Phytomedicine 52 (2019) 1–11

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

The iridoid loganic acid and anthocyanins from the cornelian cherry (*Cornus mas* L.) fruit increase the plasma L-arginine/ADMA ratio and decrease levels of ADMA in rabbits fed a high-cholesterol diet

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ARTICLE INFO

Keywords:
Loganic acid
Anthocyanins
L-arginine
ADMA
DDAH
eNOS

ABSTRACT

Background: Although fruit and vegetable-rich diets have beneficial effects on cardiovascular diseases, we have little knowledge of the impact of fruits and their constituents, iridoids and anthocyanins, on the L-arginine-ADMA-DDAH pathway. Our previous study demonstrated the modulation of those factors by the oral administration of the **c**ornelian cherry fruit.

Hypothesis/purpose: We have assessed the effects of the oral administration of two main constituents isolated from the **c**ornelian cherry fruit, iridoid loganic acid and anthocyanins, on L-arginine, its derivatives (ADMA, SDMA), metabolites (DMA, L-citrulline), and the hepatic DDAH activity and its isoform expression in rabbits fed a high-cholesterol diet. We have also analyzed eNOS expression in the thoracic aorta as well as the redox status in blood.

Study Design: In the present study, we used an animal model of diet induced atherosclerosis. For 60 days, white New Zealand rabbits were fed a standard diet, a 1% cholesterol enriched diet, or concomitantly with the investigated substances. L-arginine, ADMA, SDMA, DMA, and L-citrulline were assessed using the LC-MS/MS method. DDAH activity and redox parameters were analyzed spectrophotometrically. DDAH1 and DDAH2 isoform expressions were assessed by western blotting, mRNA expression of eNOS was quantified by real-time PCR.

Results: We demonstrated that the administration of loganic acid (20 mg/kg b.w.), and to a lesser extent of anthocyanins (10 mg/kg b.w.), caused an increase in the L-arginine level and the L-arginine/ADMA ratio. Also, both substances decreased ADMA, DMA, and L-citrulline, but not SDMA levels. Anthocyanins, but not loganic acid, enhanced the activity of DDAH in the liver. Anthocyanins also significantly enhanced both DDAH1 and DDAH2 expression, while loganic acid to a lesser extent enhanced DDAH1 but not DDAH2 expression. Both loganic acid and anthocyanins pronouncedly increased mRNA expression of eNOS in thoracic aortas. Both loganic acid and anthocyanins reversed the blood glutathione level depleted by dietary cholesterol. Cholesterol

Abbreviations: ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; GPx, glutathione peroxidase; GSH, glutathione; DMA, dimethylamine; MDA, malondialdehyde; ELISA, enzyme-linked immunosorbent assay; LC-MS, liquid chromatography–mass spectrometry; eNOS, endothelial nitric oxide synthase; HPLC, high-performance liquid chromatography; PPARs, peroxisome proliferator-activated receptors; PRMT, protein arginine methyltransferase; RONS, reactive oxygen and nitrogen species; SDMA, symmetric dimethylarginine; SOD, superoxide dismutase; UPLC, ultra-performance liquid chromatography; UPLC-DAD, ultra-performance liquid chromatography-diode array detector

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https://doi.org/10.1016/j.phymed.2018.09.175
Received 22 January 2018; Received in revised form 9 August 2018; Accepted 17 September 2018

feeding decreased the blood GPx level, and the change was not reversed by anthocyanins or loganic acid. We did not observe any significant differences in the blood levels of MDA or SOD among the groups.
Conclusion: Iridoids and anthocyanins may modulate the L-arginine-ADMA pathway in subjects fed a high-cholesterol diet.

Introduction

L-arginine and its derivatives, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), play a significant role in the regulation of ntric oxide (NO) synthesis in vessels.

The relationship between a reduced level of L-arginine and the L-arginine/ADMA ratio, or elevated ADMA and SDMA and reduced levels of NO, as well as vascular dysfunction and an increased risk of cardiovascular events in both common and various risk populations, is well documented (Notsu et al., 2015). An increase in ADMA level is an independent risk marker for all-cause mortality and CVD (cardiovascular diseases) (Zhou et al., 2017).

Also, the positive effect of fruits and vegetable consumption on the prevention and progression of diseases of the cardiovascular system is well established. The effect is reflected in new food pyramids, meta-analyses, and guidelines published by cardiology and atherosclerosis societies (Piepoli et al., 2016; Wang et al., 2014).

Nevertheless, there are only a few studies assessing the impact of food on the L-arginine-ADMA-DDAH pathway, particularly the impact of fruits and vegetables considered beneficial in the prevention of cardiovascular diseases (CVDs). In the murine model of mice fed a fatty diet, apple peel supplementation reduced plasma ADMA levels (Gonzalez et al., 2015). In human studies, it has been reported that a high dietary antioxidant intake decreases the plasma ADMA level in healthy young adults (Puchau et al., 2009), and that a higher serum level of carotenoids correlates with lower ADMA levels (Watarai et al., 2014). Besides this, Góralczyk, et al, has shown that high tea and vegetable, but not fruit intake is associated with lower plasma ADMA levels (Goralczyk et al., 2012).

There are several reports confirming the beneficial effects of fruit-derived iridoids and anthocyanins on the cardiovascular system (Tsuda, 2012; Reis et al., 2016; Viljoen et al., 2012; Leong et al., 2016). However, to the best of our knowledge, only a few studies have compared their combined effects. There are also no studies assessing their impact on L-arginine and its derivatives.

Our previous studies demonstrated the high content of iridoids, mainly loganic acid, and anthocyanins in the **c**ornelian cherry fruit. We proved the protective effect of the administration of both whole fruits and isolated iridoids and anthocyanins on atherosclerosis in rabbits with feed-induced hypercholesterolemia (Sozański et al., 2016, 2014; Kucharska et al., 2015). With the same model, we demonstrated the positive impact of the administration of whole lyophilisated **c**ornelian

