Research Article

Anthocyanins as lipoxygenase inhibitors

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Health benefits associated with diets rich in anthocyanins are ascribed to multilevel biological activities including antioxidative and anti-inflammatory effects. The present study addresses lipoxygenase inhibition as a mechanism by which anthocyanins may exert health promoting effects. The inhibitory potential of delphinidin (Dp), cyanidin (Cy), peonidin (Pn), and malvidin (Mv) glycosides, *i.e.*, 3-*O*-glucosides, 3-*O*-galactosides, and 3-*O*-arabinosides as well as their aglycons was analyzed by using soybean lipoxygenase-1 and human neutrophil granulocyte 5-lipoxygenase. The determined IC_{50} values comprised a wide range, *i.e.*, from the sub-μM level until practically no effect of inhibition (Mv and its glycosides). With IC_{50} values of 0.43 and 0.49 μM Dp 3-*O*-glucoside (Dp3glc) and Dp 3-*O*-galactoside (Dp3gal) were found to be the most effective soybean lipoxygenase-1 inhibitors; their strong inhibitory potential was also reflected by the IC_{50} values determined for these anthocyanins in the 5-lipoxygenase inhibition exhibiting 2.15 and 6.9 μM, respectively. As to the mechanism of inhibition, experiments carried out with lipoxygenase-1 revealed the uncompetitive type. Considering the powerful inhibitory properties of Dp glycosides in relation to their currently known availability in human metabolism, *in vivo* prevention of inflammatory diseases by these anthocyanins could be envisaged.

Keywords: Anthocyanins / Delphinidin 3-*O*-galactoside / Delphinidin 3-*O*-glucoside / Human neutrophil granulocyte 5-lipoxygenase / Soybean lipoxygenase-1

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1 Introduction

Lipoxygenases (linoleate oxygen oxidoreductase, EC 1.13.11.12) are widely distributed in nature [1]. They are nonheme iron enzymes which are composed of a single polypeptide chain that is folded into two domains, the mostly α-helical catalytic domain and the N-terminal β-barrel domain that is involved in membrane binding. The catalytic iron and three out of the five amino acids that serve as iron ligands are conserved within the lipoxygenase gene family. The catalytic domain converts the 1,4-*cis*, *cis* double bonds in unsaturated fatty acids to 1,3-*cis*, *trans*-5-hydroperoxides by a radical, stereo-, and regioselective mechanism [2].

Higher plants contain multiple lipoxygenases with at least eight identified in soybean, *Glycine max*. (a.o. soybean lipoxygenase-1); all of them use linoleic or linolenic acid as substrate [1]. There are five lipoxygenase enzymes in humans that are active on arachidonic acid (released from cell membranes by phospholipase A_2 activity in response to receptor signaling) which catalyze four distinct reactions, *i.e.*, 5*S*, 12*R*, 12*S*, or 15*S* oxygenation. The products are individual fatty acid hydroperoxides, members of the eicosanoids, which are, apart from phosphoinositides, sphingolipids, and fatty acids, a group of lipid signaling molecules. They have the capacity to trigger physiological responses, including cell proliferation, apoptosis, metabolism, and migration [3]. Among the human lipoxygenases, 5-lipoxygenase is the key enzyme responsible for the biosynthesis of the biologically active leukotrienes, which are potent mediators in inflammatory and allergic reactions [4]. In order to prevent resulting diseases, 5-lipoxygenase inhibition is of great therapeutic target and the research for new as well as natural inhibitors of lipoxygenase is inevitable. As to natural inhibitors, there is sufficient information about plant ingredients capable of suppressing 5-lipoxygenase activity. Apart from quinones, triterpenes, and sesquiterpenes, alkaloids and polyacetylenes, the group of polyphenols (including flavonoids) constitute the most prominent class of plant derived inhibitors of 5-lipoxygenase product synthesis [5]. Surprisingly, compared to other fla-

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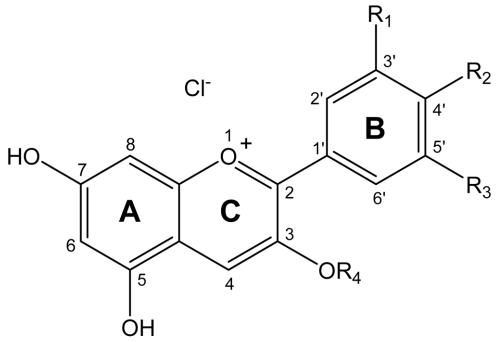
Abbreviations: Cy, cyanidin; **Cy3ara,** Cy 3-*O*-arabinoside; **Cy3gal,** Cy 3-*O*-galactoside; **Cy3glc,** Cy 3-*O*-glucoside; **Dp,** delphinidin; **Dp3ara,** Dp 3-*O*-arabinoside; **Dp3gal,** Dp 3-*O*-galactoside; **Dp3glc,** Dp 3-*O*-glucoside; **Mv,** malvidin; **Mv3glc,** Mv 3-*O*-glucoside; **PBS,** Phosphate buffer saline; **Pn,** peonidin; **Pn3ara,** Pn 3-*O*-arabinoside; **Pn3gal,** Pn 3-*O*-galactoside; **Pn3glc,** Pn 3-*O*-glucoside



