COPPER-INDUCED CHANGES OF LIPID PEROXIDATION AND HEMATO-BIOCHEMICAL PARAMETERS IN RAT BLOOD: PROTECTIVE ROLE OF FLAVONOIDS

JELENA M. MLADENOVIĆ[1], MILICA G. PAUNOVIĆ[1], MILOŠ M. MATIĆ[1], VEROLJUB S. KNEŽEVIĆ[2], BRANKA I. OGNJANOVIĆ[1,*], ANDRAŠ Š. ŠTAJN[1] and ZORICA S. SAIČIĆ[3]

[1] *Department of Biology and Ecology, Faculty of Science, University of Kragujevac,* Kragujevac, Serbia
[2] *Laboratory Diagnostics Department, Health Centre of Kragujevac,* Kragujevac, Serbia
[3] *Department of Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade,* Belgrade, Serbia

Corresponding author: branka@kg.ac.rs

Abstract – The effects of subchronic exposure to copper (Cu) on lipid peroxidation, hemato-biochemical parameters, and the possible protective role of flavonoids Quercetin and (-)-Epicatechin were studied. Male Wistar albino rats were treated with Cu (560 mg/L, *p.o.* as $CuCl_2 \cdot 2H_2O$ for 5 weeks) and Quercetin and (-)-Epicatechin (40 mg/kg BW each, *i.p.*, every third day during the last 3 weeks) alone or in combination. Cu increased the concentration of lipid peroxides, decreased the number of erythrocytes, hemoglobin and hematocrit values and increased the activities of aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase. Coadministration of Quercetin and (-)-Epicatechin with Cu lowered the process of lipid peroxidation and restored examined hemato-biochemical parameters to control values. Our results indicate that Cu induced oxidative damage in erythrocytes, which led to anemia, while Quercetin and (-)-Epicatechin showed a protective effect on the hemato-biochemical processes in the blood of rats.

Keywords: Copper; erythrocyte; flavonoids; hematological parameters; biochemical parameters; lipid peroxidation

INTRODUCTION

Copper (Cu) is an essential trace element involved in many processes responsible for normal growth and development, as an integral part of specialized cuproproteins, such as ceruloplasmin (CP), cytochrome c oxidase, dopamine β-hydroxylase, superoxide dismutase and tyrosinase (Ferenci, 2004; Kodama and Fujisawa, 2009). However, the accumulation of Cu in amounts that exceed the metabolic requirements of the organism or Cu homeostasis disorders can lead to the manifestation of its toxic effects (Fuentealba and Aburto, 2003). It is well known that redox-active metals, such as Cu, are capable of inducing oxidative stress by increasing the production of reactive oxygen species (ROS) which causes peroxidative degradation of polyunsaturated fatty acids in membrane lipids, and leads to the damage of biomolecules (Halliwell and Gutteridge, 2007).

As a result of increased anthropogenic activities, heavy metals, including Cu, are increasingly present in the environment. Cu salts are widely used as algicides, fungicides and pesticides. Acute consumption of high Cu amounts in water by humans may cause irritation of the stomach, nausea, loss of appetite and dehydration (Araya et al., 2004). The chronic use of water with increased Cu concentrations may also represent a potential risk to sensitive populations, such as children and individuals

with a genetic disorder of Cu metabolism (Brewer, 2000). Increased Cu accumulation in humans occurs mainly due to metabolic disorders of genetic origin, as in Wilson's disease, which causes hepatitis and liver cirrhosis, and nervous system and kidney disorders (Brewer, 2000; Patil et al., 2013). In addition, an excess of Cu adversely affects the cardiovascular system, leading to high blood pressure and promoting atherosclerosis (Iskra and Majewski, 2000).

Quercetin (QE) and (-)-Epicatechin (EC) belong to a group of plant polyphenolic flavonoids present in the daily diet of humans. The main sources of QE are apples, citrus fruits, broccoli, onion, berries, tea and red wine (Nutrient Data, Laboratory, 2011). EC is the most abundant flavonoid in green and black tea, cocoa products, red wine and berries (Nutrient Data, Laboratory, 2011). A diet rich in flavonoids can reduce blood pressure, the risk of cardiovascular disease, improves the liver antioxidant defense system and has a beneficial effect on symptoms of neurodegenerative disorders in Parkinson's and Alzheimer's disease (Middleton et al., 2000; Schroeter et al., 2006; Larson et al., 2012). Apart from plant sources, these flavonoids are components of supplements in an alternative therapy for the treatment of allergies, asthma, bacterial infections, arthritis, gout, eye disorders, hypertension and neurodegenerative disorders (Larson et al., 2012).

