Anthocyanins and metabolites resolve TNF-α-mediated production of E-selectin and adhesion of monocytes to endothelial cells

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ABBREVIATIONS: ACN-RF, anthocyanin-rich fraction; Cy-3-glc, cyanidin-3-glucoside;Dp-3-glc, delphinidin-3-glucoside; GA, gallic acid; HUVEC, humbelical vein endothelial cells; Mv-3-glc, malvidin-3-glucoside; PrA, protocatechuic acid; SA, syringic acid; THP-1, human monocytic cells; TNF-α, tumor necrosis factor-alpha;VCAM-1, vascular cell adhesion molecule-1.

KEYWORDS: anthocyanins; metabolites; E-selectin; VCAM-1; cell culture; atherogenesis

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Abstract

This study investigated the capacity of an anthocyanin-rich fraction (ACN-RF) from blueberry, single anthocyanins (cyanidin, delphinidin and malvidin-3-glucoside; Cy, Dp and Mv-3-glc) and related metabolites (protocatechuic, gallic and syringic acid; PrA, GA and SA) to resolve an inflammation-driven adhesion of monocytes (THP-1) on endothelial cell (HUVECs) and secretion of cell adhesion molecules E-selectin and vascular cell adhesion molecule 1 (VCAM-1).

The adhesion of THP-1 to HUVECs was induced by tumour necrosis factor α (TNF-α, 100 ng mL^{-1}). Subsequently, ACN-RF, single ACNs and metabolites (from 0.01 to 10 µg mL^{-1}) were incubated for 24 h. The adhesion was measured in a fluorescence spectrophotometer. E-selectin and VCAM-1 were quantified by ELISA. No toxicological effects were observed for the compounds and the doses tested. ACN-RF and Mv-3-glc reducedTHP-1 adhesion at all the concentrations with the maximum effect at 10 µg/ml (-60.2% for ACNs and-33.9% for Mv-3-glc). Cy-3-glc decreased the adhesion by about 41.8% at 10 µg mL^{-1}, while PrA and GA reduced the adhesion of THP-1 to HUVECs both at 1 and at 10 µg mL^{-1} (-29.5% and -44.3% for PrA, respectively, and -18.0%and -59.3% for GA, respectively). At the same concentrations a significant reduction of E-selectin, but notVCAM-1 levels, was documented. No effect was observed following Dp-3-glc and SA supplementation. Overall, ACNs and metabolites seem to resolve, in a dose-dependent manner, the inflammation-driven adhesion of THP-1 to HUVECs by decreasing E-selectin concentrations. Interestingly, Mv-3-glc was active at physiologically relevant concentrations.

1. INTRODUCTION

Anthocyanins (ACNs) are a group of abundant and widely consumed flavonoids providing the red, blue, and violet colours in fruit- and vegetable-based food products. The dietary intake of ACNs is up to 9-fold higher than that of other dietary flavonoids. Epidemiological studies have found an inverse association between the consumption of ACNs and risk of cardiovascular diseases [1-6]. Their role in prevention of cardiovascular disease is strongly linked to the protection against oxidative stress and inflammation [7-10]. Atherosclerosis is the main underlying cause of cardiovascular disease in humans. The early stage, i.e. atherogenesis, is characterized by activation of endothelial cells to express cell adhesion molecules and recruit monocytes. This process is identical to the vascular responses to tissue inflammation, which resolves when the underlying cause of inflammation (e.g. an invading infectious agent) has been removed. However, the prolonged inflammatory milieu in early atherosclerotic foci stimulates the transformation of monocytes foam cell [11].

It has been shown that ACNs prevent endothelial cell dysfunction by modulating the expression and activity of several enzymes involved in nitric oxide production [12-13]. Furthermore, recent evidence suggests that ACNs can down-regulate the expression of adhesion molecules and prevent the adhesion of monocytes to endothelial cells challenged by pro-inflammatory cytokines [12;14]. The absorption of ACNs is low (<1%), but most of them are rapidly transformed by human gut to metabolic products, reaching a plasmatic concentration much higher than that of parental ACNs, indicating their contribution in the biological activity observed should be considered [15]. We have reported that ACNs and phenolic acid-rich fractions from a wild blueberry powder counteracted the adhesion of monocyte to endothelial cells in a pro-inflammatory milieu [16]. In the same study, single ACNs and certain gut metabolites (delphinidin-3-glc and gallic acid) prevented the attachment of monocytes to endothelial cells, while malvidin-3-glc and syringic acid exacerbated the adhesion process [16].

In the present study, we investigated the capacity of the same ACNs to resolve an inflammatory process by reducing the adhesion of monocytes to activated endothelial cells and the production of vascular adhesion molecules as potential mechanisms in the atherogenesis. To this end, monocytic

(THP-1) cells were cultured with human umbilical endothelial cells (HUVECs) in the presence of the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) to promote the expression of cell adhesion molecules and interaction between the cells. TNF-α is produced by immune cells and it stimulates endothelial cells to express adhesion molecules, including E-selectin, vascular cell adhesion molecule-1 (VCAM-1) as well as chemokines (i.e. interleukin-8 and monocyte chemoattractant protein-1) that promote the recruitment of monocytes to inflamed luminal endothelium and induce their adhesion to endothelial cells at the site of activation [17]. The expression of E-selectin occurs early following stimulation of pro-inflammatory cytokines such as TNF-α in endothelial cells (4 and 6 h after stimulation and remains elevated up to 24 h) [18]. E-selectin mediates the initial attachment of free-flowing leukocytes to the arterial wall, while the expression of VCAM-1 provides a stronger interaction between leukocytes and endothelial cells and mediates the transmigration of the cells into the tissue [18-19].Cytokine-induced expression, and subsequent down-regulation after cessation of exposure, in endothelial cells occurs later for VCAM-1 than E-selectin [20]. We assessed the production of E-selectin and VCAM-1 to cover this “early” and “late” phase of the endothelial production of cell adhesion proteins.

