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Sustainability, Innovation, and Green Chemistry in the Production and Valorization of Phenolic Extracts from *Olea europaea* L.

Annalisa Romani [1], Patrizia Pinelli [1], Francesca Ieri [1] and Roberta Bernini [2,*]

[1] Dipartimento di Statistica, Informatica, Applicazioni "Giuseppe Parenti" DiSIA, Laboratorio Phytolab—Polo Scientifico e Tecnologico, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Firenze, Italy; annalisa.romani@unifi.it (A.R.); patrizia.pinelli@unifi.it (P.P.); francesca.ieri@unifi.it (F.I.)

[2] Dipartimento di Scienze Agrarie e Forestali (DAFNE), Università degli Studi della Tuscia, Via S. Camillo De Lellis, 01100 Viterbo, Italy

* Correspondence: berninir@unitus.it; Tel.: +39-761-357-452

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Abstract: This paper describes a circular economy process based on environmentally and economically sustainable procedures which was applied to the sector of olive oil processing on an industrial scale. *Olea europaea* L. tissues and by-products represent a renewable and low-cost source of polyphenols, in particular hydroxytyrosol (HTyr), a naturally occurring compound well known for its biological properties. Specifically, green leaves (GL), dried leaves (DL), and pitted olive pulp were treated with water in a pneumatic extractor to obtain the corresponding polyphenolic extracts. Three standardized fractions, named *Soft Extract Olea GL*, *Soft Extract Olea DL*, and *Soft Extract Olea HTyr* resulted after the following two steps: a separation process carried out by membrane technology, and a concentration step performed under reduced pressure and low temperature. The polyphenolic fractions showed antiradical activity and have potential industrial applications in the food, nutraceutical, pharmaceutical, feed, and agronomic fields. Novel functionalized extracts containing hydroxytyrosol methyl carbonate (HTyr-MC) were obtained from *Soft Extract Olea HTyr* through an innovative approach based on green chemistry procedures, which appear to be a promising tool to increase the applications of the polyphenolic extracts.

Keywords: *Olea europaea* L. by-products; HTyr-enriched fractions; hydroxytyrosol; hydroxytyrosol methyl carbonate; HTyr-derived compounds; circular economy; green chemistry

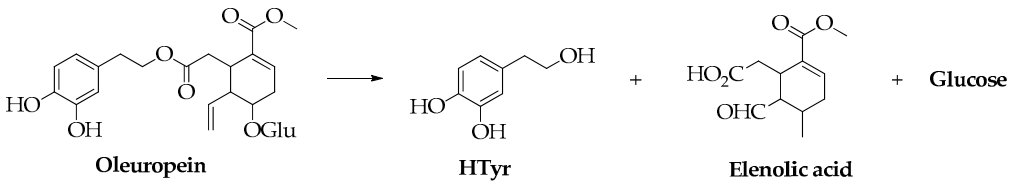
1. Introduction

Sustainability and innovation are some of the keywords of a novel economic concept, named the circular economy, based on legislative proposals suggested and adopted by the European Community to increase global competitiveness, foster economic growth, and create new jobs by saving resources and energy [1]. The aim of the circular economy is to "close the loop" of product lifecycles through greater recycling and re-use, and create benefits for both the environment and the economy [2].

This approach can be applied in almost all manufacturing fields. For example, the agro-industrial field offers a good opportunity when considering the large amounts of waste and by-products produced every year during fruit and vegetable processing. In this area, a circular economy process can be achieved through efficient small and industrial scale bioenergy plants, biorefineries, and environmentally friendly processes to obtain bioactive compounds that can be used as active ingredients for agronomic, cosmetic, food, feed, and pharmaceutical formulations [3,4]. Among these, polyphenols are excellent candidates that have many therapeutic properties. In fact, they have shown

effectiveness as antioxidants in processed foods, wine, and beverages, even at concentrations below 100 ppm; they promote health by reducing the onset of inflammation, cardiovascular illnesses, arthritis, and other free radical mediated diseases including several kinds of cancer [5]. The main sources of polyphenols are vegetables and fruits, including extra-virgin olive oil, an essential component of the Mediterranean diet [6,7]. Unfortunately, blood bioavailability of these compounds—the fraction absorbed by the human body—is low. In recent years, the food, pharmaceutical, and cosmetic industries have introduced functionalized products e.g., products containing secondary metabolites such as polyphenols, to increase prevention against the above-mentioned diseases. This is also a response to the increasing demand related to the "natural lifestyle choices" of consumers. As a result, the polyphenols market has grown rapidly over recent years and is expected to increase at an annual rate, expressed as the Compound Annual Growth Rate (CAGR), of 9% by 2020. At the same time, it is estimated that polyphenols consumption will increase from 12,200 tons to 25,000 tons, reaching a market worth of €900 million, affecting mainly three geographic areas (Asia Pacific, North America, and Europe).

