Cytoprotective, antioxidant and anti-migratory activity of Pistacia lentiscus L. supercritical carbon dioxide extract on primary human endothelial cells

Roberta Giordo  
Mohammed Bin Rashid University of Medicine and Health Sciences

Annalisa Cossu  
University of Sassari: Universita degli Studi di Sassari

Maria Cristina Porcu  
Porto Conte Research: Porto Conte Ricerche Srl

Roberto Cappuccinelli  
Porto Conte Research: Porto Conte Ricerche Srl

Grazia Biosa  
Porto Conte Research: Porto Conte Ricerche Srl

Javad Sharifi-Rad  
Shahid Beheshti University of Medical Sciences

Luca Pretti  
Porto Conte Research: Porto Conte Ricerche Srl

Gheyath K Nasrallah  
Qatar University

Gianfranco Pintus  
University of Sharjah  
See https://orcid.org/0000-0002-3031-7733

Anna Maria Posadino  
University of Sassari: Universita degli Studi di Sassari

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Abstract

Green chemistry is emerging as a useful tool for producing valuable chemicals from biomass. However, extracted compounds need to be tested for safety, quality, and efficacy before their use in humans. Here we investigate the chemical composition and biological effects of leaves *Pistacia lentiscus* L. essential oil (EO) extract obtained with supercritical carbon dioxide (SCCO$_2$). Extract’s phytoconstituents profiling was performed by GC-MS/MS, while antioxidant activity was evaluated on human primary endothelial cells (ECs) using the reactive oxygen species (ROS) probe H$_2$DCFDA. Potentil extract toxicity and protective effect against H$_2$O$_2$-induced oxidative stress were investigated using LDH-leakage and BrdU-proliferation tests. Extract’s effect on ECs migration was determined by trans-well assay. Terpenes represented the main extraction process fraction yielding 0.14% of EO. Germacrene D (11.18%), delta-cadinene (10.54%), and alpha-pinene (8.7%) were the most abundant OE molecules. Challenged with ECs, increasing extract concentrations failed to affect cell proliferation or promote cell death. ROS assessment in unstressed and H$_2$O$_2$-treated ECs demonstrated an extract dose-dependent antioxidant activity. The exposure of H$_2$O$_2$-treated ECs to increasing extract concentrations dose-dependently rescued cells from the H$_2$O$_2$-induced impairments of cell proliferation and death. Extract was able to significantly counteract fetal calf serum-induced ECs migration. For the first time, we report that a SCCO$_2$ extract obtained from PL leaves is safe on ECs and may be a useful source of valuable compounds with vasculoprotective properties.

Statement Of Novelty

Here we report that a *Pistacia lentiscus* supercritical CO$_2$ extract is safe on human primary ECs and may be a useful source of valuable compounds capable of modulating endothelial cell functions of paramount importance in preventing or counteracting cardiovascular diseases.

1. Introduction

Biomass exploitation is emerging as an important component in the production of chemicals from renewable sources [1–6]. In the regard, wild aromatic plants are invaluable sources of new potential drugs. Indeed, plant essential oils (EO) and their constituents, as well as products from secondary plants metabolism, have been widely used in the pharmaceutical, cosmetic, food and beverage industries [7]. Different beneficial properties, such as antioxidant, anti-inflammatory, antiviral, antibacterial, antidiabetic and anticancer have been reported for EO [8], which are increasingly employed in the practice of complementary therapies, such as aromatherapy [8]. Among aromatic plants, *Pistacia lentiscus* L (PL) has found extensive use in folk medicine for several therapeutic uses including anti-hypertensive, anti-inflammatory, and antiseptic [9]. PL is an aromatic bush indigenous to Italy and other Mediterranean and Middle East countries [10]. PL leaves extracts have shown antimicrobial, antifungal, and antioxidant activity [11, 12]. Mastic oil from *Pistacia lentiscus var. Chia* has also been reported to possess antioxidant and cytoprotective potential [13] and the ability to inhibit the growth and survival of human K562
leukemia cells [14]. Antiproliferative effects of PL fixed oil and its phenolic extract have also been reported on BHK21 cancer cells [15]. PL EO extraction can be performed with different techniques including steam distillation, solvent extraction, CO\textsubscript{2} extraction, maceration, enfleurage, cold press extraction, and water distillation. In this regard, supercritical CO\textsubscript{2} (SCCO\textsubscript{2}) is emerging as an excellent extraction technology since it can be performed at low temperatures, thus protecting matrices components from thermal degradation [16]. Many studies have specifically indicated that SCCO\textsubscript{2} extraction provides the most desirable solvent for separating natural products used in foods and medicines because of its inertness, non-toxicity, low cost, critical temperature, and low pressure [4, 16–18].

