SUPPLEMENTARY MATERIAL

Protective activity of an anthocyanin-rich extract from bilberries and black currants on acute acetaminophen-induced hepatotoxicity in rats

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Abstract

Acetaminophen (N-acetyl-p-aminophenol, APAP) overdose can produce fatal centrilobular hepatic necrosis in humans. The present study attempted to investigate the protective effect of an anthocyanin-rich extract from bilberries and black currants (AE) against APAP-induced acute hepatic damage in rats. Treatment with AE normalized blood activities of glutamate oxaloacetate and glutamate pyruvate transaminase and prevented APAP-induced plasmatic and tissutal alterations in biomarkers of oxidative stress, probably due to various bioproperties of the components of the extract.

Keywords: acetaminophen, hepatoprotection, anthocyanin, oxidative stress, biomarkers

Experimental

1 Animals

Male Sprague Dawley rats with an average weight of 220 ± 12 g were purchased from Harlan (Milan, Italy) and were kept for a week under environmentally controlled conditions with free access to standard food and water *ad libitum*. Experiments were carried out according to the ethical guidelines for the care of laboratory animals.

2 Chemicals and solvents

All chemicals and reagents used in this study were of analytical grade or highest grade available and purchased from Sigma Aldrich (Milan, Italy). Solvents were purchased from Carlo Erba Reagents (Milan, Italy).

3 Anthocyanin extract

The anthocyanin rich extract (AE) used in our study is a dietary supplement (Medox[®], Biolink Group AS, Sandnes, Norway) consisting of 17 purified anthocyanins (all glycosides of cyanidin, peonidin, delphinidin, petunidin, and malvidin) isolated from bilberries (*Vaccinium myrtillus*) and black currants (*Ribes nigrum*), being glucosides of cyanidin and delphinidin at least 40–50% of the total anthocyanins (Qin et al. 2009).

4 Determination of AE hepatoprotective effect

The animals were randomly divided into 4 experimental groups, each of 5 rats, which were fasted for 12 hours before first administration. APAP was dissolved in propylene glycol/water (50/50 v/v; 3g/10 ml); the AE was solubilized in water. Both drugs (APAP and AE) and the vehicles were orally (i.g.) administrated.

GROUP I: animals received only the vehicles (propylene glycol/water, 50/50 v/v, 10 ml/kg bw, and water, 10 ml/kg bw)

GROUP II: animals received a single dose of APAP (3 g/kg bw).

GROUP III: animals received AE (600 mg/kg bw) divided into two doses (each of 300 mg/kg bw, 10 ml/kg) given 12 hours before and 1 hour after the treatment with APAP (3 g/kg bw) respectively.

GROUP IV: animals received AE (600 mg/kg, 10 ml/kg) in a unique dose 1 hour after the treatment with APAP (3g/kg bw).

Twenty four hours following APAP administration, animals in all the groups were euthanized by intraperitoneal injection of sodium pentobarbital (100 mg/kg bw). Whole blood was collected in heparin-coated test tube to obtain plasma by centrifugation at 3000xg for 15 min at 4°C. Liver was removed,

washed with cold saline to remove residual blood and homogenized (Ultra Turrax, IKA Werke, Janke and Kunkel GmbH) with phosphate buffer (PBS, 75 mM, pH 7.0; 9 ml of PBS for 1 g of liver); then the homogenate was centrifuged at 3000xg for 20 min to discard cellular debris and unbroken cells.

All samples were stored at -80 °C until analyzed.

The content of proteins, both in plasma and liver homogenate, was spectrophotometrically quantified by the Bradford assay using bovine serum albumin as standard (Bradford, 1976). Bradford assay involves the binding of Coomassie Brilliant Blue G-250 to proteins. The binding of the dye to proteins causes a shift in the absorption maximum of the dye from 465 to 595 nm. Briefly, 50 µl of sample were added to 1.5 ml of Bradford reagent, and after 5 minutes the absorbance was measured at 595 nm. All analysis were performed in triplicate.

4.1 Liver Function Tests

To assess the AE protective effect on APAP-induced liver damage plasma concentrations of the transaminases GOT and GPT were measured by a colorimetric assay using a commercially available kit (Biocon Diagnostic, Germany).

4.2 Oxidative Status Biomarkers

4.2.1 Lipid Peroxidation

Lipid peroxidation was assessed by measurement of MDA and CD.

Levels of MDA (an end product of phospholipids peroxidation) was measured both in plasma and liver tissue (Sahin and Gümüslü, 2007; Tomaino et al., 2006).

