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Wild *Olea europaea* Leaves as an Alternative Source of Antioxidant and Anti-Inflammatory Compounds to Cultivated Olive Tree Leaves for Cosmetic Ingredients

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Abstract

This study assessed the potential of Oleaster leaves as a valuable cosmetic ingredient by comparing aqueous and hydroalcoholic ultrasound-assisted extracts of Oleaster leaves to those of the Olive tree. The hydroalcoholic Oleaster leaf extract showed a higher content in phenolic and flavonoid compounds, with an oleuropein and verbascoside content of 58 mg/g of dry leaves and 3.2 mg/g of dry leaves, respectively. To refine the comparison of their molecular composition, the extracts were analyzed using UHPLC-HRMS/MS. About twenty compounds, including secoiridoids, flavonoids and triterpenic acid derivatives, were annotated in both extracts, demonstrating their similarity. DPPH, CUPRAC and enzymatic SOD assays showed a good antioxidant activity with high inhibition (60–90%) for both Oleaster and Olive tree leaf extracts. The hydroalcoholic extracts at 62.5 µg/mL also exhibited 60–80% of protection against reactive oxygen species (ROS) in H_2O_2-stressed HaCaT cells, confirming this antioxidant capacity without demonstrating a severe cytotoxicity, which remained below 40%. The anti-inflammatory potential of the extracts was also demonstrated using COX-2 inhibition, which was around 70%, and by measuring the concentration of IL-8 in HaCaT cells under pro-inflammatory conditions, which decreased in the presence of extracts at a concentration of 50 pg/mL, similar to that observed for the positive control. Thus, the hydroalcoholic ultrasound extract of Oleaster leaves demonstrated its high potential to develop sustainable and active cosmetic ingredients.

Keywords: wild Oleaster; cosmetic ingredient; UHPLC-HRMS/MS; antioxidant; anti-inflammatory

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Academic Editor: Enzo Berardesca

Received: 31 March 2026
Revised: 3 June 2026
Accepted: 9 June 2026
Published: 15 June 2026

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1. Introduction

The wild Olive tree or Oleaster (*Olea europaea* var. *sylvestris*) and the cultivated Olive tree (*Olea europaea* var. *europaea*) constitute the two botanical varieties of *Olea europaea* [1]. In France, the olive tree is mainly found in the south of France, as it is one of the most widely cultivated trees in the Mediterranean Basin. It is well adapted to the mild and humid winters and hot and dry summers. The heavy rainfall in the cold season ensures substantial Olive production [2,3]. It is used for several traditional medicinal, gastronomic and industrial applications [4]. The Olive tree is well known for its fruits and particularly for their oil,

rich in unsaturated fatty acids, which have positive effects on health [5,6]. Olive tree leaves are of great interest and are used as traditional herbs due to their biological properties as antioxidants, anti-inflammatory agents, antimicrobials, hypoglycemics, antihypertensives and hypocholesterolemics [4,7–10].

Olive tree leaves have a characteristic phytochemical composition containing various bioactive compounds such as secoiridoids, flavonoids, phenolic compounds, and triterpenes [4–6,11]. Oleuropein is reported to be the main compound in the leaves and possesses significant antioxidant, anti-inflammatory and antimicrobial activities [11–13]. However, other polyphenols present (hydroxytyrosol, tyrosol, verbascoside), flavonoid aglycones (luteolin, apigenin, quercetin, diosmetin), and glycosides (luteolin-O-glucoside, luteolin-O-rutinoside, apigenin-O-glucoside) have an important impact on the biological properties of Olive tree leaves [4,6,13].

Olea europaea var. *sylvestris* is a wild variety of *Olea europaea*, better known as Oleaster. It is distinguished by both morphological and genetic features [1]. Oleaster is a shrub rather than a tree with spiny branches, more or less lanceolate leaves, and fruits that are smaller than those of the cultivated Olive tree [1]. Compared to the olive tree, the wild Oleaster is more resistant to abiotic and biotic stresses [1,14].

In a context of sustainable sourcing for developing locally produced cosmetic ingredients, both plants are grown in the Centre-Val de Loire region in France, where the cooler and rainier climatic conditions differ from those of the Mediterranean basin, but are currently changing due to global warming. The wild Oleaster seems to be more appropriate for this climate due to its higher resistance. While numerous studies have reported the phytochemical composition and biological properties of the cultivated Olive tree, there is a lack of information on those of wild Oleaster. Therefore, this study aimed to compare the phytochemical composition and biological activities of Oleaster leaf extracts with those of Olive tree leaves, in order to valorize Oleaster leaves as an active ingredient. Hydroalcoholic and aqueous ultrasound-assisted extraction were performed for both varieties. Then, to compare the leaf extracts, total phenolic and flavonoid contents were evaluated. High Performance Thin Layer Chromatography (HPTLC) and Ultra High-Performance Liquid Chromatography (UHPLC) methods were used to obtain the qualitative phytochemical profiles and were applied to the oleuropein and verbascoside quantification. UHPLC hyphenated to high-resolution mass spectrometry (UHPLC-HRMS/MS) was performed to obtain a finer annotation of the molecules present in the samples. To evaluate the antioxidant and anti-inflammatory potential of the leaf extracts DPPH and CUPRAC, superoxide dismutase (SOD) and cyclooxygenase-2 (COX-2) assays were implemented. HaCaT cell culture assays were also carried out to evaluate the cytotoxicity using the XTT assay. Lastly, antioxidant and anti-inflammatory activities protecting HaCaT were studied by measuring reactive oxygen species neutralization (DCFH-DA) and IL-8 production (ELISA), respectively.

2. Materials and Methods

2.1. Chemicals

HPLC-grade ethanol absolute (99.8%), HPLC-grade ethyl acetate (99.7%), formic acid and acetic acid were purchased from VWR (Fontenay-sous-Bois, France). Folin-Ciocalteu reagent, 2-Aminoethyl diphenylborinate (Neu, 97%), Polyethylene glycol 400, 2.2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), copper chloride, ammonium acetate (99%), neocuproine (98%), chlorogenic acid, 3,4-dihydroxyphénylalanine (L-Dopa, 98%), tyrosinase from mushroom, the SOD determination Kit (reference: 19160) and the COX 2 fluorescent inhibition assay kit (reference: MAK399) came from Merck (Saint-Quentin-Fallavier, France). Oleuropein and Verbas-

coside standards came from Extrasynthèse (Genay, France). Gallic acid (98%) standard and HPLC-grade acetonitrile were purchased from Honeywell (Saint-Germain-en-Laye, France). Aluminum chloride (99%) came from Thermo Fisher Scientific (Illkirch Graffenstaden, France). Rutin standard was purchased from Biosynth (Compton, UK). Milli-Q water was produced on a Milli-Q EQ 7000 water purification system from Merck (Darmstadt, Germany). Chemicals such as dimethylsulfoxide (DMSO; reference D4540-500ML), tocopherol (reference T3251-25G), dichlorodihydrofluorescein diacetate (DCFH-DA; reference D6883), 30% *w*/*w* hydrogen peroxide (reference H1009), dexamethasone (reference D4902-25MG), and lipopolysaccharides (LPS) from *E. coli* O55:B5 (reference L2880-25MG) were purchased from Merck (Saint-Quentin-Fallavier, France). Cell culture DMEM High Glucose serum-free medium (reference L0106-500), heat-inactivated fetal bovine serum (FBS; reference S181H-500), penicillin/streptomycin (reference L0022-100), glutamine (reference X0550-100) and phosphate buffer saline (PBS; reference L0616-500) were purchased from Biowest (Nuaillé, France). The Cell Viability assay kit CyQUANT™ XTT (reference 15960972) was purchased from Invitrogen™ Thermo Fisher Scientific (Illkirch Graffenstaden, France) and the ELISA kit for Human IL-8/CXCL8 (reference DY208) was from R&D Systems (R&D Systems, Inc., Minneapolis, MN, USA).

2.2. Plant Material and Extraction

Wild Oleaster and cultivated Olive tree were cultivated in the botanical garden of Laboheme, a French cosmetic company, located in Vierzon in the Centre-Val de Loire region in France. Olive tree and Oleaster mature leaves were harvested in March 2023 (WGS84 GPS coordinates: latitude 47.212444, longitude 2.09028). The leaves were freeze-dried for 2 days and then ground at a rotation speed of 2000 rpm using a Polymix PX-MFC 90 mill (Kinematica, Malters, Switzerland).

Ultrasound-assisted extraction of Olive tree and Oleaster leaves was performed in an ultrasonic cleaning bath (VWR, Fontenay-sous-Bois, France) at a frequency of 45 kHz. Olive tree or Oleaster leaves were introduced with a hydroalcoholic solvent (EtOH/H_2O 50/50, *v*/*v*) or distilled water with a raw material/extraction solvent ratio of 1/20 (*m*/*v*). The extraction time was 30 min [15]. Extracts were centrifuged at a rotation speed of 4000 rpm for 5 min, then filtered at 0.45 µm with a PVDF syringe. The extracts were evaporated using a rotary evaporator (Buchi, Villebon-sur-Yvette, France) and resolubilized at 5 mg/mL with an EtOH/H_2O (50/50, *v*/*v*) mixture for analysis.

To determine the dry matter percentage and the extraction yield, 1 mL of each extract was introduced into a 2 mL vial. The extract was weighed and evaporated under nitrogen flow, and the dried extracts were weighed. The procedure was performed in triplicate. The percentage of dry matter and the extraction yield were calculated using Equations (1) and (2):

$$\%\boldsymbol{dry\ matter} = \frac{\boldsymbol{m_{dry\ extract}}}{\boldsymbol{m_{liquid\ extract}}} \times \mathbf{100} \quad (1)$$

$$\boldsymbol{Yield}\ (\%) = \frac{\boldsymbol{m_{dry\ extract}}}{\boldsymbol{m_{dry\ plant}}} \times \mathbf{100} \quad (2)$$

2.3. Total Phenolic Content (TPC) Determination

Total phenolic content (TPC) was determined using the Folin-Ciocalteu assay on a 96-well plate (method described by Singleton and Rossi [16] with slight modifications). The extract concentration for the assay was 5 mg/mL. A gallic acid standard was prepared at 0.6 mg/mL to obtain a calibration range from 0.6 mg/mL to 0.05 mg/mL. Then, 20 µL of extract or gallic acid calibration solutions were deposited in the plate, and 10 µL of Folin-Ciocalteu reagent was added. After incubating for 8 min at room temperature (25 °C),

30 µL of sodium carbonate at 2% and 140 µL of purified water were added. Blanks were realized for each sample to correct the sample color without adding Folin-Ciocalteu reagent. The plate was incubated for 2 h at room temperature, and absorbance was measured at 760 nm using a CLARIOstar® Plus microplate reader (BMG Labtech, Ortenberg, Germany). The concentration was calculated in gallic acid equivalence using the calibration curve and the following Equation (3):

$$\textbf{\textit{Concentration}}\ (\textbf{mg eq gallic acid/mL}) = \frac{(\textbf{\textit{Abs}}_{\textbf{\textit{extract}}} - \textbf{\textit{Abs}}_{\textbf{\textit{blank}}}) - \textbf{\textit{b}}}{\textbf{\textit{a}}} \quad (3)$$

$Abs_{extract}$, the absorbance of Folin solution in the presence of extract, Abs_{blank}, the absorbance of the purified water in the presence of samples, a the slope and b the intercept of the gallic acid calibration curve.