Cardiovascular diseases (CVD) and vascular complications linked to diabetes, rheumatic, pulmonary and inflammatory diseases are still the leading cause of morbidity and mortality worldwide [19–22]. In this regard, oxidative-induced endothelial damage has emerged as one of the most common triggers underlying the onset and progression of several disease-associated vascular complications [23–27]. Reactive oxygen species (ROS) are generally recognized as normal byproducts of the aerobic metabolism and essential second messengers that play a crucial role in regulating vital cellular functions, including proliferation, differentiation, and migration. ROS’s physiological levels are finely tuned by the orchestrated action of ROS-generating enzymes and cellular antioxidants mechanisms [28]. However, dysregulation of the above-mentioned redox homeostasis can generate ROS increase, ultimately promoting oxidative stress, a phenomenon that has been linked to several pathological conditions [23, 25–27, 29, 30].

The linkage between oxidative stress and disease-associated vascular complications suggests that counteracting oxidative stress with antioxidants might prevent diseases occurrence or ameliorate its associated vascular complications [31]. In this light, a great deal of attention is now directed on the employment of naturally occurring antioxidants as potential candidates for disease prevention and/or treatment. However, although plant-derived compounds are recognized as useful adjuvants or therapeutic tools in preventing and treating various pathological conditions [32–34], their efficacy and safety in humans are still major concerns [35–40]. Moreover, the quality of the extracted compounds may be affected by several factors, including the technological process employed; it is therefore, essential to test their safety, efficacy, and quality before they potential therapeutic utilization [41].

To our knowledge, no previous reports have investigated the effects of PL leaves SCCO\textsubscript{2} extract on primary human ECs. In this light, the present work aims to examine the safety and potential cytoprotective, antioxidant and anti-migration properties of a SCCO\textsubscript{2} extract obtained from PL on primary human ECs as an in vitro vascular model

2. Materials And Methods

2.1 Raw material preparation and characterization.
Preparation and characterization of PL extract was performed as previously described [4]. Briefly, PL leaves samples were purchased from the Consorzio Officinerbe Sardigna (Monti, Sassari, Sardinia, Italy). Leaves were collected during full blossom and then dried to increase the ratio of essential oil to vegetable matter. This procedure also allows improving the extraction yield and preventing the risk of clogging due to the ice formation in the SCCO$_2$ extraction plant. The final moisture content was 11% (w/w). The dried leaves were milled and sieved to a particle size of ≤4 mm diameter and then vacuumed stored until use, at 4°C. All the highest purity solvents and reagents used in this work were purchased from Sigma-Aldrich, Steinheim, Germany. Chemical constituents of the oil were identified by comparison with reference compounds [Fluka, Acros Organics (Geel, Belgium) and Sigma-Aldrich (Steinheim, Germany)]

2.2 Pilot apparatus for the supercritical CO$_2$ extraction.

As previously described [4], the scale-up of the SCCO$_2$ extraction process was performed in a pilot plant (Superfluids-5/3SEP/COL; Proras S.r.l., Rome, Italy) with a maximum allowable pressure of 700 bar. This was equipped with a 5.0 L extractor vessel, three separator vessels in series and a CO$_2$ recycling system. The dried vegetable matter was loaded into the extractor in a stainless-steel basket. In all the SCCO$_2$ extractions, the CO$_2$ flow was ca. 16 L/h. The dried and triturated PL leaves were submitted to a single-step extraction process, made at 90 bars and 50°C for 4 h.

2.3 Extract Analysis.

Analysis of essential oil was carried out by gas chromatography (GC) and by gas chromatography-mass spectrometry (GC-MS) as previously reported [3]. Analytical GC was carried out in an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with HP GC ChemStation data handling system, equipped with a single injector and two flame ionization detectors (FID). A Graphpak divider (Agilent Technologies) was used for simultaneous sampling to two Supelco fused silica capillary columns (Supelco Inc., Bellefonte, PA, USA) with different stationary phases: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm I.D., film thickness 0.20 µm) and SUPELCOWAX 10 (polyethylene glycol 30 m × 0.20 mm I.D., film thickness 0.20 µm). Oven temperature was settled at 70°C, raising at 3°C min⁻¹ to 220°C and then held 15 min at 220°C; injector temperature: 250°C; carrier gas: helium, adjusted to a linear velocity of 30 cm/s; splitting ratio 1:40; detector temperature: 250°C. GC-MS analyses were carried out in an Agilent 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm I.D., film thickness 0.25 µm), interfaced with a Hewlett Packard mass selective detector 5973 (Agilent Technologies) operated by Agilent Enhanced ChemStation software. GC parameters as above; interface temperature: 250°C; MS source temperature: 230°C; MS quadrupole temperature: 150°C; ionization energy: 70 eV; ionization current: 60 µA; scan range: 35-350 u; scans/sec: 4.51. The components' identity was assigned by comparing mass spectra and retention indices for two different chromatographic stationary phases, calculated by linear interpolation to the retention of a series of n-alkanes. Experimental data were compared with corresponding data of reference oils and commercially available standards banked at a home-made library or from literature data [42, 43].
2.4 Cell culture and treatments.

