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Xanthohumol, a prenylated flavonoid from hops (*Humulus lupulus* L.), protects rat tissues against oxidative damage after acute ethanol administration

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ABSTRACT

Ethanol-mediated free radical generation is directly involved in alcoholic liver disease. In addition, chronic alcohol bingeing also induces pathological changes and dysfunction in multi-organs. In the present study, the protective effect of xanthohumol (XN) on ethanol-induced damage was evaluated by determining antioxidative parameters and stress oxidative markers in liver, kidney, lung, heart and brain of rats. An acute treatment (4 g/kg b.w.) of ethanol resulted in the depletion of superoxide dismutase, catalase and glutathione S-transferase activities and reduced glutathione content. This effect was accompanied by the increased activity of tissue damage marker enzymes (glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and lactate dehydrogenase) and a significant increase in lipid peroxidation and hydrogen peroxide concentrations. Pre-treatment with XN protected rat tissues from ethanol-induced oxidative imbalance and partially mitigated the levels to nearly normal levels in all tissues checked. This effect was dose dependent, suggesting that XN reduces stress oxidative and protects rat tissues from alcohol-induced injury.

1. Introduction

The prolonged consumption or large intake of alcohol in a short period of time causes alcoholic liver disease (ALD), a process that involves oxidative stress and a significant increase in the production of reactive oxygen species (ROS) [1]. Ethanol is metabolised by oxidative and non-oxidative pathways, and liver alcohol dehydrogenase is involved in the oxidative pathway. This enzyme converts ethanol to acetaldehyde, which, in turn, is oxidised into acetate by aldehyde dehydrogenase [2]. Xanthine oxidase, the main source of anion superoxide, is activated in this process. Ethanol also may be oxidised to acetaldehyde by the hepatic ethanol inducible cytochrome P450 system (CYP2E1), resulting in the production of ROS [3]. Furthermore, acetaldehyde oxidation by xanthine oxidase and aldehyde oxidase produces acetyl radicals that react with proteins, nucleic acids and lipids. Due to its easy diffusion through biological membranes, lipid peroxidation products are responsible for most of the pathologies related to oxidative stress. Although the adverse effects of ethanol are produced in the liver, various other tissues are also affected, but to a lesser and variable degree [2,4,5].

A system of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST), in conjunction with a non-enzymatic antioxidant system constituted by reduced glutathione, vitamin E and a broad range of antioxidant agents, such as flavonoids and phenolics, protect the cell from oxidative stress [6]. Alcohol treatment is known to deplete

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antioxidant enzymes and decrease the concentration of reduced glutathione. These effects are accompanied by an increase in the malondialdehyde and hydrogen peroxide concentrations [7].

Based on its antioxidant properties, a broad range of active compounds from plants has been recently studied as potential agents against alcohol-induced pathogenesis. The beneficial effects against ethanol intoxication are reportedly attributed to the regulation of pathways involved in oxidation, inflammation and lipid metabolism, although the mechanisms of action of these compounds remain to be elucidated [8]. Xanthohumol (XN), a hop-derived prenylated flavonoid, was identified among these compounds. The hop plant (*Humulus lupulus* L.) is a dioecious plant of the Cannabacea family that is cultivated for its female inflorescences. Hops are used in the brewing industry to add flavour, aroma, bitterness and stability to beer. *In vitro* studies have revealed that XN possesses various biological properties, such as antioxidant, anti-inflammatory and anticancer activities, suggesting a potential chemopreventive effect [9–11]. Metabolisation studies have shown that the XN molecule remains unchanged and that the antioxidant capacity measured *in vitro* is 8.9 times higher than that of trolox [12]. However, this compound has not yet been sufficiently evaluated *in vivo*.

In previous studies, we reported that XN prevents acute liver injury induced in rats by a treatment with carbon tetrachloride [13]. This model *in vivo* assay has frequently been used to evaluate the antioxidant efficiency of natural compounds, given that the toxic process has been well established to be mediated by the trichloromethyl free radical ($^{\bullet}CCl_3$) and trichloromethyl peroxy radical ($Cl_3COO^{\bullet}$) [13,14]. Although this model provides valuable information about the *in vivo* antioxidant efficiency of natural compounds, CCl_4-induced hepatotoxicity is not a general model of liver damage, and alternative models consequently need to be used to more effectively assess the antioxidant properties. Thus, the used the ALD as model of hepatotoxicity and multi-organ dysfunction, which can provide valuable information complementary to that obtained from a CCl_4-induced model of injury.

In this study we used a model of acute ethanol toxicity to determine the protective efficiency of XN on the antioxidant defence systems in the liver, kidney, lung, heart and brain for a pathological status close to that produced in humans an ethylic intoxication.

2. Materials and methods

2.1. Chemicals

Xanthohumol (96%) and all chemicals required for the biochemical assays were obtained from Sigma–Aldrich Quimica (Madrid, Spain). All other chemicals were of analytical grade.

2.2. Animals and experimental design

Male Wistar rats (200–250 g, 4 months of age) were maintained at standard environmental conditions: 22 °C with a 12 h light/dark cycle, controlled humidity and air circulation with free access to food and water. All the experiments were carried out between 9:00 and 17:00 h and, in all cases, samples were collected at 10:00 h. The appropriate guidelines of the local animal ethics committee were followed throughout all animal experiments.

