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Physical and antifungal properties of hydroxypropylmethylcellulose based films containing propolis as affected by moisture content

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ABSTRACT

Edible films based on hydroxypropylmethylcellulose (HPMC) and different concentrations of an ethanolic extract of propolis (EEP) were obtained. Film-forming dispersions (FFDs) were characterized in terms of pH, density, rheological properties, particle size distribution and ζ-potential. Dry films were evaluated in terms of their optical, mechanical and water barrier properties when equilibrated at different moisture conditions. Likewise, the antifungal activity of the films against *Aspergillus niger* and *Penicillium italicum* was studied. The incorporation of EEP affected the pure film properties by improving the water vapour permeability and giving rise to more rigid, less flexible and deformable, more opaque and coloured films with lower gloss and transparency. The composite films revealed a notable antifungal activity against the tested fungus, showing a greater inhibitory effect on *A. niger*.

1. Introduction

The main objective of applying edible coatings is to reduce moisture losses, prevent physical damage, enhance product appearance and to act as a carrier of different food ingredients such as colorants, flavours or nutrients (Franssen & Krochta, 2003). Their function can be broadened by incorporating antimicrobials to protect food products from microbial spoilage, extending their shelf-life and enhancing their safety (Cagri, Ustunol, & Ryser, 2004; Franssen & Krochta, 2003; Oussalah, Caillet, Saucier, & Lacroix, 2006; Rojas-Graü, Tapia, Rodríguez, Carmona, & Martín-Belloso, 2007). Currently, the market trend is focused on the use of natural antimicrobial compounds (Beuchat & Golden, 1989; Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2009; Tiwari et al., 2009). The most widely studied natural substances have been enzymes, nisin, organic acids, essential oils and their derivatives (Cagri et al., 2004; Eswaranandam, Hettiarachchy, & Johnson, 2004; Oussalah et al., 2006; Padgett, Han, & & Dawson, 1998; Pranoto, Rakshit, & Salokhe, 2005; Rojas-Graü et al., 2006) and more and more attention has been paid to the search for novel natural products with which to improve the functional properties of the films. Propolis has been applied in medicine and cosmetics and, more recently, to foodstuffs, due to its antibacterial, antioxidant and antifungal activities (Burdock, 1998; Ghisalberti, 1979; Tosi, Donini, Romagnoli, & Bruni, 1996).

Propolis is a natural resinous substance collected from the leaf buds of different tree species by honeybees and can be considered as a complex mixture of chemicals, whose composition depends on the constituents of the plant material and the time of collection. It usually contains resins (50%), composed of flavonoids and phenolic acids, waxes (up to 30%), essential oils (10%), pollen (5%) and various organic compounds (5%) such as Fe and Zn, vitamins (B_1, B_2, B_3 and B_6), benzoic acid, fatty acids, esters, cetons, lactones, quinones, steroids and sugars as well as natural pigments such as chlorophyll and carotenoids (Cavaco et al., 2008; Farré, Frasquet, & Sánchez, 2004; Juliano, Pala, & Cossu, 2007). Its colour varies form yellow–green to dark brown depending on its source and age (Burdock, 1998).

The antibacterial and antifungal properties of propolis have been extensively investigated and, although its chemical composition is linked to the phytogeographic origin, the activity of propolis or bee glue has always been reported (Drago et al., 2000; Kujumgiev et al., 1999; Scazzocchio, D'Auria, Alessandrini, & Pantanella, 2006). Part of the antimicrobial activity is due to polar phenolic compounds specifically of the flavonoid fraction. The propolis and its extracts (aqueous or ethanolic) have been shown to have antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella*, *Penicilium digitatum* (Soylu, Ozdemir, Ertnrk, Sahinler, & Soylu, 2008) and other fungi such as *Aspergillus niger*, *Candida albicans* and *Botrytis cinerea* (Kalogeropoulos, Konteles, Troullidou, Mourtzinos, &

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Karathanos, 2009; Mirzoeva, Grishanin, & Calder, 1997; Ozcan, 1999; Scazzocchio et al., 2006), among others.

Hydroxypropylmethylcellulose (HPMC) is a common hydrocolloid used for film formulation due to its good film-forming properties; it forms transparent, flexible films with proper sensorial characteristics (Kester & Fennema, 1985, 1986) and moderate oxygen permeability (Krochta & de Mulder-Johnston, 1997). Nevertheless, HPMC films present poor water vapour barrier properties due to its highly hydrophilic nature, which can be improved by adding hydrophobic materials such as fatty acids, natural waxes, surfactants and resins into the hydrocolloid-based films (Baldwin, Nisperos, Hagenmaier, & Baker, 1997; Villalobos, Hernández-Muñoz, & Chiralt, 2006). These compounds usually enhance the water barrier properties but have a weakening effect on the film mechanical response, as they interrupt the polymer network which is what provides most of the tensile strength (Khwaldia, Perez, Banon, Desobry, & Hardy, 2004; Pérez-Gago & Krochta, 2000; Shellhammer & Krochta, 1997).

A limited number of published papers on the incorporation of propolis into films are available, most of which deal with non-food applications. In this sense, Juliano et al. (2007) characterized polymeric films containing propolis for local delivery of propolis into the oral cavity. Based on the obtained results, the authors recommended the use of these films not only in the pharmaceutical sector but also in the agriculture and food industry. Budija, Kricej, and Petric (2008) investigated the possibility of the use of ethanolic extracts of propolis for the finishing of wood, showing that propolis could be used as an additional component in natural wood finishes based on natural resins, waxes and oils. Finally, Drapak, Bakhtinov, Gavrylyuk, Drapak, and Kovalyuk (2006) argued for the use of the propolis films in various optoelectronic device applications.

The aim of this work was to study the effect of propolis incorporation on hydroxypropylmethylcellulose film-forming dispersions and on the film properties through the characterization of different physical properties. The antifungal activity of the films against *Penicilium italicum* and *A. niger* was also studied.

2. Materials and methods

2.1. Raw materials

Hydroxypropylmethylcellulose (Methocel® E15 Food Grade) was supplied by The Dow Chemical Company (Midland, USA) and pure propolis, from Bonamel Organic S.L. (Alquería de Aznar, Spain).