Human umbilical vein endothelial cells (HUVECs), were obtained from Innoprot, (Bizkaia, Spain) and cultured as previously described [44]. Cells were cultured in 25 cm² flasks at 37°C, 90% humidity and 5% CO₂ in a complete Medium 199, supplemented with 100 U/mL penicillin, 25 µg/mL streptomycin, 0.85% amphotericin, 2 mM glutamine, 10% fetal calf serum and 10% new-born calf serum. For the experiments, ECs were used within 3 passages at an apparent confluence of 80%. The PL extract was kept in the dark at room temperature; immediately before use, a stock containing 1% extract (solubilized in the culture medium containing 1% dimethyl sulfoxide, DMSO) was prepared and sterilized by 0.45µm filter. Unless specified in the text, cells were plated in 96-well plates (Corning, Lowell, MA, USA) at a concentration of 105 cells/ml and processed for experiments in a complete medium as indicated in figure legends. Concentrations of 50, 150, and 600 µg/ml of PL extract were tested according to previous studies [4, 45]. PL extract working solution contained 0.1% DMSO, which was used as control. Potential extract toxicity was tested over a cells exposition time of 24 hrs. Potential extract protective effect against H₂O₂-induced ROS increase and oxidative cell damage was investigated by pretreating the cells for 3 hrs with different extract concentrations before exposure to H₂O₂. Potential extract anti-migratory effect against FCS-induced cell migration was investigated by pretreating the cells for 3 hrs with different extract concentrations before exposure to FCS.

2.5 Measurements of intracellular reactive oxygen species.

Intracellular ROS levels were determined using the ROS molecular probe H₂DCFDA. Within the cell, esterases cleave the acetate groups on H₂DCFDA, thus trapping the reduced form of the probe (H2DCF). Intracellular ROS oxidize H₂DCF, yielding the fluorescent product, DCF. Before the treatments, the cells were incubated for 30 min with PBS plus (PBS with 0.5 mM CaCl₂, 1 mM MgCl₂, 30 mM glucose) containing 1 µM H₂DCFDA, and then washed with PBS. Fluorescence was measured after the treatments using a GENios plus microplate reader (Tecan Mannedorf, Switzerland). The excitation and emission wavelengths used for fluorescence quantification were 485 nm and 535 nm, respectively. Treatment-induced variations in the fluorescence were measured kinetically, every minute for 15 consecutive minutes. All the fluorescence measurements were corrected for background fluorescence and protein concentrations. Using untreated cells as a reference, the antioxidant and prooxidant outcomes were evaluated by comparison of five measurements, and then expressed as a percentage of untreated controls [46]

2.6 Measurement of cell viability.

As previously described, HUVECs cell viability was assessed after the treatments by checking the leakage of the cytoplasmatic lactate dehydrogenase (LDH) from cells with a damaged membrane [47]. The amount of LDH released in the medium by death cells was assessed using the kit CytoTox-ONE™ (Promega, Madison, WI). A standard curve with definite amounts of cells (200µ/well) was made, and the release of LDH in the medium was measured after applying lysis solution (4 µl/well). Plates containing
samples were removed from the incubator and equilibrated to 22°C. Then the release of LDH from dead cells was measured by supplying lactate, NAD+, and resazurin as substrates in the presence of the enzyme diaphorase. Generation of the fluorescent resorufin product, which is proportional to the amount of LDH, was measured using a GENios plus microplate reader (Tecan Mannedorf, Switzerland) with excitation and emission of 560 nm and 590 nm, respectively. Using a standard curve, the amount of LDH release in treated and untreated cells was conversed in the number of cells per well. Data are representative of four independent experiments and are shown as the percent of untreated control cells.

2.7 Measurement of cell proliferation.

Cell proliferation was assessed after the treatments using the BrdU assay (Roche CH), a chemiluminescent immunoassay based on the determination of BrdU incorporation during DNA synthesis [48]. When cells are exposed to BrdU, the compound is incorporated into the newly synthesized DNA strands of actively proliferating cells. DNA-incorporated BrdU can be measured using anti-BrdU antibodies, allowing the assessment of the DNA synthesizing cells. BrdU was added 12 hrs before the end of the experiments, then the supernatant was removed, and cells were fixed for 30 min with a Fixing-Denaturating solution (Fix-Denat). At the end of incubation time, the Fix-Denat solution was discarded, and the cells were incubated for 90 minutes with a horseradish peroxidase-conjugated anti-BrdU antibody (anti-BrdU-POD). Following three rinsing with washing buffer, the substrate solution was added and allowed to react for 3-10 min at room temperature. Within this time window, the horseradish peroxidase catalyzes the oxidation of diacylhydrazide, and the reaction product, decaying from its excited state, yields light. Finally, a GENios Plus microplate reader (Tecan Mannedorf, Switzerland) was used to read the light emission of treated cells. Results were expressed as a means ± SD of the relative light units/sec (RLU/s) values. Data are representative of four independent experiments and are shown as the percent of untreated control cells.

