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Research Article

Morus alba Leaf Extract Attenuates Glyphosate-Induced Oxidative Stress, Inflammation and Alleviates Liver Injury in Rats

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Abstract

Background and Objective: Pesticides exposure has been reported as an environmental factor that increases the risk of developing serious health problems. Glyphosate is one of the most widely used pesticides in the world. In the present work, *in vivo* biochemical and histological study of the hepatotoxic effect of glyphosate on liver rats has been first demonstrated. In addition, *Morus alba* leaves extract have been evaluated for their protective effect against this herbicide toxicity. **Materials and Methods:** Daily dose of 100 mg kg^{-1} b.wt., of glyphosate was administered to rats by intraperitoneal injection. Then, liver functions were evaluated biochemically and histopathologically by measuring biomarkers of oxidative stress and by visualizing the effect of herbicide administration on histological sections. **Results:** Treatment of adult rats with Glyphosate solution resulted in increased serum AST and ALT levels and lipid peroxidation (MDA), altered SOD, POD and CAT activities significantly compared to the normal (control group) and increased CRP levels, a reference indicator of inflammation. Similarly, histological sections revealed vacuolar degeneration of liver cells with focal necrosis in Glyphosate treated rats. MALE treatment preserved the biochemical and histological damage caused by Glyphosate toxicity at near-normal levels. **Conclusion:** The present work concludes that MALE due to its rich phenolic composition could exert a hepatoprotective role as an antioxidant and anti-inflammatory against Glyphosate-induced oxidative stress.

Key words: Glyphosate, hepatotoxicity, inflammation, *Morus alba*, biochemical and histomorphological parameters

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Excessive use of herbicides was one of the origins of the oxidative stress phenomenon and cell injury is followed by cell damage[1-3]. Previous investigations have shown that glyphosate (GLY) and its metabolites have been detected mainly in soils and sediments, ditches and drains, rainwater, rivers and streams[4-8]. In addition, the detection of residues of this herbicide in human urine has raised the probability that many diseases are currently increasing in association with glyphosate toxicity. The liver, which represents the primary organ of detoxification, biotransformation and ROS formation, many studies have shown that glyphosate can cause significant hepatic changes[9-11] and even during subacute exposure, it can promote hepatic alterations, which could be correlated with the induction of ROS species[12]

Numerous reports have discussed the effect of glyphosate on microorganisms[13-19] have revealed the effect of glyphosate on the microbial community but have also demonstrated the resistance of some bacteria to this herbicide and even its conversion into an energy source. Kubsad *et al.*[20] concluded, briefly, that glyphosate enhances epigenetic transgenerational transmission of diseases and pathologies by germline (i.e., sperm) epimutations.

Thus, traditional medicinal plants and phytochemicals could provide a crucial role in the hepatoprotective effect against pesticide-induced liver toxicity. *Morus alba* (Moraceae) or white mulberry is already known as a medicinal plant in traditional medicine for the prevention of health disorders. *Morus alba* has been classified as a potential antioxidant. Its ROS scavenging effect comes in response to oxidative damage[21]. It has been shown that *M. alba* leaf extract can reduce liver damage *in vitro* and *in vivo* models. Oh *et al.*[22] demonstrated that the hepatoprotective effect of *Morus alba* is attributed to the presence of certain compounds isolated from the plant extract on tarcin-induced cytotoxicity in the liver. Similarly, the alcoholic extract and aqueous extract of *M. alba* have protective effects against CCl4-induced hepatotoxic patterns in rats[23,24].

Present study investigated the hepatoprotective role and molecular mechanism of *Morus alba* L. leaf extract against glyphosate-induced liver toxicity. Biochemical, histomorphological parameters and enzymatic activities in liver and plasma homogenate were measured. The mode of action seems to involve intracellular mediators such as free iron and H_2O_2 perturbations leading to calcium depletion and on the other hand modulation of the antioxidant enzyme system.

