

Glyphosate disrupts redox status and up-regulates metallothionein I and II genes expression in the liver of adult rats. Alleviation by quercetin

Nejla Soudani^{1,2}, Mariem Chaâbane^{1*}, Imen Ghorbel^{1*}, Awatef Elweji¹, Tahia Boudawara³ and Najiba Zeghal¹

¹ *Animal Physiology Laboratory, Department of Life Sciences, Sciences Faculty, University of Sfax, Sfax, Tunisia*

² *Physiology and Aquatic Environment Laboratory, Sciences Faculty, University of Tunis El Manar, Tunis, Tunisia*

³ *Histopathology Laboratory, University of Sfax, CHU Habib Bourguiba, Sfax, Tunisia*

Abstract. The present work evaluated the possible protective effects of quercetin against glyphosate-induced hepatotoxicity in adult rats. Rats were randomly divided into three groups: a control group (C), a glyphosate-treated group (Gly) and a group treated with both glyphosate and quercetin (Gly+QE). During the experimental period (15 days), glyphosate (50 mg/kg b.w.) was administered every two days by intraperitoneal way while quercetin (20 mg/kg b.w./day) was administered daily by gavage. Glyphosate-induced hepatic oxidative stress was evidenced by the increased levels of malondialdehyde, hydrogen peroxide, advanced oxidation protein products and protein carbonyls with a significant decrease in enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (non-protein thiols, glutathione, vitamin C) antioxidants. Plasma biomarkers of hepatotoxicity (AST, ALT, ALP, g-GT, albumin) were also altered. Moreover, glyphosate induced DNA damage, up-regulated metallothionein (MT I and MT II) genes expression and provoked histopathological changes in rats' liver. Quercetin supplementation to glyphosate-treated rats markedly ameliorated all the parameters indicated above as well as the liver histoarchitecture. Therefore, quercetin might have beneficial effects against glyphosate-induced hepatotoxicity in rats.

Key words: Glyphosate — Hepatic oxidative stress — Rats — Metallothionein — Quercetin

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, one way analysis of variance; AOPP, advanced oxidation protein product; AST, aspartate aminotransferase; CAT, catalase; DTNB, 5-5'-dithio-bis-2-nitrobenzoic acid; Gly, glyphosate; GPx, glutathione peroxidase; GSH, reduced glutathione; MDA, malondialdehyde; MT, metallothionein; NBT, nitro blue tetrazolium; NPSH, non-protein thiols; PCO, protein carbonyls; QE, quercetin; SOD, superoxide dismutase; TBA, thiobarbituric acid; TEP, 1,1,3,3-tetrathoxypropane; γ GT, gamma glutamyltranspeptidase.

Introduction

Herbicides are extensively used in agricultural fields in order to enhance biomass productivity, although they represent an environmental hazard, affecting non-target organisms,

including humans. Glyphosate represents one of the most commonly applied herbicides worldwide since the 1970s (Jiraungkoorskul et al. 2002). It is the active ingredient of more than 750 different broad-spectrum herbicides (Guyton et al. 2015). This herbicide is used in agricultural applications for the control of annual and perennial plants, grasses, and broad-leaved woody species. Glyphosate herbicidal action is primarily based on the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase, a key enzyme of the shikimic acid pathway present in plants, fungi and some bacteria, and implicated in the biosynthesis of aromatic amino acids. Since

* These authors contributed equally to this work.

Correspondence to: Mariem Chaâbane, Animal Physiology Laboratory, Department of Life Sciences, Sciences Faculty, University of Sfax, Sfax, Tunisia

E-mail: maryame.ch@yahoo.com

this pathway is absent in animals, glyphosate, according to Williams et al. (2000), is considered to be safe for the general population according to the manufacturer's instructions. Nevertheless, recent reports have revealed that glyphosate can be detrimental to the human health. In fact, Samsel and Seneff (2013) have recently shown that glyphosate may be a key contributor to obesity and autism epidemics in the United States, as well as to other diseases and pathologic conditions such as Alzheimer and Parkinson diseases, infertility and depression. Epidemiological data have shown also a strong and a highly significant correlation between the increased use of glyphosate and a multitude of cancers (Swanson et al. 2014).

In toxicological studies based on rodent models, glyphosate-based herbicides have been observed to elicit their toxicity through the induction of oxidative stress in the nervous system (Cattani et al. 2014), the reproductive system (de Liz Oliveira Cavalli et al. 2013), the kidneys (Wunnapuk et al. 2014), and especially, the liver, where an increased lipid peroxidation and a depleted level of glutathione (GSH), a non-enzymatic antioxidant, have been reported (El-Shenawy 2009). Metallothioneins (MTs), a class of low molecular weight proteins (6–7 kDa), represent another important part of the non-enzymatic antioxidant defense system. These proteins are involved in scavenging free radicals and have a cytoprotective role against their toxic effects (Sato and Bremner 1993). The most widely expressed isoforms in mammalian liver are MT I and MT II, which are sensitive to oxidative stress induced by some compounds such as pesticides (Sato and Sasaki 1991; Kumar et al. 2010). However, there are no scientific reports about the relation between MT I and MT II gene expression levels and oxidative stress status in mammalian hepatic tissue following glyphosate exposure.

As oxidative stress is considered to be one of the major mechanisms behind glyphosate toxicity, antioxidant therapy could be a useful therapeutic strategy for preventing its hepatotoxic effects. In this context, quercetin (QE; 3,5,7,3',4'-pentahydroxyflavone) is one of the most widely distributed flavonoids in plants belonging to the flavonol subclass. It represents an integral part of the human diet and it is mainly abundant in onions, kale, broccoli, Ginkgo Biloba, apples, berries, tea, red wine, nuts and seeds. Recently, this flavonol has an increasing scientific interest due to its outstanding health benefits, making it a promising candidate for the development of the novel functional foods and medicines (Lin et al. 2014). QE has been reported to display a broad range of biological properties like antioxidant, anticancer and anti-inflammatory activities (Lamson and Brignall 2000). Major attention has been particularly paid to its antioxidant activity and its ability to reduce oxidative stress in biological systems. Indeed, within the flavonoid family, QE is considered to be the most active scavenger of reactive oxygen species (ROS) (Boots et al. 2008) and

a potent chelator of metal ions (Ferrali et al. 2000). It has been demonstrated that the antioxidant property of QE confers a valuable therapeutic potential against various diseases such as cardiovascular diseases, renal injury and several hepatic pathologies (Jalili et al. 2006; Renugadevi and Milton Prabu 2010; Lee et al. 2013). Yet, to the best of our knowledge, there are no scientific reports about the impact of QE supplementation on the liver impairment induced by glyphosate.

Therefore, the present study aimed first to investigate the effect of glyphosate exposure on the redox status and genes expression of two MTs isoforms, namely MT I and MT II, in the liver of adult rats. Then, the potential protective effect of QE against the hepatotoxic effects induced by this herbicide was assessed.

Materials and Methods

Chemicals

Glyphosate [N-(phosphonomethyl) glycine] with a purity of 99.9%, reduced glutathione (GSH), 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB), 1,1,3,3-tetrahydroxypropane (TEP), thiobarbituric acid (TBA) and nitro blue tetrazolium (NBT) were purchased from Sigma (St. Louis; MO, USA). All other reagents of analytical grade were provided from standard commercial suppliers.

