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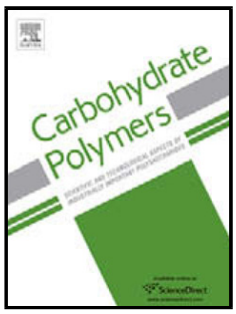

Title: Preparation and characterization of chitosan film incorporated with thinned young apple polyphenols as an active packaging material

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Highlights

1. Basic physical properties of chitosan film were increased by the addition of YAP

2. Water content, vapor permeability and mechanical properties were decreased by YAP

3. Antioxidant and antimicrobial properties of chitosan film were enhanced by YAP

4. The interactions between YAP and chitosan were likely to be non-covalent

5. Change of crystalline degree kept pace with that of thermal stability for YAP films

Preparation and characterization of chitosan film incorporated with thinned young apple polyphenols as an active packaging material

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Abstract

The objective of this study was to characterize the physical, mechanical and bioactive properties of chitosan film incorporated with thinned young apple polyphenols (YAP). The results indicated that the addition of YAP resulted in a significant increase in the thickness, density, swelling degree, solubility and opacity of chitosan film, but the water content, water vapor permeability and mechanical properties of the film were decreased. Besides, the antioxidant and antimicrobial properties of chitosan film were significantly enhanced by YAP. Both the NMR and FTIR spectra indicated the interactions between YAP and chitosan were likely to be non-covalent. Furthermore, the thermal stability of the film was decreased by YAP addition, suggested by DSC. Interestingly, the changing tendency of crystalline degree indicated by X-ray kept pace with that of thermal stability for YAP-chitosan films. Overall, YAP-chitosan film was shown a potential as a bioactive packaging material to extend food shelf-life.

Keywords: Chitosan film; Young apple polyphenols; Film characterization; Film bioactivities; Packaging material

1. Introduction

In recent years, biodegradable and biobased packaging materials have been obtained a widespread interest due to the limited natural resources, food safety and environmental problems caused by the use of petrochemical-based plastics. Some carbohydrate polymers, like starches, cellulose derivatives, chitosan and pectin that are biodegradable and edible substances, have been utilized as packaging materials targeting to extend food shelf-life (Espitia, Du, Avena-Bustillos, Soares, & Mchugh, 2014). Moreover, considerable researches have also focused on the addition of natural antimicrobial agents extracted from agricultural commodities and food product wastes into edible packaging materials (Atarés, Bonilla, & Chiralt, 2010).

Chitosan (the degree of deacetylation is 90% in this study), the most abundant biopolymer second to cellulose, is a low acetyl substituted form of chitin and a natural carbohydrate copolymer consisting of β-(1-4)-2-acetamido-D-glucose and β-(1-4)-2-amino-D-glucose units (Shahidi, Arachchi, & Jeon, 1999). It has been proved that chitosan is an excellent film forming material with a selective permeability to gasses and good mechanical properties that can be comparable to many medium-strength commercial polymers (Butler, Vergano, Testin, Bunn, & Wiles, 1996). As microbial spoilage is a major problem affecting food quality, it is promising that antioxidants are incorporated into packaging materials to delay the spoilage. Current researches concerning supplementing natural antioxidants to enhance the antioxidant functions of films include the incorporation of essential oils and the addition of phytochemicals due to their broad antimicrobial activities (Chen, Yue, &

Zhong, 2015). Essential oils have been reported to be successfully mixed into chitosan films by means of lamination, dispersion and emulsion (Prodpran, Benjakul, & Artharn, 2007). Films incorporated with cinnamon bark oil and soybean oil were shown a strong antimicrobial activity particularly against Gram-positive *L. monocytogenes* (Ma et al., 2016). The physic-mechanical properties of chitosan films containing carvacrol and grape seed extracts in different proportions showed a high potential to improve food preservation and shelf-life (Rubilar et al., 2013). Although polyphenol, a class of bioactive antioxidant, has been widely applied in food preservative field, the incorporation of it with chitosan film as a new-type and effective bio-preservative film needs to be further shed light on in terms of its structural characterization and bioactivities.

Apple, very popular and widely available, is a very significant source of polyphenols that show strong antioxidant activity (Vayndorf, Lee, & Liu, 2013). Fruit thinning is one of the most important techniques in apple growing for improving fruit quality and increasing yield (Link, 2000). In China, there are about 1.6 million tons of thinned young apples discarded in apple groves every year (Sun, Guo, Fu, Li, & Li, 2013). Thinned young apples actually are agricultural resource since the content of total polyphenols is approximately ten times higher than that in ripe apples. Furthermore, autotoxicity that plant inhibits the growth of its own kind is the major reason of the replant problem (Singh, Batish, & Kohli, 1999). Previous research suggested that replant disease of apple trees was attributed to the high concentrations of phlorizin in apple roots (Cesco et al., 2012). Thinned young apples are usually

abandoned on the ground of the groves, in which the high concentration of polyphenols maybe responsible for apple tree autotoxic behavior. Therefore, it is very important to make use of thinned young apples rather than discard them in the orchard.

Hence, the aim of this work is to prepare and characterize the chitosan film incorporated with thinned young apple polyphenols (YAP) and analyze the effect of YAP on the physical, mechanical and bioactive properties of the film to determine if the YAP-chitosan film has a potential as an active packaging material.

2. Material and methods

2.1 Materials

Chitosan with the degree of deacetylation of 90% and molecular weight of around 91000 Da was supplied by Shanghai Lanji Technology Development Co. Ltd. (China). All the chemicals in this study were of analytical grade.

2.2 Preparation and determination of YAP

YAP was extracted and separated according to our previously reported method (Sun, Guo, Fu, Li, & Li, 2013). The determination of the content of individual phenolic compound in YAP was conducted using a Thermo® Ultimate 3000 HPLC system (Thermo Electron Co. USA) and a Thermo® syncronis C18 column (250×4.6 mm I.D., 5 μm, USA). Elution with solvent A (30% acetonitrile and 70% methanol) and solvent B (1‰ trifluoroacetic acid and 5% methanol) in a step gradient way was carried out as follows: 0-3 min, 100% B at a flow rate of 0.95 mL/min; 3-19.05 min, 100-60% B

at a flow rate of 0.95 mL/min; 19.05-30.1 min, 60% B from a flow rate of 0.95 mL/min to a flow rate of 1 mL/min; 30.1-30.2 min, 60-100% B from a flow rate of 1 mL/min to a flow rate of 0.95 mL/min; 30.2-41 min, 100% B at a flow rate of 0.95 mL/min. During the run, the detection wavelength was 280 nm, and the injection volume was 20 µL.

2.3 Film preparation

Chitosan solution (2%, w/v) was prepared by dissolving into 1.0% (v/v) acetic acid aqueous solution with stirring of 800 rpm for 4h. 30% (w/w) glycerol, as plasticizer, was added into chitosan solution with stirring for 0.5h. Different concentrations of YAP (0.25%, 0.50%, 0.75%, and 1.0% (w/v)) were added into the polymer solutions. These solutions were vacuum degasified for 1 h to remove air bubbles. Finally, the respective YAP-chitosan solutions (70 ml) were cast over the petri dishes (diameter of 15 cm) for 48h at 25°C. The YAP-chitosan films were carefully peeled and stored in the desiccators containing $Mg(NO_3)_2$ saturated solutions (53% relative humidity) at 25°C for 48h for further experiments. The film solutions were freeze-dried into powder for antimicrobial assay, *in vitro* cytotoxicity assay, H^1 NMR and FTIR analysis.

2.4 Physical properties of film

2.4.1 Scanning electron microscopy (SEM)

TM3030 scanning electron microscope (Hitachi Co. Ltd., Kyoto, Japan) was used to observe the surface and cross-section morphology of the films with and without YAP. The films were imaged at a voltage of 15 kV with gold coating.

2.4.2 Thickness and density

The film thickness was measured using a digital micrometer (Mitutoyo Absolute, Tester Sangyo Co. Ltd., Tokyo, Japan). The film density was determined by the film weight and volume. The film volume was calculated according to the area and thickness of the film.