	R_1	R_2	R_3	R_4
Dp3glc	OH	OH	OH	glc
Dp3gal	OH	OH	OH	gal
Dp3ara	OH	OH	OH	ara
Dp	OH	OH	OH	H
Cy3glc	OH	OH	H	glc
Cy3gal	OH	OH	H	gal
Cy3ara	OH	OH	H	ara
Cy	OH	OH	H	H
Pn3glc	OCH_3	OH	H	glc
Pn3gal	OCH_3	OH	H	gal
Pn3ara	OCH_3	OH	H	ara
Pn	OCH_3	OH	H	H
Mv3glc	OCH_3	OH	OCH_3	glc
Mv	OCH_3	OH	OCH_3	H

Figure 1. Chemical structures of anthocyanins and anthocyanidins under study.

vonoid groups, such as flavonols [6] or isoflavones [7], information about the interaction of anthocyanins and anthocyanidins with lipoxygenase is rather scarce. Exclusively Narayan *et al*. [8] described a significant and dose dependent inhibition of soybean lipoxygenase with a deep red color pigment from carrot cell culture.

Anthocyanins are glycosylated, polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium and contain two benzoyl rings (A and B) separated by a heterocyclic (C) ring (Fig. 1). They usually appear as 3-monosides, 3-biosides, and 3-triosides as well as 3,5-diglycosides and, less often, 3,7-diglycosides joined with the sugars; glucose, galactose, rhamnose, arabinose, and xylose. Anthocyanins are known to play an important role providing the red, purple, and blue colors of many vegetables and fruits as well as products made thereof. Evidence that anthocyanins have beneficial effects for health are increasingly being reported in the scientific literature; they comprise, among others, effects of signal transduction, apoptosis, epidermal growth factor receptor, and cyclooxygenase activity in addition to antioxidative properties [9].

The occurrence of anthocyanins is widespread in plants and these compounds are consumed as part of a normal diet. Recent studies suggest that the average anthocyanin consumption ranges from 12.5 (in USA) to 82 mg/day (in Finland) [9]. The apparent bioavailability of anthocyanins is regarded to be very low with often less than 0.1% of the ingested dose appearing in the urine. Current studies show that they are little modified by gastric conditions and that amounts which are not absorbed in the stomach or small intestine are substantially modified to phenolic acids in the colon [9]. As to the small intestine, Kahle *et al*. [10] found that high amounts of blueberry anthocyanins (up to 85%) passed the small intestine unmetabolized and reached the colon after oral intake. Concerning absorption in the colon a transport efficiency of blueberry anthocyanins across Caco-2 monolayers averaging 3–4% was reported. Despite the low efficiency, this is an indication that a portion of the anthocyanins could be absorbed in the colon and fulfill the described protective effects [11].

The present study addresses lipoxygenase inhibition as a mechanism by which anthocyanins may exert health promoting effects. As an *in vitro* biochemical model, we used soybean lipoxygenase-1 [12]. Our investigations were extended by testing additionally the most effective soybean lipoxygenase-1 inhibitors for their human neutrophil granulocyte 5-lipoxygenase inhibition potential.

2 Materials and methods

2.1 Chemicals

All chemicals and solvents were of analytical grade. Solvents were redistilled before use. Water was obtained from a Millipore™ water purification unit. ACN (CH_3CN, HPLC gradient grade) and methanol were from Fisher Scientific (Leicestershire, UK), formic acid was purchased from Gruessing (Filsum, Germany). Amberlite XAD-7 was obtained from Supelco (Bellefonte, USA). Dp was from Extrasynthèse (Lyon, France), cyanidin (Cy), peonidin (Pn), and malvidin (Mv) were from Alsachim (Illkirch Graffenstaden, France). Trypan blue solution, ammonium chloride, linoleic acid, arachidonic acid, boric acid, soybean lipoxygenase-1, DTT, and calcium chloride dihydrate were ordered from Sigma (Steinheim, Germany). Phosphate buffer saline (PBS) and Biocol density gradient solution were from Biochrom (Berlin, Germany). Sodium citrate and tris(hydroxylmethyl)-aminomethane (TRIS) were obtained from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from VWR (Fontenay sous bois, France). Oleic acid and nordihydroguaiaretic acid (NDGA) were from Fluka (Buchs, Switzerland). All the other chemicals were obtained from Sigma (Steinheim, Germany).