Animals and treatment

The experiments were conducted on adult male rats of Wistar strain (220 ± 10 g) purchased from the Central Pharmacy (SIPHAT, Tunisia). The animals were allowed to acclimate under controlled humidity (40%), temperature ($22 \pm 3^\circ\text{C}$) and light conditions (12 h light/dark cycle), with free access to a commercial pellet diet (SNA, Sfax, Tunisia) and water. All the experimental procedures were conducted in strict accordance with the International Guidelines for Animal Care (Council of European Communities 1986) and approved by the Ethical Committee of Sciences Faculty, Sfax University.

After acclimatization for 1 week before the onset of the experiment, rats were randomly divided into three groups of six each. Rats of the first group (C), serving as controls, received 1 ml of distilled water by intraperitoneal (i.p.) way every two days. Rats of the second group (Gly) received every two days by i.p. way 50 mg/kg b.w. of glyphosate, dissolved in distilled water, during 15 days. Rats of the third group (Gly+QE) received every two days by i.p. way 50 mg/kg b.w. of glyphosate (dissolved in distilled water) and QE (dissolved in 2.5% dimethyl sulfoxide) administered daily by gavage at a dose of 20 mg/kg b.w./day during 15 days. Glyphosate i.p. injection started at the first day of the experiment. So,

rats received 8 doses of glyphosate during the 15 days of treatment. All groups had free access to distilled water and standard diet during the experimental period.

The dose of glyphosate (50 mg/kg b.w.) used in our experiment, which corresponded to 1/5 of LD₅₀ (median lethal dose), was chosen according to Olorunsogo and Bababunmi (1980) and WHO (1994). Concerning QE, the dose 20 mg/kg b.w./day has been reported to be effective in reducing oxidative stress in the hepatic tissue of sodium fluoride-treated rats (Nabavi et al. 2012).

At the end of the treatment period, animals of the different groups were killed by cervical decapitation to avoid stress. Blood was collected from the trunk into heparinized tubes and centrifuged at 2200 × g for 10 min. Plasma samples were drawn and stored at -20°C until analysis. Livers were dissected out, cleaned and weighed. Some samples were rinsed and homogenized (10% w/v) in Tris-HCl buffer (pH = 7.4) and centrifuged. The resulting supernatants were used for biochemical assays. Other samples were immediately removed, cleaned and used either for RNA extraction and DNA integrity evaluation or fixed in 10% buffered formalin solution and embedded in paraffin for histological studies.

Biochemical estimations

Protein quantification

Liver protein contents were measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Liver MDA assay

The liver MDA concentrations, index of lipid peroxidation, were determined spectrophotometrically according to Draper and Hadley (1990). The MDA values were calculated using TEP as standard and expressed as nmol MDA/mg protein.

Liver hydrogen peroxide content

Hydrogen peroxide (H₂O₂) content in liver tissue was determined according to the ferrous ion oxidation-xylenol orange method (Ou and Wolff 1996). Results were expressed as nmol/mg protein.

Liver advanced oxidation protein product levels

Advanced oxidation protein product (AOPP) levels were determined according to the method of Kayali et al. (2006). The concentration of AOPP for each sample was calculated using the extinction coefficient of 261 cm⁻¹·mM⁻¹ and the results were expressed as nmol/mg protein.

Liver protein carbonyls content

Protein carbonyls (PCO) were measured using the method of Reznick and Packer (1994). The carbonyl content was calculated based on the molar extinction coefficient of DNPH ($\epsilon = 2.2 \times 10^4 \text{ cm}^{-1} \cdot \text{M}^{-1}$) and expressed as nmol/mg protein.

Liver GSH content

The GSH content of the liver homogenate was determined by Ellman's method (Ellman 1959) modified by Jollow et al. (1974) based on the development of a yellow color when DTNB was added to compounds containing sulfhydryl groups. The concentration of GSH was expressed as µg/mg protein.

Liver non-protein thiols content

Liver non-protein thiol (NPSH) levels were determined by the method of Ellman (1959) and results were expressed as µmol/mg protein.

Liver vitamin C assay

Vitamin C assay was performed as described by Jacques-Silva et al. (2001). The data were expressed as µmol of ascorbic acid/mg protein.

Determination of antioxidant enzyme activities in liver

Catalase (CAT) activity was assayed by the method of Aebi (1984). Results were expressed as µmol H₂O₂ consumed/min/mg protein.

Superoxide dismutase (SOD) activity was estimated according to Beauchamp and Fridovich (1971). Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as units/mg protein.

Glutathione peroxidase (GPx) activity was measured according to Flohe and Gunzler (1984). The enzyme activity was expressed as nmol of GSH oxidized/min/mg protein.

Liver metallothionein content

Metallothionein (MT) content in liver was assayed according to the method of Viarengo et al. (1997) modified by Petrovic et al. (2001) and results were expressed as µmol GSH/g tissue.

Biomarkers of liver toxicity in plasma

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltranspeptidase (γGT) activities and albumin levels were assayed

spectrophotometrically in plasma according to the standard procedures using commercially available diagnostic kits (Biomaghreb, Tunisia, Ref. 20049, 20048, 20017, 20022 and 20094, respectively).

Liver RNA extraction

50 mg of liver tissue were used to extract total RNA using kit purchased from Invitrogen (Pure Link RNA ref 12183018A) according to the manufacturer's recommendations. To check the purity of RNA, electrophoresis was performed for its integrity and the optical density (OD) was measured. All samples must have OD values between 1.7 and 1.9 based on the 260/280 ratio.

Semi-quantitative RT-PCR

2 µg of total mRNA served to produce cDNA by reverse transcription with MMLuv reverse transcriptase using oligo (dT) as a primer in a total volume of 20 µl. Oligo (dT) primed first strand cDNA was prepared from liver RNA using MMLuv reverse transcriptase at 37°C for 60 min. PCR was performed with gene specific primers using Taq DNA polymerase (Invitrogen, France). The primers used for the gene amplification were illustrated in Table 1. Initial denaturation was performed at 94°C for 5 min, annealing from 60°C and extension at 72°C for 1 min. Expression of GAPDH, the housekeeping gene, served as the control. The number of amplification cycles was determined using individual primer sets to maintain exponential product amplification (30–35 cycles). Electrophoresis through 1% agarose gel allowed separation of the amplified PCR products. cDNA bands were stained with ethidium bromide and then visualized by ultraviolet illumination.

DNA integrity evaluation

The DNA was extracted according to the standard procedures using commercially available diagnostic kits (Pure Link Genomic DNA Invitrogen ref K 182001). To verify the extent of DNA damage in the liver, full genomic extracted DNA smear technique was performed by electrophoresis in

agarose gel which was observed under an ultraviolet lamp and then photographed.

Histological examination

Livers were placed in 10% buffered formalin solution. They were embedded in paraffin, sectioned at a thickness of 5 µm and stained with hematoxylin-eosin for histological studies. Six slides were prepared from each liver.

