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The protective effect of the *Cornus mas* fruits (cornelian cherry) on hypertriglyceridemia and atherosclerosis through PPARα activation in hypercholesterolemic rabbits

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ABSTRACT

Cornelian cherry (*Cornus mas* L.) fruits have been used in traditional cuisine and in folk medicine in various countries. This study was conducted to evaluate the constituents and impact of cornelian cherry (*C. mas* L.) fruits lyophilisate on lipid levels, PPARα protein expression, atheromatous changes in the aorta, oxido-redox state, and proinflammatory cytokines in hypercholesterolemic rabbits. The HPLC–MS method was used for determining active constituents in cornelian cherry. In a subsequent *in vivo* study the protective effect of the cornelian cherry on diet-induced hyperlipidemia was studied using a rabbit model fed 1% cholesterol. Cornelian cherry (100 mg/kg b.w.) or simvastatin (5 mg/kg b.w.) were administered orally for 60 days. Two iridoids – loganic acid and cornuside – and five anthocyanins were identified as the main constituents of the cornelian cherry. The administering of the cornelian cherry led to a 44% significant decrease in serum triglyceride levels, as well as prevented development of atheromatous changes in the thoracic aorta. Cornelian cherry significantly increased PPARα protein expression in the liver, indicating that its hypolipidemic effect may stem from enhanced fatty acid catabolism. Simvastatin treatment did not affect PPAR-α expression. Moreover, the cornelian cherry had a significant protective effect on diet-induced oxidative stress in the liver, as well as restored upregulated proinflammatory cytokines serum levels. In conclusion, we have shown loganic acid to be the main iridoid constituent in the European cultivar of the cornelian cherry, and proven that the cornelian cherry could have protective effects on diet-induced hypertriglicerydemia and atherosclerosis through enhanced PPARα protein expression and *via* regulating oxidative stress and inflammation.

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Abbreviations: LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; HMG-CoA, 3-hydroxy-3-methyl glutaryl coenzyme A; PPARα, peroxisome proliferator-activated receptor alpha; UPLC, ultra-performance liquid chromatography; Q-TOF, quadruple time of flight; TNFα, tumor necrosis factor α; IL-6, interleukin 6; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; GPx, glutathione peroxidase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel; SREBP, sterol regulatory element-binding protein; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; NF-κB, nuclear factor-kappa B; PPARγ, peroxisome proliferator-activated receptor gamma; MMP-9, matrix metalloproteinase 9; FRAP, ferric reducing/antioxidant power; AMPK, AMP-activated protein kinase.

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Introduction

Enhanced low-density lipoprotein (LDL-C) and decreased high-density lipoprotein cholesterols (HDL-C) in serum are established, independent risk factors in atherosclerosis and cardiovascular disorders. However, whether enhanced triglyceride (TG) levels are causal in atherosclerosis and cardiovascular disorders have been ambiguous for several decades. Available data, however, including recently published follow-up studies and meta-analyses implicate TG as an independent risk factor for unstable plaque formation, heart disease, and mortality in the general population (Grønholdt, 1999; Langsted et al., 2011; Miller et al., 2011). The medicinal products of choice in hypercholesterolemia are statins, and in hypertriglicerydemia, fibrates. The main hypolipidemic mechanism of action of statins has been evident since 1970s. Statins act through the inhibition of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, the key enzyme in cholesterol synthesis in the liver. By contrast, fibrates have been used in medicine for over 80 years, although their mechanism of action has been unclear for many years. The studies done in the last decade have proven that fibrates increase expression of the peroxisome proliferator-activated receptor alpha (PPARα), nuclear receptors that play an important function in the regulation of the genes involved in lipid metabolism in the liver (Gervois and Mansouri, 2012). Generally, both statins and fibrates boast efficacy, although in some patients they cause insufficient lipid-lowering effects and their administering is long-term and often causes side effects that lead to discontinuation of treatment and the need to seek alternative treatments.

The family of Cornaceae includes approximately 40 species. The best-known species of *Cornus* is the Japanese cornelian cherry (*Cornus officinalis* Sieb. et Zucc.). There are many reports that either *C. officinalis* or iridoids isolated from it – loganin and morroniside – exert positive effects on lipid metabolism and possess antidiabetic effects (Park et al., 2009a,b; Yamabe et al., 2010; Park et al., 2010, 2011). The cornelian cherry (*Cornus mas* L.) is found in southern and central Europe and southwest Asia. In various countries, cornelian cherry was used for many years in traditional cuisine as well as in folk medicine. Unfortunately, nowadays cornelian cherry is not popular. Although it is available in many European countries, nevertheless it is consumed by only few people. In comparison to *C. officinalis*, little is known about the constituents and/or the effects of cornelian cherry. There are only a few recently published reports concerning iridoid constituents (Kucharska, 2012; West et al., 2012) and the possible impact of the cornelian cherry on lipid metabolism and the development of atherosclerosis (Rafieian-Kopaei et al., 2011; Asgary et al., 2013).

The aim of our study was to assess the constituents of lyophilisate from cornelian cherries and examine their effect on lipid metabolism, PPARα protein expression, lipid peroxidation, antioxidant and proinflammatory markers, and study the histopathological changes in the thoracic aortas and livers of hypercholesterolemic rabbits.

Materials and methods

Evaluation of cornelian cherry constituents

Plant materials and sample preparation

The cornelian cherries (*C. mas* L.) were obtained from the Bolestraszyce Arboretum and Institute of Physiography, Poland, in September 2010. The plant material was authenticated by Prof. Jakub Dolatowski, and the voucher specimen (BDPA 3 967) has been deposited at the Herbarium of Arboretum and Institute of Physiography in Bolestraszyce, Poland. The lyophilisate was prepared in the Department of Fruit, Vegetable and Cereals Technology at the Wroclaw University of Environmental and Life Science. The ripe cornelian cherries were washed, frozen, and stored frozen at −20 °C until lyophilisate processing. After freezing them and removing their pits the still-frozen samples were freeze-dried. During freeze-drying the pressure was reduced to 65 Pa. The temperature in the drying chamber was −60 °C, and the heating plate reached 30 °C. About 20 g of lyophilisate was obtained from 100 g of fruit. After freeze-drying, the samples were ground into powder using a laboratory mill.

Identification of compounds by UPLC–MS/MS

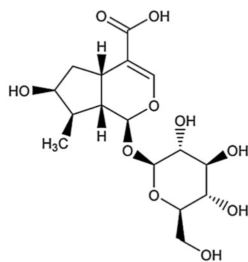
Compounds were identified through the method described by Sokół-Łętowska (2013), using the acquity ultra-performance liquid chromatography (UPLC) system coupled with a quadruple time of flight (Q-TOF) MS instrument (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) source. The instrument was operated both in positive and negative ion mode, scanning m/z from 100 to 1500 Da at a scan rate of 2.0 s/cycle. Separation was achieved on the Acquity™ BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm; Waters). Detection wavelengths were set to 245 (iridoids) and 520 nm (anthocyanins). The mobile phase was a mixture of 4.5% formic acid (A) and acetonitrile (B). The gradient program was as follows: 0–1 min, 1% B; 1–12 min, 1–75% B; 12–12.5 min, 75–100% B; 12.5–13.5 min, 100% B; 13.5–14.5 min, 1% B. The flow rate was 0.45 ml/min and the injection volume was 5 μl. The column was operated at 30 °C. The major operating parameters for the Q-TOF MS were set as follows: capillary voltage 2.0 kV, cone voltage 45 V, cone gas flow of 11 l/h, collision energy 50 eV, source temperature 100 °C, desolvation temperature 250 °C, collision gas, argon; desolvation gas (nitrogen) flow rate, 600 l/h; data acquisition range, m/z 100–1000 Da; ionization mode, negative. The data were collected with Mass-Lynx™ V 4.1 software.

