J Mater Sci: Mater Med (2010) 21:2227–2236
DOI 10.1007/s10856-010-4065-x

Physical, antibacterial and antioxidant properties of chitosan films incorporated with thyme oil for potential wound healing applications

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Received: 25 June 2009 / Accepted: 22 March 2010 / Published online: 7 April 2010

Abstract Chitosan films incorporated with thyme oil for potential applications of wound dressing were successfully prepared by solvent casting method. The water vapor permeability, oxygen transmission rate, and mechanical properties of the films were determined. Surface and cross-section morphologies and the film thicknesses were determined by Scanning Electron Microscopy (SEM). Fourier transform infrared (FT-IR) spectroscopy was conducted to determine functional group interactions between the chitosan and thyme oil. Thermal behaviors of the films were analyzed by Thermal Gravimetry (TGA) and Differential Scanning Calorimetry (DSC). In addition, the antimicrobial and the antioxidant activities of the films were investigated. The antimicrobial test was carried by agar diffusion method and the growth inhibition effects of the films including different amount of thyme oil were tested on the gram negative microorganisms of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and a gram positive microorganism of *Staphylococcus aureus*. The minimum thyme oil concentration in chitosan films showing the antimicrobial activity on all microorganisms used in the study was found as 1.2 % (v/v). In addition, this concentration showed the highest antioxidant activity due to mainly the carvacrol in thyme oil. Water vapor permeability and oxygen transmission rate of the films slightly increased, however, mechanical properties decreased with thyme oil incorporation. The results revealed that the thyme oil has a good potential to be incorporated into chitosan to make antibacterial and permeable films for wound healing applications.

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1 Introduction

Recently, the conventional wound dressings such as natural or synthetic bandages, cotton wool and gauzes which passively provide wound protection have been replaced by modern dressings such as usual textiles and other materials as films, sponges, hydrocolloids, gels and pastes that are capable of providing an optimum environment around the wound and delivering active ingredients or directly interacting with cells in the local wound environment to facilitate wound healing [1, 2]. The ideal wound dressing should (1) prevent infection, (2) remove blood and excess exudates, (3) provide or maintain moist environment, (4) allow gaseous exchange (water vapor, oxygen), (5) be thermally insulating, (6) comfortable and easily removable without causing trauma, (7) be non-toxic and non-allergenic and (8) be cost effective [3].

Chitosan has been proved to be nontoxic, biodegradable, biofunctional, biocompatible and have antimicrobial characteristics [4]. The film-forming property of chitosan has found many applications in tissue engineering and drug delivery, packaging by virtue of its mechanical strength and rather slow biodegradation [5]. Some drug-loaded chitosan films are emerging as novel drug delivery systems, and films appear to have potential for local sustained delivery of cancer chemotherapeutic agents. Chitosan also promotes favorable wound healing properties because of its rapid dermal regeneration, accelerated wound healing properties additional to its bacteriostatic effects [6, 7]. Wound healing is a complex process involving various mechanisms, such as coagulation, inflammation, matrix synthesis and deposition, angiogenesis, fibroplasia, epithelization, contraction and remodelling [8]. There are many studies showing that chitin and chitosan accelerated wound healing in many clinical cases [9–11]. In particular,

it was reported that chitin and chitosan granules enhanced reepithalization and regenerated normal skins in open wounds [9].

Essential oils are hydrophobic, aromatic, volatile liquids obtained from plant flowers, seeds, leaves, fruits and roots via most commonly distillation, expression or solvent extraction. In recent decades, the interest in essential oils for use in food ingredients, perfumes, aromatherapy and pharmaceuticals has been rised [12]. Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties due to their biologically active compounds such as carvacrol, eugenol, and thymol [13]. Cinnamon, clove, basil, lavender and rosemary oils have shown antibacterial and antifungal activity whereas lemon and rosemary oils also possess antioxidant property [14]. Hammer et al. reported that among 52 plant essential oils, lemongrass, oregano and bay showed highest antimicrobial activity at concentration of $\ll$2% (v/v) on selected microorganisms [15]. Thyme essential oil is rich in thymol and carvacrol, which yield considerable antioxidative and antimicrobial effects [16, 17]. Burt et al. reported that, thyme essential oil contains mainly carvacrol and thymol, and *p*-cymene and gamma-terpinene. However, only carvacrol and thymol displayed favorable bacteriostatic and bactericidal properties. These components are not only responsible for the antimicrobial activity but also they are the main phenolic compounds responsible for the high antioxidant capacity of thyme [18]. In addition, Braga et al. pointed that thymol can have helpful effects in controlling the inflammatory process present in many infections, which is necessary for proper wound healing [19]. Since inflammation causes many complications including wound dehiscence, infection and impaired collagen synthesis, anti-inflammatory effects of thymol would be promising when thyme essential incorporated wound healing material is used [20]. The antimicrobial activity mechanism is commonly considered as resulted by disturbing the function of the cytoplasmic membrance, disrupting the active transport of nutrients to the cell membrane, and coagulation of bacteria cell contents [12, 21]. The antimicrobial activities of some essential oils such as oregano, rosemary, and garlic oil incorporated into edible films have been investigated [13, 22, 23]. Rojas-Grau et al. observed that carvacrol and oregano essential oil in alginate apple puree edible film showed significantly greater antimicrobial activity on *E. Coli* than the activities of lemongrass oil, citral, cinnamon oil, and cinnamaldehyde incorporated [24, 25] incorporated the lemongrass oil into the partially hydrolyzed sago starch and alginate edible film and showed that lemongrass oil was effective against *E. Coli* at all studied levels [24, 25]. However, no publications in literature regarding the incorporation of thyme essential oil for the wound healing applications are available.

The main objective of this research was to assess the antibacterial property of chitosan films loaded with thyme oil having both antioxidant and antimicrobial activities. In addition, the microstructural, mechanical and physical properties such as water vapor and oxygen permeation of the films were investigated.

