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EFFECTS OF *VACCINIUM MYRTILLUS* L. EXTRACT ON TNF-α-INDUCED INFLAMMATION THROUGH HUMAN PARAOXONASES

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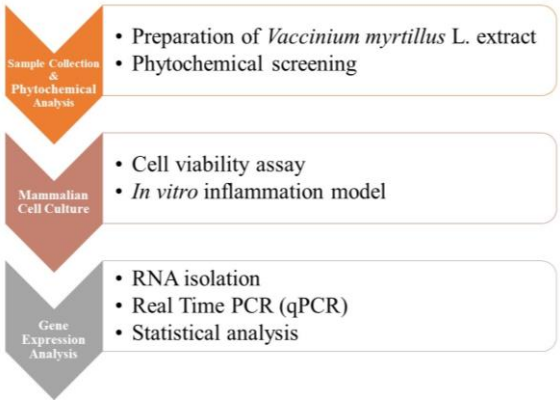
Cardiovascular diseases (CVDs) is a leading cause of death worldwide, and is characterized by a range of conditions that affect the heart and blood vessels. The extract of *Vaccinium myrtillus* L. fruit has been found to have cardioprotective effect. Human paraoxonases (*PON1*, *PON2*, *PON3*) genes play an important role in protecting against the development of CVDs by acting as antioxidants and anti-inflammatory agents.The present study aimed to investigate the chemical constituents and cardioprotective effects of *V. myrtillus* L. extract on TNF-α inflammation and oxidative stress in ECV-304 human endothelial cell line and the potential role of human paraoxonases in these effects. The results indicated that *V. myrtillus* L. extract has cardioprotective effects against inflammation and oxidative stress in ECV-304 cells, and these effects may be mediated through the upregulation of *PON1*, *PON2*, and *PON3* genes. These findings suggest that *V.myrtillus* L. may have therapeutic potential for the prevention and treatment of CVDs.



INTRODUCTION

Cardiovascular diseases (CVDs) are a group of diseases that affect the heart and blood vessels. They are the leading cause of death globally, with an estimated 17.9 million people reported to have died from CVDs in 2019. This represents 32% of all global deaths. It is stated that 85% of these deaths occur due to heart attack and stroke.[1]

Paraoxonase 1 (*PON1*), Paraoxonase 2 (*PON2*), and Paraoxonase 3 (*PON3*), are genes that encode for enzymes called paraoxonases (*PONs*). These enzymes have been found to play a role in the development of CVDs.[2,3] The paraoxonase family is also known to have antioxidant and anti-inflammatory properties.[4,5]

Vaccinium myrtillus L., also known as bilberry, is a small shrub and is native to Eurasian boreal forest.[6]

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The plant's extract has been shown to have a significant effect on the antioxidant capacity of blood, and it can reduce the oxidative stress markers in the blood.[7,8] Furthermore, the extract has been found to have anti-inflammatory properties and it can reduce the expression of pro-inflammatory genes.[9]

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that plays a key role in the development of atherosclerosis.[10,11]

The objective of this study was to investigate the cardioprotective effects of the *V. myrtillus* L. plant, which possesses antioxidant properties, on TNF-α -induced inflammation and oxidative stress in an in-vitro model using ECV-304 human endothelial cells by analysing *PON1*, *PON2* and *PON3* genes expression.

MATERIALS AND METHODS

Sample Collection

Fruit parts of *Vaccinium myrtillus* L. plant were collected in İkizdere Plateau, Rize, Turkey. Sample collection was carried out with a team of three people from the Trabzon Regional Directorate of Forestry, General Directorate of Forestry of the Republic of Turkey, and the naturally grown fruits of the *Vaccinium myrtillus* L. plant were collected.

Preparation of *Vaccinium myrtillus* L. extract

The berries of the *V. myrtillus* L. plant were used to prepare the extract. The berries were left to dry at room temperature. The dried and ground berries were extracted with 50% ethanol by maceration three times at room temperature [berries and total solvent ratio 1:10 (w/v)]. The extracts were left to rest for 24 hours and then filtered. The obtained extract was then evaporated to dryness using a rotary vacuum evaporator and stored at +4°C for use in experiments.

Phytochemical screening

The presence of phytochemicals was analyzed in the *V. myrtillus* L. extract. For the screening of alkaloids, saponins, tannins, glycosides, phenols, carbohydrates, proteins, and flavonoids, the method established by Khuda *et al.*[12] was used.

Mammalian cell culture

ECV304 (ATCC® CRL-1998 ™) human umbilical vein endothelial cell line was used for experiments. The cells were cultured in Dulbecco's Modified Eagled Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 U/mL).

Cell viability assay

MTT [3- (4,5-dimethylthiazole-2yl) – 2,5-diphenyl tetrazolium bromide] assay was performed to determine the appropriate dose range of the prepared *V. myrtillus* L. extract on ECV304 cells.