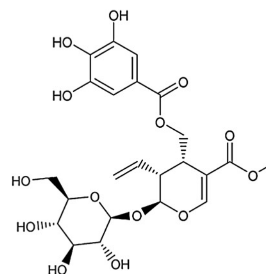
Determination of compounds by HPLC

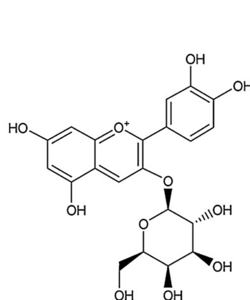
Iridoids, anthocyanins, phenolic acid, and flavonols were determined by the method described by Kucharska (2012) using the Dionex HPLC (Sunnyvale, CA, USA) system equipped with a diode array detector model Ultimate 3000, a quaternary pump LPG-3400A, an autosampler EWPS-3000SI, a thermostated column compartment TCC-3000SD, and controlled by Chromeleon v.6.8 software. Separation was performed on a Cadenza CDC18 (75 mm × 4.6 mm, 5 μm) column (Imtakt, Japan) with a guard column. Oven temperature was set to 30 °C. The mobile phase was composed of solvent A (4.5% formic acid, v/v) and solvent B (acetonitrile). The applied elution conditions were: 0–1 min, 5% B; 1–20 min, 5–25% B; 20–21 min, 25–100% B, 21–26 min, 100% B, 26–27 min, 100–5% B; 27–30 min, 5% B. The flow rate was 1.0 ml/min, and the injection volume was 20 μl. Loganic acid and cornuside were detected at 245 nm and anthocyanins at 520 nm. Iridoids were quantified as loganic acid and anthocyanins as cyanidin 3-*O*-glucoside. The results were calculated as mg of compound in 1 g dry weight of cornelian cherry fruits extract (mg/g of dw). All determinations were performed in duplicate. The chemical structures of the HPLC-identified compounds are shown in Fig. 1.

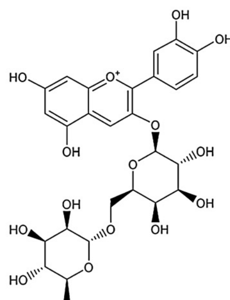
The in vivo study

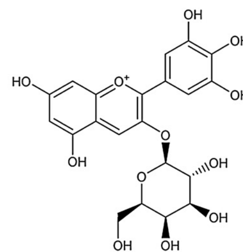
This experiment was performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and was approved by the Local Ethical Committee on Animal Research at the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław.

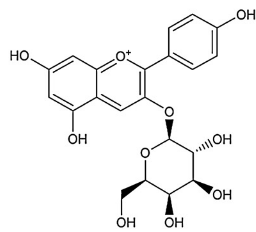












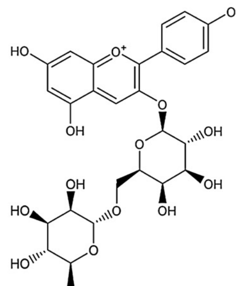


Fig. 1. The chemical structures of seven active constituents in cornelian cherry (*Cornus mas* L.).

Chemicals

Cornelian cherry lyophilisate was prepared as described above. Simvastatin was kindly delivered by Biofarm Sp z.o.o., Poland. The cholesterol was purchased from POCH S.A., Poland. Acetonitrile, formic acid, and methanol were purchased from Sigma–Aldrich (Steinheim, Germany). Acetonitrile for LC–MS was purchased from POCh (Gliwice, Poland). Loganic acid and cyanidin 3-*O*-glucoside were purchased from Extrasynthese (Lyon Nord, France).

Animals and treatment

Forty sexually mature New Zealand rabbits, aged from 8 months to 1 year were housed individually in chambers with the temperature maintained at 21–23 °C and a 12:12 h light-dark cycle. Before and during the experiment the animals had free access to water and received the same daily portion of feed (40 g/kg b.w.). After three weeks of acclimatization, the animals were randomly divided into 4 groups of 10 animals each. The animal feeding plan and scheduled administering of test substances are presented in the Table 1. For 60 consecutive days, the animals in the group P were supplied with standard feed. Groups CH, CH + CM, and CH + S were given the same feed + 1% cholesterol. Once daily, the rabbits orally ingested the following substances: groups P and CH – normal saline (negative and positive control), group CH + CM – cornelian cherry lyophilisate at a dose of 100 mg/kg. b.w., and group CH + S – simvastatin at a dose of 5 mg/kg b.w.

Just before the experiment and 60 days thereafter blood samples were collected from each animal from the marginal vein of the ear or saphenous vein. On the 60th day, the animals were euthanized with terminal anesthesia containing thiopenthal (250 mg/kg, administered intravenously). Their organs were collected, in particular the thoracic and abdominal aorta and liver for histopathological evaluations, and the liver for measurements of PPARα expression and oxido-redox state parameters.

Measurements of biochemical parameters

The serum levels of total cholesterol and triglycerides were estimated based on calorimetric, enzymatic methods. High-density

Table 1
Animal feeding plan and scheduled administering of test substances.

Control group P	Standard diet + saline p.o.
Experimental group CH	Standard diet with addition of 1% cholesterol + saline p.o.
Experimental group CH + CM	Standard diet with addition of 1% cholesterol + cornelian cherry (*Cornus mas* L.) lyophilisate 100 mg/kg b.w. p.o
Experimental group CH + SIM	Standard diet with addition of 1% cholesterol + simvastatin 5 mg/kg b.w. p.o.

lipoprotein (HDL-C) and low-density lipoprotein cholesterols (LDL-C) were measured using calorimetric, direct methods (Horiba ABX).

The atherogenic index of plasma was calculated by means of log (TG/HDL-C) and the cardiac risk ratio by means of TC/HDL-C ratio. The atherogenic coefficient was calculated by means of TC–HDL-C/HDL-C. The determination of fibrinogen in the plasma was based on the Clauss method (Q.F.A. Thrombin, Instrumentation Laboratory Co).

Measurements of proinflammatory parameters

Tumor necrosis factor α (TNFα Rabbit kit, Wuhan EIAab Science Co.) and IL-6 (Rabbit Interleukin 6, Wuhan EIAab Science Co.) were measured using ELISA methods.

Biomarkers associated with the oxido-redox state in the liver

All parameters in the liver were assessed spectrophotometrically. Malondialdehyde (MDA) level was assayed using the BIOXYTECH-MDA-586 kit (OxisResearch, USA) and expressed as μM/g of tissue. Glutathione (GSH) concentration was assayed using BIOXYTECH GSH-400 (OxisResearch, USA) and expressed as mM/mg of tissue. Superoxide dismutase (SOD) activity was measured using the Ransod kit (Randox Laboratories, UK) and expressed as U/mg of protein. Glutathione peroxidase (GPx) activity was measured using the GPx-340 kit (Randox Laboratories, UK) and expressed as U/mg of protein.

