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Iridoid–loganic acid *versus* anthocyanins from the *Cornus mas* fruits (cornelian cherry): Common and different effects on diet-induced atherosclerosis, PPARs expression and inflammation

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ABSTRACT

Background and aims: Cardiovascular benefits of fruits are attributed mainly to their (poly)phenolic constituents, especially anthocyanins. The main aim of our study is to compare effects of iridoids and anthocyanins from one fruit on diet-induced atherosclerosis. The cornelian cherry is a native or cultivated plant that grows in many European countries, used in cuisine and folk medicine. In our previous study, we showed its constituents and proved that oral administration of lyophilized fruits to hypercholesterolemic rabbits had preventive effects on atherosclerosis through the activation of PPARα expression. In this study, we have compared the effects of the main constituents of the cornelian cherry:iridoid loganic acid and anthocyanins.

Methods: Our experiment followed the model used in our previous study, in which rabbits were fed 1% cholesterol.

Results: We showed that both loganic acid (20 mg/kg b.w.) and a mixture of anthocyanins (10 mg/kg b.w.) administered orally for 60 days had a positive impact on dyslipidemia caused by cholesterol-rich diet, although the effects of anthocyanins were more pronounced. Anthocyanins decreased total and LDL-cholesterol and triglycerides and increased HDL-cholesterol. Loganic acid showed similar effects, but only the triglycerides and HDL-cholesterol changes achieved statistical significance. Anthocyanins, and to a lesser extent loganic acid, significantly decreased intima thickness and intima/media ratio in the thoracic aorta. Both substances decrease ox-LDL in the plasma. Anthocyanins significantly increased expression of PPARγ and α in the liver. Loganic acid also increased their expression, but to a lesser extent. Conversely, loganic acid showed pronounced anti-inflammatory effects, decreasing TNF-α and IL-6 activity.

Conclusions: Our results imply that both substances have a positive effect on factors contributing to the development of diet-induced atherosclerosis. Our results also indicate the potential health benefits of fruits containing anthocyanins and iridoids, and support the idea of creating composed phytopharmaceuticals containing both groups of substances.

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Abbreviations

LDL-C	low-density lipoprotein cholesterol
HDL-C	high-density lipoprotein cholesterol
TG	triglyceride
PPARα	peroxisome proliferator-activated receptor alpha
PPARγ	peroxisome proliferator-activated receptor gamma
b.w.	body weight
ox-LDL	oxidized low-density lipoproteins
AIP	atherogenic index of plasma
CRR	cardiac risk ratio
LC-MS	liquid chromatography–mass spectrometry
UPLC	ultra-performance liquid chromatography
Q-TOF	quadruple time of flight
ESI	electrospray ionization
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

1. Introduction

In modern societies, there is an increasing conviction that a diet based on natural, unprocessed plant products is a key factor in determining our health. However, scientific, evidence-based knowledge about which constituents, in which composition, and by which mechanism of action prevent the development of certain diseases, is still not fully elucidated.

Hyperlipidemia and its cardio and cerebrovascular consequences are among the leading causes of morbidity and mortality in developed countries [25]. Annual deaths in Europe from cardiovascular diseases still exceed 4 million people [36]. Yet the risk factors of such diseases may be strongly modulated by diet and lifestyle. An Interheart study of 52 countries published in *Lancet* has shown that not consuming enough fruits and vegetables is one of the nine main modifiable risk factors responsible for 90% of myocardial infarction [49].

Most animal and human studies investigating the effects of fruits on cardiovascular diseases have focused on their (poly) phenol constituents – flavonoids, especially anthocyanins. There are several previous studies showing that both anthocyanins rich fruits and isolated anthocyanins exert pronounced beneficial effects on the cardiovascular system [37,4,5]. However, the biological effects of food are often the result of the effects of their several active constituents. There is also the interesting question of whether a combination of isolated selective constituents with a proven mechanism and biological activity at a higher concentration may be a way to achieve beneficial additive or synergistic effects. To our knowledge, there are no studies that have compared the biological effects of anthocyanins and other prevalent constituents isolated from the same plants on the cardiovascular system. We know of only a few fruits containing considerable amounts of both anthocyanins and iridoids: cranberries, lingonberries, and blueberries [17]. It is noteworthy that these fruits have shown a beneficial effect on lipids, obesity, and cardiovascular diseases in previous studies [1,48,39]. However, the effects of isolated anthocyanins and iridoids from these fruits, to our knowledge, have not been compared.

The cornelian cherry contains considerable amounts of anthocyanins and iridoids. In our previous study, we showed the constituents of the European cultivar of the cornelian cherry to feature loganic acid as a predominant iridoid, cornuside as a second iridoid in lower concentration, and a mixture of 5 anthocyanins. We also demonstrated the significant preventive effects of the cornelian cherry regarding high fat diet-induced hypertrigliceridemia and the development of atherosclerosis, in a rabbit experiment. As well, we proved the activation of PPARα receptors in the liver and the modulation of both the redox system and proinflammatory cytokines [35].

As in our previous study, we have shown now the beneficial pleiotropic effect of lyophilisate from whole cornelian cherry fruits. In this study, we have attempted to compare the effects of its main compounds – iridoid loganic acid and a mixture of anthocyanins – on lipid levels, atherosclerosis development, PPARα and γ protein expression, and proinflammatory markers in a model of diet-induced hypercholesterolemic rabbits.

2. Materials and methods

2.1. Chemicals

Cholesterol and acetonitrile for LC-MS were purchased from POCH (Gliwice, Poland). Acetonitrile, formic acid, acetic acid, and methanol were purchased from Sigma-Aldrich (Steinheim, Germany). Loganic acid and cyanidin 3-*O*-glucoside were purchased from Extrasynthese (Lyon Nord, France).