2. MATERIALS AND METHODS

2.1 Reagents

Standard of cyanidin, delphinidin and malvidin-3-glucoside (Cy, Dp and Mv-3-*O*-glc) were obtained from Polyphenols Laboratory (Sandes, Norway), while those of gallic, protocatechuic, and syringic acid (GA, PrA and SA) from Sigma-Aldrich (St. Louis, MO, USA). Human Endothelial Cells Basal Medium and Human Endothelial Cells Growth Supplement were purchased from Tebu-Bio (Magenta, MI, Italy). Hanks balanced salt solution, foetal bovine serum (FBS), TNF-α were from Sigma-Aldrich (St. Louis, MO, USA). Gentamin, RPMI-1640, HEPES, Sodium Pyruvate, trypsin-EDTA were from Life Technologies (Monza Brianza, MB, Italy)while the 5-Chloromethylfluorescein Diacetate (CellTrackerTM Green CMFDA) from Invitrogen (Carlsbad, CA, USA).Hydrochloric acid and methanol were purchased from Merck (Darmstadt, Germany), while water was obtained from a Milli-Q apparatus (Millipore, Milford, MA).

2.2 Preparation and characterization of the ACN-rich fraction, single anthocyanins and metabolites

The extraction of the ACN-rich fraction from a wild blueberry powder (Future Ceuticals, Momence, IL, USA)was performed as reported by Del Bo' et al. [16]. The fraction was characterized for the content of ACNs, phenolic acids as well as other bioactives as previously published [16]. The total ACN content was 45.11 ± 0.35 mg mL^{-1} and constituted predominantly of Mv-3-glc (about 26%), Mv-3-gal (15%) followed by Dp-3-glc (9%) and Petunidin-3-glc (8%). No phenolic acids or other bioactives were detectable.

Lyophilized standards of Mv, Cy, Dp-3-*O*-glc (native compounds) and SA, PrA and GA (corresponding metabolites) are shown in **Figure 1**. The standards were prepared as previously reported [16]. These single compounds were tested since found in the blood stream of volunteers after consumption of a blueberry portion [21].

2.3 Cell culture and viability

Human umbilical vein endothelial cells (HUVECs; Tebu-Bio SrL, Magenta, MI, Italy) were cultured in endothelial cell growth medium kit containing 2% serum at 37°C and 5% CO_2 until reaching confluence (generally after 1 week). THP-1 cells were grown in a complete RPMI cell media (RPMI-1640 medium supplemented with 1% HEPES, 1% sodium pyruvate, 0.1% gentamicin, and 10% FBS at 37 °C and 5% CO_2 and maintained in culture for up to 3 months.

Cell viability was performed for each compound and concentration by Trypan blue and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, showing cells viability above 90% as previously published [16].

2.4 THP-1 adhesion to HUVECs

An aliquot of $2x10^4$ HUVECs was seeded on 0.1% gelatine pre-coated 96-well black plate and maintained at 37°C and 5% CO_2 for 24h.Subsequently, monocytic ($2x10^6$) THP-1 cells (American

Type Culture Collection, Manassas, VA, USA) were re-suspended in 1 mL serum free RPMI cell medium (containing 1% HEPES, 1% sodium pyruvate, 0.1% gentamicin) and labelled with CellTrackerTM Green CMFDA (1 µM, 30 min at 37°C and 5% CO_2). THP-1 were washed twice, re-suspended in HUVEC medium ($2x10^5$ cells mL^{-1}density) and added to HUVECs with TNF-α (100 ng mL^{-1}). After 24 h incubation (37°C, 5% CO_2) medium was removed and 200 µL of new medium, containing the single ACNs (Mv, Cy and Dp-3-glucoside) and their corresponding metabolites (SA, PrA and GA, respectively) was added at the concentrations of 0.01, 0.1, 1 and 10µg mL^{-1} for 24 h at 37°C and 5% CO_2. Then, media was collected and stored at -80°C until analysis. Cells were rinsed twice before the measure of the fluorescence (excitation: 485 nm, emission: 538 nm; mod. F200 Infinite, TECAN Milan, Italy). The level of fluorescence is associated with the number of labeled-THP-1 cells attached to the HUVECs. The results derive from three independent experiments in which each concentration was tested in quintuplicate. Data are reported as fold increase compared to the control cells without stimulation with TNF-α or bioactive compounds.

2.5 Visualization at the microscope

The adhesion of THP-1 to HUVECs was visualized at the microscope. HUVEC ($4x10^4$/well) were seeded onto 0.1 % gelatin pre-coated 12-well plate for 24 h. THP-1 ($8x10^4$/well) were stained with CellTrackerTM Green CMFDA and added with TNF-α to HUVECs as previously reported. After treatment, cells were rinsed with Hank solution in order to remove the non adherent cells and inspected with an inverted wide-field microscope with 10 × magnifications.

2.6 Determination of soluble VCAM-1 and E-selectin concentration in cell supernatant

The concentrations of soluble VCAM-1 and E-selectin, in recovered cell culture supernatants, were quantified by ELISA kits according to the manufacture’s instruction. The analyses were conducted in quadruplicate and the results derived from three independent experiments.

2.7 Statistical analysis

One-way ANOVA was applied to verify the effect of the different concentrations of ACNs and metabolites on fold increase THP-1 adhesion to HUVECs and on percentage changes in soluble VCAM-1 and E-selectin concentration. Differences between treatments was assessed by the Least Significant Difference (LSD) test with $p<0.05$ as level of statistical significance. Results are reported as mean ± standard error of mean. The statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, USA).

