doi: 10.53446/actamednicomedia.1880441

Acta Medica Nicomedia

Cilt: 9 (2026), 1-7 / Vol: 9 (2026), 1-7
https://dergipark.org.tr/tr/pub/actamednicomedia

Research Article | Araştırma Makalesi

PLANT-DERIVED EXTRACELLULAR VESICLES IMPROVE ENDOTHELIAL VIABILITY AND REDUCE CYTOTOXICITY UNDER OXIDATIVE STRESS

BİTKİ KAYNAKLI EKSTRASELÜLER VEZİKÜLLER OKSİDATİF STRES ALTINDA ENDOTELYAL HÜCRE CANLILIĞINI ARTIRIR VE SİTOTOKSİSİTEYİ AZALTIR

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ABSTRACT

Objective: Endothelial dysfunction is a central feature of cardiovascular and metabolic diseases and is closely linked to oxidative stress–induced cellular injury. Excessive reactive oxygen species production disrupts endothelial viability, membrane integrity, and redox balance. Plant-derived extracellular vesicles (PD-EVs) have emerged as biocompatible nanovesicles with antioxidant potential; however, their effects on endothelial cells under oxidative stress remain insufficiently characterized. This study aimed to evaluate the protective effects of PD-EVs on endothelial cells exposed to hydrogen peroxide–induced oxidative stress.

Methods: Extracellular vesicles were isolated from pomegranate, grape, and beetroot using differential ultracentrifugation and characterized by protein quantification, dynamic light scattering, and electron microscopy. Human umbilical vein endothelial cells (HUVECs) were exposed to hydrogen peroxide (H_2O_2) and subsequently treated with individual or combined PD-EVs. Cell viability and cytotoxicity were assessed using CCK-8 and LDH assays. Antioxidant responses were evaluated by quantitative real-time PCR analysis of *GPX1* gene expression.

Results: PD-EVs displayed nanoscale size and morphology consistent with extracellular vesicles. H_2O_2 exposure significantly reduced HUVEC viability and increased LDH release. PD-EV treatment significantly improved cell viability and reduced cytotoxicity under oxidative stress, with beetroot-derived and combined EV formulations showing the strongest effects. *GPX1* expression was suppressed by H_2O_2 but significantly restored following PD-EV treatment, indicating activation of an adaptive antioxidant response.

Conclusion: Plant-derived extracellular vesicles attenuate oxidative endothelial injury by improving cell viability, preserving membrane integrity, and restoring GPX1-associated antioxidant defenses. These findings highlight PD-EVs as a promising biocompatible, cell-free strategy for protecting endothelial cells against oxidative stress.

Keywords: Plant-derived extracellular vesicles (PD-EVs); Oxidative stress; Endothelial cells; Hydrogen peroxide (H_2O_2); Cell-free therapy

ÖZ

Amaç: Endotelyal disfonksiyon, kardiyovasküler ve metabolik hastalıkların temel bir özelliği olup oksidatif stres kaynaklı hücresel hasarla yakından ilişkilidir. Aşırı reaktif oksijen türleri (ROS) üretimi, endotel hücre canlılığını, membran bütünlüğünü ve redoks dengesini bozar. Bitki kaynaklı ekstraselüler veziküller (PD-EV'ler), antioksidan potansiyele sahip biyouyumlu nanoveziküller olarak öne çıkmaktadır; ancak oksidatif stres koşulları altında endotel hücreleri üzerindeki etkileri henüz yeterince aydınlatılmamıştır. Bu çalışmanın amacı, hidrojen peroksit ile indüklenen oksidatif stres koşullarında PD-EV'lerin endotel hücreleri üzerindeki koruyucu etkilerini değerlendirmektir.

Yöntem: Ekstraselüler veziküller nar, üzüm ve pancardan diferansiyel ultrasantrifügasyon yöntemi ile izole edilmiş; protein miktarı tayini, dinamik ışık saçılımı ve elektron mikroskopisi ile karakterize edilmiştir. İnsan umblikal ven endotel hücreleri (HUVEC'ler) hidrojen peroksit (H_2O_2) ile oksidatif strese maruz bırakılmış ve ardından tekli veya kombine PD-EV uygulamaları gerçekleştirilmiştir. Hücre canlılığı ve sitotoksisite sırasıyla CCK-8 ve LDH analizleri ile değerlendirilmiştir. Antioksidan yanıt, GPX1 gen ekspresyonunun kantitatif gerçek zamanlı PCR ile analizi yoluyla incelenmiştir.

