



Ethnopharmacological insights into *Sagrantino* grape leaves: vasoactive phytocomplexes and extracellular vesicles from an underutilised agro-waste

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ABSTRACT

Ethnopharmacological relevance: Grapevine leaves are traditionally used in Mediterranean and Middle Eastern ethnomedicine to treat circulatory disorders, inflammation, and venous insufficiency. However, cultivar-specific phytochemical profiles and their underlying vascular mechanisms remain poorly characterised.

Aim of the study: To investigate the chemical composition and vascular activity of extracts and extracellular micro- and nanovesicles (EVs) derived from leaves of the Italian *Vitis vinifera* cv. *Sagrantino*.

Materials and methods: Extracts were obtained using ultrasound-assisted extraction and Soxhlet methods with solvents of different polarity. Phytochemical profiling was performed by UHPLC-HRMS and ¹H NMR. EVs were characterised by nanoparticle tracking analysis and transmission electron microscopy and analysed by NMR. Vascular effects were assessed *ex vivo* on rat aorta rings. Modulation of vascular smooth muscle Ca_v1.2 channels was evaluated by whole-cell patch-clamp recordings.

Results: Extracts were rich in polyphenols, including flavonoid glycosides, gallotannins, ellagitannins, and cinnamic acid derivatives. All extracts induced concentration-dependent vasorelaxation in endothelium-intact aorta rings, whereas removal of the endothelium markedly reduced or reversed this effect, often leading to contraction. A hormetic response was observed at higher concentrations. Ultrasound-assisted hydroalcoholic extracts showed the strongest activity. EVs showed nanoscale morphology, contained polyphenols, sugars, fatty acids and amino acids, induced vasorelaxation, and inhibited Ca_v1.2 channel currents in a concentration-dependent manner.

Conclusions: *Sagrantino* grapevine leaves represent an underutilised source of vasoactive compounds. Their vascular effects involve both endothelium-dependent mechanisms and direct inhibition of Ca_v1.2 channels. These findings provide mechanistic support for their traditional use and highlight their potential for sustainable cardiovascular applications within a circular bioeconomy framework.

1. Introduction

Grapevine leaves (*Vitis vinifera* L.) have long been used in both culinary and medicinal traditions across Mediterranean, Middle Eastern, and Balkan cultures. Commonly consumed fresh or preserved in dishes

such as dolmathes and sarma, they have also been used in folk medicine to treat a wide range of conditions, including haemorrhage, diarrhoea, hypertension, diabetes, inflammation, varicose veins, and impaired wound healing (Lima et al., 2016; Teixeira et al., 2014). Ethnobotanical evidence consistently attributes astringent, haemostatic,

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anti-inflammatory, and vasoprotective properties to grapevine leaves, suggesting a long-standing therapeutic relevance. These traditional uses align with their rich phytochemical composition, which includes polyphenols, flavonoids, tannins, organic acids, and other bioactive constituents (Pintač et al., 2019).

Despite their recognised value, grapevine leaves remain relatively underexplored (Maia et al., 2021) compared with other viticultural by-products such as skins, seeds, and pomace, which have been extensively characterised and are already employed in nutraceutical and cosmetic applications (De Luca et al., 2023; Carullo et al., 2019, 2020). Although standardised red vine leaf extracts are approved in Europe for the treatment of chronic venous insufficiency (EMA, 2017), systematic chemical and functional investigations focusing on cultivars-specific differences - particularly among geographical restricted varieties - are still limited.

The Italian native cultivar *Sagrantino* (*Vitis vinifera* L.), typical of the Montefalco area of Umbria (central Italy), is distinguished by one of the highest polyphenol and tannin contents among Italian grape varieties (Giacosa et al., 2021). This distinctive phytochemical profile suggests that its leaves may represent a valuable yet underexplored source of bioactive compounds with potential cardiovascular benefits. However, to date, no comprehensive studies have examined the chemical composition or vascular activity of *Sagrantino* leaves.

In parallel with conventional extraction approaches, increasing attention has been directed toward plant-derived extracellular vesicles (PDEVs), which are nanoscale structures naturally enriched in lipids, proteins, metabolites, and small RNAs. Their occurrence in edible plants and their capacity to deliver bioactive molecules in biologically relevant forms make them promising candidates for nutraceutical applications and for the valorisation of agro-food residues (Alfieri et al., 2021; Lian et al., 2022; Logozzi et al., 2022).

The identification of novel health-promoting compounds from agricultural by-products is also aligned with current circular bioeconomy strategies (Liu et al., 2023; Sorrenti et al., 2023). Viticulture generates large quantities of leaves and other residues each year, most of which remain underutilised despite their significant phytochemical potential (Constantin et al., 2024). The transformation of these materials into functional bioactive ingredients represents a sustainable approach that supports both environmental and economic objectives (Muhlack et al., 2018; Capanoglu et al., 2022).

Within this framework, the present study aims to investigate the phytochemical composition and vascular effects of *Sagrantino* grapevine leaves, comparing conventional extracts obtained through green conditions with PDEVs isolated from the same plant material. By integrating ethnopharmacological knowledge with advanced chemical profiling (UHPLC-HRMS and NMR), *ex vivo* vascular assays, and electrophysiological evaluation of Cav1.2 channel modulation, this work provides new mechanistic insights into the traditional vasoprotective use of *Sagrantino* grapevine leaves and supports their valorisation as a sustainable source of cardiovascular bioactive compounds.

2. Materials and methods

2.1. Preparation of *Sagrantino* leaf extracts

Sagrantino leaves were obtained through manual collection in the Montefalco area, in the province of Perugia (Umbria, Italy). The leaves were collected between the end of October and the beginning of November 2021, stored in paper bags, and kept in the dark.

Dried and ground *Sagrantino* leaves were subjected to different extraction procedures. For ultrasound-assisted extraction (UAE), the triturated leaves were dispersed in distilled water, and the extraction was conducted in an ultrasonic bath (40 kHz, 60 °C, 1.5 h; Branson model 3800-CPXH, Milan, Italy).

The resulting solution was filtered through a Büchner funnel and freeze-dried to give the aqueous extract, referred to as FSA. The same

procedure was applied using water: absolute ethanol mixtures (90:10, 80:20, and 50:50 v/v), yielding extracts FSAE10, FSAE20, and FSAE50, respectively. Ethyl acetate was employed to obtain FSEtOAc. Hydroalcoholic extracts were concentrated under reduced pressure before lyophilisation, whereas the ethyl acetate extract was concentrated at 40 °C, resolubilised in the minimum volume of solvent, and filtered through 0.21 µm syringe filters. Another extraction was performed by using ethyl acetate in a Soxhlet apparatus under reflux for 6 h; the solvent was removed by vacuum filtration, and the resulting extract (FSEtOAcSOX) was concentrated, resolubilised in ethyl acetate, and filtered. For the ethanol reflux extraction, the leaves were treated with 250 mL of absolute ethanol for 5.5 h. The solution was then vacuum filtered using a Büchner funnel and further filtered over Celite using a Gooch crucible to yield the extract FSE. Part of this extract was fractionated by liquid-liquid partition: first with *n*-hexane, affording a fraction (FSE Hexane) that was discarded due to insolubility, and then with ethyl acetate, to obtain a fraction of intermediate polarity, the FSE EtOAc. In both cases, organic phases were dried over anhydrous sodium sulfate, vacuum filtered, and concentrated under reduced pressure at 40 °C. For each extract, the solvent was completely removed either by rotary evaporation or under a fume hood. The residues were weighed to determine the extraction yields, and the samples - typically reddish-brown and gummy in appearance - were stored at room temperature (Table S1).

2.1.1. Reverse-phase ultra-high performance liquid chromatography (RP-UHPLC)-high-resolution mass spectrometry analysis (HRMS)

Metabolite analysis was performed using a Thermo Ultimate RS 3000 UHPLC system coupled to a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer equipped with a HESI II source (Thermo Fisher Scientific, Bremen, Germany). For RP-UHPLC, a Kinetex Biphenyl column (100 mm × 2.1 mm, 2.6 µm, phenylephrinomenex®, Bologna, Italy) was utilised at a flow rate of 0.4 mL/min. The mobile phases consisted of 0.1% acetic acid in water (A) and acetonitrile with 0.1% acetic acid (B). The gradient elution program was as follows: 0-30 min, 2-30% B; 30-38 min, 30-98% B; hold at 99% B for 2 min; returning to initial conditions in 0.1 min. The column oven was maintained at 40 °C, and a 5 µL sample volume was injected.

HRMS analysis was performed in negative ion mode (ESI) using Full MS mode (*m/z* 100-1500) with data-dependent MS/MS acquisition (dd-MS², top *n* = 5). A resolution of 35,000 and 15,000 FWHM at *m/z* 200 was selected for Full MS and MS/MS, respectively. Stepped normalised collision energies of 15, 25, and 30 were applied.

The source parameters were as follows: sheath gas pressure at 50 arbitrary units, auxiliary gas flow at 13 arbitrary units, spray voltage at -3.50 kV, capillary temperature at 260 °C, auxiliary gas heater temperature at 300 °C, and S-lens RF value at 30 arbitrary units.

Metabolite annotation was carried out by comparing the MS/MS data with the *in silico* MS/MS Fiehn/Vaniya natural product library available in MS DIAL v4.80, as previously described (Sommella et al., 2017).

2.1.2. NMR analysis

NMR spectroscopy was applied to dried extracts to enable the determination of the main metabolites present.

Resonance assignments were carried out by analysing the chemical shift, multiplicity, and coupling patterns observed in the 1D-¹H NMR spectra and further confirmed by comparison with reference spectra and literature data.

For ¹H-NMR analysis, the extracts were re-dissolved in 600 µL of an appropriate deuterated solvent. Residual solvent signals were utilised as internal references for chemical shift calibration (δ = 4.79 ppm for D₂O and δ = 2.50 ppm for DMSO-*d*₆). Quantitative analysis employed 2 mM of TSP as both chemical shift and concentration reference. All ¹H NMR spectra were recorded at 298 K (25 °C) on a Bruker Avance 300 MHz or Varian 300 MHz spectrometer equipped with a BBI probe and z-axis gradient. Spectra were acquired using the presaturation pulse sequence

(zgpr) to suppress the residual water signal, with 128 transients, a spectral width of 6000 Hz and 64K data points for an acquisition time of 5.5 s. These parameters ensured complete relaxation of all resonances, enabling reliable quantification (Carullo et al., 2019).

