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Original Article

Black currant anthocyanins and their metabolites inhibit 5-lipoxygenase and low density lipoprotein oxidation

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ABSTRACT

Leukotriene formation, which is mediated by 5-lipoxygenase (5-LOX), and the oxidation of low-density lipoproteins (LDLs) have been shown to contribute to the development of atherosclerosis. Earlier studies have shown that both mechanisms are inhibited by polyphenols, especially anthocyanins. The rutinoside-conjugated anthocyanidins present in black currants (*Ribes nigrum* L.) have higher bioavailability than monosaccharide-conjugated anthocyanidins and may therefore be beneficial in reducing the risk of cardiovascular diseases. We investigated the *in vitro* inhibitory potentials of cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside against 5-LOX activity and LDL oxidation. Both anthocyanins were able to significantly inhibit 5-LOX activity and LDL oxidation. The investigated aglycones, cyanidin and delphinidin, showed a lower 5-LOX inhibitory potential, but were more potent at inhibiting LDL oxidation than their respective rutinosides. Furthermore, the inhibitory potentials of certain colonic degradation products of anthocyanins (protocatechuic acid, phloroglucinol aldehyde, vanillic acid, and gallic acid) were assessed. Gallic acid stimulated 5-LOX activity and was the only degradation product that was able to inhibit LDL oxidation. The other degradation products inhibited 5-LOX activity at high concentrations. Anthocyanins, anthocyanidins, and several of their degradation products demonstrated *in vitro* inhibition of mechanisms that contribute to atherosclerosis development.

KEYWORDS: cardiovascular health, cyanidin, delphinidin, hydroxybenzoic acid, rutinosides, *Ribes nigrum* L.

1. INTRODUCTION

Atherosclerosis plays a pivotal role in the development of cardiovascular diseases (CVD), especially ischemic heart disease (CAD), which, according to the World Health Organization (WHO), is the leading cause of death worldwide. The atherosclerotic condition is characterized by the thickening and inflammation of the innermost layer of the artery, the intima, which results in diminished arterial elasticity [1]. The risk of cardiovascular events can be reduced by managing risk factors such as hyperlipidemia or hypertension. Additionally, several studies have shown that the administration of antioxidants and a reduction in LDL oxidation decreases cardiovascular events among high-risk subjects [2]. The inhibition of 5-lipoxygenase (5-LOX) may represent a new approach for reducing CVD risk. 5-LOX initiates

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inflammation in various tissues and diseases (e.g. asthma) through the formation of leukotrienes. Furthermore, 5-LOX was found to be upregulated in atherosclerotic lesions and is believed to play a major role in atherosclerotic inflammation [3].

Several studies have shown that anthocyanins, a well-known class of flavonoids, significantly reduce CVD risk factors when participants consume anthocyanin-rich berries on a daily basis [4]. The Iowa Women's Health Study, a prospective study of 34,489 postmenopausal women, identified an inverse association between CVD occurrence and anthocyanin consumption [5]. Furthermore, anthocyanins have been shown to reduce LDL oxidation *in vitro*, and selected anthocyanins were identified as 5-LOX inhibitors *in vitro* [6-10]. Anthocyanins are red and blue pigments that can be found in a wide variety of plants, including edible plants. For example, they are responsible for the bright color of strawberries, blueberries, black currants, and red cabbage with cyanidin being the most abundant [11-14]. The bioavailability of intact anthocyanins is generally low because of the microbial degradation and/or pH shift that occurs during passage through the gut [15-17]. However, degradation products, especially phenolic acids, are readily absorbed, and this increases relative bioavailability [18, 19]. While monosaccharide-conjugated anthocyanins generally seem to be degraded, rutinoside conjugates sustain only minor losses during gastrointestinal passage [20]. The rutinoside delphinidin-3-*O*-rutinoside (Del3rut, tulipanin) and cyanidin-3-*O*-rutinoside (Cy3rut, antirrhinin), see Figure 1, are the major anthocyanins found in black currants (*Ribes nigrum* L.), with concentrations ranging from 180.3-311.4 and 70.4-138.8 mg/100 g fresh weight, respectively, in fruits and 0.089-0.689 and 0.055-0.356 g/L, respectively, in juice [14, 21]. Anthocyanin rutinosides, due to their bioavailability, are a promising nutritional complement.