Flavonoids exhibit a wide range of biological activities, including anti-oxidative, anti-allergic, antiviral, neuroprotective and cancer inhibiting, *in vitro* or in animal tissues (Verma et al., 1988; Deschner et al., 1991; Middleton et al., 2000; Ishisaka et al., 2011). In recent years attention has been devoted to their antioxidant activity. Due to their structure, flavonoids exhibit the ability to chelate transition metal ions and to "capture" and neutralize free radicals, acting as chain-breaking antioxidant (Bors et al., 1990).

The aim of this study is to investigate the effects of Cu and the protective capacity of these particular flavonoids, QE and EC, on hematological and bio-chemical parameters in the blood of rats subchronically exposed to Cu in excess.

MATERIALS AND METHODS

Chemicals

Chemicals for this study were obtained from Sigma-Aldrich Chemie GmbH (Germany) and Merck (Darmstadt, Germany). Quercetin and (-)-Epicatechin were purchased from Sigma-Aldrich Chemie GmbH (Germany). Solutions were prepared with double-distilled water. All reagents and chemicals were of analytical grade or higher purity.

Experimental animals

The study included male adult Wistar albino rats, 8 weeks old, weighing 230 ± 20 g at the beginning of the experiment. The animals were maintained in individual cages under standard laboratory conditions (temperature $22°C\pm2°C$; 12 h light-dark cycle). The animals had unlimited access to drinking water or a solution of $CuCl_2$ and standard rodent laboratory diet. The concentration of $CuCl_2$ was determined based on the oral median lethal dose for rats (LD_{50}). The amount of water and solutions they drank was measured every third day. At the end of experimental period, the animals were anesthetized with ether and sacrificed by decapitation. The experimental procedures were approved by the University Ethics Committee.

Experimental design

After a period of adaptation for one week prior to the experiment, the animals were randomly divided into 4 groups, 7 animals per group: Group 1 (Control) received saline (0.3 mL/kg BW); Group 2 (Cu) was treated with copper (as $CuCl_2 \cdot 2H_2O$ at a concentration of 560 mg/L, *p.o.*) via drinking water for 5 weeks; Group 3 (QE+EC) was treated with intraperitoneal injections (*i.p.*) of quercetin coadministered with epicatechin (40 mg QE/kg BW + 40 mg EC/kg BW, in 0.3 mL double-distilled water) every third day for the last 3 weeks of the experiment. A total of seven injec-

tions were administered; Group 4 (Cu+QE+EC) was treated with copper (as $CuCl_2 \cdot 2H_2O$ at a concentration of 560 mg/L, *p.o.*) via drinking water for 5 weeks, and with quercetin coadministered with epicatechin (40 mg QE/kg BW + 40 mg EC/kg BW, in 0.3 mL double-distilled water) *i.p.,* every third day for the last 3 weeks of the experiment. A total of seven injections were administered.

Analytical procedures

The animals were measured, anesthetized with ether and decapitated 24 h after the last injection. Blood samples were collected in K-EDTA tubes for hematological analysis or in tubes without anticoagulants for other analyses. Hematological and biochemical parameters were measured on the day of sacrifice. Hematological analysis included the number of erythrocytes (RBC), hemoglobin (Hb), hematocrit (Hct) values, as well as hematological indices (mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW)) in whole blood, and was performed by standard methods on an automated hematology analyzer (Horiba Medical ABX Micros 60, Japan).

Measurements of biochemical parameters, serum total protein (TP), albumin (Alb), and activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were performed on BioSystem BTS 330 (Spain), and serum concentrations of ceruloplasmin (CP) was performed on a Roche Hitachi 911 (Swiss) analyzer.