Preparation of nuclear fractions

Nuclear fractions were prepared using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific, 78835), following the manufacturer's instructions. Phenylmethylsulfonyl fluoride (Sigma, P7626) was added to CER I and NER to maintain the extract's integrity and function. Protein content was determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific, 23227) and the samples were stored at −80 °C.

Western blot analysis

In order to determine PPARα 10 μg of protein of each nuclear fraction was electrophoresed through 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). The proteins separated were transferred to a nitrocellulose membrane, blocked with 5% (w/v) skimmed milk in PBST (0.02% Tween 20) for 1 h and then incubated with primary antibodies to PPARα (Sigma, SAB 2104354-50UG) and β-actin (Santa Cruz Biotechnology, AC-15: sc-69879), respectively, overnight, at 4 °C. After the blots were washed, they were incubated with anti-rabbit (Sigma, A 6154) or anti-mouse (Vector laboratories, PI-2000) IgG HRP-conjugated secondary antibodies for 1 hour at room temperature. Each antigen-antibody complex was visualized using the chemiluminescent detection reagent ECL Plus Western Blotting Detection System (Amersham Biosciences, RPN2132) according to the supplier's instructions. The fluorescence signal was recorded with the Fuji Film FLA-3000 Fluorescent Image Analyzer (Raytest Isotopenmeßgeräte GmbH) using an SHG 473 nm excitation laser and a 520 nm emission filter. Fluorescent scans were analyzed using AIDA Bio-package software (Raytest Isotopenmeßgeräte GmbH).

Histopathological evaluations

Postmortem thoracic aorta, abdominal aorta, and liver specimens were isolated for histological examinations. Tissue sections were fixed in a 7% buffered formalin solution, embedded in paraffin, cut into 4 μm thick slices and stained with the hematoxylin and eosin method. Specimens were blindly examined by an experienced pathomorphologist.

Statistical analysis

Histopathological results were expressed as median, lower and upper quartiles and minimal and maximal values. The other results were expressed as mean ± standard deviation (mean ± SD). Statistical comparisons of histopathological data were performed using the Kruskall–Wallis test followed by the Dunn test for multiple comparisons. Other data were analyzed using Shapiro–Wilk's *W* test followed by an analysis of variance and a *post hoc* LSD test. Values of $p < 0.05$ were considered significant.

Results

We found two iridoids – loganic acid and cornuside – in the lyophilisate of the cornelian cherries that we investigated. We also detected five anthocyanins – delfinidin 3-*O*-galactoside, cyanidin 3-*O*-galactoside, pelargonidin 3-*O*-galactoside, cyanidin 3-*O*-robinobioside, and pelargonidin 3-*O*-robinobioside. The qualitative and quantitative composition of lyophilisate is presented in Fig. 2 and Table 2.

In the second part of experiment, we investigated the impact of orally administered cornelian cherry on dietary-induced hyperlipidemia in rabbits. Feeding for 60 days on the cholesterol rich diet-induced typical negative changes in lipid profile. The addition of cornelian cherry lyophilisate completely reversed these diet-induced changes in triglyceride levels. Cornelian cherry significantly decreased the concentration of triglycerides by 44%, compared to the group that received the cholesterol, to levels comparable in rabbits from group P, which received standard feed (Fig. 3A). As far as LDL and HDL cholesterol levels are concerned, we did not observe statistically significant changes after cornelian cherry treatment, however it increased HDL-cholesterol concentration 13.2% (Fig. 3B) and slightly decreased both total and LDL cholesterol levels compared to group CHOL (Fig. 3C and D).

The atherogenic index of plasma defined as log (triglyceride/HDL cholesterol) was significantly decreased in group CHOL + CM compared to group CHOL. Moreover, its level was insignificant in comparison with group P. Similar results were seen in group CHOL + SIM (Fig. 4A). Cardiac risk ratio defined as the total cholesterol/HDL-cholesterol ratio was not statistically different between groups CHOL and CHOL + CM. Nevertheless, the cardiac risk ratio in the CHOL + CM group was slightly lower. Simvastatin significantly restored, increased by cholesterol feeding CRR value (Fig. 4B). Similar results were obtained analyzing the atherogenic coefficient measured as TC – HDLC/HDLC (Fig. 4C).

The rabbits in the groups studied did not differ in body weight throughout the experiment (data not shown).

Cornelian cherry significantly increased expression of PPARα in the liver compared to all other groups, and this effect was independent of the provision of cholesterol rich or standard feed. Simvastatin treatment did not affect PPARα expression (Fig. 5).

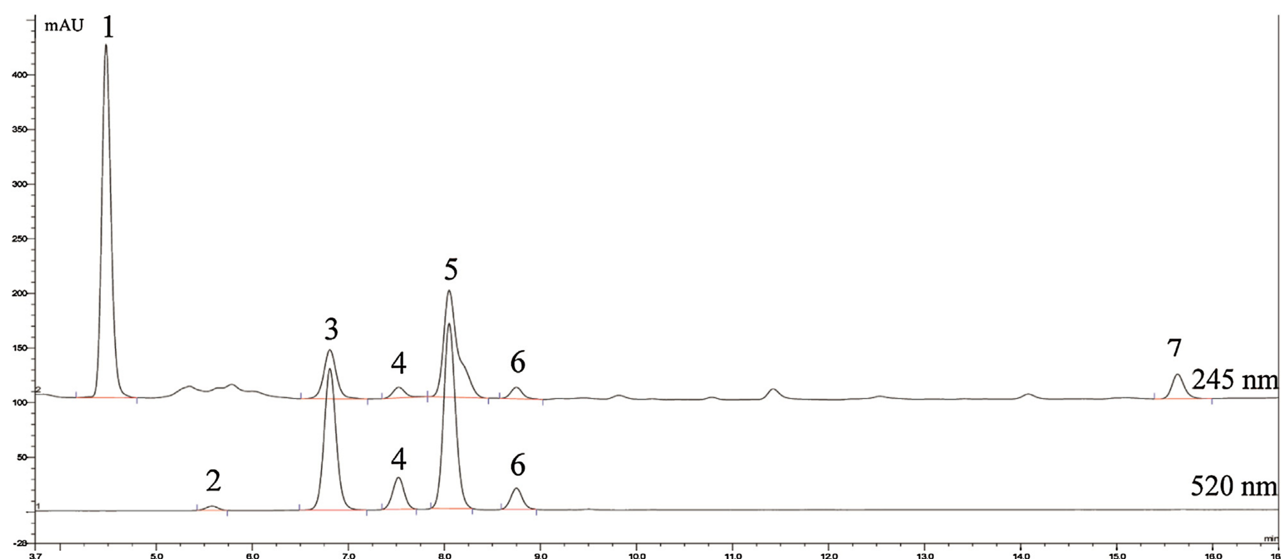


Fig. 2. HPLC-DAD chromatograms (254 nm; 520 nm) of main compounds (iridoids and anthocyanins) of lyophilizate from the cornelian cherries (*Cornus mas* L.) fruits (1: loganic acid; 2: delphinidin 3-*O*-galactoside; 3: cyanidin 3-galactoside; 4: cyanidin 3-robinobioside; 5: pelargonidin 3-galactoside; 6: pelargonidin 3-robinobioside; 7: cornuside).