2.4. Total Flavonoid Content (TFC) Determination

Total flavonoid content was determined using aluminum chloride at 1% in aqueous solution on the 96-well plate (method described by Ismail et al. [17], with slight modifications). The extract concentration for the assay was 5 mg/mL. A standard solution of rutin was prepared at 0.6 mg/mL to obtain a calibration range from 0.6 mg/mL to 0.05 mg/mL. Next, 10 µL of extract or rutin calibration solutions and 190 µL of aluminum chloride 1% solution were deposited on the plate. For blanks, 190 µL of purified water was added to the other wells instead of the aluminum chloride solution in order to correct the color of the samples. The plate was incubated for 5 min at room temperature (25 °C), and absorbance was measured at 435 nm using a CLARIOstar® Plus microplate reader. The concentration was calculated in rutin equivalence using the calibration curve and the following Equation (4):

$$\textbf{\textit{Concentration}}\ (\textbf{mg eq rutin/mL}) = \frac{(\textbf{\textit{Abs}}_{\textbf{\textit{extract}}} - \textbf{\textit{Abs}}_{\textbf{\textit{blank}}}) - \textbf{\textit{b}}}{\textbf{\textit{a}}} \quad (4)$$

$Abs_{extract}$ is the absorbance of aluminium chloride solution in the presence of extract, Abs_{blank}, the absorbance of the purified water in the presence of samples, a the slope and b the intercept of the rutin calibration curve.

2.5. Polyphenolic Profile Analysis Using HPTLC

HPTLC analyses were performed with a CAMAG (Muttenz, Switzerland) analytical system comprising an Automatic TLC Sampler ATS4 for sample deposition, an ADC2 chamber for plate pre-saturation and plate elution, a Derivatizer for compound revelation with chemical reagents and a TLC Visualizer 2 for plate visualization at visible light, 254 nm and 366 nm. Analyses were processed using WinCATS 3.2 PS 2 software.

The polyphenol analysis was performed with a glass silica gel 60 F254 plate (10 × 10 cm). The extracts were prepared at a concentration of 5 mg/mL, and a volume of 5 µL was deposited on the plate with ATS4. The plate was developed using a mixture of ethyl acetate/water/formic acid/acetic acid (100/27/11/11, $v/v/v/v$) as mobile phase in a pre-saturated ADC2 chamber. The development conditions were 20 min for the tank saturation time, 70 mm for the migration distance, and 5 min for the plate drying time. Derivatization of polyphenolic compounds with 2 mL of diphenylborinic acid aminomethylester (1 g in 100 mL of methanol) and polyethylene glycol 400 (5 g in 100 mL of methanol) reagents was carried out using the Derivatizer with respectively the green nozzle, level 3 and the blue nozzle, level 3. The plate was observed using the TLC visualizer 2 at visible light, 254 nm and 366 nm.

2.6. Analysis of Polyphenolic Content Using UHPLC-DAD

UHPLC was performed with a Thermo Fisher Scientific (Germering, Germany) Ultimate 3000 RSLC system with a binary pump and automatic sampler, a thermostated column compartment and a Diode Array Detector. The column was a Luna Omega C18 (150 × 2.1 mm; 1.6 µm) from Phenomenex (Le Pecq, France). Elution was performed at a flow rate of 0.4 mL/min using a mixture of water acidified with 0.1% formic acid (mobile phase A) and acetonitrile acidified with 0.1% formic acid (mobile phase B). The gradient was as follows: from 5% to 40% solvent B in 22 min in a linear gradient; the amount of B was then increased up to 100% from 22 min to 25 min; then maintained at 100% from 25 min to 28 min, and decreased to the initial conditions from 28 min to 28.5 min. Lastly, the column was re-equilibrated during 10.5 min with 5% of B. The oven temperature was fixed at 40 °C. Samples were prepared at a concentration of 5 mg/mL with a mixture of EtOH/H_2O (50/50; *v*/*v*). The injection volume was 2 µL. The range of DAD detection was between 190 nm and 800 nm, and the absorbance was fixed at 230 nm and 280 nm for oleuropein and verbascoside quantification, respectively. Oleuropein and verbascoside calibration stock solutions were prepared at 1.0 mg/mL, then diluted to obtain a calibration range between 0.1 mg/mL and 1.0 mg/mL and between 0.005 mg/mL and 0.1 mg/mL, respectively. The equations of the calibration curves were $\boldsymbol{y = 17.7149x - 0.1920}$ with a $\boldsymbol{R^2 = 0.9986}$ and $\boldsymbol{y = 51.7666x - 0.1043}$ with a $\boldsymbol{R^2 = 0.9996}$ for oleuropein and verbascoside quantification, respectively.

2.7. Annotation of Compounds Using UHPLC-DAD-HRMS/MS

Chromatographic separations were performed on an Ultimate 3000 RSLC system equipped with an autosampler, a binary pump, a thermostated column compartment, and a DAD detector (Thermo Fisher Scientific, Germering, Germany). The stationary phase was a Luna Omega C18 (150 × 2.1 mm; 1.6 µm) column provided by Phenomenex (Le Pecq, France). An elution gradient was applied using a mixture of acidified water (A) and acidified acetonitrile (B), both with 0.1% formic acid. The gradient was as follows: from 3% solvent B to 40% in 13 min in a linear gradient; the amount of B was then increased up to 100% from 13 min to 21 min; maintained at 100% from 21 min to 24 min, and decreased to the initial conditions from 24 min to 24.5 min. Lastly, the column was re-equilibrated during 3.5 min with 3% of B. The flow rate was 0.4 mL/min, and the injection volume was 1.5 µL for positive ionization mode mass analysis and 2.5 µL for negative ionization mode mass analysis. MS and MS/MS experiments were performed on a maXis UHR-Q-TOF mass spectrometer (Bruker, Bremen, Germany) with an electrospray ion source (ESI). MS was applied in negative and positive ionization modes, and MS/MS was applied in positive ionization mode since more fragments were produced. The nebulizing gas pressure was set at 2 bar, and the dry gas flow rate was 9 L/min at 200 °C. The capillary voltage was 4500 V and 4000 V for positive and negative ionization, respectively. Mass spectra were recorded with a range between 50 and 1650 m/z. The acquisition frequency was set at 400 ms for the positive ionization and 1000 ms for the negative ionization.

Auto MS acquisition was used for MS/MS^+ experiments with 2 precursor ions in the m/z range of 250–1200. Two collision energies were applied: 15–35 eV between 250 and 1200 m/z and 22.5–52.5 eV over 1200 m/z.

All the MS data were processed using the DataAnalysis software, version 4.4 (Bruker, Bremen, Germany). The molecular formulas were generated using the SmartFormula algorithm with an elemental composition of C, H, O to an infinite number and $N \leq 2$ with mass accuracy ≤ 5 ppm and were submitted to the SciFinder, PubChem, Lotus and KNApSAcK databases in order to propose compound annotations.

2.8. Chemical and Enzymatic Assays

2.8.1. DPPH Radical Scavenging Activity Assay

The free radical scavenging activity was determined using the DPPH radical (method described by Blois [18] with slight modifications) in triplicate. A DPPH solution was prepared at a concentration of 0.2 mM with ethanol. Trolox was used as the reference inhibitor, and two concentrations were prepared (0.1 and 1.0 mg/mL) in ethanol. Samples were dissolved in ethanol at 2.5 mg/mL and 1.0 mg/mL. In the microplate, 10 µL of samples (extracts, trolox and blank (EtOH)) were deposited, then 190 µL of DPPH solution was added. Ethanol was added to the other wells instead of the DPPH solution to correct the color of the samples. The plate was incubated in the dark for 30 min, and the absorbance was measured at 516 nm using a CLARIOstar® Plus microplate reader. The percentage of inhibition was calculated using Equation (5):

$$\%\boldsymbol{inhibition} = \frac{\left(\boldsymbol{Abs_{blank}} - \boldsymbol{Abs_{samples}}\right)}{\boldsymbol{Abs_{blank}}} \times \mathbf{100} \quad (5)$$

$Abs_{samples}$ is the absorbance of DPPH solution in the presence of the samples and Abs_{blank} the absorbance of DPPH in the presence of ethanol only. The blank with EtOH solution in the presence of samples or EtOH only was subtracted.

Trolox reference inhibitor presented a DPPH inhibition of 87.29 ± 0.58% at 1 mg/mL and of 36.75 ± 0.41% at 0.1 mg/mL.

2.8.2. CUPRAC Reducing Activity Assay

The copper-reducing capacity was determined using the $[Cu(NC)_2^{2+}]$ complex (method described by Apak et al. [19] with slight modifications) in triplicate. An ammonium acetate buffer solution, pH = 7, was prepared at 1 M. A $Cu(II)Cl_2$ solution was prepared at 10 mM in the buffer solution, while the Neocuproine was dissolved at 7.5 mM in ethanol. The three solutions were mixed at a ratio of 1/1/1 (CUPRAC solution). Trolox was used as the reference active molecule, and a calibration range was prepared between 0.05 mg/mL and 1.8 mg/mL. In the microplate, 10 µL of samples (extracts, trolox and solubilization solvent) were deposited, then 190 µL of CUPRAC solution was added. The buffer solution was added to the other wells instead of the CUPRAC solution to correct the color of the samples. The plate was incubated in the dark for 30 min, and the absorbance was measured at 450 nm using a CLARIOstar® Plus microplate reader. The copper-reducing capacity was calculated in trolox equivalence using Equation (6):

$$\boldsymbol{Concentration}\ (\mathbf{mg\ eq\ trolox/mL}) = \frac{\left(\boldsymbol{Abs_{extract}} - \boldsymbol{Abs_{blank}}\right) - \boldsymbol{b}}{\boldsymbol{a}} \quad (6)$$

$Abs_{extract}$ is the absorbance of CUPRAC solution in the presence of extract, Abs_{blank} the absorbance of the buffer solution in the presence of samples, a the slope, and b the intercept of the trolox calibration curve.

2.8.3. Enzymatic Antioxidant Assay

Enzymatic antioxidant assay was performed in triplicate using the 19160 SOD determination kit and the associated protocol from Merck (Saint-Quentin-Fallavier, France). The reagent was prepared as indicated in the kit. The enzyme solution was prepared with 15 µL of xanthine oxidase diluted in 2.5 mL of buffer solution. 1 mL of WST-1 was diluted in 19 mL of buffer solution. Chlorogenic acid was the reference inhibitor used, and two concentrations, 60 µg/mL and 12 µg/mL, were tested in ethanol. Extract samples were prepared at 100 µg/mL and 50 µg/mL in ethanol. Hence, 20 µL of extracts, purified water for the control enzyme, or reference inhibitor were deposited in the microplate, and 20 µL

of enzyme solution was added. The dilution buffer solution was added to the other wells instead of the enzyme solution to correct the color of the sample. 200 µL of the substrate WST-1 solution was added to each well. The plate was incubated in the dark for 20 min at 37 °C, and the absorbance was measured at 450 nm using a CLARIOstar® Plus microplate reader. The percentage of inhibition was calculated using the following Equation (7):

$$\%\boldsymbol{inhibition} = \frac{\left(\boldsymbol{Abs_{enzyme\ control}} - \boldsymbol{Abs_{samples}}\right)}{\boldsymbol{Abs_{enzyme\ control}}} \times \mathbf{100} \quad (7)$$

$Abs_{enzyme\ control}$ is the absorbance of enzyme and substrate in the presence of buffer solution only, and $Abs_{samples}$ the absorbance of enzyme and substrate in the presence of the samples. The blank with the dilution buffer solution in the presence of samples was subtracted.

Chlorogenic acid used as the positive reference standard presented an inhibition of 99.10 ± 0.33% at 60 µg/mL and of 67.50 ± 0.45% at 12 µg/mL.

