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DEVELOPMENT OF EDIBLE CHITOSAN FILM INCORPORATED WITH POMEGRANATE PEEL EXTRACT FOR THE PACKAGING OF BEEF

(Penghasilan Filem Kitosan yang Boleh Dimakan yang Digabungkan dengan Ekstrak Kulit Buah Delima untuk Pembungkusan Daging Lembu)

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Abstract

The aim of this research was to produce a chitosan-based edible film with pomegranate peel extract. Different chitosan concentration (1-2% w/v) and pomegranate peel extract (0%-6% w/v) were used to produce an edible film, and physical (thickness, moisture content, water-solubility, and total color change), mechanical (tensile strength and elongation at break), chemical (Fourier Transform Infrared) and antimicrobial properties of the edible films were investigated. The optimized edible film was applied to beef samples for a 7 days storage at 4 °C. When the concentration of the pomegranate peel extract increases from 1 to 6% (w/v), the thickness, water solubility, and total color difference of the film also increases. The chitosan film added with pomegranate peel extract was effective in inhibiting the *Streptococcus aureus* (SA) growth as compared to *Pseudomonas aeruginosa* (PA). In addition, an increasing in chitosan concentration (from 1% to 2%) reduced the water solubility of pomegranate peel extract film from 41.42% to 33.02%. Films with 2% (w/v) chitosan have the highest tensile strength (12.67 MPa) while the highest elongation at break (10.21%) was exhibited by the film with 1.5% (w/v) chitosan and 4% (w/v) pomegranate peel extract. The optimal concentration of chitosan and pomegranate peel extract of the film was 1.5% (w/v) and 4% (w/v), respectively. Moreover, shelf life of the beef sample was increased from 4 days to 7 days with the application of the pomegranate peel extract chitosan film.

Keywords: film, antimicrobial, chitosan, pomegranate peel extract, beef

Abstrak

Tujuan penyelidikan ini adalah untuk menghasilkan plastik yang boleh dimakan berasaskan kitosan dengan ekstrak kulit buah delima. Kepekatan kitosan yang berbeza (1-2% w/v) dan ekstrak kulit buah delima (0%-6% w/v) digunakan untuk menghasilkan plastik yang boleh dimakan, dan fizikal (ketebalan, kandungan kelembapan, kelarutan air, dan jumlah perubahan warna), mekanikal (kekuatan tegangan dan pemanjangan maksima), sifat kimia (inframerah transformasi Fourier) dan antimikrob plastik yang boleh dimakan telah dikaji. Plastik yang dioptimum, telah digunakan untuk sampel daging lembu untuk simpanan 7 hari pada 4 °C. Apabila kepekatan ekstrak kulit buah delima meningkat dari 1 hingga 6% (w/ v), ketebalan, kelarutan air, dan jumlah

perbezaan warna plastik juga meningkat. Plastik kitosan yang ditambah dengan ekstrak kulit buah delima berkesan dalam menghalang pertumbuhan *Streptococcus aureus* (SA) berbanding *Pseudomonas aeruginosa* (PA). Di samping itu, peningkatan dalam kepekatan kitosan (dari 1% hingga 2%) mengurangkan kelarutan air plastik ekstrak kulit buah delima daripada 41.42% kepada 33.02%. Plastik dengan 2% (w/v) kitosan mempunyai kekuatan tegangan tertinggi (12.67 MPa) manakala pemanjangan maksima (10.21%) dipamerkan oleh plastik 1.5% (w/v) kitosan dan 4% (w/v) ekstrak kulit buah delima. Kepekatan optimum kitosan dan ekstrak kulit buah delima adalah 1.5% (w/v) dan 4% (w/v), masing-masing. Selain itu, jangka hayat sampel daging lembu telah ditingkatkan dari 4 hari kepada 7 hari dengan aplikasi plastik kitosan-ekstrak kulit buah delima.

Kata kunci: plastik, antimikrob, kitosam, ekstrak kulit buah delima, daging lembu

Introduction

Food packaging is intentionally designed as a tool to transport, store and protect foods and goods [1]. As technologies advanced, it is utilized as a preventive measure to maintain or extend product shelf life [2]. Recent advancement such as active food packaging system has offered a great opportunity in improving food safety, quality and convenience [3]. It utilizes bioactive agents, like antioxidants and antimicrobial, to enhance product safety and quality by allowing those agents to interact with the internal environment within the package [3]. Ever since the environmental issues had become a major concern globally, researches on replacing petroleum-based packaging materials with biodegradable or edible materials have been conducted in a wide range [4]. As a result, active packaging, which is edible and biodegradable, has received much concern in the food application and packaging industries.

Edible packaging materials can be classified into three various types namely polysaccharide, protein, and lipid [5, 6, 7, 8]. Chitosan (CH) is a polysaccharide that derivate from crustaceans' shell and it is actually biodegradable as well as non-toxicity [9]. Chitosan film incorporated with essential oils such as oregano, extract of *Garcinia atroviridis* (asam keeping), and musk lime extract increased the film's antibacterial properties, potentially inhibiting the growth of microorganisms which are commonly contained in seafood especially in fish and also in meat products [10].

