

Effect of the daily ingestion of a purified anthocyanin extract from grape skin on rat serum antioxidant capacity

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ABSTRACT

The aim of this work was to study the effect of the daily ingestion of a purified anthocyanin extract from red grape skin on rat serum antioxidant capacity (ORAC) and its safety for the intestinal epithelium. The study was carried out on rats orally administered with the extract for ten days in either normal physiological conditions or exposed to a pro-oxidant chemical (CCl₄).

The oral administration of the extract significantly ($P < 0.05$) enhanced the ORAC value of the deproteinised serum of about 50% after 10 days of ingestion. Anthocyanin administration was also able to completely reverse the decrease in the serum ORAC activity induced by the CCl₄ treatment.

Experiments with Ussing chamber mounted intestine allowed to exclude any toxicity of the extract for the intestinal epithelium.

In conclusion our results demonstrate that the purified anthocyanin extract from red grape skin enhances the total antioxidant capacity of the serum in either normal physiological condition or during oxidative stress induction, revealing a protective role against the decrease in the serum antioxidant capacity induced by a pro-oxidant compound.

Key words

Anthocyanin, antioxidant capacity, ORAC, grape skin, serum

INTRODUCTION

Anthocyanins are chemically phenolic compounds belonging to the flavonoid family responsible for the colours of flowers and fruits of a great variety of plants. They have been shown to possess beneficial *in vitro* properties related to the protection against pathologies involving oxidative stress, such as cardiovascular diseases, cancer, neurodegeneration, inflammation, and viral infection (Prior and Wu, 2006). In particular, anthocyanins are potent antioxidants (Khkonen and Heinonen 2003) because of their ability to quickly reduce oxidizing species while being converted into stabilized aryloxy radicals. Moreover, they are able to chelate transition metal ions potentially involved in the development of oxidative stress (Moran et al., 1997).

In grapes (*Vitis vitifera*, L.) anthocyanins are the typical pigments of the skins of red cultivars, therefore grape skins represent an excellent source of anthocyanins. The anthocyanins identified in *V. Vinifera* spp. correspond to the 3-*O*-monoglucosides and the 3-*O*-acylated monoglucosides of the five main anthocyanidins: delphinidin, cyanidin, petunidin, peonidin, and malvidin. The antioxidant activity of red grape anthocyanins has been extensively demonstrated mainly in *in vitro* studies. Frankel et al. (1998) demonstrated the *in vitro* inhibition of the copper catalyzed oxidation of human LDL by red commercial grape juices and related this activity to the anthocyanin concentrations. Singletary et al. (2007) demonstrated the anthocyanins contained in a red grape extract to have *in vitro* breast cancer chemopreventive potential due in part to their capacity to block carcinogen–DNA adduct formation, to modulate activities of carcinogen-metabolizing enzymes, and antioxidant activity. Zenebe et al., (2003) demonstrated red wine polyphenols, including anthocyanin, to induce vasorelaxation by increased nitric oxide bioactivity.

Extracts from *V. vinifera*. are commonly used to formulate dietary antioxidant supplements. Although the dietary industry based on wine by-products is rapidly

growing, practically no scientific research is available on the *in vivo* antioxidant activity of these products and their potential effects and safety for the gastrointestinal tract.

The aim of this work was to study the *in vitro* and *in vivo* antioxidant activity and the safety for the intestinal epithelium of a purified anthocyanin extract, obtained from red grape skin by an innovative method based on supercritical CO₂ (Bleve et al., 2008) and available to be used for dietary antioxidant supplement. As previously demonstrated (Bleve et al., 2008), this purification method allowed to eliminate the solvent residue from the anthocyanin extract without any thermal or chemical degradation, obtaining a high added value product which is unchanged in its anthocyanin content. According to Bleve et al. (2008), four major anthocyanins were present in the purified extract: malvidin 3-*O*-glucoside (50%), petunidin 3-*O*-glucoside (10%), delphinidin 3-*O*-glucoside (8%), and cyanidin 3-*O*-rutinoside (5%).

