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ORIGINAL PAPER

An environmentally friendly process for the production of extracts rich in phenolic antioxidants from *Olea europaea* L. and *Cynara scolymus* L. matrices

Annalisa Romani[1] · **Arianna Scardigli**[1] · **Patrizia Pinelli**[1]

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Abstract A sustainable extractive technology followed by membrane separation methods was applied to *Olea europaea* L. (leaves and pitted olive pulp) and *Cynara scolymus* L. by-products (leaves and stems), to obtain different standardized commercial extracts for application in the functional food industry, pharmaceutical and cosmetic fields. The entire treatment, PCT registered, consists of water extraction of the aforementioned vegetal material and the following steps of fractionation: (1) microfiltration (only for *Olea*); (2) ultrafiltration (only for *Cynara*); (3) nanofiltration (only for *Olea*); (4) reverse osmosis and final concentration by evaporation at low temperature or spray-dried technique. Thanks to these steps, different fractions enriched with phenolic compounds that have an economic value can be obtained. These fractions were characterized and quantified by HPLC/DAD-ESI/MS and then tested for their antiradical and antioxidant properties. The EC_{50} values by DPPH test were 6.76×10^{-3} mM for the *Olea* green leaves (GL) soft extract, 5.44×10^{-3} mM for the *Olea* dried leaves (DL) soft extract, and 1.22×10^{-3} mM in the case of the *Olea* OH-Tyr soft extract. The EC_{50} value of the Cynara GL soft extract was 3.25×10^{-3} mM. The ORAC results were 3632 ± 110.7 μmoL TE/g for OH-Tyr *Olea* soft extract, 1410 ± 62.1 μmoL TE/g for GL *Olea* soft extract, and finally 760 ± 12 μmoL TE/g for GL Cynara soft extract, confirming the highest antioxidant activity of hydroxytyrosol.

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Keywords Microfiltration · Ultrafiltration · Nanofiltration · Reverse osmosis · Green extraction procedure · Antiradical activity

Abbreviations

PCT	Patent cooperation treaty
MF	Microfiltration
UF	Ultrafiltration
NF	Nanofiltration
RO	Reverse osmosis
OH-Tyr	Hydroxytyrosol
MCC	Monocaffeoylquinic acid
DCC	Dicaffeoylquinic acid
GL	Green leaves
DL	Dried leaves
CMF	Concentrate of microfiltration
CUF	Concentrate of ultrafiltration
CNF	Concentrate of nanofiltration
CRO	Concentrate of reverse osmosis
PES	Polyethersulfone
Soft Extract *Olea* GL	*Olea europaea* fraction deriving from green leaves
Soft Extract *Olea* DL	*Olea europaea* fraction deriving from dried leaves
Soft Extract *Olea* OH-Tyr	*Olea europaea* fraction deriving from pitted olive pulp
Soft extract Cynara GL	Cynara fraction deriving from green leaves
Spray-dried Cynara GL	Cynara powder deriving from green leaves
DPPH	(1.1-diphenyl-2-picrylhydrazil radical)
AR	Antiradical activity

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EC50	Median effective concentration (required to induce a 50% effect)
ORAC	Oxygen radical absorbance capacity
AAPH	2,2′-Azobis(2-amidinopropane) dihydrochloride
TE	Trolox equivalents
AUC	Area under the curve
HPLC/DAD	High-performance liquid chromatography/diode array detector
ESI-MS	Electrospray mass spectrometry
API	Atmosphere pressure ionization
BAT	Best available technology
EPA	Environmental protection agency

Introduction

The polyphenols from *Olea europaea* L. matrices (olive oil by-products, leaves, and pitted olive pulp) are known for their good antioxidant properties and protective biological and biomedical effects. The chemical characterization and quantitative evaluation of these minor polar compounds can be useful to obtain active principles with important applications in pharmaceutical, cosmetic, and functional food products. The main constituent of olive leaf is a phenolic secoiridoid glycoside, oleuropein, which can be broken down into elenolic acid, a powerful antibacterial molecule [1], and hydroxytyrosol, known for its important antioxidant activity [2]. *In vitro* tests have shown that flavonoids in olive leaf extracts exhibit antiradical properties [3]. Moreover, in vivo studies have demonstrated the effectiveness of olive leaf extract in lowering blood pressure, and this effect seems to be mainly ascribed to oleuropein and hydroxytyrosol [4]. The useful antioxidant properties of hydroxytyrosol may be important in the search for 'natural' replacements for 'synthetic' antioxidant food additives. A previous investigation [5] demonstrated that olive leaf phenol extract is a good antioxidant for food lipids, even at doses lower than 100 mg/kg (expressed as hydroxytyrosol) and that it has no cytotoxic effects nor does it inhibit the growth of lactic acid bacteria. For these reasons, and in view of their recognized nutraceutical activities, olive leaf phenol extracts can be used as a foodstuff ingredient. Moreover, the antimicrobial properties of phenol compounds coming from olive products have recently been investigated against *Helicobacter pylori*, as well as several food-borne pathogens [6], confirming important applications of these extracts, not only for food processing control and preservation during storage, but also for counteracting microorganisms harmful to human health.

