VIA MEDICA

ORIGINAL ARTICLE

Acta Angiol
Vol. 29, No. 3, pp. 68–75
DOI: 10.5603/aa.96599

ISSN 1234–950X
e-ISSN 1644–3276

Effect of diosmin and diosmetin on the level of pro-inflammatory factors in the endothelium artificially induced with inflammatory stimuli

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Abstract

Introduction: *Diosmin and its aglycone diosmetin are phlebotropic drugs used in the treatment of chronic venous insufficiency (CVI). Diosmin increases the elasticity and tension of blood vessel walls, exhibits an antiedematous effect, and acts as an anti-inflammatory agent. As it is commonly known that the endothelium layer plays a significant role in the physiology and pathophysiology of the cardiovascular system, this paper investigates the effect of diosmin and diosmetin on modulating the levels of pro-inflammatory factors in an endothelial cell culture (HUVEC) stimulated by lipopolysaccharide (LPS) or phorbol (PMA).*

Material and methods: *A normal human umbilical vein/vascular endothelium cell line HUV-EC-C (HUVEC) was stimulated with lipopolysaccharide (LPS) or phorbol 12-myristate-13-acetate (PMA). Cell viability was assessed using NR and MTT assays. The levels of human IL-1β, IL-6, IL-10, COX-2, and PGE2 were measured using ELISA kits.*

Results: *Depending on the agent used to initiate inflammation, different levels of factors associated with this state were obtained. Diosmetin significantly decreased the levels of pro-inflammatory IL-1β and IL-6 as well as COX-2 in PMA-treated cells. Meanwhile, diosmin did not affect the interleukins but it lowered COX-2 and increased PGE-2. Upon the LPS stimulation of HUVEC cells, diosmetin increased the levels of PGE2, IL-1β, COX-2, and nitric oxide (NO), while diosmin increased NO and IL-6.*

Conclusions: *Diosmin and diosmetin have different impacts on the levels of pro-inflammatory factors depending on the inflammation inducer. Diosmetin more effectively modulated inflammation than diosmin, suggesting that the attachment of the sugar moiety to the aglycone attenuates its activity.*

Keywords: diosmin; diosmetin; lipopolysaccharide; phorbol 12-myristate 13-acetate; endothelial cells

Acta Angiol 2023; 29, 3: 68–75

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Received: 21.07.2023 Accepted: 07.08.2023 Early publication date: 19.09.2023

Introduction

Diosmin (5,7,3-trihydroxy-4'-methoxyflavone 7-rutinoside) and its aglycone — diosmetin belong to polyphenols with a confirmed wide range of biological activities [1–3]; however the impact of these flavonoids, especially diosmin, on the cardiovascular system has the greatest significance in medicine. In vivo studies evidenced that diosmin shows a strong protective effect on blood vessels via an increase in the elasticity and tension of blood vessel walls and a reduction of venous pressure and venous stasis in the lower limb. It is also shown to be active in facilitating lymph flow in the lymphatic system and reduces the permeability of capillary walls and thus has an anti-edematous effect [4, 5]. Moreover, it alleviates oxidative stress linked with the inflammation process [6, 7]. For these reasons, diosmin is widely used to support the treatment of Chronic Venous Disease (CVD). CVD is a functional and morphological abnormality of the venous system and it is manifested by skin lesions, swollen legs, and structural changes in the vein wall, such as varicose veins and venous leg ulcers which considerably decrease the quality of life [5, 8].