MATERIALS AND METHODS

Study area: The study was carried out in the laboratory of neurophysiology, cellular pathophysiology and biomolecules valorization, Department of Biology-Faculty of Sciences of Tunis and the transmission electron microscopy unit of the Faculty of Medicine and the Department of Pathological. Anatomy of the Salah Azaiez Institute, in partnership with the National Institute of Applied Sciences and Technology, Tunisia from December, 2018 to August, 2020.

Reagents and chemicals: The Glyphosate used in this study has a commercial name Roundup Plus H.029-11 and was purchased from the company ATLAS AGRICOLE, Tunisia. Formaldehyde, bovine liver catalase, DL-epinephrine, Trichloroacetic acid (TCA) and guaiacol were acquired from Sigma Aldrich (St. Louis, MO, USA) Calcium (Réf. 20051), total proteins (Réf. 20161) ASAT (Ref.20042), ALAT (Ref. 20046), LDH (Ref. 20011), CRP (Ref. 400433) assay kits were from Bio-Maghreb (Tunisia). Butylated Hydroxy Toluene (BHT) and 2-Thio-barbituric acid (TBA) were obtained from Loba Chemie PVT.LTD. 2,7-dichlorodihydrofluorescein diacetate (H DCFDA) was purchased from Invitrogen and DAF-FM diacetate (4-amino-5-methylamino-2', 7'-Difluorofluorescein diacetate) was from Molecular Probes (Eugene or, USA).

Buffer salts (KCl, NaHCO3, Na2HPO4, NaH2PO4, K2HPO4 andKH2PO4) were purchased from Baker Inc. (Phillipsburg, USA).

Instruments: Spectrophotometer DoubleUV-1800/AT1304001 (Germany), Buchi™ Rotavapor™ R-100 (Switzerland), Microtome Leica RM 2145 microtome (Germany)

Preparation of *M. alba* extracts: The leaves of this Tunisian variety of Mulberry (*Morus alba* L.) were collected from the Rafraf region in northern Tunisia where these species grow wild. Two types of extracts were prepared: 70% acetone extract and aqueous extract. The leaves were extracted with cold (-20°C) 70% acetone. The supernatant was collected and regrouped, then concentrated to a final volume of 3 mL under vacuum using a rotary evaporator (60°C) by Fattouch *et al.*[25]. Then, the extract was freeze-dried to obtain the *M. alba* extract, which was stored at -20°C until use. Finally, rats were treated with the aqueous extract because of its performance and non-toxicity.

Determination of the total Phenolics: The Folin method was applied as reported by Elizabeth *et al.*[26]. Extracts were mixed with Folin-Ciocalteu phenolic reagent and then incubated for 10 min, followed by the addition of 7.5% sodium carbonate (Na_2CO_3). After incubation for 2 hrs, absorbance was measured by a spectrophotometer at 760 nm. Gallic acid was employed as a standard and the results were expressed as mg Gallic Acid Equivalent (GAE) per 100 g dry weight (dw).

Total antioxidant activity: The Trolox equivalent antioxidant capacity was determined by the DPPH radical scavenging assay. A 25 µL solution of extract appropriate dilution was added to 975 µL of a 40 µM methanolic solution of DPPH.

After incubation for 30 min in the dark, the Optical Density (DO) was read at 517 nm using methanol as blank. Different dilutions of Trolox were tested to set up the standard curve and the TEAC was determined in mmol Trolox equivalent/100 g dw.

Animals and treatment: Healthy adult male Wistar rats were purchased from the Pasteur Institute of Tunis, weighing 210-290 g. The *in vivo* experiments were performed in compliance with the guidelines of the Ethics Committee of the Faculty of Sciences of Tunis, Tunisia.

The animals were fed with a standard diet (standard diet in pellets-Badr Utique-TN) and water ad libitum and were maintained in the animal house of the faculty at a controlled temperature (22±2°C) with a light-dark cycle of 12 hrs.