2.2. Film-forming dispersions methodology

The ethanolic extract of propolis (EEP) was prepared as follows: 10 g of propolis was extracted with 100 mL of 96° ethanol at 20 °C for 7 days, using periodical manual shaking in dark conditions. The mixture was filtered through a Whatman No. 1 filter paper and the filtered solution was used as EEP. The EEP contained 6.7% (w/w) of dry solids.

Hydroxypropylmethylcellulose (5 wt%) was dispersed in distilled water at 80 °C for 2 h and stirred overnight at room temperature. After that, EEP was added to the hydroxypropylmethylcellulose solutions to reach final concentrations of EEP solids in the solution of 0, 0.125, 0.25, 0.5, 1 and 1.5 wt%. Thus, the mass fractions of EEP in the dried films were 0, 0.024, 0.048, 0.091, 0.167 and 0.231, respectively. These film-forming dispersions were named M, M-0.125P, M-0.25P, M-0.5P, M-1P and M-1.5P, respectively. The mixtures were emulsified at room temperature using a rotor stator homogenizer ultraturrax (DI25 Yellow Line, IKA®, Germany) at 13,500 rpm for 4 min and then degasified at room temperature by means of a vacuum pump.

2.3. Film-forming dispersions characterization

2.3.1. Density and pH

The density and pH of the film-forming dispersions was measured in triplicate at 20 °C by using a Hubbard picnometer and a pH-meter (GLP21+, Crison Instruments, Spain), respectively.

2.3.2. Rheological behaviour

The rheological behaviour of the film-forming dispersions was analysed in triplicate at 25 °C by means of a rotational rheometer (HAAKE RheoStress 1, Thermo Electric Corporation, Germany) with a type ISO 3219 Z34DIN sensor system of coaxial cylinders. Rheological curves were obtained after stabilization time of 5 min at 25 °C. Shear stress (σ) was measured as a function of shear rate ($\dot{\gamma}$) from 0 to 512 s^{-1} in the following way: 5 min to reach the maximum shear rate and 5 min to attain zero shear rate. The power law model Eq. (1) was applied to determine consistency index (K) and flow behaviour index (n). Apparent viscosities were determined at 100 s^{-1}.

$$\sigma = K\dot{\gamma}^n \tag{1}$$

2.3.3. Particle size analysis

The particle size analysis of the film-forming dispersions was carried out by using a laser scattering instrument (MasterSizer 2000, Malvern Instruments, UK). The samples were dispersed in distilled water at 2000 rpm until an obscuration rate of 10–12% was obtained. Mie theory was applied by considering the following optical properties: a refractive index of 1.50 and absorption of 0.01. Three samples of each FFD were measured in triplicate at 25 °C. The mean particle size was determined by means of the area–volume mean diameter (d_{32}), which is related to the average surface area of droplets exposed to the continuous phase per unit volume of emulsion and the volume–length diameter (d_{43}), which is the sum of the volume ratio of droplets in each size-class multiplied by the mid-point diameter of the size-class. It should be noted that d_{43} is more sensitive to the presence of large particles in an emulsion than d_{32}, hence it is often more sensitive to phenomena such as flocculation (McClements, 2005).

2.3.4. Zeta potential

Film-forming dispersions were diluted to a droplet concentration of 0.02% EEP using distilled water prior to analysis. Zeta potential (ζ-potential) was determined in triplicate by measuring the electrophoretic mobility of the dispersed particles in a charged field by using a ZetaSizer equipment (Nano-Z, Malvern Instruments, UK). The Smoluchowsky mathematical model was used by the software to convert the electrophoretic mobility measurements into zeta potential values.

2.4. Dry films

2.4.1. Preparation of films

FFDs were poured into a framed and levelled polytetrafluorethylene (PTFE) plate (ϕ = 15 cm) and were dried under natural convection at 25 °C and 60% relative humidity (RH). Film thickness was controlled by pouring in the PTFE plate the amount of FFD that will provide a surface density of solids in the dry films of 56 g/m^2 in all formulations. Dry films were peeled off from the casting surface and preconditioned prior to testing in desiccators containing over-saturated $Mg(NO_3)_2$ or NaCl at 5 and 25 °C. So the four film equilibration conditions were: 5 °C–59%, 25 °C–53%, 5 °C–76% and 25 °C–75%. The moisture contents of the films were determined after equilibration. The temperature of 5 °C was chosen to simulate the storage conditions of refrigerated coated products and 25 °C, to simulate the ambient conditions of coated products. A digital micrometer (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain) was used to measure film thickness in at least five random positions around the film.

2.4.2. Water vapour permeability

A modification of the ASTM E96-95 (McHugh, Avena-Bustillos, & Krochta, 1993) gravimetric method for measuring water vapour permeability (WVP) of flexible films was employed, using Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium). Deionised water was used inside the testing cup to achieve 100% relative humidity on one side of the film through a circular opening of 3.5 cm in diameter. Once the films were placed in the cups, the cup-film systems were placed in preequilibrated cabinets fitted with a variable-speed fan to reduce resistance to water vapour transport. The environment within the cabinets was held at constant RH using over-saturated $Mg(NO_3)_2$ and NaCl solutions, respectively. The cabinets were placed at controlled temperature of 5 °C and 25 °C. During WVP testing, the side of the film in contact with the PTFE plate was placed in contact with that part of the test cup having the highest relative humidity. The cups were weighed periodically after steady state was reached using an analytical balance (±0.0001 g).

Water vapour permeability was determined from the slope obtained from the regression analysis of weight loss data as a function of time, once the steady state was reached. The method proposed by Gennadios, Weller, and Gooding (1994) to correct the effect of concentration gradients established in the stagnant air gap inside the cup was used.

2.4.3. Scanning electron microscopy

Microstructural analysis of cross-sections of the dry films (previously conditioned in desiccators with P_2O_5 for at least 15 days) was carried out using SEM technique in a JEOL JSM-5410 (Japan) electron microscope. Pieces of 6 mm × 1 mm were cut from films and mounted in copper stubs. Samples were gold coated and observed using an accelerating voltage of 20 kV.

2.4.4. Mechanical properties

The mechanical properties were measured by using a Texture Analyser (TA-XTplus, Stable Micro Systems, UK) with a 50 N load cell equipped with tensile grips (A/TG model). Sample films were cut into 25.4 mm wide and 100 mm long strips, according to the ASTM D-882 standard (ASTM, 2001). Measures were taken at 20 °C, in samples equilibrated in the different moisture conditions.

