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ABSTRACT

Cardoon *(Cynara cardunculus)*, an herbaceous perennial plant able to grow with high productivity in dry and hot regions, as well as in unproductive soils, was used as a biomass source for the production of both bioactive compounds derived from leaves and proteins extracted from seeds. Naviglio® technology was found as an efficient method to obtain a cardoon leaf extract (CLE) characterized by high phenol content and oxygen scavenging activity. On the other hand, cardoon proteins (CPs) were demonstrated to give rise to handleable greenish films endowed with promising mechanical and barrier properties in the presence of glycerol used as plasticizer. Hence, the CLE was used to functionalize the films that were further characterized. Film microstructure observed by SEM revealed a good compatibility among CPs and CLE, showing a uniform distribution of the leaf extract components throughout the film network that reflected, in turn, an improvement in the mechanical and barrier properties of the obtained material. In addition, the CLE containing films exhibited higher hydrophobicity, as indicated by the contact angle measurement and by the evaluation of water solubility and swelling degree experiments. Finally, CLE-containing films showed a marked antioxidant activity, highlighting the potential of *Cynara cardunculus* to be exploited as a biorefinery where different low-value renewable biomass materials are turned in several higher value bio-based products.

1. Introduction

Cynara cardunculus L., commonly named cardoon, is a perennial dicotyledonous plant widely distributed in the Mediterranean area that grows naturally in harsh habitat conditions with high temperature, elevated salinity and arid summer (Benlloch-González, Fournier, Ramos, & Benlloch, 2005). This plant is part of the Asteraceae (or Compositae) family, including the globe artichoke [var. *scolymus* (L.) Fiori], the cultivated cardoon (var. *altilis*), and the wild cardoon [var. *sylvestris* (Lamk) Fiori], considered to be their common ancestor (Pesce, Negri, Bacenetti, & Mauromicale, 2017). Although native from the Mediterranean area, cardoon has been spread to several other countries like the United States of America, Mexico, Australia, and New Zealand (https://www.cabi.org/isc/datasheet/17584). Due to its natural habitat, cardoon can grow in ash and poor conditions, with high temperatures, severe drought, and in infertile stony soils (Fernández, Curt, & Aguado, 2006), and, therefore, it is a very cheap and accessible crop. Besides, cardoon is a pollinator-supporting industrial crop, so that it is beneficial for the biodiversity. The high biomass productivity of cardoon has been exploited for multiple purposes, ranging from traditional uses to industrial applications. Mauromicale, Sortino, Pesce, Agnello, and Mauro (2014) studied the potential ability of cultivated and wild cardoons to produce energy in terms of biomass, achenes, and energy yield. The authors concluded that both cultivated and wild cardoon are potential energy crops and improved the soil fertility characteristics by increasing organic matter, total nitrogen, available phosphorus, and exchangeable potassium content. The annual average outcome of cultivated cardoon is 14.6 t/ha of dry biomass, 550 kg/ha of achenes, and 275 Gj/ha yields, while for wild cardoon, the outcome is 7.4 t/ha of dry biomass, 240 kg/ha of achenes, and 138 Gj/ha of energy yield. Cardoon has been popularly used by Greeks and Romans as food

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Abbreviations

DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
TPC	total phenolic content
GAE	gallic acid equivalent
CLE	cardoon leaf extract
FFS	film forming solution
CP	cardoon protein
COC	cardoon oilseed cake
DW	dry weight
FW	fresh weight
TE	Trolox equivalent
TS	tensile strength
YM	Young's modulus
EB	elongation at break
WV	water vapor

and medicine and is part of the Mediterranean diet for the preparation of several dishes. In fact, cardoon flowers are used in soups, stews and salads (Fratianni, Tucci, Palma, Pepe, & Nazzaro, 2007), while the leaves are known for their therapeutic potential as a diuretic, choleretic, cardiotonic, antidiabetic and anti-hemorrhoidal agent (Ramos et al., 2017; Velez et al., 2012). Beside its application as functional food, cardoon biomass has been used as lignocellulosic feedstock for biodiesel and biomethane production (Pesce et al., 2017; Toscano, Sollima, Genovese, Melilli, & Raccuia, 2016, pp. 429–442). Nevertheless, cardoon has been reported as a source of bioactive compounds such as flavonoids, chlorogenic acids and anthocyanins, which have been used for medicinal and cosmetic purposes (Ierna, Sortino, & Mauromicale, 2020; Lattanzio et al., 2009; Mandim et al., 2020; Petropoulos et al., 2019). The environmental sustainability of this plant, together with its bioactive components have made cardoon a recognised key multipurpose crop in biorefinery by processing all the non-edible parts to produce a variety of interesting compounds for potential application in green chemistry (Gominho, Dolores, Lourenço, Fernández, & Pereira, 2018; Pappalardo, Toscano, Puglia, Genovese, & Raccuia, 2020; Turco et al., 2019). Thanks to its adaptability to dry regions, cardoon can be considered a good candidate as a perennial field crop able to grow on marginal lands, thus, it does not compete with food crops (Fagnano, Impagliazzo, Mori, & Fiorentino, 2015). From cardoon seeds it is possible to extract an oil characterized by a very nutritious profile, rich in unsaturated fatty acids, adapted for the production of alternative vegetable oils and herbal formulations for human consumption (Mirpoor, Giosafatto, & Porta, 2021). The remaining by-product after oil extraction is a valuable source of fibers, proteins as well as of bioactive compounds (Genovese et al., 2016). As far as the leaves, many studies have focused on the antioxidant potential of their extracts, strictly related to the polyphenol fraction, mainly composed of hydroxycinnamic derivatives, such as mono- and dicaffeoylquinic acids, and flavonoids, such as apigenin and luteolin (Dias et al., 2018; Falleh et al., 2008; Pandino, Lombardo, Mauromicale, & Williamson, 2011; Scavo, Pandino, et al., 2019). Recent studies (Barbosa et al., 2020) have indicated that cardoon leaves are rich in several polyphenol compounds, with several health benefits. As matter of fact, cardoon leaves contain antimicrobial and antioxidant compounds that have been suggested for use as natural additives for extending the shelf life of food products. Moreover, as leaves are considered cardoon by-products, they can have economic benefits if their natural antioxidants, with benefits to human health, are extracted and applied in food packaging to increase shelf life. Nevertheless, cardoon by-products and their potential for application in food packaging is still not entirely known and it would be worthy to investigate.

Several protocols for the extraction of bioactive molecules from cardoon leaves have been previously described; dried or fresh leaves are mixed with a solvent (ethanol, methanol, acetone or alcoholic solutions) and incubated with shaking (Falleh et al., 2008; Fratianni et al., 2007; Kukić et al., 2008; Pandino et al., 2011). An alternative to these traditional extraction methods could be the use of the Naviglio® extractor, based on a solid-liquid dynamic extraction. By using a suitable solvent, the generation of a negative gradient pressure between the outlet and the inlet of a solid matrix containing some extractable material, followed by a sudden restoration of the initial equilibrium conditions, induces the forced extraction of substances not chemically bonded to the principal structure of which the solid is formed (Naviglio, 2003). This would result in a shorter extraction time, higher extraction yields and preservation of integrity of the components. Naviglio® extraction has already been reported as an innovative technology for the recovery of phenolic compounds from different types of solid matrixes, emerging as a greener alternative to the latest solid-liquid extraction techniques (Naviglio, Scarano, Ciaravolo, & Gallo, 2019; Panzella et al., 2020; Scarano et al., 2020). The Naviglio® extractor is scalable up to 400 L capacity, and is economically feasible due to the following reasons: *i)* the total consumption of this equipment, mainly related to the compressed air, is very negligible, being about 50 W/h; *ii)* it does not require any increase in temperature; *iii)* the extraction method alternates static phase, where the consumption of energy is quite zero, to dynamic one in which consumption reaches the maximum (about 100 W/h). Furthermore, many studies have been done in recent years on binding and conjugating flavonoids and polyphenols with proteins in order to improve the functionality of proteins as well as for developing active protein-based films (Mirpoor, Hosseini, & Nekoei, 2017; Mirpoor, Hosseini, & Yousefi, 2017; Quan, Benjakul, Sae-leaw, Balange, & Maqsood, 2019; Taghavi Kevij, Salami, Mohammadian, & Khodadadi, 2020). Generally, biopolymers from agricultural sources are an interesting option for biodegradable/edible plastics production since agricultural industry generates a high quantity of different by-products containing biomacromolecules, such as proteins and polysaccharides, considered good candidates for the production of hydrocolloid bio-plastics. In fact, proteins from soy and different legumes as well as several carbohydrates, such as pectins, chitosan and starch are extensively used in this sector (Giosafatto, Fusco, Al-Asmar, & Mariniello, 2020). However, despite the huge worldwide production of these agricultural biomasses, they are basically used for animal feeding and on a small amount for bio-plastic production. In this respect, seed oilcakes might be considered valuable by-products for biobased materials development as they are endowed with high amount of fiber, polysaccharides and proteins that can be further utilized. In this paper the phenols were extracted from *Cynara cardunculus* leaves, comparing Naviglio® extractor and maceration methods, using ethanol as solvent. The leaf extracts from both methods were analysed in terms of phenolic content and antioxidant activity. Further towards a development of a cardoon-based biorefinery, in the present work for the first time the seed proteins obtained following the oil removal were exploited for the production of novel bio-plastics potentially able to become candidates for replacing a portion of the petroleum-derived plastics highly pollutant for the environment. In this scenario, it is worth to say that actually Novamont company (https://agro.novamont.com/en/the-innovative-agricultural-system) is trying to exploit cardoon biomass for the development of a biorefinery for the production of low environmental impact bioproducts, even though the company is not exploiting the proteins from cardoon seeds for the manufacturing of bio-plastics. The bio-plastics obtained in this study were then functionalized with cardoon leaf-derived extracts and the obtained materials were finally characterized according to their physico-chemical characteristics, such as mechanical, barrier, morphological and hydrophobicity features, as well as antioxidant properties by evaluating film radical scavenging activity.

2. Materials and methods

2.1. Materials

Ethanol (100%) was supplied by VWR International (Fontenay-sous-Bois, France), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from AppliChem GmbH (Darmstadt, Germany). Trolox was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). *N*-Hexane (99%) and Folin-Ciocalteau reagent were supplied by Carlo Erba Reagents (Val de Reuil, France). Glycerol (GLY) (~99%) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (USA). All other chemicals and reagents used were of analytical grade.

Cardoon was recovered in April from a field experiment made in Sant'Angelo dei Lombardi (Avellino, Italy), a hilly area about 700 m above sea level, characterized by cold and rainy winters and low-fertility soil. The soil was composed by 38.5% clay, 25% silt and 36.5% sand, with a pH 8.1 and a content of 0.1% N and 1.3% organic matter (Ottaiano et al., 2017). The leaves were separated from the fresh plants and stored in vacuum sealed plastic bags at −20 °C. For extract preparation, cardoon leaves were thawed at room temperature and cut into 1 cm^2 pieces. Cardoon seeds were obtained from the same field.

2.2. Cardoon leaf extract preparation

A cardoon leaf extract (CLE) was obtained by filling a filter bag (porosity 100 μm) with 40 g of cut cardoon leaves and then, by inserting it into the Naviglio® extractor chamber (Lab. model 500 cm3 capacity). Extraction was performed using 625 mL of 100% ethanol (96% v/v) at room temperature and at pressure value of 9 bar (static phase 2 min; dynamic phase 2 min, with 12 s stop piston). Liquid samples (10 mL) were collected at 2, 4, 8 and 24 h. The CLE was kept at 4 °C until it was analysed. Moreover, further 40 g of cut cardoon leaves were placed in a bottle kept under dark conditions and subjected to maceration at room temperature with constant shaking for the same times of extraction carried out with Naviglio® extractor. The CLEs were filtered through a Whatman filter paper and supernatants were kept at 4 °C until they were analysed.

