Extending beef meat shelf life: an approach using active polylactic acid films with pomegranate (*Punica granatum* L.) peel and pomegranate peel extract

Mariana A. Andrade [1,2,3], Pedro V. Rodrigues[4], Carolina Barros[4], Vasco Cruz[4], Ana Vera Machado [4,*], Cássia H. Barbosa [1,5], , Anabela Coelho [1], Rosália Furtado [1], Cristina Belo Correia [1], Margarida Saraiva [5], Fernanda Vilarinho [1], Fernando Ramos [2,3,*] and Ana Sanches Silva [2,7,8,*]

[1] Department of Food and Nutrition, National Institute of Health Dr Ricardo Jorge, Av. Padre Cruz, 1649-016 Lisbon, Portugal

[2] University of Coimbra, Faculty of Pharmacy, Coimbra, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

[3] REQUIMTE/LAQV, R. D. Manuel II, Apartado 55142, Oporto, Portugal

[4] Department of Polymer Engineering, Institute for Polymers and Composites (IPC), Campus de Azurém, University of Minho, 4804-533 Guimarães, Portugal

[5] Department of Sciences and Technology of Biomass, NOVA School of Science and Technology | FCT NOVA, NOVA University of Lisbon, Caparica Campus, 2829-516 Caparica, Portugal

[6] Department of Food and Nutrition, National Institute of Health Doutor Ricardo Jorge, Rua Alexandre Herculano 321, 4000-055 Oporto, Portugal

[7] National Institute for Agricultural and Veterinary Research (INIAV), I.P., Rua dos Lagidos, Lugar da Madalena, 4485-655, Vairão, Vila do Conde, Portugal

[8] Center for Study in Animal Science (CECA), ICETA, University of Oporto, Oporto, Portugal

Abstract: (1) Background: The search for new forms to prolong foods' shelf-life through the use of natural compounds and extracts continuous to grow among the researchers and industry. In this line of thought, the main objective of this work is to develop, characterize and evaluate the effectiveness of an active food packaging, based in polylactic acid and incorporated with natural extracts obtained from pomegranate by-products. (2) Methods: *In vitro* antioxidant methods were applied to evaluate the antioxidant capacity of the pomegranate and grape extracts. Punicalagin (A+B) and ellagic acid were determined in both extracts and films by UHPLC-DAD. The new active packaging based in PLA with pomegranate extract was carried to evaluate chemical, mechanical, optical and barrier properties. Moreover, the evaluation of the effectiveness of the new film was evaluated through the study of lipid oxidation state and microbial contamination of two high fat content foods, almonds and beef meat. (3) Results: Pomegranate peel extract (PE) with high antioxidant capacity and high content in total phenolics and flavonoids compounds was successfully obtained. The PE and the pomegranate peel (PP) were successfully incorporated into a PLA-based packaged which prevented the lipid oxidation of meat and presented antimicrobial activity against *S. aureus*. The addition of the PE and the PP to the PLA did not affect the morphology of the polymer. Although both PE and PP presented punicalagin (A+B) and ellagic acid, only ellagic acid was identified and quantified in the PLA with PE and PP. Active PLA films were not effective in delaying lipid oxidation of almonds but they showed to be significantly effective in delaying lipid oxidation of beef meat and significantly reduced the microbial growth in this food matrix over time.

Keywords: polyphenolic compounds; punicalagin; ellagic acid; natural extracts; pomegranate; polylactic acid; active food packaging.

1. Introduction

Fruits by-products are often discarded as waste, causing its potential to be lost. They are known sources of powerful bioactive compounds with several biological activities with potential health benefits [1,2]. Phenolic compounds are among these bioactive compounds. They are secondary metabolites formed by plants for their natural defense against

pathogenic organisms, predators, parasites, and UV radiation [3–5]. Plants' colors and organoleptic properties can be associated to the presence of certain phenolic compounds, which include flavonoids, catechins and phenolic acids. The production of these compounds depends on several factors such as the edaphoclimatic conditions to which the plant was exposed, harvest time, among others and also, their distribution in the plant is not homogenous, depending on the plant part [3–7].

Pomegranate (*Punica granatum* L.), native from Asia, is one of the oldest consumed fruits in the world. Only 40 % of the fruit is edible (arils) being the rest constituted by peels (50 %) and seeds (10 %), making it a major source of by-products. In traditional medicines is used for treatment of vermifuge, asthma, bronchitis, fever, inflammation, and bleeding disorders [1,8]. Its antioxidant and anticancer activities are highly documented in literature [5,9–17].

Grapes are one of the most used and consumed fruits in the world, specifically in wine production. According to FAOSTAT, the grape worldwide production in 2020 was 78 034 332 tonnes and the wine worldwide production, in 2019, was 27 025 456 tonnes [18]. Grape by-products, resulting from wine production, are usually referred to as grape pomace or wort and, it is rich in catechins, gallic acid, and procyanidins [9,19,20]. All of these compounds are known to possess powerful antioxidant activities. Their distribution in the grape is not homogeneous, being the seeds the part with the highest content in phenolic compounds (60-70%), followed by skin (28-35%) and pulp (10%) [1,21].

Food packaging' main function is to protect foods during transportation and storage, delaying its natural degradation, with no interaction with the packaged food. Active food packaging is a technology that came to revolutionize the concept of food packaging since its main goal is to prolong foods' shelf-life through the interaction of the package and food. This interaction could be made through the emission of antioxidant or antimicrobial compounds that, by the interaction with the food' surface, delay or even stop the natural degradation and even improve the organoleptic properties of the packaged food.

Lipid oxidation is still one of the main causes for food loss and one of the major enemies of the food industry [22–24]. The solution may lie in the gradual emission of active compounds with high antioxidant capacity from the package matrix to the food' surface, inhibiting the lipid oxidation of highly fatty foods [25–31].

This paper aims to evaluate the antioxidant and antimicrobial potential of natural ethanolic extracts from pomegranate and grape by-products. Moreover, the extracts that showed more promising results in the antioxidant and antimicrobial assays where further incorporated in a biodegradable polymeric matric, polylactic acid. Afterwards the films were characterized in terms of chemical, optical, mechanical and barrier properties. Moreover, the effectiveness of new active films was evaluated through the determination of the lipid oxidation status and microbial contamination of two model foods, almond and beef-meat, packaged with these films for different time periods.

2. Materials and Methods

2.1. Reagents and materials

Absolute ethanol (ACS reagent, for analysis), methanol (ACS reagent (≥99.8%) and for HPLC, ≥99.9%), chloroform (SupraSolv®, for gas chromatography ECD and FID), sodium carbonate anhydrous (ACS reagent), sodium nitrite (ACS reagent), sodium hydroxide (ACS reagent), petroleum ether (ACS reagent, bp 40-60 ° C), barium chloride dihydratate (ACS reagent), iron(II) sulfate (pro analysis), iron(III) chloride (anhydrous for synthesis), hydrochloric acid, glacial acetic acid (for HPLC, ≥99.9%), Folin-Cioucalteu's phenol reagent, were acquired to Merck (Darmstadt, Germany). n-Hexane (SupraSolv®, for gas chromatography ECD and FID) was acquired to Honeywell. Tween®40, β-carotene (≥ 93%), linoleic acid (analytical standard), gallic acid, 2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), epicatechin, aluminum chloride, trichloroacetic acid (ACS reagent, ≥ 99.0%), 2-thiobarbituric acid (≥ 98%), 1,1,3,3-tetramethoxypropane (97%), 2,2-diphenyl-1-picrylhydrazyl, xylenol orange sodium (spectrophotometric grade), were acquired to Sigma-Aldrich (Madrid, Spain). Standards punicalagin (A+B) and ellagic acid were acquired to MedChem Express. Ultra-pure water was obtained through a Milli-Q® purification system (Millipore Corp., Belford, USA).

Also, a compact stirrer Edmund Bühler™ Shaker KS 15 A (Hechingen, Germany), an Eppendorf AG 5804R centrifuge (Hamburg, Germany), a rotary evaporator Büchi model R-210 (Labortechnik, Switzerland), a Thermo Scientific Evolution 300 LC spectrophotometer, a RSLAB-6PRO Vortex, Ultra-Turrax IKA® DI 25basic, a Grindomix GM 300 (Retsch) and a Grant Instruments™ QB Series Dry Block Heating System (Cambridge, England) were used.

2.2. Extraction process

Pomegranate by-products were kindly supplied by Memória Silvestre Lda - Arilo Pomegranates, a Portuguese fruit company. The peels+mesocarp were manually separated from the seeds. The peels+mesocarp were grinded and part was freeze-dried. The extraction protocol was only applied in the natural and freeze-dried peels+mesocarp. Wine by-products (wort, in Portuguese mosto) were retrieved, after the wine production, in Sobreira, Idanha-a-Nova, Portugal, grinded and freeze, once it arrived at the laboratory.

All the extracts were obtained using absolute ethanol and the extraction method followed the method described by Andrade et al. [3]. Briefly, the by-products were mixed with ethanol in a 1:10 ratio, homogenized for 30 min and centrifuge for 10 min at 10000 rpm. The supernatant was removed for a pear-shaped flask and the extract was evaporated in the rotary evaporator, until dryness, at 35 °C. The extract was removed with the aid of a spatula, vacuum packaged and stored at -20 °C until further use.

2.3. Antioxidant activity and total content in phenolic compounds and flavonoids

The *in vitro* antioxidant activity and the total content in phenolic compounds and flavonoids was performed in the extracts obtained from the freeze drier pomegranate peels+mesocarp (PE-FD), from the natural pomegranate peels+mesocarp (PP) and from the wort extract.

2.3.1. DPPH radical scavenging activity

The applied method was initially described by Moure et al. [32] and modified by Andrade et al. [3]. Briefly, 50 μL of sample are mixed with 2 mL od a DPPH• methanolic solution (14.2 μg/mL). For the control, 50 μL of ethanol were used, instead of the sample. The mixtures were left, protected from the light, for 30 minutes. The absorbance was measured at 510 nm, in the spectrophotometer. The Inhibition Percentage (IP) of the DPPH• was calculated through the equation (1).

$$IP\ (\%) = \frac{A_b - A_s}{A_b} \times 100 \qquad (1)$$

Were, *Ab* stands for the absorbance of the control and *As* stands for the absorbance of the sample. Also, a calibration curve using Trolox as a standard was drawn. The results are expressed in mg trolox equivalents (TE)/g of sample.

2.3.2. Total Content in Phenolic Compounds

The total content in phenolic compounds was performed in accordance with the method described by Erkan et al. [33]. Briefly, 1 mL of sample was mixed with 7.5 mL of an aqueous Folin-Ciocalteu' solution (10 %, v/v), homogenized and left for 5 min. Then, 7.5 mL of an aqueous solution of sodium carbonate (60 mg/mL) were added, the samples homogenized and left to stand for 2 hours, protected from the light. The absorbance was measured at 725 nm in the spectrophotometer. Using gallic acid as a standard, a calibration curve was drawn. The results are expressed in mg of gallic acid equivalents per g of sample (mg GAE/g).

