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Title: Glyphosate disrupts redox status and up-regulates metallothionein I and II genes expression in the liver of adult rats. Alleviation by quercetin
Running title: Quercetin alleviates glyphosate hepatotoxicity
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Abstract
The present work evaluated the possible protective effects of quercetin against glyphosate-induced hepatotoxicity in adult rats. Rats received either glyphosate, quercetin or glyphosate along with quercetin during 15 days. Glyphosate (50 mg/kg bw) was administered every two days by intraperitoneal way while quercetin (20 mg/kg bw) 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, -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.

Keywords: glyphosate; hepatic oxidative stress; rats; metallothionein; quercetin

Changelog
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To Dr. Ľubica Lacinová, Editor-in-Chief

General Physiology and Biophysics

Dear editor,
We wish to thank you for your reply and for all the valuable comments that you and the reviewers have made. We are pleased to let you know that we have revised our article referred as 3042-18083 and we have brought modifications according to your comments and those of the reviewers.
So we submit our revised article entitled “Glyphosate disrupts redox status and up-regulates metallothionein I and II genes expression in the liver of adult rats. Alleviation by quercetin.” for publication in your journal.
You will therefore find below the necessary responses to your comments and those of the reviewers.

We hope that it will ensure the improvement sought by your honorable journal. See below a detailed explanation of each change and revision made.
Thanking you in anticipation for your consideration,

Dr. Mariem Chaâbane

Responses to the editor and reviewer comments are in red color.
All modifications in the revised manuscript are highlighted in yellow.

Responses to the editor comments

- Due to fact that on Figure 3 typical apoptotic DNA fragmentation was not observed, please replace "DNA fragmentation" in concerning texts by wording (depressed) DNA integrity.
- According to your recommendation, we have replaced “DNA fragmentation” in the manuscript by “depressed DNA integrity”. Please see page 17 line 316, page 31 line 604.

Responses to the reviewers’ comments

Reviewer 1:

- In your article is important and effective theme on characterisation of herbicide Gyphosate on liver damage. It was observed redox disruption (oxidative stress increase dcetected by several parammeters- MDA, hydrogen, peroxide, advanced oxidation protein products and protein carbonyl and activities of antioxidant enzymes). You observed enought outputs in plasma biomarkers of hepatotoxicity, up-regulation of metallothionein (MT I and MT II) genes expression and histopathological changes in your experimental rat model. You also tested changes of these effects by quercetin supllementatuion with improvement of liver and many other positive outputs. I am suggesting to accept your article, but you should control english writing, and correct small mistakes in text.
- As you recommended, we have revised the English language of the whole manuscript and made the necessary corrections. Please see the revised version of our manuscript.

Reviewer 2:

- The manuscript: „Glyphosate disrupts redox status and up-regulates metallothionein I and II genes expression in the liver of adult rats. Alleviation by quercetin” by Soudani et al. brings some novel data on the possibility to prevent glyphosate-induced liver damage with quercetin. However, I suggest make certain corrections to improve manuscript before publication:
- In Materials and Methods, part Animals and treatment: It should be clearly defined what was the vehicle/solution for glyfhosphate (water?), and how was this aplied to control group (i.p.?). In addition, it should be stated how was QE diluted for gavage (it is not water-soluble) and if groups

I and II (without QE treatment) received this vehicle/solution by gavage too. If not, this should be stated as the limitation of the study.

- The vehicle/solution for glyphosate was distilled water.
- Control rats received 1 ml of distilled water by intraperitoneal way every two days.
- Quercetin (20 mg/kg body weight) was dissolved in 2.5% dimethyl sulfoxide (DMSO). Groups I and II have not received this vehicle/solution by gavage.
- We have added the vehicle/solutions of glyphosate and quercetin in the Materials and Methods' section of the revised version of our manuscript (Please see page 6 lines 118-119, page 7, lines 120-121).

• In Figure 2B, the Results of gene expression should be quantified and normalized to housekeeper separately (as MT1/GAPDH; and MT2/GAPDH).

• We cannot quantify and normalize MT I and MTII genes expression because we don't have a densitometer. Indeed, we don't have means of funding to buy this machine as our country Tunisia faces an economic crisis.

• Please check if the doses of applied substances are shown correctly, e.g. dose of QE is expressed in mg/kg b.w./day but sometimes only as mg/kg b.w.

• We have changed throughout the manuscript "20 mg/kg b.w." by "20 mg/kg b.w./day". Please see page 2 line 25, page 7 line 127, page 12 lines 227 and 237, and page 13 line 255.