Rats were randomly divided into 8 groups with 6 animals per group. In all cases, an orogastric tube was used to treat rats with vehicle or XN. Group I was the normal control and was given vehicle alone (1,2-propanodiol, 2 mL/kg b.w. per day for 7 days). Group II was treated the same as Group I, but a 4.0 g/kg b.w. dose of EtOH (30% dilution) was i.p. injected into each animal on the 7th day. Groups III, IV and V were treated with Xanthohumol dissolved in 1,2-propanodiol at dose levels of 0.1, 0.2 and 0.4 mg/kg b.w., respectively, per day for 7 days, and a single dose of 4.0 g/kg b.w. EtOH (in a 30% solution) was administered i.p. on the 7th day. The range of doses used in this work was established as part of a previous study of the effect of XN using a wider range of concentrations (data not shown). Groups IIIc, IVc and Vc were the controls for Groups III, IV and V and consisted of rats pre-treated with the same XN concentrations that did not receive an EtOH injection. With the aim to check if the injection causes some alteration of measurements, as consequence of the stress induced in the animal, controls IIIc, IVc and Vc also were carried out with an injection of saline solution, but no significant differences were detected when compared with rats that not received injection. After 12 h, each rat was anesthetised by chloroform inhalation in order to draw blood and remove the tissues. The blood samples were collected by cardiac puncture and allowed to clot for 45 min at room temperature. The serum was separated by centrifugation at 600× *g* for 15 min and analysed for glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and lactate dehydrogenase (LDH). The liver, kidney, lung, heart and brain were excised, homogenised and assayed for reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS) and H_2O_2 levels, as well as for the catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST) enzyme activities according to the procedures described below.

2.3. Tissues homogenate preparation

After removal, the liver, kidney, lung, heart and brain were homogenised in a solution of 10 mM KCl, 1 mM EDTA and 100 mM phosphate buffer at pH 7.4. The homogenate was centrifuged at 12,000× *g* for 60 min. The supernatant was used as a source to assay the enzymatic markers of oxidative stress, including the SOD, CAT and GST activities. The GSH, TBARS, H_2O_2 and total protein content were also determined.

2.4. Determination of serum transaminases GOT and GPT activities

Serum GOT and GPT activities were determined using a procedure previously described by Bergmeyer and Holder [15,16]. The reaction mixture to measure the GOT activity contained 290 mM L-aspartate (600 mM L-alanine for measurement of GPT activity),

15 mM α-ketoglutarate, 0.26 mM NADH, 368 U/L of malate dehydrogenase (1080 U/L of lactate dehydrogenase for measurement of GPT activity) and 100 mM Tris–HCl at pH 7.8 in a final volume of 1 mL. Enzymatic activity was calculated based on the change in absorbance at 340 nm and is expressed as μmol/min/mg protein.

2.5. Determination of serum lactate dehydrogenase activity (LDH)

LDH activity was measured in serum using the procedure described by Kornberg [17]. A total of 100 μL of serum was incubated in the presence of 100 μL of 20 mM NADH and 100 μL of 10 mM sodium pyruvate in 100 mM sodium phosphate buffer at pH 7.4 and brought to a final volume of 3 mL. The change in the absorbance was continuously monitored at 340 nm, and the enzyme activity was calculated using a molar extinction coefficient of 6220 M^{-1} cm^{-1} and expressed as nmol NAD^+/min/mg protein.

2.6. Determination of the level of lipid peroxidation

Malonyldialdehyde (MDA) is an end product of lipid peroxidation that reacts with thiobarbituric acid to form a pink chromogen-thiobarbituric acid reactive substance (TBARS). The level of this compound was measured using a procedure previously described by Ohkawa et al. [18]. The TBARS was prepared by mixing equal volumes of 15% trichloroacetic acid and a solution of 15% 2-thiobarbituric acid in 0.25 M HCl. Two volumes of this reactive solution were added to a volume of sample, and the mixture was vigorously vortexed. Subsequently, this solution was heated to 100 °C for 20 min, cooled and centrifuged at 10,000× *g* for 15 min. The supernatant was separated and spectrophotometrically measured at 532 nm. The absorbance value is proportional to the amount of MDA formed and was calibrated to known concentrations of MDA ranging from 0 to 3.3 μM. The results are expressed as nmol of MDA/mg protein.

2.7. Determination of hydrogen peroxide

Hydrogen peroxide generation was analysed using the procedure of Pick and Keisari [19]. The reaction mixture contained the sample in addition to 180 units of horseradish peroxidase, 3.5 μM phenol red, 0.370 mM dextrose and 50 mM phosphate buffer at pH 7.6 in a final volume of 1.2 mL. The mixture was incubated for 30 min at 37 °C. Subsequently, the mixture was alkalinised with 0.3 M NaOH and centrifuged at 14,000× *g* for 3 min. The absorbance of the supernatant was measured at 610 nm to determine the hydrogen peroxide content, which is expressed as mmol/mg protein.

2.8. Determination of reduced glutathione (GSH)

Reduced glutathione content was measured using the procedure described by Ellman [20]. A volume of hepatic homogenate was mixed with a volume of 10% trichloroacetic acid and centrifuged to separate the proteins. A total of 10 μL of supernatant was added to 1 mL of 100 mM phosphate buffer at pH 8.4, 0.025 mL of 5 mM 5,5-dithio-bis-(2-nitrobenzoic acid) and 0.165 mL of distilled water. The mixture was vortexed and incubated for 15 min, and the absorbance was subsequently measured at 412 nm. The concentration of reduced glutathione is expressed as nmol GSH/mg protein.

2.9. Determination of superoxide dismutase (SOD) activity

SOD activity was measured using the method described by Marklund and Marklund [21]. The reaction mixture consisted of the sample, 0.2 M pyrogallol, 1 mM EDTA and 50 mM Tris–HCl at pH 8.2 in a final volume of 1 mL. Enzymatic activity was measured by monitoring the absorbance at 420 nm and expressed as U/mg. A unit of activity (U) was defined as the amount of enzyme that produced a 50% inhibition of pyrogallol auto-oxidation.