2.3.3. Total Content in Flavonoids

The total content in flavonoids was measured applying the method described by Yoo et al. [34]. Succinctly, 1 mL of sample was homogenized with 4 mL of ultrapure water and 300 μL of a sodium nitrite aqueous solution (5 %, w/v). The samples were left to stand for 5 minutes and, 600 μL of aluminum chloride aqueous solution (10 %, w/v) was added. The mixture was, once again homogenized and left to stand for 6 minutes. Then, 2 mL of sodium hydroxide (1 M, w/v) and 2.1 mL of ultrapure water were added. The mixtures were homogenized, and the absorbance measured at 510 nm, in the spectrophotometer. Using epicatechin as a standard, a calibration curve was drawn, and the results are expressed in mg of epicatechin equivalents per g of sample (mg ECE/g).

2.4. Identification and Quantification of Punicalagin and Ellagic Acid by UHPLC-DAD

The validation, identification, and quantification of punicalagin (A+B) and ellagic acid was performed by Ultra high-performance liquid chromatography (UHPLC) in an UPLC® ACQUITY™ (Waters, Milford, MA, EUA) equipped with a DAD detector. To obtain the best separation efficiency, two columns were tested, an ACQUITY™ UPLC® BEH C18 (2.1 × 50 mm, 1.7 μm particle size) column, and an ACQUITY™ UPLC® RP18 (2.1 × 100 mm, 1.7 μm particle size). Also, different column temperatures were tested: 20; 25; 30 and 35 °C.

The best results were achieved with the ACQUITY™ UPLC® BEH C18 column, kept at 35 °C. Samples were kept at 5 °C and the injection volume was 10 μL. Flow was kept at 0.3 mL/min. Mobile phase A was ultra-pure water acidified with glacial acetic acid at 0.1 % (v/v) and mobile phase B was acetonitrile with glacial acetic acid at 0.1 % (v/v). The gradient was as follows: 0 min, 95 % A; 1 min, 90 % A; 2.5 min, 80 % A; 3 min, 75 % A; 5 min, 90 % A.

The method was validated following the parameters: specificity, working range, linearity, Limit of detection (LoD) and Limit of quantification (LoQ), precision (repeatability and intermediate precision), and accuracy (determined by recovery assays of spiked samples of pomegranate by-products).

Mixed solutions, containing 300 μg/mL of punicalagin (A+B) and 20 μg/mL of ellagic acid diluted in methanol with 0.1 % of glacial acetic acid, were made and diluted up to six times. Calibration curves were drawn by plotting the concentration (μg/mL) versus area. Linear range, LoD and LoQ were determined. Specificity was assessed by comparison of the absorption spectra of the chromatographic peaks between the analytical standards and the samples.

2.5. Incorporation of the active compounds into PLA films

PLA Inzea F18C (Nurel Biopolymers) was used to produce two films with 3 wt.% of the PE-FD (PLA/3PE) and another with 3 % of the powder from pomegranate peels (PLA/3PP).

Prior to processing, all materials were dried in a convection drying oven at 55 °C for 4 h. Compositions of PLA with pomegranate by-products took place in a twin-screw extruder (Leistritz, Germany) at an average melt temperature of

170 °C, 150 rpm, a throughput of 5 kg/h, and an average residence time of 1.5 min. The extrudate filaments were air dried and ground into pellets in a knife mill for subsequent processing.

Prior to blown film extrusion the pellets were dried in a forced convection oven at 60 °C. The PLA films were prepared using a Periplast single screw extruder L/D= 25. The films were extruded with a screw speed of 50 rpm, at a temperature of 170 °C (first heating zone), 175 °C (second zone) and 180 °C (on the remain). A digital micrometer (No. 293- 340, Mitutoyo, Kanagawa, Japan) was used to measure the thickness measurement of the films that was 50 to 60 μm range.

2.6. Films' characterization

2.6.1. Fourier Transform Infrared spectroscopy (FTIR)

To access the structural characterization of the pomegranate by-products and films, Fourier Transformed Infrared Spectroscopy analysis was performed in a 4100 Jasco (Japan) spectrometer in attenuated total reflectance mode (ATR) at 64 scans, 8 cm^{-1} resolution in a wavelength range of 4000-600 cm^{-1}.

2.6.2. Scanning Electron Microscopy (SEM)

The morphology of the films was assessed by SEM analysis in an FEI Quanta 400 (FEI, Eindhoven, The Netherlands), after fractured in liquid nitrogen and coated with a thin gold-palladium (80/20) film.

2.6.3. Film Optical Properties

CIELab color space parameters (L* – brightness, a*-red-green and b*- yellow-blue levels) of the control and active films were measured using a Shimadzu UV2401PC reflectance spectrophotometer operating in the range of visible light between 370 to 750 nm with a 2 nm spectral resolution. The total color difference (ΔE^*) was determined according to following formula [35]:

$$\Delta E^* = \sqrt{\left(L_f^* - L_{PLA}^*\right)^2 + \left(a_f^* - a_{PLA}^*\right)^2 + \left(b_f^* - b_{PLA}^*\right)^2}$$

where L_{PLA}^*, a_{PLA}^* and b_{PLA}^*, L_f^*, a_f^* and b_f^* are the parameters of PLA control and produced films with pomegranate by-products, respectively.

The haze of the film samples was measured according to ASTM D1003-00 in a XL-211 Hazegard System. Six specimens were tested for each sample. The total light and diffuse transmittance were measured to calculate haze.

2.6.4. Water Vapor Transmission (WVT)

The WVT of the film was measured according to ASTM E96/E96M-10. Calcium chloride was used as the desiccant, the tests were conducted at 23 ± 2 °C for 16 days and the samples were weighed every day.

2.6.5. Oxygen Permeability

The films oxygen permeability was obtained through the Permeameter DP-100A from Porous Materials, Inc, with the pressure increase method. Three specimens were collected from the film samples, with a 4 cm diameter. The test was carried at 23 ± 2 °C with a pressure of 1 atm for 3 hours.

2.6.6. Mechanical properties

The storage modulus and loss factor of the films were assessed by dynamic mechanical analysis (DMA) using a DMA TRITON, with 10 mm distance between grips, in tensile mode. For this test, samples with 4 mm width were used, and a load of 1 N was applied at a frequency of 1 Hz in the temperature range of 40 to 130 °C using a heating rate of 2 °C/min.

The tensile tests were performed on a mechanical testing machine, INSTRON 5969, with a load cell of 50kN, at a speed of 100 mm/min, at 23 ± 2 °C, following ASTM D882 – 02. At least, five film specimens, with a length of 160 mm and a width of 25 mm were used. In both tests, samples were cut in the machine direction (MD).

2.7. Antimicrobial analysis of the pomegrante extract

The antimicrobial activity of the PE was measured against *Listeria monocytogenes* (Gram positive), *Staphylococcus aureus* (Gram positive), *Enterococcus faecalis* (Gram positive) and *Escherichia coli* (Gram negative). First, a Petri dish with Plate Count Agar (PCA) was flooded with the suspension of each individual microorganism. The excess liquid was removed, and, after 10 min, one cavity of 4 mm was made in the PCA, and 65 ± 1 mg of the extract was placed. The plates were incubated at 37 °C in aerobic conditions. The inhibition halo was measured at the end of 24 h.

2.8. Effectiveness of the active PLA films

For the antioxidant analysis of the PLA films, an accelerated migration assay was performed according to the method described by López-de-Dicastillo et al. [36], with a few changes. Briefly, circles of 9.08 cm^2 of the PLA films were cut and submersed in ethanol 95 % (v/v), and kept at 40 °C for 10 days, protected from the light. At the end of this period, the $DPPH^{\bullet}$ inhibition assay (see section 2.3.1), the total content in phenolic compounds and flavonoids (see section 2.3.2 and 2.3.3), and the content in punicalagin (A+B) and ellagic acid was determined.

Also, in order to total quantify the maximum the content punicalagin (A+B) and ellagic acid, the films were also submerged in methanol and kept protected from the light, at 25 and 40 °C, for 24h.

For the antimicrobial analysis of the PLA films, the ISO 22196:2011 [37] was followed. The antimicrobial analysis of the films was tested against the same microorganisms. Briefly, a single/one culture of the inoculum of each microorganism, made from stock cultures in Tryptone Soya agar (TSA), was transferred with a 1 μL loop, to a Brain Hearth Infusion Broth (BHI), the plates were incubated at 37 °C overnight and the solutions were diluted to 10^{-4}. Squares of 5 x 5 cm of each film were placed in a Petri dish, and 0.2 mL from each dilution were applied to each film and, to keep the film hydrated, squares of sterile plastic (4 x4 cm) were placed on top of each film. The plates were kept at 35 °C for 24h in a humid atmosphere in aerobiose. At the end of this time, 10 mL of Soybean Casein Lecithin Polysorbate (SCDLP) were added to each plate and stirred for 30 s. From this, serial dilutions (10^{-1} and 10^{-2}) were performed to Petri dishes and warm PCA was incorporated. After the PCA solidified, the dishes were kept at 35 °C for 48h in aerobic conditions and, at the end of the incubation period, the counts of colony forming units (CFU) were quantified.

2.9. Packaging of the model foods

According to the Portuguese National Food Composition Table, the total lipidic content of the almond is 56 g/100 g, of which 4.7 g/100 g are saturated fat acids, 34.5 g/100 g are monounsaturated fat acids, and 14.3 g/100 g are polyunsaturated fat acids, making almond one of the richest foods in unsaturated fat acids.

Almond was acquired, still in its hard shell, in a local store in Lisbon, Portugal. The hard shell was manually separated with the help of a hammer, placed in a hot bath (approximately 80 ± 1 °C) for a maximum time of 5 min and manually peeled, grinded in a Grindomix and vacuum packaged. The samples were stored at 40 ± 1 °C and at room temperature, protected from the light. The almond samples stored at 40 ± 1 °C were analyzed at the end of 2, 4, 7, 14, 21 and 30 days of storage. The almond samples stored at room temperature were analyzed at the of 7, 14 and 21 days of storage.

Regarding beef meat, according to the USDA food database, the total lipidic content is 19.07 g/100 g, of which 7.29 g/100 g are saturated fat acids, 8.48 g/100 g monounsaturated fat acids and 0.51 g/100 g are polyunsaturated fat acids. Pieces of 35 ± 1 g were also vacuum packaged and stored, protected from the light, at 4 °C. The meat oxidation state and microbiological contamination was assessed at the end of 1, 4, 6, 8 and 11 days of storage.

2.10. Lipid oxidation evaluation

2.10.1. Thiobarbituric acid reactive substances assay (TBARS)

The method originally described by Rosmini et al. [38] and slightly altered by Andrade et al. [25] was performed for the TBARS assay. Briefly, 20 mL of an aqueous solution of trichloroacetic acid (7.5 %, w/v) were added to 5 g of sample. The samples were homogenized in a compact stirrer for 1 hours, at 400-450 rpm. Using a paper filter Whatman No. 1, the samples were filtered and 2.5 mL of the filtered was mixed with 2.5 mL of an aqueous solution of 2-thiobarbituric acid (2.88 mg/mL) and kept, at 95 °C, for 30 minutes. The samples were rapidly cooled with ice for 15 min, and their absorbance was measured at 530 nm in the spectrophotometer, against the control (2.5 mL of ultrapure water + 2.5 mL of 2-thiobarbituric acid solution). A calibration curve using 1,1,3,3-tetramethoxypropane was drawn and the results are expressed in mg of malonaldehyde equivalents per kg of sample (mg MDA/kg).