• Also, you state that glyphosate was applied every second day during 15 days. It is not clear whether animals received 7 or 8 doses of glyphsate (started at day1 or 2?). Please indicate total number of doses.

• Glyphosate injection started at the first day of the experiment. So, rats received 8 doses of glyphosate during the 15 days of the experimental period. We have added this information in the revised manuscript. Please see page 7, lines 122-123.

• In Discussion, the part suggesting Nrf2 signaling to be involved in the effects of QE in the liver is very speculative as no members of this pathway have been examined in the present study. Thus, this part should be removed from Discussion.

- The part discussing the involvement of Nrf2 signaling in the hepatoproetctive effects of quercetin has been removed from the discussion as you recommended. Please see the revised version of our manuscript.

• Paper needs slight language revision.

- We have revised our manuscript for English mistakes and made the appropriate corrections. Please see the revised version of our manuscript.

• We hope that these corrections are in accordance with your recommendations and will bring the expected improvement.

Our best regards,
Dr. Mariem Chaâbane
Animal Physiology laboratory
Sfax, Tunisia

Response to reviews:
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Tables:
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Tab. 4 - download

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

Running title: Quercetin alleviates glyphosate hepatotoxicity

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Abstract

The present work evaluated the possible protective effects of quercetin against glyphosate-induced hepatotoxicity in adult rats. Rats received either glyphosate, quercetin or glyphosate along with quercetin during 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, γ-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.

Keywords: glyphosate, hepatic oxidative stress, rats, metallothionein, quercetin.

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 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 (Kumar et al. 2010; Sato 1991). 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; Lee et al. 2013; Renugadevi and Milton Prabu 2010). 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

Active ingredient glyphosate (isopropylamine salt of n-phosphonomethylglycine) with a purity of 99.9%, reduced glutathione (GSH), 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB), 1,1,3,3-tetrathoxypropane (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 ± 3°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, serving as controls, received 1 ml of distilled water by intraperitoneal (i.p.) way every two days. Rats of the second group 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 received every two days by i.p. way 50

mg/kg b.w. of glyphosate (dissolved in distilled water) and quercetin (QE) (dissolved in 2.5% dimethyl sulfoxide) administrated 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 LD_{50}, 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 malondialdehyde assay

The liver malondialdehyde (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 nmoles MDA/mg protein.

Liver hydrogen peroxide content

Hydrogen peroxide (H_2O_2) content in liver tissue was determined according to the ferrous ion oxidation-xylenol orange method (Ou and Wolff 1996). Results were expressed as nmoles/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 nmoles/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 (£ = 2.2 $\times 10^4 cm^{-1}$ M^{-1}) and expressed as nmoles/mg protein.

Liver GSH content

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

164 *Liver non-protein thiols content*

165 Liver non-protein thiol (NPSH) levels were determined by the method of Ellman (Ellman
166 1959) and results were expressed as µmoles/mg protein.

167 *Liver vitamin C assay*

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

170 *Determination of antioxidant enzyme activities in liver*

171 Catalase (CAT) activity was assayed by the method of Aebi (1984). Results were expressed
172 as µmoles H_2O_2 consumed/min/mg protein.

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

176 Glutathione peroxidase (GPx) activity was measured according to Flohe and Gunzler
177 (1984). The enzyme activity was expressed as nmoles of GSH oxidized/min/mg protein.

178

179 *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 µmoles 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 20µl. 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 ± S.D 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).

H_2O_2 production

The levels of H_2O_2 generated in the liver were increased by 44% in glyphosate group when compared to controls. In glyphosate+QE group, H_2O_2 level was significantly reduced by 23% when compared to glyphosate group without reaching control values (Table 2).

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 glyphosate 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 glyphosate 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 Figure1. 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 glyphosate-treated group increased by 71 and 75%, respectively. However, plasma ALP and γGT activities as well as albumin level decreased by 27, 50 and 21%, respectively, in glyphosate-treated 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%), γGT (+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 2 (a)]. 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 2 (b)]. Treatment with QE reduced the level of total MT by 31% as 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 4 (a)]. Nevertheless, remarkable morphological changes such as the presence of polynuclear giant cells [Figure 4 (b1)], a sinusoidal dilatation [Figure 4 (b2)], an

infiltration of inflammatory leukocytes [Figure 4 (b3)], a parenchyma dilatation [Figure 4 (b3) and (b4)], a fibrosis [Figure 4 (b2) and (b4)], a cellular degeneration and a focal hepatic necrosis [Figure 4 (b2) and (b4)] were observed in the liver of glyphosate treated rats. These histological alterations were significantly attenuated in the liver of glyphosate+QE group as compared to that of glyphosate group [Figure 4(c)].