Table 2
Content and characterization of selected constituents of lyophilizate from cornelian cherry (*Cornus mas* L.) fruits using their spectral characteristic in HPLC-DAD (λ_{max}) and negative and positive ions in UPLC-ESI/MS (MS and MS/MS).

Peak	Compound	Mean ± SD[a] (mg/100 g)	λ_{max} (nm)	Ion mode	MS (*m*/*z*)	MS/MS (*m*/*z*)
1	Loganic acid	820.4 ± 68.0	245	–	375.1316	213.0739
2	Delphinidin 3-*O*-galactoside	2.5 ± 0.8	522	+	465.1034	303.0532
3	Cyanidin 3-*O*-galactoside	123.5 ± 19.7	515	+	449.1063	287.0536
4	Cyanidin 3-*O*-robinobioside	15.1 ± 5.1	516	+	595.1664	287.0536
5	Pelargonidin 3-*O*-galactoside	87.9 ± 19.9	426, 500	+	433.1168	271.0601
6	Pelargonidin 3-*O*-robinobioside	6.7 ± 2.6	426, 501	+	579.1717	271.0601
7	Cornuside	99.1 ± 16.1	243, 271	–	541.1566	169.0136

[a] Standard deviation.

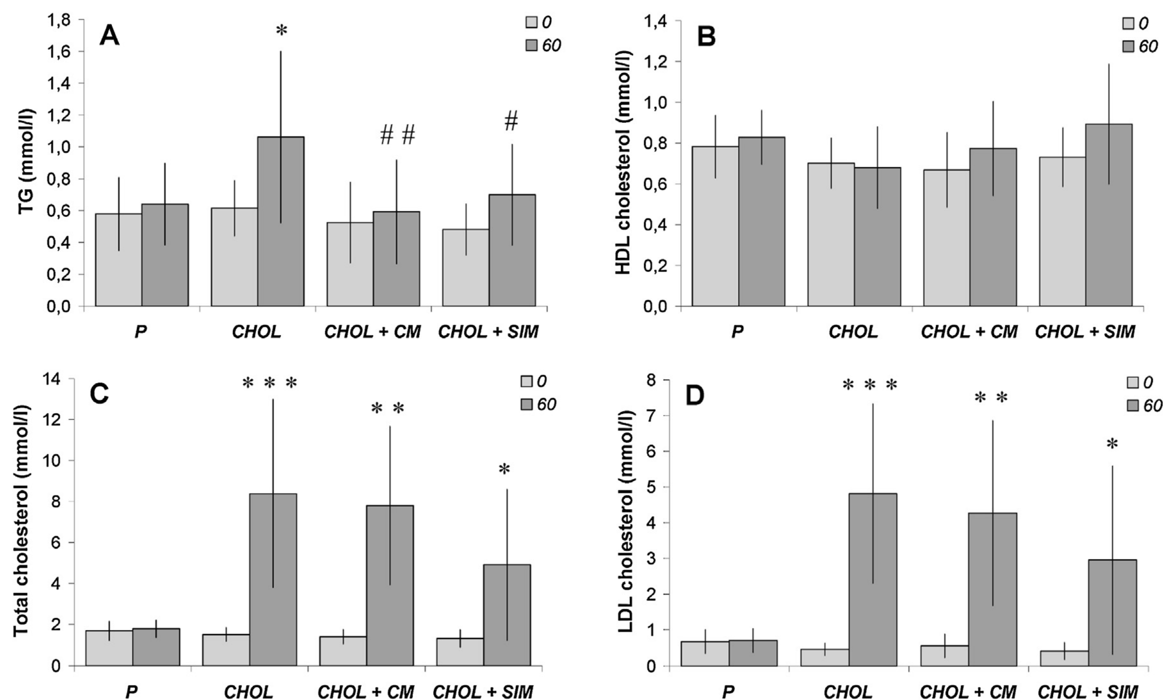


Fig. 3. Triglycerides (A), HDL cholesterol (B), total cholesterol (C), and LDL cholesterol (1D) concentrations on 0th and 60th days of the experiment. P, standard feed and vehicle-treated rabbits, CHOL, cholesterol-treated rabbits, CHOL + CM, cholesterol + cornelian cherry 100 mg/kg body weight-treated rabbits, CHOL + SM, cholesterol + simvastatin 5 mg/kg body weight-treated rabbits. Values are presented as the mean ± SD. Specific comparisons: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* P, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* CHOL.

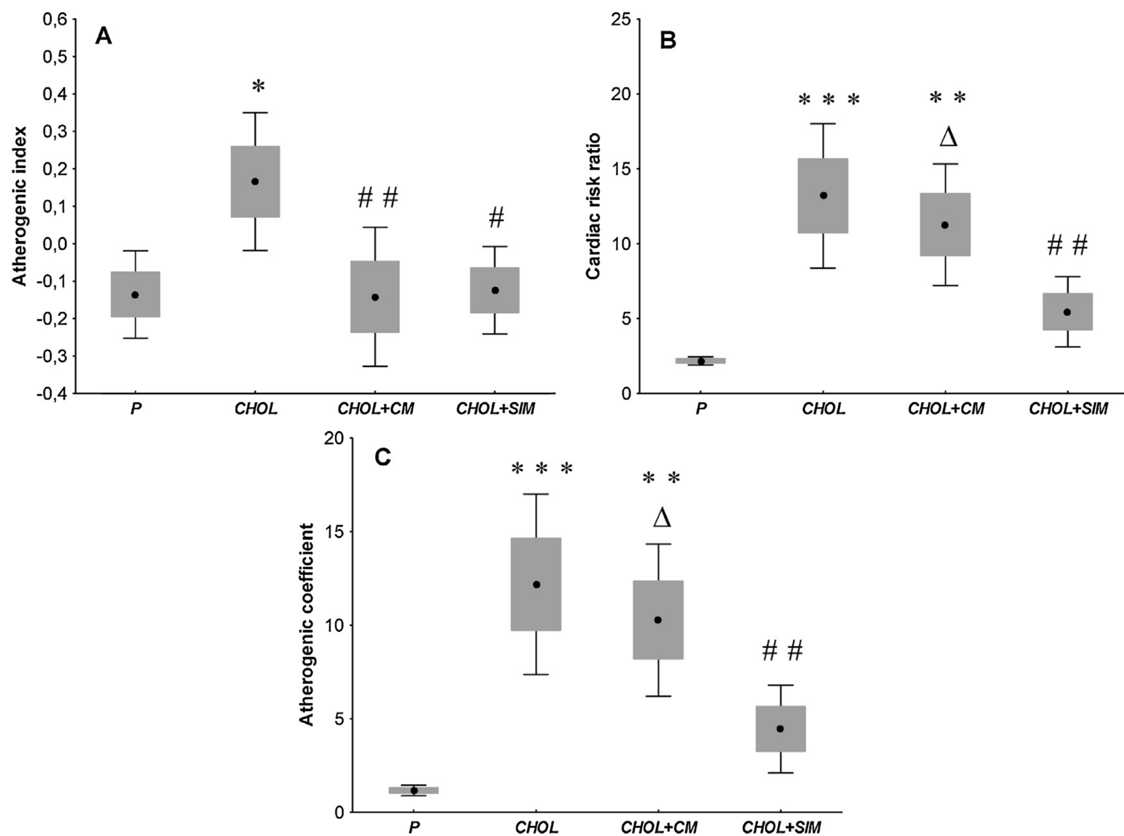


Fig. 4. Atherogenic index (A) defined as log (triglyceride/HDL cholesterol ratio), cardiac risk ratio (B) measured as total cholesterol/HDL cholesterol ratio, and Atherogenic Coefficient (C) measured as TC–HDLC/HDLC on 0th and 60th days of the experiment. P, standard feed and vehicle-treated rabbits, CHOL, cholesterol-treated rabbits, CHOL + CM, cholesterol + cornelian cherry 100 mg/kg body weight-treated rabbits, CHOL + SM, cholesterol + simvastatin 5 mg/kg body weight-treated rabbits. Values are presented as the mean ± SD. Specific comparisons: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* P, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* CHOL.