2 Materials and methods

2.1 Materials

Chitosan was supplied by Aldrich (low molecular weight chitosan, degree of deacetylation $\sim$85) acetic acid and ethanol were supplied by Merck (Germany) and the thyme oil was provided by Mecitefendi (Turkey).

2.2 Preparation of chitosan films

Chitosan film forming solution was prepared by dissolving 0.5 g low molecular weight chitosan in 25 ml of 2 wt % acetic acid solution. The thyme oil (*Thymus vulgaris*) stock solution as an antimicrobial agent [10% (v/v)] was prepared by dissolving thyme oil in ethanol and then added dropwisely into chitosan film forming solution to obtain final concentrations as 0.2, 0.4, 0.6, 0.8, 1 and 1.2% (v/v). The solutions were then casted onto polystyrene Petri dishes and vacuum dried at room temperature for 24 h and then further dried at 40°C for 5 h. The dry films obtained were peeled off and stored until analysis.

2.3 Characterization studies of the films

2.3.1 Scanning electron microscopy (SEM)

The magnified surface and cross-section pictures of the films were taken with a SEM (Philips XL 30S FEG, FEI Company, Eindhoven, the Netherlands) in Centre for Materials Research in İzmir Institute of Technology, İzmir, Turkey. The films were fractured in liquid nitrogen for cross-section analysis. Prior to observation, samples were mounted on metal grids using double-sided adhesive tape and coated with gold under vacuum.

2.3.2 Fourier transform infrared spectroscopy (FT-IR)

The spectra of chitosan films (control and those incorporated with thyme oil) and thyme oil were recorded by a FT-IR spectrometry (Shimadzu 8400S) at ambient temperature. All spectra were taken with a resolution of 2 cm^{-1}

and were averaged over 150 scans in the range of 4000–400 cm^{-1}. Films used in the infrared tests were about 0.025 mm thick and spectra of films were acquired directly. Spectrum of thyme oil was obtained by KBr discs prepared by compression under vacuum.

2.3.3 *Thermal gravimetry (TGA) and differential scanning calorimetry (DSC)*

The dynamic thermal behaviors of the chitosan films (control and those incorporated with thyme oil) were analyzed using DSC (Shimadzu DSC-50). The samples were heated from 20 to 400°C. Nitrogen gas was applied at 40 ml/min flow rate and used as a purging gas. The chitosan film samples were analyzed with TGA (Shimadzu TGA-51) under N_2 flow (40 ml/min). In TGA analysis temperature was increased from 20 to 1000°C. Heating rate was 10°C/min both for TGA and DSC.

2.4 Mechanical and physical properties of the films

2.4.1 *Mechanical properties*

The tensile strength (TS) and elongation at break (E) of the films were measured with Testometric M500-100kN (Lancashire, England) testing machine. Average film thicknesses were determined by scanning electron microscopy (SEM) as in the range of 0.022–0.027 mm. Film strips prepared in accordance with ASTM D 882 standard as 1 cm in width and 5 cm in gauge length were pulled apart at a constant head speed of 5 mm/min. Five specimens of each type of film were used for the tests.

2.4.2 *Water vapor permeability*

A vessel containing two compartments, the bottom compartment containing a deionized water bath and the upper one including a humidity probe connected to a Datalogger SK-L 200 TH, was used in the water vapor permeability studies. A humidity probe in the upper compartment records relative humidity and temperature with respect to time. The films squeezed within two O-rings were placed between two compartments. Before starting the permeation experiment, air was dried in a fixed bed Drierite column that contains anhydrous $CaSO_4$ and then continuously pumped through the upper compartment of vessel until the relative humidity in the upper compartment was lowered to 5%. Then water vapor at bottom compartment was allowed to pass through the film. The relative humidity and temperature data were taken as a function of time with 1 min interval. Permeability data was calculated using the Ficks Diffusion assuming that transport is one dimensional.

$$J = -D_{eff}\frac{\partial C}{\partial x} \tag{1}$$

where D_{eff} and C are the effective diffusivity and the concentration of the water vapor in the film, respectively. Assuming the steady state operation condition exists since the film thickness, L, is so thin and the linear equilibrium relationship between the concentrations of the water vapor in the vapor phase and the film, the flux expression can be obtained as follows by integrating Eq. (1) from $x = 0$ to L:

$$J = \frac{D_{eff}S_{eff}}{L}(P_L - P_U) \tag{2}$$

where P_L and P_U are the partial pressures of the permeant in the lower and upper compartments, respectively. The multiplication of diffusivity (D_{eff}) and solubility (S_{eff}) of the permeant in Eq. (2) equals the permeability coefficient (P_{eff}). The change in partial pressure of permeant in the upper compartment with time can be expressed as follows when the permeant (water vapor) is assumed to be an ideal gas:

$$\frac{V_U}{RT}\frac{dP_U}{dt} = \frac{D_{eff}S_{eff}}{L}(P_L - P_U)A \tag{3}$$

where V_U is the volume of the upper compartment, A is the permeation area and T is the operation temperature. If Eq. (3) is integrated with respect to time, the permeability coefficient can be calculated from the following expression:

$$\ln\frac{P_L - P_{U(0)}}{P_L - P_{U(t)}} = \frac{P_{eff}ART}{V_U L}t \tag{4}$$

$P_{U(0)}$ is the initial partial pressure of permeant in the upper compartment whereas $P_{U(t)}$ denotes the partial pressure of permeant in the upper compartment at time, t, during the operation. Water vapor permeability and transmission rate of films were calculated in the units of mol/min.cm.kPa and g/m^2 day, respectively.