Film specimens were mounted in the film-extension grip of the testing machine (grip separation was set at 50 mm) and stretched at a rate of 50 mm/min until breaking. Tensile strength (TS) and percentage of elongation (%*E*) at break, and elastic modulus (EM) were evaluated in 15 replicates of each formulation. TS, E and EM values were determined from stress–strain curves, estimated from force-deformation data.

2.4.5. Optical properties measurement

The transparency of films was measured through the surface reflectance spectra in a spectrocolorimeter (CM-3600d, Minolta Co., Tokyo, Japan) with a 10 mm diameter window. The measurements were carried out on a black and white background. The transparency was determined by applying the Kubelka–Munk theory (Hutchings, 1999) for multiple scattering to the reflection spectra. As each light flux passes through the layer, it is affected by absorption coefficient (K) and the scattering coefficient (S) (Eq. (2)). In this equation, R_∞ is the reflectance of an infinitely thick layer of the material, determined through Eqs. (3)–(5) in terms of the reflectance R of the sample layer backed by a known reflectance R_g and R_0 is the reflectance of the layer with an ideal black background. Internal transmittance (Ti) in the films was quantified by applying Eq. (6) (Judd & Wyszecki, 1975). To measure the transparency of the films five samples of each formulation were considered.

$$\frac{K}{S} = \frac{(1-R_\infty)^2}{2R_\infty} \tag{2}$$

$$R_\infty = a - b \tag{3}$$

$$a = \frac{1}{2}\left(R + \frac{R_0 - R + R_g}{R_0 R_g}\right) \tag{4}$$

$$b = (a^2 - 1)^{1/2} \tag{5}$$

$$\mathrm{Ti}^2 = (a - R_0)^2 - b^2 \tag{6}$$

2.4.6. Gloss measurements

The gloss of films was measured at 20° and 60° angles from the normal to the coating surface, according to the ASTM standard D-523 (ASTM, 1999), using a flat surface gloss meter (Multi-Gloss 268, Minolta, Germany). Measurements were taken in quintuplicate for each type of film. All results are expressed as gloss units, relative to a highly polished surface of black glass standard with a value near to 100.

2.5. Microbiological analysis

Stock cultures of *P. italicum* (CECT 2294) and *A. niger* (CECT 2574), supplied by Colección Española de Cultivos Tipos (CECT, Burjassot, Spain), were kept frozen (−25 °C) in Potato Dextrose Broth (PDB) (Scharlab, Barcelona, Spain) supplemented with 30% glycerol (Panreac, Barcelona, Spain). The fungi were inoculated on Potato Dextrose Agar (PDA) and incubated at 25 °C until sporulation. The cells were counted in a haemocytometer and diluted to a concentration of 10^5 spores/mL.

Aliquots of PDA (20 g) were poured into Petri dishes. After the culture medium solidified, diluted spore solution was inoculated on the surface and films of the same diameter as the Petri dishes (containing or not antimicrobial substance) were placed on the inoculated surface (adapted from Kristo, Koutsoumanis, & Biliaderis, 2008). Inoculated and uncoated PDA Petri dishes were used as control. Plates were then covered with parafilm to avoid dehydration and stored at 20 °C for 5 days. Microbial counts on PDA plates were examined immediately after the inoculation and at the end of the storage period. To this end, the agar was removed aseptically from Petri dishes and placed in a sterile plastic bag with 100 ml of tryptone phosphate water (Sharlab, Barcelona, Spain). The bag was homogenized for 2 min in a Stomacher blender (Bag Mixer 400, InterScience). Serial dilutions were made and then poured onto PDA. Plates were incubated for 5 days at 25 °C before colonies were counted. All tests were run in duplicate.

2.6. Statistical analysis

Statistical analysis of data was performed through a multifactor analysis of variance using Statgraphics® Plus for Windows 5.1. Multiple comparisons were performed through 95% LSD intervals.

3. Results and discussion

3.1. Characterization of the film-forming dispersions

3.1.1. Density, pH, particle size distribution and ζ-potential

The density, pH, particle size (d_{43} and d_{32}) and ζ-potential values of the film-forming dispersions are reported in Table 1. The incorporation of EEP led to a slight decrease in the density and the pH of the film-forming dispersions (FFDs). The pH decrease must be attributed to the dissociation of some EEP compounds of an acid nature in the aqueous solution.

Table 1
Density (ρ), pH, particle size (d_{43} and d_{32}) and ζ-potential of the FFDs. Mean values and (standard deviation).

FFD	ρ (kg/m^3)	pH	d_{43} (μm)	d_{32} (μm)	ζ-potential (mV)
M	1011 (2)^d	6.47	–	–	–
M-0.125P	1011 (3)^d	6.33	25 (1)^b	6.46 (0.07)^c	−1.2 (0.4)^c
M-0.25P	1009 (2)^c	5.10	27 (2)^a	11.6 (0.6)^a	−1.3 (0.2)^c
M-0.5P	1007 (4)^c	4.59	19.4 (0.9)^c	10.3 (0.2)^b	−1.7 (0.5)^{bc}
M-1P	996 (2)^b	4.39	9 (1)*	0.81 (0.05)*	−2.2 (0.2)^b
M-1.5P	993 (3)^a	4.32	11 (1)*	0.44 (0.02)*	−3.0 (0.9)^a

[a–e] Different superscripts within a column indicate significant differences among FFDs ($p < 0.05$). (*) These values are not representative, since these samples showed a bimodal distribution. The two populations, in both M-1P and M-1.5P samples, showed a mode value of 0.2 and 6 μm, respectively. The particle size range being 0.02 to 50 μm and 0.03 to 200 μm for each population.