2.2 Extraction and isolation of anthocyanins

The used anthocyanins were isolated from lowbush (wild) blueberries (*Vaccinium myrtillus*) (Dp 3-*O*-glucoside

(Dp3glc), Dp 3-*O*-galactoside (Dp3gal), Dp 3-*O*-arabinoside (Dp3ara), Mv 3-*O*-glucoside (Mv3glc)), commercially available aronia (*Aronia melanocarpa*) concentrate (Cy 3-*O*-glucoside (Cy3glc), Cy 3-*O*-galactoside (Cy3gal), Cy 3-*O*-arabinoside (Cy3ara)) and cranberry (*Vaccinium macrocarpon*) juice (Pn 3-*O*-glucoside (Pn3glc), Pn 3-*O*-galactoside (Pn3gal), Pn 3-*O*-arabinoside (Pn3ara)).

To produce phenolic crude extracts from blueberries, extraction was carried out according to Kaehkoenen *et al*. [13]. For this 2 g berries were homogenized with 20 mL of solvent (H_2O/CH_3CN/HCOOH 50:49:1) for 1 min (Ultra-Turax T25 mixer; Janke & Kunkel, Staufen, Germany). The sample was centrifuged ($1300 \times g$, 15 min) and the clear supernatant was collected. The extraction was carried out twice. The collected supernatants were evaporated and the solid residues were dissolved in 0.5% HCOOH. The purchased aronia concentrate and cranberry juice were used unextracted.

For isolation, crude blueberry extracts, aronia concentrate, and cranberry juice were further purified by a method modified by [13] using Amberlite XAD-7 column chromatography. The sample was applied to a glass column (300×40 mm), and free sugars, organic, and phenolic acids were eluted with 2% HCOOH. The anthocyanin containing fraction was obtained by elution with 50% CH_3CN (CH_3CN/H_2O/HCOOH 50:49.5:0.5) followed by a washing step with CH_3CN (CH_3CN/HCOOH 99.5:0.5).

2.3 Preparative HPLC/UV–Vis fractionation

The further fractionation of the isolated extracts was performed using preparative HPLC. The HPLC system consisted of a Rheodyne Six-Port injection system with a 1 mL sample slope (Rheodyne Europe, Alsbach, Germany), a Knauer K1001 pump and a Knauer UV–Vis detector (Knauer, Berlin, Germany) set at the detection wavelength of 520 nm. Typically, 400 μL of sample were injected and separated at a flow rate of 8 mL/min. Separation was carried out using a Waters XBridge™ Prep C18 column (150×19 mm, 5 μm particle size; Waters, Milford, MA, USA). The mobile phase consisted of 10% HCOOH (solvent A) and CH_3CN (solvent B). The gradient was as follows: isocratic elution with 2% B from 0 to 36 min; 4% B from 36 to 50 min; 6% B from 50 to 75 min; 9% B from 75 to 85 min.

The isolated anthocyanins were quantified by weighing and the yields were as follows: 2.3% Dp3glc, 1.4% Dp3gal, 2.5% Dp3ara, 0.8% Mv3glc (percentages referred to blueberry XAD extract); 0.4% Cy3glc, 0.1% Cy3gal, 0.3% Cy3ara (percentages referred to aronia concentrate); 0.3% Pn3glc, 5.1% Pn3gal, 2.4% Pn3ara (percentages referred to cranberry juice). The purity of anthocyanins was checked by HPLC/DAD analysis and individual compounds were identified using HPLC–ESI-MS/MS. Both techniques were performed according to the literature data [10, 13].

2.4 Isolation of 5-lipoxygenase enzyme from human neutrophil granulocytes

Human peripheral blood samples were obtained from healthy volunteers who gave informed consent. None of the volunteers was treated with pharmaceuticals for the last 4 wks prior taking of blood samples. Human neutrophil granulocytes were isolated with slight modifications according to [14] by Biocol density gradient and hypotonic lysis of erythrocytes. For this 45 mL of blood was mixed with PBS buffer (pH 7.4) and immediately transferred to falcon tubes containing 20 mL of Biocol solution. After centrifugation ($680 \times g$ at room temperature for 45 min) leukocytes and erythrocytes precipitated forming a pellet while thrombocytes and plasma remained in the supernatant, which was discarded. The pellet was then suspended in 10 mL PBS buffer (pH 7.4). After centrifugation at $300 \times g$ at 4°C for 10 min and removal of the supernatant, the resulting pellet was suspended in 10 mL of hypotonic lysis buffer (0.17 g NH_4Cl, 0.2 g TRIS, H_2O to 100 mL, pH 7.2) and gently shaken for 10 min at 37°C to destroy remaining erythrocytes. The suspension was centrifuged at $300 \times g$ at 4°C for 15 min. The supernatant was discarded and the pellet washed with PBS buffer ($300 \times g$, 4°C, 10 min). The lysis and washing steps were repeated until the pellet was free of erythrocytes.