Most previous studies have focused on investigating the physiological effects of the abundant glucoside anthocyanins, whereas little is known about the physiological effects of the more bioavailable rutinosides, especially in the context of CVD. This is why we examined the inhibitory potentials of delphinidin-3-*O*-rutinoside and cyanidin-3-*O*-rutinoside, along with their aglycones against 5-LOX and the initiation of LDL oxidation. As mentioned above, anthocyanin degradation products, especially phenolic acids, are formed in the gastrointestinal tract and are thereafter absorbed in the colon. Human intervention studies that examine CVD risk have focused solely on anthocyanins rather than on anthocyanins and their respective degradation products. Additionally, it is essential to investigate the impact of phenolic acids as they are well-known antioxidants [22, 23].

2. MATERIALS AND METHODS

2.1. Reagents

Human recombinant (*S. frugiperda*) 5-lipoxygenase (LOX) was purchased from Merck Millipore (Billerica, MA). Adenosine triphosphate, arachidonic acid, dithiothreitol (DTT), gallic acid, nordihydroguaiaretic acid (NDGA), phloroglucinol aldehyde, protocatechuic acid and vanillic acid were purchased from Sigma Aldrich (St. Louis, MO, USA), cyanidin and cyanidin-3-*O*-rutinoside from Phytolab (Vestenbergsgreuth, Germany) and

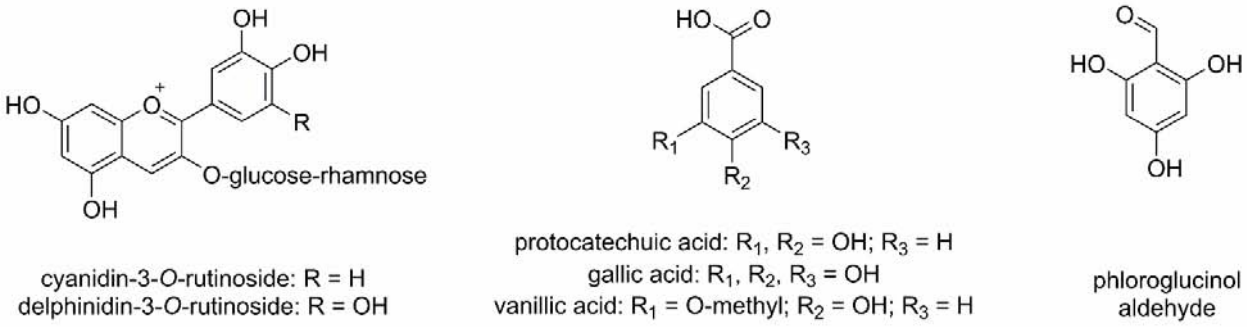

Figure 1. Structural formulas of selected black currant anthocyanins and their degradation products.

delphinidin-3-*O*-glucoside from Polyphenols A/S (Sandes, Norway). Human low-density lipoprotein was purchased from AppliChem (Darmstadt, Germany). Ascorbic acid was obtained from Th. Geyer (Renningen, Germany).

2.2. 5-Lipoxygenase activity assay

The activity of arachidonate 5-LOX was assayed spectrophotometrically according to literature with some modifications [8, 24, 25]. Reaction mixture was prepared with 5-LOX (approx. 2 U/mL) in reaction buffer containing ATP, DTT, $CaCl_2$ in final concentrations of 0.2, 0.25, and 2 mM respectively. Next, 50 µL of sample in 5% dimethyl sulfoxide (DMSO) was added to a 96-well plate (Greiner UV-Star, Frickenhausen, Germany) in triplicates, followed by 175 µL of the reaction mixture and incubated for 5 min at room temperature. Reaction was initiated at 37 °C by adding 25 µL of arachidonate (100 µM), prepared as described by literature, to reach a final reaction volume of 250 µL [26, 27]. The increase in absorbance at 236 nm was measured for 10 min every 12 s (Biotek Synergy 2, Winooski, VT). After subtraction of a blank rate, sample rates were compared to control rates. NDGA (10 µM) served as the positive control. IC_{50} and EC_{50} values for 5-LOX activity was calculated from the T/C in % after regression of the linear section of the dose-response curve with at least three concentrations in the regression line (at least one <50%).

2.3. LDL oxidation assay

Prevention of LDL oxidation was measured according to literature with modifications in a 96-well plate [28-30]. The 96-well plates were prepared by adding 20 µl of LDL solution (1 mg/ml) in phosphate buffered saline (PBS, pH 7.4) and 20 µl of either sample (end concentrations 1-200 µM based on the test substance) or water (control) to each well (Greiner UV-Star, Frickenhausen, Germany). The volume was then adjusted to 180 µl with PBS and the reaction was initiated by adding 20 µl of 50 µM $CuSO_4$ solution. Absorbance at 234 nm was monitored at 5 min intervals for 16 h. The typical lag, propagation, and decomposition phases of LDL oxidation were observed over this time period. The lag times of the samples were compared to the lag times of the solvent (negative control). Ascorbic acid (10 µM) was used as a positive control.

