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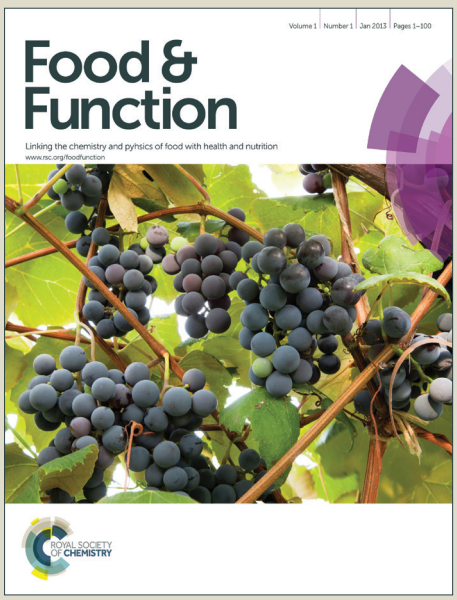

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C-Phycocyanin prevents cisplatin-induced nephrotoxicity through inhibition of oxidative stress.

Berenice Fernández-Rojas[a], Omar Noel Medina-Campos[a], Rogelio Hernández-Pando[b], Mario Negrette-Guzmán[a], Sara Huerta-Yepez[c], José Pedraza-Chaverri[a].

[a]Department of Biology, Facultad de Química, UNAM, Ciudad Universitaria, México, D.F., 04510 México. [b]Experimental Pathology Section, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga 15 sección XVI, México, D.F. 14000 México. [c]Unidad de Investigación en Enfermedades Oncológicas, Hospital Infantil de México Federico Gómez, Dr. Márquez 262, México, D.F. 06720 México. ***Corresponding author:** Phone/fax: +52 55 5622-3878. E-mail: pedraza@unam.mx

Abstract

The aim of this study was to evaluate if the antioxidant C-phycocyanin (C-PC, 5-30 mg/kg i.p.) was able to prevent cisplatin (CP, 18 mg/kg i.p.)-induced nephrotoxicity by reducing oxidative stress in CD-1 mice. Nephrotoxicity was assessed by measuring blood urea nitrogen, plasma glutathione peroxidase, plasma creatinine, renal activity of N-acetyl-β-D-glucosaminidase, apoptosis and histopathological changes. Oxidative stress was evaluated by measuring the renal content of glutathione, malondialdehyde, 4-hydroxynonenal and oxidized proteins. C-PC prevented CP-induced renal damage and oxidative stress in a dose-dependent manner. Moreover, C-PC prevented the decrease in the renal activity of the antioxidant enzymes glutathione peroxidase, glutathione reductase, glutathione-S-transferase and catalase induced by cisplatin. *In vitro* assays showed that C-PC was an effective scavenger of the following reactive species: hypoclorous acid, peroxynitrite anion, peroxyl radical, diphenyl-1-picrylhydrazyl, hydroxyl radical, superoxide anion, singlet oxygen and hydrogen peroxide. It is concluded that the protective effect of the nutraceutical C-PC against CP-induced nephrotoxicity was associated with the attenuation of oxidative stress and the preservation of the activity of antioxidant enzymes.

Keywords: C-Phycocyanin, cisplatin, nephrotoxicity, antioxidant activity, oxidative stress.

1. Introduction

There is a huge interest among consumers and food industry on products that can promote health and well-being named functional foods (FF)[1]. The beneficial action exerted by FF is due to some components called functional ingredients or nutraceuticals. They have been described as "any substance that is a food or part of a food that provides medical and/or health benefits, including the prevention and treatment of disease"[2]. In this way, C-Phycocyanin (C-PC) is a nutraceutic compound that gives the characteristic blue-green color to the FF *Spirulina maxima*. C-PC is a biliprotein composed of two subunits, α and β proteins, which are bound to the chromogen phycocyanobilin (PCB); one PCB is joined to the α subunit and two PCB are joined to the β subunit[3]. PCB is an open-chain tetrapyrrole with a chemical structure similar to biliverdin. It seems to be susceptible to biliverdin reductase, giving rise to phycocyanorubin, an analog of bilirubin[4]. It is used in the food industry, cosmetics, biotechnology and medical practice[5]. Some of its nutraceuticals properties are: antioxidant, anti-inflammatory, anti-neoplastic, and anti-diabetic, among others. In fact, its antioxidant activity is involved in the protective effect against liver, neuronal and renal damage[3,5–7]. On the other hand, cisplatin [*cis*-diammine-dichloroplatinum II (CP)] is a potent chemotherapeutic agent widely used for the treatment of various types of tumors worldwide[8] playing a major role in the treatment in many types of cancers, such as testicular, bladder, ovarian, head and neck, cervical, lung and colorectal malignant tumors[9,10]. However, its clinical use is limited by side effects, mainly nephrotoxicity which affects 30% of patients who use it[8]. Evidences suggest that the formation of reactive oxygen species (ROS), the decrease in the activity of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutahione-S-transferase (GST), inflammation, mitochondrial dysfunction and apoptosis are involved in CP-induced nephrotoxicity[11,8]. It has been found previously that C-PC prevents apoptosis in mice with CP-induced nephrotoxicity[12]. However, it has never

been studied if the C-PC protection against CP-induced nephrotoxicity is associated to the prevention of oxidative stress and the preservation of antioxidant enzymes activity. Thus, it was decided to study if the protection by the nutraceutical C-PC on the CP-induced nephrotoxicity is associated to the prevention of oxidative damage and preservation of the activity of antioxidant enzymes. In addition the *in vitro* ROS scavenging activity of C-PC used in the present study was also characterized.

2. Results and discussion

2.1 Purity of the C-PC

C-PC is a nutraceutical compound with antioxidant properties. It prevents oxidative damage and is used for human consumption as a colorant in food and milk shakes, and as an ingredient in cosmetic and pharmaceutical formulations. The α and β subunits were determined to characterize the purity of the C-PC sample used in this work. Figure 1 shows the characteristic peak absorption of PCB at 620 nm, and the two bands for α and β C-PC subunits. Purity of the C-PC used in this study was 2.5. C-PC from the same source (Parry Nutraceuticals) or from a similar purity has been used in other studies[13–15]. Also, the spectrum shows the absence of other phycobiliproteins as allophycocyanin (652 nm) and phycoerythrin (562 nm)[5].