To measure lipid peroxidation (LPO), whole blood samples with EDTA were centrifuged at 1 000 $\times g$ (+4°C) for 10 min and the plasma was removed. The erythrocytes were washed three times with an equal volume of cold saline (0.9 %, *v/v*). One milliliter of washed erythrocytes was lysed on ice in 3 mL of dH_2O (4°C) for 30 min. LPO in the hemolysate was determined using the method described by Ohkawa et al. (1979), based on the reaction of lipid peroxidation products (MDA-malondialdehydes) with thio-barbituric acid (TBA) (TBARS analysis). Hemolysate samples were extracted with 28% trichloroacetic acid and centrifuged at 1 000 $\times g$ for 10 min. The color reaction was carried out by adding 1% TBA and incubation of the samples in a warm bath at 90°C for 15 min. The absorbances were measured with a Uv-Vis Spectrophotometer (JENWAY 6105, Staffordshire, UK) and results were expressed in nmol MDA/mL erythrocytes, using a molar extinction coefficient for MDA of 1.56×10^5 $M^{-1} \cdot cm^{-1}$.

Statistical analysis

All data were evaluated using SPSS for Windows (version 13.0) software (SPSS Inc., Chicago, Il, USA). The results are expressed as mean ± standard error of the mean (SEM). Comparisons were made using either factorial analysis of variance (ANOVA) with a post hoc Bonferroni/Dunnett's multiple analysis or Kruskal-Wallis test (for comparison across several groups) and Mann-Whitney test (for comparison between two groups). Differences at $p<0.05$ were considered statistically significant.

RESULTS

The effects of treatment with Cu and the flavonoids QE and EC on average animal weight are shown in Table 1. The animals in group 1 (Control), as well as groups 3 (QE+EC) and 4 (Cu+QE+EC) gained in body weight during the 5-week experiment, while in group 2 (Cu) the body weight decreased significantly. The average amount of $CuCl_2$ solution emptied in the experimental group 2 (22.0 mL/rats/day) and in group 4 (24.3 mL/rats/day), was significantly lower than the volume of tap water drunk by the control animals (30.3 mL/rats/day) throughout the exposure (Table 1). The calculated average intake of Cu^{2+} per rat was about 5 mg/day. There was no mortality among the animals, despite a reduction in body weight that was due to the avoidance of water and food intake and probable dehydration.

A subchronic exposure to Cu significantly decreased RBC counts, Hb and Hct values compared to the control (Table 2). Coadministration of QE and

Table 1. Body weights and water consumption of control and treated groups of rats after 5 weeks of treatment.

	Experimental groups			
	Control (*n*=7)	Cu (*n*=7)	QE+EC (*n*=7)	Cu+QE+EC (*n*=7)
Initial body weight (g)	238.7 ± 13.02	232.7 ± 17.8	230.0 ± 21.3	232.9 ± 19.9
Final body weight (g)	263.3 ± 18.8	215.3 ± 15.5*	265.7 ± 20.7**	255.7 ± 17.6
Water consumption (mL/rat/day)	30.3 ± 1.5	22.0 ± 1.6*	30.8 ± 1.1**	24.3 ± 1.6*

n: number of animals in the group; Cu: copper; QE: quercetin; EC: epicatechin. Values are given as mean ± SD. *$p<0.05$, significantly different from control; **$p<0.05$, significantly different from Cu group.

Table 2. Changes in haematological parameters of control and treated groups of rats after 5 weeks of treatment.

Parameters	Experimental groups			
	Control (*n*=7)	Cu (*n*=7)	QE+EC (*n*=7)	Cu+QE+EC (*n*=7)
RBC (×10¹²/L)	6.32 ± 0.89	5.16 ± 0.77*	7.19 ± 0.42**	6.87 ± 0.35**
Hb (g/L)	135.3 ± 4.22	101.1 ± 4.27*	144.0 ± 7.79**	141.3 ± 6.8**
Hct (%)	38.41 ± 4.86	29.52 ± 4.46*	41.05 ± 2.51**	38.35 ± 2.89**
MCV (fL)	59.21 ± 2.08	56.80 ± 1.91	57.03 ± 2.96	56.10 ± 1.83
MCH (pg)	21.1 ± 2.11	19.7 ± 2.46	20.02 ± 1.24	20.57 ± 0.82
MCHC (g/L)	352.75 ± 12.62	347.87 ± 12.27	350.83 ± 7.78	368.75 ± 16.07**
RDW (%)	14.83 ± 1.73	15.95 ± 0.88	14.73 ± 1.67**	13.73 ± 0.4**

n: number of animals in the group; Cu: copper; QE: quercetin; EC: epicatechin; RBC: red blood cell; Hb: haemoglobin; Hct: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; RDW: red cell distribution width. Values are given as mean ± SD. *$p<0.05$, significantly different from control; **$p<0.05$, significantly different from Cu group.

