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Development of dense films from *Melia azedarach* polysaccharides

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ABSTRACT: The aim of this work was to prepare dense films from polysaccharides extracted from *Melia azedarach* (MA) to be used e.g. in agricultural industry. Crosslinking of MA films with glutaraldehyde (Glu) through immersion method were performed. Structural features of films were elucidated by FTIR and XRD analysis. The influence of crosslinking in mechanical properties, water uptake and water vapor permeation was evaluated. Results showed neat *Melia azedarach* film presented high elongation and elastic modulus. Hydroxyl functional groups present in MA were reacted with glutaraldheyde to render a crosslinked matrix. It was observed through FTIR analysis that OH band reduces intensity due to the formation of acetal linkage, as the crosslinking time increases. XRD evidenced structural changes in MA arrangement with the crosslinking time. Chemical crosslinking during 12 h and 24 h rendered insoluble but less deformable films. Water uptake and water vapor permeability were reduced as the crosslinking time increased.
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INTRODUCTION

Melia azedarach (MA) is a large tree of worldwide distribution, and its leaves and fruits show a variety of biological effects on insects, such as an anthelmintic, an antifeedant, and other inhibitory activities.[1–4] Moreover, many studies have demonstrated that MA is also used as a medicinal plant. It has been used as a bitter tonic, astringent, fuel, and an antiseptic. It has also been useful in fever, thirst, nausea, vomiting, and skin diseases.[5] As a consequence, chemical and biological studies on this tree have been undertaken at many regions around the world to isolate a number of tetranortriterpenoids, so called limonoids which seems to be responsible for the chemical and biological activity of MA.[6] Recently, He *et al.*[5] studied the structure and the cytotoxic activity of polysaccharides present in MA fruits. They found that the major polysaccharide present in MA fruit pulp is MPS-III. Data obtained indicated that MPS-III contains α (1→4) main chain backbone composed of arabinose, mannose in a molar ratio of 1.31 : 1.0 and has α (1→6) branch structure. MPS-III showed a strong cytotoxic activity in the BGC-823, a gastric cancer cell line. Moreover, Carpinella *et al.*[4] tested the antifeedant activity of the fruit extract of MA on a variety of herbivore and granivorous insects through choice and no-choice tests. Authors compared the bioactivity of the isolated active compounds from MA fruits: meliartenin and its interchangeable isomer 12-hydroxiamoorastatin (**1**) to azadirachtin (**2**) and toosendanin (**3**), both compounds used for comparison purposes. Authors proved MA fruit extract and its active principle, compound **1,** have interesting potential for use in pest control programs as they inhibited feeding of Epilachna paenulata Germ. (Coleoptera, Coccinellidae) and E. paenulata larvae. However, up to now, little attention has been given to the film forming ability of polysaccharides extracted from MA fruits. This might represent a useful, sustainable, and environmentally friendly way of taking advantage of this abundant raw material and its active principle for agricultural applications. In general, the development of biomaterials holds great promise to mitigate many of the sustainability problems, offering the potential of renewability, biodegradation, and a path away from harmful additives. Several scientific journals are publishing novel research articles about the exploitation of materials originating from agricultural sources i.e. produced from renewable, biological raw materials to be applied for packaging,[7–12] fuel cells,[13–15] fibers for reinforcement,[16–18] gas barrier thin films,[19–22] mulching,[23–25] and natural resources of film forming polysaccharides,[26–29] among others. MA polysaccharides might be successfully used to prepare films with insecticide activity to crops applications. The purpose of this paper involves the preparation and characterization of MA films as well as the evaluation of its properties after crosslinking. Chemical modification was carried out in order to improve water resistant. Chemical

and physical properties of films were determined through FTIR, XRD, water uptake, and water vapor permeability.