2.4.3 *Oxygen transmission rate (OTR)*

The oxygen transmission rate (OTR) of chitosan films was measured by using Systech Instruments 8001 according to standard method of ASTM D 3985 at constant temperature (23°C) and relative humidity (0% RH) conditions. The film sample was clamped in a diffusion chamber. Pure oxygen was introduced to the upper half of the chamber, while a carrier gas (nitrogen) flows through the lower half. Molecules of oxygen permeating through the film to the lower chamber are passed to the sensor by the carrier gas allowing direct measurement of OTR as cc/m^2 day.

2.5 Antimicrobial activity

Antimicrobial activities of films and thyme essential oil were determined by agar diffusion method on *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* which were obtained from BERL (Bioengineering Research Laboratory) in the Department of Chemical Engineering (İzmir Institute of Technology, Turkey). The frozen glycerol stocks were activated in Mueller-Hinton Broths at 37°C for overnight by using shaker incubator at 150 rpm. From overnight cultures of test microorganisms, approximately, five colonies were picked up by using sterile cotton swabs and dissolved in 2.5 ml phosphate-buffered saline (PBS) solution and turbidity was adjusted to McFarland 0.5. Then, the swab was streaked on the Mueller-Hinton agar plates. The film discs with 14 mm diameter were placed on the inoculated agar. Each plate was divided two equal parts and two thyme oil incorporated discs were placed on it. Then, plates were incubated at 37°C for 24 h. The clear zones around the discs were measured and recorded as inhibition zone that indicate antimicrobial property.

2.6 Antioxidant activity

Antioxidant activity of films was determined by ABTS method using SkanIt Varioscan Flash (Thermo Electron Corporation, Finland) [26]. It was based on the ability of an antioxidant component released from the films to scavenge the radical cation $ABTS^+$. The $ABTS^+$ radical was generated by a reaction between 7 mM ABTS and 2.45 mM $K_2S_8O_2$. The method was modified to detect the continuous antioxidant release from films. The release tests were performed in 12-well-plate where the cage was placed in each cell. Prior to the release test, the absorbance value of 4 ml $ABTS^+$ solution in each well was adjusted to 1 (± 0.05) at 734 nm by diluting the $ABTS^+$ solution with phosphate buffer (pH 7.4). In the release test, films were cut into 14 mm radius discs and film discs were immediately placed outside the cage in the well that had been filled with 4 ml $ABTS^+$ solution. The program was adjusted to record the absorbance values after shaking the 12-well-plate for 5 s at 37°C. The data was recorded up to the steady state was reached for each sample.

3 Result and discussions

3.1 Characterization of the films

3.1.1 Scanning electron microscopy (SEM)

The air-contacting and polystyrene petri dish-contacting surfaces were investigated (SEM) (Fig. 1). The control film was smooth, transparent and colorless. The addition of thyme oil increased roughness, opaqueness and whiteness

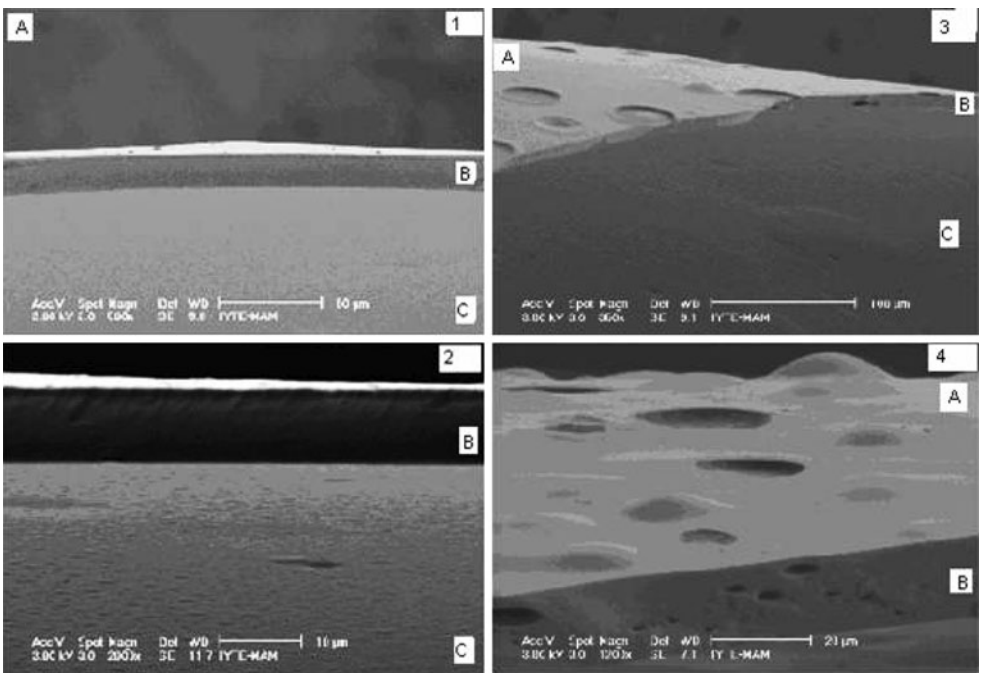

Fig. 1 Scanning electron microscopy pictures of thyme oil incorporated chitosan Films. *1* Control (pure) chitosan film, *2–3–4*: 0.4, 1, 1.2 % thyme oil incorporated chitosan films, respectively. *A* air-contacting surface, *B* cross-section, *C* petri dish-contacting surfaces

of the film. The lower surfaces of the thyme oil incorporated films had bubble like structures whereas the upper surface (air-contacting) had bursted bubbles due to the fast evaporation of thyme oil. Since no any air bubbles remained in the film after vacuum drying, these bubbles is caused by thyme oil. The bubble size and the number of bubbles also increased with increase in thyme oil concentration.

The average thickness of the films was measured by the cross sectional surface micrographs. The average thickness of the control and thyme oil incorporated films was changed in the range of 23–27 μm which showed that thyme oil concentration did not affect the film thickness significantly. The micrographs indicate that an increase in thyme oil concentration resulted in more porous structure in which pores were distributed homogeneously.