Artichoke is a perennial herbaceous plant (*Cynara cardunculus* L.) belonging to the family of *Compositae* (*Asteraceae*) and mainly cultivated in the Mediterranean area. Artichoke heads are known worldwide to be edible, whereas the leaves have been used since antiquity in popular medicine for their beneficial effects. In addition, extracts from such plants have been claimed to possess hepatoprotective and antioxidant properties due to their polyphenolic fraction [7, 8]. In particular, in the phenolic fraction, the presence of mono and di-caffeoyl ester of the quinic acid and flavonoid glycosides has been observed [9, 10].

The membrane-based techniques can be applied in order to achieve concentration and purification of natural products from their biological sources. In these last decades, methods to concentrate and purify natural products by membrane separation techniques have become a mainstream technology, with several applications, for example, in the concentration of juices, water purification and desalination, dye and sugar separation, and the recovery of valuable products [11]. Based on the size of the substances to be separated, and the resultant characteristics needed for the membrane, these techniques are generally classified into the main steps of microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. Regarding the application of membrane separation techniques to *O. europaea* L. tissues, the main examples reported in the literature have been applied to olive oil wastewaters on a laboratory scale [12, 13]. One of the main issues, that constitute a drawback for developing the membrane technologies to large-scale applications, is mainly due to the olive oil industry, which is often composed of little and dispersed factories, that cannot bear the high costs of extract production and the maintenance of industrial plants, even considering the gain, coming from the reduction of disposal costs of olive mill waste waters and other agroindustrial by-products. [14]. A very interesting study about membrane technology and the valorization of artichoke wastewaters (leaves, stems, bracts, and blanching waters) has been recently published [15]. In this work, pressure-driven membrane processes, such as ultrafiltration, nanofiltration, and reverse osmosis, have been successfully employed for the purification and concentration of bioactive compounds. Also in this case, as for *Olea* tissues and by-products in the other researches, the integrated membrane system was developed on a laboratory scale, to fractionate the wastewaters. Considering the state of the art in the membrane technology field, the present work deals about the recovery of bioactive molecules from different plant tissues (green and dried leaves, pitted olive pulp) on industrial scale.

The aim of this paper was to describe a sustainable extractive technology followed by membrane separation

methods [16, 17] applied to the previously described vegetal sources, in particular, by-products of the processing of olive oil and artichokes, in order to obtain different commercial extracts, which are useful for different applications, thanks to their antioxidant and antiradical properties. The entire treatment consisted of water extraction of the vegetal material followed by selective fractionation in four steps: (1) microfiltration, MF (only for *Olea*); (2) ultrafiltration, UF (only for *Cynara*); (3) nanofiltration, NF (only for *Olea*); and (4) reverse osmosis, RO. Thanks to these steps, different products and coproducts with an economic value can be obtained. The fractions were characterized and quantified by HPLC/DAD-ESI/MS and then tested for their antiradical and antioxidant properties. To our knowledge, this is the first example of an industrial plant in Europe that uses these technologies for the extraction of active ingredients from both *Cynara* and *Olea* tissues. The industrial plant aims at the development of a coherent industrial-scale production process, taking into account the quantities of raw material available on the local territory. Each membrane is modular and thus easily scalable for any size of production.

Materials and methods

Plant material

The *Olea europaea* samples, in detail cvs. Frantoio from Siena (Tuscany, Italy), Carboncella from Rieti (Latium, Italy), and Leccino from Foggia (Apulia, Italy) were collected during the year 2015. Leaves and pitted olive pulps were the analyzed tissues, respectively. These three geographical areas have been chosen for the presence of mills that use the same olive oil milling process (biphasic system). All the recovered fractions after the membrane-based treatment have a standardized content of active compounds. In this work, the final fractions obtained by processing the cultivar Carboncella have been shown. Dried leaves (DL) were obtained by drying the collected leaves at room temperature for 5 days.

Artichoke leaves belonging to Terom and Violetto cultivars came from two Italian regions (Tuscany and Apulia).

Solvents and reagents

All the solvents (HPLC grade) and formic acid (ACS reagent) were purchased from Aldrich Chemical Company Inc. (Milwaukee, Wisconsin, USA). Tyrosol, luteolin 7-*O*-glucoside, chlorogenic and oleuropein were obtained from Extrasynthese S.A. (Lyon, Nord-Genay, France). The 2.2-diphenyl-1-picryl-hydrazyl (DPPH) reagent was obtained from Sigma-Aldrich (St. Louis, MO, USA). The HPLC-grade water was obtained via double-distillation and purification with a Labconco Water Pro PS polishing station (Labconco Corporation, Kansas City, USA).

Extraction, pretreatment and fractionation

The green extraction was performed in a rapid extractor timatic series (from Tecnolab S.r.l., Perugia, Italy) using water (30 kg of GL or 7 kg of DL in 200 L of water; 50 kg of pitted olive pulp in 200 L of water), in a stainless steel basket at a temperature of 60 °C. This solid–liquid extraction technology was the first phase of the industrial extraction plant. The working cycle is fully automatic and alternates between a dynamic phase, obtained with a set pressure (7–9 Bar), and a static phase necessary for transferring the substance into the extraction solvent. Forced percolation is generated during the stationary phase, which, thanks to the programmable recirculation, ensures a continuous flow of solvent to the interior of the plant matrix. This process avoids over-saturation and the formation of preferential channels, thus ensuring total extraction of the active principles from the vegetal matrix. The extractions of each plant material were performed in triplicate.