Bulgular: PD-EV'ler, ekstraselüler veziküllerle uyumlu nanoskalada boyut ve morfoloji sergilemiştir. H_2O_2 uygulaması HUVEC canlılığını anlamlı düzeyde azaltmış ve LDH salınımını artırmıştır. PD-EV tedavisi, oksidatif stres koşullarında hücre canlılığını anlamlı şekilde artırmış ve sitotoksisiteyi azaltmıştır; en belirgin koruyucu etkiler pancar kaynaklı ve kombine EV gruplarında gözlenmiştir. H_2O_2 ile baskılanan GPX1 ekspresyonunun PD-EV uygulaması sonrası anlamlı şekilde geri kazanılması, adaptif bir antioksidan yanıtın aktive edildiğini göstermiştir.

Sonuç: Bitki kaynaklı ekstraselüler veziküller, hücre canlılığını artırarak, membran bütünlüğünü koruyarak ve GPX1 ilişkili antioksidan savunma mekanizmalarını yeniden düzenleyerek oksidatif endotelyal hasarı azaltmaktadır. Bu bulgular, PD-EV'lerin oksidatif strese karşı endotel hücrelerini korumada umut vadeden, biyouyumlu ve hücresiz bir terapötik strateji olduğunu ortaya koymaktadır.

Anahtar Kelimeler: Bitki kaynaklı ekstraselüler veziküller, oksidatif stres, Endotelyal hücreler, Hidrojen peroksit, hücre içermeyen tedavi yaklaşımı

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Submitted/Başvuru: 02.02.2026 • **Accepted/Kabul:** 17.02.2026 • **Published Online/Online Yayın:** 06.03.2026

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Introduction

The vascular endothelium is a highly dynamic tissue that plays a pivotal role in the regulation of vascular tone, barrier integrity, hemostatic balance, and immune cell trafficking. Endothelial dysfunction is widely recognized as an early and common pathological feature of numerous chronic diseases, including atherosclerosis, hypertension, diabetic vasculopathy, and age-related cardiovascular disorders. Among the underlying mechanisms, oxidative stress emerges as both a triggering and sustaining factor in endothelial injury. Excessive production of reactive oxygen species (ROS) leads to reduced nitric oxide (NO) bioavailability, impaired vasodilation, and the development of a pro-inflammatory and pro-thrombotic endothelial phenotype.[1]

In metabolic disorders such as diabetes, as well as during aging and chronic inflammation, several molecular mechanisms contribute to endothelial oxidative stress, including NADPH oxidase activation, mitochondrial ROS overproduction, and endothelial nitric oxide synthase (eNOS) uncoupling. In particular, diabetic microenvironments characterized by hyperglycemia, dyslipidemia, and insulin resistance have been shown to exacerbate ROS generation while suppressing NO signaling, thereby predisposing endothelial cells to functional impairment and vascular complications.[2] These observations underscore the importance of evaluating endothelial responses to oxidative stress not only to better understand disease pathogenesis but also to identify novel protective and restorative strategies.

In recent years, plant-derived extracellular vesicles (PD-EVs) have gained increasing attention as naturally occurring nanovesicles involved in intercellular and interspecies communication. PD-EVs are known to carry a diverse cargo, including lipids, proteins, nucleic acids, and plant-specific bioactive molecules such as polyphenols and secondary metabolites. Accumulating evidence suggests that PD-EVs exert biological activities associated with anti-inflammatory, antioxidant, and cytoprotective effects. Importantly, their natural origin, biocompatibility, low immunogenicity, and scalable production make PD-EVs an attractive platform for translational and therapeutic applications.[3–5] Beyond their intrinsic bioactivity, PD-EVs have also been proposed as nanocarrier systems capable of delivering therapeutic molecules and modulating immune and inflammatory responses in target cells.[3,6]

Despite growing interest in PD-EVs, their functional effects on vascular endothelial cells under oxidative stress conditions remain incompletely characterized. In particular, systematic evaluation of their impact on endothelial viability, membrane integrity, and redox homeostasis is still limited. Addressing this gap is essential for advancing our understanding of PD-EVs in vascular biology and for exploring their potential as cell-free therapeutic agents to mitigate endothelial dysfunction.