3.1.2 *Fourier transform infrared spectroscopy (FT-IR)*

Chitosan is an amino glucose characterized by a small proportion of amide groups via an amide linkage with acetic acid. Figure 2 shows the Fourier transform infrared (FT-IR) spectra of the thyme oil incorporated chitosan films for different thyme oil concentrations and pure thyme oil in the wavelength range of 4000–400 cm^{-1}. Since the grade of chitosan used in the present study was ≥80% deacetylated, the C=O stretching (amide I) peak at 1646 cm^{-1} and N–H bending (amide II) peak at 1580 cm^{-1} were observed. These peaks represented the structure of *N*-acetylglucosamine, which could be found in chitosan with a lower degree of deacetylation [27–29]. The peak at 1545 cm^{-1} was assigned to strong vibrations of secondary amide. Strong absorption bands centered at 3400 cm^{-1} were concerned with the stretching vibration of O–H and N–H bonds and those in the region of 1030–1160 cm^{-1} were assigned to the C–O bonds [13, 30]. Furthermore, in the C–H stretch region of FT-IR spectrum, the peak at 2932 cm^{-1} was assigned to the asymmetric mode of CH_2 [30, 31]. The band due to CH_2 scissoring occured at 1466 cm^{-1} [31]. These indicated that there was no major structural change in the chitosan film by incorporation of thyme oil since there were no change in the peak intensities and no shift in the wave numbers [13]. Schulz et al. assigned the most characteristic IR bands of carvacrol and thymol, which are the major compounds of thyme essential oil. According to their ATR-IR spectrum, the ring vibration bands of thymol and carvacrol were seen at 804 cm^{-1} and 811 cm^{-1}, respectively [32, 33]. In the FT-IR spectra of the present study, only the characteristic peak of carvacrol was observed at 813 cm^{-1} that is the

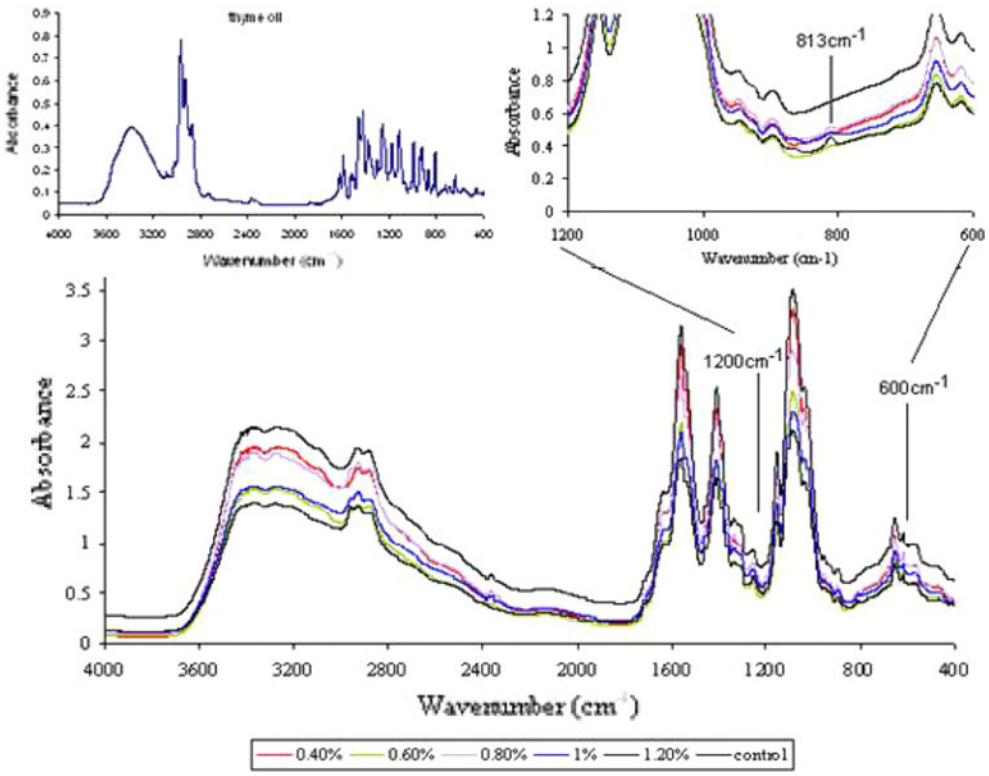

Fig. 2 FT-IR spectra of thyme oil, thyme oil incorporated chitosan films and control (pure) chitosan film (expanded spectra showing the 1200–600 cm^{-1} wavenumber region is included to depict the carvacrol peak)

evidence of the investigated thyme oil was the carvacrol-type. The spectra of chitosan films incorporated with different amounts of thyme oil shows the same pattern on their informative peaks as the control chitosan films. This indicates that there is no interaction between active groups of thyme oil with functional groups of chitosan [13].

3.1.3 *Thermal analysis by thermal gravimetry (TGA) and differential scanning calorimetry (DSC)*

Thermal behavior of the pure and thyme oil incorporated chitosan films was investigated by TGA and DSC. As shown in Fig. 3, DSC thermograms of all chitosan films exhibited the broad endothermic peaks at approximately 35–140°C and the exothermic peaks at 254–330°C. These changes corresponded to the weight loss in TGA thermograms as illustrated in Fig. 4. The endothermal effect corresponding to the weight loss at a lower temperature was attributed to the evaporation of the solvent traces (acetic acid, ethanol, water) used in the preparation of chitosan films. The exothermic peak occurred at higher temperature was attributed to polymer decomposition. According to TGA thermograms of chitosan films, weight loss due to the evaporation was affected by incorporation of thyme oil. The change in weight loss at 100°C with respect to thyme oil concentration was presented in Fig. 5.