2.2. Plant materials and samples preparation of loganic acid and anthocyanins

The cornelian cherry (*Cornus mas* L.) fruits originated from the Bolestraszyce Arboretum and Institute of Physiography, Poland. Authentication of the plant material was done by Prof. Jakub Dolatowski. The voucher specimen (BDPA 3967) has been deposited at the Herbarium of Arboretum and Institute of Physiography in Bolestraszyce, Poland. The loganic acid and anthocyanins were isolated in the Department of Fruit, Vegetable and Cereals Technology at the Wrocław University of Environmental and Life Science. The extraction, purification, and fractionation procedure, described previously by Ref. [21], was followed with slight modifications. The juice from the cornelian cherry fruits obtained with the laboratory hydraulic press was purified through an Amberlite XAD-16 resin column (Rohm and Haas, Chauny Cedex, France). Iridoids and anthocyanins were eluted with ethanol/water/acetic acid (80:19.9:0.1, v/v/v). The purified compounds were concentrated by a Rotavapor rotary evaporator (Büchi, Flawil, Switzerland) in a water bath at 40 °C, then dried in an SPT-200 vacuum dryer (ZUT Colector, Kraków, Poland) (40 °C, 0.094 MPa). The dried compounds were dissolved in a small vol. of ethanol/water/acetic acid (25:74:1, v/v/v). The dissolved sample was fractionated by polyamide (Macherey-Nagel-CC 6.6, Düren, Germany) column chromatography (150 mm × 30 mm) using ethanol/water/acetic acid (50:49.5:0.5, v/v/v) as eluent, to give three fractions. Fraction I of iridoid (compound 1) was monitored at 254 nm, fractions II (compounds 4 and 6) and III (compounds 2, 3, 5) of anthocyanins – at 520 nm (Fig. 1). Fractions II and III were connected. The resulting alcohol-water fractions were concentrated by a Rotavapor rotary evaporator and then dried in an SPT-200 vacuum dryer (ZUT Colector, Kraków, Poland) (40 °C, 0.094 MPa). The fractions were analyzed by HPLC and LC-MS.

2.3. Determination of compounds by HPLC

Iridoids, anthocyanins, phenolic acid, and flavonols were determined by the method described by Kucharska [22], using the

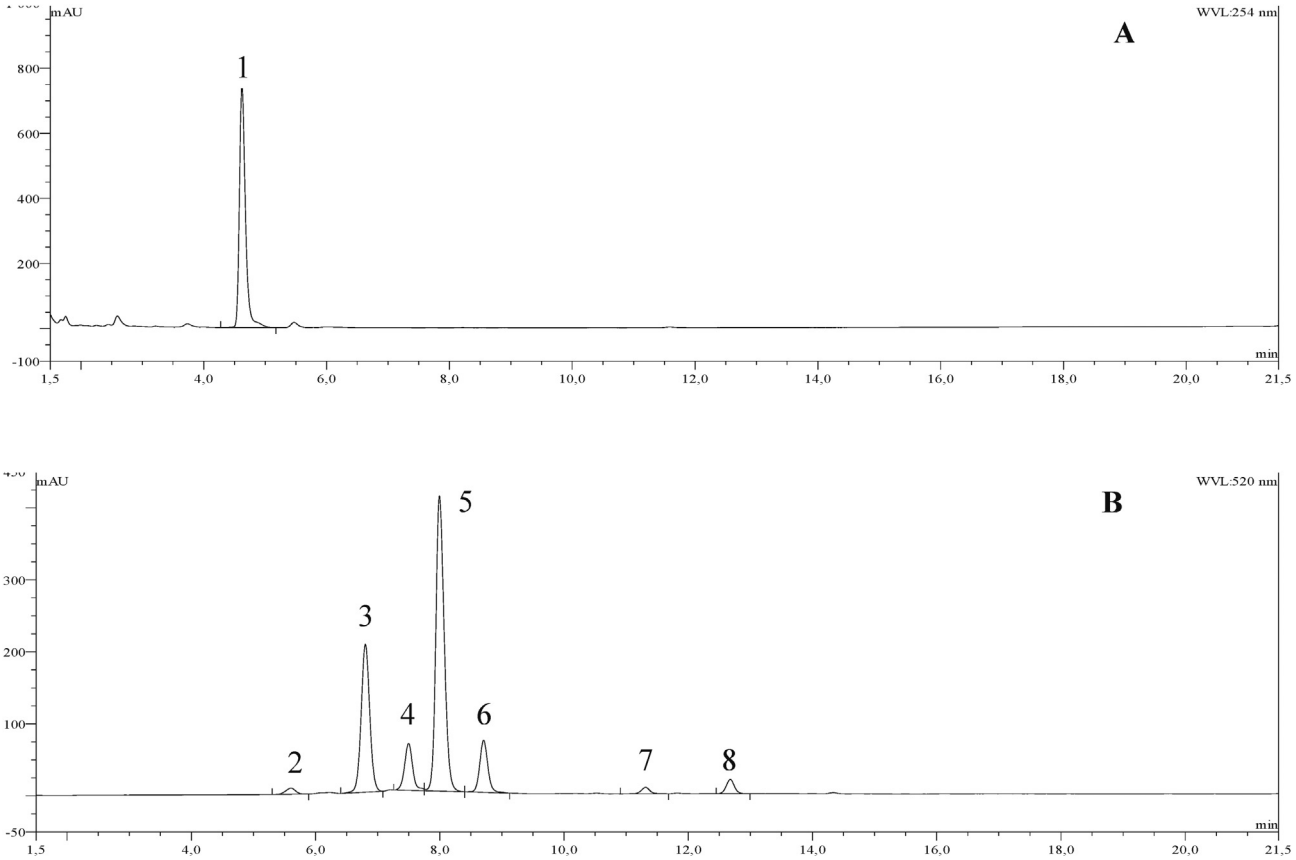

Fig. 1. HPLC-DAD fingerprint chromatograms of iridoids and anthocyanins fractions from the cornelian cherry (*Cornus mas* L.) fruits. (A) iridoids (254 nm), (B) anthocyanins (520 nm). 1: loganic acid; 2: delphinidin 3-*O*-galactoside; 3: cyanidin 3-*O*-galactoside; 4: cyanidin 3-*O*-robinobioside; 5: pelargonidin 3-*O*-galactoside; 6: pelargonidin 3-*O*-robinobioside; 7: cyanidin; 8: pelargonidin.

Dionex HPLC system (USA) equipped with the Ultimate 3000 model diode array detector. The runs were monitored at wavelengths of 245 nm (iridoids), and 520 nm (anthocyanins). Iridoid was quantified as loganic acid and anthocyanins as cyanidin 3-*O*-glucoside.

2.4. Identification of compounds by LC-MS

Compounds were identified through the method described by Sokół-Łętowska [34], 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 at a scan rate of 2.0 s/cycle.

2.5. The in vivo study

This research was conducted 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.