In vitro models based on hydrogen peroxide (H_2O_2)-induced oxidative injury in human umbilical vein endothelial cells (HUVECs) are widely used due to their reproducibility and relevance to oxidative stress–mediated endothelial damage. Exposure to H_2O_2 reliably induces reductions in cell viability, disruption of membrane integrity with increased cytotoxicity, and impairment of antioxidant defense mechanisms. Consequently, combined assessment using cell viability assays such as CCK-8/WST, cytotoxicity measurements based on lactate dehydrogenase (LDH) release, and evaluation of redox-related parameters provides a comprehensive framework for investigating protective interventions in this model.[7]

Therefore, the present study aimed to systematically investigate the effects of plant-derived extracellular vesicles isolated from pomegranate, grape, and beetroot on H_2O_2-induced oxidative injury in HUVECs. The study specifically focused on evaluating cell viability, cytotoxicity, and antioxidant defense responses, with particular emphasis on GPX1-mediated redox regulation. By integrating biochemical assays and gene expression analyses, this work sought to elucidate the potential of PD-EVs as a biocompatible, cell-free strategy for protecting endothelial cells against oxidative stress–related damage.

Methods

Isolation and characterization of Plant-Derived Extracellular Vesicles

For PD-EVs isolation, the starting plant materials were prepared prior to centrifugation steps. Fresh beetroot (*Beta vulgaris*), pomegranate, and grape samples were washed thoroughly with sterile phosphate-buffered saline (PBS) and cut into small pieces. The plant tissues were then mechanically disrupted using a laboratory blender, followed by processing with a juice extractor under sterile conditions to obtain plant juice and homogenates suitable for EV isolation. The resulting homogenates were filtered through multiple layers of sterile gauze to remove large fibrous debris, and the filtrates were collected into 50 mL conical tubes.

To remove cells and large particles, samples were subjected to sequential differential centrifugation at 4°C. Initially, samples were centrifuged at 300–500 × g for 10 min to sediment large particulates. The supernatant was carefully transferred to new tubes and centrifuged at 2,000 × g for 20 min to remove residual cellular debris. This was followed by centrifugation at 10,000 × g for 30 min, during which larger microvesicles, apoptotic bodies, and remaining membrane fragments were pelleted. After each centrifugation step, the pellet was left undisturbed, and only the supernatant was carefully collected and transferred to the subsequent step. At this stage, the supernatant represented an EV-enriched fraction containing exosome-like vesicles. To further reduce larger particles and potential bacterial contamination, the supernatant was subjected to filtration, removing

particles larger than 200 nm, consistent with the expected size range of most EVs (30–200 nm). Following filtration, the clarified supernatant was subjected to ultracentrifugation to pellet EVs. Samples were centrifuged at 100,000 × g for 70–90 min at 4°C using a CS150FNX ultracentrifuge (Hitachi Himac, Japan) with an appropriate rotor. After centrifugation, tubes were carefully removed without disturbance, and a thin, translucent EV pellet was observed at the bottom of the tubes. The supernatant was gently aspirated to avoid disrupting the pellet, which represented the crude EV fraction. To obtain a purer EV preparation, the pellet was resuspended in sterile PBS and subjected to a second ultracentrifugation step under the same conditions. Following this wash step, the supernatant was discarded, and the final EV pellet was resuspended in 1 ml PBS to obtain the final EV suspension.

Successful EV isolation was confirmed by measuring the total protein concentration using a BCA assay. Particle size distribution was assessed using a Zetasizer Nano ZS90 (v7.01; Malvern, UK). EV morphology and size were further evaluated by electron microscopy (QuadroS, Thermo Scientific, USA).

Protein Quantification by BCA Assay

The total protein concentration of extracellular vesicle (EV) preparations was determined using a bicinchoninic acid (BCA) protein assay kit, according to the manufacturer's instructions. The BCA assay is based on the formation of a purple-colored complex resulting from the reaction between cuprous ions (Cu^+) and peptide bonds under alkaline conditions, with color intensity directly proportional to protein concentration.

A standard calibration curve was generated using bovine serum albumin (BSA) prepared at the concentrations recommended by the kit protocol. EV samples and BSA standards were mixed with the working reagent and incubated at 37 °C for 1 hour. Absorbance was measured at 532 nm using a spectrophotometer. Protein concentrations of EV samples were calculated by interpolation from the standard curve.