3. RESULTS

3.1 Effect of ACN-rich fraction on monocytes adhesion process

In **Figure 2** are reported **t**he effects of ACN-RF on THP-1 adhesion to HUVECs. There was a significant increase in THP-1 cell adhesion to HUVECs following stimulation with TNF-α ($p<0.0001$), while the incubation with ACN-RF significant reduced the process ($p<0.0001$) at all the concentrations tested (from 0.01 to 10 μg mL^{-1}).The maximum effect of reduction was observed at 10 μg mL^{-1} (-60.2%) with respect to the control with TNF-α.

3.2Effect of anthocyanins and metabolic products on monocytes adhesion process

Figure 3 (A-C) shows the results on THP-1 adhesion to HUVECs after incubation with the single ACNs. The incubation with Mv-3-glc significantly decreased ($p<0.0001$) the adhesion of monocytes to HUVECs at all the concentrations tested (from 0.01 to 10 μg mL^{-1}) compared to TNF-α (**Fig. 3A**). The maximum reduction was observed for the concentration at 10 μg mL^{-1} (-33.9%; $p<0.0001$) as also reported in **Figure 4** that shows the adhesion of labelled THP-1 to endothelial cells following 24 h stimulation with TNF-α (A), TNF-α + 10 μg/mL Mv-3-glc (B) and control (C). Regarding Cy-3-glc, a significant reduction in the adhesion of THP-1 to HUVEC was observed only at 10 μg mL^{-1} (-41.8%; $p<0.01$) (**Fig. 3B**), while no significant effect was found for Dp-3-glc (**Fig. 3C**).

Figure 5 (A-C) reports the results on THP-1 adhesion to HUVECs after incubation with SA, PrA and GA (metabolites of Mv-3-glc, Cy-3-glc, and Dp-3-glc, respectively). No significant effect was observed following SA supplementation (**Fig. 5A**) in line with the results reported in **Fig. 4** that shows the adhesion of labelled THP-1 to endothelial cells following stimulation with TNF-α + 10 µg/mL SA (D). The supplementation with PrA (**Fig. 5B**) and GA (**Fig. 5C**) significantly decreased the adhesion of monocytes to endothelial cellsat 1 µg mL^{-1} (-18.0%; $p<0.05$ for GA, -29.5%; $p<0.05$ for PrA) and 10 µg mL^{-1} (-59.3%; $p<0.001$ for GA, -44.3%; $p<0.01$ for PrA) compared to TNF-α.

3.3 Effect of anthocyanins and metabolic products on soluble E-selectin and VCAM-1 levels in cell supernatant

Table 1 shows the levels of E-selectin quantified in the cell supernatant following incubation with ACNs and metabolites. There was a significant increase in E-selectin following stimulation with TNF-α compared to negative control (without TNF-α). The incubation of cells with Mv-3-glc significantly reduced ($p<0.001$) the levels of E-selectin at all concentrations tested. This reduction was not concentration dependent and the maximum effect was observed at 0.01 and 0.1 µg mL^{-1} (-66% and -67%, respectively). Cy-3-glc reduced the E-selectin concentration at 10µg mL^{-1} (-72%; $p<0.01$), PrA at 1 and 10 µg mL^{-1} (-74 and -76%; $p<0.001$, respectively), and GA at 1µg mL^{-1} (-34%; $p<0.01$) and 10 µg mL^{-1} (-40%; $p<0.01$). No effect was found after Dp-3-glc and SA incubation in line with the lack of the positive effect on the adhesion of THP-1 to HUVECs.

The levels of VCAM-1 quantified in the cell supernatant following incubation with ACNs and metabolites are reported in **Table 2**. There was a significant increase ($p<0.05$) following stimulation with TNF-α compared to negative control (without TNF-α). However, no significant effect was observed following incubation with ACNs and gut metabolites.

4. DISCUSSION

Chronic inflammation is a common factor in endothelial dysfunction and atherosclerosis [11;22]. Different cell models have been used to assess the interaction between endothelial cells and monocytic cell lines (e.g. THP-1, U937, MonoMAC) or freshly isolated leukocytes as early event in atherosclerosis. We obtained a two-fold increase in attachment of THP-1 cells to HUVECs which is in line with earlier observations with the same co-culture [23-24]). The TNF-induced attachment of monocytic U937 cells to endothelial cells seems to be in the range of a 2-3-fold increase [25-26], whereas MonoMAC cells may have higher sensitivity and response to TNF-mediated adhesion to HUVECs (i.e. 6-fold increase at 10 µg/mL TNF-α) [27] Poussin 2014).

In the last years, several studies have focused on the mechanisms through which polyphenols modulate the adhesion process and the vascular inflammation [28-29]. Here we evaluated the capacity of Mv, Cy, and Dp-3-glc, and corresponding metabolites, to resolve an inflammation-driven adhesion of THP-1 to HUVECs and the production of vascular adhesion molecules. The results obtained documented that ACN-RF and Mv-3-glc had an effect at all the concentrations tested, while Cy-3-glc, GA and PrA resolved the adhesion process only at the high concentrations (1 and 10 µg mL^{-1}).These findings are in contrast with those documented in a previous experiment, in which Mv-3-glc led to an exacerbation of the adhesion process, while Cy and PrA failed to affect the interaction between monocytes and endothelial cells [16]. In light of our results, we hypothesize that these compounds are more active in resolving than preventing the adhesion process. *In vitro* studies reported a beneficial effect on the prevention of atherogenesis only at supra-physiological concentrations in according with our findings [25-33]. However, recent *in vitro* studies showed a positive effect of ACNs, phenolic acids and gut metabolites also at physiological relevant concentrations [34-35]. For example, Kraga et al., [35] reported that Cy-3-glucoside, galattoside and arabinoside, as well as Dp and Peondin-3-glucoside and phenolic acids/gut metabolites (vanillic acid, ferulic acid, hippuric acid, 4-hydroxybenzaldehyde and PrA) decreased the adhesion of monocytes to HUVECs from 0.1 to 2 µM. The effect was also confirmed when ACNs and phenolic acids were used as a mix, suggesting an additive effect of the compounds.