A strong contribution in terms of the source of polyphenols is provided by olive tree cultivation that is particularly widespread in the Mediterranean countries (Spain, Italy, Greece, and Portugal) and in the Northern African countries (Syria, Turkey, Morocco, and Tunisia). The process of olive oil production creates a large amount of waste, including olive mill wastewaters, olive pulp, and leaves. Olive mill wastewaters are the main waste produced from three-phase olive processing while olive pulp is the main waste deriving from two-stage olive oil processing. This last technique, promoted by the European Community, was recently applied in Spain and Italy to eliminate the production of olive mill wastewaters characterized by the high level of toxicity and disposal costs [8]. Olive leaves can be considered as a waste because they derive from both olive processing and pruning practices. These raw materials are a precious source of bioactive compounds including low-molecular weight phenols [9]. In olive leaves, the main constituent is oleuropein (Scheme 1), a phenolic secoiridoid glycoside, which by enzymatic or chemical hydrolysis produces hydroxytyrosol (HTyr), elenolic acid, and glucose [10]. Among these, HTyr is an outstanding biological compound due to its properties, in particular its strong antioxidant activity [11]. These peculiar properties and the absence of genotoxicity [12] make HTyr a good candidate for use as a preservative, thus potentially replacing synthetic food and cosmetic additives such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). It is worth mentioning that these additives have recently raised concerns about their possible mutagenic and carcinogenic effects. At the same time, HTyr plays an important role in pharmaceutical applications for its beneficial health properties such as anticancer activity [13].



Scheme 1. Products of the hydrolysis of oleuropein.

In recent years, international scientific research has proven several biological effects of the polyphenols present in olive leaves and by-products. Numerous studies have shown that they exhibit a wide number of properties including antiradical and antimicrobial activities against microorganisms related to human health, food processing, and agriculture pest control [14]. For example, their effectiveness was tested against *Helicobacter pylori*, the agent responsible for peptic ulcers and some types of gastric cancer, as well as against several foodborne pathogens such as *Salmonella enteritidis* and *Staphylococcus aureus* [15,16].

Taking into account the wide applicability of these compounds, several extractive procedures have been optimized to recover low molecular weight polyphenols from *Olea* tissues and olive oil

by-products [17,18]. Among them, membrane separation technology has been recently developed to fractionate olive mill wastewaters [19–21]. This technology offers several advantages over the conventional methods, mainly in terms of low energy consumption, no additive requirements, and no phase change.

The case-study described here represents an original example of a circular economy process applied to the agricultural system of olive oil processing on an industrial scale. It concerns the valorization of *Olea europaea* L. leaves and pitted olive pulp as a source of bioactive polyphenols to produce standardized extracts to be used in the food, nutraceutical, pharmaceutical, feed, and agronomic fields. An example of green chemistry was reported in order to obtain novel functionalized extracts from HTyr-enriched fractions.

2. Materials and Methods

2.1. Reagents, Plant Material, and Instruments

Reagents, solvents, and authentic standards were supplied by Sigma-Aldrich (Milan, Italy) and were used without further purification. Hydroxytyrosol was synthesized in our laboratory as we have already described using a patented procedure [22,23].

Olea europaea leaves and pitted olive pulp (Frantoio cultivar) were collected in Tuscany (Siena, Italy), Latium (Rieti, Italy), and Apulia (Foggia, Italy) during the year 2015.

Dried leaves (DL) were obtained by two different drying processes: room temperature for 5 days and ventilated stove at 40 °C for 3 days.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 400 MHz Bruker spectrometer using chloroform-d_3, acetone-d_6, and methanol-d as solvents. All chemical shifts are expressed in parts per million (δ scale) and coupling constants are reported in Hertz (Hz).