2.6. Animals and treatment

Forty sexually mature New Zealand rabbits, aged from 8 months to 1 year were included in the study. The animals were housed in individual 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 food (40 g/kg b.w.). After 28 days of acclimatization, the animals were randomly divided into 4 groups of 10 animals each. The animal feeding plan and scheduled administration of test substances are presented in Table 1. For 60 consecutive days, animals in the P group were supplied with standard food. Groups CH, CH + LA, and CH + ANT were given the same feed +1% cholesterol. Once daily, rabbits received the following substances by gavage: groups P and CH – normal saline (negative and positive control), group CH + LA – loganic acid at a dose of 20 mg/kg b.w., and group CH + ANT – a mixture of anthocyanins at a dose of 10 mg/kg b.w.

On days 0 and 60 of the experiment, blood samples were collected from each animal from the marginal vein of the ear or

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

Control group P	Standard diet + saline p.o.
Experimental group CH	Standard diet with the addition of 1% cholesterol + saline p.o.
Experimental group CH + LA	Standard diet with the addition of 1% cholesterol + loganic acid 20 mg/kg b.w. p.o
Experimental group CH + ANT	Standard diet with the addition of 1% cholesterol + anthocyanins 10 mg/kg b.w. p.o.

saphenous vein. On day 60, animals were euthanized with terminal anesthesia using morbital (1 ml contains pentobarbital sodium-133,3 mg and pentobarbital-26,7 mg), administered intraperitoneally at a dose of 2 ml/kg b.w. Organs were collected, especially the livers for measurements of PPARα and γ expression.

2.7. Measurements of body weight and liver index

The body weight (BW) in each group was estimated on days 0, 30, and 60. The growth rate of body weight (BW%) was calculated using the formula BW% = {[BW on the last day (g)-BW on day 0 (g)]/BW on day 0 (g)} × 100. The Liver index (LI) was measured by Pooja methods. LI = [(wet weight of liver/BW]×100 [46].

2.8. Measurements of biochemical parameters

The serum levels of total cholesterol and triglycerides were estimated based on calorimetric, enzymatic methods. High-density 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 the log (TG/HDL-C) [11] and the cardiac risk ratio by means of the TC/HDL-C ratio [19].

2.9. Measurements of TNFα, IL-6, and ox-LDL

ELISA methods were used in the determination of tumor necrosis factor α (ELISA Kit for Tumor Necrosis Factor Alpha, meant for rabbits, Uscn Life Science Inc.), IL-6 (ELISA Kit for Interleukin 6, meant for rabbits, Cloud-Clone Corp.), and Oxidized Low Density Lipoprotein – ox-LDL (ELISA Kit for Oxidized Low Density Lipoprotein, meant for rabbits, Uscn Life Science Inc.)

2.10. Analysis of PPAR expression by western blotting

Nuclear fractions from rabbit livers were prepared using the NE-PER®Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific, USA). 20 μg of each nuclear fraction was added to the SDS sample buffer, boiled at 95 °C for 5 min, and subjected to SDS-PAGE on 12% gel. The resolved proteins were transferred to PVDF membrane (Milipore) using semi-dry transfer. After the transfer, the membrane was blocked with 1% casein in TBS at 4 °C, overnight, and then incubated with 1–2 μg/ml of primary antibody, PPAR-alpha (Sigma, SAB 2104354), PPAR gamma (H-100, Santa Cruz Biotechnology) and beta-actin (Santa Cruz Biotechnology, AC-15) at room temperature for 1 h, followed by secondary horseradish peroxidase-labeled antibody (DAKO). The bound antibodies were visualized using the ECL blotting detection system (Thermo Scientific, USA). The blots were scanned using Gel Doc Imaging system (BIO-RAD, USA) and the optical density of bands was analyzed with Image Lab 5.1 software.

2.11. Histopathological assessment of the thoracic aorta

After the rabbits were euthanized, the tissue sections of the thoracic aortas were washed with ice-cold sterile physiological saline and fixed in a 7% buffered formalin solution. Samples were cut into 4 μm thick slices, dehydrated, embedded in paraffin, stained with hematoxylin and eosin, and blindly examined under a light microscope by an experienced pathomorphologist. Intima and media thickness, and the intima/media ratio were evaluated according to the previous studies by Refs. [3,12].

2.12. Statistical analysis

The results were expressed as mean ± standard deviation (mean ± SD). The normality of all continuous variables was verified by the Shapiro-Wilk test. Statistical comparisons of data were performed using ANOVA, followed by a post-hoc LSD test. Values of $p < 0.05$ were considered significant.

3. Results

First, we separated and identified fractions of iridoids and anthocyanins from cornelian cherry fruits. Then, we compared impact of their oral administration on dietary-induced dyslipidemia in rabbits.

Extract of active compounds from cornelian cherry fruits was purified on an Amberlite XAD-16 resin. Next, the iridoid and anthocyanin fractions were separated on the polyamide column and monitored at 254 nm and 520 nm, respectively. Chromatograms of the compounds used in our experiment are shown in Fig. 1. One iridoid and seven anthocyanins were determined in fractions. The compounds were identified based on the analysis and comparison with standards of retention times and spectra of the individual peaks. Additionally, we confirmed the results on the basis of spectral data (UPLC-qTOF-MS/MS) and by comparison with available literature [21]. In the iridoid fraction, we identified only loganic acid, while in the anthocyanin fraction, we identified five substances: delphinidin 3-*O*-galactoside, cyanidin 3-*O*-galactoside, cyanidin 3-*O*-robinobioside, pelargonidin 3-*O*-galactoside, pelargonidin 3-*O*-robinobioside, cyanidin, pelargonidin (Table 2).

Among anthocyanins, galactoside of cyanidin and pelargonidin together accounted for over 78%. The remaining anthocyanins linked tosugars constituted over 18%, and aglycones less than 3.5%.

Rabbits fed for 60 days the cholesterol-rich diet developed typical, negative changes in lipid profile. Administration of anthocyanins significantly decreased levels of both total and LDL-cholesterol and triglycerides, as well as increased HDL cholesterol level compared to the group that received cholesterol. Loganic acid significantly decreased triglycerides and increased HDL-cholesterol levels, although its impact on total and LDL-cholesterol was slight and statistically insignificant (Fig. 2).

A cholesterol-rich diet significantly increased the levels of the atherogenic index of plasma AIP and cardiac risk ratio CRR. Anthocyanins completely reversed the AIP changes induced by the cholesterol-rich diet. Loganic acid partially reversed the AIP changes, its level in the CHOL + LA group was significantly lower compared to the CHOL group, but still higher than in the P group. CRR values in both the CHOL + ANT and CHOL + LA group were significantly lower compared to the CHOL group, but still higher than in the P control group (Fig. 3).

