Development of polylactic acid films with propolis as a source of active compounds: Biodegradability, physical and functional properties

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ABSTRACT

Active films (AFs) using polylactic acid (PLA) as a polymeric matrix containing various propolis concentrations (5, 8.5 and 13%) as the active agent (AA) were developed using a casting method. The purpose was to determine the effects of the incorporation of AA on the physical properties of the films and to evaluate the antioxidant and antimicrobial activities. Tensile strength and elastic modulus of the AFs decreased relative to the control (PLA without AA). Introducing the active substances from propolis into the PLA also affected its thermal properties (glass transition). Adding AAs to the polymer generated more opacity with a green-yellowish colour compared to the control. In addition, AFs exhibited reduced water vapor permeability as the AA concentration increased. Biodegradation assay showed that the AFs degraded faster than the control.

AFs exhibited antioxidant activity, which was measured as the ability to scavenge free radicals (DPPH and ABTS), due to the presence of bioactive compounds (phenolics). Antimicrobial activity was evaluated against *Escherichia coli* and showed a reduction over

4-log cycles. Therefore, incorporation of propolis is a useful strategy for the development of active packaging with antioxidant and antimicrobial effects, which increase the shelf-life of food products.

INTRODUCTION

The growing global environmental awareness due to packaging derived from the petrochemical industry (*i.e.*, petro-polymers) has inspired researchers to search for alternative, renewable and compostable materials that are biodegradable.[1,2]

Polylactic acid (PLA) is a compostable polymer derived from renewable sources (mainly starch and sugar beets) that is synthesized from lactic acid monomers by catalytic ring-opening polymerization.[3,4] The degradation of PLA occurs in different stages: diffusion of water into the matrix, hydrolysis of ester bonds, lowering of the molecular weight, intracellular uptake of lactic acid oligomers and catabolism. The hydrolysis rate depends on the water content and temperature and is catalyzed by the free carboxyl groups of the hydrolyzed PLA ends.[5] The type of microorganisms (*e.g.*, filamentous fungi and bacteria) available in the soil is another factor to degrade PLA into lactic and glycolic acids for use as carbon and energy sources.[6] The biodegradability of PLA is promising for a wide range of applications and competes with polyester (PET) for many food packaging functions because of its good mechanical properties. These properties include higher transparency, ease of processing and market availability.[7] However, applications of PLA for food packaging are limited by several factors, such as low glass transition temperature, weak thermal stability, low toughness and ductility, and lower barriers to oxygen, water vapour and carbon dioxide.[7–9]

The incorporation of active agents (AAs) into the food matrix may prevent microbial growth, oxidation and other degradation reactions, and the controlled release of AAs can ensure that these compounds are present throughout the food products' shelf-life.[8–10] AAs for applications in the food industry are primarily based on food-grade compounds that are preferentially derived from natural materials.[11] Several natural agents have been proposed for use in active packaging, such as chitosan[2], nisin[12], green tea[10], quercetin and oil citral[9], ginger and grape seed extracts[13], thymol[14], and yerba mate.[15] A potential natural substance that contains a high concentration of bioactive compounds is propolis. Propolis is currently used as a popular medicine for its biological properties (*i.e.*, antioxidant and antimicrobial effects).[11]

Propolis is a complex mixture of resinous (50%), gummous and balsamic substances collected by honeybees from plant sprouts, tree buds, flowers and exudates, to which bees add saliva, wax, and pollen to create the final product.[16] Its main components are flavonoids, phenolic acids, esters,

waxes, essential oils (10%), pollen (5%), and various organic compounds (5%).[17,18] The desirable properties of propolis are attributed to its flavonoids and phenolic acids (*e.g.*, phenylacetic acid and phenolic aldehydes).[19,20] The quality of propolis depends on its physicochemical properties; this is particularly true for the soluble solid because this fraction is where the major bioactive compounds are concentrated.[21,22]

Several studies have shown that propolis has potential for use in antimicrobial food packaging systems as a natural alternative agent.[11,18,23,24]

Thus, the main goal of this study was to evaluate the effects of incorporating raw propolis and its ethanolic extract as AAs on the physical properties (*i.e.*, mechanical, thermal, barrier, and optical properties and biodegradability) of the PLA polymer matrix. The antioxidant and antimicrobial activities of these active films (AFs) were also determined.

EXPERIMENTAL

Materials

PLA (Nature Works®, 7001D, Minnetonka, USA) was donated by Oxiquim S.A. (Santiago, Chile). Chloroform (high-performance liquid chromatography [HPLC] grade), methanol and ethanol were purchased from Merck (USA). Gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), Folin Ciocalteu phenol reagent, anhydrous sodium carbonate and aluminium chloride ($AlCl_3$) were purchased from Sigma-Aldrich. For biodegradability assays, soil was purchased from Anasac (Santiago, Chile). Gram-negative (G-) bacteria *Escherichia coli* O157:H7 non-toxigenic (OPS:EQAS-2003) was obtained from the Instituto Salud Publica (ISP, Chile).

Active Agents

Raw propolis samples were collected from beehives located in the Valparaíso region of Chile (V region). Two different AAs were used at different concentrations: powdered raw propolis (PWP) and ethanolic extract of propolis (EEP) at concentrations of 5, 8.5 and 13% w/w_{PLA}. For the EEP, active components from propolis were extracted using ethanol at 20 ºC in the dark for 24 h with periodic stirring using a magnetic bar. The solution was centrifuged (Hermle LaborTechnik Z36 HK, Wehingen, Germany) at 2.504 x *g* for 5 min in a 250 mL tube and filtered through Whatman N° 1 filter paper. This filtered solution was used as the EEP, and the extracts were stored away from light at -18 ºC until incorporation into the polymer matrix.

PLA film production

The PLA films containing AAs (PWP and EEP) at different concentrations were prepared using a solvent-casting technique.[7,14] First, 10 g of PLA pellets was dissolved in 100 mL of chloroform at a concentration of approximately 10% (w/v). Once the PLA was dissolved, known amounts of AAs were added. This mixture was stirred with a magnetic bar until the polymer and AAs completely dissolved. Then, 20 mL of this solution was distributed on a glass Petri dish (14.5 cm in diameter). The chloroform was allowed to evaporate at room temperature. The Petri dishes were stored in a desiccator for 48 h, and the obtained films were peeled off of the dishes and conditioned before analysis. Samples without AAs (*i.e.*, PLA controls) were prepared using the same procedure. The AFs were named according to the type and concentration of the AA added (*e.g.*, PLA/PWP5 = PLA film containing 5% PWP and PLA/EEP5 = PLA film containing 5% EEP).

Physical properties of the films

Film thickness

The film thickness was measured using a digital Mitutoyo model IDC 112 micrometre (Kawasaki, Japan), and the results were expressed as the average of ten replicates of samples taken from different locations on the material surface.

