

Protective Effects of Anthocyanins from Blackberry in a Rat Model of Acute Lung Inflammation

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Anthocyanins are a group of naturally occurring phenolic compounds related to the coloring of plants, flowers and fruits. These pigments are important as quality indicators, as chemotaxonomic markers and for their antioxidant activities. Here, we have investigated the therapeutic efficacy of anthocyanins contained in blackberry extract (cyanidin-3-*O*-glucoside represents about 80% of the total anthocyanin contents) in an experimental model of lung inflammation induced by carrageenan in rats. Injection of carrageenan into the pleural cavity elicited an acute inflammatory response characterized by fluid accumulation which contained a large number of neutrophils as well as an infiltration of polymorphonuclear leukocytes in lung tissues and subsequent lipid peroxidation, and increased production of nitrite/nitrate (NO_x) and prostaglandin E₂ (PGE₂). All parameters of inflammation were attenuated in a dose-dependent manner by anthocyanins (10, 30 mg kg⁻¹ 30 min before carrageenan). Furthermore, carrageenan induced an upregulation of the adhesion molecule ICAM-1, nitrotyrosine and poly (ADP-ribose) synthetase (PARS) as determined by immunohistochemical analysis of lung tissues. The degree of staining was lowered by anthocyanin treatment. Thus, the anthocyanins contained in the blackberry extract exert multiple protective effects in carrageenan-induced pleurisy.

Keywords: Anthocyanins; Blackberry; Carrageenan; Pleurisy; Inflammation

INTRODUCTION

The role of reactive oxygen species in the pathophysiology of inflammation is well established.

In addition to ROS, overproduction of nitric oxide due to the expression of the inducible isoform of NO synthase (iNOS) also plays an important role in various models of inflammation.^[1–3] In a number of pathophysiological conditions associated with inflammation or oxidant stress, peroxynitrite has been proposed to mediate cell damage.^[4,5] Peroxynitrite is cytotoxic via a number of independent mechanisms including (i) the initiation of lipid peroxidation, (ii) the inactivation of a variety of enzymes (most notably, mitochondrial respiratory enzymes and membrane pumps)^[4] and (iii) depletion of glutathione.^[6] Moreover, peroxynitrite can also cause DNA damage^[7,8] resulting in the activation of the nuclear enzyme poly (ADP-Ribose) synthetase, depletion of NAD and ATP and ultimately cell death.^[9]

Interventions which decrease the generation or the effects of ROS exert beneficial effects in a variety of models of inflammation including the carrageenan-induced pleurisy model used here. These therapeutic interventions include melatonin,^[10] a vitamin E-like antioxidant,^[11] a superoxide dismutase-mimetic^[12–14] and a peroxynitrite decomposition catalyst.^[15]

Current research into development of new free radical scavengers' has confirmed that food rich in antioxidants plays an essential role in the prevention of cardiovascular diseases,^[16,17] cancers^[18] and

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neurodegenerative diseases, the most well-known of which are Parkinson's and Alzheimer's diseases,^[19] as well as inflammation^[20] and problems caused by cell and cutaneous ageing.^[21] Recently, a considerable amount of epidemiological evidence have demonstrated an association between diets rich in fruit and vegetables and a decreased risk of cardiovascular disease and certain forms of cancer.^[22] It is generally assumed that the active dietary constituents contributing to these protective effects are antioxidant nutrients such as α -tocopherol and β -carotene.

In fact an antioxidant family of nutrients like the flavonoids have a good intestinal adsorption.^[23,24] However, investigations highlight an additional role of polyphenolic components of higher plants that may act as antioxidants or via other mechanisms contributing to the anticarcinogenic or cardioprotective actions.^[25,26] Anthocyanins are natural colorants belonging to the flavonoid family. They are widely distributed among flowers, fruits and vegetables. Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of flavilium salts. It has been demonstrated that, in addition to their colourful characteristics, anthocyanins possess some positive therapeutic effects, mainly linked with their strong antioxidant properties.^[27,28] In our studies, we utilized a blackberry extract, containing anthocyanins. Cyanidin-3-*O*-glucoside represents about 80% of the anthocyanin fraction, followed by small amounts of other anthocyanins, some of them only recently identified.^[29] Cyanidin-3-*O*-glucoside (kuromanin) has been found to have the highest oxygen radical absorbance capacity activity.^[27] This anthocyanin is the most ubiquitous. It represents the main anthocyanin in the edible parts of several plants. Here, we investigate the effects of anthocyanins, contained in the blackberry extract, on the inflammatory response (pleurisy) caused by injection of carrageenan in the rat. In particular, we have investigated the effects of anthocyanins, contained in the blackberry extract, on the lung injury (histology), the formation of nitrotyrosine (immunohistochemistry) as well as the increases in NO production and PARS activation caused by carrageenan in the lung.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (200–250 g; Charles River, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Blackberry Extract

Blackberry fruits were obtained as commercial products. Fruit extract was obtained by pounding in a mortar about 2 kg of fruits. The extract was centrifuged at 6000 rpm for 20 min. The juice was filtered on glass wool. The pulps were extracted overnight with about 1 l of ethyl alcohol (EtOH)/HCl (99:1 v/v) at room temperature. The filtered extract was concentrated under reduced pressure and diluted with water (1:1 v/v). Both the filtered juice and the pulp extract were applied to a C18 silica column previously activated with 10 ml of EtOH and 10 ml of water. Anthocyanins were extracted with 1 l of EtOH/HCl (99:1 v/v) and the solvent was removed under reduced pressure. The anthocyanin fraction so obtained was used for the chemical characterization^[29] and for the pharmacology study. The anthocyanin content was determined spectrophotometrically by absorbance at 518 nm and by HPLC analysis with photodiode array detector using a calibration curve obtained by analyzing different concentrations of cyanidin-3-glucoside standard.

Experimental Groups

In the treated group of animals anthocyanins contained in blackberry extract (10, 30 mg kg⁻¹) was given intraperitoneally (i.p.) 30 min before carrageenan (carrageenan + anthocyanins group). In a vehicle-treated group of rats, vehicle (saline) was given instead of anthocyanins (carrageenan group). In separate groups of rats, surgery was performed in its every aspect identical to the one in the carrageenan group, except that carrageenan was not injected (control group).