2.2.1. Naviglio® method

Filter bag (porosity of 100 μm) was filled with 40 g of cut cardoon leaves and then it was inserted into extraction chamber of Naviglio® extractor Lab. model 500 cm^3 capacity. Extractions were conducted using 625 mL of anhydrous ethanol at room temperature at pressure value of 9 bar, static phase 2 min; dynamic phase 2 min, with 12 s stop piston. Liquid samples (10 mL) were collected at 2, 4, 8 and 24 h (Naviglio, 2003). The leaf extracts (CLE) were kept at 4 °C until analysis. Ethanol was chosen as solvent for phenols extraction as described in literature (Kukić et al., 2008; Pinelli et al., 2007; Scavo, Pandino, et al., 2019).

2.2.2. Maceration method

40 g of cut cardoon leaves were used for the comparative analysis with maceration, which was placed in a bottle kept under dark conditions at room temperature with constant shaking. Samples were taken at the same times as extraction using Naviglio® extractor. The CLE were filtered through a Whatman filter paper and supernatants were kept at 4 °C until analysis.

2.3. Total phenolic content analysis

The total phenolic content (TPC) was determined using the Folin-Ciocalteau reagent as previously described (Siddiqui, Rauf, Latif, & Mahmood, 2017) with small changes. The calibration curve was plotted by mixing 100 μL of 10–250 μg/mL gallic acid solutions in ethanol with 500 μL of Folin-Ciocalteu reagent (diluted 10-fold with water) and allowed to stand at room temperature for 5 min. Then, 400 μL of 7.5% w/v Na_2CO_3 solution were added to the mixture and the absorbance was measured after 30 min at 765 nm using UV-1600PC Spectrophotometer (VWR, Leuven, Belgium). For CLEs, 100 μL of each sample were mixed with the same reagent, as performed for constructing the calibration curve and, after 3 h, the absorbance was measured to determine the total CLE phenolic contents. The results were expressed as gallic acid equivalents (GAE). All determinations were carried out in triplicate.

2.4. Antioxidant activity determination

2.4.1. ABTS radical cation decolourisation assay

ABTS activity was quantified in terms of percentage inhibition of the $ABTS^{\bullet+}$ radical cation by antioxidants in each sample. ABTS radical cation ($ABTS^{\bullet1}$) was produced by reacting 7 mM ABTS stock solution (dissolved in water) with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use (Re et al., 1999). For the study of CLE phenolic compounds, the $ABTS^{\bullet1}$ solution was diluted with water to an absorbance of 0.70 at 734 nm. After addition of 1 mL of diluted $ABTS^{\bullet1}$ solution to 10 μL of CLE, the absorbance reading was taken exactly 1 min after the initial mixing and up to 10 min using a UV-1600PC Spectrophotometer (VWR, Leuven, Belgium). CLEs were diluted two-times prior the analysis and 10 μL of ethanol in 1 mL of diluted $ABTS^{\bullet1}$ solution was used as control. Inhibition of the $ABTS^{\bullet+}$ radical cation was expressed as Scavenging effect (S) and calculated using the equation:

$$S\ (\%) = 100 \times (A_0 - A_s)/A_0 \quad (1)$$

where A_0 is the absorbance of the control (containing all reagents except the sample to be tested), and A_s is the absorbance of the tested sample. All determinations were carried out in triplicate.

2.4.2. In vitro antioxidant and free radical scavenging activity

$DPPH^{\bullet}$ radical scavenging activity was quantified in terms of percentage inhibition of a pre-formed free radical by antioxidants in each sample. 0.005% (w/v) DPPH radical was prepared in methanol as previously described (Kukić et al., 2008) with some modifications. For the study of phenolic compounds, DPPH solution was diluted with methanol to an absorbance of 0.70 at 517 nm 100 μL of CLE were mixed with 900 μL of diluted DPPH solution, shaken and left for 30 min in the dark. CLEs and film forming solutions (FFSs) were diluted two-times and 20-times, respectively, prior the analysis. Absorbance was measured at 517 nm using UV-1600PC Spectrophotometer (VWR, Leuven, Belgium); 1 mL methanol was used as blank, while 100 μL of ethanol in 900 μL of DPPH solution were used as control. Neutralisation of DPPH radical was calculated using the equation:

$$S\ (\%) = 100 \times (A_0 - A_s)/A_0 \quad (2)$$

where S is the Scavenging effect, A_0 is the absorbance of the control (containing all reagents except the sample to be tested), and A_s is the absorbance of the tested sample. Results were compared with the activity of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). All determinations were carried out in triplicate.

For the evaluation of scavenging activity of film containing CLE, 0.005% (w/v) DPPH radical was prepared in methanol. 10 mg of film were incubated in 5 mL of DPPH solution, shaken and left for 30 min in the dark. 5 mL of DPPH solution were used as control. Absorbance was measured in a 6-wells plate at 517 nm using Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

2.5. Characterization of cardoon leaf extract

The volatile triterpenes and sesquiterpenes, known for their antioxidant activity, were identified by GC-MS. The sample was prepared by dissolving 1 mg of CLE obtained by Naviglio® method in 1 mL of diethyl

ether and GC-MS analysis was carried out by a Shimazu gas chromatograph. The gas chromatograph was equipped with a 30 m × 0.25 mm fused-silica capillary column (SLB5ms) coated with 0.25 μm film of poly (5% phenyl, 95% dimethyl siloxane). The temperature was monitored from 50 °C to 280 °C. The mass spectrometer was set to scan 33–700 m/z. Samples were injected (1 μL) with a splitting ratio 1:20 and the injector temperature was set to 280 °C. The column oven was initially at 50 °C and was held for 2 min after the injection, followed by temperature ramping at 8 °C/min up to 250 °C, and 250–280 °C at 3 °C/min. The total run time was 63.33 min (Mathe, Culioli, Archier, & Vieillescazes, 2004).

The NMR analysis was performed in order to confirm the presence of the bioactive molecules observed by GC-MS analysis (see section 3.2) and for carrying out a semi-quantitative evaluation of the content of cynaropicrin respect to the other components of the CLE (see section 3.2). The NMR analysis was performed by dissolving 10 mg of CLE in 0.7 mL of deuterated chloroform. Samples were analysed on a Varian VNMRS 500 MHz NMR spectrometer at 500 MHz.

Finally, an aliquot of CLE was diluted in 70% ethanol and loaded on Vivaspin® (3 kDa and 10 kDa cut-off) ultrafiltration devices in order to determine the presence of high molecular weight molecules. The eluate fraction was recovered and dried at 60 °C overnight and the dry weight (DW) determined.

2.6. Extraction of cardoon seed proteins

Cardoon seeds were grinded at a speed of 1000 rpm for 3 min in a Knife Mill Grindomix GM 200 (Grindomix GM200, Retsch GmbH, Haan, Germany) and then defatted for 6 h by using a soxhlet apparatus (3:1, v/w hexane:grinded seeds) and, finally, the obtained cardoon oilseed cake (COC) was dried at 50 °C in an oven for 2 h. Isoelectric-precipitation technique was used for extracting cardoon proteins (CPs) from COC, according to Dapčević-Hadnađev, Hadnađev, Lazaridou, Moschakis, and Biliaderis (2018) with minor modifications. COC was suspended in water at 1:10 ratio (w/v) and 1.0 N NaOH was added under constant stirring to adjust the pH to 11. After 1 h of stirring, the suspension was centrifuged for 15 min at 5000 rpm and the pH of the collected supernatant was adjusted to 5.4 using 1.0 N HCl. Then, the precipitate was separated by another centrifugation at 5000*g* for 15 min and the obtained pellet collected and dried in an environmental chamber at 25 °C and 45% relative humidity. The protein content of the obtained CP powder was determined by the Kjeldahl's method (AACC, 2003) using a nitrogen conversion factor of 6.25.

2.7. Preparation of cardoon protein-based films

CP was dispersed in distilled water (2% w/v), the pH was adjusted to 12.0 by 1 N NaOH and the dispersion was stirred for 2 h at room temperature for complete CP dissolution. The preliminary attempts to produce CP-based films have been carried out by using 200, 300 and 400 mg of CPs added with different concentrations of GLY (10–50%, w/w protein) as plasticizer, in order to find the optimum conditions for developing handleable films.

CLE obtained as described above by Naviglio® method was dried and resuspended in ethanol up to get a final concentration of 16 mg/mL. This extract was added to the CP solution at different concentrations and the mixture was stirred for 1 h. GLY was then added to obtain a final concentration of 50% (w/w protein) and the solution was stirred for further 30 min. The prepared FFSs were cast on plastic petri dishes (8 cm diameter) and finally dried in an environmental chamber at 25 °C and 45% relative humidity (RH) for 24 h. The dried films were peeled off and conditioned at 25 °C and 50% RH, by saturated magnesium nitrate solution, for 24 h before the analyses.

2.8. Zeta potential, particle size and contact angle measurements

The effect of different concentrations of CPs, GLY and CLE on the mean hydrodynamic diameter (particle size) and the electric charge (zeta potential) values of the FFSs, as well as of CPs diluted in alkaline water (0.1 mg/mL, pH 12) by using 0.1 N NaOH, was measured by a zetasizer (Nano-ZSP, Malvern, Worcestershire, UK) at 25 °C. The effect of pH on both zeta potential and particle size of CP dissolved in water at pH 12.0 was studied by transferring 1 mL solution into the autotitrator and adjusting the pH to different values starting from pH 12.0 to pH 2.0 by adding 1.0, 0.1, and 0.01 N HCL. All measurements were performed in triplicate.

Contact angle values of the FFSs prepared in the presence or absence of CLE were determined using a homemade goniometer and parafilm (Bemis Co., Inc., Neenah, WI, USA) stripes as hydrophobic surfaces. 10 μL of each FFS were deposited on the surface of parafilm strip fixed on the horizontal stage, and the images of each FFS drop were captured using a fixed digital microscopic camera (PS Pro, China) at the moment of contact of the drop with the surface (0 time) and after 30 s. Contact angles between baseline of the FFS drops and the surfaces were then measured using Image J software. Five measurements for each FFS was reported as the average of its contact angle value.

2.9. Cardoon seed protein-based film characterization

2.9.1. Colour, opacity and density measurements

The colour parameters of CP-based films prepared in the absence or presence of different concentrations of CLEs were measured using a Mightex® HRS series compact CCD spectrometer HRS-VIS-025 (Mightex, Toronto, ON). All measurements were made at 5 random positions of each film. The colour parameters, including L as well as "a" and "b" values, indicate lightness/darkness (0–100), greenness/redness (−60 to +60) and blueness/yellowness (−60 to +60), respectively, of the materials tested. Total colour difference (ΔE) was determined by the following equation:

$$\Delta E \cdot = \cdot \sqrt{(L^{*} - \ L)^{2} + (a^{*} - \ a)^{2} + (b^{*} - \ b)^{2}} \quad (3)$$

where L* (99.94), a* (−1.07) and b* (3.74) were the colour parameter values of the standard white tile (Bai et al., 2019).

Film opacity was evaluated by a spectrophotometer according to the method described by Taghavi Kevij et al. (2020). The film specimens (4 cm × 1 cm) were attached to the cell and inserted into a spectrophotometer. The empty cell was used as a blank and the absorbance of each film specimen was read at 600 nm. The opacity of the films was calculated as a ratio between the obtained absorbance value and the film thickness (mm).

Density was determined according to the procedure described by Cruz-Diaz, Cobos, Fernández-Valle, Díaz, and Cambero (2019) with some modifications. The film samples (2 cm × 2 cm) were weighed, their thickness measured at three random places and, finally, the dry matter density was calculated as the ratio between the weight and volume of each film (thickness × film surface area). All the analyses were carried out in triplicate.

