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Effect of anthocyanin-rich sour cherry extract on the level of IL-8 in LPS-induced endothelial cell

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SUMMARY

The anthocyanin content of the Hungarian sour cherry is remarkable. Nutraceutical and pharmaceutical effects of the anthocyanins and their role in disease prevention have been studied extensively. Endothelial cells are involved in the pathogenesis of several inflammatory diseases. The objective of this work was to investigate pure sour cherry extract on human umbilical cord vein endothelial cells (HUVECs) as an inflammatory model. HUVECs were treated with 100 ng mL^{-1} lipopolysaccharide (LPS) and 50 μg mL^{-1} sour cherry extract or M199 medium as control. The optimal concentration range of the sour cherry extract was investigated and selected based on MTT assay measuring the conversion of the tetrazolium salt to formazan by mitochondrial dehydrogenases. The level of interleukine-8 (IL-8), a pro-inflammatory cytokine, was measured in Luminex MagPlex assay. LPS treatment significantly increased the secretion of IL-8. The pure sour cherry extract was able to attenuate this increment indicating the potent anti-inflammatory effect of pure sour cherry extract. Our results emphasize that pure sour cherry extract could reduce the LPS-induced inflammatory response thereby may improve endothelial dysfunction.

Keywords: *sour cherry, anthocyanin, inflammation, endothelium*

INTRODUCTION

Anthocyanins are water-soluble polyphenolic pigments that are responsible for the pigmentation of many foods, including fruits (sour cherry, blueberries, black plums, blackberries), vegetables (onion, red radish, purple cabbage), and grains (black rice, red rice, and black soybeans) (Khoo et al., 2017). Anthocyanins are a subgroup of flavonoids. Anthocyanins share a basic C-6 (A ring)-C-3 (C ring)-C-6 (B ring) carbon skeleton; it is also called the flavylium (2-phenylchromenylium) ion, with a varying number of hydroxyl groups and sugars with different degrees of methylation. The stability of anthocyanin is dependent on pH, light, temperature, and structure (Laleh et al., 2006).

The anthocyanin content of Hungarian sour cherry is outstanding, based on our preliminary investigations. They produce selective cyanidin glycosides, the main component being cyanidin-3-O-rutinoside (Homoki et al., 2016).

Several studies demonstrated that the anthocyanins can exert numerous beneficial physiological effects because of their anti-inflammatory, antioxidant, anti-obesity, anti-angiogenesis, anti-cancer, anti-diabetes, anti-microbial, neuroprotection, and immunomodulation properties (Lila, 2004).

Many studies have focused on the defensive role of anthocyanins in inflammation. An in vitro study showed that the anthocyanin-rich bilberry extract is able to attenuate the IFN-α-induced overexpression of MCP-1, IL-6, and TNF-α in human monocytic THP-1 cells. (Roth et al., 2014). Another in vitro study emphasized the protective effect of purified sour cherry anthocyanin extract on cytokine-induced inflammatory caco-2 monolayers (Nguyen et al., 2018).

In the present study, the effect of sour cherry anthocyanin extract was investigated in an inflammatory model. Because human umbilical cord vein endothelial cells (HUVECs) have been extensively used in studies of the physiology and pathophysiology of various diseases, including the inflammatory process (Medina-Leyte et al., 2020), we applied HUVECs as an inflammatory model. The inflammatory response was induced by lipopolysaccharide (LPS), an endotoxin from the outer membrane of bacteria, which is routinely used to trigger inflammation.

MATERIALS AND METHODS

Preparation of Hungarian sour cherry (*Prunus cerassus*)

Extraction and purification of sour cherry have been performed as described previously (Nemes et al., 2018). The analytical characterization and determination of flavonoids and proanthocyanidins was carried out using by UHPLC. The investigated compounds were separated using by Phenomenex Kinetex column (2.6 μ, XB.C18, 100 X 4.6 mm) and gradient elution. Methanol and 3% Formic acid in water was used as aluent. Flow rate was 0.7 mL min^{-1}. UV–vis detection was used at 535 nm wavelength. Hungarian sour cherry cultivar, "*Újfehértői fürtös*", was applied in our in vitro experiments

Cell culture conditions

The human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords. Umbilical cords were obtained from the Department of Obstetrics and Gynaecology, Clinical Centre, University of Debrecen, Debrecen, Hungary. The HUVECs were isolated using by enzymatic digestion and maintained according to the method previously described (Biro et al., 2020). In our experiments, the M199 medium was used as a control. 100 ng mL^{-1} lipopolysaccharide (LPS)

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was used in cell culture to generate an inflammatory model.

Determination of cellular viability

The viability of the cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay measuring the conversion of the tetrazolium salt to formazan by mitochondrial dehydrogenases. Cells were seeded to 96-well plates at a density of 20 000 cells per well in quadruplicates and were treated with anthocyanin extract of different concentrations (1, 5, 10, 50, 100, 500, 1000 µg mL^{-1}) and without anthocyanin extract (control group) for 24 hours. The medium was removed, and the cells were then incubated with MTT reagent for 3 hours; subsequently, the formazan crystals were dissolved in 100 µL solubilizing solution. The concentration of formazan crystals was determined colorimetrically at 465 nm by using Clariostar microplate reader (BMG Labtech, Ortenberg, Germany). Cell viability at different anthocyanin extract concentrations was expressed relative to 100% of the control group.