2.10.2. Fat extraction

For the determination of the peroxide value and the *p*-anisidine value, the fat of the samples had to be extracted. Briefly, 10 ± 1 g of sample was mixed with 100 mL of petroleum ether and agitated in the compact stirrer at 350-400 rpm for 1 hour. Then, the samples were filtered using a paper filter Whatman No. 4 with 1 g of sodium carbonate anhydrous. The ether was removed using the rotary evaporator at 35 °C.

2.10.3. Peroxide Value Determination

For the peroxide determination, the method described by Shantha & Decker [39]. First, to prepare an iron (II) chloride solution, 50 mL of an aqueous solution of barium chloride (8 mg/mL) was slowly pored under constant stirring, in a 50 mL of an aqueous solution of iron (II) sulfate. Still under constant stirring, 2 mL of hydrochloric acid (10 N) were added, and the solution was left to stand to allow the deposition of the barium chloride. The supernatant was moved to an amber flask and stored for a maximum time period of 1 week, protected from the light. Then, 50 mg of fat were agitated with the vortex for 2-4 sec. with 9.8 mL of a chloroform-methanol solution (70-30%, v/v). Then, 50 μL of a xylenol orange solution (10 mM) were added, the solution was again homogenized in the vortex, and 50 μL of the iron(II) chloride solution were added, the solution was mixed, and, after 5 min, the absorbance was measured at 560 nm, in the spectrophotometer. The peroxide value was calculated through the equation (2) and the results are expressed in milliequivalents of oxygen per kilogram of sample (meq O2/kg).

$$PV = \frac{(As-Ab)\times m}{55.84 \times m_0 \times 2} \quad (2)$$

Where, *As* stands for the samples' absorbance, *Ab* stands for the blanks' absorbance, *m* stands for the slope of the iron (III) calibration curve, *m0* stands for the samples' mass in g, and 55.84 is the atomic weight of iron.

2.10.4. Determination of the *p*-anisidine value

The *p*-anisidine value determination followed the method described by British Standard method BS 684-2.24 [40]. Briefly, to 50 mg of fat 25 mL of *n*-hexane were added. The solution was placed in the ultrasounds for 5 min to make sure that all of the fat was dissolved. The absorbance of the solution was measured at 350 nm, against *n*-hexane. Then, to 5 mL of sample, 1 mL of a *p*-anisidine in acetic acid (2.5 mg/mL) was added. The samples were placed, for 10 min, in the dark, and their absorbance was measured against the control. The *p*-anisidine value (AV) was calculated by the equation (3).

$$AV = \frac{25(1.2As-As0)}{\mathrm{m}} \quad (3)$$

Where, *As* stands for the absorbance of the sample at the end of 10 min, *AS0* stands for the absorbance of the sample at 0 min, and *m* the sample' weigh in g.

2.11. Evaluation of the lipid oxidation and microbial growth in meat with active PLA films

In order to fully evaluate the potential of the active PLA, raw beef meat was also used as a model food to evaluate the antioxidant and antimicrobial potential of the active PLA films. Beef meat was chosen due to its short shelf-life and because its normal degradation is generally due to the action of microorganisms.

Briefly, 25 ± 1 g of raw beef meat were vacuum packaged and stored for a maximum period of 11 days at 4 °C, protected from the light. The lipid oxidation of the meat was evaluated through the TBARS assay using the method described in the section 2.10.1. The total microorganisms count at 30 °C was performed using the automated test TEMPO® Aerobic Count-AFNOR BIO 12/35-05/13.

2.12. Statistical analyses

All the assays were performed in triplicate. The results are expressed in means ± standard deviation. The statistical analysis was performed in the software IBM® SPSS® Statistics, version 27.0.1.0, using a one-way analysis of variance (ANOVA) and ANOVA with repeated measures. The differences among mean values were processed by the Tukey test.

3. Results

3.1. Extracts' antioxidant capacity and total content in phenolic compounds and flavonoids

The antioxidant capacity of the extracts is compiled in Table 1. PE-FD presented the highest inhibition percentage (175.3 ± 0.38 mg TE/g) and content in total phenolic compounds (221.5 ± 0.62 mg GAE/g) and total flavonoids (31.39 ± 0.61 mg ECE/g). The extract obtained from wort presented a low inhibition percentage and a low content in phenolic compounds. The total content in flavonoids was not possible to be determined since the solution kept precipitating. This associated with the difficulty in obtaining a workable extract, pomegranate extract and dried by-products were chosen to be incorporated into the PLA active packaging.

Table 1. Antioxidant capacity of the extracts.

Samples	IP (%)	mg TE/g	TPC (mg GAE/g)	TFC (mg ECE/g)
PE-FD	92.42 ± 0.2[a]	175.3 ± 0.38[a]	221.5 ± 0.62[a]	31.39 ± 0.61[a]
PE-N	88.48 ± 0.31[b]	167.8 ± 0.58[b]	151.6 ± 0.47[b]	17.72 ± 1.08[b]
Wort	4.810 ± 0.14[c]	10.03 ± 0.27[c]	15.85 ± 0.16[c]	*

Legend: PE-FD – Pomegranate Freeze-dried extract; PE-N – Natural pomegranate extract; IP – inhibition percentage; TE – Trolox equivalents; TPC – Total content in phenolic compounds; GAE – Gallic acid equivalents; TFC – Total content in flavonoids; ECE – Epicatechin equivalents.

*Not determined.

Comparing these results with the literature and recent review paper by Andrade et al. [1], PE-FD presented, in general, higher antioxidant capacity and total content in phenolic compounds. The DPPH radical inhibition percentage is similar to the inhibition percentage reported by Rashid et al. [41] (97.3 %) in the pomegranate extract obtained with ultrasonic assisted extraction with ethanol 70% (v/v). However, in the other pomegranate extract obtained though maceration by the same authors, the inhibition percentage is much lower (48.7 %). Lower inhibition DPPH radical percentages were also found by in the hydroethanolic extract by Selahvarzi et al. [42] (72.11 %). El-Hadary and Taha [43] found similar inhibition percentages in pomegranate peel extract obtained with methanol 80% (v/v).

The phenolics content of both pomegranate extracts was higher than the results reported by Zago et al. [44], Rashid et al. [41], and by Selahvarzi et al. [42] (66.14 mg GAE/g; 37. 52 mg GAE/g and 2.70 mg GAE/g, respectively). However, higher content in phenolic compounds was reported by Rashid et al. [41] (277.6 mg GAE/g) in a pomegranate extract obtained with ethanol 70% (v/v) by ultrasonic assisted extraction. Other solvents, such as methanol and acetone, can be more efficient in extracting bioactive compounds than ethanol. However, these may raise safety concerns [45].

3.2. Films' characterization

3.2.1. FTIR & SEM

As can be seen in Figure 1, the PLA spectrum matches the PLA spectrum presented by Yuniarto et al. [46] and Bodbodak et al. [47]. The incorporation of the PE and the PP seemed to have no effect on the PLA structure. Bodbodak et al. [47] only reported a slight shifting in the C-OH and carbonyl groups, at 1750 – 1754 cm^{-1}, with the addition of the pomegranate peel extract, which was not observed in this study. Also, Miletićet al. [48] and Dai et al. [49] also reported no changes in PLA-based nanofibers with pomegranate seed oil and PLA active films with pomegranate peel extract, respectively. The change in 1716 cm^{-1} peak intensity for both PLA/3PE (PLA incorporated with 3% of PE) and PLA/3PP (PLA incorporated with 3% of PP) confirms the presence of pomegranate by-products into the film samples.

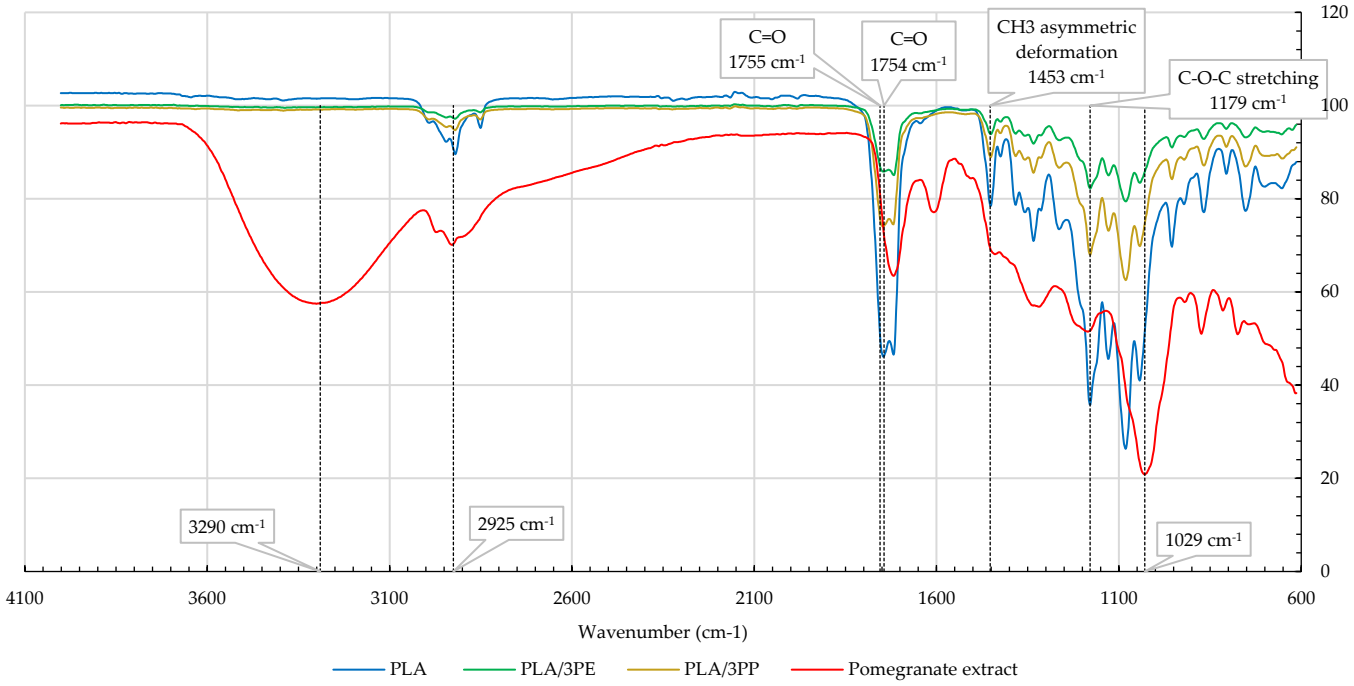

Figure 1. FTIR spectrum of the pomegranate extract (PE), control (PLA) and active films (PLA/3PE and PLA/3PP).