High Performance Liquid Chromatography/Diode Array Detector (HPLC/DAD) analyses were carried out using a HP 1200 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with an analytical column Lichrosorb RP18 250 × 4.60 mm i.d, 5-μm (Merck Darmstadt, Germany). The eluents were H_2O adjusted to pH = 3.2 with HCOOH (solvent A) and CH_3CN (solvent B). A four-step linear solvent gradient was used starting from 100% of solvent A up to 100% of solvent B, for 88 min at a flow rate of 0.8 mL·min^{-1} [24]. Phenolic compounds found in the extracts were identified by comparing retention times and UV/Vis spectra with those of the authentic standards. Each compound was quantified at the selected wavelength (240, 280, 330, and 350 nm) using a five-point regression curve built with the available standards: tyrosol, hydroxytyrosol, oleuropein, chlorogenic acid, and luteolin 7-*O*-glucoside. All results are reported in Table 1.

Table 1. Total polyphenols and oleuropein present into *Olea europaea* GL and DL (Frantoio cultivar) extracts.

	Samples	Extracted Leaves (%)	Extraction Time (min)	Total Polyphenols and Oleuropein [1,2]
entry 1	GL	15	30	13.6 (38.5)
entry 2	GL	15	60	17.8 (43.9)
entry 3	DL, rt, 15 days	15	30	12.3 (19.5)
entry 4	DL, rt, 15 days	15	60	16.1 (22.0)
entry 5	DL, ventilated stove, 40 °C, 3 days	15	30	11.4 (26.0)
entry 6	DL, ventilated stove, 40 °C, 3 days	15	60	16.8 (41.0)

[1] Total polyphenols are mg/g for GL, mg/g dry weight for DL; [2] Oleuropein percentage is reported within brackets. All results are the mean of three analyses with standard deviations <5%.

2.2. Extraction of Low-Molecular Weight Phenolic Compounds

Olea europaea L. extracts were obtained according to a patented eco-friendly and economically sustainable process [25,26]. The extractions were carried out in a pneumatic extractor (Timatic series,

Tecnolab S.r.l., Spello, Perugia, Italy) using water as the extractive solvent. In detail, 30 kg of green leaves (GL), 30 kg of dried leaves (DL), or 50 kg of pitted olive pulp required 200 L of water. The process was carried out in a stainless steel basket at 70 °C for 30 or 60 min. The working cycle was fully automatic and alternated 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 was generated during the stationary phase, which thanks to the programmable recirculation, ensured a continuous flow of the solvent within the plant matrix. This avoided any over-saturation or formation of preferential channels, thus ensuring the total extraction of polyphenols from the plant materials. The extractions were performed in triplicate. In the pre-treatment phase, the pH was lowered from the original value of 5.7 to 3.5 by adding concentrated HCl and citric acid (1% v/v) in order to inactivate the polyphenol oxidase present in the aqueous raw extracts and avoid any enzymatic oxidation. This was also done to create the optimal conditions for the subsequent addition of the pectinase enzyme when the processed sample was pitted olive pulp.

2.3. Fractionating of the Phenolic Extracts

The fractionating of the extracts was done with membrane technology, notably by Microfiltration (MF), Ultrafiltration (UF), Nanofiltration (NF), and Reverse Osmosis (RO). The membranes were characterized by different molecular weights with cut-off and filtration degrees. During the manufacturing process, the MF stage was carried out with tubular ceramic membranes in titanium oxide while the UF, NF, and RO stages were conducted with spiral wound module membranes in polyether sulfone (PES) [26]. At the end of each working cycle, the membrane modules were automatically washed to remove any sediments settled on the membranes and to reduce the membrane fouling. In particular, the MF membrane was washed with tap water for 5 min, a basic solution for 30 min, then an acidic medium until reaching neutral pH and finally with distilled water; NF and RO membranes were simply washed first with tap water and then with distilled water [25,26].

2.4. Isolation of HTyr-Enriched Fractions

Each fraction derived from the MF, UF, and RO stages was concentrated by using a scraper evaporator series provided with a heat-pump (C&G Depurazione Industriale Company, Firenze, Italy) (Figure 1). Specifically, the boiler was kept at P = −1 bar. The depression was guaranteed by the presence of a vacuum pump which allowed the evaporation of the aqueous solution at low temperatures (35 °C) and the minimization of energy consumption. Three standardized extracts were obtained upon completion of the process: *Soft Extract Olea GL* from GL; *Soft Extract Olea DL* from DL, and *Soft Extract Olea HTyr* from pitted olive pulp.