2.4. Stability of anthocyanins and anthocyanidins

The stability of anthocyanins and anthocyanidins under general assay conditions was also investigated. The compounds were incubated at pH = 7.4 and room temperature for 0, 5, 10, 20, 30, 40, 50, and 60 min, then stabilized with 10% formic acid and analyzed according to literature [13].

2.5. Statistical analysis

Results of LDL-oxidation assay and 5-LOX inhibition assay are expressed as mean ± standard deviation of at least three independent experiments. For statistical analyses Microsoft Excel 2010 was used and normal distribution was assumed but not tested due to low sample sizes. Homogeneity of variance was tested with the F-test at a significance level of 0.05. Subsequently, a one-sided Student's t-test was used to determine significant differences between means of sample and control. Asterisks reflect the level of significance: * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Outliers were identified with Nalimov's test.

3. RESULTS AND DISCUSSION

3.1. Inhibition of 5-lipoxygenase

The activity of 5-LOX was determined by measuring absorbance at 236 nm when 5-LOX was incubated with arachidonate and either a test substance or control solvent. Nordihydroguaiaretic acid (NDGA, 10 µM) was used as a positive control. A previously published procedure was modified so that the assay could be performed with 96-well plates. The observed 5-LOX inhibitory potential of the positive control was comparable to what has previously been reported [31]. Along with NDGA, delphinidin-3-*O*-glucoside (Del3glu, 10 µM) was used as a positive control with respect to literature [8]. An experimental IC_{50} value of 10 µM for Del3glu was determined which was slightly higher than the IC_{50} value of 2.15 µM reported by literature [8]. A possible explanation for this difference is the different sources of 5-LOX between literature and this study. In the study from literature 5-LOX was

extracted from neutrophil granulocytes [8], whereas we used human recombinant 5-LOX from *Spodoptera frugiperda*. The 5-LOX inhibitory potential of the anthocyanins Del3rut and Cy3rut was assayed at concentrations up to 20 µM. The compounds showed comparable concentration dependent 5-LOX inhibition with IC_{50} values of 18.2 ± 0.1 and 17.1 ± 3.4 µM, respectively, see Table 1. This indicates that the additional hydroxyl group in delphinidin did not influence 5-LOX inhibition. The inhibitory potentials of Del3rut and Cy3rut, when compared to that of Del3glu, were slightly lower, which suggests that saccharides had a minor influence on 5-LOX inhibition. A probable explanation for the observation that the disaccharides had less of an inhibitory effect than the monosaccharides may be steric hindrance of the bigger residues. However, testing the aglycones to evaluate the role of the saccharides revealed contradictory results. While an IC_{50} value of 36 ± 14 µM was determined for cyanidin, which was tested at concentrations up to 100 µM, delphinidin showed weak inhibition of 5-LOX activity over the same concentration range. These findings suggest that anthocyanidins are weaker 5-LOX inhibitors or less stable than their respective anthocyanins. An HPLC analysis revealed that Cy3rut was mostly stable under assay conditions while cyanidin degraded steadily over the studied time period (data not shown), as was expected based on the literature [32, 33]. It was assumed that delphinidin would also readily degrade under assay conditions of the present study; however, this was not investigated. The degradation of anthocyanidins can lead to several degradation products, including their B-ring metabolites protocatechuic acid (PCA), gallic acid (GA) and vanillic acid (VA), all of which were tested for their 5-LOX inhibitory potential at concentrations up to 300 µM, see Figure 2. PCA, with an IC_{50} value of 263 ± 30 µM, showed the highest inhibitory potential of the tested phenolic acids. The inhibitory potential of VA was slightly lower than that of PCA; however, an IC_{50} value for VA could not be determined because the 50% inhibition threshold was not reached at the tested concentrations. At 300 µM, VA exerted inhibition of 41.6%. The testing of higher concentrations caused problems with the plate reader's upper detection limit. Interestingly, GA did not inhibit, but rather activated 5-LOX with an EC_{50} value of 199 ± 31 µM. The influence of the A-ring metabolite phloroglucinol aldehyde (PGA) on 5-LOX activity was also tested. PGA exerted significant ($p < 0.05$), concentration-dependent 5-LOX inhibition and its inhibitory potential was comparable to that of VA. However, an IC_{50} value could not be determined due to

Table 1. IC_{50} or EC_{50} values for 5-lipoxygenase (5-LOX) inhibition by the tested compounds. These values were determined based on at least three independent experiments. Percentages in brackets depict the 5-LOX activity at the highest concentration tested.