EXPERIMENTAL

Materials and Methods

Raw Material. Fruits of MA were collected from a seeding station of San Luis, San Luis Province, Argentina. The extraction of polysaccharides from *Melia azedarach* fruits was made following the procedure reported by He *et al.*[5] in order to retain the active principle for insecticide applications. Fruit of MA (100 g) was washed with distilled water at 40°C during 2 h. Then, the fruits were placed in a flask containing 700 mL of distilled water and stirred for 12 h at 80°C. The fruit pulp of MA was filtered and defatted with ethanol (50 mL) for 4 h. The aqueous extract was then filtered and concentrated at 80°C to reduce a 50% the initial volume. After centrifugation the crude polysaccharide was treated with absolute ethanol to decolorize the solution. Hydrolysis of polysaccharides from fruit pulp of MA was performed by using 0.1*M* NaOH during 4 h at 60°C. Then the solution was allowed to cool and it was neutralized with 0.1*M* HCl. Basic hydrolysis was performed in order to completely isolate polysaccharides from residues of cell wall compounds.[30] After, polysaccharides were precipitated with ethanol, filtered, and redissolved into water. The precipitation step was repeated three times. Finally, hydrolyzed polysaccharides were dried at 60°C during 24 h and grinded in a Rolco mill.

Purity of MA Polysaccharide. The purity of MA polysaccharide was measured by applying Bradford assay in order to quantify the amount of protein residue present in MA extracts. The Bradford method involves the binding of Coomassie Brilliant Blue G-250 to protein. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm, and it is the increase in absorption at 595 nm which is monitored. This assay is very reproducible and rapid with the dye binding process virtually complete in ~2 min with good color stability for 1 h.[31] UV-Vis U-2001 Hitachi spectrophotometer was used to quantify protein residue.

Films Preparation. Aqueous solution of polysaccharides at 2 wt % (50 mL) was stirred at 40°C for 2 h. 1% v/v of glycerin (GLY, Biopack Argentina) was early added to the solution as a plasticizer. Then, the solution was casted on a leveled polycarbonate-plate (11 cm diameter) and kept in an oven at 60°C for 12 h. To control film thickness, the quantity of each film forming solution poured onto a plate was calculated so that the solid content (polysaccharide and glycerin) was the same (50 mL for control film forming solution). Following drying step, the film was peeled off the plate and thickness was measure using a Köfer micrometer (precision ± 1 μm). Specimens for different characterizations were then cut. Five thickness measurements were taken on each tensile testing specimen along the length of the strip with the mean used in tensile strength calculations. Similarly, three measurements were taken on each water vapor samples, and the mean values were used for calculations.

Crosslinking of Films. Crosslinking of *Melia azedarach* films were performed by immersing polysaccharide film in an acetone-hydrochloric acid solution of glutaraldehyde for 12 and 24 h at ambient conditions. Crosslinking solution was prepared by dissolving 5 wt % of glutaraldehyde (GA, Sigma-Aldrich) and 1 wt % of hydrochloric acid (Sigma-Aldrich) in Acetone (Biopack, Argentina). The modified films were called MA-12 h and MA-24 h, regarding with the crosslinking time, i.e., 12 h and 24 h, respectively. MA nomenclature was used to refer to neat *Melia azedarach* films. Figure 1 shows mechanism of crosslinking reaction using GA as the crosslinking agent. All neat and crosslinked MA films were no transparent, dark-brown colored, and handling materials.

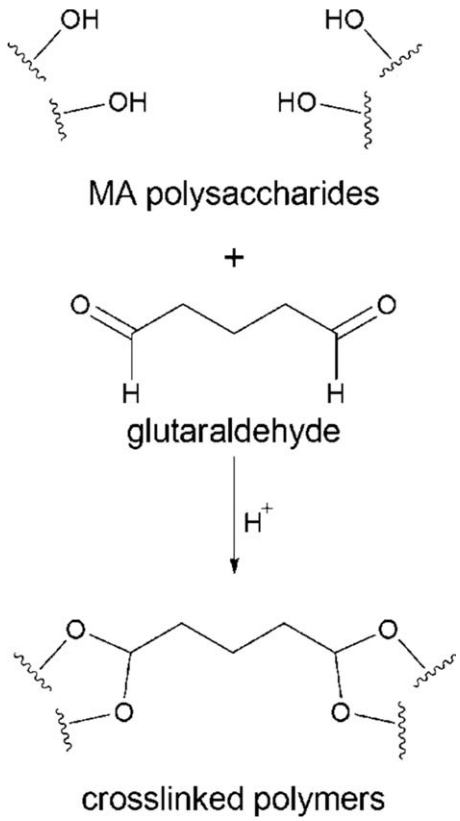

Figure 1. Scheme of GA crosslinking reaction.

FTIR Spectroscopy. FTIR spectra were determined by the diffuse reflectance (DRIFTS) mode using a Nicolet PROTEGE 460 Spectrometer and the transmission mode using a Varian 640 Spectrometer. The operational range was 400–4000 cm^{-1}. The number of scans for each sample was 64.