2.1.3. Preparation of *Sagratino* leaf vesicles and NMR analyses

Dried *Sagratino* leaves were soaked in distilled water for 24 h. Extraction was performed using a household juice extractor, and the resulting leaf juices were centrifuged at 500xg for 10 min. Supernatants were filtered through 100 µm filters. The filtrates were sequentially centrifuged at 2,000xg for 20 min to remove cell debris, followed by centrifugation at 15,000xg for 30 min to deplete the microvesicles-enriched fraction.

Nanovesicles were then collected by ultracentrifugation at 110,000×g for 90 min using a Sorvall WX Ultracentrifuge Series (Thermo Fisher Scientific, Rome, Italy). The resulting pellet was lyophilised to enhance preservation and facilitate resuspension. The lyophilised material was resuspended either in a modified Krebs-Henseleit physiological solution (KHS) for the evaluation of vasoactive properties or in distilled water for physicochemical characterisation.

Sample preparation involved the use of an Ultrasonic Processor (SONICS Vibra cell, probe sonicator, 130 Watts, 20 kHz), where vesicles were solubilised in a 1:1 ratio with methanol. Microvesicles and nanovesicles fractions were subjected to different sonication durations to optimise their dissolution.

2.1.4. Nanoparticle tracking analysis

The size distribution and concentration of extracellular vesicle (EV) samples were evaluated using the NanoSight NS300 (Malvern Instruments, Worcestershire, UK). For each sample, five videos, each approximately 60 s in duration, were recorded. Data acquisition and analysis were performed with the NTA 3.0 software (Malvern Instruments), which tracks individual particles frame by frame. Particle tracking was based on the Brownian motion of each vesicle, using the Stokes-Einstein equation: $D^{\circ} = kT/6\pi\eta r$, where D° is the diffusion coefficient, $kT/6\pi\eta r$ is the frictional coefficient of the particle, for the special case of a spherical particle of radius r moving at uniform velocity in a continuous fluid of viscosity η , k is Boltzmann constant, and T is the absolute temperature (Castelli et al., 2023).

2.1.5. Transmission electron microscopy of *Sagratino* leaf vesicles

After isolation, *Sagratino* leaf vesicles were resuspended in distilled water, and 3 µL of each sample was loaded onto formvar-coated 300 mesh Cu grids (Ted Pella Inc., Redding, CA, USA) for 2 min at room temperature. After removing the excess of sample with filter paper, 3 µL of 1% uranyl acetate (Polysciences Inc., Warrington, PA, USA) in distilled water was added for 30 s, blotted again with filter paper, and air dried. Finally, the samples were analysed using a Tecnai G2 Spirit transmission electron microscope (FEI Company, Hillsboro, OR, USA), operating at an accelerating voltage of 120 kV, equipped with a TVIPS TemCam-F216 CMOS camera.

2.2. Assessment of *Sagratino* leaf extract vasoactivity

2.2.1. Statement on animal care

All the study procedures were carried out in strict accordance with the European Union Guidelines for the Care and Use of Laboratory Animals (European Union Directive, 2010/63/EU) and received approval from the Animal Care and Ethics Committee of the University of Siena, as well as the Italian Department of Health (7DF19.N.SJQ on 12 April 2024). Male Wistar rats (weighing 220–470 g; $n = 46$) were obtained from Charles River Italia (Calco, Italy) and housed in an animal facility at $25 \pm 1^{\circ}\text{C}$ with a 12:12 h light:dark cycle, provided with standard chow and water *ad libitum*.

2.2.2. Preparation of aorta rings and functional tests

Animals were anaesthetised with an isoflurane (4%) and O_2 gas mixture using Fluovac (Harvard Apparatus, Holliston, Massachusetts, USA), decapitated and exsanguinated. The tail was immediately isolated and placed in an external solution for the subsequent isolation of the main caudal artery (see below). Following the opening of the abdominal and thoracic cavity, the thoracic aorta was gently removed along with adhered PVAT, washed from blood, and placed in KHS at 4°C containing (final concentrations expressed in mM): NaCl 118; KCl 4.75; KH_2PO_4 1.19; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.19; NaHCO_3 25; glucose 11.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.5; saturated with carbogen (95% O_2 , 5% CO_2) to obtain a pH of 7.4. The thoracic aorta was cut into 6–10 rings of 3 mm length using a tissue slicer (2Biological Instruments, Besozzo, Italy). Where necessary, the endothelium was removed by gently rubbing the lumen of the ring with a forceps tip. Rings were mounted in organ baths between two parallel, L-shaped, stainless-steel hooks, one fixed in place and the other connected to an isometric transducer (WPI, Berlin, Germany) and subjected to 1 g passive tension based on preliminary experiments where higher or lower passive tensions gave rise to unstable and not sustained contractions (Ramunno et al., 2024). Preparations were allowed to equilibrate for 60 min to overcome the mechanical stress suffered during preparation. During this equilibration phase, KHS was renewed every 15 min and passive tension, if decreased by more than 25% within the first 15 min, was restored to its initial value. The mechanical activity of aortic rings was converted into an electrical signal using an isometric transducer, amplified, and differentiated with an operational amplifier. The signal was then converted from analogue to digital for analysis using a computerised acquisition system (LabChart 7.3.7; ADInstrument, Castle Hill, Australia).

After 60 min of initial equilibration, rings were pre-contracted by a sub-maximal concentration (0.3 µM) of the α_1 -adrenergic receptor selective agonist phenylephrine. Once the contraction reached a plateau, 10 µM acetylcholine was added; a relaxation of less than 10% denoted the absence, while one of more than 75% the presence of a functional endothelium. Preparations were then washed for at least 60 min, once every 15 min, before performing the electromechanical coupling test.

Preparations were challenged by 60 mM KCl for 15 min and then washed with KHS for at least 60 min, every 15 min, to restore muscle tone to basal values before proceeding with the various experimental protocols (Carullo et al., 2021).

2.2.3. Experimental protocols

2.2.3.1. Effect of *Sagratino* leaf extracts on phenylephrine- and KCl-induced contractions. In this series of experiments, the extracts were solubilised in DMSO (final concentration $<0.33\%$) except FSA, FSAE10, and FSAE20, solubilised in water, and FSAE50 in a 50:50 (v/v) water: absolute ethanol solution, matching the extraction solvent ratio. Preliminary experiments indicated that 1–100 µg/mL is the effective concentration range. Each extract was cumulatively added to aortic rings precontracted by 0.3–0.6 µM phenylephrine, in both endothelium-intact and endothelium-deprived preparations. Alternatively, endothelium-deprived aortic rings were stimulated by 25–30 mM or 60 mM KCl before the addition of cumulative concentrations of the extracts. At the end of each concentration-response curve, the NO donor sodium nitroprusside (100 µM) in phenylephrine-precontracted rings, or the $\text{Ca}_v1.2$ channel blocker nifedipine (1 µM) followed by sodium nitroprusside in KCl-precontracted rings, was added to confirm the functional integrity of vascular smooth muscle. Contractile responses were expressed as a percentage of the initial tension induced by phenylephrine or KCl. Data acquisition and analysis were performed using LabChart 7.3.7 Pro software (PowerLab; ADInstruments) (Medana et al., 2024).

2.2.3.2. Isolation of vascular smooth muscle cells. Vascular smooth muscle cells were enzymatically isolated from the main caudal artery at

37 °C in 2 mL external solution (composition in mM: 110 NaCl, 5.6 KCl, 10 HEPES, 20 glucose, 1.2 MgCl₂, and 5 sodium pyruvate, pH adjusted to 7.4 with NaOH) containing 20 mM taurine, 0.1 mM CaCl₂, collagenase type XI (1.2–1.3 mg/mL), BSA (1 mg/mL), and trypsin inhibitor (1 mg/mL), continuously bubbled with a 95% O₂–5% CO₂ gas mixture, as previously described (Fusi et al., 2002). The final cell suspension (4 mL), stored at 4 °C in external solution containing 20 mM taurine, 0.1 mM CaCl₂, and 1 mg bovine serum albumin, was used at least 30 min after preparation.

For electrophysiological experiments, 2–5 µL of cell suspension was placed into a ~250 µL recording chamber on a phase-contrast inverted microscope (TE 300 Eclipse, Nikon, Japan). Cells were continuously superfused with external solution containing 0.1 mM CaCl₂ using a peristaltic pump (LKB 2132, Bromma, Sweden) at a flow rate of 400 µL/min. Only phase-dense, well-adhered cells with smooth contours were selected for recordings.

2.2.3.3. Patch-clamp recordings. Borosilicate glass pipettes (1.5 mm OD; WPI, Berlin, Germany) were pulled using a PC-10 puller and fire-polished with an MF-90 microforge (Narishige, Japan) to a final resistance of 2–4 MΩ. Ba²⁺ currents through Cav1.2 channels (I_{Ba}) were recorded in voltage-clamped myocytes under the whole-cell configuration (Fusi et al., 2016) using an external solution containing 5 mM Ba²⁺ and 30 mM tetraethylammonium chloride. The pipette solution contained (in mM): 100 CsCl, 10 HEPES, 11 EGTA, 2 MgCl₂, 1 CaCl₂ (pCa 8.4), 5 Na-pyruvate, 5 succinic acid, 5 oxaloacetic acid, 3 Na₂ATP, and 5 phosphocreatine, adjusted to pH 7.4 with CsOH.

Currents were evoked by 250-ms depolarising pulses to 10 mV from a holding potential (V_h) of –50 mV at 0.067 Hz. Data acquisition began once the current amplitude stabilised (typically 5–10 min after whole-cell access). Under these conditions, I_{Ba} remained stable for at least 20–30 min (Mugnai et al., 2014). K⁺ currents were blocked by 30 mM TEA in the external solution and Cs⁺ in the pipette solution. Leak and residual outward currents were subtracted offline using 10 µM nifedipine, which completely inhibited I_{Ba}.