Evaluation of the level of IL-8 on Luminex MagPlex assay8

HUVECs were seeded into a 6-well plate (500 000 cells/well) and were treated with M199 Medium as control and 100 ng mL^{-1} LPS with or without 50 µg mL^{-1} anthocyanin extract for 24 hours. The supernatant was collected and centrifuged for 10 min 10 000 min^{-1} and stored at -80 °C. The levels of IL-8 were assessed using by MILLIPLEX MAP Human cytokine/chemokine Magnetic Bead Panel (HCYTOMAG-60K-09, EMD Millipore Corp., Billerica, MA, USA).

Statistical analysis

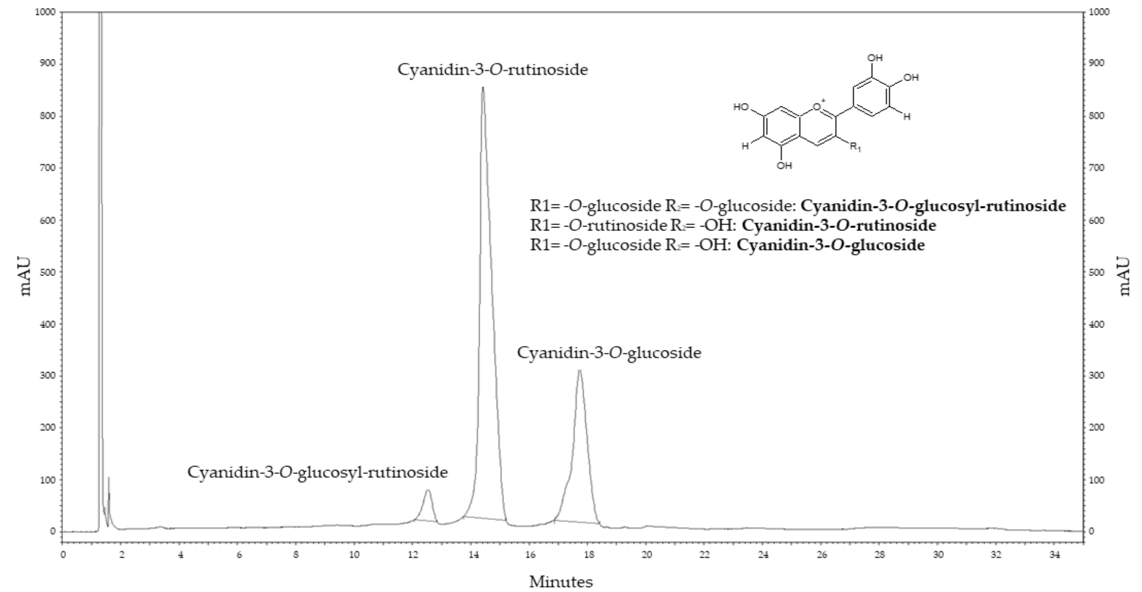
For multiple comparisons, results were analyzed by ANOVA followed by modified t-test for repeated measures according to Bonferroni’s method. Data were presented as mean±SEM. Differences were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Main compounds of the purified sour cherry extract

Based on our previous study (Nemes et al., 2018), the main anthocyanin components of the sour cherry were found as cyanidin-3-*o*-rutinoside, cyanidin-3-*o*-glucoside, and cyanidin-3-*o*-glucosyl-rutinoside (*Figure 1*).

Figure 1: **UHPLC chromatogram of sour cherry extract**



The optimal anthocyanin containing sour cherry extract concentration was determined by using MTT-assay. We found that the high anthocyanin containing extract did not decrease the viability of HUVECs up to 500 µg mL^{-1} (*Figure 2*). These data suggest that relatively high concentrations of the anthocyanin containing extract can be applied without the possibility of cytotoxic effects. Moreover, we assessed that the highest anthocyanin containing extract concentration that significantly reduced the cell viability of HUVECs was 1000 µg mL^{-1} Given that the maximum of the main component (cyanidin 3-*o*-rutinoside) of the anthocyanin extract in blood is close to 50 µg mL^{-1} (Kay et al., 2017), that concentration was selected for further investigations.