In our experimental conditions, the reduction of adhesion of THP-1 to TNF-α-activated HUVECs after supplementation with ACNs and metabolites can be attributed to different non-specific and/or specific complex mechanisms of action. Further insight into the mechanisms can be gained by high content screening and transcriptomics of inflammatory and oxidative stress pathways as used in co-culture studies of monocytes and HUVECs [36]. Inhibition of NF-κB activity could have reduced the synthesis of numerous cytokines by decreasing the levels of inflammation at endothelial level. In this regard, the inhibition of pro-inflammatory cytokines such as TNF-α and the reduction of leukocyte adhesion to endothelial cells are key mechanisms in the control of atherogenesis and atherosclerosis. Moreover, ACNs have a pivotal role in the modulation of mitogen-activated protein kinase pathways implicated in several cellular processes including proliferation, differentiation, apoptosis, cell survival, cell motility, metabolism, stress response and inflammation [8]. Alternatively, the use of ACNs and phenolic acids may repress the secretion of chemokine (C-C motif) ligand 2 (MCP-1), which pilots the migration of monocytes toward the intracellular cleft between adjacent endothelial cells, or reduce the production of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin that regulate the recruitment of monocytes into atherosclerosis-prone area. In our experimental conditions, we found that the alleviating effects on cell adhesion, induced by the single compounds, were associated with changes in the levels of E-selectin, but not VCAM-1 levels. We found that Mv-3-glc was more effective in reducing the production of E-selectin compared to the other compounds tested. In fact, the decrease was observed both at low and high concentrations, while for Cy-3-glc, PrA and GA the effects were detected only at the high doses. The increased E-selectin production at high concentration may be due to a stimulation of the cells as also shown in a previous study where Mv-glc led to an exacerbation of the adhesion process [16]. Dp-3-glc and SA supplementation did not show any reduction in line with the lack of an effect on THP-1 adhesion to HUVECs. Conversely, different studies report changes in the expression/levels of VCAM-1, ICAM-1, other than E-selectin, following ACNs and metabolites supplementation; most of them showed a beneficial effect only at supra-physiological concentrations. For example, Ferrari et al., [38] demonstrated that Cy-3-glc (20 μM) counteracted the acute pro-inflammatory effects of TNF-α in HUVECs, reduced leukocyte recruitment from microcirculation, and decreased the gene expression levels of E-selectin and VCAM-1. Huang et al., [39] reported that the supplementation with different concentrations of Mv-3-glc (1-100µM) inhibited the TNF-α-induced inflammatory response in a concentration-dependent manner and reduced the production of MCP-1, ICAM-1 and VCAM-1 in endothelial cells. Nizamutdinova and colleagues [40] found that ACNs from black soybean seed coats (rich in Cy, Dp and Petunidin-3-glucoside) reduced TNF-α-mediated VCAM-1 induction in a concentration-dependent manner (10, 50, and 100 µg/mL), but not ICAM-1 in HUVEC. Amin et al., [41] showed that simulated human vascular endothelial cells with oxidized-LDL and co-treated with Cy-3-glc (0.1, 1, and 10 µM concentrations) significantly reduced VCAM-1 protein production. In addition, phenolic acids affected the expression and the levels of adhesion molecules. Warner et al., [42] tested the capacity of 20 different phenolic acids to reduce the secretion of VCAM-1 in activated TNF-α endothelial cells showing a significant effect for PrA in a concentration-dependent manner (1-100 µM). Similar results were also found following vanillic, isovanillic, ferulic, hyppuric acids and derivates supplementation [37;41-42].

5. CONCLUSIONS

In conclusion, this study documented the capacity of Mv-3-glc, Cy-3-glc, PrA and GA to reverse an atherogenic condition. This reduction can be explained by a significant decrease in the adhesion of monocytes to endothelial cells and in the production of E-selectin, but not VCAM-1 in the present short-term incubation period. Mv-3-glc seems the most potent anti-atherogenic compound since it actives both at supraphysiological and physiological concentrations.

6. ACKNOWLEGMENTS

Cristian Del Bo’ designed the study, performed the experiments and wrote the first draft of the manuscript. Mirko Marino performed the analysis and reviewed the manuscript. Peter Moller and Patrizia Riso critically revised the manuscript. Marisa Porrini supported the research, supervised the analysis and critically revised the manuscript.