Recordings were acquired using an Axopatch 200B amplifier (Molecular Devices, CA, USA) and a DigiData 1200A interface. Signals were low-pass filtered at 1 kHz, digitised at 3 kHz, and corrected for cell capacitance and series resistance (70–75%).

2.3. Statistical analysis

Data analysis was performed using LabChart 7.3.7 Pro (PowerLab; ADInstruments), pCLAMP 9.2.1.8 (Molecular Devices Corporation, Sunnyvale, CA, USA), and GraphPad Prism version 5.04 (GraphPad Software Inc., Boston, MA, USA). Data are reported as mean ± S.E.M.; n is the number of rings or cells analysed, obtained from three to five animals.

Statistical analyses and significance as measured by one-way or two-way ANOVA followed by Bonferroni post-test, Student's *t*-test for unpaired samples (two-tailed), repeated measures ANOVA followed by either Dunnett or Bonferroni post-test, were obtained using GraphPad Prism version 5.04 (GraphPad Software Inc.). A *P* value < 0.05 was considered statistically significant.

The pharmacological response to drugs, described in terms of potency (IC₅₀ value, i.e. the drug concentration that caused a decrease of muscle tone equal to 50% of the maximum response, respectively) and efficacy (E_{max}, i.e. the maximal response achieved with the highest concentration tested), was obtained by nonlinear regression analysis.

2.4. Chemical reagents

The chemicals used were acetylcholine, nifedipine, phenylephrine (Sigma Chimica, Milan, Italy), and sodium nitroprusside (Riedel-De Haen AG, Seelze, Hannover, Germany). All other substances were of

analytical grade and used without further purification. Phenylephrine was solubilised in 0.1 M HCl. Nifedipine was dissolved directly in ethanol. Extracts were dissolved in DMSO, distilled water, and an ethanol/water solution. Vesicles were dissolved in KHS.

Absolut ethanol ≥99.8%, *n*-hexane, and ethyl acetate (analytical grade) were obtained from Merck Life Science S.r.l. (Milan, Italy) e Alfa Aesar (Segrate, Italy).

3. Results

3.1. Chemical characterisation of *Sagratino* leaf extracts via HRMS and ¹H NMR

HRMS analyses of *Sagratino* leaf extracts revealed the presence of sugars, gallotannins and ellagitannins, glycosylated flavonoids (mainly quercetin and catechin derivatives), and cinnamic acid derivatives (ferulic and caffeic acids) (Table 1). MS and UV chromatograms confirmed the complex metabolite profiles of the samples, particularly in the FSEtOAc and FSEtOAcSOX fractions, where numerous classes of secondary metabolites were annotated based on accurate mass, fragmentation patterns, and UV absorbance. Galloyl derivatives showed key *m/z* fragments at 169 (gallic acid) and 125 (gallic acid – CO₂), punicalin was characterized by fragments at *m/z* 601 (–180, loss of hexose), and 301 (ellagic acid); punicalagin isomers, detected as doubly charged [M–2H]² ions, showed fragments at *m/z* 781 (loss of HHDP/ellagic unit), 601 (gallagic residue), and 301 (ellagic acid). Some compounds, sharing the same fragment ion at *m/z* 301.0324 with molecular formula C₁₅H₁₀O₇, derived from losses and cleavages of hexose and pentose moieties: these were tentatively assigned as quercetin-derivatives. Glycosylated derivatives of kaempferol yielded the aglycone fragment at *m/z* 285 with molecular formula C₁₅H₁₀O₆. The last-eluting peaks corresponded to flavonoid aglycones (Table 1, Fig. S1 and Table S2). Of note, extracts obtained from hydroalcoholic mixtures showed a similar mass profile, with the most representative metabolites reported in Table 1. Interestingly, changing the extraction method slightly modified the chemical composition of FSEtOAc and FSEtOAcSOX, which presented specific metabolites known for their beneficial properties. Among them, the most relevant included narirutin, hesperidin, quercetin glycosides like quercetin-3-*O*-glucuronide or quercetin-3-*O*-hexoside, and quercetin 3-*O*-galactoside (hyperoside).

¹H-NMR spectroscopy of FSA, FSAE10, FSAE20, and FSAE50 (Table 2) confirmed the presence of several compounds previously identified by UHPLC-HRMS analyses. Furthermore, the qualitative analysis demonstrated that all the compounds listed in the Table were consistently present across all samples. Fig. S2 shows the ¹H NMR spectra of the extracts in DMSO-*d*₆ (~2.50 ppm). DMSO was selected as the solvent due to its suitability for the analysis of polar compounds and complex natural mixtures. The spectra appeared largely superimposable, with major metabolites identified in all of them.

3.2. Effects of *Sagratino* leaf extracts on phenylephrine-induced contraction in rat thoracic aorta rings

All extracts obtained from *Sagratino* leaves were evaluated for vasorelaxant activity on aorta rings, with or without an intact endothelium, precontracted by 0.3–0.6 µM phenylephrine. Fig. 1 shows the concentration-response curves to the extracts FSA, FSAE10, FSAE20, and FSAE50 along with the solvent DMSO. In endothelium-intact rings, cumulative addition of the extracts at the plateau of phenylephrine-induced contraction caused a significant concentration-dependent relaxation (Fig. S3A and B). Notably, increasing the ethanol content in the extraction process correlated with greater, though not significantly different (*p* = 0.2363; see Table 3), vasodilatory efficacy. However, at concentrations exceeding 10 µg/mL, a hormetic behaviour resulting in a reversal of vasorelaxation was observed (Fig. S3A and C).

The vasorelaxant activity was shown to be endothelium-dependent,

Table 1

UHPLC-HRMS qualitative analysis of the major components in FSA, FSAE10, FSAE20, FSAE50, FSE, FSE EtOAc, and FSEtOAcSOX *Sagratino* leaf extracts.

Metabolites identified in FSA, FSAE10, FSAE20, FSAE50, FSE extracts			
Compound	[M-H] ⁺ / [M-2H] ²⁺	Compound	[M-H] ⁺ / [M-2H] ²⁺
Citric acid	191.0194	1,3,6-Tri-O-galloylhexose_IV	635.0883
Galloyl-hexoside	331.0577	Myricetin 3-O-beta-L-galactopyranoside	479.0844
HHDP-hexose	481.0693	1,3,6-Tri-O-galloylhexose_V	635.0883
Gallic acid	169.0133	Quercetin 3-O-glucuronide	477.0669
Galloyl-hexoside_I	331.0577	Ellagic Acid	300.9987
HHDP galloyl hexose	633.0739	Quercetin-3-O-Rutinoside	609.1458
Punicalin β	781.0593	Naringenin-7-O-hexoside	433.1143
Protocatechuic acid	153.0184	Quercetin 3-O-hexoside	463.0895
Punicalin β_I	781.0593	Quercetin 3-O-glucuronide	477.0669
HHDP galloyl hexose_I	633.0739	Quercetin-3-arabinoside	433.0780
Epigallocatechin	305.0673	Eryodictol	287.0568
Pedunculagin (di-HHDP-hexose)	783.0629	Kaempferol 7-O-hexoside	447.0943
HHDP galloyl hexose_II	633.0740	Kaempferol-3-O-glucorhamnoside	593.1523
Puniculagin	541.0262**	Quercetin-3-arabinoside_I	433.0780
HHDP galloyl hexose_III	633.0738	Kaempferol 7-O-hexoside_I	447.0943
Hamamelitannin	483.0773	1,2,3,6-Tetragalloylglucose	787.1018
Puniculagin_I	541.0262**	Myricetin	317.0309
Pedunculagin (di-HHDP-hexose)_I	783.0629	Kaempferol 3-alpha-L-arabinopyranoside	417.0833
HHDP galloyl hexose_IV	633.0740	Apigenin 8-C-hexoside	431.0996
Puniculagin_II	541.0266**	Kaempferol 3-alpha-L-arabinopyranoside_I	417.0833
2-hydroxycinnamic acid	163.0393	Phloridzin	435.1304
p-Coumaric acid hexoside	325.0929	Hesperidin	609.1816
Pedunculagin (di-HHDP-hexose)_II	783.0629	Quercetin	301.0359
1,3,6-tri-O-galloylhexose	635.0881	Kaempferol	285.0402
Eriodictyol-7-O-hexoside	449.1094	Naringenin	271.0613
Puniculagin	541.0266**	Apigenin	269.0453
HHDP galloyl hexose_V	633.0740	Luteolin	285.0402
1,3,6-tri-O-galloylhexose_I	635.0881	Isorhamnetin	315.0517
1,3,6-tri-O-galloylhexose_II	635.0880	Syringetin	345.0612
1,3,6-tri-O-galloylhexose_III	635.0882		
Metabolites identified in FSEtOAc and FSEtOAcSOX extracts			
Compound	[M-H] ⁺ / [M-2H] ²⁺		
Ferulic acid	193.0501		
Diosmetin-O-hexoside	461.1025		
Naringenin-glucoside	417.1025		
Limonin-hexoside	537.2024		
Narirutin	579.1718		
Hesperidin	609.1829		
Dydimin	591.1718		
Quercetin-3-O-glucuronide	477.0669		
Quercetin-3-O-hexoside	463.0895		
Quercitrin	447.0928		
Hyperoside (quercetin 3-O-galactoside)	463.0876		
Luteolin-7-glucoside	447.3721		
Vicenin II	593.1556		
Quercetin-O-hexoside-O-rhamnoside-O-glucoside	825.2286		
Neodiosmin	609.1809		

I: isomer; **: double charged.

as it was absent in endothelium-deprived rings, where, conversely, a contractile activity, though not significantly different ($p = 0.3163$; see Table 3), was observed, except at the highest concentration assessed (300 $\mu\text{g/mL}$), which caused a return to control values or even vasorelaxation.

When distilled water replaced DMSO to dissolve FSA, FSAE10, and FSAE20 extracts, and a 1:1 water: absolute ethanol mixture was used for FSAE50, comparable trends were observed, although with reduced statistical significance (Fig. 2). No statistically significant differences were found among the four extract vasorelaxant or vasoconstricting efficacies (phenylephrine + endothelium $p = 0.3803$, and -endothelium $p = 0.4337$, respectively; see Table 3).