2.9.2. Fourier transform infrared (FTIR) spectroscopy

The interactions between CLE and CP in the film matrix were investigated by recording the FT-IR spectra using FTIR Nicolet 5700 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Infrared spectra analysis was performed using the Omnic software in the range of 4000–500 cm^{-1} with a spectral resolution of 2 cm^{-1}.

2.9.3. Scanning electron microscopy analyses

Film microstructure was examined by both surface and cross section analysis using a field emission scanning electron microscope (FE-SEM, FEI Nova NanoSEM450, Thermo Fisher, Scientific, MA, USA). In order to

prepare the films for cross-sectional analysis, each sample was cryo-fractured in liquid nitrogen, then coated with a thin layer of gold and platinum using a vacuum sputter coater and, finally, observed at an accelerating voltage of 5 kV.

2.9.4. Mechanical properties and thickness

Film thickness was measured from the average of the thickness determined at five different locations of each film using an electronic digital micrometer (IP65 Alpa Metrology Co., Pontoglio, Italy, sensitivity 0.001 mm). Film tensile strength (TS), Young's modulus (YM) and elongation at break (EB) were determined according to ASTM D882 - 18 (1997) method using an Instron universal testing instrument (Model 5543 A, Instron Engineering Corp., Norwood, MA, USA), with a 1000 N load cell. The film strips (8 cm × 1 cm) were fixed between two grips of the instrument with an initial grip distance and crosshead speed of 40 mm and 5 mm/min, respectively.

2.9.5. Hydrophilicity properties

Film moisture content was evaluated by the method of Roy and Rhim (2020) by weighing each film sample (2 cm × 2 cm) before and after drying in an oven at 105 °C for 24 h. Moisture content was calculated as the percentage of the film weight loss with respect to the initial weight of the film.

Water solubility of the films was measured according to the procedure of Adilah, Jamilah, Noranizan, and Hanani (2018) with some slight modifications. The initial weight (W_i) of each film sample (2 cm × 2 cm) was obtained by oven drying at 105 °C for 24 h. The dried films were then immersed in 30 mL of distilled water and stirred in a shaker incubator at 25 °C for 24 h. After that, the final weight (W_f) of the samples were obtained by separating the non-soluble parts of the films and drying in oven at 105 °C for another 24 h. Finally, water solubility was calculated using the following equation:

$$\text{Water solubility } (\%) = [(W_i - W_f)/W_i] \times 100 \quad (4)$$

Film swelling ratio was measured according to the method of Roy, Rhim, and Jaiswal (2019). The initial weight (Wi) of each film sample (2 cm × 2 cm) was measured and, then, the films were immersed in 30 mL distilled water at 25 °C for 1 h. After that, the excess water was drained with filter paper, the surface water of films was dried with an absorbent paper and, finally, the films were weighed again (W_s). Film swelling ratio was calculated using the following equation:

$$\text{Swelling ratio } (\%) = (W_s - W_i) \times 100/Wi \quad (5)$$

The surface wettability of film samples was studied using a home-made water contact analyser. Each film strip was placed on the horizontal stage and then made in contact with 10 μL of distilled water. The image of the water drop was immediately captured using a fixed digital microscopic camera (PS Pro, China). The contact angle between water drop and the film surface was measured by an Image J software and five measurements were performed to calculate the average of the contact angle value of each film sample.

2.9.6. Gas and water vapor permeability

Film permeability to WV water vapor, O_2 and CO_2 was measured in duplicate for each film, according to the modified method ASTM D3985 - 05 (2010), ASTM F 2476-05 (2005), ASTM F1249-13, 2013 using a MultiPerm instrument (ExtraSolutions s.r.l., Pisa, Italy). After conditioning the film samples for 24 h at 50% RH, they were placed into the aluminium masks and their exposed surface area was reduced to 5 cm^2.

2.9.7. Statistical analysis

The experiments were always performed in triplicate and in a completely randomized design. In order to determine the significant difference between treatments, one-way analysis of variance (ANOVA) and Duncan's multiple range tests ($p < 0.05$) were done using the Statistical Package for the Social Sciences (SPSS19, SPSS Inc., Chicago, IL, USA) software. Pearson correlation (r) to measure the strength of the linear relationship between TPC and antioxidant activity were calculated using the same software.

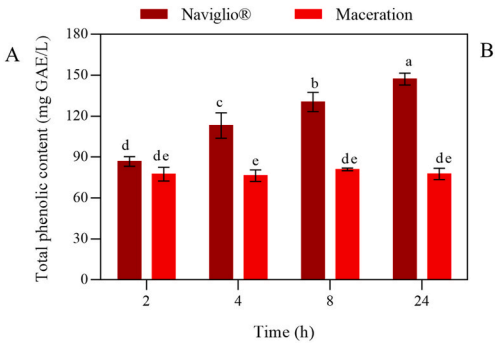
3. Results and discussion

3.1. Cardoon leaf extract preparation and evaluation of phenol content and antioxidant activity

CLE was obtained by two different methods: conventional maceration and Naviglio® extraction. Both processes were carried out using 100% ethanol as solvent, collecting samples after 2, 4, 8 and 24 h. The extracts were analysed and compared in terms of total phenol content (TPC) using Folin-Ciocalteau assay. Naviglio® extracts were characterised by a higher TPC than maceration extracts regardless the extraction time (Fig. 1, A). In particular, Naviglio® CLE showed an increase in the TPC over the time, from 86.8 ± 3.5 mg GAE/L (corresponding to 140 mg GAE/100 g (fresh weight FW) of leaves at 2 h to 147.2 ± 4.4 mg GAE/L (corresponding to 230 mg GAE/100 g (FW fresh weight) of leaves at 24 h.

These results are in agreement with TPC of leaves of several artichoke cultivars, ranging from 141.7 ± 20.5 to 264.5 ± 44.7 mg GAE/100 g (FW fresh weight) of leaves extracted using hydro methanolic solution (Rouphael et al., 2016). On the contrary, TPC did not change significantly in leaf samples obtained by the maceration method at the different extraction times. In particular, the highest value of TPC obtained by maceration after 24 h was lower than that detected after 2 h by Naviglio® extraction. Furthermore, no further increase in TPC was observed over 24 h extraction by both methods.

It is worth noting that, by using Naviglio® extractor, the values of mg GAE/L and mg/mL of extract showed a positive correlation with time increase ($r = 0.915$), since an increase in TPC content was achieved by prolonging the extraction time. In fact, the amount of GAE/g (dry weight DW) in the extracts did not significantly change with the time (Fig. 1, B). On the contrary, in the case of CLE obtained by maceration, no linear correlation was determined between mg GAE/L and mg/mL ($r = 0.067$): the highest amount of GAE/g (DW dry weight) was achieved after 2 h and significantly decreased thereafter, indicating an increase of components in the extracts other than phenols. Naviglio® extraction has been recently applied reported as a green technology for the to the recovery of phenolic compounds from different sources types of solid matrix (Naviglio et al., 2019; Panzella et al., 2020). Up to 69.9 ± 7.3 mg GAE/g of extract have been obtained from the flowering aerial parts of *Schizogyne sericea* using both water and ethanol as solvents (Caprioli et al., 2017), whereas Posadino et al. (2018) have reported the extraction of phenols from wine waste (*Cagnulari* Grape Marc) using water: ethanol (60:40 v/v) as solvent with a recovering of 4000 mg/L ± 0.05 TPC containing specific anthocyanins. Moreover, the extraction of 9210.4 ± 45.8 mg GAE/L has been reported from crushed, dried and shredded grapes using water as solvent (Gallo et al., 2019) and aqueous vine shoot extracts, resulting into high phenolic content, have been obtained by Naviglio® method (Sánchez-Gómez, Zalacain, Alonso, & Salinas, 2014). Djeridane et al. (2006) reported that TPC in plants of Asteraceae family was higher than that detected in several other plants reported in literature, probably due to the ability to grow in harsh habitat conditions. Polyphenols extraction from cardoon leaves has been carried out by using different solvents such as ethanol, methanol or hydroalcoholic mixtures (Barbosa et al., 2020). According to Ramos et al. (2014), 22.6 GAE/g (DW dry weight) of extract were obtained from *C. cardunculus* L. var. altilis using methanol/water/acetic acid (49.5:49.5:1) after removing the lipophilic fraction. Similarly, 14.79 mg GAE/g (DW dry weight) of extract were obtained by using methanol (Falleh et al., 2008), and 50 mg GAE/g (DW dry weight) of extract were obtained by subsequent extraction with ethanol (Kukić et al., 2008). These differences in TPC reported in literature may be related to



B

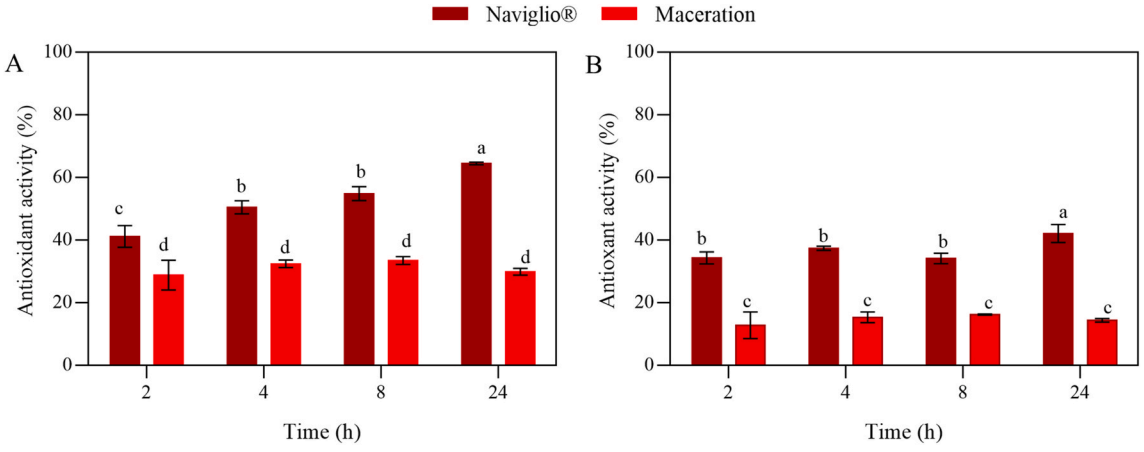
Sample	Naviglio® Extraction		Maceration Extraction	
	mg/mL extract	mg GAE/ g DW extract	mg/mL extract	mg GAE/ g DW extract
2 h	1.6	58.0	1.0	80.8
4 h	1.9	61.1	1.2	64.2
8 h	2.0	68.2	1.3	64.9
24 h	2.9	53.8	1.6	51.9

Fig. 1. A) Total phenolic content in cardoon leaves extracted by Naviglio® and maceration methods. CLE were analysed by using the Folin-Ciocalteau reagent. Values with different small letters (a–e) are significantly different (Duncan's multiple range tests, $p < 0.05$). B) Comparison of extraction efficiency of the two methods. Gallic acid equivalent (GAE), dry weight (DW). Further experimental details are given in the text.

intrinsic factors, such as genetics, as well as extrinsic ones, including geographical location, handling methodologies, storage, and extraction procedures (Fratianni et al., 2007). In addition, the distribution of secondary metabolites, such as polyphenols, may be dependent on the life cycle stage of the plant (Del Baño et al., 2003). The values of GAE/g (DW dry weight) detected in this work by using Naviglio® extractor fall within the range reported in literature, demonstrating the applicability of this method to the cardoon leaves.

Antioxidant activity of samples collected at different extraction times with both methods was evaluated using ABTS radical cation decolourisation and DPPH radical scavenging activity assays. Both methodologies are based on the reaction of the radicals ($DPPH^{\bullet}$ and $ABTS^{\bullet 1}$) with the antioxidant molecules which can be determined by spectrophotometric analysis (Dawidowicz, Wianowska, & Olszowy, 2012; Re et al., 1999). ABTS assay is commonly used for both hydrophilic and hydrophobic antioxidants, whereas DPPH is more efficient for hydrophobic systems as it is dissolved in organic solution (Bitencourt, Fávaro-Trindade, Sobral, & Carvalho, 2014; Floegel, Kim, Chung, Koo, & Chun, 2011). Regardless the assay method applied, Naviglio® extracts showed higher scavenging activity than the samples obtained by maceration (Fig. 2).