As an alternative to maintaining food quality, the invention of an antimicrobial edible packaging method has been proposed [7, 8]. Constitutes of fruits and plants that possess antimicrobial properties have also been studied in the development of edible packaging [5]. Whey protein edible film added with 6.0% anise oils was inhibit the growth of the molds (*Aspergillus flavus* and *Penicillium* sp.) which is found on dried fish [11]. To summarise, edible films containing an antimicrobial compound extracted from fruits and plants can help food products last longer [12].

Pomegranate is an ancient fruit that has been cultivated for over a thousand years [13]. Pomegranate fruit is a rounded shape with dark reddish skin [14]. It is normally consumed as fresh or in commercial products, whereby peels are usually disposed of as waste. Pomegranate extracts have been used for millennia for their anti-infective properties, with activity more recently being attributed to their rich composition of ellagitannins and other secondary polyphenolic compounds [15]. It has an antimicrobial effect against *Staphylococcus aureus*, and *Pseudomonas aeruginosa* which are commonly found in fresh meat, especially on beef [16].

Fresh meat, such as beef, is an important protein source. The shelf life of fresh beef is 3 to 5 days at 4˚C [17]. Beef is highly susceptible to microbial degradation and lipid oxidation attribute to its intrinsic parameters, such as high moisture and fat content, availability of nutrients, and optimal pH range [18, 19]. The degradation of meat quality causes a heavy off-odour, discoloration, and slime development, which cause a major problem with customer acceptance [17]. Reduction of microbial spoilage has been a challenge in the meat industry as the contamination can be originated from the animal or processing environment [20]. In the retail shop, the common preservation of

fresh beef is kept in the chill condition and wrapped with a polyethylene bag. Currently, there is scarce information on the application of edible film incorporated with plant extract as a preservation method on fresh beef.

Hence, the present study aims at developing a chitosan film which will be incorporated with pomegranate peel extract (PPE). Based on mechanical, physical, chemical, and antimicrobial activity, the optimal concentration of chitosan and pomegranate peel extract will be determined. The optimal film will be proceeding to the storage test in order to determine the effect of chitosan-pomegranate peel extract edible film on fresh beef meat samples.

Material and Methods

Extraction of pomegranate peel

The drying method of pomegranate peel was conducted with some modification [21]. Pomegranate fruits were washed with water before being sliced in half with a knife. Arils were then removed manually from the peel. The peels were cut into small pieces and dried in a hot air oven at 55 °C for 22 to 24 hours. The dried peels were then sieved after being ground into a fine powder with a food grinder. The extraction method of pomegranate peel extract was conducted with some modifications [22]. Pomegranate peel powder was firstly soaked in 80% methanol in a 1:4 ratio at room temperature for 24 hours. This is because methanol is more widely used than ethanol and has a boiling point of 78.4 °C compared to 64.7 °C for ethanol. Methanol extract needs a lower temperature in the rotary evaporator to evaporate the solvent, resulting in less damage to the extract. Methanol is safe to consume since it evaporates during the process. Subsequently, the solution was filtered with cotton wool and collected in a conical flask. Then, a rotary evaporator was used to evaporate the filtrates at 50 °C. All the condensation of waste was collected in a waste collector. The process of evaporation was completed when no excess condensed vapour. Finally, the extract was stored in a glass bottle at 0 °C until further analysis.

Preparation of CH-PPE edible film

The solvent casting method was used to produce the chitosan-pomegranate peel extract edible film [23]. Chitosan powder (1%, 1.25%, 1.5%, 1.75% and 2% (w/v)) was dissolved in 1% acetic acid to prepare 30 g for each solution. Chitosan solution was then left to stir overnight using magnetic stirrer at low speed. Followed by filtration with the use of a vacuum filter and Whatman No.3 filter paper. Glycerol (0.5% v/v) has been added as a plasticizer into the chitosan solution and stirred for 15 minutes to get a homogenous suspension. Tween 80 (0.05% v/v) was incorporated as an emulsifying agent. After stirring for 15 minutes, pomegranate peel extract was incorporated into chitosan solution and the final concentrations of 0%, 1%, 2%, 3%, 4%, 5% and 6% (w/v) were achieved. The chitosan-pomegranate peel extract (CH-PPE) film-forming solution was subjected to homogenization at 9,000 rpm for 4 minutes. Lastly, 25 mL of homogenous film-forming solution were cast on a sterile petri dishes (90 mm × 15 mm) and dried in a ventilated oven for 22-24 hours at 40˚C. Once the films dried, peeled off from the petri dishes and kept in desiccators at room temperature for further experimental analysis.

Physical properties of CH-PPE edible film

Thickness

Edible film's thickness was determined with minor modifications [24]. The thickness (mm) of the chitosan-pomegranate peel extract edible film was determined using a hand-held micro-meter at five random locations of each film.