Table 3. Changes in biochemical parameters of control and treated groups of rats after 5 weeks of treatment.

Parameters	Experimental groups			
	Control (*n*=7)	Cu (*n*=7)	QE+EC (*n*=7)	Cu+QE+EC (*n*=7)
TP (g/L)	60.9 ± 2.6	59.0 ± 2.9	61.4 ± 3.5	60.3 ± 5.1
Alb (g/L)	12.7 ± 1.5	12.0 ± 1.4	12.8 ± 1.9	11.5 ± 1.8
CP (mg/L)	54.4 ± 10.7	37.9 ± 12.2*	45.7 ± 11.4	52.7 ± 12.1
AST (U/L)	141.8 ± 14.7	216.2 ± 18.4*	147.2 ± 13.7**	138.8 ± 18.7**
ALT (U/L)	50.6 ± 6.3	70.1 ± 12.3*	55.2 ± 8.9	61.4 ± 18.1
LDH (U/L)	1084.7 ± 158.3	2012.2 ± 114.2*	1097.7 ± 81.8**	998.3 ± 142.9**

n: number of animals in the group; Cu: copper; QE: quercetin; EC: epicatechin; TP: total protein; Alb: albumin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; CP: ceruloplasmine. Values are given as mean ± SD. *$p<0.05$, significantly different from control; **$p<0.05$, significantly different from Cu group.
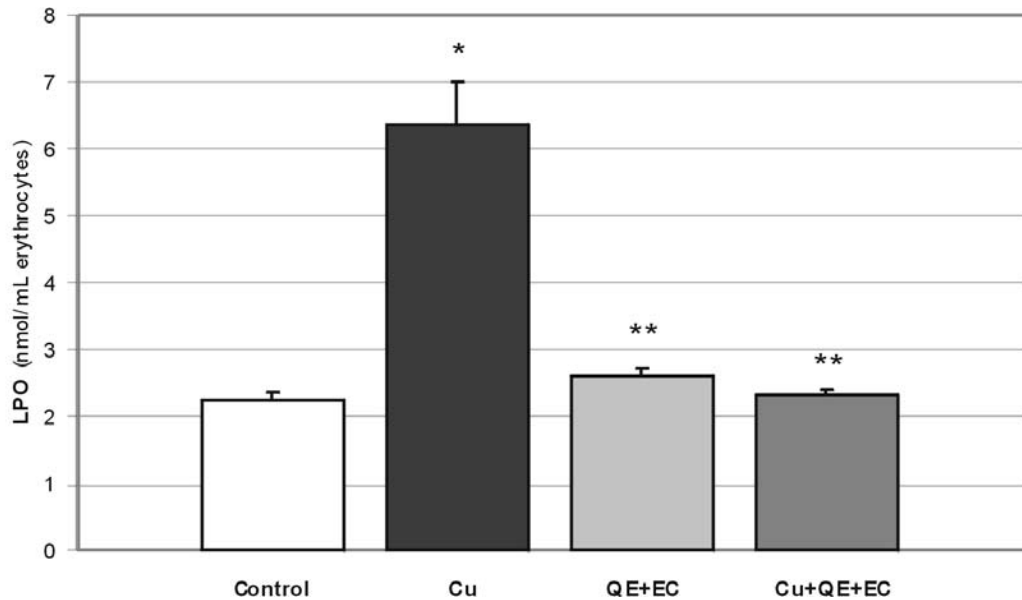
Fig. 1. LPO values in erythrocytes of control and treated groups of rats after 5 weeks of treatment Cu: copper; QE: quercetin; EC: epicatechin. Values are given as mean ± SD ($n = 7$); $^*p<0.05$, significantly different from control; $^\#p<0.05$, significantly different from Cu group.

EC with Cu significantly increased the values of the examined parameters compared to the Cu group. We noticed a slight a decrease in the values of the hematological indices MCV, MCH and MCHC in the Cu group compared to the control. However, the MCHC value was significantly higher in the Cu+QE+EC group compared to the Cu group.