Changes in lipid peroxidation and ox-redox status in the livers were measured by MDA concentrations and both enzymatic and non-enzymatic oxidative markers. MDA concentration in the group that received the cholesterol increased significantly in comparison to group P. The addition of cornelian cherry significantly decreased the MDA level in group CHOL + CM compared to group CHOL, although it was still higher than in group P. Simvastatin treatment completely reversed the increased MDA level caused by the administering of cholesterol (Fig. 6A). Significant depletion of glutathione concentration was seen in the group treated with cholesterol. Cornelian cherry treatment significantly increased GSH level in comparison to group CHOL. However, the glutathione concentration in group CHOL + CM was still higher than in group P. Simvastatin completely prevented a decrease in glutathione concentration (Fig. 6B). No significant changes in GPx and SOD activities were noticed between the experimental groups (Fig. 6C and D).

Cornelian cherry significantly reversed the increased serum level of IL-6 caused by the administering of cholesterol (Fig. 7A). A similar result was obtained in the case of TNFα, whose level on the 60th day of the experiment was even lower than the baseline (Fig. 7B).

No significant changes in the fibrinogen plasma level were observed in the groups examined (Fig. 8).

After 60 days of the experiment, there were no visible atherosclerotic changes in the aortae of rabbits in the control group, whereas the cholesterol-treated rabbits developed pronounced atheromatous changes in the thoracic and abdominal aortae. The typical atherosclerotic plaque was composed of inflamed cells, proliferated fibroblasts, assembling macrophages and foam cells, fibrin, and amorphous masses. Administering both cornelian cherry and simvastatin significantly prevented the formation of atheromatosis in the thoracic aorta. In the abdominal aorta simvastatin significantly reversed atheromatosis development, whereas cornelian cherry showed a moderate, but not statistically significant, decrease compared to the cholesterol- treated rabbits (Figs. 9 and 10). Half of the rabbits in group, CHOL + CM showed no pathological changes in either the aortae, and in the other half of the animals the changes were limited to slight damages in endothelium

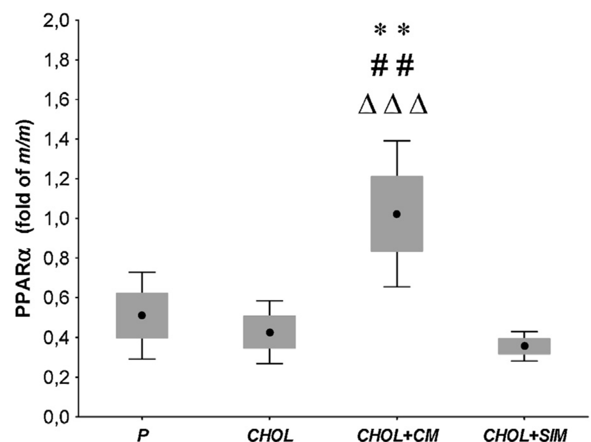


Fig. 5. Liver PPAR-α expression. P, standard feed and vehicle-treated rabbits, CHOL, cholesterol-treated rabbits, CHOL + CM, cholesterol + cornelian cherry 100 mg/kg body weight-treated rabbits, CHOL + SM, cholesterol + simvastatin 5 mg/kg body weight-treated rabbits. Values are presented as the mean ± SD. Specific comparisons: $^{**}p < 0.01$ *vs.* P, $^{\#\#}p < 0.01$ *vs.* CHOL, $^{\Delta\Delta\Delta}p < 0.001$ *vs.* CHOL + SIM.

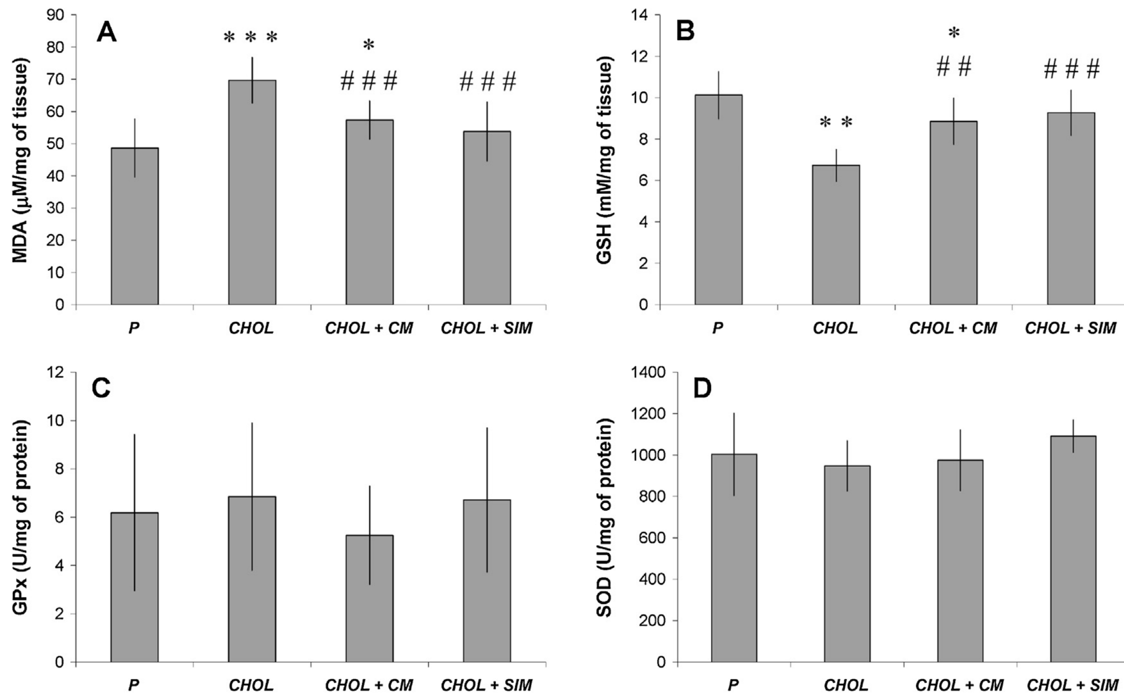


Fig. 6. Malondialdehyde concentrations (A), glutathione concentrations (B), GPX activities (C), and SOD activities (D) in the liver. P, standard feed and vehicle-treated rabbits, CHOL, cholesterol-treated rabbits, CHOL + CM, cholesterol + cornelian cherry 100 mg/kg body weight-treated rabbits, CHOL + SM, cholesterol + simvastatin 5 mg/kg body weight-treated rabbits. Values are presented as the mean ± SD. Specific comparisons: $^{*}p < 0.05$, $^{***}p < 0.001$ *vs.* P, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* CHOL.