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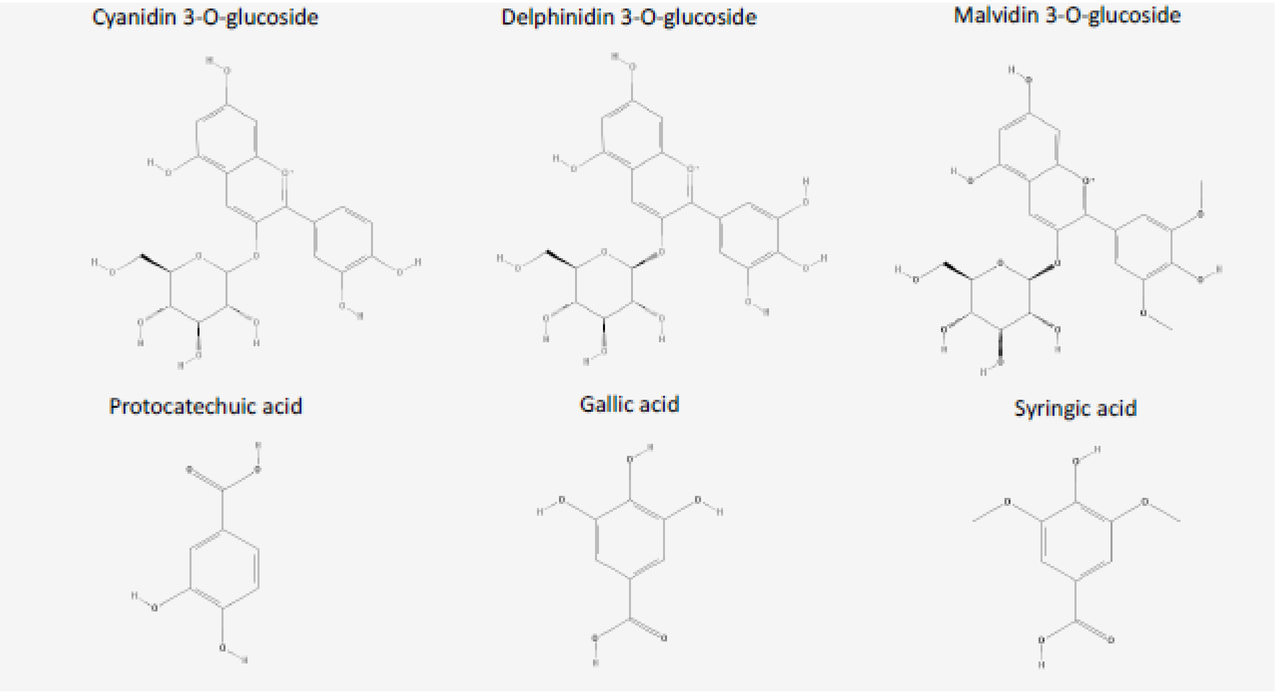
FIGURE CAPTION

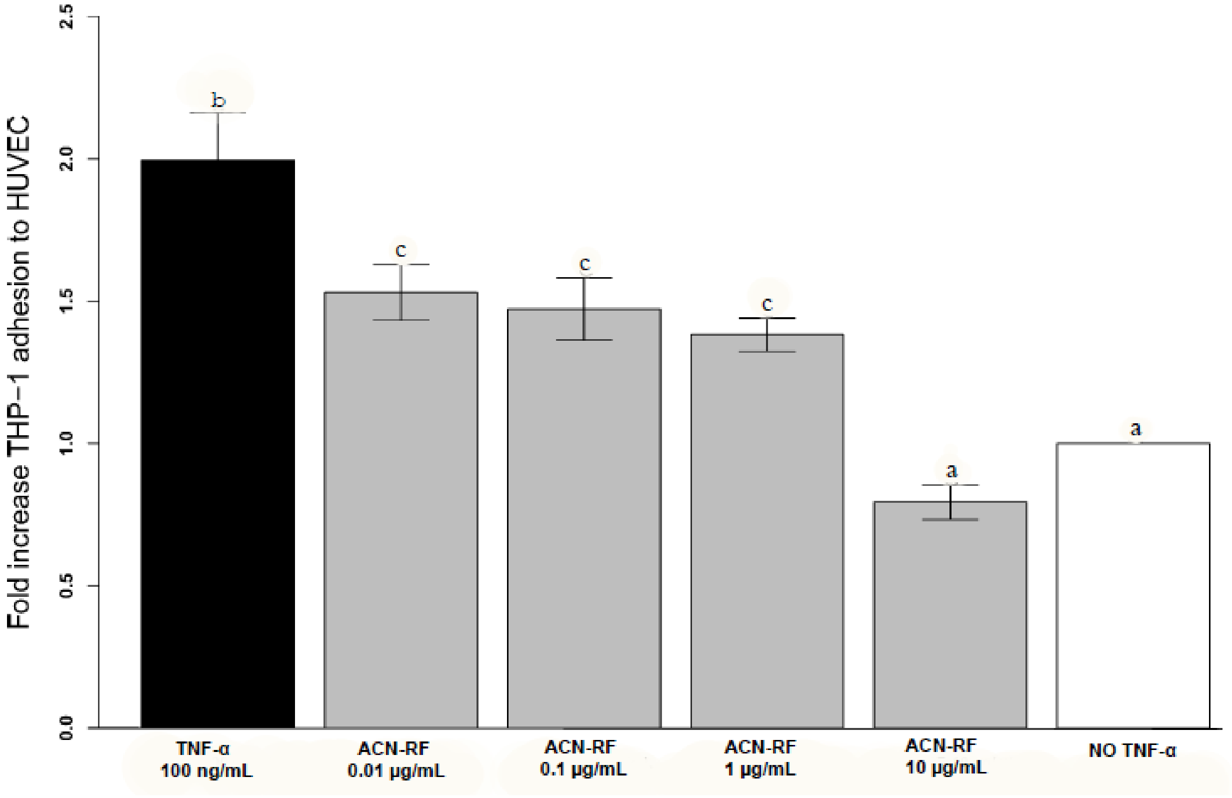
Figure 1- Chemical structure of anthocyanins and their metabolites used in this study



Legend: *Mv-3-glc*, malvidin-3-glucoside; *Cy-3-glc*, cyanidin-3-glucoside; *Dp-3-glc*, delphinidin-3-glc; *SA*, syringic acid; *PrA*, protocatechuic acid; *GA*, *g*allic acid;