Colour measurement

The AF colour was analysed on a Minolta CR-410 Chroma Meter colorimeter (Minolta, Osaka, Japan) using the CIE*Lab* scale, obtaining the parameters L^* (lightness) and chromaticity (a^* and b^*). The standard tile (L^* = 97.11; a^* = -0.03; b^* = 1.96) was used as the background with a D65 illuminant and 2° observer. Measurements were taken at random positions above the film surface. The total colour differences (ΔE^*) induced by AA (PWP and EEP) incorporation in contrast to the PLA control film were calculated by applying Eq. (1):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

Opacity

Absorbance measurements were used to evaluate changes in the opacity of the AFs. The absorbance value of each film was obtained on a UV/visible spectrophotometer (6715 UV/Vis Jenway, Dunmow, England) at a wavelength of 600 nm.[25] Film samples (1 cm x 4.5 cm) were placed into the equipment compartment for measurement. Finally, the opacity of each sample was calculated using Eq. (2):

$$Opacity = [Abs_{600}/e] \quad (2)$$

where Abs_{600} = absorbance at 600 (nm), and e = film thickness (mm).

Fourier transform infrared (FT-IR) spectroscopy analysis

The FT-IR spectra of different films were recorded on a Bruker Alpha spectrometer (Wismar, Germany) equipped with an attenuated total reflection diamond crystal accessory (Bruker, Platinum). Spectra were obtained with a resolution of 4 cm^{-1} in a wavenumber range from 4000 to 400 cm^{-1} with 60 scans. Spectral analysis was performed using Opus® Software, version 7.

Thermal properties

Differential scanning calorimetry (DSC) was performed using a Mettler Toledo 822e instrument (Greifense, Switzerland) under a nitrogen atmosphere. Eight milligrams of the sample was sealed in an aluminium pan, heated from 0 °C to 200 °C at a heating rate of 10 °C/min and immediately cooled to 0 ºC at the same rate. For the second scan, the samples were heated under the same conditions. Calibration was performed using an indium sample. The glass transition temperature (Tg), melting temperature (Tm) and melting enthalpy (ΔHm) were calculated by integrating the respective peaks (second heating).

Mechanical properties

The tensile strength, elongation percentage at break and elasticity modulus were measured at room temperature on a Zwick Roell (Ulm, Germany) dynamometer model BDO-FBO 0.5TH according to ASTM D882. Strips (14 cm x 2.5 cm) of each film were properly cut. All samples were previously conditioned at 25 °C for 48 h at a relative humidity (RH) of 50%. Tests were performed at a crosshead speed of 50 mm/min until breaking.

Water vapour permeability (WVP)

The film WVP was gravimetrically measured based on the ASTM E96-95 standard method described by Rubilar *et al.*[26] with some modifications. Film samples were cut and mounted on glass cups (transfer area = 2.986 x 10^{-4} m^2) containing a saturated solution of potassium hydroxide (8.2%, RH_2). Silicon sealant was used to seal the films onto the glass cups. The cups were weighed and introduced into a 25 ºC desiccator containing a saturated salt solution of potassium sulphate (97.3%, RH_1) to expose the films to high *RH*. This side of the films was in contact with the test cup side with the lower *RH*. Weight measurements were taken for each cup at 0, 2, 4, 6, 24, 36, 48, 60 and 72 h. Weight gain was plotted over time to obtain straight lines (R^2> 0.9991). The water vapour

transmission rate (WVTR) was determined as the ratio between the slope of the weight gain curve and the film area:

$$WVTR = F/A \quad (3)$$

where *F* is the slope of the weight gain against the time curve (g/h), and A is the exposed film area (m^2).

For WVP, Eq. (4) was used:

$$WVP = (WVTR \times e)/[S \times (RH_1 - RH_2) \times 3600] \quad (4)$$

where *e* is the film thickness, *S* is the saturation pressure at 25 ºC (3159 Pa) and $(RH_1\text{-}RH_2)$ is the difference in *RH* between the exterior and interior exterior capsule.[14]

Biodegradation test

The biodegradability was assessed using the method described by Gónzalez and Alvarez Igarzabal[14], with slight modifications. Equal masses of each film (PLA, PLA/PWP and PLA/EEP) were buried in soil for 314 days in a closed environment. The most relevant physicochemical properties of the soil were as follows: C/N ratio of <0.50, pH 6.7 and electrical conductivity of 3 dS/m. Film samples were cut into probes of equal size (8 cm x 5 cm), dried in an oven at 40°C for 12 h and weighed (w_0). Then, the films were buried at a depth of 10 cm from the soil surface to ensure aerobic degradation conditions. This assay was performed at 25 ± 2 °C and 44 ± 4% *RH*, which was maintained by periodically adding water. Fluctuations in soil moisture were gravimetrically charted using the standard method of oven drying. The film samples were taken from the soil at different times (an average of 15 days), cleaned by wiping gently with a brush, dried in an oven at 40 °C for 3 h and weighed (w_b) to assess the average weight loss. All determinations were performed in triplicate. Weight loss (% w_L) was calculated using Eq. (5):

$$\%\, w_L = [(w_0 - w_b)/w_0] \times 100 \quad (5)$$

Extraction of bioactive compounds from PLA matrixes with AAs

Bioactive compounds were extracted from the AFs according to Byun *et al*.[27] and Tongnuanchan *et al*.[28] The films (0.5 g) were cut into small pieces and mixed with 10 mL of methanol. Then, the mixtures were vigorously vortexed for 1 min and allowed to stand at room temperature. Then, the extractive solutions from the films were placed in contact with the solvent for various times (0 to 2160 min). The obtained supernatant extracts, at various times, were used to determine the bioactive compounds content (*i.e*., the total phenol content [TPC] and total flavonoid content [TFC]) and

respective antioxidant activity (*i.e.*, DPPH and ABTS radical scavenging), and to correlate the concentration of the AAs to the observed activity.

Total Phenolic Content release from AFs

Phenol within the AFs was analyzed using Folin-Ciocalteu's spectrophotometric method according to Bodini *et al.*[11] with slight modifications. A 0.5 mL sample of supernatant extract (obtained as described above) was transferred to test tubes containing 2 mL of Folin-Ciocalteu reagent (1:10). After stirring, the tubes were left standing for 3 min; then, 3 mL of sodium carbonate (10%) was added, and the tubes were filled with 25 mL of distilled water and left standing away from light for 2 h. The absorbance was determined in a 6715 UV/Vis Jenway spectrophotometer at 760 nm. The TPC results were expressed as mg of gallic acid equivalents (GAE)/g of the film.