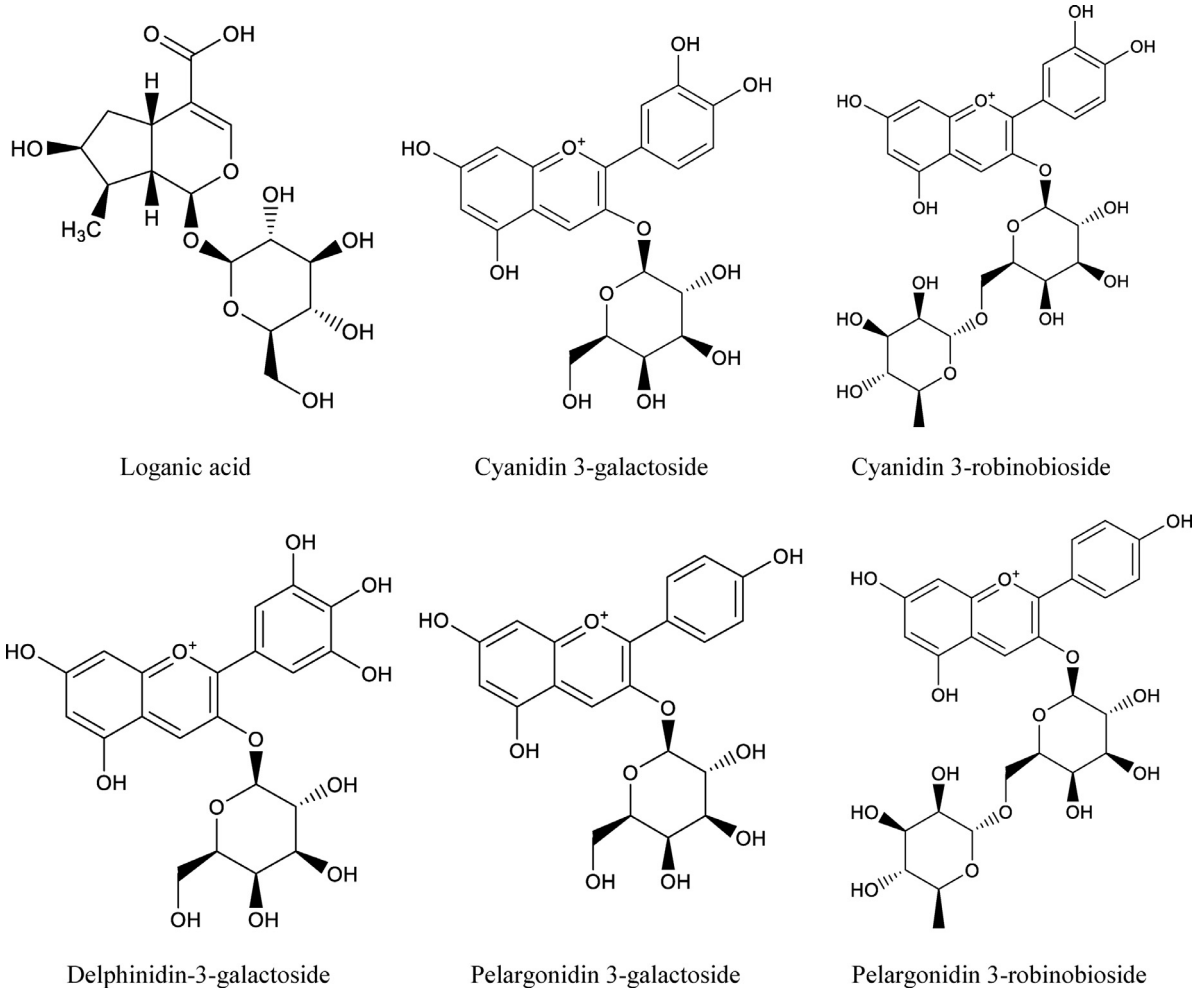

Fig. 1. The chemical structures of loganic acid and anthocyanins isolated from cornelian cherry (*Cornus mas* L.) fruits.

cherry fruit on the L-arginine-ADMA-DDAH pathway (Sozański et al., 2017).

In the present study, we have attempted to examine the effects of the main constituents of the **c**ornelian cherry fruit, an iridoid (loganic acid) and anthocyanins, on L-arginine, its derivatives (ADMA, SDMA) and metabolites (dimethylamine - DMA, L-citrulline) in the blood. We have also assessed the hepatic activity of dimethylarginine dimethylaminohydrolase (DDAH) and nuclear protein expression of its isoform DDAH1 and DDAH2, because DDAH1 is the main enzyme responsible for ADMA metabolism. Moreover, we have examined mRNA expression of eNOS in the thoracic aorta as well as redox status in the blood.

Materials and methods

Chemicals and materials

Acetonitrile for liquid chromatography-mass spectrometry-LC-MS was 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). Cholesterol was obtained from Coel (Krakow, Poland).

2,3,4,5,6-Pentafluorobenzoyl chloride (PFBoylCl), hydrochloride salts of unlabelled dimethylamine (D0-DMA), hexadeuterodimethylamine (D6-DMA, declared as 99 at% ^{2}H) and sodium carbonate (Na_2CO_3) were purchased from Sigma–Aldrich (Steinheim, Germany). Isotope labelled L-Arginine:HCl (D7, 98%) and Asymmetric Dimethylarginine (2,3,3,4,4,5,5-D7, 98%) were obtained from Cambridge Isotope Laboratories (Tewksbury, United States). Methanol, water, and formic acid were obtained from J. T. Baker (Deventer, the Netherlands).

Plant materials and samples preparation of loganic acid and anthocyanins

Cornelian cherry (*Cornus mas* L.) fruits were provided by the Bolestraszyce Arboretum and Institute of Physiography, Poland. The plant material was authenticated and the voucher specimen (BDPA 3 967) has been deposited at the Herbarium of the Arboretum and Institute of Physiography in Bolestraszyce, Poland. The investigated substances, loganic acid and a mixture of anthocyanins (Fig. 1), were isolated by the Department of Fruit, Vegetable and Plant Nutraceutical Technology at the Wrocław University of Environmental and Life Sciences. Details of the procedures of extraction, purification, and fractionation have been described previously (Kucharska et al., 2015; Sozański et al., 2016). As a result of the purification of the Amberlite XAD-16 resin column (Rohm and Haas, Chauny Cedex, France) and fractionation by polyamide (Macherey-Nagel-CC 6.6, Düren, Germany) column chromatography (150 mm × 30 mm), two fractions were obtained (I and II). Fraction I of iridoid (compound LA) was monitored at 254 nm, fraction II of anthocyanins (compounds Cy3gal, Cy3rob, Pg3gal, and Pg3rob) at 520 nm (Fig. 2). The fractions were analyzed and identified by LC-MS (Fig. 3). Fraction I contained approximately 690 μg/mg of loganic acid, fraction II contained approximately 585 μg/mg of anthocyanins, the amounts and purity of compounds were detected by high-performance liquid chromatography.

Quantification of compounds by HPLC-PDA

Iridoids and anthocyanins were assayed using the method described by Kucharska et al. (2017), with a Dionex HPLC system (Germering, Germany) equipped with the Ultimate 3000 model diode array detector. The Cadenza Imtakt column C5–C18 (75 4.6 mm, 5 m) was used. The column temperature was 30 °C. The mobile phase was composed of solvents: A (4.5% aq. formic acid, v/v), and B (100% acetonitrile). The elution system was as follows: 0–1 min 5% B in A, 20 min 25% B in A, 21 min 100% B, 26 min 100% B, 27 min 5% B in A. The flow rate of the mobile phase was 1.0 ml/min and the injection volume was 20 μl. 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.

Identification of compounds by UPLC-qTOF-MS/MS

Compounds were identified with the method described by Kucharska et al. (2017), 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. Separation was achieved with an Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm; Waters). The column temperature was 30 °C. The mobile phase was a mixture of 2.0% aq. formic acid v/v (solvent A) and acetonitrile (solvent B). Gradient conditions are presented in Supporting Information Table S1. The instrument was operated both in the positive and the negative ion mode, scanning *m/z* from 100 to 1500 at a scan rate of 2.0 s/cycle.