Anthocyanins	
Del3rut	18 ± 0 µM
Cy3rut	17 ± 3 µM
Anthocyanidins	
Delphinidin	n.d.
Cyanidin	36 ± 14 µM
Phenolic acids	
GA	199 ± 31 µM[a]
PCA	263 ± 30 µM
PGA	> 300 µM (56.0 ± 2.8%)
VA	> 300 µM (58.6 ± 13.1%)

[a]activating effect, EC_{50}; n.d. = not determinable due to poor inhibitory effects (< 50%).

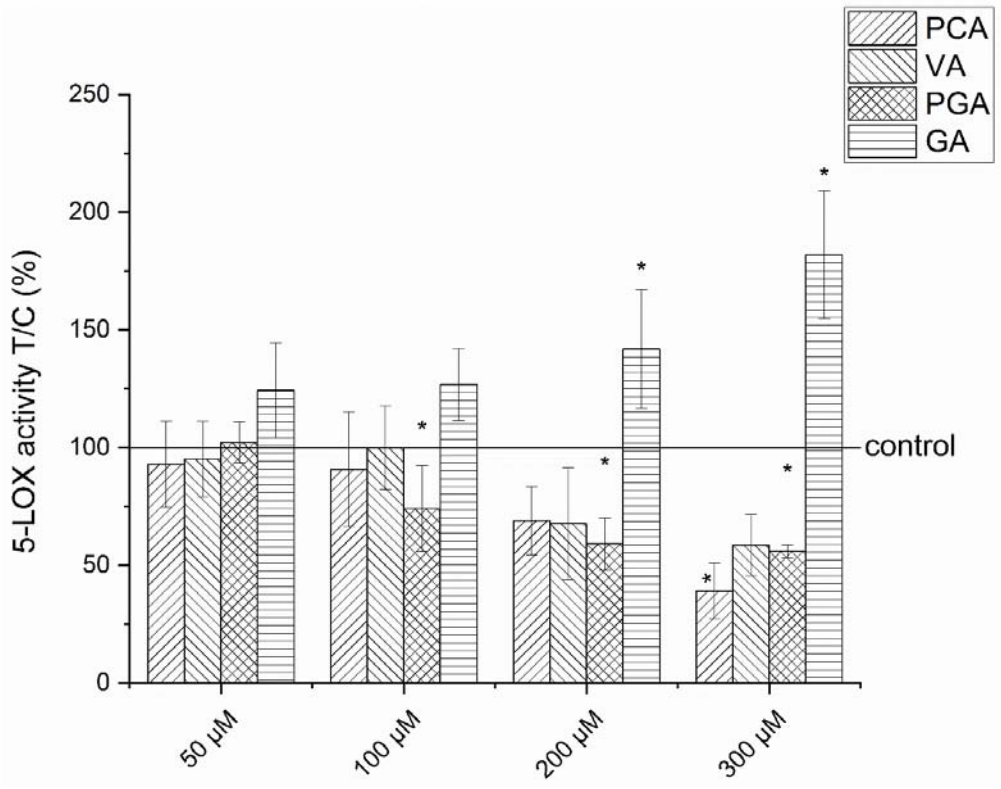

Figure 2. Inhibition of 5-lipoxygenase (5-LOX) by the anthocyanin degradation products protocatechuic acid (PCA), vanillic acid (VA), gallic acid (GA), and phloroglucinol aldehyde (PGA) at 50, 100, 200, and 300 µM. Data are presented as the mean ± standard deviation of at least three independent experiments. The significance of differences between the means of samples and controls was assessed with a Student's t-test. Significance levels: $p < 0.05$ - *.

inhibition of 44% at the highest tested concentration (300 µM).

PCA is the only phenolic acid that has already been reported to be a weak inhibitor of 5-LOX, with an observed IC_{50} value of 160 µM [34]. The IC_{50} value determined in this study was in the same range (263 ± 30 µM). In another study, PCA showed no inhibitory effect on 13-LOX, a lipoxygenase present in plants [35]. The same study found that VA is a weak inhibitor of 13-LOX (IC_{50} value of 85.6 µM), but not of 5-LOX. Additionally, GA has been reported to be a weak inhibitor of 12-LOX with an IC_{50} value of 393 µM [36]. Thus, to the best of our knowledge, only PCA has been reported to influence 5-LOX activity. However, our results show that other anthocyanin degradation products can exert significant inhibitory effects as well. With the exception of GA, the degradation products showed weak 5-LOX inhibition in the order of PCA > VA ≈ PGA. A possible explanation for the 5-LOX-activating effect of GA in this study is its pro-oxidative potential under certain circumstances leading to oxidation of Fe^{2+} in the catalytic subunit activating 5-LOX [37-40].