Total Flavonoid Content release from AFs

The TFC in the AF was analyzed using the spectrophotometric method described by Chang *et al.*[25] with slight modifications. A 0.5 mL sample of supernatant extract (obtained as described in section 2.5) was transferred to test tubes containing 0.1 mL of $AlCl_3$ (10%) and 1.5 mL of methanol. After stirring, the tubes were left standing for 3 min, and then, 0.1 mL of potassium acetate (1mol) was added. Subsequently, the tubes were filled with 2.8 mL of distilled water and left standing away from light for 30 min. The absorbance was determined in a 6715 UV/Vis Jenway spectrophotometer at 415 nm. The TFC results were expressed as mg of quercetin equivalents (QE)/g of the film.

Functional properties of the films

Antioxidant activities

The antioxidant activities of the AFs were evaluated using DPPH and ABTS radical scavenging assays.

DPPH free radical scavenging method

The activity of the films was measured using the DPPH method according Bitencourt *et al.*[29] with slight modifications. A 0.5 mL sample of supernatant extract (obtained as described in section 2.5) was added to 2 mL of DPPH (0.12 mM) and kept in the dark for 30 min. Then, the absorbance was determined at 517 nm using a 6715 UV/Vis Jenway spectrophotometer. The antioxidant activity was calculated using Eq. (6):

$$\%DPPH\ scavenging = \left[\left(Abs_{DPPH} - Abs_{sample}\right)/Abs_{DPPH}\right] \times 100 \quad (6)$$

ABTS free radical scavenging method

The activity of the films was measured using the ABTS method according Bitencourt *et al*.[29] Initially, a solution containing ABTS radicals (7 mM) and potassium persulfate (2.45 mM) (1:1) was kept in the dark for 16 h. Then, an aliquot of the solution was diluted with methanol to an absorbance of 0.700 ± 0.002 at 734 nm using a 6715 UV/Vis Jenway spectrophotometer to obtain the ABTS working solution. One millilitre of supernatant extract (obtained as described in section 2.5) was added to 1 mL of ABTS solution, and the mixture was incubated in the dark at room temperature for 7 min. Subsequently, the absorbance was determined at 734 nm using a spectrophotometer. The antioxidant activity was calculated using Eq. (7):

$$\%\ ABTS\ scavenging = \left[\left(Abs_{ABTS} - Abs_{sample}\right)/Abs_{ABTS}\right] \times 100 \quad (7)$$

Antimicrobial activity

The antimicrobial activity of the AFs (*i.e*., PLA/PWP and PLA/EEP) was evaluated using *E. coli* as a test microorganism according to ASTM E 2149-10, as described in López De Dicastillo *et al*.[30] with slight modifications. The bacterial inoculum was diluted using a sterile buffer solution (composition of 1 L: 0.15 g of KCl, 2.25 g of NaCl, 0.05 g of $NaHCO_3$, 0.12 g of $CaCl{\cdot}H_2O$, and a pH of 7.0) until the solution reached an absorbance of 0.30 ± 0.01 at 600 nm (exponential phase), as measured spectrophotometrically. The solution, which had a concentration of 10^8 colony-forming units per millilitre (CFU/mL), was diluted with the buffer solution to obtain a final working concentration of 10^6 CFU/mL. Each 0.5 g AF sample was cut into small pieces and placed in separate sterile tubes, maintaining contact with 10 mL of buffer containing 10^6 CFU/mL. Films without AAs constituted the PLA control group and were used as blanks to observe PLA antimicrobial effects. Serial dilutions of sterile buffers were prepared and placed in Petri dishes containing tryptic soy broth culture medium. Colonies were counted after incubation at 37 ºC for 18 h. The antimicrobial activity was expressed as % reduction and log (*cycles*) reduction using Eqs. (8) and (9), respectively:

$$\%\ reduction = \left[(CFU/ml)_{control} - (CFU/ml)_{film}\right]/\left[(CFU/ml)_{control}\right] \quad (8)$$

$$\log(cycles) = \log_{10}(control) - \log_{10}(film) \quad (9)$$

Statistical analysis

Differences between the PLA control and AFs (*i.e.*, PLA/PWP and PLA/EEP) were assessed using one-way multivariate analysis of variance (MANOVA) in Statgraphics (USA). Differences between the means were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Physical properties of the films

The thickness and mechanical properties of the films are shown in Table 1. The AAs exerted an important effect on every film. Moreover, the majority of the AFs showed significant differences ($p < 0.05$) from the PLA control. The thicknesses of the films with different concentrations of PLA/PWP and PLA/EP increased by approximately 21.5% and 52.5%, respectively. The thickness was related to the filmogenic solution volume/plate area ratio efficiency for all formulations.[29] In addition, AAs with higher dry matter contents resulted in films with greater thicknesses.[15]

The optical properties (*i.e.*, colour and opacity) of the films are shown in **Table 2.** A reduction in lightness values was observed, indicating that the films became darker, and the films also tended to become more green and yellow in colour, as indicated by lower a^* and higher b^* values. This behaviour was more evident in films containing higher concentration of AAs (8.5 and 13%). Similar results were obtained by Giteru *et al*.[9] for kafirin films with citral and quercetin essential oils; their films were darker, redder and yellower than the control film. Figure 1 shows the visual appearances of AFs containing high concentrations of AAs (13%). Greater significant ($p < 0.05$) differences in total colour (ΔE^*) were obtained for the PLA/PWP13 and PLA/EEP13 films: 36.66 and 36.37, respectively. A similar appearance (yellowish) was obtained by De Araujo *et al*.[24] for films made from cassava starch with incorporated EEP due to the presence of chlorophyll and carotenoids. For the opacity index (Table 3), higher values and significant differences ($p < 0.05$) were observed for films with higher AA concentrations than the PLA (control); these differences were associated with decreases in the amount of light passing through the AFs. Thus, the addition of AAs decreased the transparency and increased the opacity of the AFs. However, this new attribute may help preventing oxidative deterioration in packaged foods caused by exposure to visible and UV light, leading to nutrient losses, discolouration and off–flavours.[31]

FT-IR spectroscopy was used to investigate the incorporation of AAs into the PLA matrix. The FT-IR spectra of PLA (control) and the AFs are shown in Figure 2. The characteristic peaks of PLA at 2860-3000 cm^{-1} (–CH– stretching bands), 1700-1760 cm^{-1} (carbonyl group, –C=O), 1358-1451 cm^{-1}