2.2 *In vitro* antioxidant activity

First, the *in vitro* ROS scavenging properties of C-PC was characterized. This nutraceutical was challenged with several ROS synthesized *in vitro* that were scavenged even up to 90% for peroxyl radical ($ROO^{\bullet}$), hydroxyl radical ($OH^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$) as shown in Figure 2. In fact, C-PC scavenges all ROS tested in a concentration-dependent way. The concentration that reduces the ROS tested by 50% (IC_{50}) values is summarized in Table 1. C-PC was more efficient to scavenge hypoclorous acid (HClO) than the reference scavenging compound and less efficient to scavenge the other ROS tested. The decreasing order of

scavenging efficiency was: HClO> peroxynitrite anion ($ONOO^-$)> $ROO^\bullet$> diphenyl-1-picrylhydrazyl radical ($DPPH^\bullet$)> $OH^\bullet$> $O_2^{\bullet-}$> singlet oxygen ($^1O_2$)> hydrogen peroxide (H_2O_2). Our results are consistent with the ability of C-PC to scavenge HClO, $ONOO^-$, $ROO^\bullet$, $DPPH^\bullet$, $OH^\bullet$ and $O_2^{\bullet-}$ reported in earlier works[16,3,17]. Nevertheless, this is the first time that the ability of C-PC to scavenge H_2O_2 and $^1O_2$ is reported. Based on the antioxidant properties of C-PC, it was decided to investigate whether C-PC prevents CP-induced nephrotoxicity by reducing oxidative stress markers and by preservation of the activity of antioxidant enzymes in an *in vivo* model in CD-1 mice.

2.3 *In vivo* experimental model

2.3.1 Determination of renal function

Nephrotoxicity was characterized by a marked increase in both blood urea nitrogen (BUN) and creatinine in plasma induced by a single dose of CP (18 mg/kg i.p.) and was confirmed by the determination of N-acetyl-*β*-D-glucosaminidase (NAG) and plasma GPx and by the histopathological studies. There was an increase (2 to 3-fold) of BUN ($P<0.05$) and creatinine ($P<0.001$) in mice treated with CP compared to control mice. Damage was prevented when mice were pre-treated with C-PC at doses of 10 (Figure 3A, $P<0.01$) and 30 mg/kg (Figure 3B, $P<0.05$), but not with 5 mg/kg. Moreover, NAG is a lysosomal renal enzyme found in proximal tubular cells and its urinary excretion increases when these cells are damaged, thereby, NAG is a specific marker of tubular damage. NAG activity in renal homogenates decreased 34% in the CP-treated group. Another marker of tubular damage is plasma GPx that is synthesized in renal proximal tubules. The decrease in plasma GPx in CP-treated group indicates a fair damage at the CP target site. Plasma GPx activity was reduced by 43.5% in the CP group compared to the control one (Figure 3D, $P<0.05$), evidencing kidney damage. These alterations were prevented by C-PC in a dose-dependent manner. These parameters were unchanged in the C-PC group (Figure 3). Our data show that C-PC was

able to prevent CP-induced nephrotoxicity which was evaluated by four markers (plasma creatinine, BUN, renal activity of NAG and plasma GPx).

2.3.2 Histopathological analysis

Light microscopy revealed structural alterations specifically in proximal convoluted tubules in the CP-treated mice, these were characterized by cellular edema, death, and detached cells (Figure 4). The automated morphometry analysis showed 52±5% of proximal tubules with these histological abnormalities, and the surface area of damaged epithelium was of 58±8% [Figure 4, $P<0.05$ vs control (CT)]. These alterations were prevented by 30 mg/kg of C-PC (11%, $P<0.001$ vs CP). No histological abnormalities were seen in the C-PC group (30 mg/kg). C-PC prevented not only renal functional damage (BUN, plasma GPx, plasma creatinine and renal activity of NAG) but also structural damage (histopathological changes such as necrosis, edema and vacuolization). Pre-treatment of C-PC reduced the above described renal injury markers in a dose-dependent manner. These data are consistent with the protection of C-PC observed in the functional renal markers described in the section 2.3.1.

2.3.3 Apoptosis detection

Terminal-deoxynucleotidyltransferase mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick end labeling (TUNEL)-positive tubular cells in kidneys of mice treated with CP are shown in Figure 5. Strongly stained nuclei were observed depicting early apoptosis in some tubules. TUNEL positive cells were clearly decreased by 30 mg/kg of C-PC (Figure 5). Quantitative data, expressed as percentage of TUNEL-positive cells compared to control, revealed that TUNEL positivity was 2-fold higher in the CP group ($P<0.05$), while this increase was prevented by 30 mg/kg of C-PC ($P<0.007$ vs CP, Figure 5). These results are according with Lim et al.[12] who found that C-PC prevented CP-induced apoptosis.

2.3.4 Oxidative stress markers

The redox status was evaluated by the activity of antioxidant enzymes and by markers of oxidative stress that were altered in the group treated with CP. This group showed a marked decrease of 25% of glutathione (GSH) content ($P<0.001$), the main water-soluble antioxidant. Also, it was found an increase in the content of malondialdehyde (MDA) (2.3-fold, $P<0.001$), 4-hydroxy-2-nonenal (4-HNE) (4.6-fold, $P<0.05$) and oxidized proteins (1.4-fold, $P<0.001$, Figure 6) compared to the control. These data agree with previous studies[18,19] and suggest that oxidative damage is involved in the mechanisms of CP induced nephrotoxicity. These changes were prevented by C-PC in a dose-dependent manner ($P<0.05$ vs CP).

2.3.5 Antioxidant enzymes

The activity of the antioxidant enzymes GPx, GST, CAT and GR was decreased in mice from the CP-group by 40, 23, 40 and 11%, respectively ($P<0.001$ vs CT, Figure 7). The protective effect was associated with the prevention of CP-induced alterations in both oxidative stress markers and activity of antioxidant enzymes in a dose-depend way. GSH is both a ROS scavenger and a cofactor of the antioxidant enzymes GPx and GST. Thus, an imbalance of GSH homeostasis increases ROS levels; leading to oxidative damage. Our results demonstrate that all markers of kidney damage were reduced to values close to baseline by a single dose of C-PC with better results at doses of 10 and 30 mg/kg 1 hour before CP. Moreover, CP decreased the activity of GPx, GR, GST and CAT. Similar results have been reported by Chakraborty et al.[20], Guerrero-Beltrán et al.[19], and Chirino and Pedraza-Chaverri[21]. C-PC prevented this decrease in a dose-dependent manner. These data suggest that the renoprotective effect of C-PC is mediated at least in part, by the preservation in the activity of the antioxidant enzymes and support the hypothesis that the mechanism of CP induced nephrotoxicity is related to the depletion of renal antioxidant enzymes.