Carrageenan-induced Pleurisy

Rats were lightly anesthetized with isoflurane and subjected to a skin incision at the level of the left sixth intercostal space. The underlying muscles were dissected and 0.2 ml saline alone or containing 2% λ -carrageenan was injected into the pleural cavity. The skin incision was closed with a suture and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were sacrificed under CO₂ vapor. The chest was carefully opened and the pleural cavity washed with 2 ml of saline solution with heparin (5 U/ml) and indomethacin (10 μ g/ml). The exudate and washing were removed by aspiration and the total volume measured. Exudates contaminated with blood were discarded. The results were calculated by subtracting the volume injected (2 ml) from the total volume of fluid recovered. The leucocytes in the exudate were suspended in phosphate buffer saline and counted

with optical microscope by Burker's chamber after vital Trypan Blue stain.

Measurement of Nitrite/nitrate (NO_x)

NO_x production, an indicator of NO synthesis, was measured in pleural exudate. At the first nitrate in the supernatant was incubated with nitrate reductase (0.1 U/ml) and NADPH (1 mM) and FAD (50 μM) at 37°C for 15 min. Then followed another incubation with LDH (100 U/ml) and sodium pyruvate (10 mM) for 5 min. The nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H₂O and 1% sulphanilamide in 5% concentrated H₂PO₄; vol. 1: 1) to 100 μl samples. The optical density at 550 nm (OD₅₅₀) was measured using ELISA microplate reader (SLT-Labinstruments Salzburg, Austria). Nitrate concentrations were calculated by comparison with OD₅₅₀ of standard solutions of sodium nitrate prepared in saline solution. Levels of NO_x are expressed as ng/rat.

Measurement of PGE₂ Concentration (PGE₂)

The amount of PGE₂ in the pleural exudate was measured by enzyme immunoassay according to the manufacturer's instruction (Cayman Chemical). Levels of PGE₂ are expressed as ng rat⁻¹.

Myeloperoxidase Activity

Myeloperoxidase activity, an indicator of polymorphonuclear leukocyte accumulation, was determined as previously described.^[30] At the specified time following injection of carrageenan, lung tissues were obtained and weighed and each piece homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM hydrogen peroxide. The rate of change in absorbance was measured spectrophotometrically at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 37°C and was expressed in U g⁻¹ of wet tissue.

Malondialdehyde Levels

Malondialdehyde levels in the lung tissue were determined as an indicator of lipid peroxidation as previously described.^[31] Lung tissue collected at the specified time, was homogenised in 1.15% (w/v) KCl solution. A 100 μl aliquot of the homogenate was added to a reaction mixture containing 200 μl of 8.1%

(w/v) sodium dodecyl sulfate (SDS), 1.5 ml of 20% (v/v) acetic acid (pH 3.5), 1.5 ml of 0.8% (w/v) thiobarbituric acid and 600 μl distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3,000g for 10 min. The absorbance of the supernatant was measured using spectrophotometry at 650 nm. Levels of malondialdehyde are expressed as μM 100 mg⁻¹ of wet tissue.

Histological Examination

Lung biopsies were taken 4 h after injection of carrageenan. Lung biopsies were fixed for 1 week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Tissue sections (thickness) were then deparaffinized with xylene, stained with hematoxylin and eosin. All sections were studied using light microscopy (Dialux 22 Leitz).

Immunohistochemical Localization of Nitrotyrosine

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or reactive oxygen species, was determined by immunohistochemistry as previously described.^[32] At the end of the experiment, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). The sections were then incubated overnight with 1:1000 dilution of primary anti-nitrotyrosine monoclonal antibody (DBA, Milan, Italy) or with control solutions including buffer alone or non-specific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy).

Immunohistochemical Localization of ICAM-1 and PAR

Immunohistochemical staining was performed on 7 μm thick sections of unfixed lung. Sections were cut in with a SLEE and London cryostat at -30°C, transferred onto clean glass slides and dried overnight at room temperature. Sections were permeabilized with acetone at -20°C for 10 min and

rehydrated in PBS (phosphate buffered saline, 150 mM NaCl, 20 μ M sodium phosphate pH 7.2) at room temperature for 45 min. Sections were incubated overnight with: (1) ICAM-1 (hamster anti-mouse CD54) at a dilution 1:500 in PBS v/v or (2) with anti-poly (ADP-ribose) (PAR) monoclonal antibody (1:500 in PBS v/v). Sections were washed with PBS, and incubated with secondary antibody for 2 h at room temperature. Specific labeling was detected with a avidin–biotin peroxidase complex.

Materials

The PGE₂ enzyme immunoassay kit was obtained from Cayman Chemical. Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, CA, USA). Primary anti-nitrotyrosine antibody was purchased from Upstate Biotech (DBA, Milan, Italy). Reagents and secondary and nonspecific IgG antibody for immunohistochemical analysis were from Vector Laboratories (DBA, Italy, Milan). Primary monoclonal anti-poly (ADP-ribose) antibody was purchased by Alexis. All other reagents and compounds used were obtained from Sigma Chemical Company. Primary monoclonal anti-intercellular adhesion molecule-1 antibody was purchased by PharMingen (San Diego, USA). All other reagents and compounds used were obtained from Sigma Chemical Company.

Statistical Analysis

All values in the figures and text are expressed as mean \pm standard error of the mean of n observations, where n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. Data sets were examined by one-way analysis of variance (ANOVA) followed by a Bonferroni *post-hoc* test for multiple comparisons. A p -value less than 0.05 was considered significant.

RESULTS

Effects of Anthocyanins in Carrageenan-induced Pleurisy

Histological examination of lung sections revealed significant tissue damage (Fig. 1A). When compared with lung sections taken from saline-treated animals (data not shown). Histological examination of lung sections from rats treated with carrageenan showed edema, tissue injury as well as infiltration of the tissue with neutrophils (Fig. 1A). Anthocyanins at