The role of the endothelium layer in the physiology and pathophysiology of the cardiovascular system is still a subject of interest [9, 10]. It is known that a properly functioning endothelium layer in normal conditions not only constitutes an anticoagulant layer, but also maintains the local balance between pro- and anti-inflammatory factors. Dysfunctions of this layer, e.g. inappropriate activation or over-activation, can lead to local changes in the stability and functionality of this barrier and, consequently, not only to disturbances in homeostasis in the vessels themselves but also to multiple organ failure. Initiation of endothelial inflammation is a known factor contributing to the development of e.g. atherosclerosis [11] since endothelial cells function both as target and effector cells during inflammation [12]. Besides significant pathological changes in the expression of adhesive molecules such as VCAM-1 or ICAM-1, subendothelial retention of lipoproteins also changes the recruitment of specific blood cells and adhesion to endothelial cell membranes. Moreover, microenvironmetal changes in the production of specific pro-inflammatory cytokines like IL-10 or IL-1β as well as compounds or intermediary factors of the arachidonic pathway (COX-2, PGE2, or NO) are also observed [13]. These all factors released by endothelial cells after induction with various stimuli can both be a process of defense of the blood vessel epithelium or lead to its clinically manifested dysfunction [12]. The regulating and balance-keeping factors released by endothelial cells have been quite well studied; however, factors directly controlling systemic endothelial inflammation are still in the discovery phase [14]. Analysis of the level of inflammatory factors released by endothelial cells in the presence of diosmin or diosmetin may therefore help to indicate a target for potential control or modulation of inflammation and the unfavorable phenomena occurring in blood vessels. Therefore, the aim of the present paper was to investigate the influence of popular phlebotonics diosmin and diosmetin on the modulation/regulation of artificially induced inflammation in a human endothelial cell culture model. Bacterial lipopolysaccharide (LPS) or phorbol ester (PMA) were used as a stimuli to induce inflammation.

Material and methods

Cell line

A normal human umbilical vein/vascular endothelium cell line HUV-EC-C (HUVEC) (ATCC® CRL-1730™) was used in this study. The cells were cultured as monolayers in 25 cm^2 culture flasks (NuncTM, Roskilde, Denmark) coated with PureColTM ultrapure collagen type I (INAMED Biomaterials, Fremont, CA, USA) at a 3.1 mg/ml concentration (about 12 $\mu g/cm^2$). The cell line was maintained in CS-C medium (Sigma) supplemented with 75 μg/ml of endothelial cell growth factor (ECGF) (Sigma, St Louis, MO, USA) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin (Sigma) at 37°C in a humidified atmosphere with 5% CO_2.

Experimental design

The cells were cultured in 24-well (for ELISA, nitric oxide level, and staining) or 96-well (viability) plates (NuncTM). The cells were seeded on the well bottom at a density of 1×10^5 cells/ml. After 24 h of inoculation, the medium was renewed, and the cells were stimulated with lipopolysaccharide (LPS) from Escherichia coli serotype 0111:B4 (Sigma) (10 μg/ml for 2 h) or phorbol 12-myristate-13-acetate (PMA) (Sigma) (1 μg/ml for 30 min). The concentrations were selected based on literature data and our previous experiments. After cell stimulation, the medium was renewed, and diosmin (5 μM) or diosmetin (5 μM) (for ELISA, staining, and NO analysis) or the compounds in the range of 0–100 μM (for toxicity tests) were added. Then, the culture was conducted for a further 24h. After that time, culture supernatants were collected and analyzed for the levels of cytokines (IL-1β, IL-6), serine protease (uPA), and its receptor (uPAR). In turn, MMP activity was determined in the supernatants from the well bottom and inserts for comparison.

Both diosmin and diosmetin stock solutions were prepared by solubilization in a mixture of DMSO/culture medium (1:1). The stock solution concentration was

10 mM. At the highest working concentration of both compounds used in the research (100 μM), the DMSO concentration did not exceed 0.5%. In our previous papers, we showed no toxicity and no interference of this DMSO amount with the results.

Neutral Red (NR) uptake assay

The cells were grown in 96-well multiplates for 24 h in 100 μl of CS-C culture medium with the supplements and diosmin (5 μM) or diosmetin (5 μM). Subsequently, the medium was discarded and 0.4% NR (Sigma) solution medium was added to each well. The plate was incubated for 3 h at 37°C in a humidified 5% CO_2/95% air incubator. After incubation, the dye-containing medium was removed, the cells fixed with 1% $CaCl_2$ in 4% paraformaldehyde, and the incorporated dye was solubilized using 1% acetic acetate in a 50% ethanol solution (100 μl). The plates were gently shaken for 20 min at room temperature, and the extracted dye absorbance was measured spectrophotometrically at 540 nm using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