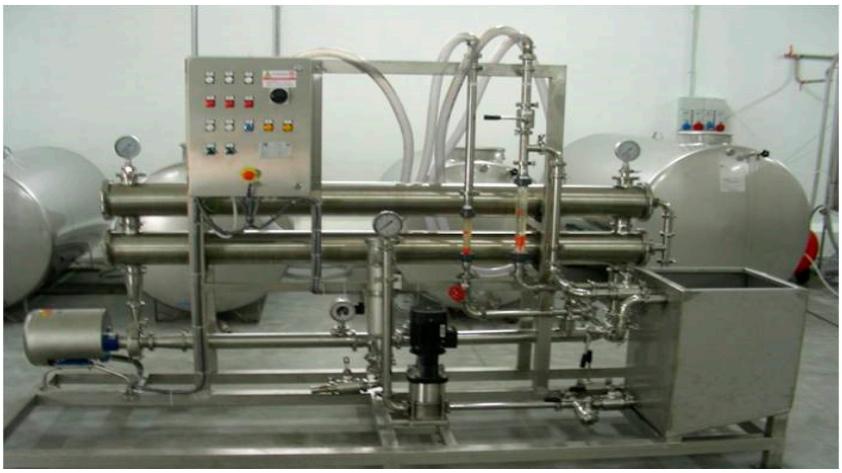


Figure 1. The separation system based on the membrane technology.

2.5. Evaluation of the Antiradical Activity of Soft Extract Olea GL, Soft Extract Olea DL, and Soft Extract Olea HTyr

The antiradical capacity (AR) of *Soft Extract Olea GL*, *Soft Extract Olea DL*, and *Soft Extract Olea HTyr* was evaluated by using the 1,1-diphenylpicrylhydrazil radical (DPPH) assay [27]. The data were expressed in percentages according to the following relationship: $AR\% = 100 \times (A_0 - A_{20})/A_0$, where A_0 and A_{20} were the absorbance of DPPH at 517 nm at the initial time and after 20 min, respectively.

2.6. Functionalization of the HTyr-Enriched Fraction (Soft Extract Olea HTyr)

Methanol or ethanol (5 mL) was added to 100 mg of *Soft Extract Olea HTyr*. After the removal of the precipitate represented by the inorganic salts, the filtered solution containing standardized HTyr was recovered. The alcoholic solvent was removed under reduced pressure; then, the residue was treated with dimethyl carbonate (DMC) at the reflux temperature in the presence of a catalyst: 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) or sulfuric acid for 3 or 7 h [28]. The yields were calculated by HPLC analysis at 280 nm to be 90% and 92%, respectively.

3. Results and Discussion

Olea europaea GL and DL of Frantoio cultivar were the first matrices utilized to recover low-molecular weight phenolic compounds. The extractions were repeated in triplicate on 15% *v*/*v* on fresh weight and dry weight for 30 and 60 min in a pneumatic extractor (see the Experimental Section). The experimental conditions are summarized in Table 1. As reported in the last column, the total polyphenol content (expressed as mg/g) and the percentage of oleuropein varied according to the starting material status, namely GL or DL. Prolonging the extraction time from 30 to 60 min resulted in an increase of both parameters, and was observed in all cases (compare *entry 1* to *entry 2*; *entry 3* to *entry 4*; *entry 5* to *entry 6*). However, these results may not justify a production of high extraction volumes given the low cost of the raw materials.

As previously stated, pitted olive pulp was also used as a raw material, being a consistent olive oil by-product deriving from two-stage olive processing. The industrial process to obtain standardized phenolic extracts included three steps: (1) extraction of low-molecular weight phenolic compounds from raw materials; (2) fractionation of the phenolic extracts; (3) concentration of the fractions. The first step was carried out in a pneumatic extractor, as previously described, and was followed by an enzymatic pre-treatment. 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 allowed both the release of the bioactive compounds from the *Olea* material and the optimized recovery of HTyr. The fractionation of the phenolic extracts was then performed by an innovative separation system based on membrane technology defined as BAT (Best Available Technology) and recognized by the EPA (Environmental Protection Agency) [25,26]. This separation system consisted of MF, NF, and RO stages. The third step of the industrial process was carried out in order to increase the concentration of bioactive compounds in the final extracts. It was performed in a heat-pump evaporator using the fractions derived from MF, NF, and RO. Three standardized extracts were obtained: *Soft Extract Olea GL* from GL; *Soft Extract Olea DL* from DL; and *Soft Extract Olea HTyr* from pitted olive pulp. For each extract, the total polyphenols and the content of hydroxytyrosol, secoiridoid, elenolic acid derivatives, flavonoids, verbascoside, and lignans were quantified by HPLC/DAD analysis (Table 2). As shown, the content of total polyphenols of *Soft Extract Olea GL* and *Soft Extract Olea HTyr* was very similar (24% and 29% *w*/*w*, respectively) whereas that of *Soft Extract Olea DL* was significantly lower (about 6% *w*/*w)* [29]. In particular, *Soft Extract Olea HTyr* was rich in HTyr and HTyr derivatives (96.5% of the total polyphenols); *Soft Extract Olea GL* contained secoiridoids as the main components and among them, oleuropein accounted for 67.2% of the total polyphenols. As expected, *Soft Extract Olea DL* contained secoiridoids in only 19.2% of the total polyphenols, showing the occurrence of a great loss of oleuropein during the drying process.