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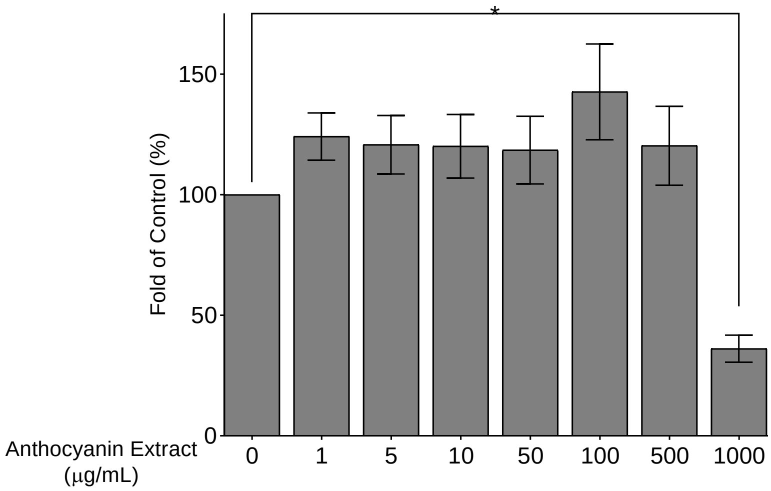
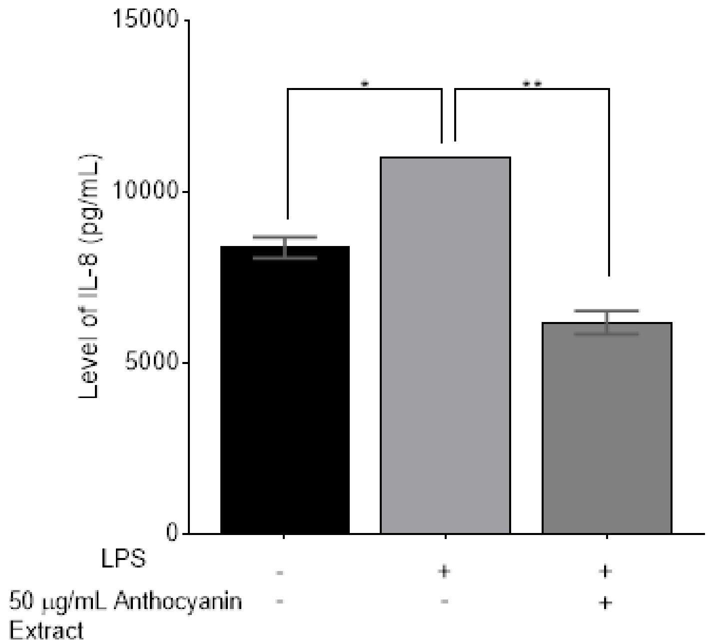
Figure 2: **MTT-assays. The viability of HUVECs, was monitored followed up to 24 hours. The remaining reduction capacity of the cells were expressed in the percentage of the control samples. Data are expressed as the mean ± SEM of four individual experiments. *means a significant difference between the data sets p<0.05**



Numerous studies have demonstrated the anti-inflammatory effects of anthocyanins (Vendrame et al., 2015) and IL-8 is one of the primary mediators of the inflammatory response. It plays a crucial role in the recruitment of the immune cells, primarily neutrophils, to the site of inflammation (Selders et al., 2017). We investigated the effect of the anthocyanins-rich sour cherry extract on the level of IL-8 increased due to the LPS-induced inflammatory response. As expected, administration of LPS significantly increased the level of IL-8. Anthocyanin extract was able to reduce this increment (*Figure 3*), indicating the significant anti-inflammatory effect of our anthocyanin containing sour cherry extract.

Figure 3: **After LPS-treatment, the level of IL-8 increased significantly compared to the control indicated by * (P<0.05). ** (P<0.005) indicates the significant changes in level of IL-8 in LPS-induced inflammation and the anthocyanin extract treatment compared to the level of IL-8 in LPS-induced inflammation without anthocyanin extract. Data are expressed as the mean ± SEM of three individual experiments**



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CONCLUSIONS

Plants produce several chemically-highly diverse secondary metabolites, which may be suitable for exerting positive effects on human diseases (Ncube et al., 2015). The object of this study was to investigate the effect of the anthocyanin-rich sour cherry extract on LPS-induced inflammatory response in HUVECs. Firstly, the main compounds of purified sour cherry extract were determined by UHPLC liquid chromatography. Subsequently, the non-cytotoxic concentration of anthocyanin extract was determined by using MTT-assay. Finally, we investigated the effect of anthocyanin extract on the level of IL-8, a pro-inflammatory cytokine, in LPS-induced HUVECs. A recent study reported the Protective effect of pure sour cherry anthocyanin extract on cytokine-induced inflammatory caco-2 monolayers (Nguyen et al., 2018). The authors showed that the pure sour cherry anthocyanin extract was able to decrease the TNF-α-induced increased release of IL-8. In accordance with it, the anthocyanin-rich sour cherry extract significantly decreased the level of this pro-inflammatory cytokines. Several studies have demonstrated that anthocyanins can inhibit the cyclooxygenase-2 (COX-2) enzyme, thereby exerting an anti-inflammatory effect (Seeram et al., 2001). We hypothesize that the decrease in IL-8 by the anthocyanin-rich sour cherry extract is due to the inhibition of COX-2 enzyme by the anthocyanins. However, further studies are needed to elucidate the mechanisms of action of anthocyanin extract. Our results suggest that anthocyanin extract may exert anti-inflammatory effect and have therapeutic potential in inflammation-caused endothelial dysfunction.

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