2.10. Determination of catalase (CAT) activity

CAT activity was measured using the method described by Aebi [22]. The reaction mixture contained the sample, 10 mM H_2O_2 and 50 mM phosphate buffer at pH 7.0 in a final volume of 1 mL. The rate of decomposition of H_2O_2 was measured at 240 nm, and catalase activity is expressed as nmol H_2O_2 decomposed/min/mg protein using an extinction coefficient of 32.54 M^{-1} cm^{-1} for hydrogen peroxide.

2.11. Determination of glutathione S-transferase (GST) activity

GST activity was measured according to Habig et al. [23]. The reaction mixture contained sample, 0.1 mL of 1 mM GSH, 20 μL of 50 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 0.1 M phosphate buffer at pH 6.5 in a final volume of 3.0 mL. Enzyme activity was measured based on the change in the absorbance at 340 nm using a molar extinction coefficient of 9.6 μM^{-1} cm^{-1} and expressed as nmol CDNB–GSH conjugate formed/min/mg protein.

2.12. Determination of protein concentration

Protein concentration was measured using the method described by Lowry et al. [24] with bovine serum albumin as a standard.

2.13. Statistical analysis

Data were expressed as mean ± standard deviation (SD) and *p*-values were considered significant for $p < 0.05$. Differences within the experimental groups were established by one or two-way analysis of variance (ANOVA) followed by *F*-Fisher post hoc test when appropriate. All tests were executed using the SPSS statistical software (version 19).

3. Results and discussion

The aim of this study was to evaluate the efficiency of XN as a protective agent against oxidative damage induced

Table 1
Effect of xanthohumol on serum GOT, GPT and LDH activities.

	GOT μmol/min/mg protein	GPT μmol/min/mg protein	LDH μmol/min/mg protein
Control (Group I)	5.91 ± 0.23	2.95 ± 0.11	1.37 ± 2.03
EtOH (Group II)	12.53 ± 0.61*	5.32 ± 0.22*	2.03 ± 0.11*
XN 0.1 + EtOH (Group III)	9.51 ± 0.35*,#	4.63 ± 0.19*,#	2.02 ± 0.09*
XN 0.2 + EtOH (Group IV)	6.89 ± 0.21*,#	3.52 ± 0.17*,#	1.75 ± 0.08*,#
XN 0.4 + EtOH (Group V)	6.05 ± 0.19#	3.11 ± 0.15#	1.45 ± 0.08*,#

Each value represents the mean ± SD ($n = 6$).
* Significantly different from control ($p < 0.05$).
# Significantly different from EtOH treated rats ($p < 0.05$).

in rat tissues after acute intoxication due to ethanol administration. Our experimental design is based on previous studies in which acute ethanol-induced oxidative damage constituted a valid procedure to determine the protective effect of compounds of therapeutic interest [25–27].

The mechanisms of intoxication for acute and chronic alcohol treatment are known to differ. For instance, Yang et al. [34] reported that although CYP2E1 plays a major role in chronic ethanol-induced oxidative stress, the initial oxidative process for acute alcohol treatment is independent of CYP2E1. In addition, the effect of ethanol in the cardiovascular system reportedly depends on the pattern of ethanol drinking, i.e., acute or chronic. While chronic ethanol intake induces hypertension, acute treatment induces hypotension, which suggests different mechanisms of action [28].

The experimental conditions used in the present study coincided with a binge-drinking model. This model, based on the ethanol administration via i.p. injection, was designed to achieve blood ethanol levels, behavioural effects and physiological changes comparable to those observed in human binge drinking and has been used to evaluate the effect of ethanol in various tissues, such as liver [25,29–31], brain [32,33], lung, kidney [27] and vascular tissues [28]. Acute ethanol intake was shown to produce oxidative stress in all cases.

Despite the considerable interest in the binge ethanol treatment of rats because this approach mimics human binge drinking, the number of studies that have evaluated antioxidant protective efficiency of natural compounds using this assay model are limited.

The dose of ethanol used in this study was based on previous studies [25,32,34–36], and our preliminary experiments (data not shown).

To evaluate the *in vivo* protective efficiency of XN, we examined the effect of XN pre-treatment on the response of the antioxidant defence system and the level of oxidative stress in various rat tissues after acute ethanol consumption. A dose of 4.0 g/kg b.w. EtOH produced a peak blood concentration of 0.4%, which did not affect the survival of rats. The effect of this acute treatment on GOT, GPT and LDH is shown in Table 1.

The serum GOT, GPT and LDH of binge ethanol-treated rats were significantly elevated compared to the control ($p < 0.05$); increases of 212% for GOT, 180% for GPT and 148% for LDH were estimated. These data reveal that binge alcohol treatment resulted in cellular damage in the liver, although the membrane permeability of other tissues may have also changed as consequence of the effect of ROS generated during ethanol metabolism. The protective effect of the XN pre-treatment was significant for the dose-dependent normalisation of the serum GOT, GPT and LDH activities compared to the ethanol only-treated group. Notably, XN alone did not produce significant effects compared to the control (groups IIIc, IVc and Vc, data not shown); however, XN pre-treatment at 0.4 mg XN/kg b.w. protected GOT, GPT and LDH activities by 97.8%, 93.2% and 87.9%, respectively, compared to control rats. Table 1 shows the clear dose-dependent protective effect of XN against cellular damage produced by ROS.

The above data are consistent with the observed effect of ethanol and XN pre-treatment on lipid peroxidation. Table 2 shows a 6.7-fold increase in the level of lipid peroxidation of livers stressed by ethanol. This effect does not quantitatively coincide with the other assayed tissues. The lipid peroxidation in the liver increased approximately 3-fold over the value of the control, but the increase was only 50%, 13% and 7% for lung, heart and brain, respectively. This significant increase in liver lipid peroxidation when compared with other tissues has been previously reported, being supported by the fact that nearly 60–80% of ingested alcohol is metabolised in the liver, which makes this organ

Table 2
Effect of ethanol administration and xanthohumol treatment on TBARS (nmol MDA/mg protein) concentration in different rat tissues.