MTT assay

Cell viability after incubation with diosmin or diosmetin was determined in a standard spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells grown in the 96-well multiplates in 100 μl of culture medium were incubated for 24h with the flavonoids. After that time, an MTT solution (5 mg/ml, 25 μl/well) (Sigma) was added, and further incubation was conducted for the next 3 h. The yellow tetrazolium salt was metabolized by viable cells to purple formazan crystals. The reaction was catalyzed by mitochondrial succinyl dehydrogenase. The crystals were solubilized overnight in a 10% sodium dodecyl sulfate (SDS) in a 0.01M HCl mixture. The product was quantified spectrophotometrically by absorbance measurement at 570 nm wavelength using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

Nitric oxide (NO) measurement

Nitrate, i.e., a stable end product of NO, was determined in the culture supernatants with a spectrophotometric method based on the Griess reaction. Briefly, the cells were induced with LPS or PMA and incubated for 24 h with diosmin or diosmetin. Thereafter, 100 μl of the supernatant was plated in 96-well flat-bottomed plates in triplicate and incubated with 100 μl of Griess reagent (1% sulphanilamide/0.1% N-(1-naphthyl)ethylenediamine dihydrochloride) (Sigma) in 3% H_3PO_4 (POCH Gliwice, Poland) at room temperature for 10 min. The optical density was measured at 550 nm using a microplate reader (Molecular Devices Corp., Emax, Menlo Park, CA, USA). A standard curve was achieved using 0.5–25 μM sodium nitrite ($NaNO_2$) for calibration.

May-Grünwald-Giemsa (MGG) staining

The MGG staining performed in this study facilitates only visualization of morphological changes in cells induced by the culture conditions. The cells at a density of 1×10^5 cells/ml were cultured in Petri dishes (35 mm). After treatment with LPS or PMA, the medium was changed, and the cells were incubated with diosmin or diosmetin for 24 h. After incubation, the cells were fixed with methanol for 5 min and stained with the May-Grünwald dye diluted in an equal volume of water for 2 minutes. Thereafter, the dye was removed and the Giemsa stain, previously diluted (1 vol. Giemsa: 19 vol. water), was added for 20 min. The dishes were rinsed three times with distilled water and dried. The observation was performed under a light microscope (Olympus BX51, Tokyo, Japan).

ELISA assay

The levels of human IL-1β, IL-6, IL-10, COX-2, and PGE2 (Elabscience, Houston, TX, USA) were measured immunoenzymatically (ELISA) using commercially available kits according to the manufacturer's instructions. Briefly, 100 μl of samples were added to appropriate plate wells. After incubation (2 h) and a series of washing, enzyme-conjugated secondary antibodies (100 μl) were added to the wells and incubated for 1 h. After washing, detection was performed by adding 100 μl of the enzyme-substrate to the wells. After 30 min of incubation, the color reaction was stopped by adding 2M H_2SO_4 to each well. The optical density of the end product was determined using a microplate reader (Molecular Devices Corp., Emax) at 450 nm. The concentrations of the cytokines in the analyzed samples of supernatant were calculated on the basis of a standard curve. The detection limit was 4.69 pg/ml (IL-1β, IL-6 and IL-10), 0.19 ng/ml (COX-2), and 18.75 pg/ml (PGE2).

Statistical analysis

The results are presented as means ± SD of three independent experiments ($n = 3$). The data were analyzed using a one-way analysis of variance ANOVA followed by Dunnett's multiple comparison post-hoc test. Differences were considered significant at $p \leq 0.05$.

Results and Discussion

Diosmin has long been widely used for the treatment of CVD because it relieves the symptoms, improves the

quality of patient's life, and is well tolerated [15]. It has already been shown that this polyphenol may exhibit anti-inflammatory properties by modulation of the local level of soluble factors (cytokines, chemokines), thus inducing the development of e.g. inflammation in tissue [16–18]. In our research, we artificially induced inflammation in umbilical vein endothelial cells (HUVEC) to estimate changes in the level of basic inflammatory factors after diosmin/diosmetin treatment.

The initial investigation involving the determination of the direct biological activity of diosmin and diosmetin on human umbilical vein cells (HUVEC) was based on toxicological analyses. The effect of these factors on cellular metabolism was checked with the MTT method (Fig. 1) and the stability of cell membranes was checked using the Neutral Red (NR) uptake assay (Fig. 2).