Fig. 3 depicts the concentration–response curves for the extracts FSE, FSE EtOAc, FSEtOAc, and FSEtOAcSOX alongside DMSO, on phenylephrine-induced contractions. In endothelium-intact rings, cumulative application of these extracts elicited a significant concentration-dependent relaxation. No statistically significant differences were found among the four extract efficacies ($p = 0.1248$; see Table 3). FSE EtOAc, at concentrations exceeding 30 $\mu\text{g/mL}$, exhibited a modest hormetic behaviour characterised by reduced spasmolysis. In endothelium-deprived preparations, the extracts FSE, FSE EtOAc, and FSEtOAcSOX potentiated the active tone, only FSE and FSE EtOAc showing a hormetic effect, as at 300 $\mu\text{g/mL}$: vessel tone reverted to control values or even below. The extraction methodology appeared to influence the biological activity of the extracts. In fact, as shown in Fig. 3C, the vasorelaxant effect of the UAE-derived extract (FSEtOAc) was only partially affected by endothelium removal. The Soxhlet-derived extract (FSEtOAcSOX), however, displayed a pharmacological profile like that of FS and FSE EtOAc, as contraction instead of relaxation was recorded in endothelium-deprived rings. Though the statistical analysis did not find any significant differences ($p = 0.2092$; see Table 3), the extract FSEtOAc emerged as the most interesting in the series.

3.3. Effects of *Sagratino* leaf extracts on KCl-induced contraction in rat thoracic aorta rings

The extracts were evaluated on endothelium-deprived aorta rings, precontracted by either 25–30 mM or 60 mM KCl, to differentiate between potassium channel opening effects and calcium channel antagonistic activity. Fig. 4 displays the concentration–response curves of FSA, FSAE10, FSAE20, FSAE50, FSE, FSE EtOAc, FSEtOAc, and FSEtOAcSOX recorded under both depolarisation conditions. The cumulative application of these extracts during the plateau phase of KCl-induced active tone elicited a concentration-dependent contractile response that was more pronounced at lower depolarisation levels (25–30 mM KCl; Fig. S3D and E). In rings precontracted by 25–30 mM KCl, a mild, but not significant, concentration-dependent relaxation was observed with both FSEtOAc and FSEtOAcSOX at concentrations $\geq 3 \mu\text{g/mL}$. This effect, however, was significantly different from that observed with FSE ($p < 0.05$). Notably, extracts obtained using a mixture of absolute ethanol and water were associated with a more pronounced, though not statistically significant, contractile effect compared to those extracted with either absolute ethanol ($p = 0.7862$; see Table 3) or water alone ($p = 0.4211$). In rings precontracted by 60 mM KCl, no statistically significant differences were detected in both series (FSA, FSAE10, FSAE20, and FSAE50 $p = 0.5543$; FSE, FSE EtOAc, FSEtOAc, and FSEtOAcSOX $p = 0.7006$).

3.4. Characterisation of *Sagratino* leaf vesicles via NTA, transmission electron microscopy, and chemical description via ¹H NMR

NTA analysis was employed to characterise the size distribution and concentration of EVs isolated from *Sagratino* leaves. As shown in Fig. 5A and B, the EVs exhibited the typical profile of PDEVs. The microvesicles fraction displayed a broader and slightly right-shifted size

Table 2¹H NMR qualitative analysis of the major components in FSA, FSAE10, FSAE20, and FSAE50 *Sagratino* leaf extracts.

Compounds	¹ H ppm	Multiplicity	FSA	FSAE10	FSAE20	FSAE50	FSE	FSEtOAc	FSEtEtOAcSOX
Kaempferol	8.01	d	-	+	+	+	+	+	+
Trans-cinnamic acid	7.72	s	-	-	-	+	+	+	+
Tryptophane	7.70	m	+	+	+	-	-	+	+
Trans-p-coumaric acid	7.59-7.51	m	+	+	+	+	+	+	+
Trans-fertaric acid	7.56	d	+	+	-	-	+	+	+
Flavanes, flavonoids	7.56-7.47	s (ArH)	+	+	+	+	+	+	+
Caftaric acid	7.52	d	+	+	+	+	+	+	+
Phenylalanine	7.42	m	-	+	+	-	-	+	+
Myricetin	7.36	s	-	-	+	+	+	+	+
Cinnamic acid	6.81-6.76	d	+	+	+	+	+	+	+
Trans-resveratrol	6.47	m	+	+	+	+	+	+	+
Caffeic acid	6.41	s	+	+	+	+	+	+	+
Quercetin	6.40	d	+	+	+	+	+	+	+
cis-Ferulic acid	6.94-5.97	m	+	+	+	+	+	+	+
Catechin	6.21	d	+	+	+	+	+	+	+
Disaccharides	5.30-5.09	m	+	+	+	+	+	+	+
β-Rhamnose	4.89	d	+	+	+	+	+	+	+
Ascorbic acid	4.43	n.a.	+	+	+	+	+	+	+
Malic acid	4.25	dd	+	+	+	+	+	+	+
Tartaric acid	3.98	d	+	+	+	+	+	+	+
Fructose	4.22	d	+	+	-	-	-	+	+
α-Glucose	3.55	m	+	+	-	-	-	+	+
β-Glucose	3.20	dd	+	+	-	-	-	+	+
GABA	2.80	m	+	+	-	-	-	+	+
Malate, citrate	2.90-2.70	m	+	+	+	+	+	+	+
Aspartate	2.86	d	+	+	+	+	+	+	+
Shikimic acid	2.70	dd	+	+	+	+	+	+	+
Alanine	1.51	d	+	+	+	+	+	+	+
Threonine	1.33	d	+	+	+	+	+	+	+
Valine	1.05	d	+	+	+	+	+	+	+
Fatty acids	1.03	s	+	+	+	+	+	+	+
Isoleucine	1.01	d	+	+	+	+	+	+	+
Leucine	0.96	d	+	+	+	+	+	+	+

+: identified; -: not identified.

distribution (mean diameter 207.7 ± 1.1 nm; Fig. 5A), while the nanovesicles fraction presented a narrower distribution (mean diameter 172.8 ± 0.5 nm; Fig. 5B). The upper size limit (D90) of the nanovesicles (253.1 ± 3.0 nm) was lower than that of the microvesicles (307.7 ± 11.7 nm), consistent with the enrichment of larger particles in the latter fraction. Polydispersity was higher in microvesicles (68.1 ± 1.9 nm) than in nanovesicles (61.8 ± 0.6 nm). Particle concentration was significantly greater in nanovesicles ($1.83 \pm 0.06 \times 10^{14}$ particles/mL) compared to microvesicle preparation ($8.66 \pm 0.68 \times 10^{13}$ particles/mL). No significant secondary peaks were detected in the size distribution profiles, indicating the absence of vesicle aggregation. The vesicle preparations appeared well-dispersed and stable under the applied analytical conditions.

Transmission electron microscopy (TEM) was employed to further investigate the morphology of *Sagratino*-derived vesicles. TEM analysis revealed numerous typically rounded, well-defined, and intact vesicles, mostly below 200 nm in diameter, consistent with the characteristics of nanovesicles (Fig. 5C–H). At higher magnification, these vesicles exhibited well-preserved ultrastructural features, including a continuous, unbroken bilayer membrane, distinguishable as a thin white filament surrounding their electron-dense content. These findings confirm that the vesicles isolated from *Sagratino* leaves are nanosized and display morphological features consistent with exosome-like vesicles.

To gain insights into the chemical composition of the vesicles, ¹H NMR spectroscopy was applied under the same conditions adopted for the extracts. Although the concentrations of metabolites present in the vesicles limited the acquisition of a high-quality ¹H NMR spectrum, it was possible to reveal the presence of multiple classes of bioactive compounds in both microvesicle and nanovesicle fractions. The spectra showed characteristic signals attributable to polyphenols, glycosylated flavonoids, sugars, amino acids, and fatty acids. Representative spectra are reported in Fig. 5I and J.

3.5. Effects of *Sagratino* leaf vesicles on phenylephrine- or KCl-induced contraction in rat thoracic aorta rings

The vesicles were assessed on either endothelium-intact or -deprived aorta rings, precontracted by 0.3–0.6 μM phenylephrine. In endothelium-intact rings, cumulative addition of microvesicles and nanovesicles at the plateau of phenylephrine-induced active tone resulted in a concentration-dependent relaxation (Fig. 6A and B and S4A): their efficacy was not statistically different ($p = 0.9020$; see Table 3). However, at concentrations exceeding 1×10^{11} particles/mL, a hormetic response was observed, characterised by a return toward control values.

In endothelium-deprived preparations, vasorelaxation was observed only at the highest concentration tested (1×10^{12}) (Fig. S4B; $p = 0.9770$; see Table 3).

In a second series of experiments, the vesicles were evaluated on endothelium-deprived aorta rings, precontracted by either 25–30 mM or 60 mM KCl. Fig. 6C and D displays the concentration-response curves of microvesicles and nanovesicles, respectively, recorded under both depolarisation conditions. The cumulative application of these vesicles during the plateau phase of KCl-induced active tone elicited a concentration-dependent contractile response that was more pronounced at lower depolarisation levels (25–30 mM KCl; Fig. S4C). In rings challenged by nanovesicles, the curves constructed in rings depolarised by moderate and high KCl concentrations were significantly different ($p = 0.0269$).