In the pretreatment phase, to avoid the polyphenol oxidation, the pH was lowered from the original value of 5.7–3.5, in order to inactivate the polyphenol oxidase enzyme present in the aqueous raw extract, and to create the optimal conditions for the subsequently addition of the pectinase enzyme when the processed sample was ‘pitted olive pulp’. The pH was lowered by adding concentrated HCl and citric acid (1% p/V). In particular, to reduce the effect of membrane clogging by the solids present in the pitted olive pulp, the commercial enzyme complex Pectinex SMASH XXL (Novo Nordisk, Franklinton, N.C.) extracted from *Aspergillus niger* was employed. The preliminary enzymatic phase allows both the releasing of the bioactive compounds from the *Olea* stuff and the optimized recovery of the hydroxytyrosol (OH-Tyr).

This innovative separation process performed with physical technologies [16–18] can be defined as BAT (Best Available Technology) and EPA (Environmental Protection Agency) recognizes it. The studied technology consists of an integrated system of all the filtration steps: Micro (MF), Ultra (UF), Nano (NF), and Reverse Osmosis (RO). The different filtration steps are characterized by different molecular weights, with cutoff and filtration degrees. During the manufacturing process, the MF step is carried out with tubular ceramic membranes in titanium oxide and the UF, NF, and RO steps are performed with spiral wound module membranes in polyethersulfone (PES). This design maximizes the surface area in a minimum amount of space. Less expensive but more sensitive to pollution, this ecofriendly system consists of consecutive layers of

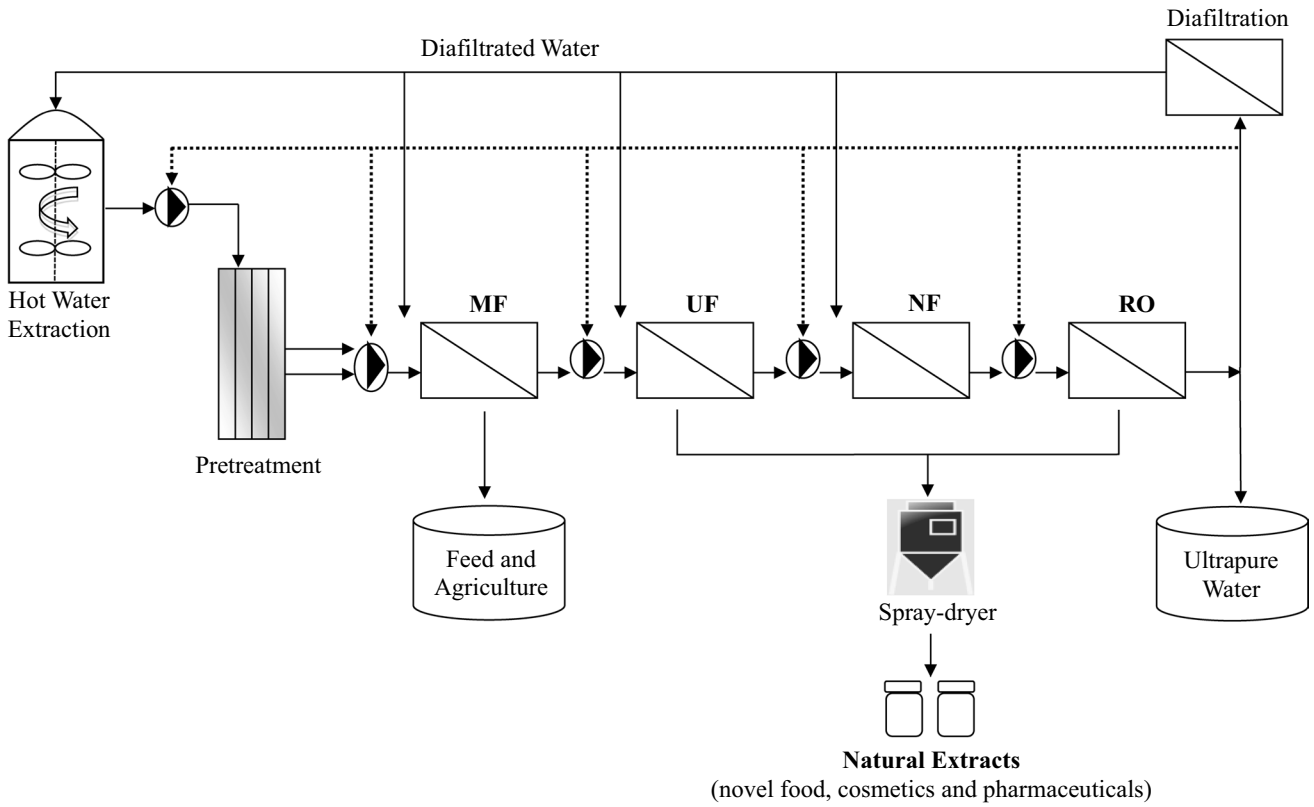

Fig. 1 Industrial plant scheme of the sustainable process, for the recovery of phenolic fractions and pure water from *Olea europaea* L. and *Cynara scolymus* L. matrices, using membrane technologies: microfiltration, MF (only for *Olea*), ultrafiltration, UF (only for *Cynara*), nanofiltration, NF (only for *Olea*) and reverse osmosis (RO)

large membranes and supporting material in an envelope-type design rolled up around a perforated steel tube [19]. The diagram of the sustainable industrial plant is shown in Fig. 1.

