Antimicrobial, Mechanical, Barrier, and Thermal Properties of Starch–Casein Based, Neem (*Melia azardirachta*) Extract Containing Film

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ABSTRACT: The Starch–Casein-based edible films containing with or without neem (*Melia azadirachta*) extract was prepared. The neem based free films were also heat pressed and all of them were assessed for inhibition of pathogenic organisms namely *E. coli, Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Pseudomonas spp.,* and *Salmonella*, using disc diffusion assay. The Glass-transition temperature (T_g) and thermal properties of the films were determined with the help of DSC and DMA. Tensile strength (TS), elongation at break (EAB), water vapor transmission rate (WVTR), and oxygen transmission rate (OTR) of the films were also determined. Incorporation of neem extract to edible film did not affect any of the physical properties except microbial, and the films were effective in inhibiting the growth of pathogens, since the inhibition zones varied from 15 mm as large as 24 mm. However, the heat-pressed films containing neem extract led higher the T_g, TS, and modulus, while the EAB was marginally affected, indicating the toughening of the film and as expected, the heat pressing of films decreased the WVTR and marginally affected OTR.
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Key words: edible films; glass-transition temperature; neem extract; pathogenic organisms; toughening

INTRODUCTION

Neem is cultivated mostly in India and to some extent in Burma. Every part of the plant, particularly the bark and leaves, is used as medicine in India. Neem is labeled as "indigenous drugs of India." Chatterjee and Roes[1] reported on clinical evidence that the neem leaves extract had antibacterial effect against protozoa, crysipelas, *ancro fula*, and skin diseases like ring worm, scabies, periphigus, etc.

Active packaging films used in food industry contain antimicrobial agents namely antibiotics, biocide, and chemicals and are very important in food industry.[2,3] However, because of adverse effect of synthetic chemicals on environment and health, the consumers prefer packaging material containing natural preservatives or phytochemicals as antimicrobial agent[4–6] Grower et al.[2] incorporated nisin into methyl cellulose film and determined its ability to inhibit the growth of *Listeria monocytogenes* on the surface of vacuum-packaged meat. Other researchers[7,8] incorporated antimicrobial agent to Soy protein and corn zein films to inhibit Gram-negative bacteria. Incorporation of antimicrobial agent to edible films has an added advantage of being biodegradable, the subject of many review articles.[9–11]

Chen et al.[8] attempted to prepare an antimicrobial film containing chitosan and methylcellulose as well as either sodium benzoate or potassium sorbate as antimicrobial agent. Although the film was found to inhibit fungi as judged from inhibitory zones on agar media, the release of the antimicrobial from the film was too high. Antimicrobial components have also been used in traditional films such as low-density polyethylene; for example, the fungicide imazalil and the antimicrobial grape fruit seed extract have been recently investigated.[12]

The preparation and evaluation of mechanical and barrier properties of edible starch–protein-based films have been reported in an earlier study.[13] In continuation to our previous work, this study deals with the effect of neem as antimicrobial agent in edible films. The edible film containing neem was heat pressed to study the thermal properties testing DSC and DMA in addition to mechanical and barrier properties.

MATERIALS AND METHODS

Preparation of the film forming solution, film casting, and drying and measurement of film thickness were carried out as described earlier.[13] Casein (50 g) was dissolved in 20–30% aqueous ammonia solution in a 2-L round-bottom flask to which water was added.

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The solution of casein was heated to 85–95°C while stirring. The stirrer was a glass rod with a teflon blade and was remotely controlled by a motor-driven pulley. When the casein solution became clear and transparent, 50 g corn starch slurry was added to the reaction vessel. The stirring and heating was continued till a homogenous blend of starch and Casein was obtained. Propylene glycol (10 mL) was added to ensure flexibility to the film. EDTA (1 g) was also added to the reaction vessel as compatibiliser. The aqueous blend of Casein and starch, along with propylene glycol as plasticizer and EDTA as compatibiliser, was the film-forming solution. A clean glass plate bounded on all the four sides having depth varying from 0.25 to 1 in. was used for the preparation film. Any vegetable oil could be used as mold releasing agent. The blend solution obtained was poured on to the glass plate, and a uniform spread was obtained using spirit level. The glass plates were dried for 24–48 h in a dust-free chamber. The free film was obtained by peeling off the dried film from the glass plate, and film thickness was measured according to ASTM 065 method.

