Research article

CD39/NTPDase-1 EXPRESSION AND ACTIVITY IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS ARE DIFFERENTIALLY REGULATED BY LEAF EXTRACTS FROM *Rubus caesius* AND *Rubus idaeus*

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Abstract: Many experimental studies have demonstrated the favorable biological activities of plants belonging to the genus *Rubus*, but little is known of the role of *Rubus* leaf extracts in the modulation of the surface membrane expression and activity of endothelial apyrase. The aim of this study was to assess the influence of 1–15 µg/ml *Rubus* extracts on CD39 expression and enzymatic activity, and on the activation (ICAM-1 expression) and viability of human umbilical vein endothelial cells (HUVEC). The polyphenolic contents

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and antioxidative capacities of extracts from dewberry (*R. caesius* L.) and raspberry (*R. idaeus* L.) leaves were also investigated. The techniques applied were flow cytometry (endothelial surface membrane expression of ICAM-1 and CD39), malachite green assay (CD39 activity), HPLC-DAD (quantitative analysis of polyphenolic extract), ABTS, DPPH and FRAP spectrometric assays (antioxidant capacity), and the MTT test (cell viability). Significantly increased CD39 expressions and significantly decreased ATPDase activities were found in the cells treated with 15 μg/ml of either extract compared to the results for the controls. Neither of the extracts affected cell proliferation, but both significantly augmented endothelial cell ICAM-1 expression. The overall antioxidant capacities of the examined extracts remained relatively high and corresponded well to the determined total polyphenol contents. Overall, the results indicate that under in vitro conditions dewberry and raspberry leaf extracts have unfavorable impact on endothelial cells.

INTRODUCTION

The proper functioning of platelets, leukocytes, and endothelial cells (EC) is crucial for maintaining hemostasis [1]. Endothelial cells employ three biochemical systems to control platelet reactivity and regulate blood fluidity. Two of these produce the autacoids nitric oxide (NO) and prostacyclin upon interaction with agonists, while the third is based on CD39, an integral component of the endothelial cell surface. CD39 exerts Ca^{2+}/Mg^{2+}-dependent diphosphohydrolase (ATPDase, apyrase) activity [2, 3]. ATPDase regulates the concentrations of extracellular tri- and diphosphate nucleosides, which appear in the blood as a consequence of blood cell lysis, tissue damage, and the secretory response of blood platelets. The product of this phosphate removal reaction is AMP, which is subsequently hydrolyzed to adenosine via another antigen expressed on endothelial cells, CD73, which shows ecto-5'-nucleotidase activity. Increased adenosine concentrations downregulate platelet aggregation and endothelial cell activation, and suppress the production of prostaglandins and NO [4–6]. CD39 loses its enzymatic properties after endothelial activation with TNF-α, but its expression on the cell surface remains unaltered [7]. Endothelial activation may lead to increased expression of such typical cellular adhesion molecules as ICAM-1 (CD54), VCAM-1 and E-selectin, so these antigens are frequently monitored as indicators of the influence of polyphenolic compounds on endothelial cells [8, 9]. Polyphenols are well known for their ability to improve endothelial functions, mainly by modulating NO metabolism and suppressing the expression of adhesion molecules. Nevertheless, very little is known about the role of polyphenols in the regulation of endothelial apyrase function [8, 10].

The cardioprotective action of *Rubus* L. is mainly described with regard to its fruit extracts: *R. ideaus* L. fruit extract has been shown to exert anticoagulant and fibrinolytic activity in vitro [11]. Raspberry juice consumption is known to diminish oxidative stress and regulate plasma triglyceride and cholesterol levels in hypercholesterolemic golden Syrian hamsters [12]. However, the data concerning the cardioprotective action of polyphenolic compounds extracted from *Rubus* L. leaves requires further study.

Leaves of various *Rubus* plants have a long history of widely application in folk medicine. For decades, *R. suavissimus* S. Lee leaves have been used in China to lower blood pressure, treat diabetes, and improve kidney function [13]. Leaf tea brewed from *R. idaeus* L. is recommended in traditional medicine as a remedy for upper airway inflammation and fevers, and as a facilitator of child birth [14]. Experimental data indicates that polyphenolic extracts obtained from the leaves of *Rubus* spp. possess anticancer, antioxidant, antimicrobial, and relaxant properties [14–16]. Methanol extracts from *R. idaeus* L. leaves exert strong relaxant effects in cells from guinea pig ileum under in vitro conditions [14]. Furthermore, these extracts demonstrated cytotoxic effects when incubated with human laryngeal carcinoma and colon adenocarcinoma culture cells [15]. A leaf extract from *R. ulmifolius* S. also demonstrated antioxidant properties and antimicrobial activity against *Helicobacter pylori* strains [16].

The aim of this study was to assess the influence of 1–15 μg/ml *Rubus* extracts on CD39 expression and enzymatic activity. We also assessed its influence on the activation of human umbilical vein endothelial cells (HUVEC), measured in terms of ICAM-1 expression, and the effect on the viability of HUVEC. In order to better describe the profile and characteristics of the polyphenolic extracts used, we also monitored the antioxidant activity and cytotoxicity of the examined extracts with respect to endothelial cells.

Our outcomes demonstrate that the two extracts do not affect HUVEC proliferation or viability, and that they upregulate CD39 expression when applied at the concentrations of 1–15 μg/ml. However, they do activate endothelial cells and diminish ATPDase activity at higher concentrations. Therefore, even though raspberry and dewberry leaves seem to be promising sources of polyphenols with antioxidative properties, their inhibitory effects on apyrase activity and their ability to suppress the triggering of cell activation clearly indicate that an overall adverse influence on HUVEC.