In particular, all the samples obtained by Naviglio® extractor displayed up to two and three fold the antioxidant activity with respect to the samples obtained by maceration, measured with either DPPH or ABTS assays, respectively. Furthermore, Naviglio® extract showed an increasing antioxidant activity as the extraction time increased, ranging from 41.2% ± 3.4 at 2 h of extraction to 64.5% ± 0.4 at 24 h of extraction using DPPH assay. These results reflect the same trend observed for the TPC determination, since the antioxidant activity of Naviglio® extract evaluated over the extraction time correlates with the TPC measured in the extract, showing highly significant correlation coefficients (r) (0.994 and 0.71) in both DPPH and ABTS assays. The observed correlation between TPC and antioxidant activity supports the hypothesis that this class of compounds directly contributes to the free



C

Sample	Naviglio® Extraction		Maceration Extraction	
	µg TE/g FW	µmol TE/ 100g FW	µg TE/g FW	µmol TE/ 100g FW
2 h	912.4	364.5	652.9	306.7
4 h	1107.8	442.6	728.3	333.0
8 h	1199.4	479.2	750.5	305.5
24 h	1401.9	560.1	676.0	372.3

Fig. 2. Antioxidant activity of cardoon leaf extracts obtained by Naviglio® and maceration methods detected following DPPH (A) and ABTS (B) assays. Values with different small letters (a–d) are significantly different (Duncan's multiple range tests, $p < 0.05$)). Comparison of antioxidant activity of the extracts are expressed as ratio of Trolox equivalents (TE) and fresh weight (FW) of extracts (C).

radical scavenging activity of the extracts.

It is worthy to note that the extract obtained by maceration does not follow the trend exhibited by the Naviglio® extract, showing an almost constant scavenging activity over the time (Fig. 2), in agreement with the TPC data. The antioxidant activity values measured in Naviglio CLEs are consistent with those reported in literature for the extracts of leaves of other species of *Cynara* genus. A radical scavenging activity in the range of 28.7%–94% has been reported for Green Globe and Violet artichoke (*Cynara scolymus* L.) varieties (Ben Salem et al., 2017; Biel, Witkowicz, Piątkowska, & Podsiadło, 2020). When referred to the Trolox standard, the antioxidant activities of the samples obtained with Naviglio® extractor were higher than those reported for methanolic extracts of leaves from *Cynara cardunculus* var. *ferocissima* (Madeira cardoon), expressed in the same way (176 μmol eq. Trolox/100 g) (Gouveia & Castilho, 2012).

3.2. Characterization of cardoon leaf extract

The characterization of the components of the Naviglio® extracts at 24 h was carried out by GC-MS and ^{1}H NMR spectroscopy. The panel a) of Fig. 3 shows a typical chromatogram of the extract, where the peaks were identified by comparing their mass spectra with the NIST14s database and with literature data (Mathe et al., 2004; Ramos et al., 2013). The GC-MS chromatogram provides qualitative information on the chemical composition of the CLE. The volatile pentacyclic triterpenes and sesquiterpenes, known for their antioxidant activity, are the main families of lipophilic components previously identified in the CLE (Ramos et al., 2013; Scavo, Rial, et al., 2019). In particular, cynaropicrin ($C_{19}H_{22}O_6$) and grosheimin ($C_{15}H_{18}O_4$) (RT = 35.61–37.25 min) have been identified in higher amount in the leaves than in other parts of cardoon and globe artichoke (Eljounaidi et al., 2015; Ramos et al., 2013; Rouphael et al., 2016). Grosheimin has also been previously reported in globe artichoke (Bernhard, 1982). Since the structures of the two sesquiterpene lactones are similar, it was not possible to identify their specific molecular fragmentation. Fatty acids (RT = 19.17 min), long-chain aliphatic alcohols (RT = from 19.50 to 22 min) and some aromatic compounds were also detected in traces. In particular, linoleic acid was identified, as also confirmed by ^{1}H NMR characterization (Fig. 3, panel b) (Ramos et al., 2013; Sobolev, Brosio, Gianferri, & Segre, 2005). The signal at 31.26 min is ascribable to squalene, whereas the signals corresponding to pentacyclic triterpenes fall in the range 23.45–29.73 min. Finally, the signals of hydrophobic long chain alkanes is visible at the range 40–42.5 min. Pentacyclic triterpenes have been identified as the main liphophilic constituents of *C. cardunculus* L. var. *altilis*, although less present in the leaves, where they represent only 8% of total detected compounds. On the other hand, fatty acids are reported to be mainly concentrated in the leaves, especially the saturated ones (Ramos et al., 2013). Furthermore, Rouphael et al. (2016) reported a wide range of phenolic compounds, including flavonoids, hydroxycinnamic acids, tyrosols, and lignans, in the extracts of leaves of different cultivars of artichoke. The characterization by ^{1}H NMR spectroscopy confirmed the presence of lupeol (Reynolds et al., 1986), and pheophytins (Sobolev et al., 2005) (Fig. 3, panel b). The cynaropicrin, a well known bioactive molecule endowed with antioxidant activity, was clearly identified by means of ^{1}H NMR in the extract, because of its typical signals. A semi-quantitative evaluation of its content respect to all other components was carried out because it was possible to integrate the signals of cynaropicrin by taking as a reference the signal of -CH2 protons at 4′ position, having a theoretical integral value of 2 and an observed experimental integral value of 0.85. When considering that the integral value of all protons present in the spectrum (range 0.5–6.5 ppm) is 84.14, it results that the proton of cynaropicrin correspond to the 11% of the protons present in the mixture (Fig. 3, panel c).

Finally, CLE was further analysed by ultrafiltration to determine the average molecular weight of its components. No retention of high molecular weight molecules was determined after ultrafiltration with neither 10 kDa nor 3 kDa cut-off membranes, indicating that CLE contained molecules smaller than 3 kDa.

3.3. Cardoon protein-based films

3.3.1. Zeta potential and particle size of film forming solutions

The data reported in Fig. 4 show that CPs were positively charged in the acidic pH range, since the detected zeta potential was found to increase from a value of about −40.00 mV at pH 12 to that of about −30 mV at pH 7.0 and 0 mV just under pH 4.0, indicating that the electrostatic repulsion pattern was gradually modified as a result of the gradual deprotonation of carboxyl groups and protonation of the amino groups of each CPs present in the sample. As far as the CP particle size, it also varied, like zeta potential, as a function of pH. In fact, at pH $\leq$ 5 high molecular mass protein species were present in the solution, likely because the isoelectric point of CPs was between pH 4 and 5 where the zeta potential value was around 0 mV. CP zeta potential and particle size were measured also in FFSs prepared at pH 12 by varying the concentrations of both proteins and GLY, used as plasticizer. All the FFSs tested were quite stable, regardless of protein and GLY concentration, zeta potential value fluctuating between −28 mV and −33 mV (Table 1).

As far as the particle size, it did not change by varying the GLY content, but it significantly increased by enhancing CP concentration, likely as a consequence of protein aggregation. However, the observed size distribution in the range of 300–400 nm is in agreement with previous results obtained with protein-based FFSs prepared by using other oilcakes (Mirpoor et al., 2020, 2021).

3.3.2. Thickness and mechanical properties of cardoon protein-based films

FFSs previously analysed for the particle size and zeta potential have been used for the preparation of bio-plastics by the casting method. It is worthy to note that the minimum GLY concentration to obtain handleable films under the described experimental conditions was 30% (w/w of CP), since the films cast in the absence or lower amount (10 and 20%) of plasticizer were brittle and not able to be peeled off from the plates. The handleable films were thus characterized for their thickness and mechanical properties (e.g. tensile strength, elongation at break and YMYoung's modulus) (Fig. 5).

As expected, the film thickness was found to increase by enhancing CP concentration independently on GLY amount present in the FFSs. Conversely, all the parameters characterising the mechanical properties of the films changed by varying both CP and plasticizer concentrations. In fact, TS tensile strength and YM Young's modulus decreased whereas EB elongation at break increased by enhancing GLY amounts at all the CP concentrations used. On the other hand, the investigated mechanical properties increased as function of CP amount. This effect was the result of the well known capacity of the plasticizer to increase the free volume and the polymer mobility by decreasing the attractive intermolecular forces into the protein matrix. Porta, Di Pierro, Roviello, and Sabbah (2017) also observed a similar effect for bitter vetch protein-based films prepared with two different plasticizers, such as GLY and spermidine. Sun et al. (2019) demonstrated that such phenomenon occurs also with carbohydrate-based materials, since they reported that chitosan/starch blended films had a greater extensibility when plasticizing components were added. In conclusion, among the different films produced, the one obtained from 400 mg of CP and 50% GLY was selected for the further investigations, possessing the highest EB elongation at break (154.19 ± 8.25) and acceptable TS tensile strength (1.69 ± 0.33) and YM Young's modulus (53.59 ± 6.89) (Fig. 5).

3.3.3. Zeta potential, particle size, contact angle and antioxidant activity of cardoon protein-based film forming solutions containing cardoon leaf extract

FFSs, prepared with 400 mg CPs and 50% GLY, were added with different amounts of CLE in order to test their zeta potential, particle size, contact angle and antioxidant activity as well as the properties of the derived films. Regarding the particles, it should be mentioned that

