Mechanical, Physical, Antioxidant, and Antimicrobial Properties of Gelatin Films Incorporated with Thymol for Potential Use as Nano Wound Dressing

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Abstract: The aim of this study was to determine the properties of gelatin films incorporated with thymol. Gelatin films were prepared from gelatin solutions (10% w/v) containing thymol (1, 2, 4, and 8% w/w), glycerol (25% w/w) as plasticizer, and glutaraldehyde (2% w/w) as cross-linker. Cross-likened films showed higher tensile strength, higher elongation at break, lower Young's modulus, lower water solubility, lower swelling, lower water uptake, and lower water vapor permeability. Incorporation of thymol caused a significant decrease in tensile strength, increase in elongation at break, decrease in Young's modulus, increase in water solubility, decrease in swelling and water uptake, and increase in water vapor permeability slightly. The films incorporated with thymol exhibited excellent antioxidant and antibacterial properties. The antibacterial activity of the films containing thymol was greatest against *Staphylococcus aureus* followed by *Bacillus subtilis* followed by *Escherichia coli* and then by *Pseudomonas aeruginosa*. Thus, gelatin films-containing thymol can be used as safe and effective source of natural antioxidant and antimicrobial agents with the purpose of evaluating their potential use as modern nano wound dressing.

Keywords: antibacterial, gelatin films, physical properties, thymol, wound dressing

Practical Application: This study clearly demonstrates the potential of gelatin films incorporated with thymol as natural antioxidant and antimicrobial nano film. Such antimicrobial films exhibited excellent mechanical, physical, and water activities and could be used as antibacterial nano wound dressing against wounds burn pathogens.

Introduction

Gelatin is a natural biopolymer derived from controlled hydrolysis of the fibrous insoluble collagen present in the bones and skin and has good biocompatibility and biodegradability. It is an effective biomaterial for using as wound dressing since it can absorb wound exudates and provide moist environment to a wound leading to acceleration of wound healing (Gomez-Guillen and others 2011). The best moist wound dressing must prevent infection, remove blood and excess exudates, provide or maintain moist environment, allow gaseous exchange, be thermal insulation, comfortable and easily removable, and be nontoxic and nonallergic (Boateng and others 2008). However, gelatin itself has no antimicrobial activity to prevent wound infection. Silver nanoparticles (Vimala and others 2010) and Zinc oxide nanoparticles (Li and others 2010) successfully incorporated to gelatin films as antimicrobial agent. Natural phenolic monoterpenes derived from herbal medicines also can be added to the gelatin films as antimicrobial agent to improve its antimicrobial activity.

Thymol (2-isopropyl-5-methyl phenol) is a phenolic monoterpenes in the essential oils derived from genera *Origanum*, *Thymus*, *Coridithymus*, *Thymbra*, *Satureja*, and *Lippia* that has been used for many generations as food preservative (Baser 2008). Antioxidant (Huang and others 2011), antibacterial (Xu and others 2008; Garcia-Garcia and others 2011), antifungal (Rao and others 2010; Ahmad and others 2011), and antiparasite (Tasdemir and others 2006) activities of thymol were confirmed. Although advances in chemical and pharmacological evaluation of thymol have occurred in the recent past however, several useful features of thymol have remained unknown. In this study the gelatin films with antioxidant and antimicrobial activities were prepared from gelatin solutions containing different thymol concentrations. Antioxidant activity of the gelatin films incorporated with thymol was examined using 2′-azino-di (3-ethylbenzthiazoline-6-sulfonate) (ABTS) decolorization. The gelatin films containing thymol individually tested against 2 Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and 2 Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) commonly found in human pathogenesis. The main objective of the present study was to examine the gelatin films grafted with thymol as antioxidant and antimicrobial agents with the purpose of improving their potential use as antioxidative and antimicrobial nano wound dressing.

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Materials and Methods

Preparation of gelatin film forming solution

To prepare gelatin film forming solutions, 10 g of bovine gelatin powder (Merck, Germany) dissolved into 100 mL of distilled water (10% based on the volume of the mixed solvent) at ambient temperature and temperature increased to 60 °C using a Hotplate stirrer and the mixture was stirred for 30 min at this temperature. After cooling to 37 °C, thymol (Fluka, Germany) with different concentration (1, 2, 4, and 8% based on the weight of the gelatin powder) as antimicrobial agent and glycerol (25% w/w based on the weight of the gelatin powder) (Merck, Germany) as plasticizer were added to gelatin solutions and stirred for 10 min. Thereafter, glutaraldehyde (2% w/w based on the weight of the gelatin powder) (Merck, Germany) added to the solution as cross-linker. The gelatin solution was homogenized at 2000 rpm for 10 min using a homogenizer. The dissolved air in the gelatin solution was removed by a vacuum pump for 30 min at room temperature (Ahmad and others 2012; Nunez-Flores and others 2012).