MATERIALS AND METHODS

Chemicals

HPLC standards (chlorogenic, gallic, elagic, caffeic, *p*-coumaric, sinapic and ferulic acids, and (+)catechin, quercetin and methyl gallate), L-ascorbic acid, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), potassium persulfate, 2,2'-azinobis(3-ethylbenzo-thiazoline-6 sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri-2-pyridyl-*s*-triazine (TPTZ),

ethylenediaminetetraacetic acid (EDTA), vanillin, formic acid, polyvinylpolypyrrolidone (PVPP), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), N,N-dimethylformamide (DMF), bovine serum albumin (BSA), adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'-diphosphate sodium salt (ADP), trizma base, tetramisole hydrohloride, calcium chloride ($CaCl_2$), bicinchoninic acid kit for protein determination, propidium iodide, and human tumor necrosis factor alpha (TNFα) were purchased from Sigma-Aldrich. Sodium azide was obtained from POCH. Human umbilical vein endothelial cells (HUVEC), CM 200 medium, LSGS kit, trypsin with EDTA, and trypsin neutralizer were purchased from GIBCO. Plastic disposable 6-well dishes and 75-cm^2 flasks were purchased from TPP. Plastic 96-well dishes were obtained from NUNC. Anti-human mAb CD39/R-PE, mouse IgG1/R-PE isotype control, anti-human CD54/FITC, and mouse IgG1/FITC were obtained from Ancell. HPLC-grade acetonitryle was from J.T. Baker. The Malachite Green Phosphate Assay Kit was from BioAssay Systems. The water used for solution preparation and glassware washing was passed through a Thermolyne Barnstead Easy Pure UF water purification unit.

Plant materials
Leaves of raspberry (*R. idaeus* L.) and dewberry (*R. caesius* L.) were collected in September 2009 in the central region of Poland (Lodz). Harvested leaves were air-dried at 60ºC for 4 h in a heating cupboard, then ground to a fine powder and stored in glass bottles for further phenolic extraction.

Preparation of phenolic extracts
Leaf powder (10 g) was extracted with 100 ml of 70% aqueous acetone for 30 min at room temperature with continuous stirring, and centrifuged. The pellet was re-extracted twice in 100 ml of extraction solvent for 15 min. The combined extracts were evaporated under vacuum at ≤ 40ºC in a Rotavapor RII (Büchi) and then diluted with water to 50 ml. Crude extracts were extracted 3 times (dewberry leaves) or 4 times (raspberry leaves) with 50 ml of trichloromethane to remove lipid compounds. The concentrated water phases were lyophilized using Alpha 1-2 LD Plus. The respective yields for the dewberry and raspberry leaf extracts were 1.68 g and 1.93 g. These were stored at 4ºC for use in subsequent experiments. For chemical analysis, the dry extracts were diluted to 10 mg/ml with 10% DMSO.

Spectrophotometric analysis of phenolic compounds
The total phenolic content was determined using Folin-Ciocalteu reagent according to the procedure described by Bordonaba and Terry, and the results were expressed as mg gallic acid equivalents per g of extract (mg GAE/g) [17]. The total flavan-3-ol content was estimated using the vanillin assay, and the results were expressed as mg catechin equivalents per g of extract (mg CE/g) [18]. After acid depolymerisation, the proanthocyanidins were determined to their corresponding anthocyanidins as described by Rösch et al. [19]. The

proanthocyanidin content was calculated using the molar extinction coefficient of cyanidin (ε = 17360 L $\cdot$ mol $^{-1}$ $\cdot$ cm^{-1} and molar mass = 287 g $\cdot$ mol^{-1}) and was expressed as mg of cyanidin equivalents (CYE) per g of extract.

Analysis of phenolic compounds using HPLC-DAD
Hydrolyzable tannin content was estimated via HPLC after acid hydrolysis of gallotannins into methyl gallate and ellagitannins into ellagic acid according to Hartzfeld et al., with some modifications [20]. Briefly, 5–10 mg samples of dry extract were weighed into 25 ml Pyrex screw top tubes with Teflon cap liners, and 4 ml of methanol was added, followed by 0.4 ml of concentrated sulfuric acid. The samples were placed in a heating block that had previously been heated to 85ºC. They were allowed to react for 20 h. After cooling, 0.4 ml of ethanolamine (commercial preparation, 100% ethanolamine) was added to the mixture and the volume was adjusted to 10 ml with distilled water. Prior to HPLC analysis, the samples were centrifuged for 3 min (13,000 rpm/min) and filtered through a 0.45-µm syringe filter Minisart RC4 (Sartorius). Methyl gallate (detection at 280 nm) and ellagic acid (detection at 254 nm) were analyzed directly via HPLC, and respectively quantified as methyl gallate and ellagic acid equivalents. The phenolic profiles and the methyl gallate and ellagic acid contents were determined using an analytical reversed-phase HPLC system (Waters) with a 2707 autosampler and 1525 binary HPLC pump coupled to a 996 photodiode array detector (2998), controlled by Waters Breeze 2 software. Separation was performed on a SYMMETRY C18 (250 mm x 4.6 mm, 5 µm) column according to Dyrby et al. [21]. The binary mobile phase consisted of water and formic acid in a 90:10 (v/v) ratio for solvent A, and water, acetonitrile and formic acid in a 49:50:10 (v/v/v) ratio for solvent B. The separation of phenolic compounds was performed using the following gradient program with a flow rate of 1 ml/min: 0 min, 88% A + 12% B; 26 min, 70% A + 30% B; 40–48 min, 0% A + 100% B; 48–50 min, 88% A + 12% B. Detection was performed by scanning from 200 to 550 nm. The detector was set at 280 nm for hydroxybenzoic acid derivatives and flavanols, 320 nm for hydroxycinnamic acid derivatives and 360 nm for flavonols. Peak identification involved comparing retention times and diode array spectral characteristics with standards.
After removing tannins from the extracts using insoluble PVPP [22], the remaining non-tannin phenolics were identified by analytical reversed-phase HPLC as for the phenolic profiles above. The phenolic acids present in the extracts were fractionated into free and bound forms according to Zadernowski et al. and Nardini et al. with some modifications, and then identified using HPLC as described above [23, 24]. A 100 mg amount of dry extract dissolved in 5 ml of water and acidified to pH 2 using 1 M HCl was extracted five times with 10 ml diethyl ether for 2 min at room temperature. The diethyl ether extract of free phenolic acids was combined with anhydrous sodium sulfate and evaporated to dryness under vacuum at $\leq$ 40ºC. The water phase was mixed with 0.5 ml EDTA (446.7 mg/5 ml of water), 0.5 ml ascorbic acid (1.21 g/5 ml of water) and