Atherosclerotic animal model

The protocol of the animal study was approved by the Local Ethical Committee on Animal Research at the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław, and

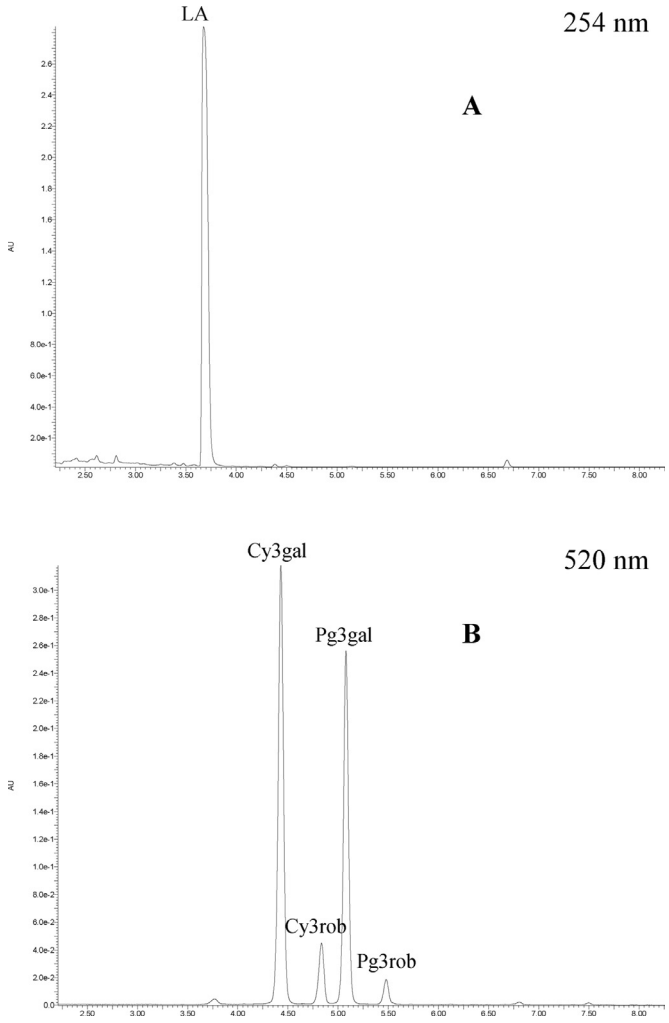

Fig. 2. UPLC-DAD chromatograms of fraction I (A) and fraction II (B). LA, loganic acid; Cy3gal, cyanidin 3-O-galactoside; Cy3rob, cyanidin 3-O-robinobioside; Pg3gal, pelargonidin 3-O-galactoside; Pg3rob, pelargonidin 3-O-robinobioside.

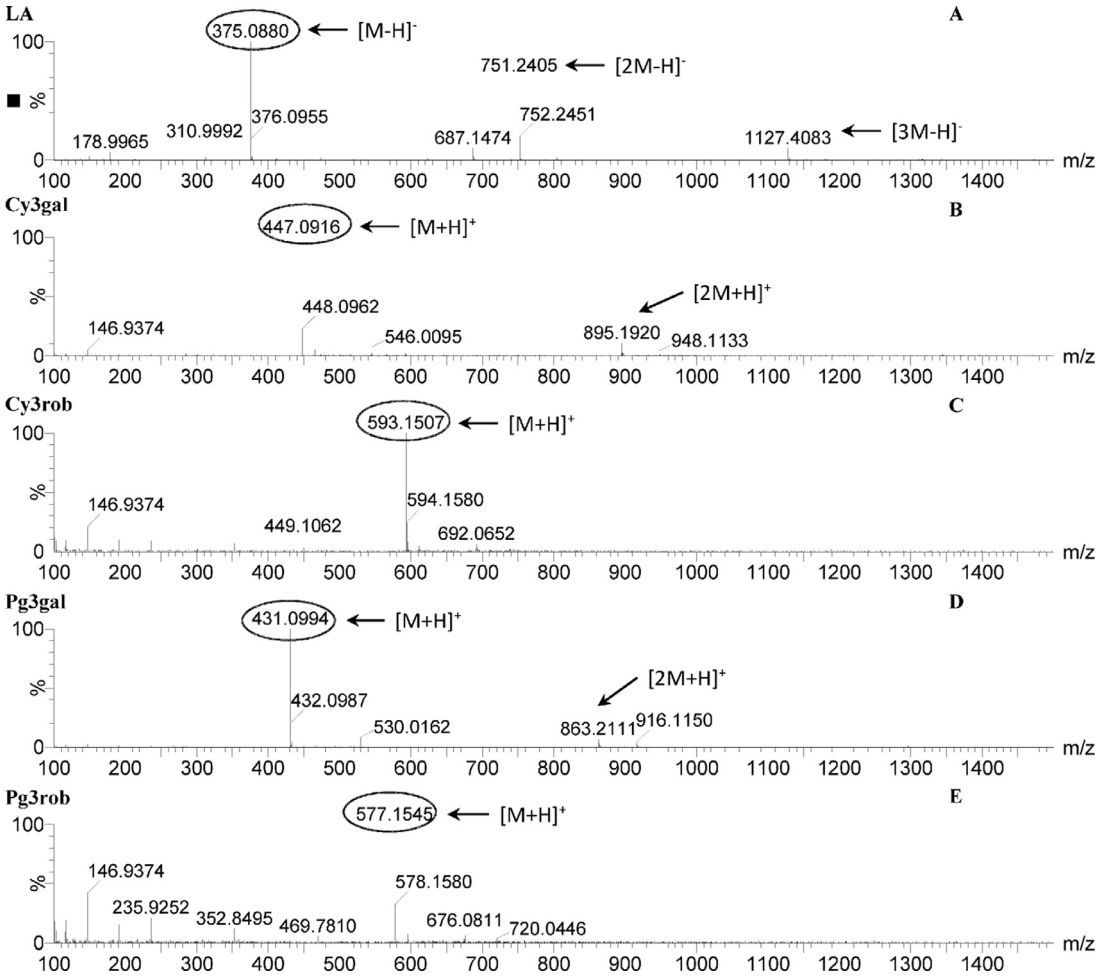

Fig. 3. MS spectra of compounds of fraction I in negative ionization mode (A) and fraction II in positive ionization mode (B – E). LA, loganic acid; Cy3gal, cyanidin 3-O-galactoside; Cy3rob, cyanidin 3-O-robinobioside; Pg3gal, pelargonidin 3-O-galactoside; Pg3rob, pelargonidin 3-O-robinobioside.

was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

The animals and schedule of feeding and administration of investigated substances were described in detail in our previous publication (Sozański et al., 2016). In short: 40 New Zealand rabbits were enrolled in the study (aging from 8 to 12 months). Animals were given the same daily portion of laboratory feed (40 g/kg b.w.) and had unrestricted access to water. The adaptation period was three weeks. Afterwards, the animals were randomly divided into 4 groups of 10 animals each. The P group (negative control) was given standard feed, the CH group (positive control) administered the same feed + 1% cholesterol. CH + LA and CH + ANT groups received the same feed + 1% cholesterol and received loganic acid at the dose of 20 mg/kg b.w., or anthocyanins at the dose of 10 mg/kg b.w., respectively. The hyper-cholesterolemic diet and compounds were administered for 60 consecutive days. On days 0 and 60, blood samples were collected from each animal from the marginal vein of the ear or the saphenous vein. On day 60, the rabbits 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 2 ml/kg b.w.