2.4.3 Solubility and swelling degree

The films were cut into 2cm×2cm pieces for the determination of solubility and swelling degree. The pieces were dried at 105°C to constant weight to obtain the initial dry mass (M_1). Then, they were placed in 100 ml beakers with 50 ml distilled water covered with plastic wraps and stored at 25°C for 24 h. Next, the films were dried superficially with filter papers and dried at 105°C to constant weight to obtain the final dry mass (M_2). Then, the solubility was calculated using the following equation:

$$\text{Film solubility} = \frac{M_1 - M_2}{M_1} \times 100\% \quad (1)$$

The films were put into 50ml beakers with 30ml distilled water for 24h at 25°C after weighing the films (M_1). The wet films were then dried superficially with filter papers, followed by weighing the wet films (M_2). The swelling degree was calculated using the following equation:

$$\text{Film swellng degree } = \frac{M_2 - M_1}{M_1} \times 100\% \quad (2)$$

2.4.4 Water vapor permeability (WVP) and water content

WVP was determined according to the method provided by Talja et al. (2008) with some modifications. The films were cut into pieces (6 cm×6 cm) before sealed onto a

special aluminum cup (internal diameter: 6 cm, external diameter: 9 cm, exposed area: 28.27 cm^2, depth: 1.3 cm) containing anhydrous calcium chloride and ensured that the gap between the film and anhydrous calcium chloride was less than 6mm. Then the cup was placed into a desiccator with distilled water at room temperature. The cup was weighed every 2 h using an analytical balance with a precision of 0.1 mg (Mettler-Toledo Instrument (Shanghai) Co. Ltd., China). The WVP was determined using the following equation:

$$\mathrm{WVP} = (md)/(At \triangle p) \quad (3)$$

where WVP is the water vapor permeability ($g \cdot m^{-1}s^{-1} Pa^{-1}$), *m* is the mass increase (g), *t* is the time of permeation (s), *A* is the exposed area, *d* is the film thickness (m^2) and *⊿p* is the saturated vapor pressure of water at the test temperature (25°C, pa).

The film pieces (2 cm×2 cm) were prepared and weighed (M_1) for water content according to Jimenez et al. (2012) with some modifications. The pieces were dried at 105°C to a constant mass (M_2). Then, the water content was determined using the following equation:

$$\text{Water content} = \frac{M_1 - M_2}{M_1} \times 100\% \quad (4)$$

2.4.5 Film color and opacity

The film color was determined using a Konica® colorimeter (CR-300, Japan). The white tile was used as standard during the color measurement. Lightness (L^*) and chromaticity parameters a^* (red-green) and b^* (yellow-blue) were used to characterize the film color in the Hunter Lab scale (CIE Lab scale). Total color difference (*⊿E)* was calculated using the following equation:

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{0.5} \quad (5)$$

The film opacity was determined according to the method of Park et al. (2004). The absorbance value was measured at 600 nm using a spectrophotometer (UV 2100, Unico Instruments Co. Ltd., Shanghai, China). Then, the opacity was calculated using the following equation:

$$O = Abs_{600}/L \quad (6)$$

where, O is the opacity, Abs_{600} is the absorbance value at 600 nm and L is the film thickness (mm).

2.5 Mechanical properties

The mechanical properties of the films were measured by use of Universal Testing Machine according to the method of Bangyekan et al. (2006), including tensile strength (σ) and percentage of elongation at break (E). The films were cut into 1.5cm×15cm strips for the determination of mechanical properties. Each film strip was fixed between the molds with an initial grip separation of 5 cm and the extension speed of 1 mm/s. The tensile strength (σ) and percentage of elongation at break (E) were calculated using the following equations:

$$\sigma = F_{max}/A \quad (7)$$

$$E = \frac{\Delta L}{L_0} \times 100\% \quad (8)$$

where F_{max} is the maximum load (N), A is the initial cross-sectional area (m^2), ΔL is the extension of film strips (m) and L_0 is the initial length (m).

2.6 Bioactivities assay

2.6.1 Antioxidant activity

The antioxidant activity of the films was evaluated by the scavenging free radical DPPH• following the method of Blois (1958). The DPPH• scavenging activity was calculated using the following equation:

$$\text{DPPH} \bullet \text{ scavenging activity} = \left[1 - \frac{A_i - A_j}{A_0}\right] \times 100\% \quad (9)$$

where, A_0 is the absorbance value of methanolic solution of DPPH•, A_i is the absorbance value of mixture of methanolic DPPH• and film solutions, and A_j is the absorbance value of mixture of methanol and film solutions.

2.6.2 Antimicrobial activity

Bacteria strains: *Escherichia coli* were obtained from China General Microbiology Culture Collection Center (Beijing, China). *Staphylococcus aureus* and *Listeria monocytogenes* were collected from the College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an, China.

Yeasts strains: *Saccharomyces cerevisiae, baker's yeast* and *tropical candida* were collected from the College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an, China.

Moulds strains: *Colletotrichum fructicola, Botryosphaerial dothidea* and *Alternaria tenuissima* were collected from the College of Plant Protection, Northwest A&F University, Xi'an, China.

For the culture of *E. coli, S. aureus* and *L. monocytogenes*, Luria-Bertani medium, Nutrient Agar medium and Trypticase Soy Broth (TSB) supplemented with yeast extract (TSBYE) medium were used, respectively. Each type of bacteria was cultured at 37°C and transferred at least 2 times prior to use. For the culture of yeast and

moulds strains, potato dextrose agar (PDA) medium were used. Yeast strains were cultured at 28°C and moulds strains were cultured at 25°C.

The antimicrobial activities against bacteria and yeasts were measured using a paper disk diffusion assay. 100 μL of test strain with 10^6 CFU/mL was spread uniformly on the surface of agar plate. A filter paper with a diameter of 8 mm was immersed in different concentrations of YAP-chitosan film solutions and then placed on each plate. The diameters (mm) of inhibition zones were then measured after incubation for 24 h.

The antimicrobial activity against moulds was measured by a mycelium growth rate method: the 5 mm diameter of mould cake was inoculated in a medium containing different concentrations of YAP-chitosan film. The growth diameters were measured after incubation for 144 h.

2.7 Characterization of YAP-chitosan film

2.7.1 FTIR analysis

The preliminary structures of the films with and without YAP were characterized by Fourier Transform Infrared (FTIR) spectrometry (EQUINX55, Brucher, Germany) through KBr module. The FTIR spectra were determined at resolution of 4 cm^{-1} with 16 scans in the range of 4000 to 670 cm^{-1}.

2.7.2 H^1 NMR analysis

The chitosan film, YAP-chitosan films and YAP were dissolved in D_2O (the concentration of polymer was 10 mg/mL) at room temperature. The H^1 NMR spectra of samples were recorded on a Bruker® AVANCE 600 spectrometer (Rheinstetten,

Germany) according to one previously reported method (Lee, Woo, Ahn, & Je, 2014)

2.7.3 X-ray diffraction analysis

The crystal structures of YAP-chitosan films were measured by a Rigaku® X-ray diffractometer (RINT2000, Tokyo, Japan) at a voltage of 40 kV and 100 mA. The scattered radiation was detected in the angular range 2θ=10-35° with a scanning speed of 5 °/min.

2.7.4 Thermal stability analysis

Differential scanning calorimetry (DSC) analysis was performed using a Q1000 DSC system (TA instruments, New Castle, USA). 10mg of the film pieces were sealed in a standard aluminum pan heated at a constant rate of 10°C/min from 0 to 350°C at the nitrogen atmosphere.

2.8 Statistical analysis

The results were expressed as mean values and standard deviation (SD). The experiment data were subjected to variance analysis and the Tukey's test using SPSS 18.0 at 5% significance level (p<0.05).

3. Results and discussions

3.1 Phenolic compositions and contents in YAP

The individual phenolic compositions in YAP were determined using HPLC method and summarized in Fig. 1. The content of total polyphenols in YAP was 72.72%, in which chlorogenic acid accounted the most for 39.34%, followed by phlorizin (18.56%). There was a small amount of procyanidin B2, epicatechin, caffeic

acid, rutin and quercetin-3-galactoside.