Preparation of gelatin films

To casting the films, 10 mL of gelatin film forming solutions containing different thymol concentrations were transferred into the polyester Petri dish (Farazbin Kimia Co., Tehran, Iran, the radius of 74 mm) and placed for 24 h at room temperature and then for 12 h at 37 °C and 45% relative humidity in an environmental chamber until films were dried. The dry films obtained were peeled off and stored until analysis. Thicknesses of films were measured to the nearest 0.01 mm with a digital micrometer (The L.S. Starrett Co. Ltd., , UK) and the average was taken 145 ± 5 μm.

Mechanical properties of gelatin films

The mechanical properties of films were achieved according to American Society for Testing and Materials 638-02a (ASTM D 638-02a) in texture analyzer (TA.XT stable Micro System UK). Gelatin films containing different concentration of thymol transferred to a closed container with relative humidity of 65% (saturated sodium nitrite vapor) and left for equilibrium for 48 h before mechanical testing. Films were cut into the rectangles with length of 60 mm, width of 10 mm, and thickness 0.14 mm. The tensile strength test was then performed by stretching the film at pretest, test, and posttest speeds of 1, 1, and 10 mm/s, respectively. The net length between the jaws for all films was almost constant at about 20 mm. The texture analyzer runs at auto force mode with the trigger force of 5 g (0.049 Newton). From stress–strain curves, 3 parameters were calculated: 1) tensile strength (TS) was calculated as maximum stress, 2) Young's modulus (E) the initial slope of the stress–stain curves at the linear part, 3) elongation at break (EAB) where the film is torn (Ahmad and others 2012; Nunez-Flores and others 2012):

$$TS(N/m^2) = (\text{Breaking force}/\text{Cross} - \text{sectional area of sample})$$

$$EAB(\%) = [(\text{Increase in length at breaking point} / \text{Initial length})] \times 100$$

$$E(MPa) = [(F/A_0)]/[(\Delta L/L_0)]$$

where E is the Young's modulus (modulus of elasticity), F the force exerted on an object under tension, A_0 to original cross-sectional area through which the force is applied, ΔL the amount by which the object changes, L_0 the original length of the object (http://en.wikipedia.org/wiki/young-modulus). The area of film used for each experiment was 6 × 1 cm^2. However, 2 cm of the film was within the jaws, so the initial length of the film was taken as 4 cm^2. All tests are the means of at least 3 measurements.

Water solubility of gelatin films

For solubility determination, one piece of films (20 mm × 20 mm × 0.14 mm) placed in an oven at 104 °C for 24 h and initial dry weight (W_i) was calculated. Then, the dried films immersed into 100 mL Erlenmeyer flask containing 50 mL of distilled water and placed inside the shaker for 24 h at 25 °C (Incubator with inventilator, Pars Azma Co. Tehran, Iran). Thereafter, the films were taken out and transferred to the oven of 104 °C for 24 h and the final dry weight (W_f) was calculated. The cross-linking or weight remaining percentage can be determined using following formula: cross-linking (%) = $[(W_f / W_i)] \times 100$. The weight losing or solubility percentage (S%) was determined using following formula (Ahmad and others 2012; Nunez-Flores and others 2012): S (%) = $[(W_{i-} W_f) / W_i] \times 100$. All tests are the means of at least 3 measurements.

Swelling test of gelatin films

The films were dried in an air-circulating oven at 104 °C for 24 h until reached to constant weight before swelling test (W_i). Square were cuts with the dimension of 20 mm × 20 mm × 0.14 mm used for swelling experiment. Each sample was immersed into a 100 mL Erlenmeyer flask containing 50 mL of the distilled water. The samples were kept at room temperature for the duration of swelling experiment (24 h). Each sample was taken out of the flask after 24 h, wiped between filter papers to remove the excess surface water and were weighed (W_f). The swelling percentage (SW%) was calculated using the following equation (Ahmad and others 2012): SW (%) = $[(W_{f-} W_i) / W_i] \times 100$. All tests are the means of at least 3 measurements.

Water uptake of gelatin films

The water uptake was determined for all films as follow. The films (20 mm × 20 mm × 0.14 mm) dried in desiccators at concentrated H_2SO_4 (relative humidity = 0%) for 3 d to constant weight were achieved (W_i). Then films transferred into desiccators at 100% relative humidity (sodium sulfate solution) at 37 °C for 1 wk and allowed to absorb water, and then weighed after the equilibrium state reached (W_f). The water uptake percentage calculated using the following equation (Ahmad and others 2012): water uptake (%) = $[(W_{f-} W_i) / W_i] \times 100$. All tests are the means of at least 3 measurements.