Moisture content

The moisture content of chitosan-pomegranate peel extract (CH-PPE) edible film was measured [10]. The obtained film was first cut into a square with a 2 cm x 2 cm diameter and its initial mass was weighed (W_0). It was subsequently placed in a plastic cup and placed in a 60°C oven for 24 hours to remove moisture before a constant mass was reached. After drying, films were cooled to room temperature, and the final mass (W_1) of edible films was determined, and its weight was recorded. The calculation of moisture content of the edible film was determined as the following equation 1.

$$Moisture\ content = (W0 - W1)/W0\ x\ 100\% \quad (1)$$

Water solubility

The water solubility of CH-PPE edible film was obtained, with modifications [25]. At room temperature, each dried edible film was immersed in 25 mL distilled water for 2 hours. The free water was removed, and the films were dried for 24 hours at 60°C in an oven. The final mass of dried edible films (W_2) was measured after it was brought to room temperature. Calculation of water solubility was determined as the following equation 2.

$$Water\ solubility = \frac{W1-W2}{W1} \times 100\% \quad (2)$$

Color

The edible film's color was determined [26]. Colorimeter was firstly calibrated with a white and black calibrate plate. After calibration, 64 mm diameter of CH-PPE edible film was placed on the ColorFlez sample cup. The sample was rotated 90° after each reading. The film color was expressed in L*, a*, and b* values. L* represents the darkness of black at L=0 and the brightness of white at L=100; a* indicates red and green hue whereby the negative value signifies green hue and the positive value signifies red hue; the value of b* represents yellow/blue whereby yellow at the positive value and blue at a negative value. The color index (ΔE) that identifies the total color differences between each sample was determined as the following equation.

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (3)$$

Mechanical properties of CH-PPE edible film

Tensile strength and elongation at the breakpoint of CH-PPE edible film were determined using the method of which conformance to ASTM D882-02 method [27]. The film was cut into 20 mm × 70 mm strips. The strips were placed into the film-extension grips and clamped with a 50 mm initial grip distance. The crosshead was set to a speed of 20 mm/min.

Chemical properties of CH-PPE edible film

The absorbance spectra of CH-PPE edible film were measured using Fourier Transform Infrared (FTIR) spectrometry (Nicolet^{tm} iS^{tm} 5 Spectrometer, Thermo Scientific, Malaysia**)** connected to OMNIC Specta Software in transmission mode, which was slightly modified [28]. A tiny probe was used to fix the films to the sample holder. All spectra were then obtained with a resolution of 4 cm^{-1} as the average of 20 scans in the range of 4000 cm^{-1} to 500 cm^{-1} against a background spectrum from an empty cell.

Antimicrobial properties of CH-PPE edible film

The antimicrobial activity of the chitosan-pomegranate peel extract edible film against *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA) was determined using the agar disc diffusion method [29]. *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA) were pre-cultured overnight at 37°C in sterile nutrient broth. The turbidity of the bacterial suspensions was adjusted to 0.5 Mcfarland standard in a final inoculum density of 1.5×10^8 CFU/ml [30]. A 100 µL inoculum of each bacteria strain was spread evenly on Mueller-Hilton Agar (MHA) under aseptic conditions.

CH-PPE edible film was cut into 6mm diameter disc, then placed on the prepared MHA plate. A 6mm sterile paper disc (Whatman No.3 filter paper) was dipped in 80% methanol and dried in laminar flow as a negative control. Positive controls for *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA) were streptomycin and chloramphenicol, respectively. Finally, plates were incubated for 24 hours at 37°C. A ruler was used to measure the total diameter of inhibition, and the inhibition zone was measured in millimetres (mm).

Storage test of beef: Pre-treatment of beef

Freshly cut sirloin tip side of beef was acquired at a local supermarket. The beef meat was roughly cut into 10 g with 2 cm × 3 cm. These pieces were then wrapped with chitosan-pomegranate peel extract (CH-PPE) edible film (10 cm × 2 cm) and covered the entire meat surface. Beef meats without wrapping of CH-PPE edible film was controlled sample while beef meats wrapped with 1.5% CH + 4.0% PPE edible film was treatment sample. All meat samples were wrapped in a

polyethylene plastic bag and kept in 4˚C until further experimental analysis.

pH value of beef

The pH value of beef samples was obtained with slight modification [31]. Before the test, the pH meter was calibrated with pH 4, 7, and 10 buffer solutions. A stomacher was used to homogenise a beef sample of about 10 g in 100 mL sterile peptone water for 1 minute. The test was carried out during each day of storage (day 0, 1, 2, 3, 4, 5, 6, and 7).

Total plate count

The total plate count (TPC) test was conducted with some modifications [32]. To make the stock solution, a 10 g beef from treatment sample was homogenised for 1 minute in 100 mL sterile peptone water using a stomacher. Serial dilutions (10^{-1}-10^{-6}) were prepared in the test tube to enumerate the colonies in the range of 30 to 300. A 100 µL of bacteria inoculum of each dilution was spread evenly on plate count agar (PCA) by using the spread plate technique. All plates were incubated for 18 to 24 hours at 37°C. The TPC was conducted during each day of storage (day 0, 1, 2, 3, 4, 5, 6, and 7). The colony count was expressed in log CFU/mL.