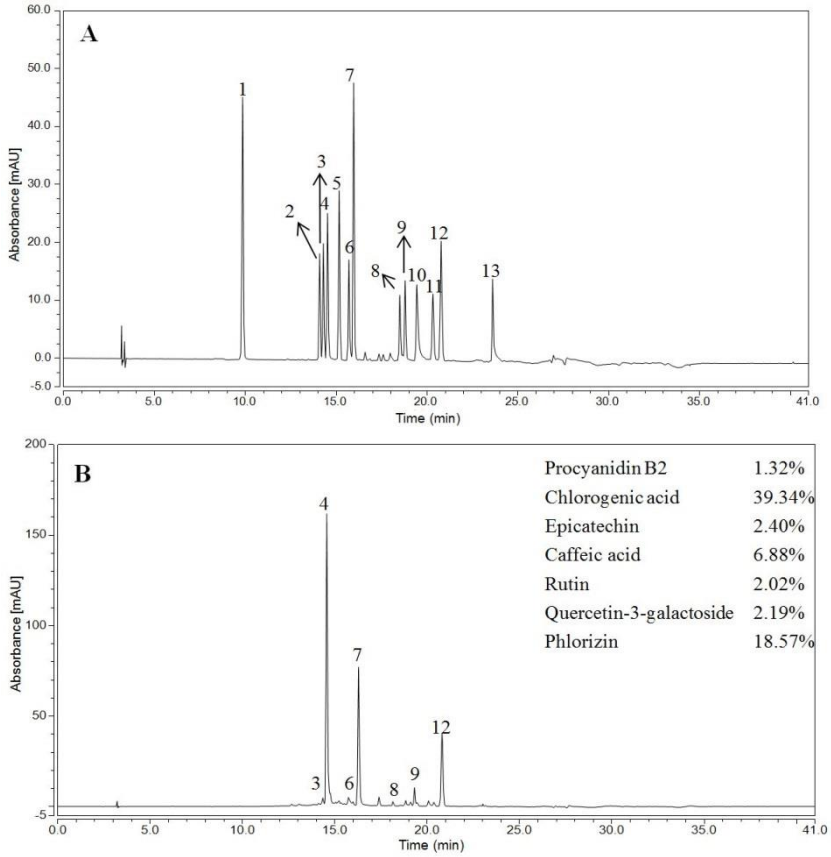

Fig. 1 HPLC chromatograms of 13 phenolic standards (**A**) and YAP (**B**). Individual peaks: 1. Gallic acid, 2. Catechin, 3. Procyanidin B2, 4. Chlorogenic acid, 5. 4-Hydroxyl benzoic acid, 6. Epicatechin, 7. Caffeic acid, 8. Rutin, 9. Quercetin-3-galactoside, 10. Ellagic acid, 11. Quercetin-3-rhamnoside, 12. Phlorizin, 13. Quercetin. The contents of individual phenolic compounds in YAP were summarized in the table inset in (**B**).

3.2 Physical properties of YAP-chitosan film

The chitosan films incorporated with different concentrations of YAP were photoed and presented in Fig. 2A, where the colour appearance of the films became darker with the YAP concentration increasing. The SEM images showed that there were no differences in the external appearance between the control and YAP-chitosan films, all of which were very smooth and uniform, indicating that polyphenols were well-distributed into the chitosan matrix (Fig. 2B and C).

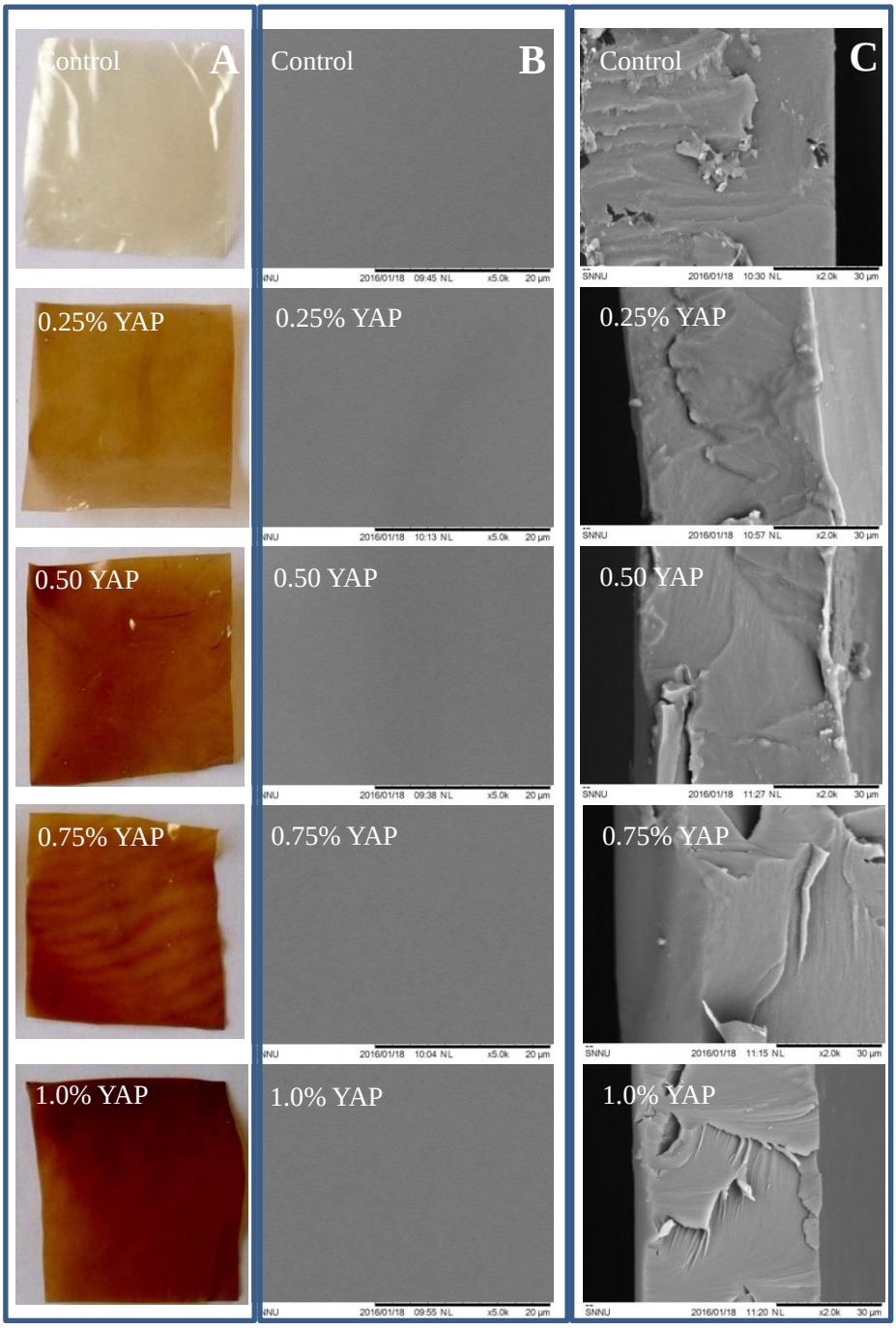

Fig. 2 The photographs (**A**) and SEM micrographs of surface (**B**) and cross-section (**C**) of chitosan films incorporated with different concentrations of YAP.

The effects of YAP on the thickness, density, water content, swelling degree and solubility of films are shown in Table 1.

Table 1 Physical properties of YAP-chitosan films.