Statistical analysis

All data were presented as mean ± SD and were analyzed using the statistical package program Stat view 5 Software for Windows (SAS Institute, Berkley, CA). One way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test as a post hoc test was performed for statistical comparison between groups. When comparison between two groups was required, we have used Student unpaired *t*-test. Differences were considered significant if $p < 0.05$.

Results

Estimation of lipid peroxidation

In the liver of glyphosate-treated rats, MDA levels significantly increased by 69% compared to those of control group. Co-treatment with QE (20 mg/kg b.w./day) decreased the hepatic MDA content by 23% when compared to glyphosate group without reaching control values (Table 2).

H₂O₂ production

The levels of H₂O₂ generated in the liver were increased by 44% in glyphosate group when compared to controls. In Gly+QE group, H₂O₂ level was significantly reduced by 23% when compared to glyphosate group without reaching control values (Table 2).

Table 1. Primer sequences for PCR amplification

Genes	Primer sequences	Accession n ^o	Length (bp)	Reference
<i>MTI</i>	Forward : 5'-CACCGTTGCTCCAGATTCAC-3'	RATMETI	238	Dai et al. 2013
	Reverse: 5'-GCAGCAGCACTGTTTCGTCAC-3'			
<i>MTII</i>	Forward : 5'-ATCTCCAAGTCCCGCCTCC-3'	XM 001062488	198	
	Reverse: 5'-TGCACTTGTCGGAAGCCTCT-3'			
<i>GAPDH</i>	Forward: 5'-CCTCTCTCTTGCTCTCAGTAT-3'	NM 017008	341	Alok et al. 2010
	Reverse: 5'-GGGTTCCATGGTGAAGTCAAC-3			

Table 2. MDA, H₂O₂, AOPP and PCO levels in liver of control (C) and treated rats with glyphosate (Gly) or glyphosate with quercetin (Gly+QE)

Parameter (nmol/mg protein)	Group		
	C	Gly	Gly+QE
MDA	1.538 ± 0.264	2.612 ± 0.267***	2.005 ± 0.213**, ^{£££}
H ₂ O ₂	0.085 ± 0.002	0.125 ± 0.016***	0.103 ± 0.013**, [£]
AOPP	0.146 ± 0.014	0.291 ± 0.014***	0.212 ± 0.033***, ^{£££}
PCO	2.214 ± 0.017	3.433 ± 0.018***	2.656 ± 0.021**, ^{£££}

Values were expressed as means ± SD. The number of determinations was $n = 6$. ** $p < 0.01$, *** $p < 0.001$ vs. control group; [£] $p < 0.05$, ^{£££} $p < 0.001$ vs. glyphosate group. MDA, malondialdehyde; H₂O₂, hydrogen peroxide; AOPP, advanced oxidation protein products; PCO, protein carbonyls; QE, quercetin.

Markers of protein oxidative damage in the liver

In the glyphosate group, a significant increase of liver AOPP and PCO levels by 93 and 55%, respectively, was observed when compared to those of controls. The administration of QE at 20 mg/kg b.w./day to glyphosate-treated rats ameliorated AOPP and PCO levels by 28 and 22% respectively, when compared to Gly group, without reaching control values (Table 2).

Non-enzymatic antioxidant status in the liver

Our results showed a significant decrease in the levels of GSH (-42%), NPSH (-41%) and vitamin C (-44%) in rats exposed to glyphosate, when compared to controls. Supplementation of QE resulted in a partial recovery in the levels of these non-enzymatic antioxidants which increased by 38, 28 and 41%, respectively, when compared to Gly group (Table 3).

Enzymatic antioxidant status in the liver

Antioxidant enzyme activities of SOD, GPx and CAT in control and treated groups are represented in Figure 1. Glyphosate treatment led to a significant decrease in SOD, GPx and CAT activities by 45, 37 and 32% compared to those of control group. Treatment with QE restored partially the

activities of these antioxidant enzymes which increased by 30, 37 and 29%, respectively, when compared to glyphosate-treated rats.

Effects of glyphosate on biochemical markers of liver toxicity in plasma

AST and ALT activities in plasma of Gly group increased by 71 and 75%, respectively. However, plasma ALP and γ GT activities as well as albumin level decreased by 27, 50 and 21%, respectively, in Gly group when compared to those of controls (Table 4). Oral administration of QE (20 mg/kg b.w./day) ameliorated the levels of AST (-22%), ALT (-29%), ALP (+14%), γ GT (+33%) and albumin (+18%) when compared to glyphosate-treated rats without reaching control values.

Total MT level and MTI and MTII genes expression in the liver

There was a significant increase of total MT level by 70% in liver of rats treated with glyphosate when compared to those of controls (Figure 2A). The expression of MT I and MT II mRNA was also increased in the liver of glyphosate-treated groups as compared to that of control group (Figure 2B). Treatment with QE reduced the level of total MT by 31% as

Table 3. Non-enzymatic antioxidant levels (GSH, NPSH and vitamin C) in liver of control (C) and treated rats with glyphosate (Gly) and glyphosate with quercetin (Gly+QE)

Parameter	Group		
	C	Gly	Gly+QE
GSH (μ g/mg protein)	1.383 ± 0.070	0.796 ± 0.081***	1.096 ± 0.205**, ^{££}
NPSH (μ mol/mg protein)	1.723 ± 0.165	1.022 ± 0.063***	1.232 ± 0.106**, ^{££}
Vitamin C (μ mol/mg protein)	1.860 ± 0.160	1.048 ± 0.128***	1.476 ± 0.183**, ^{£££}

Values were expressed as means ± SD. The number of determinations was $n = 6$. ** $p < 0.01$, *** $p < 0.001$ vs. control group; ^{££} $p < 0.01$, ^{£££} $p < 0.001$ vs. glyphosate group. GSH, reduced glutathione; NPSH, non-protein thiols; QE, quercetin.

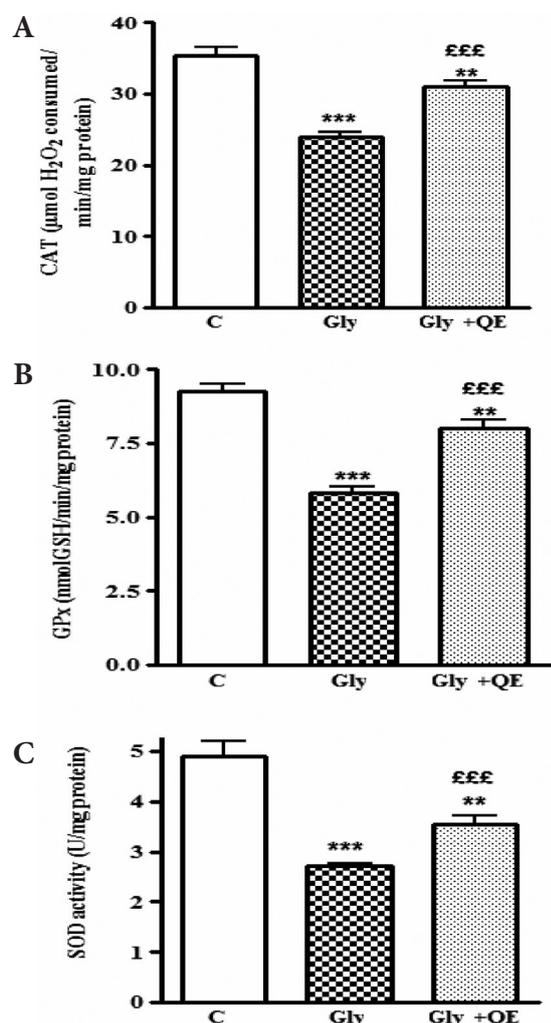


Figure 1. Antioxidant enzyme activities of superoxide dismutase (SOD; A), glutathione peroxidase (GPx; B) and catalase (CAT; C) in the liver of control (C) and treated rats with glyphosate (Gly) or glyphosate along with quercetin (Gly+QE). Values are means \pm SD for six rats in each group. ** $p < 0.01$, *** $p < 0.001$ vs. C group; $^{\text{***}}$ $p < 0.001$ vs. Gly group.