2.8.4. COX-2 Inhibiton Assay

COX-2 inhibition was determined in triplicate using the MAK399 kit and the associated protocol from Sigma-Aldrich (St. Louis, MO, USA). The reagent was prepared as indicated in the kit. For each well, 66 µL buffer solution, 1 µL of COX probe, 2 µL of COX factor previously diluted 200-fold with buffer solution and 1 µL of COX-2 human recombinant were mixed. The substrate, arachidonic acid, was prepared by diluting 2-fold with NaOH and buffer solution and diluting 10-fold with purified water. Celecoxib, prepared according to the supplier's protocol, was used as an inhibitor reference and was diluted 5-fold in the buffer solution. Extract samples were prepared at 1.0 mg/mL, and the oleuropein standard at 0.18 mg/mL in ethanol. In the microplate, 10 µL of extracts, inhibitor, buffer solution (for enzyme control) and solvent were deposited. 70 µL of the mix reagents was added to each well. Lastly, 20 µL of substrate was added. The fluorescence was measured for 15 min at 25 °C with λ_{Ex} = 535 nm/λ_{Em} = 587 nm using a CLARIOstar® Plus microplate reader. The percentage of inhibition was calculated using the following Equation (8):

$$\%\boldsymbol{inhibition} = \frac{\boldsymbol{a_{enzyme\ control}} - \boldsymbol{a_{sample}}}{\boldsymbol{a_{enzyme\ control}}} \times \mathbf{100} \quad (8)$$

$a_{enzyme\ control}$ is the slope of the linear part of the kinetic response of the control and a_{sample} the slope of the linear part of the kinetic response of the sample measured using fluorescence.

Celecoxib diluted 5-fold, used as the positive reference, presented an inhibition of 95.45 ± 0.16%.

2.9. Epidermis Keratinocyte Cell Line HaCaT Assays

To measure reactive oxygen species neutralization and IL-8 secretion, immortalized human keratinocyte cell line HaCaT (PromoCell® GmbH, Heidelberg, Germany) was cultured in transparent 96-well microplates (Sarstedt, reference 83.3924.300) with DMEM high glucose medium supplemented with 10% heat-inactivated FBS, 2% L-glutamine and 1% Penicillin-Streptomycin mixture. HaCaT cells were seeded at a concentration of 1×10^5 cells/mL and maintained at 37 °C under 5% CO_2 atmosphere. The medium was changed twice a week. The cell confluence at the time of the experiments was approximately 90%. All biological analyses were performed at passages 10 to 15.

2.9.1. HaCaT Cell Viability

Cell viability, in the presence of Olive tree and Oleaster serial dilutions of hydroalcoholic leaf extracts (500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL), was evaluated after

24 h, 48 h and 72 h incubation cycles with the Cell Viability assays kit CyQUANT™ XTT (Invitrogen™, reference 15960972 from Thermo Fisher Scientific, Illkirch Graffenstaden, France) according to the manufacturer's instructions.

2.9.2. Reactive Oxygen Species Neutralization on HaCaT Cells

The intercellular production of ROS levels was determined using 2,7-dichlorofluorescein diacetate (DCFH-DA). The permeable DCFH-DA is oxidized by ROS to the highly fluorescent compound 2,7-dichlorofluorescein (DCFH). HaCaT cells were seeded in black 96-well microplates (Sarstedt Lumox®, reference 94.6120.096) as described previously and treated with serial dilutions of Olive tree or Oleaster hydroalcoholic leaf extracts (500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL) and tocopherol (25 mM) as a positive reference for 24 h at 37 °C under 5% CO_2 atmosphere [20]. HaCaT cells were washed with phosphate buffer saline (PBS), and DCFH-DA (20 mM) was added to the cells and incubated for 45 min. The cells were then stimulated by an H_2O_2 solution (100 mM in PBS) to generate oxidative stress during 20 min. After stimulation, DCFH fluorescence was detected with λ_{Ex} = 488 nm/λ_{Em} = 535 nm, using a Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, Illkirch Graffenstaden, France). Fluorescence intensity was directly proportional to the concentration of free radical compounds, and the percentage of ROS was measured according to Equation (9). The assay was performed in triplicate.

$$\%\boldsymbol{ROS\ neutralization} = \frac{\boldsymbol{Fluorescence\ (control)} - \boldsymbol{Fluorescence\ (sample)}}{\boldsymbol{Fluorescence\ (control)}} \times \mathbf{100} \quad (9)$$

2.9.3. Measurement of IL-8 Secretion

HaCaT cells were seeded in 96-well microplates at a density of 1×10^5 cells/mL at up to 90% confluence and then further cultured in fresh DMEM medium containing 500 µg/mL, 250 µg/mL, 125 µg/mL and 62.5 µg/mL of previously filtered, sterilized solutions of Olive tree or Oleaster hydroalcoholic leaf extracts. Lipopolysaccharide (LPS) at 10 ng/mL was used as a cytokine/inflammation generator, and dexamethasone (DXM) as a cytokine/inflammation suppressor. After 24 h, supernatants were collected and further used in the analysis of newly secreted interleukins. IL-8 was quantified using a sandwich ELISA assay kit (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's instructions [21]. The assay was performed in triplicate.

2.10. Statistical Analysis

All chemical, except UHPLC quantification, enzymatic and cellular experiments were performed in triplicate (n = 3). Data are presented as means ± standard deviation (SD). The differences between the different samples or between the samples and the positive controls were analyzed using one-way analysis of variance (ANOVA) and Tukey's post hoc test. Multiple comparisons were conducted, and a p-value of <0.05 was accepted as the level of statistical significance. The statistical analysis was performed using XLsat 017 software (version 19.02.44125 from Lumivero, Bordeaux, France).

3. Results and Discussion

3.1. Extraction and Yields

Several studies in the literature have demonstrated the benefits of ultrasound-assisted extraction for isolating phenolic compounds from Olive tree leaves [22]. Aqueous and hydroalcoholic solvents are best suited for this purpose. Thus, hydroalcoholic (EtOH/H_2O, 50/50, v/v) and aqueous ultrasound-assisted extractions were performed for Olive tree and Oleaster leaves. The percentages of dry matter and extraction yield were calculated, and the results are presented in Table 1.

Table 1. Dry matter and extraction yield of Oleaster and Olive tree leaf extracts. Data are presented as mean ± SD (n = 3). Means followed by a different letter are significantly different at $p < 0.05$.

	Extraction Solvent	%Dry Matter	Yield (%)
Oleaster	EtOH/H_2O	1.76 ± 0.36 ^{ab}	31.9 ± 6.5 ^{a}
	H_2O	1.85 ± 0.05 ^{a}	32.6 ± 4.9 ^{a}
Olive tree	EtOH/H_2O	1.04 ± 0.48 ^{b}	17.7 ± 8.1 ^{a}
	H_2O	1.26 ± 0.03 ^{ab}	21.0 ± 2.4 ^{a}

Dry matter and extraction yield values of around 1.8% and 32%, respectively, were obtained for Oleaster. Olive tree extracts gave results of around 1.1% for dry matter and 18% for extraction yield. Given the high standard deviation observed across the three replicates, which could be explained by the hygroscopic nature of the extracts, Oleaster and Olive tree leaf extracts lead to the recovery of similar amounts of compounds. Hydroalcoholic solvents such as methanol or non-toxic ethanol/water mixtures have been shown to be the best choice for extracting both lipophilic and hydrophilic phenols from Olive tree leaves [22]. Sahin et al. optimized the ultrasound extraction of Olive tree leaves in Turkey using a response surface design and demonstrated that the optimal conditions were a 500 mg solid to 10 mL solvent ratio, 60 min of extraction time and 50% EtOH composition with an ultrasound-assisted frequency of 50 Hz [15]. The yields they obtained were between 16% and 19%, which are in accordance with our results obtained with the olive tree and Oleaster. Their extraction time was longer than ours, yet after 20 min, they observed a slow extraction and only a slight increase in polyphenol concentrations, which confirms that 30 min may be sufficient to recover most of the compounds.

3.2. Quantification of Total Phenolic (TPC) and Flavonoid (TFC) Contents

Phenolic compounds and flavonoids are the main compounds in *Olea europaea* leaves [4–6,11]. These compounds are of great interest to human health as they present antioxidant and anti-inflammatory properties [23–25]. Thus, TPC and TFC were determined for Olive tree and Oleaster leaf aqueous and hydroalcoholic extracts. Figure 1 shows the results obtained in gallic acid (GA) equivalence for TPC and rutin equivalent for TFC.

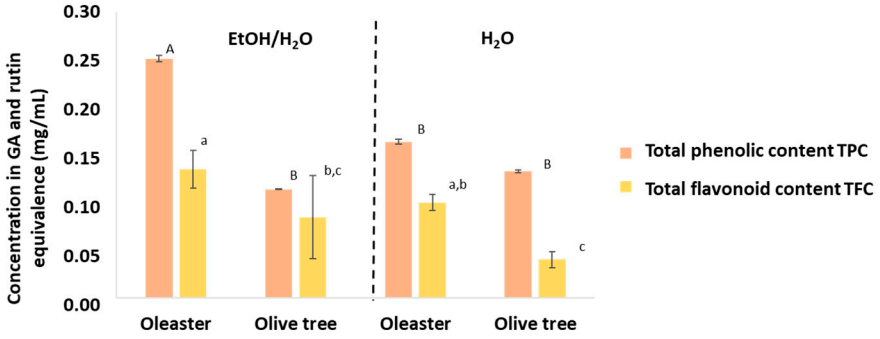


Figure 1. TPC and TFC of hydroalcoholic and aqueous leaf extracts for Oleaster and Olive tree. Data are presented as mean ± SD (n = 3). Means followed by a different letter (upper-case letters for TPC and lower-case letters for TFC) are significantly different at $p < 0.05$.

Results show that with hydroalcoholic extraction, polyphenol and flavonoid concentrations were higher for Oleaster leaf extracts than Olive tree ones, with 0.25 mg/mL against 0.11 mg/mL respectively for polyphenol concentration and 0.14 mg/mL against 0.08 mg/mL respectively for flavonoid concentration. For the aqueous extraction, polyphenolic content was similar for Oleaster and Olive tree leaf extracts, with no significant difference between the obtained values. Concerning the flavonoid content, Oleaster leaf

extract with 0.10 mg/mL was richer than the Olive tree leaf extract with 0.04 mg/mL. Comparing the extraction solvent for Oleaster leaf extracts, hydroalcoholic extraction allowed a better extraction of polyphenolic compounds, while flavonoid content seemed to be similar. For Olive tree leaf extracts, similar results were obtained for TPC and TFC for both solvents.

Under similar extraction conditions, Sahin et al. obtained polyphenol concentrations between 14 and 20 mg/g of dry leaves for hydroalcoholic extraction and between 8 and 10 mg/g of dry leaves for aqueous extraction [15]. Our results, in mg/g of dry leaf, ranging from 50.3 mg/g of dry leaves for Oleaster hydroalcoholic leaf extract to 22.8 mg/g of dry leaves for Olive tree hydroalcoholic leaf extract, were higher than those. Alcántara et al. compared conventional extraction to ultrasound-assisted extraction, and the results obtained on TPC and TFC suggest that conventional extraction was more effective compared to ultrasound-assisted extraction regardless of solvent type. TPC ranged from 21.1 to 25.4 mg GAE/g of dry extract for Olive leaves with the conventional extraction method [26]. Flavonoid contents of 20 mg/g of dry extract and 15 mg/g of dry extract for hydroalcoholic and aqueous extraction, respectively, were rather similar to those obtained for Oleaster and Olive tree extracts in this study. Indeed, TFC were 27.1 mg/g of dry extract and 16.9 mg/g of dry extract for Oleaster and Olive tree leaf hydroalcoholic extraction and 20.0 mg/g of dry extract and 8.8 mg/g of dry extract for Oleaster and Olive tree leaf aqueous extraction, respectively.

