugar, generally glucose, joined to phenolic acids. Tannins are categorized as GRAS food additives commonly used to protect food nutrients against oxidative degradation and microbial spoilage (Chung et al., 1998; El Gharras, 2009; Sung et al., 2012), rendering them technically and physiologically useful in the packaging field. Antibacterial actions of tannins have been reported as bacteriostatic and bactericidal against harmful bacteria including *Escherichia, Listeria, Pseudomonas, Salmonella, Staphylococcus,* and *Streptococcus* (Sung et al., 2012). The ability of tannin components to cause the bacterial colonies to disintegrate probably results from their interference with the bacterial cell wall, thereby inhibiting the microbial growth.

Although the use of some individual polyphenolic compounds such as tannic, gallic, caffeic, and ferrulic acid to strengthen gelatin-based films via protein–polyphenol interaction has been reported (Cao et al., 2007; Jongjareonrak et al., 2008; Yan et al., 2011; Zhang et al., 2010a, 2010b), few studies concerning the use of vegetable tannins themselves as modifiers of gelatin films for food packaging have been conducted. The production and characterization of antioxidant edible tuna gelatin films added with aqueous extracts of murta with acceptable water, tensile, and optical barrier properties has been reported by Gómez-Guillén et al. (2007). The increased antioxidant activity of films incorporated with murta extract was counterbalanced by the decreased mechanical properties due to the greater polyphenol–protein interactions. The inclusion of polyphenolic borage extract into sole films was also studied (Gómez-Estaca et al., 2009). Obtained films exhibited a pronounced increase of their antioxidant properties, with minor modifications of their physicochemical properties such as decrease of the breaking force and increase of film opacity. Taylor et al. (2012) explored the use of quebracho tannin as cross-linking agent for gelatin in leather applications. Authors reported that after using tannin the melting point and viscosity of samples increased. Wu et al. (2013) have recently reported the effect of adding green tea extract (GTE) rich in polyphenols into fish skin gelatin films. Authors stated that increasing GTE in film formulation enhanced the antioxidant activity and indirectly affected functional properties due to interactions between gelatin and GTE. In our previous work, we studied the effect exerted by hydrolysable chestnut tannin (CT) on some properties of pigskin gelatin (G) films (Peña et al., 2010). Mechanical and thermal behavior varied as a function of the content of tannin showing optimum values for films modified with 10% w/w tannin. The transparency of films with tannin was maintained while ultraviolet (UV) resistance

and water repellency were enhanced. Nonetheless, such improvements were counterbalanced by the increased stiffness and brittleness of the resultant films, making them hardly suitable for packaging purposes.

The addition of plasticizers modifies the three-dimensional organization decreasing attractive intermolecular forces and increasing chain mobility, resulting in films with increased extensibility and flexibility. The use of glycerol as a modifier of gelatin–tannin films relies on its well-known plasticizing efficiency for proteinaceous matrices (Andreuccetti et al., 2009; Bergo and Sobral, 2007; Cao et al., 2009) as well as on its water solubility, which ensures tannin and gelatin compatibility during the dissolution stage of the casting processing method.

Accordingly, the present study analyses the influence of the addition of glycerol on some relevant properties of gelatin–chestnut films such as color and light barrier properties, tensile properties, water solubility, and in vitro antioxidant activity according to its 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (RSA). The antibacterial effect against *Escherichia coli* and *Staphylococcus aureus* was also tested.