In the present paper the *in vitro* and *in vivo* antioxidant activity of the anthocyanin extract was determined by the ORAC (Oxygen Radical Absorbance Capacity) assay which is still one of the few methods that combines both inhibition percentage and inhibition time of the reactive species action by antioxidants in a single quantity.

The *in vivo* antioxidant properties of the extract were studied by measuring the total antioxidant capacity (ORAC) of the deproteinised serum of rats orally administrated with the extract for ten days. The total antioxidant capacity of blood plasma or serum by the ORAC method represents an integrated measure of the non enzymatic antioxidant defence mechanisms of the organism (Cao et al., 1998). The effects of the anthocyanin extract ingestion were analyzed in either normal physiological conditions or after treatment with a single dose of CCl₄ (~~2 ml/kg under skin~~) in order to investigate the possible protective action of the extract administration against oxidative stress induced by the exposure to a pro-oxidant agent. CCl₄ is a well established hepatotoxin (Szymonik-Lesiuk et al., 2003). However, various studies demonstrated that liver is not

the only target organ of CCl₄ and that this compound causes free radical generation also in other tissues such as kidneys, heart, lung, testis, brain, and blood (Ohta et al 1997; Ozturk et al, 2003).

The potential effect of the extract on intestinal cells functionality was assessed *in vitro*, by exposing the jejunum tract of rat intestine to the extract and monitoring transepithelial electric resistance (R_{te}) and lactate dehydrogenase (LHD) release.

The results give more insight on the *in vivo* antioxidant activity and safety for the gastrointestinal tract of a purified red grape anthocyanin extract, and point out the protective effect of the prolonged daily ingestion of anthocyanins on the serum antioxidant capacity observed during exposure to a pro-oxidant compound.

MATERIALS

The anthocyanin purified extract was provided by Pierre Chimica S.r.l., Galatina, Lecce, Italy and Dip. of Innovation Engineering, University of Salento Lecce, Italy. According to Bleve et al. (2008) four major anthocyanins were present in the extract: malvidin 3-*O*-glucoside (50%) petunidin 3-*O*-glucoside (10%), delphinidin 3-*O*-glucoside (8%), and cyanidin 3-*O*-rutinoside (5%). 0.16 g of extract were obtained per one gram of grape skin wet weight.

Male Wistar rats weighing 200 to 250 g were purchased from Harlan Italy s.r.l. (UD, Italy). All chemicals were reagent grade and were purchased from Sigma (St. Louis, U.S.A.).

METHODS

All experiments were carried out in accordance with the European Guidelines on Laboratory Animal Care and had the approval by the Italian Ministry of Health.

ORAC assay of the anthocyanin extract

The ORAC protocol utilized in this work refers to the method described by Ou et al. (2001) and further modified by Davalos et al. (2004) which uses fluorescein instead of β -phycoerythrin as fluorescent probe. Briefly, the diluted anthocyanin-rich extract (20 μ l) and the fluorescein solution (1200 μ l, 120 nM in phosphate buffer, pH 7.4) were pre-incubated for 15 min at 37 °C; then the peroxy radical generator AAPH (600 μ l, 40mM in phosphate buffer, pH 7.4) was added. The fluorescence of the mixture was recorded every minute for 80 min ($\lambda_{\text{ex}}=535$; $\lambda_{\text{em}}=560$ nm). Trolox (1, 2, 4 and 8 μ M final concentration) was used as antioxidant standard for the calibration curve construction. For each fluorescent decay curve (fluorescence versus time) (Fig. 1) the area under curve (AUC) was calculated as follows:

$$\text{AUC} = \sum_{i=0}^n f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i . For each sample the net AUC was calculated by subtracting the AUC corresponding to the blank (fluorescein + AAPH without the antioxidant solution). The AUC value was interpolated on the calibration curve. The ORAC values were expressed as micromoles of trolox equivalents.