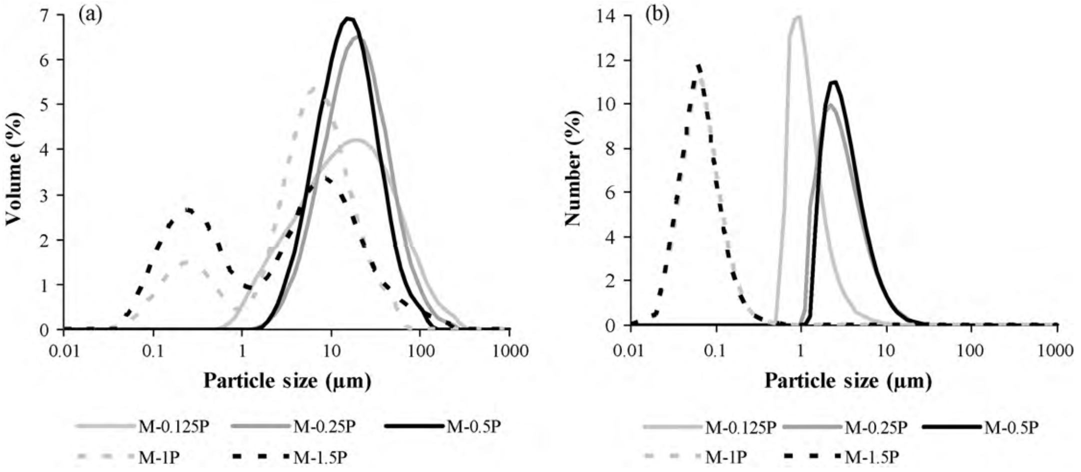

Fig. 1. Particle size distributions of FFDs in terms of volume (a) and number (b) percentages for different EEP concentrations.

The typical average particle size distributions of these FFDs are plotted in Fig. 1. Particle size distributions were monomodal up to formulations containing 0.5% EEP (M-0.5P) (Fig. 1a). Higher EEP content promoted bimodal distributions in terms of volume percentage but apparently monomodal in terms of the number of particles, which indicates there is a very small number of big particles and most particles were smaller when the ratio of EEP increased in the film-forming dispersions (Fig. 1b). This was reflected in Table 1, where the overall decrease of both the mean diameters and the difference between d_{43} and d_{32} when the EEP content increased was observed ($p < 0.05$). The decrease in the mean particle size could be related with the presence of amphiphilic compounds in the EEP extract such as fatty acids, which act as emulsifiers when they reach a critical concentration in the aqueous FFDs.

Dispersed particles showed a slight negative charge as deduced from ζ-potential (ranging from −1.2 to −3 mV), as can be observed in Table 1. The EEP contents of over 0.5% led to a higher ($p < 0.05$) negative charge of the dispersed particles, which indicates that as the EEP ratio increased in the system, an arrangement of compounds occurs in the organic phase. The greater amount of surfactant compounds adsorbs on the surface of the organic aggregates favouring dispersion of non-polar compounds while directing their ionisable/polar groups to the aqueous phase.

3.1.2. Rheological behaviour

Rheological data were fitted to the Ostwald de Waale model and the model parameters (n and K) are shown in Table 2, together with the r^2 coefficient, and the apparent viscosity (η_{ap}) values at 100 s^{-1} shear rate. All FFDs showed a slight shear thinning behaviour, with n values around 0.97 and no thyxotropic effects were observed from the comparison of the up and down curves. The incorporation of the EEP in the pure HPMC dispersion promotes slight changes in the rheological pattern of the FFD towards a more marked shear thinning behaviour and more consistent fluids (higher K values) when EEP concentration was lower than 1%. As shown in Table 2, the apparent viscosity values increased ($p < 0.05$) in comparison to the viscosity of pure HPMC dispersion as a new dispersed phase appears in the system. Nevertheless, viscosity significantly decreased ($p < 0.05$) when the EEP content reached 1%, which coincides with the decrease of the mean particle size and the presence of a very a small number of big particles (bimodal distributions in terms of volume %) and with the significant increase of the particle surface charge.

Viscosity of dispersion can be explained in terms of the different particle size, shape and its distribution, as well as the interactions among components that give rise to different degrees of solvent immobilisation (Barreto et al., 2003). These interactions are promoted when the interfacial surface area increases (greater number of smaller particles) in the FFDs. In this sense, the lowest viscosity values of dispersions with 1% and 1.5% of EEP could be explained by the promotion of EEP–polymer interactions, since HPMC molecules may adsorb on the more hydrophilic surface of

Table 2
Ostwald de Waale model parameters (n and K) and apparent viscosity (η_{ap}) at a shear rate of 100 s^{-1} of the FFDs. Mean values and (standard deviation).

FFD	n	K (Pa s)^n	r^2	η_{ap} (100 s^{-1}) (Pa s)
M	0.9862 (0.0004)^f	0.129 (0.002)^b	0.9999	0.1215^b
M-0.125P	0.9851 (0.0012)^e	0.158 (0.005)^d	0.9994	0.1474^d
M-0.25P	0.9788 (0.0007)^d	0.162 (0.004)^e	0.9997	0.1472^d
M-0.5P	0.9683 (0.0008)^b	0.168 (0.004)^f	0.9996	0.1451^d
M-1P	0.9631 (0.0006)^a	0.148 (0.003)^c	0.9997	0.1245^c
M-1.5P	0.9699 (0.0005)^c	0.093 (0.003)^a	0.9998	0.0813^a

[a–f] Different superscripts within a column indicate significant differences among FFDs ($p < 0.05$).

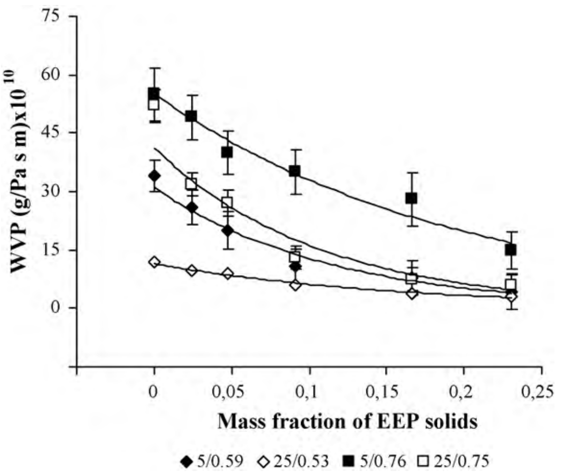

Fig. 2. Development of WVP as a function of the EEP content in the film (points) and fitted curves (lines) for the different equilibration conditions. Mean values and LSD intervals.

the new small-lipid aggregates. So, the obtained results suggest that from a critical amount of EEP enough surfactants are present in the system to favour dispersion of EEP components with an ordered compound arrangement in the organic aggregates where polar-surfactant compounds are located at the interface.