MATERIALS AND METHODS

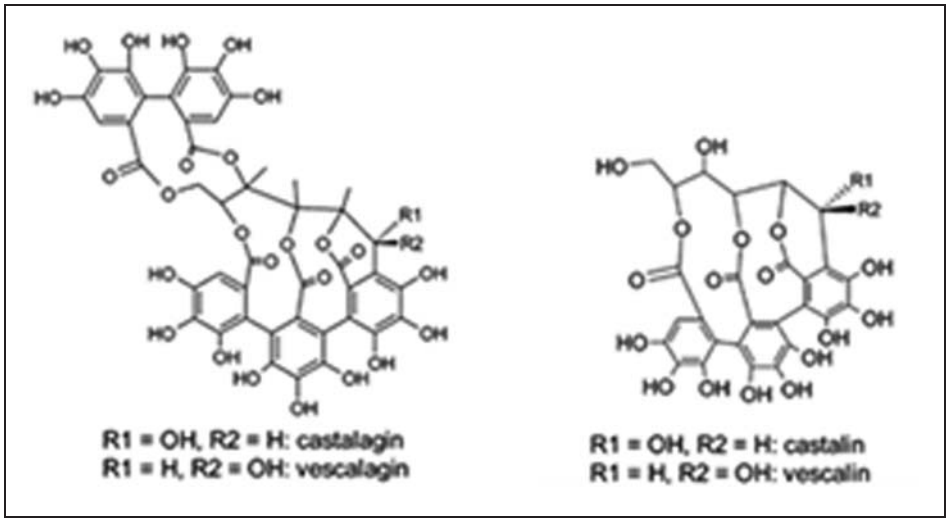
Materials

Commercial pigskin gelatin (G) type A (isoelectric point, Ip 7) was sourced by Sigma and employed as received without any further treatment. Commercial hydrolysable CT was kindly supplied by Agrovin Products (Spain). Main components of CT, as reported by the supplier, are depicted in Scheme 1. Glycerol (Gly, purity 99%) was obtained from Panreac. Ultrapure water was obtained from Millipore purification system (Milli Q, water resistivity 0.066 μS/cm).

Preparation of films

Gelatin powder (2.5 g) was hydrated with 50 ml of distilled water at room temperature (around 25 °C) with constant stirring for 30 min. Subsequently, the hydrated gelatin was heated up to 50 ± 5 °C and different concentrations of glycerol (5–30% w/w on dry gelatin basis) were added to the film-forming solution. Then, the pH of the aqueous solution was adjusted to 11 using a 1 N NaOH solution and maintained at 50 ± 5 °C for 10 min. Afterwards it was poured into polystyrene Petri plates and dried at room conditions (25 °C, 60% RH) to obtain films with average thickness of 100 ± 10 μm. Gelatin films containing glycerol were labeled as GGlyX, where X corresponds to the weight percentage of glycerol.

Gelatin films incorporated with CT were obtained on a similar basis. Tannin was added to achieve 10% w/w (on dry gelatin basis). This value was fixed based on results of our previous work (Peña et al., 2010) where it was verified that undesirable free tannin remained in gelatin films containing CT > 10% w/w. During tannin addition, the initial pH of gelatin solution was adjusted to 11 with a 1 N NaOH solution so as to prevent gel formation. Subsequently, glycerol was included and the solution was stirred for another 30 min. Afterwards films were cast under similar conditions to those used for GGlyX films. Gelatin films with 10% w/w tannin and glycerol were labeled as GGlyXCT10, where X corresponds to the weight percentages of glycerol. Control gelatin films were



Scheme 1. Chemical structures of chestnut ellagitannins.

prepared under similar conditions without adding any tannin or glycerol. Samples were conditioned at $25 \pm 2\,^{\circ}C$ and 50% RH in a hydrothermal chamber (Dycometal CCK-81, Barcelona, Spain) before testing.

Measurements

Water uptake (WU). WU tests were performed gravimetrically. Samples (square shape, $2.5 \times 2.5\,cm^2$) were dried until constant weight in an oven to remove moisture before testing and this weight was taken as the initial one (w_0). After this, samples were immersed in 30 ml of ultrapure water at $25 \pm 2\,^{\circ}C$. The soaked samples were removed after 24 h, blotted on filter paper to remove liquid excess, and reweighed (w_h) to determine the weight gain. The percentage of WU at the equilibrium (WU) was expressed as

$$WU(\ \%) = [(w_h - w_0)/w_0] \times 100 \qquad (1)$$

Measurements were taken by triplicate for each composition.

Fourier transform infrared spectroscopy (FTIR). FTIR spectra of the films were obtained with a Thermo Scientific Nicolet 6700 spectrometer (Wisconsin, EEUU). The acquisition conditions were 600–$4000\,cm^{-1}$ spectral range, 20 scans, and a resolution of $4\,cm^{-1}$.

X-Ray diffraction patterns (XRD). XRD patterns were collected on a Philips PW1710 X-ray diffractometer (Eindhoven and Almelo, The Netherlands) equipped with a CuKα radiation source ($\lambda = 1.5418$ Å) operating at 40 kW and 20 mA. Data were recorded in the range of $2\theta = 2$–40° at a scanning rate of $1^{\circ}\,min^{-1}$.

Color and light barrier properties. Color parameters and opacity were determined by using a Lovi Bond Colorimeter RT500 (Amesbury, United Kingdom) with an 8 mm diameter measuring area. Film specimens were measured against the surface of a standard white plate, and the CIELAB color space was used to obtain the color coordinates. Lightness (L*), redness (+a*), or greenness (–a*), and yellowness (b*) or blueness (–b*) and opacity (%) values were reported as the average of three measurements.

Tensile tests. Tensile tests were carried out in a Miniature Materials Tester MiniMat2000 (Rheometric Scientific Ltd, Leatherhead, UK). Probes were cut with a pneumatic die cutter. Probe dimensions and test conditions were set according to ASTM D1708-93 standard and each film thickness was measured at five random positions using a micrometer (Mitutoyo, Japan) with an accuracy of ±0.001 mm.