6 ml of 8 M NaOH stored under nitrogen for 4 h at room temperature. The reaction mixture was then acidified with 6 M HCl to pH 2 and extracted with diethyl ether as described above. The ether extracts of phenolic acids comprised phenolic acids released from ester bonds. Following this, the water phase was heated with 4 ml 12 M HCl for 30 min at 95ºC, cooled to room temperature, and extracted with diethyl ether as described above. The ether extracts of phenolic acids were found to comprise phenolic acids released from glycosidic bonds. Each phenolic acid fraction residue was dissolved in 1 ml of methanol and analyzed directly using HPLC.

Determination of antioxidant capacity
The dewberry and raspberry leaf extracts were assessed for antioxidant capacity using the following assays:
(i) 2,2'-azino-bis(3-ethylbenzo-thiazoline-6 sulfonic acid) radical scavenging activity (ABTS);
(ii) 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH); and
(iii) ferric reducing antioxidant power (FRAP) assay [21, 25–27].
Trolox was used as a standard for all three methods and the antioxidant capacity was expressed as mmoles of Trolox equivalents (TE) per 1 g of extracts.

Maintaining HUVEC culture
HUVEC were grown in culture medium (CM200) supplemented with fetal bovine serum (2%), hydrocortisone (1 μg/ml), human epidermal growth factor (10 ng/ml), basic fibroblast growth factor (3 ng/ml) and heparin (10 μg/ml) at 37ºC in a humidified 95% air/5%CO_2 incubator. When the cultures reached confluence, the cells were detached using an EDTA–trypsin solution and collected by centrifugation (6 min, 100 x g). The cells were then re-suspended in fresh medium and seeded onto sterile plates. HUVEC used in all of the experiments were between passages 3 and 6. In all of the experiments, DMSO was applied to control samples to make sure that the same concentration of the solvent occurred in the control and examined samples (to which we added the DMSO solutions of polyphenol that had been used to treat the cells). Depending on the extract concentration used in the experiments, the final DMSO concentration in the medium was within the range 0.001–0.02%.

Cell proliferation
Cell proliferation was measured using an MTT assay [28]. Briefly, endothelial cells were cultured in a 96-well dish at a density of 1 x 10^4 cells/well for 24 h. To evaluate the proliferative effect of extracts, the cells were preincubated with increasing concentrations of dewberry and raspberry extracts (0.1–100 μg/ml) for 24 h. The cells were then washed and fresh medium containing 0.5 mg/ml MTT was added to each well. After 2 h incubation at 37ºC, 100 μl 20% SDS in 25% DMF was added to release the formed formazan crystals from the cells. The absorbance was measured at 560 nm against a reagent blank.

Apoptosis detection
HUVEC were cultured at a density of 5 x 10^4 cells/well in a 6-well dish to confluence for 48 h and then treated with various concentrations of extracts (1–15 µg/ml). Following 24 h incubation with the dewberry or raspberry extract, the cells were detached from the plate, centrifuged (6 min, 100 x g) and stained for 15 min with Annexin V FITC and propidium iodide (PI) in binding buffer (0.01 M HEPES, 0.14 M NaCl and 2.5 mM $CaCl_2$, pH 7.4). After staining, the cells were analyzed using a Becton Dickinson FACS CANTO Cytometer. The annexin V-positive cells were identified as early apoptotic, whereas the annexin V-positive and PI-positive cells were identified as late apoptotic.

Analysis of the ATPDase Activity of CD39
The NTPDase activity of CD39/NTPDase-1 in the presence of the polyphenolic extracts was determined by measuring inorganic phosphate release from ATP, as previously described [29]. The solution preparation, enzyme reactions and standard curve were done with disposable sterile phosphate-free plastic labware. Cells (5 x 10^4 cells/ well) were treated with different concentrations of extracts (1–15 µg/ml) for 24 h. Cells treated with 10 nM TNFα for 2 h before measurement were used as a negative control.
NTPDase activity was determined in cells washed twice with phosphate-free Tris buffer, pH 7.5. The enzymatic reaction was assayed in 1 ml of phosphate-free Tris buffer containing 5 mM $CaCl_2$ along with 5 mM tetramisole to eliminate the effect of alkaline phosphatase. ATP was added to each well to a final concentration of 3 mM and incubated with the cells for 15 min at 37ºC. The reaction was stopped by transferring 80 µl of reaction buffer to a 96-well plate and subsequently adding 20 µl of Malachite Green Reagent. The calibration curve and Malachite Green Reagent were prepared according to the instructions of the Malachite Green Kit manufacturer. The absorbance was read at 640 nm.
After incubation with ATP, the reaction buffer was discarded and the cells were lysed with 1 M NaOH, then a tenfold dilution was prepared. The protein concentration was assayed according to the BCA procedure [30], using bovine serum albumin (BSA) as a standard. The results were presented as the concentration of Pi (nmol), released in the course of ATP and ADP hydrolysis, expressed per 1 mg of protein.