3.2. Characteristics of the films

3.2.1. Water vapour permeability

The average thickness of the pure HPMC films (M) and EEP composite films, which was used to determine WVP, was of 50 μm (SD = 2 μm). Fig. 2 shows the WVP values as a function of the EEP content for the different equilibration conditions (RH and temperature). Water vapour permeability values of pure HPMC films were in the range of those reported by other authors (Sánchez-González et al., 2009) in similar experimental conditions and, as expected, they showed poor moisture barrier properties, greatly dependent on the RH gradient and temperature, although the incorporation of EEP led to a significant ($p < 0.05$) decrease in the WVP, also depending on the RH gradient and temperature.

Fig. 3 shows the moisture content of the equilibrated films as a function of the EEP mass fraction in the film, where the decrease in water content when EEP increased can be observed for all the equilibration conditions. Nevertheless, it is remarkable that when moisture content was referred to the HPMC basis (g water/g HPMC) no significant change in water content was detected until 9.1% EEP in the film was reached, whereas a significant and similar decrease was obtained for 16.7% and 23.1% of EEP in the film. This decrease was greater for low RH conditions (74% for both temperatures) than at high RH (12–14%). These results seem to indicate that, whereas at low EEP content the polymer matrix is mainly responsible for water sorption and no notable interactions with the EEP compounds seem to take place, at the highest EEP contents, these compounds modify the water–HPMC relationships, thus reducing the ability of the polymer chains to bond water molecules. This fact coincides with the different behaviour observed for the more concentrated FFD in EEP and points to the binding of HPMC chains to the EEP components, limiting their capacity for water interactions. The degree of the polymer binding was greater at low moisture contents in the films and seems to reduce in line with the water gain in the matrix. In this sense, it is remarkable that the temperature effect on water sorption, which is related to the sorption enthalpy, was very similar at high water contents (samples equilibrated at 75% and 76% RH) for all samples containing different ratios of EEP. Nevertheless, it is heavily reduced at low water content (samples equilibrated at 53% and 59% RH) for the highest ratios of EEP in the films. This indicates the great decrease of the water sorption enthalpy in these samples which points to the promotion of the hydrophobicity of the matrix, associated to the above mentioned high degree of HPMC binding to the EEP compounds, which blocks the HPMC active points.

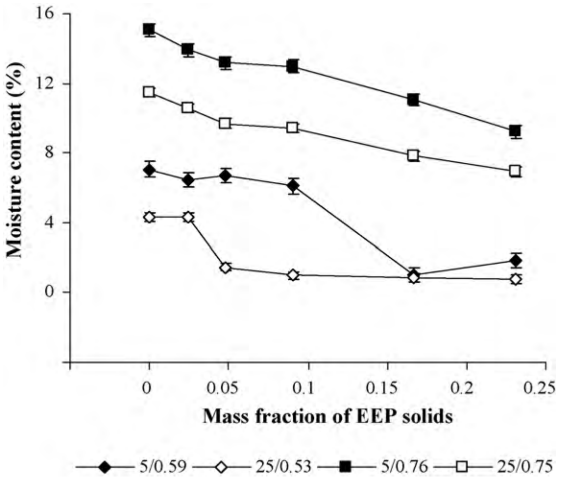

Fig. 3. Moisture content of films as a function of the EEP content in the film equilibrated at different conditions. Mean values and LSD intervals.

An increase in moisture content promotes WVP in the film and this can be clearly seen when Figs. 2 and 3 are observed. In hydrophilic films, the permeability increases at high moisture content due to the swelling and plasticization of the polymer network caused by moisture sorption, which induce a less dense structure where chain extremities are more mobile and water diffusivity and permeation become easier (Morillon, Debeafort, Blond, Capelle, & Voilley, 2002). The water transfer through the film occurs in three steps: first, water vapour condenses and dissolves on the high water concentration side of the film; second, water molecules move through the film, driven by a concentration or activity gradient; third, water evaporates from the other side of the films (Krotcha & Miller, 1997). Therefore, the affinity between the water and the film material and the resistance of water movement in the polymeric network matrix affect the permeability process (Larotonda, Matsui, Sobral, & Laurindo, 2005; Muller, Laurindo, & Yamashita, 2009; Muller, Yamashita, & Laurindo, 2008). In the experiments for WVP determination, the moisture gradient established in the film was more abrupt for the 53/59–100% RH gradient although, in this case, the mean moisture content was lower than when the films were submitted to a RH gradient of 75/76–100%. At the same time, the increase in temperature promotes molecular diffusion, but decreases the water sorption capacity, which has the opposite effect on molecular mobility. In the cases where temperature greatly influenced water sorption, this masked the temperature effect on the water molecule diffusion and, so, on WVP.

So, different effects must be taken into account to interpret the WVP behaviour of the films: the water–polymer interactions in the film affected by the EEP presence, and the temperature–water sorption relationship which affects water sorption-diffusion behaviour, determining the WVP.

3.2.2. Microstructure

The final structure of the film depends on the arrangement of the different components in the FFDs and its evolution during film drying, affected by the interactions between them. Fig. 4 shows SEM micrographs of the cross-sections of the films, where the effect of the EEP incorporation on the microstructure can be observed. Whereas a continuous and homogeneous structure was observed