Particle Size Analysis by Dynamic Light Scattering (DLS)

The size distribution of PD-EVs was analyzed by DLS using a Zetasizer instrument (Zetasizer Nano ZS, Malvern Instruments, UK). Prior to measurement, EV suspensions were diluted 1:10 with ultrapure distilled water to obtain an appropriate particle concentration and to minimize multiple scattering effects. Measurements were performed at 25°C using standard settings recommended by the manufacturer. Data were recorded as hydrodynamic diameter and expressed as the mean particle size.[8]

Electron Microscopy Analysis of Extracellular Vesicles

For ultrastructural characterization PD-EVs suspensions were deposited onto Formvar/carbon-coated copper grids (300 mesh; approximate grid hole size ~63 µm) and allowed to adsorb for 10–15 min at room temperature. Excess liquid was gently removed using filter paper, and the grids were air-dried under sterile conditions. No additional contrasting or staining was applied unless otherwise stated. Prepared grids were examined using an S-TEM microscope (QuadroS, Thermo Scientific, USA) operated under standard imaging conditions. Representative micrographs were acquired to assess EV morphology and size distribution. [9]

Cell Culture and Experimental Design

The experimental study was conducted using human umbilical vein endothelial cells (HUVECs). Cells were cultured and expanded at 37°C in a humidified incubator with 5% CO_2 and atmospheric oxygen levels under standard culture conditions. For experiments, cells were seeded into 96-well and 48-well culture plates. Seeding densities were adjusted to $1.0–1.2 \times 10^4$ cells/well for 96-well plates and $2.0–2.5 \times 10^4$ cells/well for 48-well plates. After seeding, cells were allowed to adhere and reach 50–70% confluence by incubation for 16–24 h prior to treatment. Acute oxidative stress was induced using hydrogen peroxide (H_2O_2). Briefly, culture medium was replaced with phenol red–free DMEM/F12 containing 200 µM H_2O_2, and cells were incubated for 30 min. Following oxidative stress induction, the H_2O_2-containing medium was removed and replaced with fresh complete medium.

For treatment, plant-derived extracellular vesicles (PD-EVs) were added to the culture medium at a final concentration of 10 µg EV protein/mL. EV-containing fresh medium was applied to both control and H_2O_2-injured groups. Cells were then incubated for 48 h, after which cell proliferation, viability, and recovery from oxidative injury were evaluated using the respective assays.

The experiment was designed with the following main groups, with at least three biological replicates and three technical replicates per group:

- Group 1 – Normoxic control (vehicle-treated, no EVs)
- Group 2 – Normoxia + pomegranate-derived EVs (P-EVs)
- Group 3 – Normoxia + grape-derived EVs (G-EVs)
- Group 4 – Normoxia + beetroot-derived EVs (B-EVs)
- Group 5 – Normoxia + combined pomegranate, grape, and beetroot EVs (PGB-EVs)
- Group 6 – Hydrogen peroxide (H_2O_2) control
- Group 7 – H_2O_2 + P-EVs (10 µg/mL)
- Group 8 – H_2O_2 + G-EVs (10 µg/mL)
- Group 9 – H_2O_2 + B-EVs (10 µg/mL)
- Group 10 – H_2O_2 + PGB-EVs (10 µg/mL each)

Cell Viability Assay (CCK-8)

The same experimental setup was used to evaluate cell viability using the Cell Counting Kit-8 (CCK-8) assay. Following the completion of oxidative stress induction and EV treatments, CCK-8 reagent was added to each well at a volume corresponding to 10% of the culture medium, according to the manufacturer's instructions. Plates were incubated at 37 °C for 1 h, and absorbance was measured at 450 nm using a microplate reader.

The obtained absorbance values were compared among normoxic control, oxidative stress, and EV-treated groups to assess whether plant-derived EVs exert protective effects on cell viability under oxidative stress conditions.

Lactate Dehydrogenase (LDH) Cytotoxicity Assay

Cell cytotoxicity was evaluated using an LDH Cytotoxicity Detection Kit by measuring LDH activity released into the cell culture supernatant. The assay is based on the LDH-catalyzed conversion of lactate to pyruvate, during which NAD^+ is reduced to $NADH/H^+$. In the presence of the catalyst diaphorase, $NADH/H^+$ transfers hydrogen ions to the yellow tetrazolium salt INT, resulting in the formation of a red formazan product. The amount of formazan produced is directly proportional to the total LDH activity in the culture supernatant.

Culture supernatants were incubated with the reaction mixture for 30 min at room temperature, and absorbance was measured at 490 nm. Spectrophotometric values were compared between experimental and control groups to determine cytotoxic effects and the potential protective role of plant-derived EVs.

qRT-PCR Analysis

For gene expression analysis, total RNA isolation (A.B.T.™ RNA Purification Kit) was performed. Then, cDNA was synthesized from isolated RNA using the cDNA Synthesis Kit (A.B.T.™ One-Step cDNA Synthesis Kit) according to the manufacturer's instructions. Quantitative real-time PCR reactions were prepared by combining RealQ Plus Master Mix Green (Ampliqon, Odense, Denmark), gene-specific primers (Table 1), and the synthesized cDNA, and loaded into 96-well PCR plates. Amplification was carried out under the following thermal cycling conditions: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing/extension at 60 °C for 60 s. Real-time PCR analyses were performed using a Roche LightCycler 480 II system (Roche Molecular Diagnostics, Mannheim, Germany). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to the reference gene Actin Beta (ACTB), with data analysis conducted using Roche LC480 software. Primer sequences for all target genes are listed in Table 1.