Water vapor permeability of gelatin films

The water vapor permeability (WVP) was determined according to the ASTM E96-95 method. The films were conditioned for 24 h at 25 °C and 75% relative humidity before WVP determination. Film samples were mounted on an aluminum cup (height and diameter of 2.1 and 5.6 cm, respectively). The cup was filled with 20 g of silica gel and covered with a film specimen. The cup was placed at 25 °C and 75% relative humidity in desiccators. The weight of the cup was measured at 3 h intervals during 1 d. Simple linear regression was used to estimate the slope of mass change compared with time plot. The WVP was calculated using the following formula (Nunez-Flores and others 2012): WVP (g mm kPa^{-1} m^{-2} h^{-1}) = $[(WVTR \times T)] / \Delta P$,

where water vapor transmission rate (WVTR) is the slope per film area (g m^{-2} h^{-1}), T the film thickness (mm), and ΔP the partial water vapor pressure difference (kPa) between the 2 sides of the film (4.2449 kPa at 30 °C).

Color analysis of gelatin films

Color values of the films were evaluated by measuring the L* (lightness/brightness), a* (redness/greenness), and b* (yellowness/blueness) values using a Fujifilm digital camera (A202, FinePix, China) installed at a 30 cm constant distance from the film surface and then the images were analyzed by Photoshop software 8. The lamp and the camera were placed in a box (50 × 50 × 60 cm) with interior white walls. The axis of the camera lens was perpendicular to the surface. The angle between the film surface and light source was 45°. Illumination was achieved using a 40-Watt fluorescent light lamp (Natural Daylight, Cixing, China). Total color difference (ΔE) was determined using following formulas (Ahmad and others 2012; Nunez-Flores and others 2012):

$$\Delta E = [(L^*_{uncross-linked} - L^*_{sample})^2 + (a^*_{uncross-linked} - a^*_{sample})^2 + (b^*_{uncross-linked} - b^*_{sample})^2]^{0.5}$$

Antioxidant activity of gelatin films

Antioxidant activity of the films was determined by decolorization method with 2, 2′-azino-di (3-ethylbenzthiazoline-6-sulfonate) (ABTS, Sigma, Germany) (Re and others 1999). The method was modified to detect the continuous antioxidant release from films. The release tests were performed in 24 well plates. Briefly, films cuts (10 mm × 10 mm × 0.14 mm) from different parts of the films containing 1, 2, 4, and 8% of the thymol were added to 2.0 mL of diluted ABTS radical solution (7 mM ABTS and 2.54 mM potassium persulfate, A734 = 1 ± 0.1). Films without thymol were used as blank. The program was adjusted to record the absorbance values after shaking the 24 well plates for 30 s using a plate reader (BioTek Elx 808, Winooski, Vt., U.S.A.). The data recorded up to steady state were reached for each sample. A standard curve of ascorbic acid ranging from 0.44 to 15.76 mg/mL was prepared. Antioxidant activity was expressed as mg ascorbic acid equivalents per gram of films using standard curve.

Antibacterial assay of gelatin films using disk diffusion

All microorganisms obtained from the Persian type culture collection (PTCC), Tehran, Iran. The films were individually tested against 2 Gram-negative bacteria (*P. aeruginosa* PTCC 1074 [ATCC 9027 and *E. coli* PTCC 1330 [ATCC 8739]) and 2 Gram-positive bacteria (*S. aureus* PTCC 1112 [ATCC 6538] and *B. subtilis* PTCC 1023 [ATCC 6633]). To investigate the antimicrobial activity of the films using disk diffusion, 30 mm diameter disks were cut from different parts of the films and sterilized by autoclaving for 30 min at 120 °C (Bauer and others 1996). Bacterial suspensions with a turbidity equivalent to a McFarland 0.5 standard were prepared (10^8 CFU/mL) and then diluted to 10^5 CFU/mL with Luria-Bertani (LB). The adjusted bacterial suspensions (0.1 mL) spread onto the nutrient agar plates containing LB (Farazbin Kimia Co., Tehran, Iran). Subsequently, the disks placed in direct contact with the agar medium. Plates inverted and incubated at 37 °C for 24 h (Incubator with inventilator, Pars Azma Co. Tehran, Iran). Films without thymol under the same condition were used as control. The diameters of clear inhibition zones, including the diameter of the disk, measured using a ruler were used to evaluate antibacterial potential of films.

Antibacterial assay of gelatin films using colony counting

The bacteria colony counting assays were carried according to Clinical and Laboratory Standards Institute (CLSI). Bacteria strains suspended in LB media and the densities adjusted to 0.5 McFarland standards at 570 nm (10^8 CFU/mL) and then diluted to 10^5 CFU/mL with LB. A sample film with 30 mm diameter was placed in a 10 mL liquid culture containing 10 μL microbe cultures. Then, the sample was incubated at 37 °C for 24 h (Shaking Incubator, Shin Saeng, Fine Tech, Korea). From the incubated samples, a 100 μL solution was taken and diluted with the appropriate dilution factor and the final diluted microbe solution was plated and distributed onto nutrient agar plate (Farazbin Kimia Co., Tehran, Iran). The plate cultured with the films without thymol under the same condition was used as control. All plates were incubated at 37 °C for 24 h and the numbers of colonies that formed were counted. The antibacterial efficacy of the films was calculated according to the following equation (Maneerung and others 2008): Colony reduction (%) = [(Number of colony in test samples − Number of colony in control)/ Number of colony in test samples] × 100.