Deformation at break (ε_u) expressed as percentage of elongation, elastic modulus (E), and tensile strength (TS) was calculated from the resulting stress–strain curves. At least five specimens were tested for each sample and the average was calculated.

Determination of DPPH RSA. Film samples were immersed in liquid nitrogen and subsequently crushed and grounded with a pestle. A precise amount of crushed film (0.4 g) was mixed with 4 ml of methanol in a caped tube, stirred vigorously, and let to stand overnight (about 24 h). Afterwards, the tubes were centrifuged at 5000 r/min for 10 min (Centrifuge type 4–15, Sartorius AG, Gottingen, West Germany) and the supernatant was extracted and reserved for DPPH RSA. RSA was determined according to the method of Yen and Hsieh (1995) with slight modifications. A volume of 400 µl of each solution was mixed with 2 ml of a 0.06 mM solution of DPPH in methanol. Mixtures were mixed vigorously and allowed to stand in the dark for 30 min at room temperature. The reduction of DPPH radical was measured at 517 nm using an UV–Visible spectrophotometer (Agilent 8453, China). The control was conducted in the same manner but methanol was used instead of sample. DPPH RSA was calculated as follows

$$RSA\ (\%) = (1 - (A_{517sample}/A_{517control})) \times 100 \qquad (2)$$

where $A_{517sample}$ is the absorbance of sample and $A_{517control}$ is the absorbance of the control. The control was conducted in the same manner, except that distilled water was used instead of sample. A lower absorbance of the reaction mixture indicated a higher DPPH RAS. All determinations were made in triplicate.

In vitro *antibacterial activity.* The antibacterial activity tests included food-borne spoilage and pathogenic bacteria such as Gram-negative *E. coli* O157:H7 (32158, American Type Culture Collection (ATTC)) and Gram-positive *S. aureus* (25923, ATTC) were achieved on Mueller–Hinton agar. Vegetative cells of each microorganisms previously cultivated on Mueller–Hinton agar for 24 h at $37 \pm 0.5\,^{\circ}C$ were suspended in double-distilled sterile water, and the suspension was standardized according to FDA (1998) method. Antibacterial activity test on films was assessed using the agar diffusion method (Ponce et al., 2008). The concentration was adjusted to 0.5 of Mac Farland scale (10^5–10^6 CFU/ml) for measuring antimicrobial activity with disc diffusion assay. Films were cut into a disc

shape of 8 mm radius using a circular knife and then placed on Mueller–Hinton (Merck, Darmstadt, Germany) agar plates, which had been previously seeded with inoculums of tested bacteria. The plates were then incubated at $37 \pm 0.5\,^{\circ}C$ for 24 h and, afterwards, examined for width of inhibition. The radius of the growth inhibition zone surrounding the film discs was accurately measured with a manual caliper (Mitutoyo, Japan). Bacterial suspensions without films were used as positive controls, whereas films without bacterial strains were used as negative controls. All determinations were performed in triplicate.

Statistical analysis. The analysis of variance was used to evaluate the significance of the differences between factors and levels. Comparison of the means was carried out by employing a Tukey's test to identify which groups were significantly different from other groups. The least significant difference was $P < 0.05$.

RESULTS AND DISCUSSION

Color and light barrier properties

Opacity and color attributes such as lightness (L*), redness/greenness (a*), and yellowness/blueness (b*) values of gelatin-based films incorporated with 10% w/w CT and different concentrations of glycerol are presented in Table 1. While control gelatin film (without CT nor glycerol) was visually colorless and transparent, the addition of 10% w/w CT greatly influenced color parameters evidenced by the higher tendency to both, redness (a*) and yellowness (b*) ($P < 0.05$) (Table 1). It is commonly found that the addition of plant extracts alters the original color of protein-based films to a certain extent and the magnitude of such is determined by the type and concentration of polyphenols which are believed to confer yellow-brown coloration (Tongnuanchan et al., 2012). Decreased lightness (L*) and concomitant increased opacity values were found in films incorporated with CT compared with control film ($P < 0.05$) (Table 1). Taking into account that the chromaticity components of the color space CIELab were obtained on a standard white plate, a decrease of L* values with respect to the control film may reflect a decrease in transparency or a gain of color of the films. In this sense the incorporation of light brownish CT contributed to strengthen the color of the films. Similar results were already reported in fish gelatin films incorporated with antioxidants such as borage extract (Gómez-Estaca et al., 2009), murta leaves (Gómez-Guillén et al., 2007), buthylated hydroxytoluene and α-tocopherol (Jongjareonrak et al., 2008), and GTE (Wu et al., 2013). The addition of 10% w/w Gly into GCT10 films greatly reduced ($P < 0.05$) a* value, increased slightly b* while causing a significant reduction in opacity and, consequently an increment in lightness ($P < 0.05$), suggesting that such glycerol level had a dilution effect in accordance with results reported by other authors (Ramos et al., 2013; Sobral et al., 2005). The addition of further glycerol resulted in a sharp increase in film opacity coincidently with a reduction in L* ($P < 0.05$) indicating darker films (Table 1). It is well known that light transmission of films depends on many factors such as thickness, the presence of a dispersed phase within the matrix with a particle size bigger than the wavelength of the visible light, as well as the presence of interactions between film components (Martucci et al., 2012). Since no significant differences in thickness values were detected with glycerol content (e.g. mean thickness $100 \pm 10\,\mu m$) and polyphenol concentration was constant it was postulated that the reduction in film transparency is more likely associated with a light scattering effect due to some degree of phase separation. Nonmiscible phase promotes opacity as a function of the differences in the refractive index of the phases and the concentration and particle size of the dispersed phase (De la Caba et al., 2011). During the drying stage, gelation takes place through gelatin–tannin–glycerol intermolecular interactions which contribute to the formation of a cohesive network (Gómez-Estaca et al., 2009; Jongjareonrak et al., 2008; Peña