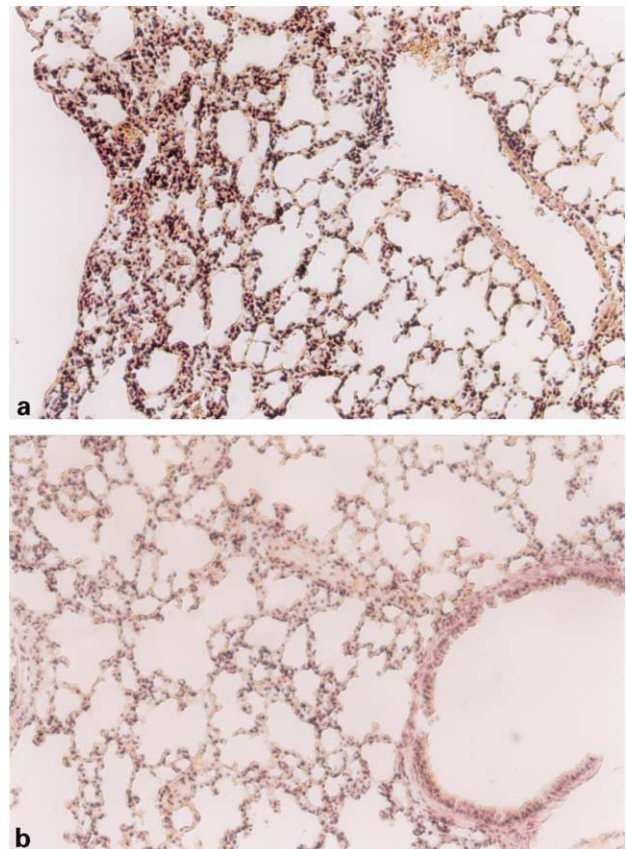


FIGURE 1 Effect of anthocyanins on lung injury. The lung section from a carrageenan-treated rat (A) shows interstitial hemorrhage and polymorphonuclear leukocyte accumulation. The lung section from a carrageenan-treated rat that had received anthocyanins, contained in blackberry extract (30 mg kg⁻¹) (B) shows reduced interstitial hemorrhage and less cellular infiltration. Figure is representative of at least three experiments performed on different experimental days.

the highest dose tested (30 mg/kg, intraperitoneally, i.p.), significantly reduced the degree of injury as well as the infiltration of polymorphonuclear leukocytes (Fig. 1B). Furthermore, the injection of carrageenan into the pleural cavity of rats elicited an acute inflammatory response characterized by the accumulation of fluid (oedema) that contained large amounts of polymorphonuclear leukocytes (Table I). Neutrophils also infiltrated in the lung tissues (Table I) and this was associated with lipid peroxidation of lung tissues as evidenced by an increase in the levels of malonyldialdehyde (Table I). Oedema, neutrophil infiltration in lung tissue and lipid peroxidation were attenuated in a dose-dependent fashion by the intraperitoneal injection of anthocyanins (10, 30 mg/kg, $n = 10$) (Table I).

Effects of Anthocyanins on the Expression of Adhesion Molecules (ICAM-1)

Staining of lung tissue sections obtained from saline-treated rats with anti-intercellular adhesion molecule-1 antibody showed a specific staining along

TABLE I Effects of anthocyanins (10,30 mg/kg, i.p.) on carrageenan-induced inflammation

	Volume exudate (ml)	PMNs infiltration (million cells/rat)	MPO (U/g)	MDA (μ M/100mg)
Sham + vehicle	0.053 \pm 0.029	6.3 \pm 2.606	206.5 \pm 58.34	32.43 \pm 4.557
Sham + anthocyanins (10 mg/kg)	0.060 \pm 0.030	5.92 \pm 2.223	208.2 \pm 59.46	35.9 \pm 1.212
Sham + anthocyanins (30 mg/kg)	0.066 \pm 0.033	6.47 \pm 2.772	202.9 \pm 56.04	31.9 \pm 5.086
CAR + vehicle	1.625 \pm 0.090*	85.97 \pm 6.21*	2962 \pm 177.3*	72.77 \pm 5.77*
CAR + anthocyanins (10 mg/kg)	0.91 \pm 0.012**	50 \pm 1.155**	2366 \pm 79.10**	53.27 \pm 1.936**
CAR + anthocyanins (30 mg/kg)	0.582 \pm 0.128**	36.04 \pm 10.90**	1949 \pm 221.8**	40.73 \pm 3.031**

Data are means \pm SE means of 10 rats for each group. * $p < 0.01$ vs. sham. ** $p < 0.01$ vs. carrageenan.

vascular wall (Fig. 2A1) but not in the bronchial epithelium (Fig. 2A), demonstrating that intercellular adhesion molecule-1 is constitutively expressed. At 4 h after carrageenan injection, the staining intensity substantially increased along the vascular wall (Fig. 2B) as well in the bronchial epithelium (Fig. 2B1). Significantly less positive staining for intercellular adhesion molecule-1 was found in the lungs of carrageenan-treated rats that received intraperitoneal injection of anthocyanins (30 mg/kg) (Fig. 2C and C1). To verify the binding specificity for intercellular adhesion molecule-1 some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections indicating that the immunoreaction was positive in all the experiments carried out.

Effects of Anthocyanins on Nitrotyrosine and PARS

At 4 h after carrageenan injection, lung sections were taken in order to determine the immunohistological staining for nitrotyrosine or PARS. Sections of lung from saline-treated rats did not reveal any immunoreactivity for nitrotyrosine (Fig. 3A) or for PAR (Fig. 4A) within the normal architecture. A positive staining for nitrotyrosine (Fig. 3B) and for PARS (Fig. 4B) was found primarily localized in alveolar macrophages and in airway epithelial cells of carrageenan treated animals. Anthocyanins (30 mg/kg, i.p.) reduced the staining for both nitrotyrosine and PARS (Figs. 3C and 4C). In order to confirm that the immunoreaction for the nitrotyrosine was specific some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for PARS, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections indicating that the immunoreaction was positive in all the experiments carried out.

Effects of Anthocyanins on the Release of NOx and PGE₂

In pleural exudate obtained from rats at 4 h after carrageenan injection, NOx (Fig. 5A) and PGE₂ (Fig. 5B) production were significantly ($p < 0.01$) increased (48.4 \pm 7.8 nmol/rat and 2.9 \pm 0.43 ng/rat, respectively) over a basal level (12.6 \pm 0.86 nmol/rat and 0.32 \pm 0.20 ng/rat, respectively). The production of NOx was significantly ($p < 0.05$) reduced in a dose dependent manner in rats treated with anthocyanins (10, 30 mg/kg) (Fig. 5A). The production of PGE₂ was significantly ($p < 0.05$) reduced in rats treated with anthocyanins (30 mg kg⁻¹) (Fig. 5B).

DISCUSSION

ROS have been implicated in a wide variety of diseases. Evidence for increased oxidant stress in lung inflammation is emerging.^[33,34] Recently it has been pointed out that neutrophil activation represents an important source of ROS.^[35] Furthermore, there is much evidence that the production of ROS such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contributes to tissue damage.^[3,36-38] Recently, much attention has focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on the mechanisms of their action.^[39] Phenolic compounds, which are widely distributed in plants, were considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living systems.^[40] Anthocyanin pigments are widely distributed in the human diet through crops, beans, fruits and vegetables,^[41] suggesting that we ingest considerable amounts of anthocyanin pigments from plant-based daily diets. The pigments may play an important role as dietary antioxidants for prevention of oxidative damage caused by active oxygen radicals in living systems.

