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Original Article

Evaluation of phenolic compounds as cross-linkers to improve the qualities of halal gelatin from milkfish scales (*Chanos chanos*)

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Abstract

Gelatin is a versatile substance extensively used in medical and pharmaceutical industries for many applications, including capsule shells, X-ray film, infusion for plasma substitute, and the fabricating of artificial tissue. Fish scale gelatin is a profitable alternative source as a halal material despite its inferior quality. An addition of phenolic cross-linker may enhance the qualities of fish scale gelatin. The aim of this study was to determine the ability of phenolics to act as cross-linkers for fish scale gelatin and to identify the factors affecting this process. Gelatin production from fish scales (*Chanos chanos*) was carried out through basic pre-extraction and acidic pre-extraction. Thereafter, the gelatin was reacted with 10 different phenolics (phenol, pyrocatechol, resorcinol, α-naphthol, vanillin, L-tyrosine, curcumin, gallic acid, quercetin, and tannic acid). The resultant gelatins were characterized by infrared spectrum, X-ray diffraction pattern, swelling index, degree of cross-linking, viscosity, gel strength, mechanical profile, thermal profile, and water vapor permeability. Gelatin with the most favorable characteristics was further investigated for the effects of acidity (pH 4, 7, and 10) and cross-linker concentrations (2.5–10%). The findings revealed the formation of cross-linkage through shifted vibrational peaks of amide A, amide B, and amide II in the infrared spectrum. Shifted X-ray diffraction peaks in the gelatin with phenol addition also indicated the formation of cross-linkage. Significant improvement in the gelatin characteristics, such as swelling index, degree of cross-linking, viscosity, gel strength, mechanical profile, thermal profile, and water vapor permeability, could be attributed to the addition of phenolics cross-linkers. The highest improvement was observed in gelatin added with basic tannic acid 10%. Gelatin cross-linked with basic tannic acid 10% had a moisture content of 9.24±0.14%, swelling index of 323±17%, degree of cross-linking of 69.99±0.84%, viscosity of 8.48±0.23 cP, gel strength of 151.5±6.9 Bloom, melting temperature of 213.5°C, tensile strength of 7.00±0.54 $N.cm^{-2}$, elongation at the break of 114.08±14.63%, elastic modulus of 58.45±8.20 $N.cm^{-2}$ and water vapor permeability of 0.57±0.07 $g.mm.m^{-2}.h^{-1}. kPa^{-1}$. The qualities of tannic acid-cross-linked gelatin films and film-forming gel increased when manufactured under basic conditions in comparison to acidic or neutral conditions. Furthermore, increasing the quantity of tannic acid to 10% improved the overall characteristics as compared to non-cross-linked gelatin. In conclusion, tannic acid has the ability to cross-link the fish gelatin, thereby enhancing its qualities.

Keywords: *Chanos chanos*, piscine gelatin, halal, cross-linking, oxidized phenolic

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Introduction

Gelatin is a prominent substance that has been utilized extensively in the medical, pharmaceutical, and food industries since it has several non-substitutable advantages. Gelatin provides elastic texture, water binding, thickening, stabilizing, emulsifying, and foaming. The texture is stable at room temperature but melts once applied at body temperature, a characteristic not found in other polymers [1]. Thus, gelatin is a suitable material for the production of capsule shells, vaginal ovules, skin gels, wound patches, X-ray film, and food jelly and emulsion. The material is also commonly utilized in nanoencapsulation, a method aiming to enhance drug delivery. Gelatin is non-toxic in nature since the material is a collagen derivative, making it a suitable ingredient for the manufacturing of artificial implants [2,3]. Gelatin could also be used as a plasma substitute in intravenous infusion. In previous studies, peptides derived from hydrolyzed fish gelatin have been reported to have a potential in diabetes treatment due to their blood glucose and blood pressure lowering effects [4,5].

Gelatin is produced by hydrolyzing collagen from animal tissues under acidic or basic conditions, mainly sourced from pigs and cattle, with smaller amounts derived from fish, camels, and poultry. Porcine gelatin dominates the global market but it is strictly forbidden for Muslim and Jewish consumers. Bovine gelatin is forbidden in Hindu societies, and in Islam, it must come from animals slaughtered according to religious guidelines [6]. Fish gelatin is considered halal by Muslims, as it does not require religious slaughter and is accepted by most religions and communities. Various fish species from both oceanic and freshwater sources have been utilized for gelatin production, where processing waste such as skin, bones, and scales were often used [7]. However, fish gelatin exhibits inferior qualities compared to mammalian gelatin, including reduced gel strength, viscosity, and mechanical properties. This decrease in quality is attributed to the extraction temperature and the low concentrations of proline and hydroxyproline in fish gelatin, which affect the compactness and integrity of the helical structure [5,8].

Fish gelatin quality can be enhanced by adding cross-linkers, which improve gel stability, functional properties, thermal properties, and mechanical properties. The addition of a cross-linker catalyzes the binding between gelatin molecules, forming a polymeric network. Lysine, an amino acid, is the main target for cross-linking, with cross-links formed by the condensation of primary amines and active phenolic groups [9,10]. In previous studies, glutaraldehyde is frequently used as a cross-linker for proteins, but its manufacturing process can leave hazardous residues [5,8]. On the contrary, phenolics have low toxicity and can used as cross-linkers in gelatin production [11,12]. Phenolics are the most varied group of phytochemicals, offering a broad range of structures from simple monophenols with one or two hydroxyl groups to complex polyphenolics with additional non-hydroxyl groups. Phenolics can form a cross-linkage through covalent bonds (oxidized derivatives), ionic bonds, hydrogen bonds, or hydrophobic interactions. Covalent cross-links, the strongest chemical bonds, enhance the structural integrity of gelatin. The covalent bonds between the amine of gelatin and the oxidized phenolics are formed in a meta- or para-orientation [13,14]. Several phenolics, including tannic acid, caffeic acid, rutin, gallic acid, and ferulic acid, have been used as cross-linkers, where they could improve the bonds between polymers, contributing to thicker and more rigid characteristics [11,12]. However, there is a lack of research investigating the impact of acidity and structure on the cross-linking reaction.

The aims of the present study were two-fold. The first aim was to identify which phenolics act as the most effective cross-linker for gelatin, indicated by the characteristics of the cross-linked gelatins. Various phenolics were used, ranging from simple monophenols to complex polyphenols. The second aim was to investigate the optimum acidity and cross-linker concentration affecting gelatin quality. The effects of these parameters have not been reported for piscine gelatin, highlighting the novelty of this study.

Methods

Study design

This study employed a controlled post-test-only approach. The effect was assessed or evaluated by comparing the results of the experimental groups against the non-cross-linked group. The research comprised three stages: gelatin extraction, assessment of phenolics as crosslinkers, and evaluation of acidity and crosslinker concentration effects. The ethical clearance was waived since the fish samples utilized in this research were collected as fishing industry waste. This research did not involve living organisms as the subject of the research.