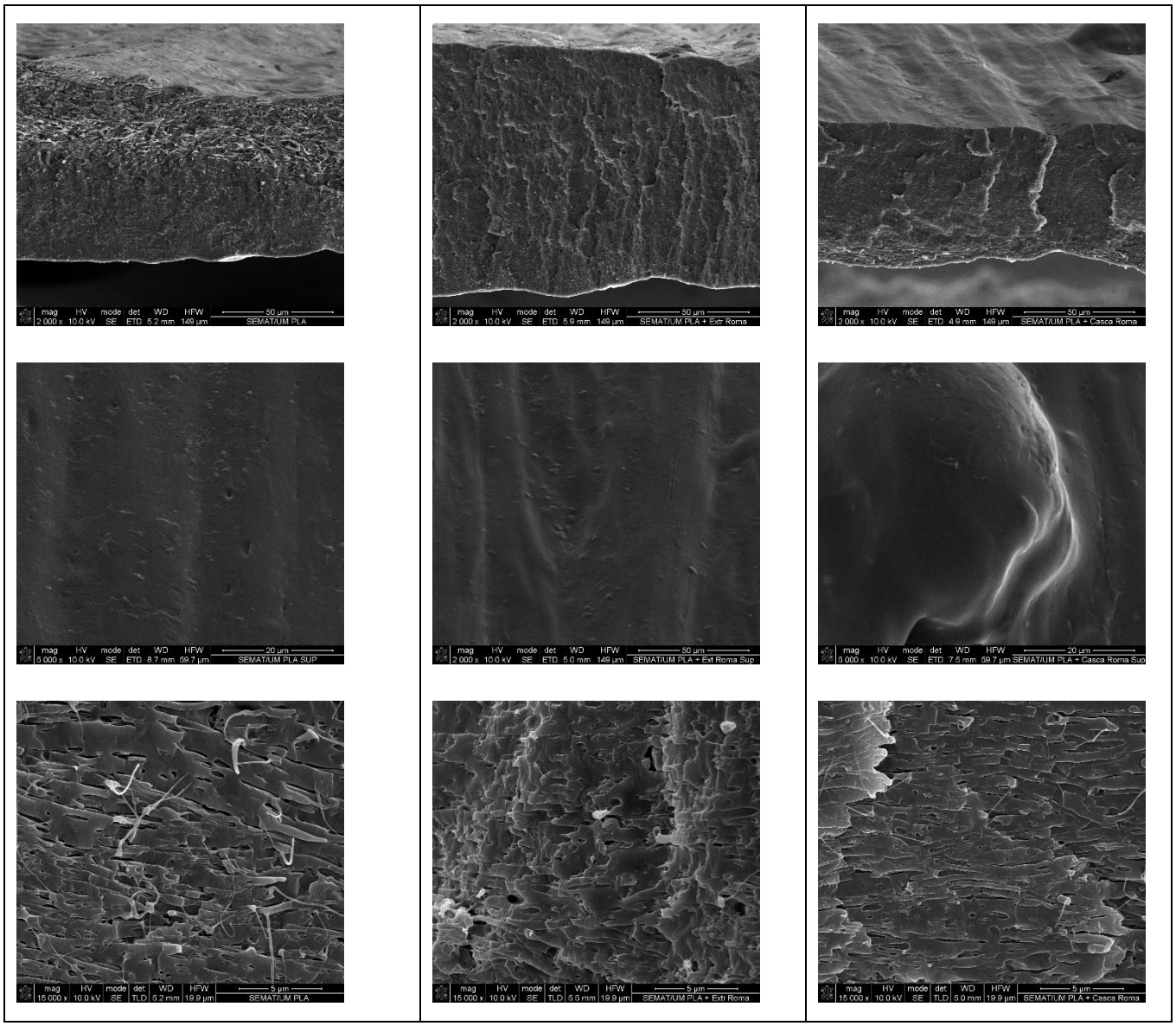

Figure 2. Photographs of the control (PLA) and the active (PLA/3PE and PLA/3PP) films, and the respective SEM images.

Observing the SEM images in Figure 2, for the control and active PLA films, a clear distinction can be seen among the control PLA, the PLA/3PE and PLA/3PP. The PLA scanning shows similar results to previous studies [49]. PLA/3PE presents a more homogenized distribution than the PLA/3PP, which presents layers indicating a non-homogenous distribution of the pomegranate peel. Also, roughness of PLA/3PP surface is more pronounced due to the presence of larger pomegranate peel particles embedded in the PLA matrix.

3.2.2. Water Vapor and Oxygen permeability and optical properties

In terms of the films' optical properties (Table 2), PLA/3PE presented a higher variation in terms of L*, a* and b* when compared to the PLA, than the PLA/3PP. The film that came closest to the color of the standard film (neat PLA) was PLA/3PP, as it can be seen by the coordinates ΔL*, Δa* and Δb*. The L* parameter (lightness) showed low values for all the samples with PLA/3PE having the lowest value of 40.84. The a* parameter, related to the green-red axis, showed a slight increase with the incorporation of pomegranate extract, contrary to the incorporation of pomegranate peel which maintained this parameter compared to the standard sample. The b* parameter, related to the blue-yellow axis showed an increase from 4.65 to 8.77 with the incorporation of pomegranate extract, whereas the incorporation of PE only increased to 6.485. These small differences in the values of the Cielab coordinates do not significantly change the color

of these films. The Cielab coordinates indicate that the films have a grayer color, although they visibly have a more brownish color. Overall, the change in color is more pronounced for the PLA/3PE sample, due to an even distribution of the additive, compared to PLA/3PP.

PLA Haze, 17.5 ± 0.2 %, slightly increased with the incorporation of pomegranate extract, 19.0 ± 0.1 % and pomegranate peel 19.2 ± 0.4 %.

Table 2. Optical properties of the control (PLA) and active (PLA/3PE and PLA/3PP) films.

Samples	L*	a* [red (+a) or green (−a)]	b* [yellow (+b) or blue (−b)]	ΔL*	Δa*	Δb*	ΔE*
PLA	47.33	0.410	4.65	0	0	0	0
PLA/3PE	40.84	0.600	8.77	-6.49	0.19	4.12	7.69
PLA/3PP	46.21	0.50	6.49	-1.12	0.085	1.84	2.15

Legend: PLA/3PE – PLA with 3 % of pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel.

Regarding barrier properties, the WVT tests were only performed for PLA and PLA/3PE.The PLA film presented a WVT de 1.99 ± 0.57 g/m²h having the film of PLA/3PE obtained a very similar result of 1.94 ± 0.64 g/m²h. The incorporation of pomegranate extract did not alter the water vapor barrier properties of the PLA film.

3.2.3. Mechanical properties

The tensile test results can be seen in Figure 3 in MD direction. The sample with the highest modulus and strength was the neat PLA. The values of the tensile test results relative to the other samples are much lower except for the percentage of deformation at break which increased considerably for the PLA/3PE sample. It seems that the introduction of low mass molecules acts as a slip agent, allowing the polymer molecules to move, which increases the plastic region of the material. The incorporation of both pomegranate extract and peel clearly translates into a loss in stiffness.

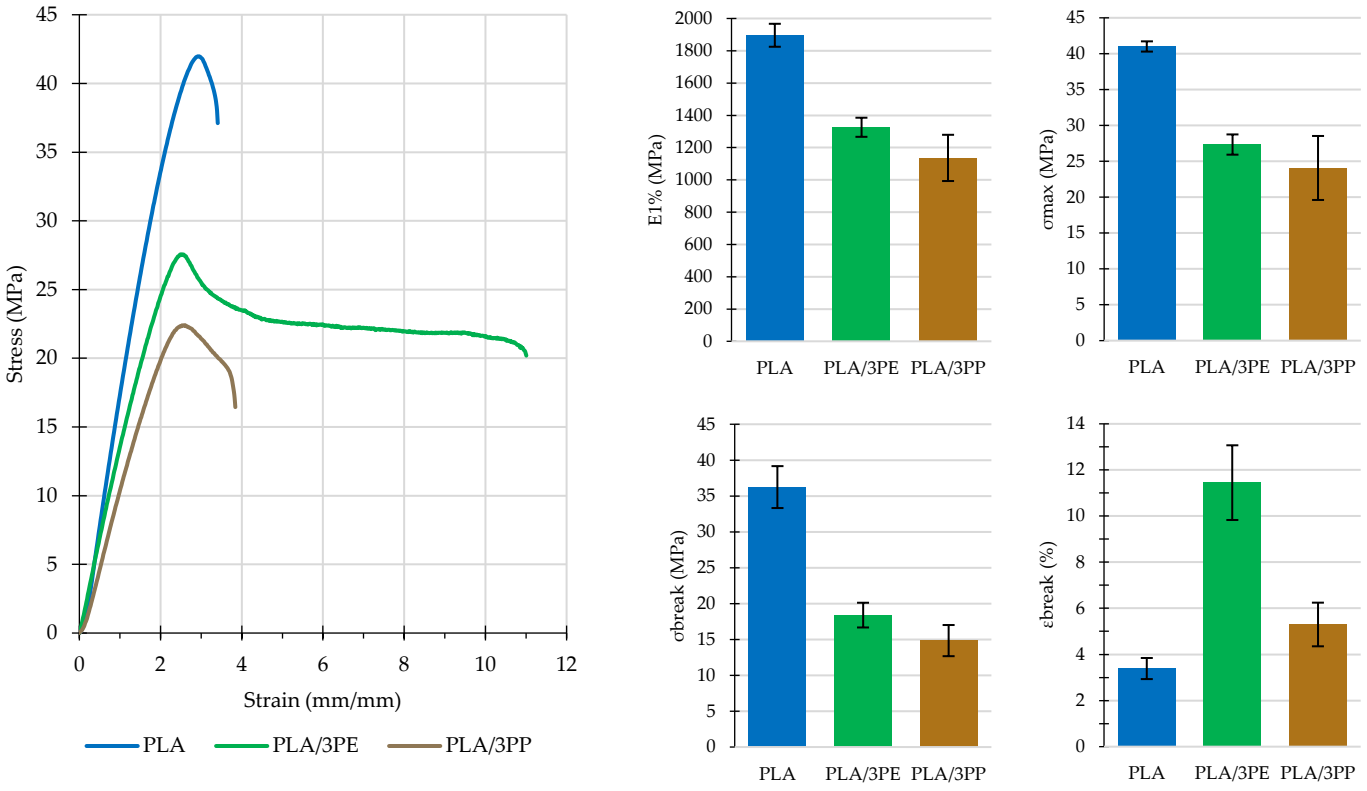

Figure 3. Representative stress vs strain curves of PLA, PLA/3PE and PLA/3PP in MD direction (left line chart), and respective mechanical indexes (right bar chart).