Encapsulation of neem

Neem leaves (100 g) were soaked in 1 L water containing 100 mL of propylene glycol in a 5-L stainless steel vessel and digested for about 4 h. There after, the extract was filtered through a double fold of muslin cloth. The filtrate was mixed with film forming solution and blended for about two minutes at 90°C. The resultant blend was homogenized using a homogenizer (Virtis, Gardner, NY) and used for casting of edible films. The casted films were then subsequently dried.

Heat-pressed films

The dry neem-free film of size 100 × 200 cm and thickness 100 μm was placed on tissue paper smeared with vegetable oil as releasing agent and medium hot iron box (temperature not exceeding 150°C) was placed on the film and pressed for 2 min. This operation was repeated five times taking care that it was not excessively heated and there was no color change.

Microbiological studies

Organisms: *E. coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, and *Salmonella typhimuruin* were obtained from Institute of microbial technology (IMTECH), Chandigarh, India. *Pseudomonas* sp was isolated from water within the laboratory.

Disc diffusion assay

The test organisms, except *L. monocytogenes*, were grown in nutrient broth at 37°C. One hundred microliters of the inoculum was uniformly spread on nutrient agar. *L. monocytogenes* was grown on Listeria identification agar base with supplement at 37°C. The overnight culture was diluted with 10 mL distilled water, and 100 μL of the diluted inoculum was uniformly spread on the same medium. Circular discs of 1 cm diameter were punched from the test films using a punching device and placed on the bacterial lawns and incubated at 37°C for 24 h. The plates were visually examined for inhibition zones around the discs, and diameter of the zone was measured using a vernier caliper.

Image analysis

The dried neem-free film of size 100 × 200 cm and thickness 2 mm was cut and embedded in low-viscosity resin solution (paraffin wax) and then cut on a sorwall MY 2-B ultra cut microtome for examination in a reflected fluorescence microscope system (Olympus Japan) linked to image analysis software (micro image lite version 4.0).

Mechanical and barrier properties

Mechanical properties

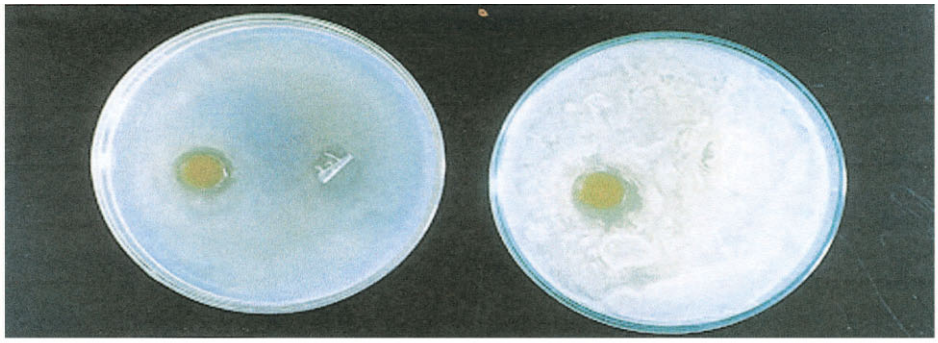
The tensile strength and elongation at break of the film were determined using ASTM D 882 method. The specimen was cut to strips of size 14 × 160 mm from cured free film. The specimens were conditioned for 24 h at 60% RH and were tested in Universal testing machine (Imtron Model 4302, Instran, UK)

Water vapor transmission rate (WVTR)

WVTR was determined using free film as per method ASTM D 96. Free films were cut to obtain a circular test specimen of 12 cm diameter. Test specimen was fastened to payne cup containing 8 mL of distilled water, using C-Type clamp. Area of the film for water vapor to permeate was of 10 cm^2. The cups were kept in desiccators over fused $Cacl_2$. The cups were weighed every 24 h, till a constant water loss was obtained. WVTR was calculated as per ASTM method. Inside the cup the RH will be 100%, but outside the cup (i.e., in the desiccators), the RH will be zero.