Analysis of CD39/ATPDase and ICAM-1 expression
HUVEC were cultured at a density of 5 x 10^4 cells/ well in 6-well plates to confluence for 48 h, and then treated with various concentrations of extracts (1–15 µg/ml). Following a 24-h incubation with polyphenolic extract, the cells were detached from the plate, centrifuged for 6 min at 100 x g and stained with monoclonal antibodies specific to human antigens: CD39 R-PE or CD54 FITC (ICAM-1) diluted in a staining solution (PBS, 1% BSA, 0.01% sodium azide) for 30 min. Mouse IgG1 (R-PE or FITC) isotype was used as a negative control. After

staining, the cells were washed twice, suspended in staining buffer and subjected to FACS analysis using an LSR II Flow Cytometer (Becton Dickinson).

Statistical analysis

Means ± SE or median and interquartile ranges (IQR) from lower (LQ, 25%) to upper quartile (UQ, 75%) are given for all of the parameters. The Shapiro-Wilk test was used to verify whether the data were normally distributed. Levene's test was used to verify the homogeneity of variances. The significance of differences between samples and controls (untreated cells) was determined with ANOVA for repeated measures, followed by multiple comparison paired tests: either the paired Student t test or the Wilcoxon signed-rank test with Bonferroni's correction for multiple comparisons. The Pearson correlation coefficient was calculated to estimate the dependence between changes in the extract concentrations and the investigated parameters.

RESULTS

Polyphenolic content and the antioxidant activity of extracts obtained from dewberry and raspberry leaves

The phenolic compound profile of the dewberry and raspberry leaf extracts is presented in Tables 1 and 2. The tannins were the dominant phenolic compounds found in both extracts, respectively constituting 9.7% and 13.2% by weight in the raspberry and dewberry leaf extracts. The most common tannins were ellagitannins, which constituted over 80% of the total tannin content. After removing the tannins from the samples using PVPP, only 10% of total polyphenols remained in solution, the most common of which were found to be hydroxycinnamic acid derivatives. Unfortunately, flavonol compounds were also bound to the PVPP (Table 2).

Table 1. Characteristics of the dewberry and raspberry leaf extracts

	Dewberry	Raspberry
Polyphenol content (mg/g of dry extracts)		
Total polyphenols[a]	301.8 ± 10.8	269.2 ± 4.9
Total flavanols[b]	3.8 ± 0.2	8.8 ± 0.4
Total proanthocyanidins[c]	3.2 ± 0.2	6.7 ± 0.2
Ellagitannins[d]	117.6 ± 1.1	78 ± 0.5
Gallotannins[e]	11.5 ± 2.5	12.3 ± 1.3
Antioxidant capacity – TEAC (mmoles of Trolox/g of dry extracts)		
ABTS	3.5 ± 0.1	2.5 ± 0.1
DPPH	3.5 ± 0.1	2.3 ± 0.1
FRAP	2.6 ± 0.1	2.16 ± 0.04

Mean ± SE, n ≥ 3. [a]determined with Folin-Ciocalteu reagent as gallic acid equivalents. [b]determined with vanillin reagent as (+)catechin equivalents. [c]determined using acid hydrolysis as cyanidin equivalents. [d]determined using HPLC at 254 nm by acid hydrolysis as elagic acid. [e]determined using HPLC at 280 nm by acid hydrolysis as methyl gallate

Table 2. Phenolic profiles of the extracts determined via HPLC before and after PVPP-mediated tannin removal

	Contents (mg/g of dry extracts)			
	Dewberry		Raspberry	
	Before PVPP	After PVPP	Before PVPP	After PVPP
HBA and flavanols[a]	165.9 ± 0.6	3.3 ± 0.2	149.2 ± 0.7	3.9 ± 0.2
HCA[b]	27.7 ± 1.2	17.1 ± 0.3	22.1 ± 0.04	17 ± 0.2
Flavonols[c]	8.4 ± 0.03	0	34.8 ± 0.6	0
Total	202 ± 0.6	20.4 ± 0.4	206.1 ± 0.1	20.9 ± 0.2

Mean ± SE, n ≥ 3. [a]determined at 280 nm as gallic acid equivalents. [b]determined at 320 nm as chlorogenic acid equivalents. [c]determined at 360 nm as quercetin equivalents. HBA – hydroxybenzoic acids, HCA – hydroxycinnamic acids.

Table 3. Content of free and released phenolic acids in dewberry and raspberry leaf extracts

Phenolic acid	Content (mg/g of dry extracts)			
	Dewberry		Raspberry	
	Free phenolic acids	Released phenolic acids	Free phenolic acids	Released phenolic acids
Caffeic	n.d.	n.d.	0.64 ± 0.11	7.21 ± 0.51[1]
Chlorogenic	n.d.	n.d.	0.97 ± 0.17	n.d.
Ellagic	0.39 ± 0.07	0.72 ± 0.19[1] (3.06 ± 0.19)[2]	0.30 ± 0.06	0.45 ± 0.04[1] (4.65 ± 0.61)[2]
Ferulic	n.d.	0.44 ± 0.09[1]	0.10 ± 0.02	0.49 ± 0.04[1]
Gallic	n.d.	7.43 ± 0.92[1]	n.d.	0.50 ± 0.04[1]
p-coumaric	0.04 ± 0.01	0.53 ± 0.03[1]	0.07 ± 0.01	0.95 ± 0.06[1]
Sinapic	n.d.	0.10 ± 0.02[1]	0.08 ± 0.01	0.08 ± 0.01[1]