ECV304 cells were counted and adjusted to 1×10^5 cells/mL. Ten µl of 6 different stock solutions (1000, 500, 200, 100, 50, and 10 µg/mL) of the extract were added to wells as 100, 50, 20, 10, 5, and 1 µg/mL and 90 µL medium including cells were added to each well to be 1×10^5 cells/mL. Plates were incubated for 48 hours in a humidified environment at 37˚C in a 5% CO_2 incubator. After incubation 10 µl MTT (5 mg/mL) was added to each well for 4 hours incubation. Then, 80 µL of the supernatant in the wells was withdrawn and 100 µL of 50% solution of sodium dodecyl sulfate (SDS, pH 5.5) dissolved in isopropyl alcohol was added. After 24 hours incubation, the resultant color was measured at 570 nm on Multiskan™ GO Microplate Spectrophotometer. As controls, only the cells incubated with the medium were used. Effects of the extract on cell viability comparing with the control were calculated[13,14].

In vitro inflammation model

An *in vitro* inflammation model was generated by stimulating ECV304 cells with 10 ng/mL TNF-α.[15] Four different groups were created for the experiments: 1) Control group without TNF-α stimulation and untreated with the extract, 2) Only TNF-α stimulation on ECV304 cells, 3) ECV304 cells stimulated with TNF-α and treated with a 20 µg/mL concentration of *V. myrtillus* L. extract, 4) Only treatment with *V. myrtillus* L. extract as 20 µg/mL. Treatment with the extract for group 3 and 4 was performed on the second day for 24 hours. After incubation, inflammation was induced for group 2 and 3 with 10 ng/mL TNF-α for 24 hours.

RNA isolation

RNA isolation was performed by using RNeasy-Mini kit (QIAGEN, Germany) according to the manufacturer's instructions. The resulting RNA was stored at –80°C until quantitative polymerase chain reaction (qPCR) analysis.

Real Time PCR (qPCR)

To analyze the expression levels of *PON1*, *PON2* and *PON3* genes, qPCR analysis with Roche LightCycler Nano instrument was performed using 100 ng of RNA from each group, using the GoTaq 1-Step RT-qPCR System (Thermo Fisher Scientific, ABD) kit. The expression of the *GAPDH* gene in the cells was used for normalization. The sequences of the primers are shown in Table 1.

Table 1

Primer sequences of target genes

Gene Name	Oligonucleotide sequence (5′–3′)
PON1	f: TGCCATAGAAACTGAGGCCAT
	r: GGTCAGCATTCATTGTTGAGCT
PON2	f: ATGGGGCGGCTGGTGGCT
	r: TTAGAGTTCACAATACAAGGCTCTG
PON3	f: TGCCACCAGAGACCACTA
	r: AGAAGAGCACCTGAGTCC
GAPDH	f: AGGGCTGCTTTTAACTCTGGT
	r: CCCCACTTGATTTTGGAGGGA

Statistical analysis

The statistical analysis of the results was conducted using the Statistical Product and Service Solutions (SPSS) program. Statistically significant differences were analyzed using Student's t-test. Analyses were considered statistically significant at a *p*-value of < 0.05.

RESULTS

Phytochemical analysis results

According to the results of the phytochemical screening,[12] it was found that the *V. myrtillus* L. extract contains glycosides, tannins, flavonoids, and carbohydrates as the major secondary metabolites (Table 2).

Table 2

Phytochemical Screening of the *V. myrtillus* L. extract. *Bricket Red (more than 2% sugar)

Phytochemicals	Chemical Tests	Results
Alkaloids	Hager's test	-
Saponins	Foam test	-
Flavonoids	General Test	+
Tannins	Alkaline Reagent Test	+
Glycosides	Keller Killiani Test	+
Carbohydrates	Benedict's Test	+ *
Proteins	Xanthoproteic Test	-

Cell viability results

The effect of V. myrtillus L. extract against ECV-304 cells was investigated using the MTT cytotoxicity test. It showed that 20 µg/mL concentration had the highest proliferative effect on ECV304 cells at a rate of 3.14 ± 110.4%. Other concentrations of the extract (10, 50 and 100 µg/mL) showed 4.43±97.5%, 5.5 ± 106.9% and 3.14 ± 106% viability effects on ECV304 cells, respectively. Accordingly, cell viability rates were determined in the control group and *V. myrtillus* L. extract groups at increasing concentrations, and the optimum dose was determined as 20 µg/mL (Fig. 1).

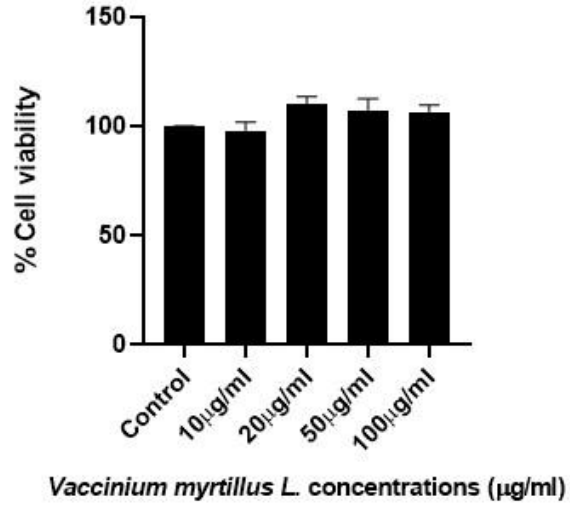

Fig. 1 – Effects of *V. myrtillus* L. extract on ECV304 cells at different concentrations for 48 hours on % cell viability (***$P < 0.001$, vertical bars show standard deviation values).