	Control (Group I)	EtOH (Group II)	0.1 XN + EtOH (Group III)	0.2 XN + EtOH (Group IV)	0.4 XN + EtOH (Group V)
Liver	0.115 ± 0.006[a]	0.771 ± 0.038*,a	0.513 ± 0.021*,#,a	0.365 ± 0.018*,#,a	0.211 ± 0.011*,#,a
Kidney	0.334 ± 0.015[b]	0.972 ± 0.047*,b	0.662 ± 0.029*,#,b	0.483 ± 0.022*,#,b	0.398 ± 0.019*,#,b
Lung	0.243 ± 0.011[c]	0.377 ± 0.018*,c	0.361 ± 0.016*,#,c	0.322 ± 0.015*,#,a	0.323 ± 0.016*,#,c
Heart	0.251 ± 0.012[c]	0.285 ± 0.014*,d	0.271 ± 0.013*,#,d	0.265 ± 0.013*,#,c	0.253 ± 0.014#,d
Brain	0.654 ± 0.031[d]	0.702 ± 0.031*,a	0.660 ± 0.032*,#,b	0.658 ± 0.008*,#,d	0.655 ± 0.032#,e

Each value represents the mean ± SD ($n = 6$).
Different letters in the same column represent statistically significant differences ($p < 0.05$).
* Significantly different from control ($p < 0.05$).
# Significantly different from EtOH treated rats ($p < 0.05$).

Table 3
Effect of ethanol administration and xanthohumol treatment on H_2O_2 concentration (mmol/mg protein) in different rat tissues.

	Control (Group I)	EtOH (Group II)	0.1 XN + EtOH (Group III)	0.2 XN + EtOH (Group IV)	0.4 XN + EtOH (Group V)
Liver	0.48 ± 0.02^{a}	$1.32 \pm 0.06^{*,a}$	$1.18 \pm 0.05^{*,\#,a}$	$0.75 \pm 0.03^{*,\#,a}$	$0.61 \pm 0.03^{*,\#,a}$
Kidney	0.98 ± 0.04^{b}	$1.66 \pm 0.07^{*,b}$	$1.41 \pm 0.07^{*,\#,b}$	$1.09 \pm 0.05^{*,\#,b}$	$1.05 \pm 1.05^{*,\#,b}$
Lung	1.38 ± 0.05^{c}	$3.00 \pm 0.12^{*,c}$	$2.21 \pm 0.11^{*,\#,c}$	$2.19 \pm 0.11^{*,\#,c}$	$2.05 \pm 0.11^{*,\#,c}$
Heart	1.75 ± 0.08^{d}	$1.79 \pm 0.07^{*,b}$	$1.78 \pm 0.06^{*,d}$	$1.77 \pm 0.07^{*,\#,d}$	$1.75 \pm 0.06^{\#,d}$
Brain	1.27 ± 0.05^{c}	$1.32 \pm 0.05^{*,a}$	$1.28 \pm 0.05^{*,\#,a}$	$1.28 \pm 0.06^{*,\#,e}$	$1.27 \pm 0.06^{\#,e}$

Each value represents the mean ± SD ($n = 6$).
Different letters in the same column represent statistically significant differences ($p < 0.05$).
* Significantly different from control ($p < 0.05$).
# Significantly different from EtOH treated rats ($p < 0.05$).

more vulnerable than others to alcohol-induced oxidative stress [37]. Furthermore, the presence of a high concentration of oxidisable fatty acids and iron in liver significantly contributes to ROS production. Similarly, oxidative stress also significantly affects the kidney because it is involved in the excretion of metabolised products [38]. Pre-treatment with low (0.1 mg/kg b.w.) or high (0.4 mg/kg b.w.) doses of XN significantly decreased the level of lipid peroxidation. A low XN dose decreased lipid peroxidation level by 33.5% in liver, 31.9% in kidney, 4.2% in lung, 4.9% in heart and 7.4% in brain compared with the controls, which lacked XN pre-treatment. Pre-treatment with a high dose of XN decreased the level of peroxidation, by 72.6% in liver and 50.05% in kidney, but this decrease was only 14.4% lung, 0.7% in heart and 7.2% in brain. These results suggest that pre-treatment with XN strongly reduces lipid peroxidation in tissues more affected by ROS, because the effect was not significant in tissues (lung, heart and brain) where the oxidative changes were small. The effect of binge ethanol treatment on the generation of hydrogen peroxide was studied to confirm this point.

Acute and chronic alcohol exposure are well documented to increase the generation of ROS, given that hydrogen peroxide one of the main compounds responsible for the generation of oxidative stress in many tissues. Hydrogen peroxide can be generated as a by-product of EtOH oxidation by Cyt P450 and alcohol dehydrogenase because this peroxide is specifically and directly involved in EtOH cytotoxicity [8].

Our results (Table 3) clearly show that binge ethanol treatment increased hydrogen peroxide concentration by a factor of 2.75 in liver, 1.69 in kidney and 2.17, in lung; the hydrogen peroxide generation was negligible in heart and brain. These results fit well with the observed effects of binge EtOH treatment on lipid peroxidation, suggesting that the oxidative stress is more relevant only in tissues where the EtOH metabolisation is more intense. The efficiency of XN pre-treatment is manifested by a significant dose-dependent decrease in the hydrogen peroxide concentrations in liver, kidney and lung, while the changes in heart and brain were unremarkable.