3.2. Inhibition of low density lipoprotein oxidation

The inhibition of LDL oxidation was assessed after the reaction was initiated with 5 µM copper sulfate. The lipid peroxidation lag time observed in the samples was compared versus a negative (solvent) and a positive control (ascorbic acid, 10 µM). The negative control showed lag times between 58 and 87 min (mean 75 ± 10 min, n = 10), see Figure 3, while the positive control ascorbic acid prolonged mean lipid peroxidation lag time by 59 minutes (134 min absolute). The anthocyanin Cy3rut prolonged the lag time

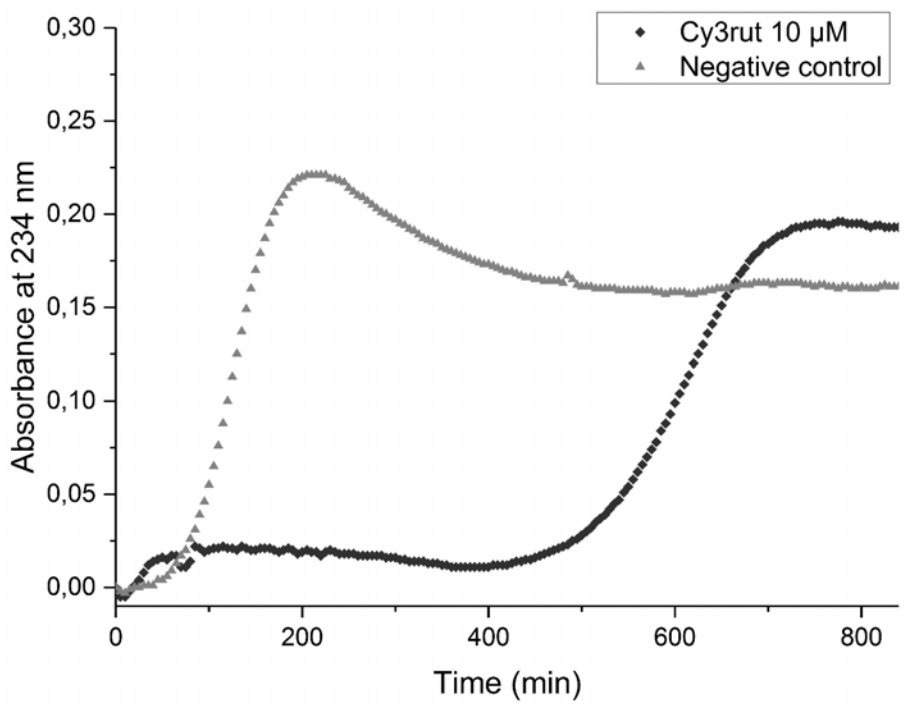

Figure 3. Diene formation, based on absorbance at 234 nm, as an indicator of low density lipoprotein (LDL) oxidation when an LDL solution was incubated with a control solvent (negative control) and cyanidin-3-*O*-rutinoside (10 µM).

significantly ($p < 0.001$) more than the positive control. At a concentration of 10 µM, Cy3rut prolonged the mean lag time by 360 min (435 min absolute), see Figure 3. Measurements at higher concentrations (> 10 µM) exceeded the upper limit of the detector and hence the results could not be evaluated. Lower concentrations of Cy3rut did not noticeably prolong the lag time. In comparison, aglycone cyanidin prolonged the mean lag time by 412 min at a concentration of 5 µM ($p < 0.001$). No LDL oxidation was observed during the entire 16 h monitoring time when the concentration of cyanidin was increased to 10 µM. As with Cy3rut, no prolongation was observed at lower concentrations of cyanidin. A more concentration-dependent pattern was observed with Del3rut and the aglycone delphinidin compared to Cy3rut and cyanidin, see Figure 4, within similar ranges of lag time prolongation. Del3rut prolonged the mean LDL oxidation lag time by 117 and 446 mins at concentrations of 5 µM and 10 µM ($p < 0.01$), respectively. The aglycone delphinidin prolonged the mean lag time by 67 min already at a concentration of 5 µM ($p < 0.001$), and no oxidation was observed during the entire 16 h monitoring time when the concentration was increased to 10 µM. The potentials of Cy3rut, Del3rut, cyanidin, and delphinidin to prolong LDL oxidation seem to be significantly higher than that of the positive control, ascorbic acid, at 10 µM. The obtained results indicate that the anthocyanidins are more potent inhibitors of metal-catalyzed LDL oxidation than their respective rutinosides. This relation has already been identified in other *in vitro* studies that showed that the antioxidative potential of anthocyanidins decreased as the size of the conjugated saccharide increased. Furthermore, our results show that there is no difference between the delphinidin and cyanidin anthocyanins in terms of antioxidative potential, a finding that corroborates previous literature [41, 42]. However, an analysis of the stability of these substances under the 5-LOX assay conditions indicated that the aglycones are not stable at these conditions (pH 7.4), a finding which makes it difficult to interpret the results.