CP induced renal damage by accumulation in the tubular epithelium, specifically in the S3 segment of the proximal tubule[21]. It causes an increase in both BUN and creatinine and a decrease in glomerular filtration rate[22]. Several mechanisms are involved in the CP-induced nephrotoxicity such as inflammation, mitochondrial dysfunction, necrosis, apoptosis and oxidative stress[23–25]. Some studies have demonstrated the role of ROS as a major cause of kidney damage induced by CP, the species involved in oxidative and nitrosative damage are: $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$, $ONOO^-$, $ROO^{\bullet}$ and $^1O_2$[21]. It was clearly showed in the present study that all these ROS are scavenged by C-PC (see Table 1 and Figure 2). These data strongly suggest that the ROS scavenging properties of C-PC are involved in the renoprotective effect against CP-induced damage. Our data are also consistent with the renoprotective effect of the alga *Spirulina platensis* and C-PC in different animal models[26,27,12,28]. Besides, C-PC prevents oxidative damage in other organs of different animal models in which oxidative stress is involved. In fact, Ou et al.[29] demonstrate that C-PC prevents hepatic and pancreatic oxidative damage in mice treated with alloxan, Sun et al.[30] showed the protective effect of C-PC in oxidative stress in the lung by paraquat, and Pentón-Rol et al.[6] demonstrated the reduction of oxidative damage in the brain of rats with experimental autoimmune encephalomyelitis.

A recent investigation conducted by Lim *et al*.[12] showed that C-PC (50 mg/kg i.p.) given one hour before the injection of CP (12 mg/kg i.p.) in mice prevents nephrotoxicity by reducing apoptosis. Moreover, Mohan *et al.*[31] showed that the algae *Spirulina platensis* at dose of 1,000 mg/kg bw for 8 days prevents the CP (6 mg/kg i.p.) induced nephrotoxicity, oxidative stress and the decrease in the activity of antioxidant enzymes in rats. Nevertheless, this is the first time in which it has been shown that C-PC prevents the CP-induced oxidative damage (evaluated by the renal content of protein carbonyl, GSH, MDHA, 4-HNE) and decrease of the activity of antioxidant enzymes (GPx, CAT, GR and GST) in kidney homogenates.

Probably, the prevention by CP-C is due to PCB mimics bilirubin. Both compounds inhibit a the $O_2^{\bullet-}$ producing enzyme NADPH oxidase[26]. Interestingly, there is a recent epidemiological evidence that patients with Gilbert syndrome (harmless increased bilirubin in up to 5%) are at greatly reduced risk for coronary diseases, hypertension, atherosclerosis, and others illnesses[32]. However, bilirubin has high cost and therefore the use of C-PC is an attractive alternative.

In summary, this is the first time that demonstrates the antioxidant effect of C-PC on oxidative stress in the nephrotoxicity induced by CP. The protection was associated with the preservation in antioxidant enzymes. Further experiments are required to evaluate if additional mechanisms are involved in the protective effect of C-PC against CP-induced nephrotoxicity in mice.

3. Materials and methods

3.1 Reagents

C-PC was a generous gift from Parry Nutraceuticals (EID Parry (I) Ltd., India). CP (Cat. No. P4394, Lt 026K1291), 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), fluorescein, DL-penicillamine, diethylenetriaminepentaacetic acid (DTPA), $DPPH^{\bullet}$, terephtalic acid (TA), ascorbic acid, Amplex red, horseradish peroxidase (HRP), pyruvate, dimethylthiourea (DMTU), nicotinamide adenine dinucleotie reduced form (NADPH), lipoic acid, GSH, 1-chloro-2,4-dinitrobenzene (CDNB) and p-nitrophenyl-N-acetyl-*β*-D-glucosaminide, tetramethoxypropane, monochlorobimane, buffered formalin, 1-methyl-2-phenylindole, methanesulfonic acid, dimethylsulfoxide (DMSO), 1,3-diphenylisobenzofuran (DPBF), nordihydroguaiaretic acid (NDGA), oxidized glutathione (GSSG), p-aminobenzoic acid, sodium dodecyl sulfate (SDS) and polyacrilamyde were from Sigma-Aldrich (St. Louis, MO, USA). Dihydrorhodamine 123 (DHR-123) was from Cayman Chemical Co. (Ann Arbor, MI, USA). Trolox was from EMD

Millipore (Billerica, MA, USA). Ethylenediaminetetraacetic acid, disodium salt (Na_2EDTA), sodium hypoclorite (NaClO) and H_2O_2 were from JT Baker (Xalostoc, Edo. Mexico, Mexico). Commercial kits for the measurement of BUN and plasma creatinine concentration were from Spinreact (Girona, Spain). *In Situ* Cell Death Detection Kit, POD (Cat. No. 11 684 817 910) for apoptosis detection was obtained from Roche Applied Science (Mannheim, Germany). 3,3'-diaminobenzidine (DAB) was from Dako (Carpintería, CA, USA). All other reagents were analytical grade and commercially available.

3.2 Purity determination of C-PC

To determine the purity of C-PC, spectrophotometric readings at wavelengths of 280, 620 and 652 nm were taken (250-700 nm) and the ratio A620/A280 was calculated[33]. Besides, SDS polyacrilamide gel electrophoresis (SDS-PAGE) analysis was performed according to Pentón-Rol *et al*.[6] on 15% polyacrylamide gel at 60 V for 3.5 h.

3.3 *In vitro* antioxidant activity

The experiments were performed in C-PC and reference compounds in a Synergy HT multimode microplate reader (Biotek Instruments, Winooski, VT, USA). The data are expressed as IC_{50} (μg/mL). The reference compounds used were: NDGA (for $O_2^{\bullet-}$), DMTU (for $OH^{\bullet}$), penicillamine (for $ONOO^-$), trolox (for $ROO^{\bullet}$), lipoic acid (for $^1O_2$ and HClO), pyruvate (for H_2O_2) and vitamin C (for $DPPH^{\bullet}$).

3.3.1 $O_2^{\bullet-}$ scavenging assay

Scavenging activity of C-PC was determined by evaluating its ability to decrease formazan production from NBT induced by $O_2^{\bullet-}$ generated by the xanthine-xanthine oxidase system. Briefly, 100 μL of a solution composed of C-PC (0-22.5 μg/mL) or 50 mM phosphate buffer pH 7.0 (0% scavenging tube) or NDGA (0-1.6 μg/mL) were added to 800 μL of the reaction mixture (90 μM xanthine, 16 mM Na_2CO_3, 22.8 μM NBT and 18 mM phosphate buffer, pH

7.0). The reaction started by adding 100 µL of xanthine oxidase (168 U/L). Finally, optical density at 295 nm and 560 nm was read to measure uric acid and $O_2^{\bullet -}$, respectively[34].