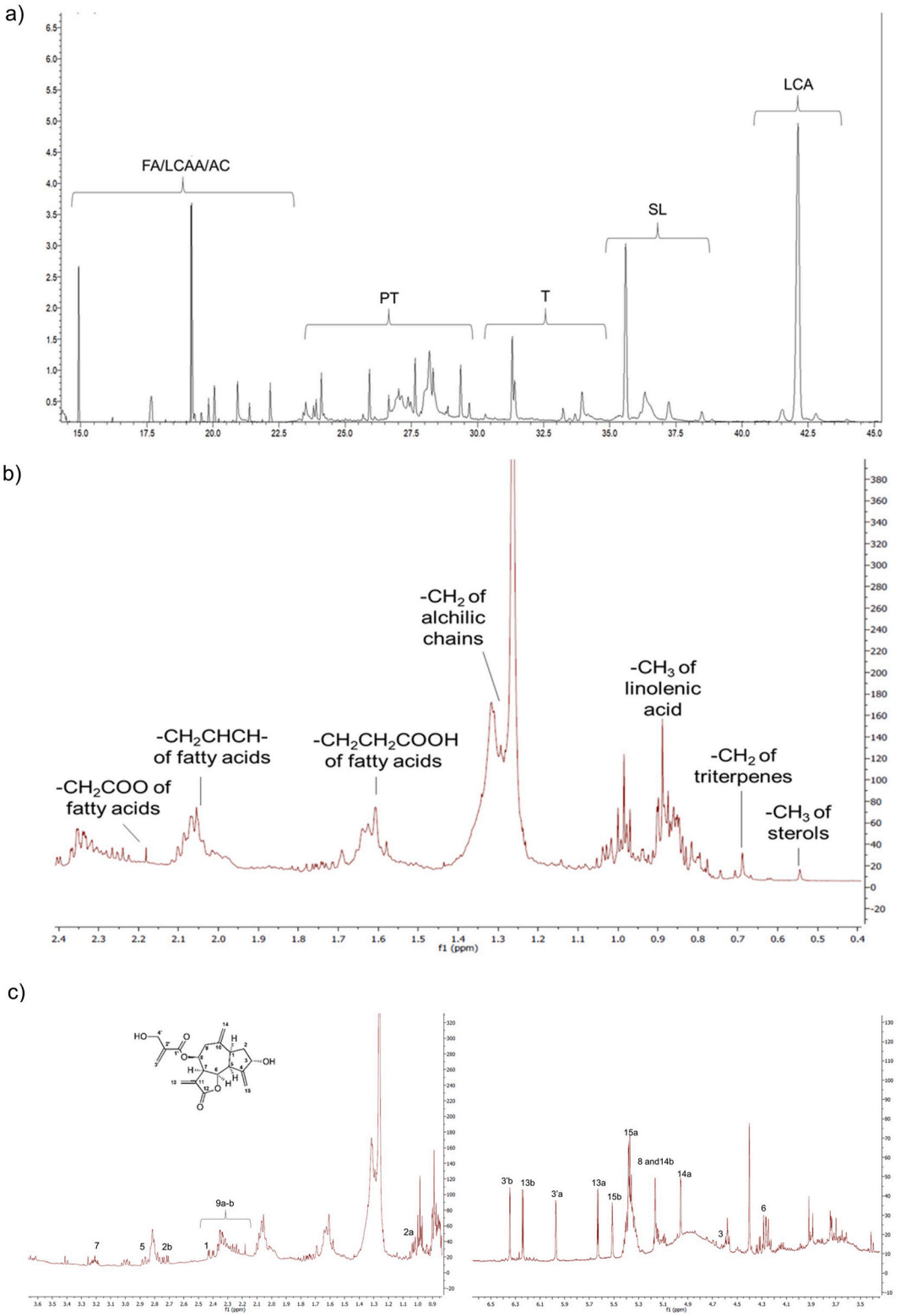


Fig. 3. a) GC-MS chromatogram; b) ^{1}H NMR spectrum of a cardoon leaf extract obtained by Naviglio® method. The GC-MS peaks represent the total ion current (TIC) of the compounds: FA = fatty acids; LCAA = long chains aliphatic alcohols, AC = aromatic compounds; PT = pentacyclic triterpenes; T = triterpene; SL = sesquiterpene lactones; LCA = long chain alkanes. ^{1}H NMR spectrum was detected in the range of 0.4–2.4 ppm. Further experimental details are given in the text. c) Signals of cynaropicrin in the range of 0.9–3.6 ppm (on the left) and signals of cynaropicrin in the range of 3.5–6.5 ppm (on the right). ^{1}H NMR (500 MHz, CD3OD): 1 δ: 2.43, dt; 2a δ: 1.09, ddd; 2b δ:2.07, dt; 3 δ:4.62, tt; 5 δ:2.84,dd; 6, δ:4.27,dd; 7 3.27, tt; 8 and 14b δ:5.17,ttt; 9a-b δ:2.25–2.45, dd; 13a δ:5.62,d; 13b, δ:6.25,d; 14a δ:4.96,d; 15a δ: 5.43,t; 15b δ:5.52,t; 3′a δ:5.9,m; 3′b δ:6.35, m.

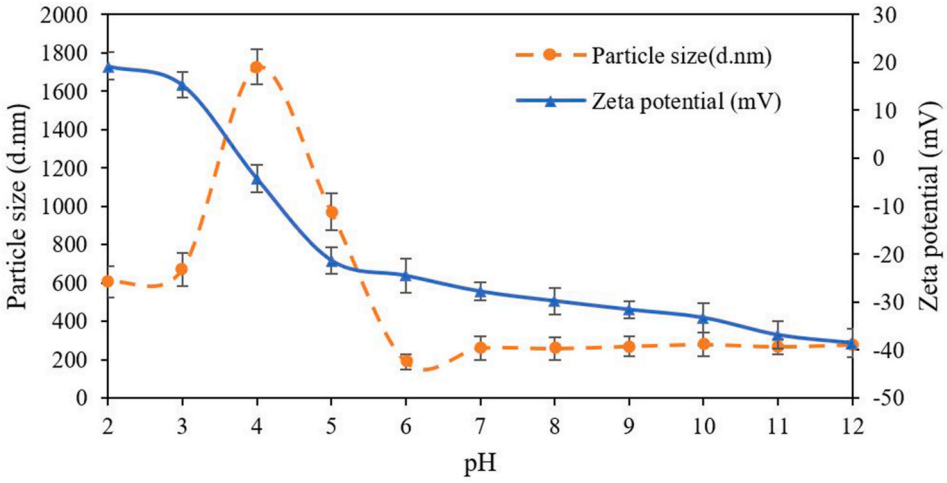


Fig. 4. Cardoon protein zeta potential and particle size measurements vs different pH values.

Table 1
Effect of different concentrations of cardoon proteins (CPs) and glycerol (GLY) on particle size and zeta potential on CP film forming solutions prepared at pH 12[a].

CPs *(mg)*	GLY *(% w/w)*	Particle size *(d. nm)*	Zeta Potential *(mV)*	PDI
200	30	312.7 ± 3.7^{c}	−31.50 ± 1.49 ^{cd}	0.44 ± 0.03^{c}
	40	309.2 ± 3.5^{c}	−31.63 ± 0.58 ^{cd}	0.43 ± 0.02^{c}
	50	310.6 ± 2.7^{c}	−32.56 ± 1.42^{d}	0.42 ± 0.02^{c}
300	30	379.3 ± 7.1^{b}	−29.63 ± 1.61 ^{bc}	0.52 ± 0.04^{b}
	40	379.0 ± 10.2^{b}	−29.70 ± 0.85 ^{bc}	0.51 ± 0.02^{b}
	50	381.8 ± 16.5^{b}	−29.45 ± 1.52 ^{ab}	0.53 ± 0.03^{b}
400	30	436.2 ± 4.8^{a}	−28.51 ± 0.40^{a}	0.60 ± 0.02^{a}
	40	435.0 ± 6.3^{a}	−28.78 ± 0.86^{a}	0.61 ± 0.04^{a}
	50	433.3 ± 5.9^{a}	−29.17 ± 0.46^{a}	0.60 ± 0.02^{a}

[a] Different small letters (a–d) indicate significant differences among the values reported in each column (Duncan's multiple range tests, $p < 0.05$). Polydispersion index (PDI). Further experimental details are given in text.

they consist of different kinds of proteins, some fibres and phenols (coming from the seed oilcakes), glycerol used as plasticizer and finally, as far as the activated FFSs, the CLE. From the performed experiments, it is possible to note that particle size of CPs FFS increased by incorporation of higher concentration of antioxidant in the FFS compared to the control sample, as reported in Table 2. These results could be attributed to the interactions between the CP functional groups and the phenolic hydroxyl groups of antioxidants that formed larger CPs polymers that increase the polydispersity index (PDI), that is an indicator of relative variance in the particle size distribution. These results are in agreement with a linear increase in polydispersity index. The protein surface charges were also affected by the addition of antioxidants and all the FFSs were stable with negative zeta potential higher than −28 mV shows the high stability of solution (Bhattacharjee, 2016). As shown in Table 2, zeta potential of CPs FFS decreased as a function of increasing the concentration of antioxidants due to the participation of negative functional groups of CPs in protein-polyphenols interaction and/or interactions between the CPs surface and CLE that can modify the surface charge of the proteins. The same results were reported for changing the surface charge of soy protein isolate and whey protein isolate based films loaded by curcumin by Chen, Li, and Tang (2015) and Taghavi Kevij et al. (2020), respectively.

The contact angle of the different FFSs was measured on parafilm, the semi-transparent, flexible film composed of a proprietary blend of waxes and polyolefins currently used in research laboratories. The results reported in Fig. 6 clearly show that the addition of increasing amounts of CLE significantly decreased the contact angle value of the CP FFS in comparison to the control sample, thus indicating that the hydrophobicity of CP FFS significantly increased in the presence of increasing CLE concentrations (Fig. 6).

The higher hydrophobicity of the FFS prepared in the presence of CLE might be dependent on the decrease in the number of free hydrophilic groups of CPs involved in the interactions between protein and CLE components (Fathi, Almasi, & Pirouzifard, 2019).

Furthermore, FFS antioxidant activity was preliminarily evaluated using the DPPH radical scavenging activity assay with the aim of choosing the right amount of CLE to graft in the CP-based films. CP containing FFS had a marked antioxidant activity also in the absence of CLE and, whereas the antioxidant activity of the samples added with an amount of CLE up to 3% were at the same level of the control, it progressively increased in the presence of 5, 10, 15 and 30% (w/w) CLE (Fig. 7). For this reason, CP-based films were manufactured by preparing FFSs containing 15% and 30% CLE.

3.3.4. FT-IR spectra of CP-based films incorporated wit CLE

The FT-IR spectra of CP-based films incorporated or not with CLE are shown in Fig. 8. The sharp peaks in the spectrum of neat CP-based film in the range of 1649 cm^{-1} are attributed to the amide I (C=O stretching vibrations) and in the range of 1544 cm^{-1} to the amide II (N–H bending with C–N groups stretching vibrations) are the regions that employed to study the secondary structure properties of proteins (Mohammadian et al., 2019). The FT-IR results of the films incorporated with different amounts of CLE showed a significant shift in the position of these peaks to the lower wavenumbers. These alterations in the peak positions could be attributed to new molecular arrangement in the film matrix due to the interactions between the polypeptide chain of the protein and CLE molecules (Moghadam, Salami, Mohammadian, Khodadadi, & Emam-Djomeh, 2020). Moreover, there are the characteristic peaks around 2878 cm^{-1} and 3270 cm^{-1} in the CP-based films, that correspond to the aliphatic C–H stretching vibrations of CH_2 functional groups and O–H stretching overlapping N–H stretching vibrations, respectively. The incorporation of CLE in the CP-based films matrix caused a considerable shift in the position of these peaks (Chentir et al., 2019; Taghavi Kevij et al., 2020). A common peak observed in the spectra of all the

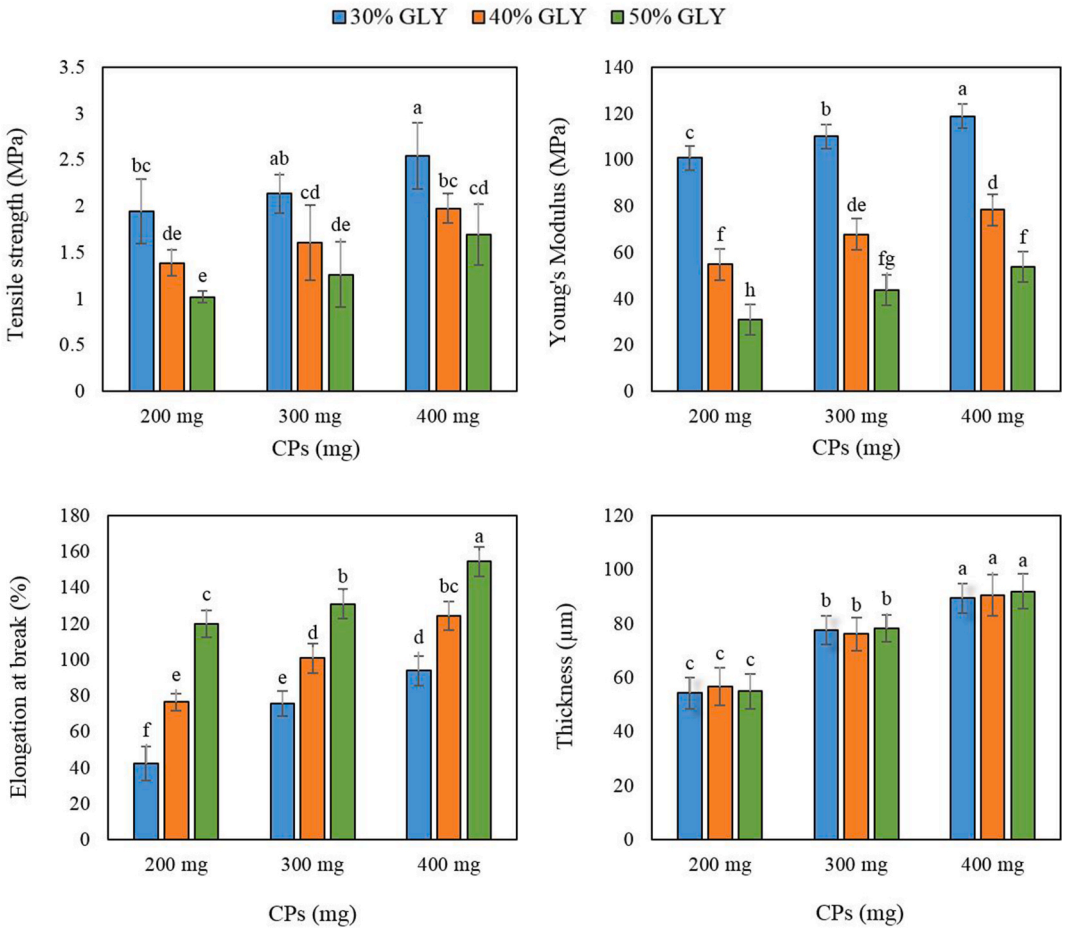


Fig. 5. Effect of different amounts of cardoon proteins (CPs) on the thickness and mechanical properties of films prepared at pH 12 and containing different glycerol (GLY) concentrations. Different small letters (a–g) indicate significant differences among the values reported in each bar (Duncan's multiple range tests, $p < 0.05$). Further experimental details are given in the text.