Statistical analysis

All *data* are representative of at least 3 independent experiments. Data *are expressed as the means* plus standard deviations. The significant differences between treatments were analyzed by one-way analysis of variance (ANOVA) and the Duncan tests at $P < 0.05$ using statistical package for the social sciences (SPSS, Abaus Concepts, Berkeley, Calif., U.S.A.) and Prism 5 (Graph Phad, San Diego, Calif., U.S.A.) softwares.

Results and Discussion

Mechanical properties of gelatin films

The mechanical properties of gelatin films are summarized in Table 1. Tensile strength, elongation at break, and Young's modulus are the parameters that relate mechanical properties of films to their chemical structures. Cross-linking caused a significant increase in tensile strength ($P < 0.05$), a significant increase in elongation at break ($P < 0.05$), and a significant decrease in Young's modulus ($P < 0.05$). Incorporation of thymol especially at higher concentration caused a significant decrease in tensile strength ($P < 0.05$), a significant increase in elongation at break ($P < 0.05$), and a significant decrease in Young's modulus ($P < 0.05$).

Gelatin films were mainly stabilized by the weak bond including hydrogen bond and hydrophobic interaction. Cross-linking with glutaraldehyde by inserting covalent bond between gelatin strands leads to a significant increase to tensile strength and rigidity of films (Akin and Hasirci 1995; Rujitanaroj and others 2008). However, thymol incorporation especially at higher concentrations caused a significant decrease in films tensile strength. Addition of thymol possibly resulted in the lowered interaction between gelatin monomers, and may hinder polymer chain-to-chain interactions and reduce cross-linking (Table 1) and consequently, the decrease in rigidity with the simultaneous decrease in elasticity of film was gained (Fabra and others 2008; Limpisophon and others 2010). Study on the properties of gelatin films incorporated with citrus oil (mainly contains limonene) clearly indicated that films incorporated with essential oil showed the lower tensile strength but

Table 1–Mechanical properties and cross-linking percent of gelatin films incorporated with thymol.

Films	Tensile strength (N m^{-2})[1]	Elongation (%)[2]	Young's modulus (MPa)[3]	Cross-linking[4] (%)
Uncross-linked	2.9 ± 0.32^{b}	57 ± 7^{e}	10.7 ± 0.7^{a}	35.5 ± 3^{c}
Cross-linked	4 ± 0.4^{a}	125 ± 9^{d}	8.7 ± 0.2^{b}	62.5 ± 1.5^{b}
Cross-linked + Thymol 1%	1.7 ± 0.3^{c}	145 ± 8^{bc}	2.4 ± 0.3^{c}	70 ± 1.3^{a}
Cross-linked + Thymol 2%	1.4 ± 0.15^{c}	150 ± 6^{b}	2.2 ± 0.21^{c}	69 ± 2^{a}
Cross-linked + Thymol 4%	1.3 ± 0.17^{cd}	160 ± 7^{ab}	2 ± 0.17^{cd}	68 ± 1.6^{a}
Cross-linked + Thymol 8%	1.2 ± 0.2^{d}	170 ± 5^{a}	1.8 ± 0.14^{d}	67 ± 1.5^{a}

[a–e]Mean values with different letters within a column are significantly different by Duncan's multiple range test at ($P < 0.05$).
[1]Tensile strength was expressed as maximum stress in Newton per square.
[2]Elongations at break where the film is torn in percent.
[3]Young's modulus was expressed as the initial slope of the stress–stain curves at the linear part in megapascal.
[4]Cross-linking was expressed as weight remaining of films in distilled water after 24 h.

Table 2–Water solubility, swelling, water uptake, and water vapor permeability of gelatin films incorporated with thymol.

Films	Water solubility[1] (%)	Swelling[2] (%)	Water uptake[3] (%)	WVP[4] (g mm kPa^{-1} m^{-2} h^{-1})
Uncross-linked	64.3 ± 2.5^{a}	707 ± 15^{a}	143 ± 4^{a}	0.27 ± 0.05^{b}
Cross-linked	27.7 ± 1.6^{c}	390 ± 17^{b}	128 ± 3^{b}	0.20 ± 0.01^{c}
Cross-linked + Thymol 1%	30 ± 1.3^{b}	385 ± 12^{bc}	126 ± 4^{b}	0.21 ± 0.05^{c}
Cross-linked + Thymol 2%	31 ± 1.0^{b}	375 ± 8^{c}	124 ± 3^{b}	0.23 ± 0.04^{bc}
Cross-linked + Thymol 4%	32 ± 1.8^{b}	362 ± 5^{c}	119 ± 2^{bc}	0.27 ± 0.03^{b}
Cross-linked + Thymol 8%	33 ± 2^{b}	348 ± 7^{d}	115 ± 3^{c}	0.34 ± 0.05^{a}