The resulting pellet, which mainly contained neutrophil granulocytes, was suspended in 2 mL of PBS buffer. To check cell vitality and cell concentration, 50 μL leukocytes suspension and 10 μL of 0.4% Trypan blue solution were mixed on a glass object carrier and examined with a light microscope (Nikon microscopes, Duesseldorf, Germany) at $1000\times$ magnification. Dead cells appear larger and dark because of the absorption of Trypan blue solution, whereas vital cells remain smaller, lighter, and more granulose. The vitality of the cells was determined to be over 90%. Leukocytes concentration was determined using cell diluted suspension distributed into a Neubauer chamber (Assistant, Germany) and a light microscope with $100\times$ magnification. For the bioassay the cell concentration was determined to be 5000 cells/μL PBS buffer. The suspension was sonicated in a Bandelin Smarex TK 52 ultrasonic bath (Bandelin Electronics, Berlin, Germany) for 20–30 s at 20 kHz to release the cytosolic 5-lipoxygenase into the solution. The solution was centrifuged at $8500 \times g$ for 30 min at room temperature and the supernatant was directly used as a source of enzyme. Protein concentration was estimated by the method of Lowry [15] using BSA as standard.

2.5 Enzyme assays

2.5.1 Soybean lipoxygenase-1

The assay was carried out by monitoring the appearance of linoleic acid *cis, trans*-hydroperoxide derivative at 234 nm (product, $\varepsilon = 25$ cm^2/μmol) in a 10 mm pathlength cuvette (Hellma, Müllheim, Germany) at room temperature for

10 min (spectrophotometer: Cary 50 Bio, Varian). The reaction was followed continuously by recording the absorbance every 30 s at pH 9.0 using oxygen saturated 0.1 M borate buffer. Two microliters assay mixture contained 10 μL of enzyme (protein stock solution: 0.5 mg/mL; specific activity: 131 000 units/mg protein), 10–200 μL of anthocyanins (anthocyanidins) (stock solutions: 2–200 mM in MeOH (0.1% HCl)/0.1 M borate buffer (pH 9.0)), not more than 0.1% MeOH, and was filled up with 0.1 M borate buffer (pH 9.0). The reaction was initiated by the addition of 100 μL of substrate (1 mM linoleic acid). The control sample (enzyme activity) received equal volumes of MeOH (0.1% HCl) without the inhibitor (anthocyanins and anthocyanidins). The rate (v) was determined from the curves shown as example in Fig. 2 by extrapolating the initial velocity over 90 s. The remaining activity was calculated by comparing the rates (v) of the uninhibited *versus* inhibited reaction. Oleic acid served as positive control for inhibition of lipoxygenase. All results are an average of at least three sets of measurements.

2.5.2 Human neutrophil granulocyte 5-Lipoxygenase

Human neutrophil granulocyte 5-lipoxygenase was assayed according to the method of Aharony and Stein [16] by following the increase in absorbance at 236 nm due to the formation of 5-HETE (product, $\varepsilon = 28$ cm^2/μmol) in a 10 mm pathlength cuvette (Hellma, Müllheim, Germany) at 37°C for 5 min (spectrophotometer: Cary 50 Bio, Varian). The reaction was followed continuously by recording the absorbance every 30 s. The substrate, arachidonic acid, was prepared according the method of Axelrod *et al*. [17]. The standard reaction mixture (500 μL total volume) contained 100 μL of human neutrophil granulocyte protein solution (specific activity: 0.22 μmol/min/mg protein), 2.5–25 μL of Dp-3-glc and Dp-3-gal (stock solution: 2 mM in MeOH (0.1% HCl)/0.1 M borate buffer (pH 9.0)), not more than 0.1% MeOH, 75 μL of cofactors (25 μL each of 5 mM DTT, 4 mM ATP, and 6 mM $CaCl_2$), and was filled up with 0.1 M PBS buffer (pH 7.4). The reaction was started by the addition of 10 μL of substrate (10 mM arachidonic acid). The control sample (enzyme activity) received equal volumes of MeOH (0.1% HCl) without the inhibitor (Dp3glc and Dp3gal). The rate (v) was determined from the curves shown as example in Fig. 2 by extrapolating the initial velocity over 90 s. The remaining activity was calculated by comparing the rates (v) of the uninhibited *versus* inhibited reaction. NDGA served as positive control for inhibition of lipoxygenase. All results are an average of at least three sets of measurements.