Histomorphometric measures of the thoracic aorta and representative sections of the aortic segments are presented in Fig. 4. The cholesterol-fed diet resulted in both a significant thickening of the intima layer and an increase in intima/media ratio, and in a moderate thickening of the media-layer. Treatment with both anthocyanins and, to a lesser extent, loganic acid, prevented changes in the thoracic aorta, significantly decreasing intima thickness and intima/media ratio. In the anthocyanins-fed group, values were close to the control group.

We observed an increase in the plasma ox-LDL levels between the control and cholesterol groups. Both loganic acid and anthocyanins diminished the ox-LDL level to a level lower compared to the CHOL group, but still higher than in the control group (Fig. 5).

We did not observe differences in body weights among the examined groups after 60 days. The growth rate of body weight between 0 and 60 days was slightly higher in rabbits fed cholesterol

Table 2
Identification of iridoid and anthocyanin fractions from cornelian cherry (*Cornus mas* L.) fruits, using their spectral characteristic in HPLC-DAD (retention time, relative area) negative and positive ions in UPLC-ESI/MS (MS and MS/MS).

Peak[a]	Ret. Time (min)	Compound	Rel. area (%)	Ion mode	MS (m/z)	MS/MS (m/z)
Iridoid						
1	4.44	Loganic acid	>98.5	–	375 $[M-H]^-$	213 $[M\text{-}162\text{-}H]^-$
Anthocyanins						
2	5.60	Delphinidin 3-*O*-galactoside	1.3	+	465 $[M+H]^+$	303 $[M-162+H]^+$
3	6.80	Cyanidin 3-*O*-galactoside	26.6	+	449 $[M+H]^+$	287 $[M-162+H]^+$
4	7.50	Cyanidin 3-*O*-robinobioside	8.0	+	595 $[M+H]^+$	449 $[M\text{-}146+H]^+$ 287 $[M-162+H]^+$
5	7.99	Pelargonidin 3-*O*-galactoside	51.6	+	433 $[M+H]^+$	271 $[M-162+H]^+$
6	8.71	Pelargonidin 3-*O*-robinobioside	8.9	+	579 $[M+H]^+$	433 $[M-146+H]^+$ 271 $[M-162+H]^+$
7	11.32	Cyanidin	1.2	+	287 $[M+H]^+$	–
8	12.68	Pelargonidin	2.4	+	271 $[M+H]^+$	–

[a] Peaks are shown in Fig. 1.

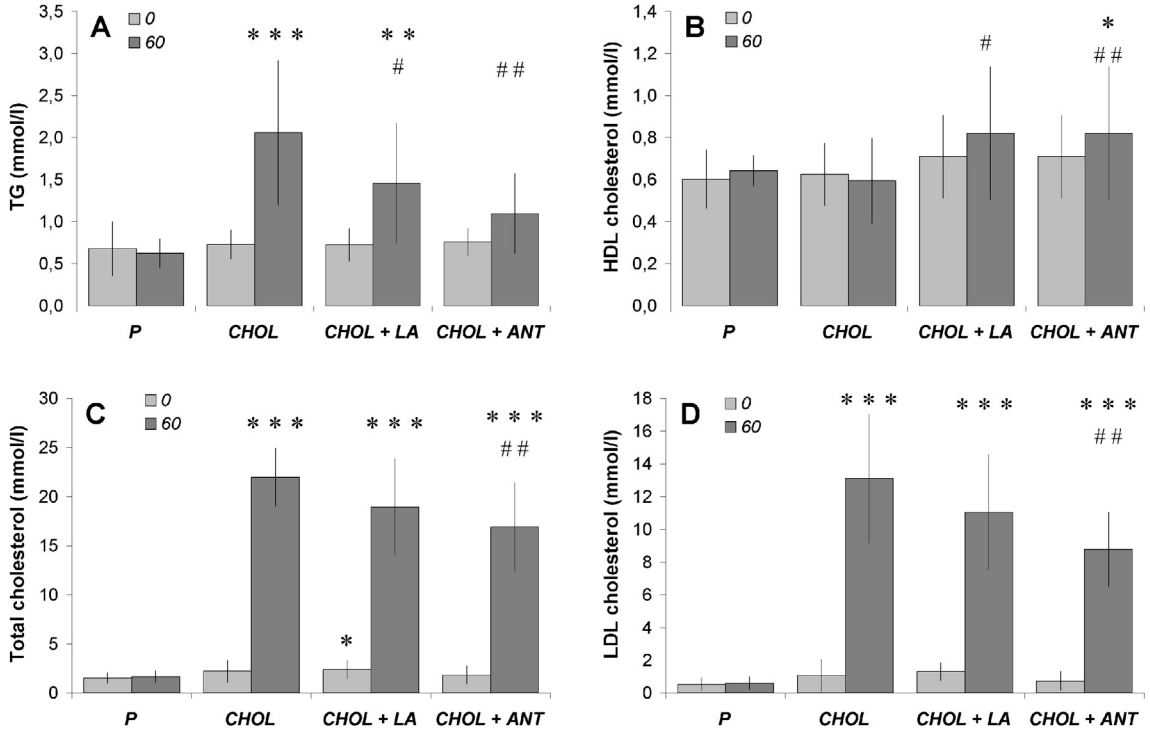

Fig. 2. Serum lipid levels. (A) triglycerides, (B) HDL cholesterol, (C) total cholesterol, and (D) LDL cholesterol levels on days 0 and 60 of the study. P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values are presented as mean ± SD. Specific comparisons: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* P; #$p < 0.05$, ##$p < 0.01$ *vs.* CHOL.