297 (–C–H–), 1039-1266 (–C–O– stretching) and 867 cm^{-1} (–C–C–) appeared in all samples[1,32,33].
298 However, spectra from the AFs contained extra peaks at 1450, 1515, 1633, 1683 and 1690 cm^{-1},
299 which corresponded to lipids, aromatic rings, $–CH_3$, $–CH_2–$ and flavonoids, respectively.[34,35] These
300 bands were more evident and intense for high concentrations of AAs (8.5 and 13%) because these
301 compounds and functional groups are found in propolis.[34,35] The composition of propolis (*i.e.*,
302 resins, phenolic acid and their esters) has polar characteristics and interacts with the hydrophilic
303 groups along the backbones of the polymer molecules.[15] These spectra illustrate the presence of
304 propolis in the PLA matrix, demonstrating the successful incorporation of the AAs.
305
306 The thermal properties of PLA and AFs are shown in Table 3. The control film exhibited a glass
307 transition temperature (T_g) of 50 ºC and a melting temperature (T_m) of 150 ºC. The T_g values
308 obtained for the AFs decreased slightly compared to the control, and only the PLA/PWP13 films
309 showed a significant reduction, likely due to the plasticizing effect of PWP (T_g = 38.6 ºC). These
310 phenomena result from an increase in amorphous zones (greater polymer chain mobility).[1] The
311 reduced T_g values may be a product of the wax and essential oils present in propolis (AA), which
312 act as plasticizers.[24,36] The incorporation of AAs into polymers can increase the free volume in the
313 matrix and, consequently, enhance the polymer chain mobility (*i.e.*, decrease the crystallinity
314 degree), potentially altering the polymer's thermal properties.[7,8,37] It was not possible to measure the
315 degree of crystallinity because the crystallization process was obscured by the presence of AAs.
316 A slight decrease in T_m was also observed as the amount AAs increased in the AFs PLA/PWP8.5,
317 PLA/PWP13, PLA/EPP8.5 and PLA/EEP13 films compared to the control film. This behaviour can
318 be explained by the lack of homogeneity of the films, and the differences were more evident in
319 films with higher AA concentrations (8.5 and 13%). These films also contained increased
320 amorphous zones and, therefore, lower amounts of crystals due to the incorporation of AAs.[1]
321 Similarly, the lower melting enthalpy (ΔH_m) of all the AFs can be explained by the incorporation of
322 AAs, which disrupted the regularity of the chain structures in the polymer and increased the spacing
323 between the chains[38], thereby decreasing the crystallinity. Additional melting peaks in the
324 thermograms of the PLA films containing AAs are more noticeable than those in pure PLA, which
325 may be related to the reorganization of the crystal structure. These molecules can act as plasticizers
326 at the interface between amorphous and crystalline parts, affecting the actual melting point, or co-
327 crystallize with the polymer during film casting, introducing defects and, thus, changing the melting
328 temperature.[7] A decrease in the crystallinity degree after AA incorporation also affected the
329 mechanical properties by enhancing the polymer chain mobility.[1,13,35] The incorporation of AAs
330 (*i.e.*, PWP and EEP) modified the mechanical properties of the PLA films (Table 1). The elasticity

modulus and tensile strength decreased, and these differences were more significant for PWP. However, similar values were obtained at higher concentrations (13%) for both AAs. The decrease in the elasticity modulus could be attributed to AAs plasticizing effect due to its smaller chain length as previously referred. However, the percentage of elongation of the AFs was not affected when AAs were incorporated into the films, and only the PLA/EEP13 film showed a substantial increase in this parameter. The interactions between the polymeric matrix and the phenolic compounds present in the AAs caused changes in mechanical properties.[15,29] The effect on the behaviour of the polymeric matrix depends on the type and concentration of AAs and the interaction between them.[17,24]

Chang-Bravo *et al*.[15] obtained similar results for these properties using carrageenan films with Cuban red propolis as an active compound. These film samples demonstrated decreases in the tensile strength and elasticity modulus (48% and 32%, respectively) compared to the control (carrageenan without propolis). De Araujo *et al*.[24] obtained the same results using cassava starch films with incorporated EEP: decreases in the tensile strength and Young's modulus of approximately 50% compared to the control (cassava starch without propolis). Bodini *et al*.[11] and Pastor *et al*.[17] reported changes in the tensile strength and elastic modulus due to the incorporation of propolis extract into different matrixes (gelatine and hydroxypropyl methylcellulose, respectively) due to the production of discontinuous areas. In contrast, Kanatt *et al*.[39] determined that the presence of phenolic compounds containing -OH groups that can form hydrogen bonds with PLA increased the tensile strength upon the addition of EEP.

The effects of AAs on the WVP of the films are shown in Table 3. The permeability values decreased significantly as the AA content increased compared to the control (2.39×10^{-11} g m/m^2 Pa s). These results revealed improvements in the barrier property for AFs with the highest concentrations of AAs: 1.85×10^{-11} g m/m^2 Pa s and 1.36×10^{-11} g m/m^2 Pa s for PLA/PWP13 (reduction about 22.5%) and PLA/EEP13 (43.1%), respectively. This reduction can be explained by the hydrophobic nature of the AAs (propolis), which reduced the film hygroscopicity by interrupting water molecule penetration through the films.[23,24] In addition, the reduction of the WVP values could be explained by the interactions between the PLA matrix and phenolic compounds of the AAs, which could reduce the free spaces in the polymer. Thus, the passage of vapour was restricted and water sorption was inhibited.[29,40] Similar behaviour was obtained by De Araujo *et al*.[24] for films of cassava starch with EEP, which showed decreased WVP values when the films contained 1% extract.

A biodegradability assay was performed for 314 days, and the weight losses from the film samples are shown in Figure 3. The weight losses were approximately 4% for the PLA films (control) and higher for the AFs, with values between 2.5-5% for concentrations of PLA/PWP films and 9-24% for concentrations of PLA/EEP films. The PLA films did not show surface discolouration, cracks or pitting, unlike the AFs. Discolouration, small holes in the surface and fragmentation were more evident in PLA/PWP and PLA/EEP films due to microbial growth (shown in Figure 4). The presence of microorganisms in these films was expected due to the higher content of nutrients from the propolis, which facilitated the growth of microorganisms (e.g., fungus and bacteria).[6] During biodegradation, water also diffuses into the polymer matrix, causing swelling and enhancing biodegradation.[40]

PLA biodegrades slowly in soils under ambient conditions (25 °C and 45-50% RH), likely because of the slow rate of hydrolysis at low temperature and water content and the relative scarcity of PLA-degrading organisms.[4] The total degradation period of PLA depends on several factors: molecular weight, type of specimen (e.g., film, powder, or plate), enantiomeric composition (related to crystallinity), microbial capacity and environmental conditions (e.g., humidity, temperature, and pH).[34,41] After 50 days, all AFs exhibited more rapid weight loss compared with the control. Nevertheless, the AFs containing PWP showed a constant weight loss from day 100 until the end of the test. This result is probably related with other compounds present in its composition, such as wax, that retards the biodegradation effect.[42] On the other hand, the AFs containing EEP showed an increase in the weight loss during the same period, due to the higher availability of the nutrients present in the films, which were rapidly degraded by the microorganisms.