compared to glyphosate-treated rats and down-regulated the genes expression of MT I and MT II.

Effect of glyphosate on oxidative DNA damage

As shown in Figure 3, agarose gel electrophoresis showed undetectable DNA laddering in the liver of control rats. A smear (hallmark of necrosis) on agarose gel, indicating random DNA degradation, was observed through the lane of DNA liver samples of glyphosate-treated rats. However, rats treated with QE and glyphosate showed a slight decrease in DNA smearing as compared to that of glyphosate-treated rats.

Histological examination

The effect of glyphosate exposure on the liver structural integrity was evaluated by histological analysis. Light microscopic examination indicated a normal structure of the liver in controls (Figure 4A). Nevertheless, remarkable morphological changes such as the presence of polynuclear giant cells (Figure 4B), a sinusoidal dilatation (Figure 4C), an infiltration of inflammatory leukocytes (Figure 4D), a fibrosis (Figure 4C and E), a cellular degeneration and a focal hepatic necrosis (Figure 4C and E) were observed in the liver of glyphosate-treated rats. These histological alterations were significantly attenuated in the liver of Gly+QE group as compared to that of Gly group (Figure 4F).

Discussion

Glyphosate, one of the most commonly applied herbicides worldwide, has been previously described to alter the hepatic redox status in rats (El-Shenawy 2009). Thus, antioxidant therapy could be considered as a good strategy to mitigate liver damage induced by this herbicide. Therefore, the present study was designed to examine whether co-treatment with QE, a plant flavonoid with potent antioxidant action,

Table 4. Enzyme activities and albumin level in the plasma of control (C) and treated rats with glyphosate (Gly) and glyphosate with quercetin (Gly+QE)

Parameter	Group		
	C	Gly	Gly+QE
ALT (IU/l)	26.501 \pm 1.378	46.500 \pm 4.549***	33.166 \pm 4.167** ^{££}
AST (IU/l)	113.333 \pm 18.018	193.666 \pm 7.174***	151.500 \pm 14.761** ^{£££}
ALP (IU/l)	375.833 \pm 14.427	273.166 \pm 21.3461***	310.333 \pm 38.629** [£]
γ GT (IU/l)	0.055 \pm 0.004	0.025 \pm 0.002***	0.038 \pm 0.009** ^{££}
Albumin (g/l)	32.250 \pm 1.172	25.500 \pm 1.516***	30.041 \pm 1.122** ^{£££}

Values are expressed as mean \pm SD. The number of determinations was $n = 6$. ** $p < 0.01$, *** $p < 0.001$ vs. C group; $^{\text{£}}$ $p < 0.05$, $^{\text{££}}$ $p < 0.01$, $^{\text{£££}}$ $p < 0.001$ vs. Gly group. AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; γ GT, gamma glutamyltranspeptidase; QE, quercetin.

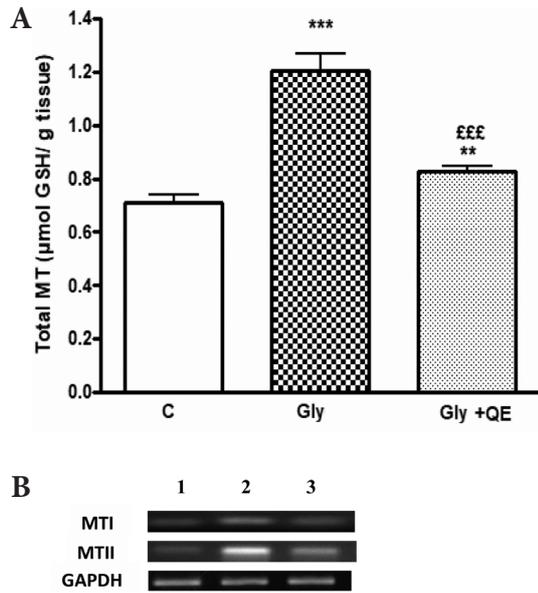


Figure 2. Levels of total MT (A) and genes expression (B) of metallothioneins MT I and MT II mRNA in the liver of control (C) and treated rats with glyphosate (Gly) or glyphosate along with quercetin (Gly+QE). ** $p < 0.01$, *** $p < 0.001$ vs. C group; ^{£££} $p < 0.001$ vs. Gly group.

could potentially have a protective effect against glyphosate-induced hepatotoxicity.

In general, one of the most established mechanisms of pesticides toxicity is their ability to induce oxidative stress through an overproduction of ROS which react with cellular biomolecules. Biological membranes contain high amounts of polyunsaturated fatty acids, which are particularly susceptible to peroxidative attacks. The results of the present study showed that glyphosate treatment induced hepatic oxidative damage, as evidenced by the increased levels of MDA, the end product of lipid peroxidation, in the liver tissue of rats exposed to this herbicide. Our results were consistent with the previous findings of El-Shenawy (2009) in liver of rats treated with sub-lethal doses of glyphosate. Furthermore, other works have shown elevated MDA levels in tissues like liver, brain, testes and plasma of glyphosate-based herbicides-treated rats (Astiz et al. 2009a, 2009b). The enhanced lipid peroxidation observed under our experimental conditions might be ascribed to an excessive generation of ROS resulting from glyphosate exposure.

At the cellular level, ROS are produced *via* several mechanisms. As mitochondria are the primary intracellular sites of oxygen consumption, they may be the primary sites of ROS generation such as H₂O₂. In addition to the rise of MDA levels, we have recorded a significant increase of H₂O₂ levels in the liver of glyphosate-treated rats, suggesting mitochondria as a potential target of this pesticide

toxicity. In fact, glyphosate has been reported to be a mitochondrial toxin which uncouples mitochondrial oxidative phosphorylation (Bradberry et al. 2009). Once formed, H₂O₂ readily diffuses through cellular membranes to reach sites distant from where it was generated. In the presence of transition metals, it can be reduced to the hydroxyl radical, which in turn interacts with membrane lipids to initiate lipid peroxidation and provokes tissue damage.