3.6. Effect of *Sagratino* leaf vesicles on caudal artery myocyte *I_{Ba}*

A biophysical analysis was carried out to elucidate the interaction between *Sagratino* leaf microvesicles and nanovesicles and vascular smooth muscle Cav1.2 channels. The addition of cumulative

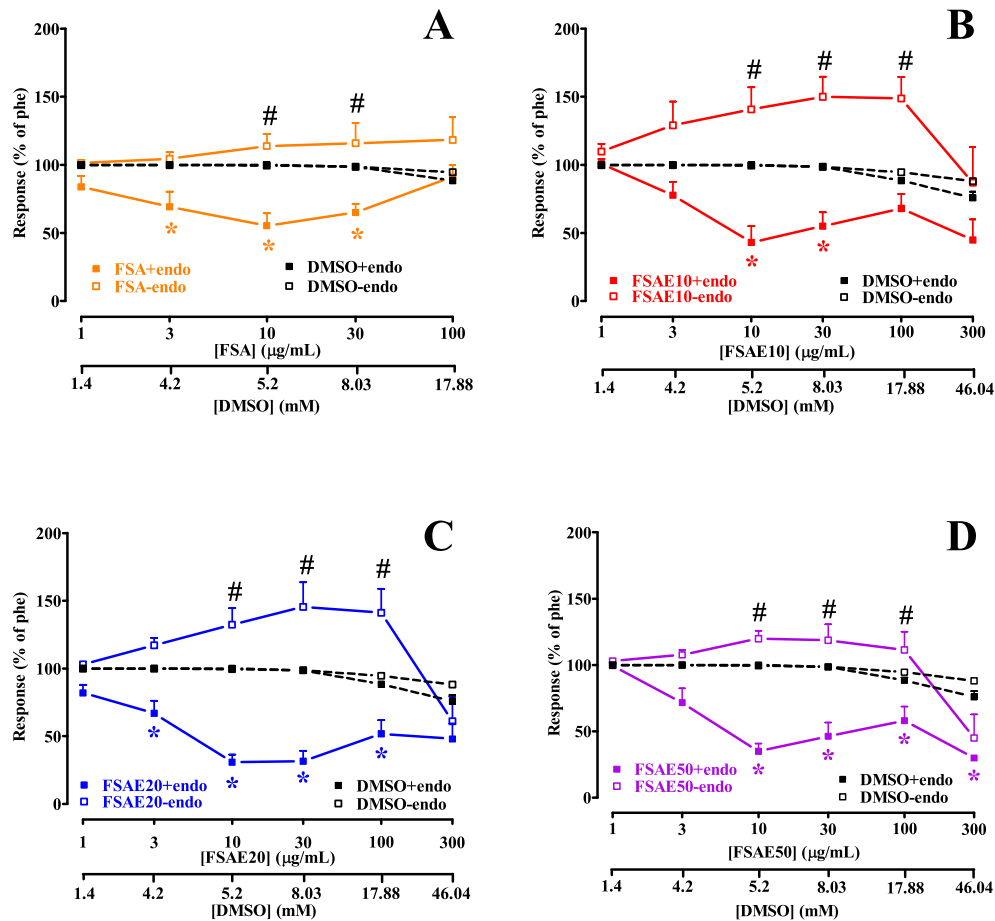


Fig. 1. Effects of *Sagrantino* leaf extracts dissolved in DMSO on phenylephrine-induced contraction of rat aorta rings. Concentration-response curves to (A) FSA, (B) FSAE10, (C) FSAE20, and (D) FSAE50 in rings with (+endo) or without endothelium (-endo) pre-contracted by 0.3–0.6 μ M phenylephrine. DMSO data are the same throughout the panels. In the ordinate scale, response is reported as a percentage of the initial tension induced by phenylephrine (phe). *Sagrantino* cv. leaves extracted in water (FSA), or in water: absolute ethanol (v/v) 90:10, 80:20, and 50:50 (FSAE10, FSAE20, and FSAE50, respectively). Data are mean $\pm$ S.E.M. (n = 3–10). *p < 0.05 vs. DMSO and #p < 0.05 vs. +endo, two-way ANOVA and Bonferroni post-test.

Table 3

Vasorelaxant and vasoconstricting efficacy of *Sagrantino* leaf extracts and vesicles.

Solvent	Extract	Phenylephrine		KCl	
		+endothelium	-endothelium	25/30 mM	60 mM
DMSO	FSA	−44.8 $\pm$ 9.4 (7)	+18.4 $\pm$ 16.5 (7)	+44.8 $\pm$ 12.4 (15)	−3.8 $\pm$ 1.5 (5)
DMSO	FSAE10	−56.8 $\pm$ 12.0 (8)	+49.9 $\pm$ 14.8 (9)	+63.3 $\pm$ 71.8 (6)	−2.7 $\pm$ 7.9 (5)
DMSO	FSAE20	−69.4 $\pm$ 5.8 (8)	+45.4 $\pm$ 18.2 (10)	+167.8 $\pm$ 46.7 (5)	+16.2 $\pm$ 12.1 (5)
DMSO	FSAE50	−65.1 $\pm$ 5.9 (8)	+19.8 $\pm$ 6.0 (9)	+152.6 $\pm$ 108.0 (9)	+38.6 $\pm$ 36.6 (7)
H ₂ O	FSA	−46.3 $\pm$ 4.9 (31)	+29.2 $\pm$ 12.9 (5)	N.A.	N.A.
H ₂ O	FSAE10	−64.6 $\pm$ 6.6 (3)	+25.9 $\pm$ 25.9 (3)	N.A.	N.A.
H ₂ O	FSAE20	−65.6 $\pm$ 16.9 (3)	−10.2 $\pm$ 23.3 (3)	N.A.	N.A.
H ₂ O:EtOH	FSAE50	−61.1 $\pm$ 6.9 (3)	+12.5 $\pm$ 6.9 (3)	N.A.	N.A.
DMSO	FSE	−65.2 $\pm$ 9.5 (5)	+20.2 $\pm$ 16.4 (10)	+69.7 $\pm$ 13.5 (5)	+29.9 $\pm$ 14.1 (4)
DMSO	FSE EtOAc	−56.7 $\pm$ 9.2 (8)	+34.4 $\pm$ 25.2 (7)	+44.2 $\pm$ 17.3 (7)	+37.8 $\pm$ 6.1 (5)
DMSO	FSEtOAc	−60.5 $\pm$ 3.6 (10)	−59.7 $\pm$ 8.1 (3)	−23.8 $\pm$ 12.2 (5)	+32.1 $\pm$ 29.8 (3)
DMSO	FSEtOAcSOX	−42.4 $\pm$ 4.9 (8)	+37.5 $\pm$ 69.9 (3)	−9.3 $\pm$ 7.7 (4)	+12.3 $\pm$ 13.2 (3)
KHS	MV	−62.0 $\pm$ 38.0 (3)	−68.7 $\pm$ 9.1 (6)	+105.3 $\pm$ 10.6 (5)	+33.2 $\pm$ 7.9 (5)
KHS	NV	−59.1 $\pm$ 5.4 (8)	−68.3 $\pm$ 1.7 (3)	+196.7 $\pm$ 123.0 (3)	+43.2 $\pm$ 6.5 (3)

Data represent mean $\pm$ S.E.M., with the number of replicates in parentheses. MV: microvesicles; NV: nanovesicles; N.A.: not assessed.

concentrations of microvesicles caused a concentration-dependent inhibition of I_{Ba} (Fig. S5A) with an E_{max} value of about 80%, and an IC_{50} value of $1.64 \pm 0.40 \times 10^{11}$ particles/mL (n = 5; Fig. 7A). The current-voltage relationships show that 1×10^{11} microvesicles significantly decreased the peak inward current over a membrane potential range

from −10 mV to 50 mV, shifting the peak of the curve toward more hyperpolarised potentials, without affecting the activation threshold (approximately −30 mV) (Fig. 7B). Current amplitude inhibition was partially reversed upon microvesicle washout.

Fig. S5B and 7C illustrate the effect of increasing nanovesicle

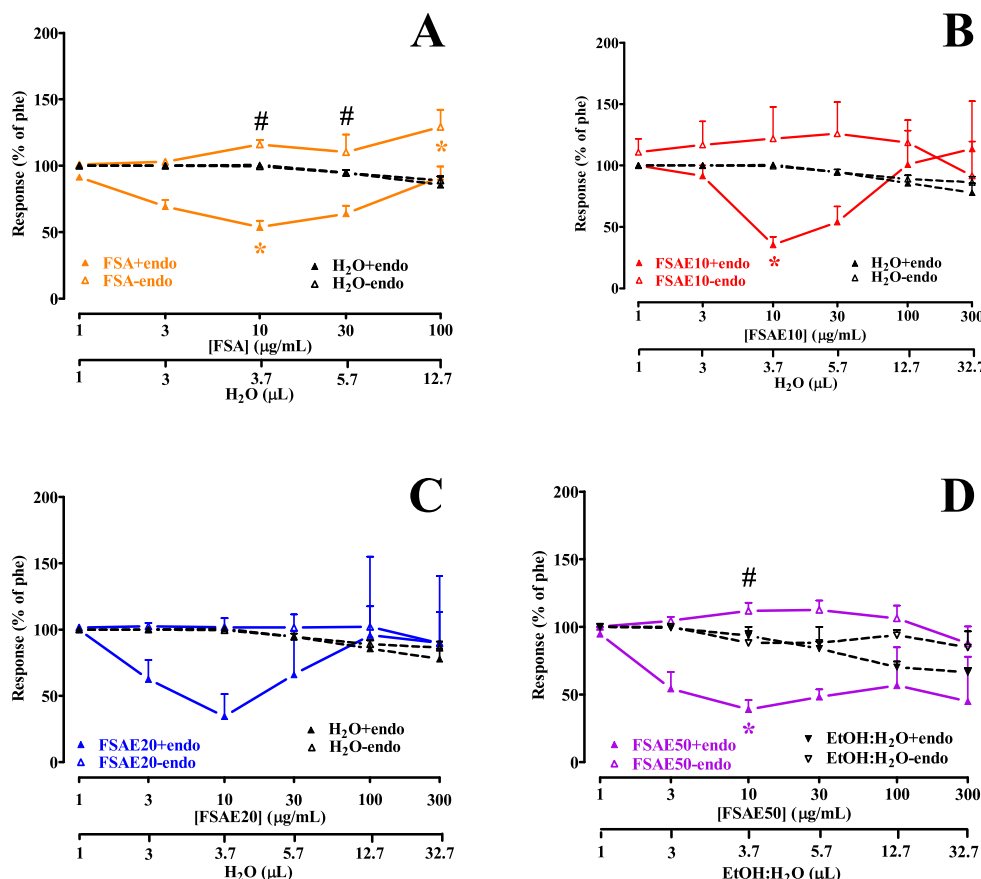


Fig. 2. Effects of *Sagrantino* leaf extracts dissolved in water or water: absolute ethanol on phenylephrine-induced contraction of rat aorta rings. Concentration-response curves to (A) FSA, (B) FSAE10, (C) FSAE20, and (D) FSAE50 in rings with (+endo) or without endothelium (-endo) pre-contracted by 0.3–0.6 μ M phenylephrine. Distilled water data are the same throughout panels A–C. In the ordinate scale, response is reported as a percentage of the initial tension induced by phenylephrine (phe). *Sagrantino* cv. leaves extracted in water (FSA), or in water: absolute ethanol (v/v) 90:10, 80:20, and 50:50 (FSAE10, FSAE20, and FSAE50, respectively). Data are mean $\pm$ S.E.M. ($n = 3$ –31). * $p < 0.05$ vs. solvent and # $p < 0.05$ vs. +endo, two-way ANOVA and Bonferroni post-test.

concentrations on I_{Ba} . Nanovesicles inhibited the current in a concentration-dependent manner, with an E_{max} of about 93%, and an IC_{50} of $5.17 \pm 2.17 \times 10^{10}$ particles/mL. The current-voltage relationships show that 3×10^{10} nanovesicles significantly decreased the peak inward current in the range from 0 to 30 mV, shifting the apparent peak by 5 mV in the hyperpolarising direction without affecting the threshold (approximately -30 mV) (Fig. 7D). Current amplitude inhibition was reversible upon washout.