Mean ± SE, n ≥ 3. [1]Phenolic acids released from ester bonds; [2]content of ellagic acid released from glycosidic bonds; n.d. – not detected

A total of 5 phenolic acids were quantified in the dewberry leaf extracts and 7 in the raspberry leaf extracts. Most phenolic acids were found to be present in bound form, the main ones being gallic acid in dewberry extract (58.5% of the total phenolic acids) and caffeic acid in raspberry extract (47.6%; Table 3). Dewberry leaf extract was found to have 1.1 times the polyphenol content ($p < 0.05$), 1.5 times the elagitannin content ($p < 0.0001$), 1.1 times the hydroxbenzoic acid ($p < 0.001$) and 3 times the hydroxycinnamic acid content ($p < 0.01$) of raspberry leaf extract. Raspberry leaf extract was found to have 4 times more flavonols ($p < 0.0001$), and 2 times more gallotannins and proanthocyanidins than dewberry ($p < 0.0001$). The dewberry leaf extract had 1.2 times the antioxidant capacity of the raspberry leaf extract according to the FRAP method and 1.4 to 1.5 times according to the ABTS and DPPH methods ($p < 0.001$).

Effect of polyphenolic extracts on cell proliferation

As shown in Fig. 1, the treatment of human umbilical vein endothelial cells with 0.1–100 µg/ml dewberry leaf extract for 24 h resulted in a dose-dependent decrease in the number of cells, with a significant reduction in the viable cell number (30%; $p < 0.01$) at the highest extract concentration. Raspberry extract was found to be a stronger cell viability inhibitor, decreasing the number of living cells by up to 26% ($p < 0.05$) at a concentration of 25 µg/ml (Fig. 1).
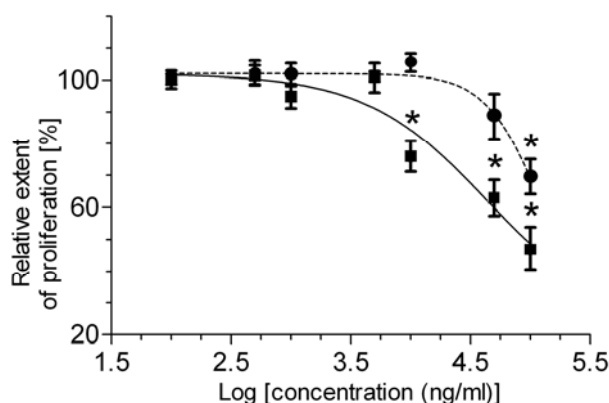

Fig. 1. The effects of leaf extracts on HUVEC proliferation. Data shown as means ± SE. Using the MTT assay, cell viability was assessed in HUVEC exposed to dewberry leaf extract (●) or raspberry leaf extract (■). The significance of differences was estimated with the paired Student t test with Bonferroni's correction for multiple comparisons. Dewberry extract (n = 10): $\mu_{Control} \neq \mu_{100\ \mu g/ml}$ ($p < 0.01$). Raspberry extract (n = 8): $\mu_{Control} \neq \mu_{25\ \mu g/ml}$ ($p = 0.01$); $\mu_{Control} \neq \mu_{50\ \mu g/ml}$ ($p = 0.05$); $\mu_{Control} \neq \mu_{100\ \mu g/ml}$ ($p = 0.001$).

Apoptosis detection

Preincubation of HUVEC for 24 h with dewberry or raspberry extracts at low concentrations (≤ 5 µg/ml) had a minor effect on the progression of apoptosis (Table 4). At higher extract concentrations (≥ 10 µg/ml), 24 h treatment of the cells with the extracts resulted in a 1.4- to 2-fold increase in the fraction of

Table 4. The effects of extracts on HUVEC viability

Extract concentration [µg/ml]	Raspberry		Dewberry	
	AV/PI-positive cells* [%]	AV-positive cells [%]	AV/PI-positive cells [%]	AV-positive cells [%]
0	22 ± 2.0	2.8 ± 0.3	20.0 ± 1.9	2.6 ± 0.3
1	20.5 ± 2.7	2.8 ± 0.6	22.1 ± 1	4.1 ± 1.0
2.5	21 ± 2.5	3.9 ± 0.9	22.6 ± 1.4	2.9 ± 0.6
5	23 ± 3.8	3.5 ± 0.8	24.8 ± 4.5	4 ± 1.3
10	26 ± 3.2	4.0 ± 0.7	30.9 ± 6.8	5.4 ± 0.8
15	31.4 ± 4.3	4.8 ± 0.8	38.9 ± 6.7	4.7 ± 0.9

Data shown as means ± SE, n = 6. *For the purposes of this study, AV/PI-positive cells were defined as the cells that were positive for both AV and PI staining.

annexin V-positive cells (early apoptotic cells) and a 1.1- to 1.9-fold increase in the fraction of annexin V/PI-positive cells (i.e. late apoptotic cells) as compared to the untreated cells. However, these changes in cell viability were not statistically significant (Table 4).

ATPDase activity of CD39
Raspberry extract decreased CD39 activity in a dose-dependent manner, but the changes were only significantly different from the controls at a concentration of 15 µg/ml: 48.56 ± 5.1 nmol P_i/mg protein for extract-treated cells, and 65.36 ± 6.3 nmol P_i/mg protein for untreated cells ($p < 0.05$; Fig. 2). Some downregulation of CD39 activity was observed after incubation with 10 µg/ml dewberry extract, but significant differences were only seen compared to the controls when 15 µg/ml extract was applied: 41.25 ± 6.5 nmol Pi/mg protein for extract-treated cells, and 53.43 ± 7.2 nmol Pi/mg protein for untreated cells ($p < 0.05$; Fig. 2).