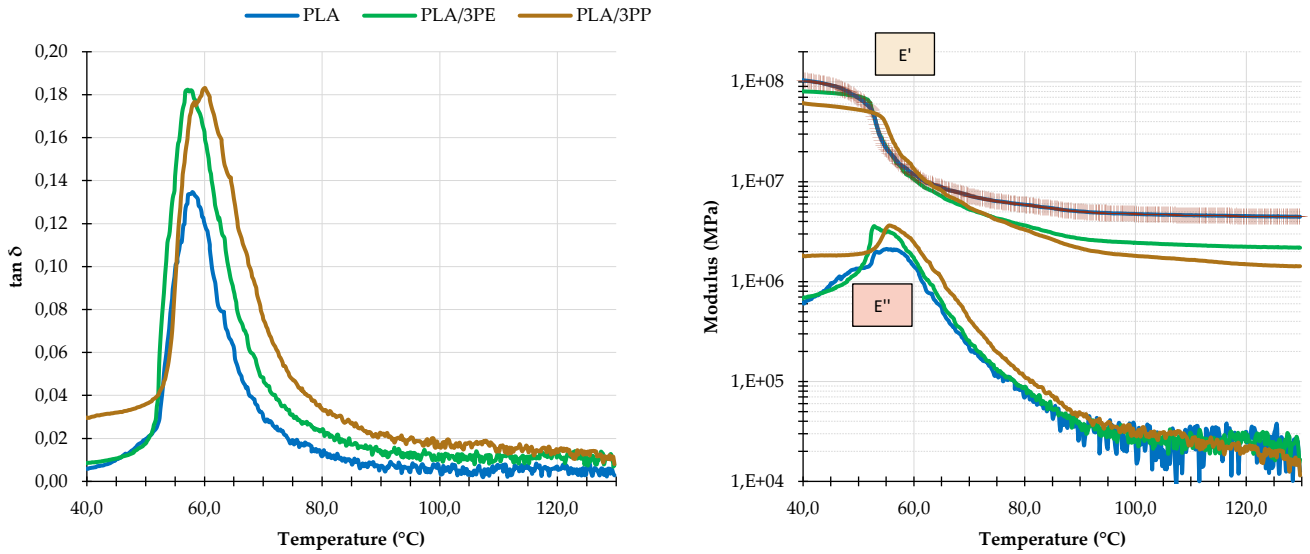

Figure 4. DMA curves of PLA, PLA/3PE and PLA/3PP in MD direction: tan δ (left) and storage (E') and loss (E'') modulus (right).

Through dynamic mechanical analysis (Figure 4) it is possible to observe that both pomegranate extract and peel have distinct effects on the polymeric matrix. DMA results show a well-defined peak around 57 °C, which is related to molecular relaxation of PLA chains (T_g). The presence of large particles (PLA/3PP) shifts the peak to higher temperature (60 °C), indicating that the peel particles restrict molecular mobility. On the other hand, pomegranate extract only

increases the value of tan δ, showing that PLA/3PE has higher loss module than PLA itself. These results are in agreement with tensile tests, where pomegranate extract has a positive effect on PLA ductile behavior, allowing PLA molecules to move more freely. Also, pomegranate by-products lower the storage modulus (E') over the temperature range, decreasing the stiffness of the polymer matrix.

3.3. Antioxidant capacity of the PLA films

The antioxidant capacity and the total content in phenolic compounds and flavonoids of the active PLA can be observed in Table 3. PLA/3PE significantly presented a higher inhibition percentage and a higher content in total phenolic compounds than the PLA/3PP. PLA/3PP presented a significantly higher content in flavonoids.

Table 3. Antioxidant capacity and total content in phenolic compounds and flavonoids of the active PLA films.

Samples	IP (%)	μg TE/dm²	TPC (μg GAE/dm²)	TFC (μg ECE/ dm²)
PLA/3PE	13.34 ± 0.36[a]	24.06 ± 0.67[a]	14.64 ± 0.27[a]	13.88 ± 1.04[a]
PLA/3PP	7.19 ± 0.46[b]	12.64 ± 0.85[b]	11.59 ± 0.37[b]	28.77 ± 0.77[b]

Legend: PLA/3PE – PLA incorporated with 3 % of pomegranate extract; PLA/3PP – PLA incorporated with 3 % of pomegranate peel; IP – inhibition percentage; TE – Trolox equivalents; AAC – Antioxidant Activity Coefficient; TPC – Total content in Phenolic compounds; GAE – Gallic acid Equivalents; TFC – Total content in Flavonoids; ECE – Epicatechin Equivalents.

Bodbodak et al. [47] developed active nanofiber based in PLA with a pomegranate peel extract by electrospinning that showed a DPPH radical inhibition percentage between 50 and 60 %, in the nanofibers with 5 and 10 % of extract. The inhibition percentages reported by these authors were higher than the reported in the present study, but the applied methodology for the migration assay did not follow the same procedure. Miletić et al. [48] also reported higher antioxidant activities of PLA-based nanofibers with 5 and 10 % of pomegranate seed oil (43.53 and 40.94 %, respectively). Dai et al. [49] also reported high antioxidant activity in PLA based films with pomegranate peel extract. These authors concluded a concentration dependent effect, i.e., the higher the extract percentage in the PLA films, the higher the inhibition percentage.

3.4. Identification and Quantification of punicalagin (A+B) and ellagic acid by UHPLC-DAD

The chromatographic parameters of the method to identify and quantify punicalagin (A+B) and ellagic acid can be observed in Table 4. Also, Table 5 presents the content in punicalagin (A+B) and ellagic acid of the PE, PP, PP-FD and active films (PLA/3PE and PLA/3PP).

Punicalagin is one of the major phenolic compounds present in the pomegranate peel and mesocarp, with reported anti-inflammatory, antioxidant and antimicrobial activities [45,50]. Being a water-soluble compound, punicalagin can be spontaneous hydrolyzed into ellagic acid [45]. Ellagic acid is known for its antioxidant, antimutagenic and anticancer activities [51,52].

Table 4. Validation parameters of the UHPLC-DAD method for the identification and quantification of punicalagin (A+B) and ellagic acid, in the pomegranate extracts and in the active films.

Analytes	Calibration curve	R^2	Linear range (μg/mL)	LoD (μg/mL)	LoQ (μg/mL)	Recovery	Repeatability	Precision
Punicalagin (A+B)	y = 27894x - 242255	0.9999	75 - 300	3.07	9.31	97.89%	6.30%	7.27%
Ellagic acid	y = 120247x - 261405	0.9990	8 - 20	0.58	1.77	118.48%	3.89%	5.70%

Legend: LoD – Limit of Detection; LoQ – Limit of Quantification

PE presented the highest content in punicalagin and ellagic acid, followed by the freeze-dried PP. El-Hadary and Taha [43] found lower content of ellagic acid (125.61 mg/kg) in pomegranate peel extract obtained with methanol 80 %. The authors did not quantify the punicalagin (A+B).

Table 5. Quantification of punicalagin (A+B) and ellagic acid, in the pomegranate extract and by-products and in the active films.

Samples	Punicalagin (A+B)	Ellagic Acid
PE	85.84 ± 0.15 mg/g	6.67 ± 0.01 mg/g
PP	12.15 ± 0.55 mg/g	0.77 ± 0.03 mg/g
PP-FD	44.22 ± 0.46 mg/g	1.94 ± 0.03 mg/g
PLA/3PE 40 °C, ethanol 95 %, 10 days	< LoD	0.30 ± 0.01 mg/dm^2
PLA/3PP 40 °C, ethanol 95 %, 10 days	< LoD	0.31 ± 0.01 mg/dm^2
PLA/3PE 40 °C, methanol, 24 h	< LoD	0.40 ± 0.03 mg/dm^2
PLA/3PP 40 °C, methanol, 24 h	< LoD	0.43 ± 0.01 mg/dm^2
PLA/3PE 25 °C, methanol, 24 h	< LoD	0.39 ± 0.00 mg/dm^2
PLA/3PP 25 °C, methanol, 24 h	< LoD	0.27 ± 0.01 mg/dm^2

Legend: LoD – Limit of Detection; PE – Pomegranate extract; PP – Pomegranate Peel; PP – Pomegranate Peel Freeze-Dried; PLA/3PE – PLA with 3 % of pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel

Comparing the results found in the active PLA films, both PLA/3PE and PLA/3PP presented similar content in ellagic acid. Punicalagin was not determined in the food simulant for fatty foods (ethanol 95%, v/v) nor in methanol (extraction solvent), this might be due to the high molecular weight of punicalagin (1 084.71 g/mol) [45] which prevents the migration of the molecule to the food simulant/solvent (punicalagin might be entrapped in the polymeric matrix) or due to the hydrolyzation of punicalagin into ellagic acid.

3.5. Antimicrobial analysis of the extract and the active PLA films

Regarding the antimicrobial activity of the extract, the results can be observed in Figure 3. Inhibition hallows can be observed for the *S. aureus* (20 mm radius), *E. coli* (15 mm radius) and *Enterococcus* spp. (6 mm radius). No inhibition was observed against *L. monocytogenes*.

Antimicrobial activity against *S. aureus* and *E. coli* of extracts obtained from pomegranate peels are reported in the literature [53–56]. Three of these studies also reported antimicrobial activity of pomegranate peel extracts against *L. monocytogenes*, which was not observed in this study. The main differences between the pomegranate extract from the present study and the other extracts are the extraction processes. Although the extract developed by Harini et al.[53],

using 100 % ethanol as solvent, was similar to the extraction procedure in this study, the ratio between the pomegranate peels and solvent is different as well as the extraction time (30 min for 16 h). The other pomegranate extracts were obtained with different solvents and different temperatures. These differences can explain the lack of antimicrobial activity against *L. monocytogenes*.

The antimicrobial results of the active PLA can be observed in Table 6. The PLA/3PE and PLA/3PP presented antimicrobial activity against *S. aureus*. Also, a decrease in the count of bacteria in the *L. monocytogenes* with PLA/3PE seem to indicate a potential antimicrobial activity against this microorganism. However, neither the PLA/3PE and the PLA/3PP presented antimicrobial activity against the other two strains.

Bodbodak et al. [47] and Dai et al. [49] reported antimicrobial activity of nanofibers and active films with pomegranate peel extracts against *S. aureus* and *E. coli.* Mushtaq et al. also reported antimicrobial activity of an aqueous pomegranate peel extract against, not only *E. coli* and *S. aureus*, but also against *Proteus vulgaris*, *Pseudomonas perfringens*, *Micrococcus luteus*, *E. faecalis* and *Salmonella typhii*.