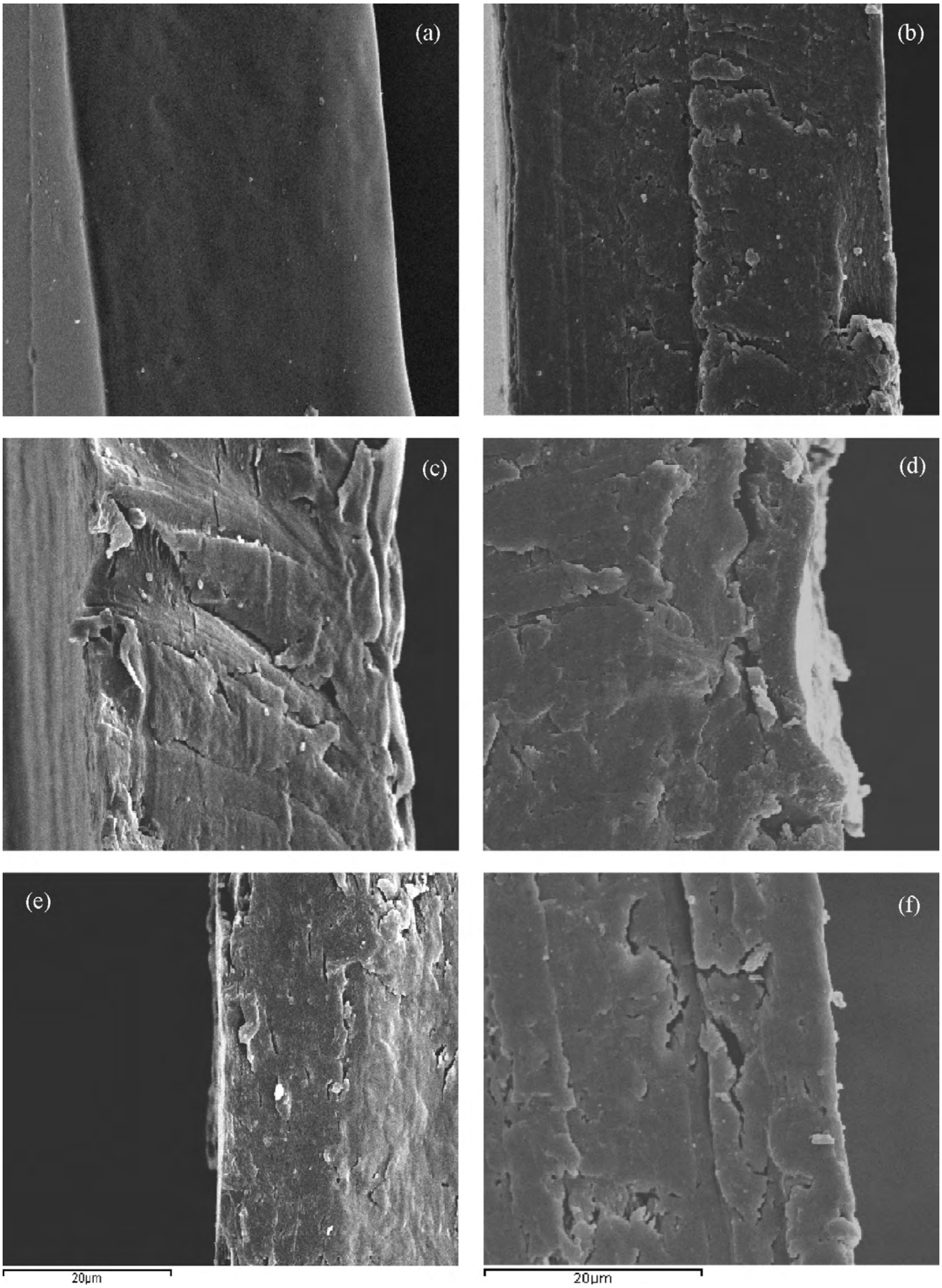

Fig. 4. SEM micrographs of the cross-sections of the different films (a) M, (b) M-0.125P, (c) M-0.25P, (d) M-0.5P, (e) M-1P and (f) M-1.5P.

for the pure HPMC film (Fig. 4a), in agreement with previous works (Sánchez-González et al., 2009; Villalobos, Chanona, Hernández, Gutiérrez, & Chiralt, 2005), the incorporation of EEP (Figs. 4b–f) led to coarser structures where, in some parts crystalline zones seem to be appreciated, as deduced from the observed film fracture behaviour. This could greatly contribute to reduce the WVP, thus improving the water barrier properties. In this sense, Drapak, Bakhtinov, Gavrilyuk, Prylutskyy, and Kovalyuk (2006), Drapak, Gavrylyuk, Kaminskii, and Kovalyuk (2008) reported that organic films prepared from an alcohol solution of propolis showed a crystal structure X-ray diffraction pattern and did not lose their capacity of crystallization when chemically interacting with other tested components.

3.2.3. Mechanical properties

Fig. 5 shows the percentage of elongation (%*E*) and tensile strength (TS) at break and elastic modulus (EM) for films equilibrated at different moisture contents under different conditions. Elastic modulus represents the stiffness of the material. Tensile strength represents film resistance to elonga-

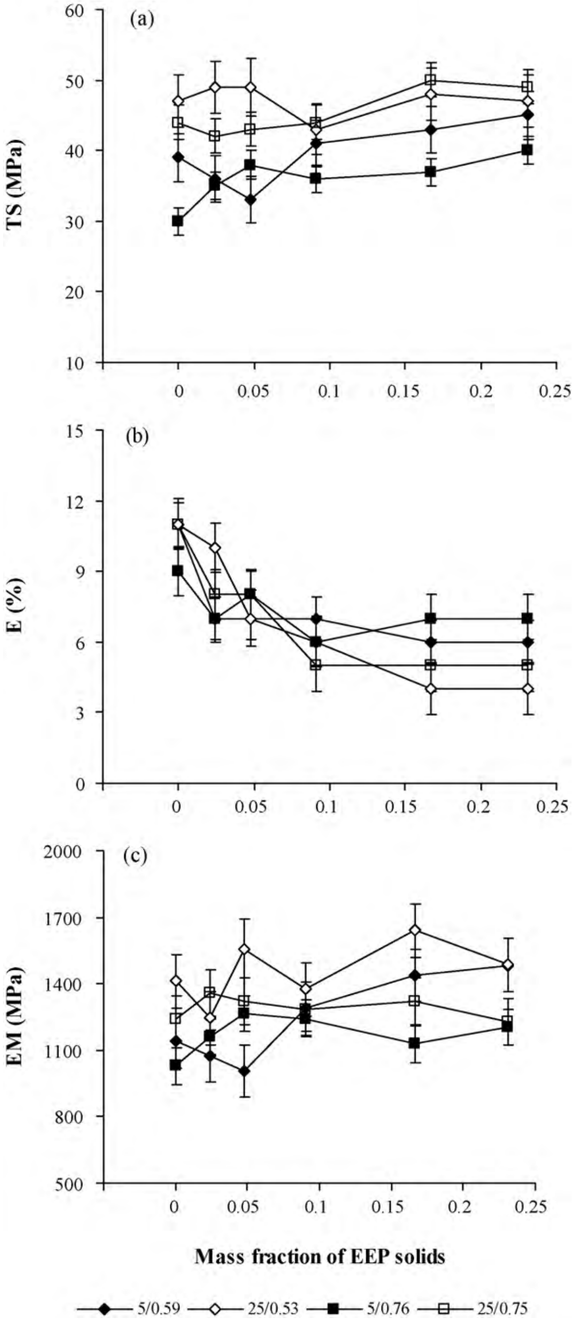

Fig. 5. Development of mechanical properties of the films: tensile strength (a), percentage of elongation (b) and elastic modulus (c) as a function of the EEP content in the film equilibrated at different moisture conditions. Average values and LSD intervals.

tion till fracture, this being a measure of the film's capacity for stretching.