Film samples	Thickness (mm)	Density ($g \cdot cm^{-3}$)	Swelling degree (%)	Solubility (%)	Water content (%)
Control	0.070 ± 0.002^{a}	1.129 ± 0.045^{a}	263.76 ± 44.07^{e}	16.68 ± 0.47^{a}	29.58 ± 1.45^{a}
0.25%YAP	0.079 ± 0.003^{b}	1.204 ± 0.013^{b}	353.92 ± 11.21^{d}	19.93 ± 0.76^{b}	25.90 ± 0.78^{b}
0.50%YAP	0.092 ± 0.003^{c}	1.264 ± 0.004^{c}	555.93 ± 30.79^{c}	25.36 ± 0.52^{c}	23.37 ± 1.44^{c}
0.75%YAP	0.103 ± 0.004^{d}	1.323 ± 0.011^{d}	746.54 ± 16.91^{b}	32.99 ± 1.27^{d}	20.87 ± 0.60^{d}
1.0%YAP	0.112 ± 0.002^{e}	1.382 ± 0.007^{e}	866.79 ± 10.17^{a}	40.56 ± 0.93^{e}	16.94 ± 2.03^{e}

Values are expressed as mean ± standard deviation. Different letters in the same column indicate significant differences ($p<0.05$).

The thickness and density of films increased significantly ($p<0.05$) with the YAP concentration increasing, in agreement with one previous study in which chitosan film was incorporated with tea polyphenols (Peng, Wu, & Li, 2013). With the increase in polyphenols concentration, the interactions between polyphenols and chitosan (including hydrogen bonding and hydrophobic force) increased, causing tighter binding of polyphenols and chitosan (Zhang, Yang, Tang, Hu, & Zou, 2008). By this way, polyphenols might act as a bridge, binding with more than one chitosan molecules due to the existence of polyhydroxyl groups in the molecular structures; therefore, the distance between chitosan molecules became shorter, inducing the film structure more compact and thus increased thickness and density. Swelling degree and water solubility are two important characters of a film, affecting its water resistance property. The control films showed the lowest swelling degree and solubility. A significant ($p<0.05$) increase in water solubility and swelling degree were observed for the YAP-chitosan films. The high values of swelling degree and water solubility of the YAP-chitosan films may be attributed to the hydrophilic groups of polyphenols that can easily interact with water molecules (Mathew, Brahmakumar, & Abraham,

2006). The addition of YAP elevated the capacity of the matrix to bind with water and thus improved its hydrophily.

One of the main functions of the preservative films is separating foods from surrounding atmosphere vapor to prevent or retard food deterioration. WVP is an important parameter to evaluate the permeation of water vapor at a given temperature. To keep foods fresh, the WVP value should be maintained as low as possible. The results in this study showed that YAP had a significant decreasing effect (p<0.05) on the WVP of chitosan films (Fig. 3A). The WVP of films was decreased from 10.68×10^{-11} to 7.50×10^{-11} $g\cdot m^{-1}s^{-1}Pa^{-1}$ as the concentration of YAP increased from 0 to 1.0%, suggesting that the addition of YAP could enhance the water barrier properties of the film. The increase in the water barrier properties may be attributed to the increased thickness and density of chitosan film induced by YAP (Table 1), because of which, the water vapor molecules are more easily blocked outside (or it takes a longer time for water vapor molecules to permeate the film).

Moreover, the control films possessed the highest water content. After the addition of 0.75% YAP and 1.0% YAP, the water content of YAP-chitosan films decreased by 19.45% and 42.73%, respectively. The declining tendency for water content of chitosan films after incorporation of YAP is in accordance with one previous observation, in which addition of tea polyphenols also caused a decrease in this parameter (Peng, Wu, & Li, 2013). Polyphenols may establish the interactions with chitosan molecules through the potential hydrogen bonding, which may limit the interactions between hydrophilic groups of chitosan and water molecules due to the

competitive binding effect (Siripatrawan, & Harte, 2010; Sascha, Rawel, & Jürgen, 2004). Therefore, water molecules that bound with chitosan become less, resulting in the lower water content.

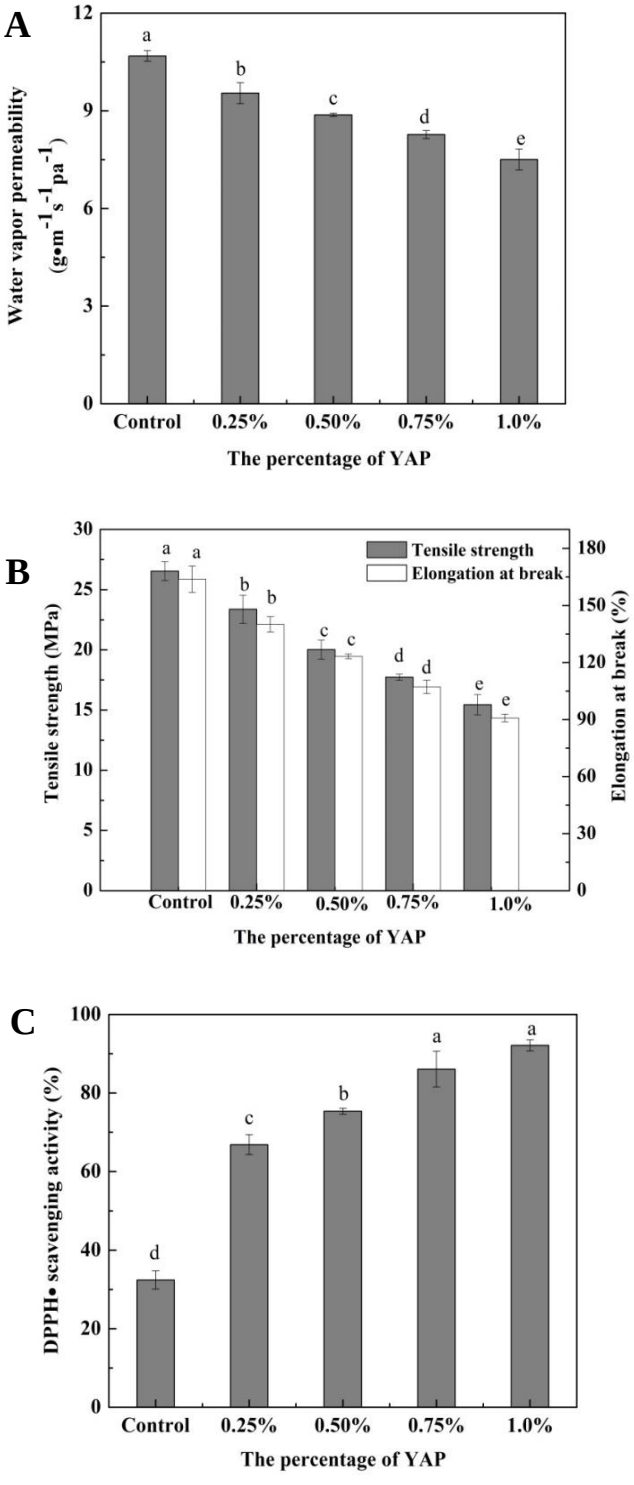

Fig. 3 The effects of YAP on the water vapor permeability (**A**), mechanical properties (**B**) and antioxidant activity (**C**) of films. Different letters indicate significant differences ($p < 0.05$).

Color and opacity are two important parameters for film appearance that affects consumer acceptance degree. The values of L^*, a^*, b^*, ΔE and opacity are shown in Table 2. All the parameters were observed to be affected by YAP. The control films had a slightly yellow appearance with higher transparency compared to the other groups. After the addition of YAP, the opacity and a^* values were significantly ($p<0.05$) increased, while the L^* value decreased, indicating a tendency towards redness and darkness. This may help to protect the packaged foods from visible and ultraviolet light that lead to nutrient losses, discoloration and off-flavour (Rubilar et al., 2013). It should be noted that with the increase of YAP concentration, the films became darker, redder, more yellow and lower transparency, which may also be attributed to the original color of YAP.

Table 2 Color and opacity of YAP-chitosan films.

Film samples	L^*	a^*	b^*	ΔE	Opacity ($A \cdot mm^{-1}$)
Control	85.61±0.99^a	-0.41±0.05^e	11.00±0.37^d	16.36±0.55^d	0.71±0.15^e
0.25%YAP	64.90±2.20^b	10.84±1.61^d	33.17±1.42^c	47.48±2.85^c	1.69±0.04^d
0.50%YAP	56.19±1.12^c	20.40±1.71^c	44.63±3.71^b	63.72±3.48^b	2.59±0.25^c
0.75%YAP	49.35±0.94^d	28.12±1.84^b	54.20±2.84^a	77.82±3.36^a	3.74±0.13^b
1.0%YAP	38.15±1.02^e	34.13±1.54^a	43.67±6.52^b	81.18±4.59^a	4.25±0.24^a

Values are expressed as mean ± standard deviation. Different letters in the same column indicate significant differences ($p<0.05$).