Gene expression analysis results

Gene expression analysis of *PON1*, *PON2* and *PON3* genes in TNF-mediated inflamed ECV304 cells, after addition of *V. myrtillus* L. to inflamed cells and control cells, was performed by Real Time PCR method. The results are presented in the Fig. 2. The results of gene expression analysis performed in our study show that *V. myrtillus* L. extract has a protective effect against TNF-α-induced inflammation.

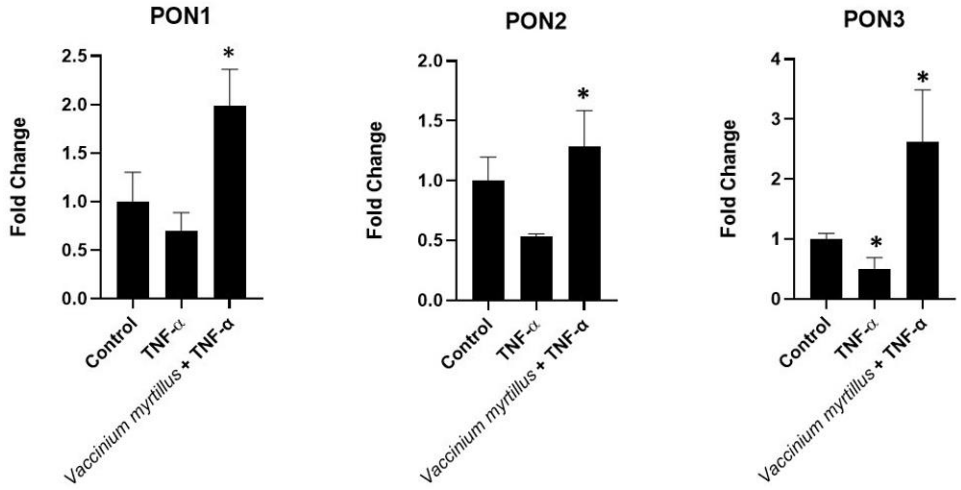

Fig. 2 – Gene expression levels of *PON1*, *PON2* and *PON3* genes with TNF-α-induced inflammation and effect of *V. myrtillus* L. extract on gene expression.

DISCUSSION

It is a widely accepted fact that *PON* functions as a cardioprotective agent in atherosclerosis and similar vascular disorders.[16] Experiments involving mice deficient in *PON1* provide strong evidence for the atheroprotective properties of *PON1*.[17,18] A significant reduction in plasma *PON1* activity promotes the atherogenic process in experimental models of hypercholesterolemia.[19] Overexpression of *PON2* in ApoE-deficient mice leads to the attenuation of atherosclerosis development, improvement in serum antioxidant capacity, and restoration of the anti-inflammatory function of high-density lipoprotein.[20] Hepatic inflammation and abnormalities in mitochondrial function, including respiration defects and increased

superoxide production, were observed in mice that lacked *PON3*.[21]

In clinical studies, bilberry fruit, juice, and powder have demonstrated anti-inflammatory and antioxidant effects. In another study, adults at high risk for cardiovascular disease who consumed bilberry juice exhibited reduced levels of circulating inflammation markers. These findings suggest a positive correlation between bilberry supplementation and a reduction in inflammation markers.[22] According to Lehtonen *et al*.,[23] overweight and obese women who consumed whole bilberries for 33-35 days experienced a reduction in plasma TNF-α levels.

Previous studies in the literature have demonstrated the protective effects of *V. myrtillus* L. extract against inflammation mediated by TNF-α, which is consistent with the findings of our study.[24,25] The observed increase in paraoxonase enzyme levels in diabetic rats treated with *V. myrtillus* L. extract suggests a potential protective effect of the extract.[26]

Based on our findings, *V. myrtillus* L. extract appears to have a potential cardioprotective effect by mitigating TNF-α-induced inflammation. Nonetheless, further investigations are required to elucidate the underlying mechanisms by which *V. myrtillus* L. extract confers these benefits and to establish the optimal dosage and administration for its use as a dietary supplement.

CONCLUSION

The findings of the presented suggest that the tested concentrations of *V. myrtillus* L. extract have a low proliferative effect on ECV304 cells, indicating that the extract could potentially be used preventively or therapeutically in cardiovascular diseases. The extract's antioxidant and anti-inflammatory properties are believed to be due to its flavonoid content. Additionally, the expression analysis of *PON1*, *PON2*, and *PON3* genes indicated increased expression in the experimental groups that treated with the *V. myrtillus* L. extract, providing further evidence of the extract's antioxidant and anti-inflammatory properties.

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