HPLC analysis

HPLC/DAD analysis

The HPLC/DAD analyses were performed with an HP 1100 L liquid chromatograph equipped with HP DAD (Agilent Technologies, Palo Alto, CA). In detail, the analytical column used for the *Olea* samples was a LiChrosorb RP18 250 4.60 mm, 5 μm (LichroCART, Merck Darmstadt, Germany) maintained at 26 °C. The eluents were H_2O adjusted to pH 3.2 by HCOOH and CH_3CN. A four-step linear solvent gradient was used, starting from 100% H_2O up to 100% CH_3CN, for an 88-min period at a flow rate of 0.8 ml/min, in accordance with a previous paper [20].

For the analyses of Cynara extracts, a Luna C18 column 150 × 3.0 mm, 5 μm (Phenomenex) operating at 27 °C was used. The eluents were H_2O adjusted to pH 3.2 by HCOOH and CH_3CN. A three-step linear solvent gradient was performed starting from 100% H_2O up to 100% CH_3CN, with a flow rate of 0.6 ml/min for a 30-min period, in line with a previous paper [21].

HPLC/ESI–MS analysis

The HPLC–MS analyses were performed using an HP 1100L liquid chromatograph equipped with a DAD and 1100 MS detectors. The interface was an HP 1100 MSD API-electrospray (Agilent Technologies). Mass spectrometer operating conditions were the following: gas temperature 350 °C at a flow rate of 10.0 L/min, nebulizer pressure 30 ψ, quadrupole temperature 30 °C, and capillary voltage 3500 V. The mass spectrometer operated in positive and negative ionization mode at 80–120 eV, for both ionization modes.

Qualitative and quantitative analysis

The identity of the phenolic compounds was ascertained using data from the HPLC/DAD and HPLC/MS analyses by comparing and combining their retention times, UV/Vis,

and mass spectra with those of authentic standards. Each compound was quantified by HPLC/DAD using a five-point regression curve built with the available standards. Calibration curves with $r^2 \geq 0.9998$ were considered. In all cases, the actual concentrations of derivatives were calculated after making corrections for changes in molecular weight.

In particular, for the *O. europaea* extracts, a HPLC/DAD quantitative analysis of each compound was performed using specific calibration curves, built with the specific standards. More specifically, secoiridoid molecules were calculated at 280 nm using oleuropein as a reference; elenolic acid derivatives at 240 nm using oleuropein; hydroxytyrosol, lignans, and derivatives were calibrated as tyrosol at 280 nm; verbascoside and other hydroxycinnamic derivatives were calibrated at 330 nm using chlorogenic acid as a reference; and, finally, flavonoids were calibrated with the specific pure compound (luteolin 7-*O*-glucoside) at 350 nm. For the *Cynara scolymus* extracts, chlorogenic acid, mono- and di-caffeoylquinic acids were calibrated at 330 nm with chlorogenic acid as a reference; cynarin was calibrated at 330 nm with the pure standard, and flavonoids at 350 nm with luteolin 7-*O*-glucoside. The determining of the polyphenol content was carried out in triplicate. The results (see Tables) are recorded as mean values with the standard deviation.

Antiradical activity (DPPH method)

The extracts previously analyzed by HPLC were then used for the DPPH·(1.1-diphenyl-2-picrylhydrazil radical) assay. The antiradical capacity of the extracts was estimated according to a previously reported procedure [22], with slight modifications. More specifically, the extracts were opportunely diluted and an amount equal to 1:1 added to an ethanol solution of DPPH (0.025 mg/mL). Measurements were carried out at 517 nm with a DAD 8453 spectrophotometer (Agilent Technologies) at time 0 and then every 2 min. for the following 20 min. The antiradical activity (AR%) was calculated through the relationship: $[AR\% = 100\ (A_0 - A_{20})/A_0]$, where A_0 and A_{20} were the absorbance of DPPH, at time 0 and 20 min., respectively, after adding the diluted extract. The EC_{50} of the extracts was determined through the use of five-point linearized curves [AR%-ln (concentration in polyphenols)], built determining AR% for five different dilutions of each extract and then by calculating the molar concentration in polyphenols of the solution that inhibits the DPPH activity to 50%.

Antioxidant activity (ORAC method)

The original method [23] was applied with few modifications [24]. The final reaction mixture for the assay (2 mL) was prepared as follows: 1650 ml 0.05 mM fluorescein sodium salt in 0.075 M sodium phosphate buffer, pH 7.0, 200 mL diluted sample or 50 mM Trolox. The control was 0.075 M-Na phosphate buffer, pH 7.0. Fluorescence was read every 5 min. at 37 °C using an LS-5 spectrofluorometer (Perkin-Elmer, Norwalk, CT, USA) at 485 nm excitation, 520 nm emission. When stability was reached, the reaction was initiated with 150 ml 5.55 mM AAPH, and fluorescence was read up to a value of zero. The ORAC value is expressed as μmol Equivalents Trolox (TE) per gram and is calculated according to the formula:

$$ORAC\ (\mu\text{moL TE/g}) = [(A_s - A_b)/(A_t - A_b)] \times K \times a \times h$$

where A_s is the area under the curve (AUC) of fluorescein in the sample, calculated with the ORIGIN 2.8 integrating program (Microcal Software), A_t is the AUC of the Trolox, A_b is the AUC of the control, k is the dilution factor, a is the concentration of the Trolox in mmol/L, and h is the ratio between the liters of extract and the grams of plant extracts (*Olea* and *Cynara* soft extracts).