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, 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

296 works have shown elevated MDA levels in tissues like liver, brain, testes and plasma of
297 glyphosate-based herbicides-treated rats (Astiz et al. 2009 a,b). The enhanced lipid
298 peroxidation observed under our experimental conditions might be ascribed to an excessive
299 generation of ROS resulting from glyphosate exposure.

300 At the cellular level, ROS are produced via several mechanisms. As mitochondria are
301 the primary intracellular sites of oxygen consumption, they may be the primary sites of ROS
302 generation such as H_2O_2. In addition to the rise of MDA levels, we have recorded a significant
303 increase of H_2O_2 levels in the liver of glyphosate-treated rats, suggesting mitochondria as a
304 potential target of this pesticide toxicity. In fact, glyphosate has been reported to be a
305 mitochondrial toxin which uncouples mitochondrial oxidative phosphorylation (Bradberry et
306 al. 2009). Once formed, H_2O_2 readily diffuses through cellular membranes to reach sites
307 distant from where it was generated. In the presence of transition metals, it can be reduced to
308 the hydroxyl radical, which in turn interacts with membrane lipids to initiate lipid
309 peroxidation and provokes tissue damage.

310 It is widely accepted that lipid peroxidation products, such as MDA, are implicated in
311 cell genotoxicity (Singh et al. 2011). In the present study, the enhanced hepatic MDA levels
312 caused by glyphosate have been suggested to provoke DNA damage in rats' hepatocytes.
313 Indeed, as revealed by agarose gel electrophoresis, glyphosate treatment resulted in DNA
314 shearing reflecting the genotoxic potential of this pesticide. On the other hand, pesticide
315 biotransformation often results in the generation of ROS, which are highly toxic and cause

316 oxidative damage to DNA. So, it is possible that the depressed DNA integrity observed in
317 hepatocytes of glyphosate-treated rats could be due to the direct reaction of ROS, generated
318 by the metabolism of the herbicide, with the DNA of exposed animals. Our results were in
319 line with data reported by Astiz et al. (2009) after sub-chronic exposure of rats to glyphosate.
320 Moreover, Roundup®, a glyphosate-based herbicide, has been previously demonstrated to
321 provoke DNA lesions in the kidney and the liver of mice (Peluso et al. 1998).

322 Administration of QE along with glyphosate significantly modulated lipid
323 peroxidation and decreased H_2O_2 generation, indicating its important role in providing
324 protection against oxidative damage. QE is recognized as a potent free radical scavenger able
325 to inhibit the lipid peroxidation process. According to Moridani et al. (2003), the advanced
326 diffusion of this flavonoid through biological membranes allows it to scavenge oxyradicals at
327 numerous sites of the lipid bilayer. Moreover, QE is able to react with ROS, decreases DNA
328 damage and prevents the tumorigenic processes (Murota and Terao 2003).

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

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

335 To counteract the deleterious effects of oxidative stress, mammalian cells are equipped
336 with enzymatic and non-enzymatic antioxidant systems which work together to combat
337 oxidative stress. Within the first line of cellular defense, there are the key enzymatic
338 components which limit the effects of oxidant molecules in tissues by means of their free
339 radical scavenging properties. In the current work, glyphosate treatment decreased
340 significantly hepatic activities of SOD, CAT and GPx. This result suggested that the
341 inhibition of antioxidant enzyme activities might be considered as a potential mechanism by
342 which this herbicide could induce oxidative stress.

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

347 The second line of cellular defense against oxidative stress is offered by non-
348 enzymatic antioxidants with low molecular weight. In this regard, NPSH are among the most
349 important non-enzymatic antioxidants which present a variety of functions in the reduction
350 and detoxification processes. In our study, a marked depletion in the hepatic contents of
351 NPSH and GSH was observed following glyphosate exposure, which might be attributed to
352 their consumption in removing H_2O_2 and other peroxides produced in excess due to oxidative
353 stress. GSH reduction in glyphosate-treated rats might also explain the decreased hepatic
354 levels of vitamin C which is known to enter the cell mainly in its oxidized form where it is

355 reduced by GSH. This vitamin is a hydrophilic reducing agent which directly reacts with
356 ROS, such as superoxide radicals, as well as various lipid hydroperoxides more efficiently
357 than any other water soluble antioxidant (Briviba and Sies 1994). So, the observed reduction
358 in vitamin C level could also be ascribed to its increased utilization in trapping the oxy-
359 radicals resulting from glyphosate exposure.