3.3.2 $OH^{\bullet}$ scavenging assay

$OH^{\bullet}$ was generated by the Fenton reaction[35]. Twenty µL of a solution of C-PC (10-22.5 µg/mL) or distilled water (0% scavenging) or DMTU (0-10 µg/mL) were added to 180 µL of the reaction mixture (0.2 mM ascorbic acid, 0.2 mM $FeCl_3$, 0.208 mM Na_2EDTA, 1 mM H_2O_2, 1.4 mM TA, and 20 mM phosphate buffer, pH 7.4). The mixture was incubated for 30 min at room temperature to obtain a fluorescent product that was measured at excitation and emission wavelengths of 326 nm and 432 nm, respectively.

3.3.3 $ONOO^-$ scavenging assay

This anion was synthesized according to Cervantes *et al*.[34] and its concentration was determined using $\varepsilon_{302\ nm}$ = 1,670 M^{-1} cm^{-1}. DHR-123 was used as an indicator of the presence of the anion. In brief, 25 µL of a solution of C-PC (0-22.5 µg/mL) or 0.1 M phosphate buffer pH 7.4 (0% scavenging tube) or penicillamine (0-15 µg/mL) were added to 225 µL of the reaction mixture (100 µM DTPA, 14 µM DHR-123, and 50 µM $ONOO^-$). In the absence of an antioxidant, DHR-123 is oxidized to rhodamine-123, a fluorescent compound which is measured at excitation and emission wavelenghts of 500 and 536 nm, respectively.

3.3.4 $ROO^{\bullet}$ scavenging assay

The scavenging activity of the sample was determined by the stability of the fluorescence of fluorescein by $ROO^{\bullet}$. Briefly, a solution of 25 µL of C-PC (0-56.3 µg/mL) or 75 mM phosphate buffer, pH 7.4 (0% scavenging tube) or trolox (0-2.5 µg/mL) was added to the solution composed of 23 nM fluorescein and 19 mM AAPH. Fluorescence was measured at excitation and emission wavelengths of 485 nm and 520 nm, respectively for 1.5 h at 37°C. At the end of the assay the area under the curve (AUC) was obtained by Gen 5 software (Biotek Instruments).

3.3.5 $^1O_2$ scavenging assay

$^1O_2$ was generated from HClO and H_2O_2 as previously described[34]. The reaction consisted of 25 µL of C-PC (0-50 µg/mL) or 45 mM phosphate buffer pH 7 (0% scavenging tube) or lipoic acid (0-150 mg/mL) to 275 µL of the reaction mixture (1 mM H_2O_2, 30 µM HClO and 1 µM DPBF). $^1O_2$ causes a reduction in fluorescence of DPBF that was determined at excitation and emission wavelengths of 410 nm and 455 nm, respectively.

3.3.6 H_2O_2 scavenging assay

The ability of C-PC to scavenge H_2O_2 was conducted using Amplex Red. The assay was performed with 20 µL of C-PC (2.2-50 µg/mL) or 50 mM phosphate buffer pH 7.4 (0% scavenging tube) or pyruvate (0-6.1 µg/mL) and 50 µL of 5 µM H_2O_2 and 100 µL of the reaction mixture (0.1 mM Amplex Red, HRP 0.2 U/mL). Amplex Red is oxidized in the presence of H_2O_2 to produce resorufin, a fluorescent compound that is measured using excitation and emission filters of 530/25 and 590/35, respectively[34].

3.3.7 HClO scavenging assay

The ability of C-PC to scavenge HClO was determined using *p*-aminobenzoic acid which reacts with HClO to produce the fluorescent compound 3-chloro-4-aminobenzoic acid[36]. Briefly, 30 µL of C-PC (0-9 µg/mL) or lipoic acid (0-16.6 mg/mL) or buffer (0% scavenging tube) were mixed with 270 µL of the reaction mixture (0.05 mM HClO, 0.1 mM *p*-aminobenzoic acid in 50 mM KH_2PO_4/KOH buffer, pH 7.4) and fluorescence was determined at excitation and emission wavelengths of 280 nm and 340 nm, respectively.

3.3.8 DPPH$^\bullet$ scavenging capacity

The activity of C-PC to scavenge DPPH$^\bullet$ was performed by the method described by Coballasa-Urrutia *et al.*[37]. Briefly, 10 µL of C-PC (0-22.5 µg/mL) or vitamin C (0-103 µg/mL) or distilled water (0% scavenging tube) were mixed with 290 µL of 100 mM DPPH$^\bullet$ (in ethanol).

After incubation in the dark for 20 minutes the sample was centrifuged at 100 x g for 10 min and the absorbance at 517 nm was measured.

3.4 *In vivo* experimental model

Fifty four CD1 male mice (30-35 g, 8-9 week-old) were fed with standard chow and water *ad libitum*. They were randomly distributed in 6 groups of 4 mice each: the first group received only the vehicle (saline solution) by intraperitoneal (i.p.) route (control, CT), the second group received C-PC 30 mg/kg i.p., the third one received a single dose of CP (18 mg/kg i.p.), and the fourth, fifth and sixth groups received different doses of C-PC (5, 10 and 30 mg/kg i.p., respectively) one hour before CP administration (18 mg/kg, i.p.)[38]. Seventy two hours after CP administration, mice were anesthetized with pentobarbital (70 mg/kg, i.p.) and euthanized by exsanguination sectioning the axilary artery. Blood was collected in eppendorf tubes with heparin. Right kidneys were immediately dissected and frozen by immersion in liquid nitrogen and prepared the homogenate (1:10 m:v) in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7 and used for NAG, oxidative stress markers, and antioxidant enzymes was used. Left kidneys were sagittally sectioned and fixed by immersion in 10% buffered formalin for histological studies and apoptosis detection. We followed the guidelines of the Official Mexican Standard Care and Use of Laboratory Animals (NOM-062-ZOO-1999) and the Mexican Official Standard NOM-087-ECOL-SSA1-2002-Infectious Bio Hazardous Waste - Classification and Specifications Handling. This protocol was approved by the local ethics committee (FQ/CICUAL/043/12).

3.4.1 Determination of renal function

BUN and plasma creatinine were determined with commercial kits. Activity of NAG was determined in renal homogenates with citrate buffer 0.1 M, pH 4.4 with p-nitrophenyl-N-acetyl-β-D-glucosaminide as a substrate (30 mM) using a p-nitrophenyl (PNP) standard curve. Reaction mixture was incubated for 15 min at 37°C and the reaction was stopped with sodium

carbonate buffer, pH 10.4. The optical density at 405 nm was measured. The data were expressed as U/mg of protein where one unit of NAG was defined as the amount of enzyme that releases 1 μmol of PNP in the assay conditions[19]. Plasma GPx activity was assessed by the disappearance of NADPH at 340 nm in a coupled reaction containing 1 mM GSH, 0.2 mM NADPH and 1 U/mL of GR. Protein concentration was determined by the Lowry method.