It was shown that diosmin at a concentration above 10 μM reduced the metabolic activity of the cells by 9% in comparison to the untreated control, while diosmetin had an opposite effect and, from the concentration of 10 μM, stimulated the metabolic activity in the cells by 8.5%, compared to the untreated control. In turn, both substances at a concentration exceeding 10 μM disturbed the stability of cell membranes and reduced the viability of the cells in the culture by more than 15% at the concentration of 100 μM, compared to the untreated control.

The detailed microscopic observation of the morphology of epithelial cells after MGG staining (Fig. 3) showed that diosmetin at 5 μM induced no significant morphological changes relative to the untreated cells in the control conditions; however, treatment of the control cells with diosmin caused slight cytoplasm contraction and separation of the cells from each other.

Although some adverse effects of diosmin at 5 μM were observed on cell morphology, we decided to continue the investigation with this concentration. Preliminary experiments showed the lower concentration was not effective and the differences between control and cells treated with diosmin/diosmetin were hard to estimate.

To assess the anti-inflammatory activity of investigated compounds, inflammation in endothelial cells was induced using LPS and PMA LPS is known to initiate the inflammatory signaling pathway by activation of the NF-κB factor. This takes place through phosphorylation and subsequent degradation of IκB-α. Consequently, the active form of NF-κB is released from this complex. The active form of NF-κB regulates gene transcription of nitric oxide synthase (NOS), COX-2, or selected pro-inflammatory cytokines [19]. In turn, PMA is an important factor activating protein kinase C (PKC). PKC controls the function of other proteins by phosphorylation of the serine and threonine hydroxyl groups

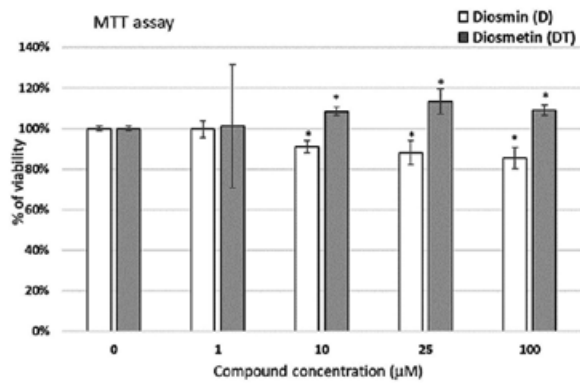

Figure 1. Cell metabolic activity determined by means of the MTT assay. The analysis was performed after 24h culture of human umbilical vein endothelial cells (HUVEC) with diosmin or diosmetin (0–100 μM)

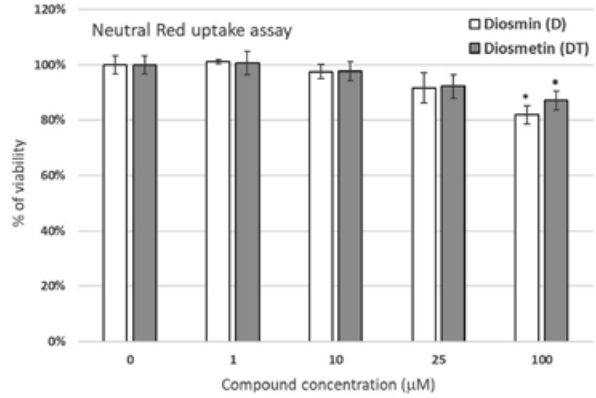

Figure 2. Neutral Red (NR) uptake assay performed after 24h culture of human umbilical vein endothelial cells (HUVEC) with diosmin or diosmetin (0–100 μM)

found in these proteins. Their role is to modulate events within the cell membrane, regulate transcription, or mediate immune response processes. Their impact on the induction of inflammation is therefore different.

The concentration of LPS and PMA, which demonstrated no toxicity in the assumed experimental time interval, was based on our previous studies. As can be seen (Fig. 3) treatment of the cells with LPS did not cause any morphological changes; however, for PMA-treated cells adverse effects including cytoplasm contraction, separation of the cells from each other and vacuolization appeared in single cells were observed.