4. Discussion

This study demonstrates that *Sagrantino* vine leaves, an underutilised by-product of winemaking, represent a valuable source of vasoactive phytochemicals and PDEVs, aligning with circular economy strategies aimed at converting agro-waste into health-promoting resources. The integration of chemical profiling, vascular reactivity studies, and electrophysiological analyses provides a mechanistic basis for the traditional use of grapevine leaves in Mediterranean, Middle Eastern, and Balkan ethnomedicine, where they have long been employed for circulatory disorders, inflammation, varicosities, and wound healing. The dual approach - solvent-based extraction and vesicle isolation - highlights both the richness of the phytochemical profile and the innovative role of plant-derived nanostructures in modulating vascular function.

The results clearly show that the bioactive compounds present in grapevine leaves can be efficiently recovered using sustainable

extraction methods (Singh et al., 2023). Mild conditions, such as low temperature, short processing times, and eco-friendly solvents, proved crucial for preserving phenolic integrity and bioactivity. UAE enhanced yield and protected thermolabile compounds, while solvent polarity and solid-to-liquid ratio strongly influenced extraction efficiency (De Luca et al., 2023).

HRMS and ^1H NMR analyses revealed a complex metabolite profile, dominated by polyphenols, glycosylated flavonoids (quercetin, kaempferol, and catechin derivatives), ellagitannins, amino acids, and cinnamic acid derivatives. These findings are consistent with previous literature (Singh et al., 2023; Acero et al., 2025), reinforcing the hypothesis that *Sagrantino* leaves retain a potent phytochemical profile comparable to that of grape skins, seeds, and pomace, as previously reported (Carullo et al., 2020; Saadaoui et al., 2023). This rich polyphenolic and antioxidant matrix offers a credible mechanistic foundation for the *ex vivo* vasoactive effects observed here, as these compound classes are known to modulate endothelial NO bioavailability, redox balance, and Ca^{2+} -dependent smooth muscle function (Figueiredo et al., 2017; Carullo et al., 2019).

Ex vivo assays on isolated rat aorta rings revealed that *Sagrantino* leaf extracts promote a concentration- and endothelium-dependent relaxation, consistent with polyphenols-induced stimulation of endothelial NO synthase, hyperpolarising pathways, and redox modulation (Fusi et al., 2003; Figueiredo et al., 2017). Interestingly, several extracts exhibited a biphasic, hormetic pattern at higher concentrations (>10 μ g/mL),

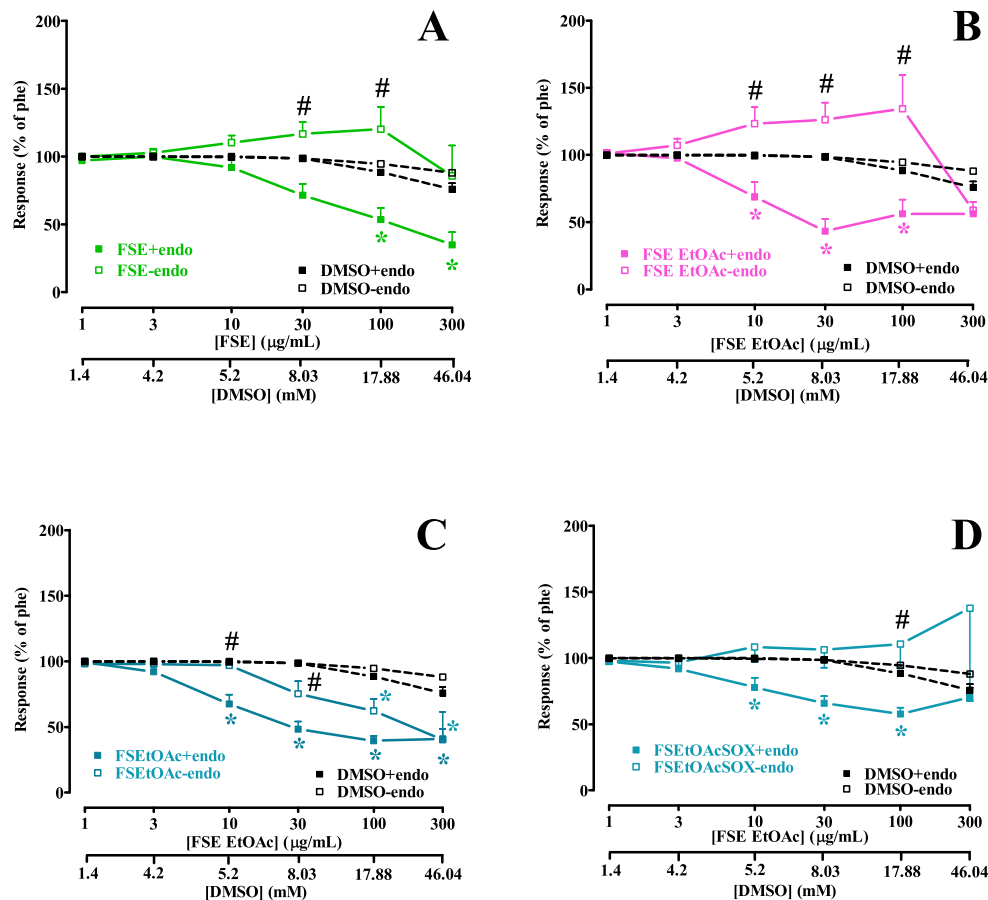


Fig. 3. Effects of *Sagraantino* leaf extracts on phenylephrine-induced contraction of rat aorta rings. Concentration-response curves to (A) FSE, (B) FSE EtOAc, (C) FSEtOAc, and (D) FSEtOAcSOX in rings with (+endo) or without endothelium (-endo) pre-contracted by 0.3–0.6 μ M phenylephrine. DMSO data are the same throughout panels. In the ordinate scale, response is reported as a percentage of the initial tension induced by phenylephrine (phe). Data are mean $\pm$ S.E.M. ($n = 3$ –11). *Sagraantino* cv. leaves extracted in absolute ethanol (FSE), in ethyl acetate (FSE EtOAc), in ethyl acetate using UAE (FSEtOAc) or Soxhlet (FSEtOAcSOX). * $p < 0.05$ vs. DMSO and # $p < 0.05$ vs. +endo, two-way ANOVA and Bonferroni post-test.

characterised by a reversal of the relaxant effect. Similar behaviour has been reported for pomace and red wine extracts (Carullo et al., 2020; Medana et al., 2024), highlighting the complexity of polyphenol-vascular interactions, where excessive exposure may trigger paradoxical vasoconstriction, oxidative imbalance, or activation of contractile pathways (Perrone and D'Angelo, 2025).

Extracts obtained from hydroalcoholic mixtures, particularly those prepared via UAE, displayed superior vasorelaxant activity, suggesting that green extraction conditions favour the preservation and recovery of bioactive molecules otherwise sensitive to temperature or prolonged processing (Barba et al., 2016; De Luca et al., 2023). Conversely, the contractile response observed in endothelium-denuded rings, especially with hydroalcoholic extracts, suggests that in the absence of NO-mediated buffering, polyphenols and tannins may directly activate smooth muscle pro-contraction mechanisms, such as Rho kinase signalling or Ca^{2+} sensitisation, mirroring previous findings on grape pomace extracts (Carullo et al., 2019; Medana et al., 2024). Noticeably, among these extracts, FSEtOAc showed an interesting pharmacological profile, as it was the only one capable of relaxing vessels independently of the presence of an intact endothelium, as well as under moderate depolarising conditions.

When tested on endothelium-deprived rings precontracted by KCl, most extracts elicited a contractile response, particularly under lower depolarisation conditions (i.e., in rings stimulated by 25–30 mM KCl). This suggests a possible K^{+} channel blockade or non-specific smooth

muscle activation.

A particularly innovative aspect of this work is the demonstration that EVs naturally present in *Sagraantino* leaves exhibit pronounced and mechanistically distinct vascular effects. NTA and TEM confirmed that EVs possess well-defined morphology and size distribution, consistent with exosome-like particles described in other plant matrices (Lian et al., 2022; Buratta et al., 2024). Their ^1H NMR spectra demonstrated the presence of polyphenols, sugars, fatty acids, and amino acids, supporting their role as natural nanocarriers of vasoactive metabolites.

Functionally, both microvesicles and nanovesicles elicited a comparable concentration-dependent vasorelaxation in endothelium-intact aortic rings. However, at concentrations exceeding 1×10^{11} particles/mL, a hormetic response emerged, characterised by a decrease in spasmolytic activity. In endothelium-deprived rings, the vesicles were inactive at lower concentrations, although vasorelaxation was evident at the highest concentration tested (1×10^{12} particles/mL), as already observed for most *Sagraantino* leaf extracts. The effect of EVs on depolarised vascular preparations was in line with that caused by the extracts, the elicited contraction being more evident in the presence of moderate KCl concentrations.