It is widely accepted that lipid peroxidation products, such as MDA, are implicated in cell genotoxicity (Singh et al. 2011). In the present study, the enhanced hepatic MDA levels caused by glyphosate have been suggested to provoke DNA damage in rats' hepatocytes. Indeed, as revealed by agarose gel electrophoresis, glyphosate treatment resulted in DNA shearing reflecting the genotoxic potential of this pesticide. On the other hand, pesticide biotransformation often results in the generation of ROS, which are highly toxic and cause oxidative damage to DNA. So, it is possible that the depressed DNA integrity observed in hepatocytes of glyphosate-treated rats could be due to the direct reaction of ROS, generated by the metabolism of the herbicide, with the DNA of exposed animals. Our results were in line with data reported by Astiz et al. (2009) after sub-chronic exposure of rats to glyphosate. Moreover, Roundup®, a glyphosate-based herbicide, has been previously demonstrated to provoke DNA lesions in the kidney and the liver of mice (Peluso et al. 1998).

Administration of QE along with glyphosate significantly modulated lipid peroxidation and decreased H₂O₂ generation, indicating its important role in providing protection against oxidative damage. QE is recognized as a potent free radical scavenger able to inhibit the lipid peroxidation process. According to Moridani et al. (2003), the advanced diffusion of this flavonoid through biological membranes allows it to scavenge oxyradicals at numerous sites of the lipid bilayer. Moreover, QE is able to react with ROS, decreases DNA damage and prevents the tumorigenic processes (Murota and Terao 2003).

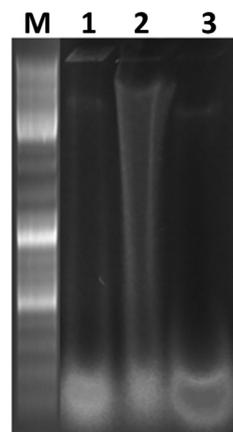


Figure 3. Agarose gel electrophoresis of depressed DNA integrity. DNA isolated from experimental liver tissues was loaded into 1% agarose gel. M, marker (3 kb DNA ladder); Lane 1, DNA isolated from control liver sample; Lane 2, DNA isolated from glyphosate liver sample; Lane 3, DNA isolated from glyphosate+quercetin liver sample.

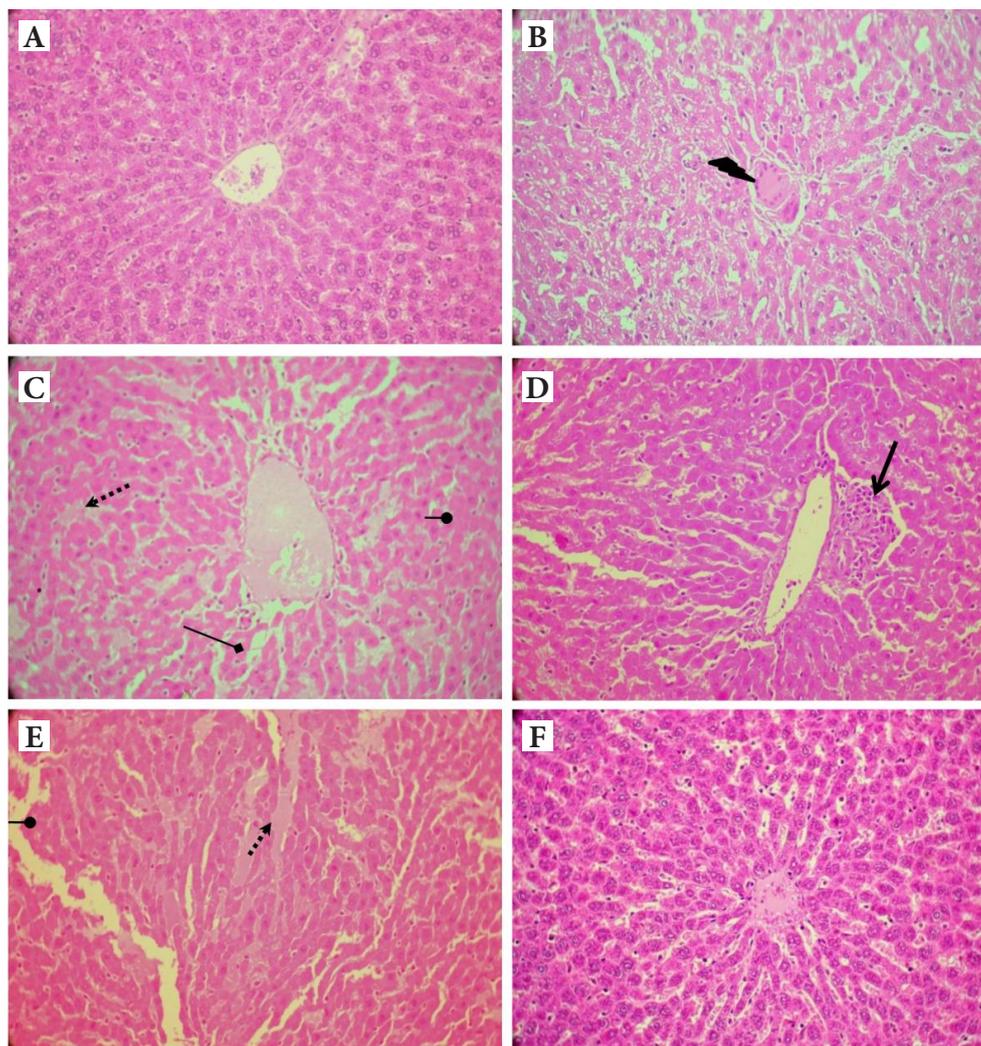


Figure 4. Liver histological sections of control (A) and treated rats with glyphosate (B, C, D, E) or glyphosate along with quercetin (F). Optic microscopy: H&E, magnification $\times 200$. Arrows indicate: \blacktriangledown polynuclear giant cells, \blacktriangledown sinusoidal dilatation, \blacktriangleleft infiltration of inflammatory leukocytes, \cdots fibrosis, \blacktriangledown cellular degeneration and focal hepatic necrosis.

Likewise, free radicals attack not only lipids and DNA but also proteins. In the present study, our results revealed a significant increase of hepatic PCO and AOPP levels in glyphosate-treated group compared with the control group.

Interestingly, QE prevented the increase in the hepatic levels of protein oxidation biomarkers. In fact, QE is recognized to have a powerful oxygen radicals scavenging activity and protects proteins against oxidative stress in methimazole-treated rats (Santi et al. 2014).

To counteract the deleterious effects of oxidative stress, mammalian cells are equipped with enzymatic and non-enzymatic antioxidant systems which work together to combat oxidative stress. Within the first line of cellular

defense, there are the key enzymatic components which limit the effects of oxidant molecules in tissues by means of their free radical scavenging properties. In the current work, glyphosate treatment decreased significantly hepatic activities of SOD, CAT and GPx. This result suggested that the inhibition of antioxidant enzyme activities might be considered as a potential mechanism by which this herbicide could induce oxidative stress.

Treatment with QE significantly enhanced the activities of enzymatic antioxidants which substantiated its strong capacity to scavenge ROS. In fact QE, a potent antioxidant, is known to modulate the activities of different enzymes through its interaction with various biomolecules (de David et al. 2011).