In the LPS or PMA pre-incubated cells treated with diosmin, further contraction of the cytoplasm and cell separation were visible. Moreover, in the case of the pre-incubation with PMA, vacuolization was pronounced in individual cells. In turn, in the LPS or PMA pre-incubated cells treated with diosmetin sharpening of cell shapes and the appearance of vacuoles in the cells treated with PMA was found.

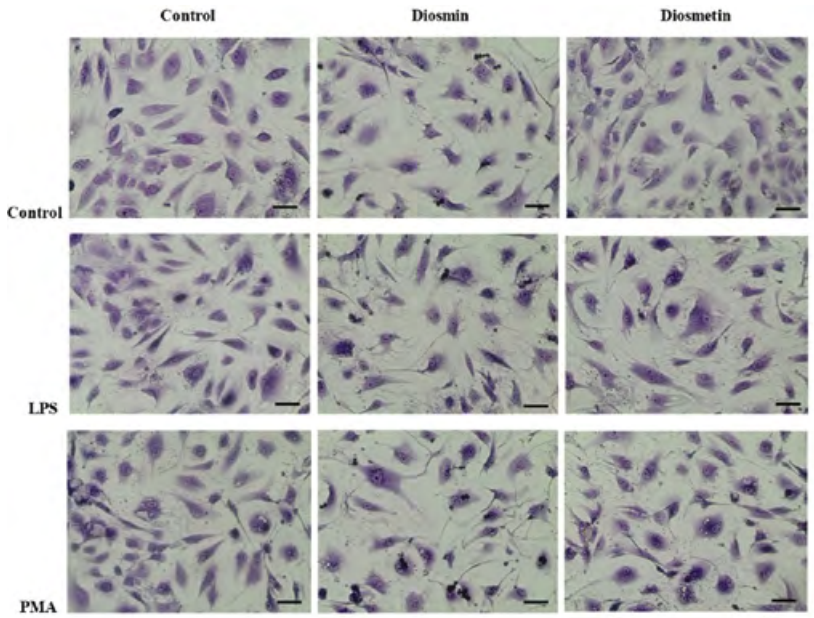

Figure 3. May-Grünwald-Giemsa (MGG) staining of human umbilical vein endothelial cells (HUVEC) incubated with diosmin or diosmetin (5 μM) for 24 h, prior to 2-h pre-treatment with *E. coli* LPS (10 μg/ml) or 30-min treatment with PMA (1 μg/ml). Magnification 200×. Bar = 20 μm

To assess the anti-inflammatory effect of diosmin or diosmetin, the level of IL-1β, IL-6, and IL-10 was monitored in endothelial cell culture pre-treated with LPS or with PMA (Fig. 4).

As can be observed, diosmin/diosmetin induced the production of cytokines but this was not surprising because all exogenous factors disturb cell homeostasis. Moreover, it should be also noted that the up-regulation of IL-1β and IL-6 was accompanied by an increase of IL-10; therefore, the balance between pro-inflammatory and anti-inflammatory cytokines has been almost maintained. Such an increased level of cytokines does not need to be considered as an adverse effect. Inflammation is necessary to initiate local adaptive immunity in endothelial cells which may lead to the induction of an epithelial-derived defense reaction that would bring our experimental conditions closer to in vivo reality. It also means that the epithelium was reactive and sensitive to experimental conditions.

The stimulation of inflammation with LPS or PMA resulted in a significant increase in the level of IL-6 (approx. 10-fold and 12-fold, respectively) in comparison to the untreated control and simultaneously, the level of IL-10 was only 3–3.5 — fold higher. No positive effect of diosmin on the level of cytokines in LPS/PMA-induced cells was found and even it slightly increased the production of IL-6. In turn, diosmetin lowered the level of IL-6 (approx. 2-fold) and slightly lowered IL-10 in PMA-induced cells and therefore, the balance between these factors was improved. Diosmetin did not affect the level of these interleukins in the cells pre-incubated with LPS; however, it significantly induced IL-1β.

The influence of diosmin on interleukins was also reported by Ali et al., who showed that diosmin treatment significantly reduced plasma concentrations of IL-1β and IL-6 in diabetic rats [17]. A similar effect was described by Carballo-Villalobos et al. [18].