In the present study, we demonstrated that anthocyanins contained in blackberry extract (1) reduces the development of carrageenan-induced pleurisy, (2) reduces morphological injury and neutrophil infiltration in carrageenan-induced

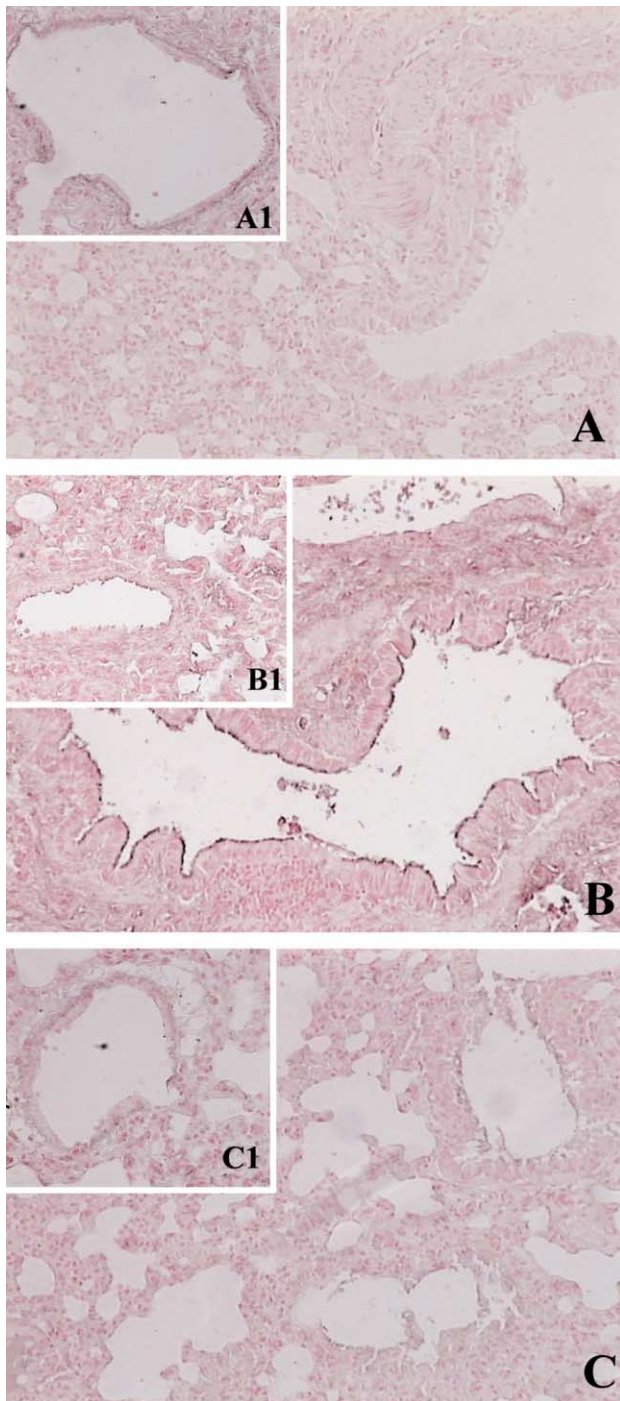


FIGURE 2 Immunohistochemical localization of intercellular adhesion molecule-1 in the rat lung. Staining was absent in control tissue (A, A1). At 4 h following carrageenan injection, intercellular adhesion molecule-1 immunoreactivity was localized mainly to macrophages and some epithelial cells (B, B1). There is a marked reduction in the immunostaining in the lungs of carrageenan-treated rats pre-treated with anthocyanins, contained in blackberry extract (30 mg kg^{-1}) (C, C1). Figure is representative of at least three experiments performed on different experimental days.

inflammation, (3) reduces nitrotyrosine immunostaining, an indicator of nitrosative stress in inflammation, (4) reduces intercellular adhesion molecule-1 immunostaining, an indicator of

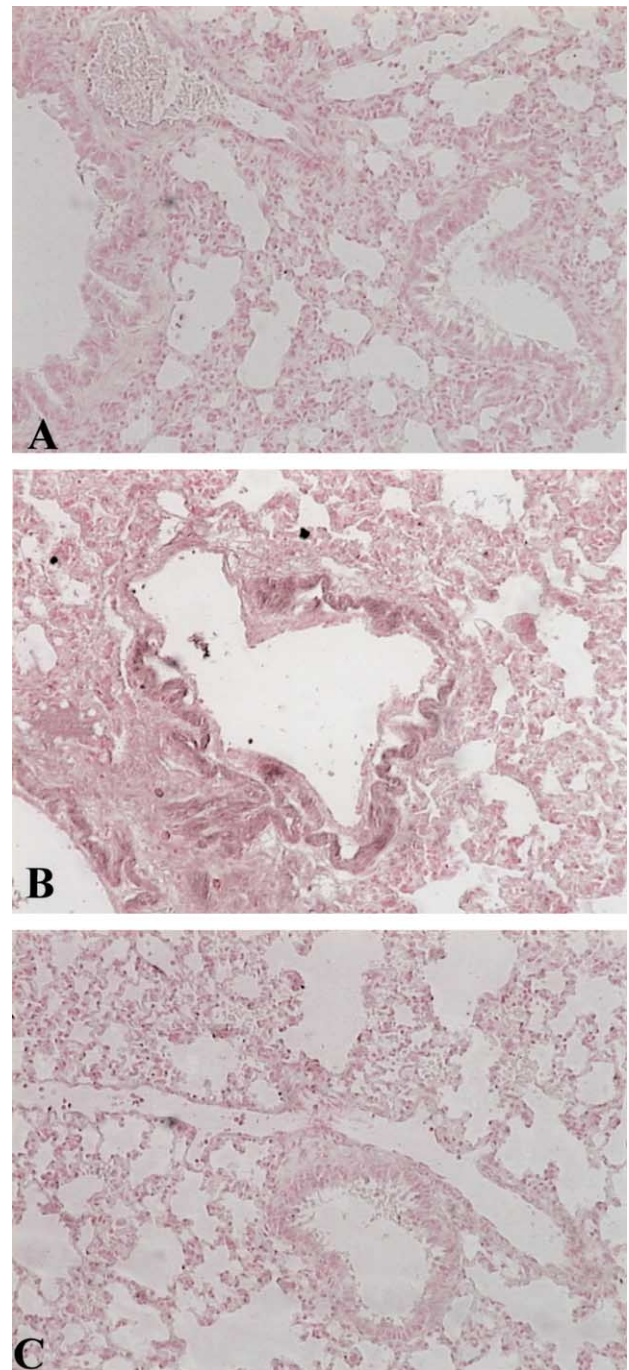


FIGURE 3 Effect of anthocyanins on nitrotyrosine formation. Immunohistochemical localization of nitrotyrosine in the rat lung. Staining was absent in control tissue (A). At 4 h following carrageenan injection, positive staining for nitrotyrosine (typical are as are indicated by arrows) was observed (B). There was a marked reduction in the immunostaining in the lungs of carrageenan-treated rats pre-treated with anthocyanins, contained in blackberry extract, (30 mg kg^{-1}) (C). Figure is representative of at least three experiments performed on different experimental days.

neutrophil infiltration and (5) reduces PAR immunostaining. What then, is the mechanism by which anthocyanins protects the lung against this inflammatory injury?