Functional properties (antioxidant and antimicrobial) of the films

Figure 5 presents the results obtained for the release of bioactive compounds (TPC, Figure 5a and TFC, Figure 5b) from the PLA polymer matrix. As the AA concentration increased, the amount of polyphenolic compounds incorporated in the films also increased, as reflected in the increased release of these compounds. All samples exhibited similar behaviour, namely, "exponential growth to a maximum" profile and release that was proportional to the nominal concentration of PWP or EEP incorporated into the PLA matrix. The maximum release of phenolic compounds was obtained at 124 min; for PLA/PWP containing different concentrations (5, 8.5 and 13%), values of 8.01, 28.41 and 88.59 mg GAE/g of film were obtained, respectively (Figure 5a). For PLA/EEP films at different concentrations (5, 8.5 and 13%), the maximum release occurred after 240 min: 6.76, 20.41 and 93.03 mg GAE/g of film, respectively (Figure 5b). As expected, the amount of phenolic compounds released increased with the AA concentration.

Kanatt *et al*.[39]obtained lower values when monitoring the release of phenolic compounds from chitosan-polyvinyl alcohol films that contained aqueous extracts of mint and pomegranate peel extract. The highest release of TPC was observed at 37 ºC and 1440 min; the films released approximately 22 mg catechin/g of film with mint extract and 20 mg catechin/g of film with pomegranate peel extract. The release of bioactive compounds was also studied by Mascheroni *et al*.[43], who evaluated the migration of propolis from PLA and found that polyphenols were released from matrixes in relevant quantities. Bodini *et al*.[11] obtained similar results for gelatine films containing an ethanol extract of propolis (40 g/100 g of gelatine), which exhibited a mean TPC value of 50 mg GAE/g of film over 182 days. However, De Araujo *et al*.[24] reported lower TCP values for cassava starch films containing different concentrations of EEP (0.5, 0.75 and 1%). The range observed for these films was between 3 and 7 mg GAE/g of film. Siripatrawan & Vitchayakitti[23] studied chitosan films with different concentrations of hydroalcoholic propolis extract (2.5, 5, 10 and 20%), and the TPC results obtained for these films ranged between 4.2 and 6.1 mg GAE/g of sample.

The TFC results revealed that the maximum releases occurred after 720 min for PLA/PWP and 1440 min for PLA/EEP. The amounts of TFC releases for PLA/PWP containing different concentrations (5, 8.5 and 13%) were 2.23, 7.23 and 31.12 mg GAE/g of film, respectively (Figure 5b). AFs containing EEP as an AA (5, 8.5 and 13%) showed values of 1.88, 7.02 and 29.98 mg QE/g of film, respectively, at 1440 min (Figure 5b). The rate of phenol compound release from the PLA matrix was slightly slower for PWP than for EEP. Contrary behaviour was observed for flavonoid compounds, and the release was slower in PLA/EEP films. The controlled release of bioactive compounds into food contributes to extending its shelf life. Since oxidation is commonly initiated on the food surface, antioxidant-releasing packaging is a promising means to protect food surfaces from rancidity.[9,18,31] The number of bioactive compounds released from the PLA matrix increased as the storage time and concentration increased. Other factors that affect the bioavailability and functionality of the AAs include the polymer characteristics, concentrations and polymer-agent interactions.[15]

As expected, and shown in Figures 5c and 5d, the antioxidant activity of PLA films increased significantly as the AA concentration in the polymeric matrix increased. The AAs containing bioactive compounds (*i.e*., phenols and flavonoids) are responsible for the antioxidant activity of the films, and this capacity was proportional to the AA concentration. The PLA/PWP films exhibited maximum DPPH and ABTS radical scavenging values of 58% (Figure 5c) and 80% (Figure 5d).

These two methods can determine the presence of lipophilic and hydrophilic compounds with antioxidant activity.[29] The films with the highest AA contents showed the highest antioxidant activities, and PLA/EEP films showed higher DPPH radical scavenging ability (62%) (Figure 5c); additionally, its ABTS radical scavenging ability was approximately 95% (Figure 5d). The bioactive compounds present in PWP and EEP agents acted as antioxidants by trapping free radicals, but flavonoids can also chelate metals.[15] The two major mechanisms involved in the deactivation of radicals are: i) by hydrogen atom transfer (HAT); and ii) by single electron transfer (SET). DPPH and ABTS radical scavenging methods utilize both HAT and SET mechanisms.

The antioxidant activity plateaued according to both methods used and was independent of the contact time between AFs/methanol. The maximum scavenging activity for DPPH occurred at 720 min for all films, whereas that for ABTS was observed at 90 min for all films (Figures 5c and 5d). The decrease in the time to maximum activity could be due to the use of the ABTS method with lipophilic and hydrophilic compounds.[29]

Bitencourt *et al*.[29] analysed the antioxidant activity of gelatine films containing an ethanolic extract of curcuma. The results obtained showed that films contained 2% extract showed 79% inhibition of DPPH radicals and 57% inhibition of ABTS radicals. De Araujo *et al*.[24] studied cassava starch films containing EEP at different concentrations (0.5, 0.75 and 1%) and reported antioxidant activity between 8.5 to 13μmol TE/g of film. Similar results were found by Lopéz De Dicastillo[30] for methylcellulose films with murta fruit extract. The activities of the films were proportional to the antioxidants released to the solution from the active compounds (*i.e.*, contact time).

The antimicrobial properties of PLA containing AAs against *E. coli* were compared to that of the PLA control and are shown in Table 4. The PLA control did not show any inhibition of the tested bacteria. In contrast, the AFs (*i.e.*, PLA/PWP and PLA/EEP) showed antimicrobial activity against *E. coli*. AFs with the highest AAs content (13%) presented the highest antimicrobial capacity, with an approximately four-log reduction for this bacterium (PLA/PWP13 and PLA/EEP13 films). These results showed that propolis as an AA has antimicrobial activity and can ensure food safety.[20,44] To exhibit effective antimicrobial activity, AFs must present a log reduction higher than 2 log cycles[17]. The antimicrobial activity of propolis extract against bacteria can be attributed to the presence of phenolic compounds that inhibit bacterial growth by inhibiting the bacterial RNA polymerase and disrupting the bacterial cell membrane and cytoplasm, leading to cell death.[23,40,45,46]

De Araujo *et al*.[24] analysed the activities of starch films with EEP against *Staphylococcus aureus* and *E. coli*; however, whether the films were most active against Gram-positive (G+) or G- bacteria remains uncertain. In contrast, Siripatrawan & Vitehayakitti[23] evaluated the antimicrobial activity of chitosan films with propolis extract. Their results showed that films were more effective against G+

than G- bacteria. Similar behaviour was reported by Bodini *et al*.[11], who analysed the inhibition activity of gelatine films containing EEP against *S. aureus*. Their disc diffusion study revealed growth inhibition of approximately 25-29 mm for the highest concentration (2 g EEP/g of gelatine).