In addition to the increased concentration of ROS produced by EtOH intoxication alcohol-related diseases are known to deplete GSH. This thiol plays a pivotal role in the defence mechanisms against ROS, reacting directly with oxygen radicals and electrophilic metabolites to protect essential thiol groups against oxidation, which act as a substrate for GSH-related enzymes involved in antioxidative defence processes. Table 4 shows that acute EtOH administration produces a relevant decrease in the GSH concentration in liver (near 68%), kidney (44%), lung (31%), heart (33%) and brain (12%). The pre-treatment of rats with XN protect, in a dose-dependent manner, against GSH depletion induced by acute EtOH administration. The level of GSH concentration in livers of rats pre-treated with a dose of 0.4 mg/kg b.w. reaches 91.2% of the control level in the absence of EtOH. The level of protection reached values near 90% for kidney and lung, but the depletion of GSH was very small in these cases, and the protective effect of XN is less relevant that in liver. The marked decrease in liver GSH of rats treated with EtOH and the significant differences among the GSH level in different tissues coincides with previously reported studies in rats receiving chronic ethanol treatment [39]. The intense GSH depletion in liver compared to other tissues may be explained by the high rate of hepatic EtOH metabolisation, which depletes GSH by damaging the inner mitochondrial membrane [40]. Our result shows that XN partially prevents ethanol-induced GSH depletion in a dose-dependent manner.

SOD catalyses the decomposition of superoxide into hydrogen peroxide and water, making it one of the more

Table 4
Effect of ethanol administration and xanthohumol treatment on GSH concentration (nmol/mg protein) in different rat tissues.

	Control (Group I)	EtOH (Group II)	0.1 XN + EtOH (Group III)	0.2 XN + EtOH (Group IV)	0.4 XN + EtOH (Group V)
Liver	18.831 ± 1.38^{a}	$6.053 \pm 0.220^{*,a}$	$6.410 \pm 0.315^{*,\#,a}$	$10.185 \pm 0.478^{*,\#,a}$	$17.184 \pm 0.851^{*,\#,a}$
Kidney	9.445 ± 0.482^{b}	$5.271 \pm 0.262^{*,b}$	$5.662 \pm 0.251^{*,\#,b}$	$6.541 \pm 0.315^{*,\#,b}$	$7.809 \pm 0.350^{*,\#,b}$
Lung	1.088 ± 0.104^{c}	$0.746 \pm 0.029^{*,c}$	$0.748 \pm 0.035^{*,c}$	$0.776 \pm 0.033^{*,\#,c}$	$0.976 \pm 0.035^{*,\#,c}$
Heart	1.127 ± 0.078^{c}	$0.751 \pm 0.030^{*,c}$	$0.750 \pm 0.037^{*,c}$	$1.011 \pm 0.047^{*,\#,d}$	$1.026 \pm 0.051^{\#,c}$
Brain	3.761 ± 0.225^{d}	$3.293 \pm 0.161^{*,d}$	$3.296 \pm 0.164^{*,d}$	$3.504 \pm 0.157^{*,\#,e}$	$3.358 \pm 0.155^{*,\#,d}$

Each value represents the mean ± SD ($n = 6$).
Different letters in the same column represent statistically significant differences ($p < 0.05$).
* Significantly different from control ($p < 0.05$).
# Significantly different from EtOH treated rats ($p < 0.05$).

Table 5
Effect of ethanol administration and xanthohumol treatment on SUPEROXIDE DISMUTASE activity (U/mg protein) in different rat tissues.

	Control (Group I)	EtOH (Group II)	0.1 XN + EtOH (Group III)	0.2 XN + EtOH (Group IV)	0.4 XN + EtOH (Group V)
Liver	25.06 ± 1.21[a]	6.66 ± 0.29[*,a]	12.52 ± 0.58[*,#,a]	16.65 ± 0.81[*,#,a]	20.14 ± 0.98[*,#,a]
Kidney	11.12 ± 0.51[b]	4.48 ± 0.21[*,b]	5.41 ± 0.21[*,#,b]	5.74 ± 0.21[*,#,b]	8.33 ± 0.25[*,#,b]
Lung	4.52 ± 0.22[c]	2.39 ± 0.11[*,c]	2.54 ± 0.12[*,#,c]	3.32 ± 0.15[*,#,c]	4.16 ± 0.16[*,#,c]
Heart	3.70 ± 0.18[d]	1.61 ± 0.08[*,d]	1.92 ± 0.11[*,#,d]	2.56 ± 0.11[*,#,d]	2.94 ± 0.11[*,#,d]
Brain	3.57 ± 0.17[d]	2.45 ± 0.11[*,c]	2.77 ± 0.14[*,#,c]	2.89 ± 0.13[*,#,d]	3.19 ± 0.12[*,#,d]

Each value represents the mean ± SD ($n = 6$).
Different letters in the same column represent statistically significant differences ($p < 0.05$).
[*] Significantly different from control ($p < 0.05$).
[#] Significantly different from EtOH treated rats ($p < 0.05$).

Table 6
Effect of ethanol administration and xanthohumol treatment on CATALASE activity (nmol H_2O_2 decomposed/min/mg protein) in different rat tissues.