Experimental design of *in vivo* rat exposure

Male Wistar rats weighing 200 to 250 g were kept in a temperature- and humidity-controlled room with 12-hour light/dark cycles. They were given free access to tap water and standard rat chow.

In the first exposure experiment 16 rats were divided in two groups: 1) control animals (not exposed to any treatment); 2) anthocyanin exposed animals, orally administered by micropipette to an anthocyanin extract solution at the dosage of 0.6 mg of anthocyanin per kg of body weight. The animals were daily exposed to the extract for ten days in order to experimentally simulate a prolonged exposure. Thereafter, they were sacrificed at the 11th day of the experiment.

In the second exposure experiment 32 rats were divided into four groups (eight animals for each group) composed as follows: 1) control animals; 2) animals daily exposed for ten days by oral administration to the anthocyanin extract (as reported above); 3) animals who received one dose of a subcutaneous injection of CCl₄ at the eighth day of the experiment; 4) animals daily exposed to the extract as above who received in addition one dose of a subcutaneous injection of CCl₄ at the eighth day of the experiment. Control and anthocyanin exposed animals received one dose of a subcutaneous injection of oil (vehicle of CCl₄) at the eighth day of the experiment. According to Tirkey et al. (2005), CCl₄ was applied only at 8th day of the experiment in order to reduce the risk of mortality following a prolonged exposure to the drug.

For the second exposure experiment an orthogonal two factor experimental design was chosen: factor (A) “anthocyanin exposure” which included two levels (“not exposed” and “exposed”), and factor (B) “CCl₄ exposure” which included two levels (“not exposed” and “exposed”).

Blood sample preparation and ORAC assay

At the 11th day of the experiment the animals were anaesthetized with chloroform prior to sampling and then killed by decapitation. Blood was collected in a micro-tube within 5 sec after decapitation. Then, the blood was allowed to clot at room temperature for 30 min. The clot was removed by centrifuging at 2,000 g for 10 minutes in a refrigerated centrifuge and the resulting supernatant was collected. The serum was immediately diluted with saturated ammonium sulfate (1:4 v/v), incubated for 10 min and then centrifuged at 11,500 rpm for 30 min at 4°C. The supernatant was removed as deproteinized fraction that preserves the water-soluble antioxidants within the sample. The supernatant was utilized for the ORAC assay, as above described.

Tests on rat intestine: R_{te} measurements and LDH release assay

With the aim of evaluating the potential effects of the anthocyanin extract on the intestinal mucosa functionality, segments of rat jejunum intestine were placed in direct contact with the extract for a 3-hour incubation time. The epithelial barrier integrity was monitored by means of transepithelial electric resistance (R_{te}) measurements. The LDH release was measured during and after the exposure respectively, as significant indicator of cell damage (Sannino et al., 2006). 3-hour incubation time is expected to approximate the real time of contact *in vivo* where food passes through the duodenum and jejunum. The jejunum represents the gastrointestinal segment where most digestion and absorption occurs.

The experiments were carried out on native tissues mounted in an Ussing chamber set up (Clarke, 2009). This experimental approach has advantages in surveillance of the mucosa functionality compared to other *in vitro* techniques (Smith, 1996).

Four male Wistar rats (weighing about 250g) were sacrificed for the experiment. Two adjacent segments (1.5 cm length) of the jejunum tract were isolated from each