The degradation products VA (20-200 µM), PGA (5-100 µM), and PCA (5-20 µM) did not

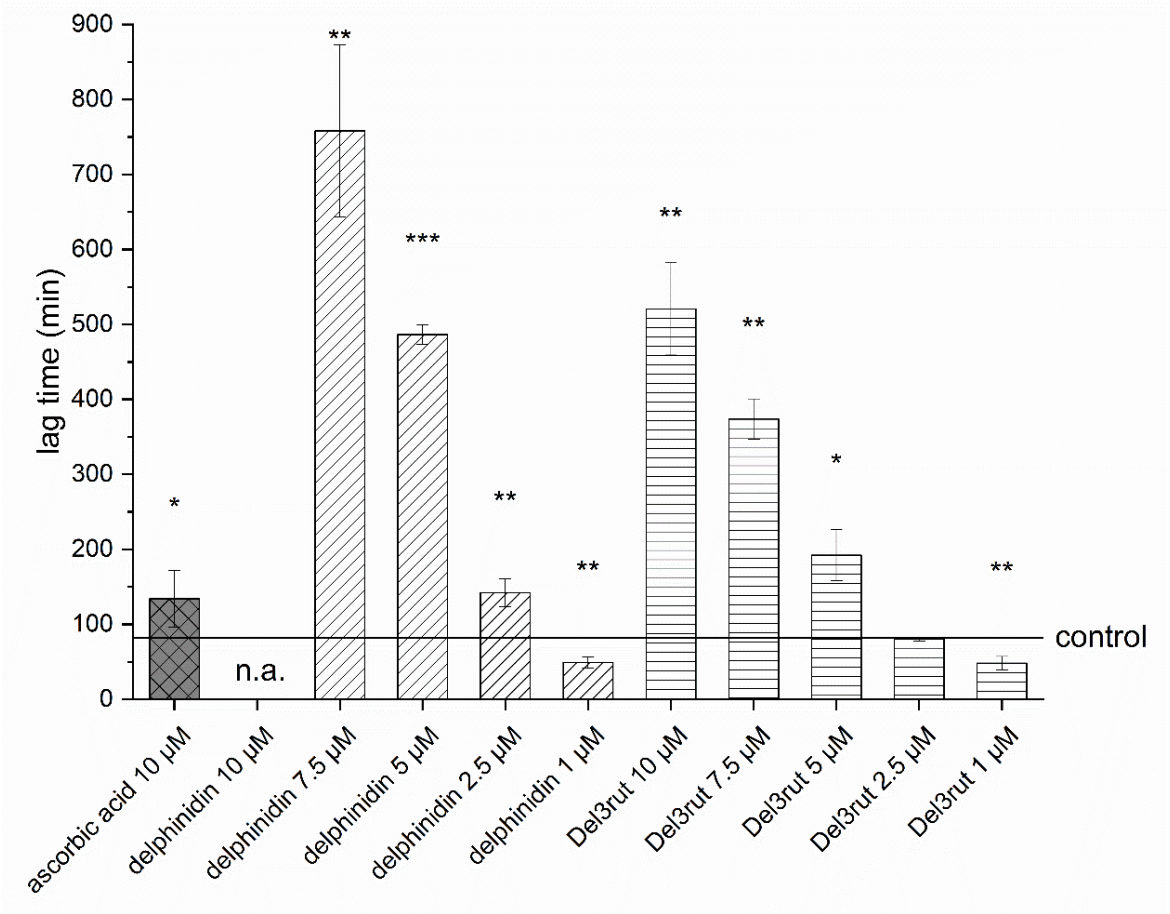

Figure 4. Lag times of low density lipoprotein (LDL) oxidation (diene formation) for delphinidin-3-rutinoside and delphinidin (1-10 µM each). Data are presented as the mean ± standard deviation of at least three independent experiments. The significance of differences between the means of samples and controls was assessed with a Student's t-test. Significance levels: $p < 0.05$ - *; $p < 0.01$ - **; $p < 0.001$ - ***.