Olive tree cultivar, geographical origin, maturity stage, climate, leaf lifetime, as well as sampling, harvest time and storage are typical pre-harvest and post-harvest agronomical and technological factors affecting the phenolic compounds and overall chemical composition of Olive leaves [22]. Several studies have shown that ultrasound-assisted extraction (UAE) is able to improve the extraction yield of phenolic compounds from Olive leaves, by markedly shortening the extraction time without provoking significant changes in the structural molecular properties and functionality of most biophenols [22].

According to the results obtained and compared to the literature, the extraction methods developed and the raw materials of the olive tree and more particularly Oleaster cultivated in the Centre-Val de Loire Région, allowed the recovery of similar or higher polyphenol and flavonoid contents.

3.3. Polyphenolic Fingerprint and Oleuropein and Verbascoside Content Assessment

To obtain a first polyphenolic fingerprint of the different extracts, HPTLC analysis was carried out for Oleaster and Olive tree leaves for both extraction solvents. Standards such as rutin and quercetin for flavonoid compounds and chlorogenic acid for phenolic acids were also deposited on the plate. The polyphenol plate revealed with NEU-PEG reagents and observed at 366 nm is shown in Figure 2. Different spots are present all along the elution zone for Oleaster and Olive tree leaf extracts. As suggested by the color of rutin and quercetin standards, the orange and yellow spots obtained for both plants could be characteristic of flavonoid compounds. Blue spots could indicate the presence of phenolic acids such as chlorogenic acid derivatives. Qualitatively, a greater number of spots are observed on the elution band of the hydroalcoholic extract of Oleaster than on the other bands. Indeed, at $Rf < 0.2$, dark blue spots are observed, which are also present in the aqueous extract of Oleaster. The phenolic compounds and flavonoids eluting at $0.4 < Rf < 0.7$ appear to be common to Olive tree leaf extracts. Finally, at the top of the plate ($Rf > 0.8$), yellow spots are again detected more clearly in the hydroalcoholic extract of Oleaster. For both Oleaster and Olive tree leaves, hydroalcoholic extractions seemed to be more efficient than aqueous extractions.

To confirm HPTLC observations, UHPLC-DAD analyses were performed on the different extracts. Chromatograms are represented in Figure 3 at 280 nm. Samples were prepared at 5 mg/mL for analysis. For both extractions, similar profiles were obtained.

While Oleaster and Olive tree leaf extracts have the same phytochemical profile, some peak intensities are different. The peak intensity is higher for Oleaster leaves than for Olive tree leaves for both extraction solvents. Moreover, the peak intensity is higher with hydroalcoholic extraction than with aqueous extraction for both raw materials.

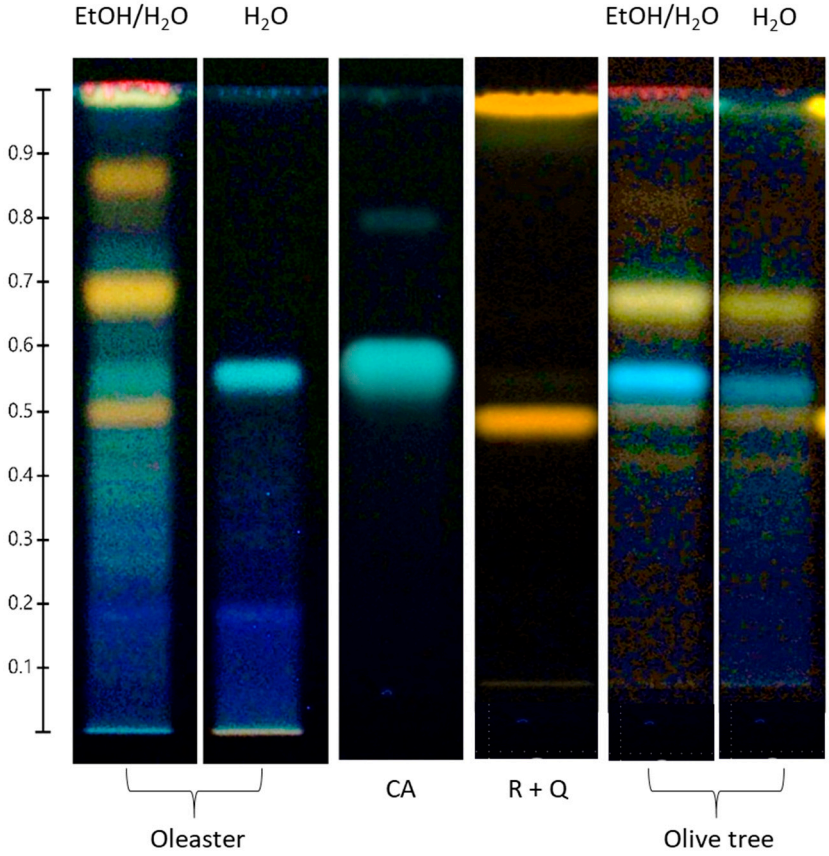
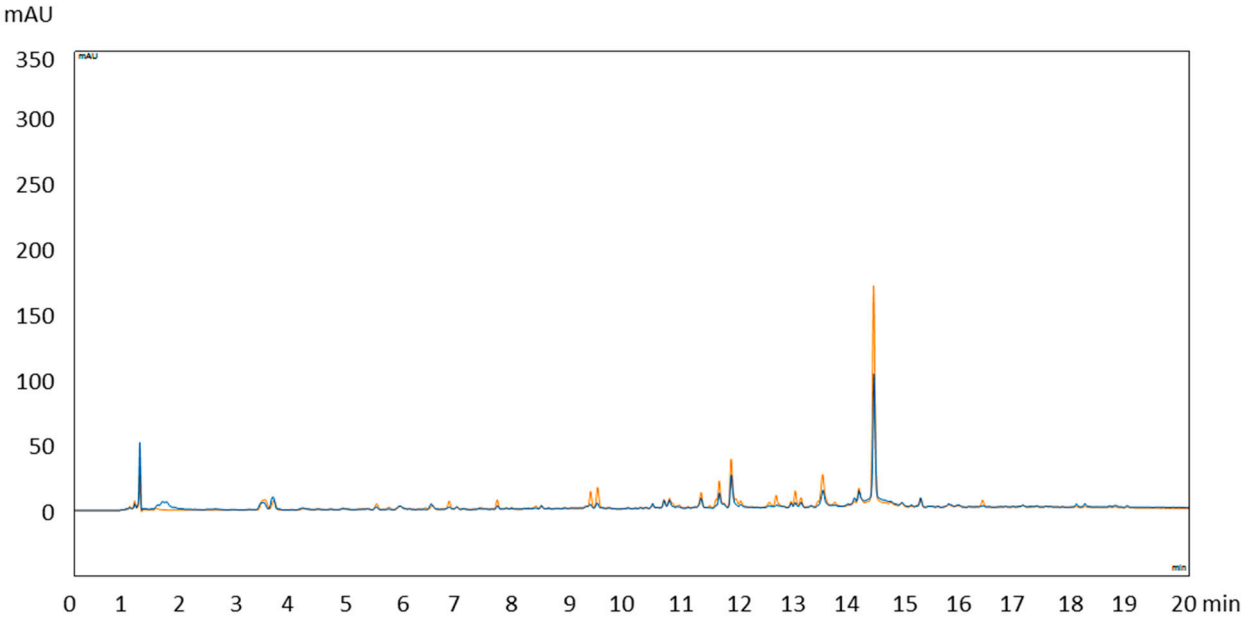


Figure 2. HPTLC analysis of hydroalcoholic and aqueous Oleaster and Olive tree leaf extracts. NEU-PEG derivatization and 366 nm. Chlorogenic acid (CA), Rutin (R) and Quercetin (Q) were used as standards.

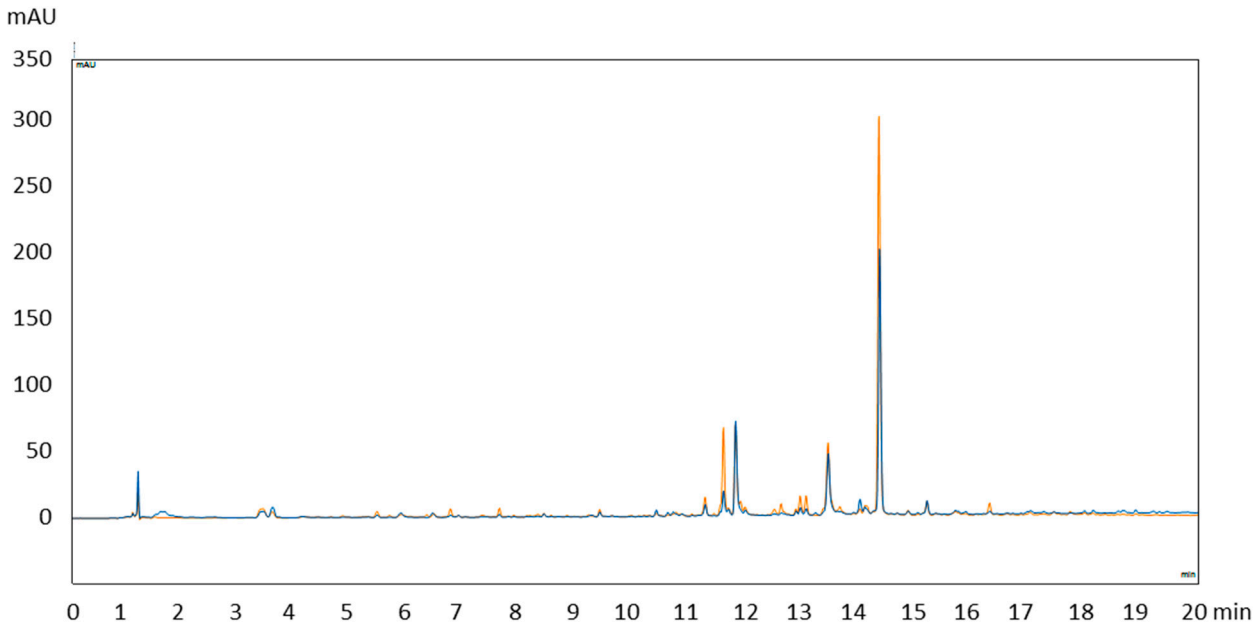
Oleuropein and verbascoside are compounds that are specific to *Olea europaea*, considered as phytochemical markers. They possess antioxidant and inflammatory properties, amongst others. Oleuropein, which is described on several occasions as the predominant compound in hydroalcoholic extracts of Olive leaves [27], is the main peak of this chromatogram with a retention time of 14.40 min. Verbascoside is eluted with a retention time of 11.56 min. Both compounds were unequivocally identified by HRMS/MS and analysis of the corresponding standard molecules. Quantification of these molecules was performed using UHPLC-DAD at 230 nm, which is the maximum wavelength of oleuropein and at 280 nm for verbascoside. Results obtained for extracts analyzed at 5 mg/mL are shown in Table 2.

Table 2. Oleuropein and verbascoside concentrations in Oleaster and Olive tree leaf extracts.

	Oleaster		**Olive Tree**	
	EtOH/H_2O	**H_2O**	**EtOH/H_2O**	**H_2O**
Oleuropein (µg/mL)	916	543	554	347
Oleuropein (mg/g of dry leaves)	58	35	20	14.5
Verbascoside (µg/mL)	50	15	16	11
Verbascoside (mg/g of dry leaves)	3.2	0.98	0.56	0.46



(**a**)



(**b**)

Figure 3. Chromatograms of Oleaster (orange) and Olive tree (blue) leaf with aqueous (**a**) and hydroalcoholic (**b**) extractions at 280 nm. Analyses were performed using the Luna Omega C18 column (150 × 2.1 mm; 1.6 µm) and a mixture of acetonitrile and water, both acidified with formic acid as mobile phase.