Results

Three main fractions were produced from the *Olea* vegetal material using the sustainable extraction technology followed by membrane separation, as described in the Introduction and Materials and Methods sections.

The concentrates from microfiltration (CMF), nanofiltration (CNF), and finally, from reverse osmosis (CRO) were obtained using green olive leaves (GL) and dried leaves (DL) as starting materials. The HPLC/DAD/ESI–MS quali–quantitative analyses were performed for each sample obtained from the industrial plant. Hydroxytyrosol and derivatives, secoiridoids and elenolic acid derivatives, hydroxycinnamic acids, verbascoside, flavonoids, lignans, and trace amounts of others phenolic compounds were characterized and quantified.

By way of example, the chromatographic profile registered at 280 and 240 nm of the fraction OLEA CRO GL is reported in Fig. 2, with the list of each identified compound. The quantitative data of each polyphenol in the raw fractions of the industrial plant are reported in Table 1, as a mean value of three sampling (±SD).

The concentrations of CNF and CRO from GL are very similar, with a value in total polyphenols of 3.9% *w/V*.

Table 1 also illustrates the concentrated fractions, or soft extracts, obtained by using a heat pump evaporator (Vacuum Evaporators-Scraper Series, C&G Depurazione Industriale srl, Firenze, Italy), being the initial extracting materials not only GL and DL, but also pitted olive pulp. This last fraction was called *Olea* OH-Tyr since the main compounds are hydroxytyrosol (OH-Tyr) and its derivatives.

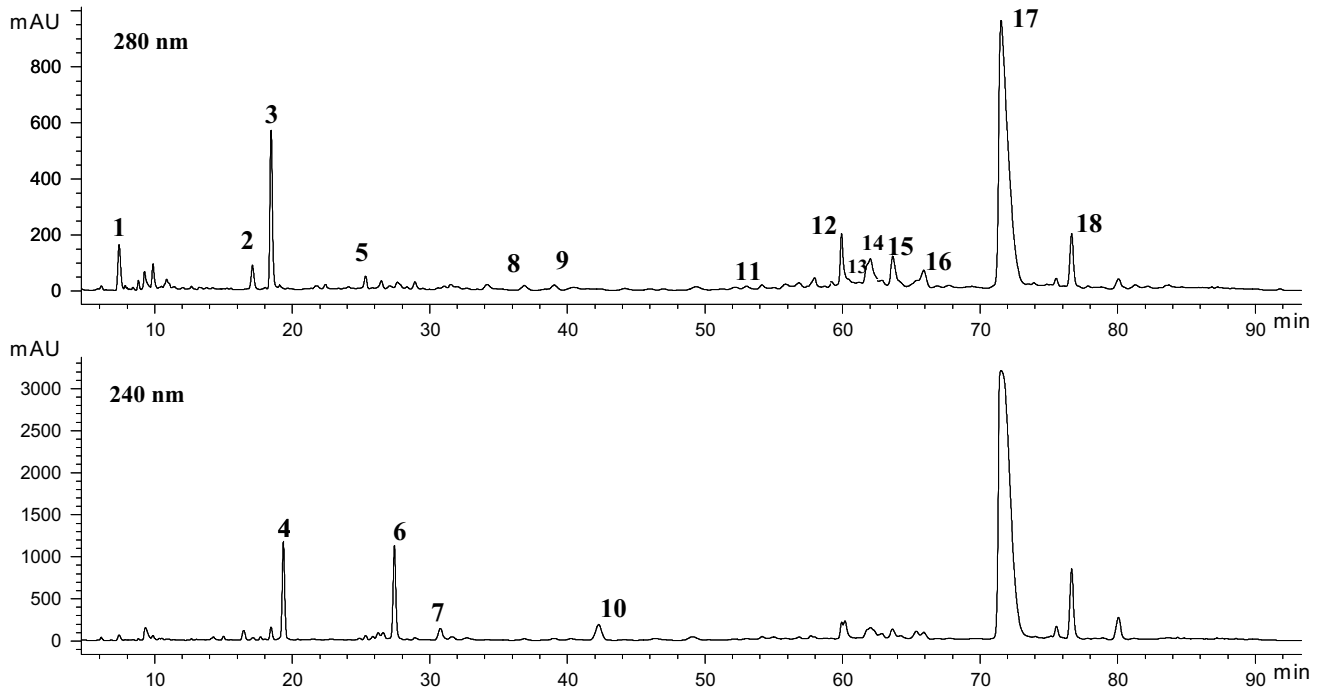

Fig. 2 Chromatogram of *Olea* CRO GL. *Peaks*: *1*. hydroxytyrosol derivative; *2*. hydroxytyrosol; *3*. hydroxytyrosol glucoside; *4*. oleoside; *5*. esculin; *6*. dimethyl elenolic acid diglucoside; *7*. elenolic acid glucoside; *8*. Lignan derivative; *9*. hydroxycinnamic derivative; *10*. elenolic acid glucoside derivative; *11*. *β*-OH-verbascoside; *12*. verbascoside; *13*. luteolin 7-*O*-glucoside; *14*. pinoresinol; *15*. verbascoside isomer; *16*. acetoxypinoresinol; *17*. oleuropein; *18*. oleuropein isomer