3.2 Mechanical and physical properties of the films

3.2.1 *Mechanical properties*

Tensile strength, elongation, and Young's modulus are parameters that relate mechanical properties of films to their chemical structures. Tensile strength expresses the maximum stress developed in a film during tensile testing [25]. Incorporation of thyme oil caused a significant

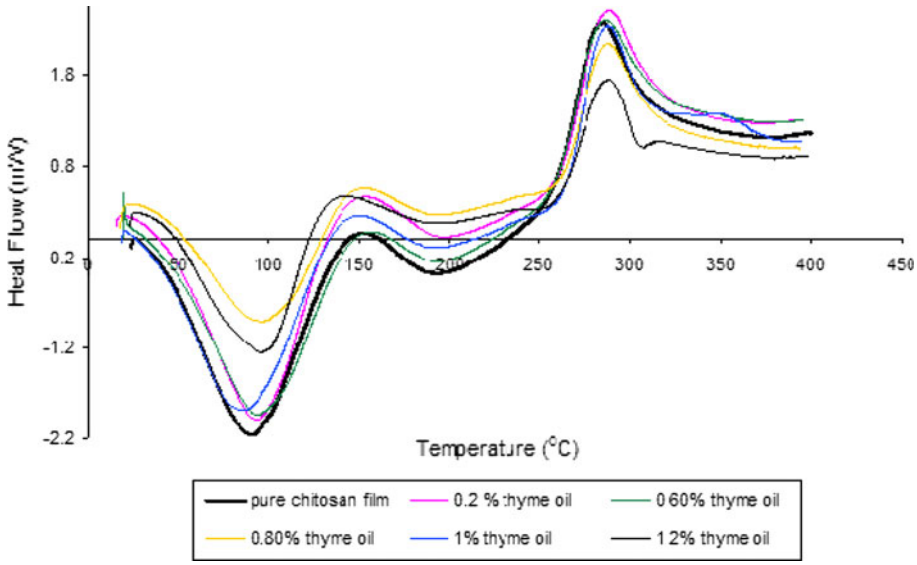

Fig. 3 DSC thermograms of thyme oil incorporated and control (pure) chitosan films

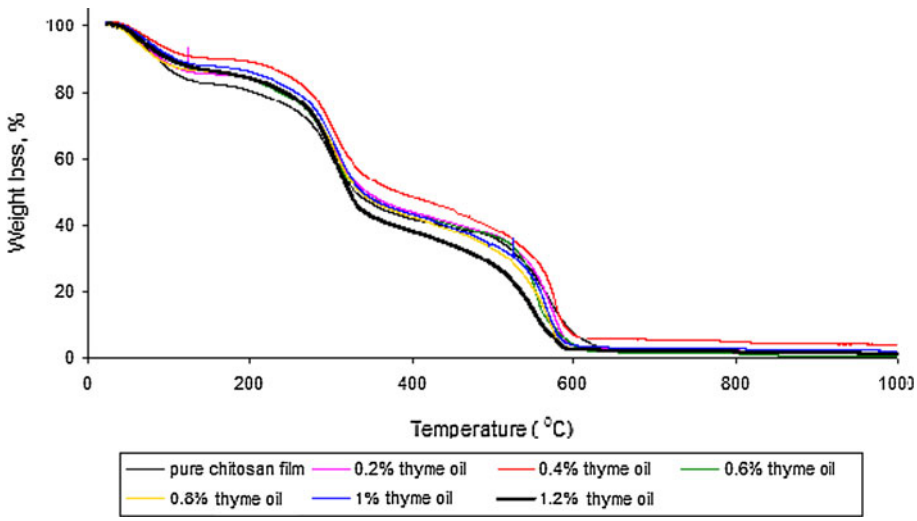

Fig. 4 TGA thermograms of thyme oil incorporated and control (pure) chitosan films

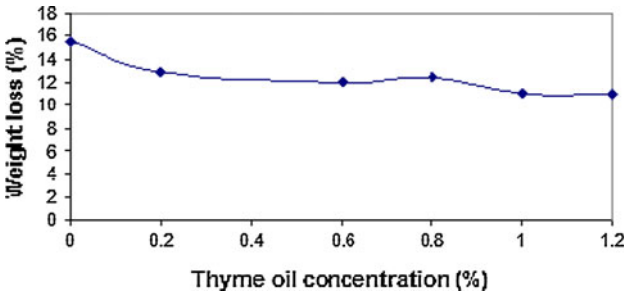

Fig. 5 Weight loss (%) in thyme oil incorporated chitosan films at 100°C

reduction ($p < 0.05$) in tensile strength of the films (Table 1). This result confirms the outcome of the report by Cagri et al. (2001) who had concluded earlier that incorporation of additives other than crosslinking agents generally lowers TS value [34]. However, the thyme oil concentration has not significant effect on tensile strength statistically. Elongation at break is a measure of the film's stretch ability prior to breakage. Percent elongation at break was calculated based on the length extended and original length of the films. As expected, in the range of concentrations studied, the percent elongation decreased when concentration of thyme oil was increased in chitosan film due to increase in pore sizes and porosity of the films. The percent elongation of control film was 4.8 ± 0.28 and decreased to 1.8 ± 0.22 of 1.2 % thyme oil concentration in film. Young's modulus was estimated at the point of highest tangent and yield stress by visual examination of the departure from linearity of the plot. Similar to the tensile strength, Young's modulus also reduced significantly by the incorporation of thyme oil ($p < 0.05$) as dense structures become stiffer and have higher modulus values than the porous structures.

Data obtained from the experiments were assured statistically by one-way ANOVA followed by the Tukey's multiple comparison tests.