3.4.2 Histopathological analysis

The kidneys were dehydrated in ascending concentrations of ethanol, embedded in paraffin and sectioned to a 4 μm thickness, subsequently stained with hematoxylin and eosin (H&E). Slides were observed in the light microscope (40X) and the histological alterations of at least 30 randomly chosen fields/slice by automated morphometry using a Leica Qwin Image Analyzer (Cambridge, England). With this equipment the percentage of damaged convoluted proximal tubules was obtained considering the histopathological changes such as swelling, vacuolization, desquamation, and death cell. In addition, the total area of the tubular epithelium was measured in microns, then the area of damaged and death cells were determined and its percentage with respect to total area was calculated.

The histological profile of 20 proximal tubules randomly selected per rat (three rats per experimental group) was recorded, using a Leica Qwin Image Analyzer (Cambridge, UK).

3.4.3 Apoptosis detection

DNA fragmentation as an apoptosis marker was evaluated by TUNEL in renal tissue samples. 3 μm sections were deparaffinized and antigens were recuperated by boiling for 20 min in 0.01% sodium citrate solution, pH 6.0. Endogenous peroxidase activity was blocked with 3% H_2O_2 solution in methanol for 30 minutes. Antibody non-specific binding was inhibited by incubation in a 2% solution of normal swine serum in phosphate buffer saline (PBS) for 60 min. The enzyme terminal transferase was subsequently added in a 1:100 mixture in buffer

solution (including fluorescein-conjugated oligonucleotides) to the tissues and incubated for 50 min at 37°C in the dark. Tissues were washed 5 times for 5 min in PBS 1X and subsequently incubated for 30 min with the anti-fluorescein antibody at 37°C. After washing, color was developed by adding 3,3'-diaminobenzidine and monitored under the light microscope. Slides were scanned with Aperio CS (San Diego, CA, USA) digital pathology equipment and TUNEL-positive cells were digitally analyzed in the renal cortex with the Aperio ScanScope System (San Diego, CA, USA).

3.4.4 Oxidative stress markers

MDA, 4-HNE, oxidized proteins and GSH content were measured in kidney homogenates. MDA content was determined using tetramethoxypropane as standard curve or renal homogenized in a solution of acetonitrile: methanol (3:1) with 10 mM 1-methyl-2-phenylindole in the presence of concentrated HCl and incubated for 45 min at 40°C. After that, samples were centrifuged at 3,000 g for 5 min, the optical density of the supernatant was measured at 586 nm. Values were reported as nmol of MDA/mg of protein[25]. The content of 4-HNE was evaluated with the same technique used to measure MDA, but employing methanesulfonic acid with Fe (III) instead of HCl[19]. Renal GSH content was obtained by forming fluorescent adducts with 1 mM monochlorobimane (33 µL) in the presence of 1 U/L glutathione S transferase (GST) (33 µL)[39]. It was employed a standard curve of GSH (33 µL) in Krebs-Henseleit solution, pH 7.4. Adducts were measured in a Synergy HT multimode microplate reader at 385 and 478 nm excitation and emission, respectively. Values are reported as mmol/mg protein.

3.4.5 Antioxidant enzymes

The activity of GPx on renal homogenates was assessed using the technique previously described to measure plasma GPx activity with some modifications. Briefly, 400 µL of the reaction mixture was mixed with 50 µL of kidney homogenate or 50 mM phosphate buffer, pH

7.4 and 15 µL of 2.5 mM H_2O_2. To determine the activity of GST, 20 µL of kidney homogenate was employed with a solution containing 910 µL of 50 mM phosphate buffer pH 6.5, 20 µL of 2 mM GSH and 50 µL of 1 mM CDNB. GR activity was measured based on the disappearance of NADPH at 340 nm. Fifty µL of renal homogenate were mixed with 950 µL of the reaction mixture (1.25 mM GSSG, 0.1 mM NADPH, 0.5 mM Na_2EDTA in 100 mM phosphate buffer, pH 7.6). CAT activity was determined by the disappearance of 30 mM H_2O_2 (725 µL) in the presence of 25 µL kidney homogenate at 240 nm. Data were expressed as *k*/mg protein[40].

3.5 Statistical analysis

All the values are expressed as mean±standard error of the mean (SEM). Results of scavenging ability were expressed as the ability of the sample to scavenge 50% of each ROS (IC_{50}). The lower the IC_{50} values the higher the scavenging ability of the compound. Values were determined by interpolation using the least squares method calculated from three independent experiments. One-way ANOVA and Bonferroni analysis were used to compare the *in vivo* data and the Student t test was used to compare the results of the histological analysis using the statistical program SigmaPlot v 11.0 (Systat Software, San Jose, CA, USA). $P<0.05$ was considered significant.

4. Conclusion

This is the first report of the prevention of oxidative stress and decrease in the activity of antioxidant enzymes in CP-induced nephrotoxicity by a single dose of the nutraceutical C-PC. The protective effect of C-PC against CP-induced nephrotoxicity was associated with the ROS scavenging ability of C-PC. Our data suggest that the human consumption of C-PC may be useful for the prevention and/or treatment of kidney diseases associated to oxidative stress. Further pre-clinical investigation should be performed.

5. Acknowledgements

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Caption

Figure 1. Evaluation of C-PC purity. **A**: Absorption spectra of C-PC (250–700 nm). The characteristic absorption peak of phycocyanobilin (PCB) at 620 nm is observed. **B**: SDS-PAGE. The gel shows molecular weight markers (MW, 7 µL) and the sub-units α and β of C-PC (C-PC; 8 µg of protein). The ratio A620/A280 is 2.5.

Figure 2. C-PC scavenging activity. **A**: HClO, **B**: $ONOO^-$, **C**: $ROO^\bullet$, **D**: $DPPH^\bullet$, **E**: $OH^\bullet$, **F**: $O_2^{\bullet-}$, **G**: $^1O_2$, **H**: H_2O_2, HClO, hypochlorous acid; $ONOO^-$, peroxynitrite anion; $ROO^\bullet$, peroxyl radical; $DPPH^\bullet$, 2,2-diphenyl-1-picrylhydrazyl; $OH^\bullet$, hydroxyl radicals; $O_2^{\bullet-}$, superoxide anion; $^1O_2$, singlet oxygen; H_2O_2, hydrogen peroxide. Data are show as mean±SEM; n = 3.

Figure 3. Protective effect of C-PC against renal damage induced by CP in CD-1 mice. **A**: Plasma creatinine, **B**: Blood urea nitrogen (BUN), **C**: N-acetyl-β-D-glucosaminidase (NAG) in renal homogenates, **D**: Glutathione peroxidase (GPx) in plasma. CT: control, CP: Cisplatin 18 mg/kg i.p., C-PC 5, 10, 30: Phycocyanin 5, 10, 30 mg/kg i.p., respectively. Data are mean±SEM; n=5-6. *P<0.05 vs CT, **P<0.05 vs CP.