Measurements of plasma levels of L-arginine, ADMA, SDMA, DMA, and L-citrulline

Preparation of calibration standards

The analysis was performed according to the method described by Wiśniewski et al. (2017). Briefly, internal standards of D7 ADMA, D7 Arg and D6 DMA (10 µl aliquots of a 20 µM, 100 µM, 50 µM in water, respectively) and 10 µl of Na_2CO_3 (20 mM) were added to 100 µl of calibration samples and mixed by vortexing for 1 min. Precipitation solution 500 µl of acetonitrile and 10 µl of 10% PFBoylCl in acetonitrile were added to calibration samples in this order. Then, samples were mixed by vortexing for 5 min at 25 °C. After centrifugation (7 min, 15,000 RCF, 4 °C), an aliquot (100 µl) was transferred into autosampler glass vials with 100 µl of water for the LC-MS analysis.

Sample preparation

Plasma (100 µl aliquots) was pipetted into 1.5 ml polypropylene tubes. 10 µl of internal standard solution (IS D6- DMA (50 µM); D7-ADMA (20 µM); D7-Arg (100 µM)) and 10 µl of Na_2CO_3 (20 mM) were added to plasma samples. Then, samples were mixed by vortexing for 1 min at 1100 RPM. Derivatization was performed by adding the precipitation solution 500 µl of acetonitrile and 10 µl of 10% PFBoylCl in acetonitrile to plasma samples. Then, samples were mixed by vortexing for 5 min at 25 °C. After centrifugation (7 min, 15,000 RCF, 4 °C), 100 µl of the supernatant was transferred into autosampler glass vials with 100 µl of water for the LC-MS analysis.

The LC-MS/MS method

The LC-tandem MS method in the ESI mode was performed on the triple quadrupole mass spectrometer Agilent Technologies 6420 Triple Quad LC/MS, linked with the HPLC system Agilent Technologies 1260 Infinity (Santa Clara, United States). The Acquity UPLC HSS T3 1.8 µm,

1.0 × 50 mm column from Waters (Dublin, Ireland) was used. Gradient elution was performed using 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) at a flow rate of 0.2 ml/min. Gradient conditions are shown in Supporting Information Table S2.

The following transitions were used for quantitative analyses in the multiple reaction monitoring mode (MRM) after fragmentation with argon (see Supporting Information Table S3). Parent ions and fragment ions with the energy of fragmentation used for the chromatographic analysis of Arg, Arg D7, ADMA, D7 ADMA, SDMA and L-citrulline, DMA, DMA and D6 are presented in Supporting Information Table S3.

The DDAH activity

The activity of DDAH in liver homogenates was estimated using the colorimetric method (spectrophotometer MARCEL S350 PRO, Marcel sp. z o.o., Poland). The method is based on the rate of L-citrulline formation and was described in detail in our previous study (Trocha et al., 2014). The DDAH activity was presented as μm of L-citrulline/gram of protein/minute at 37 °C.

Analysis of DDAH expression by western blotting

Rabbit liver tissues were homogenized in a RIPA lysis buffer and after centrifugation the clear supernatants were mixed with 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 the 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 μg/ml of primary antibody, DDAH1 (Origene TA334438), DDAH2 (Origene, TA344265) and beta-actin (Santa Cruz Biotechnology, AC-15) at room temperature for 1 h, followed by secondary horseradish peroxidase-labeled antibody (DAKO). The bounded antibodies were visualized using the ECL West Pico blotting detection system (Thermo Scientific, USA).

RNA isolation, reverse transcription, and real-time PCR

Total RNA was isolated from studied tissue samples with the RNeasy Fibrous Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To eliminate genomic DNA contamination, on-column DNase digestion was performed using RNase-Free DNase Set (Qiagen, Hilden, Germany). The quantity and purity of RNA samples