animal, cut along the intestine longitudinal axis to produce a mucosal sheet and mounted vertically in an Ussing chamber. After mounting, each half chamber was filled with 6 ml Krebs-buffer (containing in mM: NaCl 124, KH₂PO₄ 1.25, MgCl₂ 1.8, KCl 1.75, CaCl₂ 1.6, NaHCO₃ 26, glucose 10), bathing the intestinal tissue on both the mucosal and serosal side. The exposed tissue surface area was 0.6 cm². The Krebs-buffer was continuously oxygenated with O₂/CO₂ (95/5%) and stirred by gas flow in the chambers. The equilibrium between CO₂ and NaHCO₃ maintained the pH of the Krebs buffer at the constant value of 7.4. The temperature of the Krebs solution was kept constant at 28°C all through the experiment. Tissues were connected to an automatic short-circuit current device (WPI's DVC-1000) by four Ag/AgCl electrodes which made contact with the bathing solutions via agar-Ringer filled cartridges. Transepithelial voltage (V_{te}) was measured with respect to the mucosal bath (grounded); the short circuit current (I_{sc}) was measured by passage of sufficient current through Ag/AgCl electrodes to reduce the spontaneous V_{te} to zero automatically (the resistance of the chamber fluid was subtracted automatically). R_{te} was measured by pulsed current injection (20 $\mu\text{A} \cdot \text{cm}^{-2}$, 500 ms) through the tissue. The resulting V_{te} deflection was measured.

For each experiment the two adjacent jejunum segments were treated as follows: segment (1) was taken as a control, i.e. simply bathed with physiological solution, segment (2) was exposed to the extract (0.02 mg/ml final concentration) from the luminal side of the epithelium. V_{te} and I_{sc} , which are expression of the ion transport function of the intestine, were constantly monitored as tissutal viability index. All through the experiments V_{te} and I_{sc} showed the constant value of 1.5 ± 0.2 mV and 25 ± 2.1 $\mu\text{A} \cdot \text{cm}^{-2}$ respectively, which are typical for this tract of rat intestine (Madsen et al., 1996).

At the end of the experiment, the lactate dehydrogenase (LDH) leakage into the mucosal and serosal bathing solutions was monitored. LDH activity was measured according to Wroblewski and La Due (1955) and was expressed as Enzymatic Units per milliliter (Uml^{-1}). Triplicate determinations were performed for each sample.

Statistical analysis

Data were statistically analyzed by Student t test (for the first anthocyanin *in vivo* exposure experiment) and by two ways ANOVA and Newman-Keuls post test (for the second *in vivo* exposure experiment). The homogeneity of variance was tested by Cochran's test prior to applying ANOVA. Data are reported as means \pm S.E.M.

RESULTS

***In vitro* ORAC activity of the purified extract**

The *in vitro* antioxidant activity of the purified anthocyanin extract, as determined by the ORAC assay, was 370 ± 18.3 ORAC per gram of grapes skin wet weight. When the antioxidant activity was referred to the anthocyanin concentration of the extract, it corresponded to 355 ± 17.5 ORAC/mg anthocyanin. The samples showed good antioxidant properties in terms of *in vitro* peroxy radical scavenging activities compared to other grape skin extracts reported in literature (Yilmaz and Toledo, 2003).

Effect of the anthocyanin administration on rat serum antioxidant capacity

The total antioxidant capacity of blood plasma is mainly derived from small molecules such as ascorbic acid, α -tocopherol, β -carotene, and from macromolecules such as transferrin and ceruloplasmin. In order to better investigate the contribution of

anthocyanin ingestion to the serum antioxidant capacity, the protein contribution to the total antioxidant capacity was excluded by performing the ORAC measurement on a serum deproteinized fraction.

With the aim to evaluate the potential effect of the prolonged daily ingestion of anthocyanins on the total serum antioxidant capacity, the rats were orally administrated with the purified extract for ten days. The daily intake of red grape anthocyanins utilized in the present work (0.6 mg of anthocyanin per kg of body weight) represents a fraction (about 20-25%) of the total average daily intake of anthocyanins per kg of body weight in humans according to Kühnau (1976).

As observed in Tab.1 the rats orally administrated for ten days with the purified anthocyanin extract showed a significant increase in the antioxidant capacity of the deproteinized serum of about 50%.

Effect of the anthocyanin administration on rat serum antioxidant capacity in pro-oxidant conditions.