Statistical analysis of data

All analyses were carried out in triplicate forms (n=3) except the storage test, which was conducted in duplicate (n=2). All results were reported as mean ± standard deviation. Tukey test was conducted to determine the significant differences among all treatments of CH-PPE films. All significant differences were set as 95% interval ($p \leq 0.05$). A pair T-test was performed to analyse the significant difference of total plate count and pH value between the two treatment groups of the beef sample from the three independent trials.

Results and Discussion

Physical properties of films

Thickness is an essential parameter in characterization of film because it affects the mechanical properties of films. The thickness of CH-PPE edible film was affected by concentration of the pomegranate peel extract (PPE), as shown in Table 1. The addition of PPE increased the thickness of CH-PPE edible film from 0.05 mm to 0.16 mm, as shown in Table 1. The thickness of 1.5% CH + 5% PPE edible film was 0.16 mm, and there was no statistical difference ($p > 0.05$) to a thickness of 1.5% CH + 6% PPE edible film.

This observation was consistent with the findings who discovered that the incorporation of antimicrobial agent increased the thickness of the chitosan film [25]. The CH-PPE edible film was thicker compared to the films used in previous research. The film-forming methods used in the research have an effect on the difference in film thickness 30 g of 1% (w/v) CH film-forming solution was cast on a 17.8 cm x 17.8 cm glass plate, produced 0.01 mm chitosan film [25]. The thickness of chitosan films was increased from 0.017 mm to 0.020 mm by adding lauric arginate and cinnamon oil in concentrations ranging from 0.1% (v/v) to 1% (v/v). 25 mL of film-forming solution was poured into a 90 mm 15 mm sterile petri dish in this experiment. Thus, as a smaller container was used for film casting in this analysis, a thicker film may result.

To prevent moisture transfer, it is important to maintain the edible film's moisture content at a similar level to the product's [33]. The moisture level and total soluble solid content of edible film affect its application on food. Table 1 shows the moisture content of CH-PPE film with various concentration of pomegranate peel extract ranging from 1% to 6%. The moisture content of the chitosan film was reduced from 28.01% to 7.47% after the addition of PPE (1% to 6%). A similar analysis revealed that when carvacrol and grape seed essence are applied to chitosan film, the moisture content decreases [34].

The film's moisture content can be determined by total void volume filled by water molecules in the polymeric structure of the chitosan film [35]. From the result in Table 1, the presence of pomegranate peel extract lowered the film's moisture content. It is suggested that the addition of PPE has occupied the space between the microstructural networks of chitosan film. As a result, lesser hydrogen bonds were formed between a water molecule and a functional group of chitosan, such as

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Water solubility is an essential parameter that indicates the film's integrity in an aqueous environment. It is also one of the essential factors that determines the edible film's biodegradability [25]. Films with high-water solubility possess lower water resistance and vice versa. The requirement of film water solubility is different in various food applications. A film with a high-water solubility is better for releasing antimicrobial agents into the surrounding food, while a film with a low water solubility is better for longer storage periods, particularly for food products with a lot of moisture or water activity [37]. The chitosan film's water solubility is influenced by the functional group in the chitosan and water holding capacity of plasticizer [38].

The addition of bioactive compounds disrupts the polymer chain's positioning and hydrogen bonding of chitosan film, resulting in a change in the film's solubility [39]. Table 1 also presented the effect of PPE concentration on the water solubility of CH-PPE edible film. From the observation of the result shown in Table 1, the increasing order of water solubility on CH-PPE edible film resulted from the increase of PPE concentration. Additional PPE from 0% to 6% has caused a significant change of water solubility that increased from 27.29% to 41.68%. The addition of PPE to chitosan can cause bonding to break and the structure of the molecule to change, and the increasing water solubility [25]. The hydrophilic compound in PPE contributed to the increased hydrophilicity of the film. By increasing hydrophilic properties, the affinity of polymeric molecules to water could be improved [40].

The appearance of a product can affect the consumer's acceptance. Color is one of the main parameters that affect product appeal. Thus, the determination of the film's color profile is critical in this case. Table 2 showed the effect of PPE concentration on the color profile of CH-PPE edible film in terms of its lightness (L*), red-green (a*) and yellow-blue (b*), and total color difference (ΔE). The lightness (L*) value of CH-PPE edible films was reduced when PPE was added from 0% to 6%. An increment in both the a* parameter and decrement of b* parameter was observed, indicating an increase of redness and a reduction of yellowness of CH-PPE edible film, respectively, with an increasing PPE concentration when compared to control film (1.5% CH + 0% PPE).

The reduction of lightness (L*), from 31.16 to 14.18, may be attributed to the color nature of PPE, which showed dark reddish-brown color visually. The a* parameter of the control film has shown in negative value (-0.77), which indicated the exhibition of green color. The polyphenol in PPE, anthocyanins that exhibits red color is suggested to the contribution of the increment of a* parameter, which intensifies redness to the film [41]. As the concentration of PPE increased, the color of PPE masks the original color of chitosan film and leads to the decrement of yellowness of CH-PPE edible film. With the addition of PPE from 0 to 6% in 1.5% CH film, the total color difference (E) has increased from 0 to 17.56. It reduced the transparency and yellowness but intensified the redness of CH-PPE edible film.