X-ray Diffraction. Wide angle X-ray diffractions (WAXD) studies were carried out using an equipment Rigaku model D-Max III C, lamp of Cu Ka and filter of Nickel. The 2θ operational range was from 0° to 30°. The *d*-spacing of each HCMMM was determined by Bragg's equation (eq. 1).

$$n\lambda = 2d\,sen\,\theta \quad (1)$$

where: d is the average intercatenary distance, n is the integer determined, λ is the wavelength of the X-ray (nm), and θ is the Bragg's angle.

Mechanical Properties. Mechanical properties were measured with a Comten Industries Series 94 VC instrument (USA) at a

constant traction speed of 5 mm/min following the ASTM D882 requirements. To ensure complete relaxation of the polymeric structures and to standardize the experimental procedure, the mechanical properties were measured at room temperature ($T = 25°C$) and at a relative humidity of 40% 24 h after the film casting. The results are the average values from three samples of each membrane. Tensile strength (σ) was calculated by dividing the maximum load by the initial cross-sectional area of the specimen. Percentage elongation at break (% ε) was calculated as the change percentage of the initial gage length of specimen ($l_0 = 40$ mm) at the point of sample failure. Finally, elastic module (E) was calculated from the slope of the stress–strain curve when a linear relationship between the stress and strain was observed. The stress–strain relationship is given by the following equation:

$$\sigma = E * \varepsilon \tag{2}$$

where: $\sigma = F/A$, $\varepsilon = \Delta l/l_0$, and E is the Young's modulus [units N m^{-2} = Pa].

Water Uptake. Water uptake was determined gravimetrically. Weights of completely dried samples were measure directly. Film specimens were introduced into bottles containing 20 mL of distilled water and shaken at ambient temperature (25°C). At predetermined times (12, 24, 36, 48 h) films were removed from the medium, blotted to remove excess water, and immediately weighed. This procedure was repeated until the films reached constant weight (equilibrium water uptake).[32] The water uptake of the crosslinked films was calculated according to the following equation:

$$WU = \frac{W_t - W_o}{W_o} \tag{3}$$

where: WU = water uptake (g/g film), W_t = weight of swollen film at a time "t", W_o = weight of dried film.

Water Vapor Permeability. Water vapor transmission rate (WVTR) was determined gravimetrically using a modified ASTM Method E 96-95. Film specimen was mounted on acrylic permeation cell comprises two chambers. Upper chamber is in contact with water vapor pressure, while the bottom chamber is filled with an adsorbent material. The film specimen is in between both chambers acting as a barrier. The driven force of the global process is the difference of water vapor pressure at both sides of the film specimen. Once the permeation cell is assembled, the all system is placed into a chamber with temperature and relative humidity control. The operational conditions are fixed at 37 ± 2°C and 98% relative humidity (RH). Water vapor permeability (WVP) (ng m m^{-2} s^{-1} Pa^{-1}) was calculated from:

$$WVP = \frac{WVTR \times L}{\Delta P} \tag{4}$$

where: WVTR (ng m^{-2} s^{-1}) was measured through a film specimen; L (m) was mean film thickness, ΔP (Pa) was partial water vapor pressure difference across the two sides of the film specimen.

Statistical Analysis

Mechanical properties, water uptake, and water permeability results were subjected to one-way analysis of variance (one-way ANOVA). Comparison of means was carried out by Duncan's new multiple range tests at a confidence level of 95%. All statistical calculations were performed using SPSS for Windows® (SPSS, Chicago, IL). The experimental results are presented as mean values with standard deviations.

RESULTS AND DISCUSSION

Purity of MA Polysaccharides

Several spectroscopic methods are used to determine the concentration of protein in a solution. Among them, the Bradford method is faster, involves fewer mixing steps, does not require heating, and gives a more stable colorimetric response than other assays. Because of those advantages, the Bradford assay using bovine serum albumin (BSA) as standard was used for determining protein content in MA polysaccharides.[33] Results were obtained from averaging at least three experiments for MA sample. MA polysaccharide presented a protein residue of 1.3 ± 5 wt % which is in good agreement with the value reported by He *et al.*[5] Protein residues lower than 2% are considered acceptable.