2.8 Migration Assay.

HUVEC migration assays were performed in 24 wells trans-well as previously described [49]. Near confluent cells were serum-starved overnight, and then 40,000 cells were plated onto the top chamber of the cell culture inserts. Cells in the upper part of the chamber were treated with PL extract in serum free medium (Medium 199 with antibiotics), whereas the complete medium was added to the lower chamber. The assembled cell culture chamber was then incubated at 37°C, 5% CO₂ for 12 hrs. At the end of incubation time, the upper surfaces of the membranes were gently wiped with cotton swabs to remove the non-migratory cells. The membranes were then fixed with paraformaldehyde, PAF (4%), stained with the fluorophore Hoechst 33342 (10 µg/ml), and the fluorescence was measured using a GENios plus microplate reader (Tecan Mannedorf, Switzerland). The excitation and emission wavelengths used for fluorescence quantification were 340 nm and 485 nm, respectively.

2.9 Statistical analysis.
One-way analysis of variance (ANOVA), followed by a post-hoc Newman-Keuls for multiple comparisons, was used to detect differences among studied groups. All statistical analyses were performed using GraphPad Prism version 9.00 for Windows (GraphPad Software, San Diego, CA, USA), and p-values < 0.05 were considered statistically significant.

3. Results

3.1 Essential oils composition

Percentages of individual components of PL extract were calculated based on gas chromatography (GC) peak areas without flame ionization detection (FID) response factor correction. Chromatogram and main chemical components of PL essential oil are reported respectively in figure 1 and Table 1. The analytical results were consistent with those reported in the literature concerning terpene compounds, representing the more important fraction with antioxidant activity. The total essential oil yield, after 4 hours of extraction was 0.14% and the main abundant constituents included germacrene D (11.18%), delta-Cadinene (10.54%), alpha-pinene (8.7%), beta-caryophyllene (5.74%), myrcene (4.5%), beta-phellandrene (4.33%), terpin-4-ol (4.306), epi-alpha-muurolol (3.262) and beta-pinene (3.00%). The complete profiling of the essential oil components is reported in Table 1.
Table 1
Chemical components of the *Pistacia Lentiscus L.* SCCO₂ extract obtained by GC-GM

| n  | RIK | Tr (min) | Compound             | %    |
|----|-----|----------|----------------------|------|
| 1  | 926 | 5.01     | Tricyclene           | 0.096|
| 2  | 931 | 5.11     | Alpha-Thujene        | 0.219|
| 3  | 938 | 5.29     | Alpha-Pinene         | 8.692|
| 4  | 953 | 5.66     | Camphene             | 0.453|
| 5  | 977 | 6.29     | Sabinene             | 2.857|
| 6  | 980 | 6.39     | Beta-Pinene          | 3.025|
| 7  | 993 | 6.76     | Myrcene              | 4.562|
| 8  | 1007| 7.17     | Alpha-Phellandrene   | 2.170|
| 9  | 1019| 7.55     | Alpha-Terpinene      | 1.661|
| 10 | 1028| 7.79     | Para-Cymene          | 0.649|
| 11 | 1032| 7.95     | Beta-Phellandrene    | 4.332|
| 12 | 1051| 8.56     | Beta(E)-Ocimene      | 0.135|
| 13 | 1059| 8.83     | Isopentyl N-Butanoate| 0.245|
| 14 | 1062| 8.94     | Gamma-Terpinene      | 2.533|
| 15 | 1090| 10       | Terpinolene          | 0.830|
| 16 | 1093| 10.12    | 2-Nonanone           | 0.248|
| 17 | 1101| 10.43    | Linalool             | 0.388|
| 18 | 1107| 10.63    | Isopentyl Isovalerate| 0.207|
| 19 | 1180| 13.47    | Terpin-4-Ol          | 4.306|
| 20 | 1191| 13.98    | Alpha-Terpineol      | 0.874|
| 21 | 1253| 16.49    | N.I.                 | 0.440|
| 22 | 1256| 16.61    | N.I.                 | 0.218|
| 23 | 1286| 17.95    | Bornyl Acetate       | 0.118|
| 24 | 1294| 18.32    | 2-Undecanone         | 1.362|
| 25 | 1302| 18.65    | N.I.                 | 0.110|

Retention indices (RIK), retention times (Tr) and chromatographic area percentages (%) of the most abundant compounds found in the essential oil extract obtained by supercritical CO₂ (SCCO₂) extraction at 90 bar, 50°C from *Pistacia lentiscus L.*
| n  | RIK  | Tr (min) | Compound                           | %    |
|----|------|----------|------------------------------------|------|
| 26 | 1351 | 20.61    | Alpha-Cubebene                     | 0.262|
| 27 | 1377 | 21.69    | Alpha-Copaene                      | 1.330|
| 28 | 1390 | 22.29    | Beta-Cubebene                      | 0.353|
| 29 | 1392 | 22.38    | Beta-Elemene                       | 1.288|
| 30 | 1408 | 23.04    | N.I.                               | 0.138|
| 31 | 1420 | 23.48    | Beta-Caryophyllene                 | 5.738|
| 32 | 1429 | 23.85    | Beta-Gurjunene                     | 0.213|
| 33 | 1438 | 24.18    | N.I.                               | 0.746|
| 34 | 1451 | 24.71    | N.I.                               | 0.759|
| 35 | 1454 | 24.82    | Alpha-Humulene                     | 2.097|
| 36 | 1461 | 25.1     | Allo-Aromadendrene                 | 0.907|
| 37 | 1463 | 25.2     | Muurola-4(14),5-Diene<Cis>         | 0.198|
| 38 | 1474 | 25.66    | Cadina-1(6),4-Diene<Trans>        | 1.297|
| 39 | 1478 | 25.82    | Gamma-Muurolene                    | 2.610|
| 40 | 1482 | 26       | Germacrene-D                       | 11.182|
| 41 | 1486 | 26.15    | Beta-Selinene                      | 0.279|
| 42 | 1491 | 26.36    | Muurola-4(15),5-Diene<Trans>      | 1.025|
| 43 | 1495 | 26.55    | Alpha-Selinene                     | 1.507|
| 44 | 1499 | 26.73    | Alpha-Muurolene                    | 2.313|
| 45 | 1503 | 26.88    | N.I.                               | 0.294|
| 46 | 1506 | 26.99    | N.I.                               | 0.183|
| 47 | 1509 | 27.09    | Beta-Bisabolene                    | 0.924|
| 48 | 1513 | 27.25    | Gamma-Cadinene                     | 1.186|
| 49 | 1525 | 27.68    | Delta-Cadinene                     | 10.542|
| 50 | 1533 | 27.97    | Trans-Cadina-1(2)-4-Diene          | 0.791|
| 51 | 1538 | 28.16    | Alpha-Cadinene                     | 0.234|