Table 1 Tensile strength, elongation at break and Young's Modulus of thyme oil incorporated chitosan films

Thyme oil (%)	Tensile strength (MPa)	% Elongation at break	Young's Modulus (MPa)
0 (control)	51.20 (±1.94)	4.8 (±0.28)	34.08 (±3.12)
0.2	38.22 (±3.12)	3.6 (±0.25)	26.95 (±2.25)
0.4	41.91 (±2.83)	3.6 (±0.22)	27.81 (±2.32)
0.6	31.05 (±1.95)	3.2 (±0.24)	21.26 (±2.51)
0.8	34.37 (±1.51)	2.7 (±0.25)	25.38 (±2.12)
1.0	34.57 (±4.29)	1.9 (±0.20)	22.31 (±1.17)
1.2	32.94 (±3.32)	1.8 (±0.22)	19.32 (±2.44)

3.2.2 *Water vapor permeability*

To determine the effect of thyme oil addition onto water vapor permeability of chitosan films, water vapor permeation experiments were carried on. Permeabilities were calculated using Eq. (4) The water vapor permeabilities of the thyme oil incorporated chitosan films were demonstrated in Table 2. As it was seen in Table 2, the water vapor permeability of chitosan film was slightly increased by incorporating the thyme oil to the films. This behavior could be explained by the formation of porous structure with the addition of thyme oil supported by SEM data. Zivanovic et al. have found that the water vapor permeability of chitosan films enrinched with oregona oil decreased due to hydrophobicity of the oils [35]. However, in another study the addition of essential oils into alginate based edible films did not significantly changed the water vapor permeability of the films [25].

In this study, the water vapor transmission rate of control and thyme oil incorporated chitosan films was determined in the range of 200–300 g/m^2 day. In the literature studies, water vapor transmission rate was reported in the broad range of 76–9360 g/m^2 day depending on the material used for wound healing applications [36, 37]. However, there are no clinical data suggesting ideal standards value that works in practice as far as we know.

3.2.3 *Oxygen transmission rate*

Oxygen transmission rate of the control and thyme oil loaded chitosan films in dry state were measured and tabulated in Table 3. Oxygen transmission rate of the films increased with an increasing oil concentration due to the microstructural changes in the film becoming porous with the addition of oil. Oxygen transmission rate of the pure chitosan film was 1.24 cc per m^2 per day. The highest oxygen permeability was obtained as 4.61 cc per m^2 per day for 1.2% (v/v) thyme oil incorporation which

Table 2 Water vapor permeability of thyme oil incorporated chitosan films

Thyme oil concentration (v/v)	Water vapor permeability × 10^{10} (mol/min.cm.kPa)
0 (control)	6.51
0.2	6.49
0.4	7.19
0.6	8.33
0.8	8.11
1	7.98
1.2	8.10

Table 3 Oxygen transmission rate of thyme oil incorporated chitosan films

Thyme oil concentration (v/v)	Oxygen transmission rate (cc/m^2/day)
0	1.24
0.2	1.25
0.4	1.90
0.6	2.46
0.8	4.62
1.0	4.34
1.2	4.61

suggests the promising applications of thyme oil loaded chitosan film as a wound dressing.

3.3 Antimicrobial activity

The antimicrobial activities of thyme essential oil incorporated chitosan films were evaluated for four different pathogens given in Table 4. Positive inhibition was observed when thyme oil added chitosan films were tested. The inhibition at contact area of the films with agar surface was investigated in order to see any potential antimicrobial effects of pure chitosan films. The antimicrobial tests showed that the minimum thyme essential oil concentration which prevents the growth of all selected microorganisms was 1.2%(v/v). *E. coli* was found as more sensitive to the thyme essential oil than the others. However, the highest antimicrobial effect was achieved for *K. pneumoniae* at 1.2 % (v/v) thyme essential oil concentration. The mode of antimicrobial action would be related with the hydrophobicity of essential oil due to the hydrophobic phenolic constituents such as carvacrol and thymol in thyme oil. The hydrophobic essential oil participates in the lipid layer of the cell membrane. This structural distortion would cause deterioration of the membrane and would increase membrane permeability [19, 21, 38]. In addition, Braga et al. postulated that the phenolic components such as thymol and carvacrol suppress the calcium and potassium transport by partitioning in the lipid phase of the membrane and subsequently altering the the local environment of calcium channels [19]. In the antimicrobial tests, although the growth medium does not resemble the wound exudates, it simulates the worst case scenario where the number of microorganisms is much greater than that possibly present at a wound surface. The culture conditions in the antimicrobial test provide the optimum environment in terms of medium composition, pH, and temperature for the growth of test microorganisms. In these conditions the maximum microbial growth and activity are achieved. Therefore, it would be appropriate to conclude that, if significant antimicrobial activity is observed at these extreme conditions, higher activity is expected in real cases because wound surface and exudates do not exhibit optimum growth conditions for the microorganisms. Additionally, the natural defense mechanism during healing process also helps to eliminate the growth of these microorganisms.

Table 4 Antimicrobial activity of chitosan films incorporated with different thyme essential oil concentrations

Bacteria	Thyme oil % (v/v)	Observation at 24 h	
		Inhibitory zone (mm)	Contact area*
Escherichia coli	0 (control)	0	+
	0.2	0	+
	0.4	0	+
	0.6	0	+
	0.8	15.5	+
	1	16	+
	1.2	17	+
Klebsiella pneumoniae	0 (control)	0	+
	0.2	0	+
	0.4	0	+
	0.6	0	+
	0.8	0	+
	1	16	+
	1.2	19	+
Pseudomonas aeruginosa	0 (control)	0	+
	0.2	0	+
	0.4	0	+
	0.6	0	+
	0.8	0	+
	1	0	+
	1.2	16	+
Staphylococcus aureus	0 (control)	0	+
	0.2	0	+
	0.4	0	+
	0.6	0	+
	0.8	0	+
	1	15.5	+
	1.2	16	+

* contact area is the part of the agar directly underneath film pieces

+: represents an inhibitory effect

3.4 Antioxidant activity

The antioxidant release profiles were obtained by modified ABTS method. The test was based on the scavenging of the free radical because of the phenolic compound of thyme oil, i.e. carvacrol, releasing from the films. This indirect method is easy, time saving, and very informative. The antioxidant measurements actually reflects the release