FTIR Analysis

FTIR spectra were performed in order to visualize typical polysaccharide bands and chemical crosslinking of *Melia Azedarch* films. According to He *et al.*[5] the extract obtained from mesocarp of MA fruits contained 99% of carbohydrate and 1% of protein after hot water extraction and ethanol precipitation. The crosslinking reaction takes place when glutaraldehyde (GA) reacts with the –OH groups of MA polysaccharides during the immersion time.[13,32,34] The degree of crosslinking of the membranes is strongly dependent on the reaction time.[35] In this work 12 and 24 h crosslinking time were assayed. Spectra for MA, MA-12 h, and MA-24 h were recorded. All spectra showed the characteristic peaks of polysaccharide structure, broad stretching intense band at 3400–3000 cm^{-1} for the hydroxyl group and weak C—H stretching band at 2927 cm^{-1}. Other typical band appears at 1038 cm^{-1}, common in all polysaccharides, due to the coupling of C—O or the C—C stretching modes with the C—O—H bending modes.[11] On the other hand, a band observed at 1648 cm^{-1} is attributed to the stretching band of amide groups present in protein residues. This band was also observed for other polysaccharides.[33] By the crosslinking with GA, the intensity of —OH bands of MA became much weaker because of the conversion of —OH into —C—O—C— (acetal linkage) as shown in Figure 2. The modes due to the C—O—C bridge may also contribute at the frequency of 1038 cm^{-1} as well as the modes due to aliphatic C—H groups contribute to the frequency of 2854 cm^{-1}. When the reaction time increases, more —OH groups are converted into —C—O—C— groups. As a consequence, the intensity of the —OH band in MA-12 h and MA-24 h decreases as well as aliphatic C—H band increases regarding to MA-unmodified film.

XRD Analysis

To analyze structural changes in MA based film, X-ray diffraction patterns of the films with different crosslinking times were studied and they are shown in Figure 3. 2θ angles and d-spacing values are also showed in Table I. All diffractograms showed a predominant amorphous pattern due to the presence of at least

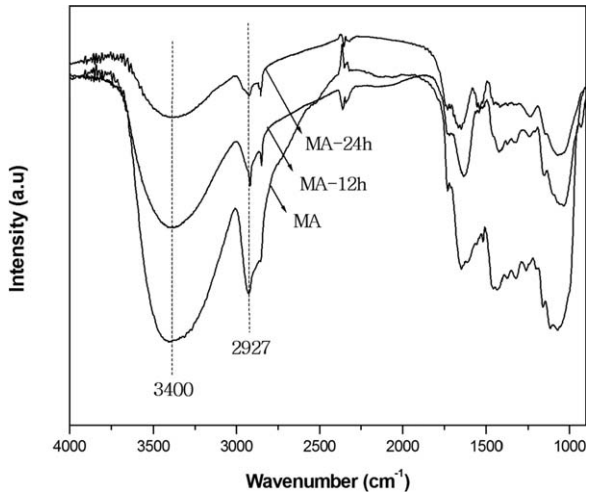

Figure 2. FTIR spectra of MA films.

three broad bands. In MA film it can also be distinguished several narrower peaks lying on broad bands. Amorphous bands have been called A, B, and C band, from lower to higher 2θ angles, respectively. In MA diffraction pattern A, B, and C bands are centered at about $2\theta = 10.17$, 16.15, and 21.42, respectively. Furthermore, lying on "B" band it was observed two narrower peaks at $2\theta = 13.95$ and 16.45, respectively; while on "C" band it was noted only one narrow peak at $2\theta = 29.35$. Results mentioned above allow to state that neat MA film not only presents amorphous regions but also ordered ones. This is due to the formation of a secondary structure of polysaccharide macromolecules which takes place through molecular interactions between polysaccharide functional groups. According to Rees and Welsh,[35] molecular interactions that can take place to build secondary structures are H-bonding, dipole and ionic interactions. On the other hand, crosslinking reaction caused structural changes in the macromolecular arrangement of polysaccharide chains, sometimes called "interruptions". These interruptions can modify the secondary building structure or construct a new dimensional arrangement known as tertiary structure.[36,37] Diffraction patterns of MA-12 h and MA-24 h