2.5.3 Determination of the type of inhibition using Pn and soybean lipoxygenase-1

The presence of an inhibitor changes the Michaelis–Menton constant (K_m) and/or the velocity (v) of the reaction

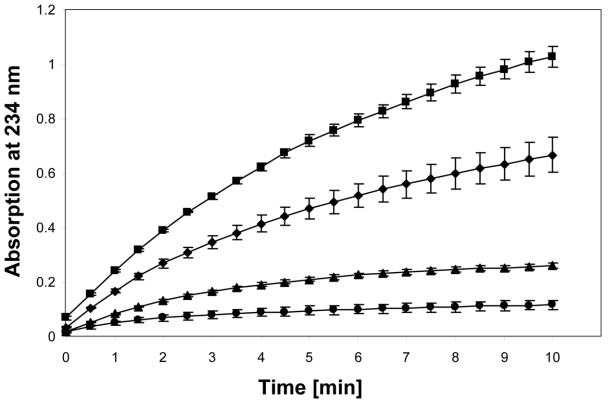


Figure 2. Time course of lipoxygenase catalyzed reaction. Dp3gal was assayed for soybean lipoxygenase-1 activity for 10 min in oxygen saturated 0.1 M borate buffer (pH 9.0). The reaction was started by the addition of 50 μM linoleic acid. Formation of the hydroperoxide products of lipoxygenase assay was followed at 234 nm. The concentrations of Dp3gal were 0 (|), 0.1 (■), 0.3 (♦), and 8 μM (▲). The values are the means ($n = 3$) ± SD.

[18]. The amount of change depends on the concentration of inhibitor used (I) as well as the affinity of the inhibitor for the enzyme denoted by the inhibitor constant, K_i. A large value for the inhibitor constant indicates low affinity and *vice versa*. To study the inhibition patterns and any structure–activity relationships in greater detail, the type of inhibition (competitive, uncompetitive, noncompetitive) of soybean lipoxygenase-1 by Pn was examined. For this, Lineweaver–Burk double reciprocal plots of $1/S$ against $1/v$ in the absence and presence of the different inhibitor concentrations were constructed, where S is the substrate concentration and v is the activity of enzyme. For each concentration of the inhibitor (three different concentrations in each case) plots were constructed for a substrate range of 25–500 μM. The arrangement of the family of trend lines obtained from the plots gave an indication of the type of inhibition. The K_m and V_{max} values were obtained from the intercepts on the $1/S$ axis and the $1/v$ axis, respectively, both of the Lineweaver–Burk plot.

To calculate the value of K_i, a secondary plot was constructed by plotting the reciprocal of the maximal velocity ($1/V_{max}$) against the inhibitor concentration where the intercept on the inhibitor axis gave the value for K_i.

2.6 Statistical analysis

Mean values and SD were calculated for all data (each experiment was carried out in triplicate). Datasets were analyzed by one-way ANOVA with *post-hoc* Bonferroni's multiple comparison test. Statistical significance was defined as a significance level of $p \leq 0.05$. Due to the very limited sample number a pretest was performed to test the normal distribution of the residuals. Therefore, the residuals of

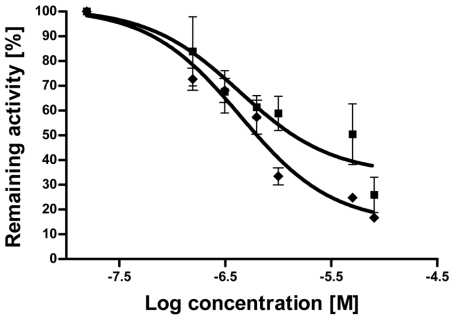


Figure 3. Dose–response curves for the inhibition of soybean lipoxygenase-1 by Dp3glc (■) and Dp3gal (♦). Both compounds were assayed for soybean lipoxygenase-1 activity for 10 min in oxygen saturated 0.1 M borate buffer (pH 9.0). The reaction was started by the addition of 50 μM linoleic acid. Formation of the hydroperoxide products of lipoxygenase assay was followed at 234 nm. All the tested compounds inhibited lipoxygenase in a concentration dependent manner. The values are the means ($n = 3$) ± SD.

each data group were calculated and the ratio of range to SD was analyzed according to David *et al*. [19]. On one dataset a reciprocal transformation was performed for normal distribution of the residuals and subsequent ANOVA analysis. Due to the limited number of data, p values should be interpreted very cautiously.

3 Results

Anthocyanins, *i.e*., Dp3glc, Dp3gal, Dp3ara, Cy3glc, Cy3gal, Cy3ara, Pn3glc, Pn3gal, Pn3ara, and Mv3glc as well as their corresponding aglycons (Fig. 1) were tested for their lipoxygenase inhibitory potential. In order to understand the interaction and the nature of inhibition, we have chosen soybean lipoxygenase-1, with its known crystal structure, as the fundamental model in our experiments. In addition, the inhibitory potential of Dp3glc and Dp3gal (evaluated as the most effective inhibitors) against human neutrophil granulocyte 5-lipoxygenase was examined.