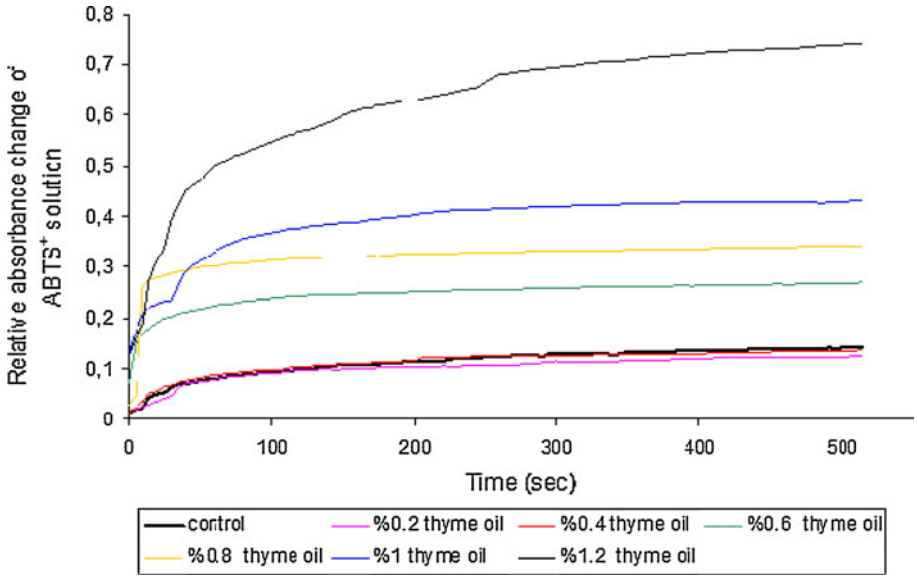

Fig. 6 Release kinetics of phenolic compounds from thyme oil incorporated chitosan films

studies of oil into environment. Because the determination of the absorbance change of $ABTS^{+}$ free radical against time also demonstrates the release of oil from the dressing into the surrounding environment since the antioxidant activity and the amount of oil that is released are directly proportional. Figure 6 shows the antioxidant release profiles of control and oil incorporated chitosan films. However, release tests showed that the chitosan film contained some antioxidant activity itself. Incorporation of 0.2 and 0.4% (v/v) thyme oil to the chitosan film did not affect the antioxidant activity significantly. However, the addition of 1 and 1.2% (v/v) thyme oil caused the four and eight times greater antioxidant activity than the control chitosan film. The strong antioxidant activity of 1.2% thyme oil incorporated chitosan film would be mainly due to the phenolic compound, carvacrol, in thyme essential oil. The antioxidant activities of thymol and carvacrol have been previously reported using various testing systems [39, 40]. According to the antioxidant activity test, the antioxidative compounds were released within 100 s except 1.2% thyme oil incorporated chitosan for which it was approximately 300 s. In addition, high swelling of thyme oil enriched chitosan films was observed during the antioxidant release test. This property is desirable combined with the high water vapor permeability during wound healing since maintaining moisture balance controls the exudate. Moisture retaining dressings like hydrogels aid in maintaining moisture and prevent desiccation that conduct healing [41].

4 Conclusion

In this study, the effect of thyme oil addition into chitosan, which is known for its beneficial effects for wound healing, was studied on the physical, antibacterial and antioxidant properties of the films. The antibacterial test results demonstrated that the minimum thyme oil concentration in chitosan films showing the antimicrobial activity on all microorganisms used in the study was found as 1.2% (v/v). In addition, this concentration showed the highest antioxidant activity due to mainly the carvacrol in thyme oil. Water vapor permeability and oxygen transmission of films slightly increased, however, mechanical strength decreased with thyme oil incorporation. The films showed both antibacterial and antioxidant activities besides a slight increase in water vapor and oxygen transmission rates with the addition of thyme oil into chitosan were obtained. Since these properties are the most important properties for ideal wound healing materials, thyme oil incorporated chitosan films could be used as a potential wound healing materials. Further studies are carried out in our group for the cytotoxicity of the films.

References

1. Ovington LG. Advances in wound dressings. Clin Dermatol. 2007;25:33–8.
2. Yudanova TN, Reshetov IV. Modern wound dressings: manufacturing and properties. Pharm Chem J. 2006;40(2):85–92.
3. Boateng JS, Matthews KH, Stevens HNE, Eccleston GM. Wound healing dressings and drug delivery systems: a review. J Pharm Sci. 2008;97:2892–923.
4. Pranoto Y, Rakshit SK, Salokhe VM. Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin. LWT-Food Sci Technol. 2005;38:859–65.
5. Tien CL, Lacroix M, Ispaz-Szabo P, Mateescu MA. *N*-acetylated chitosan: hydrophobic matrices for controlled drug release. J Control Release. 2003;93:1–13.