Table 1 HPLC/DAD quantitative analyses of *Olea* plant fractions, concentrate fractions and powders (spray-dried)

	Plant fractions (g/L)				Concentrated fractions (mg/g)			Spray-dried (mg/g)	
	GL *Olea* CMF	GL *Olea* CNF	GL *Olea* CRO	DL *Olea* CRO	Soft extract *Olea* OH-Tyr	Soft extract *Olea* GL	Soft extract *Olea* DL	*Olea* GL	*Olea* DL
Hydroxytyrosol derivatives	0.29 ± 0.10	4.69 ± 0.67	6.18 ± 0.58	3.63 ± 0.64	279.89 ± 18.24	24.69 ± 3.47	25.21 ± 1.56	23.55 ± 0.03	15.98 ± 0.96
Secoiridoid der.	2.74 ± 1.75	25.13 ± 8.88	26.62 ± 8.14	2.44 ± 1.74	nd	164.19 ± 1.47	11.09 ± 0.45	78.18 ± 16.70	25.41 ± 11.20
Elenolic acid der.	0.82 ± 0.28	4.05 ± 1.33	4.15 ± 0.45	1.05 ± 0.37	0.51 ± 0.04	28.34 ± 0.43	7.54 ± 0.40	16.98 ± 1.17	9.30 ± 4.46
Hydroxycinnamic derivatives	0.03 ± 0.02	0.24 ± 0.13	0.30 ± 0.67	0.21 ± 0.12	7.83 ± 0.25	1.42 ± 0.06	4.30 ± 0.31	1.26 ± 0.88	1.49 ± 0.61
Flavonoids	0.15 ± 0.09	0.56 ± 0.18	0.83 ± 0.13	0.29 ± 0.21	nd	1.27 ± 0.01	1.00 ± 0.41	4.38 ± 1.63	3.08 ± 1.05
Verbascoside	0.09 ± 0.03	0.99 ± 0.31	0.83 ± 0.23	0.71 ± 0.49	1.69 ± 0.17	6.76 ± 0.10	5.85 ± 1.05	4.13 ± 0.44	2.27 ± 0.37
Lignans	nd	3.18 ± 1.16	nd	nd	nd	17.48 ± 0.01	2.65 ± 0.23	nd	nd
Total Polyphenols	4.12 ± 2.12	38.84 ± 10.31	38.91 ± 8.24	8.33 ± 2.51	289.93 ± 18.70	244.15 ± 5.54	57.63 ± 4.42	128.48 ± 20.84	57.53 ± 18.66

Data are mean values of triplicate samples (±SD)

CMF concentrate of microfiltration, *CNF* concentrate of nanofiltration, *CRO* concentrate of reverse osmosis, *GL* green leaves, *DL* dried leaves, *nd* not detected

Table 2 HPLC/DAD quantitative analyses of different plant fractions from *Cynara* leaves (CUF and CRO), and two concentrated fractions from CRO: soft extract and spray-dried from green leaves

	CUF Cynara GL mg/L	CRO Cynara GL mg/L	Soft extract Cynara GL mg/g	Spray-dried Cynara GL mg/g
MCC	1.07 ± 0.58	65.19 ± 13.28	6.61 ± 1.34	14.23 ± 0.48
DCC	2.81 ± 1.19	3.96 ± 5.60	7.64 ± 0.69	7.63 ± 0.20
Chlorogenic acid	2.04 ± 0.47	34.00 ± 7.38	11.93 ± 1.72	12.36 ± 0.03
Cynarin	0.50 ± 0.43	28.94 ± 14.89	1.62 ± 0.01	4.41 ± 0.34
Flavonols	0.23 ± 0.06	10.11 ± 5.39	1.09 ± 0.27	3.48 ± 0.56
Total Polyphenols	6.57 ± 1.92	142.21 ± 9.58	28.90 ± 4.02	42.10 ± 0.42

Data are mean values of triplicate samples (±SD)

CUF concentrate of ultrafiltration, *CRO* concentrate of reverse osmosis, *GL* green leaves