Table 1. Gene Names ande primer sequences used for qRT- PCR analysis

Gene/ Full Names	Accession Number	Primer Sequences (5' → 3') (F: forward, R: reverse)
GPX1 / glutathione peroxidase 1	NM_000581.4	F- CCAGTCGGTGTATGCCTTCT R- TCTTGGCGTTCTCCTGATGC
HPRT1 / hypoxanthine phosphoribosyltransferase 1	NM_000194.3	F-TGACCTTGATTTATTTTGCATACC R-CGAGCAAGACGTTCAGTCCT

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (version 23; IBM Corp). Data normality was assessed using the Shapiro–Wilk test. Depending on the distribution of the data, either the Student's t-test or the Mann–Whitney U test was used for pairwise group comparisons. Statistical significance was defined as ns (not significant), $p < 0.05$, $*p < 0.01$, and $**p < 0.001$. All experiments were conducted with three biological replicates and independently repeated on at least three separate occasions. Data are presented as mean ± standard deviation (SD), with figures showing representative results from independent experiments.

Results

Characterization of Plant-Derived Extracellular Vesicles

Total protein concentrations of plant-derived extracellular vesicles (EVs) were determined using the BCA assay. According to the analysis, pomegranate-derived EVs exhibited the highest protein content (346.74 ± 56.97 µg/mL), followed by grape-derived EVs (122.62 ± 21.34 µg/mL) and beetroot-derived EVs (102.54 ± 11.45 µg/mL).

Particle size characterization was performed using a Zetasizer instrument. Dynamic light scattering analysis revealed mean hydrodynamic diameters of 227.0 ± 6.96 nm for pomegranate EVs (Figure 1A), 234.93 ± 12.29 nm for grape EVs (Figure. 1B), and 109.13 ± 1.25 nm for beetroot EVs (Figure. 1C).

These findings indicate that all EV preparations fell within the expected nanoscale size range. Furthermore, electron microscopy analysis (Figure 2) confirmed that EVs displayed appropriate morphology and size, consistent with extracellular vesicle characteristics.

CCK-8 Cell Viability Assay

In this study, the potential protective and restorative effects of PD-EVs on cell viability were evaluated in an acute oxidative stress model established in human umbilical vein endothelial cells (HUVECs) using hydrogen peroxide (H_2O_2). Cell viability was assessed by the CCK-8 assay. Exposure of HUVECs to H_2O_2 resulted in a significant reduction in cell viability compared with untreated control cells ($*p<0.05$), confirming the successful induction of oxidative stress–mediated cellular damage.

In the absence of H_2O_2, treatment with pomegranate-, grape-, and beetroot-derived EVs resulted in a significant increase in HUVEC viability compared with untreated control cells ($p<0.05$). Among the single EV treatments, beetroot-derived EVs induced the greatest increase in cell viability. Notably, the combined EV formulation (pomegranate + grape + beetroot; PGB-EVs) produced the highest viability values ($**p<0.01$), exceeding those observed with individual EV treatments (Figure 3).

Exposure to H_2O_2 markedly reduced cell viability compared with normoxic controls. Treatment with PD-EVs significantly improved cell viability in H_2O_2-

challenged HUVECs compared with the H_2O_2-only group (*p<0.05). This recovery effect was most pronounced in cells treated with beetroot-derived EVs and the combined EV formulation, indicating enhanced protection against oxidative stress–induced endothelial injury.