6. Shi C, Zhu Y, Ran X, Wang M, Su Y, Cheng T. Therapeutic potential of chitosan and its derivatives in regenerative medicine. J Surg Res. 2006;133:185–92.
7. Berger J, Reist M, Mayer JM, Felt O, Gurny R. Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. Eur J Pharm Biopharm. 2004;57:5–52.
8. Alemdaroglu C, Degim Z, Celebi N, Zor F, Ozturk S, Erdogan D. An investigation on burn wound healing in rats with chitosan gel formulation containing epidermal growth factor. Burns. 2006; 32:319–27.
9. Kweon DK, Song SB, Park YY. Preparation of water-soluble chitosan/heparin complex and its application as wound healing accelerator. Biomaterials. 2003;24:1595–601.
10. Wittaya-Areekul S, Prahsarn C. Development and in vitro evaluation of chitosan-polysaccharides composite wound dressing. Int J Pharm. 2006;313:123–8.
11. Ishihara M, Nakanishi K, Ono K, Sato M. Photocrosslinkable chitosan as a dressing for wound occlusion and accelerator in healing process. Biomaterials. 2002;23:833–40.
12. Burt S. Essential oils: their antibacterial properties and potential applications in foods−a review. Int J Food Microbiol. 2004; 94:223–53.
13. Pranoto Y, Rakshit SK, Salokhe VM. Physical and antibacterial properties of alginate-based edible film incorporated with garlic oil. Food Res Int. 2005;38(3):267–72.
14. Prabuseenivasan S, Jayakumar M, Ignacimuthu S. In vitro antibacterial activity of some plant essential oils. BMC Complem Altern Med. 2006;6:39–46.
15. Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. J Appl Microbiol. 1999; 86:985–90.
16. Marino M, Bersani C, Comi G. Antimicrobial activity of the essential oils of *Thymus Vulgaris* L. measured using a bioimpedometric method. J Food Prot. 1999;62(9):1017–23.
17. Sacchetti G, Maietti S, Muzzoli M, Scaglianti M, Manfredini S, Radice M, et al. Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals, and antimicrobials in foods. Food Chem. 2005;91:621–32.
18. Burt SA, Vlielander R, Haaqsman HP, Veldhuizen EJ. Increase in activity of essential oil components carvacrol and thymol against *Escherichia Coli* O157:H7 by addition of food stabilizers. J Food Prot. 2005;68(5):919–26.
19. Braga PC, Sasso MD, Culici M, Bianchi T, Bordoni L, Marabini L. Anti-inflammatory activity of thymol: inhibitory effect on the release of human neutrophil elastase. Pharmacol. 2006;77(3): 130–6.
20. Busti AJ, Hooper JS, Amaya C, Kazi S. Effects of perioperative anti-inflammatory and immunomodulatory therapy on surgical wound healing. Pharmacotherapy. 2005;25:1566–91.
21. Lambert RJW, Skandamis PN, Coote PJ, Nychas GJE. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol. 2001;91:453–62.
22. Quattara B, Simard RE, Piette G, Begina A, Holleyc RA. Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. Int J Food Microbiol. 2000;62:139–48.
23. Seydim AC, Sarikus G. Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. Food Res Int. 2006;39:639–44.
24. Maizura M, Fazilah A, Norziah MH, Karim AA. Antibacterial activity and mechanical properties of partially hydrolyzed sago starch-alginate edible film containing lemongrass oil. J Food Sci. 2007;72(6):324–30.
25. Rojas-Grau MA, Avena-Bustillos RJ, Olsen C, Friedman M, Henika PR, Martin-Belloso O, et al. Effects of plant essential oils and oil compounds on mechanical, barrier and antimicrobial properties of alginate–apple puree edible films. J Food Eng. 2007;81:634–41.
26. Altiok E, Ulku S, Bayraktar O. Recovery of proanthocyanidin from waste of Turkish traditional product, Pekmez (molasses). Food Sci Technol Res. 2007;13:321–6.
27. Balau L, Lisa G, Popa MI, Tura V, Melnig V. Physico-chemical properties of chitosan films. Cent Eur J Chem. 2004;2(4):638–47.
28. Lawrie G, Keen I, Drew B, Chandler-Temple A, Rintoul L, Frederics P, et al. Interaction between alginate and chitosan biopolymers characterized using FT-IR and XPS. Biomacromolecules. 2007;8:2533–41.
29. Nunthanid J, Puttipipatkhachorn S, Yamamoto K, Peck GE. Physical properties and molecular behavior of chitosan films. Drug Dev Ind Pharm. 2001;27(2):143–57.
30. Xiao C, Zhang J, Zhang Z, Zhang L. Study of blend films from chitosan and hydroxypropyl guar gum. J Appl Polym Sci. 2003;90:1991–5.
31. Dhanikula AB, Panchagnula R. Development and characterization of biodegradable chitosan films for local delivery of paclitaxel. AAPS J. 2004;6(3):1–12.
32. Schulz H, Ozkan G, Baranska M, Kruger H, Ozcan M. Characterisation of essential oil plants from Turkey by IR and Raman spectroscopy. Vib Spectrosc. 2005;39:249–56.
33. Schulz H, Quilitzsch R, Kruger H. Rapid evaluation and quantitative analysis of thyme, origano and chamomile essential oils by ATR-IR and NIR spectroscopy. J Mol Struct. 2003;661–662:299–306.
34. Cagri A, Ustunol Z, Ryser ET. Antimicrobial, mechanical, and moisture barrier properties of low pH whey protein-based edible films containing *p*-aminobenzoic or sorbic acids. J Food Sci. 2001;66(6):865–70.
35. Zivanovic S, Chi S, Draughon AF. Antimicrobial activity of chitosan films enriched with essential oils. J Food Sci. 2005; 70:45–51.
36. Lee YM, Kim SS, Park MH, Song KW, Sung YK, Kang IK. β-Chitin-based wound dressing containing silver sulfurdiazine. J Mater Sci. 2000;11(12):817–23.
37. Wu P, Fisher AC, Foo PP, Queen D, Gaylor JDS. In vitro assessment of water vapour transmission of synthetic wound dressings. Biomaterials. 1995;16(3):171–5.
38. Ultee A, Bennink MHJ, Moezelaar R. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Appl Environ Microbiol. 2002;68(4): 1561–8.
39. Lacroix M, Smoragiewicz W, Pazdernik L, Kone MI, Krzystyniak K. Prevention of lipid radiolysis by natural antioxidants from rosemary (*Rosmarirzus oficinalis* L.) and thyme (*Thymus vulgaris* L.). Food Res Int. 1997;30(6):451–62.
40. Schwarz K, Ernst H. Evaluation of antioxidative constituents from thyme. J Sci Food Agric. 1996;70:217–23.
41. Kumar S, Wong PF, Leaper DJ. What is new in wound healing? Turk J Med Sci. 2004;34:147–60.