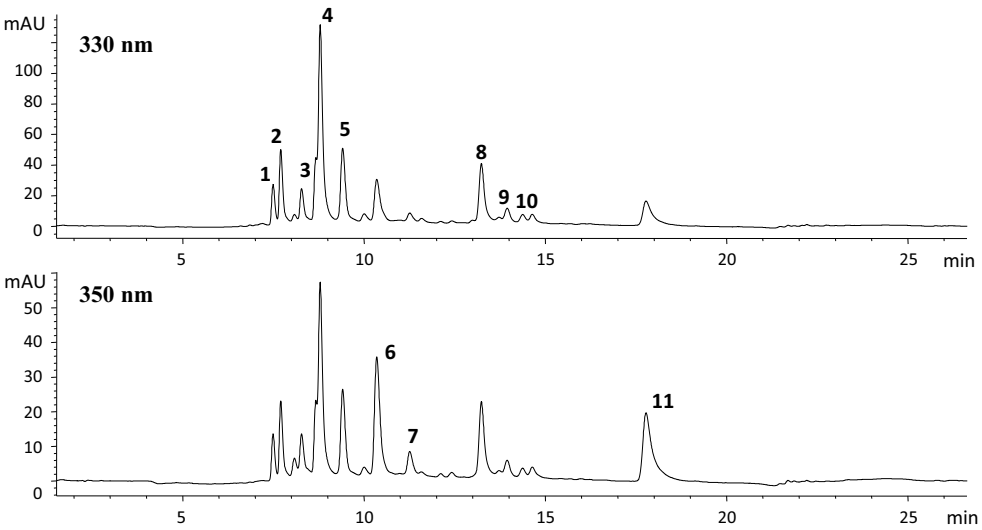

Fig. 3 Chromatogram of *Cynara* GL soft extract. *Peaks*: 1-*O*-caffeoylquinic acid; *2*. 3-*O*-caffeoylquinic acid; *3*. caffeoylquinic acid; *4*. chlorogenic acid; *5* cynarin; *6*. luteolin 7-*O*-rutinoside; *7*. luteolin 7-*O*-glucoside; 8. dicaffeoylquinic acid; *9*. dicaffeoylquinic acid; *10*. dicaffeoylquinic acid; *11*. luteolin

The total polyphenols of the soft extracts obtained from green leaves and pitted olive pulp are very similar, 24.4–28.9% *w/w*, whereas that coming from the dried leaves is 5.7% *w/w*. By applying a spray drying process, a powder with a final concentration of 5.7% *w/w* has been obtained (44.2% of secoiridoids; 27.8% of hydroxytyrosol derivatives; 16.2% of elenolic acid derivatives; 5.3% of flavonoids; 3.9% of verbascoside, and 2.5% of hydroxycinnamic acid derivatives). Table 1 also shows the spray-dried composition of GL extract, with a final concentration of 12.8% *w/w* (60.8% of secoiridoids; 18.3% of hydroxytyrosol derivatives; 13.2% of elenolic acid derivatives; 3.4% of flavonoids; 3.2% of verbascoside; 1% of hydroxycinnamic acid derivatives).

The industrial plant described in this paper can also work with *Cynara scolymus* (artichoke) tissues (leaves and stems) without the nanofiltration equipment. Table 2 shows the HPLC/DAD quantitative analysis of the different plant fractions obtained by processing artichoke leaves, in particular, the concentrate of ultrafiltration (CUF) of Cynara green leaves (GL), the concentrate of reverse osmosis (CRO), the soft extract obtained by heat pump evaporation of CRO, and the spray-dried. All these fractions contain hydroxycinnamic acid derivatives (mono-caffeoylquinic acids, MCC; di-caffeoylquinic acids, DCC; chlorogenic acid, cynarin) and flavonoids (luteolin glycosides and the aglycone). Figure 3 shows the chromatographic profile, recorded at 330 and 350 nm, of the Cynara GL soft extract, with the list of each identified compound. The concentrations of CUF and CRO from GL are strongly different, with a value in total polyphenols of 0.7 and 14% *w/V*, respectively.

Cynara GL soft extract has a concentration of 2.8% *w/w* (41.3% chlorogenic acid; 26.4% DCC; 22.9% MCC; 5.6% cynarin; 3.8% flavonoids), and the spray-dried Cynara GL has a concentration of 4.2% *w/w* (33.8% MCC; 29.3% chlorogenic acid; 18.1% DCC; 10.5% cynarin; 8.3% flavonoids).

Artichoke's stems can be considered an interesting by-product of the agricultural artichoke practices, usually not fully evaluated. However, CUF and CRO fractions obtained by processing these tissues in industrial plants have a total

polyphenolic concentration of 8.5 and 3.94 mg of antioxidants per liter, as this source is also very rich in fibers.

Recently, the system uses industrial solutions of scalding of artichoke heads, which after three cycles of processing are saturated in caffeoyl derivatives with an average concentration of about 0.8–1.2 g/L of total polyphenols.

Antiradical and antioxidant activities by DPPH and ORAC assays

The antiradical activities of *Olea* fractions, evaluated using a DPPH solution (3.16×10^{-4} mM), show the following EC_{50} values: 6.76×10^{-3} mM for the *Olea* GL soft extract (green leaves), 5.44×10^{-3} mM for the *Olea* DL soft extract (dried leaves), and 1.22×10^{-3} mM in the case of the *Olea* OH-TYR soft extract (from pitted olive pulps). The EC_{50} value of the Cynara GL soft extract is 3.25×10^{-3} mM. Concerning the ORAC assay, according to the formula reported into Materials and Methods section, the obtained results are the following: 3632 ± 110.7 μmoL TE/g for OH-Tyr *Olea* soft extract, 1410 ± 62.1 μmoL TE/g for GL *Olea* soft extract, and finally 760 ± 12 μmoL TE/g for GL Cynara soft extract.

Discussion

Concerning the *Olea* extracts, three geographical areas with mills that use the same olive oil milling process (biphasic system) have been considered for the sampling of the by-products (leaves and pitted olive pulps). All the recovered fractions after the membrane-based treatment have a standardized content of active compounds. In this work, the final fractions obtained by processing the cultivar Carboncella are reported.

As shown in Table 1, GL *Olea* CMF fraction is more diluted than the others (CNF and CRO) because of the higher cutoff of the membrane, allowing for the passage of the main part of the molecules, and then concentrated in the following nanofiltration and reverse osmosis steps. It is worth of noting that even so, this fraction can still be used for animal feed and agriculture applications.