The highest oleuropein concentration was found in Oleaster hydroalcoholic leaf extraction, with a quantification of 916 µg/mL. In aqueous extraction, the oleuropein concentration decreased to 543 µg/mL. For the Olive tree leaves, a higher oleuropein extraction was also achieved with the EtOH/H_2O mixture than with water. For both extractions, the quantity of oleuropein was higher in Oleaster leaf extracts than in Olive tree ones. Slightly lower amounts of oleuropein are described by Giacometti et al., with 12 mg/g of dry leaves obtained for Olive tree leaf using hydroalcoholic ultrasound-assisted extraction [28]. Clodoveo et al. [22] report Oleuropein content values ranging from 0.073 to 210.65 mg/g

dw. The oleuropein content can be influenced by agronomic variables and the extraction method. In general, the oleuropein concentration was significantly higher in the cold season and in the tropical regions than in the hot season and the temperate regions. Oleuropein concentration changes with the season, with higher content in spring and is known to degrade into other compounds during the maturation of Olive fruits and leaves [6].

The amount of verbascoside recovered was lower than that of oleuropein in Oleaster and Olive tree leaf extracts. The highest quantity of verbascoside, with a concentration of 50 µg/mL, was obtained in hydroalcoholic extraction for Oleaster leaves. In comparison with the literature, where 0.30 mg/g of dry leaves of verbascoside in Olive tree is reported, our results were slightly higher [28]. Values found in the literature range from 0.005 to 4.4 mg/g dw, which are in accordance with our results [22].

The mature leaves harvested in March in Région Centre-Val de Loire appear to be a valuable source of oleuropein, verbascoside and other polyphenolic and flavonoid compounds. Moreover, intensified extraction technologies such as ultrasound-assisted extraction allow efficient extraction and a high compound recovery, reducing the volume of extraction solvent, saving time and cost.

3.4. Compound Identification by UHPLC-DAD-HRMS/MS

The initial screening analysis reported above showed that hydroalcoholic extracts of Oleaster and Olive tree leaves contained higher diversity and concentration of polyphenolic compounds than aqueous extracts. Thus, hydroalcoholic extracts of both varieties were analyzed using UHPLC-DAD-HRMS/MS to perform the annotation of the main compounds. The exact masses of the compounds were obtained, and molecular formulae were proposed with a mass tolerance between 2 and 5 ppm for the MS and MS/MS. Comparison of the negative and positive ionizations was used to confirm the proposed molecular formulae. MS/MS was applied in positive ionization mode to obtain compound fragmentation. The annotation was done for each compound using the literature and public databases (Pubchem, Lotus, KNApSAcK). The retention time, m/z of molecular and fragment ions, proposed molecular formulae and proposed annotations are presented in Table 3. MS and MS/MS spectra recorded in positive ionization mode were given as Supplementary Data.

Compound 1 was eluted at 6.44 min. Negative and positive ionization modes showed m/z 609.1465 and 611.1600, respectively, corresponding to the molecular formula $C_{27}H_{30}O_{16}$. MS/MS^+ fragments were obtained at m/z 449.1078 and 287.0557, corresponding respectively to two losses of hexose (162 u). The m/z 287.0557 corresponded to the genin whose molecular formula was $C_{15}H_{10}O_6$, which could be associated with luteolin or kaempferol molecules. In view of the experimental UV spectra (λ_{max} of 266 and 344 nm) and the yellow spots shown by the polyphenol HPTLC analysis, the proposed annotation for the genin was luteolin. Thus, luteolin-di-O-glucoside was the proposed annotation for Compound **1** [29].

Compound **2** at 7.60 min presented the same m/z (611.1607 in MS^+ and 609.1466 in MS^-) and proposed molecular formula of $C_{27}H_{30}O_{16}$ as Compound **1**. Fragment ions at m/z 465.1029 and m/z 303.0507 were obtained after losses of 146 u and 162 u, corresponding to deoxyhexose and hexose, respectively. For m/z 303.0507, $C_{15}H_{10}O_7$ was the proposed molecular formula, which matched with quercetin. The proposed annotation of the compound was rutin [30].

Table 3. Characterization of Oleaster and Olive tree leaf hydroalcoholic extracts by UHPLC-DAD-HRMS/MS.

#	Retention Time (min)	Meas. *m/z* $[M+H]^+$	Error (ppm)	MS/MS^+ (Intensity)	Molecular Formula	λ_{max} (nm)	Compound Annotation	References
1	6.44	611.1600	1.1	611.1607 (100) 449.1078 (65) 287.0557 (86)	$C_{27}H_{30}O_{16}$	266, 344	Luteolin-di-O-hexoside	[27]
2	7.60	611.1608	−0.3	611.1610 (20) 465.1029 (49) 303.0507 (100)	$C_{27}H_{30}O_{16}$	252, 353	Rutin	[11,30]
3	7.69	595.1658	−0.1	595.1655 (89) 449.1079 (100) 287.0558 (91)	$C_{27}H_{30}O_{15}$	221, 270, 344	Luteolin-O-rutinoside	[6,11]
4	7.89	625.2121	1.0	625.2138 (7) 479.1497 (22) 325.0925 (100) 163.0397 (86)	$C_{29}H_{36}O_{15}$	219, 330	Verbascoside	[6,11,31]
5	7.96	595.1651	1.1	595.1653 (64) 449.1066 (2) 287.0549 (100)	$C_{27}H_{30}O_{15}$	254, 346	Luteolin-O-rutinoside	[6,11]
6	7.96	449.1086	1.1	449.1074 (61) 287.0561 (100)	$C_{21}H_{20}O_{11}$	331	Luteolin-O-glucoside	[6,11,32,33]
7	8.22	725.2282	0.8	725.2283 (85) 579.1698 (73) 433.1119 (10) 271.0612 (100)	$C_{33}H_{40}O_{18}$	269, 345	Ligustroflavone	[6,11]
8	8.51	579.1718	−1.7	579.1708 (71) 433.1135 (100) 271.0615 (66)	$C_{27}H_{30}O_{14}$	258, 345	Apigenin-O-rutinoside	[6,11,33]
9	8.56	703.2432	1.7	541.1910 (14) 379.1354 (52) 361.1284 (10) 225.0754 (20) 137.0595 (100)	$C_{31}H_{42}O_{18}$	230, 280	Oleuropein-O-hexoside	[11,34]
10	8.75	609.1811	0.5	609.1808 (86) 463.1234 (75) 301.0709 (100)	$C_{28}H_{32}O_{15}$	314	Diosmetin-O-rutinoside	[6,11,35]

Table 3. *Cont.*

#	Retention Time (min)	Meas. *m*/*z* [M+H]$^+$	Error (ppm)	MS/MS$^+$ (Intensity)	Molecular Formula	λ_{max} (nm)	Compound Annotation	References
11	8.75	579.1708	0.1	579.1711 (62) 433.1131 (5) 271.0613 (100)	$C_{27}H_{30}O_{14}$	269, 345	Apigenin-O-rutinoside	[6,11,33]
12	8.88	449.1087	−1.9	449.1076 (100) 287.0564 (86)	$C_{21}H_{20}O_{11}$	267, 334	Luteolin-O-hexoside	[6,11,32,33]
13	8.90	433.1138	−2.0	433.1128 (46) 271.0614 (100)	$C_{21}H_{20}O_{10}$	267, 336	Apigenin-O-hexoside	[6,11,36]
14	9.19	463.1241	−1.2	463.1229 (42) 301.0710 (100)	$C_{22}H_{22}O_{11}$	352	Diosmetin-O-hexoside	[6,11,35]
15	9.61	541.1920	−0.8	379.1391 (11) 361.1290 (74) 225.0759 (18) 137.0613 (100)	$C_{25}H_{32}O_{13}$	230, 280	Oleuropein	[6,11,34]
16	10.02	541.1924	−1.6	379.1393 (91) 361.1286 (65) 225.0748 (85) 137.0618 (100)	$C_{25}H_{32}O_{13}$	230, 280	Oleuropein isomer	[6,11,34]
17	11.07	287.0554	−1.2	287.0547 (100) 153.0179 (17)	$C_{15}H_{10}O_6$	254, 347	Luteolin	[6,11,33]
18	18.71	473.3627	−0.3	473.3635 (16) 455.3528 (20) 409.3469 (100) 203.1794 (53)	$C_{30}H_{48}O_4$	195	Maslinic acid or isomer	[6,11]
19	18.81	473.3626	0.0	473.3612 (23) 455.3504 (26) 409.3452 (42) 205.1585 (100)	$C_{30}H_{48}O_4$	195	Maslinic acid or isomer	[6,11]

Compound **3** was detected in positive ionization mode with $[M+H]^+$ ion at m/z 595.1668. The proposed molecular formula is $C_{27}H_{30}O_{15}$. Fragments with m/z 449.1079 and 287.0558 were obtained, arising from the losses of 146 u and 162 u, respectively. For the genin fragment ion at m/z 287.0558, the proposed molecular formula was $C_{15}H_{10}O_6$. Mass information associated with λ_{max} suggested luteolin. The putative annotation of the molecule was luteolin-O-rutinoside. An isomer of this compound was detected with an Rt at 7.96 min (Compound **5**). The same m/z and molecular formula were found. For the fragmentation, the genin fragment was mainly obtained at m/z 287.0549 with a loss of 308 u corresponding to a rutinoside fragment.

Compound **4** with m/z 625.2121 suggested a molecular formula of $C_{29}H_{36}O_{15}$. Fragment ions at m/z 479.1497 corresponding to a loss of deoxyhexose, m/z 325.0925 and m/z 163.0397 corresponding to a loss of hydroxytyrosol and hexose, respectively, were observed. The proposed annotation was verbascoside [31].

Compound **6** was detected at 7.96 min, and the m/z obtained was 449.1086. Only one fragment corresponding to a loss of hexose was observed at m/z 287.0561 with a molecular formula of $C_{15}H_{10}O_6$. The genin matched with the luteolin molecule. The proposed molecular formula was $C_{21}H_{20}O_{11}$, and luteolin-O-hexose was the proposed identification [32,33]. The same annotation was done for Compound **12** at 8.88 min because the same m/z, molecular formula and fragmentation were found. Thus, Compounds **6** and **12** should be isomeric derivatives of luteolin-O-hexose.

Compound **7** was detected with an m/z of 725.2282. Fragmentation ions in MS/MS^+ were obtained with m/z 579.1698 and m/z 433.1119, corresponding to two losses of deoxyhexose and with m/z 271.0612 corresponding to a loss of hexose. $C_{15}H_{10}O_5$ was the proposed molecular formula for the m/z 271.0612, and it corresponded to apigenin. The proposed molecular formula was $C_{33}H_{40}O_{18}$, and the proposed compound annotation was ligustroflavone (apigenin-O-dehoxyhexose-O-hexose).

Compounds **8** and **11**, eluted at 8.51 min and 8.75 min, respectively, were detected with m/z 579.1718 and m/z 579.1708 and the same molecular formula of $C_{27}H_{30}O_{14}$. For both compounds, similar fragmentations were found with m/z 433.1135 and m/z 271.0615 corresponding to losses of deoxyhexose and hexose, respectively. The genin of the compound corresponded to apigenin, and the proposed annotation was apigenin-O-rutinoside and its isomer [33].