Table 1. The effect of pomegranate peel extract concentration on the thickness, moisture content, water-solubility of CH-PPE edible film

PPE (%) in Chitosan Film	Thickness, Mm	Moisture Content (%)	Water Solubility (%)
0	0.05 ± 0.01^{a}	28.01 ± 0.12^{f}	27.29 ± 0.66^{a}
1	0.08 ± 0.01^{b}	15.03 ± 0.78^{e}	33.28 ± 1.82^{b}
2	0.09 ± 0.01^{b}	13.79 ± 0.11^{d}	34.18 ± 1.94^{b}
3	0.12 ± 0.01^{c}	11.84 ± 0.39^{c}	36.86 ± 2.50^{cd}
4	0.13 ± 0.01^{c}	10.66 ± 0.25^{b}	37.62 ± 1.95^{cd}
5	0.16 ± 0.01^{d}	9.70 ± 0.02^{b}	40.40 ± 2.15^{d}
6	0.16 ± 0.01^{d}	7.47 ± 0.12^{a}	41.68 ± 2.40^{d}

[a-f]Means ± standard deviations with different superscript letters within the same column indicate significant difference between formulations ($p \leq 0.05$) according to Tukey's test.

Table 2. The effect of pomegranate peel extract concentration on the color, and mechanical properties of CH-PPE edible film

PPE (%) in Chitosan Film	*L**	*a**	*b**	Δ*E*	Tensile Strength, MPa	Elongation at Break, %
0	31.16 ± 0.31^{e}	-0.77 ± 0.02^{a}	4.75 ± 0.05^{e}	0.00 ± 0.00^{a}	5.02 ± 0.08^{a}	15.60 ± 0.87^{e}
1	27.06 ± 0.25^{d}	0.04 ± 0.05^{b}	4.73 ± 0.09^{e}	5.36 ± 0.65^{b}	7.78 ± 0.15^{b}	14.30 ± 0.10^{d}
2	27.01 ± 0.30^{d}	0.75 ± 0.04^{c}	3.90 ± 0.03^{d}	5.78 ± 0.43^{b}	10.07 ± 0.35^{c}	11.77 ± 0.35^{c}
3	25.93 ± 0.62^{c}	1.14 ± 0.02^{d}	3.50 ± 0.06^{c}	4.75 ± 0.43^{b}	10.54 ± 0.07^{c}	10.84 ± 0.32^{bc}
4	25.65 ± 0.26^{c}	1.68 ± 0.01^{e}	3.28 ± 0.08^{c}	4.96 ± 0.36^{b}	11.93 ± 0.4^{d}	10.21 ± 0.36^{c}
5	19.56 ± 0.11^{b}	1.82 ± 0.05^{e}	1.99 ± 0.02^{b}	12.21 ± 0.32^{c}	10.83 ± 0.69^{c}	7.57 ± 0.15^{b}
6	14.18 ± 0.11^{a}	2.14 ± 0.05^{f}	1.34 ± 0.17^{a}	17.56 ± 0.31^{d}	5.10 ± 0.77^{a}	5.83 ± 0.35^{a}

$^{a\text{-}f}$Means ± standard deviations with different superscript letters within the same column indicate significant difference between formulations ($p \leq 0.05$) according to Tukey's test.

Mechanical properties of films

The mechanical properties of a film can be used to determine its ability to protect product samples during storage, transportation, and handling. The films' tensile strength and elongation at break were tested at various PPE concentrations. The tensile strength (TS) and elongation at break (% E) of 1.5% CH-PPE edible film with varying PPE concentrations from 0 to 6% were shown in Table 2. Tensile strength of CH-PPE edible film was also improved significantly from 5.02 MPa to 10.54 MPa with the increasing concentration of PPE from 0% to 4%. Moreover, 1.5% CH + 4.0% PPE edible film showed the highest tensile strength (11.93 MPa) at all films. However, the films' tensile strength started to drop from 11.93 MPa to 5.10 MPa when the concentration of the PPE increase from 4% to 6%.

The addition of PPE into the film has improved the tensile strength of the edible film by enhancing the formation of the hydrogen bonds between pomegranate peel extract and chitosan and yielded a compact structure of the films. Besides, a brittle film and uneven surface of the film may result from low tensile strength. It may due to the higher content of PPE was difficult to disperse and form homogenous CH-PPE edible film, therefore, reduced its intermolecular interaction. The tensile strength of 1.5% CH + 6% PPE film was similar to the tensile strength of 1.5% CH + 0% PPE film (5.02 MPa).

On the other hand, a reduction of %E was revealed by the increment of PPE concentration ranging from 1% to 6%, which reduced %E from 15.60% to 5.83%. Among all films, the results of %E were all significantly different from each other. However, the %E of 1.5% CH + 3% PPE (10.84%) edible film had no significantly different to the %E of 1.5% CH + 2% PPE (11.77%) edible film and 1.5% CH + 4% PPE (10.21%) edible film. Thus, as the concentration of the PPE increases, the value of the elongation factor decreases. The optimal concentration of chitosan and pomegranate peel extract of the edible film was 1.5% (w/v) and 4.0% (w/v), respectively.