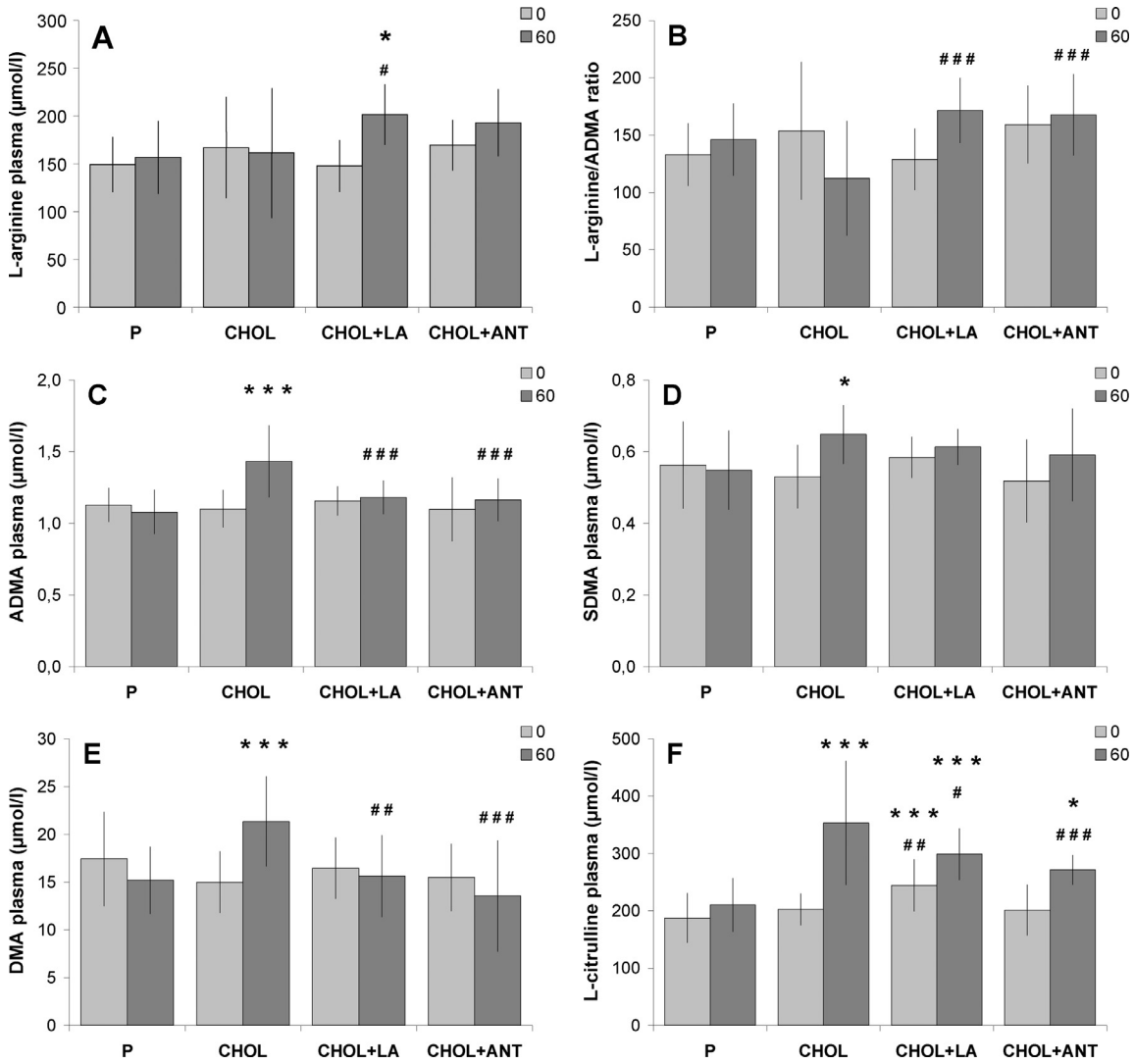

Fig. 4. L-arginine (A), L-arginine/ADMA ratio (B), ADMA (C), SDMA (D), DMA (E), and L-citrulline (F) in plasma on day 0 and day 60 of the experiment. P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values presented as the mean ± SD. Specific comparisons: * $p < 0.05$, *** $p < 0.001$ vs. P, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. CHOL.

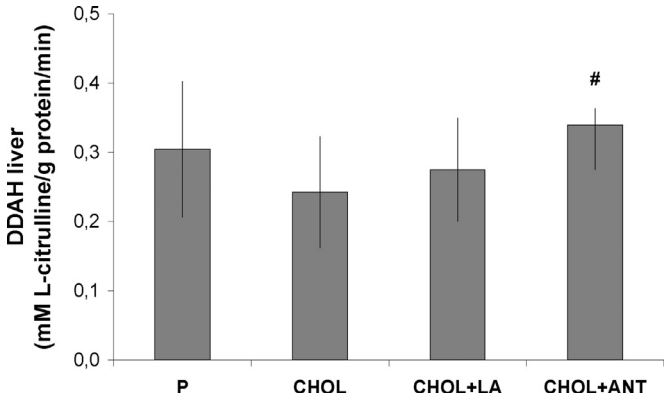

Fig. 5. Liver DDAH activity on day 60 of the experiment. P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values presented as the mean ± SD. Specific comparisons: # $p < 0.05$ vs. CHOL.

were assessed by measuring the absorbance at 260 and 280 nm with a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) as described in the protocol. The mRNA expression of eNOS was determined by quantitative real-time PCR with 7500 Real Time PCR System and Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The reactions were performed with the RT^2 qPCR Primer Assay for Rabbit eNOS, cat no. PPN00740A and for Rabbit GAPDH, cat no. PPN00377A (Qiagen, Hilden, Germany). All the reactions were performed in triplicates under following conditions: activation of polymerase at 50 °C for 2 min, initial denaturation at 94 °C for 10 min, and 40 cycles of denaturation at 94 °C for 15 s followed by annealing and elongation at 60 °C for 1 min. The specificity of the PCR was determined by melt-curve analysis for each reaction. The relative mRNA expression of eNOS was calculated with the ΔΔCt method.

The measurement of redox parameters in blood

Spectrophotometrical methods were used for the assessment of redox parameters. The malondialdehyde (MDA) level in plasma was assayed using the BIOXYTECH-MDA-586 kit (OxisResearch, USA) and expressed as M/L. The glutathione (GSH) concentration in erythrocyte lysate was measured using BIOXYTECH GSH-400 (OxisResearch, USA) and expressed as μM/g of hemoglobin. Superoxide dismutase (SOD) activity in erythrocyte lysate was assayed using the Ransod kit (Randox Laboratories, UK) and expressed as UI/g hemoglobin. Glutathione peroxidase (GPx) activity was measured using a GPx-340 kit (Randox Laboratories, UK) in heparinised blood and expressed as IU/g of hemoglobin.

The statistical analysis

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.

Results

We have studied the effects of the oral administration of loganic acid and the mixture of anthocyanins, isolated from the **c**ornelian cherry fruit on plasma levels of L-arginine, its derivatives (ADMA, SDMA) and metabolites (DMA, L-citrulline), DDAH activity and its isoform expression in the liver, eNOS expression in thoracic aorta and

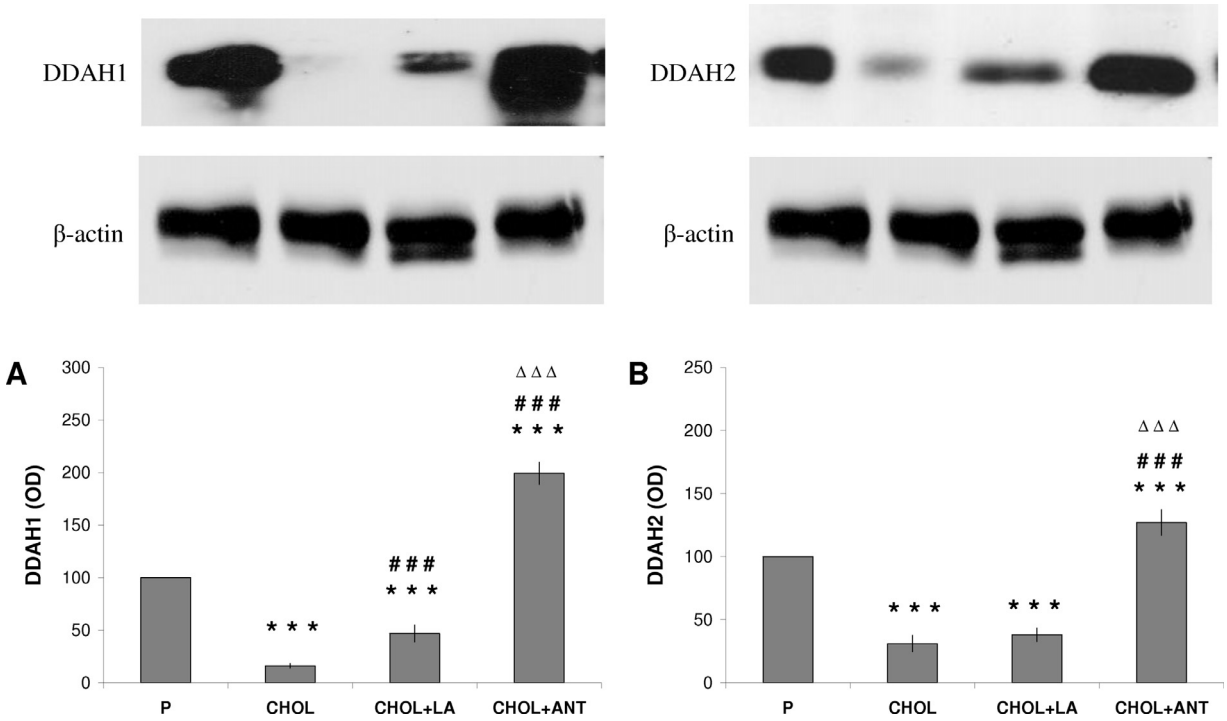

Fig. 6. Liver DDAH1 (A) and DDAH2 (B) isoform expression on day 60 of the experiment. P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values presented as the mean OD (optical density) ± SD. Specific comparisons: *** $p < 0.001$ *vs*. P, ### $p < 0.001$ *vs*. CHOL, ΔΔΔ $p < 0.001$ *vs*. CHOL + LA.

redox status in the blood.