In the further part of our study, the influence of diosmin/diosmetin on cyclooxygenase 2 (COX-2) and the other markers of inflammation including prostaglandin E2 (PGE2) and NO in cells with induced inflammation were investigated. Cyclooxygenases (COXs) are intracellular enzymes that catalyze the conversion of arachidonic acid to various forms of prostaglandins (PGs), thromboxanes, and hydroxyeicosatetraenoic acids. Various mitogens may induce inflammation mediated by COX-2 and then changes in COX-2 activity are closely related to PGE2 and NO levels. COX-2 is considered to be linked with a lot of symptoms in CVD including inflammation, pain, increased angiogenesis, and vascular permeability [9, 10].

As can be seen in Figure 5, in the control conditions, diosmin and diosmetin did not significantly change the level of COX-2 produced by the HUVEC cells. On the other hand, both compounds lowered the level of COX-2 in PMA pre-treated cells in a significant manner. In these conditions, diosmetin showed higher inhibitory activity than diosmin. In LPS-stimulated cells, no effect for diosmin was noted, meanwhile diosme-

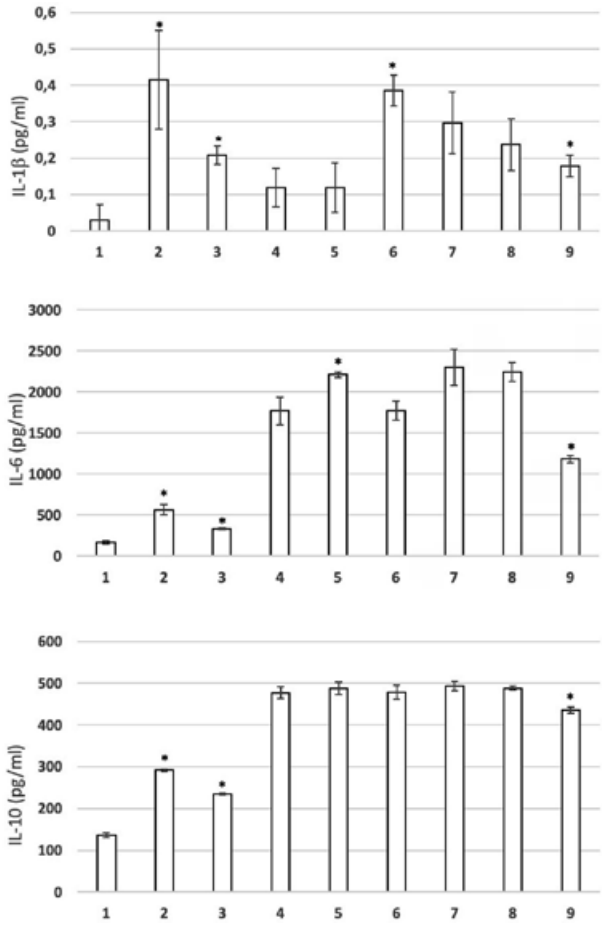

Figure 4. Effect of diosmin or diosmetin (5 μM) on interleukin level (A- IL-1β, B- IL-6, C-IL-10) in endothelial cells (HUVEC) pre-incubated with lipopolysaccharide (LPS) or phorbol 12-myristate-13-acetate (PMA). 1–3: Control, Value after cell treatment with diosmin, Value after cell treatment with diosmetin; 4–6: similarly to 1–3 but the cells were pre-incubated with LPS; 7–9: similar to 1–3 but the cells were pre-incubated with PMA. The data are mean ± SD (n = 3). One-way analysis of variance ANOVA followed by Dunnett's multiple comparison post-hoc tests.; *p-value of ≤ 0.05 vs. the respective control was considered significant