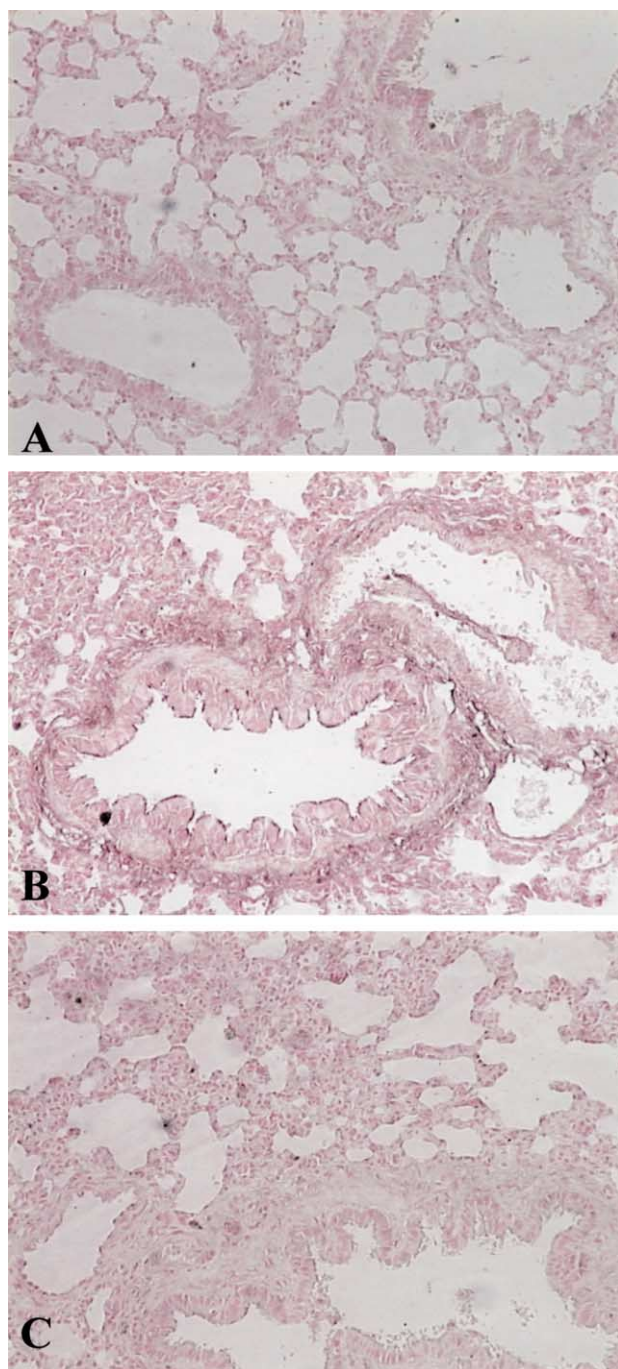


FIGURE 4 Immunohistochemical analysis for PAR antibody. Staining was absent in control tissue (A). Sections obtained from carrageenan-treated rats showed intense positive staining for poly-(ADP-ribose) polymerase (B). The degree of staining for poly-(ADP-ribose) polymerase was markedly reduced in pleural macrophages obtained from carrageenan-treated rats which were pre-treated with anthocyanins, contained in blackberry extract (30 mg kg^{-1}) (C). Figure is representative of at least three experiments performed on different experimental days.

A mechanism by which anthocyanins attenuates inflammation is by reducing peroxynitrite formation by simply removing O_2^- before it reacts with NO. This is important since the pro-inflammatory and cytotoxic effects of peroxynitrite are numerous.^[15,42]

Peroxynitrite also nitrosates tyrosine residues in proteins and nitrotyrosine formation along with its detection by immunofluorescence has been used as a marker for the detection of the endogenous formation of peroxynitrite.^[43] Using nitrotyrosine as a marker for the presence of peroxynitrite has been challenged by the demonstration that other reactions can also induce tyrosine nitration, e.g. the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine.^[44] Thus, increased nitrotyrosine staining is considered, as an indicator of "increased nitrosative stress" rather than a specific marker of the generation of peroxynitrite.^[44] We have found that nitrotyrosine is indeed present in lung sections taken after carrageenan injection and that anthocyanins reduced the staining in these tissues. In addition, injection of carrageenan in the pleural cavity of rats induces the expression of iNOS that in turn release large amounts of pro-inflammatory NO_x .^[1,3,45] This study demonstrates that anthocyanins attenuate the NO formation production in the exudates from carrageenan-treated rats (Fig. 2A). Thus, the reduction of the NO formation by anthocyanins may contribute to the attenuation by this agent of nitrotyrosine in the lung from carrageenan-treated rats (Fig. 5C).

Based on these findings, we conclude that carrageenan evoked, in part, a superoxide-driven peroxynitrite formation that was, in turn, responsible for the formation of nitrotyrosine. The fact that there is residual nitration can be explained by the presence of neutrophils in the lung. ROS can also cause DNA single-strand damage which is the obligatory trigger for PARS activation^[46] resulting in the depletion of its substrate NAD^+ *in vitro* and a reduction in the rate of glycolysis. Since NAD^+ functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD^+ depletion leads to a rapid fall in intracellular ATP and, ultimately, cell injury.^[46] Furthermore, substantial evidence exists to support the fact that PARS activation is important in inflammation.^[46] PARS inhibitors such as nicotinamide and 3-aminobenzamide attenuate both acute and chronic inflammatory processes.^[46-48] As shown in Fig. 6C anthocyanins reduced PARS immunostaining. In light of the role of PARS in inflammation, it is possible that PARS inhibition by anthocyanins accounts for its anti-inflammatory response.