Feeding a cholesterol-rich diet compared to the baseline caused a decrease in the L-arginine/ADMA ratio but not in the L-arginine level alone, and induced an increase in ADMA, SDMA, DMA, and L-citrulline levels.

The administration of loganic acid and, to lesser extent, of anthocyanins, increased the L-arginine level (Fig. 4A). The level in the CHOL + LA and in the CHOL + ANT groups was lower compared to the CHOL group, although it did not reach a level of statistical significance in the latter case. Both loganic acid and anthocyanins pronouncedly increased the L-arginine/ADMA ratio (Fig. 4B). Both compounds decreased the ADMA (Fig. 4C), but not the SDMA level (Fig. 4D). Finally, both anthocyanins, and—to a lesser extent—loganic acid, significantly decreased levels of DMA (Fig. 4E) and L-citrulline (Fig. 4F).

Administration of the high-cholesterol diet caused a slight decrease in DDAH activity compared to the negative control fed the standard diet. Administration of anthocyanins, though not loganic acid, slightly reversed these changes, enhancing the activity of DDAH in the liver compared to cholesterol-fed animals (Fig. 5).

In analyzing expression of DDAH1 and DDAH2 isoforms, more pronounced differences were found. The cholesterol-fed diet resulted in a significant decrease in both DDAH1 and DDAH2 isoform expressions. Treatment with anthocyanins prevented those changes significantly increasing expression of both DDAH1 and DDAH2 (Fig. 6A). Loganic acid exerted less pronounced effects, decreasing expression of DDAH1 isoform and causing no significant effect on DDAH2 expression (Fig 6B).

The cholesterol-fed diet resulted in a significant decrease of mRNA expression of eNOS. Treatment with both loganic acid and anthocyanins prevented those changes, significantly increasing expression of eNOS to levels even higher than in the negative control group, treated with standard feed (Fig 7).

Cholesterol feeding caused a significant depletion of the glutathione level in the blood, which was completely reversed by both loganic acid and anthocyanins. The glutathione levels in the CHOL + LA and in the CHOL + ANT groups were higher than in the CHOL group, and comparable to the P group, fed the standard diet (Fig 8A).

Cholesterol feeding caused a moderate increase of MDA level compared to the baseline. We did not observe any significant differences in MDA levels among the groups examined after 60 days of treatment (Fig. 8B).

GPx activity was depleted by cholesterol feeding. The administration of neither loganic acid, nor athocyanins, reversed these changes (Fig. 8C).

SOD levels at the baseline were not equal among groups. Cholesterol feeding caused a moderate decrease of SOD activity compared to the baseline. We did not observe any significant differences in SOD activity between the groups examined after 60 days of treatment (Fig. 8D).

Discussion

The most important finding of our study is that both iridoid loganic acid and anthocyanins isolated from edible fruit of the **c**ornelian cherry had a positive impact on the key substrate (L-arginine) and inhibitors (ADMA, SDMA) of nitric oxide synthases, and may influence NO bioavailability in blood vessels through the L-arginine-ADMA pathway in subjects with feed-induced atherosclerosis. The key results are that the administration of both investigated substances increased the L-arginine level and the L-arginine/ADMA ratio, and decreased ADMA but not SDMA levels in the blood. Anthocyanins and loganic acid also significantly increased mRNA expression of eNOS in thoracic aortas. Anthocyanins slightly increased hepatic DDAH activity and significantly increased mRNA expression of DDAH1 and DDAH2 isoforms, as DDAH1 is the main isoform responsible for ADMA catabolism. Secondly, both loganic acid and anthocyanins reversed the glutathione level in blood depleted by dietary cholesterol.

In this study, we investigated the plant constituents commonly consumed by humans, anthocyanins–abundant in many red, purple, and blue fruits and vegetables (Tsuda, 2012), and loganic acid, which belongs to iridoids, a subclass of monoterpenoids, substances present in the green parts of plants, some fruits, and a few animal sources. This study followed the animal model of feed-induced atherosclerosis used in the previous study on the **c**ornelian cherry fruit, in which we also demonstrated that the oral administration of whole lyophilisated **c**ornelian cherry fruit increased the L-arginine level and the L-arginine/ADMA ratio, and also decreased ADMA and SDMA levels. These findings were consistent with the effects observed in the current experiment on isolated substances. Many studies have demonstrated the beneficial effects of both anthocyanins and some iridoids on CVDs (Reis et al., 2016; Leong et al., 2016; Bulotta et al., 2014). Importantly, we demonstrated that consumption of the above mentioned various constituents isolated from the same edible fruit may positively modulate factors contributing to NO bioavailability in vessels, which may suggest the additive or synergistic effects of anthocyanins and iridoids on vascular functioning.

Although the high-cholesterol diet used in this study caused only a slight decrease in the L-arginine level, the L-arginine/ADMA ratio decreased significantly and administration of either of the investigated substances increased the L-arginine level and the L-arginine/ADMA ratio significantly. L-arginine is a conditionally essential amino-acid that is synthesized endogenously from L-citrulline. It is also consumed in large quantities in the western diet (Lüneburg et al., 2011; Böger, 2014). Some plant foods, including fruits, may also contain L-arginine, although to the best of our knowledge we found no data concerning the amino-acid composition of the **c**ornelian cherry fruits we investigated. In the current study, we have demonstrated that isolated loganic acid and anthocyanins from the **c**ornelian cherry exerted similar effects on the L-arginine level as the whole lyophilisated fruits used in our previous study (Sozanski et al., 2014). That excludes exogenous arginine (supposedly contained in the **c**ornelian cherry) as a principal factor responsible for the described effects.

Moreover, the decrease in ADMA and increase in the Arg/ADMA ratio with a concomitant increase in mRNA expression of eNOS in the thoracic aorta in animals fed either loganic acid or anthocyanins suggests an enhancement in NO bioavailability in vascular endothelium. The Arg/ADMA ratio is regarded as indicator of NO bioavailability and correlates with the risk of atherogenesis (Brinkmann et al., 2015). These findings are also consistent with our previous results showing that both substances can diminish dyslipidemia and decrease the intima thickness

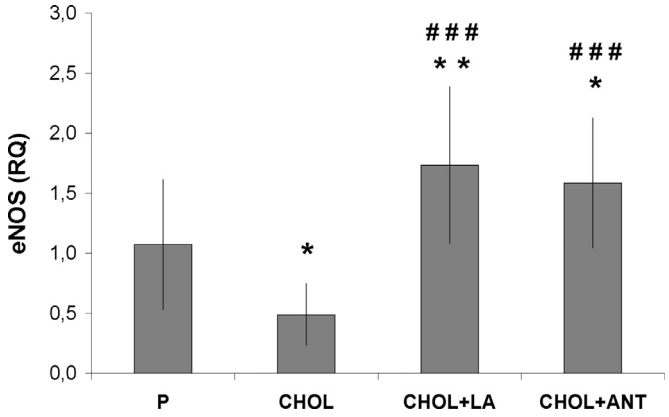

Fig. 7. eNOS mRNA expression in aorta on day 60 of the experiment. P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values presented as the mean RQ (relative quantity) ± SD. Specific comparisons: * $p < 0.05$ *vs.* P, ** $p < 0.01$ *vs.* P, ### $p < 0.001$ *vs.* CHOL.