Oxygen transmission rate (OTR)

OTR was determined using free film as per method ASTM D 1434–66. OTR is the quantity of oxygen gas passing through a unit area of the parallel surfaces of the film per unit time under the conditions of test. Test specimen used is of circular shape of size 10 cm diameter. The diffusion cell is unclamped and opened. A thin layer of sealing grease is applied around the



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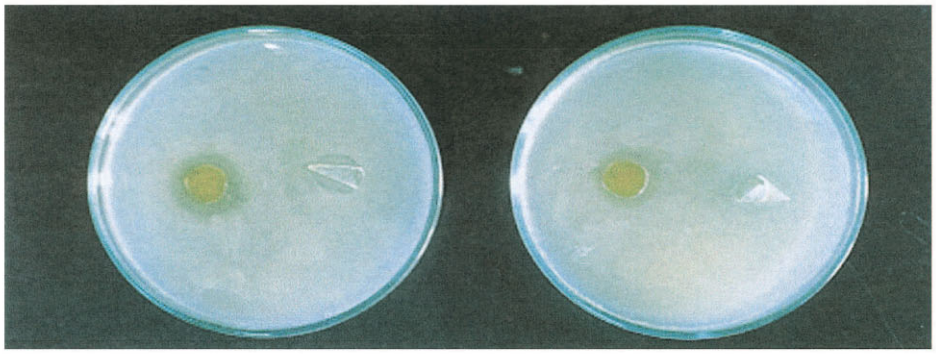

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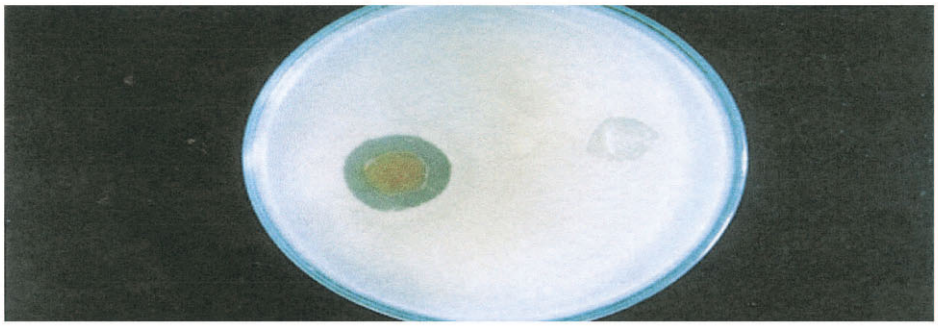

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Figure 1 Inhibition zones to different species (a) *E. coli*, (b) *Staphylococcus aureus*, (c) *Bacillus cereus*, (d) *Pseudomonas* spp., and (e) *Salmonella typhimurium* MTCC inhibited by neem containing heat-pressed film. The right side of the disc is control film showing no inhibition zone. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

raised rim of the lower half of the diffusion cell. The test specimen is then removed from the desiccator and placed upon the greased surface. Then, taking care to avoid wrinkles or creases, the upper half of the diffusion cell is lowered into place and both the halves are clamped lightly together. Before starting the experiment, zero voltage level (E_{on}) is set. After establishing E_{on} value, the flow switch is put on and oxygen

TABLE I
Antimicrobial Activity of Neem Films Using Disc (10 mm) Diffusion Assay

Test organisms	Inhibition zone diameter (mm)		
	Heat pressed	Without heat pressed	Control
E.coli	23	24	No inhibition
Staphylococcus aureus	15	20	No inhibition
Bacillus cereus	18	21	No inhibition
Pseudomonas spp.	15	18	No inhibition
Salmonella typhimurium MTCC-98	20	24	No inhibition
Listeria monocytogenes MTCC-657	No cfu	No cfu	No inhibition

cfu, colony forming units.

purged. Sensor output current, as indicated by the strip chart recorder, should increase gradually, ultimately stabilizing at a constant value. The constant value of the voltage on the strip chart recorder shall be recorded and labeled Eel.

OTR is calculated using formula given as below:

$$\text{OTR} = \frac{(S_o - S_o) \times Q}{An \times R_1}$$

where A is the specimen area, Q is the calibration constant (given), and R_1 is the value of load resistant (given). OTR is expressed in mol/m^2s.

Thermal properties

Glass-transition temperature (T_g): The T_g values of the films were determined using DSC 2010 (TA Instruments, USA) equipped with an intracooler. The measurements were made using a sealed empty pan as the reference material and N_2 as a flushing agent over the head. The instrument was calibrated with Indium. Samples (5–10 mg) were weighed into standard aluminum pans, sealed, and heated from −50 to 100°C at the rate of 5°C/min. The measurements were made in triplicate. The data were analyzed using TA universal thermal analyzer software.