Materials

The samples used in this research were scales waste of milkfish (*Chanos chanos*, 65–75 cm in length) collected from the Makassar Industrial Area (KIMA). The fresh fish scales were cleaned by removing any meat or fat leftover and oven-drying at 40°C for 48 hours. The milkfish scales were considered halal since they originated from the ocean. Sodium hydroxide and hydrochloric acid solutions were procured from Merck. Albumin, biuret reagent, oxidant reagent (chloramine-T), colorimetric reagent (p-dimethylaminobenzaldehyde), phenol, resorcinol, α-naphthol, L-tyrosine, and curcumin, were also procured from Merck. Pyrocatechol, hydroxyproline, vanillin, gallic acid, quercetin, tannic acid, anhydrous calcium chloride, cotton, ninhydrin reagent, and 10% isopropanol were purchased from Sigma-Aldrich. Deionized water was obtained Onemed. Except for the fish scales, all materials were purchased in analytical grade quality and used without pre-treatment.

Extraction and characterization of gelatin from milkfish scales

The gelatin extraction followed the methods reported previously with minor adjustments [15]. The fish scale sample (100 grams) was soaked in 700 mL sodium hydroxide 0.1 N for 45 minutes at room temperature (20–25°C). Thereafter, the scale was filtered and rinsed three times with deionized water. The scales residue was soaked in 700 mL of 1 N hydrochloric acid for 45 minutes at room temperature (20–25°C) before being collected and rinsed three times with deionized water. The produced soft scales residue was then soaked with 700 mL of deionized water. The mixture pH was neutralized (pH=7) using hydrochloric acid 0.1 N or sodium hydroxide 0.1 N. The mixture was heated at 90°C for eight hours. To reduce the water contents, the resulting concentrated liquid was oven-heated at 60°C for around 48 hours. The dried gelatine was ground into fine powder. Extraction efficiency was calculated by the percentage of dried gelatin weight relative to the dried fish scale weight. The resultant gelatin was characterized by pH, total protein content, hydroxyproline content, moisture content, and ash content. The pH was determined based on potentiometry using a pH meter [16]. The total protein content of gelatin was determined using the Biuret method [15]. The hydroxyproline content was determined based on the oxidation reaction of hydroxyproline with chloramine-T [16]. The moisture content was determined as the percentage loss in weight of the sample after oven-heating 5,000 mg of the sample at 105°C for 20 hours [16].

Assessment of different phenolics as cross-linking agents for fish gelatin

The cross-linked gelatins were produced following the previously published methods with minor adjustments [11,17]. Phenolics were selected based on their chemical structures, ranging from simple to complex structures, such as phenol, pyrocatechol, resorcinol, α-naphthol, vanillin, L-tyrosine, curcumin, gallic acid, quercetin, and tannic acid. Milkfish scale gelatin (3,800 mg) was dissolved in deionized water (40 mL) and was stirred on a magnetic stirrer at 50°C at 1,000 rpm for 30 minutes. Cross-linkers were prepared by dissolving phenolic compounds (200 mg) in sodium hydroxide 0.1 N (6 mL). The pH was adjusted to 10 using hydrochloric acid 0.1 N or sodium hydroxide 0.1 N, and the mixture was allowed to stand for 24 hours. The cross-linker solution was mixed with the gelatin solution and the pH was readjusted to 10 using sodium hydroxide 0.1 N. The mixture was adjusted to 60 mL and homogenized at 1,000 rpm for 120 minutes. The mixture was poured into a non-stick container (14 × 5 cm) and oven-dried at 60°C for around 48 hours until a film sheet was formed. To produce the film-forming gel, the same protocol was used but without the drying stage. The non-cross-linked gelatin was produced using the same procedure, with a gelatin weight of 4,000 mg and without the addition of phenolic compounds.

Preparation of tannic acid-cross-linked gelatin

Tannic acid was found as the optimum cross-linker and selected for further investigation. The cross-linking protocol employed was reported previously with several adjustments [11,17]. The cross-link reaction was carried out at pH 4, 7, and 10, where the resultant gelatin was labeled as acidic tannic, pH-neutral tannic, and basic tannic, respectively. Each gelatin was varied in tannic acid concentrations of 2.5%, 5,0%, and 10,0% relative to the solid composition. The sample labels assigned according to the tannic acid concentration and pH are presented in **Table 1**. The milkfish scale gelatin was dissolved in 40 mL of deionized water and stirred on a magnetic stirrer at 50°C and 1,000 rpm. The tannic acid was dissolved in 6 mL of sodium hydroxide 0.1 N, hydrochloric acid 0.1 N, or deionized water. The pH was adjusted to 10, 4, or 7, then left to stand for 24 hours. The tannic acid solution was added to the gelatin solution and the pH was adjusted to 10, 4, or 7. Adjustments of the pH were carried out by the addition of hydrochloric acid 0.1 N or sodium hydroxide 0.1 N. The mixture was made up to 60 mL and stirred at 1,000 rpm for 120 minutes. The mixture was poured into an anti-stick container (14 × 5 cm) and dried in an oven at 60°C for 48 hours, resulting in a film sheet. The same procedure was utilized to produce a film-forming gel without oven-drying.

Table 1. Formula of a film of gelatin cross-linked with tannic acid

Label	Gelatin (mg)	Tannic acid (mg)	HCl 0.1 N (mL)	NaOH 0.1 N (mL)	pH
Basic tannic 5%	3,800	200	NA	6	10
Acidic tannic 5%	3,800	200	6	NA	4
pH-neutral tannic 5%	3,800	200	NA	NA	7
Basic tannic 2.5%	3,900	100	NA	6	10
Basic tannic 10%	3,600	400	NA	6	10
Basic gelatin	4,000	NA	NA	6	10
Acidic gelatin	4,000	NA	6	NA	4
pH-neutral gelatin	4,000	NA	NA	NA	7

NA: not applicable

Characterizations of cross-linked gelatin

The dried cross-linked gelatin films were analyzed for their moisture contents, swelling indices, degrees of cross-linking, mechanical properties (tensile strength, elongation at the break, and elastic modulus), water vapor permeabilities, infrared spectra, thermograms, and X-ray diffraction patterns. The viscosity and gel strength were evaluated for film-forming gel samples. The moisture content was determined using the same procedure previously stated.

The infrared spectrum was determined using a Fourier-Transform Infrared spectrometer featuring attenuated total reflection (Shimadzu IRSpirit with QATRs) [18]. To assess the thermal and X-ray diffraction properties among samples produced from different phenolics, differential scanning calorimetry (PerkinElmer DSC 4000) and powder X-ray diffractometer (Rigaku Miniflex II) were used, respectively. Meanwhile, thermograms and X-ray diffraction patterns of tannic acid-cross-linked samples were generated using simultaneous thermal analyzer 449 F5 Jupiter and Shimadzu MAXima X XRD-7000, respectively [11]. The swelling index was determined using gravimetric techniques by immersing the sample into deionized water. A gelatin cross-linked film (1 × 1 cm) was weighed before (w_0) and after (w_{120}) the immersion. The percentage of the swelling index was calculated using the formula = $((w_{120}-w_0)/w_0) \times 100\%$.

The cross-linking degree was determined by reacting ninhydrin with the primary amine group. The film sample (500 mg) was heated in a water bath at 10°C for 20 minutes after reacting with 500 µL of ninhydrin reagent in a screw-cap test tube. After being left to reach room temperature, deionized water (2,500 µL) and isopropanol 10% (5,000 µL) were added. The UV-Vis spectrophotometer was utilized to measure the absorbance of the solution at 570 nm [19]. The percentage of cross-linking degree was calculated using the formula = ((absorbance blank–absorbance cross-linked)/absorbance blank) × 100%.