Table 1. Opacity and color parameters of gelatin–10% w/w chestnut tannin films plasticized with varying glycerol contents

Formulation	Opacity (%)	L*	a*	b*
G	10.70 ± 0.91 d	93.31 ± 0.20 d	−1.90 ± 0.12 d	5.60 ± 1.10 d
GCT10	29.10 ± 2.29 a	56.45 ± 1.40 a	15.07 ± 0.73 a	44.86 ± 0.97 a
GGly10CT10	19.03 ± 0.88 b	70.85 ± 4.32 b	6.17 ± 3.15 b	49.01 ± 3.08 a
GGly20CT10	30.60 ± 3.92 a	58.37 ± 3.65 a	17.61 ± 2.09 a	48.97 ± 4.38 a
GGly30CT10	43.51 ± 1.78 c	55.92 ± 2.01 a	19.54 ± 1.02 a	42.07 ± 0.96 a

Average ± standard deviation. Averages with different superscripts for each parameter are significantly different ($P < 0.05$) according to Tukey's test.

et al., 2010). Even when glycerol is uniformly dispersed into GCT10 matrix, plasticizer might migrate from the bulk to the surface because of the binding limitations between the matrix and glycerol. This was experimentally assessed for films containing Gly $\geq$30% w/w, which exhibited undesirable greasy surfaces, suggesting that such plasticizer content was higher than the critical value beyond which phase separation occurs. The reduction in light transmission could be beneficial for food preservation since light induces undesirable lipid oxidation.

FTIR and XRD

Interactions between polyphenols, gelatin, and glycerol could play an important role in determining the properties of the resultant films. Different biopolymers might react with polyphenols to diverse extents, depending on the polyphenolic constituents of plant extracts. Therefore, FITR spectra were used to explore the involvement of hydrogen bonding interactions between components. Representative spectra of gelatin, glycerol, gelatin–glycerol, and gelatin–glycerol–tannin films are illustrated in Figure 1. Neat gelatin film was characterized by typical bands located at 3288, 1631, 1544, and 1243 cm^{-1} corresponding to amide A (NH stretching coupled with hydrogen bonding), amide I (representative of C=O stretching vibration), amide II (representative of NH bending coupled with CN stretching), and amide III (NH bending), respectively (Bergo and Sobral, 2007; Ramos et al., 2013). In the glycerol-added gelatin film, the characteristic C–O stretching band of glycerol was clearly observed at 1045 cm^{-1} (Bergo and Sobral, 2007), and its intensity increased with glycerol content (results nor shown), as expected. The amide I band indicated differences in the secondary structure of polypeptide chains (Ramos et al., 2013). The exact location of the amide I depends on the hydrogen bonding and conformation of the protein structure. In general, most proteins have mixed secondary structures (α-helix, β-sheet, or a random structure), thus the amide I band often shows several components or shoulders (Jongjareonrak et al., 2008; Ramos et al., 2013). No substantial variations in the position of amide I was observed, suggesting that the inclusion of CT in the formulation has minor effect on protein conformation. The most noticeable effect detected was the increased absorption in amide II, attributed to an out-of-phase combination of CN stretch and in-plane NH deformation modes of the peptide group (Jongjareonrak et al., 2008) and amide V, attributed to skeletal C–N–C vibrations which suggest the existence of hydrogen interactions of glycerol and tannin with the skeletal C–N–C group of gelatin (Yan et al., 2011). The amide II band is generally considered to be much more sensitive to hydration than to secondary structure changes (Jongjareonrak et al., 2008). Since in this study FTIR spectra were performed in dry state, changes in amide II could be related to possible alterations of the secondary structure of gelatin induced by tannin, as already reported for gelatin–α-tocopherol films (Jongjareonrak et al., 2008). As regards amide V band, such interaction should affect the percentage of triple-helical collagen-like structure in CT and Gly-incorporated gelatin films. The potential modification of certain physicochemical properties of gelatin films added with 10% w/w CT will depend on the extent of such interactions as reported previously for tuna skin films incorporated with borage extract (Gómez-Estaca et al., 2009).