Table 2. Polyphenols present into the standardized extracts obtained by the industrial plant. [1]

	Compounds	*Soft Extract Olea GL*	*Soft Extract Olea DL*	*Soft Extract Olea HTyr*
entry 1	Hydroxytyrosol derivatives	24.69 ± 3.47	25.21 ± 1.56	279.89 ± 18.24
entry 2	Secoiridoid derivatives	164.19 ± 1.47	11.09 ± 0.45	nd
entry 3	Elenolic acid derivatives	28.34 ± 0.43	7.54 ± 0.40	0.51 ± 0.04
entry 4	Flavonoids	1.42 ± 0.06	4.30 ± 0.31	7.83 ± 0.25
entry 5	Verbascoside	1.27 ± 0.01	1.00 ± 0.41	1.69 ± 0.17
entry 6	Lignans	6.76 ± 0.10	5.85 ± 1.05	nd
entry 7	Total Polyphenols	244.15 ± 5.54	57.63 ± 4.42	289.93 ± 18.70

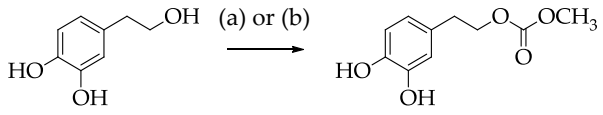
[1] Data expressed in mg/g, nd = not detected.

An economic estimate of the described industrial platform was calculated considering 79,300 kg of processed olives which produced 66,600 kg of pitted olive pulp. After extraction, membrane separation, and concentration processes, a total of 2940 kg of extracts were obtained (yield: 4.4%). The production costs and market prices of the different extracts increased proportionally to the HTyr percentage. In particular, the production cost varied from 11.34 to 16.34 €/Kg and the market price varied from 30 to 100 €/Kg. The estimated net income per year for the platform was about €70,000.

Soft Extract Olea GL, *Soft Extract Olea DL*, and *Soft Extracts Olea HTyr* were tested for their antiradical activity by the DPPH assay. The data evidenced a high activity for all fractions: 74.5%, 80.9%, and 97.8%, respectively. In consideration of this, all extracts are of industrial interest. *Soft Extract Olea GL* and *Soft Extract Olea DL* can be employed in the food industry to preserve meat and bakery products [30,31], whereas *Soft Extract Olea HTyr*, rich in HTyr, can be used in pharmaceutical, nutraceutical, and cosmetic applications in combination with chestnut tannin extracts [32].

In this paper, we described an original approach towards the utilization of HTyr-enriched extracts to increase their potential applications. Specifically, we reported an example of functionalization of these extracts by green chemistry procedures. As described in the Experimental Section, *Soft Extract Olea HTyr* was treated with DMC and a catalyst (Scheme 2). HTyr present in *Soft Extract Olea HTyr* was converted into hydroxytyrosol methyl carbonate (HTyr-MC), previously obtained in our laboratory from pure HTyr under similar conditions [28]. The efficiency of the functionalization of HTyr results are shown in Figure 2 which represents the HPLC profile of *Soft Extract Olea HTyr* before and after the process.