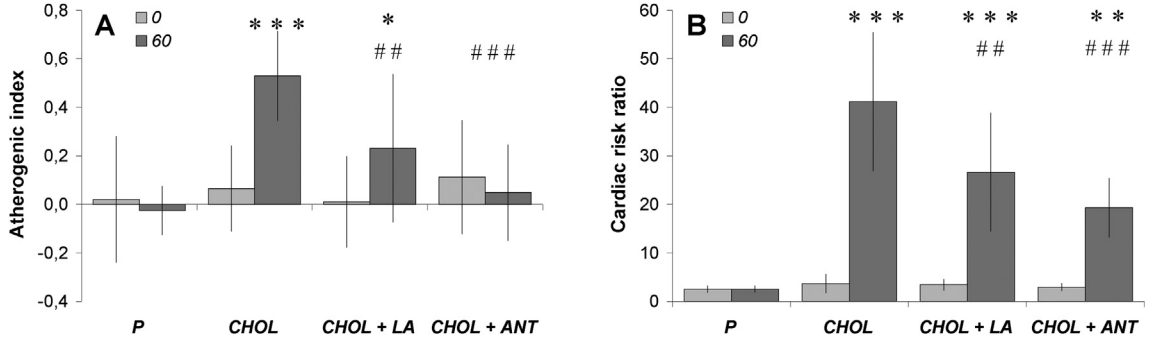

Fig. 3. Atherogenic indices in the blood. (A) atherogenic index calculated as log (triglyceride/HDL cholesterol ratio) and (B) cardiac risk ratio defined as total cholesterol/HDL cholesterol ratio on days 0 and 60 of the experiment. P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values are presented as mean ± SD. Specific comparisons: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* P; ##$p < 0.01$, ###$p < 0.001$ *vs.* CHOL.

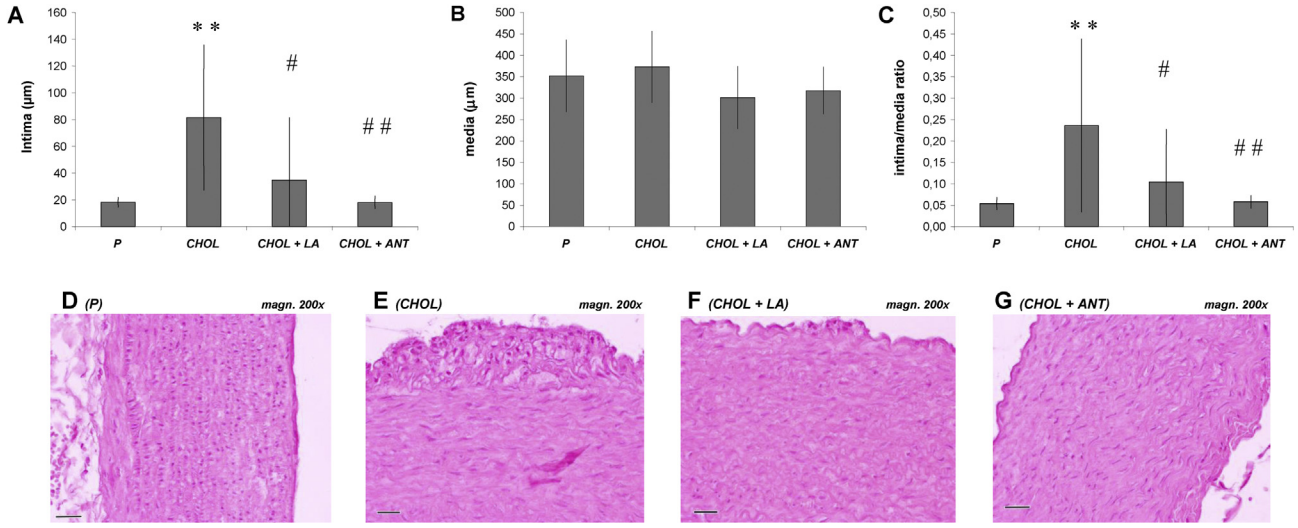

Fig. 4. Intima and media thickness, and the intima/media ratio in the thoracic aorta. (A) intima thickness, (B) media thickness, and (C) the intima/media ratio. Values are presented as mean ± SD. Specific comparisons: $^{**}p < 0.01$*vs.* P; #$p < 0.05$, ##$p < 0.01$ *vs.* CHOL. Representative cross-sections of thoracic aorta segments, stained with hematoxylin-eosin in the (D) P group - rabbit nr 8, (E) CHOL group – rabbit nr 14, (F) CHOL + LA group – rabbit nr 20, and (G) CHOL + ANT group – rabbit nr 33.

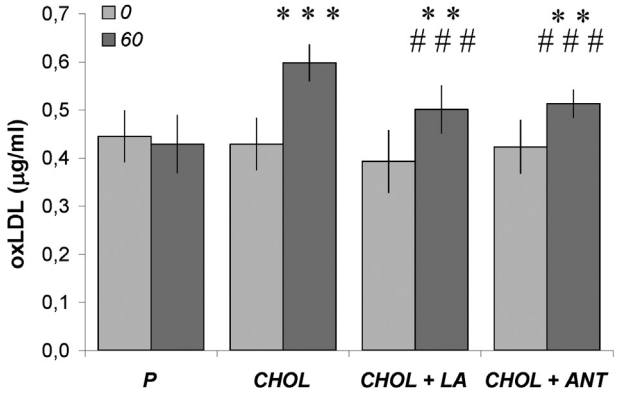

Fig. 5. ox-LDL levels in the plasma. Results on days 0 and 60 of the study. P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values are presented as mean ± SD. Specific comparisons: $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* P; ###$p < 0.001$ *vs.* CHOL.

and significantly lower in those receiving anthocyanins. The liver weight and liver index (LI) increased in the cholesterol-fed group compared to the control group. Administration of both loganic acid and anthocyanins partially restored those cholesterol-induced changes, although this did not result in statistical significance (Table 3).

Generally, our experiment showed that cholesterol feeding decreased while the substances we investigated increased PPARs expression. Anthocyanins significantly increased, by 193%, the expression of PPARγ in the liver compared to cholesterol-fed animals, and was much higher than in all other groups, including the control group. Loganic acid increased PPARγ expression by 67,6%. Both anthocyanins and loganic acid increased PPARα expression by 123% and 85,2%, respectively (Fig. 6).

The marked differences between the anti-inflammatory properties of anthocyanins and loganic acid were seen while assessing their impact on TNFα and IL-6 activities. Loganic acid significantly reversed the increased serum level of both cytokines caused by administration of cholesterol. Anthocyanins moderately diminished IL-6 activity, but did not significantly affect TNFα activity (Fig. 7).

4. Discussion

The main conclusion of our study is that both substances have a positive, but partially different, effect on factors contributing to the development of atherosclerosis. The key findings are that both investigated compounds – iridoid loganic acid and a mixture of anthocyanins – had a positive impact on dyslipidemia and atherosclerosis caused by a cholesterol-rich diet, although the effects brought on by anthocyanins were more pronounced. Both constituents diminished oxidation of LDL. Secondly, anthocyanins significantly increased the expression of PPARγ in the liver, loganic acid also enhanced its expression but to a much lesser extent. Both anthocyanins and loganic acid increased the expression of PPARα. Lastly, loganic acid, though not anthocyanins, showed anti-inflammatory effects – decreasing TNFα and IL-6 activity.