To link FTIR results with structural changes, X-ray diffractograms of GGlyCT and control films were obtained and results are shown in Figure 2. Neat gelatin film exhibited low intensity diffraction peak at $2\theta = 8.1^\circ$, ascribed to the triple-helical crystalline structure of the renatured collagen in pigskin gelatin (Bigi et al., 2004; Martucci et al., 2012). Glycerol addition induced a slight reduction in intensity and a significant shift toward the lower angles of the characteristic diffraction peak, i.e. from 8.1° for neat gelatin to 7.3° for gelatin containing 30% w/w Gly. Structural changes in gelatin films plasticized with glycerol have already been observed by others and related to the ability of glycerol to disrupt gelatin chain-to-chain interactions (Bergo and Sobral, 2007; Martucci and Ruseckaite, 2009; Rivero et al., 2010; Thomazine et al., 2005; Vanin et al., 2005). The incorporation of 10% w/w CT into gelatin–glycerol formulations resulted in a noticeable reduction in the peak intensity at $2\theta = 8.1^\circ$ of the

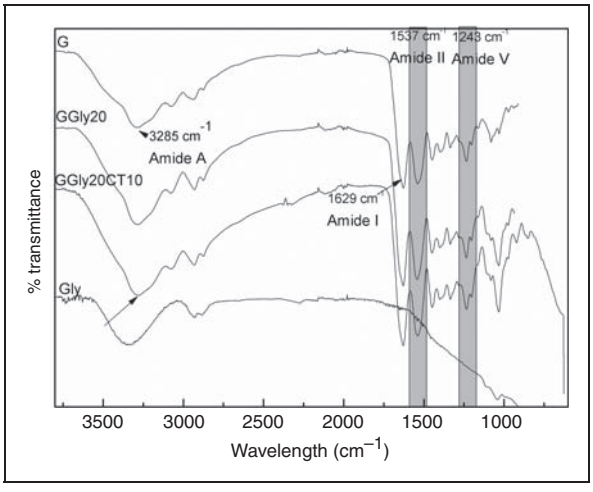


Figure 1. FTIR spectra of neat gelatin (G), gelatin–20% w/w glycerol (GGly20), gelatin–20% w/w glycerol–10% w/w chestnut tannin (GGly20CT10), and pure glycerol.

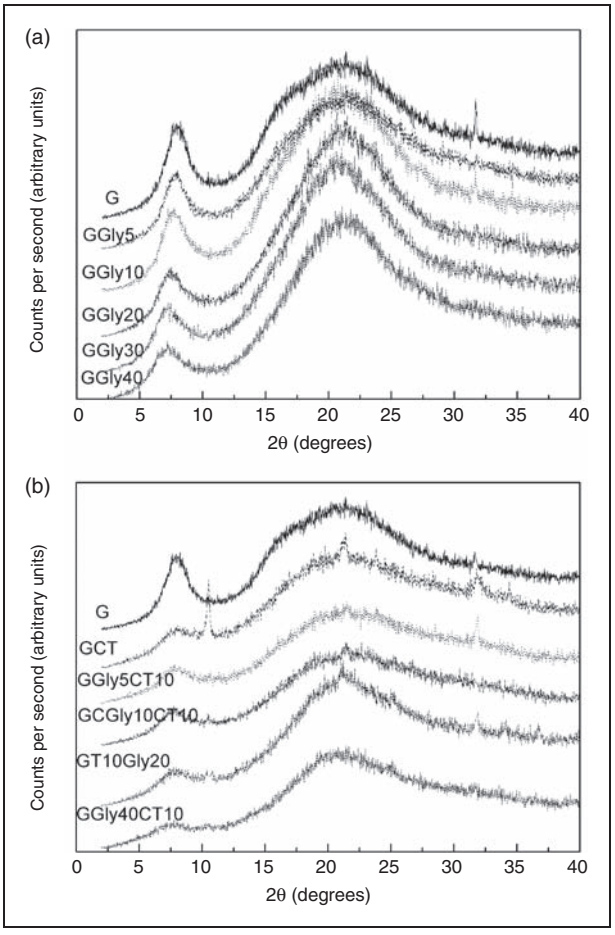


Figure 2. XRD patterns: (a) gelatin–glycerol films and (b) gelatin–glycerol films incorporated with 10% w/w CT.

resultant films (Figure 2(b)). Interestingly, the XRD of the GCT film exhibited a new diffraction peak at about $2\theta = 10.1^{\circ}$, which was absent in glycerol-plasticized gelatin films (Figure 2(a)). Since the tannins used in this work are amorphous this new peak evidences that the presence of low tannin content may induce new crystalline structures in CT-modified gelatin films (Peña et al., 2010). The disappearance of this new diffraction band with the addition of glycerol may be due to the enlarged intermolecular hydrogen interactions in gelatin due to glycerol (plasticizing effect) which restrain the potential tannin–gelatin interactions, thus preventing crystallization. It is anticipated that these interactions might influence tensile properties and WU of the produced films.