Gel strength was measured using a Texture Analyzer (CT3 Brookfield) in Bloom units. Viscosity was determined by measuring the efflux time of 6.67% gelatin cross-linked in film-forming gel form at 40°C using a viscometer (Cannon-Fenske Routine Calibrated size 100) [15,16]. Density was calculated using a Pycnometer (Iwaki) in g/mL at 40°C. Viscosity (cP) was calculated using the formula = efflux time (s) × density (g/mL) × viscometer constant (mm^2s^2).

The mechanical profiles were determined based on the tensile strength, elongation at the break, and elastic modulus characteristics, which were then analyzed on a Universal Test Machine

(A&D Tensilon RTF). Tensile strength was determined by dividing the ultimate with the initial cross-sectional area. The elongation at the break was determined by dividing the extension at rupture with the initial length. The elastic modulus was calculated from the slope of the initial linear range of the stress-strain plot, as suggested previously [17,20].

The water vapor permeability was determined by quantifying the amount of water vapor that passed through the permeable film. The film was determined by the thickness (t) using a screw micrometer and was attached to the 80 × 10 mm (area=A) rectangular open section of the airtight-waterproof test cells. The test cell was filled with 20 mL of ionized water and 2 grams of cotton. The test cell's total weight was taken as the initial weight (w_0). The test cell was placed in an incubator with one kilogram of anhydrous calcium chloride, and the temperature was set to 37°C. After 24-hour storage, the test cell was weighed (w_{24}). ΔP represents the difference in partial water vapor pressure (kPa) between the two sides of the film (4.2449 kPa at 30°C) [20,21]. Water vapor permeability ($g.mm.m^{-2}.h^{-1}.\ kPa^{-1}$) was calculated using the formula = $(((w_0-w_{24}) \times t)/A \times h \times \Delta P)$. All of the tests were conducted with a minimum of three replications, with the exception of the water vapor permeability test, which required a minimum of five replications.

Statistical analysis

Data from replicated protocols were presented as mean ± standard deviation (SD). The statistical analysis was performed using a one-way analysis of variance (ANOVA) and continued with the Duncan's post-hoc test. Statistical significance was considered achieved if $p<0.05$. All statistical analyses were carried out on SPSS version 26 (IBM, New York, USA).

Results

Extraction efficiency and characteristics of milkfish scale gelatin

Following the cleaning process, the scales appeared colorless. The extraction efficiency was 32.37±1.03%. The gelatin that was extracted in this study had the following characteristics: pH of 5.33±0.21, protein content of 80.79±0.80%, hydroxyproline content of 7.94±0.16%, moisture content of 8.89±0.15%, and ash content of 1.65±0.08%.

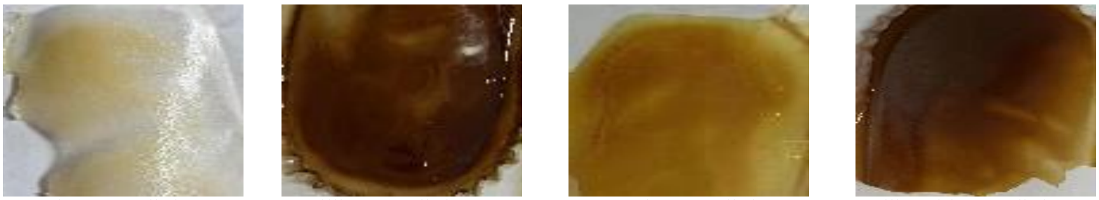
Effect of phenolic types as gelatin cross-linkers

The resultant cross-linked gelatins appeared in brown to black colors, deriving from the phenolic oxidation (**Figure 1**). The infrared spectra show the vibrational peaks of amide A at 3,310–3,270 cm^{-1}, amide B at 3,080–2,899 cm^{-1}, amides I at 1,700–1,600 cm^{-1}, amide II at 1,550–1,400 cm^{-1}, and amide III at 1,240–670 cm^{-1} (**Figure 2**). The thermograms show a shift in the endothermic peak, indicating an increase in melting temperatures (**Figure 3A**). The melting temperature of the gelatin film increased in the following order: non-cross-linked (208.6°C), catechol (214.0°C), naphthol (218.5°C), gallic (257.5°C), phenol (259.8°C), resorcinol (261.7°C), curcumin (261.7°C), tyrosine (262.1°C), vanillin (264.8°C), tannic (264.8°C), and quercetin (285.7°C). Furthermore, some compounds like gallic acid (179.7°C) and tannic acid (195.1°C) exhibit exothermic baseline, indicative of the crystallization process (**Figure 3A**). The X-ray diffraction pattern had a large broadening diffraction peak that indicates the semicrystalline structure (**Figure 3B**). The change in the 2θ peak signifies a structural alteration caused by the interaction of oxidized phenolic with gelatin. The degree of crystallization (based on crystalline peaks to overall peaks ratio) increases with the addition of phenolic cross-linkers, primarily gallic acid and tannic acid (**Table 2**).

The physicochemical properties of the produced gelatins are presented in **Table 3**. The lowest moisture content was observed in tannic acid-cross-linked gelatin (9.26±0.43%). The lowest swelling indices were observed in gelatin cross-linked with tannic acid, quercetin, and gallic acid with values of 416±43, 447±10, and 366±9%, respectively. The highest cross-linking degrees were observed in gelatin cross-linked with vanillin, curcumin, gallic acid, quercetin, and tannic acid with values of 43.62±1.84, 43.67±3.06, 44.05±2.84, 43.79±2.06, and 41.88±2.85%, respectively. Gallic acid cross-linker yielded a gelatin gel with the highest viscosity (8.08±0.26 cP), while the addition of phenol, catechol, and resorcinol did not significantly change the viscosity. The highest gel strength was reached when the tannic acid was used as a cross-linker (142.1±6.5 Bloom).

The influence of the type of phenolic cross-linkers on the mechanical properties and water vapor permeability of the gelatin films is presented in **Table 4**. Tannic acid had the highest tensile strength (6.64±0.62 N.cm^{-2}), the highest elastic modulus (58.24±2.40 N.cm^{-2}), the lowest elongation at the break (82.96±3.78%), and the lowest water vapor permeability (0.69±0.04 g.mm.m^{-2}.h^{-1}. kPa^{-1}).

Phenol cross-linker Resorcinol cross-linker Catechol cross-linker α- Napthol cross-linker



Tyrosine cross-linker Vanillin cross-linker Curcumin cross-linker Gallic acid cross-linker

Quercetin cross-linker Tannic acid cross-linker Non-cross-linked gelatin

Figure 1. Milkfish scale-based gelatin films were produced with different phenolics as cross-linkers.