To provide a negative control, the cells were exposed to 10 ng/ml of TNFα for 2 h, which resulted in significantly decreased CD39 activity: from 56.31 ± 5.3 nmol P_i/mg protein in the untreated cells to 43.77 ± 5.2 nmol P_i/mg protein in the TNFα-stimulated cells ($p < 0.01$).
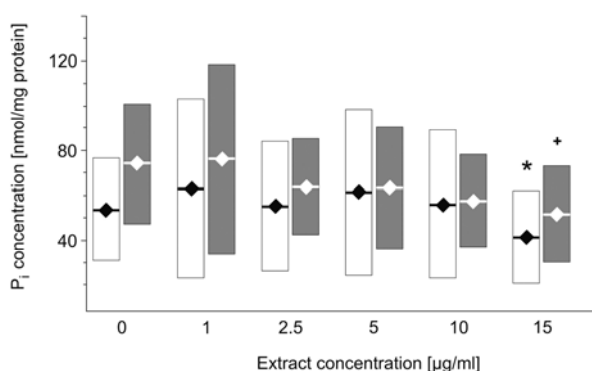

Fig. 2. The effects of leaf extracts on HUVEC CD39/ATPDase activity. Data shown as means + SD, n = 10. Using the Malachite Green Assay, CD39/ATPDase activity was assessed in HUVEC exposed to dewberry leaf extract (white box) or raspberry leaf extract (grey box). The significance of differences was estimated using the paired Student t test with Bonferroni's correction for multiple comparisons. Dewberry extract: $\mu_{Control} \neq \mu_{15 \,\mu g/ml}$ (*p < 0.05); raspberry extract: $\mu_{Control} \neq \mu_{15 \,\mu g/ml}$ ($^{+}$p = 0.01).

CD39/NTPDase expression
Preincubation of HUVEC with dewberry or raspberry extracts for 24 h was associated with an increase in the fraction of CD39-positive cells in a concentration-dependent manner. Incubation with raspberry extract significantly upregulated the expression of CD39 on HUVECs (by up to 21% at 5 µg/ml, p = 0.01; by up to 43% at 10 µg/ml, p < 0.01; and by up to 37% at 15 µg/ml, p < 0.05; Fig. 3). However, significantly increased CD39 expression

was seen at higher dewberry extract concentrations: by up to 19% for 10 µg/ml (p = 0.01) and 27% for 15 µg/ml (p < 0.0001; Fig. 3).
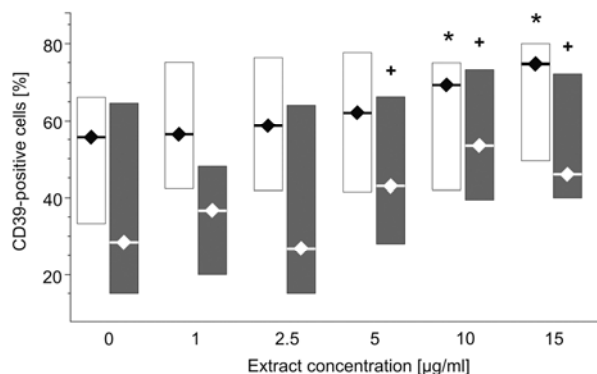

Fig. 3. The effects of leaf extracts on CD39 antigen expression on HUVEC. Data shown as median and IQR. Using flow cytometry, CD39 antigen expression was assessed for HUVEC exposed to dewberry leaf extract (white box) or raspberry leaf extract (grey box). The significance of differences was estimated using the paired Student t test or Wilcoxon matched-pairs signed rank test with Bonferroni's correction for multiple comparisons. Dewberry extract (n = 14): $\mu_{Control} \neq \mu_{10\ \mu g/ml}$ (*p = 0.001), $\mu_{Control} \neq \mu_{15\ \mu g/ml}$ (*p < 0.0001). Raspberry extract (n = 7): $\mu_{Control} \neq \mu_{5\ \mu g/ml}$ (⁺p = 0.01); $\mu_{Control} \neq \mu_{10\ \mu g/ml}$ (⁺p = 0.01); $\mu_{Control} \neq \mu_{10\ \mu g/ml}$ (⁺p = 0.01).