Tensile properties

TS, Young's modulus (E), and elongation at break (EAB) of pigskin gelatin films added with 10% w/w CT along with 10, 20, and 30% w/w glycerol are presented in Table 2. Films with glycerol >30% w/w were not able to be analyzed due to migration of plasticizer, as explained earlier. Nonplasticized gelatin films exhibited the typical characteristics of brittle and rigid materials: high Young's modulus (E) and TS at expenses of a reduced EAB (Peña et al., 2010; Rivero et al., 2010). The inclusion of 10% w/w CT incremented greatly TS and E values compared to neat gelatin film, ca. from 80 ± 10 MPa for neat gelatin up to 106 ± 10 MPa for GCT10 film ($P < 0.05$) (Table 2). The increase in TS and stiffness of gelatin films incorporated with polyphenolic compounds could be attributed to the ability

Table 2. Tensile properties and water uptake at the equilibrium (WU_{eq}) of gelatin–glycerol systems with and without 10% w/w CT

Formulation	E (MPa)	TS (MPa)	ε_u (%)	WU_{eq} (%)
0% w/w CT				
G	3150 ± 200 a	80 ± 10 a	3.3 ± 0.7 a	240 ± 9 a
GGly10	2600 ± 260 a,b	75 ± 2 a	4.1 ± 0.2 a,b	290 ± 2 b
GGly20	2370 ± 230 b	70 ± 2 a	5.4 ± 0.8 b	350 ± 10 c
GGly30	1400 ± 240 c	40 ± 2 b	5.6 ± 0.5 b	420 ± 10 d
10% w/w CT				
GCT10	4260 ± 240 d	106 ± 10 c	3.3 ± 0.7 a	145 ± 5 e
GGly10CT10	2470 ± 250 b	67 ± 7 a,d	4.5 ± 1.0 a,b	210 ± 10 f
GGly20CT10	2000 ± 200 b,c	53 ± 8 b,d	4.6 ± 0.8 a,b	240 ± 12 a
GGly30CT10	1650 ± 120 c	45 ± 2 b	5.2 ± 0.8 b	250 ± 9 a

Average ± standard deviation. Averages with different superscripts for each parameter are significantly different ($P < 0.05$) according to Tukey's test.

of CT to interact with gelatin via hydrophobic interactions and hydrogen bonds, leading to film strengthening (Cao et al., 2007; Jongjareonrak et al., 2008; Peña et al., 2010; Wu et al., 2013). Extensibility did not vary since no significant differences in EAB values were detected ($P > 0.05$) (Table 2). This feature determines that the films cannot be folded without cracking.

Plasticization by glycerol is expected to overcome these limitations by forming gelatin–plasticizer hydrogen bonds, replacing or disrupting gelatin–gelatin and gelatin–tannin strong interactions and so leading to more ductile films (Arvanitoyannis, 2002; Bergo and Sobral, 2007; Rivero et al., 2010). Tannin-free films incorporated with glycerol up to 20% w/w displayed insignificant variations in tensile parameters ($P > 0.05$) while further glycerol addition greatly reduced ($P < 0.05$) TS and E values rather than those of the nonplasticized counterpart (Table 2). Results agreed with those previously reported by others for gelatin films from different sources plasticized with glycerol (Bergo and Sobral, 2007; Rivero et al., 2010; Vanin et al., 2005). On the other hand, films incorporated with CT showed lower TS and E values ($P < 0.05$) in comparison with those determined for tannin-free films at the same glycerol level. A decrease in breaking force was already observed for sunflower protein films incorporated with polyphenols (Orliac et al., 2002), catfish and sole gelatin films incorporated with borage extract (Gómez-Estaca et al., 2009), and sole gelatin films added with murta extract (Gómez-Guillén et al., 2007). Authors ascribed this result to a weakening of protein–protein interactions which stabilize the protein network, in favor of new interactions among the film components, i.e. gelatin, glycerol, and tannin. This finding agreed well with the reduction in the intensity of the characteristic XRD diffraction peak of gelatin observed earlier (Figure 2(b)). Results differ from other studies concerning gelatin–polyphenols films (Cao et al., 2007; Wu et al., 2013). Comparison between experimental data was not reliable due to variations in the chemical composition of polyphenol, different source of gelatin, and test conditions (Cao et al., 2007). Again, no significant differences in EAB among control and gelatin films incorporated with CT were verified ($P > 0.05$). Therefore, glycerol is not useful to increase the flexibility of gelatin–CT films.