Table 2. Degree of crystallinity of film gelatin cross-linked with phenolics

Phenolics cross-linkers	Degree of crystallinity (%)
Phenol	61.20
Catechol	61.23
Resorcinol	61.13
α-Naphthol	62.93
Tyrosine	61.51
Vanillin	64.92
Curcumin	64.13
Gallic acid	67.38
Quercetin	63.49
Tannic acid	67.06
Non-cross-linked	61.04

Table 3. Characteristics of film gelatin cross-linked with phenolics

Phenolics cross-linkers	Moisture content (%)	Swelling index (%)	Cross-linking degree (%)	Viscosity (cP)	Gel strength (Bloom)
Phenol	9.64±0.28 $^{a, b, c}$	Dissolve	32.92±1.40 ^{b}	6.49±0.26 ^{e}	131.8±4.8 $^{a, b}$
Catechol	10.21±0.16 ^{d}	1435±47 ^{f}	33.22±1.40 ^{b}	6.70±0.27 ^{e}	133.6±6.5 $^{a, b}$
Resorcinol	9.90±0.11 $^{c, d}$	1155±106 ^{e}	31.09±1.89 ^{b}	6.97±0.27 $^{d, e}$	134.3±6.6 $^{a, b}$
α-Naphthol	10.16±0.33 ^{d}	1246±97 ^{e}	34.75±1.98 ^{b}	7.28±0.48 $^{c, d}$	136.6±5.1 $^{a, b}$
Tyrosine	10.23±0.17 ^{d}	994±78 ^{d}	31.86±1.66 ^{b}	7.36±0.29 $^{c, d}$	135.0±5.3 $^{a, b}$
Vanillin	9.66±0.31 $^{a, b, c}$	721±67 ^{c}	43.62±1.84 ^{a}	7.48±0.29 $^{b, c, d}$	137.0±3.9 $^{a, b}$
Curcumin	9.39±0.17 $^{a, b}$	576±55 ^{b}	43.67±3.06 ^{a}	7.62±0.21 $^{a, b, c}$	139.4±5.7 $^{a, b}$
Gallic acid	9.80±0.13 $^{b, c, d}$	416±43 ^{a}	44.05±2.84 ^{a}	8.08±0.26 ^{a}	140.3±4.9 $^{a, b}$
Quercetin	9.38±0.30 $^{a, b}$	447±10 ^{a}	43.79±2.06 ^{a}	7.99±0.22 $^{a, b}$	141.2±6.6 $^{a, b}$
Tannic acid	9.26±0.43 ^{a}	366±9 ^{a}	41.88±2.85 ^{a}	7.97±0.39 $^{a, b}$	142.1±6.5 ^{a}
Non-cross-linked	10.24±0.13 ^{d}	Dissolved	Not applicable	6.45±0.31 ^{e}	130.2±5.2 ^{b}

Different letters indicate statistically significant differences (p<0.05)

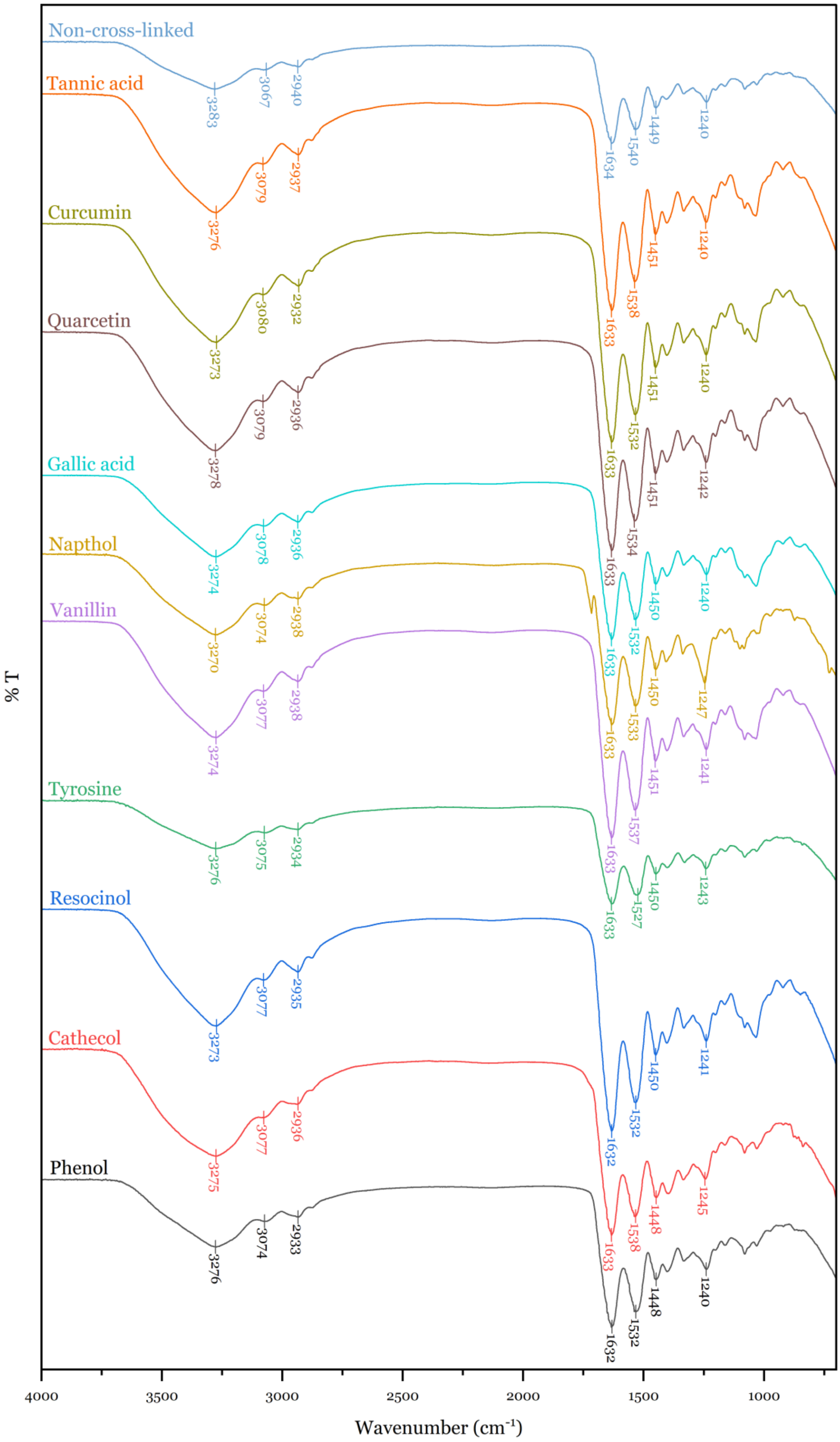


Figure 2. Infrared spectrum of milkfish scale-based gelatin cross-linked with different phenolics.