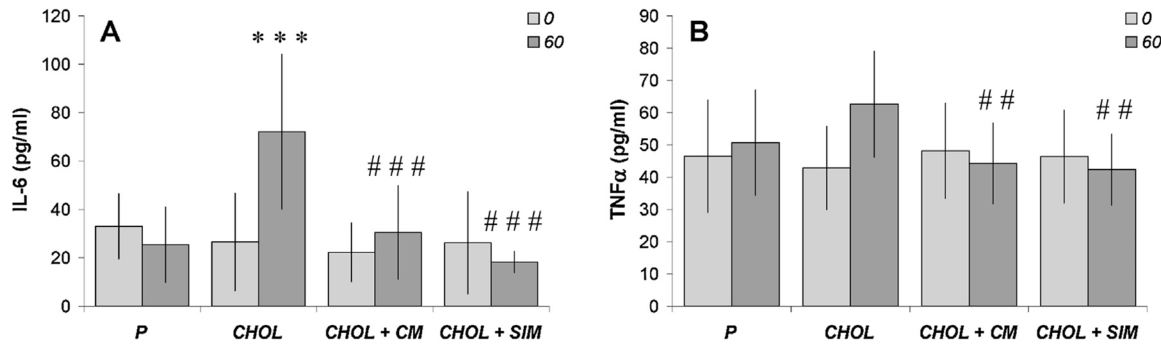


Fig. 7. IL-6 (A) and TNFα (B) activities in the plasma. P, standard feed and vehicle-treated rabbits, CHOL, cholesterol-treated rabbits, CHOL + CM, cholesterol + cornelian cherry 100 mg/kg body weight-treated rabbits, CHOL + SM, cholesterol + simvastatin 5 mg/kg body weight-treated rabbits. Values are presented as the mean ± SD. Specific comparisons: $^{***}p < 0.001$ *vs.* P, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* CHOL.

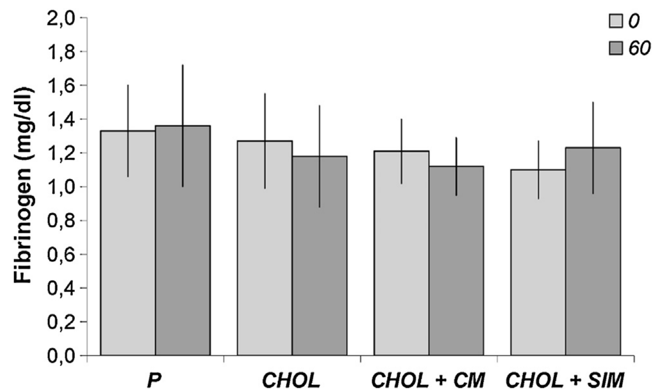


Fig. 8. Fibrinogen levels in the plasma. P, standard feed and vehicle-treated rabbits, CHOL, cholesterol-treated rabbits, CHOL + CM, cholesterol + cornelian cherry 100 mg/kg body weight-treated rabbits, CHOL + SM, cholesterol + simvastatin 5 mg/kg body weight-treated rabbits. Values are presented as the mean ± SD.

and inner layer and small subendothelial deposits of lipids. In group CHOL + SIM, most of the animals developed no injuries in their aortae, and the changes observed referred mainly to cell adhesion to the endothelium and its slight injuries.

Discussion and conclusion

The most important results of our study are the following: (1) we found loganic acid to be a main iridoid constituent of liophylisate in the European cultivar of the cornelian cherry, (2) oral administering of this lyophylisate significantly decreased triglycerides in serum, diminished the formation of atherosclerotic changes in the aorta, had a protective effect on lipid peroxidation, the redox system, and proinflammatory cytokines in hypercholesterolemic rabbits, (3) The cornelian cherry significantly increased expression of PPARα in the liver. This suggests that the beneficial effect of the cornelian cherry on lipid turnover in a hyperlipidemic diet is a result of increased liver fatty acid catabolism, and (4) Simvastatin treatment did not affect PPAR-α expression.

Some of the limitations of this data should be considered. The disadvantages of our experiment are the small sample of animals, which might influence the statistical power of the results, especially differences in the cholesterol levels among the groups examined. Moreover, we decided to compare the cornelian cherry's effects to simvastatin, though the effects of the cornelian cherry observed in this study were more similar to fibrates, with a significant decrease of triglycerides and only a slightly beneficial effect on cholesterol levels. These effects have not been observed in previous studies. Nevertheless, we obtained interesting additional data about the impact of simvastatin on PPARα expression, the redox system, and inflammatory cytokines.

Iridoids and anthocyanins are considered the major bioactive compounds found in the *Cornus* genus (Vareed et al., 2006). We found loganic acid to be a main iridoid in lyophilisate. To our knowledge, only one study by West et al. has described loganic acid as a constituent of cornelian cherries (West et al., 2012), and it has rarely been found in much lower amounts as a non-predominant constituent in other *Cornus* species (Yamabe et al., 2007). Additionally, we revealed that cornuside is a second iridoid. This constituent was also found in other Cornaceae, especially *C. officinalis* (Du et al., 2008).

We also detected 5 anthocyanins: 3 galactosides of delfinidin, cyanidin, and pelargonidin, and 2 robinobiosides of cyanidine and pelargonidine. Among those, two anthocyanins (cyanidin 3-*O*-galactoside and pelargonidin 3-*O*-galactoside) were dominant. Anthocyanins are the most diverse group of plant flavonoids. They are pharmacologically polyvalent natural products exerting several positive effects. Previous studies have proven their strong antioxidant, antihypertonic, anti-obesity and lipid-lowering properties (Tsuda, 2012). Antidiabetic effects of anthocyanins have been also shown in earlier reports. Anthocyanins decreased blood glucose level and peripheral insulin resistance both in animals models of diabetes and obesity (Sarikaphuti et al., 2013) and human cross-sectional study (Jennings et al., 2014). It has been shown that the total content of anthocyanins in fruits of various *Cornus* species is higher than in other native or cultivated edible small fruits and vegetables (Vareed et al., 2006; Pantelidis et al., 2007), although content may differ greatly between cultivars (Popović et al., 2012). The qualitative composition found in our cultivar was different from cultivars from Asia (Tural and Koca, 2008).

In the second part of our experiment, we assessed the *in vivo* effects of the cornelian cherry in hypercholesterolemic rabbits. We found a significant positive effect of cornelian cherries on triglycerides and slightly beneficial effects on cholesterol concentrations, accompanied by reduced atheromatous lesions in the aorta. Analysis of the aortic atherosclerotic lesions proved that the cornelian cherry reduced both the amount of atherosclerotic plaque and prevented the infiltration of inflammatory cells and the accumulation of foam cells. Our results are in accordance with the study by Jayaprakasam et al., who discovered the positive impact of pure anthocyanins mixture isolated from cornelian cherries on obesity and lipid accumulation in the liver in high-fat-fed C57BL/6 mice (Jayaprakasam et al., 2006). Another study by Rafieian-Kopaei et al. revealed a similar result regarding LDL and HDL cholesterol and triglycerides serum concentrations in rabbits by administering whole dried cornelian cherries from Iranian cultivars, though the total cholesterol increased over 30%. The constituents of the cornelian cherry and the mechanism of action was not examined in that experiment (Rafieian-Kopaei et al., 2011). In another recently published study Asgary et al. consumption of the Iranian cultivar of the cornelian cherry by dyslipidemic children and adolescents decreased either the total and LDL cholesterol or triglycerides, as well as increased HDL-cholesterol compared to baseline results. However, no significant differences were seen comparing cornelian cherry-treated and control subjects on a standard diet (Asgary et al., 2013). The possible positive effects of the *Cornus* species are also supported by previous studies describing their impact on lipid metabolism from both whole extract of *C. officinalis* Sieb. et Zucc. fruits and their isolated constituents, especially loganin and morroniside (Park et al., 2009a,b; Yamabe et al., 2010).