WU

Gelatin films incorporated with glycerol are highly swellable as indicated by the high WU values at the equilibrium $(WU)_{eq}$ registered (Table 2). It is mainly due to the hydrophilic nature of several α-amino acids constituting the protein chains and the substantial amount of hydrophilic plasticizer added (Arvanitoyannis, 2002; Martucci and Ruseckaite, 2009; Thomazine et al., 2005; Vanin et al., 2005). WU reduced significantly ($P < 0.05$) with the incorporation of 10% w/w CT, for the same glycerol content (Table 2). However, the WU in these films was still substantial, i.e. $145 \pm 5\%$ to $250 \pm 9\%$, for 0 and 30% w/w Gly, respectively (Table 2), indicating that materials were still water sensitive. These data are consistent with values reported previously for gelatin–tannic acid films (Zhang et al., 2010a, 2010b). Therefore, it may be concluded that films modified with CT had the least ability to entrap water. Results of WU suggest that hydrogen interactions between gelatin and tannin are still efficient to restrict the water binding capacity of the films by blocking hydrophilic groups in the protein chain (Cao et al., 2007; Peña et al., 2010; Zhang et al., 2010a, 2010b), but have negligible consequences on film strength, as revealed by tensile test results.

Antioxidant and antimicrobial activity

Antioxidant packaging is a major category of active packaging and very promising technique for extending food product shelf life. Moreover, enriching films with antioxidants allows nutritional and esthetic quality aspects to be enhanced without affecting the integrity of the food product (Guilbert et al., 1996). The DPPH radical scavenging capacity is an important method to measure the antioxidant property of a wide range of biopolymers (Wang et al., 2012; Zhang et al., 2012). For example, Wu et al. (2013) employed this method for the evaluation of antioxidant activity of an active film from silver carp skin gelatin incorporated with GTE, and more recently, Li et al. (2014) have analyzed the antioxidant capacity of several natural antioxidant in gelatin matrices. Figure 3 showed the DPPH RSA of gelatin-based films after adding CT at various glycerol levels. The addition of 10% w/w CT increased significantly ($P < 0.05$) the RSA (87.1 ± 0.002 and $0.033 \pm 0.002\%$ for GCT10 and G films with no glycerol added, respectively). This result suggests that CT contributed substantially to the antioxidant activity of gelatin films. It has been determined that the antioxidant effect of tannins is mainly due to the RSA of phenolic compounds. The antioxidant activity of phenolic compounds is mainly explained by their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Haslam, 1989). This activity did not vary substantially ($P > 0.05$) with the addition of up to 20% w/w glycerol but decreased with further adding plasticizer. Tongnuanchan et al. (2012) observed films based on fish skin gelatin incorporated with essential oils containing 30% w/w glycerol

exhibited higher antioxidant activity than those with 20% w/w glycerol because of the more loosen films structure which favored the release of the active agent. The loss of plasticizer already observed for GGlyCT10 films containing glycerol ≥30% w/w might be the reason of such discrepancy with reported data.

Inhibitory activity of control and CT-added gelatin films against *S. aureus* and *E. coli* were measured based on the clear zone surrounding a film disc and the results of inhibition zone (mm) are shown in Figure 4. After incubation the inhibition radius around each film disc (colony-free perimeter) was measured with a digital caliper and the inhibition area was then calculated in mm^2 (Table 3). Control films showed no inhibition ($P > 0.05$) against none of the pathogens analyzed (Table 3, Figure 4), in accordance to results previously informed for bovine gelatin films (Kavoosi et al., 2013). For films containing 10% w/w, CT inhibition effect toward *E. coli* was evident but no activity was observed against *S. aureus* (Table 3). No variations in the antibacterial activity of films were observed with the addition of glycerol suggesting that CT had similar solubility in both plasticized and nonplasticized films. It was already pointed out that inhibition zone could be affected by the solubility of the active agent, diffusion range in the agar, lost during processing (it can affect the dose) (Sung et al., 2012). However, according to the Swiss norm 195920-ASTM E 2149-01, any compound showing zone inhibition of >1 mm is considered as a good antibacterial agent. Consequently, gelatin films incorporated with 10% w/w could be applied to protect foods against *E. coli*.