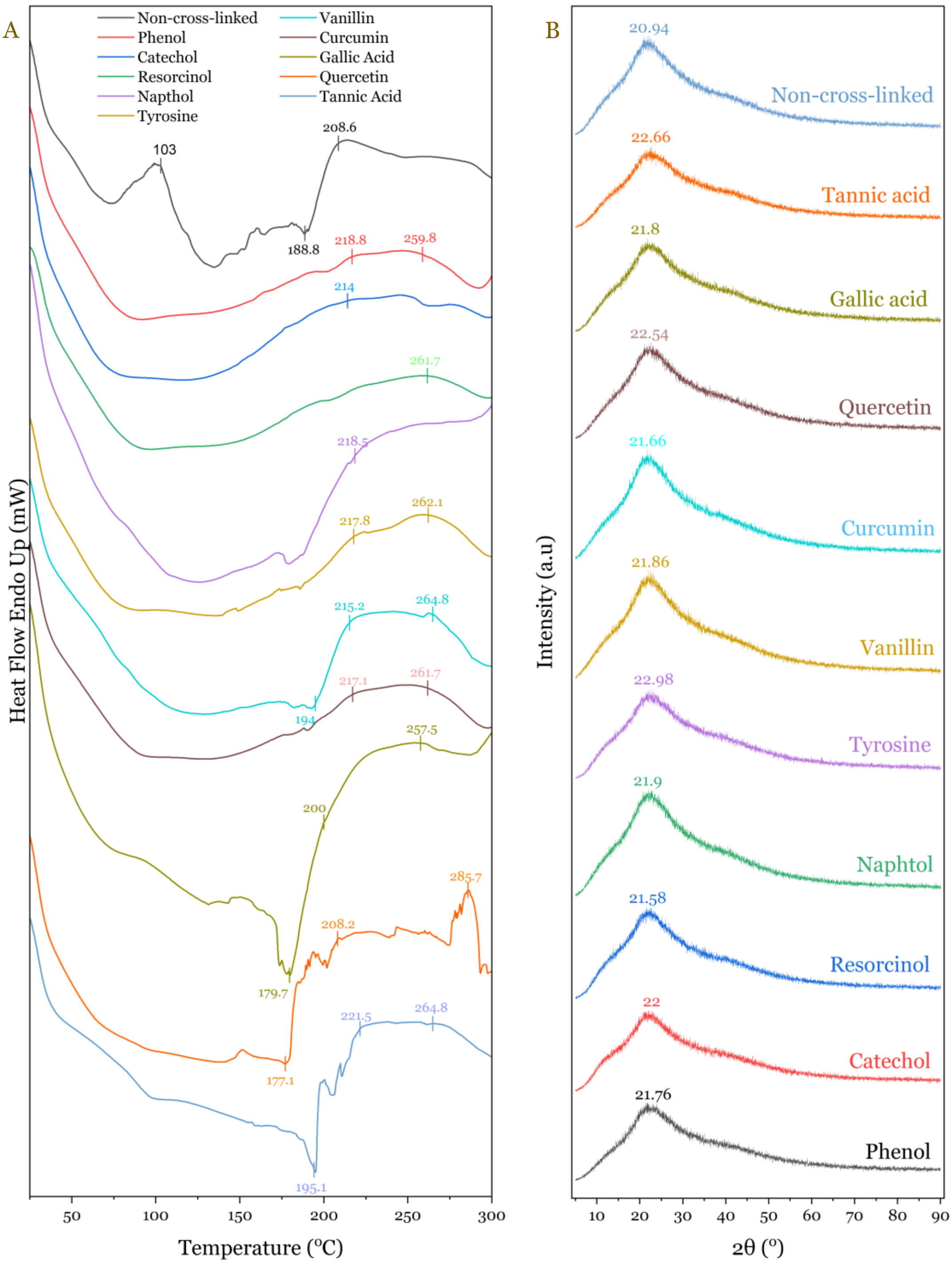


Figure 3. Differential scanning calorimetry (DSC) thermogram (A) and X-ray diffraction pattern (B) of milkfish scale-based gelatin film cross-linked with different phenolics.

Effect of pH and cross-linker concentration

Photographed images of the gelatin films produced with pH and cross-linker concentration variations are presented in **Figure 4.** In basic conditions, the addition of tannic acid turns the solution into brown, consequently resulting in a brown film. In acidic or pH-neutral conditions, the reaction of tannic acid with gelatin produced a haze flocculant precipitate, whereas the film appeared in an opaque light brown color. The infrared spectra indicated the presence of molecular vibration at 3,310–3,270 cm^{-1} for amide A, 3,080-2,899 cm^{-1} for amide B, 1,700–1,600 cm^{-1} for amide I, 1,550–1,400 cm^{-1} for amide II and 1,240–670 cm^{-1} for amide III (**Figure 5**). The assignments of the spectral peaks were in accordance with the suggestions of previous studies [18,22]. A shift in the endothermic peak was observed in the thermogram, which was an indication of a potential increase in melting temperature (**Figure 6**). The melting temperatures of the non-cross-linked groups were 228.6, 235.2, and 254.1°C for basic, acidic, and pH-neutral gelatins, respectively. Meanwhile, the melting temperatures of the cross-linked groups were 256.6, 270.6, 282.4, 282.9, and 289.8°C for basic tannic 2.5%, acidic tannic 5%, pH-neutral tannic 5%, basic tannic 5%, basic tannic 10%. Moreover, the gelatin film cross-linked with tannic acid had exothermic valleys suggesting the crystallization phase, which was most noticeable in basic tannic groups, irrespective of the tannic acid concentration. The X-ray diffraction patterns had large broadening diffraction peaks that indicated the semicrystalline structure. The shift in the 2θ peak at 20.9–22.22 was an indication of structural modifications resulting from the interaction of tannic acid with gelatin (**Figure 6**). The degree of crystallization of gelatin films was also slightly increased by the addition of a tannic acid cross-linker compared to non-cross-linked films (**Table 5**).

Table 4. Mechanical and water vapor permeability profile of film of gelatin cross-linked with phenolics

Phenolics cross-linkers	Tensile strength ($N.cm^{-2}$)	Elongation at break (%)	Elastic modulus ($N.cm^{-2}$)	Water vapor permeability ($g.mm.m^{-2}.h^{-1}. kPa^{-1}$)
Phenol	3.60 ± 0.21^{f}	158.81 ± 10.38^{e}	22.79 ± 4.17^{e}	0.91 ± 0.02^{g}
Catechol	$4.03 \pm 0.17^{e, f}$	155.91 ± 11.71^{d}	28.57 ± 2.44^{d}	$0.88 \pm 0.04^{f, g}$
Resorcinol	$4.22 \pm 0.22^{d, e}$	143.59 ± 6.48^{d}	28.51 ± 1.29^{d}	0.90 ± 0.02^{g}
α-naphthol	$4.71 \pm 0.32^{c, d}$	120.61 ± 7.91^{c}	$34.14 \pm 0.71^{c, d}$	$0.87 \pm 0.03^{e, f, g}$
Tyrosine	3.55 ± 0.16^{f}	122.98 ± 5.41^{c}	22.09 ± 1.88^{e}	$0.84 \pm 0.03^{e, f}$
Vanillin	4.92 ± 0.19^{c}	122.64 ± 9.00^{c}	35.3 ± 2.39^{c}	$0.82 \pm 0.03^{d, e}$
Curcumin	5.63 ± 0.49^{b}	$109.39 \pm 6.19^{b, c}$	46.23 ± 6.60^{b}	$0.77 \pm 0.03^{b, c}$
Gallic acid	5.78 ± 0.40^{b}	98.57 ± 7.18^{b}	49.17 ± 4.34^{b}	$0.78 \pm 0.05^{c, d}$
Quercetin	5.93 ± 0.20^{b}	$95.79 \pm 9.83^{a, b}$	51.11 ± 2.66^{b}	$0.73 \pm 0.05^{a, b}$
Tannic acid	6.64 ± 0.62^{a}	82.96 ± 3.78^{a}	58.24 ± 2.40^{a}	0.69 ± 0.04^{a}
Non-cross-linked	2.72 ± 0.21^{g}	180.06 ± 9.07^{f}	13.78 ± 1.51^{f}	1.21 ± 0.05^{h}