Compound **9** with m/z 703.2432 and $C_{31}H_{42}O_{18}$ as the molecular formula was detected at 8.56 min. Several fragments were obtained in MS/MS^+. The first fragment was m/z 541.1910 arising from the loss of hexose (162 u). Then, a second loss of hexose was identified with m/z 379.1354. H_2O and hydroxytyrosol molecules were annotated as losses leading to the formation of fragment ions at m/z 361.1284 and m/z 225.0754, respectively. The proposed annotation was oleuropein-O-hexoside, confirmed through UV spectra and the last fragment with m/z 137.0595, which is characteristic of oleuropein fragmentation [34].

Compound **10** was detected with m/z 609.1811 and $C_{28}H_{32}O_{15}$ as the molecular formula. Fragment ions with m/z 463.1234 and m/z 301.0709 were due to the losses of deoxyhexose and hexose, respectively. The proposed molecular formula $C_{16}H_{12}O_6$ for m/z 301.0709 corresponded to diosmetin. The proposed annotation was diosmetin-O-rutinoside [35].

Compound **13** was a derivative compound of apigenin with a proposed molecular formula of $C_{21}H_{20}O_{10}$. Only a loss of 162 u was observed with a m/z 271.0614, and the proposed molecular formula was $C_{15}H_{10}O_5$. Thus, the proposed annotation was apigenin-O-hexoside [36].

Compound **14** presented a similar fragmentation pattern to that of Compound **10**. The proposed molecular formula of Compound **14** was $C_{22}H_{22}O_{11}$, and only a loss of 162 u,

corresponding to a loss of hexose, was observed to produce the m/z 301.0710 fragment. Thus, the proposed annotation was diosmetin-O-hexose.

Compounds **15** and **16**, eluted at 9.61 min and 10.02 min, had a similar m/z 541.192, molecular formula $C_{25}H_{32}O_{13}$ and fragmentation. The fragments m/z 379.1391 and m/z 361.1290 corresponded to hexose and H_2O losses. A loss of hydroxytyrosol led to the formation of the fragment ion at m/z 225.0759. A characteristic fragment was detected at m/z 137.0613. The proposed annotation was oleuropein for Compound **15** and an isomer for Compound **16** [34].

Compound **17** was detected with a m/z 287.0554 and $C_{15}H_{10}O_6$ as the molecular formula. A fragment ion was detected at m/z 153.0179 corresponding to a loss of 134 u. This fragmentation pathway and the UV spectra (λ_{max} = 254, 347 nm) are characteristic of the luteolin molecule [33].

Compounds **18** and **19** eluted at 18.71 min and 18.81 min, had a similar m/z 473.3627, molecular formula of $C_{30}H_{48}O_4$ and fragmentation pattern. Several fragments were identified at m/z 455.3528 corresponding to H_2O loss, m/z 409.3469 due to acidic function loss and m/z 203.1794. The proposed annotations were maslinic acid and an isomer [6,11].

In the negative ionization mode only, i.e., without MS/MS acquisition, a compound was detected at Rt 12.48 min and m/z 269.0448, with $C_{15}H_{10}O_5$ as molecular formula. No fragment was observed on the full scan MS, and the UV spectrum showed λ_{max} = 285, 331 nm. These elements are characteristic of the apigenin molecule [33].

Two other compounds were detected only in the negative ionization mode at 20.69 and 20.79 min, and with the same m/z 455.3522 and $C_{30}H_{48}O_3$ as molecular formula. No fragmentation was observed on the full scan MS. The proposed annotations were oleanic acid and betulinic acid [6,11].

All these compounds were present in both Olive tree and Oleaster hydroalcoholic leaf extracts, indicating the similarity in the phytochemical composition of the two raw materials.

Michel et al. investigated the chemical composition of diverse organs (leaf, stem and root) from two *O. europaea* varieties (i.e., Koroneiki and Chetoui) using UHPLC-ESI-HRMS/MS [11]. They found that leaf samples were mainly characterized by the presence of numerous flavonoids, including quercetin, luteolin, apigenin and diosmetin glycosides. Among them, luteolin and apigenin derivatives were the most abundant, with the detection of three luteolin rutinoside isomers and three luteolin glucoside isomers.

Kabbash et al. compared the metabolic profiling of Olive tree leaf hydroalcoholic extracts from twelve different cultivars cultivated in Egypt and collected in spring and autumn [6]. In spring, almost all the studied cultivars were characterized by high oleuropein and various phenolic compounds, while in autumn, the leaf extracts were a rich source for bioactive pentacyclic triterpenes. These results support the molecular composition observed for the mature leaves harvested in March, used in this study.

According to the literature, tyrosol and hydroxytyrosol compounds are also specific markers in Olea europaea leaves [5,9,37], but these compounds were not found in the Oleaster and Olive tree leaf extracts. As shown by Mechri et al. [37], tyrosol and hydroxytyrosol concentrations decrease when water stress decreases. The Centre-Val de Loire region is not considered a dry region, and rainfall is relatively high [38]. These climate conditions could have an impact on the quantity of compounds, particularly on the lack of tyrosol and hydroxytyrosol.

3.5. Bioassay Activity

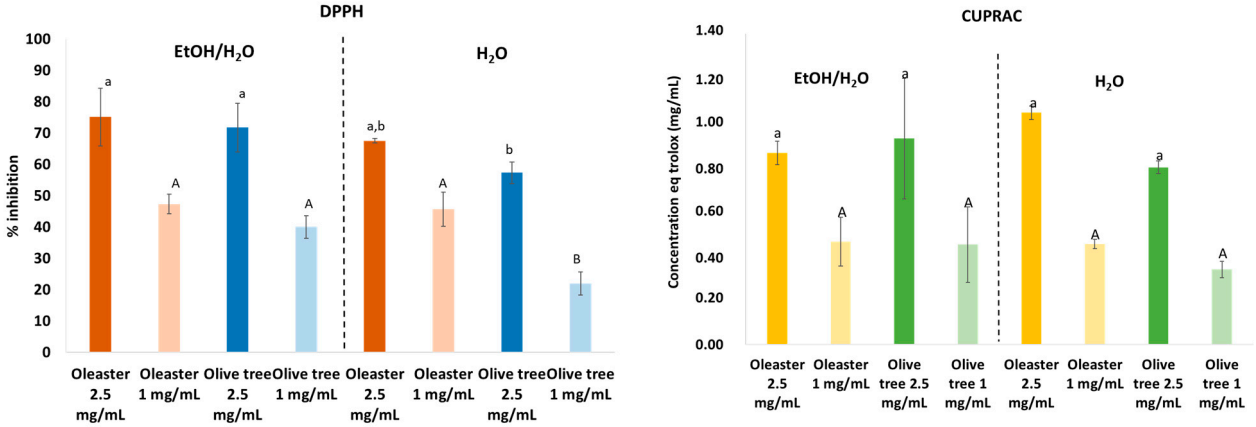
3.5.1. Antioxidant Assays: DPPH and CUPRAC Assays

An excessive reactive oxygen species (ROS) concentration is present during oxidative stress and can be harmful to health, as ROS are implicated in the development of cancer and

degenerative diseases [39–42]. Antioxidant molecules are compounds that prevent or delay the oxidation of chemical substances and can fight free radicals, even at low concentrations. To determine if a molecule or an extract has an antioxidant potential, the DPPH radical scavenging activity assay is commonly used [42,43]. An antioxidant can react with DPPH radical by electron transfer or by donating H atoms, causing a change in the color of the solution from purple to yellow.

The CUPRAC assay is another assay used to determine the antioxidant activity of compounds. This assay is based on the capacity of antioxidant molecules to reduce a blue Cu(II) complex to a yellow Cu(I) complex [43,44].

The first evaluation of the potential of the extracts as antioxidants was assessed with the DPPH radical scavenging activity assay and the CUPRAC reducing activity assay. The results are shown in Figure 4.



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

Figure 4. DPPH (**a**) and CUPRAC (**b**) assays of Oleaster and Olive tree leaf hydroalcoholic and aqueous extracts at two concentrations (2.5 mg/mL and 1.0 mg/mL). Data are presented as mean ± SD (n = 3). Means followed by a different letter (lower-case letters for 2.5 mg/mL and upper-case letters for 1 mg/mL) are significantly different at $p < 0.05$.

For the DPPH assay, considering standard deviation and with a concentration of 2.5 mg/mL, nearly 70% of inhibition was obtained for each extract. With a concentration of 1 mg/mL, between 50% and 20% of inhibition was obtained. The inhibition of the DPPH radical decreased as the concentration of the extracts was lower, regardless of the extraction condition. A slight increase in the percentage of inhibition was observed for hydroalcoholic extraction in comparison to aqueous extraction for both raw materials. At 1 mg/mL, the antioxidant activity was higher for Oleaster aqueous leaf extract than for the Olive tree one, while no significant difference was observed for hydroalcoholic extracts. In similar extraction conditions, the literature reports a percentage of inhibition close to 90% for hydroalcoholic and aqueous extractions [15]. These results are slightly higher than ours (almost 70% of inhibition), but in the literature, the extract concentrations are not mentioned. In accordance with our results, Nasser et al. demonstrated higher DPPH inhibition for Oleaster leaf water extract than Olive tree, while for methanolic extract, Olive tree extract showed higher DPPH inhibition [10]. Yang et al. studied the impact of seasonal variation on olive tree leaves collected during one year by evaluating the antioxidant activity of Olive tree leaf water and methanolic extracts. Extracts presented DPPH inhibition around 60% for samples tested at 1 mg/mL without any significant difference in methanol and water extract among the 12 months [6]. The antioxidant capacity changes through leaf maturation, with mature leaves showing more antioxidant activity than young leaves [22].

For the CUPRAC assay, given the standard deviation, similar antioxidant activity was observed for all the extracts evaluated at the same concentration. A concentration in trolox equivalence between 0.8 mg/mL and 1 mg/mL was obtained for extracts tested at 2.5 mg/mL, while a concentration around 0.5 mg/mL trolox equivalent was obtained for extracts tested at 1.0 mg/mL. Very few studies have assessed the antioxidant activity of Olive tree leaf extracts using the CUPRAC assay. Ronca et al. evaluated the antioxidant activity of different Olive leaf extracts performed with different solvents. Results obtained for CUPRAC assays ranged from 0.22 to 0.43 mmol Trolox g^{-1} sample [45].

Antioxidant activity is generally correlated with the polyphenol and flavonoid contents in the extracts. Oleuropein, luteolin, apigenin and their derivatives play a key role in antioxidant activity [45,46]. The Oleaster and Olive tree leaf extracts developed in this study present a high content of oleuropein and numerous derivatives of luteolin and apigenin compounds, which could explain the high antioxidant activity observed. The phytochemical composition and antioxidant activity of Olive fruit and leaf byproducts make these bioactive extracts attractive ingredients to be in cosmetic formulations [47].

3.5.2. Enzymatic Antioxidant and Anti-Inflammatory Activities

To complete the antioxidant in vitro evaluation, a superoxide dismutase (SOD) inhibitor assay was also performed to determine the antioxidant capacity enzymatically. This assay is based on a free radical mechanism. SOD is an oxidoreductase that catalyzes the dismutation of O_2^- to O_2 and H_2O_2 in the organism [39]. In this assay, xanthine oxidase generates ROS as O_2^-, and a water-soluble tetrazolium salt (WST-1) is used as an indicator of O_2^- production [37]. This reaction produces the water-soluble formazan reduction, and the colorless liquid turns yellow. If the samples are antioxidant, there is competition between extracts and WST-1 for O_2^-. WST-1 is not reduced, and no coloration is observed.