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

365 Another interesting group of non-enzymatic antioxidants, metallothioneins (MTs),
366 plays a central role in the cellular defense against oxidative stress. Because of their high thiol
367 content, these proteins can effectively scavenge several types of ROS (Miles et al. 2000). It
368 has been demonstrated that MT genes are readily induced by oxidative stress resulting from
369 pesticides intoxication (Miles et al. 2000). In our experimental study, we have found in the
370 liver of glyphosate-treated rats a significant increase of total MT. MT I and MT II gene
371 expression levels were also significantly up-regulated. The observed induction of MTs under
372 our experimental conditions reflected probably an adaptive response of the liver tissue to the
373 toxicological manifestations induced by this herbicide. To our knowledge, the present study is
374 the first attempt evaluating the effect of glyphosate on the genes expression of the two

375 isoforms MT I and MT II in rat by means of semi quantitative PCR. The possible mechanism
376 of MT protection against glyphosate liver injury might be due to the twenty cysteine residues,
377 constituting a part of its structure, which are involved in quenching hydroxyl and superoxide
378 radicals (Chiaverini and De Ley 2010).

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

381 In the present study, the impairment of the enzymatic and non-enzymatic antioxidant
382 status caused by glyphosate exposure could enhance the susceptibility to oxidative damage in
383 hepatic cells and contribute to hepatocellular dysfunction. Liver enzymes such as AST, ALT,
384 ALP and γGT are considered to be the important markers of hepatic function. In our findings,
385 we demonstrated that glyphosate, administered to rats, provoked a marked increase in plasma
386 AST and ALT activities, indicating hepatocellular damage. The excessive production of free
387 radicals and lipid peroxides might cause an alteration of membrane permeability leading to
388 the leakage of these cytosolic enzymes from the hepatic tissue. When QE was administered to
389 glyphosate-treated rats, transaminase activities in plasma were decreased significantly,
390 suggesting that QE probably prevented the leakage of these marker enzymes by keeping the
391 structural integrity of the liver. This hepatoprotective effect of QE may be mainly due to its
392 anti-lipoperoxidative, antioxidant and membrane stabilizing properties as reported by
393 Renugadevi and Milton Prabu (2010) in cadmium-treated rats co-treated with QE. On the
394 other hand, glyphosate administration led to a significant decrease in the plasma activities of

ALP and γGT. 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, γGT 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 hepathic tissue revealed that glyphosate treatment caused abnormal ultrastructural changes including the presence of polynuclear giant cells, suggesting a cell cycle disturbance, a sinusoidal and a parenchyma 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.

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Figures' captions

Figure. 1 Antioxidant enzyme activities SOD (a), GPx (b) and CAT (c) in the liver of control and treated rats with glyphosate or glyphosate along with QE. Values are means ± SD for six rats in each group.

Glyphosate and glyphosate + QE groups vs control group: $^{**}p<0.01$; $^{***}p<0.001$.

Glyphosate + QE group vs glyphosate group: $^{£££}p<0.001$.

Figure. 2 Levels of total MT (a) and genes expression (b) of MT I and MT II mRNA in the liver of control and treated rats with glyphosate or glyphosate along with QE.

Glyphosate and glyphosate + QE groups vs control group: $^{**}p<0.01$; $^{***}p<0.001$.

Glyphosate + QE group vs glyphosate group: $^{£££}p<0.001$.

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; (1) Lane 2: DNA isolated from glyphosate liver sample (2); Lane 3: DNA isolated from glyphosate + QE liver sample (3).

Figure. 4 Liver histological sections of control (a) and treated rats with glyphosate (b1, b2, b3 and b4) or glyphosate along with QE (c). Optic microscopy: HE x200

Arrows indicate:

612  polynuclear giant cells,  sinusoidal dilatation,  infiltration of inflammatory

613 leukocytes,  parenchyma dilatation,  fibrosis,  cellular degeneration and focal

614 hepatic necrosis.