Different letters indicate statistically significant differences ($p<0.05$)

Basic gelatin

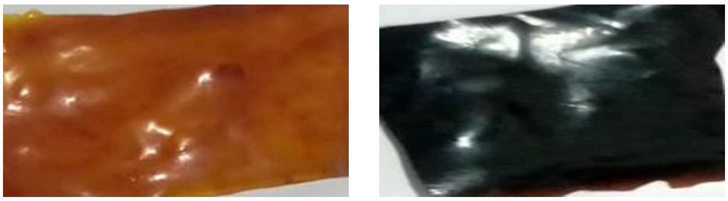
Acidic gelatin

pH-neutral gelatin

Basic tannic 5%

Acidic tannic 5%

pH-neutral tannic 5%



Basic tannic 2.5%

Basic tannic 10%

Figure 4. Film of gelatin cross-linked with tannic acid at different acidities and concentrations.

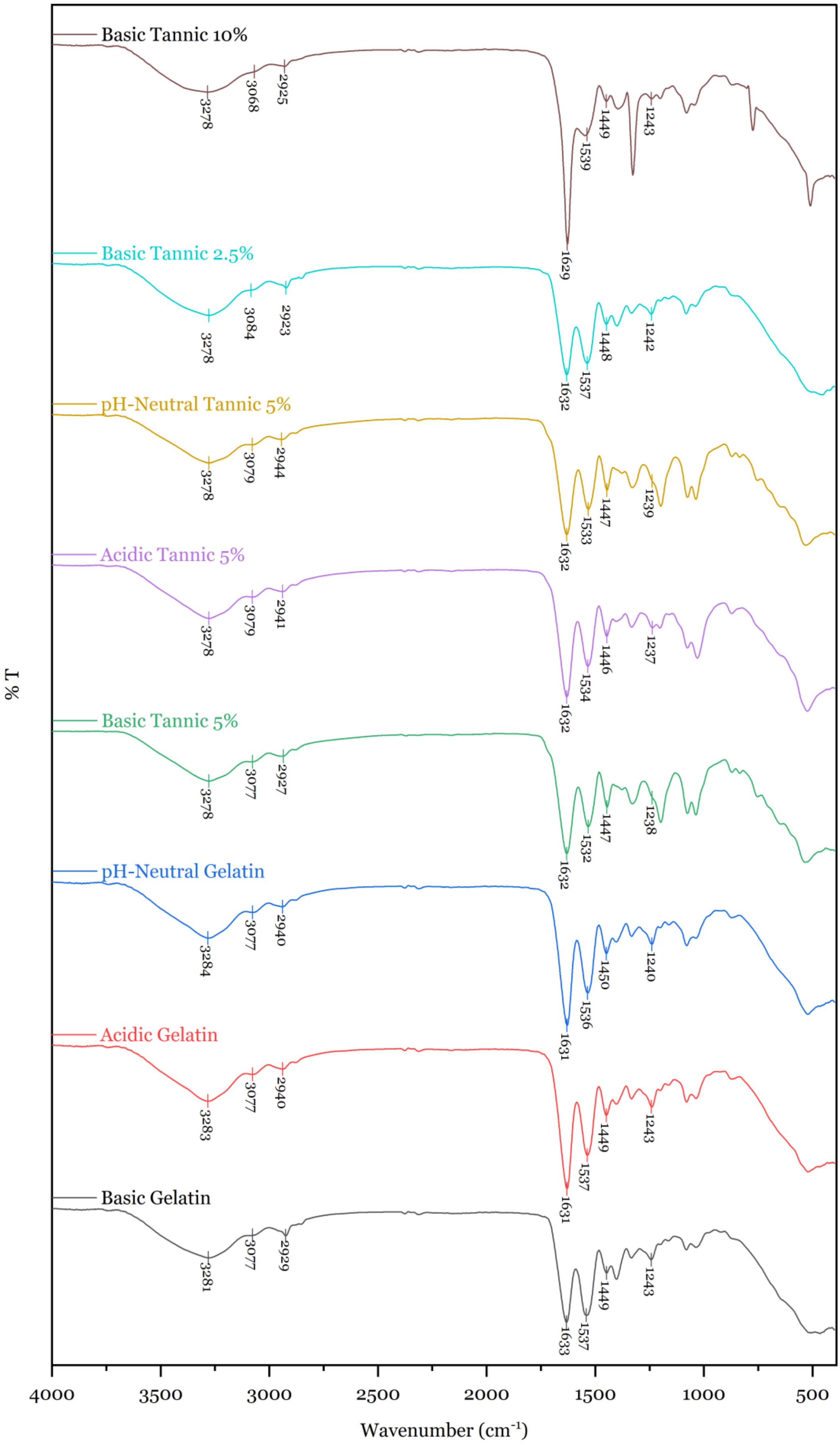


Figure 5. Infrared spectrum of film of gelatin cross-linked with tannic acid at different acidities and concentrations.