	Control (Group I)	EtOH (Group II)	0.1 XN + EtOH (Group III)	0.2 XN + EtOH (Group IV)	0.4 XN + EtOH (Group V)
Liver	469.2 ± 19.2[a]	225.5 ± 10.27[*,a]	272.3 ± 13.32[*,#,a]	303.2 ± 13.12[*,#,a]	379.5 ± 17.23[*,#,a]
Kidney	215.5 ± 9.32[b]	128.7 ± 5.43[*,b]	145.5 ± 6.98[*,#,b]	180.2 ± 8.98[*,#,b]	194.5 ± 8.25[*,#,b]
Lung	27.8 ± 1.11[c]	19.4 ± 0.77[*,c]	20.1 ± 0.85[*,#,c]	27.5 ± 1.31[#,c]	28.0 ± 1.22[#,c]
Heart	25.5 ± 1.21[c]	13.2 ± 0.66[*,d]	17.6 ± 0.81[*,#,d]	21.2 ± 0.88[*,#,d]	24.3 ± 1.02[#,d]
Brain	6.5 ± 0.29[d]	7.1 ± 0.32[*,e]	7.0 ± 0.25[*,d]	6.8 ± 0.29[*,#,e]	6.5 ± 0.23[#,e]

Each value represents the mean ± SD ($n = 6$).
Different letters in the same column represent statistically significant differences ($p < 0.05$).
[*] Significantly different from control ($p < 0.05$).
[#] Significantly different from EtOH treated rats ($p < 0.05$).

relevant regulators of ROS concentration. SOD is known to be inactivated by prolonged ethanol administration, and although it has been studied in several organs, the liver enzyme is more strongly inactivated. Acute ethanol intake has also been reported to significantly decrease SOD activity in brain [32,33], liver [29,25,30], lung and kidney [27]. Our data (Table 5) show that binge ethanol intake significantly decreased the SOD activity in liver by 73.5%, in kidney by 59.7%, in lung by 47.8%, in heart by 56.5% and in brain by 31.4% compared with normal control. The level of inactivation of liver SOD is higher than in other organs. This observation coincides with the results obtained by other authors using a model of chronic ethanol intake [41]. Although SOD activity is enhanced in the presence of a low concentration of ROS, the overproduction of free radicals during EtOH intoxication results in enzyme inactivation [42]. The pre-treatment with XN prevented this inactivation in a dose-dependent manner: a dose of 0.4 mg XN/kg b.w. significantly increased the SOD activity to values of 80.36% in liver, 74.6% in kidney, 92.03% in the lung, 79.45% in the heart and 89.35% in the brain compared with the controls in the absence of EtOH treatment. This effect may be mediated by the direct interaction of EtOH or by an indirect process mediated by acetaldehyde from EtOH metabolisation [43].

At physiological conditions, SOD and CAT act synergistically to fine-tune the ROS levels. Our results in Table 6 reveal that the inhibitory effect of EtOH on CAT activity and the protection of XN from this inhibition are similar to those observed for SOD. Furthermore, the activity levels for each tissue studied are coincident for both enzymes, and the high levels of activity in liver and kidney are remarkable. CAT activity measured in rats pre-treated with 0.4 mg XN/kg b.w. reached 80.8% in liver, 90.2% in kidney, 99.2% in lung, 95.3% in heart and 89.3% in brain compared to the controls, evidencing the high efficiency with which XN protected cells from acute EtOH intoxication.

Finally, we measured the glutathione S-transferase (GST) activity in tissues. This enzyme is involved in ROS elimination together with SOD and CAT. Plant extracts with antioxidant properties have been reported to up-regulate GST in rats [8]. GST is also involved in the ethanol detoxification process; the GST activity was shown to be significantly lower in mouse primary hepatocytes after ethanol exposure [44]. Our results (Table 7) clearly demonstrate the protective effect of XN in all examined tissues,

Table 7
Effect of ethanol administration and xanthohumol treatment on glutathione S-transferase activity (nmol CDNB–GSH conjugate formed/min/mg protein) in different rat tissues.

	Control (Group I)	EtOH (Group II)	0.1 XN + EtOH (Group III)	0.2 XN + EtOH (Group IV)	0.4 XN + EtOH (Group V)
Liver	0.416 ± 0.019[a]	0.197 ± 0.009[*,a]	0.215 ± 0.011[*,#,a]	0.241 ± 0.011[*,#,a]	0.282 ± 0.014[*,#,a]
Kidney	0.068 ± 0.003[b]	0.028 ± 0.001[*,b]	0.035 ± 0.001[*,#,b]	0.055 ± 0.003[*,#,b]	0.067 ± 0.003[#,b]
Lung	0.041 ± 0.002[c]	0.017 ± 0.001[*,c]	0.021 ± 0.001[*,#,c]	0.029 ± 0.001[*,#,c]	0.031 ± 0.001[*,#,c]
Heart	0.082 ± 0.004[d]	0.006 ± 0.0003[*,d]	0.008 ± 0.0003[*,#,d]	0.010 ± 0.0003[*,#,d]	0.015 ± 0.0002[*,#,d]
Brain	0.022 ± 0.001[e]	0.018 ± 0.001[*,c]	0.019 ± 0.001[*,c]	0.021 ± 0.001[#,e]	0.021 ± 0.007[#,e]

Each value represents the mean ± SD ($n = 6$).
Different letters in the same column represent statistically significant differences ($p < 0.05$).
[*] Significantly different from control ($p < 0.05$).
[#] Significantly different from EtOH treated rats ($p < 0.05$).

especially at a dose of 0.4 mg/kg b.w. The significant differences between the GST activities of tissues coincide with previous studies [45–47] and are attributable to their different metabolic roles [5].

The results presented here led us to conclude that XN administration to rats protects against acute alcohol-induced oxidative damage. Additionally, the fact that in absence of ethanol all the parameters measured in rats only treated with XN were coincident with control (Group I), strongly suggest the absence of toxicity of XN at assayed concentration. The prevention of EtOH toxicity by XN pre-treatment is evidenced by the inhibition of lipid peroxidation, the protection from tissue degradation and the maintenance of normal values for the chemical and biochemical markers of oxidative stress. In previous work [13] we shown from histopathological studies that XN protect against severe hepatic necrosis induced by carbon tetrachloride, an alteration similar to described in liver of EtOH intoxicated rats [29,30], suggesting that XN can exert similar protective effect against histopathological alteration induced by acute administration of EtOH. However, despite all the information accumulated, the exact mechanism by which XN exerts its antioxidant effect remains unclear, and further studies are necessary in order to implement the therapeutic use of XN to protect tissues from ethanol-induced oxidative injury.