Thermal degradation temperature: DMA measurement was carried out using Eplexor (Gabo instruments, Germany) in tensile mode. The sample was prepared according to ASTM D 882. A stress in tensile mode was applied to the sample at 5 Hz, and the stress was measured at a strain of 40%. The modulus (E'), loss modulus (E''), and loss tangent (tan $\delta = E'/E''$) accompanying the heating of the samples were measured.

Statistical analysis

The standard deviation (SD) of three replicate was calculated using software statistica 99 and the values are expressed as Mean ± SD.

RESULTS AND DISCUSSION

Antimicrobial properties

The effectiveness of neem-based films in terms of antimicrobial activity has been depicted in Figure 1 and mentioned in Table I. The antimicrobial activity of the films with and without heat press was tested against *E. coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Pseudomonas spp.*, and *Salmonella typhimurium*. The films showed inhibitory zones against all the test organisms. However, the film which was not heat pressed showed larger inhibition zones against test organisms as compared with the heat-pressed ones, indicating higher antimicrobial activity of the earlier against pathogenic organisms. The activity was maximum against *L. monocytogenes*, which did not show any colony forming units on test plates within 24 h. The film showed greater activity against *E. coli* and *S. typhimurium* as compared with that against *S. aureus*, *Pseudomonas spp.*, and *Bacillus cereus*.

The control film without neem extract was found to have a very little activity against any of the test organisms. The results indicated that the neem-based films

TABLE II
Data[a] for Tensile Strength (TS), Elongation at Break (EAB), Water Vapour Transmission Rate (WVTR), and Oxygen Transmission Rate (OTR) of Edible Films (n = 3)

Composition	TS (MPa)	EAB (%)	WVTR (g cm^{-2} day^{-1})	OTR (mL m^2 day^{-1})
Edible film	5.86 ± 0.2	68.0 ± 2.3	0.168 ± 0.003	91.5 ± 1.0
Edible film with neem	5.93 ± 0.2	65.0 ± 2.3	0.167 ± 0.003	91.3 ± 1.0
Heat-pressed edible film with neem	9.37 ± 0.3	55.0 ± 2.1	0.197 ± 0.004	56.8 ± 0.5
Synthetic films				
LDPE[b]	16.5 ± 0.9	>1000	0.02 ± 0.006	29.8 ± 5.1
PE[b]	50.7 ± 8.2	73.0 ± 27	0.038 ± 0.035	10.20 ± 0.01
PVDC[b]	65.6 ± 10.8	18 ± 5	0.002 ± 0.001	8.6 ± 0.001

[a] Mean ± SD.
[b] LDPE, low density poly ethylene; PE, polyethylene; PVDC, poly vinylidine chloride.

TABLE III
Thermal Analysis Data[a] for Edible Films (n = 3)

Composition	T_g DSC	T_g DMA	ΔH value	Degradation temp. (DMA)	Modulus DMA (MPa)
Edible film	52 ± 1.0	58 ± 1.0	45.8 ± 1.0	202 ± 3.0	2.5
Edible film with neem	54 ± 1.0	59 ± 1.0	49.5 ± 1.0	205 ± 3.0	2.6
Heat-pressed edible film with neem	82 ± 1.0	84 ± 1.0	1.58 ± 3.0	242 ± 4.0	4.5

[a] Mean ± SD.

with/without heat pressing could effectively control the microorganisms on test agar.

Barrier properties

Water vapor transmission and oxygen transmission rates of different edible films (Table II) showed that the incorporation of neem extract had no effect on WVTR and OTR properties. However, in the case of heat-pressed film, a drastic decrease (about 70%) in WVTR and a marginal decrease in OTR (about 8%) were observed. Hence, edible film to be tried for modified atmosphere preservation should have low WVTR with optimum permeability of O2 and CO_2 to allow respiration/breathing. This unique property is imparted to the films by protein i.e., casein component.[14] These films are also known as breathable films.[15] Considering this aspect, the heat-pressed edible film can be considered as a breathable film, since heat pressing did not affect OTR while WVTR was considerably decreased.