The second line of cellular defense against oxidative stress is offered by non-enzymatic antioxidants with low molecular weight. In this regard, NPSH are among the most important non-enzymatic antioxidants which present a variety of functions in the reduction and detoxification processes. In our study, a marked depletion in the hepatic contents of NPSH and GSH was observed following glyphosate exposure, which might be attributed to their consumption in removing H_2O_2 and other peroxides produced in excess due to oxidative stress. GSH reduction in glyphosate-treated rats might also explain the decreased hepatic levels of vitamin C which is known to enter the cell mainly in its oxidized form where it is reduced by GSH. This vitamin is a hydrophilic reducing agent which directly reacts with ROS, such as superoxide radicals, as well as various lipid hydroperoxides more efficiently than any other water soluble antioxidant (Briviba and Sies 1994). So, the observed reduction in vitamin C level could also be ascribed to its increased utilization in trapping the oxy-radicals resulting from glyphosate exposure.

The improvement in the hepatic GSH status following QE supplementation to glyphosate-treated rats indicated that this flavonoid is a strong inducer of GSH content. Our results were in accordance with those of Molina et al. (2003) who have shown that QE protects liver against ethanol-induced oxidative damage in mouse by enhancing the hepatic level of GSH.

Another interesting group of non-enzymatic antioxidants, metallothioneins (MTs), plays a central role in the cellular defense against oxidative stress. Because of their high thiol content, these proteins can effectively scavenge several types of ROS (Miles et al. 2000). It has been demonstrated that MT genes are readily induced by oxidative stress resulting from pesticides intoxication (Miles et al. 2000). In our experimental study, we have found in the liver of glyphosate-treated rats a significant increase of total MT. MT I and MT II gene expression levels were also significantly up-regulated. The observed induction of MTs under our experimental conditions reflected probably an adaptive response of the liver tissue to the toxicological manifestations induced by this herbicide. To our knowledge, the present study is the first attempt evaluating the effect of glyphosate on the genes expression of the two isoforms MT I and MT II in rat by means of semi quantitative PCR. The possible mechanism of MT protection against glyphosate liver injury might be due to the twenty cysteine residues, constituting a part of its structure, which are involved in quenching hydroxyl and superoxide radicals (Chiaverini and De Ley 2010).

MT induction in the hepatic tissue of glyphosate-treated rats was significantly attenuated by QE which could be attributed to its direct ROS scavenging capacity.

In the present study, the impairment of the enzymatic and non-enzymatic antioxidant status caused by glyphosate exposure could enhance the susceptibility to oxidative

damage in hepatic cells and contribute to hepatocellular dysfunction. Liver enzymes such as AST, ALT, ALP and γ GT are considered to be the important markers of hepatic function. In our findings, we demonstrated that glyphosate, administered to rats, provoked a marked increase in plasma AST and ALT activities, indicating hepatocellular damage. The excessive production of free radicals and lipid peroxides might cause an alteration of membrane permeability leading to the leakage of these cytosolic enzymes from the hepatic tissue. When QE was administered to glyphosate-treated rats, transaminase activities in plasma were decreased significantly, suggesting that QE probably prevented the leakage of these marker enzymes by keeping the structural integrity of the liver. This hepatoprotective effect of QE may be mainly due to its anti-lipoperoxidative, antioxidant and membrane stabilizing properties as reported by Renugadevi and Milton Prabu (2010) in cadmium-treated rats co-administered with QE. On the other hand, glyphosate administration led to a significant decrease in the plasma activities of ALP and γ GT. The latter enzyme is involved in the catabolism of extracellular GSH, providing amino acids to be assimilated and reutilized as precursors for intracellular GSH synthesis (Lee et al. 2004). Inhibition of this enzyme activity could account for the low cytoplasmic GSH levels recorded following glyphosate exposure.

In addition, albumin, the most abundant plasma protein synthesized by the liver, represents another biomarker of hepatic function (Chaâbane et al. 2015). The observed reduction of plasma albumin level in glyphosate-treated group could be attributed to the direct interaction with albumin. In fact, it has been previously demonstrated that glyphosate binds to human serum albumin causing an alteration of the protein secondary structure (Yue et al. 2008).

Administration of QE attenuated glyphosate-induced hepatic oxidative injury as shown by the increased levels of ALP, γ GT and albumin, reflecting its hepatoprotective effect which might be related to its antioxidant potential.

To substantiate the biochemical findings, a histological examination of the liver was undertaken. In fact, histopathological examination of the hepatic tissue revealed that glyphosate treatment caused abnormal ultrastructural changes including the presence of polynuclear giant cells, suggesting a cell cycle disturbance, a sinusoidal dilatation, a fibrosis, a cellular degeneration and a focal hepatic necrosis. These histological changes might be due to the formation of highly reactive radicals and subsequent lipid peroxidation induced by this herbicide.

Administration of QE to glyphosate-treated rats was quite appreciable as it reduced the histological alterations. This effect could be attributed to the antioxidant and free radical scavenging properties of QE, which significantly reduced the oxidative insult leading to the reduction of pathological changes and restoration of normal physiological functions.

Conclusion

Results from the present study clearly confirmed that glyphosate induced redox status unbalance and up-regulated metallothionein (MT I and MT II) genes expression in the liver of adult rats. Our findings demonstrated also that QE administration had a marked protective effect against glyphosate hepatotoxicity which seemed to be related to its strong antioxidant activity. Further studies are needed to elucidate the exact signaling pathways targeted by QE and mediating its hepatoprotective action in response to glyphosate-induced hepatic oxidative damage in rats.

Conflict of interest. The authors declare that they have no conflict of interest.

Acknowledgment. This work was supported by the Ministry of Higher Education and Scientific Research [UR 13/ES-35], Tunisia. The authors are thankful to Mr. Chedli Tmar for the laboratory animals' maintenance and to Mrs. Raoudha Ben Amar Abdennadher for her skillful technical assistance.