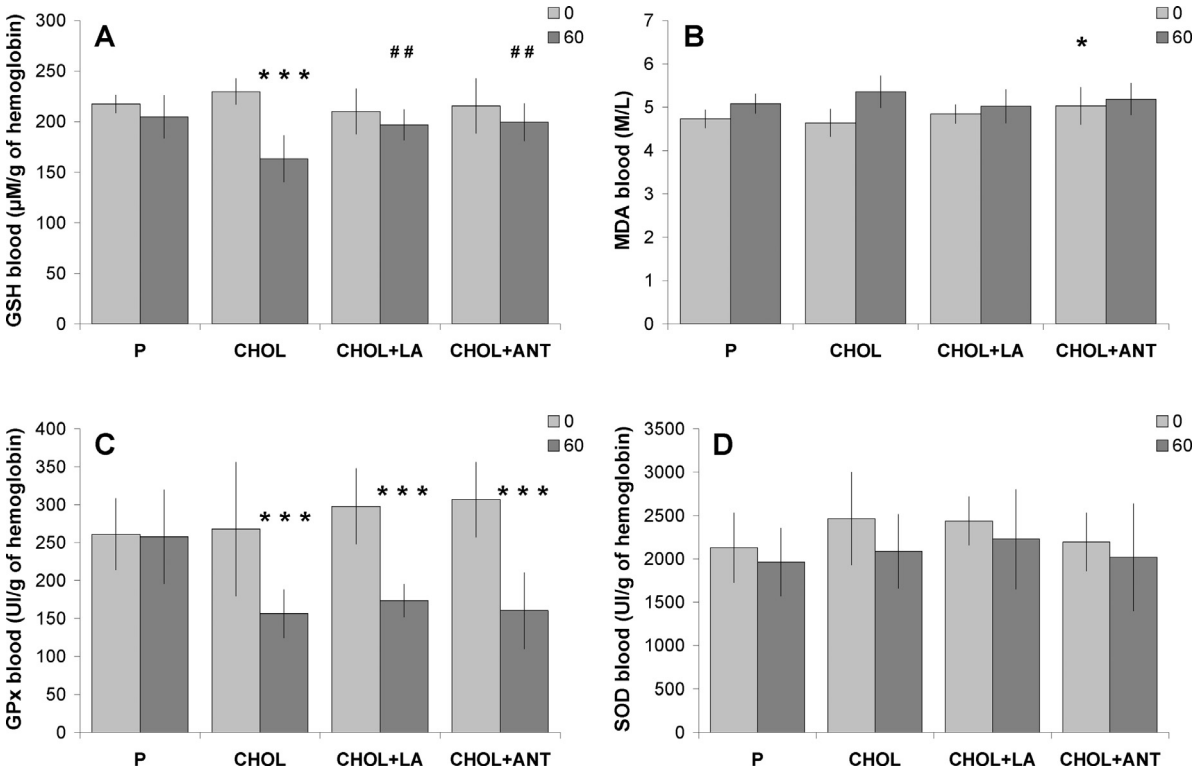

Fig. 8. Glutathione (GSH) concentrations (A), malondialdehyde (MDA) concentrations (B), glutathione peroxidase (GPx) activities (C), and superoxide dismutase (SOD) activities (D) in blood. P, standard feed and vehicle-treated rabbits; CHOL, cholesterol feed and vehicle-treated rabbits; CHOL + LA, cholesterol feed + loganic acid 20 mg/kg b.w. treated rabbits; CHOL + ANT, cholesterol feed + anthocyanins 10 mg/kg b.w. treated rabbits. Values presented as the mean ± SD. Specific comparisons: * $p < 0.05$, *** $p < 0.001$ vs. P, ## $p < 0.01$ vs. CHOL.

and the intima-media-ratio in the thoracic aorta, and decrease inflammatory markers in high-cholesterol fed rabbits (Sozanski et al., 2016). Increased intima thickness correlates with vascular dysfunction (Daiber et al., 2017). Notsu et al. (2015) found that a low Arg/ADMA ratio was independently correlated with intima-media thickness (IMT) in the carotid artery of community-dwelling people, and the Arg/ADMA ratio may serve as a sensitive marker of atherosclerosis. Correlation between IMT and ADMA has also been reported by other authors (Wang et al., 2018). This allows one to assume that changes in L-arginine and ADMA observed in our study may contribute significantly to the anti-atherosclerotic effect of both investigates substances. We also previously demonstrated an increased expression of liver PPAR alpha and gamma caused by both the cornelian cherry fruit and their isolated constituents. Those observations may be consistent with the described possible multidirectional interplay between PPARs and eNOS (Maccalini et al., 2017).

We have also investigated DDAH, the hepatic enzyme primarily responsible for the metabolism of methylated arginines, particularly ADMA. We demonstrated a moderate increase in DDAH activity caused by anthocyanin supplementation, and no significant effect of loganic acid. Then we analyzed the expression of DDAH1 and DDAH 2 isoforms, because DDAH1 is the main isoform responsible for ADMA metabolism. Although both anthocyanins and loganic acid significantly increased DDAH1 expression, the effect of anthocyanins was much more pronounced. Moreover, anthocyanins but not loganic acid increased expression of DDAH2. This suggests that activation of DDAH was the key reason of the decrease in ADMA caused by anthocyanins, although in the case of loganic acid the results obtained are equivocal. They may suggest that increased metabolism of ADMA was not the key or lone cause of its decrease in response to loganic acid. We should take into account that the decrease in ADMA may be also mediated by decreased ADMA synthesis or its decreased effect on NOS, rather than through its degradation (LiVolti et al., 2011). DDAH is also evolutionarily older than NOS, which may suggest other functions of methylated arginines. DDAH independently interacts with many other enzymes, such as protein kinases (Murphy et al., 2016) involved in pathways, regulating glucose and lipid metabolism. Thus, an interplay between ADMA and DDAH levels may be affected by other factors.