Retention indices (RIK), retention times (Tr) and chromatographic area percentages (%) of the most abundant compounds found in the essential oil extract obtained by supercritical CO$_2$ (SCCO$_2$) extraction at 90 bar, 50°C from *Pistacia lentiscus* L.
| n  | RIK  | \(\text{Tr (min)}\) | Compound                | %  |
|----|------|----------------------|-------------------------|----|
| 52 | 1544 | 28.4                 | N.I.                    | 0.340 |
| 53 | 1550 | 28.61                | Elemol                  | 0.178 |
| 54 | 1556 | 28.86                | N.I.                    | 0.116 |
| 55 | 1559 | 28.98                | Elemicin                | 0.100 |
| 56 | 1565 | 29.21                | E-Nerolidol             | 0.117 |
| 57 | 1576 | 29.65                | Spatutenol              | 0.248 |
| 58 | 1582 | 29.87                | Caryophyllene Oxide     | 0.251 |
| 59 | 1584 | 29.97                | N.I.                    | 0.155 |
| 60 | 1590 | 30.19                | N.I.                    | 0.052 |
| 61 | 1613 | 31.07                | N.I.                    | 0.104 |
| 62 | 1616 | 31.17                | N.I.                    | 0.158 |
| 63 | 1627 | 31.58                | N.I.                    | 1.247 |
| 64 | 1631 | 31.7                 | Gamma-Eudesmol          | 0.236 |
| 65 | 1642 | 32.11                | Epi-Alpha-Muurolol      | 3.262 |
| 66 | 1646 | 32.25                | Alpha-Muurolol          | 0.828 |
| 67 | 1649 | 32.36                | Beta-Eudesmol           | 0.121 |
| 68 | 1654 | 32.56                | Alpha-Cadinol           | 2.626 |
| 69 | 1683 | 33.64                | Epi-Alpha-Bisabolol     | 0.506 |
| 70 | 1685 | 33.71                | Alpha-Bisabolol         | 0.148 |
| 71 | 1694 | 34.05                | N.I.                    | 0.086 |

Retention indices (RIK), retention times (Tr) and chromatographic area percentages (%) of the most abundant compounds found in the essential oil extract obtained by supercritical \(\text{CO}_2\) (SCCO\(_2\)) extraction at 90 bar, 50°C from *Pistacia lentiscus* *L*.

3.2 Biological activity

Using primary human endothelial cells as an in vitro vascular model, we investigated the PL extract ability to modulate different biological cell functions, including proliferation, migration, and intracellular redox status.

3.2.1 PL extract does not affect endothelial cell viability and proliferation
Compounds safety is of paramount importance for their potential therapeutic employment; therefore, we first investigated the potential toxicity of the obtained extract by assessing its effects on cell viability, cell proliferation, and intracellular ROS production. Indeed, intracellular ROS generation is closely related to cell survival, proliferation and apoptosis [50, 51]. Based on previously reported data concerning the effect of PL essential oil on different cultured cells [45], we evaluated the possible harmful effects of three concentrations [50, 150, and 600 µg/ml] of the obtained PL extract on HUVEC viability and proliferation. To this end, cells were treated for 24 hrs with the PL extract, then cell viability and proliferation were assessed as reported in the material and methods sections. As reported in Figure 2A, the PL extract had no toxic effect at any of the tested concentrations indicating safety of both the extract and the extraction process applied. Likewise, the data in figure 2B indicate that the tested extract concentrations failed to induce any detrimental effects on HUVEC proliferation, further confirming the extract's safety.