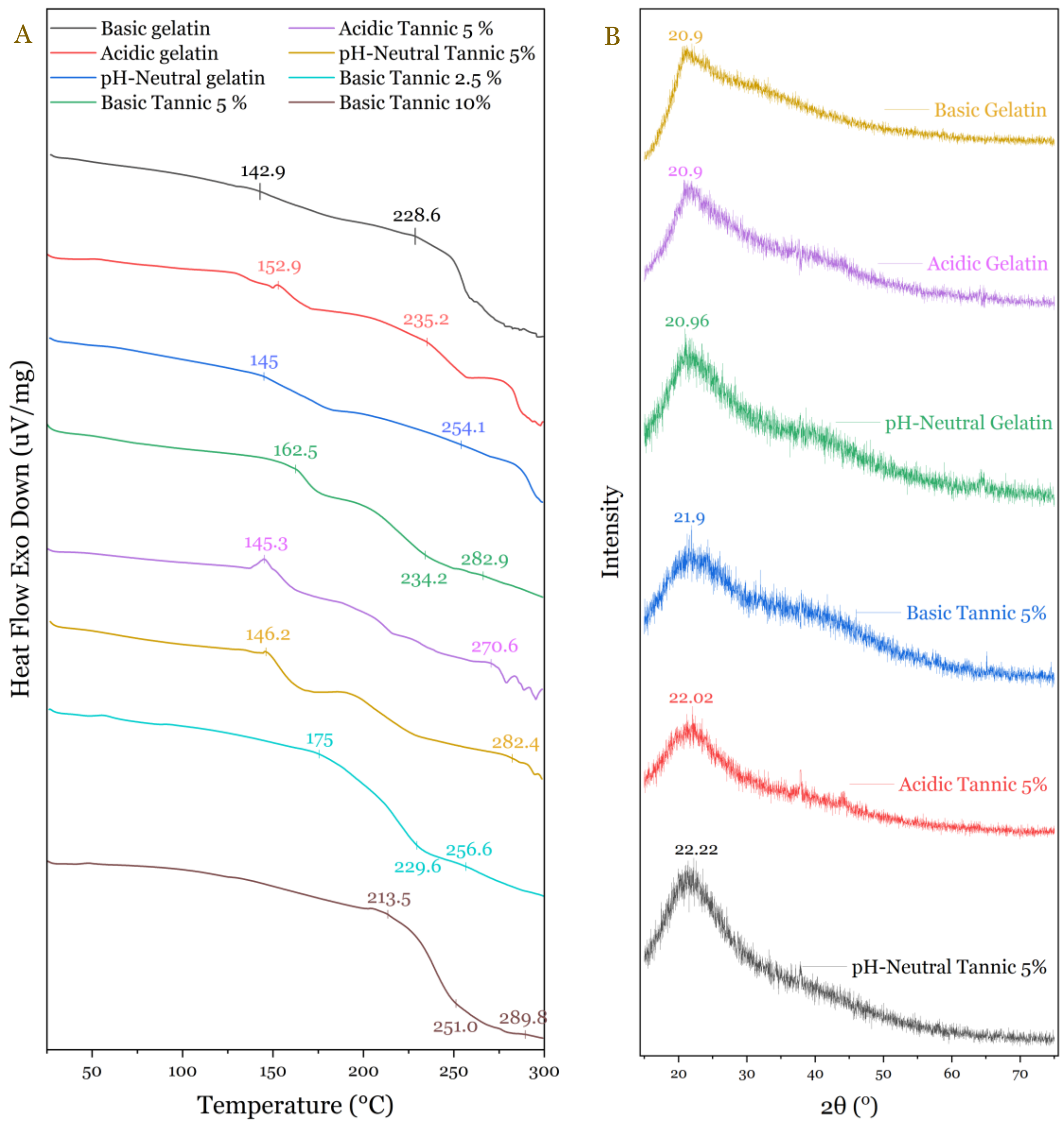


Figure 6. Differential scanning calorimetry (DSC) thermograms (A) and X-ray diffraction patterns (B) of film of gelatin cross-linked with tannic at different acidities and concentrations.

Table 5. Degree of crystallinity of tannic acid-cross-linked gelatin film

Sample	Degree of crystallinity (%)
Basic tannic 5%	57.51
Acidic tannic 5%	56.52
pH-neutral tannic 5%	55.82
Basic gelatin	51.86
Acidic gelatin	53.90
pH-neutral gelatin	54.69

The effects of pH levels and tannic acid concentration on the physicochemical properties of the gelatin are presented in **Table 6**. Basic tannic 10% had the lowest moisture content (9.24±0.14%), the highest cross-linking degree (69.99±0.84%), the highest viscosity (8.48±0.23 cP), and the highest gel strength (151.5±6.9 Bloom). Swelling indices were observed the lowest in the basic tannic 10% sample, where the values were found to correspond to the tannic acid concentration (1052±51, 420±34, and 323±17% for tannic acid addition with 2.5%, 5%, and 10% concentrations, respectively).

The effects of pH and cross-linker concentration on mechanical and water vapor permeability profiles are presented in **Table 7**. The highest tensile strength (7.00±0.54 N.cm^{-2}) and the lowest elongation at the break (114.08±14.63%) were observed in basic tannic 10%. Basic tannic acid 10% had the highest elastic modulus and the lowest water vapor permeability of 58.45±8.20 N.cm^{-2} and 0.57±0.07 g.mm.m^{-2}.h^{-1}. kPa^{-1}, respectively.

Table 6. Physicochemical properties of milkfish gelatin produced with different pH levels and tannic acid concentrations

Group	Moisture content (%)	Swelling index (%)	Cross-linking degree (%)	Viscosity (cP)	Gel strength (bloom)
Basic tannic 5%	9.46±0.23 ^{a}	420±34 ^{b}	49.74±3.00 ^{b}	7.82±0.41 ^{b}	144.7±4.9 $^{a, b}$
Acidic tannic 5%	9.62±0.20 $^{a, b, c}$	618±47 ^{c}	34.86±4.45 ^{c}	NA *	132.5±5.6 $^{a, b*}$
pH-neutral tannic 5%	9.53±0.22 $^{a, b}$	621±66 ^{c}	35.12±2.67 ^{c}	NA *	138.6±3.4 $^{a, b*}$
Basic tannic 2.5%	9.95±0.2 $^{b, c}$	1052±51 ^{d}	34.73±1.97 ^{c}	7.27±0.17 ^{c}	141.3±5.8 ^{b}
Basic tannic 10%	9.24±0.14 ^{a}	323±17 ^{a}	69.99±0.84 ^{a}	8.48±0.23 ^{a}	151.5±6.9 ^{a}
Basic gelatin	10.07±0.21 ^{c}	Dissolve	NA	6.12±0.25 ^{d}	132.0±9.8 ^{c}
Acidic gelatin	10.00±0.26 ^{c}	Dissolve	NA	6.16±0.26 ^{d}	131.4±9.0 ^{c}
pH-neutral gelatin	10.59±0.41 ^{d}	Dissolve	NA	6.36±0.24 ^{d}	134.6±6.4 ^{c}

NA: not applicable

* The film-forming gel, consisting of hazy flocculant precipitates

Values with different alphabets in the same column are statistically significant at $p<0.05$ based on Duncan's post-hoc test

Table 7. Mechanical and water vapor permeability profile of milkfish gelatin film produced with different pH levels and tannic acid concentrations

Groups	Tensile strength (N.cm^{-2})	Elongation at break (%)	Elastic modulus (N.cm^{-2})	Water vapor permeability (g.mm.m^{-2}.h^{-1}. kPa^{-1})
Basis tannic 5%	6.29±1.03 $^{a, b}$	126.21 ±15.93 $^{a, b}$	55.88±2.26 $^{a, b}$	0.71±0.06 ^{b}
Acidic tannic 5%	6.18±0.74 $^{a, b}$	134.76 ±12.33 $^{a, b}$	52.62±6.99 ^{b}	0.87±0.06 ^{c}
pH-neutral tannic 5%	6.34± 0.80 $^{a, b}$	132.12 ±9.88 $^{a, b}$	52.90±4.60 ^{b}	0.85±0.05 ^{c}
Basic tannic 2.5%	5.24±0.91 ^{b}	140.22 ±20.92 $^{a, b}$	43.26±6.66 ^{c}	0.90±0.06 ^{c}
Basic tannic 10%	7.00±0.54 ^{a}	114.08 ±14.63 ^{a}	58.45±8.20 ^{a}	0.57±0.07 ^{a}
Basic gelatin	2.82±0.34 ^{c}	151.98 ±17.87 ^{b}	20.10±0.78 ^{d}	1.14±0.06 ^{d}
Acidic gelatin	2.52±0.31 ^{c}	152.86 ±11.46 ^{b}	21.94±2.98 ^{d}	1.17±0.06 ^{d}
pH-neutral gelatin	3.20±0.53 ^{c}	147.94 ±10.36 ^{b}	18.79±1.84 ^{d}	1.19±0.07 ^{d}