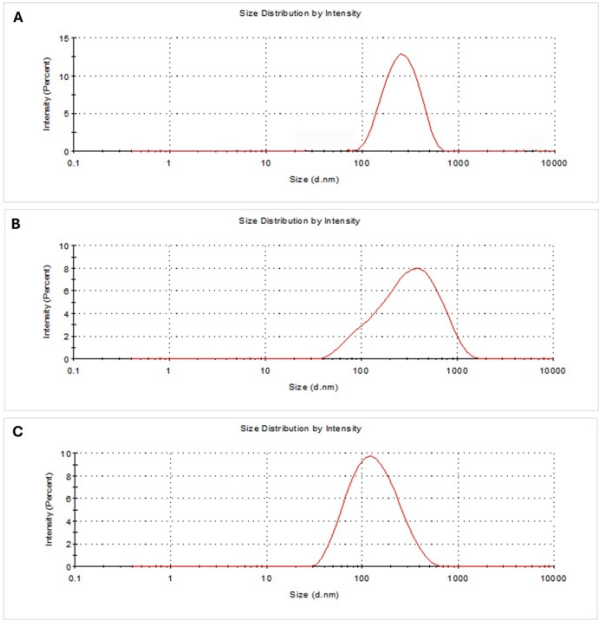

Figure1. Size distribution analysis of plant-derived extracellular vesicles (EVs). Dynamic light scattering (DLS) analysis was performed using a Zetasizer to determine the hydrodynamic size distribution of EVs isolated from different plant sources. Size distribution by intensity is shown for (A) pomegranate-derived EVs, (B) grape-derived EVs, and (C) beetroot-derived EVs

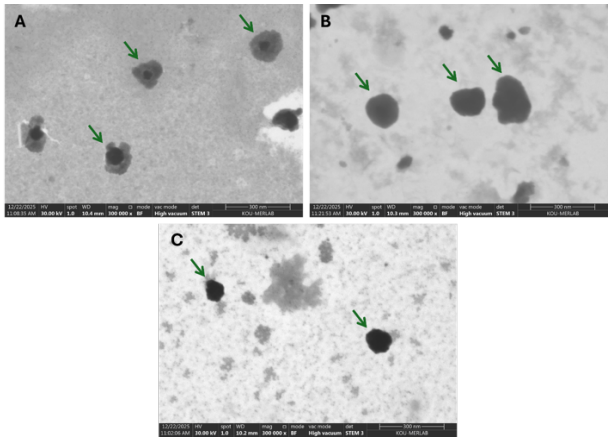

Figure 2. Morphological characterization of plant-derived extracellular vesicles (EVs) by electron microscopy. Representative scanning transmission electron microscopy (S-TEM) images showing the morphology of extracellular vesicles isolated from (A) pomegranate, (B) grape, and (C) beetroot samples. EVs appear as spherical to rounded nanostructures with sizes consistent with extracellular vesicles (indicated by green arrows). Scale bar: 300 nm.

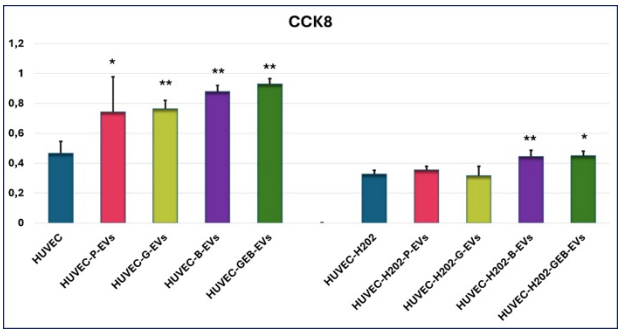

Figure 3. CCK-8 analysis of HUVEC viability following plant-derived EV treatment under oxidative stress. Cell viability was evaluated in HUVECs treated with pomegranate-, grape-, and beetroot-derived EVs under normoxic and H_2O_2-induced oxidative stress conditions. Each treatment group was statistically compared with its corresponding control group. Data are presented as mean ± SD. *p<0.05.

LDH Cytotoxicity Assay Results

H_2O_2–induced oxidative injury in HUVECs was evaluated by measuring LDH release into the culture supernatant as an indicator of cell membrane damage. As expected, H_2O_2 treatment resulted in a marked increase in LDH levels compared with the control group, confirming significant membrane disruption and successful establishment of the oxidative stress model.

In groups not exposed to H_2O_2, single applications of PD-EVs from pomegranate, grape, and beetroot did not cause a significant increase in LDH release, indicating that these EVs were not cytotoxic to HUVECs. Similarly, the triple EV combination (NÜP) exhibited LDH levels comparable to the control group, further supporting the biocompatibility of plant-derived EVs.

In H_2O_2-treated groups, application of plant-derived EVs reduced LDH release to varying extents. P-EVs and G-EVs partially attenuated the H_2O_2-induced increase in LDH levels, whereas B-EVs demonstrated a more pronounced protective effect, significantly reducing LDH release compared with the H_2O_2-only group (*p<0.05). Notably, the triple EV combination PGB-EVs exerted the strongest membrane-protective effect, resulting in a highly significant reduction in LDH levels relative to the H_2O_2 group (***p<0.001) (Figure 4).