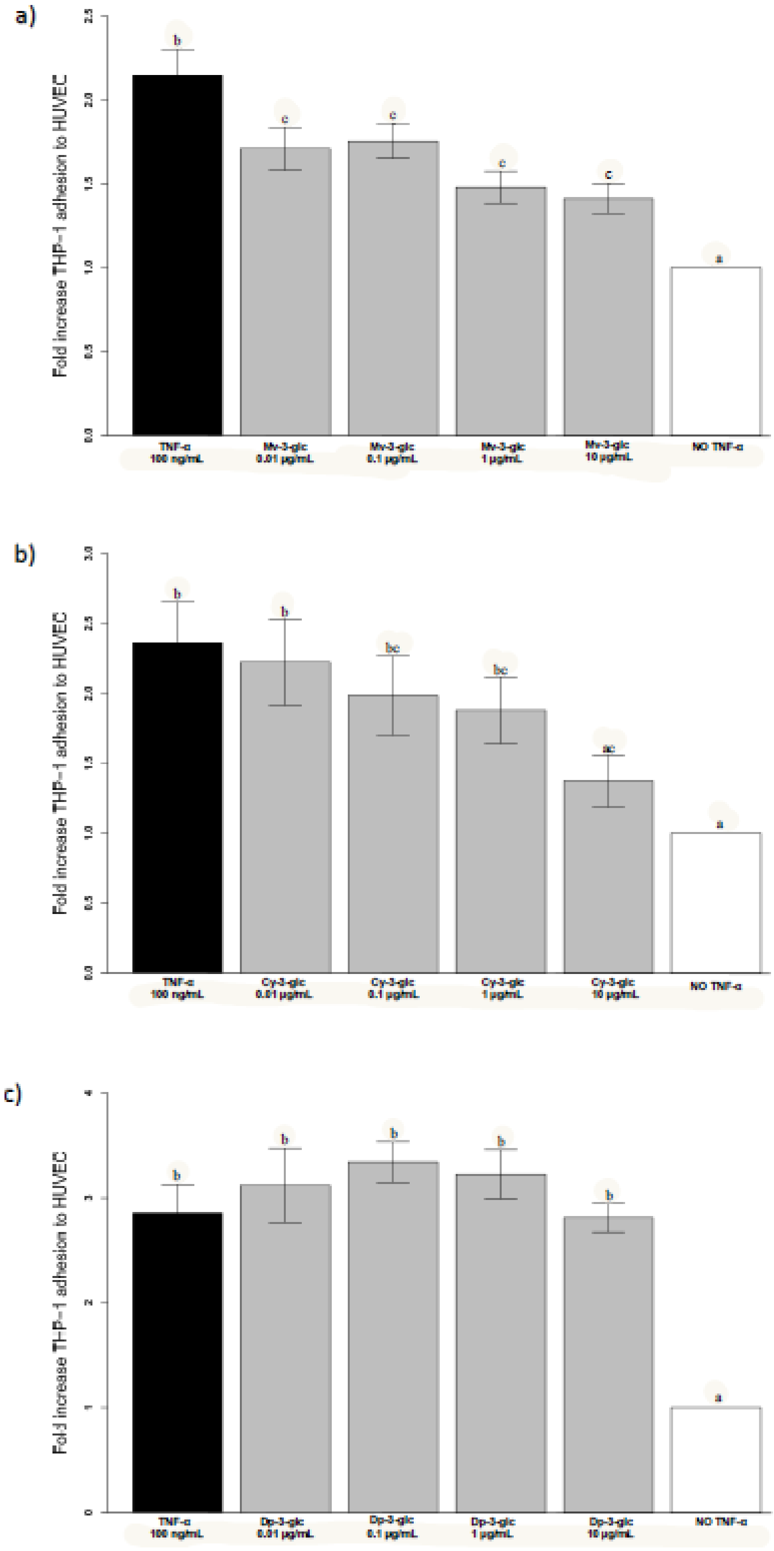
Figure 2- Effect of *ACN-RF* (0.02 and 18.9 µM, expressed as Mv-3-glc as the main compound) on THP-1 adhesion to HUVECs. Results are expressed as mean ± standard error of mean. [a,b,c]Bar graphs reporting different letters are significantly different ($p \leq 0.05$).

Figure 2



Legend: *TNF-α* tumor necrosis factor alpha, *ACN-RF* anthocyanin-rich fraction, *NO TNF-α* (control).

Figure 3- Effect of **A)** *Mv-3-glc* (0.02-18.9 µM), **B)** *Cy-3-glc* (0.03–25.9 µM) and **C)** *Dp-3-glc* (0.02–19.9 µM) on THP-1 adhesion to HUVECs. Results are expressed as mean ± standard error of mean. ^{a,b,c}Bar graphs reporting different letters are significantly different ($p \leq 0.05$).



Legend: *TNF-α,* tumor necrosis factor alpha; *Mv-3-glc,* malvidin-3-glucoside; *Cy-3-glc,* cyanidin-3-glucoside; *Dp-3-glc,* delphinidin-3-glc; *NO TNF-α* (control).

Figure 4 Visualization of THP-1 adhesion to HUVEC following 100 ng mL^{-1} of TNF-α (**a**), TNF-α +10 μg mL^{-1} of Mv-3-glc (**b**), TNF-α + 10 μg mL^{-1} of SA (**c**), and NO TNF-α (**d**).

Legend: *TNF-α,* tumor necrosis factor alpha; *Mv-3-glc,* malvidin-3-glucoside; *SA,* syringic acid; *NO TNF-α* (control). Round yellow cells represent THP-1 cells adhered to HUVECs. The black arrows indicate an example of adhered THP-1, while the red arrows indicate HUVECs.