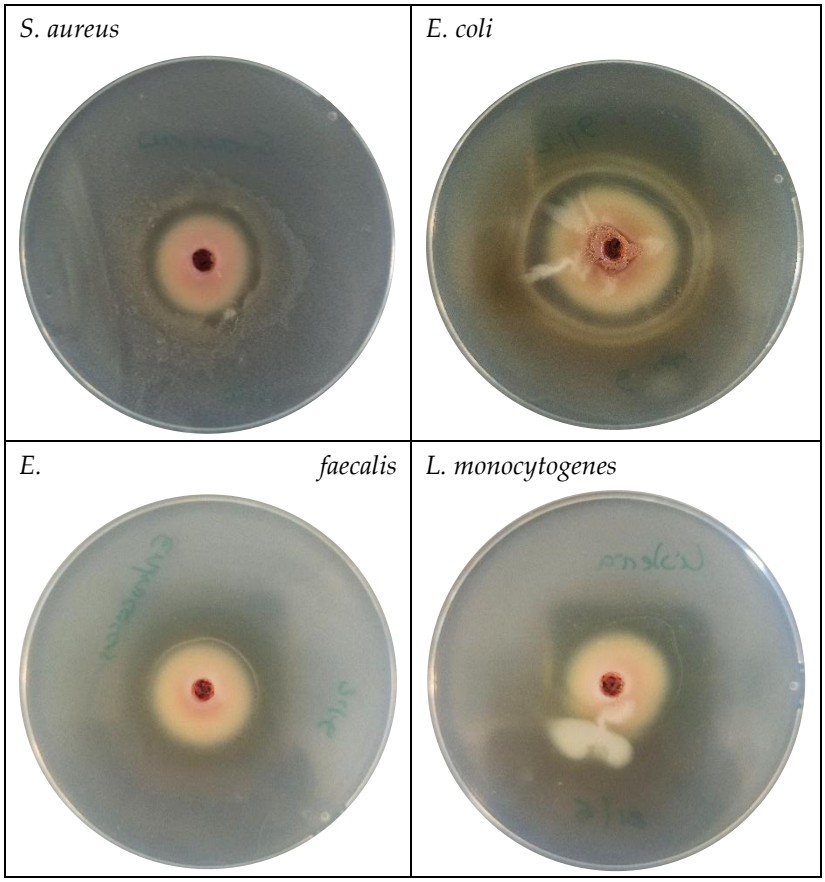

Figure 3. Antimicrobial activity of the pomegranate extract.

Table 6. Antimicrobial activity of the control (PLA) and active (PLA/3PE and PLA/3PP) films.

Samples	Dilution	*S. aureus*		*L. monocytogenes*		*E. coli*		*E. faecalis*	
		UFC	Log/cm²	UFC	Log/cm²	UFC	Log/cm²	UFC	Log/cm²
PLA	1	>300	-	>300	-	>300	-	>300	-
	10^{-1}	>300		>300		>300		>300	
	10^{-2}	>300		>300		>300		73	
PLA/3PE	1	15	0.97	>300	-	>300	-	>300	-
	10^{-1}	1.5		>300		>300		>300	
	10^{-2}	0		144		>300		>300	
PLA/3PP	1	4	0.49	>300	-	>300	-	>300	-
	10^{-1}	0.5		>300		>300		>300	
	10^{-2}	0.5		>300		>300		>300	

Legend: PLA/3PE – PLA with 3 % of pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel.

3.6. Lipid oxidation of the almond packaged with the active films

The MDA eq. values (Tables 7 and 8) of the almond packaged with the PLA significantly increased over time. A significant rise of the MDA eq. value occurs until the 4th day of accelerated storage, in the almonds' packaged with the PLA, PLA/3PE and PLA/3PP. Then, in the 4th day of accelerated storage, the almond packaged with the PLA/3PP presented a significantly MDA eq. value than the almond packaged with the PLA. In the 21st day of storage, the PLA/3PE presented a significantly lower MDA eq. value than the PLA and PLA/3PP. At the 30th day of accelerated storage, only the PLA/3PP showed significantly higher MDA eq. values than the PLA and PLA/3PE. Looking at the TBARS results from the almond stored at room temperature, no significant differences can be observed between the three types of packaging. However, the MDA eq. value significantly increased in the almond packaged with the PLA/3PP.

Table 7. Results of the TBARS assay, determination of the peroxide value and the *p*-anisidine value of the almond packaged with control and active PLA, stored at 40 °C (accelerated assay).

Storage days	PLA			PLA/3PE			PLA/3PP		
	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value
0	3.53 ± 0.16^{Aa}	0.037 ± 0.01^{Aa}	35.37 ± 0.21^{Ac}	3.53 ± 0.16^{Aa}	0.04 ± 0.01^{Aab}	35.37 ± 0.21^{Ab}	3.53 ± 0.16^{Aa}	0.04 ± 0.01^{Aab}	35.37 ± 0.21^{Acd}
2	6.89 ± 0.35^{Ab}	0.041 ± 0.018^{Aa}	33.91 ± 0.37^{Ac}	6.76 ± 0.82^{Ab}	0.035 ± 0.00^{Aa}	29.02 ± 0.55^{Ba}	6.54 ± 0.79^{Ab}	0.033 ± 0.00^{Aab}	30.7 ± 0.54^{Cbc}
4	11.65 ± 0.65^{Af}	0.035 ± 0.003^{Aa}	36.57 ± 0.36^{Ac}	12.01 ± 0.39^{ABc}	0.033 ± 0.003^{Aa}	39.95 ± 3.19^{Ab}	12.92 ± 0.85^{Be}	0.03 ± 0.002^{Aa}	39.11 ± 2.54^{Ade}
7	8.89 ± 1.18^{Acd}	0.086 ± 0.001^{Ab}	34.35 ± 0.08^{Ac}	9.99 ± 1.22^{Ac}	0.062 ± 0.004^{Bc}	48.88 ± 0.2^{Bc}	8.27 ± 1.28^{Ac}	0.077 ± 0.001^{Cc}	40 ± 0.2^{Cde}
14	9.63 ± 0.31^{Ade}	0.034 ± 0.001^{Aa}	34.96 ± 2.39^{Ac}	10.78 ± 1.46^{Ac}	0.051 ± 0.004^{Bbc}	34.75 ± 4.18^{Ab}	10.96 ± 0.4^{Ad}	0.047 ± 0.002^{Bb}	44.17 ± 6.53^{Ae}
21	10.63 ± 0.62^{Aef}	0.17 ± 0.001^{Ac}	27.91 ± 0.32^{Ab}	6.21 ± 0.12^{Bb}	0.199 ± 0.006^{Bd}	28.99 ± 0.24^{Ba}	8.02 ± 0.24^{Cc}	0.188 ± 0.013^{ABd}	15.93 ± 0.25^{Ca}
30	7.76 ± 0.69^{Ac}	0.037 ± 0.003^{Aa}	21.39 ± 1.1^{Aa}	10.86 ± 2.81^{Ac}	0.037 ± 0.002^{Aab}	27.22 ± 0.94^{Ba}	11.07 ± 1.76^{Bd}	0.046 ± 0.003^{Bab}	25.59 ± 0.19^{Bb}

Legend: PLA – Polylactic acid; PLA/3PE – PLA with 3 % of Pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel

In the peroxide assay results (Tables 7 and 8), there are no significant differences in the almond packaged with the PLA over time, either in the accelerated assay and in the assay at room temperature. However, in the accelerated assay, the active films presented significantly lower peroxide values than the control, at the 7th storage day. In the almond stored at room temperature, there are no significant differences between the control PLA and the PLA/3PE and PLA/3PP.

Table 8. Results of the TBARS assay, determination of the peroxide value and the p-anisidine value of the almond packaged with control and active PLA, stored at room temperature (23 °C).

Storage days	PLA			PLA/3PE			PLA/3PP		
	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value	mg MDA eq/kg	meq O_2/kg	*p*-anisidine value
0	3.53 ± 0.16^{Aa}	0.037 ± 0.009^{Aa}	35.37 ± 0.21^{Ad}	3.53 ± 0.16^{Aa}	0.037 ± 0.009^{Aa}	35.37 ± 0.21^{Ab}	3.53 ± 0.16^{Aa}	0.037 ± 0.009^{Aa}	35.37 ± 0.21^{Ab}
7	6.61 ± 0.98^{Ab}	0.053 ± 0.005^{Ab}	31.28 ± 0.67^{Ac}	5.96 ± 0.45^{Ab}	0.06 ± 0.009^{Ab}	40.5 ± 0.41^{Bb}	5.73 ± 0.53^{Ab}	0.065 ± 0.007^{Ab}	29.86 ± 0.43^{Ca}
14	6.44 ± 0.04^{Ab}	0.041 ± 0.001^{Aab}	27.58 ± 1.81^{Ab}	6.73 ± 0.24^{Ab}	0.042 ± 0.002^{Aab}	37.21 ± 4.73^{Bb}	7.09 ± 0.56^{Ac}	0.039 ± 0.001^{Aa}	31.12 ± 2.27^{ABa}
21	6.29 ± 0.27^{Ab}	0.138 ± 0.004^{Ac}	24.48 ± 0.48^{Aa}	6.24 ± 0.51^{Ab}	0.144 ± 0.006^{Bc}	24.16 ± 0.57^{Aa}	6.89 ± 0.08^{Ac}	0.153 ± 0.004^{Bc}	29.53 ± 0.3^{Ba}

Legend: PLA – Polylactic acid; PLA/3PE – PLA with 3 % of Pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel

In the accelerated assay, the PLA/3PE and the PLA/3PP showed lower p-anisidine values than the PLA at the end of 2 storage days. Also, the PLA/3PP showed significant lower p-anisidine values at the end of 21 storage days. In the almond stored at room temperature, PLA/3PP showed lower p-anisidine value than the PLA at the end of the 7th day of storage.

The results seem to indicate that the PLA/3PP is more efficient in preventing the lipid oxidation that the PLA/3PE. However, in the three lipid oxidation assays, both films accelerated the lipid oxidation process instead of delaying it, with the exception of the 7th day of storage in the assay at room temperature and on the 21st day of storage. These results indicate that this type of packaged is not indicated to storage almond for long time periods, since they cannot prevent the formation of primary and secondary lipid oxidation products.

Nevertheless, the *in vivo* antioxidant potential of pomegranate extracts is reported in the scientific literature. For example, El-Hadary and Taha [43] applied pomegranate peel extract to sunflower, soy bean and corn oil and submitted the oils to 70 °C for 10 days. The pomegranate peel extract, when compared to the control, significantly decreased the peroxide value content and the *p*-anisidine value of the oils in the 10-day period.

3.7. Lipid oxidation and antimicrobial analysis of the meat packaged with active films

Regarding the lipid oxidation of the meat packaged with the PLA films (Figure 4), both active PLA films were significant effective in delaying meats' lipid oxidation, in comparison to PLA. Between the two active films, PLA/3PP was more effective than the PLA/3PE. Contrary to the results obtained in the almond assays, a clear decrease can be observed in the malonaldehyde equivalents over time.

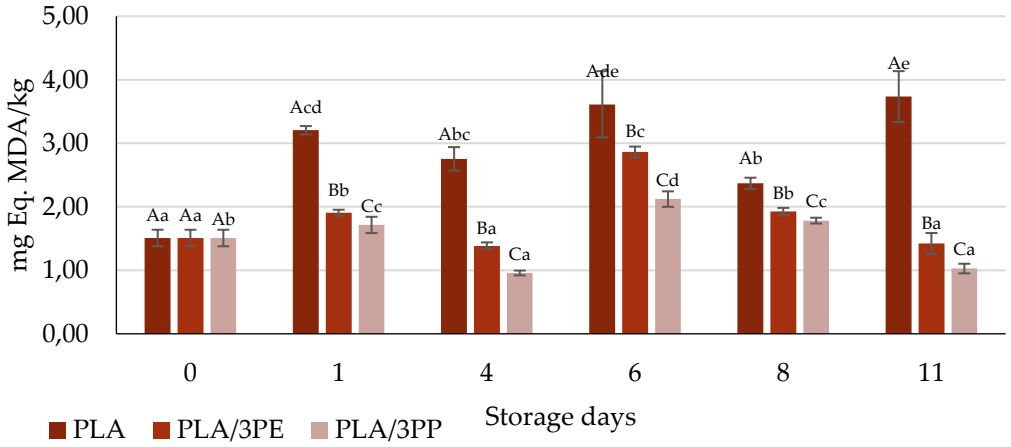

Figure 4. Results of the TBARS assay of the beef-meat packaged with the control (PLA) and the active (PLA/3PE and PLA/3PP) films.