After having demonstrated the increase of rat serum antioxidant capacity following daily ingestion of the anthocyanin extract, we addressed the effect of the anthocyanin administration on serum ORAC in *pro-oxidant* conditions.

When the animals were treated with the pro-oxidant agent CCl_4 , the serum antioxidant capacity significantly ($P < 0.01$) decreased (Fig.2). Interestingly, when the animals were orally administrated with the anthocyanin extract before treatment with CCl_4 , the effect of CCl_4 was completely reversed resulting not statistically different from control animals (Fig.2). Two way ANOVA analysis showed that either anthocyanin administration or CCl_4 treatment had a significant ($P < 0.01$) effect on serum ORAC.

R_{te} measurement and LDH assay

With the aim of evaluating the potential effects of the anthocyanin extract on the intestinal mucosa functionality, different segments of rat jejunum intestine were placed in direct contact with the extract for a 3-hour incubation time. The concentration utilized (0.02 mg/ml final concentration) *in vitro* is consistent with the concentration used in *in vivo* extract oral administration and takes into account the dilution that the ingested substances undergo through the gut lumen. The gastrointestinal epithelium normally functions as a selective barrier that permits the absorption of nutrients, electrolytes, and water. The epithelial barrier function is provided by the epithelial cells and the tight junctions that connect them. The integrity of tight junctions is fundamental for the functional integrity of the tissue and can be electrophysiologically expressed by the R_{te}.

In Fig.3 R_{te} values through the course of the experiment are reported as percentage of the initial value (corresponding to $20.2 \pm 1.2 \Omega\text{cm}^2$), measured before the incubation of the epithelium with the extract. It can be observed that the exposure of the rat intestine with the extract does not significantly affect the R_{te} values, if compared to the control (segment perfused only with physiological solution). The statistical significance of the data was tested by Dunnett's test after transforming percentage data in corresponding arcsen values. These results suggest that the extract does not significantly affect the integrity of gastrointestinal epithelium or alter its permeability in *in vitro* conditions.

Cytosolic LDH is widely used as a sensitive measure of cellular damage such as cell membrane injury (Whittle and Steel, 2002). The results of LDH activity measured in the mucosal and serosal perfusates, collected after the 3-hour period of incubation of rat intestine, are reported in Table 2. No significant release of LDH is detected for the anthocyanin extract exposed segments either in the serosal or mucosal bathing solution with respect to the control tissues, as stated by Dunnett's test.

Discussion

In the present paper the *in vivo* effect of a purified anthocyanin extract from red grape skin on the total antioxidant capacity of deproteinised rat serum was demonstrated after a prolonged daily ingestion in either normal physiological conditions or after treatment with a single dose of CCl₄. Experiments with Ussing chamber mounted intestine allowed to exclude any significantly toxic effect of the extract on the integrity and functionality of gastrointestinal epithelium of the experimental animals.

In normal physiological condition we demonstrated that the oral administration of the purified anthocyanin extract significantly ($P < 0.05$) enhanced the oxygen radical absorbance capacity of the deproteinised serum of about 50% after 10 days of ingestion. It is known that the contribution of the non protein fraction to the antioxidant capacity of the serum is about 10-20 % in adult rats (Cao et al., 1996), being higher in the newborn and decreasing significantly during growth. Therefore, according to Cao et al. (1996), the observed ORAC increase should correspond to an increase of the total antioxidant capacity of the entire serum of about 5-10%. Increasing the serum antioxidant status has been implicated as a possible preventative means to reduce the development of cardiovascular disease (Kaplan and Aviram, 1999; Salonen et al., 2000), diabetes (Vendemiale et al., 1999) and cancer (Willett, 2001). Recently, researchers have determined a correlation between increased *ex vivo* blood antioxidant status and risk of cardiovascular disease and cancer (Durak et al., 2001; Ching et al., 2002).