Interestingly, results revealed that CT was more effective to Gram-negative bacteria rather than to Gram-positive. Min et al. (2007) reported that CT have increased growth-inhibitory and bactericidal effect against *E. coli* O157:H7 than mimosa tannin because of the higher hydroxylation degree of the active agents in CT, consisting mainly of vescalagin and castalagin molecules (Scheme 1), which have three OH groups compared with mimosa tannin which has only two OH groups. Although the inhibition of CT against Gram-negative bacteria is still

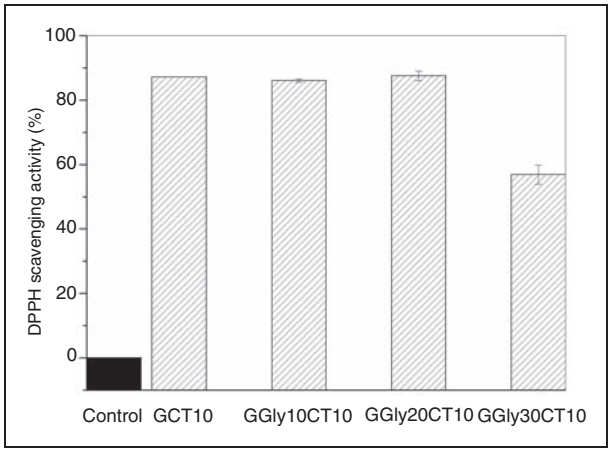


Figure 3. DPPH radical scavenging activity of gelatin–10% w/w CT films plasticized with varying amounts of glycerol.

Table 3. Inhibition halos obtained by the agar diffusion method against *E. coli* and *S. aureus*

	Inhibition halo (mm)	
Formulation	*E. coli*	*S. aureus*
G	8.0 ± 1.0a	8.0 ± 0.8a
GCT10	10.0 ± 2.1a	8.6 ± 0.1a
GGly20CT10	10.8 ± 0.4a	8.8 ± 1.0a

Average ± standard deviation. Averages with different superscripts for each parameter are significantly different ($P < 0.05$) according to Tukey's test.

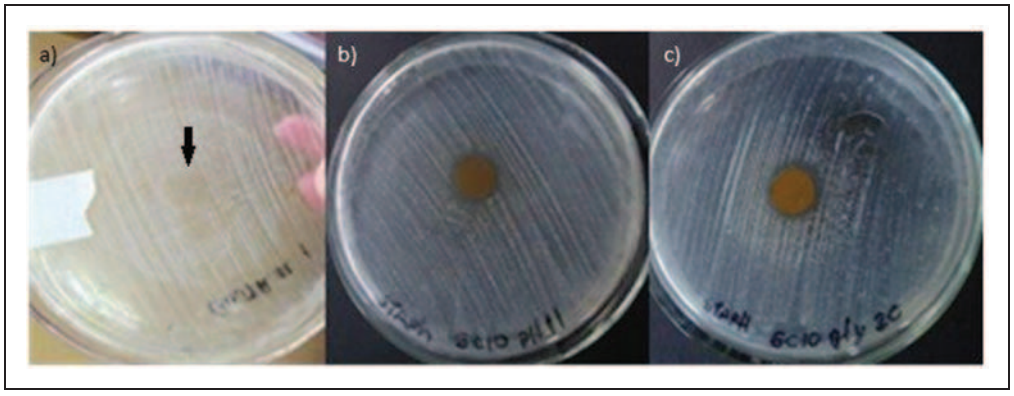


Figure 4. Inhibition halos obtained by the agar diffusion method against *E. coli* for (a) gelatin, (b) GCT10, and (c) GGly20CT10 films.

unknown, Chung et al. (1998) proposed a plausible explanation based on the well-known chelating ability of tannin using tanninc acid as a source of hydrolysable tannin. In their study, the growth inhibition against *E. coli* was restored by the supplementation of additional iron, thus indicating that some tannins chelate iron. Similarly, tannins can chelate divalent cations such as Mg^{2+} and Ca^{2+} which stabilize the lipopolysaccharide membrane of Gram-negative bacteria, facilitating the active agent to reach the target cytoplasmic membrane of Gram-negative bacteria where cytotoxicity occurs.

CONCLUSIONS

This study demonstrated that gelatin films incorporated with 10% w/w of natural and nontoxic hydrolysable CT and plasticized by glycerol exhibited reduced WU, enhanced antioxidant activity, and moderate growth-inhibitory activity against *E. coli* O157:H7, without significantly compromising the tensile properties. The enhanced water repellency was associated with the presence of hydrogen interactions between gelatin, tannin, and glycerol as evidenced by FTIR. Additional work is necessary to improve the flexibility and the processing ability of these films to meet similar requirements than the synthetic polymer counterpart usually applied in active packaging systems. Moreover, moisture uptake, water permeation studies, and thermal behavior of materials are performed in order to complete the characterization of the obtained materials. The potential application of gelatin films incorporated with harmless CT can introduce direct benefits to the food industry by improving safety and microbial quality to the packed product.

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