Lipid-lowering agents may act through several mechanisms. In this study, we found a pronounced increase in PPARα protein expression in the liver of cornelian cherry supplemented rabbits. Enhanced expression was revealed compared to both standard and hypercholesterolemic diet-fed control groups. Similar results to those in our study were described by Park et al. (2009a) and Yamabe et al. (2010) examining *C. officinalis* and loganin isolated from it. In our study, simvastatin did not affect PPARα expression. This result is opposite to earlier reports suggesting that the pleiotropic effects of simvastatin involve anti-inflammatory actions through PPARα activation (Paumelle et al., 2006; Rinaldi et al., 2011). PPARα belongs to the nuclear receptors and is a ligand-activated transcription factor that influences the expression of multiple genes contributing to the regulation of lipid catabolism and inflammatory processes (Fruchart, 2009). Both the endogenous and exogenous activators of PPARα possess lipid-lowering properties mainly through their ability to induce β-oxidative fatty acids catabolism in the liver (Gervois and Mansouri, 2012). The other possible mechanisms responsible for the beneficial effects of PPARα activation on lipids comprise enhanced expression of apolipoprotein A1 and A2 and increased HDL-mediated cholesterol efflux from macrophages (Fruchart, 2009). In our study, increased PPARα expression was associated with a significant decrease in TG and the atherogenic index, as well as a nonsignificant increase of HDL-cholesterol. We also observed a concomitant decrease in pro-inflammatory cytokines levels. Similar effects to those in our study were described in patients with atherosclerosis after treatments with fenofibrate (Madej et al., 1998). The anti-inflammatory action of PPARα agonists has been shown in vascular cells *via* the inhibition of inflammatory cytokines IL-1, IL-6, TNFα, and adhesion molecules–vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Staels et al., 1998; Delerive et al., 1999; Gervois and Mansouri, 2012). Additionally, PPARα ligands, through the repression of nuclear factor-kappa B (NF-κB) signaling, diminish expression of cyclooxygenase-2 (Delerive et al., 1999). Through an impact on lipid distribution and inflammation in the arteries, PPAR-α may attenuate several stages of atherosclerotic plaque formation. Moreover, PPARα agonists may protect against the proliferation of smooth muscle cells in vessels (Fruchart, 2009) and decrease the secretion of matrix metalloproteinase 9

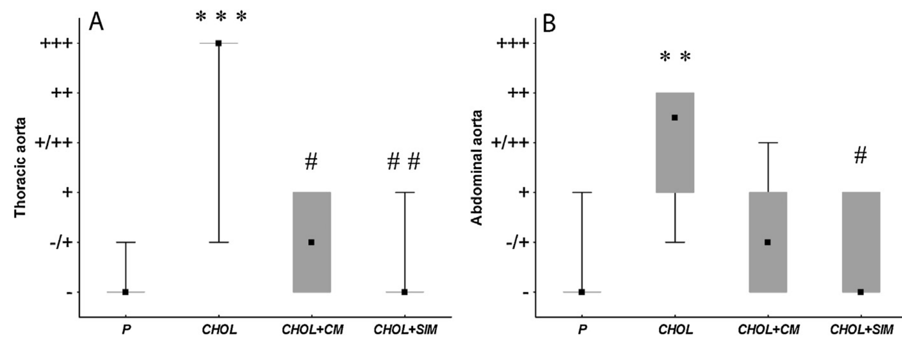


Fig. 9. Histopathological changes in the thoracic (A) and abdominal (B) aorta in rabbits in experimental groups (HE staining). Scoring: /−/, no visible endothelium damage; /+/, lipids accumulated under endothelium; /++/, lipids accumulated under endothelium, fibrin, moderate infiltration of inflamed cells; /+++/, lipids accumulated under endothelium, fibrin, infiltration of inflamed cells and fibroblast, glycosaminoglycans. P, standard feed and vehicle-treated rabbits, CHOL, cholesterol-treated rabbits, CHOL + CM, cholesterol + cornelian cherry 100 mg/kg body weight-treated rabbits, CHOL + SM, cholesterol + simvastatin 5 mg/kg body weight-treated rabbits. Values are presented as median, lower and upper quartiles, and minimal and maximal values. Specific comparisons: $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* P, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* CHOL

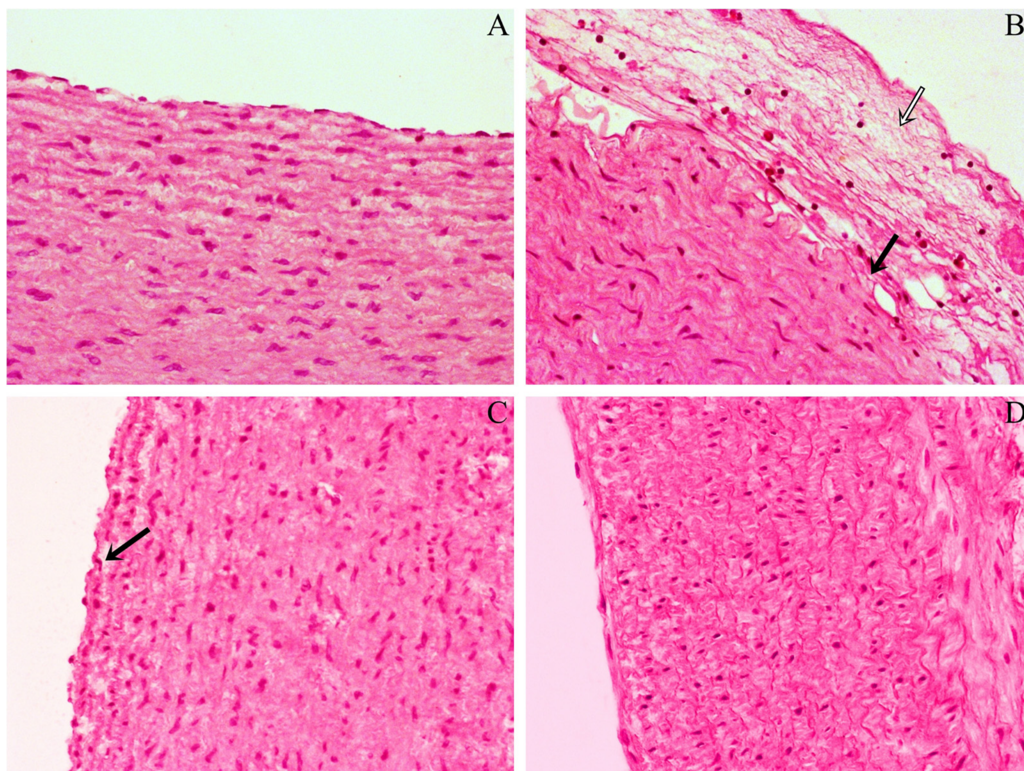


Fig. 10. Representative photos showing histological cross-sections of thoracic aortas with hematoxylin and eosin (HE) staining, 200× (A) control group (rabbit nr 5). Normal endothelium and media; (B) group CHOL (rabbit nr 12). The black arrow shows undermined epithelium and the white arrow shows a thick layer composed of fibrin, deposits of amorphous masses, infiltrated fibroblasts, lymphocytes, and macrophages; (C) CHOL + CM group (rabbit nr 22). The black arrow shows slightly undermined epithelium. (D) CHOL + SIM group (rabbit nr 37). Normal endothelium and media.