The results from the microbiological assays of the meat can be observed in Table 8. While the meat packaged with the PLA showed significant growth of microorganisms at the 4th storage day and again at the 11th storage day, as opposed to the meat packaged with the active PLA (PLA/3PE and PLA/3PP). The microbiological growth in the meat packaged with the active PLA presented a significant growth at the 8th storage day, indicating that both the extract and the pomegranate peel incorporated in the PLA presented antimicrobial activity. The microbiological assay results support the hypothesis that both the PE and PP are viable additives to be applied to meat and meat products to delay its lipid oxidation and to assure meat and meat products safety.

The results of the present study are in agreement with the scientific literature, since the antimicrobial activity of the pomegranate by-products and its extracts is reported against both Gram positive and Gram negative bacteria [57–60].

Table 8. Results of the microbiological assays regarding the meat packaged with the control (PLA) and the active (PLA/3PE and PLA/3PP) films.

Storage days	**PLA (CFU/g)**	**PLA/3PE (CFU/g)**	**PLA/3PP (CFU/g)**
0	2.5×10^6	2.5×10^6	2.5×10^6
1	3.0×10^6	2.2×10^6	1.4×10^7
4	9.0×10^8	3.2×10^6	8.6×10^6
6	8.1×10^8	2.8×10^6	4.2×10^6
8	3.3×10^8	1.2×10^7	1.7×10^7
11	8.3×10^9	3.3×10^7	2.9×10^7

Legend: CFU - colony forming unit; PLA – Polylactic acid; PLA/3PE – PLA with 3 % of Pomegranate extract; PLA/3PP – PLA with 3 % of pomegranate peel

4. Conclusions

A pomegranate peel extract with high antioxidant capacity and a high content in total phenolic compounds and flavonoids, was successfully obtained with a simple and economic solvent-extraction method, using only ethanol (a food grade solvent) as an extraction solvent. On the other hand, the ethanolic extract obtained from wort did not present high antioxidant activity. A chromatographic method for the identification and quantification of punicalagin (A+B) and

ellagic acid was successfully validated by UHPLC-DAD system. The components were quantified in the pomegranate peel, natural and freeze-dried, and in the pomegranate extract.

Pomegranate extract (PE) and pomegranate peel (PP) were successfully incorporated into active polylactic acid-based packages (PLA/3PE and PLA/3PP). The addition of the PE and the PP seemed to have no significant interference in the morphologic characteristics of the PLA. It was only possible to quantify ellagic acid in the active films both in the food simulant (ethanol 95%, v/v) and extraction solvent (methanol). PE presented antimicrobial activity against *S. aureus, E. coli* and *E. faecalis* but the active films only presented antimicrobial activity against *S. aureus*, suggesting that the PLA traps the antimicrobial compounds.

Regarding the lipid oxidation, the active films were extremely efficient in protecting the beef-meat against lipid oxidation and microbiological growth, when comparing to the control PLA. The same cannot be concluded in the almond assays, indicating that this new active packaging is more suitable to extend the shelf life of meat and meat products.

Author Contributions: Conceptualization, MA, AVM and ASS; methodology, MA, PVR, CB, VC, CHB, AC and RF; software, MA, PVR, CB; validation, AVM, CBC, MS, FV,ASS and FR; formal analysis, MA, AVM, and ASS; investigation, MA; resources, AVM, ASS, MS, FR; data curation, MA, PVR, CB, and VC; writing—original draft preparation, MA, PVR, CB, VC; writing—review and editing, AVM, CHB, CBC, MS, FV, FR, ASS; visualization, AVM, FR, ASS; supervision, AVM, FR, ASS; project administration, ASS and FR; funding acquisition, ASS and FR. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: This paper was carried out under the MobFOOD Project (POCI-01-0247-FEDER-024524 and LISBOA-01-0247-FEDER-024524), funded by POCI (Operational Programme "Competitiveness and Internationalization") and POR Lisboa (Lisbon Regional Operational Programme), through ANI, and by the Programa de Cooperación Interreg-A España–Portugal (POCTEP) 2014–2020 (project 0377_IBERPHENOL_6_E). Cássia H. Barbosa is grateful for her Ph.D. Grant 2021.08154.BD funded by Foundation for Science and Technology (FCT), Portugal. The authors also would like to thank Talho Girassol, LDA, for kindly supplying the beef meat samples and to Dr. Sidney Tomé for his contribute for the statistical analysis.

References

1. Andrade, M.A.; Lima, V.; Sanches Silva, A.; Vilarinho, F.; Castilho, M.C.; Khwaldia, K.; Ramos, F. Pomegranate and grape by-products and their active compounds: Are they a valuable source for food applications? Trends Food Sci. Technol. 2019, 86, 68–84.
2. Martínez, R.; Torres, P.; Meneses, M.A.; Figueroa, J.G.; Pérez-Álvarez, J.A.; Viuda-Martos, M. Chemical, technological and in vitro antioxidant properties of mango, guava, pineapple and passion fruit dietary fibre concentrate. Food Chem. 2012, 135, 1520–1526.
3. Andrade, M.A.; Ribeiro-Santos, R.; Costa Bonito, M.C.; Saraiva, M.; Sanches-Silva, A. Characterization of rosemary and thyme extracts for incorporation into a whey protein based film. LWT - Food Sci. Technol. 2018, 92, 497–508.
4. Hill, L.E.; Gomes, C.; Taylor, T.M. Characterization of beta-cyclodextrin inclusion complexes containing essential oils (trans-cinnamaldehyde, eugenol, cinnamon bark, and clove bud extracts) for antimicrobial delivery applications. LWT - Food Sci. Technol. 2013, 51, 86–93.
5. Dai, J.; Mumper, R.J. Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. Molecules 2010, 15, 7313–7352.

6. Almela, L.; Sánchez-Muñoz, B.; Fernández-López, J.A.; Roca, M.J.; Rabe, V. Liquid chromatograpic–mass spectrometric analysis of phenolics and free radical scavenging activity of rosemary extract from different raw material. J. Chromatogr. A 2006, 1120, 221–229.
7. Dvaranauskaite, A.; Venskutonis, P.; Raynaud, C.; Talou, T.; Viškelis, P.; Sasnauskas, A. Variations in the essential oil composition in buds of six blackcurrant (Ribes nigrum L.) cultivars at various development phases. Food Chem. 2009, 114, 671–679.
8. Reddy, M.; Gupta, S.; Jacob, M.; Khan, S.; Ferreira, D. Antioxidant, Antimalarial and Antimicrobial Activities of Tannin-Rich Fractions, Ellagitannins and Phenolic Acids from Punica granatum L. Planta Med. 2007, 73, 461–467.
9. Özalp Özen, B.; Soyer, A. Effect of plant extracts on lipid and protein oxidation of mackerel (Scomber scombrus) mince during frozen storage. J. Food Sci. Technol. 2018, 55, 120–127.
10. Kim, N.D.; Mehta, R.; Yu, W.; Neeman, I.; Livney, T.; Amichay, A.; Poirier, D.; Nicholls, P.; Kirby, A.; Jiang, W.; et al. Chemopreventive and adjuvant therapeutic potential of pomegranate (Punica granatum) for human breast cancer. Breast Cancer Res. Treat. 2002, 71, 203–217.
11. Deng, Y.; Li, Y.; Yang, F.; Zeng, A.; Yang, S.; Luo, Y.; Zhang, Y.; Xie, Y.; Ye, T.; Xia, Y.; et al. The extract from Punica granatum (pomegranate) peel induces apoptosis and impairs metastasis in prostate cancer cells. Biomed. Pharmacother. 2017, 93, 976–984.
12. Shirode, A.B.; Kovvuru, P.; Chittur, S. V.; Henning, S.M.; Heber, D.; Reliene, R. Antiproliferative effects of pomegranate extract in MCF-7 breast cancer cells are associated with reduced DNA repair gene expression and induction of double strand breaks. Mol. Carcinog. 2014, 53, 458–470.
13. Pantuck, A.J.; Leppert, J.T.; Zomorodian, N.; Aronson, W.; Hong, J.; Barnard, R.J.; Seeram, N.; Liker, H.; Wang, H.; Elashoff, R.; et al. Phase II Study of Pomegranate Juice for Men with Rising Prostate-Specific Antigen following Surgery or Radiation for Prostate Cancer. Clin. Cancer Res. 2006, 12, 4018–4026.
14. Rettig, M.B.; Heber, D.; An, J.; Seeram, N.P.; Rao, J.Y.; Liu, H.; Klatte, T.; Belldegrun, A.; Moro, A.; Henning, S.M.; et al. Pomegranate extract inhibits androgen-independent prostate cancer growth through a nuclear factor- B-dependent mechanism. Mol. Cancer Ther. 2008, 7, 2662–2671.
15. Seeram, N.P.; Aronson, W.J.; Zhang, Y.; Henning, S.M.; Moro, A.; Lee, R.; Sartippour, M.; Harris, D.M.; Rettig, M.; Suchard, M.A.; et al. Pomegranate Ellagitannin-Derived Metabolites Inhibit Prostate Cancer Growth and Localize to the Mouse Prostate Gland. J. Agric. Food Chem. 2007, 55, 7732–7737.
16. Kasimsetty, S.G.; Bialonska, D.; Reddy, M.K.; Ma, G.; Khan, S.I.; Ferreira, D.; Blalonska, D.; Reddy, M.K.; Ma, G.; Khan, S.I.; et al. Colon cancer chemopreventive activities of pomegranate ellagitannins and Urolithins. J. Agric. Food Chem. 2010, 58, 2180–2187.
17. Nuñez-Sánchez, M.A.; Dávalos, A.; González-Sarrías, A.; Casas-Agustench, P.; Visioli, F.; Monedero-Saiz, T.; García-Talavera, N. V.; Gómez-Sánchez, M.B.; Sánchez-Álvarez, C.; García-Albert, A.M.; et al. MicroRNAs expression in normal and malignant colon tissues as biomarkers of colorectal cancer and in response to pomegranate extracts consumption: Critical issues to discern between modulatory effects and potential artefacts. Mol. Nutr. Food Res. 2015, 59, 1973–1986.
18. Food and Agriculture Organization of the United Nations (FAO) FAOSTAT Available online: https://www.fao.org/faostat/en/#data/QCL (accessed on Apr 1, 2022).
19. de Campos, L.M.A.S.; Leimann, F. V.; Pedrosa, R.C.; Ferreira, S.R.S. Free radical scavenging of grape pomace extracts from Cabernet sauvingnon (Vitis vinifera). Bioresour. Technol. 2008, 99, 8413–8420.
20. Maier, T.; Schieber, A.; Kammerer, D.R.; Carle, R. Residues of grape (Vitis vinifera L.) seed oil production as a valuable source of phenolic antioxidants. Food Chem. 2009, 112, 551–559.