Mechanical properties

Mechanical properties namely tensile strength (TS) and elongation at break (EAB) of edible and synthetic films (Table II) together with showed that the incorporation of neem extract into edible film had no effect on mechanical properties. However, TS of heat-pressed films was significantly higher (55%) than that of the control ones. This is in agreement to that of modulus determined by DMA (Table III). No significant decrease in EAB elongation

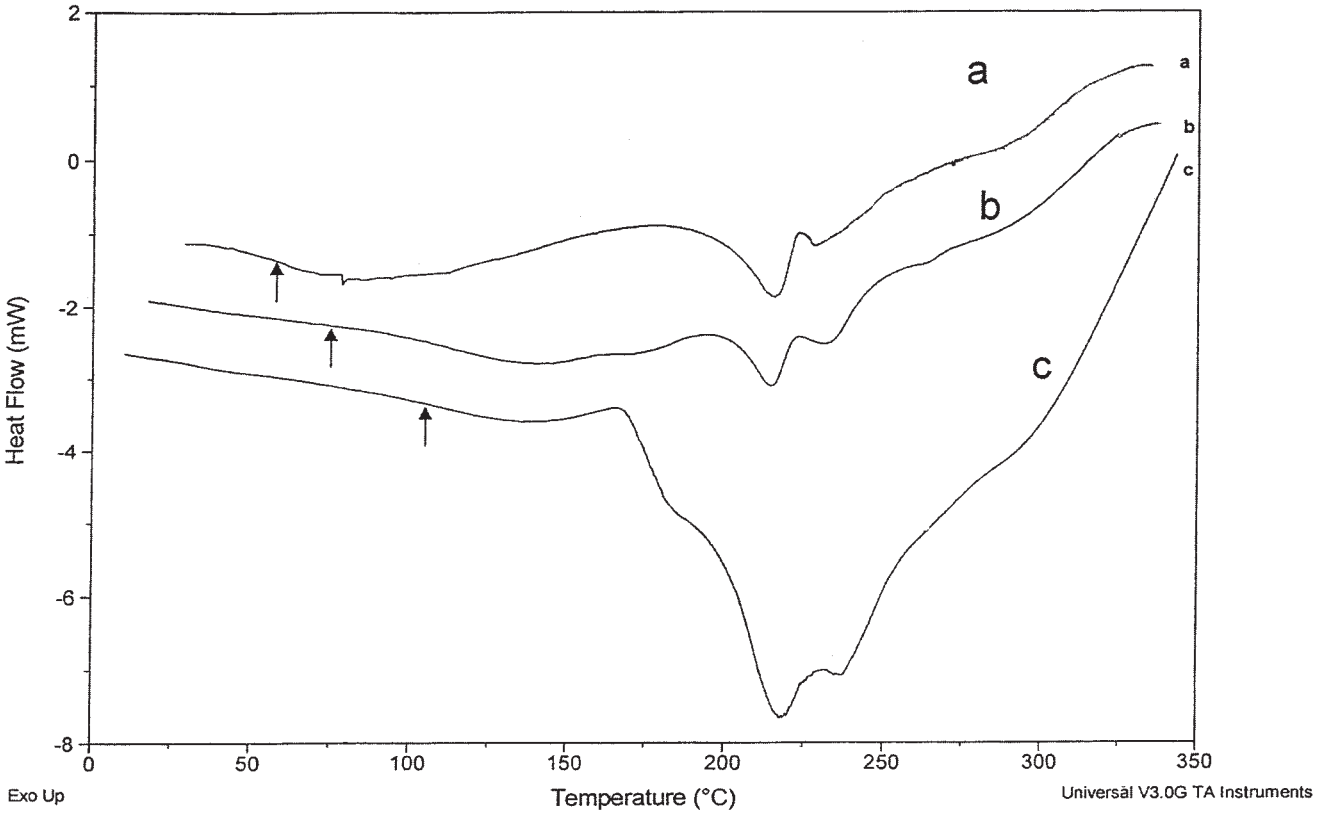

Figure 2 DSC thermogram showing glass-transition temperature (↑) of (a) control, (b) neem containing, and (c) neem containing heat-pressed film.