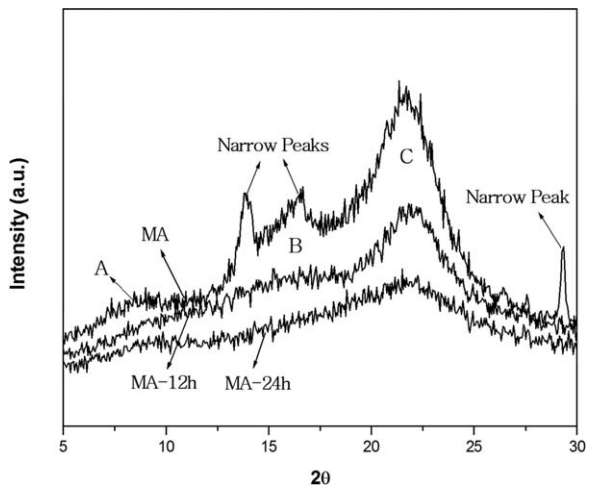

Figure 3. X-ray diffraction of MA films

Table I. *d*-Spacing of MA Films Amorphous Bands

Film	Band	2θ	*d*-spacing (Å)
MA	A	10.17	8.69
	B	16.15	5.48
	C	21.42	4.15
MA-12h	A	11.66	7.58
	B	17.44	5.08
	C	21.77	4.08
MA-24h	A	10.20	8.67
	B	-	-
	C	21.12	4.20

depicted the influence of crosslinking by vanishing of narrower peaks and increasing wideness of amorphous bands. This result can be associated with the secondary structure modification. Moreover, it was observed a transition from three to one less intense amorphous band as the crosslinking time increased. Glutaraldehyde prevents molecular packing of polysaccharide chains by rigidizing polymeric matrix. This avoids the formation of ordered regions and explains the disappearance of narrower peaks.

Mechanical Properties

Tensile strength (σ) is the ability of a material to resist under mechanical stress until it breaks and is one of the most important measured properties of materials used in structural applications. Elongation-at-break (%ε) of a material is the percentage increase in length that occurs before it breaks under tension. Elastic modulus (EM) represents the rigidity of the material.[38,39] Values of these parameters were obtained from stress–strain curves for at least three samples of each film. Results presented in Table II showed a marked effect of cosslinking in mechanical parameters. It was observed a decrease in σ, % ε and EM with increasing crosslinking time. These results were expected considering crosslinking of a polymeric matrix restricts molecular movement of polymer chains, and acts as points of rupture when an external force is applied.[40] Neat MA film showed better mechanical performance. On the other hand, MA-12 h presented lowest values of tensile strength and elastic modulus; however, it showed high elongation at break which represents an interesting performance for a flexible film. An increase in "σ and ME" in MA-24 h regarding to MA-12 h, demonstrated higher crosslinking time render more rigid films with lower elongation at break and mechanical behavior similar to brittle materials.

Table II. Mechanical Properties of MA Films

Film	σ (MPa)	% ε	EM (MPa)
MA	15.01 ± 3.55^{a}	10.76 ± 2.84^{d}	174.02 ± 10.55^{f}
MA-12h	0.12 ± 0.06^{b}	8.55 ± 2.50^{d}	2.13 ± 0.91^{g}
MA-24h	0.60 ± 0.30^{c}	4.08 ± 1.80^{e}	20.62 ± 11.82^{h}

Values in the same column with different letters are significantly different ($P < 0.05$).

Table III. Water Uptake of MA Films

	MA		MA-12h		MA-24h	
t_c (h)	Film weight (g)	WU (g g^{-1} film)	Film weight (g)	WU (g g^{-1} film)	Film weight (g)	WU (g g^{-1} film)
0	0.0125	–	0.0185	0.00	0.0293	0.00
12	-	–	0.0558	2.02 ±0.28[a]	0.0840	1.87 ±0.31[a]
24	-	-	0.0578	2.12 ±0.38[a]	0.0856	1.92 ±0.36[a]
36	-	-	0.0608	2.28 ±0.54[a]	0.0866	1.96 ±0.40[a]
48	-	-	0.0607	2.28 ±0.54[a]	0.0890	2.04 ±0.48[a]

Values in the same row with different letters are significantly different ($P < 0.05$).

MA films depicted tensile strength and elongation at break values similar to other polysaccharides such as alginate (6–41 MPa and 0.9–1.2% ε),[41] starch 30% Gly (11–30 MPa and 2.8–21%ε)[42] and pectin 0.6% Gly (14 MPa and 8.7% ε),[43] among others.