CONCLUSIONS

This study showed the effects of two types of AAs (PWP and EEP) incorporated into a PLA film matrix on the physical (mechanical, thermal, barrier), functional (antioxidant and antimicrobial) properties and biodegradability, which should be considered for future applications of these materials in active packaging composed of biopolymers.

The incorporation of natural constituents (AAs), such as propolis, is a useful strategy for the development of AFs with improved antioxidant and antimicrobial properties. In addition, this technique is becoming a very promising method for extending the shelf life of food products that is consistent with the preferences of consumers for more natural food products with few or no preservatives and sustainable packaging.

CONFLICT OF INTEREST

All authors declare that they do not have any conflicts of interest regarding the work being submitted.

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FIGURE CAPTIONS

Fig. 1. Visual appearances of the PLA film (control) and AFs with PWP (13%) and EEP (13%) obtained using the casting method.

Fig. 2. FT-IR spectra of the powder raw propolis (PWP), PLA film (control) and AFs containing the highest concentrations (PLA/PWP13 and PLA/EEP13).

Fig. 3. Biodegradability assays of the PLA film (control) and all AFs (PLA/PWP and PLA/EEP) containing different concentrations of AAs.

Fig. 4. Visual appearances of the PLA film (control) and all AFs (PLA/PWP and PLA/EEP) containing different concentrations of AAs after being buried for 314 days (biodegradation assay).

Fig. 5. Release of bioactive compounds (TPC and TFC) from AFs (PLA/PWP and PLA/EEP) and antioxidant activities of all AFs containing different concentrations of AAs: (**a**) Release of TPC from AFs, (**b**) Release of TFC from AFs, (**c**) DPPH radical scavenging activity, and (**d**) ABTS radical scavenging activity. ●PLA/PWP5; ▼PLA/PWP8.5; ■ PLA/PWP13; ● PLA/EEP5; ▼ PLA/EEP8.5; ■ PLA/EEP13.

TABLE CAPTIONS

Table 1. Thicknesses and mechanical properties of the PLA film (control) and AFs (PLA/PWP and PLA/EEP) containing different concentrations of AAs.

Table 2. Colour parameters (L^*, a^*, and b^*), colour differences (ΔE^*) and opacities of the PLA film (control) and AFs (PLA/PWP and PLA/EEP) containing different concentrations of AAs.

Table 3. Thermal and barrier properties of the PLA film (control) and AFs (PLA/PWP and PLA/EEP) containing different concentrations of AAs.

Table 4. Antimicrobial activities of PLA film (control) and AFs (PLA/PWP and PLA/EEP containing different concentrations of AAs) against *E. coli.*

601 **GRAPHICAL ABSTRACT**

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606 **Table 1.** Thicknesses and mechanical properties of the PLA film (control) and AFs
607 (PLA/PWP and PLA/EEP) containing different concentrations of AAs.

		Mechanical properties		
	Thickness	Elasticity modulus	Tensile strength	Percentage of elongation
Films	(μm)	(N/mm^2)	(N/mm^2)	(%)
PLA (control)	96 ± 7^{a}	1371.2 ± 287.3^{a}	48.5 ± 6.2^{a}	3.3 ± 0.6^{a}
PLA/PWP5	$104 \pm 15^{a,b}$	539.7 ± 162.2^{b}	15.4 ± 1.2^{b}	4.2 ± 1.0^{a}
PLA/PWP8.5	$121 \pm 11^{b,c}$	765.7 ± 202.1^{c}	$17.1 \pm 1.4^{b,c}$	3.8 ± 0.6^{a}
PLA/PWP13	$125 \pm 27^{b,c}$	335.1 ± 142.7^{d}	16.4 ± 2.5^{b}	5.9 ± 2.5^{a}
PLA/EEP5	136 ± 22^{c}	$1073.9 \pm 308.0^{a,e}$	35.7 ± 2.9^{d}	4.6 ± 1.1^{a}
PLA/EEP8.5	150 ± 23^{d}	802.02 ± 157.9^{e}	28.7 ± 3.1^{e}	4.2 ± 0.8^{a}
PLA/EEP13	153 ± 28^{d}	339.1 ± 135.2^{d}	$22.8 \pm 5.1^{c,f}$	12.1 ± 3.2^{b}

608 **For each parameter, mean values marked with the same letter do not differ significantly ($p<0.05$).*
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635 **Table 2.** Thermal and barrier properties of the PLA film (control) and AFs (PLA/PWP and
636 PLA/EEP) containing different concentrations of AAs.

	Thermal properties			Barrier property
	T_g^A	T_m^B	ΔH_m^C	WVP^D x 10^{-11}
Films	**(ºC)**	**(ºC)**	**(J/g)**	**(g m/m² Pa s)**
PLA (control)	50.1 ±7.5[b]	150.4 ± 0.4[a,b]	22.4 ± 3.1[b]	2.39 ± 0.11[a]
PLA/PWP5	48.3 ± 5.4[a,b]	148.1 ± 3.8[a,b]	21.3 ± 8.9[b]	2.05 ± 0.08[b]
PLA/PWP8.5	43.4 ± 6.7[a,b]	148.3 ± 3.6[a,b]	19.4 ± 0.4[a,b]	1.88 ± 0.21[b,c]
PLA/PWP13	38.6 ± 7.4[a]	149.8 ± 3.2[a,b]	18.7 ± 0.4[a,b]	1.85 ± 0.31[b,c]
PLA/EEP5	46.7 ± 2.4[a,b]	151.2 ±1.4[b]	24.2 ± 2.4[b]	1.77 ± 0.03[c]
PLA/EEP8.5	43.7 ± 3.4[a,b]	149.7 ± 0.9[a,b]	21.6 ± 4.7[b]	1.71 ± 0.12[c]
PLA/EEP13	49.8 ± 4.8[b]	146.8 ± 1.4[a]	11.4 ± 6.5[a]	1.36 ± 0.03[d]

637 [*]*For each parameter, mean values marked with the same letter do not differ significantly (p<0.05).*
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639 [A]transition temperature
640 [B]melting temperature
641 [C]melting enthalpy
642 [D]water vapor permeability
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Table 3. Colour parameters (L^*, a^*, and b^*), colour differences (ΔE^*) and opacities of the PLA film (control) and AFs (PLA/PWP and PLA/EEP) containing different concentrations of AAs.