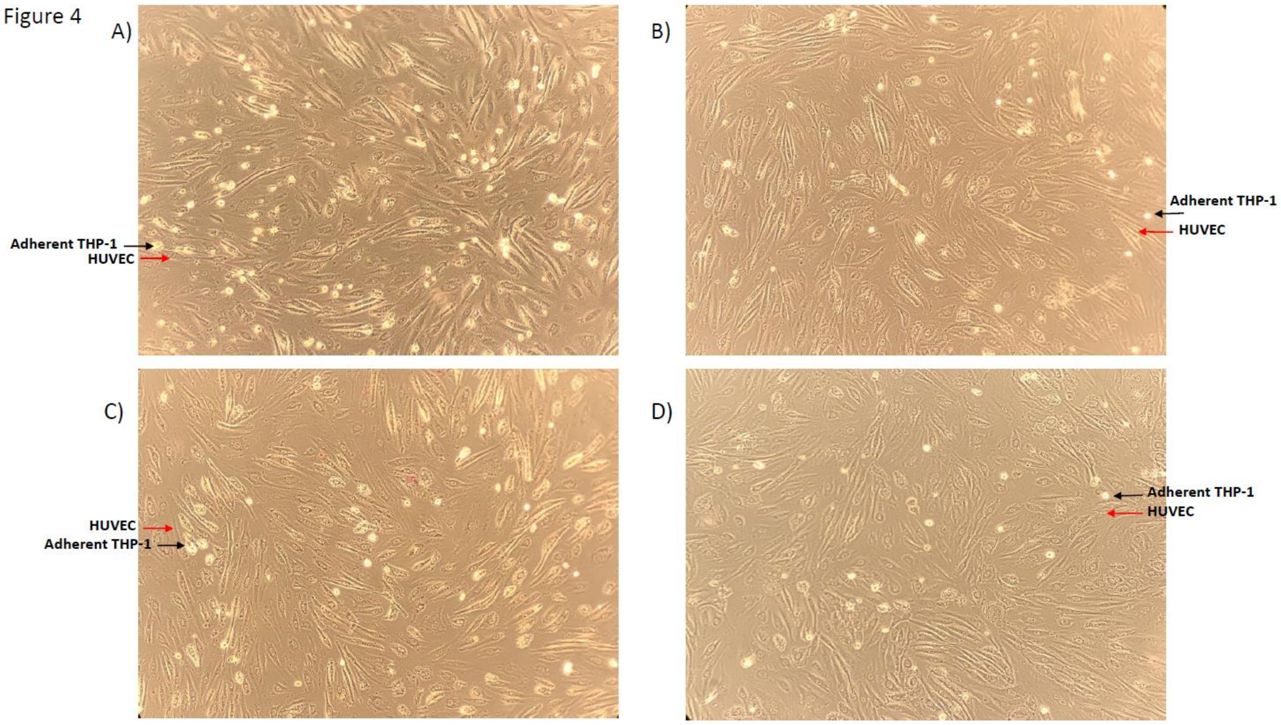
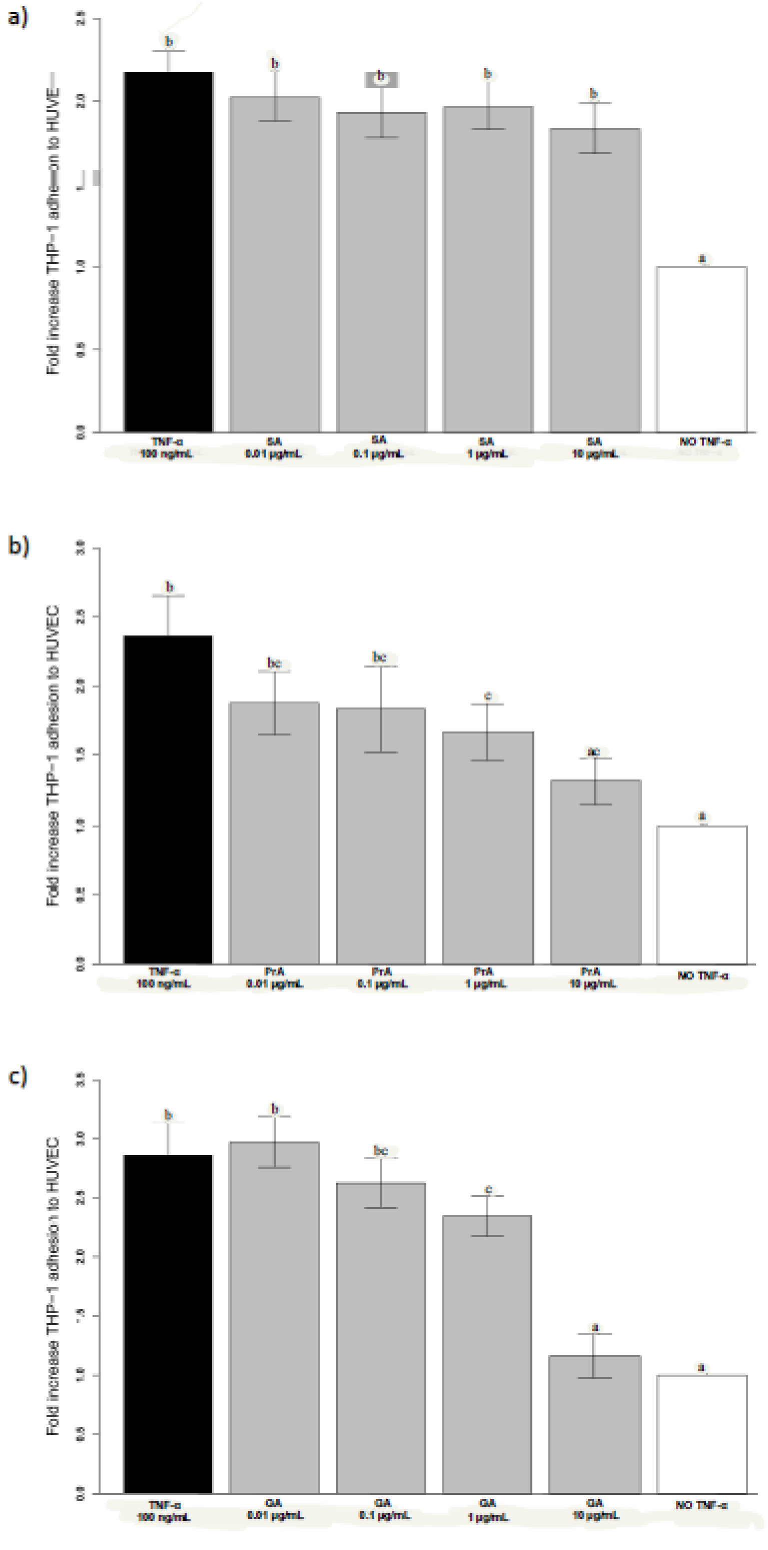