Water Uptake

Polysaccharides are biopolymers able to absorb huge amount of water due to the polarity of their functional groups. Water uptake is a method performed to measure water swelling capacity of a material. Nevertheless, chemical or physical crosslinking is used to improve water resistance and reduce water swelling.[32] Even though crosslinked films are not soluble in water, swelling was observed after placing them in aqueous media. Results are shown in Table III. It was observed MA uncrosslinked film was water soluble whereas the crosslinked MA films swell water but not dissolved after 48 h of immersing time. The amount of water absorbed was 1.12 times average higher in MA-12 h than MA-24 h. However, the difference was not statistically significant. Similar results were obtained by Kulkarni *et al.*,[44] who crosslinked Na-Alginate beads using GA at different exposure times. It was concluded that, swelling of the polymeric beads decreases with increasing exposure time to the crosslinking agent. On the other hand, Remuñan-López *et al.*[32] studied the effect of crosslinking time in chitosan glutamate and sodium alginate films. It was found that longer exposure time of the films to the crosslinking agent increased the degree of crosslinking and hence decreased the equilibrium water uptake in alginate films.

Water Vapor Permeation

Permeability depends on the solubility and diffusivity of water within the polymeric matrix. The WVP of MA films as function of crosslinking time is showed in Figure 4. Even when crosslinked films are water insoluble, they are water vapor permeable. Crosslinking of MA films resulted in a significant decrease in WVP regarding to the unmodified system. This behavior is associated with the combined effect of the rigidity in crosslinked polymeric matrix showed in Table II and the decrease in water solubility observed in Table III. WVP values of MA films are similar to those reported for other biopolymers such as chitosan (0.0675 ng m m^{-2} s^{-1} Pa^{-1}),[45] alginate (5 ng m m^{-2} s^{-1} Pa^{-1}),[41] starch (0.537 ng m m^{-2} s^{-1} Pa^{-1}),[42] and pectin (1.58 ng m m^{-2} s^{-1} Pa^{-1}).[43] Even when crosslinking is performed, films keep hydrophilic.[38] Crosslinking causes rigidification of polymeric matrix and prevents not only water absorption but also water vapor permeation. This is due to a restriction in the segmental movement of polymer chains; hence, diffusion of vapor molecules is energetically hindered. As a consequence, the permeability is reduced as the degree of crosslinking increases.[46,47]

CONCLUSIONS

In this work nonconventional, not even exploited and renewable *Melia Azedarch* fruits were used to obtain new polysaccharides able to form films. MA films were prepared and characterized in order to be applied in agricultural issues such as insecticide or mulching films. Pure MA polysaccharides were strength resistant as well as flexible. However, crosslinking of MA films with glutaraldehyde (GA) at two different crosslinking times rendered less resistant and flexible films as the crosslinking time increased. Crosslinking was performed in order to reduce water absorption and solubility in MA polysaccharides. Crosslinking reaction was followed by FTIR analysis. It was observed that OH bands reduce intensity as the crosslinking time increases due to the formation of acetal linkage. XRD analysis showed that crosslinking of MA produced completely amorphous and water resistant films. It was also proved by WVP measurements in which water vapor barrier increases as the crosslinking time increased.

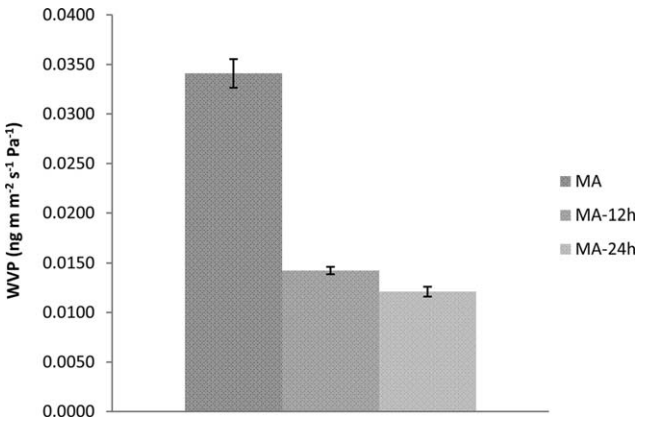

Figure 4. WVP of MA films. Error bars are SD. Each value was significantly different ($P < 0.05$).

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