	Colour				Opacity
Films	L^*	a^*	b^*	ΔE^*	**(nm/mm)**
PLA (control)	98.25 ± 0.09[a]	-0.11 ± 0.04[a]	2.38 ± 0.06[a]		2.24 ± 0.03[a]
PLA/PWP5	91.64 ± 0.42[b]	-3.52 ± 0.54[b]	21.30 ± 2.02[b]	20.33	7.27 ± 0.88[b]
PLA/PWP8.5	88.65 ± 0.39[c]	-3.64 ± 0.11[b]	28.93 ± 1.07[c]	28.45	8.68 ± 0.03[c]
PLA/PWP13	84.34 ± 0.66[d]	-2.54 ± 0.15[c]	36.22 ± 0.77[d]	36.66	9.62 ± 0.27[c]
PLA/EEP5	93.94 ± 0.50[e]	-4.06 ± 0.20[b]	19.82 ± 1.50[b]	18.39	7.90 ± 0.21[b,c]
PLA/EEP8.5	91.63 ± 0.65[b]	-4.97 ± 0.13[d]	28.49 ± 1.93[c]	27.37	8.70 ± 0.61[c]
PLA/EEP13	89.10 ± 1.28[c]	-4.91 ± 0.69[d]	37.25 ± 2.82[d]	36.37	14.85 ± 1.78[d]

[*] *For each parameter, values marked with the same letter do not differ significantly ($p<0.05$).*

651 **Table 4.** Antimicrobial activities of PLA film (control) and AFs (PLA/PWP and PLA/EEP
652 containing different concentrations of AAs) against *E. coli*.
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	***E. coli* reduction**	
Films	**CFU/mL**	***Log* cycles**
PLA (control)	1.8×10^7	-
PLA/PWP5	2.8×10^6	1.18
PLA/PWP8.5	1.5×10^6	1.38
PLA/PWP13	1.5×10^4	3.45
PLA/EPP5	2.0×10^6	1.47
PLA/EPP8.5	1.9×10^6	1.50
PLA/EPP13	1.6×10^4	3.57

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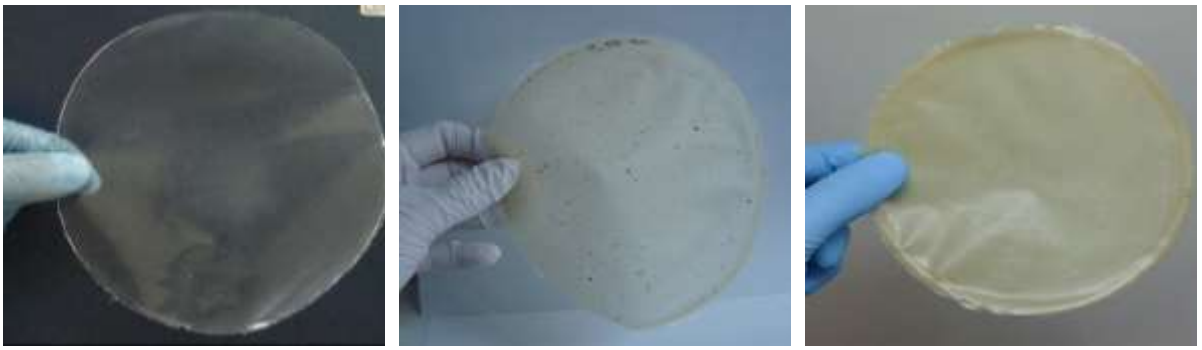
657 **Fig. 1**



PLA control **PLA/PWP13** **PLA/EEP13**

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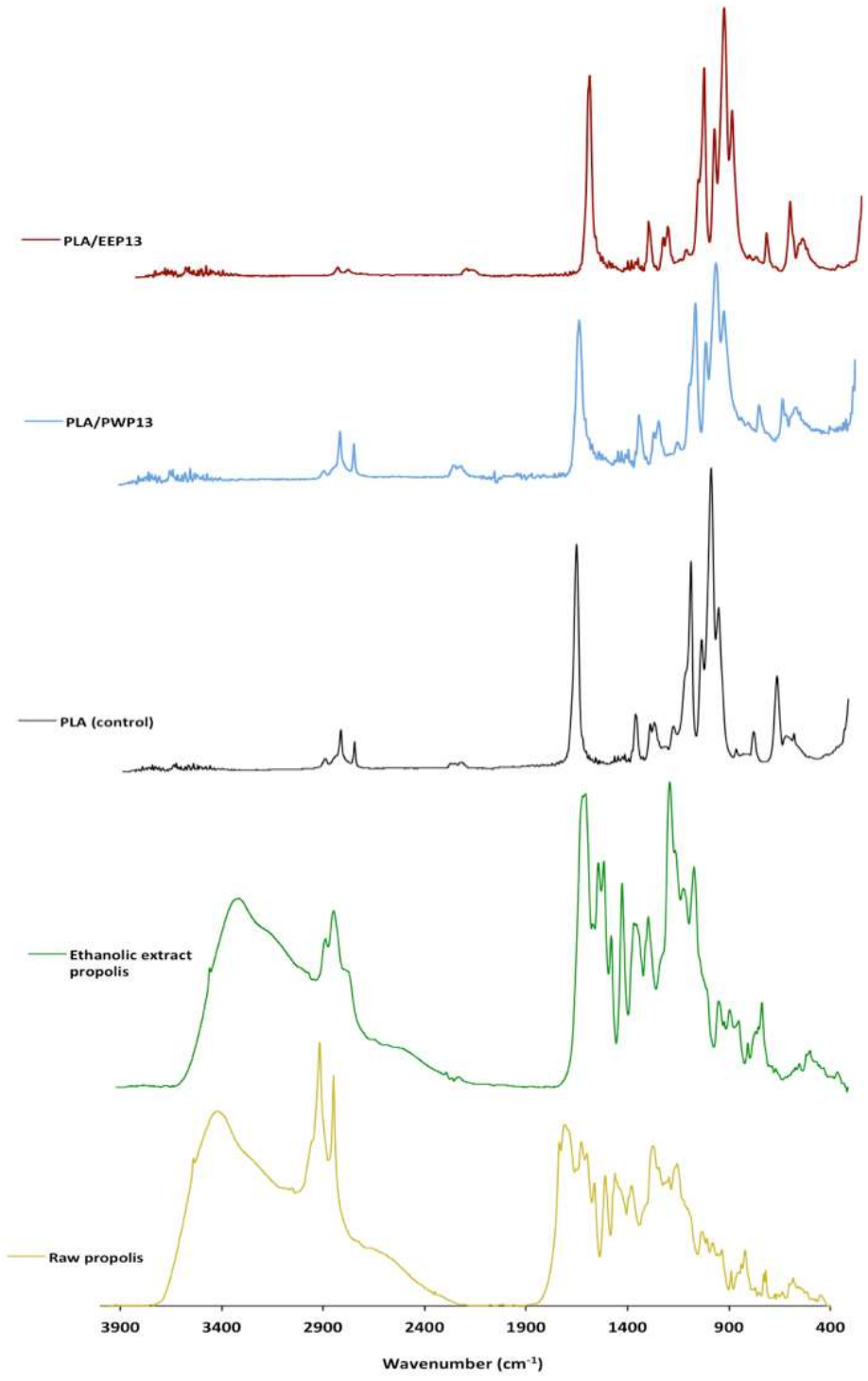
661 **Fig. 2**