Figure 4. Representative histology and automated morphometry study. **A**: Normal kidney histology in control mouse. **B**: Cellular edema (asterisks), death (arrows) and detached cells (arrow head) of proximal tubular epithelium from mouse treated with CP. **C**: In contrast, occasional tubules show damage (asterisks) in a mouse treated with C-PC30+CP. **D**: There are not histological abnormalities in the kidney from mouse treated with 30 mg/kg of phycocyanin. **E**: Automated morphometry showed a high percentage of damaged convoluted proximal tubules in animals treated with CP than in mice that received C-PC30+CP. **F**: The percentage of the damaged epithelium in injured proximal tubules is higher in CP treated mice than in animals that received C-PC30+CP. **P<0.05 vs CP.

Figure 5. Immunohistochemical detection of apoptosis by TUNEL (40X). **C**: Sections of renal cortex of mice treated with CP show nuclei intensely stained (arrows) which indicate tubular

cells in apoptosis. **D**: Apoptosis was prevented in the C-PC30 group. Basal staining was just observed in renal sections from **A**: CT and **B**: C-PC30 groups. **E**: Quantitative analysis confirms a remarkable protection of C-PC against CP-induced apoptotic death. Data are mean±SEM. **P<0.01 vs CP.

Figure 6. Oxidative stress markers in renal homogenates. **A**: Glutathione (GSH), **B**: Malondialdehyde (MDA), **C**: 4-hydroxy-2-nonenal (4-HNE), **D**: Protein carbonyl. CT: control, CP: Cisplatin 18 mg/kg i.p., C-PC 5, 10, 30: Phycocyanin 5, 10, 30 mg/kg i.p., respectively. Data are mean±SEM; n=4-9. *P<0.05 vs CT, **P<0.05 vs CP.

Figure 7. Activity of antioxidant enzymes in renal homogenates. **A**: Glutathione peroxidase (GPx), **B**: Glutathione-S-transferase (GST), **C**: Glutathione reductase (GR), **D**: Catalase (CAT). CT: control, CP: Cisplatin 18 mg/kg i.p., C-PC 5, 10, 30: Phycocyanin 5, 10, 30 mg/kg i.p., respectively. Data are mean±SEM; n=4-9. *P<0.05 vs CT, **P<0.05 vs CP.

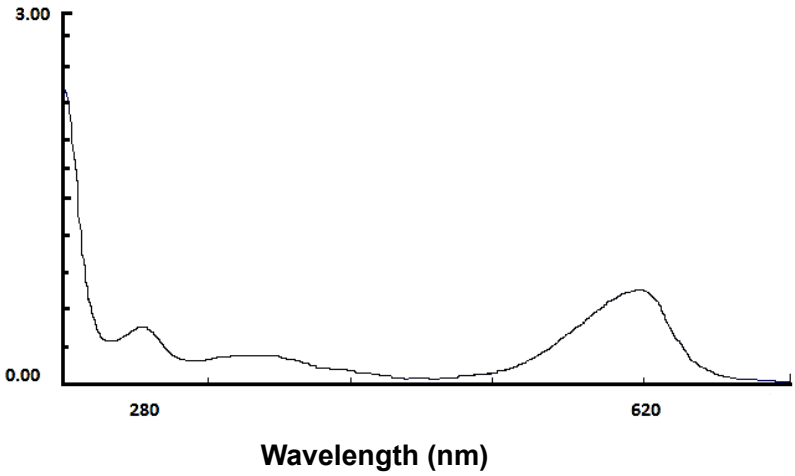
Table 1. Scavenging ability of C-phycocyanin (C-PC) and reference compounds. Data are expressed as IC_{50} (μg/mL).

Compound	HClO	$ONOO^-$	$ROO^\bullet$	$DPPH^\bullet$	$OH^\bullet$	$O_2^{\bullet-}$	$^1O_2$	H_2O_2
C-PC	4.9±0.2	5±0.6	20±6	197±7	334±9	450±5	11,670±1	21,758±2302
Lipoic acid	1148±132	-	-	-	-	-	214±85	-
Penicillamine	-	1.01±0.26	-	-	-	-	-	-
Trolox	-	-	1.19±0.03	-	-	-	-	-
Vitamin C	-	-		51.15±4.19	-	-	-	-
DMTU	-	-	-	-	2.6±0.62	-	-	-
NDGA	-	-	-	-	-	0.18±0.02	-	-
Pyruvate	-	-	-	-	-	-	-	0.28±0.001

HClO, Hypoclorous acid; $ONOO^-$, peroxynitrite anion; $ROO^\bullet$, peroxyl radical; $DPPH^\bullet$, diphenyl-1-picrylhydrazyl; $OH^\bullet$, hydroxyl radical; $O_2^{\bullet-}$, superoxide anion; $^1O_2$, singlet oxygen; H_2O_2, hydrogen peroxide. NDGA, nordihydroguaiaretic acid; DMTU, dimethylthiourea. IC_{50}= ability of the sample to scavenge 50% of each reactive oxygen species. Data are mean±SEM; n = 3.