To measure MDA levels in liver tissue we used the thiobarbituric acid method. Briefly, 400 µL of homogenate were added with 2.5 mL of 8% trichloroacetic acid; the sample was centrifuged at 3000 xg for 10 min at 4°C. The supernatant was collected, added with 2 ml of thiobarbituric acid (0.67%, w/v) and incubated for 15 min at 95°C. After cooling at room temperature, the absorbance was measured at 523 nm (Shimadzu UV-1601, Spectrophotometer, Japan).

The determination of MDA in plasma was performed by using a commercially available kit purchased from Calbiochem (Germany). Briefly, 200 µl of plasma were added with 650 µl of N-methyl-2-phenylindole solubilized in acetonitrile (10.3 mM) and 150 µl of methansulfonic acid (15.4 M); the mixture was incubated at 45°C for 40 minutes. The reaction was stopped by placing the tubes on ice and the absorbance was measured at 586 nm (Shimadzu UV-1601, Spectrophotometer).

A calibration curve was prepared using 1'1'3'3'-tetramethoxypropane as standard (in the range from 2.5 to 40 µM). The results were expressed as nmoles of MDA/mg protein for liver tissues and as nmoles of MDA/ml for plasma.

Furthermore, the levels of CD (a marker of early lipid peroxidation) in liver tissue were determined according to the method reported by Sahin and Gümüřlü (2007). Five hundred μl of liver homogenate were extracted with 3 ml of chloroform:methanol 2:1 (v/v), and the extract was evaporated to dryness with a rotavapor, and then re-dissolved in 1 ml of cyclohexane. The absorbance of the cyclohexane solution was measured at 234 nm. The results were expressed as nmol/mg protein (molar extinction coefficient = $2.52 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).

4.2.2 Thiol Group Analysis

The liver tissue concentrations of total thiol groups (T-SH), NP-SH groups and P-SH groups were measured according the method described by Kayali et al. (2006).

To determine T-SH groups, aliquots of 250 μl of tissue homogenate were added with 750 μl of 0.2 M TRIS buffer (pH 8.2), 50 μl of 0.01 M 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and 3.950 ml of absolute methanol. The blank sample (without DTNB) was prepared in a similar manner. The reaction mixture was incubated for 15 minutes at room temperature. Then, the samples were centrifuged at 3000xg for 15 min at 25°C. The absorbance of the supernatant was read at 412 nm. To determine the content of NP-SH groups, 250 μl of homogenate were added with 125 μl of trichloroacetic acid (TCA; 20% w/v) and 125 μl of distilled water. The samples were shaken for 10 min and then centrifuged for 15 min at 3000xg at room temperature. Two hundred μl of the supernatant were mixed with 400 μl of TRIS buffer (0.4 M, pH 8.9) and 10 μl of DTNB (0.01 M). The absorbance at 412 nm was read within 5 min following the addition of DTNB.

In both cases the concentration of SH groups was calculated as nmoles of SH for mg protein (molar extinction coefficient: $13.1 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}$).

The concentration of P-SH groups was calculated by subtracting NP-SH from T-SH (Kayali et al., 2006).

4.2.3 Protein oxidation

The determination of levels of proteins CG in plasma and liver tissue was performed according to the method described by Levine et al., (1990) with slight modifications (Di Rosa et al., 2011; Tomaino et al., 2006).

Aliquots (150 μl) of liver homogenate or plasma were added with 500 μl of TCA (20% w/v) and centrifuged at 3000xg for 5 min at 4°C; the supernatant was removed and the pellets was used to determine the content of protein carbonyl groups. Pellet was resuspended in 500 μl of 2,4-dinitrophenylhydrazine (DNPH; 10 mM in 2 M HCl) and kept at room temperature for 1 hour. Then the proteins were reprecipitated with 500 μl of TCA (20% w/v) and centrifuged at 3000xg for 5 min at 4°C; the pellet was washed 3 times with ethanol:ethyl acetate (1:1 v/v). The resulting precipitate was dissolved

in 600 μL of guanidine (6 M in phosphate buffer 20 mM, pH 2.3). The CG content was determined from the absorbance at 366 nm. The results were expressed as nmol/mg protein (molar absorption coefficient: 22,000 $\text{M}^{-1} \text{cm}^{-1}$).

5. Statistical Analysis All data were represented as mean $\pm$ SD. Significant difference between groups were statistically analyzed using one-way analysis of variance (ANOVA). *P* values less than 0.05 were considered as significant.

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