Elongation at breakpoint accounts for the extensibility and elasticity of material [42]. The value of elongation at breakpoint might relate to the moisture content of the film. The decrease of %E resulted from the increasing concentration of plant extracts incorporated in chitosan films may be owing to the decreasing moisture content in the films [12].

Chemical properties of films

The interaction between chitosan and pomegranate peel extract (PPE) was studied using FTIR analysis [28]. Figure 1 has depicted the spectra of CH-PPE edible film incorporated with PPE at varying levels. At the peaks of 1021 cm^{-1}, 2154 cm^{-1}, and 2874 cm^{-1}, all spectra showed similar patterns. The absorption peak at (i) 1350 cm^{-1} to 1000 cm^{-1} represented the C=O and C-N stretching vibrations [33], (ii) the peak around 2154 cm^{-1} indicated the stretching of alkyne bond (-C=C-), (iii) a weak band 2874 cm^{-1} represent the stretching of C-H and O-H bond [43] (iv) absorption peak at 1550 cm^{-1} corresponded to N-H stretching of amide I, (v) peak at 1400 cm^{-1} was assigned as a carboxylic group (-COO--) [28], (vi) broadband at 3500 cm^{-1} to 3200 cm^{-1} indicated the stretching vibration of N-H and O-H bond, as well as intermolecular hydrogen bonding of chitosan molecules [28].

The intensification of the absorption peak at the region of 1350 cm^{-1} to 1000 cm^{-1} be may be owing to the high content of PPE that contain a considerable amount of polyphenols, such as punicalagin and punicalin, which consists of hydroxyl, carbonyl, and aromatic group [44]. CH-PPE edible film with concentration between 4% to 6% PPE showed absorption peaks at 1400 cm^{-1} and 1550 cm^{-1}. This may due to the phenolic compounds were insufficient to be detected when PPE concentration was at 3% and below.

Glycerol has intensified the absorption peak of flaxseed mucilage film in the region of 3500 cm^{-1} to 3200 cm^{-1}, which represents O-H bond stretching [45]. Two new peaks at 2883.1 cm^{-1} and 1100 cm^{-1} to 920 cm^{-1} region that signifies that stretching vibration of C-H bond and C=O group appeared upon the incorporation of glycerol into flaxseed mucilage-based film. These three peaks indicated the vibration of the functional group in glycerol. Besides, the stretching vibration of methyl and methylene group ($-CH_2$) of Tween 80 shared the same absorption peak with glycerol at 2800-2960 cm^{-1} region [46].

By comparing the FTIR spectra to the result of the present study, a similar absorption band and peak were observed at 3281 cm^{-1}, 2874 cm^{-1}, and 1350 cm^{-1} to 1000 cm^{-1} region. However, the amplitude of the absorption band and peak at 3281 cm^{-1} and 2874 cm^{-1} region were similar among all CH-PPE edible films. This may vary due to the concentration of glycerol, and Tween 80 added into the CH-PPE edible films was fixed at 0.5% (v/v). As a result, the fixed concentration of glycerol and Tween 80 did not contribute to the intensification or shift or absorption band and peak.

Polar functional groups in organic compounds, such as hydroxyl, amines, carbonyl, and carboxylic groups, can increase their water solubility [47]. These compounds enhance the polarity of water and thus improved the hydrophilicity of film when incorporated in. From the result, the higher amplitude of absorption peak in the hydroxyl group, carbonyl group, amide group, and the carboxylic group were obtained as the concentration of PPE was increased.

Antimicrobial properties of films

An anti-microbial activity of chitosan film incorporated with pomegranate peel extract (PPE) at various concentrations against predominant bacteria found on beef meat sample was assessed using circular discs of the chitosan-pomegranate peel extract edible film with a diameter of 6 mm. Table 3 has shown the inhibitory effect of CH-PPE film against *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA). According to the findings, CH-PPE edible film was more effective at inhibiting SA growth. Control film (1.5% CH + 0% PPE) has similar inhibitory properties against the growth of tested microorganisms. The highest antimicrobial effect of CH-PPE film against PA was recorded at the 1.5% CH + 6% PPE edible film, of which the inhibition zone reached 12.16 mm. This was followed by 1.5% CH + 5% PPE edible film and 1.5% CH + 4% PPE edible film that exhibited an inhibition diameter of 12.06 mm and 11.56 mm, respectively.

In the case of *Staphylococcus aureus* (SA), the inhibition zones fell in the range of 12.39 mm to 15.33 mm as the PPE concentration increased. The highest effective concentration of PPE against *Staphylococcus aureus* (SA) was 6% (15.33). The inhibition zone is given by 4%, and 5% PPE incorporated film was 14.89 mm and 15.11 mm, respectively. However, there was

no significant difference ($p > 0.05$) among the addition of 4%, 5%, and 6% PPE into chitosan film.