The effects of several plants on human systems may stem from the activities of their different constituents and the combination of selective constituents with a proven mechanism and biological

Table 3
Body weight on day 60, growth rate of body weight between days 0 and 60 of the experiment (BW%), liver weight, and liver index.

Group	Body weight on day 60 (g)	The growth rate of body weight (BW%)	Liver weight (g)	Liver index
P	3202 ± 264.36	1.94 ± 3.79	93.8 ± 17.87	2.81 ± 0.45
CHOL	3328 ± 323.55	4.08 ± 3.89	113.6 ± 16.96*	3.55 ± 0.49*
CHOL + LA	3334 ± 184.52	1.30 ± 2.21	104.81 ± 19.27	3.14 ± 0.53
CHOL + ANT	3267 ± 603.62	−1.24 ± 6.64#	106.8 ± 24.52	3.27 ± 0.57

P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values are presented as the mean ± SD. Specific comparisons: *$p < 0.05$ *vs.* P; #$p < 0.05$ *vs.* CHOL.

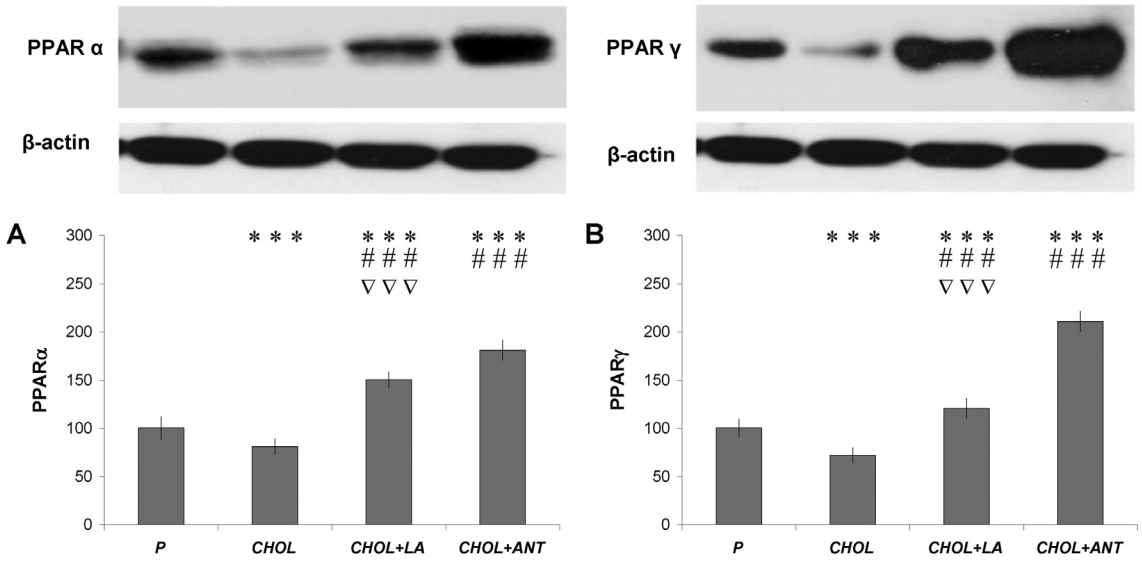

Fig. 6. Liver PPARs expressions. (A) Liver PPAR-α and (B) PPAR-γ. P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values are presented as mean ± SD. Specific comparisons: ***$p < 0.001$ *vs.* P; ###$p < 0.001$ *vs.* CHOL; ΔΔΔ $p < 0.001$ *vs.* CHOL + ANT.

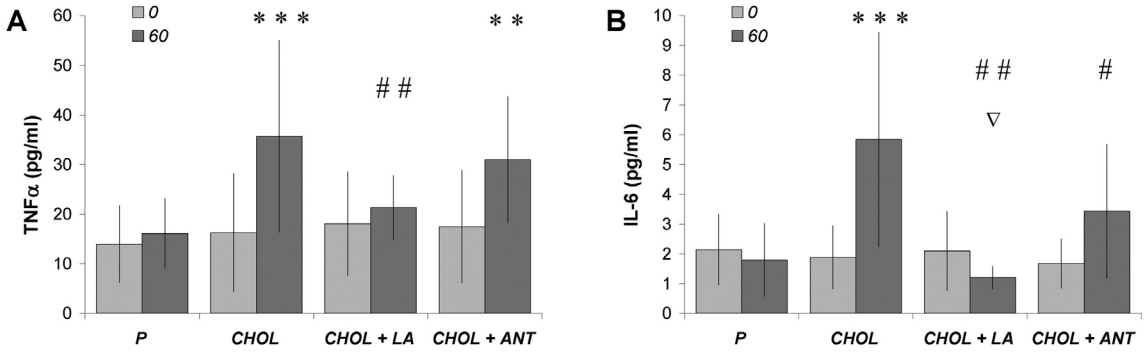

Fig. 7. TNFα and IL-6 activities in the plasma. (A) TNFα and (B) IL-6 on days 0 and 60 of the experiment. P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values are presented as mean ± SD. Specific comparisons: **$p < 0.01$, ***$p < 0.001$ *vs.* P; #$p < 0.05$, ##$p < 0.01$ *vs.* CHOL; Δ $p < 0.05$ *vs.* CHOL + ANT.

activity may be a way to achieve beneficial additive or synergistic effects. So far, the cardiovascular benefits of plants are attributed mainly to their flavonoid constituents, especially anthocyanins. Among many studies supporting this hypothesis, large population-based, follow-up studies have shown that habitual, generous consumption of anthocyanins (though not most other flavonoid subclasses) is associated with a reduced risk of developing cardiovascular diseases, especially hypertension [4] and myocardial infarction [5]. At the same time, anthocyanins-rich plants contain other biologically active substances. In the cornelian cherry cultivar we investigated, there are mainly iridoids. Until recently, we have had knowledge of only a few other fruits containing both considerable amounts of anthocyanins and iridoids. These are: cranberries, lingonberries, and blueberries [17]. It is worth noting that these plants have been shown to have a beneficial effect on lipids, obesity, and cardiovascular diseases in previous studies [1,48,39]. However, the effects of isolated anthocyanins and iridoids from those plants, to our knowledge, have not been compared.