It is known that after ingestion anthocyanins quickly appear as intact molecules in the plasma (Wu et al., 2002; Mazza et al., 2002; Bub et al., 2001). Therefore, it is possible to attribute the observed increase in the antioxidant activity to the increased anthocyanin concentration in the plasma. The use of a purified extract in our experiment allows to exclude the contribution of other antioxidants also present in the red grape skin (such as other polyphenols) to the observed serum antioxidant activity increase. Moreover, it

allows also to exclude any interference with the plasma concentration of other antioxidants like the known effect of fructose on urate plasma concentration (Lotto and Frei, 2004). In fact, it is known that the ingestion of fructose, often contained in many not purified anthocyanin extracts or in juices, can increase the plasma concentration of urate which is one of the main components of deproteinized serum antioxidant capacity (Lotto and Frei, 2004). With respect to the oral intake of red grape juice reported in other works (Bitsch et al., 2004), the use of the purified anthocyanin extract in our experiments allows to ascribe the observed changes in the antioxidant capacity of plasma only to the anthocyanin contribution. In previous studies carried out on single anthocyanin administration, the anthocyanin plasma levels and the serum antioxidant activity returned to the basal level within two hours (Bitsch et al., 2004; Kay and Holub, 2002). What is not known is if anthocyanins are accumulated in tissues when consumed over an extended period of time. In the present work the serum antioxidant capacity, measured at the 11th day of experiment after a prolonged exposure to a purified anthocyanin extract for ten days, was still increased. This result seems to suggest a possible accumulation of anthocyanin in plasma when daily ingested for a prolonged period of time. However, this represents an intriguing hypothesis that need to be tested in a future work.

Moreover, in the present paper the *in vivo* effect of the purified anthocyanin extract from red grape skin on the total antioxidant capacity of the deproteinised serum was demonstrated after treatment with a pro-oxidant agent, CCl₄. The hepatotoxicity of CCl₄ is well documented; however various studies demonstrated that liver is not the only target organ of CCl₄ and that this compound causes free radical generation also in other tissues such as kidneys, heart, lung, testis, brain, and blood (Ohta et al., 1997; Ozturk et al., 2003). This compound generates *in vivo* trichloromethyl radicals that stimulate a sequence of reactions culminating in the initiation of the peroxidation of membrane

lipids. It is known that CCl₄ causes an elevation in the serum content of alanine transaminase and aspartate transaminase which is indicative of damage to the liver and other organs of the body (Reinke et al., 1988). It is also known that treatment of rats with anthocyanin for 4 weeks before CCl₄ administration caused progressively less hepatotoxicity of CCl₄ alone, as evidenced by the decreased serum content of alanine transaminase and aspartate transaminase relative to the CCl₄-treated anthocyanin-free group (Obi et al., 1998). In the present paper for the first time a decrease of the serum oxygen radical absorbance capacity (ORAC) induced by CCl₄ is reported. Surprisingly, when the animals were pretreated with the purified anthocyanin extract for ten days the inhibitory effect of CCl₄ was completely reversed. Considering that the oxygen radical absorbance capacity (ORAC) measures the capacity of plasma/serum to inhibit peroxy radical-induced oxidations (Cao et al., 1993), the obtained results suggest a protective role of the daily consumption of anthocyanin against the peroxy radicals induced by CCl₄. In general, peroxy radicals are the predominant free radical found in biological systems (Prior et al., 2005) and the exposure to several agents, such as chemicals, drugs, and pollutants are known to induce peroxy radicals in the organisms. Therefore, by extrapolation it is possible to argue a potential protective effect of the anthocyanin daily ingestion against oxidative stress induced by peroxy radicals coming from different sources.

CONCLUSIONS

Our results demonstrate that the purified anthocyanin extract from red grape skin enhances the total antioxidant capacity of the serum in either normal physiological condition or during oxidative stress induction, revealing in this case a protective role against the decrease in the serum antioxidant capacity induced by a pro-oxidant

compound. This work contributes to increase the knowledge of the effects of daily consumption of anthocyanin on the antioxidant defences of the organism.