Analysis of ICAM-1-positive (CD54-positive) cells
To evaluate the influence of dewberry and raspberry leaf extracts on endothelial cell activation, the fraction of ICAM-1-positive (CD54-positive) cells was measured in non-stimulated and TNF-stimulated cell cultures after 24 h incubation. The fraction of ICAM-1-positive (CD54-positive) cells increased in a dose-dependent manner for both the raspberry and dewberry extracts at concentrations between 1 and 15 µg/ml. Significant changes in comparison to the control cells were observed at concentrations as low as 2.5 µg/ml, with the number of ICAM-1-positive (CD54-positive) increasing by 14% for raspberry (p = 0.01) and 11% (p < 0.05) for dewberry-treated cells. The maximum increase in the fraction of ICAM-1-positive (CD54-positive) cells in comparison to control cells was observed at the highest extract concentration: an 85% increase in raspberry extract-treated cells (p = 0.0001) and a 77% increase in dewberry extract-treated cells (p = 0.01; Fig. 4A).
After 1 h of incubation with 10 ng/ml TNFα, the percentage of ICAM-1-positive (CD54-positive) cells was elevated to 74% ± 8.55 in comparison to 35.5% ± 3.54 in non-stimulated cells (p < 0.01). Moreover, both extracts significantly potentiated the endothelial cell activation evoked by TNFα. Significant changes were seen in the TNF-stimulated cell cultures incubated with 5 µg/ml (by 19%; p < 0.01), 10 µg/ml (44.7%; p < 0.01) and 15 µg/ml (59%; p = 0.01) raspberry leaf extract, compared to controls. Dewberry leaf extract significantly increased TNF action at 10 µg/ml (31%; p < 0.01) and 15 µg/ml (57%; p < 0.01; Fig. 4B).
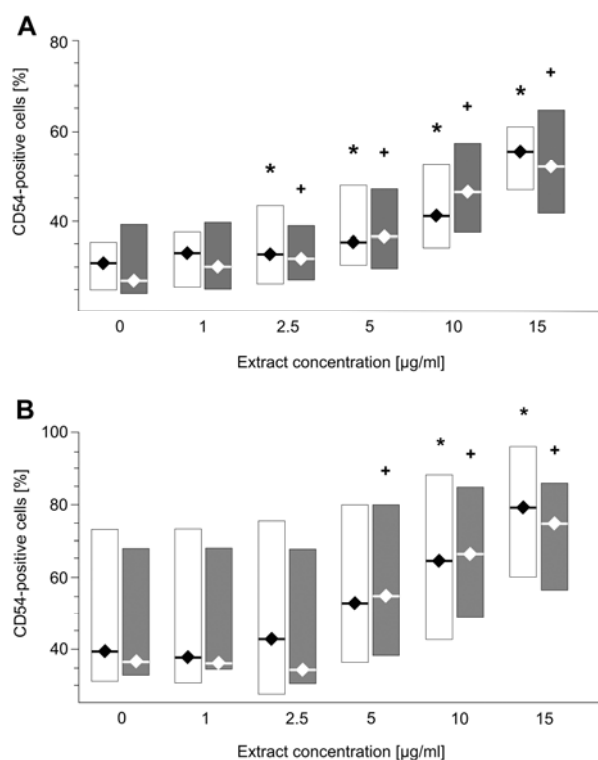
Fig. 4. The effects of leaf extracts on ICAM-1 (CD54) antigen expression in resting and TNF-α-activated HUVEC. Data shown as median and IQR. Using flow cytometry, ICAM-1 antigen expression was assessed for resting (A) or TNF-α-activated (B) HUVEC exposed to dewberry leaf extract (white box) or raspberry leaf extract (grey box). The significance of differences was estimated using Wilcoxon's matched-pairs signed rank test with Bonferroni's correction for multiple comparisons. A – Resting HUVEC: dewberry extract (n = 9): $\mu_{Control} \neq \mu_{2.5~\mu g/ml}$ (*p = 0.05); $\mu_{Control} \neq \mu_{5~\mu g/ml}$ (*p = 0.01); $\mu_{Control} \neq \mu_{10~\mu g/ml}$ (*p = 0.05); $\mu_{Control} \neq \mu_{10~\mu g/ml}$ (*p = 0.01). Raspberry extract (n = 10): $\mu_{Control} \neq \mu_{2.5~\mu g/ml}$ (+p = 0.01); $\mu_{Control} \neq \mu_{5~\mu g/ml}$ (+p = 0.01); $\mu_{Control} \neq \mu_{10~\mu g/ml}$ (+p = 0.001); $\mu_{Control} \neq \mu_{15~\mu g/ml}$ (+p = 0.0001). B – TNF-α-activated HUVEC: dewberry extract (n = 7): $\mu_{Control} \neq \mu_{10~\mu g/ml}$ (*p < 0.05); $\mu_{Control} \neq \mu_{15~\mu g/ml}$ (*p < 0.01). Raspberry extract (n = 7): $\mu_{Control} \neq \mu_{5~\mu g/ml}$ (+p = 0.05); $\mu_{Control} \neq \mu_{10~\mu g/ml}$ (+p = 0.01); $\mu_{Control} \neq \mu_{15~\mu g/ml}$ (+p = 0.001).

DISCUSSION

Endothelial dysfunctions contribute to the development and progression of cardiovascular diseases. Endothelial cells line the luminal surface of all blood vessels, and they play a key role in the regulation of vascular homeostasis by producing factors that act locally in the vessel wall and lumen. Epidemiological data indicates that consuming food and drinks rich in polyphenols reduces the incidence and severity of cardiovascular disorders [31, 32]. Some studies suggest that the cardioprotective influence of polyphenols is related to their

ability to prevent the oxidation of low-density lipoprotein and influence platelet dysfunction and smooth muscle cell migration and proliferation, while others report that it may be based on their ability to modulate endothelial function, i.e. to promote the anti-inflammatory response in endothelial cells [33–35].

This study shows the effects of extracts from raspberry and dewberry leaves on CD39/NTPDase expression and activity in human umbilical vein endothelial cells (HUVEC). It also assesses their influence on the activation (measured as ICAM-1 expression), proliferative properties, and viability of HUVEC, particularly in the context of their polyphenolic profile and characteristics, and the antioxidative capacity of the tested extracts. Our results indicate that even though the examined extracts are a source of polyphenols with antioxidative properties and do not affect HUVEC viability and apoptosis, they may significantly stimulate the ICAM-1 expression and markedly inhibit CD39 activity.

Generally, dewberry extract contains significantly more polyphenols than raspberry extract. However, the amount of polyphenolic compounds in each examined extract (302 ± 11 mg GAE/g of extract for dewberry and 269 ± 5 for raspberry) was considerably higher compared to the values detected in the ethanol extract of raspberry, as described by Venskutonis et al. (between 4.8 and 12.0 mg GAE/g of extract). The main phenolic acid in dewberry was gallic acid (ca. 60% of the total phenolic acids), whereas in raspberry, it was caffeic acid (up to 50%). However, although Oszmiański et al. concur that chlorogenic acid is present in raspberry extract, they report that ellagic acid is the main phenolic acid present the extract, and they have also identified *p*-coumaroyl-glucoside, feruloyltartaric acid and caffeoyltartaric acid in an ethanol extract from raspberry leaves [36]. It was also previously demonstrated that raspberry leaf extract is a source of kaempferol, quercetin glycosides and rutin [36–39]. The amount of polyphenols in our examined extracts was found to be proportional to their total antioxidant capacity, which was higher in dewberry than in raspberry leaf extract. Although the antioxidant capacity of dewberry leaf extract had not been described earlier, Serteser et al. evaluated the antioxidant activities, such as free radical scavenging, hydrogen peroxide scavenging and metal-chelating, of a 50% water-methanol extract of dewberry obtained from the whole plant [40]. However, the antioxidant activity of raspberry leaf extract was reported on earlier with the use of DPPH and ABTS assays by Venskutonis et al. [37].