21. Ribeiro-Santos, R.; Carvalho-Costa, D.; Cavaleiro, C.; Costa, H.S.; Albuquerque, T.G.; Castilho, M.C.; Ramos, F.; Melo, N.R.; Sanches-Silva, A. A novel insight on an ancient aromatic plant: The rosemary (Rosmarinus officinalis L.). Trends Food Sci. Technol. 2015, 45, 355–368.
22. Gutiérrez, J.B. Ciencia bromatológica: principios generales de los alimentos; Ediciones Díaz de Santos: Madrid, 2000;
23. Márquez-Ruiz, G.; García-Martínez, M.C.; Holgado, F. Changes and Effects of Dietary Oxidized Lipids in the Gastrointestinal Tract. Lipid Insights 2008, 2, 11–19.
24. Mariutti, L.R.B.; Bragagnolo, N. Influence of salt on lipid oxidation in meat and seafood products: A review. Food Res. Int. 2017, 94, 90–100.
25. Andrade, M.A.; Barbosa, C.H.; Souza, V.G.L.; Coelhoso, I.M.; Reboleira, J.; Bernardino, S.; Ganhão, R.; Mendes, S.; Fernando, A.L.; Vilarinho, F.; et al. Novel Active Food Packaging Films Based on Whey Protein Incorporated with Seaweed Extract: Development, Characterization, and Application in Fresh Poultry Meat. Coatings 2021, 11, 229.
26. Andrade, M.A.; Ribeiro-Santos, R.; Guerra, M.; Sanches-Silva, A. Evaluation of the Oxidative Status of Salami Packaged with an Active Whey Protein Film. Foods 2019, 8, 387.
27. Castro, F.V.R.; Andrade, M.A.; Sanches Silva, A.; Vaz, M.F.; Vilarinho, F. The Contribution of a Whey Protein Film Incorporated with Green Tea Extract to Minimize the Lipid Oxidation of Salmon (Salmo salar L.). Foods 2019, 8, 327.
28. Vilarinho, F.; Andrade, M.; Buonocore, G.G.; Stanzione, M.; Vaz, M.F.; Sanches Silva, A. Monitoring lipid oxidation in a processed meat product packaged with nanocomposite poly(lactic acid) film. Eur. Polym. J. 2018, 98, 362–367.
29. Brody, A.L.; Strupinsky, E.R.; Kline, L.R. Ative Packaging for Food Applications; CRC Press, 2001;
30. Silva, A.S.; Freire, J.M.C.; Franz, R.; Losada, P.P. Mass transport studies of model migrants within dry foodstuffs. J. Cereal Sci. 2008, 48, 662–669.
31. Umaraw, P.; Munekata, P.E.S.; Verma, A.K.; Barba, F.J.; Singh, V.P.; Kumar, P.; Lorenzo, J.M. Edible films/coating with tailored properties for active packaging of meat, fish and derived products. Trends Food Sci. Technol. 2020, 98, 10–24.
32. Moure, A.; Franco, D.; Sineiro, J.; Domínguez, H.; Núñez, M.J.; Lema, J.M. Antioxidant activity of extracts from Gevuina avellana and Rosa rubiginosa defatted seeds. Food Res. Int. 2001, 34, 103–109.
33. Erkan, N.; Ayranci, G.; Ayranci, E. Antioxidant activities of rosemary (Rosmarinus Officinalis L.) extract, blackseed (Nigella sativa L.) essential oil, carnosic acid, rosmarinic acid and sesamol. Food Chem. 2008, 110, 76–82.
34. Yoo, K.M.; Lee, C.H.; Lee, H.; Moon, B.; Lee, C.Y. Relative antioxidant and cytoprotective activities of common herbs. Food Chem. 2008, 106, 929–936.
35. Martins, P.C.; Latorres, J.M.; Martins, V.G.; Machado, A.V. Effect of starch nanocrystals addition on the physicochemical, thermal, and optical properties of low-density polyethylene (<scp>LDPE</scp>) films. Polym. Eng. Sci. 2022, 62, 1786–1796.
36. López-de-Dicastillo, C.; Gómez-Estaca, J.; Catalá, R.; Gavara, R.; Hernández-Muñoz, P. Active antioxidant packaging films: Development and effect on lipid stability of brined sardines. Food Chem. 2012, 131, 1376–1384.
37. ISO Measurement of antibacterial activity on plastics and other non-porous surfaces. In Norma ISO 22196; Geneva, Switzerland, 2011 ISBN 9782832214961.
38. Rosmini, M.R.; Perlo, F.; Pérez-Alvarez, J.A.; Pagán-Moreno, M.J.; Gago-Gago, A.; López-Santoveña, F.; Aranda-Catalá, V. TBA test by an extractive method applied to 'paté.' Meat Sci. 1996, 42, 103–110.
39. Shantha, N.C.; Decker, E.A. Rapid, Sensitive, Iron-Based Spectrophotometric Methods for Determination of Peroxide Values of Food Lipids. J. AOAC Int. 1994, 77, 421–424.
40. British Standard Method BS 684-2.24-1998: Methods of analysis of fats and fatty oils. Other methods. Determination of anisidine value. 1998.

41. Rashid, R.; Wani, S.M.; Manzoor, S.; Masoodi, F.A.; Altaf, A. Nanoencapsulation of pomegranate peel extract using maltodextrin and whey protein isolate. Characterisation, release behaviour and antioxidant potential during simulated invitro digestion. Food Biosci. 2022, 50, 102135.
42. Selahvarzi, A.; Ramezan, Y.; Sanjabi, M.R.; Namdar, B.; Akbarmivehie, M.; Mirsaeedghazi, H.; Azarikia, F. Optimization of ultrasonic-assisted extraction of phenolic compounds from pomegranate and orange peels and their antioxidant activity in a functional drink. Food Biosci. 2022, 49, 101918.
43. El-Hadary, A.E.; Taha, M. Pomegranate peel methanolic-extract improves the shelf-life of edible-oils under accelerated oxidation conditions. Food Sci. Nutr. 2020, 8, 1798–1811.
44. Zago, G.R.; Gottardo, F.M.; Bilibio, D.; Freitas, C.P.; Bertol, C.D.; Dickel, E.L.; Santos, L.R. dos Pomegranate (Punica granatum L.) peel lyophilized extract delays lipid oxidation in tuscan sausages. Ciência Rural 2020, 50.
45. Xu, J.; Cao, K.; Liu, X.; Zhao, L.; Feng, Z.; Liu, J. Punicalagin regulates signaling pathways in inflammation-associated chronic diseases. Antioxidants 2022, 11.
46. Yuniarto, K.; Purwanto, Y.A.; Purwanto, S.; Welt, B.A.; Purwadaria, H.K.; Sunarti, T.C. Infrared and Raman studies on polylactide acid and polyethylene glycol-400 blend. In Proceedings of the AIP Conference Proceedings; 2016; Vol. 1725, p. 020101.
47. Bodbodak, S.; Shahabi, N.; Mohammadi, M.; Ghorbani, M.; Pezeshki, A. Development of a Novel Antimicrobial Electrospun Nanofiber Based on Polylactic Acid/Hydroxypropyl Methylcellulose Containing Pomegranate Peel Extract for Active Food Packaging. Food Bioprocess Technol. 2021, 14, 2260–2272.
48. Miletić, A.; Pavlić, B.; Ristić, I.; Zeković, Z.; Pilić, B. Encapsulation of Fatty Oils into Electrospun Nanofibers for Cosmetic Products with Antioxidant Activity. Appl. Sci. 2019, 9, 2955.
49. Dai, L.; Li, R.; Liang, Y.; Liu, Y.; Zhang, W.; Shi, S. Development of Pomegranate Peel Extract and Nano ZnO Co-Reinforced Polylactic Acid Film for Active Food Packaging. Membranes (Basel). 2022, 12, 1108.
50. Venusova, E.; Kolesarova, A.; Horky, P.; Slama, P. Physiological and immune functions of punicalagin. Nutrients 2021, 13.
51. Shivashankara, A.R.; Venkatesh, S.; Bhat, H.P.; Palatty, P.L.; Baliga, M.S. Can Phytochemicals be Effective in Preventing Ethanol-Induced Hepatotoxicity in the Geriatric Population? An Evidence-Based Revisit. In Foods and Dietary Supplements in the Prevention and Treatment of Disease in Older Adults; Elsevier, 2015; pp. 163–170.
52. Baliga, M.S.; Shivashankara, A.R.; Venkatesh, S.; Bhat, H.P.; Palatty, P.L.; Bhandari, G.; Rao, S. Phytochemicals in the Prevention of Ethanol-Induced Hepatotoxicity. In Dietary Interventions in Liver Disease; Elsevier, 2019; pp. 79–89.
53. Harini, K.; Chandra Mohan, C.; Ramya, K.; Karthikeyan, S.; Sukumar, M. Effect of Punica granatum peel extracts on antimicrobial properties in Walnut shell cellulose reinforced Bio-thermoplastic starch films from cashew nut shells. Carbohydr. Polym. 2018, 184, 231–242.
54. Alexandre, E.M.C.; Silva, S.; Santos, S.A.O.; Silvestre, A.J.D.; Duarte, M.F.; Saraiva, J.A.; Pintado, M. Antimicrobial activity of pomegranate peel extracts performed by high pressure and enzymatic assisted extraction. Food Res. Int. 2019, 115, 167–176.
55. Panichayupakaranant, P.; Tewtrakul, S.; Yuenyongsawad, S. Antibacterial, anti-inflammatory and anti-allergic activities of standardised pomegranate rind extract. Food Chem. 2010, 123, 400–403.
56. Martínez, L.; Castillo, J.; Ros, G.; Nieto, G. Antioxidant and Antimicrobial Activity of Rosemary, Pomegranate and Olive Extracts in Fish Patties. Antioxidants 2019, 8, 86.
57. Giannelli, M.; Lacivita, V.; Posati, T.; Aluigi, A.; Conte, A.; Zamboni, R.; Del Nobile, M.A. Silk Fibroin and Pomegranate By-Products to Develop Sustainable Active Pad for Food Packaging Applications. Foods 2021, 10, 2921.
58. Chen, J.; Liao, C.; Ouyang, X.; Kahramanoğlu, I.; Gan, Y.; Li, M. Antimicrobial Activity of Pomegranate Peel and Its Applications on Food Preservation. J. Food Qual. 2020, 2020, 1–8.

59. Yuan, G.; Lv, H.; Tang, W.; Zhang, X.; Sun, H. Effect of chitosan coating combined with pomegranate peel extract on the quality of Pacific white shrimp during iced storage. Food Control 2016, 59, 818–823.
60. Licciardello, F.; Kharchoufi, S.; Muratore, G.; Restuccia, C. Effect of edible coating combined with pomegranate peel extract on the quality maintenance of white shrimps (Parapenaeus longirostris) during refrigerated storage. Food Packag. Shelf Life 2018, 17, 114–119.