3.3 Mechanical properties of YAP-chitosan film

The effects of different concentrations of YAP on the mechanical properties of chitosan film are presented in Fig. 3B. The presence of YAP caused significant

(p<0.05) differences in the tensile strength and elongation at break (p<0.05). Specifically, the addition of YAP with the concentration from 0.25% to 1.0% decreased the tensile strength from 23.37 MPa to 16.10 MPa and decreased the elongation at break from 140.09% to 93.13%. Tensile strength represents the maximum strength that a film can withstand, and elongation is the measurement of the ability of a film to stretch. Chitosan composition, film network microstructure and intermolecular forces play an important role in the mechanical properties of chitosan film. Besides, the mechanical property also strongly depends on the crystallites of film. The decrease of mechanical properties may result from the decrease in crystalline structure in the chitosan matrix. It has been reported that the incorporation of polyphenols into chitosan film may interrupt the ordered crystalline structure formation in the chitosan matrix, weakening the intermolecular hydrogen bonding, hindering the polymer-polymer chain interactions and providing the flexible domains within the films, and thus resulted in the decreased mechanical properties (Ahmed, Mulla, & Arfat, 2016; Ying, Creber, Peppley, & Bui, 2003).

3.4 Bioactivities of YAP-chitosan film

3.4.1 DPPH• scavenging activity

Fig. 3C showed the DPPH• scavenging activity of the control and YAP-chitosan films. The control films were shown a low DPPH• scavenging activity, which was similar to a previous study (Yen, Yang, & Mau, 2008). The DPPH• scavenging activity significantly (p<0.05) increased with the addition of YAP. The antioxidant

activity of 1.0% YAP-chitosan films increased by nearly 3 folds compared to the control films.

3.4.2 Antimicrobial activity

The antimicrobial activity of YAP-chitosan films was carried out in the disc diffusion method by use of three bacteria *(Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*), three moulds (*Colletotrichum fructicola*, *Botryosphaerial dothidea* and *Alternaria tenuissima*) and three yeasts (*Saccharomyces cerevisiae*, *Baker's yeast* and *Tropical candida*). The inhibitory effects of YAP-chitosan films on the growth of the three kinds of microorganisms are shown in Fig. 4 and Table S1. As shown, YAP-chitosan films had the concentration-dependent antimicrobial activities against bacteria and moulds (Fig. 4A-F), but no activity against yeasts (Fig. 4G-I). According to the inhibition and growth zone diameters (Table S1), the orders of the antimicrobial activities of YAP-chitosan film against bacteria and moulds were *E. coli*< *S. aureus* < *L. monocytogenes* and *C. fructicola*< *B. dothidea*< *A. Tenuissima*, respectively. Previous studies have demonstrated that chitosan could inhibit the growth of a wide variety of fungi and bacteria, and is more effective against Gram-positive than Gram-negative bacteria (Jeon, Park, & Kim, 2001; Sayari et al., 2016). The antibacterial activity of chitosan may be due to the free amino groups. The free amino groups may bind to cell surface, disturbing the cell membrane, and thus cause death of the cell by inducing leakage of intracellular components (Chung & Chen, 2008). It has been reported that bioactive phenolic compounds can exert the physiological changes of microbial cell membrane, and

eventually result in bacteria death (Kabir, Katayama, Tanji, & Nakamura, 2014). Therefore, the antimicrobial activities of YAP-chitosan films may be the outcome of both functions of chitosan and polyphenols. Because the main microorganisms that cause foods spoilage are bacteria and moulds (Elgayyar, Draughon, Golden, & Mount, 2001), although YAP-chitosan films hardly showed inhibitory activity against yeast (Fig.4G-I), the positive antibacterial and antimould properties indicate that YAP-chitosan films may be applied as antimicrobial packaging materials in improving food shelf-life, if the problem of permeation of polyphenols from the film materials into foods can be solved as the commercial packaging materials.

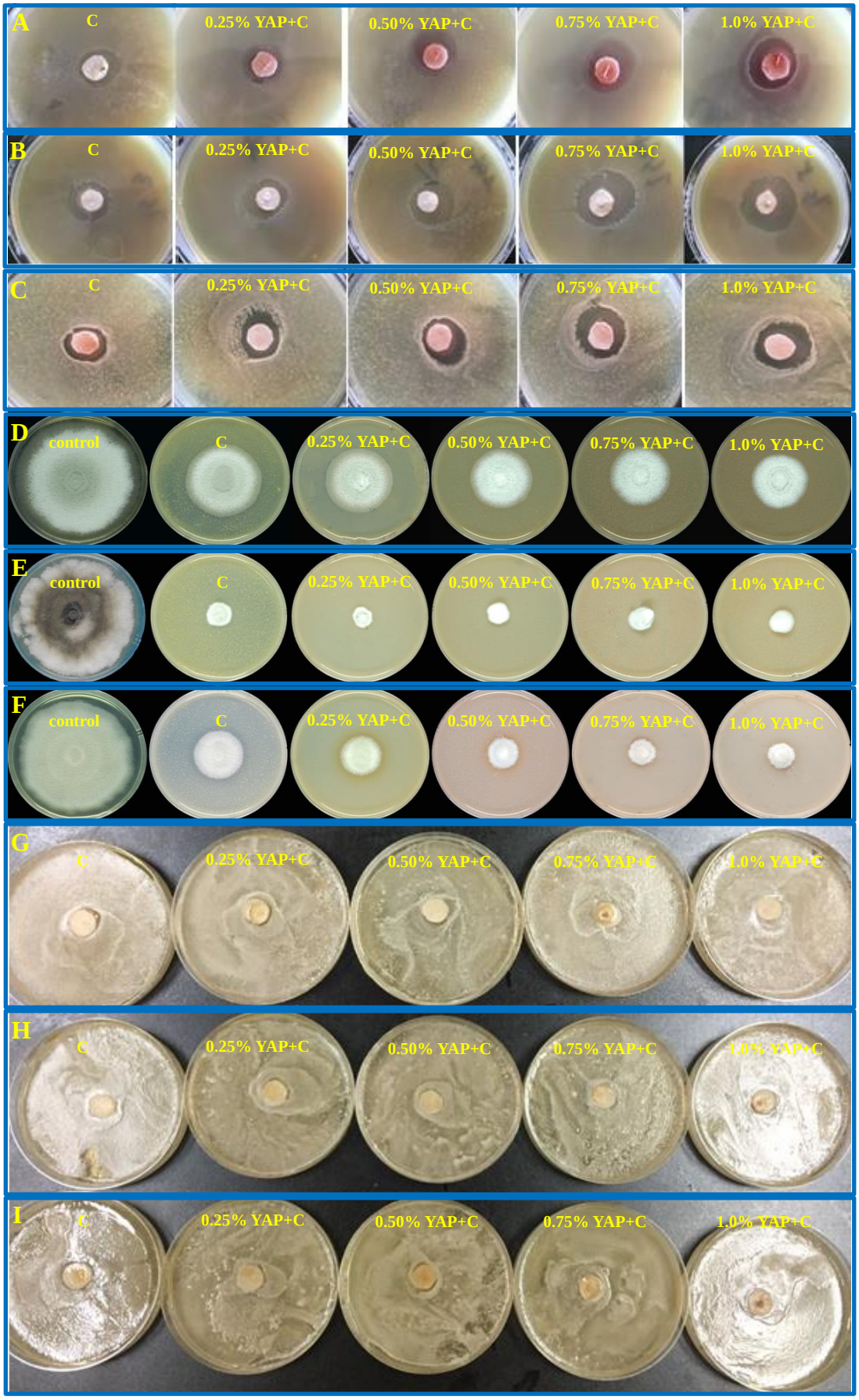

Fig. 4 Inhibitory effects of YAP-chitosan films on the growth of *Escherichia coli* (**A**), *Listeria monocytogenes* (**B**), *Staphylococcus aureus* (**C**), *Colletotrichum fructicola* (**D**), *Alternaria tenuissima* (**E**), *Botryosphaerial dothidea* (**F**), *Saccharomyces cerevisiae* (**G**), *Baker's yeast* (**H**) and *Tropical candida* (**I**). C represents chitosan.