Chitosan exhibits antimicrobial effect on several Gram-negative bacteria such as *Salmonella typhimurium, Escherichia coli, Vibrio parahaemolyticus,* and *Pseudomonas fluorescens*, as well as Gram-positive bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Bacillus megaterium, Lactobacillus brevis and, Lactobacillus bulgaricus* and *Lactobacillus plantarum* [48, 49]. The inhibitory effect of chitosan was attributed to the cationic amino group of chitosan to bind to the anionic groups of these microorganisms [50]. Also, the antimicrobial effect of pomegranate peel extract (PPE) has been investigated and reported in the literature.

Gallic acid, flavonols, ellagitannins, anthocyanin, procyanidins, and ellagic acid are among the polyphenol compounds contained in pomegranate peel [41]. This phenolic compound has a number of biological functions, including antimicrobial, antioxidant, anti-inflammatory, and anticancer. Among them, ellagitannins (punicalagins and punicalin) are particularly high in amount. Punicalagins have remarkable antimicrobial activity against SA and PA [51]. By disrupting the bacterial cell membranes, phenolic compounds can inhibit the survival and proliferation of bacteria. Additionally, phenolic compounds can deactivate pathogenic microorganisms through adhesive binding, protein and cell wall adhesion, enzyme deactivation, and migration into the cell wall and/or DNA [6].

Storage of beef

The pH value is crucial in determining the quality of meat. Before slaughtering process, initial pH value of the meat is between the values of pH 6.8 to pH 7.3. An unstressed animal would have a pH value of 5.4 to 5.8 at rigor mortis, while a stressed animal would result in DFD (dark, firm, dry) meat with a higher pH value of 5.9 to 6.5 [52]. Figure 2 has indicated the pH change of both the control and sample groups during the storage period. The pair t-test value was 0.001 ($p<0.05$), indicating that the pH values in the control and sample groups were significantly different.

Initial pH value of beef in the present study was 5.59. The finding which reported the initial pH value of fresh beef at 5.87 [31]. During the 7 days of storage at 4 °C, the pH of beef meat increased significantly ($p <0.05$). The pH of beef sample in the control group was risen faster than the pH of beef in the treatment group. The pH value of beef samples in the control group varied from the value of 5.59 to 7.45 whereas the pH value of treatment beef sample at day 7 was 6.25. However, the findings of the current study were in line with the observation of [31], where an increment of pH value at both control and treatment group during the storage period.

The total plate count of beef meat in both the control and treatment beef samples were illustrated in Figure 3. The perforated black line represented the maximum limit of acceptable microbial load on fresh beef meat. Both control and treatment beef samples showed an increasing trend from 3.77 log CFU/mL to 8.06 log CFU/mL and 3.77 log CFU/mL to 7.02 log CFU/mL in microbial load during the storage period at 4 °C from day 0 to day 7. On day 0, initial value of the total plate count (TPC) on beef meat was 3.77 log CFU/mL. As expected, the treatment beef sample showed a reduction ($p \leq 0.05$) in total plate count than control group. The antimicrobial effect of CH-PPE edible film was attributed to the punicalagins in PPE. By inhibiting the growth of PA and SA, the total plate count can be reduced partly.

Beef meat with > 7 log CFU/mL of spoilage microorganisms is unacceptable and unsafe for human consumption [54]. For the control beef sample, the total plate count has reached the limit point at day 4 and unfit for consumption. With the application of CH-PPE edible film, the TPC of beef sample was 4.17 log CFU/mL at day 4 where a 2.8 log reduction was achieved, and the shelf life of beef has been effectively extended from day 4 to day 7. A similar study has reported that milk protein-based film incorporated pimento essential oil has shown a reduction of *E. coli* counts on beef meat on day 5 [32]. In comparison, CH-PPE edible film is an effective tool in retaining the quality and prolonging the shelf life of beef.

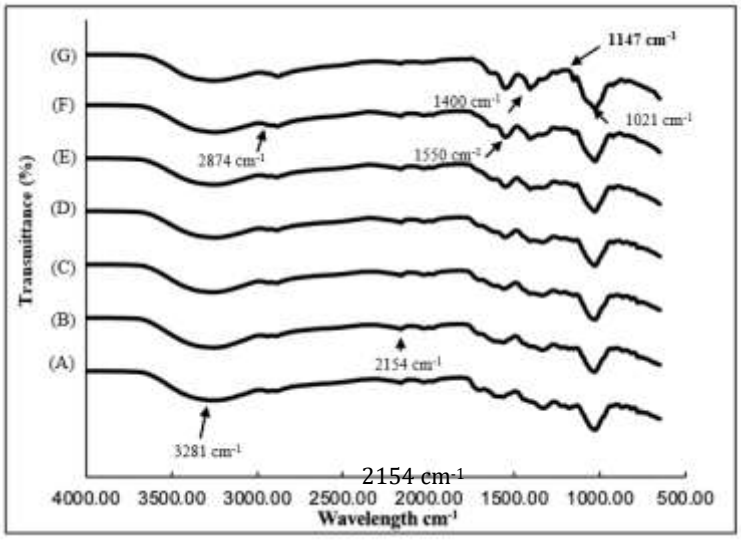

Figure 1. Spectra of CH-PPE edible films (A) 1.5%CH+0%PPE (B) 1.5%CH+1%PPE (C) 1.5%CH+2%PPE (D) 1.5%CH+3%PPE (E) 1.5%CH+4%PPE (F) 1.5%CH+5%PPE (G) 1.5%CH+6%PPE