Fig. 1

A



B

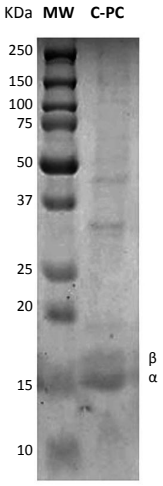


Fig. 2

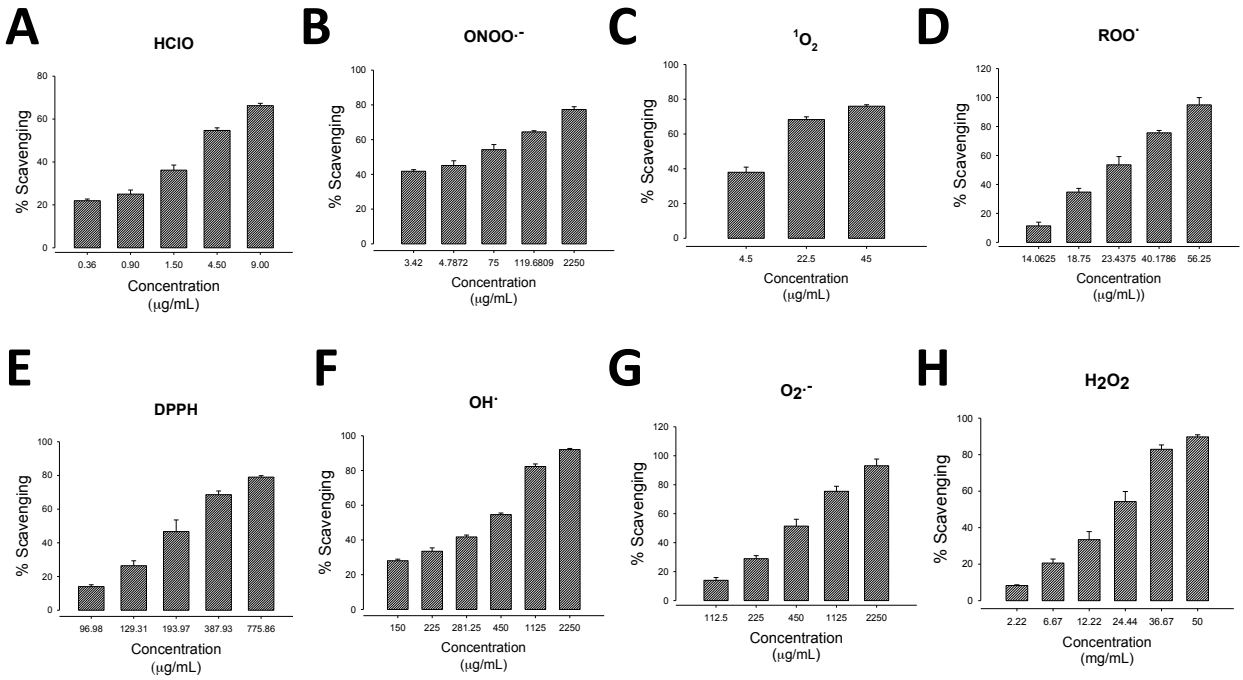


Fig. 3

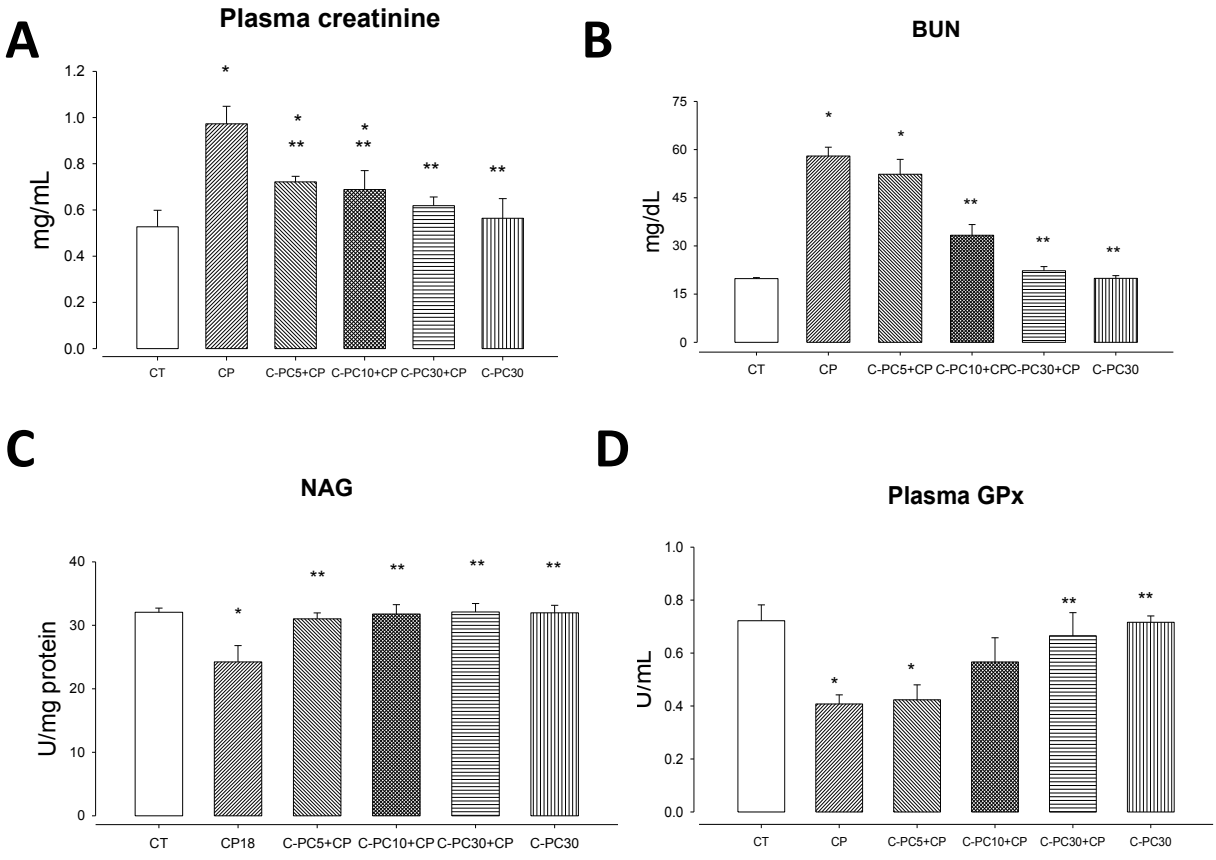