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3.5 Characterization of YAP-chitosan film

3.5.1 FTIR analysis

FTIR spectroscopy was performed to investigate the intermolecular interaction between chitosan and YAP that was related to the physical and mechanical characters of films, and the spectra are shown in Fig. 5A. The broad peaks between 3500 and 3000 cm^{-1} were the stretching vibration of free hydroxyl group and it always overlaps with the stretching of N-H bonds in amino group. The peak at 2941 cm^{-1} and 2885 cm^{-1} were attributed to the vibration absorbance of C-H. Besides, the peaks at 1650 cm^{-1}, 1545 cm^{-1}, 1389 cm^{-1} and 1143 cm^{-1} corresponded to C=O, N-H bending vibration, C-N stretching and C-O-C band stretching, respectively (Bourtoom & Chinnan, 2008; Shahzadi et al., 2016; Wang, Yan, Men, Jin, & Jiang, 2013). It should be noted that there were no obvious reflectance peaks for YAP (green line in Fig. 5A) at the wavelengths where chitosan film showed characteristic peaks, suggesting that the effects of FTIR spectrum of YAP itself on that of chitosan could be excluded.

After addition of YAP into chitosan film, no additional peaks and no significant wavelength shift were observed, indicating that no covalent bonds between YAP and chitosan were detected (Lee, Woo, Ahn, & Je, 2014); therefore, the interaction between the two compounds was more likely to be physical response. It was observed that the broad peak at 3259 cm^{-1} became more flattened with the YAP concentration

increasing, indicating the decreased stretching of free –OH and/or –NH due to the binding interactions between polyphenols and chitosan. It has been reported that the hydrogen bonds between –OH groups of polyphenols with –OH or –NH groups of chitosan contribute to the interactions between the two compounds (Peng, Wu, & Li, 2013). The hydrogen bonds affect the electron distributions in –OH and –NH, inducing the weakened internal stretching of bonds between oxygen and hydrogen atoms and that between nitrogen and hydrogen atoms in the corresponding groups (Nakamoto, Margoshes, & Rundle, 1955). Similarly, due to the interactions between polyphenols and chitosan, including hydrogen bonding and hydrophobic force (Peng, Wu, & Li, 2013), the changes of internal bonds between atoms occurred in certain functional groups, which induced the peaks located at 2941 cm^{-1}, 2885 cm^{-1}, 1650 cm^{-1} becoming less discernible.

3.5.2 NMR analysis

The chitosan films in the absence and presence of YAP were further characterized by H^1 NMR spectroscopy (Fig. 5B). It is suggested that the chitosan film control had a peak at 1.975 ppm corresponding to the methyl protons of the acetylated glucosamine residues. Peaks at 3.001, 3.448 3.686 ppm indicated the C2 proton of glucosamine residue, C3 and C4 protons of the pyranose ring (Lee, Woo, Ahn, & Je, 2014). After addition of YAP, the typical H^1 NMR peak of chitosan was observed as well and no new peak appeared, indicating that there were no covalent bonds between YAP and chitosan. This conforms to the results of FTIR that the two compounds might interact through non-covalent forces. It has been reported that the covalent

interactions between polyphenols and polysaccharides usually induce the changes of intermolecular and/or intramolecular bonds of polysaccharides, and this can be reflected by the changes of NMR spectra of macromolecules, such as peak intensity and number (Lee, Woo, Aha, & Je, 2014). However, the non-covalent interactions (such as physical binding) between the two compounds do not alter the internal chemical bonds of polysaccharides (Le Bourvellec, Bouchet, Renard, 2005), which normally results in the characteristic chemical shifts of carbohydrates (around 3.0-5.3 ppm) unchanged because of the relatively weaker interactions (Prakash, Iturmendi, Grelard, Moine, & Dufourc, 2016; Khoo, Abas, Abdullah, Tohit, & Hamid, 2014). Besides, there were some characteristic peaks of polyphenols at 6.0-7.5 ppm (Prakash, Iturmendi, Grelard, Moine, & Dufourc, 2016) in the spectrum of chitosan film with YAP, indicating that YAP bound with the film successfully. The incorporation of YAP into chitosan film caused the increase in the antioxidant, antimicrobial and cytotoxicity activities of the film due to the existence of phenolic compounds (Kabir, Katayama, Tanji, & Nakamura, 2014).

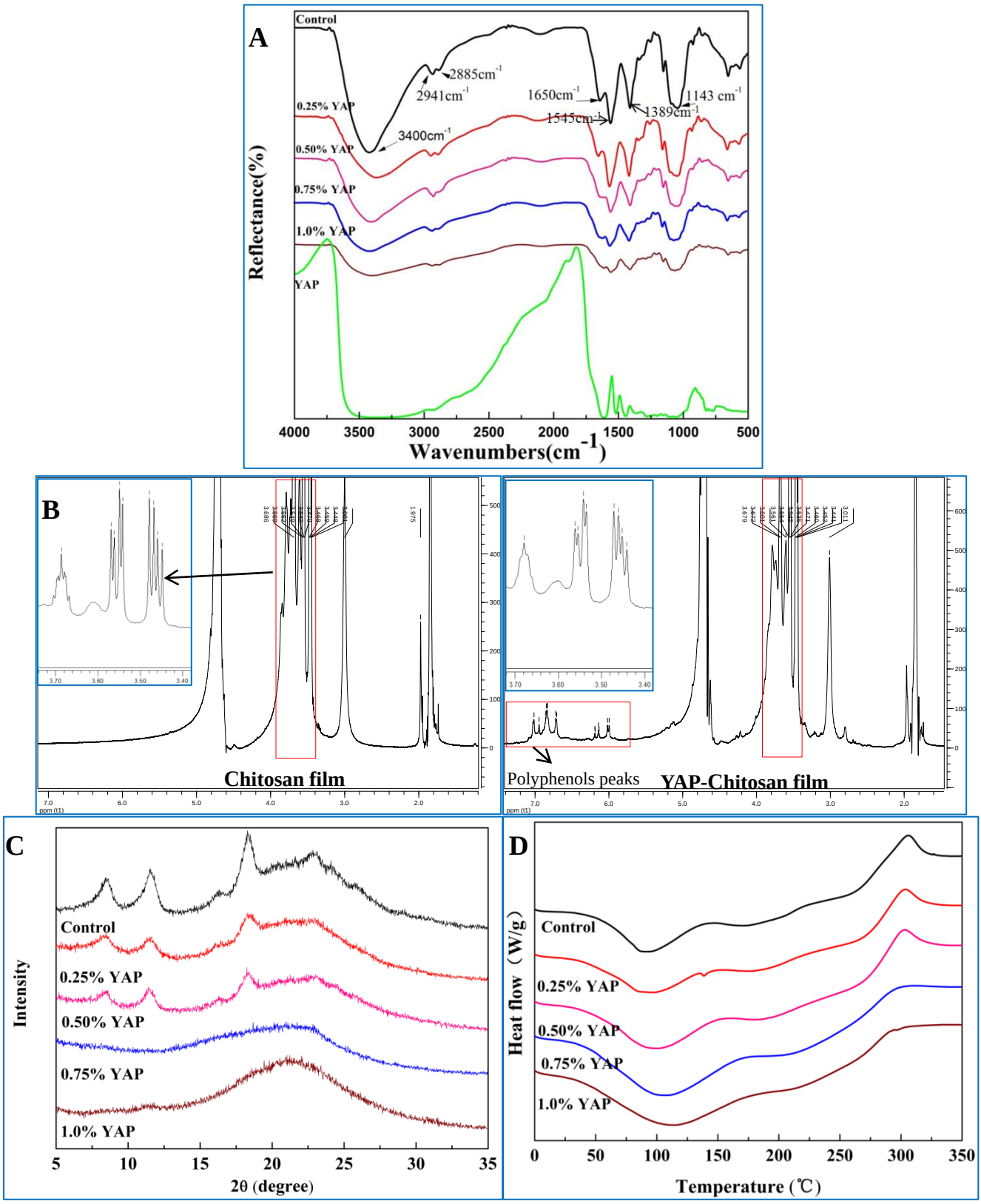

Fig. 5 Characterization of YAP-chitosan films: FTIR spectra (**A**), H^1 NMR spectra (**B**), X-ray diffraction patterns (**C**) and DSC thermograms (**D**).