Figure 5- Effect of **A)** *SA* (0.05-50.5 μM), **B)** *PrA*(0.03–64.9 μM) and **C)** *GA* (0.03–58.8 μM) on THP-1 adhesion to HUVECs. Results are expressed as mean ± standard error of mean. ^{a,b,c}Bar graphs reporting different letters are significantly different ($p \leq 0.05$).



Legend: *TNF-α*, tumor necrosis factor alpha; *SA*, syringic acid; *PrA*, protocatechuic acid; *GA*, *g*allic acid; *NO TNF-α* (control).

Table 1: Effect of ACNs and metabolites on the levels of E-selectin

	Compounds					
Concentrations	Mv-3-glc	Cy-3-glc	Dp-3-glc	SA	PrA	GA
0.01 µg mL^{-1}	107±15^{a}	311±13^{a}	308±11^{a}	299±15^{a}	290±13^{a}	304±15^{a}
0.1 µg mL^{-1}	104±16^{a}	297±15^{a}	299±22^{a}	297±15^{a}	257±12^{a}	321±11^{a}
1 µg mL^{-1}	186±12^{a}	300±14^{a}	295±12^{a}	297±16^{a}	83±15^{b}	206±10^{b}
10 µg mL^{-1}	149±24^{a}	83±10^{b}	315±16^{a}	295±14^{a}	74±18^{b}	188±17^{b}
(TNF-α) 100 ng mL^{-1}	316±16^{b}	307±11^{a}	318±12^{a}	316±16^{a}	307±11^{a}	318±12^{a}
(TNF-α) 0 ng mL^{-1}	59±9.0^{c}	64±10^{c}	65±4.6^{b}	59±9.0^{b}	64±10^{c}	65±4.6^{c}

Data derived from three different experiments and each concentration tested in triplicate. Each ACN and metabolite was tested in presence of TNF-α stimulus. Results are expressed as mean ± SEM. *Mv-3-glc*, malvidin-3-glucoside; Cy-3-glc, cyanidin-3- glucoside,*Dp-3-glc*, delphinidin-3-glc;*SA*, syringic acid, *PrA*, protocatechuic acid; *GA*, gallic acid;*TNF-α*, tumor necrosis factor alpha. ^{a,b,c}Data with different letters are significantly different (*p* <0.05). Concentration range: 0.02-18.9 µM for Mv-3-glc, 0.02–19.9 µM for Dp-3-glc, 0.02–20.6 µM for Cy-3-glc, 0.25 and 50.5 µM for SA, 0.32–64.9 µM for PrA and 0.29–58.8 µM for GA.

Table 2: Effect of ACNs and metabolites on the levels of VCAM-1

	Compounds					
Concentrations	Mv-3-glc	Cy-3-glc	Dp-3-glc	SA	PrA	GA
0.01 µg mL^{-1}	13.16±0.78	15.10±0.35	15.98±0.76	15.43±0.41	14.38±0.17	16.98±1.76
0.1 µg mL^{-1}	13.64±0.04	14.56±0.23	15.80±1.10	16.59±0.28	14.83±0.53	14.99±1.90
1 µg mL^{-1}	14.15±0.33	14.65±0.20	16.94±0.51	18.85±0.23	15.28±0.42	16.64±0.71
10 µg mL^{-1}	14.38±0.11	15.10±0.24	16.30±0.40	17.45±0.29	16.19±0.37	16.26±0.80
(TNF-α) 100 ng mL^{-1}	15.74±1.14	15.17±1.08	16.97±1.81	15.74±1.14	15.17±1.08	16.97±1.81
(TNF-α) 0 ng mL^{-1}	11.04±0.37*	10.99±0.35*	11.27±0.28*	11.04±0.37*	10.99±0.35*	11.27±0.28*

Data derived from three different experiments and each concentration tested in triplicate. Each ACN and metabolite was tested in presence of TNF-α stimulus. Results are expressed as mean ± SEM. *Mv-3-glc*, malvidin-3-glucoside; Cy-3-glc, cyanidin-3- glucoside, *Dp-3-glc*, delphinidin-3-glc; *SA*, syringic acid, *PrA*, protocatechuic acid; *GA*, gallic acid; *TNF-α*, tumor necrosis factor alpha.*Significantly different (*p* <0.05). Concentration range: 0.02-18.9 µM for Mv-3-glc, 0.02–19.9 µM for Dp-3-glc, 0.02–20.6 µM for Cy-3-glc, 0.25 and 50.5 µM for SA, 0.32–64.9 µM for PrA and 0.29–58.8 µM for GA.