References

- Aebi H (1984): Catalase in vitro. *Methods. Enzymol.* **105**, 121–126
[https://doi.org/10.1016/S0076-6879\(84\)05016-3](https://doi.org/10.1016/S0076-6879(84)05016-3)
- Alok K, Ram L, Singh G, Nagesh B (2010): Cell death mechanisms in the early stages of acute glutamate neurotoxicity. *Neurosci. Res.* **66**, 271–278
<https://doi.org/10.1016/j.neures.2009.11.009>
- Astiz M, de Alaniz MJT, Marra CA (2009a): Effect of pesticides on cell survival in liver and brain rat tissues. *Ecotoxicol. Environ. Saf.* **72**, 2025–2032
<https://doi.org/10.1016/j.ecoenv.2009.05.001>
- Astiz M, de Alaniz MJT, Marra CA (2009b): The impact of simultaneous intoxication with agrochemicals on the antioxidant defense system in rat. *Pestic. Biochem. Physiol.* **94**, 93–99
<https://doi.org/10.1016/j.pestbp.2009.03.005>
- Beauchamp C, Fridovich I (1971): Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**, 276–287
[https://doi.org/10.1016/0003-2697\(71\)90370-8](https://doi.org/10.1016/0003-2697(71)90370-8)
- Boots AW, Haenen GR, Bast A (2008): Health effects of quercetin: from antioxidant to nutraceutical. *Eur. J. Pharmacol.* **585**, 325–337
<https://doi.org/10.1016/j.ejphar.2008.03.008>
- Bradberry SM, Proudfoot AT, Vale JA (2004): Glyphosate poisoning. *Toxicol. Rev.* **23**, 159–167
<https://doi.org/10.2165/00139709-200423030-00003>
- Briviba K, Sies H (1994): Non enzymatic antioxidant defense systems. In: *Natural Antioxidants in Human Health and Disease* (Ed. B. Frei), pp. 107–128, Academic Press, San Diego
- Cattani D, de Liz Oliveira Cavalli VL, Heinz Rieg CE, Domingues JT, Dal-Cim T, Tasca CI, Mena Barreto Silva FR, Zamoner A (2014): Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: Involvement of glutamate excitotoxicity. *Toxicology* **320**, 34–45
<https://doi.org/10.1016/j.tox.2014.03.001>
- Chaâbane M, Soudani N, Benjeddou K, Turki M, Ayadi Makni F, Boudawara T, Zeghal N, Ellouze Ghorbel R (2015): The protective potential of *Nitraria retusa* on penconazole-induced hepatic injury in adult rats. *Toxicol. Environ. Chem.* **97**, 1253–1264
<https://doi.org/10.1080/02772248.2015.1093633>
- Chiaverini N, De Ley M (2010): Protective effect of metallothionein on oxidative stress-induced DNA damage. *Free. Radic. Res* **44**, 605–613
<https://doi.org/10.3109/10715761003692511>
- Council of European Communities (1986): Council instructions about the protection of living animals used in scientific investigations. *OJEC (JO 86/609/CEE)* **358**, 1–18
- Dai S, Yina Z, Yuanb G, Lua H, Jiaa R, Xua J, Song X, Li L, Shua Y, Liang X, He C, Lva C, Zhang W (2013): Quantification of metallothionein in the liver and kidney of rats by subchronic lead and cadmium in combination. *Environ. Toxicol. Phar.* **36**, 1207–1216
<https://doi.org/10.1016/j.etap.2013.10.003>
- de David C, Rodrigues G, Bona S, Meurer L, González-Gallego J, Tu-ón MJ, Marroni NP (2011): Role of quercetin in preventing thioacetamide-induced liver injury in rats. *Toxicol. Pathol.* **39**, 949–957
<https://doi.org/10.1177/0192623311418680>
- de Liz Oliveira Cavalli VL, Cattani D, Heinz Rieg CE, Pierozan P, Zanatta L, Benedetti Parisotto E, Wilhelm Filho D, Mena Barreto Silva FR, et al. (2013): Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells. *Free. Radic. Biol. Med.* **65**, 335–346
<https://doi.org/10.1016/j.freeradbiomed.2013.06.043>
- Draper HH, Hadley M (1990): Malondialdehyde determination as index of lipid peroxidation. *Methods. Enzymol.* **186**, 421–431
[https://doi.org/10.1016/0076-6879\(90\)86135-1](https://doi.org/10.1016/0076-6879(90)86135-1)
- Ellman GL (1959): Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70–77
[https://doi.org/10.1016/0003-9861\(59\)90090-6](https://doi.org/10.1016/0003-9861(59)90090-6)
- El-Shenawy NS (2009): Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate. *Environ. Toxicol. Pharmacol.* **28**, 39–85
<https://doi.org/10.1016/j.etap.2009.06.001>
- Ferrali M, Signorini C, Ciccoli L, Bambagioni S, Rossi V, Pompella A, Comporti M (2000): Protection of erythrocytes against oxidative damage and autologous immunoglobulin G (IgG) binding by iron chelatorfluorbenzoil-pyridoxalhydrazone. *Biochem. Pharmacol.* **59**, 1365–1373
[https://doi.org/10.1016/S0006-2952\(00\)00273-2](https://doi.org/10.1016/S0006-2952(00)00273-2)
- Flohe L, Gunzler WA (1984): Assays of glutathione peroxidase. *Methods. Enzymol.* **105**, 114–121
[https://doi.org/10.1016/S0076-6879\(84\)05015-1](https://doi.org/10.1016/S0076-6879(84)05015-1)
- Guyton KZ, Loomis D, Grosse Y, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Scoccianti C, Mattock H, Straif K (2015): Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. *Lancet. Oncol.* **16**, 490–491
[https://doi.org/10.1016/S1470-2045\(15\)70134-8](https://doi.org/10.1016/S1470-2045(15)70134-8)
- Jacques-Silva MC, Nogueira CW, Broch LC, Flores EMM, Rocha JBT (2001): Diphenyl diselenide and ascorbic acid changes