3.5.3 X-ray diffraction analysis

The X-ray spectra of film samples are displayed in Fig. 5C. Previous studies have proved that there was a semicrystallinity character in chitosan films. The X-ray diffraction analysis was carried out to additionally reveal the effect of YAP on the crystal structures of chitosan films. There are three forms of chitosan: noncrystalline, hydrated crystalline and anhydrous crystalline (Shahzadi et al., 2016). In the control films, the chitosan was in a crystalline state with four main diffraction peaks. Specifically, there was a diffraction peak at around 8.44° observed in the control films, in accordance with one previous study (Ziani, Oses, Coma, & Mate, 2008). The diffraction peak at around 11.54° was attributed to the anhydrous crystalline, and the diffraction peak at around 18.34° was the hydrated crystal character (Mathew, Brahmakumar, & Abraham, 2006). Additionally, the diffraction peak at 22.8° observed was a typical fingerprint for common chitosan films (Srinivasa, Ramesh, Kumar, & Tharanathan, 2004). After YAP was added to the chitosan films, significant ($p< 0.05$) changes were observed. When the YAP concentration increased to 0.50%, the diffraction peaks still existed but became more flattened and less discernible, suggesting a lower crystallinity in the film. As the YAP concentration increased to 0.75%, the diffraction peaks disappeared and became more mobile. Therefore, YAP induced a conversion of chitosan films from crystalline to an amorphous structure. As discussed, the mechanical properties of a film strongly depend on the crystallites in its film structure. In the YAP-chitosan films, the interactions between YAP and chitosan may hinder the inter- and intramolecular hydrogen bonds formation in chitosan itself

because of the competitive effect of hydrogen bonds between chitosan and YAP (this could be shown from FTIR results as well), resulting in a low crystallinity. Thus, the amorphous structures in chitosan films that appeared after the addition of YAP leaded to lower mechanical properties (Fig. 3B) of films in this study as described above.

Table 3 DSC thermal parameters (peak temperature (T_m) and peak area (ΔH)) of YAP-chitosan films.

Film samples	Peak temperature (°C)	Peak area ($J \cdot g^{-1}$)
Chitosan	305.13 ± 0.86^{a}	158.78 ± 0.89^{a}
0.25%YAP-chitosan	303.41 ± 0.20^{b}	132.75 ± 0.57^{b}
0.50%YAP-chitosan	300.99 ± 0.70^{c}	119.60 ± 0.17^{c}
0.75%YAP-chitosan	297.05 ± 0.61^{d}	101.5 ± 1.23^{d}
1.0%YAP-chitosan	296.65 ± 0.57^{d}	99.41 ± 0.84^{d}

Values are expressed as mean ± standard deviation. Different letters in the same column indicate significant differences ($p<0.05$).

3.5.4 DSC analysis

DSC analysis was used to measure the enthalpy change (ΔH) and the peak melting temperature (T_m) of the films. DSC analysis of the YAP-chitosan films are shown in Fig. 5D and Table 3. The thermograms showed that peak 1, an endothermic peak at around 100 °C related to water composition, and that peak 2, a major degradation exothermic peak at around 300°C was associated with the depolymerization and pyrolytic decomposition of the polysaccharide backbone (Martins, Cerqueira, & Vicente, 2012). The addition of YAP had negative influence on thermal stability of chitosan films, which can be demonstrated from peak 2. The peak temperature and peak area decreased with the increase of YAP concentration, indicating a declining tendency of thermal stability. The decrease in thermal stability may be attributed to

chemical etching caused by bond disrupt, chain scission or chemical degradation of macromolecules (Pankaj et al., 2014). Also, the thermal stability of macromolecules is related to the crystalline structure in them, meaning that higher crystal degree corresponds to higher thermal stability, because more energy (heat) is required to break the higher crystalline structure. Our results indicated the changing tendency of crystalline degree kept pace with that of thermal stability of YAP-chitosan film with the YAP concentration increasing, supporting this statement. Additionally, it was also found in the control and YAP-chitosan films that the decreased thermal stability was associated with the decreased mechanical properties (Fig. 3B) of the film samples, consisting with the previous findings for VE-chitosan films. VE addition decreased the thermal stability of films, which was also supportively reflected by the reduced mechanical properties of films (Park & Zhao, 2004).

4. Conclusion

Thinned young apple is a rich source of antioxidant compounds, in which chlorogenic acid and phlorizin accounted for a large proportion of total polyphenols. Then, the current study discussed the effects of YAP as an antioxidant on chitosan film properties. With the addition of YAP, the thickness, density, swelling degree, solubility and opacity of chitosan film were significantly increased, but the water content and vapor permeability were decreased, suggesting that water barrier property of the film was enhanced. Besides, the YAP-chitosan films were shown antimicrobial effects on both bacteria and moulds, but no effect on yeast. Both the NMR and FTIR

spectra of YAP-chitosan films suggested that the interactions between YAP and chitosan were likely to be non-covalent. Interestingly, the changing tendency of crystalline degree kept pace with that of thermal stability for all the tested chitosan films. Conclusively, the modified colour, increased bioactivities and enhanced water barrier properties of chitosan film, as well as the slight changes of mechanical properties at low concentration of polyphenols indicate that chitosan film incorporated with YAP can be a promising alternative to synthetic materials, potentially contributing to food shelf-life extension.

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References

Ahmed, J., Mulla, M. Z., & Arfat, Y. A. (2016). Thermo-mechanical, structural characterization and antibacterial performance of solvent casted polylactide/cinnamon oil composite films. *Food Control*, *69*, 196-204.

Atarés, L., Bonilla, J., & Chiralt, A. (2010). Characterization of sodium caseinate-based edible films incorporated with cinnamon or ginger essential oils. *Journal of Food Engineering, 100*, 678-687.

Bangyekan, C., Aht-Ong, D., & Srikulkit, K. (2006). Preparation and properties evaluation of chitosan-coated cassava starch films. *Carbohydrate Polymers*,

63, 61-71.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature, 181*, 1199-1200.

Bourtoom, T., & Chinnan, M. S. (2008). Preparation and properties of rice starch–chitosan blend biodegradable film. *LWT - Food Science and Technology, 41*, 1633–1641.

Butler, B. L., Vergano, P. J., Testin, R. F., Bunn, J. M., & Wiles, J. L. (1996). Mechanical and barrier properties of edible chitosan films as affected by composition and storage. *Journal of Food Science, 61*, 953-956.

Cesco, S., Mimmo, T., Tonon, G., Tomasi, N., Pinton, R., Terzano, R., Neumann, G., Weisskopf, L., Renella, G., Landi, L., & Nannipieri, P. (2012). Plant-borne flavonoids released into the rhizosphere: impact on soil bio-activities related to plant nutrition. A review. *Biology and Fertility of Soils, 48*, 123-149.

Chen, H., Yue, Z., & Zhong, Q. (2015). Physical and antimicrobial properties of spray-dried zein–casein nanocapsules with co-encapsulated eugenol and thymol. *Journal of Food Engineering, 144*, 93-102.

Chung, Y. C., & Chen, C. Y. (2008). Antibacterial characteristics and activity of acid-soluble chitosan. *Bioresource Technology, 99*, 2806-2814.

Elgayyar, M., Draughon, F. A., Golden, D. A., & Mount, J. R. (2001). Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. *Journal of Food Protection, 64*, 1019-1024.