In this study, we monitored the expression and enzymatic activity of CD39. This molecule improves blood fluidity by decomposing ATP and ADP, which leads to reduced platelet and neutrophil activation. This is the first report that dewberry and raspberry leaf extracts may upregulate CD39 expression in a dose-dependent manner. On the other hand, both extracts at their highest tested concentrations (15 μg/ml) decreased the enzymatic activity of CD39. The influence of polyphenolic compounds isolated from various plants on the expression and enzymatic activity of CD39 was described earlier. Kaneider et al. report that thrombin was found to downregulate human endothelial apyrase activity, which was then restored by incubation with resveratrol and quercetin [41].

Schmatz et al. also observed increased platelet ATPDase activity in streptozotocin-diabetic rats in vitro, after treatment with resveratrol, gallic and caffeic acid, but unlike Kaneider et al., they noticed that quercetin and rutin may diminish ATPDase activity [41, 42]. In line with our studies, Ashraf et al. note that methanol extract obtained from *Cymbopogan jawarancusa*, *Asparagus officinalis*, *Tribulus terrestris*, *Rubia cordizolla*, *Echimops echimatus* and *Portulaca oleracea* also exerted an inhibitory action on ATPDase obtained from chicken liver [43]. Furthermore, the inhibition of ectonucleotidase activity by antiplatelet drugs such as ticlopidine and clopidogrel has been also observed in endothelial cells [44].

The fact that preincubation of HUVEC with raspberry and dewberry leaf extracts both elevated the CD39 antigen level and decreased ATPDase activity may be a result of HUVEC activation. Both Robson et al. and our results show that HUVEC activation induced by TNFα may decrease ATPDase activity [45]. Recently, Garcia-Hernadez et al. reported increased expression of the CD39 antigen on immune cells obtained from patients with type 2 diabetes. As this expression was delayed and prolonged, it was considered a marker of chronically activated cells [46].

One of the markers of endothelial activation is ICAM-1, which is an adhesive molecule for monocytes, granulocytes and T lymphocytes, expressed in a basal state on the surface of quiescent endothelial cells. Inflammatory cytokines such as TNF-α, IL-1, INFγ and bacterial endotoxins enhance ICAM-1 expression, which reaches a maximum 24 h after stimulation and remains unchanged for the next 48 h [47]. The results of this study demonstrate that dewberry and raspberry leaf extracts may enhance ICAM-1 expression in non-stimulated and TNF-α-stimulated HUVEC. Most studies concerning the effect of polyphenols on HUVEC activation have shown that these compounds are able to suppress the expression of ICAM-1 when it has been elevated by inflammatory cytokines or pathological conditions [8, 48]. Interestingly, Mochizuki et al. have demonstrated that the influence of quercetin derivatives on enhanced ICAM-1 expression in TNF-α-stimulated HACEs may depend on their specific chemical structures. Quercetin and quercetin-3-sulfate suppressed ICAM-1 expression significantly, while quercetin-3-glucuronide showed no effect [49]. The ability of polyphenols to activate cells was shown by Kołodziej and Kiderlen, who suggest that tannins may be responsible for cell activation [50]. Our results may add support in this regard as dewberry and raspberry were found to be rich sources of tannins.

The next step was to study the effect of dewberry and raspberry extracts on the proliferation and apoptosis of human endothelial cells. Neither extract was toxic to endothelial cells when used at a concentration between 1–15 µg/ml. Several studies have reported on the effects of extracts isolated from *Rubus* spp. on cell proliferation and apoptosis. Although raspberry leaves are known to exert a cytotoxic effect on human laryngeal carcinoma (HEp2) and colon adenocarcinoma (SW 480) cell lines, SW 480 cells are more susceptible to the

extract [51]. The ethyl acetate fraction of *R. aleaefolius* Poir. root ethanol extract demonstrates significant hepatoprotective activity by decreasing the incidence of apoptosis [52]. On the other hand, total root alkaloids of *R. aleaefolius* Poir. were found to inhibit hepatocellular carcinoma growth both in vivo and in vitro [53]. A similar action was described for total saponins obtained from *R. parvifolius* L., where the extract inhibited proliferation of malignant melanoma cells in vivo and in vitro, and exerted antitumor activities through promoting tumor cell apoptosis [54].

Extracts from dewberry and raspberry leaves are a rich source of polyphenols, which made up almost 30% of the dry extracts, and both possess antioxidant capacity. Although the raspberry leaf extract was revealed as more abundant in flavonols and proanthocyanidins than the dewberry leaf extract, the latter seems to be a stronger antioxidative agent. This study is the first to demonstrate that despite the lack of anti-proliferative and pro-apoptotic effects, the dewberry and raspberry leaf extracts exhibit adverse effects on endothelial cell metabolism by overall cell activation and by decreasing ATPDase activity. Such contradictory results may indicate that the assessment of the biological activity of polyphenolic compounds should be the subject of further multiparametric analysis.

Conflict of interest. The authors confirm that there is no conflict of interest.

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