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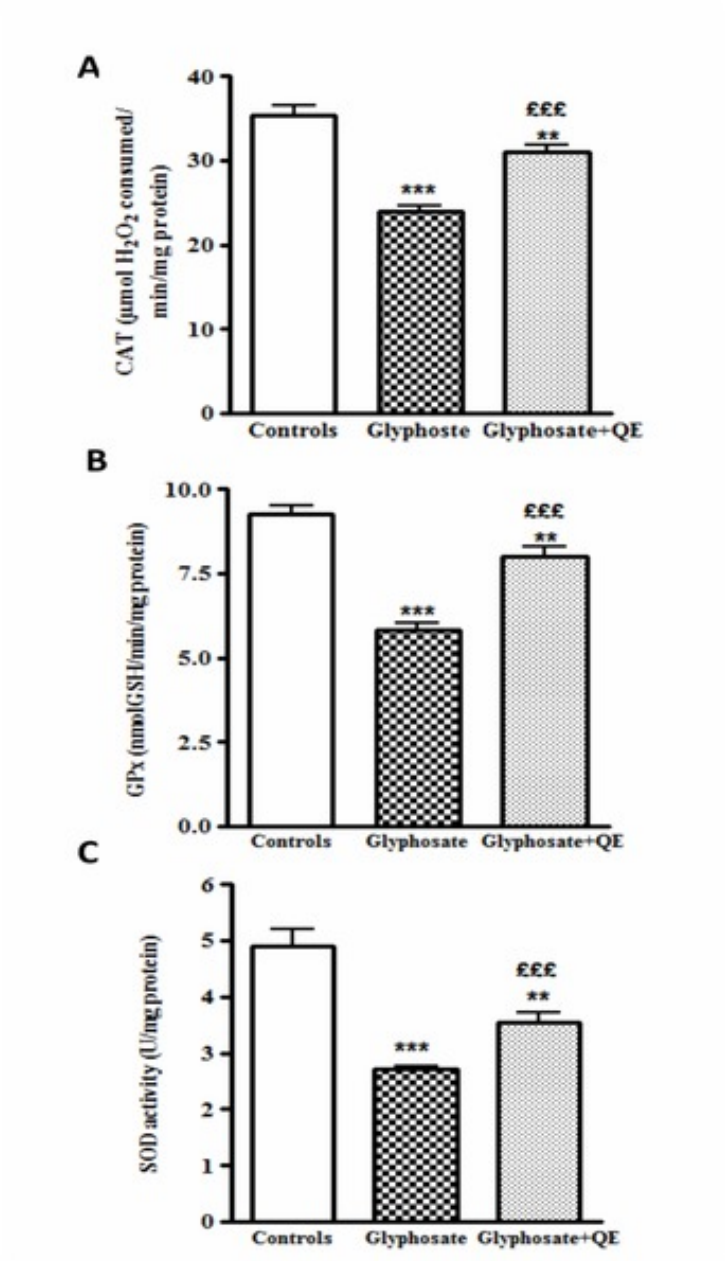

Figure 1

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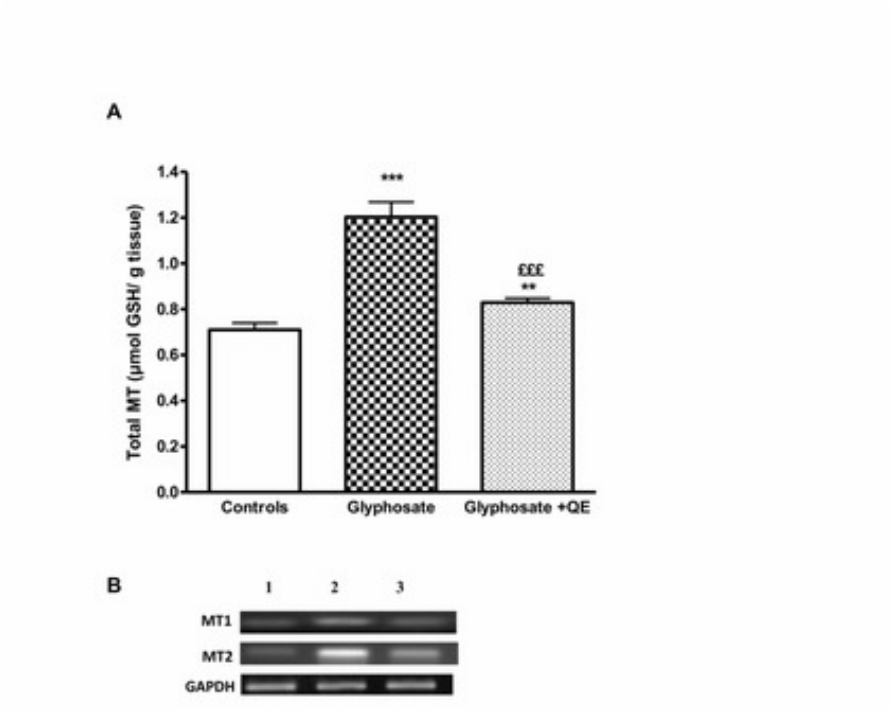