Table 2
Effect of different concentrations of cardoon leaf extract (CLE) on Z-average and zeta potential of cardoon proteins (400 mg) film forming solutions prepared at pH 12 in the presence of 50% glycerol (GLY)[a].

CLE (% w/w)	Mean particle size (d.nm)	Zeta potential (mV)	PDI (%)
0	411.00 ± 8.20[c]	−34.35 ± 1.41[c]	0.47 ± 0.01[c]
15	441.4 ± 10.08[b]	−31.69 ± 0.95[b]	0.52 ± 0.03[b]
30	512.10 ± 12.22[a]	−28.84 ± 1.12[a]	0.58 ± 0.02[a]

[a] Different small letters (a–d) indicate significant differences among the values reported in each column (Duncan's multiple range tests, $p < 0.05$). Polydispersion index (PDI). Further experimental details are given in text.

investigated samples at around 1040 cm^{-1} is related to OH group of glycerol, indicating the incorporation of glycerol into the film matrix (Moghadam et al., 2020).

3.3.5. *Physicochemical, morphological and mechanical properties of cardoon protein-based films containing cardoon leaf extract*

Films containing different ratios of CP/CLE amounts were manufactured to investigate the effects of CLE on the formation of CP-based materials. To this aim FFSs containing a constant presence of 400 mg of total mass were prepared by increasing the percentage of CLE and parallely decreasing the CP amount. The data reported in Table 3 indicate that thinner films were obtained by reducing the content of CPs until 70% and a concurrent increase in the CLE amount to 30%.

At the same time the mechanical performance of the obtained materials was found to get worse having been observed a progressive decrease of all the parameters (TS, tensile strength, elongation at break EB and YM Young's modulus). Therefore, these findings suggested to test the influence of increasing CLE concentrations on the films manufactured with a constant CP amount (400 mg). Fig. 9 shows that the film thickness slightly increased as a function of CLE amount present in the film matrix, probably due to the interaction of CLE component(s) with the CP polymeric chains, via

Hydrogen bonding and hydrophobic forces (Arciello et al., 2021). In fact, polyphenols may lead to protein crosslinking, thus, increasing the film thickness. These findings were consistent with the results of Hanani, Yee, and Nor-Khaizura (2019) and Moghadam et al. (2020) who observed the effects of pomegranate peel powder on fish gelatin and mung bean protein films, respectively. In addition, both TS tensile strength and YM Young's modulus of films containing 15% CLE were found markedly increased, whereas only a lower, but significant, reduction of the elongation at break EB value was detected in the films prepared in the presence of the same amount of CLE compared to the control samples. However, it should be noted that, by enhancing CLE concentration to 30%, both ~~TS~~ tensile strength and ~~YM~~ Young's modulus values decreased, whereas elongation at break EB slightly increased, with respect to the values detected with films containing 15% CLE. These findings suggest that hydrogen bonding and/or hydrophobic interactions between some CLE components, possibly polyphenols, and CP reactive groups might be responsible for the observed reinforcement of the film network at lower CLE concentrations (Moghadam et al., 2020). Conversely, higher amounts of CLE active molecules could reduce TS tensile strength and increase the flexibility of the CP-based films due to

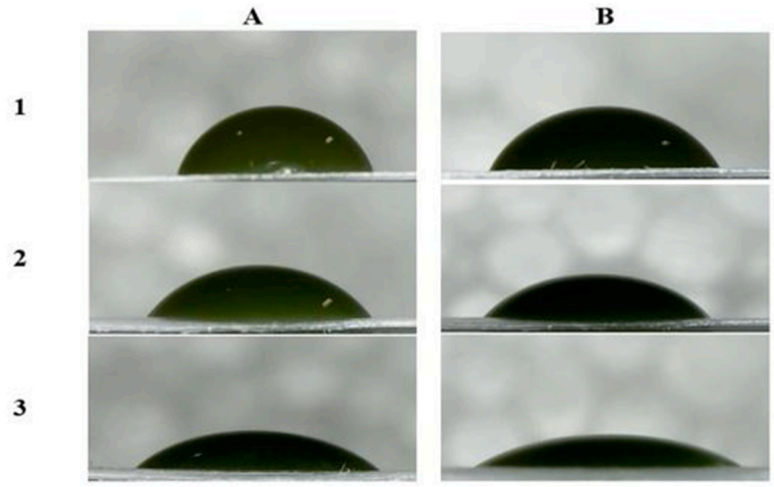


Fig. 6. Images of drops on parafilm surface, captured immediately (A) and after 30 s (B), and contact angle (ɵ) values of film forming solution (FFS), containing cardoon protein (CP) and 50% glycerol, prepared at pH 12 in the absence (1) or presence of 15% (2) and 30% (3) of cardoon leaf extract (CLE). Different small letters (a–c) indicate significant differences among the values reported in each column (Duncan's multiple range tests, $p < 0.05$). Further experimental details are given in the text.

FFS	0 *(sec)*	30 *(sec)*
CP	66.20 ± 0.12 [a]	58.60 ± 0.31 [a]
CP + 15% CLE	50.33 ± 1.15 [b]	41.53 ± 0.63 [b]
CP+ 30% CLE	40.70 ± 1.65 [c]	28.50 ± 1.27 [c]

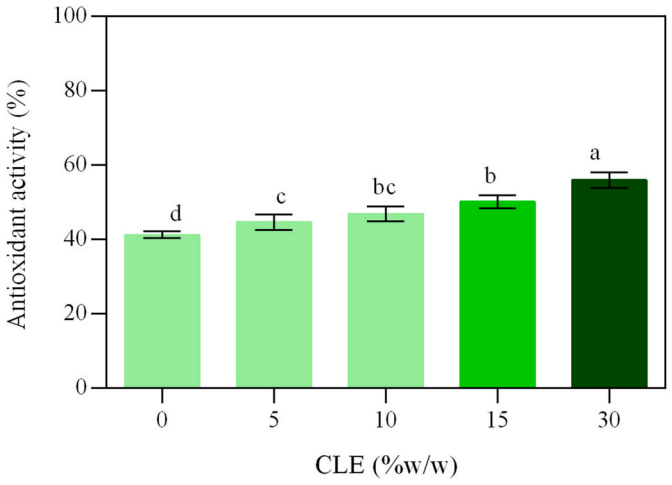


Fig. 7. Antioxidant activity of cardoon protein-based film forming solutions containing different concentrations of cardoon leaf extract (CLE) determined by DPPH assay. Values with different small letters (a–d) are significantly different (Duncan's multiple range tests, $p < 0.05$). Further experimental details are given in the text.

their possible plasticizing effects, as previously reported for bitter vetch protein-based films added with different polyphenol containing extracts (Arabestani, Kadivar, Shahedi, Goli, & Porta, 2016) or for whey protein- and fish gelatin-based films in which curcumin and mango peel extracts were, respectively, incorporated (Adilah et al., 2018; Taghavi Kevij et al., 2020). The films functionalized with 15% CLE were slightly less flexible than the ones prepared in the absence of the phenolic components showing a lower elongation at break, however a significant increase in tensile strength and Young's modulus was observed as a result of CLE incorporation. The same observations were reported by Arciello et al. (2021) who studied whey protein-based bio-plastics prepared with the addition of phenolic extracts from Pecan nuts.

Furthermore, it is well known that color parameters and opacity of the films play a key role in consumers' willingness to choose packed food products, and, in addition, these parametes can affect food quality, specially for the foods that are sensitive to the light (Baek, Kim, & Song, 2018). As shown in Fig. 10 and inferred from the data reported in Table 4, all the CP-based films had low L*, b* and a* values determining a dark greenish-yellow color of the films. The presence of CLE in the CP-based films increased their opacity and the a* and b* values by increasing its concentrations. The higher a* and b* values of films obtained in the presence of higher amount of CLE indicate that the films were more close to green and yellow colors, compared to the control film, probably due to the natural pigments present in CLE. Moreover, also the lightness (L* value) of the film samples was reduced by increasing CLE concentrations. The density of CP-based films significantly increased by increasing CLE concentration, suggesting that the formation of hydrogen and hydrophobic bonds between proteins and CLE components increased by increasing CLE concentrations and led to a more compact film microstructure. Similar behaviour has been previously reported by Riaz et al. (2018) who observed an increasing trend in the density of chitosan-based films by increasing its polyphenols content.

As ar fas as the structural morphology, the films prepared in the absence of CLE exhibited some irregularities and large pores in both surface and matrix (Fig. 10, B1 and C1**),** whereas the microstructural images of the CLE containing films appeared more homogeneous, continuous and smooth. On the other hand, the film prepared in the presence of 15% CLE seems smoother and more compact than that cast with 30% CLE especially in the cross-section where several holes, voids and discontinuities are quite visible (Fig. 10, B2 and C2 and B3 and C3).

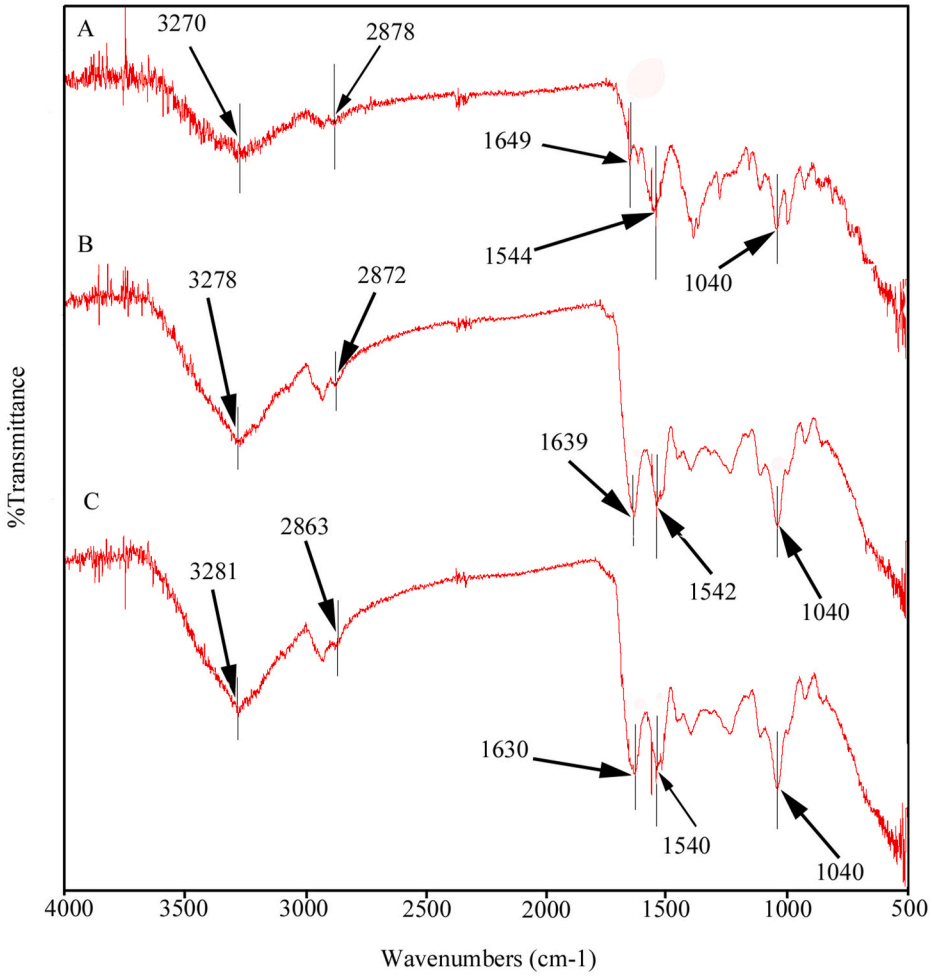


Fig. 8. FT-IR spectra of films prepared in the absence (A) and the presence of 15% (B) and 30% (C) of cardoon leaf extract (CLE).