Besides attenuating ROS production and PARS activation, anthocyanins also reduced the development of oedema, neutrophil accumulation and lipid peroxidation and had an overall protective effect on the degree of lung injury as assessed by histological examination. A possible mechanism by which anthocyanins attenuates polymorphonuclear leukocyte s infiltration is by down-regulating adhesion molecules such as intercellular adhesion molecule-1.

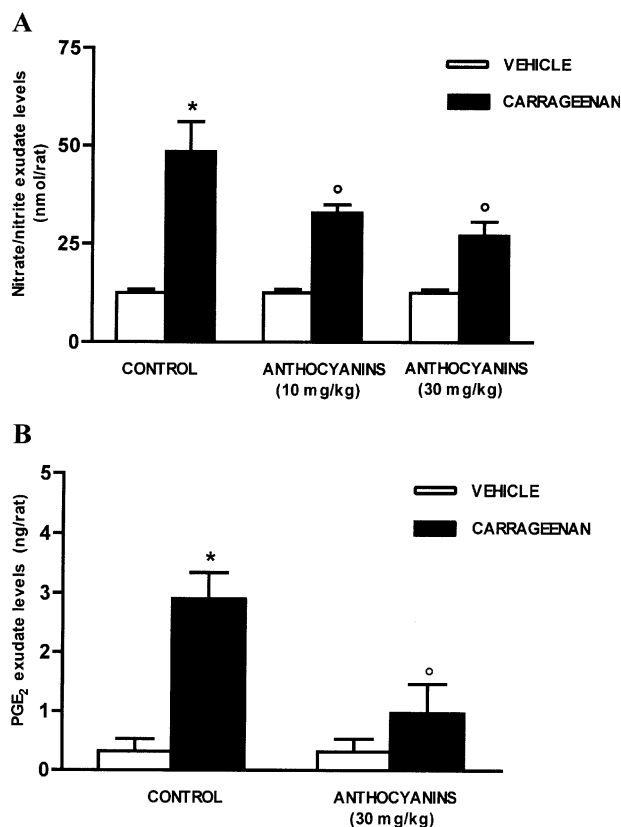


FIGURE 5 Nitrate/nitrite production (A) in supernatant obtained from pleural exudate. Anthocyanins, contained in blackberry extract (10, 30 mg kg⁻¹), significantly reduced the NO_x production PGE₂ production (B) in supernatant obtained from pleural exudate. Anthocyanins, contained in blackberry extract (30 mg kg⁻¹), significantly reduced the PGE₂ production. Data are means ± SE mean of 10 rats for each group. **p* < 0.001 vs. control; ^o*p* < 0.05 vs. carrageenan.

Therefore, there is good evidence in this and in other models of inflammation that an enhanced formation of prostanoids following the induction of COX-2 contributes to the pathophysiology of local inflammation and chronic^[49,50] and also that selective inhibitors of COX-2 exert potent anti-inflammatory effects.^[51,52] We demonstrate here that the increase in the levels of PGE₂ caused by injection of carrageenan into the pleural cavity is reduced in the exudate of anthocyanins-treated rats. The enhanced formation of PGE₂ is secondary to the expression of COX-2 protein, as (i) there was no increase in the expression of COX-1 protein^[53] after carrageenan injection and (ii) selective inhibitors of COX-2 activity including NS-398 (nimesulide) and SC-58125 (Celecoxib) marked abolish the increase in PGE₂ caused by injection of carrageenan into the pleural space.^[54–56] Thus, we propose that anthocyanins reduced the PG production caused by injection of carrageenan and that this may be an additional anti-inflammatory mechanism.

In conclusion, this study demonstrated that the anthocyanins contained in blackberry extract,

attenuates the pleurisy caused by carrageenan administration in the rat. We speculate that the observed anti-inflammatory effects of anthocyanins may be dependent on a combination of the following pharmacological properties of this agent: (1) anthocyanins scavenges and inactivates superoxide anions, which would prevent the formation of peroxynitrite. This, in turn, prevents the activation of PARS and the associated tissue injury. (2) In addition to superoxide anions, anthocyanins also scavenges other ROS, including hydroxyl radicals. It is not clear whether anthocyanins is able to scavenge peroxynitrite. (3) In addition, anthocyanins reduces the recruitment of polymorphonuclear leukocyte s into the inflammatory site. This effect of anthocyanins is very likely secondary to the prevention by anthocyanins of endothelial oxidant injury and, hence, preservation of endothelial barrier function. These results support the view that the overproduction of reactive oxygen or nitrogen free radicals contributes to acute inflammation.

References

- [1] Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) "Nitric oxide: physiology, pathophysiology and pharmacology", *Pharmacol. Rev.* **43**, 109–142.
- [2] Nathan, C. (1996) "Nitric oxide as a secretory product of mammalian cells", *FASEB J.* **6**, 3051–3064.
- [3] Cuzzocrea, S., Zingarelli, B., Gilard, E., Hake, P., Salzman, A. and Szabó, C. (1998) "Anti-inflammatory effects of mercaptoethylguanidine, a combined inhibitor of nitric oxide synthase and peroxynitrite scavenger, in carrageenan-induced models of inflammation", *Free Radic. Biol. Med.* **24**, 450–459.
- [4] Crow, J.P. and Beckman, J.S. (1995) "The role of peroxynitrite in nitric oxide-mediated toxicity", *Curr. Topics Microbiol. Immunol.* **196**, 57–73.
- [5] Miller, M.J.S., Thompson, J.H., Zhang, X.J., Sadowska-Krowicka, H., Kakkis, J.L., Munshi, U.K., Sandoval, M., Rossi, J., Elobi-Childress, S., Beckman, J., Ye, Y.Z., Rodi, C.P., Manning, P., Currie, M. and Clark, D.A. (1995) "Role of inducible nitric oxide synthase expression and peroxynitrite formation in guinea pig ileitis", *Gastroenterology* **109**, 1475–1483.
- [6] Phelps, D.T., Ferro, T.J., Higgins, P.J., Shankar, R., Parker, D.M. and Johnson, M. (1995) "TNF-alpha induces peroxynitrite-mediated depletion of lung endothelial glutathione via protein kinase C", *Am. J. Physiol.* **269**, L551–L559.
- [7] Inoue, S. and Kawanishi, S. (1995) "Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide", *FEBS Lett.* **371**, 86–88.
- [8] Salgo, M.G., Bermudez, E., Squadrito, G. and Pryor, W. (1995) "DNA damage and oxidation of thiols peroxynitrite causes in rat thymocytes", *Arch. Biochem. Biophys.* **322**, 500–505.
- [9] Szabó, C. and Billiar, T.R. (1999) "Novel roles of nitric oxide in hemorrhagic shock", *Shock*, 1–9, Review.
- [10] Cuzzocrea, S., Zingarelli, B., Gilard, E., Hake, P., Salzman, A.L. and Szabó, C. (1997) "Protective effect of melatonin in carrageenan-induced models of local inflammation", *J. Pineal Res.* **23**, 106–116.
- [11] Cuzzocrea, S., Costantino, G., Mazzon, E. and Caputi, A.P. (1999) "Beneficial effects of raxofelast (IRFI 016), a new hydrophilic vitamin E-like antioxidant, in carrageenan-induced pleurisy", *Br. J. Pharmacol.* **126**, 407–414.