In our previous study on whole lyophilized cornelian cherry, we showed its constituents: two iridoids – loganic acid and cornuside – (with loganic acid in much higher concentration), and five anthocyanins. We also proved the preventative effects of whole fruits on diet-induced dyslipidemia and atherosclerosis [35]. In the present study, we have made an attempt to elucidate the differences and similarities between the main compounds of cornelian cherry – anthocyanins and iridoids – regarding their effects on diet-induced dyslipidemia. We have attributed the possible effects of cornelian cherry fruits to their main constituents, i.e. iridoid loganic acid and anthocyanins, based on the previous studies of both other researchers [44,10] and our team [22,35,21]. We used doses of compounds based on our previous research on whole lyophilized fruits, although it is also possible to achieve these doses by consumption of fresh or lyophilized fruits. A higher dose of loganic acid than anthocyanins was established to partially reflect its higher content in cornelian cherry fruits.

To the best of our knowledge, our study is likely one of the first to examine in one survey anthocyanins and iridoid from the same plant, and show their similar impact on serum lipids, in animals fed a hypercholesterolemic diet. Although the lipid-lowering effects of anthocyanins were stronger than the effects exerted by loganic acid, the trend in lipidogram changes produced by both constituents was similar. These results, bearing in mind the beneficial effects showed in our previous study on cornelian cherry, may imply the additive or synergistic effect of anthocyanins and iridoids. It

also suggests that both constituents may contribute to the prevention of the development of atherosclerosis. Moreover, comparison of effects on lipids of isolated loganic acid and anthocyanins with the effects of whole lyophilized fruits in our previous study suggests that the effects of both isolated compound were stronger, and support the hypothesis that they are the main active constituents of cornelian cherry fruits.

Both investigated compounds of cornelian cherry also significantly decreased lipid indexes – AIP and CRR – predicting the risk of atherosclerosis and cardiac diseases. AIP and CRR indexes seem to be more sensitive in predicting the risk of developing cardiovascular diseases than the evaluation of single lipid levels. Estimating those indexes and other risk factors seems to be important, as well, especially because half of all cardiovascular events develop even when simple plasma lipid levels are within the normal range [2]. Anthocyanins exerted more pronounced effects than loganic acid, especially in decreasing the AIP to levels similar to the control group. Moreover, CRR value in the loganic acid-treated group was significantly lower than in the cholesterol-treated group, despite the fact that the total cholesterol level did not reach statistically significant differences between the groups.

The results of dyslipidemia regression in anthocyanins and loganic acid-fed animals were confirmed by an observed decrease in intima and media thickness and the intima/media ratio in the thoracic aorta in both groups. Thickening of the arterial wall, especially of the intima layer, is regarded as a risk-marker of atherosclerosis and is described as one of the earliest vascular pathologies observed in microscopic assessment during atherogenesis, a Type I lesion according to the American Heart Association-AHA classification [32].

The body weight of the rabbits after 60 days of experiment did not differ significantly between the groups examined – although the growth rate of the body weight between 0 and 60 day was a little higher in cholesterol-fed rabbits, and lower in animals receiving anthocyanins. This effect of anthocyanins is consistent with previous studies showing positive impact of their consumption on obesity [37,15].

We also found that both loganic acid and anthocyanins diminished the oxidation of LDL-cholesterol brought on by a cholesterol rich-diet. Formation of ox-LDL is one of the key events in atherogenesis that causes endothelial cell dysfunction, foam cells formation, and smooth muscle proliferation, as well as induces inflammation and activates the immune system [28,50]. Downregulation of ox-LDL by iridoid and anthocyanins may be the simple result of their impact on lipid levels, but our research also suggests that consumption of plants rich in those substances may positively impact the early stages of atherosclerosis.

Aside from dyslipidemia and LDL oxidation, there are several other risk factors contributing to the development of cardiovascular diseases. We have focused on the impact of investigated substances on PPARα and γ receptors expression, because they play a crucial role in the regulation of lipid and glucose metabolism [14,16]. Moreover, upregulation of PPARs exerts other pleiotropic effects, especially by diminishing the inflammation and secretion of metalloproteinases in atherosclerotic plaques, which increase stability and resistance to ruptures [14,41]. PPARs are also probably one of the more promising receptors that could be modulated by new antilipidemic drugs.

In our previous research on whole fruits, we showed the pronounced up-regulation of PPARα activity associated with a positive impact on blood lipid levels and proinflammatory cytokines. Now, we have proven that anthocyanins, and to a lesser degree iridoids, affect PPARs expression in feed-induced dyslipidemic animals. Anthocyanins exerted a pronounced increase in PPARγ and PPARα protein expression in the liver by 193% and 123%, respectively. These effects of anthocyanins on PPARs were correlated with positive changes in serum lipids, but not proinflammatory cytokines levels. A significant decrease of TNFα and IL-6 was observed (as described below) only in loganic acid-supplemented rabbits. Our knowledge about the influence of anthocyanins on PPAR receptors expression is inadequate. So far, anthocyanins' mechanism of action was mainly attributed to cholesterol efflux from macrophages and foam cells. That effect was observed in cell cultures after the addition of pure anthocyanins, or serum from patients treated with anthocyanins [45,29]. Only one *in vitro* study by Jia et al. suggests that cyanidin may act as a PPARα agonist [18]. *In vivo*, Vendrame et al. show that anthocyanins-rich wild blueberry enhanced the expression of genes related to lipid metabolism – including PPARγ – in abdominal adipose tissue in Zucker rats [39]. Nevertheless, blueberries also probably contain other active constituents like iridoids, and the isolated constituents were not examined in that study.

There is a possible link between our results, which showed the impact of anthocyanins on PPARs, and observations made in previous studies that attributed the lipid lowering properties of anthocyanins to their ability to induce cholesterol efflux from macrophages. Chinetti et al. [7] showed that PPARα upregulation increases the expression of the cell-surface scavenger receptor CLA-1/SR-BI in macrophages. Activation of those receptors correlates with HDL-mediated efflux of unestrified cholesterol from macrophages and foam cells, decreasing fatty streaks accumulation in atherosclerotic plaques and initiating cholesterol transport from peripheral tissues to the liver, with its subsequent elimination.

In our study – as described above – loganic acid also increased the expression of PPARα and PPARγ, but to a lesser degree than anthocyanins. These effects are consistent with previous findings made by Refs. [47,26] showing that iridoids isolated from asiatic Corni Fructus (*Cornus officinalis* L.) up-regulated PPARα expression in the livers in type 2 diabetic *db/db* mice.