demonstrate LDL oxidation inhibiting effects in the assay. PCA had pro-oxidative effects, as a decreased lag time was observed at all tested concentrations. GA significantly ($p < 0.001$) prolonged the mean lag time by 72 and 291 min at 5 and 10 µM, respectively, but did not affect lag time at lower concentrations, see Figure 5. Previous studies have tested the degradation product VA in different antioxidative assays but have not found any antioxidative potential, which is consistent with our findings [23, 43]. The assay presented in this paper assesses how a certain compound can prevent LDL oxidation based on the stabilization of the catalyst Cu^{2+} and the regeneration of LDL-incorporated antioxidants such as tocopherols [28]. Polyphenols are generally able to prevent oxidation through mechanisms such as the chelation of catalytic metal ions, which preferably involves the B-ring, or acting as a reducing agent in self-radicalization and quinoid stabilization [44]. This rule is applicable to certain hydroxybenzoic acids. However, VA, which has a single hydroxy group and a methoxy group, does not meet the structural requirements for radical stabilization or metal chelation and did not show any antioxidative effect in our assay. In comparison, PGA and PCA possess the structural prerequisite but did not exert antioxidative effects in the assay as well. PGA has not been tested for its antioxidative potential before, whereas PCA has been identified as an antioxidant with either a light or strong influence depending on the deployed assay [23, 43]. Further mechanistic studies of PCA in literature revealed a weak potential to

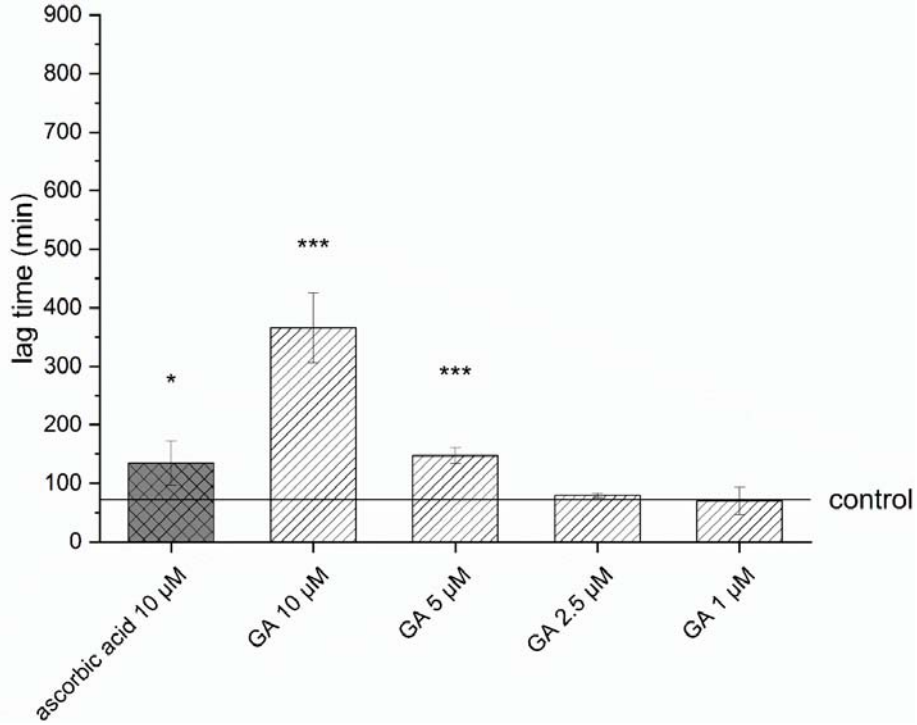

Figure 5. Lag time of low density lipoprotein (LDL) oxidation (diene formation) for gallic acid (GA, 1-10 µM). Data are presented as the mean ± standard deviation of at least three independent experiments. The significance of differences between the means of samples and controls was assessed with a Student's t-test. Significance levels: $p < 0.05$ - *; $p < 0.001$ - ***.