Fig. 4

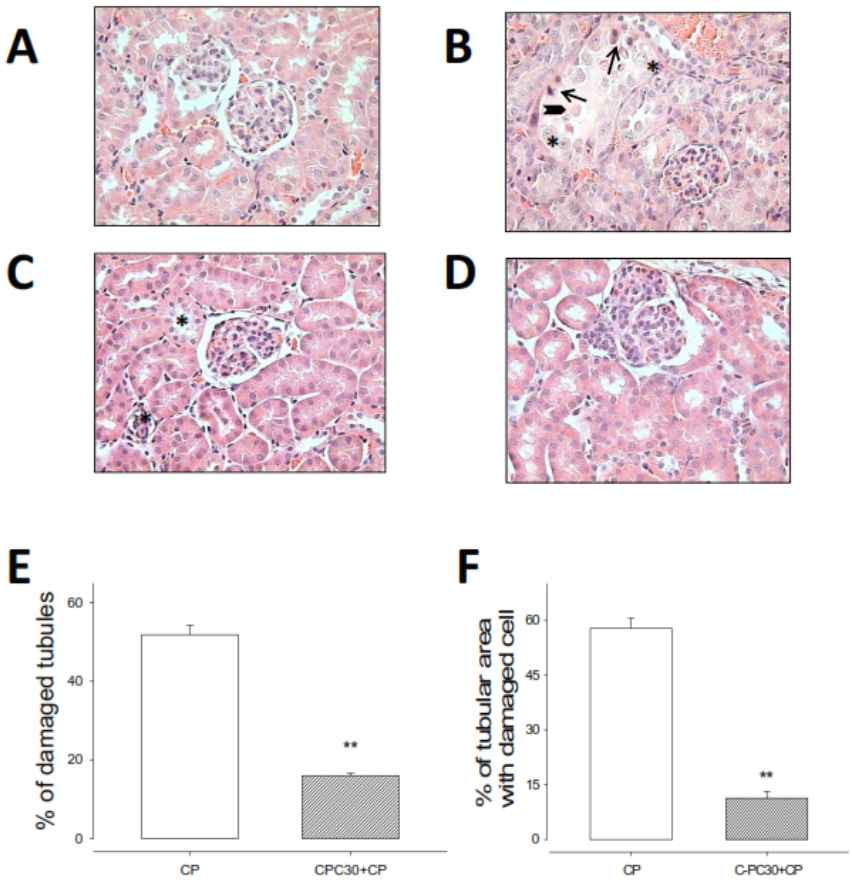


Fig. 5

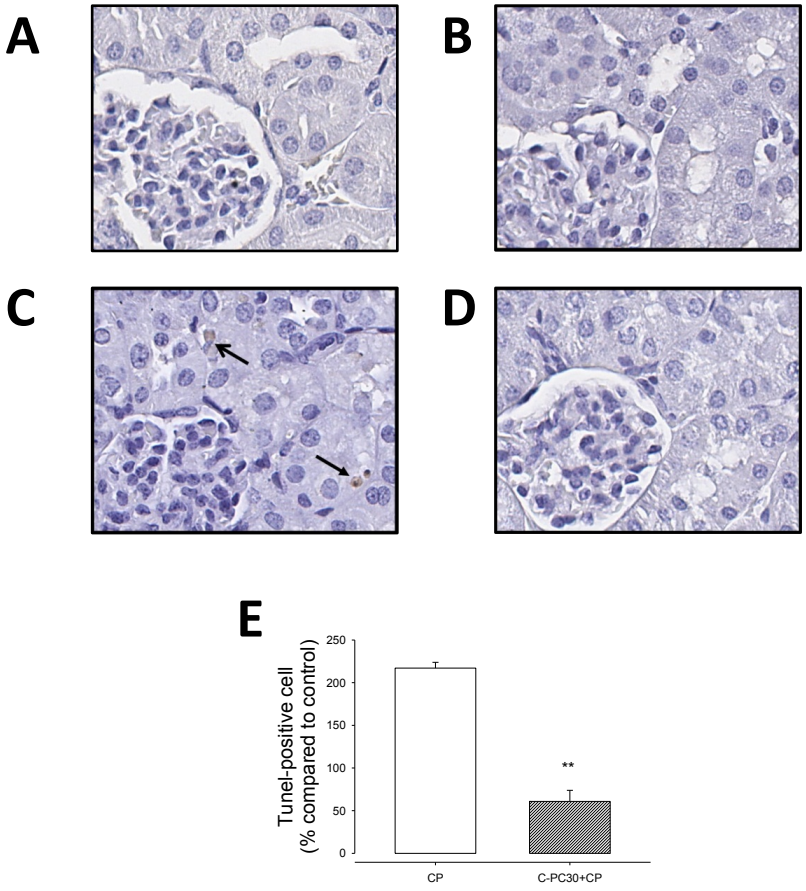


Fig. 6

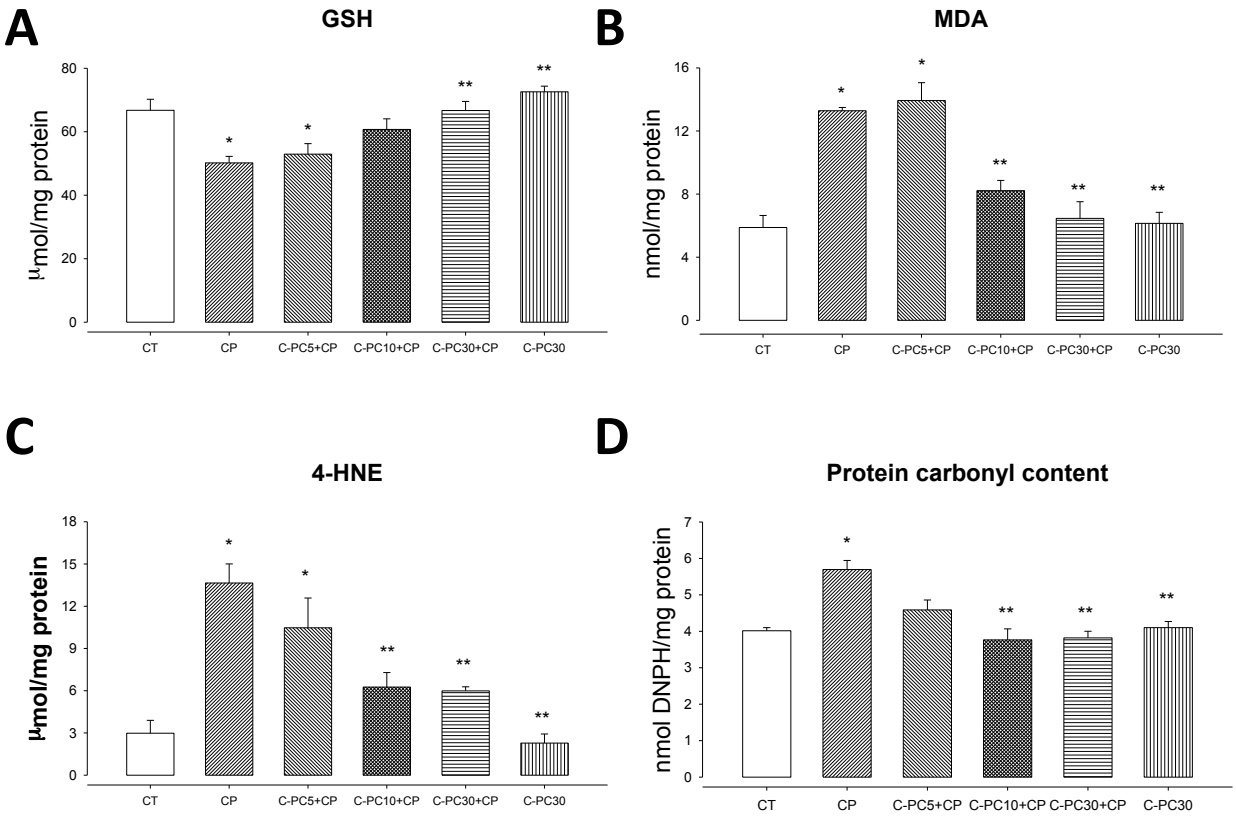


Fig. 7