(MMP-9) in human monocytic cells (Shu et al., 2000). In atherosclerotic lesions, produced by foam cells, matrix metalloproteinases degrade the extracellular matrix. This increases plaque instability and promotes plaque rupture and thrombus formation, responsible for clinical acute cardiovascular events (Gervois and Mansouri, 2012).

It should be considered that the main constituents of the *Cornus* genus may function due to various mechanisms of action, which may also not be directly connected with PPAR activation. Kang et al. has revealed that cornuside, found in both *C. officinalis* and *C. mas*, including our cultivar, dilates vascular smooth muscle *via* endothelium dependent nitric oxide (NO)/cGMP signaling (Kang et al., 2007a). Cholesterol efflux from macrophages and foam cells is the possible explanation of anthocyanins' influence on lipid metabolism (Xia et al., 2005; Qin et al., 2009). Moreover, the observable effects of biological functioning of whole fruits may stem either from the sum of individual constituents' functioning, or from the prevailing functioning of one of the constituents.

In hyperlipidemic subjects oxidative stress and lipid peroxidation contribute heavily to the development of atherosclerosis and its complications. We show a marked decrease in MDA and an increase in glutathione concentrations in the livers of either cornelian cherry or simvastatin-treated rabbits. Analyzing GPx levels a slight but insignificant decrease was seen in the cornelian cherry group, whereas no changes were noted in the simvastatin group. Previous *in vitro* studies showed the strong antioxidant capacities of cornelian cherries, although those capacities differed between the

cultivars analyzed (Samec and Piljac Žegarac, 2011; Popović et al., 2012).

Although cornelian cherries possess marked *in vitro* antioxidant potentials correlated with high anthocyanins and phenol content, many concomitantly investigated cultivars of other plants possessed either higher antioxidant activities or superior anthocyanins and total phenol contents (Pantelidis et al., 2007; Celep et al., 2012). This raises the question of whether the cornelian cherry is one of the anthocyanin rich fruits and whether its health benefits are similar to other berries. Or, does it possess other unique biological activities? Moreover, *in vitro* antioxidant properties may not exactly correlate with *in vivo* antioxidant functioning, which requires the activation of the recipient's ox-redox system.

The cornelian cherry's *in vivo* antioxidant effect and ability to protect against lipid peroxidation were seen in described above study by Rafieian-Kopaei et al. (2011), whose findings are consistent with ours. They are also consistent with similar protective effects concerning the oxidative stress of *C. officinalis* (Peng et al., 1998) and iridoids isolated from *C. officinalis* – loganin and morroniside – in diabetic mice and renal mesangial cell cultures exposed to advanced glycation end products (Xu et al., 2006; Park et al., 2010, 2011; Yamabe et al., 2010). Nevertheless, we found no surveys assessing *in vivo* loganic acid impact on the ox-redox system. Anthocyanins, the second main constituents of the *Cornus* genus, and generally flavonoids, also possess antioxidant properties. Human studies show correlations between anthocyanins intake and postprandial increases in serum antioxidant capacity (Mazza et al., 2002).

Aside from the factors described above, inflammation contributes significantly to the development and consequences of atherosclerosis (Libby 2012). We investigated two cytokines involved in systemic inflammation. TNFα is a key proinflammatory cytokine involved in several stages of inflammation. IL-6 exerts both pro- and anti-inflammatory effects and may partially inhibit TNFα synthesis (Di Santo et al., 1997). We found significantly lower concentrations of either IL-6 or TNFα in both the cornelian cherry and simvastatin groups when compared to the cholesterol-treated rabbits. These results are consistent with the anti-inflammatory properties of PPARα, which act *via* the inhibition of inflammatory cytokines and adhesion molecules. Expressed by endothelial cells, adhesion molecules VCAM-1 and ICAM-1 play an important role in the early phase of atherosclerosis development (Libby 2012). Asgary et al. found decreased levels of adhesion molecules in children after administering the Iranian cultivar of the cornelian cherry (Asgary et al., 2013). The similar *in vitro* effect of cornuside isolated from *C. officinalis* was observed by Kang et al. The addition of cornuside to human umbilical vein endothelial cell cultures diminished the mRNA expression of VCAM-1 and ICAM-1, as well as decreased TNFα induced NF-kB activation (Kang et al., 2007b).

Lastly, we examined fibrinogen levels. Fibrinogen is the main coagulation protein in blood that contributes to regulation of blood viscosity and platelet aggregation. Several studies have shown a positive correlation between fibrinogen level and coronary heart disease and strokes (Fibrinogen Studies Collaboration, 2005). In a previous study, Asgary et al. found that cornelian cherry powder at a dose of 1 g/kg b.w. significantly decreased fibrinogen levels (Asgary et al., 2010). In this study, we did not confirm these results. Instead, we found no statistically significant differences in fibrinogen levels among the groups we examined, although we used lyophlisate in a different daily dose of 100 mg/kg b.w.

To summarize, we have shown loganic acid to be a predominant constituent of the cornelian cherry and demonstrated the pronounced protective qualities of the cornelian cherry regarding diet-induced hypertrigliceridemia and the development of atherosclerosis through the activation of PPARα receptors in the liver. Future studies should focus on the constituents of the cornelian cherry, especially loganic acid and their effect on lipid metabolism.

Author contributions

Tomasz Sozański contributed to the conception and design of the study, participation in acquisition of all laboratory data, literature search, analysis and interpretation of data collected, main contribution in drafting of the article, and final approval and guarantor of manuscript. Alicja Z Kucharska contributed to the acquisition of laboratory data – identification and determination of compounds by HPLC–MS/MS, drafting in manuscript part of the methods section concerning HPLC–MS/MS, and critical revision of manuscript and its approval to be submitted. Antoni Szumny contributed to the acquisition of laboratory data – identification and determination of compounds by HPLC–MS/MS and critical revision of manuscript and its approval to be submitted. Jan Magdalan contributed in literature search, analysis and interpretation of data collection, and critical revision of manuscript and its approval to be submitted. Katarzyna Bielska contributed to the acquisition of laboratory data – determination of PPARα, drafting in manuscript part of the methods section concerning PPARα, and critical revision of manuscript and its approval to be submitted. Merwid-Ląd Anna contributed in the literature search, analysis and interpretation of data collection and critical revision of manuscript and its approval to be submitted. Woźniak Anna contributed in feeding and caring for the animals, blood and organs collection and critical revision of manuscript and its approval to be submitted. Dzimira Stanisław contributed to the histopathological evaluations and critical revision of manuscript and its approval to be submitted. Piórecki Narcyz contributed in providing cornelian cherry fruits and in critical revision of manuscript and its approval to be submitted. Trocha Małgorzata contributed to the literature search, analysis and interpretation of data collected, drafting of the article and to the critical revision of manuscript and its approval to be submitted.

Conflict of interest

None.

Acknowledgment

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