chelate metal ions, which explains why no antioxidative effect was observed in our study [45]. Additionally, phenolic substances are, under certain circumstances, able to generate reactive oxygen species (ROS) in the presence of oxygen and catalytic metals [46, 47]. PCA seems to act as either an antioxidant or a weak pro-oxidant based on the testing system applied. GA is already renowned for its antioxidative potential [48]. Taking results from literature into account, an antioxidative effect was expected at concentrations lower than 5 µM. In the present study however, the inhibition of LDL oxidation was mostly observed when the concentration of the inhibiting compound was 5 µM. This indicates a threshold concentration that is consistent with the catalytic Cu^{2+} concentration of 5 µM. However, the physiological concentrations of these compounds from anthocyanin-rich foods and extracts are significantly lower than those used in our assay. In this context, c_{max} values of 73.4 nM and 46.3 nM for Del3rut and Cy3rut, respectively, were observed in a study from literature after the consumption of a black currant extract [49]. In regard to this, the antioxidative effects that were found within the present study cannot exclusively explain the anti-atherosclerotic mechanisms of an anthocyanin-rich diet. Evidence suggests that in the context of CVD anthocyanins not only act as direct antioxidative or anti-inflammatory agents but also on a cellular level [50]. Thus, studying the effects of black currant anthocyanins and phenolic acids on e.g. 5-LOX expression, Nrf2 regulation, or NO production could deliver more answers to whether these rutinosides and their degradation products also affect atherosclerosis on a cellular level. As phenolic acids not only derive from anthocyanins but other polyphenols as well and can be found in foods, anthocyanin-rich foods are not the only source of these antioxidants [51-53]. In this context, a c_{max} of 25 nM was found for the anthocyanin degradation product GA after the consumption of bilberry extract [19] and after the consumption of Assam black tea a c_{max} of 2.1 µM

was observed [54]. This indicates that GA plasma concentrations can reach levels that are in the LDL oxidation inhibiting range when GA is obtained from different food sources. In general, the tested substances that exerted antioxidative effects exhibited lag times that were 2.7-3.9 times longer than that of ascorbic acid at 10 µM. Thus, these substances possess a higher potential to prevent LDL oxidation than ascorbic acid in the frame of this study. The anthocyanins and degradation products used for this investigation represent the biological activity of some of the key constituents contained within the nutritional supplement 'MEDOX®' (MedPalett AS, Sandnes, Norway), a bilberry and blackcurrant extract consisting of highly purified anthocyanins.

4. CONCLUSION

In conclusion, the anthocyanins Del3rut and Cy3rut, which are present in black currants, were able to inhibit 5-LOX activity at concentrations in the µM range. Their inhibitory potentials were similar to those of the positive controls NDGA and Del3glu. The results of aglycones were difficult to interpret due to their degradation during the assay. Cyanidin showed inhibition of 5-LOX whereas delphinidin did not. The degradation products PCA, VA, and PGA showed weak 5-LOX inhibitory potentials while GA seemed to activate 5-LOX. Furthermore, the anthocyanin rutinosides Cy3rut and Del3rut were able to significantly delay LDL oxidation at µM concentrations; the potentials of their aglycones were slightly higher, which is in accordance with the literature. Interestingly, GA was the only anthocyanin degradation product that showed LDL oxidation inhibiting potential.

Taken together, anthocyanin rutinosides from black currants, their aglycones, and some degradation products were able to inhibit pivotal mechanisms of atherosclerosis *in vitro*. The inhibitory potentials of the anthocyanin rutinosides were comparable to those of other anthocyanins [8]. Due to higher bioavailability, anthocyanin rutinosides may be more beneficial for reducing CVD risk than monosaccharide anthocyanins. Additionally, the degradation products that form in the colon during the digestion of anthocyanins may significantly contribute to the inhibitory potential of anthocyanin rutinosides. However, further studies on the role of anthocyanins and their degradation products in the reduction of CVD risk factors are required to fully understand how the consumption of red fruits may lower the relative risk of CVD.

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CONFLICT OF INTEREST STATEMENT

This study was funded by Evonik Nutrition & Care GmbH, Hanau, Germany. No other conflict of interest exists besides this funding. Some authors are employees of Evonik Nutrition & Care GmbH.

ABBREVIATIONS

5-LOX	:	5-lipoxygenase
CAD	:	coronary artery disease
CVD	:	cardiovascular diseases
Cy3rut	:	cyanidin-3-*O*-rutinoside
Del3rut	:	delphinidin-3-*O*-rutinoside
GA	:	gallic acid
NDGA	:	nordihydroguaiaretic acid
PCA	:	protocatechuic acid
PGA	:	phloroglucinol aldehyde
VA	:	vanillic acid

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