These values were in the range of those reported by other authors working with films based on pure HPMC (Li et al., 2002; Sánchez-González et al., 2009; Sebti, Chollet, Degraeve, Noel, & Peyrol, 2007).

A multifactor ANOVA was carried out to analyse the effect of moisture of the films and the EEP content on the mechanical properties. Results showed that the EEP did not significantly affect TS and EM parameters but elongation at break significantly decreased when the EEP solids increased in the films. Nevertheless, moisture content did not affect the film stretching capacity although it did have significant effect on TS and EM. In general, both parameters showed a slight decrease when moisture content increased, but this tendency was not reproduced at every EEP concentration, probably due to the variability in the values.

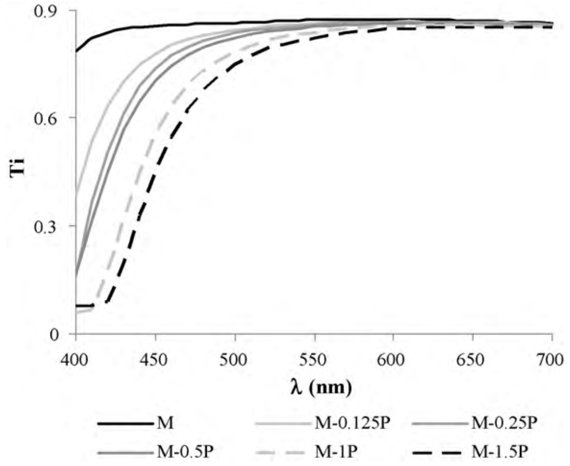

Fig. 6. Spectral distribution of transmittance (Ti) as a function of the EEP content for samples equilibrated at 53% RH and 25 °C as a function of the EEP content.

The mechanical response of the material is mainly affected by the spatial distribution of the different constituents, their physical state and by the interactions established between them. Usually, the incorporation of a dispersed phase caused a decrease in the load parameters (elastic modulus and tensile strength at break), as has been previously reported by different authors in lipid based composite films (Brindle & Krochta, 2008; Fabra, Talens, & Chiralt, 2008; Monedero, Fabra, Talens, & Chiralt, 2009; Sánchez-González et al., 2009; Vargas, Albors, Chiralt, & González-Martínez, 2009). This has been attributed to the presence of structural discontinuities which reduces the film matrix resistance to fracture. In the obtained films, the incorporation of EEP did not produce a weakening effect in the matrix since no decrease in the EM and TS was observed when the EEP increased. This could be due to the interactions of EEP compounds with the HPMC and to the formation of some crystalline zones, as commented on above. The EEP components (resins, balsams containing flavonoids, phenolic acids or their esters) have polar characteristics and can interact with the hydrophilic groups along the cellulose backbone of the HPMC molecules. These interactions can result in stronger interfacial adhesion between these and the EEP which leads to a more efficient resistance to the mechanical stress. Crystal formation can reduce the film flexibility and stretchability which was observed through the development of elongation at break when the EEP content increased in the film (Fabra, Jiménez, Atarés, Talens, & Chiralt, 2009).

3.2.4. *Optical properties*

Film transparency was evaluated through the development of the internal transmittance Ti obtained from the Kubelka–Munk analysis. An increase in the Ti values is related to more homogeneous and transparent films, while a decrease in these values implies film matrices with greater opacity. Fig. 6 shows the spectral distribution of Ti for samples equilibrated at 53% RH and 25 °C. Similar behaviour was observed in the other moisture equilibration conditions. As can be observed, pure HPMC films were highly transparent (high Ti values) in agreement with previous studies (Sánchez-González et al., 2009; Villalobos et al., 2005). The presence of the EEP significantly ($p < 0.05$) decreased the HPMC film transparency (increased opacity) due to the light scattering phenomena provoked by the presence of aggregates (with a different refractive index) distributed throughout the film network. These aggregates are arranged in apparent crystalline structures as has

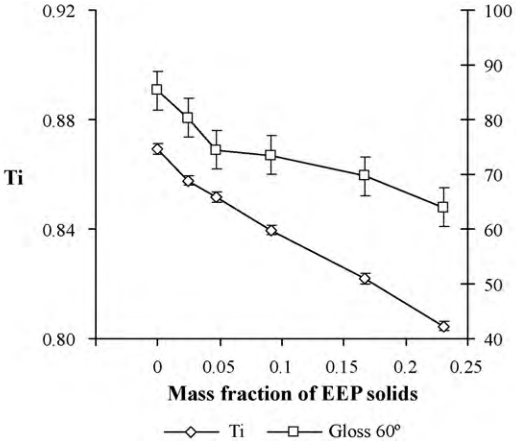

Fig. 7. Development of film transparency (Ti) at 530 nm and gloss at 60° as a function of the EEP content for all samples equilibrated at the different conditions. Average values and LSD intervals.

been observed in the microstructure observations. As no significant effect of moisture content on the film transparency was observed as a function of EEP content, Fig. 7 shows the development of Ti at 530 nm *vs* EEP content for all the samples equilibrated at the different conditions. The promotion of film opacity, which is typical when there is a dispersed phase with a refractive index other than the continuous polymer matrix, can be observed.

The gloss values of the films measured at an incidence angle of 60° are also shown in Fig. 7. Again, no significant effect of the film moisture content was observed for a determined EEP content and, so, the mean values and LSD intervals were plotted. The composite films became less glossy when the concentration of EEP increased in the film. As can be observed, pure HPMC films were highly glossy in agreement with previous studies (Sánchez-González et al., 2009; Villalobos et al., 2005). The loss of gloss must be attributed to the presence of irregularities on the film surface (Ward & Nussinovitch, 1996). Thus, the decrease in the gloss in line with the increase in EEP content could be explained by an increase in the surface roughness of the composite films. This roughness appears to be due to the presence of aggregates on the top of the film. The increase in EEP concentration leads to a great number of irregularities which contribute to reduce the gloss.