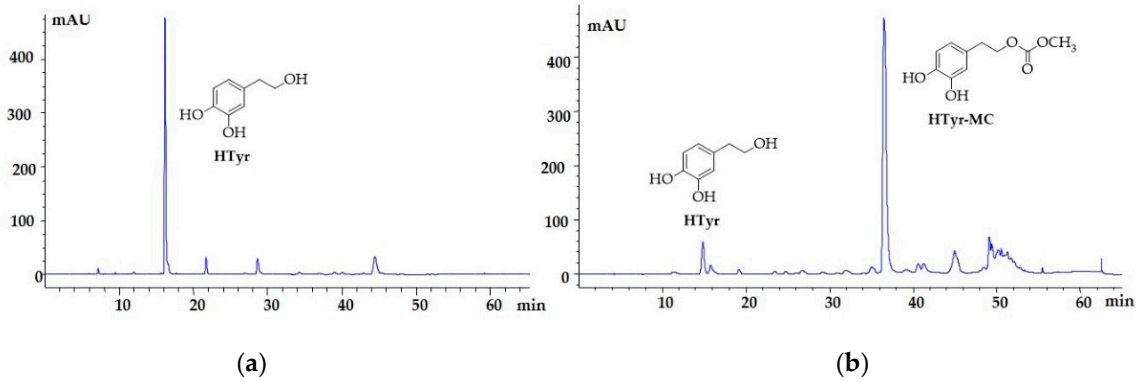
As is well known, DMC is an eco-friendly chemical that is able to act both as a solvent [33] and a reagent [34] depending on the experimental conditions. The selective introduction of the methyl carbonate moiety into HTyr reduces the hydrophilicity, while the free catecholic moiety guarantees the antioxidant activity. In vitro tests confirmed the efficiency of HTyr-MC as a radical scavenger [28] and the lack of cytotoxicity makes it possible to be used as a preservative for food applications [35]. Recently, HTyr-MC was added into poly(vinyl alcohol) (PVA) matrices to obtain novel PVA-based binary films useful for packaging applications, to preserve food susceptible to oxidation [36]. Anticancer assays performed on melanoma (M14), pulmonary (H125), colon (WiDr), and promyelocytic leukaemia (HL60) cell lines revealed that HTyr-MC was more effective than HTyr in cell growth inhibition and apoptosis induction [37]. Moreover, HTyr-MC was previously used as a synthetic precursor of a large panel of novel HTyr-derived compounds [38–42].



Soft Extract Olea HTyr **Novel functionalized extracts**

(a) DMC/DBU, reflux, 3 h; (b) DMC/H_2SO_4, reflux, 7 h

Scheme 2. Obtaining the functionalized *Soft Extract Olea HTyr*.



(**a**) (**b**)

Figure 2. HPLC/DAD chromatograms of *Soft Extract Olea HTyr.* (**a**) Before and (**b**) after the functionalization.

Due to these various applications of HTyr-MC, obtaining functionalized extracts containing this compound from *Olea europaea* by-products appears to be a target for increasing the potential applications of these extracts as an integrated step of the circular economy process applied to the olive oil processing sector.

4. Conclusions

Olive oil by-products and olive leaves produced from pruning practices represent an attractive source of biologically active compounds, and green chemistry procedures can play an effective role to increase their potential applications. In this study, *Olea europaea* green leaves, dried leaves, and pitted olive pulp were used to obtain different polyphenolic extracts useful for industrial applications and for the production of novel molecules. The technology optimized to prepare these extracts is environmentally and economically sustainable and applicable on an industrial scale. The extracts could be employed to design and market a great number of stabilized or enhanced products, such as baked foods, cosmetics, and supplements for human health. Novel functionalized extracts were prepared by applying green chemistry semi-synthesis techniques from HTyr-enriched fractions, offering additional potential uses.

The results described in this paper highlight the possibility of the creation of a multifunctional platform based on the reuse of agricultural waste and agro-industrial by-products by sustainable processes, according to the circular economy concept, in order to "close the loop" of product lifecycles.

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Author Contributions: Annalisa Romani designed the industrial plant for the optimization of polyphenol high-scale extraction and separation via membrane-technology. Patrizia Pinelli performed the analytical characterization of the polyphenols of the extracts. Francesca Ieri, an expert of the industrial plant, estimated the economic impact. Roberta Bernini planned, designed, and performed the experiments for the functionalization of HTyr-enriched extracts. All authors have contributed to writing the paper. Roberta Bernini and Annalisa Romani have done the critical revision and editing.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

HTyr	Hydroxytyrosol
HTyr-MC	Hydroxytyrosol methyl carbonate
GL	Green leaves
DL	Dried leaves
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 HTyr	Olea europaea fraction deriving from pitted olive pulp
HPLC/DAD	High Performance Liquid Chromatography/Diode Array Detector

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