As a representative example, the time course for the inhibition of soybean lipoxygenase-1 activity in the presence of 0 to 8 μM Dp3gal is shown in Fig. 2.

All tested anthocyanins and anthocyanidins except for the Mv derivatives – they revealed in the applied model no inhibitory activity at all (data not shown) – inhibited soybean lipoxygenase-1 in a concentration-dependent manner as shown for Dp3glc and Dp3gal as representative examples in Fig. 3. Dp derivatives exhibited IC_{50} values ranging from 0.43 to 0.49 mM (in detail: Dp3glc: 0.43 ± 0.07 μM, Dp3gal: 0.46 ± 0.16 μM, Dp3ara: 0.49 ± 0.06 mM, Dp: 0.27 ± 0.06 mM), followed by Cy derivatives with IC_{50} values varying from 0.18 to 1.24 mM (in detail: Cy3glc: 0.25 ± 0.04 mM, Cy3gal: 0.18 ± 0.02 mM, Cy3ara: 1.24 ± 0.36 mM, Cy: 0.58 ± 0.36 mM) and Pn derivatives

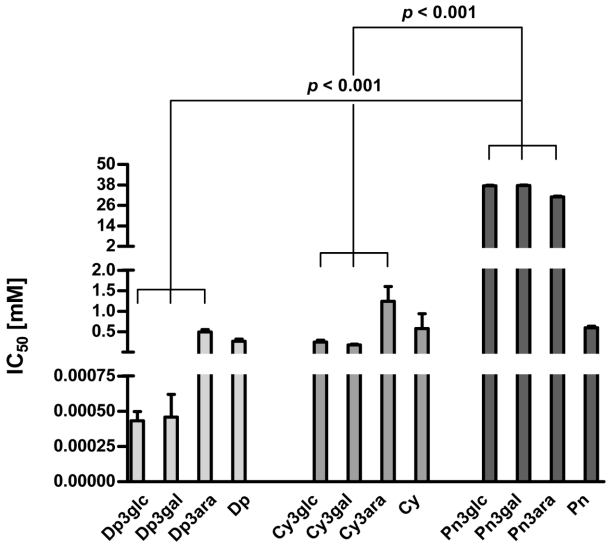


Figure 4. IC_{50} values (mM) of the inhibition of soybean lipoxygenase-1 by anthocyanins and their corresponding aglycons. The IC_{50} values are the means ($n = 3$) + SD. Significance tests were carried out by one-way ANOVA with *post-hoc* Bonferroni's multiple comparison test.

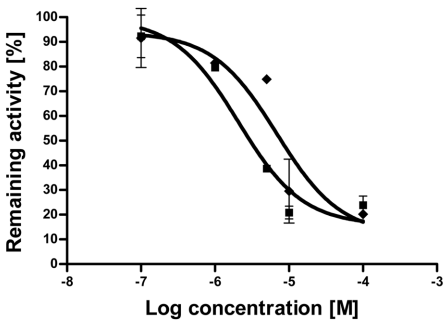


Figure 5. Dose–response curves for the inhibition of human neutrophil granulocyte 5-lipoxygenase by Dp3glc (■) and Dp3gal (♦). Both compounds were assayed for human neutrophil granulocyte 5-lipoxygenase activity for 5 min in oxygen saturated 0.1 M PBS buffer (pH 7.4). The reaction was started by the addition of 10 μL of 10 mM arachidonic acid. Formation of 5-HETE was followed at 236 nm. Dp3glc and Dp3gal inhibited lipoxygenase in a concentration dependent manner. The values are the means ($n = 3$) ± SD.

with the highest IC_{50} values, ranging from 0.59 to 37.7 mM (in detail: Pn3glc: 37.5 ± 0.33 mM, Pn3gal: 37.7 ± 0.25 mM, Pn3ara: 31 ± 0.43 mM, Pn; 0.59 ± 0.04 mM). A schematic overview of the IC_{50} values determined in our study is given in Fig. 4. The statistical analysis revealed significantly lower ($p < 0.001$) IC_{50} values for the unmethylated anthocyanins (in detail: Dp3glc, Dp3gal, Dp3ara, Cy3glc, Cy3gal, Cy3ara) than the methylated Pn glycosides (in detail: Pn3glc, Pn3gal, Pn3ara).