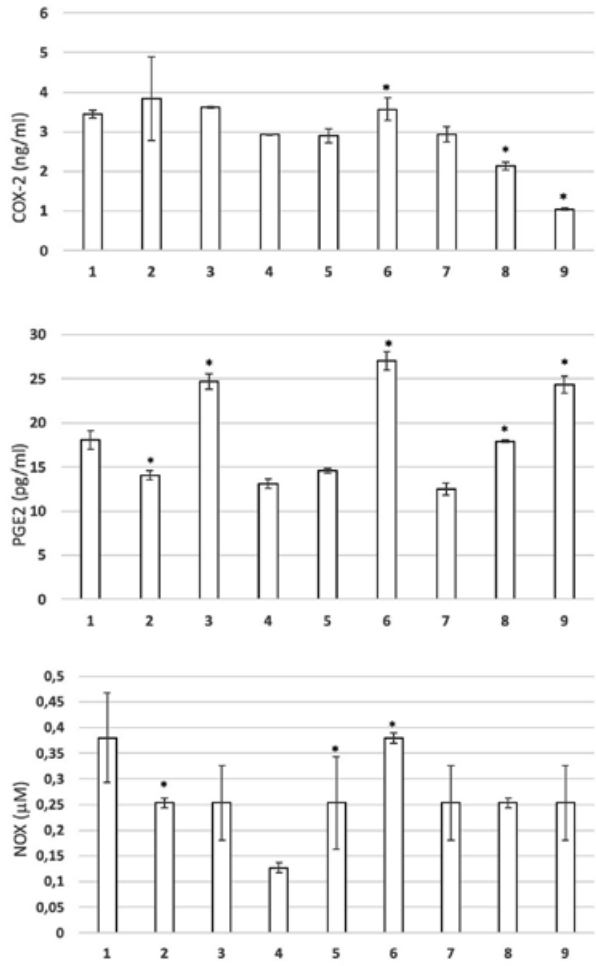

Figure 5. Effect of diosmin or diosmetin (5 μM) on COX-2, PGE2, and NO release by endothelial cells (HUVEC) pre-incubated with lipopolysaccharide (LPS) (10 μg/ml for 2 h) or phorbol 12-myristate-13-acetate (PMA) (1 μg/ml for 30 min). 1–3: Control, Value after cell treatment with diosmin (5 μM), Value after cell treatment with diosmetin (5 μM); 4–6: similarly, to 1–3 but the cells were pre-incubated with LPS; 7–9: similar to 1–3 but the cells were pre-incubated with PMA. The data are mean ± SD (n = 3). One-way analysis of variance ANOVA followed by Dunnett's multiple comparison post-hoc test.; *p-value of ≤ 0.05 vs. the respective control was considered significant

tin stimulated slightly the release of this enzyme. It should be observed that the PMA, LPS, and diosmin reduced the level of PGE2 in relation to control. In contrast, diosmetin turned out to be a potent inducer of PGE2, both in cells with as well as without induced inflammation. A moderate increase of PGE2 was also observed in PMA-induced cells after diosmin treatment. The decrease of NO was observed for all investigated conditions compared to the control, with the exception of LPS cells treated with diosmetin, where NO was on the same level as in HUVEC cells. LPS lowered NO the most effectively and surprisingly diosmin and diosmetin increased NO levels in LPS-induced cells.

Our study confirmed the results reported by Berkoz et al., who found that diosmin decreased NO and PGE2 production through inhibition of the expression of phosphorylated-ERK, p38, and p-IκB-α [19].

To sum up, the influence of diosmin/diosmetin on changes of investigated factors in cells with induced inflammation varied depending on the inducer; however, it seems that aglycone form more effectively modulates the inflammation. It significantly decreased the level of pro-inflammatory IL-1β and IL-6 as well as COX-2 in PMA-treated cells. Meanwhile, diosmin did not affect the interleukins but it lowered COX-2.

Interestingly, we found that decreases in COX-2 were accompanied by increases in PGE2 and it suggested the production of PEG2 in such conditions maybe connected with COX-1 activity. It is in line with the literature data. In vitro studies proved that diosmetin stimulated PGE2 via COX-1 more effectively than via COX-2 [20].

Our investigation showed the activity of both tested flavonoids varies depending on the presence or absence of inflammation as well as the inflammatory stimuli and it may depend not only on typical already known pathways but also on other factors of signal transducers differently expressed in different experimental conditions. The study confirmed the conclusions formulated by Lopez-Posadas et al., who stated, that the activity of flavonoids strongly depends on experimental conditions. Depending on the agent used to initiate inflammation, different levels of factors associated with this state were obtained [21].

Conflict of interest

None.

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