As occurred with transparency and gloss, colour coordinates and whiteness index were not affected ($p > 0.05$) by the moisture content of the film. Thus, Fig. 8 shows the development of colour coordinates of the films containing different amounts of EEP for all the samples equilibrated at the different moisture conditions. As can be observed, clarity (L^*) and whiteness index (WI) decreased when the EEP content rose at the same time as colour saturation (C^*_{ab}) increased. Hue slightly decreased (change from yellow to red) with increasing EEP mass fraction. This is a consequence of the presence of coloured substances in the EEP such as carotenoids, which usually absorb blue light as can be observed in Fig. 8.

3.2.5. Antifungal activity

3.2.5.1. Penicillium italicum. The inhibitory effect of HPMC films enriched with propolis on *P. italicum* is shown in Table 3. No significant differences were observed between growth of *P. italicum* on control PDA plates and plates coated with pure HPMC film during storage. Valencia-Chamorro, Palou, del Río, and Pérez-Gago (2008) did not observe any antifungal effect of HPMC films on *Penicillium* species, either. *P. italicum* population increased from 1.5 to approximately 6 log UFC/cm^2 at the end of the storage period. This result coincides with previous studies.

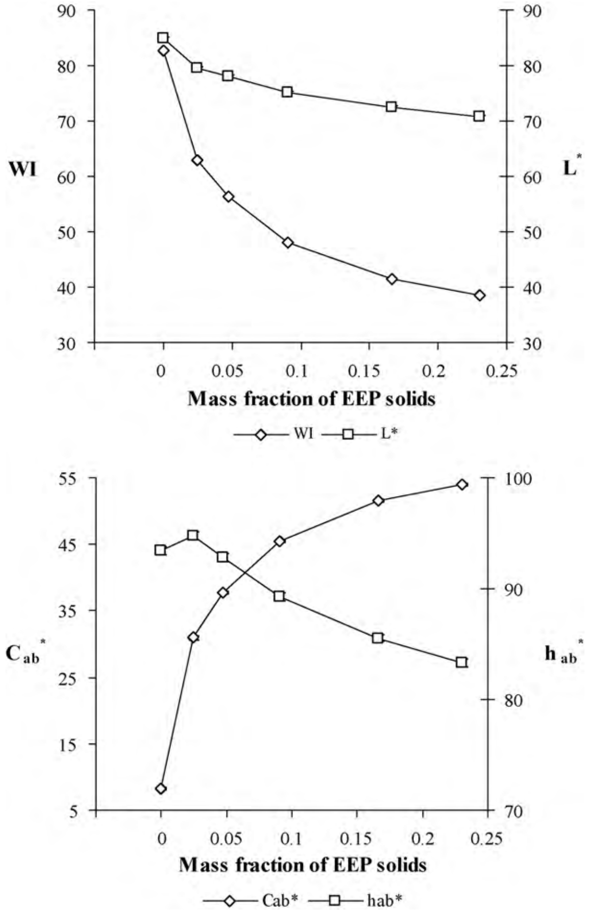

Fig. 8. Development of whiteness index (WI), clarity (L^*), chrome (C^*_{ab}) and hue (h^*_{ab}) calculated from R_∞ for all samples equilibrated at the different conditions, as a function of the EEP content in the film. Average values and LSD intervals.

Growth data indicate that composite films were effective at reducing microbial growth and the antimicrobial effectiveness increased when the propolis concentration rose, but a complete inhibition of fungal growth was not possible at the concentrations of propolis being tested. In fact, using the highest propolis concentration, a 2 log fungal reduction was achieved in comparison with control. The lowest amounts (0.024 and 0.048 mass fraction of EEP solids) of propolis led to a fungal reduction of approximately 0.5–1 log after 5 days at 20 °C, which was not considered an effective antimicrobial activity.

3.2.5.2. Aspergillus niger. The inhibitory effect of HPMC films enriched with propolis on *A. niger* is shown in Table 3. Pure HPMC

Table 3
Inhibitory effect of HPMC films enriched with propolis on *Penicillium italicum* and *Aspergillus niger* inoculated on PDA plates and stored 5 days at 20 °C. Mean values and (standard deviation).

FFD	Antifungal reduction (log UFC/cm^2)	
	Penicillium italicum	*Aspergillus niger*
M	0.24 (0.2)^{a}	0.44 (0.03)^{a}
M-0.125P	0.57 (0.14)^{a}	0.53 (0.06)^{ab}
M-0.25P	1.27 (0.04)^{b}	0.56 (0.07)^{ab}
M-0.5P	1.90 (0.05)^{b}	0.74 (0.13)^{b}
M-1P	1.92 (0.13)^{bc}	1.97 (0.2)^{c}
M-1.5P	2.02 (0.13)^{c}	2.84 (0.13)^{d}

$^{a–d}$ Different letters in the same column indicate significant differences among formulations ($p < 0.05$).

films were ineffective at reducing *A. niger* growth, as no significant differences exist between fungal growth on control and HPMC PDA plates, coinciding with previous studies with methylcellulose, another derivative of cellulose (Türe, Eroglu, Soyer, & Özen, 2008).

Propolis incorporation into HPMC films significantly ($p < 0.05$) reduced fungal population of *A. niger* which agrees with previous studies carried out by Ozcan (1999) and Soylu et al. (2008). The highest levels of propolis led to a reduction of 2.84 log of the fungal growth; 2 log reduction being the minimum to consider an effective antimicrobial activity. The fungal counts at the end of the storage period in these films were lower than 1.9 log UFC/cm^2.

4. Conclusions

Properties of HPMC films were affected by the presence of the EEP, which appears to be arranged in the dry film as crystalline structures. These formations were very effective in the improvement of the water barrier properties. The reduction of WVP for the highest propolis content in the film ranged between 56% and 88%, the higher the moisture content, the greater the WVP reduction caused by the EEP incorporation. EEP in the films gave rise to more resistant, more rigid and less flexible films that showed greater opacity and lower gloss than pure HPMC films, showing colour. The development of optical properties was dependent on propolis concentration. Films containing propolis showed a notable antifungal activity against the tested fungi, which was more intense against *A. niger* than against *Penicillium* specie. The incorporation of propolis into hydropropylmethylcellulose edible films preserved the antifungal properties of this natural compound and so, it could be used to control the fungal decay in foodstuffs. Future research will focus on studying the direct application of these films to food preservation.

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