3.2.2 PL extract showed antioxidant effect against H₂O₂-induced oxidative stress

The antioxidant properties of PL leaves and fruits compounds have been reported in vitro by measuring their scavenging ability or assessing their anti-lipid peroxidation potential [52, 53]. However, whether PL SCCO₂ extract is able to exert antioxidant effects in biological models by protecting cells from oxidative stress remains to be elucidated. Therefore, we sought to investigate whether the obtained PL extract could counteract oxidative stress in human primary endothelial cells oxidatively stressed with H₂O₂. For this purpose, H₂DCFDA-loaded cells were pretreated for 3 hrs with the indicated extract concentrations and then incubated for 6 hrs in the presence or absence of 75 µM H₂O₂. The data derived from five pooled measurements were expressed as percentages of the untreated cells and compared with the vehicle control (0.1% DMSO). As depicted in figure 3A, exposure of H₂O₂-treated cells to increasing concentrations of PL extract showed a significant dose-dependent antioxidant effect compared to cells treated with only H₂O₂. We next wondered whether the PL extract per se could exert any antioxidant or prooxidant effect in the absence of oxidative insults. As reported in figure 3B, the exposure of unstressed cells to increasing extract concentrations induced a significant antioxidant effect at 150 and 600 µg/ml, while failing to affect the intracellular redox state at 50 µg /ml. These findings are in agreement with the cell viability and proliferation results showing no extract toxicity up to 24 hrs of cell treatment (Fig. 2A-B)

3.2.3 PL extracts showed protective effect against H₂O₂-induced oxidative stress

Oxidative-induced endothelial damage appears to trigger and sustain cardiovascular diseases [51]. Therefore, a great deal of research is now focused on finding natural antioxidants capable to prevent or counteract CVD-associated ROS increases. To determine whether the observed PL extract antioxidant effect could be protective against the H₂O₂-induced oxidative damage, we measured HUVEC viability by assessing the cellular membrane integrity. To this end, cells were exposed for 3 h to increasing concentrations of PL extract and then incubated for 24 h in the absence or presence of 75 µM H₂O₂. Cell were then processed for cell viability as reported in material and methods. As indicated in figure 4, cells exposition to increasing doses of PL extract provided a significant dose-dependent cytoprotective effect.
with respect to the H2O2-induced cell damage. Indeed, all the tested PL concentrations were able to significantly counteract H2O2-induced cell damage. Consonant with these findings are the data in figure 3B, reporting the extract's ability to dose-dependently prevent the detrimental effect on HUVEC proliferation elicited by H2O2.

3.2.4 PL extracts showed antimigratory effect against serum-induced migration

Migration, the cell movement in response to chemical and/or mechanical signals, beside playing an essential role in physiologic processes, such as embryonic development, wound healing, tissue regeneration, it is also a critical process in pathological conditions such as tumor growth, blinding eye diseases, diabetic retinopathy, and arthritis [54]. Endothelial cell migration is an essential step of the angiogenic process; indeed, endothelial cells, which normally are maintained in a quiescent state, are stimulated to degrade the basement membrane and migrate into the perivascular stroma in response to either proangiogenic factors or by the downregulation of antiangiogenic factors [55]. For this reason, we investigated the ability of PL extract to modulate endothelial cells migration using the matrigel transfilter cell invasion assays, a modification of the Boyden chamber migration assay [56]. These three-dimensional assays are based on the migration of endothelial cells, placed on top of a filter containing 8 µm diameter pores, which allow only active passage of the cells towards an attractant placed in the lower chamber [57]. Migrated endothelial cells through modified Boyden chambers were measured by reading the fluorescent product of Hoechst 33342 resulted from the metabolism of live cells migrated through the micropores of the upper chamber. Figure 5 demonstrated that the PL extract dose-dependently counteracted the serum-induced cell migration, eliciting a significant reduction at the doses of 150 and 600.