Table 3 shows the effects of treatment on biochemical parameters. Exposure to Cu led to a slight decrease of TP and Alb, and a significant decrease in CP serum level compared to the control. Coadministration of QE+EC with Cu raised CP to the level of the control.

The Cu treatment caused a significant increase in the activities of AST, ALT and LDH as compared to the control. Coadministration of QE+EC with Cu reversed these changes to the values measured in control.

The data in Fig. 1 show that subchronic treatment with Cu led to a significant increase in LPO in the rats' erythrocytes when compared to the control.

Co-treatment of QE+EC with Cu restored the LPO level to nearly those measured in the control group.

DISCUSSION

Although Cu is an essential element for a number of biological processes, prolonged exposure to elevated concentrations may have adverse effects (Fuentealba and Aburto, 2003). In this study, we investigated the effects of Cu and the influence of the flavonoids QE and EC on hematological and biochemical parameters in the blood of rats after subchronic exposure to Cu in excess.

Our results show that in animals subchronically exposed to Cu their body weight decreased, which can be a predictor of poor general health. The decrease in body weight may indicate an excessive breakdown of tissue proteins. Similarly, the results of Bataineh et al. (1998) showed that long-term consumption of Cu salts causes growth disorders and weight loss in animals. On the other hand, animals co-treated with EC, QE and Cu continuously gained weight.

Exposure to Cu significantly decreased RBC, Hb and Hct and led to anemia. Similar results were obtained in other studies on animals (Bozynski et al., 2009; Nikolic et al., 2013). The research of Fernandes et al. (1988) showed that the incubation of an erythrocyte suspension with Cu^{2+} causes lipid peroxidation and hemolysis as a consequence of oxyhemoglobin oxidation by Cu. The erythrocytes are more susceptible to oxidative damage than other cells because they are constantly exposed to ROS and their membranes are rich in unsaturated fatty acids (Clemens and Waller, 1987). It was found that toxic Cu concentrations reduce deformability of erythrocytes and increase membrane permeability and osmotic fragility of cells, resulting in reduced erythrocyte survival (Adams et al., 1979). The observed reduced number of RBC, Hb and Hct after Cu exposure in our study is probably due to the oxidative damage of erythrocytes and increased erythrocyte destruction.

Coadministration of QE+EC with Cu showed protective effects on hematological parameters. In the study of Chouhan et al. (2011), QE exerted beneficial effect on hematological parameters in rats treated with fluoride. Martinez et al. (2012) found that the presence of EC prevents protein oxidation and positively affects membrane fluidity and erythrocyte morphology, thereby preventing hemolysis resulting from peroxidation. These results suggest that the protective effects of QE and EC are likely due to their ability to capture ROS and thereby prevent erythrocyte damage and hemolysis.

Metabolic processes in rats exposed to Cu were also affected, which is reflected in the disturbance of biochemical parameters. CP, a cuproprotein that is synthesized in the liver, is the major carrier of Cu in the blood. Reduced levels of CP are typically associated with a reduced level of Cu in the serum of patients with Wilson's disease, but reduced CP with normal or increased levels of serum Cu may suggest increased levels of Cu not bound to CP (the so-called free copper or non-ceruloplasmin-bound copper) (Kodama and Fujisawa, 2009). An elevated level of Cu in circulation may be the result of a sudden Cu release from the hepatocytes, caused by liver damage

or intoxication with Cu (Patil et al., 2013). Treatment with Cu in the present study significantly decreased CP but did not lead to significant changes in the serum level of Cu (data not shown) as compared to the control, which may indicate the release of non-ceruloplasmin-bound Cu from damaged hepatocytes. Coadministration of QE+EC with Cu reversed the changes in protein levels and CP, suggesting protective effects of these flavonoids on hepatocytes.

Cu treatment increased the activities of AST, ALT and LDH in the serum. Transaminases are widely distributed in tissues, while their concentrations in the serum are low. Since the liver is the organ of Cu accumulation, these changes may indicate liver damage and, due to the distortion of functional integrity of hepatocyte cell membranes, the release of these enzymes in serum and their enhanced activity. Increased transaminase (AST and ALT) activities in serum serve as parameters in the diagnosis of hepatitis and hemolytic anemia in humans and also indicate liver damage in animal (Fuentealba and Aburto, 2003; Gaetke and Chow, 2003). George and Chandrakasan (1997) reported a significant increase in LDH activity in the serum of rats as a result of hepatic fibrosis.