Figure 2

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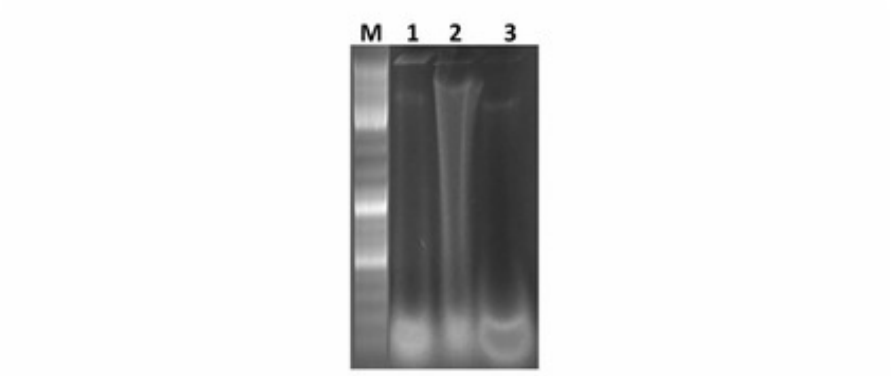

Figure 3

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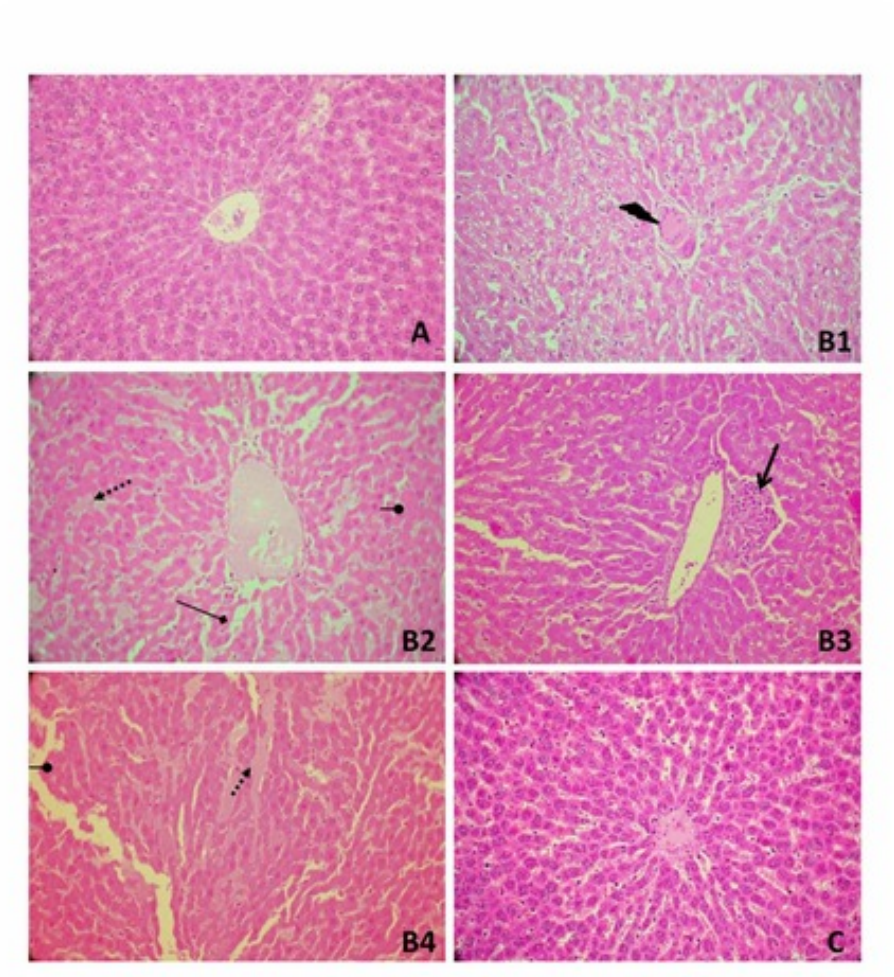

Figure 4