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Table 1. Effect of the anthocyanin administration on rat serum antioxidant capacity. The Oxygen Radical Absorbance Capacity (ORAC) of deproteinized serum, expressed as ORAC (micromoles Trolox Equivalents)/ml of serum, was measured on control and anthocyanin administrated rats. The animals were daily exposed (by oral administration) to an extract amount corresponding to 0.6 mg of anthocyanin per kg of body weight as described in the method section. n = number of animals. Data are reported as mean \pm E.S. * t-Student.

	ORAC/ml	n	P*
Control rats	0.574 \pm 0.083	8	
Anthocyanin exposed rats	0.883 \pm 0.089	8	0.0236

Table 2. Lactate dehydrogenase (LDH) release by rat intestinal cells after 3 hour incubation with the extract. The release of LDH has been measured both in the mucosal and serosal perfusates, as explained in the method section. The reported results are expressed as mean \pm SEM (n=3) of three independent experiments.

	Control (mU/ml)	+anthocyanin extract (mU/ml)
Mucosal solution	5.90 \pm 1.11	4.09 \pm 0.65
Serosal solution	5.55 \pm 0.65	4.40 \pm 1.10

Captions to figures

Fig.1. Fluorescent decay curves of trolox (1 μ M, 2 μ M, 4 μ M, 8 μ M), utilized as standard, and anthocyanin extract sample. Representative determination.

Fig. 2. Effect of the anthocyanin administration on rat serum antioxidant capacity in either physiological condition or after treatment with CCl₄. The Oxygen Radical Absorbance Capacity (ORAC) of deproteinized serum was measured on: 1) control animals; 2) animals daily exposed to the anthocyanin extract; 3) animals exposed to CCl₄; 4) animals exposed at first to anthocyanin and then to CCl₄. Data are reported as mean \pm S.E.M. Data were analyzed by two way ANOVA and Newman Keules post test.
* P<0.05; ** P<0.01

Fig.3. Transepithelial electrical resistance (R_{te}) of rat jejunum, monitored during the exposure to the anthocyanin extract. Data are expressed as percentage of the initial value measured before the incubation with the extract. The time of contact of about 3 hours is expected to approximate the real time of contact *in vivo*. Data are reported as mean \pm SEM of three independent experiments.