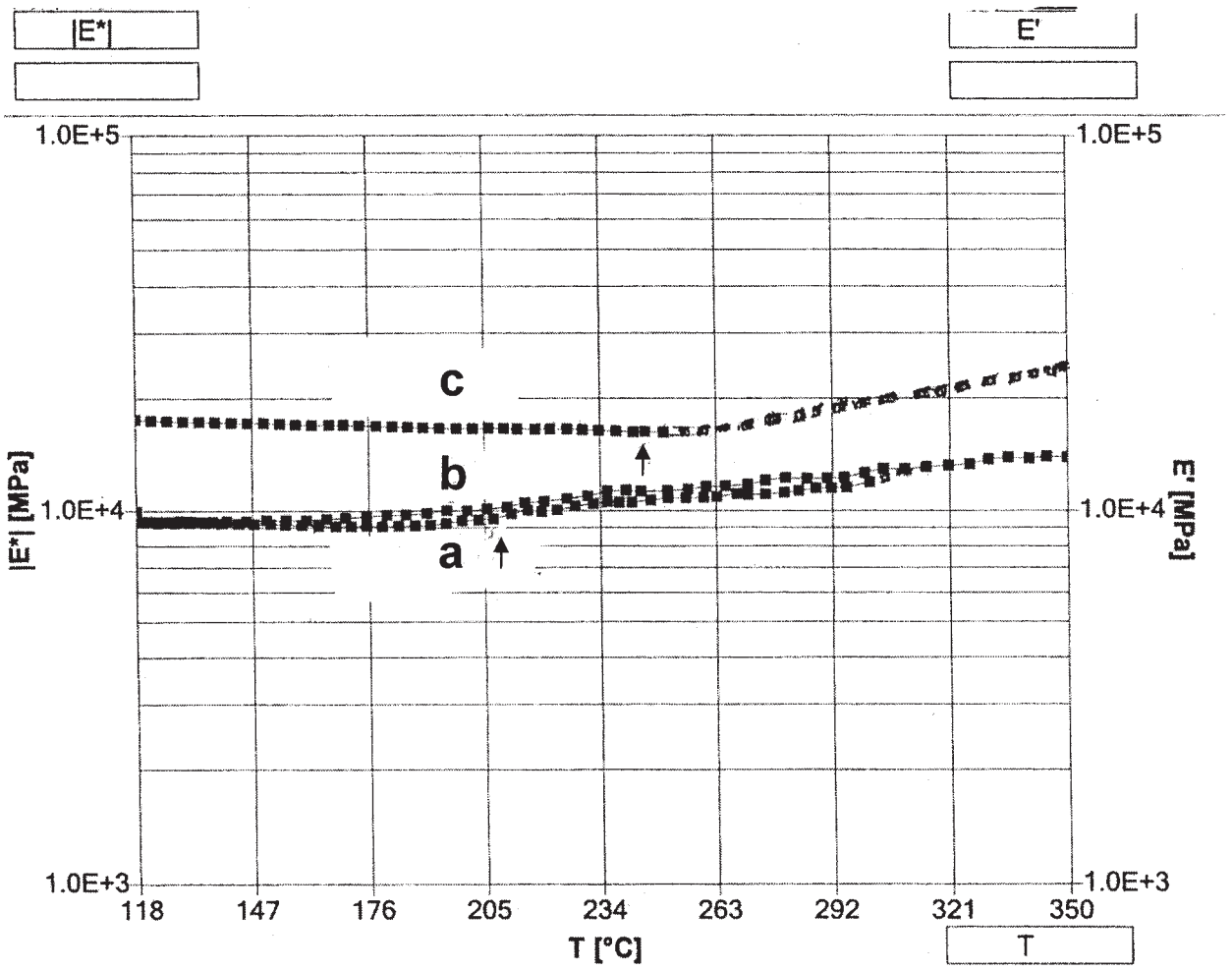

Figure 3 DMA thermogram showing degradation temperature (↑) of (a) control, (b) neem containing, and (c) neem containing heat-pressed film.