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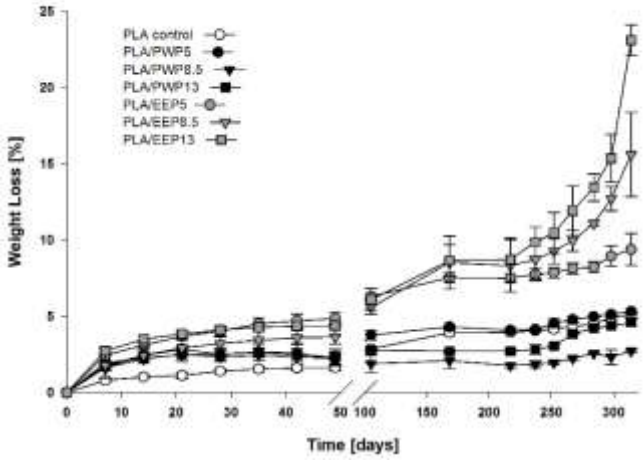
664 **Fig. 3**



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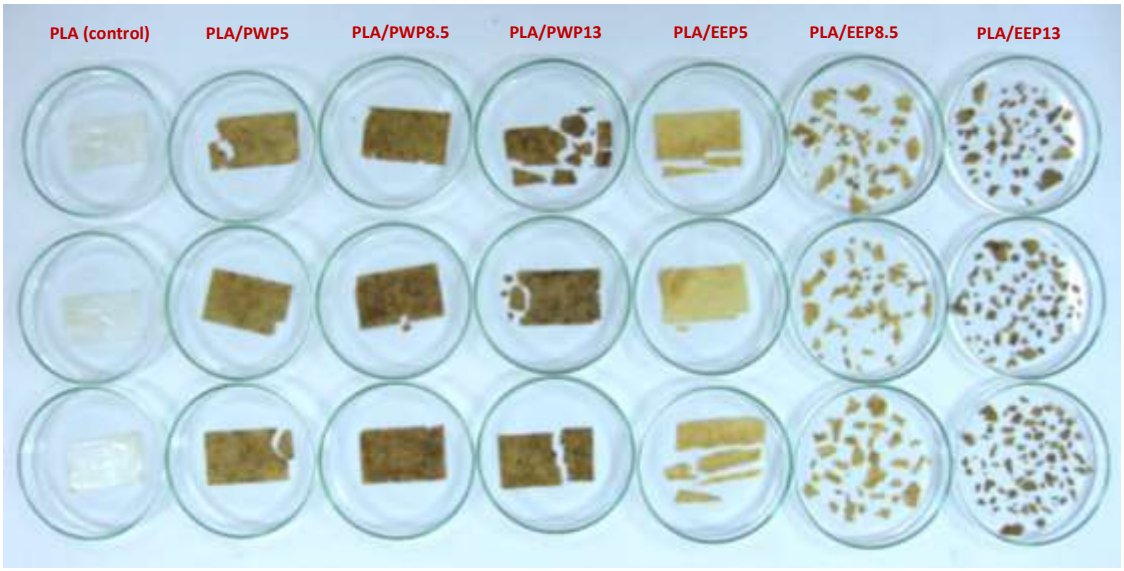
668 **Fig. 4**



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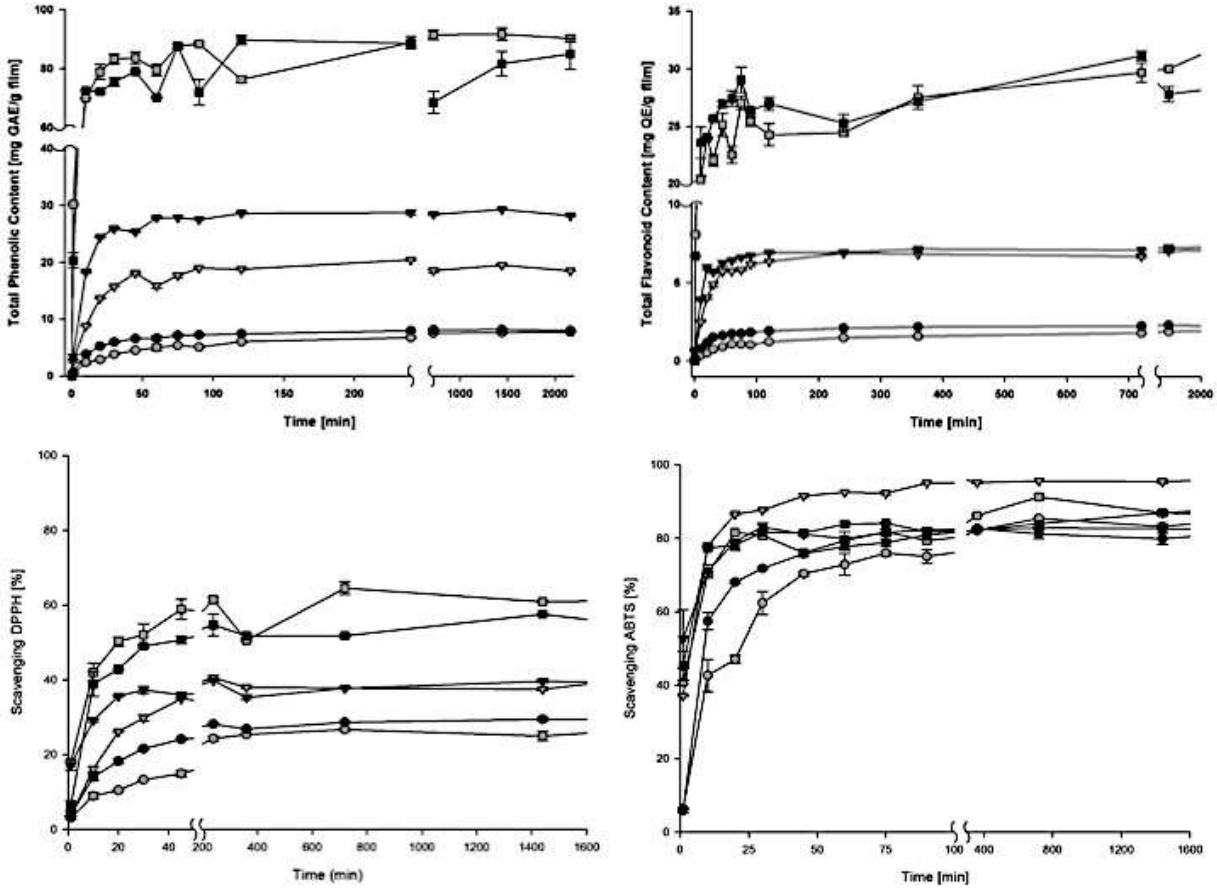
672 **Fig. 5**



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