Rats were randomly divided into four groups of six animals each: Group 1 received a standard diet (control). Group 2 received an injection (i.p., intraperitoneal) of glyphosate-based solution (100 mg kg^{-1} b.wt.). Group 3 received an injection of aqueous extract of *Morus alba* (100 ug mL^{-1}) (i.p., intraperitoneal) and finally and group 4 received an injection of glyphosate and MALE. The rats were treated daily for two weeks. At the end of the treatment time, the animals were anaesthetized with Ketamine (80 mg mL^{-1} kg^{-1} b.wt.) and then sacrificed, the blood was collected from the jugular vein. And the liver was collected, homogenized and processed for biochemical and histological parameters.

Biochemical parameters assessment

Determination of hepatic markers: Lactate dehydrogenase (LDH), alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) activities were determined using commercial. kits (Biomaghreb, Tunisia) based on kinetic method following the manufacturer's procedure.

Determination of lactate dehydrogenase (LDH): Extracellular lactate dehydrogenase activity was assessed by a spectrophotometric method by using a commercial kit from Biomaghreb, Tunisia. The estimation of LDH activity was performed by measuring the oxidation of NADH and the results were expressed relative to the control.

Total protein determination: The determination of total. protein was performed according to the method described by Hay *et al.*[27]. At acidic pH, a blue-colour complex of soluble proteins with copper was quantified by spectrophotometry at 546 nm.

Antioxidant enzyme activity (CAT, SOD and POD): Catalase activity (CAT) was evaluated by measuring the initial rate of H_2O_2 disappearance at 240 nm[28]. Peroxidases (POD) and Superoxide dismutase (SOD) activities were assessed using a modified epinephrine assay by the method of Weydert *et al.*[29].

Determination of liver lipoperoxidation: The level of lipid peroxidation in the liver was carried out with the MDA measurement method according to De Las Heras Rosa *et al.*[30]. An aliquot of liver homogenate was mixed with a BHT-TCA solution containing 1% BHT and 20% TCA. After centrifugation, the supernatant was combined with a second solution containing 0.5 N HCl and TBA (120 mmol mL^{-1}) and heated at 80°C for 10 min. After cooling, the absorbance of the resulting chromophore was measured at 532 nm using a Double UV-visible spectrophotometer. Malondialdehyde contents were represented as millimoles of MD per milligram of protein with an extinction coefficient of 1.56105 mol L^{-1} cm.

Histological preparation: At the end of the treatment, the rats were sacrificed and tissue samples (liver, kidney, brain and heart) were recovered and fixed in a 4% formalin solution for 24 hrs, then dehydrated in an ascending series of alcohol, clarified by using xylene and incorporating it into kerosene. The kerosene was sectioned in 5 µm slices and stained with Hematoxylin-Eosin (HE) for microscopic examination. The scoring system was used to evaluate liver damage by Krishna[31], Klopfleisch[32].

Determination of C-reactive protein: CRP determination was performed according to the protocol of Sproston NR *et al.*[33] using a kit purchased from Biomaghreb-Tunisia by Photometric measurement of turbidity caused by the antigen-antibody reaction antigen-antibody reaction in the endpoint method at 340 nm.

Statistical analysis: The data were represented as mean±Standard Error of the Mean (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests, which was performed with GraphPad Prism software version 6 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when the p level was less than 0.05.

RESULTS

Quantification of total polyphenols and antioxidant activities of *Morus alba* leaf extracts: Total phenol compounds of *Morus alba* leaf extracts were 2267.62±68.64 gallic acid equivalent/100g/were shown in Table 1, Values were expressed as mean±SEM (n = 3), The highest DPPH inhibition rate is recorded with 0.8 mM equivalent Trolox. *Morus alba* leaf extracts have a high antioxidant capacity and can act as a free radical scavenger.