Values with different alphabets in the same column are statistically significant at $p<0.05$ based on Duncan's post-hoc test

Discussion

The extracted piscine gelatin had a pH of 5.33±0.21 with protein, hydroxyproline, moisture, and ash contents of 80.79±0.80, 7.94±0.16, 8.89±0.15, and 1.65±0.08%, respectively. Furthermore, the milkfish gelatin had a viscosity of 6.36±0.24 cP and a gel strength of 134.6±6.4 Bloom. The extraction process employed high-temperature heating, which induced extensive collagen hydrolysis and resulted in poor-quality gelatin. The gelatin complies with the specifications set for type A gelatin, including a pH range of 3.8–5.5, a moisture content of approximately 8–13%, and an ash content of 0.3–2% [23]. Moreover, type A gelatin is characterized by a viscosity ranging between 1.5–7.5 cP and a gel strength between 50–300 Bloom [23]. The characteristics are similar to those of piscine gelatin in general, where they were reported with protein content ranging from 71.1% to 89.7% and hydroxyproline content ranging from 5.2% to 9.5% [6,7]. However, the gel strength of the milkfish gelatin in the present study was still lower compared to those produced from porcine skin (300 Bloom) or bovine skin (225 Bloom).

The cross-linking of gelatin with phenolics was achieved between the phenolic reactive sites of the phenolic compounds and the amino groups in gelatin to form C-N covalent bonds. In general, phenolics are spontaneously oxidized under basic conditions and subsequently form quinone groups. Herein, phenolic oxidation was observed by the darker-colored gelatin samples in basic conditions.

An infrared spectrum of the non-cross-linked film indicates the characteristic peaks for gelatin, including amide A, amide B, amide I, and amide II. In gelatin films added with phenolics, these peaks were shifted, indicating the formation of cross-linkage. Moreover, the cross-linking between gelatin and oxidized phenolics was identified by a downshift in the peaks assigned to amides A, B, and II, together with a reduction in the intensity of the amide II peak

[12]. The formation of cross-linkage between gelatin and oxidized phenolics was corroborated by the increase in melting temperature, as indicated by the differential scanning calorimetry (DSC) thermogram. According to a previous report, the shifted endothermic peak in the DSC thermogram indicates the presence of cross-links through the strengthening of covalent bonds, hydrogen bonds, and hydrophobic interactions [12]. Some films produced in the present study had exothermic valleys in the DSC thermogram that suggested the presence of crystalline phases. The exothermic valleys were most pronounced in samples cross-linked with gallic acid and tannic acid. Gelatin contains a semicrystalline structure that is inherently generated from an alpha-helix arrangement, in addition to the amorphous structure [24]. The X-ray diffraction pattern herein showed a large broadening diffraction peak, confirming its semicrystalline structure [25]. The X-ray diffraction pattern of water molecules can be neglected due to the fact that the entire film consists of approximately 10% water. Gelatin films cross-linked with complex phenolic structures, such as gallic acid and tannic acid, were observed to have high crystallinity degrees. The 2θ peak of the cross-linked gelatin exhibited a slight upshift in comparison to the non-cross-linked film. In line with a previous study, cross-linkage in gelatin causes a reduction in the space between the atomic trellis planes [11].

The type of phenolic influences the physicochemical characteristics of gelatin cross-linked film or film-forming gel, such as the swelling index, cross-linking degree, viscosity, and gel strength. Herein, some cross-linked gelatin films had a slightly higher moisture content compared to the non-cross-linked group. All gelatin films, except for the phenol group, were not dissolved in water, but they absorbed water and swelled. An increase in cross-linking degree reduced the swelling index. The rigid cross-link structure hinders water penetration into the film, causing decreased water uptake [12]. Further, increasing the degree of cross-linking led to improved viscosity and gel strength. As compared with non-cross-linked gelatin, the addition of complex phenolic also improved the mechanical and water vapor permeability characteristics including tensile strength, elongation at break, and elastic modulus. In the present study, tannic acid was found to have a significant impact on improving the physicochemical and mechanical properties of the gelatin film. We also found that simple phenolic structures such as phenol, catechol, and resorcinol, which contain one or two hydroxyl groups, exhibited a low effect on improving the characteristics. More complex phenolic structures (such as curcumin, gallic acid, quercetin, and tannic acid) and those with additional aromatic, carboxylate, amine, and aldehyde groups (such as α-naphthol, tyrosine, and vanillin) exhibited higher effects compared to simple phenolics.

In the present study, varying the pH of tannic acid-cross-linked gelatin resulted in the sample appearance with different colors, where darker colors were observed in basic conditions. This confirmed the spontaneous oxidation of tannic acid in basic conditions, thus facilitating higher cross-linkage formation. The cross-linking formation was observed through the shifted infrared spectral peaks assigned to amide A, amide B, and amide II, reduced crystalline peaks in X-ray diffraction and shifted endothermic peaks in DSC thermogram. The cross-linking degree was found to be concentration-dependent in basic conditions, with basic tannic 10% having the highest value. The cross-linking formation with tannic acid, particularly in basic conditions, could be attributed to the improvement of overall physicochemical characteristics. Tannic acid is a strong cross-linking agent owing to the possession of multiple phenol groups, which can bind to multiple polymer strands of gelatin. Under basic conditions, tannic acid induces the spontaneous oxidation of hydroxyl groups into a quinone group. The oxidative derivatives of tannic acid would chemically react with the primary amino group of lysine in the gelatin backbone, producing a covalent bond.

The cross-linking of phenolics with milkfish gelatin offers a promising option as a halal material alternative to major sources like porcine and bovine and has the potential to be utilized in the field of pharmaceuticals and medicines. The material produced in the present study meets the necessary requirements for a wide range of manufacturing applications, including capsule shells, nanoencapsulation matrices for drug delivery, gel bases, and others [26,27]. Human albumin, an intravenous solution that is forbidden (haram) for Muslims, can be substituted with cross-linked fish gelatin as a plasma expander, which is considered permissible (halal) [27,28]. Wound healing can be facilitated by applying films and hydrogels that utilize a gelatin-cross-linked matrix as a tissue adhesive [29,30]. Despite the strength and potential of the milkfish gelatin revealed in the present study, interpretation of the findings should be taken with caution. The limitations of the study include the absence of other important characterizations such as contact angle, wettability, cytotoxicity, and lifetime of the gelatin film and gel. Moreover, prior to its application in therapy and commercialization, phenolic cross-linked gelatin needs to pass preclinical and clinical toxicity testing.

Conclusion

The film and film-forming gel were enhanced by cross-linking gelatin with phenolics, as indicated by the improved swelling index, degree of cross-linking, viscosity, gel strength, mechanical profile, endothermic peak, and water vapor permeability. The most optimum improvement of the characteristics was observed in piscine gelatin cross-linked with tannic acid. The characteristics can be improved by further increasing the pH level and tannic concentration. The gelatin films manufactured with 10% tannic acid in basic condition reached the highest overall physicochemical and mechanical properties. Cross-linked gelatin offers the potential of being a halal raw material alternative to porcine and bovine in the production of capsule shells, nanoencapsulation matrices, gel bases, and other types of medicines.

Ethics approval

Not required.

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

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