Transparency document

The Transparency document associated with this article can be found in the online version.

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References

[1] G. Testino, Alcoholic diseases in hepato-gastroenterology: a point of view, Hepatogastroenterology 55 (2008) 371–377.
[2] J.H. Wang, R.G. Batey, J. George, Role of ethanol induction of hepatic stellate cell function, World J. Gastroenterol. 12 (2006) 6926–6932.
[3] J.I. Cohen, L.E. Nagy, Pathogenesis of alcoholic liver disease: interactions between parenchymal and non-parenchymal cells, J. Digest. Dis. 12 (2011) 3–9.
[4] W. Luczaj, S. Stankiewicz-Kranc, E. Milewska, W. Roskowska-Jakimiec, E. Skrzydlewska, Effect of sweet grass against oxidative stress in rat liver and serum, Food Chem. Toxicol. 50 (2012) 135–140.
[5] A.J. Cederbraun, Y. Lu, D. Wu, Role of oxidative stress in alcohol-induced liver injury, Arch. Toxicol. 83 (2009) 519–548.
[6] M.F. Molina, I. Sanchez-Reus, I. Iglesias, J. Benedi, Quercetin, a flavonoid antioxidant, prevents and protect against ethanol- induced oxidative stress in mouse liver, Biol. Pharm. Bull. 26 (2013) 1398–1402.
[7] S.C. Pandanaboina, S.R. Kondeti, S.L. Rajbanshi, P.N. Kunala, S. Pandanaboina, M.M. Pandanaboina, R. Wudayagiri, Alterations in antioxidant enzyme activities and oxidative damage in alcoholic rat tissues: protective role of *Thespesia populnea*, Food Chem. 132 (2012) 150–159.
[8] R.-B. Ding, K. Tian, L.-L. Huang, C.-W. He, Y. Jiang, Y.-T. Wang, J.-B. Wang, Herbal medicines for the prevention of alcoholic liver disease: a review, J. Ethnopharmacol. 144 (2012) 456–457.
[9] P.J. Magalhaes, S.M. Almeida, A.M. Carvalho, L.M. Gonçalves, J.G. Pacheco, J.M. Cruz, L.F. Guido, A.A. Barros, Influence of malt on the xanthohumol and isoxanthohumol behavior in pale and dark beers: a micro-scale approach, Food Res. Int. 44 (2011) 351–359.
[10] M.R. Peluso, C.L. Miranda, D.J. Hobbs, R.R. Proteau, J.F. Stevens, Xanthohumol and related prenylated flavonoids inhibit inflammatory cytoquine production in LPS activated THP-1 monocytes: structure-activity relationships and in silico binding to myeloid differentiation protein-2(MD-2), Planta Med. 76 (2010) 1536–1543.
[11] E.C. Colgate, C.L. Miranda, J.F. Stevens, T.M. Bray, E. Ho, Xanthohumol, a prenylflavonoid derived from hops induces apoptosis and inhibits NF-kappaB activation in prostate epithelial cells, Cancer Lett. 246 (2007) 201–209.
[12] J.F. Stevens, J.E. Page, Xanthohumol and related prenylflavonoids from hops and beer: to your good health!, Phytochemistry 6 (2004) 1317–1330.
[13] C. Pinto, A.L. Duque, B. Rodriguez-Galdon, J.J. Cestero, P. Macias, Xanthohumol prevents carbon tetrachloride-induced acute liver injury in rats, Food Chem. Toxicol. 50 (2012) 3405–3412.
[14] M. Kujawska, E. Ignatowicz, M. Murias, M. Ewertowska, K. Mikolajczyk, J. Jodynis-Liebert, Protective effect of red beetroot against carbon tetrachloride- and N-nitrosodiethylamine-induced oxidative stress in rats, J. Agric. Food Chem. 57 (2009) 2570–2575.
[15] U.H. Bergmeyer, N. Holder, Stimation of GOT activity in serum, Ann. Biol. Clin. 44 (1986) 497–501.
[16] U.H. Bergmeyer, N. Holder, Determination of GPT activity in blood, Ann. Biol. Clin. 44 (1986) 481–489.
[17] A. Kornberg, Lactic dehydrogenase of muscle, in: S.P. Colowick, N.O. Kaplan (Eds.), Methods in Enzymology, vol. 1, Academic Press, New York, 1955, pp. 441–443.
[18] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.
[19] E. Pick, Y. Keisari, A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture, J. Immunol. Methods 38 (1980) 161–170.
[20] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.
[21] S. Marklund, G. Marklund, Involvement of superoxide anion radical in autooxidation of pirogallol as convenient assay for superoxide dismutase, Eur. J. Biochem. 47 (1974) 469–474.
[22] H. Aebi, Catalase, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Academic Press, New York/London, 1974, pp. 673–684.
[23] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases: the first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130–7139.
[24] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[25] A.Q. Khan, S. Nafees, S. Sultana, Perillyl alcohol protects against ethanol induced acute liver injury in Wistar rats by inhibiting oxidative stress, NFκ-B activation and proinflammatory cytoquine production, Toxicology 279 (2011) 108–114.
[26] Kalaz, E.B. Evran, B. Develi, S. Erata, G.O. Uysal, M. Koçak-Toker., Effect of binge ethanol treatment on prooxidant-antioxidant balance in rat heart tissue, Pathophysiology 19 (2012) 49–53.
[27] R.B. Scott, K.S. Reddy, K. Husain, E.C. Schorff, L.P. Rybak, S.M. Somani, Dose response of ethanol on antioxidant defense system of liver, lung, and kidney in rat, Pathophysiology 7 (2000) 25–32.
[28] A. Yogi, G.C. Callera, A.S. Mecawi, M.E. Batalhao, E.C. Carnio, J. Antunes-Rodrigues, R.H. Queiroz, R.M. Touyz, C.R. Tirapelli, Acute ethanol intake induces superoxide anion generation and mitogen-activated protein kinase phosphorylation in rat aorta: a role for angiotensin type 1 receptor, Toxicol. Appl. Pharmacol. 264 (2012) (2012) 470–478.
[29] H-Y. Park, S.K. Ha, H. Eom, I. Choi, Narirutin fraction from citrus peels attenuates alcoholic liver disease in mice, Food Chem. Toxicol. 55 (2013) 637–644.
[30] D. Mladenovic, M. Ninkovic, V. Aleksic, T. Sljivancanin, D. Vucevic, V. Todorovic, M. Stankovic, T. Stanojlovic Radosavljevic, The effect of calorie restriction on acute ethanol-induced oxidative and nitrosative liver injury in rats, Environ. Toxicol. Pharmacol. 36 (2013) 296–302.
[31] K. Mallikarjuna, K.R. Shanmugan, K. Nishant, M.-C. Wu, C.W. Hou, C.H. Kuo, K.S. Reddy, Alcohol-induced deterioration in primary