[a–d]Mean values with different letters within a column are significantly different by Duncan's multiple range test at ($P < 0.05$).
[1]Water solubility was expressed as weight losing percent of films in distilled water after 24 h.
[2]Swelling was expressed as weight gaining of films in distilled water after 24 h.
[3]Water uptake was expressed as weight gaining of films in distilled water after 24 h.
[4]Water vapor permeability was expressed as g mm kPa^{-1} m^{-2} h^{-1}.

higher elongation at break rather than the control films without incorporated essential oil (Tongnuanchan and others 2012). The gelatin films have good mechanical properties and these properties changed slightly in the presence of thymol.

Solubility determination of gelatin films

The solubility (weight losing after 24 h) percentages of the films are summarized in Table 2. Cross-linking caused a significant decrease ($P < 0.05$) in water solubility. Gelatin is water-soluble and it may partially dissolve and lose fibrous structure to high ambient humidity especially for long period of time. However, cross-linking can stabilize gelatin structure and decrease its solubility in aqueous medium (Akin and Hasirci 1995; Rujitanaroj and others 2008). Incorporation of thymol especially at higher concentrations caused a significant increase ($P < 0.05$) in solubility of the films. Addition of thymol in films may hinder polymer chain-to-chain interactions and reduce cross-linking and consequently, increase the solubility of the films. Some study reported a significant increase in the gelatin-chitosan films solubility in the presence of essential oil (Gomez-Estaca and others 2010). Generally, the effects of the additives on the solubility of films depend on the type of compounds and concentrations and their hyrophilicity and hydrophobicity index (Nunez-Flores and others 2012). Hydrophilic compounds could increase while hydrophobic compounds could decrease films solubility (Rhim and others 2000; Ahmad and others 2012). The cross-linked gelatin films have low solubility and this solubility increased slightly but not very large in the presence of thymol.

Swelling capacity and water uptake of gelatin films

The results of swelling and water uptake capacity of the films were summarized in Table 2. Cross-linking caused a significant decrease ($P < 0.05$) in swelling and water uptake capacity of the films. Incorporation of thymol especially at higher concentration caused a significant decrease ($P < 0.05$) in swelling and water uptake of the films. Gelatin is a hydrophilic material that is expected to absorb molecule of water. However, cross-linking within the gelatin chain networks may be responsible to the lower swelling and water uptake capacity (Vimala and others 2010; Singh and Pal 2012). The higher cross-linking restricts the water penetration for swelling and water uptake. The porous uncross-linked films showed higher swelling capacity because of presence of porosity in their network structures that allow more water to enter inside the film. However, incorporation of thymol reduced swelling capacity of the cross-linked films due to its hydrophobicity. Generally, the effects of the additives on the swelling of films depend on the type of compounds and their hyrophilicity and hydrophobicity index (Ahmad and others 2012; Nunez-Flores and others 2012). Hydrophilic compounds could increase while hydrophobic compounds could decrease films swelling (Vimala and others 2010; Singh and Pal 2012). Gelatin films have excellent swelling and water uptake capacity and these properties reduced slightly in the presence of thymol due to hydrophobicity of the thymol.

Water vapor permeability of gelatin films

The results of WVP of the films are summarized in Table 2. The WVP in the cross-linked gelatin film significantly decreased ($P < 0.05$) rather than uncross-linked films. Incorporation of thymol especially at higher concentration caused a significant increase ($P < 0.05$) in WVP of the films. The water vapor transfer process in films depends on the hydrophilic–hydrophobic ratio of the film constituents and the degree of cross-linking. Gelatin is a hydrophilic material that is expected absorb water vapor and so caused the increase in WVP. However, cross-linking within the gelatin chain networks may be responsible to the lower water vapor transfer. The higher cross-links restrict the water vapor penetration across the films (Nunez-Flores and others 2012). Thymol at higher concentration may hinder polymer chain-to-chain interactions and reduce cross-linking (Table 1) and consequently, the increase in WVP was occurred. Essential oils or their components also have different ability to attract water to the film network. The WVP of gelatin films incorporated with lemongrass oil decreased while the WVP of gelatin films incorporated with bergamot oil increased (Ahmad and others 2012). The WVP of chitosan films

Table 3–Color parameters of gelatin films incorporated with thymol.