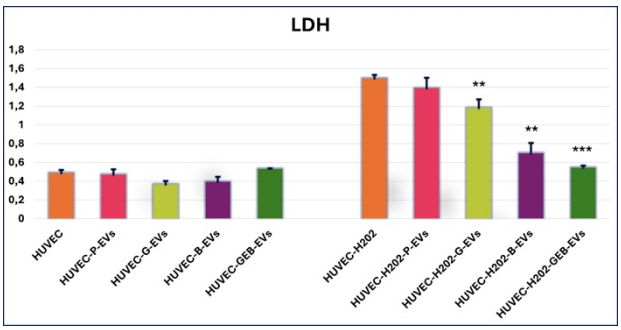

Figure 4. LDH analysis of HUVEC cytotoxicity following plant-derived EV treatment under oxidative stress. LDH release was assessed in HUVECs treated with pomegranate-, grape-, and beetroot-derived EVs under normoxic and H_2O_2-induced oxidative stress conditions. Each treatment group was statistically compared with its corresponding control group. Data are presented as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

Effects of EV treatments on *GPX1* gene expression in an H_2O_2-induced oxidative stress model in HUVECs

To evaluate the effects of extracellular vesicle (EV) treatments under normoxic conditions and following H_2O_2-induced oxidative stress, *GPX1* gene expression was analyzed by quantitative real-time PCR. EV treatments significantly modulated the expression of the antioxidant-related gene *GPX1* in H_2O_2-challenged HUVECs (Figure 5).

Under normoxic conditions, *GPX1* expression levels were low in all EV-treated groups compared with untreated HUVECs. Following H_2O_2 exposure, *GPX1* expression was markedly reduced. Treatment with plant-derived EVs significantly increased *GPX1* expression in H_2O_2-injured cells, with pomegranate-, beetroot-, and combined EV groups showing the most pronounced increases.

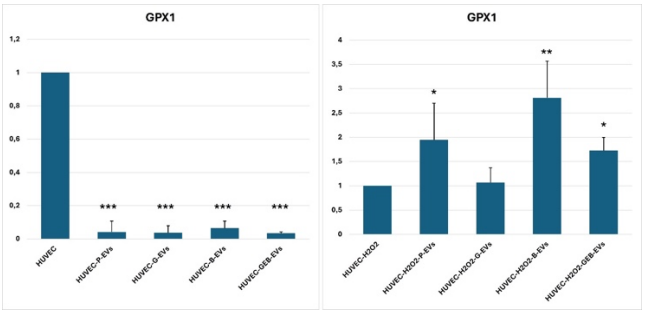

Figure 5. Effects of plant-derived extracellular vesicles on *GPX1* gene expression under normoxic and oxidative stress conditions. Relative mRNA expression levels (fold change) of *GPX1* in HUVECs under normoxic conditions (left panel) and following H_2O_2-induced oxidative stress (right panel). Gene expression was normalized to *HPRT* and calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

Discussion

In the present study, we investigated the protective effects of plant-derived extracellular vesicles (PD-EVs) isolated from pomegranate, grape, and beetroot on endothelial cells subjected to H_2O_2-induced oxidative stress. Our findings demonstrate that PD-EVs improve endothelial cell viability, reduce cytotoxicity, and modulate antioxidant defense responses in a condition-dependent manner, with a particular impact on *GPX1* expression. Notably, the combined EV formulation exerted the most pronounced protective effects, suggesting complementary and potentially synergistic interactions among EV cargos derived from different plant sources.

Oxidative stress–induced endothelial dysfunction is a central pathological event in the development of cardiovascular and metabolic diseases. Exposure of HUVECs to H_2O_2 is a well-established in vitro model that mimics excessive reactive oxygen species (ROS) production, leading to reduced cell viability, membrane damage, and impaired redox homeostasis. In line with previous reports, H_2O_2 treatment in our study resulted in decreased cell viability and increased LDH release, confirming successful induction of oxidative injury.[7]

Treatment with PD-EVs significantly attenuated these deleterious effects. CCK-8 analysis revealed that all EV formulations improved cell viability under oxidative stress, while LDH assays demonstrated reduced membrane damage, indicating a cytoprotective effect. Importantly, PD-EVs did not induce cytotoxicity under normoxic conditions, supporting their biocompatibility. These observations are consistent with accumulating evidence that plant-derived exosome-like nanovesicles are non-toxic, well tolerated by mammalian cells, and capable of modulating cellular stress responses.[6]