Our results also raise the question of whether the investigated substances directly impact the L-arginine-ADMA-DDAH pathway, or whether the observed changes are only a consequence of the impact on redox stress and inflammation. There is growing evidence that RONS, proinflammatory cytokines and oxLDL interplay with eNOS, indirectly decreasing DDAH activity and subsequently increasing the ADMA level, or directly causing eNOS uncoupling, which in turn causes an increase of RONS as well as direct cellular damage through increased O_2^- (Daiber et al., 2013). In the present study, we have demonstrated that the high-cholesterol feed caused a depletion of the blood glutathione level, and that the administration of either loganic acid or anthocyanins restored its levels significantly. This hypothesis may be supported by the findings of Li Volti et al. (2011), specifically that in *ex vivo* isolated aorta segments from db/db mice the increased contraction in phenylephrine—attributed to a reduced basal NO production—was not improved by the addition of silibinin, which caused a decrease in both blood and aorta ADMA levels. Moreover, the endothelium-independent vasodilation to the NO donor, nitroprusside sodium, was also impaired in that study. This suggests a different mechanism than changes in ADMA responsible for endothelial dysfunction, pointing to the increased generation of oxygen free radicals as a possible cause of NO scavenging or reduced sensitivity to NO.

As mentioned above, in the present study we have observed an increase in mRNA expression of eNOS in the thoracic aorta in both loganic acid and anthocyanins treated animals. Several previous studies have demonstrated that polyphenols might increase the expression and activity of eNOS, including a decrease in its uncoupling, thus improving NO signaling and vasodilation ability (Forte et al., 2016). Several

animal (Gonzalez et al., 2015) and human (Moreno-Luna et al., 2012) studies have shown that the effect might be mediated by effects on ADMA. Our finding that iridoids may also enhance NO bioavailability in blood vessels is consistent with the previous finding that cornuside, another iridoid found in the Cornaceae family, may dilate the smooth muscle of vessels in a manner dependent on endothelial NO/cGMP signaling. *Ex vivo* cornuside induced relaxation of the phenylephrine-precontracted isolated rat aortas, and that effect was negated by removal of endothelium or preincubation with NO inhibitors. Those effects were also confirmed *in vitro*. Cornuside enhanced production of cGMP in human umbilical vein endothelial cells (HUVECs) in a dose dependent manner, and the effect was also inhibited by preincubation with NO blockers (Kang et al., 2007). We did not examine cornuside, because it is a **c**ornelian cherry constituent present in much smaller amounts compared to loganic acid. However, higher amounts of cornuside are found in the Asiatic *Cornus officinalis* and may be present in the diet of some populations.

Aside from iridoids and anthocyanins, possible modulation of ADMA by an isolated single plant constituent was confirmed by *in vitro* and *in vivo* studies demonstrating that epigallocatechin—a flavonoid from green tee—prevented the ADMA-mediated endothelial dysfunction induced by a single injection of native LDL in rats, or by incubation of endothelial cell cultures with oxLDL (Tang et al., 2006). Moreover, in another study epigallocatechin protected *in vitro* human brain microvascular endothelial cells from ADMA-induced injury, through attenuation of ROS production, MDA expression, and apoptosis (Li et al., 2016). The impact of isolated plant constituents on ADMA was also found for silibinin—a flavonolignan found in milk thistle, which administrated intraperitoneally to dm/dm mice caused a decrease in the serum and aortic ADMA levels associated with the improvement of endothelium, (NO)-dependent vasodilation to acetylcholine in isolated *ex vivo* aorta segments (LiVolti et al., 2011). Those findings corroborate our results and point to the possible importance of some single constituents of plants in regulation of vascular functioning.

Analyzing the drawbacks of our study, we did not investigate markers of lipid metabolism, although we have done so in previous publications where both the model of feed-induced atherosclerosis and the positive impact of the investigated substances on plasma lipids were proven. Another limitation of our study is that we investigated animals, and although metabolic pathways in rabbits are more similar to humans than those of rodents, the results of our study should be replicated in human studies. Comparing the results of studies on whole fruits and on their isolated constituents, the significant differences among them were that a decrease of SDMA was seen only in the first study on whole fruits, and that an increase in DDAH was significant in the study on whole fruits, whereas in the present study it was demonstrated mainly in anthocyanins treated animals.

In summary, our study demonstrates that consumption of iridoid loganic acid or of anthocyanins isolated from the cornelian cherry fruit might modulate the L-arginine-ADMA pathway in subjects fed a high-cholesterol diet, and increases the mRNA expression of eNOS in thoracic aorta. Consumption of both investigated compounds may also raise the blood glutathione level. These findings suggest that various constituents of a diet may have an impact on the development and progression of CVDs through the modulation of factors affecting NO bioavailability. The mechanism of action responsible for the observed increase in L-arginine and decrease in the ADMA level is not fully understood, although examining isolated constituents we excluded the contribution of arginine, contained in many food products, as a cause of the observed effects. In the case of anthocyanins we found that they have a significant impact on the activity of DDAH and expression of DDAH isoforms, which may contribute to a decrease in ADMA through its increased metabolism. We have also pointed to the possible contribution of the blood redox system in L-arginine - ADMA changes. Our study adds a small piece of the puzzle of how a fruit and vegetable-rich diet may affect the pathological vital cycle involving redox stress, inflammation, and NO depletion, causing vascular dysfunction that, with concomitant dyslipidemia, leads to atherosclerosis and subsequent CVDs. It may also support the idea of assessing L-arginine and ADMA blood levels in populations at risk of CVDs.

Conflicts of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Competing interests statement

None.

Authors' contributions

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1. The main conception and design of the study
2. Participation in acquisition of laboratory data
3. Literature search
4. Analysis and interpretation of data collected
5. Main contribution in drafting of the article
6. Final approval and guarantor of manuscript

Alicja Z. Kucharska

1. Plant materials and samples preparation of loganic acid and anthocyanins
2. Acquisition of laboratory data – identification and determination of compounds by
3. UPLC-MS/MS and HPLC-PDA
4. Drafting in manuscript part of the methods section concerning plant materials, samples preparation and UPLC-MS/MS and HPLC-PDA
5. Critical revision of manuscript and its approval to be submitted

Jerzy Wiśniewski

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2. Drafting in manuscript part of the methods section concerning determination of L-arginine and its derivatives by HPLC-MS/MS
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2. Drafting in manuscript part of the methods section concerning eNOS

mRNA expressions
3. Critical revision of manuscript and its approval to be submitted

Piotr Dzięgiel

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Jan Magdalan

1. Literature search
2. Analysis and interpretation of data collected
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Beata Nowak

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2. Analysis and interpretation of data collected
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Dorota Szumny

1. Acquisition of laboratory data
2. Analysis and interpretation of data collected
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1. Literature search
2. Analysis and interpretation of data collected
3. Critical revision of manuscript and its approval to be submitted

Narcyz Piórecki

1. Providing cornelian cherry fruits
2. Critical revision of manuscript and its approval to be submitted

Adam Szeląg

1. Literature search
2. Analysis and interpretation of data collected
3. Critical revision of manuscript and its approval to be submitted

Małgorzata Trocha

1. Literature search
2. Analysis and interpretation of data collected
3. Drafting of the article
4. Critical revision of manuscript and its approval to be submitted

Acknowledgments

This study was financially supported by the statutory means of the Wrocław Medical University (ST.A080.17.035). Publication was supported by the Wrocław Centre of Biotechnology, Leading National Research Centre program (KNOW) for the years 2014–2018.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.09.175.

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