Essential oil (%)	L*[1]	a*[2]	b*[3]	ΔE[4]
Uncross-linked	55 ± 1.9^{a}	-7.4 ± 1.7^{c}	1.7 ± 0.9^{c}	–
Cross-linked	46 ± 1.6^{b}	3.4 ± 1.2^{b}	44 ± 1.6^{a}	45 ± 1.7^{b}
Cross-linked + Thymol 1%	46 ± 1.3^{b}	4 ± 1.6^{b}	44 ± 1.7^{a}	45 ± 2^{b}
Cross-linked + Thymol 2%	44 ± 1.5^{bc}	5 ± 2.5^{ab}	43 ± 1.2^{a}	48 ± 2.2^{ab}
Cross-linked + Thymol 4%	42 ± 1.4^{c}	7 ± 1.6^{a}	41 ± 2^{a}	49 ± 2^{a}
Cross-linked + Thymol 8%	41 ± 1.0^{d}	8.8 ± 1.3^{a}	40 ± 1.4^{b}	50 ± 1.3^{a}

[a–d]Mean values with different letters within a column are significantly different by Duncan's multiple range test at ($P < 0.05$).
[1]Lightness.
[2]Redness.
[3]Yellowness.
[4]Color difference as compared with the color of uncross-linked film.

enriched with oregano oil (mainly contains carvacrol and thymol) decreased due to hydrophobicity of the oils (Zivanovic and others 2005). The WVP of chitosan films incorporated with thyme oil slightly increased (Altiok and others 2010). The addition of essential oils into alginate based films did not significantly change the WVP of the films (Rojas-Grau and others 2007). The final WVP capacity is the balance between hydrophobicity/hyrophilicity of all compounds in the films. Our results indicated that gelatin films have good WVP and this property was increased to some extent but not large in the presence of thymol.

Color parameters of gelatin films

The results of color parameters of the films were summarized in Table 3. The L* value in the cross-linked gelatin films significantly decreased rather than uncross-linked films ($P < 0.05$). The a* value, b* value, and ΔE value in the cross-linked film significantly increased rather than uncross-linked films ($P < 0.05$). Incorporation of thymol caused a little decrease in L* value and b* value of the films ($P > 0.05$). Incorporation of thymol caused a significant increase in a* value and ΔE of the films ($P > 0.05$).

According to our results, cross-linking of gelatin films caused significant change in color parameters from white to yellow. The change in color of the films upon cross-linking with glutaraldehyde is caused by the formation of aldimine linkages between the free amino groups of lysine and hydroxylysine residues of gelatin and the aldehyde groups of glutaraldehyade (Akin and Hasirci 1995; Rujitanaroj and others 2008). However, incorporation of thymol especially at higher concentrations caused an increase in the whiteness along with a decrease in the yellowness of gelatin films. These effects could be related to the level of cross-linking. Thymol at higher concentration may hinder polymer chain-to-chain interactions and reduce cross-linking (Table 1) and the increase in whiteness with the simultaneous decrease in yellowness of cross-linked films was occurred.

Previous study demonstrated that the effects of essential oil on the color of gelatin films depend on the type and concentrations of essential oil incorporated (Ahmad and others 2012; Tongnuanchan and others 2012). Study on the gelatin films incorporated with bergamot (mainly contains geraniol, linalool, and limonene) and lemongrass (mainly contains citral) oils reported a clear lowered light transmission in the visible range compared to gelatin films without the oils (Ahmad and others 2012). However, other study reported that transparency of films was not significantly affected by the addition of palm oil (Pranoto and others 2005). The incorporation of cinnamon oil in chitosan-based films considerably increased its total color difference, whereas such pronounced effect was not found with thyme and clove oil (Hosseini and others 2009). Accordingly, gelatin films have good color and this property remained unchanged in the presence of thymol.

Table 4–Antioxidant activity of gelatin films incorporated with thymol.

Films	ABTS (mg AAE / g film)[1]
Uncross-linked	0 ± 0^{e}
Cross-linked	0 ± 0^{e}
Cross-linked + Thymol 1%	1.6 ± 0.33^{d}
Cross-linked + Thymol 2%	2.6 ± 0.3^{c}
Cross-linked + Thymol 4%	4.4 ± 0.6^{b}
Cross-linked + Thymol 8%	6.8 ± 0.6^{a}

[a–e]Mean values with different letters within a column are significantly different by Duncan's multiple range test at ($P < 0.05$).
[1]The antioxidant activity was expressed milligram ascorbic acid equivalent (AAE) per gram of films incorporating different concentrations of thymol.