- [12] Cuzzocrea, S., Zingarelli, B., Costantino, G. and Caputi, A.P. (1999) "Beneficial effects of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), a superoxide dismutase mimetic, in carrageenan-pleurisy", *Free Radic. Biol. Med.* **26**, 26–33.
- [13] Cuzzocrea, S., Mc Donald, M.C., Filipe, H.M., Costantino, G., Mazzon, E., Santagati, S., Caputi, A.P. and Thiemermann, C. (2000) "Effects of tempol, a membrane-permeable radical scavenger, in a rodent model of carrageenan-induced pleurisy", *Eur. J. Pharmacol.* **390**, 222–290.
- [14] Salvemini, D., Mazzon, E., Dugo, L., Riley, D.P., Serraino, I., Caputi, A.P. and Cuzzocrea, S. (2001) "Pharmacological manipulation of the inflammatory cascade by the superoxide dismutase mimetic, M40403", *Br. J. Pharmacol.* **132**, 815–827.
- [15] Salvemini, D., Wang, Z.Q., Stern, M.K., Currie, M.G. and Misko, T.P. (1998) "Peroxynitrite decomposition catalysts: novel therapeutics for peroxynitrite-mediated pathology", *Proc. Natl Acad. Sci. USA* **95**, 2659–2663.
- [16] Bagchi, D., Bagchi, M., Stohs, S.J., Das, D.K., Ray, S.D., Kuszynski, C.A., S., C.A., Joshi, S. and Pruess, H.G. (2000) "Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention", *Toxicology* **148**, 187–197.
- [17] Haenen, G.R., Paquay, J.B., Korthouwer, R.E. and Bast, A. (1997) "Peroxynitrite scavenging by flavonoids", *Biochem. Biophys. Res. Commun.* **236**, 591–593.
- [18] Dragsted, L.O., Strube, M. and Larsen, J.C. (1993) "Cancer-protective factors in fruits and vegetables: biochemical and biological background", *Pharmacol. Toxicol.* **72**, 116–135.
- [19] Okuda, T., Yoshida, T. and Hatano, T. (1992) "Antioxydant effects of tannins and related polyphenols", In: Huang, M.T., Ho, C.T. and Lee, C.Y., eds, *Phenolic Compounds in Food and their Effects on Health, II* (American Chemical Society, Washington, DC), pp 87–97.
- [20] Crespo, M.E., Galvez, J., Cruz, T., Ocete, M.A. and Zarzuelo, A. (1999) "Anti-inflammatory activity of diosmin and hesperidin in rat colitis induced by TNBS", *Planta Medica* **65**, 651–653.
- [21] Deters, A., Dauer, A., Schnetz, E., Fartasch, M. and Hensel, A. (2001) "High molecular compounds (polysaccharides and proanthocyanidins) from *Hamamelis virginiana* bark: influence on human skin keratinocyte proliferation and differentiation and influence on irritated skin", *Phytochemistry* **58**, 949–958.
- [22] Singletary, K.W. and Meline, B. (2001) "Effect of grape seed proanthocyanidins on colon aberrant crypts and breast tumors in a rat dual-organ tumor model", *Nutri. Cancer* **39**, 252–258.
- [23] Nemeth, K., Plumb, G.W., Berrin, J.G., Juge, N., Jacob, R., Naim, H.Y., Williamson, G., Swallow, D.M. and Kroon, P.A. (2003) "Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the adsorption and metabolism of dietary flavonoid glycosides in humans", *Eur. J. Nutr.* **42**, 29–42.
- [24] Miyazawa, T., Nakagawa, K., Kudo, M., Muraishi, K. and Someya, K. (1999) "Direct intestinal adsorption of red fruit anthocyanins, cyaniding-3-glucoside and cyaniding-3,5-diglucoside, into rats and humans", *J. Agric. Food Chem.* **47**, 1083–1091.
- [25] Huang, M.T. and Ferrano, T. (1992) "Phenolic compounds in food and cancer prevention", In: Huang, M.T., Ho, C.T. and Lee, C.Y., eds, *Phenolic Compounds in Food and Health II: Antioxidants and Cancer Prevention* (American Chemical Society, Washington, DC) pp 8–34.
- [26] Newmark, H.L. (1992) "Plant phenolic compounds as inhibitors of mutagenesis and carcinogenesis", In: Huang, M.T., Ho, C.T. and Lee, C.Y., eds, *Phenolic Compounds in Food and their Effects on Health II: Antioxidants and Cancer Prevention* (American Chemical Society, Washington, DC), pp 48–52.
- [27] Wang, H., Cao, G. and Prior, R.L. (1997) "Oxygen radical absorbing capacity of anthocyanins", *J. Agric. Food Chem.* **45**, 304–309.
- [28] Wang, S.Y. and Lin, H. (2000) "Antioxidant activity in fruits and leaves of blackberry, raspberry and strawberry varies with cultivar and developmental stage", *J. Agric. Food Chem.* **48**, 140–146.
- [29] Dugo, P., Mondello, L., Errante, G., Zappia, G. and Dugo, G. (2001) "Identification of anthocyanins in berries by narrow-bore high performance liquid chromatography with electrospray ionization detection", *J. Agric. Food Chem.* **49**, 3987–3992.
- [30] Mullane, K.M., Kraemer, R. and Smith, B. (1985) "Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium", *J. Pharmacol. Methods* **14**, 157–167.
- [31] Ohkawa, H., Ohishi, N. and Yagi, K. (1979) "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction", *Anal. Biochem.* **95**, 351–358.
- [32] Cuzzocrea, S., Mazzon, E., Dugo, L., Serraino, I., Ciccolo, A., Centorrino, T., De Sarro, A. and Caputi, A.P. (2001) "Protective effects of *n*-acetylcysteine on lung injury and red blood cell modification induced by carrageenan in the rat", *FASEB J.* **15**, 1187–1200.
- [33] Rahman, I., Skwarska, E. and Macnee, W. (1997) "Attenuation of oxidant/antioxidant imbalance during treatment of exacerbations of chronic obstructive pulmonary disease", *Thorax* **52**, 565–568.
- [34] Repine, J.E., Bast, A. and Lankhrst, I. (1997) "The Oxidative Stress Study Group. Oxidative stress in chronic obstructive pulmonary disease", *Am. J. Respir. Crit. Care Med.* **156**, 341–357.
- [35] Dawson, J., Sedgwick, A.D., Edwards, J.C. and Lees, P.A. (1991) "Comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse", *Int. J. Tissue React.* **13**, 171–185.
- [36] Salvemini, D., Wang, Z.Q., Wyatt, P., Bourdon, D.M., Marino, M.H., Manning, P.T. and Currie, M.G. (1996) "Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation", *Br. J. Pharmacol.* **118**, 829–838.
- [37] Cuzzocrea, S., Zingarelli, B., Gilard, E., Hake, P., Salzman, A.L. and Szabó, C. (1997) "Protective effect of melatonin in carrageenan-induced models of local inflammation", *J. Pineal Res.* **23**, 103–116.
- [38] Wei, X.Q., Charles, I.G., Smith, A., Ure, J., Feng, G.J., Huang, F.P., Xu, D., Muller, W., Moncada, S. and Liew, F.Y. (1995) "Altered immune responses in mice lacking inducible nitric oxide synthase", *Nature* **375**, 408–411.
- [39] Nijveldt, R.J., Van Nood, E., Van Hoorn, D.E., Boelens, P.G., Van Norren, K. and Van Leeuwen, P.A. (2001) "Flavonoids: a review of probable mechanisms of action and potential applications", *Am. J. Clin. Nutr.* **74**, 418–425.
- [40] Youdim, K.A., Mcdonald, J., Kalt, W. and Joseph, J.A. (2002) "Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults (small star, filled)", *J. Nutr. Biochem.* **13**, 282–288.
- [41] Harborne, J.B. and Grayer, R.J. (1988) "The anthocyanins", In: Harborne, J.B., ed, *The Flavonoids* (Chapman and Hall, London), pp 1–20.
- [42] Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. (1990) "Apparent hydroxyl radical production by peroxynitrite: implication for endothelial injury from nitric oxide and superoxide", *Proc. Natl Acad. Sci. USA* **87**, 1620–1624.
- [43] Beckman, J.S. (1996) "Oxidative damage and tyrosine nitration from peroxynitrite", *Chem. Toxicol. Res.* **9**, 836–844.
- [44] Eiserich, J.P., Hristova, M., Cross, C.E., Jones, A.D., Freeman, B.A., Halliwell, B. and Van Der Vliet, A. (1998) "Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils", *Nature* **391**, 393–397.
- [45] Cuzzocrea, S., Mazzon, E., Calabrò, G., Dugo, L., De Sarro, A., Van De Loo, F.A.J. and Caputi, A.P. (2000) "Inducible nitric oxide synthase knock out mice exhibit a resistance to carrageenan-induced local inflammation", *Am. J. Respir. Crit. Care Med.* **162**, 1859–1866.
- [46] Szabó, C. and Dawson, V.L. (1998) "Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion", *Trends Pharmacol. Sci.* **19**, 287–298.