Table 3
Effect of different cardoon protein (CP)/cardoon leaf extract (CLE) ratios on the thickness and mechanical properties of films prepared at pH 12[a].

CP/CLE *(% w/w)*	TS *(MPa)*	EB *(%)*	YM *(MPa)*	Thickness *(μm)*
100	1.86 ± 0.23^{a}	141.21 ± 6.14^{a}	62.39 ± 7.74^{a}	86.84 ± 5.92^{a}
90/10	1.64 ± 0.31^{a}	132.83 ± 5.27^{a}	56.47 ± 6.31^{ab}	77.31 ± 5.47^{a}
80/20	1.29 ± 0.17^{ab}	107.62 ± 7.41^{b}	49.93 ± 4.71^{bc}	65.72 ± 6.28^{ab}
70/30	1.17 ± 0.06^{b}	92.43 ± 6.08^{b}	34.15 ± 5.73^{c}	49.32 ± 4.88^{c}

[a] Tensile strength (TS), elongation at break (EB), Young's module (YM). Different small letters (a–c) indicate significant differences among the values reported in each column (Duncan's multiple range tests, $p < 0.05$). Further experimental details are given in text.

3.3.6. Water resistance of cardoon protein-based films containing cardoon leaf extract

The moisture content, solubility, swelling ratio and contact angle values of CP-based films containing different amount of CLE are reported in Fig. 11. It is well known that the moisture content of the protein-based films is related to the free volume occupied by water molecules that is in turn influenced by the conformation of the protein and the number of exposed polar groups, and the consequent surface polarity of the polymeric matrix (Sarıcaoglu & Turhan, 2020). The CP-based films revealed a declining tendency for moisture content after incorporation of CLE components, probably due to their hydrophobic properties that limited the water retention in the film matrix (Emam-Djomeh, Moghaddam, & Yasini Ardakani, 2015; Sarıcaoglu & Turhan, 2020). Similar results were obtained by Shams, Ebrahimi, & Khodaiyan (2019) who studied the effects of the orange peel extract added to nanocomposite films made with whey protein and gelatin. Also the reduced tendency for water content of chitosan films observed after incorporation of apple peel polyphenols (Riaz et al., 2018) seems in agreement with the present results.

Furthermore, since both water solubility and swelling of the protein-based films are considered as important factors for their possible applications, mainly in the humid enviroment (Batista, Araújo, Peixoto Joele, Silva, & Lourenço, 2019; Haghighi et al., 2019), also the effect of CLE on these film features was analysed. The data reported in Fig. 11 clearly indicate that both water solubility and swelling ratio of the CLE containing films significantly decreased in comparison with the control CP films. In fact, the hydrophobic nature of several molecules present in CLE might be responsible for the formation of a more compact protein matrix able to maintain the integrity of the films upon their immersion in water, as well as for the reduced interactions among protein and water molecules.

In agreement with these results, Nur Hanani, Aelma Husna, Nurul Syahida, Nor Khaizura, and Jamilah (2018) showed that the water solubility of gelatin/polyethylene bilayer films markedly improved after their enrichment with different fruit peels.

Finally, it is well known that the contact angle is a valid indicator of hydrophobicity of the film surface and that this parameter is affected by different factors such as heterogeneity, surface roughness, particle and

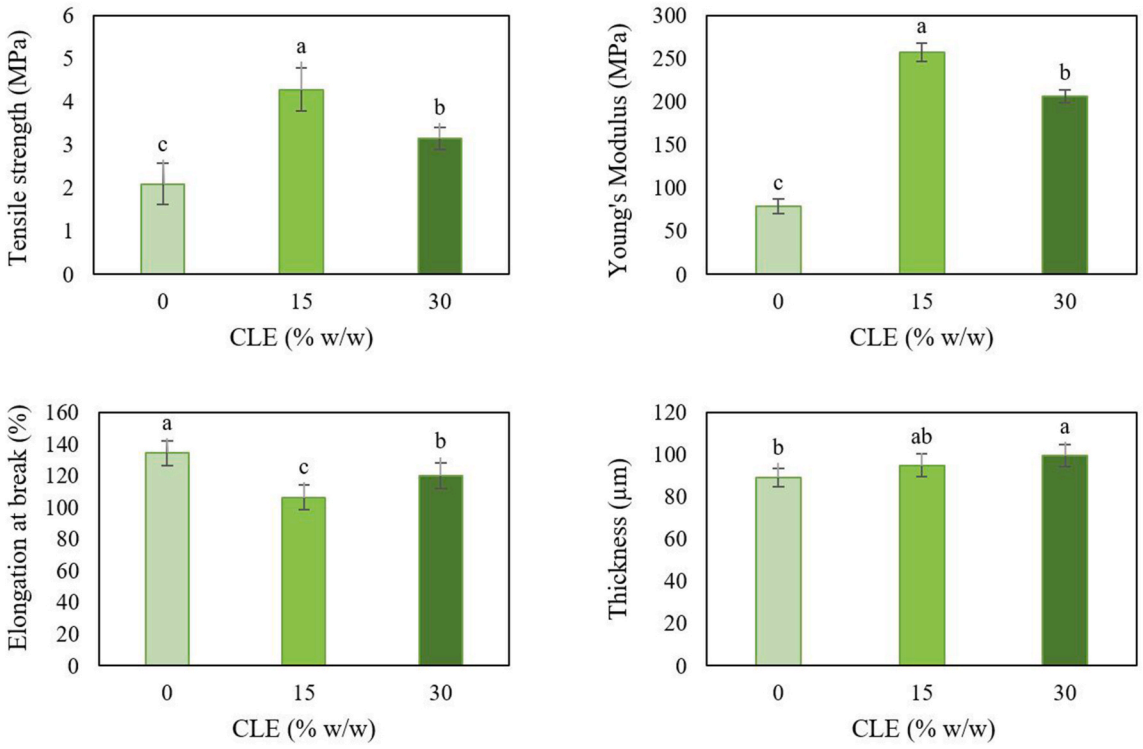


Fig. 9. Thickness and mechanical properties of cardoon protein-based films containing different concentrations of cardoon leaf extract (CLE). Values with different small letters (a–c) are significantly different (Duncan's multiple range tests, $p < 0.05$). Further experimental details are given in the text.

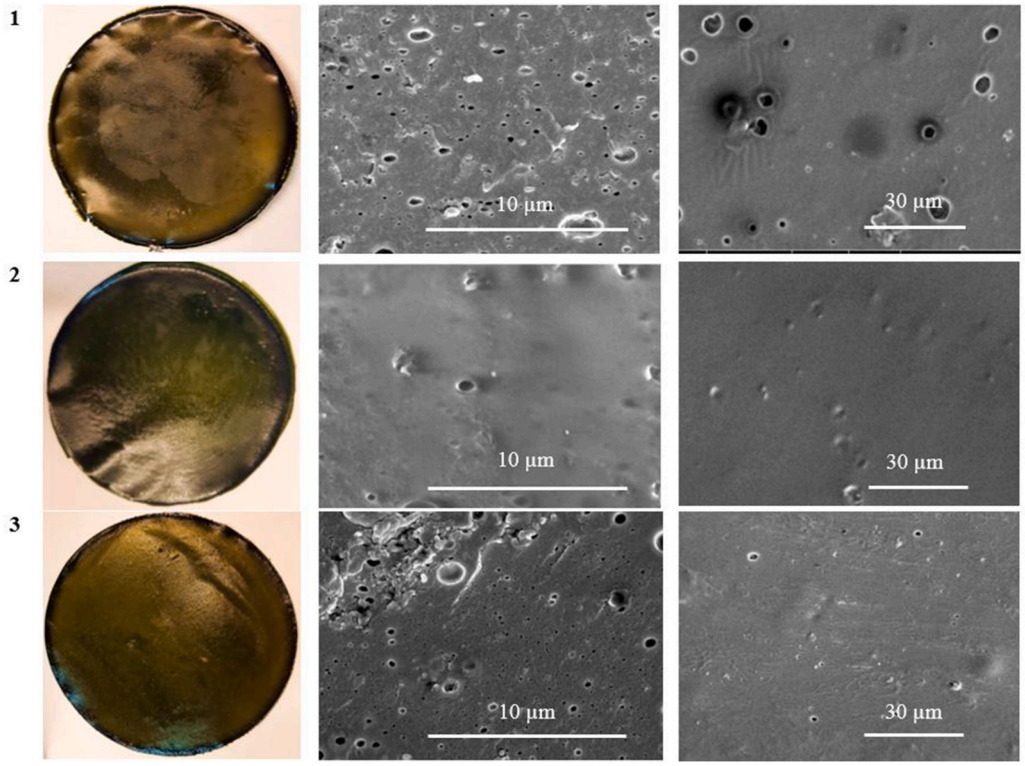


Fig. 10. Images of films (A), prepared in the absence (1) and presence of either 15% (2) or 30% (3) of cardoon leaf extract, and of their SEM cross sections (B, magnification 8000 ×) and surfaces (C, magnification 4000 ×). Further experimental details are given in the text.

pore size of the film matrix (Abdelhedi et al., 2018; Hebbar, Isloor, & Ismail, 2017). As shown in Fig. 11, the incorporation of CLE into CP-based films significantly increased their contact angle values compared to those of control films. These findings suggest that the presence of CLE components in the CP film matrix might increase the smothness of the film surface leading to an increase in the

Table 4
Colour parameters, opacity and density of cardoon proteins-based films containing different concentrations of cardoon leaf extracts (CLE).

CLE %, w/w)	L	a	b	ΔE	Opacity (mm^{-1})	Density (g/cm^3)
0	28.42 ± 0.87[a]	−2.25 ± 0.10[a]	13.82 ± 0.69[c]	72.22 ± 1.54[c]	14.89 ± 0.17[c]	1.19 ± 0.01[c]
15	23.64 ± 1.03[b]	−2.81 ± 0.07[b]	15.51 ± 0.84[b]	77.20 ± 1.08[b]	16.14 ± 0.35[b]	1.28 ± 0.02[b]
30	20.44 ± 0.57[c]	−3.29 ± 0.15[c]	20.04 ± 0.56[a]	81.16 ± 1.12[a]	17.61 ± 0.28[a]	1.34 ± 0.01[a]

[a]L, a and b values indicate lightness/darkness (0–100), greenness/redness (−60 to +60) and blueness/yellowness (−60 to +60), respectively; ΔE, total color difference. Values with different small letters (a-c) are significantly different (Duncan's multiple range tests, $p < 0.05$). Further experimental details are given in the text.

hydrophobicity of their surface, as a consequence of the interactions between CPs and CLE components able to decrease the number of protein hydrophilic groups. Increasing of the surface hydrophobicity of mung bean protein-based films enriched with pomegranate peel powder was previously reported by Moghadam et al. (2020).