Among the single-source EVs, beetroot-derived EVs exhibited relatively stronger protective effects, which may be attributed to their enrichment in antioxidant-related metabolites such as betalains and polyphenols. Previous studies have demonstrated that Beta vulgaris–derived exosome-like nanovesicles possess intrinsic antioxidative activity, promote angiogenesis, and modulate gene expression in endothelial and stromal cells.[10] More recent omics-based analyses further revealed that beetroot-derived nanovesicles carry bioactive proteins, lipids, and microRNAs capable of regulating oxidative stress– and inflammation-related pathways in human cells.[5]

Previous HUVEC-based in vitro studies have demonstrated that endothelial responses to biological interventions can be reliably evaluated using a combination of cell viability assays (such as WST-1/CCK-8) and RT-PCR–based gene expression analyses, supporting the robustness of this methodological approach.[11] In the present study, analysis of *GPX1* gene expression provided important insight into the redox-modulating effects of plant-derived extracellular vesicles (PD-EVs) under both normoxic and oxidative stress conditions.

Under normoxic conditions, *GPX1* expression was significantly reduced in EV-treated groups compared with untreated HUVECs, indicating that PD-EVs do not induce constitutive or unnecessary activation of antioxidant defense mechanisms in the absence of oxidative stress. This finding suggests that plant-derived EVs maintain redox homeostasis without triggering basal antioxidant overexpression, which may be advantageous for preserving physiological cellular signaling.

In contrast, exposure to H_2O_2 markedly impaired endogenous antioxidant capacity, as reflected by suppressed *GPX1* expression. Notably, treatment with PD-EVs significantly restored *GPX1* expression in H_2O_2-challenged HUVECs, with pomegranate-, beetroot-, and combined EV formulations exerting the strongest effects. These results indicate that PD-EVs promote an adaptive and context-dependent antioxidant response, selectively enhancing GPX1 expression under oxidative stress conditions.

GPX1 is a critical antioxidant enzyme that catalyzes the reduction of hydrogen peroxide to water using glutathione, thereby limiting reactive oxygen species accumulation and protecting cells from oxidative damage. Similar restoration of GPX and SOD activities has been reported in HUVECs treated with natural

antioxidant compounds and plant-derived bioactives under H_2O_2-induced stress.[7] Our findings extend these observations by demonstrating that whole EV formulations, rather than isolated phytochemicals, can effectively re-establish endogenous antioxidant gene expression programs.

Notably, the combined EV formulation (pomegranate, grape, and beetroot) induced the highest *GPX1* expression levels under oxidative stress and was associated with the most pronounced improvements in cell viability and membrane integrity. This suggests a synergistic effect arising from the diverse molecular cargo of EVs derived from different plant sources. Pomegranate-derived EVs are enriched in polyphenols such as ellagic acid and have been shown to exert potent antioxidant and anti-inflammatory effects in mammalian disease models.[12] Similarly, grape-derived exosome-like nanovesicles have been reported to protect epithelial and endothelial cells against oxidative and photo-induced damage by modulating proliferation, differentiation, and ROS-related signaling pathways.[13] The convergence of these complementary bioactivities likely underlies the superior protective efficacy observed with the combined EV treatment.

Collectively, our findings support the concept that PD-EVs function as biologically active nanocarriers capable of activating endogenous cytoprotective pathways in endothelial cells. Rather than acting solely as passive antioxidant reservoirs, PD-EVs appear to modulate transcriptional programs related to redox balance, cell survival, and membrane integrity. This study provides further evidence that plant-derived EVs represent a promising, scalable, and cell-free therapeutic platform for mitigating oxidative endothelial injury. Future studies should focus on detailed cargo profiling and mechanistic dissection of EV uptake pathways to better define the molecular determinants of their protective effects.

Compliance with Ethical Standards

Ethics committee approval was not required for this study, as all experiments were conducted in vitro using commercially available human umbilical vein endothelial cells (HUVEC) and plant-derived materials. No human participants or animals were involved in the study.

Conflict of Interest

The authors declare that there are no conflicts of interest related to this study.

Author Contributions

ZSH, NS, RK; Conceptualization, Investigation, Writing – review and editing, ZSH, BÖD, ÖSU, YY; Methodology, ZSH, BÖD, ÖSU, YY; Data curation, ZSH, ÖSU, GG; Formal analysis, ZSH, NS, RK, BÖD, GG; Writing original draft, ZSH, GG; Supervision. All authors have read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Financial Disclosure

This study did not receive any specific financial support from public or commercial organizations. The study was conducted with the infrastructural support of Kocaeli University and Bahçeşehir College Kocaeli Campus.

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