Oleuropein is described in the literature as an antioxidant compound mainly involved in the antioxidant activity of Olive tree extract [11,12]. Thus, to evaluate the contribution of oleuropeine in the antioxidant activity of the crude extracts, the percentage of inhibition of Oleaster and Olive tree leaf extracts at 100 and 50 µg/mL was evaluated, as well as for the oleuropein standard at a concentration of 18 µg/mL, which corresponds to the oleuropein concentration in 100 µg/mL of Oleaster hydroalcoholic extract. This concentration was chosen because it was the highest oleuropein concentration that was determined in the different extracts. As shown in Figure 5, the percentage of enzyme inhibition was nearly 90% for Oleaster and Olive tree leaf extracts at 100 µg/mL. The percentage of inhibition decreased slightly to between 70% and 80% at 50 µg/mL. For oleuropein, nearly 90% of inhibition was obtained, which is the same result as that obtained for the hydroalcoholic leaf extract of Oleaster. A high antioxidant activity was observed for all extracts (both hydroalcoholic and aqueous) without any significant difference, probably due to the high contribution of the antioxidant activity of oleuropein, but also to the antioxidant activity of other phenolic compounds present in the extracts [24]. These results were supported by Sahin et al., showing that Olive tree leaf extract is more antioxidant than vitamins C and E, due to the synergy among flavonoids, oleuropeosids and phenols [27].

The cyclooxygenase 2 (COX-2) enzyme is responsible for the production of prostaglandin from arachidonic acid that promotes inflammation in the tissues [48]. According to the literature, *Olea europaea* leaves can inhibit the COX-2 enzyme and the inflammatory effect [13,49]. A COX-2 inhibitor assay was carried out on each hydroalcoholic and aqueous extract. Results are presented in Figure 6. An anti-inflammatory effect was observed with an inhibition close to 70% for Oleaster and Olive tree leaf extracts for both extraction solvents. Oleuropein was also tested at a concentration of 180 µg/mL, which is the oleuropein equivalence in 1 mg/mL of Oleaster hydroalcoholic extract. An inhibition of nearly 50%

was found for the oleuropein standard, which may explain in part the anti-inflammatory activity of Oleaster and Olive tree leaf extracts.

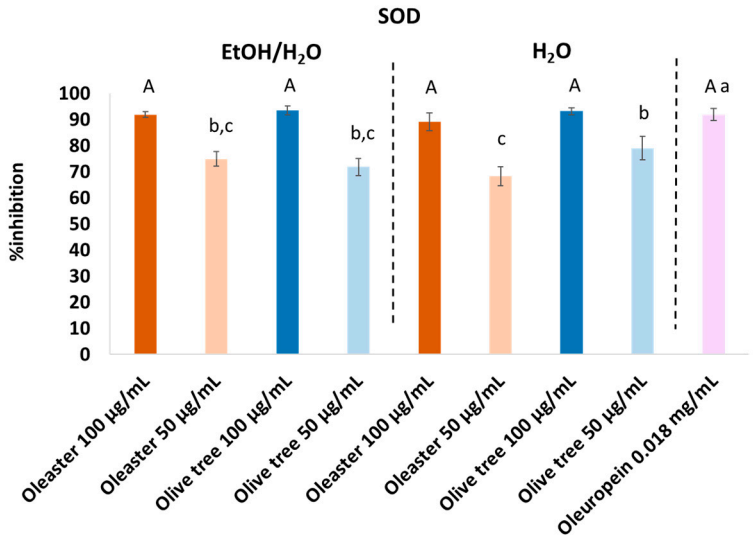


Figure 5. SOD assay of Oleaster and Olive tree leaf hydroalcoholic and aqueous extracts at 100 µg/mL and 50 µg/mL. Data are presented as means ± SD (n = 3). Means followed by a different letter (upper-case letters for 100 µg/mL and lower-case letters for 50 µg/mL) are significantly different at $p < 0.05$.

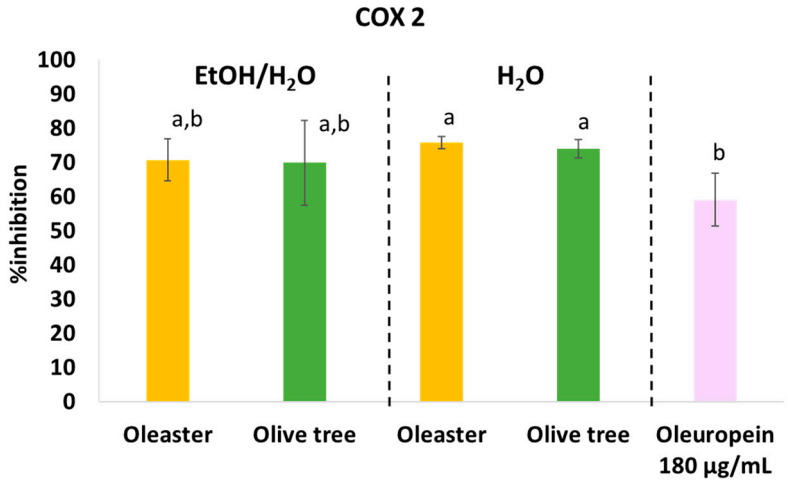


Figure 6. COX-2 assay of Oleaster and Olive tree leaf hydroalcoholic and aqueous extracts at 1.0 mg/mL. Data are presented as means ± SD (n = 3). Means followed by a different letter are significantly different at $p < 0.05$.

The presence in the extracts of other molecules such as verbascoside, flavonoid and terpenic compounds known to have an anti-inflammatory capacity [24,50] can contribute to the high percentage of inhibition of the extracts. Romero-Marquez et al. [44] studied Olive leaf extracts from Spain, Italy, Greece, Portugal, and Morocco. They characterized their anti-cholinergic, anti-inflammatory, and antioxidant activities and showed that secoiridoids contributed to the COX-2 inhibitory activity and antioxidant capacity. PLS-DA assigned to oleuropein and its derivatives a significantly high VIP score, and these findings were further supported by Pearson's correlation analysis. The luteolin-7-O-glucoside content had the greatest VIP score and Pearson's correlation value in the flavonoid family with respect to COX-2 inhibitory activity [46,51].

3.5.3. Antioxidant and Anti-Inflammatory Activity on HaCat Cells

Before evaluating the activity of Oleaster and Olive tree leaf extracts on HaCaT cells, different concentrations of extracts were evaluated on HaCaT cell viability during 24 h, 48 h and 72 h. Specifically, cell viability was analyzed by mitochondrial activity using an XTT assay, and the results are presented in Figure 7. The effect of the different concentrations of Oleaster and Olive tree leaf extracts on cell viability was compared with the positive control, which did not induce cell death.

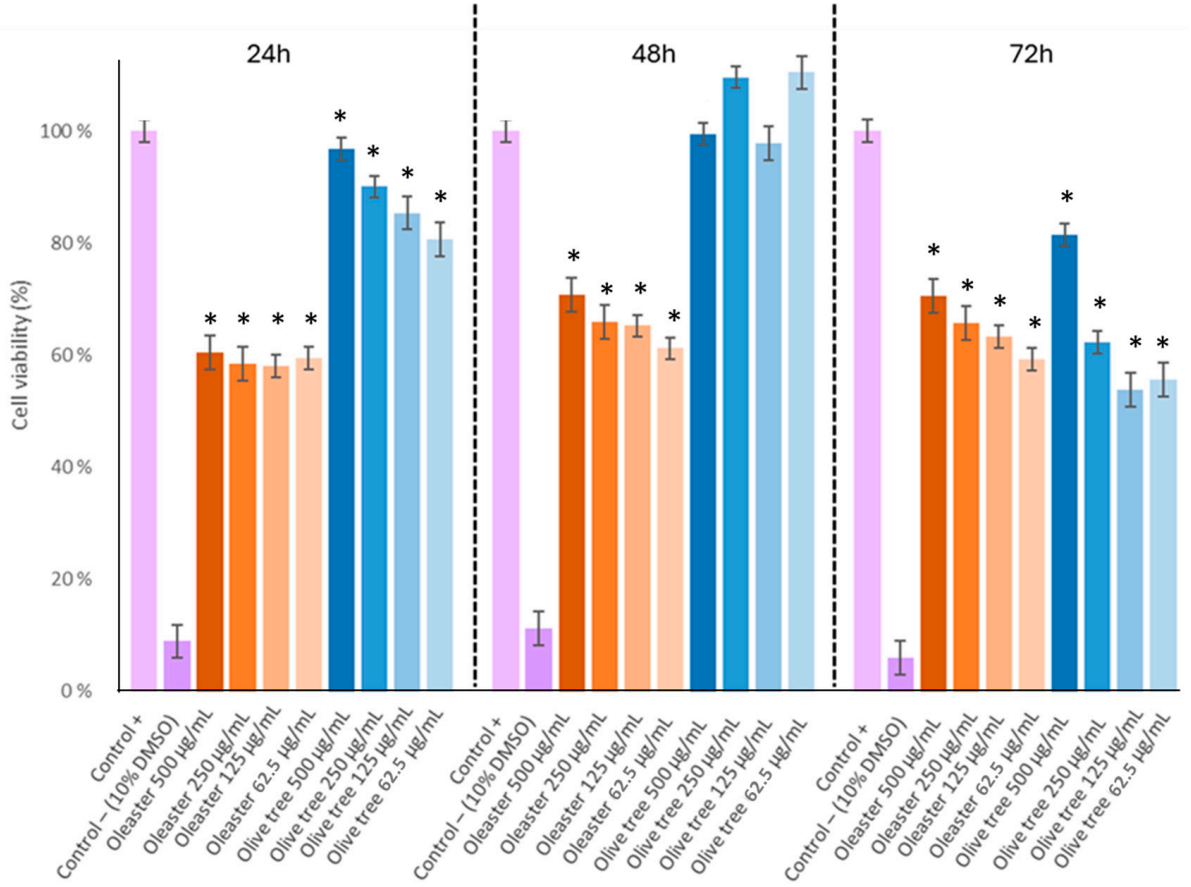


Figure 7. Effect of different concentrations of Olive tree and Oleaster leaf hydroalcoholic extracts on HaCaT cell viability during 24 h, 48 h and 72 h incubation cycles. Extract conditions marked with * were significantly different from the control+ condition with $p < 0.05$.

After 24 h of incubation, all treatments had an impact on cell viability. Cell viability obtained with Oleaster leaf extract was approximately 60%, while that obtained with Olive tree leaf extract reached 80%. After 48 and 72 h of incubation, the cell viability remained constant in the presence of Oleaster leaf extract. Cell viability in the presence of Olive tree leaf extract was not significantly different from that of the control after 48 h, but then decreased to 60% after 72 h. Regardless of the extract used and the incubation time, cell viability does not decrease when the extract concentration increases from 62.5 µg/mL to 500 µg/mL. Although cell viability may decrease by up to 60%, the fact that it remains stable for 72 h and that high extract concentrations do not result in increased toxicity makes these results encouraging for crude extracts. In the literature, the cytotoxicity of two different oleuropein-enriched extracts from *O. europaea* fruits and leaves (O20 = 20% oleuropein and O30 = 30% oleuropein + 10% triterpenes) was evaluated on the HaCaT cell line. The HaCaT cell line viability did not decrease after exposure to different concentrations of O20 and O30, showing a viability of around 100% until a concentration of 100 µg/mL. Nevertheless,

at the highest concentration tested (1000 µg/mL), the cell viability decreased to 61.05 and 42.06%, respectively, after exposure to O20 and O30 extracts [47].

Given these results, the duration of treatment to evaluate antioxidant and anti-inflammatory activity on HacaT cells did not exceed 24 h, and the tested extract concentration remains from 500 to 62.5 µg/mL.