Electrophysiological assays revealed that both vesicle populations inhibited $Cav1.2$ channels in vascular smooth muscle cells, with partial reversibility upon washout. As the patch-clamp experiments were performed in the whole-cell configuration, which causes the dialysis of the cytoplasm, it is reasonable to speculate that the vesicles may block

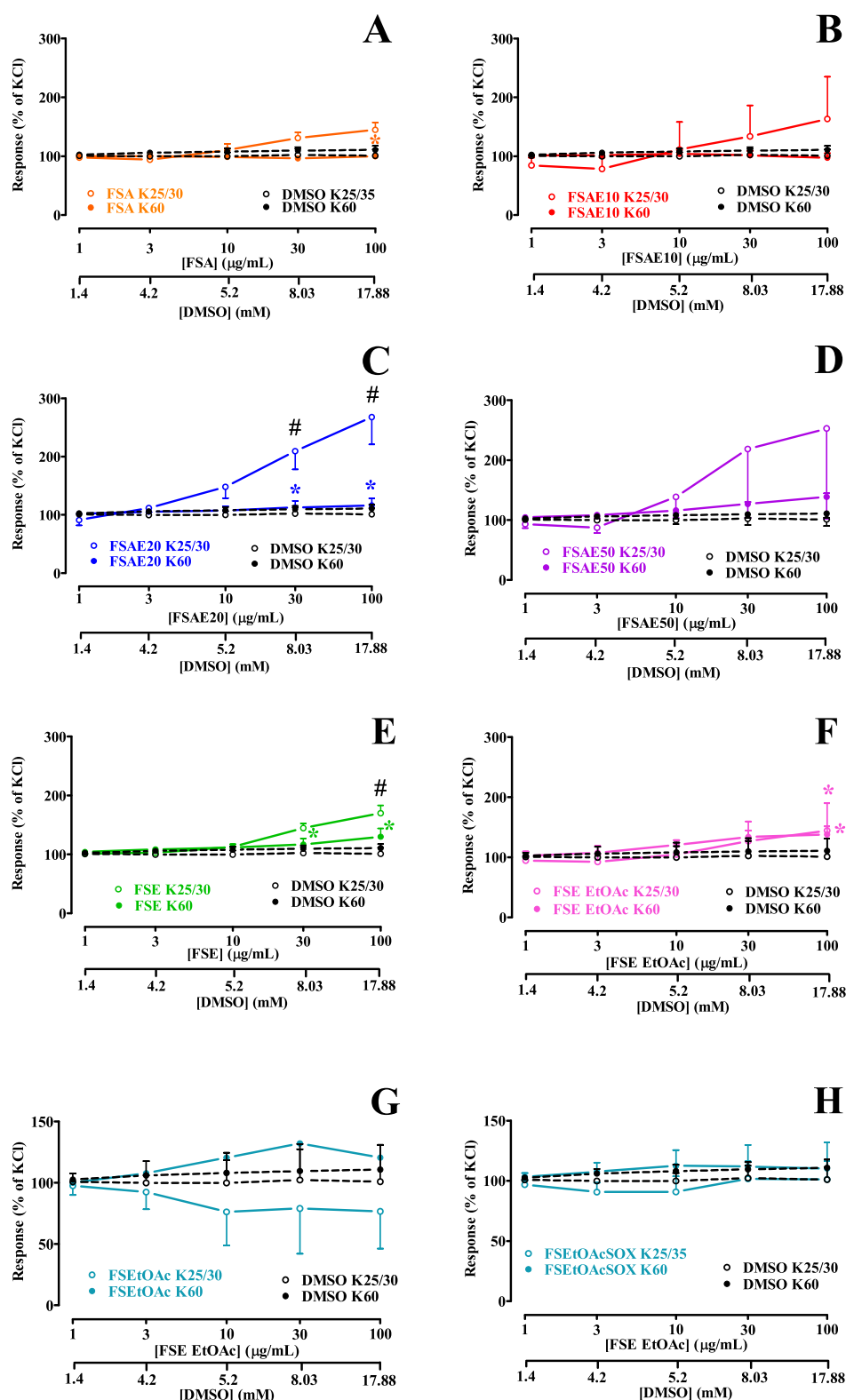


Fig. 4. Effects of *Sagrantino* leaf extracts on KCl-induced contraction of rat aorta rings. Concentration-response curves to (A) FSA, (B) FSAE10, (C) FSAE20, (D) FSAE50, (E) FSE, (F) FSE EtOAc, (G) FSEtOAc, and (H) FSEtOAcSOX on endothelium-deprived preparations pre-contracted by either 25–30 mM (K25/K30) or 60 mM KCl (K60). In the ordinate scale, response is reported as a percentage of the initial tension induced by KCl. *Sagrantino* cv. leaves extracted in water (FSA), in water: absolute ethanol (v/v) 90:10, 80:20, and 50:50 (FSAE10, FSAE20, and FSAE50, respectively), in absolute ethanol (FSE), in ethyl acetate (FSE EtOAc), in ethyl acetate using UAE (FSEtOAc) or Soxhlet (FSEtOAcSOX). Data are mean $\pm$ S.E.M. (n = 3–15). *p < 0.05 vs. DMSO and #p < 0.05 vs. K60, two-way ANOVA and Bonferroni post-test.

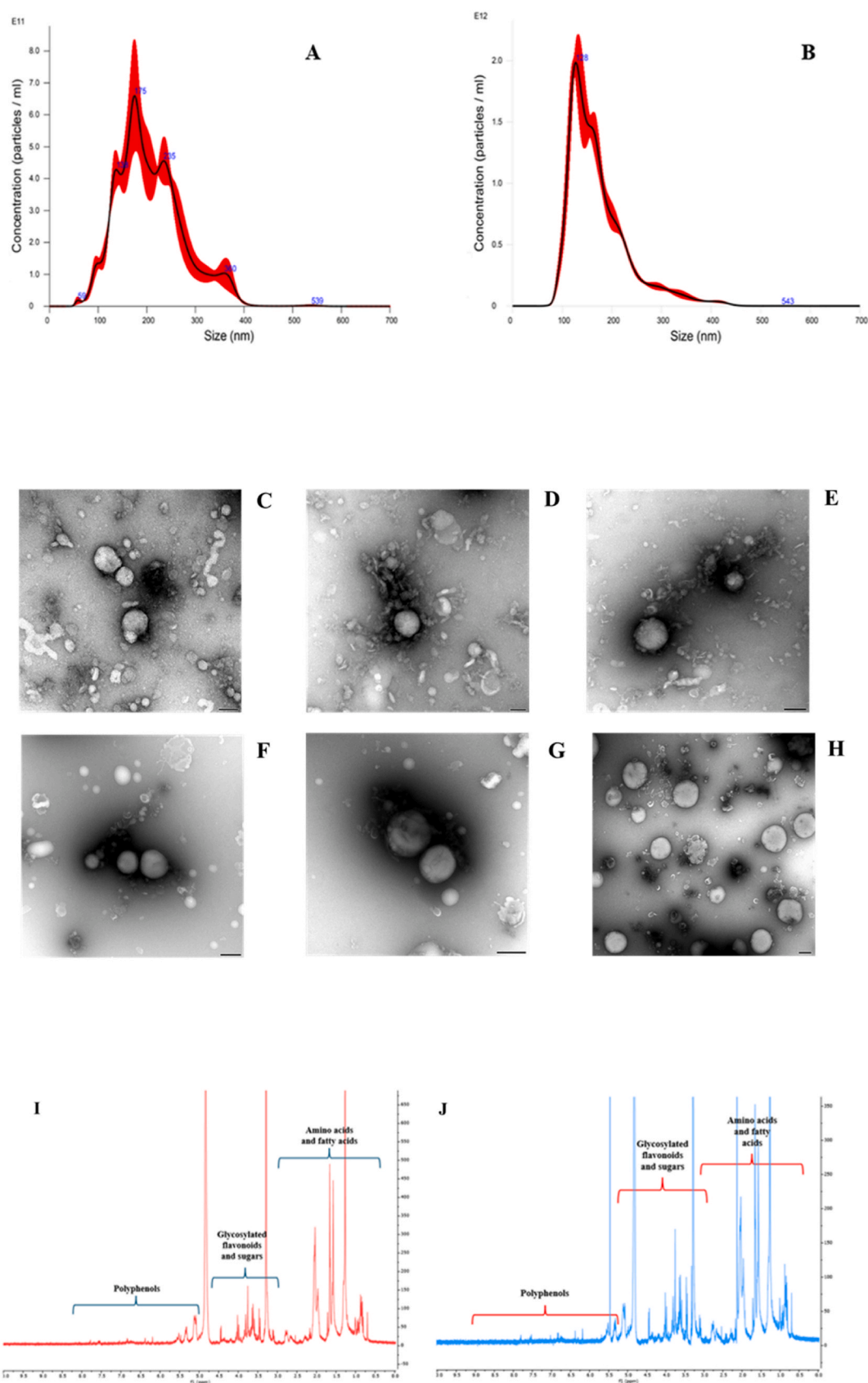


Fig. 5. Analysis of size distribution, concentration, and components of *Sagrantino* leaf vesicles. (A,B) NTA profile of *Sagrantino* leaf (A) microvesicles and (B) nanovesicles. (C-H) Transmission electron microscopy images of *Sagrantino* leaf vesicles. (C-E) Typical rounded structure and nanometer size of microvesicles. (F-H) Typical rounded structure and nanometer/micrometer size of nanovesicles. Scale bar C, D: 100 nm, E: 200 nm, F-H: 500 nm. (I,J) Representative ^1D NMR spectrum of *Sagrantino* leaf (I) nanovesicles and (J) microvesicles.