Antioxidant activity of gelatin films

Antioxidant activity of the gelatin films incorporated with different thymol concentrations was determined by ABTS method and expressed as mg ascorbic acid equivalent per gram of films (Table 4). According to the results obtained, the gelatin films without thymol showed no activity against the ABTS decolonization. The gelatin film containing different thymol concentrations decolorize ABTS dose-dependently. Accordingly, gelatin films incorporated with thymol significantly showed antioxidant activity in a dose-dependent manner. Antioxidant activity was increased with increasing concentration of thymol in the films. Antioxidant properties of chitosan films incorporated with thyme oil (mainly contains thymol and carvacrol) for potential wound healing applications was confirmed (Altiok and others 2010). In addition, antioxidant activity of fish skin gelatin film incorporated with citrus essential oils was established (Tongnuanchan and others 2012). The antioxidant activities of samples are mainly due to their redox properties, which can play an important role in neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The total phenolic content and related total antioxidant capacity of some medicinal plant infusions was analyzed, which indicated that there was a significant linear correlation between total phenol content and antioxidant capacity (Katalinic and others 2006; Huang and others 2011). Thus antioxidant activity of gelatin films impregnated with thymol could be related to thymol content. Thymol gradually released from the films to the ABTS solution and decolorized it.

Antibacterial assay of gelatin films

Antibacterial of gelatin films incorporated thymol expressed by disk diffusion method and viable colony counting assay. The results of disk diffusion are summarized in Table 5. The initial diameter of all films was fixed at 30 mm. The diameters of clear inhibition zones, including the diameter of the disk, were used for antibacterial activity analysis. According to the results obtained, all the gelatin films without thymol showed no activity against the tested bacteria. For the gelatin films containing different

Table 5–The antibacterial activity of gelatin films incorporated with thymol.

Films	Inhibition zone diameter (mm)[1]			
	B. Subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*
Uncross-linked	0 ± 0^{e}	0 ± 0^{e}	0 ± 0^{e}	0 ± 0^{e}
Cross-linked	0 ± 0^{e}	0 ± 0^{e}	0 ± 0^{e}	0 ± 0^{e}
Cross-linked + Thymol 1%	33 ± 1.2^{d}	31 ± 0.9^{d}	31 ± 1^{d}	30 ± 0.7^{d}
Cross-linked + Thymol 2%	38 ± 1^{c}	37 ± 0.8^{c}	33 ± 1.2^{c}	31 ± 0.8^{c}
Cross-linked + Thymol 4%	42 ± 1.5^{b}	41 ± 1.1^{b}	34 ± 1.6^{b}	31 ± 1.1^{b}
Cross-linked + Thymol 8%	46 ± 1.4^{a}	45 ± 1.3^{a}	38 ± 1.7^{a}	34 ± 1.5^{a}

[a–e]Mean values with different letters within a column are significantly different by Duncan's multiple range test at ($P < 0.05$).
[1]Antibacterial activity was expressed as diameter of bacterial growth inhibition zone in the presence of films with different thymol concentration.

thymol concentrations inhibitory zones were obvious (Figure 1). The antibacterial activity of gelatin film containing different thymol concentrations was greatest against *B. subtilis* and *S. aureus* followed by *E. coli* and then *P. aeruginosa*. The results of disk diffusion indicated that thymol are more effective to Gram-positive bacteria rather than to Gram-negative bacteria. According to Swiss norm (SN) 195920-ASTM E 2149-01, any compound show zone inhibition of > 1 mm is considered as a good antibacterial agent. Therefore, we can conclude that the gelatin films incorporated with thymol exhibited excellent activity against all the 4 bacteria.

The results of colony counting and reduction percentage were summarized in Table 6. According to the results obtained, the antibacterial activity of the gelatin films containing different thymol concentrations were greatest against *S. aureus* followed by *B. subtilis* followed by *E. coli* and then by *P. aeruginosa* (Figure 2). The results of colony counting also indicated that thymol is more effective to Gram-positive bacteria rather than to Gram-negative bacteria. Antimicrobial properties of gelatin film from the skin of unicorn leatherjacket incorporated with essential oils were reported (Ahmad and others 2012). Also, antibacterial properties of chitosan films incorporated with thyme oil for potential wound healing applications were established (Altiok and others 2010). Furthermore, antimicrobial activity of soy edible films incorporated with thyme and oregano essential oils on fresh ground beef patties were confirmed (Emiroglu and others 2010).

The antibacterial activity of phenolic monoterpenes is related with the attack on the phospholipids present in the cell membranes, which causes increased permeability and leakage of cytoplasm, or in their interaction with enzymes located on the cell wall (Burt 2004; Emiroglu and others 2010). Thus, the resistance of Gram-negative bacteria to the phenolic monoterpenes likely lay in the protective role of their cell wall lipopolysaccharide or outer membranes proteins, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide layer (Solorzano-Santos and Miranda-Novales 2012). However, at higher concentrations this polysaccharide layer can be disrupted by essential oils. Phenolic monoterpenes have the ability to disrupt lipid structure of the cell wall of bacteria, leading to destruction of cell membrane, cytoplasmic leakage, cell lysis, and ultimately cell death (Emiroglu and others 2010). The decrease in pH that occurs due to cell membrane disruption resulted in a loss of control of cellular process such as ATP biosynthesis, DNA transcription, and protein synthesis (Oussalah and others 2007). Phenolic monoterpenes also penetrate into mitochondrial membrane, leading to the greater