Oxidative stress and excessive production of intracellular ROS are responsible for a variety of conditions, including skin aging and skin inflammation. Therefore, the Olive tree and Oleaster leaf extracts were tested to evaluate their ability to neutralize intracellular ROS formation in H_2O_2-stressed HaCaT cells. The 2,7-dichlorofluorescein diacetate (DCFH-DA) probe was used as an indicator of intracellular ROS formation. In this assay, the ROS scavenging activities of Olive tree and Oleaster leaf hydroalcoholic extracts were compared to α-tocopherol (25 µM) as ROS scavenging active control. The results displayed in Figure 8 show that all the tested concentrations of Olive tree extract provided about 80% of protection against ROS, which is in the same range of values as the positive control α-tocopherol. Oleaster presented a slightly lower protection, ranging from 40% to 60% of protection against ROS. These in-cellulo results confirm our in-vitro enzymatic (SOD) and colorimetric (DPPH and CUPRAC) antioxidant assay results, which indicated the good antioxidant activity of the extracts.

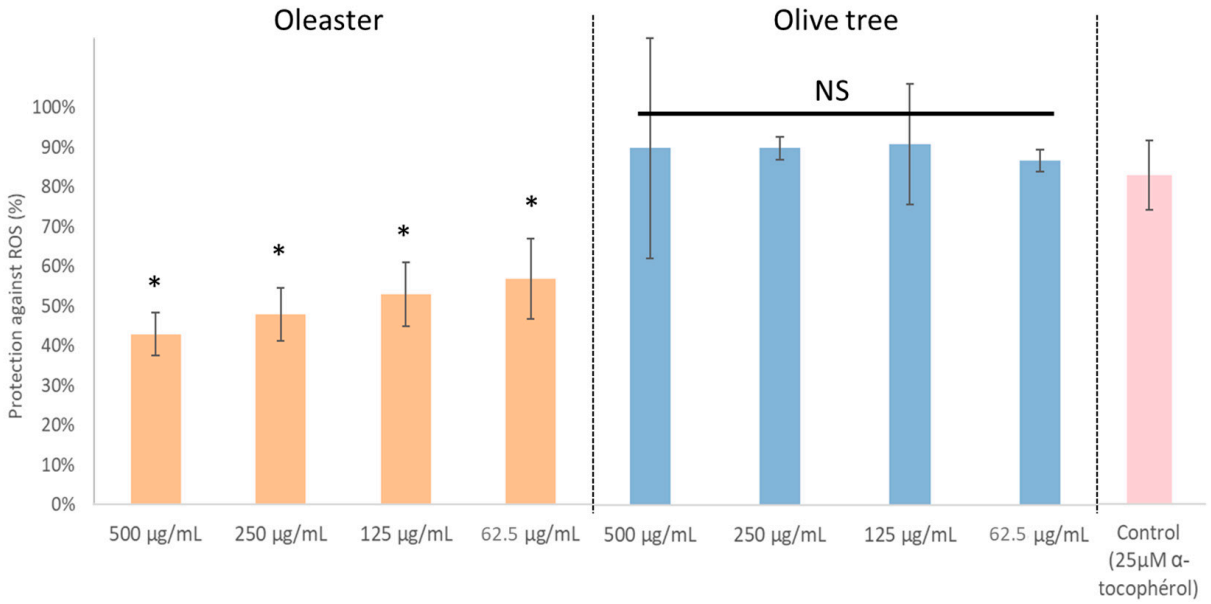


Figure 8. Protection against reactive oxygen species (ROS) in H_2O_2-stressed HaCaT cells by Olive tree and Oleaster leaf hydroalcoholic extracts. Results are expressed as percentages of total ROS neutralization in HaCaT cells calculated by comparison with H_2O_2-stressed cells. Extract conditions marked with * were statistically different from the tocopherol control condition with $p < 0.05$, while NS indicates No Significant difference.

In addition to antioxidant properties, the potential of Olive tree and Oleaster leaf extracts as skin anti-inflammatory and protection agents was evaluated. Their effects were measured on the inhibition of IL-8 production on HaCaT cells pretreated with each extract separately and afterward treated with pro-inflammatory LPS. As shown in Figure 9, cells treated with LPS exhibit significant production of IL-8, whereas those pre-treated with DXM show secretion of only around 50 pg/mL. When the cells were pre-treated with Oleaster or Olive tree extracts, a global reduction in pro-inflammatory IL-8 was observed for all the concentrations tested.

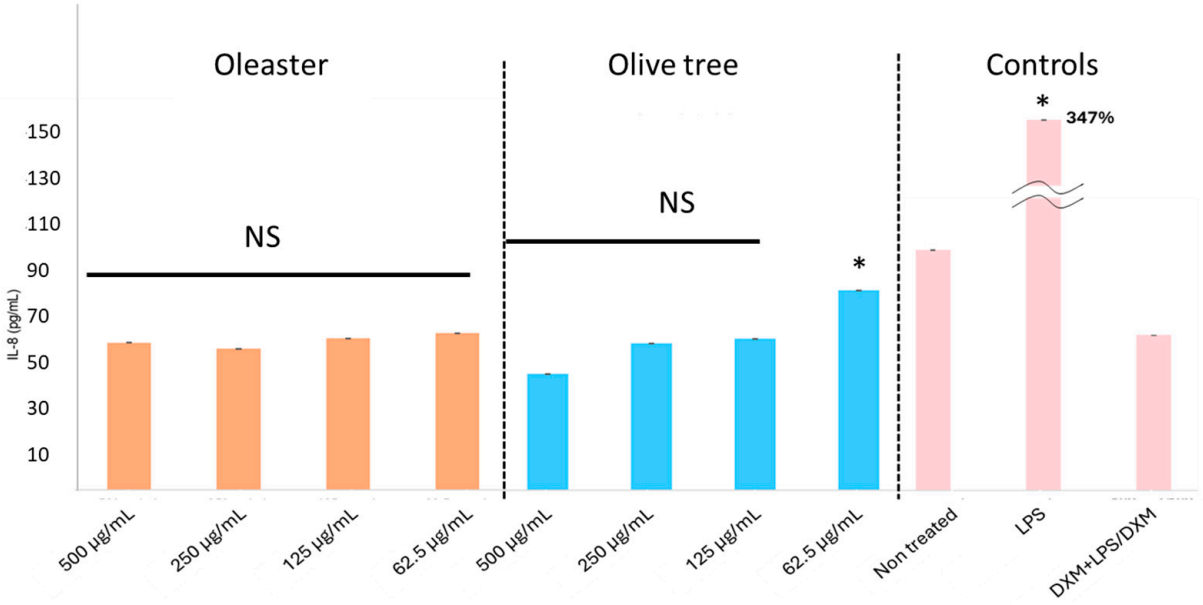


Figure 9. Concentration of IL-8 in HaCaT cells under pro-inflammatory conditions and the anti-Inflammatory effect of different concentrations of Olive tree and Oleaster leaf hydroalcoholic extracts. The results are expressed in picogram/mL (pg/mL) of secreted IL-8 quantified by ELISA assay for each experimental condition. Extract conditions marked with * were statistically different from the DXM control condition with $p < 0.05$, while NS indicates No Significant difference.

In fact, all concentrations tested for Oleaster leaf extract and those greater than or equal to 125 µg/mL for Olive tree leaf extract induce IL-8 production of approximately 50 pg/mL. This level of IL-8 production is similar to that observed for treatment with DXM, indicating the high efficiency of the extracts to protect cells under pro-inflammatory conditions.

No studies evaluating the antioxidant and anti-inflammatory activity of *Olea europaea* leaf on HaCaT cells were found in the literature, making this study probably the first one. The activity of Olive tree and Oleaster leaf hydroalcoholic extracts observed on the HaCaT cells confirms the results obtained from the chemical and enzymatic activity tests. The advantage of these cell-based tests is that they provide a more accurate picture than chemical and enzymatic tests of what might happen on the skin when a cosmetic product is applied. The results obtained in this study on crude extracts of Olive tree and Oleaster leaves suggest that the lowest concentration tested (62.5 µg/mL) was sufficient to protect HaCaT cells from oxidative and inflammatory conditions, while minimizing the risk of cytotoxicity.

As previously mentioned, the antioxidant and anti-inflammatory activities of Olive leaf extracts are generally linked to their content in oleuropein and flavonoids such as luteolin derivatives. Our ultrasound-assisted hydroalcoholic extracts of Olive tree and Oleaster leaves have a relatively high oleuropein content and contain numerous luteolin derivatives, which may explain their antioxidant and anti-inflammatory activities.

4. Conclusions

The aim of this study was to evaluate whether Oleaster, grown in the Centre-Val de Loire Region in France, and which is more resistant to the region's climatic conditions than the Olive tree, could be considered as a local source of interest for developing new cosmetic active ingredients. Thus, the study compared the phytochemical content, the antioxidant and anti-inflammatory properties of aqueous and hydroalcoholic ultrasound-assisted extracts of Oleaster leaves to those of the Olive tree.

The hydroalcoholic extract of Oleaster proved to be richer in phenolic and flavonoid compounds, with higher oleuropein and verbascoside contents. The chemical composition of the extracts determined using HPLC-MS/MS is fairly similar, with the presence of oleuropeine, luteoline, apigenin, diosmetin and maslinic acid derivatives for both species.

Furthermore, strong antioxidant and anti-inflammatory properties have been demonstrated, with inhibition of more than 60% of DPPH, CUPRAC, SOD and COX-2 activity, for both Oleaster and Olive tree leaf extracts. In addition, at concentrations that do not cause severe cytotoxicity (62.5 µg/mL), hydroalcoholic extracts exhibited 60–80% of protection against ROS in H_2O_2-stressed HaCaT cells and under inflammatory conditions, kept IL-8 concentrations at a low level (50 pg/mL) comparable to that obtained through treatment with the reference compound dexamethasone.

This study demonstrates for the first time that compared to Olive tree leaves, already used in cosmetic products, Oleaster leaves represent a more sustainable resource and offer at least equivalent, if not greater, potential for the development of high-value cosmetic ingredients.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cosmetics13030154/s1, MS and MS/MS spectra recorded in positive ionization mode were given as supplementary data.

Author Contributions: Conceptualization, E.V.-P., M.R. and E.D.; methodology, E.V.-P., M.S., E.L.R., C.C. and E.D.; validation, E.V.-P., C.C. and E.D.; investigation, E.L.R., M.S., E.V.-P., C.C. and E.D.; resources, M.R. and M.P.; data curation, E.L.R., M.S., E.V.-P., C.C. and E.D.; writing—original draft preparation, E.L.R. and M.S.; writing—review and editing, E.L.R., M.S., E.V.-P., C.C., M.R. and E.D.; supervision, E.V.-P. and E.D.; project administration, E.V.-P., M.R., M.P. and E.D.; funding acquisition, E.V.-P., M.R., M.P. and E.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by BPI France and the Centre-Val de Loire Region, through the "Berry Beauté" project grant number DOS0202343/00.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be addressed to the corresponding author.

Acknowledgments: The authors thank the SALSA platform for UHPLC-HRMS/MS analyses. We also thank the projects CHemBio (FEDER-FSE 2014-2020-EX003677), Valbiocosm (FEDER-FSE 2014-2020-EX003202), Techsab (FEDER-FSE 2014-2020-EX011313), QUALICHIM (APR-IA-PF 2021-00149467), Project ESTIM-ICOA (CPER/FEDER-FSE+ 2021-2027-00022860), the RTR Motivhealth (2019-00131403) and the Labex programs SYNORG (ANR-11-LABX-0029) and IRON (ANR-11-LABX-0018-01) for their financial support of ICOA, UMR 7311, University of Orléans, CNRS.

Conflicts of Interest: E.L.R. is employed by IES Ingredients. M.R. is employed by Sweetmom and M.P is employed by Uvalia. The companies had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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