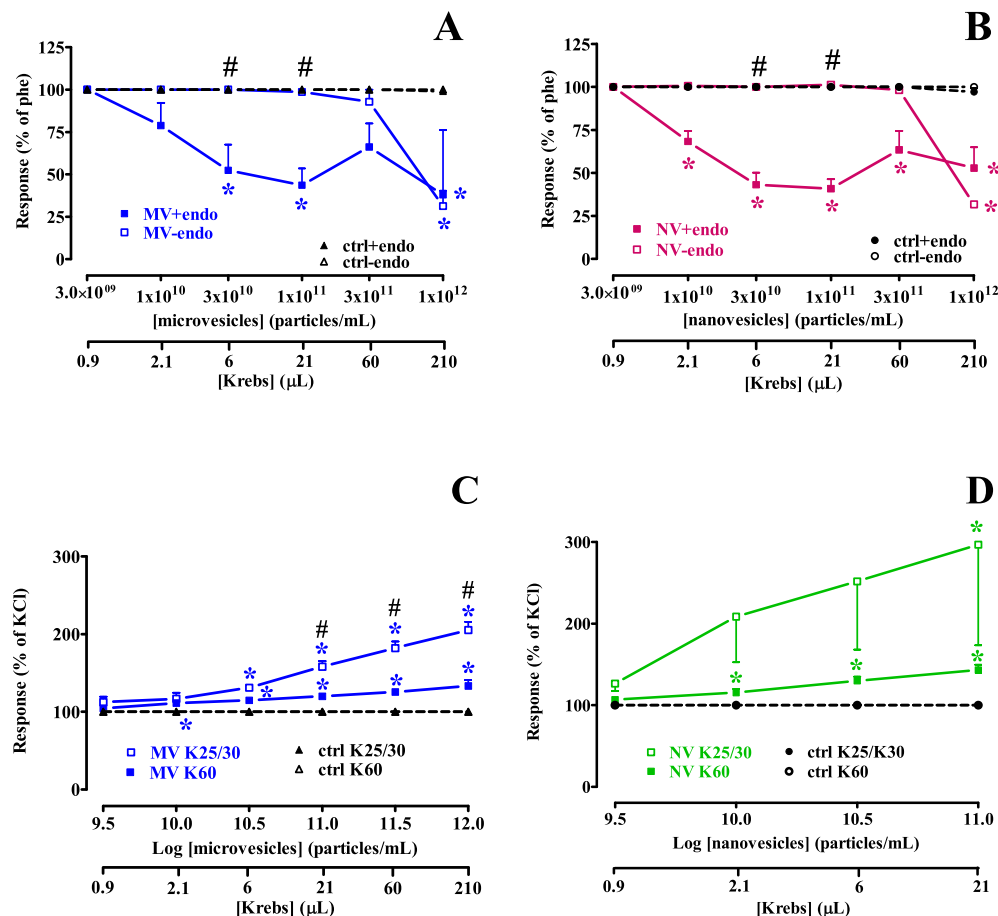


Fig. 6. Effects of microvesicles and nanovesicles on either phenylephrine- or KCl-induced contraction of rat aorta rings. (A,B) Concentration-response curves to (A) microvesicles (MV) and (B) nanovesicles (NV) in rings with (+endo) or without endothelium (-endo) pre-contracted by 0.3–0.6 μ M phenylephrine. In the ordinate scale, response is reported as a percentage of the initial tension induced by phenylephrine (phe). Data are the mean $\pm$ S.E.M. ($n = 6$ –8). * $p < 0.05$ vs. KHS (ctrl) and # $p < 0.05$ vs. +endo, two-way ANOVA and Bonferroni post-test. (C,D) Concentration-response curves to (C) microvesicles and (D) nanovesicles on endothelium-deprived preparations pre-contracted by either 25–30 mM (K25/K30) or 60 mM KCl (K60). In the ordinate scale, response is reported as a percentage of the initial tension induced by KCl. Data are mean $\pm$ S.E.M. ($n = 3$ –5). * $p < 0.05$ vs. control, and # $p < 0.05$ vs. K60, two-way ANOVA and Bonferroni post-test.

Cav1.2 channels through a direct interaction with the channel protein rather than via activation or inhibition of intracellular pathways. Finally, the observed hyperpolarising shift in the current-voltage relationship, without alteration of the activation threshold, suggests that vesicles may affect the voltage sensitivity of the channel.

This mechanistic insight positions plant EVs as bioactive entities capable of modulating Ca^{2+} -dependent vascular tone, expanding their role beyond passive carriers and aligning with emerging evidence on cross-kingdom bioactivity (Fang and Liu, 2022; Di Giulio et al., 2023).

Together, these results underscore that both conventional extracts and EVs contribute to vascular relaxation through complementary mechanisms: endothelial stimulation, likely NO-mediated, and, at least in part, direct smooth muscle modulation via Cav1.2 channel inhibition. This integrative mechanistic insight strengthens the pharmacological basis of grapevine leaf use in traditional medicine and supports their valorisation as sustainable cardiovascular health resources.

Beyond their ethnopharmacological relevance, these findings have broader implications for the circular bioeconomy. Viticulture generates large amounts of leaves that are typically discarded, yet these residues contain potent vasoactive compounds and functional EVs, supporting their repurposing for nutraceutical applications. Nonetheless, limitations include the absence of quantitative metabolite profiling, restriction to a single vascular district, lack of *in vivo* validation, and potential variability in vesicle composition due to growth or isolation conditions.

Future studies should quantify the principal bioactives, explore bioavailability and metabolism *in vivo*, assess inter-cultivar variability, and investigate the capacity of PDEVs to enhance the delivery of specific phytochemicals.

5. Conclusions

This study provides mechanistic evidence supporting the vascular benefits historically attributed to grapevine leaves and highlights the *Sagrantino cultivar* as a sustainable source of cardiovascular bioactives. Combining traditional ethnopharmacological knowledge with the synergy of phytochemicals and EVs reveals a new dimension of their biological activity. Our findings show that this agricultural residue can be efficiently repurposed, offering a scalable circular economy model with environmental, economic, and health benefits.

CRedit authorship contribution statement

Alice Panti: Data curation, Formal analysis, Investigation, Writing – original draft. **Fabio Fusi:** Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Writing – original draft. **Francesca De Rosa:** Data curation, Formal analysis, Investigation. **Eduardo Maria Sommella:** Data curation, Investigation. **Gabriele Carullo:** Data curation, Formal analysis, Investigation, Writing – review & editing.

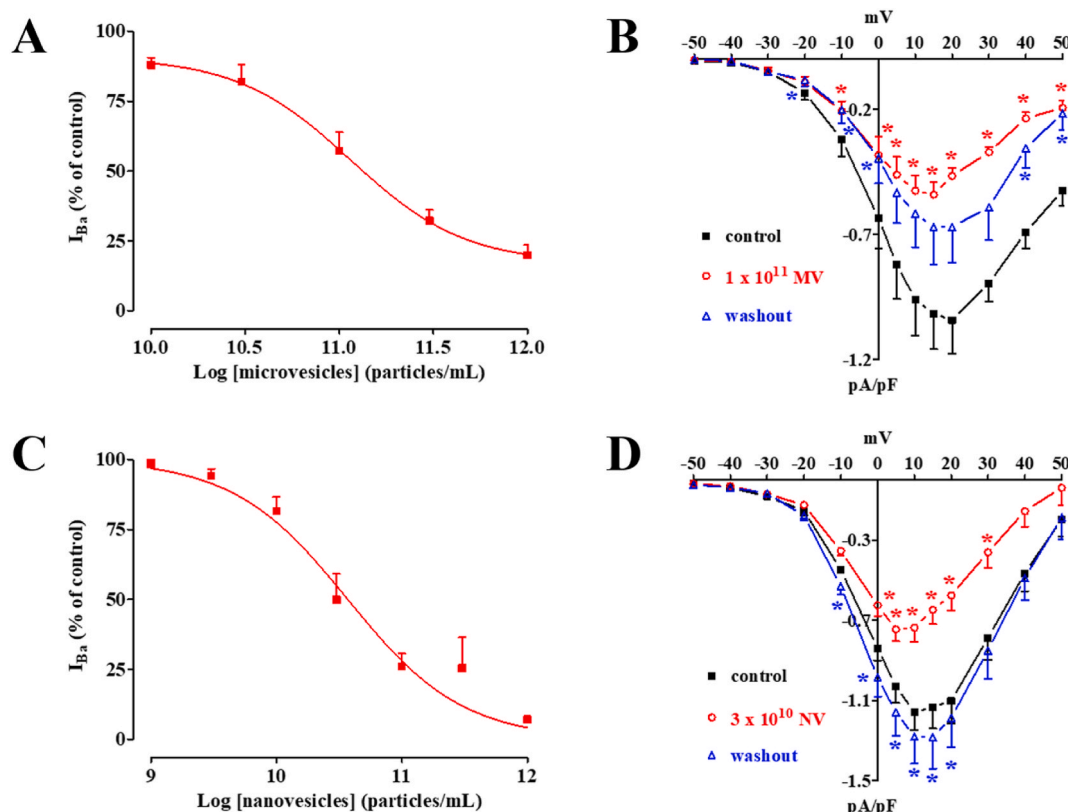


Fig. 7. Effect of microvesicles and nanovesicles on I_{Ba} of caudal artery myocytes. I_{Ba} was elicited with 250 ms depolarising steps from a V_h of -50 mV every 15 s. (A,C) Concentration-dependent effect of (A) microvesicles and (C) nanovesicles. On the ordinate scale, current amplitude is reported as a percentage of the control. The curve shows the best fit of the points. Data points are the mean $\pm$ S.E.M. ($n = 3-7$). (B,D) Current-voltage relationships, recorded from a V_h of -50 mV, constructed before the addition (control), in the presence of (B) 1×10^{11} *Sagratino* leaf microvesicles (MV) or (D) 3×10^{10} *Sagratino* leaf nanovesicles (NV), and after vesicle washout. Data points are the mean $\pm$ S.E.M. ($n = 6-8$). * $p < 0.05$ vs. control, repeated measures ANOVA and Dunnett post-test.

Elisabetta Francesca Strangis: Investigation. **Francesca Aiello:** Data curation, Formal analysis, Methodology. **Antonella Aloï:** Formal analysis, Investigation. **Mariangela Gentile:** Formal analysis, Investigation. **Giuseppe Campiani:** Writing – review & editing. **Francesco Puoci:** Conceptualization. **Mariantonia Logozzi:** Methodology, Supervision. **Massimo Valoti:** Conceptualization, Resources. **Simona Saponara:** Conceptualization, Data curation, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2026.122033>.

Data availability

Data will be made available on request.

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