The main phenolic compounds in all the fractions, the secoiridoids, show the lowest quantity in the CRO fraction obtained from DL due to the decomposition of oleuropein, one of the main secoiridoid compounds, during the drying process itself. The low concentration of secoiridoids is still evident in the concentrated fractions or soft extracts from olive leaves after their drying process. To partially avoid the secoiridoid loss, and, particularly, in order to increase the percentage of secoiridoids in the DL fraction, a spray drying process can be applied to obtain a powder. The main marketable fractions have been produced by evaporation of CNF and CRO to obtain the previously described soft extracts with a different polyphenolic composition, depending on the initial extracting material (olive leaves and pitted olive pulp). It is worth noting that the soft extract *Olea* OH-Tyr is composed almost exclusively by hydroxytyrosol and derivatives, 96.5% of the total polyphenols. Another marketable fraction can be obtained from appropriate mixing of the concentrated fractions of CNF and CRO of pitted olive pulp, in order to obtain a standard concentration in polyphenolic compounds, generally ranging from 2.3 to 4.5%*w/w*, depending on how the membrane filtration process lasted long. Concerning the final waste after extraction of *Olea* tissues, the residue can undergo a bio-digestion process to obtain biogas and fertilizers, as already happen in highly innovative platforms [25].

The industrial plant described in this paper can also work with *Cynara scolymus* (artichoke) tissues (leaves and stems) without the nanofiltration equipment, which was instead a fundamental step in the *Olea* fractionation, to guarantee the recovery of a hydroxytyrosol (MW 154) enriched fraction.

In this case, the CUF fraction can be used to dilute the CRO, thereby lowering the production costs with an appropriate final concentration of total antioxidants, in order to obtain a standard extract, similar to what was carried out to optimize the extracts of the *Olea* tissues. This plant is an industrial prototype that operates in Apulia district (Italy), and to our knowledge, it is the first plant in Europe that uses these technologies for the extraction of active ingredients from *Cynara* tissues.

Concerning the final waste after extraction of *Cynara* tissues, the residue after the membrane extraction and fractionation is completely depleted or devoid of phenolic compounds, particularly bitter, and then, it may constitute an interesting raw material for feeding farmed animals and ruminants, even with integration of other plants (forage, alfalfa, clover, etc.).

Antiradical and antioxidant activities by DPPH and ORAC assays

The DPPH test is not meaningful to define the different activities between the fractions obtained from the two different species, and then a second antioxidant assay with the ORAC procedure has been carried out for the soft extracts. For this purpose, the soft extracts have been diluted to a final concentration of 1 mg/mL of polyphenols, to make a comparison among the different samples.

Both the in vitro procedures confirm the powerful antioxidant activity of hydroxytyrosol, since among *Olea* samples, the fraction enriched in OH-Tyr has the lowest EC50 value (1.22×10^{-3} mM for the *Olea* OH-Tyr soft extract) and the highest ORAC result.

Due to the previously reported biological activities and their antiradical properties, the individual extracts or their combination allows for specific applications as new formulations in different industrial fields. In particular, associations between different natural extracts or molecules derived from other plant species are under study, mainly in the pharmaceutical and food industry, since the synergies of natural phenolic ingredients used as a source of bioactive compounds to control pathogenic bacteria have been recently investigated [25].

Given the high antioxidant activity of the OH-Tyr, the plant fractions enriched with this molecule are used for the stabilization of bakery products in substitution of the synthetic ascorbic acid and other preservatives. The dose used in biscuits and rusks is 250 ppm of *Olea* OH-Tyr fraction. In a recent patent, the mixture of fractions from *Olea*, *Cynara*, and antimicrobial tannins from chestnut and grapes seeds is proposed as a new ingredient in the meat stabilization and for the decrement of the mycotoxins in food [26].

Therefore, the fractions enriched with OH-Tyr can be used in pharmaceutical, nutraceutical and cosmetic applications, whereas those with lower phenol concentrations can be employed in the food industry, to preserve meat and bakery products [27–29].

Interestingly, due to acting as blood pressure regulators and modulators of triglyceride and cholesterol levels, both olive and artichoke leaf fractions could be used alone or in combination in new formulations of nutraceutical beverages and powders for food supplements [30]. In particular, via use of concentrated solutions and powdered extracts, enriched foods, cosmetics, and food supplements can be designed for the prevention and treatment of aging-related diseases.

Conclusions

Despite the fact that by-products of plant food processing represent a major disposal problem for the relative industry, they are also promising sources of compounds which could be exploited for their favorable technological or nutritional properties. Another important issue, in order to utilize natural antioxidant substances in food, pharmaceutical or cosmetic industries, methods to extract the phenolic compounds from agroindustrial by-products without using organic solvents must be devised. In this paper, standardized fractions enriched with antioxidant compounds were obtained from *Olea* tissues (leaves and pitted olive pulp) and *Cynara* by-products (leaves and stems) through an environmentally friendly process based on a water extraction and membrane separation technology.

The innovative process of separation used, allowed for obtaining individual extracts, which, both alone and in combination, have specific applications due to their biological activities for obtaining new formulations in different application fields, including the pharmaceutical, cosmetic, food, and functional food industries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This study does not contain any experiment involving human or animal subjects.

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