Table 3. Antimicrobial activity of CH-PPE edible film

PPE (%) in Chitosan Film	**Inhibition Zone (mm)**	
	***Pseudomonas aeruginosa* (PA)**	***Staphylococcus aureus* (SA)**
0	6.07 ± 0.04^{a}	6.12 ± 0.06^{a}
1	10.28 ± 0.20^{b}	12.39 ± 0.10^{b}
2	10.38 ± 0.14^{b}	13.72 ± 0.27^{c}
3	10.56 ± 0.20^{b}	14.11 ± 0.10^{c}
4	11.56 ± 0.10^{c}	14.89 ± 0.20^{d}
5	12.06 ± 0.10^{cd}	15.11 ± 0.10^{d}
6	12.16 ± 0.17^{d}	15.33 ± 0.16^{d}

[a-e]Means ± standard deviations followed by different superscript letters within the same column indicate a significant difference between formulations ($p \leq 0.05$) according to Tukey's test.

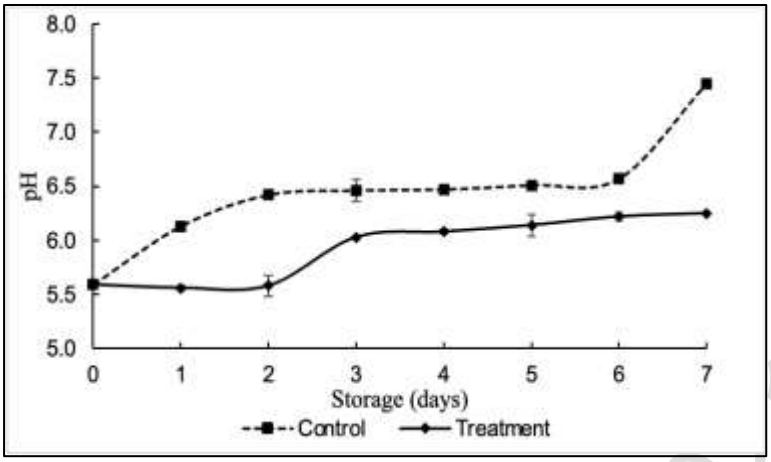

Figure 2. The change of pH throughout the 7 storage days at 4 °C

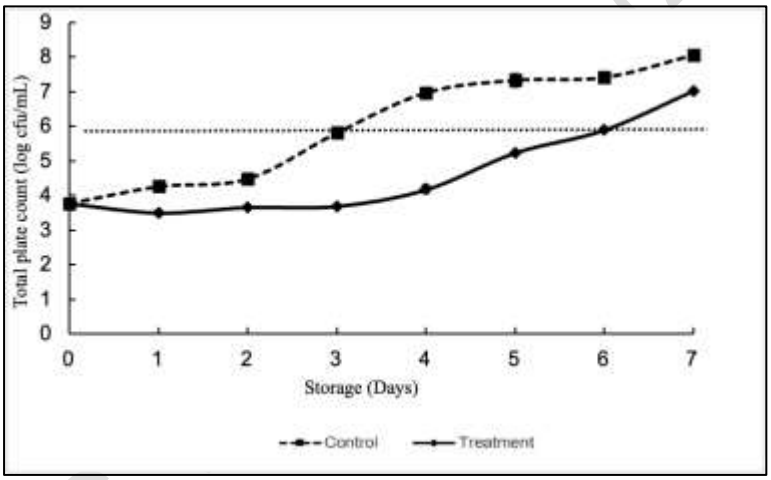

Figure 3. Total plate count of beef meat at both control and treatment samples during 7 storage days at 4 °C

Conclusion

The pomegranate peel extract and chitosan concentration had a significant impact on the edible film's physical, mechanical, chemical, and antimicrobial properties. The best combination of the pomegranate peel extract and chitosan in this study was 4.0% PPE and 1.5% chitosan. Increasing concentration of pomegranate peel extract enhance film's thickness, water solubility, and antimicrobial properties against *Pseudomonas aeruginosa* and *Staphylococcus aureus* while the addition of chitosan improved film's thickness and moisture content, but decrease film's water solubility. The CH-PPE edible film is an important tool for preserving the quality of beef and extending its shelf life. With the application of CH-PPE edible film, the TPC of beef meat was 4.17 log CFU/mL at day 4 where a 2.8 log reduction was achieved, and the shelf life of beef has been effectively extended from day 4 to day 7. The edible film with enhanced mechanical and antimicrobial properties had the potential to be developed for active food packaging applications. Furthermore, additional research should be conducted to investigate other applications to various meat products and microorganisms for the strength and effectiveness of chitosan-based edible film containing pomegranate peel extract.

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