Fig.1

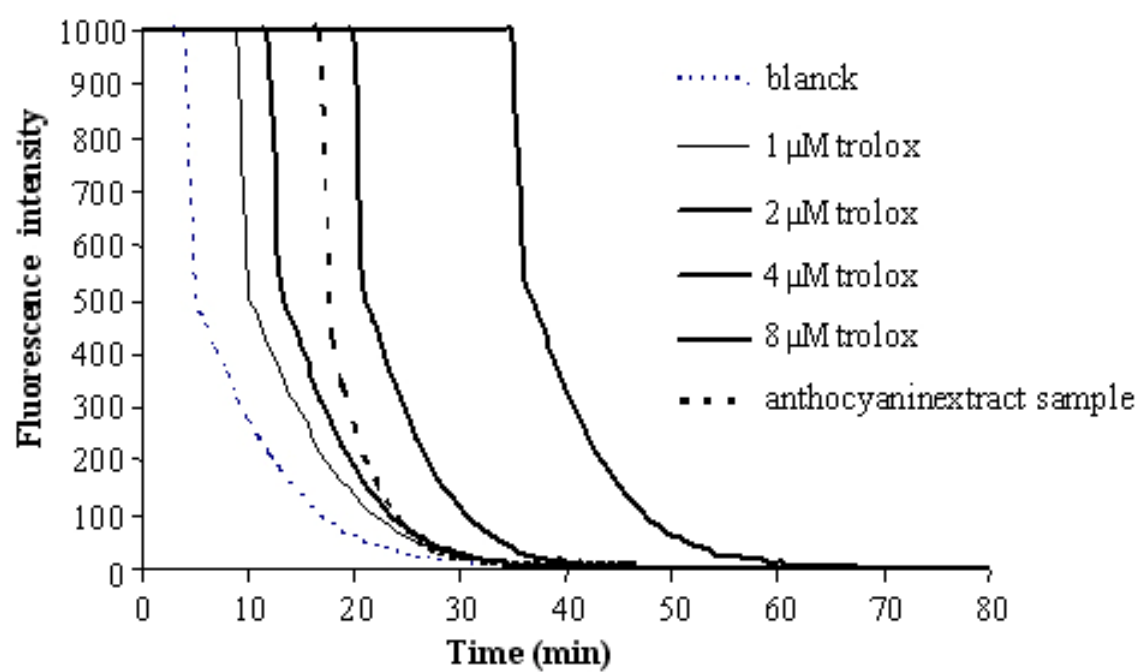


Fig.2

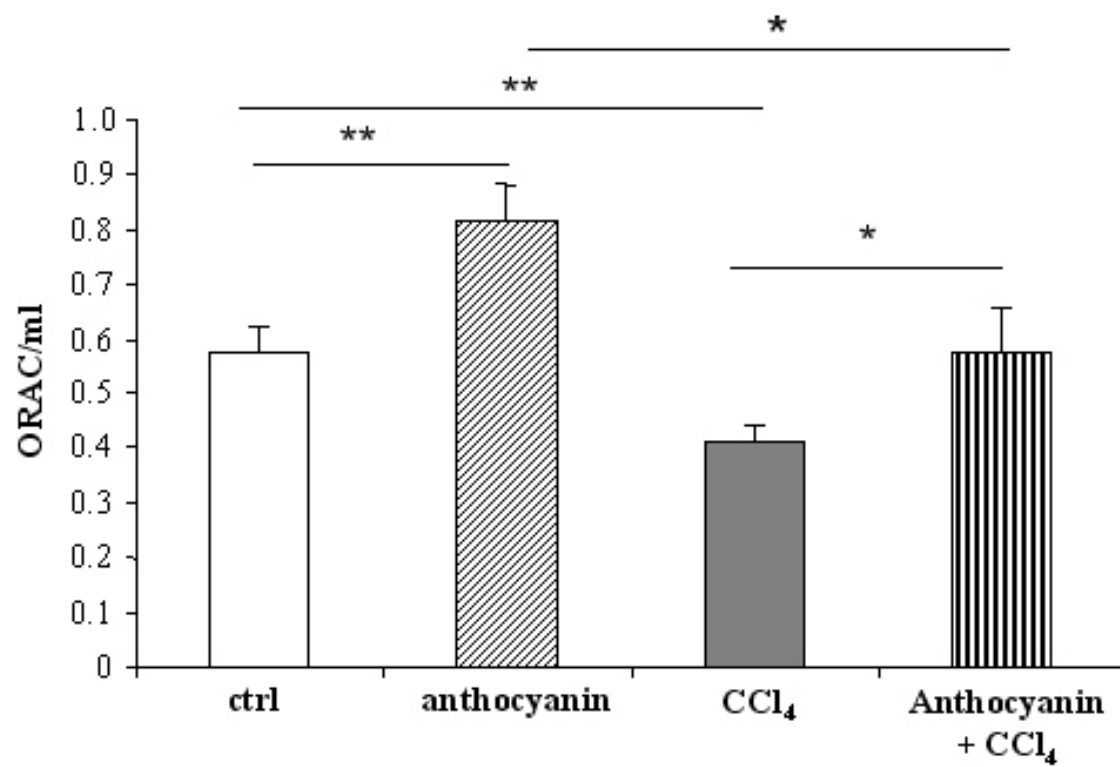


Fig.3