4. Discussion

The genus *Pistacia* belongs to the Anacardiaceae family and comprises about 70 genera and over 600 species [9, 58]. The species PL is a dioecious evergreen shrub or small tree, from 1 to 5 m high, with a strong smell of resin, growing in dry and rocky areas in Mediterranean Europe. The aromatic resin, ivory colored, is harvested as a spice from the cultivated mastic trees. The genus *Pistacia* has shown many interesting biological activities in vivo and in vitro, such as antimicrobial activity [59], antifungal and anticancer activity [60]. It has also been shown to inhibit pro-inflammatory substances production and protect the cardiovascular system by lowering total serum cholesterol, low-density lipoprotein, and triglycerides in rats. In addition, it resulted able to preserve low-density lipoproteins from oxidation in humans.[61, 62]. Although the antioxidant, antimicrobial and anti-inflammatory activities of the PL. gum and the fixed oil have been previously tested [12, 63–66]; this is the first report showing the effects of the SCCO₂ technique-obtained PL essential oil on primary human endothelial cells. However, we need to clarify that we cannot discriminate whether the biological effects observed in this work are attributable to a single molecule's type present in the analyzed PL extract or to an entire chemical class of secondary metabolites, the terpenes (beta-caryophyllene, germacrene D, alpha-pinene, myrcene, beta-phellandrene, and alpha-humulene). A great number of plants produce terpenes and many studies have demonstrated
how this fraction represents the more important component with antioxidant activity [67]. In this regard, an interesting review shows how the terpene, lycopene, may protect against atherosclerosis, inhibiting ROS production in vitro and protecting LDL from oxidation [68]. Furthermore, several studies showed that sesquiterpene lactones, another class of terpenes, possess strong anti-inflammatory, anti-tumor and antimicrobial activities [69–71]. Since then, this class of phytochemicals has attracted the attention of researchers towards their potential medicinal properties. Several sesquiterpene lactones such as artemisinins, thapsigargins, parthenolide etc., have demonstrated the ability to inhibit angiogenesis in vitro and in vivo by suppressing HUVECs proliferation and migration, microvessel formation, and vascular endothelial growth factor (VEGF) expression. In addition, they resulted also able to prevent osteolytic bone metastasis by suppressing the growth and migration of mammary carcinosarcoma cells [72–74]. Therefore, based on the literature data, we are of the opinion that our current data might be the result of the synergistic effect produced by the components present in the extract, for which antioxidant and anti-inflammatory activities have been already reported [63, 75, 76]. This synergic mechanism is not fully understood, and it may involve the compounds’ action toward different pharmacological targets. In the present study, we investigated the SCCO$_2$-obtained PL extract cytoprotective, antioxidant, and antimigratory potential, three aspects essential for maintaining an optimal vascular structure and functionality. According to previously reported data [45], HUVECs were treated with increasing PL extract concentrations [50, 150 and 600 µg/ml], and no effect on the cell viability and proliferation was observed, indicating that the applied process was able to provide a safe extract that lack of cell cytotoxicity. Then we investigated potential biological properties harbored by the obtained extract. To this end, the same PL extract concentrations were employed to assess their antioxidant potential against H$_2$O$_2$-induced oxidative stress in primary human endothelial cells, along with their possible protective effect toward oxidative stress-induced cell death. All the tested extract doses were able to counteract H$_2$O$_2$-induced ROS increase and oxidative-induced cell death, confirming that the possible synergistic effect of its components conveys antioxidant properties to the PL extract. In recent years, it was established that natural antioxidants might modulate HUVECs migration [77]. As already mentioned, PL extract GC–MS data demonstrated a predominance of terpenes (monoterpenes and sesquiterpenes). In this regard, previous studies showed that sesquiterpenes owned anti-inflammatory properties [78,79], and the contained-monoterpene in plant-derived EO effectively inhibited carrageenan-induced edema and neutrophil migration [78]. Cell migration is a highly integrated, multi-step process that plays an important role in the progression of various diseases, including cancer, atherosclerosis, and arthritis. In this regard, the two higher PL concentrations tested were able to significantly reduce serum-induced HUVECs migration, suggesting a direct effect of the extract on the migration process. These results were in line with those related to PL extract antioxidant activity as a confirmation of the link between oxidative stress and endothelial cells migration. In fact, ROS produced via NADPH oxidase activation stimulate various redox signaling pathways leading to angiogenic responses, including endothelial cell migration. In particular, activated VEGF increases ROS production via Rac1-dependent NADPH oxidase, and ROS in turn are involved in VEGF-induced autophosphorylation of VEGFR-2 [55]. We know that further investigations are needed to better understand the molecular events elicited by PL extract on this cellular
model. Nevertheless, our data provide novel insight concerning the use of SCCO$_2$ as useful green chemistry process to obtain valuable products harboring essential biological processes from biomass.

5. Conclusions

Whether PL extract obtained by SCCO$_2$ is safe and possesses biological activities against CVD remains to be investigated. Here we report that SCCO$_2$-derived PL extract is safe, shows cytoprotective effects against oxidative damage, and modulates ROS production and HUVECs migration. Our results indicate SCCO$_2$ as a secure green chemistry process to exploit biomasses to obtain precious material containing molecules capable of modulating cell functions of paramount importance in preventing or counteracting CVD.

Credit Authorship Contribution Statement

RG, GP, AMP: Conceptualization. RG, AC, MCP, RC, GB, JSR, AMP: Methodology. RG, AC, MCP, RC, GB, AMP: Investigation. GKN, LP, GP: resources. RG, GP, AMP: writing—original draft preparation. RG, AC, MCP, RC, GB, JSR, GKN, LP, GP, AMP: writing—review and editing. GP: supervision. LP, GP: project administration. GKN, LP, GP: funding acquisition

Declarations

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

The data presented in this study are available in this article.

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Figures

Figure 1

Chromatogram of the SCCO$_2$ extract obtained at 90 bar, 50°C from *Pistacia lentiscus L.* leaves.
Figure 2

Effect of *Pistacia lentiscus* L extract on HUVEC viability and proliferation. Cells were exposed for 24 h in the absence (CTRL) or presence to the indicated concentrations of *Pistacia lentiscus* L extract. Cell viability and proliferation were assessed as reported in the "Materials and Methods" section. CTRL, untreated cells; PL, *Pistacia lentiscus* L. extract. Values are shown as mean ± SD and expressed as a percentage of the vehicle CTRL equal to 100% (0.1% DMSO), (n = 4).
Figure 3