antioxidant and glutathione family enzymes reversed by exercise training in the liver of old rats, Alcohol 44 (2010) 523–529.
[32] A. Boveris, S. Llesuy, L.A. Azzalis, L. Giavarotti, K.A. Simon, V.B.C. Junqueira, E.A. Porta, L.A. Videla, E.A. Lissi, In situ rat brain and liver spontaneous chemiluminiscence after acute ethanol intake, Toxicol. Lett. 93 (1997) 23–28.
[33] M. Enache, V. van Waes, E. Vinner, M. Lhermitte, S. Maccari, M. Darnaudery, Impact of an acute exposure to ethanol on the oxidative stress status in the hippocampus of prenatal restraint stress adolescent male rats, Brain Res. 1191 (2008) 55–62.
[34] P. Yang, Z. Wang, Y. Zhan, T. Wang, M. Zhou, L. Xia, X. Yang, J. Zhang, Endogenous A_1 adenosine receptor protects mice acute ethanol-induced hepatotoxicity, Toxicology 309 (2013) 100–106.
[35] R.J. Ward, M.A. Colivicchi, A.R. Schol, F. Lallemand, P. De Witte, C. Ballini, L.D. Corte, D. Dexter, Neuro-inflammation induced in the hippocampus of binge drinking rat may be mediated by elevated extracellular glutamate content, J. Neurochem. 111 (2009) 1119–1128.
[36] M. Glover, Cheng Deng, S. Pruett, The role of glucocorticoids in the immediate vs. delayed effects of acute ethanol exposure on cytokine production in a binge drinking model, Int. Immunopharmacol. 11 (2011) 755–761.
[37] C.S. Lieber, Relationship between nutrition, alcohol use, and liver disease, Alcohol Res. Health 27 (2003) 220–231.
[38] Z. Meng, B. Zhang, Oxidative damage of sulfur dioxide inhalation on brains and liver of mice, Environ. Toxicol. Pharmacol. 13 (2003) 1–8.
[39] K. Husain, B.R. Scott, S.K. Reddy, S.M. Somani, Chronic ethanol and nicotine interaction on rat tissue antioxidant defense system, Alcohol 25 (2001) 89–97.
[40] P.Y. Lam, P.Y. Chiu, H.Y. Leung, N. Chen, P.K. Leong, K.M. Ko, Schisandrin B co-treatment ameliorates the impairment on mitochondrial antioxidant status in various tissues of long-term ethanol treated rats, Fitoterapia 81 (2010) 1239–1245.
[41] P. Yao, K. Li, Y. Jin, F. Song, S. Zhou, X. Sun, A. Nüssler, L. Liu, Oxidative damage after chronic ethanol intake in rat tissues: prophylaxis of *Ginkgo biloba* extract, Food Chem. 99 (2006) 305–314.
[42] G.N. Landis, J. Tower, Superoxide dismutase evolution and life span regulation, Mech. Ageing Dev. 126 (2005) 157–170.
[43] D.B. Rosemberg, R.F. Da Rocha, E.P. Rico, A. Zanotto-Filho, R.D. Dias, M.R. Bogo, C.D. Bonan, J.C.F. Moreira, F. Klamt, D.O. Souza, Taurine prevents enhancement of acetylcholinesterase activity induced by acute ethanol exposure and decreases the level of markers of oxidative stress in zebrafish brain, Neuroscience 171 (2011) 683–692.
[44] M.A. Gyamfi, Y.J.Y. Wan, The effect of ethanol, ethanol metabolizing enzyme inhibitors, and vit E on regulating glutathione, glutathione S-transferase, and S-adenosylmethionine in mouse primary hepatocyte, Hepatol. Res. 35 (2006) 53–61.
[45] M. Sidhu, M. Sharma, M. Bhatia, Y.C. Awasthi, R. Nath, Effect of chronic cadmium exposure on glutathione S-transferase and glutathione peroxidase activities in Rhesus monkey: the role of selenium, Toxicology 83 (1993) 203–213.
[46] Z.S. Saicic, M.O. Simovic, B. Korac, D. Blagojevic, B. Buzadzic, M.B. Spasic, V.M. Petrovic, Activity of antioxidant defense enzymes and glutathione content in some tissues of the Belgrade (b/b) Laboratory Rat, Comp. Biochem. Physiol. C 117 (1997) 173–179.
[47] B. Yurt, I. Celik, Hepatoprotective effect and antioxidant role of sun, sulphited-dried apricot (*Prunus armeniaca* L.) and its kernel against ethanol-induced oxidative stress in rats, Food Chem. Toxicol. 49 (2011) 508–513.