The most effective soybean lipoxygenase-1 inhibitors Dp3glc and Dp3gal also inhibited the activity of human neutrophil granulocyte 5-lipoxygenase (Fig. 5) in a concen-

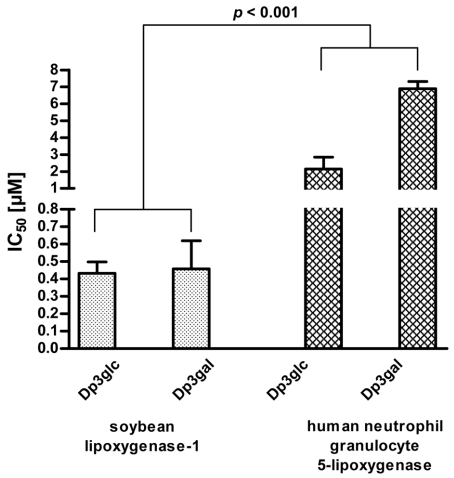


Figure 6. IC_{50} values (µM) for the inhibition of soybean lipoxygenase-1 and human neutrophil granulocyte 5-lipoxygenase by Dp3glc and Dp3gal. The IC_{50} values are the means ($n = 3$) + SD. Significance tests were carried out by one-way ANOVA with *post-hoc* Bonferroni's multiple comparison test.

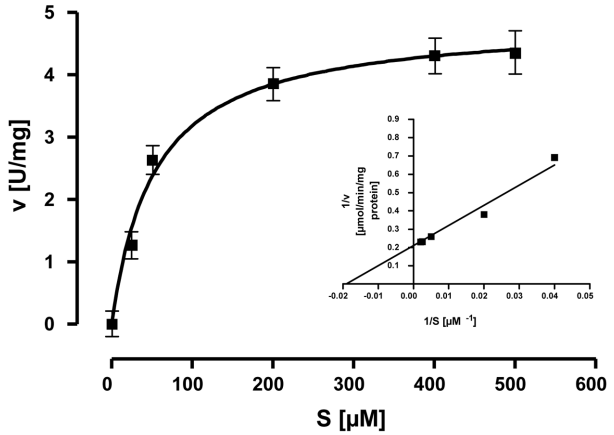


Figure 7. Combination graph used to characterize soybean lipoxygenase-1 activity (without addition of inhibitor) consisting of Michaelis–Menten plot [mean values ($n = 3$) ± SD) and inserted Lineweaver–Burk plot (mean values ($n = 3$)].

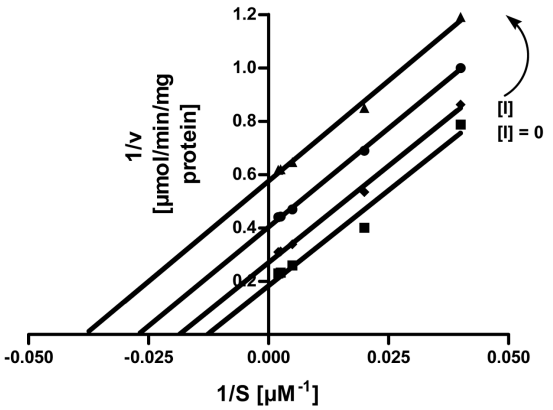


Figure 8. Lineweaver–Burk plot of soybean lipoxygenase-1 inhibition by Pn [mean values ($n = 3$)]. The assay was carried out with enzyme (10 µL of a 0.5 mg/mL protein stock solution; specific activity: 131 000 units/mg protein) in the absence and in the presence of Pn concentrations. The concentrations of Pn were 0 (■), 500 (♦), 1000 (●), and 1500 µM (▲). The linoleic acid concentration was varied from 25–500 µM. A K_m value of 78.4 µM and a V_{max} value of 5.47 µmol/min were observed in the absence of the inhibitor, whereas with the increasing concentration of the inhibitor [*I*] at 500, 1000, and 1500 µM, the K_m values were 53.8, 36.8, and 26.2 µM and the V_{max} values were 3.71, 2.48, and 1.74 µmol/min, respectively.

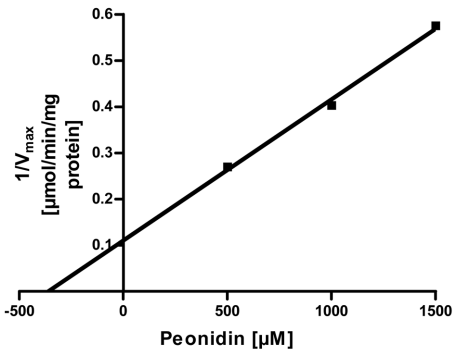


Figure 9. Determination of K_i of Pn. The $1/V_{max}$ values described from the double reciprocal plot are plotted against the Pn concentration in order to determine the K_i value of Pn.

tration-dependent manner; their high soybean lipoxygenase-1-inhibiting potential was also reflected on 5-lipoxygenase inhibition revealing IC_{50} values of 2.15 ± 0.7 µM (Dp3glc) and 6.9 ± 0.42 µM (Dp3gal). In general, the IC_{50} values obtained with human neutrophil granulocytes 5-lipoxygenase were significantly higher ($p < 0.001$) than those determined using soybean lipoxygenase-1 (Fig. 6).