We also found a pronounced anti-inflammatory effect of loganic acid measured as decrease of TNFα and IL-6. We measured these two proinflammatory cytokines because their increase is observed in atherosclerosis. Downregulation of these cytokines may help prevent atherotrombosis [6,31]. Inflammation – both systemic and local in atherosclerotic plaques – is an important risk factor for cardiovascular diseases, contributing to the initiation, development, and complications of atherosclerosis [51,9,24]. Inflammation regulates the composition and stability of atherosclerotic plaques, modulates thrombotic complications, and correlates with outcomes in acute coronary syndromes and with risk of myocardial infarction [51,23].

Comparing the anti-inflammatory effects of loganic acid in the present study with the effects of whole lyophilisated fruits in our previous study, we suggests that the main anti-inflammatory effects of the cornelian cherry fruit should be attributed to its iridoids fraction. We did not find any previous studies investigating the impact of loganic acid on inflammatory outcomes during atherosclerosis. Nevertheless, the anti-inflammatory effect of loganic acid in our study is consistent with the potential of iridoids and iridoid-containing plant species described in previous studies [40]. Some of those plants have been used in traditional medicine to treat inflammatory related disease. *Cornus officinalis* and its isolated iridoids, such as loganin and cornuside, showed anti-inflammatory effects in both *in vitro* models [20,8,43] and *in vivo* animal models with induced diabetes [47,27].

Knowledge of the *in vivo* anti-inflammatory properties of the cornelian cherry's isolated iridoids is limited but promising. Recio et al. have proven the anti-inflammatory effects of loganic acid and loganine in carrageenan-induced mouse paw edema and TPA-induced mouse ear edema tests [30]. The strong *in vitro*

inhibitory effect of loganic acid on fMLP-stimulated activation of human neutrophils – measured by superoxide generation – was described in another study by Wei et al. [43].

Anthocyanins moderately diminished IL-6 activity but did not show an anti-inflammatory effect measured as TNFα activity. These results of our study are ambiguous and a bit surprising compared with previous *in vitro* and *in vivo* findings [38]. We hypothesize that this discrepancy may be a result of 1) the low doses of anthocyanins used in our study; 2) their low gastrointestinal absorption and high metabolism in both the gut and liver; 3) the possible differences between *in vitro* effects of anthocyanins on, e.g. stimulated macrophages and expected *in vivo* modulation of the cytokines level; 4) Others have examined composed extracts from plants, attributing observed effects to anthocyanins. Yet, those extracts could have also contained other active constituents.

It is also worth mentioning that the described effects of the investigated substances may be related not only to native compounds but also to their metabolites and interactions with gut microflora. Regarding anthocyanins, there are several studies showing interactions between them and the microflora. On one hand, anthocyanins may act as a prebiotic, on the other hand, intestinal enzymes and gut microbiota metabolize anthocyanins into several phenolic acids and aldehydes. This changes the bioavailability of anthocyanins and the appearance of metabolites – formed both in the intestines and in the liver – may contribute to the observed biological effects of ingested compounds [33,13]. Anthocyanins are readily absorbed from gastrointestinal tract, appear in the blood within a few minutes, and reach maximum concentration in 15 min to 2 h. They are found in both blood and urine as intact, phenolic acid and aldehyde derivatives, as well as glucuronide, methylated or sulfate conjugates [33]. Moreover, a study on apolipoprotein E (ApoE)-deficient mice proved that the administration of pure anthocyanins may increase bile acid excretion, which is an important pathway for removing circulation cholesterol from the body [42].

Here the limitations of this study should be considered. We have examined an important but single PPARs pathway involved in lipid and glucose metabolism. It will be essential in further studies to also examine other pathways involved in lipid metabolism, atherogenesis, and vascular dysfunction, such as factors involved in NO synthesis, cholesterol synthesis and circulation, or regulation of redox stress. We have also shown differences in anthocyanins and iridoids, proving the anti-inflammatory effects of the latter in blood. However, we did not examine the effects of loganic acid on inflammation in tissues, especially the arteries, a target organ in atherogenesis. Nevertheless, these findings provide a basis for further investigations.

Anthocyanins are regarded as safe substances. Iridoids are also generally considered safe, although the toxicity data are still limited for some of them [40]. In the course of our experiment we observed no adverse effects in the animals treated with anthocyanins or loganic acid.

Our study, we assume, is one of the first comparing anthocyanins and iridoids from the same plant and their impact on diet-induced dyslipidemia. We have shown that both anthocyanins and iridoids from the cornelian cherry have a positive impact on the serum lipids in cholesterol-fed animals. We have also found that they may affect both different and similar factors contributing to the development of atherosclerosis. Anthocyanins significantly increased the expression of PPAR receptors, especially PPARγ. Loganic acid also increased their expression but to a lesser extent. Conversely, loganic acid, though not anthocyanins, showed anti-inflammatory effects by decreasing proinflammatory cytokines activity. These results may suggest the potential health benefits of fruits containing both considerable amounts of anthocyanins and iridoids. The establishment of this link supports the idea of creating composed phytopharmaceuticals containing both group of substances.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

T. Sozański: conception and design of the study, participation in acquisition of all laboratory data, literature search, analysis and interpretation of data, main contribution in drafting the article, final approval and guarantor of manuscript.

A. Z. Kucharska: Plant materials and samples preparation of loganic acid and anthocyanins, acquisition of laboratory data. Identification and determination of compounds by HPLC-MS/MS, drafting of methods section on plant materials, samples preparation, and HPLC-MS/MS, critical revision of manuscript and approval of submission.

A. Rapak: Acquisition of laboratory data, determination of PPARs, drafting of methods section concerning PPARs, critical revision of manuscript and approval of submission.

D. Szumny: Acquisition of laboratory data, drafting of the article, critical revision of manuscript and approval of submission.

M. Trocha: Literature search, analysis and interpretation of data, critical revision of manuscript and approval of submission.

A. Merwid-Ląd: Literature search, analysis and interpretation of data, critical revision of manuscript and approval of submission.

S. Dzimira: Histopathological evaluations, histomorphometric measures of the intima and media thickness and the intima/media ratio, critical revision of manuscript and approval of submission.

T. Piasecki: Feeding and caring for the animals, blood and organs collection, critical revision of manuscript and approval of submission.

N. Piórecki: Provision of cornelian cherry fruits, critical revision of manuscript and approval of submission.

J. Magdalan: Literature search, analysis and interpretation of data, critical revision of manuscript and approval of submission.

A. Szeląg: Analysis and interpretation of data, critical revision of manuscript and approval of submission.

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