Espitia, P. J. P., Du, W. X., Avena-Bustillos, R. D. J., Soares, N. D. F. F., & Mchugh, T.

H. (2014). Edible films from pectin: Physical-mechanical and antimicrobial properties - A review. *Food Hydrocolloids, 35*, 287-296.

Jeon, Y. J., Park, P. J., & Kim, S. K. (2001). Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydrate Polymers, 44*, 71-76.

Jimenez, A., Fabra, M. J., Talens, P., & Chiralt, A. (2012). Effect of re-crystallization on tensile, optical and water vapour barrier properties of corn starch films containing fatty acids. *Food Hydrocolloids, 26*, 302-310.

Kabir, F., Katayama, S., Tanji, N., & Nakamura, S. (2014). Antimicrobial effects of chlorogenic acid and related compounds. *Journal of the Korean Society for Applied Biological Chemistry, 57*, 359-365.

Khoo, L. T., Abas, F., Abdullah, J. O., Tohit, E. R. M., & Hamid, M. (2014). Anticogulant activity of polyphenolic-polysaccharides isolated from Melastoma malabathricum L. *Evidence-Based Complementary and Alternatifve Medicine*, Article ID 614273.

Le Bourvellec, C., Bouchet, B., & Renard, C. M. G. C. (2005). Non-covalent interaciton between procyanidins and apple cell wall material. Part III: Studty on model polysaccharides. *Biochimica et Biophysica Acta-General Subject, 1725*, 10-18.

Lee, D. S., Woo, J. Y., Ahn, C. B., & Je, J. Y. (2014). Chitosan-hydroxycinnamic acid conjugates: Preparation, antioxiant and antimicrobial activity. *Food Chemistry, 148*, 97-104.

Link, H. (2000). Significance of flower and fruit thinning on fruit quality. *Plant Growth Regulation, 31*, 17-26.

Ma, Q. M., Zhang, Y., Critzer, F., Davidson, P. M., Zivanovic, S., & Zhong, Q. X. (2016). Physical, mechanical, and antimicrobial properties of chitosan films with microemulsions of cinnamon bark oil and soybean oil. *Food Hydrocolloids, 52*, 533-542.

Martins, J. T., Cerqueira, M. A., & Vicente, A. A. (2012). Influence of α-tocopherol on physicochemical properties of chitosan-based films. *Food Hydrocolloids, 27*, 220-227.

Mathew, S., Brahmakumar, M., & Abraham, T. (2006). Microstructural imaging and characterization of the mechanical, chemical, thermal, and swelling properties of starch-chitosan blend films. *Biopolymers, 82*, 176-187.

Nakamoto, K., Margoshes, M., & Rundle, R. E. (1955). Streching frequencies as a function of distances in hydrogen bonds. *Journal of the Ameirican Chemical Society, 77*, 6480-6486.

Pankaj, S. K., Bueno-Ferrer, C., Misra, N. N., O'Neill, L., Tiwari, B. K., Bourke, P., & Cullen, P. J. (2014). Physicochemical characterization of plasma-treated sodium caseinate film. *Food Research International, 66*, 438-444.

Park, P. J., Je, J. Y., & Kim, S. K. (2004). Free radical scavenging activities of differently deacetylated chitosans using an ESR spectrometer. *Carbohydrate Polymers, 55*, 17-22.

Park, S., & Zhao,Y. Y. (2004). Incorporation of a high concentration of mineral or

vitamin into chitosan-based films. *Journal of Agricultural & Food Chemistry, 52*, 1933-1939.

Peng, Y., Wu, Y., & Li, Y. Y. (2013). Development of tea extracts and chitosan composite films for active packaging materials. *International Journal of Biological Macromolecules, 59*, 282-289.

Prakash, S., Iturmendi, N., Grelard, A., Moine, V., & Dufourc, E. (2016). Quantitative analysis of Bordeaux red wine precipitates by solid-state NMR: Role of tartrates and polyphenols. *Food Chemistry, 199*, 229-237.

Prodpran, T., Benjakul, S., & Artharn, A. (2007). Properties and microstructure of protein-based film from round scad (Decapterus maruadsi) muscle as affected by palm oil and chitosan incorporation. *International Journal of Biological Macromolecules, 41*, 605-614.

Rohn, S., Rawel, H. M., & Kroll, J. (2004). Antioxidant activity of protein-bound quercetin. *Journal of Agricultural & Food Chemistry, 52*, 4725-4729.

Rubilar, J. F., Rui, M. S. C., Silva, H. D., Vicente, A. A., Khmelinskii, I., & Vieira, M. C. (2013). Physico-mechanical properties of chitosan films with carvacrol and grape seed extract. *Journal of Food Engineering, 115*, 466-474.

Sayari, N., Sila, A., Abdelmalek, B. E., Abdallah, R. B., Ellouzchaabouni, S., Bougatef, A., & Balti, R. (2016). Chitin and chitosan from the Norway lobster by-products: Antimicrobial and anti-proliferative activities. *International Journal of Biological Macromolecules, 87*,163-171.

Shahidi, F., Arachchi, J. K. V., & Jeon, Y. J. (1999). Food applications of chitin and

chitosans. *Trends in Food Science & Technology, 10*, 37-51.

Shahzadi, K., Wu, L., Ge, X., Zhao, F., Li, H., Pang, S., Jiang, Y., Guan, J., & Mu, X. (2016). Preparation and characterization of bio-based hybrid film containing chitosan and silver nanowires. *Carbohydrate Polymers, 137*, 225-232.

Singh, H. P., Batish, D. R., & Kohli, R. K. (1999). Autotoxicity: concept, organisms, and ecological significance. *Critical Reviews in Plant Sciences, 18*, 757-772.

Siripatrawan, U., & Harte, B. (2010). Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. *Food Hydrocolloids, 24*, 248–252.

Srinivasa, P. C., Ramesh, M. N., Kumar, K. R., & Tharanathan, R. N. (2004). Properties of chitosan films prepared under different drying conditions. *Journal of Food Engineering, 63*, 79-85.

Sun, L. J., Guo, Y. R., Fu, C., Li, J. J., & Li, Z. (2013). Simultaneous separation and purification of total polyphenols, chlorogenic acid and phlorizin from thinned young apples. *Food Chemistry, 136*, 1022-1029.

Talja, R. A., Helén, H., Roos, Y. H., & Jouppila, K. (2008). Effect of type and content of binary polyol mixtures on physical and mechanical properties of starch-based edible films. *Carbohydrate Polymers, 71*, 269–276.

Vayndorf, E. M., Lee, S. S., & Liu, R. H. (2013). Whole apple extracts increase lifespan, healthspan and resistance to stress in Caenorhabditis elegans. *Journal of Functional Foods, 5*, 1235-1243.

Wan, Y., Creber, K. A. M., Peppley, B., & Bui, V. T. (2003). Ionic conductivity of

chitosan membranes. *Polymer, 44*, 1057-1065.

Wang, L., Dong, Y., Men, H. T., Tong, J., & Zhou, J. (2013). Preparation and characterization of active films based on chitosan incorporated tea polyphenols. *Food Hydrocolloids, 32*, 35-41.

Yen, M. T., Yang, J. H., & Mau, J. L. (2008). Antioxidant properties of chitosan from crab shells. *Carbohydrate Polymers, 74*, 840-844.

Zhang L., & Kosaraju S. L. (2007). Biopolymeric delivery system for controlled release of polyphenolic antioxidants. *European Polymer Journal, 43*, 2956-2966.

Zhang, Y. Y., Yang, Y., Tang, K., Hu, X., & Zou, G. L. (2008). Physicochemical characterization and antioxidant activity of quercetin-loaded chitosan nanoparticles. *Journal of Applied Polymer Science, 107*, 891-897.

Ziani, K., Oses, J., Coma, V., & Mate, J. I. (2008). Effect of the presence of glycerol and Tween 20 on the chemical and physical properties of films based on chitosan with different degree of deacetylation. *Lwt-Food Science and Technology, 41*, 2159-2165.