- deposition of selenium and ascorbic acid in liver and brain of mice. *Pharmacol. Toxicol.* **88**, 119–125
<https://doi.org/10.1034/j.1600-0773.2001.d01-92.x>
- Jalili T, Carlstrom J, Kim S, Freeman D, Jin H, Wu TC, Litwin SE, David Symons J (2006): Quercetin-supplemented diets lower blood pressure and attenuate cardiac hypertrophy in rats with aortic constriction. *J. Cardiovasc. Pharmacol.* **47**, 531–541
<https://doi.org/10.1097/01.fjc.0000211746.78454.50>
- Jiraungkoorskul W, Upatham ES, Kruatrachue M, Sahaphong S, Vichasri-Grams S, Pokethitiyook P (2002): Histopathological effects of Roundup, a glyphosate herbicide, on Nile tilapia (*Oreochromis niloticus*). *Science* **28**, 121–127
- Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR (1974): Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology* **11**, 151–169
<https://doi.org/10.1159/000136485>
- Kayali R, Cakatay U, Akcay T, Altug T (2006): Effect of alpha-lipoic acid supplementation on markers of protein oxidation in post mitotic tissues of ageing rat. *Cell. Biochem. Funct.* **24**, 79–85
<https://doi.org/10.1002/cbf.1190>
- Kumar A, Ahmad I, Shukla S, Singh BK, Patel DK, Pandey HP, Singh C (2010): Effect of Zn and PQ co-exposure on neurodegeneration: modulation of oxidative stress and expression of metallothioneins, toxicant responsive and transporter genes in rats. *Free Radic. Res.* **44**, 950–965
<https://doi.org/10.3109/10715762.2010.492832>
- Lamson DW, Brignall MS (2000): Antioxidants and cancer, part 3: quercetin. *Altern. Med. Rev.* **5**, 196–208
- Lee CC, Shen SR, Lai YJ, Wu SC (2013): Rutin and quercetin, bioactive compounds from tartary buckwheat, prevent liver inflammatory injury. *Food Funct.* **4**, 794–802
<https://doi.org/10.1039/c3fo30389f>
- Lee DH, Blomhoff R, Jacobs DR, Jr (2004): Is serum gamma glutamyltransferase a marker of oxidative stress? *Free Radic. Res.* **38**, 535–539
<https://doi.org/10.1080/10715760410001694026>
- Lin SY, Wang YY, Chen WY, Chuang YH, Pan PH, Chen. J (2014): Beneficial effect of quercetin on cholestatic liver injury. *J. Nutr. Biochem.* **25**, 1183–1195
<https://doi.org/10.1016/j.jnutbio.2014.06.003>
- Lowry OH, Rosebrugh NJ, Farr AL, Randall RJ (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
- Miles AT, Hawksworth GM, Beattie JH, Rodilla V (2000): Induction, regulation degradation, and biological significance of mammalian metallothioneins. *Crit. Rev. Biochem. Mol. Biol.* **35**, 35–70
<https://doi.org/10.1080/10409230091169168>
- Molina M, Sanchez-Reus I, Iglesias I, Benedi J (2003): Quercetin, a flavonoid antioxidant, prevents and protects against ethanol induced oxidative stress in mouse liver. *Biol. Pharm. Bull.* **10**, 1398–1402
<https://doi.org/10.1248/bpb.26.1398>
- Moridani MY, Pourahmad J, Bui H, Siraki A, O'Brien PJ (2003): Dietary flavonoid iron complexes as cytoprotective superoxide radical scavengers. *Free Radic. Biol. Med.* **34**, 245–253
[https://doi.org/10.1016/S0891-5849\(02\)01241-8](https://doi.org/10.1016/S0891-5849(02)01241-8)
- Murota K, Terao J (2003): Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism. *Arch. Biochem. Biophys.* **417**, 12–17
[https://doi.org/10.1016/S0003-9861\(03\)00284-4](https://doi.org/10.1016/S0003-9861(03)00284-4)
- Nabavi SM, Nabavi S.F, Eslami S, Moghaddam AH (2012): In vivo protective effects of quercetin against sodium fluoride-induced oxidative stress in the hepatic tissue. *Food Chem.* **132**, 931–935
<https://doi.org/10.1016/j.foodchem.2011.11.070>
- Olorunsogo OO, Bababunmi EA (1980): Interference of herbicides with mitochondrial oxidative phosphorylation: the N⁻ (phosphonomethyl) glycine model. *Toxicol. Lett.* **5**, 148
- Ou P, Wolff SP (1996): A discontinuous method for catalase determination at near physiological concentrations of H₂O₂ and its application to the study of H₂O₂ fluxes within cells. *J. Biochem. Biophys. Methods* **31**, 59–67
[https://doi.org/10.1016/0165-022X\(95\)00039-T](https://doi.org/10.1016/0165-022X(95)00039-T)
- Peluso M, Munnia A, Bolognesi C, Parodi S (1998): 32P-post labeling detection of DNA adducts in mice treated with the herbicide roundup. *Environ. Mol. Mutagen* **31**, 55–59
[https://doi.org/10.1002/\(SICI\)1098-2280\(1998\)31:1<55::AID-EM8>3.0.CO;2-A](https://doi.org/10.1002/(SICI)1098-2280(1998)31:1<55::AID-EM8>3.0.CO;2-A)
- Petrovic S, Ozretic B, Krajnovic-Ozretic M, Bobinac D (2001): Lysosomal membrane stability and metallothioneins in digestive gland of mussels (*Mytilus galloprovincialis* Lam.) as biomarkers in a field study. *Mar. Pollut. Bull.* **42**, 1373–1378
[https://doi.org/10.1016/S0025-326X\(01\)00167-9](https://doi.org/10.1016/S0025-326X(01)00167-9)
- Renugadevi J, Milton Prabu S (2010): Quercetin protects against oxidative stress-related renal dysfunction by cadmium in rats. *Exp. Toxicol. Pathol.* **62**, 471–481
<https://doi.org/10.1016/j.etp.2009.06.006>
- Reznick AZ, Packer L (1994): Oxidative damage to proteins: spectrophotometric method for carbonyl. *Methods. Enzymol.* **233**, 357–363
[https://doi.org/10.1016/S0076-6879\(94\)33041-7](https://doi.org/10.1016/S0076-6879(94)33041-7)
- Samsel A, Seneff S (2013): Glyphosate's suppression of cytochrome P450 enzymes and amino acid biosynthesis by the gut microbiome: Pathways to modern diseases. *Entropy* **15**, 1416–1463
<https://doi.org/10.3390/e15041416>
- Santi A, Baldissarelli J, Murussi CR, Dias GR, de Menezes CC, Zanini D, Abdalla FH, Thomé GR, Martins CC, Schetinger MR, Loro VL (2014): Effects of quercetin on oxidative stress biomarkers in methimazole-induced hypothyroid rats. *Exp. Clin. Endocrinol. Diabetes* **122**, 533–539
<https://doi.org/10.1055/s-0034-1376968>
- Sato M, Bremner I (1993): Oxygen free radicals and metallothionein. *Free. Radic. Biol. Med.* **14**, 325–337
[https://doi.org/10.1016/0891-5849\(93\)90029-T](https://doi.org/10.1016/0891-5849(93)90029-T)
- Sato M, Sasaki M (1991): Enhanced lipid peroxidation is not necessary for induction of metallothionein-I by oxidative stress. *Chem. Biol. Interact.* **78**, 143–154
[https://doi.org/10.1016/0009-2797\(91\)90010-5](https://doi.org/10.1016/0009-2797(91)90010-5)
- Singh S, Kumar V, Thakur S, Banerjee BD, Chandna S, Rautela RS, Grover SS, Rawat DS, Pasha ST, Jain SK, Ichhpujani RL, Rai A (2011): DNA damage and cholinesterase activity in occupational workers exposed to pesticides. *Environ. Toxicol. Pharmacol.* **31**, 278–285
<https://doi.org/10.1016/j.etap.2010.11.005>

- Swanson NL, Leu A, Abrahamson J, Wallet B (2014): Genetically engineered crops, glyphosate and the deterioration of health in the United States of America. *J. Organic Systems* **9**, 6–37
- Viarengo A, Ponzano E, Dondero F, Fabbri R (1997): A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic mollusks. *Mar. Environ. Res.* **44**, 69–84
[https://doi.org/10.1016/S0141-1136\(96\)00103-1](https://doi.org/10.1016/S0141-1136(96)00103-1)
- WHO (1994): Glyphosate. *Environmental Health Criteria* **159**, p. **161**, Geneva
- Williams GM, Kroes R, Munro IC (2000): Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Regul. Toxicol. Pharmacol.* **31**, 117–165
<https://doi.org/10.1006/rtph.1999.1371>
- Wunnapuk K, Gobe G, Endre Z, Peake R, Gricea JE, Roberts MS, Buckleyf NA, Liu X (2014): Use of a glyphosate-based herbicide-induced nephrotoxicity model to investigate a panel of kidney injury biomarkers. *Toxicol. Lett.* **225**, 192–200
<https://doi.org/10.1016/j.toxlet.2013.12.009>
- Yue Y, Zhang Y, Zhou L, Qin J, Chen X (2008): In vitro study on the binding of herbicide glyphosate to human serum albumin by optical spectroscopy and molecular modeling. *J. Photochem. Photobiol.* **90**, 26–32
<https://doi.org/10.1016/j.jphotobiol.2007.10.003>

Received: June 9, 2018

Final version accepted: November 5, 2018

First published online: March 1, 2019