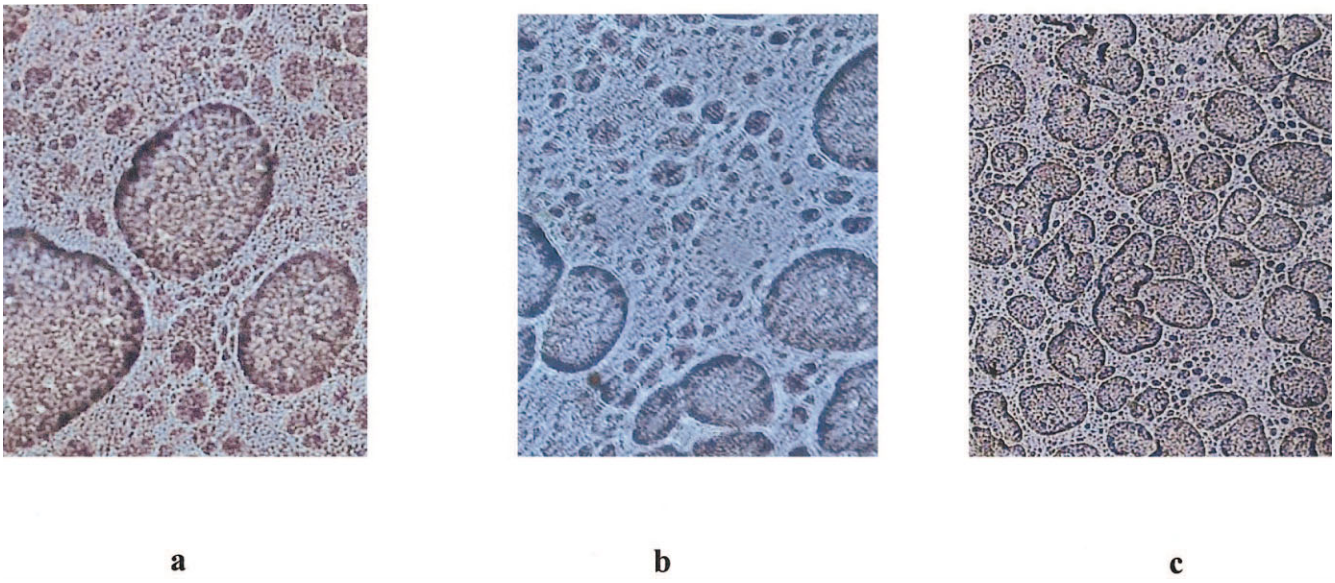

Figure 4 LT images of (a) control, (b) neem containing, and (c) neem containing heat-pressed film. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(7%) was observed. This type of behavior generally results in toughening of the material, causing a drastic increase in TS, and a marginal increase or decrease in EAB.[16] In normal case the increase in TS results in proportional decrease in EAB. Hence, the heat pressing treatment for 10 min at 130–140°C, gives to edible film had a toughening effect. The mechanical properties of the heat-pressed film was some what comparable with those of the synthetic ones, which were not biodegradable and breathable (Table II).

Thermal analysis

The DSC and DMA thermograms of different edible films have been shown in Figures 2 and 3, and thermal data are given in Table III. It can be seen from Figure 2 and Table III that the incorporation of neem extract had no effect on T_g values; however, film with and without heat press showed T_g values of 100 and 42°C, respectively. There was almost 100% increase in the T_g value in case of heat-pressed film, indicating that the film integrity and the bonding of the protein–starch particle had improved through heat pressing. This also indicated the toughening of the film and correlated well with the mechanical properties. A marginal increase in T_m values for different edible films was observed (Table III); however, ΔH also increased, indicating greater requirement of heat for melting of heat-pressed film because of toughness. The film behaved like any typical biological film in the sense that the after melting underwent degradation/charring.[17,18] This phenomenon was also observed in DMA experiment. The DMA isotherms (Fig. 3) showed that, upto certain temperature, there was no appreciable increase in modulus; however, thereafter modulus drastically increased, indicating degradation or charring of films. The heat-pressed film showed degradation temperature as 242°C, while that for edible film with and without neem was about 205°C. The T_g (i.e., decrease in modulus) was not sharp eventhough the modulus data (not shown) indicated it to be 52 and 98°C, respectively, for films with and without heat pressing and is in agreement with the T_g values obtained using DSC (Table III).

Image analysis

Microscopic observation of the edible film (Fig. 4) revealed the size, shape, and distribution domains. It can be seen that the incorporation of neem extract had no effect on the morphology of films; however, the heat pressing resulted in better dispersion of protein–starch particles as compared with film without heat pressing. In case of heat-pressed film, the particle size was about 2 μm and the distribution was uniform, while in case of film which was not heat pressed, with and without neem, the particle size was about 4 μm and the distribution was uneven. Manson and Sperling[15] reported the relationship between morphology and modulus. The microscopic observation suggested that the film integrity and bonding of particles were better in case of heat pressed as compared with that which was not heat pressed and correlated well with the tensile and modulus data (Table II).

CONCLUSIONS

Heat pressing of the edible film with the help of hot iron box caused a drastic decrease in WVTR, while the OTR was only marginally affected. Application of heat had a toughening effect on the edible film, since a considerable improvement in tensile properties was also observed. Thermal stability of heat-pressed film was enhanced, since T_g and degradation temperature were increased by more than 50%. Incorporation of neem to the edible film resulted in satisfactory antimicrobial properties and these properties were only marginally affected by heat pressing. The film can be tried as breathable one in modified atmosphere packaging.

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