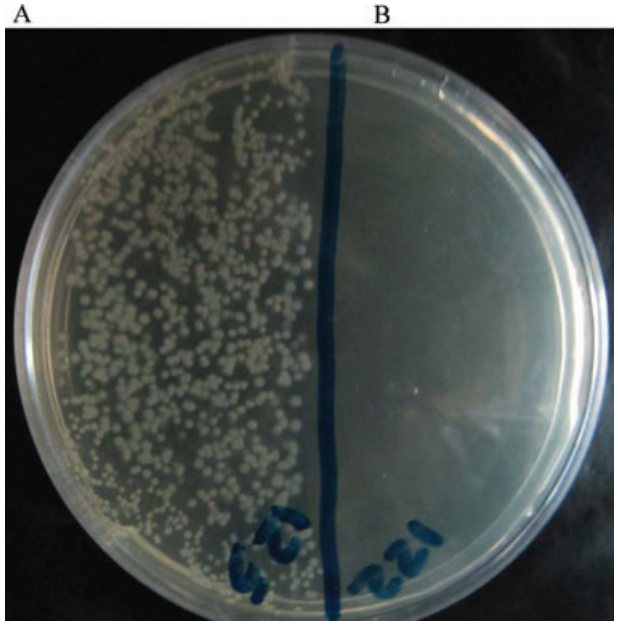

Figure 2–Antibacterial activity of gelatin film incorporated with thymol against *S. aureus*. Antibacterial activity was expressed as number of viable colony in the presence of gelatin film (A) and thymol (8%) containing gelatin film (B).

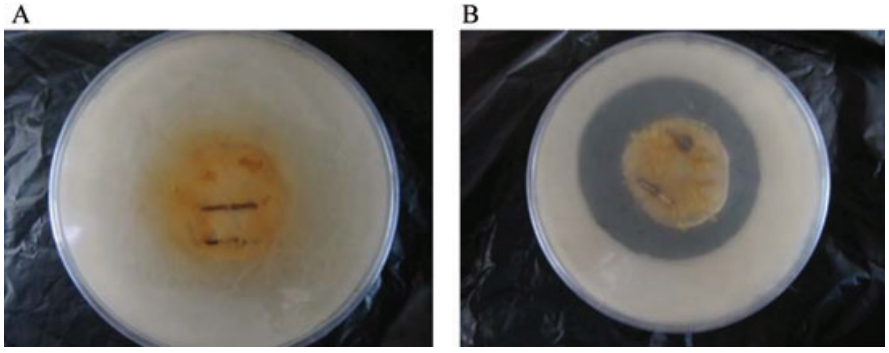

Figure 1–Inhibition zone of *S. aureus* growth on a bacterial plate included gelatin film (A) and thymol (8%) containing gelatin film (B).

Table 6–Antibacterial activity of gelatin films incorporated with thymol.

Films	Colony reduction (%)[1]			
	B. Subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*
Uncross-linked	0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}
Cross-linked	0 ± 0^{e}	0 ± 0^{e}	0 ± 0^{e}	0 ± 0^{e}
Cross-linked + Thymol 1%	55 ± 3.5^{c}	60 ± 2.6^{d}	16 ± 2.8^{d}	3 ± 1.2^{d}
Cross-linked + Thymol 2%	76 ± 2.5^{c}	97 ± 2.8^{c}	35 ± 2.6^{c}	14 ± 2.7^{c}
Cross-linked + Thymol 4%	96 ± 4.8^{b}	99 ± 2.7^{b}	68 ± 3.2^{b}	36 ± 3.4^{b}
Cross-linked + Thymol 8%	100 ± 4.2^{a}	100 ± 2.6^{a}	100 ± 2.8^{a}	93 ± 4.2^{a}

[a–e]Mean values with different letters within a column are significantly different by Duncan's multiple range test at ($P < 0.05$).
[1]Antibacterial activity was expressed as bacterial growth reduction in the presence of films with different thymol concentration.

permeability of organelle and the potassium ion leakage process. The leakage of ions especially potassium out of a cell is a clear indication of membrane damage and cell death (Xu and others 2008; Garcia-Garcia and others 2011).

Conclusions

Cross-linked gelatin films rather than uncross-linked one showed higher tensile strength, higher elongation at break, lower Young's modulus, lower water solubility, lower swelling and water uptake and lower WVP. Incorporation of thymol caused a significant decrease in tensile strength, increase in elongation at break, decrease in Young's modulus, and increase in water solubility, decrease in swelling and water uptake, and increase in WVP slightly. Gelatin films incorporated with thymol exhibited excellent antioxidant and antibacterial properties. Thus, gelatin films containing thymol can be used as safe and effective source of natural antioxidant and antimicrobial agents with the purpose of evaluating their potential use as modern nano wound dressing. However, further *in vivo* research is needed to make their use in practical applicability

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