QE+EC administered alone did not cause significant differences in the activities of these enzymes compared to the control group. However, coadministration of QE+EC with Cu decreased ALT and significantly reduced AST and LDH activities compared to the Cu group. This is consistent with the results of other studies in which QE inhibited the increase of AST, ALT and LDH activities in serum by reducing the LPO in the liver (Tokyol et al., 2006) and myocardium of animals (Matouk et al., 2013; Milton et al., 2013). These results indicate that QE and EC are able to preserve the functional integrity of hepatocyte cell membranes and to prevent leakage of these enzymes into the blood.

Cu is essential for numerous biological functions, including the synthesis of phospholipids in cell membranes that maintains the integrity of the cell (Ferenci, 2004). However, as earlier studies have

shown, an excess of Cu in rat diet may cause LPO and damage of the membrane (Dillard and Tappel, 1984; Burkitt, 2001; Gaetke and Chow, 2003). Fernandes et al. (1988) reported increased LPO after incubation of human erythrocyte suspension with Cu^{2+} ions. The Cu^+ ion generated by the reduction of Cu^{2+} in the presence of superoxide anion ($O_2^{\bullet-}$) catalyzes the formation of hydroxyl radicals ($\bullet$OH) that readily enter into further reactions (Halliwell and Gutteridge, 2007). $O_2^{\bullet-}$, $\bullet$OH, or lipid peroxyl radicals can cause LPO, a decrease in membrane potential, and an increase in permeability to H^+ and other ions, which eventually leads to the release of contents from cells (Halliwell and Gutteridge, 2007). Cu in our study significantly increased LPO in erythrocytes, indicating oxidative damage of cell membranes and the occurrence of oxidative stress in erythrocytes.

Our data indicate that QE+EC inhibited Cu-induced LPO. Previous studies have shown that flavonoids can block LPO due to their structure that enables them to chelate metal ions or scavenge ROS as hydrogen- or electron-donating compounds (Middleton et al., 2000; Mira et al., 2002). They thus can be helpful in conditions and diseases caused by increased metal concentrations or in conditions that are the result of oxidative stress. Mira et al. (2002) showed that QE and catechin chelate Cu^{2+} ions, due to their hydroxyl groups with interaction probably between the 5-hydroxyl and 4-oxo groups. It has also been reported that catechins have an antioxidant effect on iron-induced LPO due to iron chelation (Sugihara et al., 2001).

LPO could be inhibited by ROS scavenging, and QE as well as EC appears to be an extremely efficient radical scavenger (Middleton et al., 2000). The antioxidant properties of QE and EC may contribute to the improvement of cells' antioxidant defense. Filipe et al. (2001) showed that QE inhibited Cu-induced LPO in human plasma. These authors have also shown that there was a synergy of QE with endogenous urate in antioxidant action against Cu-induced LPO, and that the flavonoids were able to protect urate from oxidative degradation. Dietrich-Muszalska et al. (2012) have shown that EC was more effec-

tive than QE in reducing LPO in human plasma *in vitro*. In an *in vitro* study of Boadi et al. (2003), it was shown that in reducing LPO, combinations of flavonoids provide better antioxidant protection than the individual treatments.

According to Bors et al. (1990), three structural group of flavonoids are responsible for metal chelating and radical scavenging: i) the O-dihydroxy structure in the B-ring, which is the radical target site for all flavonoids with a saturated C-2/C-3 bond; ii) the C-2/C-3 double bond in conjugation with a 4-oxo function, which is responsible for electron delocalization from the B-ring; and iii) the additional presence of both 3- and 5-hydroxyl groups for strongest radical absorption.

Our data suggest that Cu in excess exerted prooxidant effects on hematological and biochemical processes in the blood and caused oxidative damage in erythrocytes. Treatment with QE and EC could protect against oxidative damage in the blood of rats subchronically exposed to Cu in excess. The cytoprotective role of QE and EC can be ascribed to their ability to chelate the ions of transition metals and scavenge ROS. They managed to inhibit LPO, thus demonstrating a significant role in alleviating the manifestations of Cu toxicity.

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