3.3.7. Film barrier properties of cardoon protein-based films containing cardoon leaf extract

The resistance to water vapor (WV) permeation of CP-based films prepared in the presence or absence of two different CLE concentrations is reported in Table 5. CP-based films containing 15% CLE exhibited WV water vapor permeability values lower than those observed by testing CP films prepared in CLE absence, while WV water vapor permeability was found significantly increased with respect to control samples in the films obtained in the presence of CLE double concentration (30%). The enhancement of WV water vapor barrier properties of films containing a lower CLE amount could be attributed to the decrease of the free space among the CP chains due to the formation of hydrogen bonds and/or hydrophobic interactions among protein and CLE components that could probably decrease the penetration and diffusion of WV water vapor through the film matrix.

Conversely, the addition of higher amounts of CLE to the CP-based FFS probably prevented an homogeneous distribution of the same components in the film matrix, thus determining an opposite effect on the WV water vapor permeability of the resulting films. In this regard, Moghadam et al. (2020) reported that WV water vapor barrier properties of mung bean protein-based films increased in the presence of pomegranate peel powder, whereas Adilah et al. (2018) did not observe any significant change in WV water vapor permeability of the fish gelatin films by adding increasing amounts of mango peel.

Furthermore, as shown in Table 5, even the permeabilities to both O_2 and CO_2 of the CP films containing CLE were observed to significantly decrease with respect to those of control films, probably as a consequence of the lower number of pores observed by SEM in the film surface and cross-section images (Fig. 10). More in particular, the interactions between CP and CLE components might be responsible for the reduced diffusion paths of the gas molecules through the film networks (Laufer, Kirkland, Cain, & Grunlan, 2013). However, also the O_2 and CO_2

Table 5
Water vapor (WV) and gas permeability of cardoon protein (CP)-based films containing different concentrations of cardoon leaf extract (CLE)[a].

CLE (% w/w of CP)	WV	O_2	CO_2
	(cm^3 mm m^{-2} d^{-1} kPa^{-1})		
0	0.05 ± 0.01[a]	2.24 ± 0.01[a]	6.53 ± 0.42[a]
15	0.02 ± 0.01[b]	1.19 ± 0.06[b]	1.89 ± 0.37[b]
30	0.08 ± 0.01[c]	1.64 ± 0.08[c]	3.47 ± 0.82[c]

[a] Different small letters (a–c) indicate significant differences among the values reported in each column (Duncan's multiple range tests, $p < 0.05$). Further experimental details are given in text.

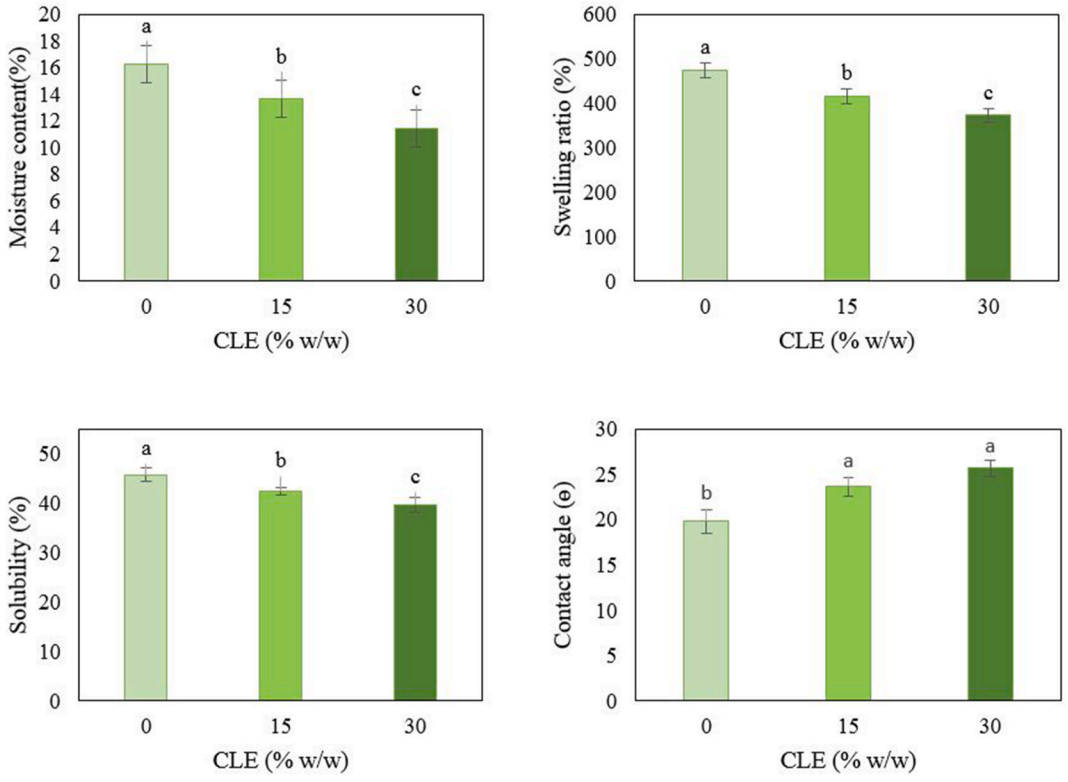


Fig. 11. Moisture content, water solubility, swelling and contact angle of cardoon protein-based films containing different concentrations of cardoon leaf extract (CLE). Values with different small letters (a–c) are significantly different (Duncan's multiple range tests, $p < 0.05$). Further experimental details are given in the text.

permeability values of CP films containing higher aumounts of CLE were found to be higher than those containing 15% CLE. Similar findings were obtained by Galus and Kadzińska (2016) who reported an increase in gas permeabilities by increasing almond and walnut oil concentrations in the whey protein films. Therefore, also these data seem to reflect the microstructure of the prepared films, since an increase in pores and voids in the film network was observed in the films prepared with the highest (30%) CLE concentration (Fig. 10, B3). Similar results were reported by Bai et al. (2019) who studied the effects of different amounts of quercetin on carboxymethyl chitosan based films. Nevertheless, it is worthy to note that the behaviour in terms of barrier properties of the films containing CLE is not far from that observed for another protein-based bio-plastic, such as the one produced by using whey proteins functionalized with a phenolic extract (30% w/w of proteins) obtained from Pecan nutshell (Arciello et al., 2021). In fact, the authors found out that the permeabilities towards water vapor and the two types of gases performed by the whey protein film prepared with the extract were found to be significantly lower compared to those of the unfunctionalized one. The authors concluded that these results are useful for a potential application of such bio-plastics in the food sector, since, to keep food fresh, the water vapor permeability value should be maintained as low as possible. Furthermore, a higher O_2 barrier property is also important since oxygen causes, for example, the rancidity of fatty acids (Arciello et al., 2021).

3.3.8. Antioxidant activity of cardoon protein-based films containing cardoon leaf extract

CP-based films containing CLE were tested for DPPH radical scavenging activity over time. Fig. 12 shows that the films freshly manufactured in the absence of CLE exhibited a marked antioxidant activity and that the addition of 30% CLE improved this property since the observed scavenging activity against DPPH radical of the films increased form 30% up to 60%. The antioxidant activity of all films remained quite stable after 30 days at room temperature, suggesting their potential exploitation as active packaging for shelf life extension of foodstuffs. Similar results were obtained by Moghadam et al. (2020) who described a range of scavenging activity from 14% to 65% for mung bean protein-based films enriched with different amounts of pomegranate peel powder, whilst Adilah et al. (2018) and Hanani et al. (2019) reported an enhancement of the scavenging activity up to 89% and up to 72% for fish gelatin films added with mango kernel extract and pomegranate peel powder, respectively. From Fig. 12 it also possible to note that over a period of 70 days the antioxidant activity decreased roughly of 25% for all the bio-plastics tested, likely because of the oxidation of the phenolic compounds responsible of conferring the films with this biological activity. Nevertheless, the materials functionalized with the highest amount of CLE seem to be still endowed with the highest antioxidant activity (45%) even after a period of 70 days, suggesting their possible application in protecting some foods from the oxidation.

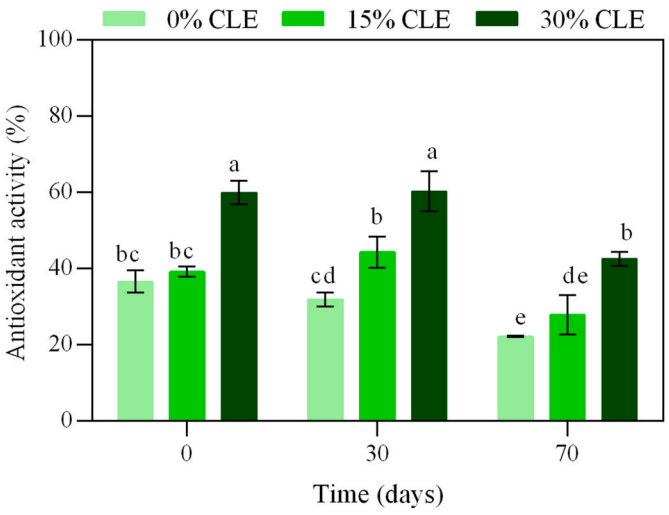


Fig. 12. Effect of different concentrations of cardoon leaf extract (CLE) on the antioxidant activity of cardoon protein-based films measured by DPPH assay at different times of storage. Values with different small letters (a–e) are significantly different (Duncan's multiple range tests, $p < 0.05$). Further experimental details are given in the text.

4. Conclusions

The objective of this paper was to valorise different *Cynara cardunculus* segments by extracting bio-based products useful for an integrated application. A method for recovery of a functional cardoon leaf extract (CLE) was set up and its composition analysis revealed the presence of several low molecular weight molecules (such as two sesquiterpene lactone, cynaropicrin and grosheimin) of potential attractiveness as bioactive compounds. In addition, proteins extracted from cardoon seeds were tested as raw material for producing, in the presence of glycerol, manipulable bio-plastics endowed with promising mechanical and barrier features, as well as antioxidant properties. Towards the development of a *Cynara cardunculus* biorefinery, the obtained CLE was added to the protein-based film forming solutions and the characterization of the derived films showed a significant improvement of all the properties of the manufactured material. The CLE-containing films appeared smoother and more homogenous, and showed also a higher and lasting antioxidant activity that conferred a higher value to the obtained bio-plastics. Therefore, all the presented data envisage *Cynara cardunculus* as a potential biomass resource for the development of a plant biorefinery devoted to the production of innovative bio-based products according to the principles of the circular bio-economy. In particular, the produced bio-plastics may be exploited for extending the shelf-life of different kinds of foodstuffs due to the fact that, besides showing good mechanical and barrier properties, they are endowed with antioxidant features that are of a paramount importance for food protection. Nevertheless, a potential use of such biomaterials as mulching sheets is also advisable.

Author contributions

Seyedeh Fatemeh Mirpoor: Investigation; Methodology; Formal analysis; Writing-original draft. Simona Varriale: Investigation; Methodology; Formal analysis; Writing-original draft. Raffaele Porta: Supervision; Funding acquisition; Conceptualization; Writing review and editing. Daniele Naviglio: Methodology; Investigation. Mariachiara Spennato: Methodology; Investigation. Lucia Gardossi: Methodology; Investigation. C. Valeria L. Giosafatto: Supervision; Conceptualization; Writing review and editing. Cinzia Pezzella: Supervision; Conceptualization; Writing review and editing.

Declaration of competing interest

None.

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