In case of Pn (selected due to practical reasons, *i.e.*, as the most available compound among the inhibitors) the type of inhibition was deduced from Lineweaver–Burk double reciprocal plots (Fig. 7) using soybean lipoxygenase-1 test. Parallel lines were obtained, attesting an uncompetitive inhibition type and therefore exhibiting affinity for the enzyme–substrate complex (Fig. 8). The equilibrium constant for inhibitor binding, K_i, was determined as 361 µM by plotting a secondary plot ($1/V_{max}$ values obtained from the Lineweaver–Burk plot *versus* concentration of inhibitor [*I*]), which is illustrated in Fig. 9.

4 Discussion

The IC_{50} values of the inhibitors under study varied in a great range, *i.e.*, from 0.43 to 37.7 mM. The two most effective anthocyanins, Dp3glc and Dp3gal, exhibited both with soybean lipoxygenase-1 and human neutrophil granulocyte 5-lipoxygenase a higher inhibitory potential than baicalein – a well-known powerful flavonoid 5-lipoxygenase inhibitor [5] (IC_{50} in rat and human leucocytes, 7.13–9.5 µM). Conjugation of Dp and Cy with glucose and galactose

increased the inhibitory potential. These findings are in agreement with recently published data [5] where the conjugated myricetin 3-*O*-glucuronide showed with 2.2 μM a lower IC_{50} value than its aglycon (IC_{50}: 13 μM). Nonetheless, conjugation with arabinose led in all assays to a slight increase of IC_{50} values. In case of the Pn derivatives each glycosilation decreased the inhibitory potential compared with the aglycon, which is in agreement with earlier findings of a study with phenolic compounds [6], where glycosylation led to a loss of inhibition potential.

In general, the inhibitory activity toward soybean lipoxygenase-1 decreased in the following order: Dp derivatives > Cy derivatives > Pn derivatives > Mv derivatives. Hence, the IC_{50} values of the unmethylated anthocyanins (Dp3glc, Dp3gal, Dp3ara, Cy3glc, Cy3gal, Cy3ara) were significantly lower than those of the corresponding methylated (Pn) glycosides (Pn3glc, Pn3gal, Pn3ara) (the Mv derivatives showed no measurable inhibitory activity at all). However, the inhibitory potential of the aglycons was in the same order (Dp > Cy > Pn > Mv), albeit without any significance. This observation may be due to the different antioxidant activities of anthocyanins and anthocyanidins connected with their varying number of free hydroxyl groups. Rahman *et al*. [20] have shown that the potency of antioxidant activity of anthocyanins is affected by aglycon structure as well as attached sugar moiety, leading to the following order of activity against the superoxide radical as well as peroxynitrite: Dp > Cy = Mv > Pn. Although we did not measure antioxidant activity of the tested compounds, these findings fit well, except for Mv, within our results. Drawing an overall conclusion about the structure–activity relationship for lipoxygenase inhibition during our assays, vicinal hydroxyl groups at ring B and conjugation with glucose and galactose at C3 appeared to be crucial determinants. Hence, there is a distinct difference to flavonols such as quercetin, for which high antioxidative capacity maintained after conjugation at the C3 position but loss of lipoxygenase inhibitory activity was observed [21].

As to the postulated uncompetitive nature of inhibition, uncompetitive inhibitors bind exclusively to the enzyme–substrate complex with or no affinity to the free enzyme itself. This could be a reaction of Pn with an enzyme-bound lipid radical species (more likely a peroxyl radical). If the peroxyl or alkyl radical leaves the active site, this in theory would leave the active site iron in the reduced (inactive) form, thus, essentially reducing the amount of active enzyme available in the reaction. Literature data shows no result or discussion that would confirm this hypothesis and therefore it would take some further experimental investigation to support this. The uncompetitive nature of inhibition was also shown elsewhere for, *e.g.*, vanillic acid (–)-epicatechin and sclerotiorin, each of them phenolic compounds [6, 22].

In conclusion, our results show that dietary compounds, above all Dp3glc and Dp3gal (rich sources, bilberry and black currant), exhibit strong lipoxygenase inhibitory properties *in vitro*. To confirm these properties *in vivo*, the anthocyanins obviously have to reach the target cells unmodified, because metabolic transformation has been shown to have a profound effect on bioactivity of the flavonoids [21, 23]. Indeed, anthocyanins were found to be the only group of flavonoids being absorbed unmodified as intact glycosides [9].

Considering the low IC_{50} values in relation to the currently known availability in human metabolism [9], *in vivo* prevention of inflammatory diseases by these anthocyanins could be envisaged.

Of course, due to the known instability of anthocyanins at pH 7.4, it has still to be clarified which exact structures excert the activity under *in vivo* conditions. Nonetheless, our control tests performed at pH 7.4 (data not shown) revealed the occurrence of the corresponding chinoid anhydrobases.

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