Antioxidant dose-response effect of *Pistacia lentiscus* L extract against H$_2$O$_2$-induced oxidative stress. A) Dose-response effect of *Pistacia lentiscus* extract on intracellular ROS levels in HUVEC treated with 75µM of H$_2$O$_2$. Cells were exposed for 3 hrs to the indicated concentrations of PL extract and then incubated for 6 hrs in the absence or presence of 75 µM H$_2$O$_2$. B) Dose-response effect of *Pistacia lentiscus* L extract on intracellular ROS levels in unstressed HUVECs. Cells were exposed for 3 hrs to the indicated concentrations of *Pistacia lentiscus* L extracts and then incubated for 6 hrs before ROS determination. Intracellular ROS levels were assessed, as reported in the “Materials and Methods” section. CTRL, untreated cells; H$_2$O$_2$, hydrogen peroxide; PL, *Pistacia lentiscus* L extract. Values are shown as mean ± SD and expressed as a percentage of the vehicle CTRL equal to 100% (0.1% DMSO). *Significantly different from the CTRL (p < 0.05). #, Significantly different from H2O2 (p < 0.05), (n = 5).
3.2.3 PL extracts showed protective effect against \( \text{H}_2\text{O}_2 \)-induced oxidative stress

Oxidative-induced endothelial damage appears to trigger and sustain cardiovascular diseases [51]. Therefore, a great deal of research is now focused on finding natural antioxidants capable to prevent or counteract CVD-associated ROS increases. To determine whether the observed PL extract antioxidant effect could be protective against the \( \text{H}_2\text{O}_2 \)-induced oxidative damage, we measured HUVEC viability by assessing the cellular membrane integrity. To this end, cells were exposed for 3 h to increasing concentrations of PL extract and then incubated for 24 h in the absence or presence of 75 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \). Cell were then processed for cell viability as reported in material and methods. As indicated in figure 4, cells exposition to increasing doses of PL extract provided a significant dose-dependent cytoprotective effect with respect to the \( \text{H}_2\text{O}_2 \)-induced cell damage. Indeed, all the tested PL concentrations were able to significantly counteract \( \text{H}_2\text{O}_2 \)-induced cell damage. Consonant with these findings are the data in figure 3B, reporting the extract's ability to dose-dependently prevent the detrimental effect on HUVEC proliferation elicited by \( \text{H}_2\text{O}_2 \).
Cytoprotective dose-response effect of *Pistacia lentiscus* L extract against H2O2-induced cell death. Dose-response effect of *Pistacia lentiscus* L extract on cell viability in HUVEC treated with 75µM of H2O2. Cells were exposed for 3 hrs to the indicated concentrations of *Pistacia lentiscus* L extracts and then incubated for 24 hrs in the absence or presence of 75 µM H2O2. Cell viability was assessed as reported in the “Materials and Methods” section. CTRL, untreated cells; H2O2, hydrogen peroxide; PL, *Pistacia lentiscus* L extract. Values are shown as mean ± SD and expressed as a percentage of the vehicle CTRL equal to 100% (0.1% DMSO). *Significantly different from CTRL (p < 0.05). #, Significantly different from H2O2 (p < 0.05), (n = 4).
3.2.4 PL extracts showed antimigratory effect against serum-induced migration

Migration, the cell movement in response to chemical and/or mechanical signals, beside playing an essential role in physiologic processes, such as embryonic development, wound healing, tissue regeneration, it is also a critical process in pathological conditions such as tumor growth, blinding eye diseases, diabetic retinopathy and arthritis [54]. Endothelial cell migration is an essential step of the angiogenic process; indeed, endothelial cells, which normally are maintained in a quiescent state, are stimulated to degrade the basement membrane and migrate into the perivascular stroma in response to either proangiogenic factors or by the downregulation of antiangiogenic factors [55]. For this reason, we investigated the ability of PL extract to modulate endothelial cells migration using the matrigel transfilter cell invasion assays, a modification of the Boyden chamber migration assay [56]. These three-dimensional assays are based on the migration of endothelial cells, placed on top of a filter containing 8 μm diameter pores, which allow only active passage of the cells towards an attractant placed in the lower chamber [57]. Migrated endothelial cells through modified Boyden chambers were measured by reading the fluorescent product of Hoechst 33342 resulted from the metabolism of live cells migrated through the micropores of the upper chamber. Figure 5 demonstrated that the PL extract dose-dependently counteracted the serum-induced cell migration, eliciting a significant reduction at the doses of 150 and 600.

Figure 5

Antimigratory dose-response effect of *Pistacia lentiscus* L extract against serum-induced cell migration. Dose-response effect of *Pistacia lentiscus* L extract on cell migration in HUVEC treated with 10% fetal calf serum. Cells were exposed for 3 hrs to the indicated concentrations of *Pistacia lentiscus* L extracts in serum free medium, then cell migration was determined for 12 hrs as reported in the “Materials and Methods” section. CTRL, untreated cells; PL, *Pistacia lentiscus* L extract. Values are shown as mean ± SD
and expressed as a percentage of the vehicle CTRL equal to 100% (0.1% DMSO). *Significantly different from CTRL (p < 0.05), (n = 4).