- [47] Cuzzocrea, S., Zingarelli, B., Gilard, E., Hake, P., Salzman, A.L. and Szabó, C. (1998) "Protective effects of 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthase in carrageenan-induced models of local inflammation", *Eur. J. Pharmacol.* **34**, 67–76.
- [48] Szabo, C., Lim, L.H.K., Cuzzocrea, S., Getting, S.J., Zingarelli, B., Flower, R.J., Salzman, A.L. and Perretti, M. (1997) "Inhibition of poly (ADP-ribose) synthetase exerts anti-inflammatory effects and inhibits neutrophil recruitment", *J. Exp. Med.* **186**, 1041–1049.
- [49] Salvemini, D., Manning, P.Z., Zweifel, B.S., Seibert, K., Connor, J., Currie, M.G., Needleman, P. and Masferrer, J.L. (1995) "Dual inhibition of nitric oxide and prostaglandin production contributes to the antiinflammatory properties of nitric oxide synthase inhibitors", *J. Clin. Investig.* **96**, 301–308.
- [50] Sautebin, L., Ialenti, A., Ianaro, A. and Di Rosa, M. (1998) "Relationship between nitric oxide and prostaglandins in carrageenin pleurisy", *Biochem. Pharmacol.* **55**, 1113–1117.
- [51] Schreck, R., Rieber, P. and Baeuerle, P.A. (1991) "Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1", *EMBO J.* **10**, 2247–2258.
- [52] Wadleigh, D.J. and Herschman, H.R. (1999) "Transcriptional regulation of the cyclooxygenase-2 gene by diverse ligands in murine osteoblasts", *Biochem. Biophys. Res. Commun.* **264**, 865–870.
- [53] Cuzzocrea, S., McDonald, M.C., Mazzon, E., Siriwardena, D., Serraino, I., Dugo, L., Britti, D., Mazzullo, G., Caputi, A.P. and Thiemermann, C. (2000) "Calpain inhibitor I reduces the development of acute and chronic inflammation", *Am. J. Pathol.* **157**, 2065–2079.
- [54] Cuzzocrea, S., Mazzon, E., Sautebin, L., Dugo, L., Serraino, I., De Sarro, A. and Caputi, A.P. (2002) "Protective effects of Celecoxib on lung injury and red blood cells modification induced by carrageenan in the rat", *Biochem. Pharmacol.* **63**, 785–795.
- [55] Bartus, R.T., Elliott, P.J., Hayward, N.J., Dean, R.L., Harbeson, S., Straub, J.A., Li, Z. and Powers, J.C. (1995) "Calpain as a novel target for treating acute neurodegenerative disorders", *Neurol. Res.* **17**, 249–258.
- [56] Harada, Y., Hatanaka, K., Kawamura, M., Saito, M., Ogino, M., Majima, M., Ohno, T., Yamamoto, K., Taketani, Y., Yamamoto, S. and Katori, M. (1996) "Role of prostaglandin H synthase-2 in prostaglandin E₂ formation in rat carrageenin-induced pleurisy", *Prostaglandin* **51**, 19–33.

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