Evaluation of the subacute toxicity of glyphosate on body weight and organs: The remarkable changes in body weight of the rats began from the fourth day of treatment between groups treated with glyphosate alone or in conjugation with *Morus alba* compared to the control group ($p<0.05$) in Table 2. Weight gain decreased significantly both in the groups treated with Gly alone at 100 mg^{-1} kg^{-1} b.wt., or in conjugation with *Morus Alba* extract at 100 μg^{-1} kg^{-1} b.wt., compared to the control group ($p<0.05$) a significant difference in weight loss was also observed but less pronounced in the group treated with phenolic extract alone. The weight loss observed after glyphosate treatment indicates a state of decreased food consumption which affects the body condition. Such a decrease in weight but less severe was even noticed in the group that received the phenolic extract.

The absolute weight of the organs or the relative weight of the organs of the liver, brain, heart and kidney showed a significant decrease in the 100 mg kg^{-1} Gly group ($p<0.05$, Table 2), which suggests that the overt toxicity of Gly is primarily towards growth and development at the dosages studied.

Histological evaluation of liver tissue: Histopathological changes in the liver of Gly-treated rats are shown in Fig. 1 subdivided into Fig 1(b and d). The control rats, shown in Fig. 1a, presented normal hepatic morphology with distinct hepatocytes with prominent nuclei and no tissue damage. In contrast, liver sections of Gly-treated rats showed severe structural damage characterized by periportal expansion and degeneration of hepatocytes, necrosis, vascular congestion and sinusoidal dilatation in Fig. 1(b-d). However, the livers of the co-treated rats with Gly/MA in Fig. 1d had to recover histology than the rats from Gly-only treated rats in Fig. 1b.

Protective effect of MA against glyphosate-induced liver toxicity and LDH levels: The results in Fig. 2 indicate that the LDH release level was significantly (*) doubled in the Glyphosate intoxicated group compared to the control group 433.1 U mg^{-1} of protein (0.0392). Glyphosate induces toxicity and destruction of hepatocytes manifested by the increase of levels of LDH release, thus highlighting an excessive rate of cell death. Whereas phenolic extract treatment restored LDH level ($p = 0.19460$) compared to the control group but not significantly. Restoration of LDH level indicates that MA can prevent hepatotoxicity and cell death induced by glyphosate.

Modulation of antioxidant enzymes activities: The evaluation of the effects of the injection of Glyphosate-based solution followed by the curative treatment with the phenolic extract of *Morus alba* on antioxidant enzymes gave the results presented in Fig. 3.

In Fig. 3a, it can be seen that an alteration of the CAT activity expressed by a remarkable and significant increase (**) of the released rate of this enzyme 0.01666 mM min^{-1} mg^{-1} protein ($p = 0.0047$) in the group treated with Glyphosate alone compared to the control lot, thus indicating the suffering of the hepatic cells, while the double-treated group shows a non-significant Catalase activity close to that recorded in the control lot.

The third group that received only the phenolic extract showed a non-significantly (*) low rate of release of this enzyme compared to the control.

In Fig. 3b, which evaluates the impact of the treatment with Glyphosate and the phenolic extract of *Morus alba* on the activity of peroxidase, current results showed a decrease in the activity of this antioxidant enzyme in the group that received only the Glyphosate-based solution 1.646×10^{-6} mmol min^{-1} mg^{-1} protein ($p = 0.1415$), this alteration was effectively corrected in the double-treated group (Gly+MA) in such a way as to re-establish the activity of peroxidase at a threshold close to normal. 2.53 10^{-6} mmol $min^{-1}mg^{-1}$ protein ($p = 0.8568$). And finally, in Fig. 3c, with the evaluation of the

Table 1: Total phenolics and Trolox equivalent antioxidant capacity (DPPH scavenging activity) of white Tunisian mulberry leaves

Morus alba total phenolics (mg GAE/100g dw)	*Morus alba* TEAC (mmol Trolox /100g dw)
2267.62±68.64	138.23±5.23

Histopathological evaluation

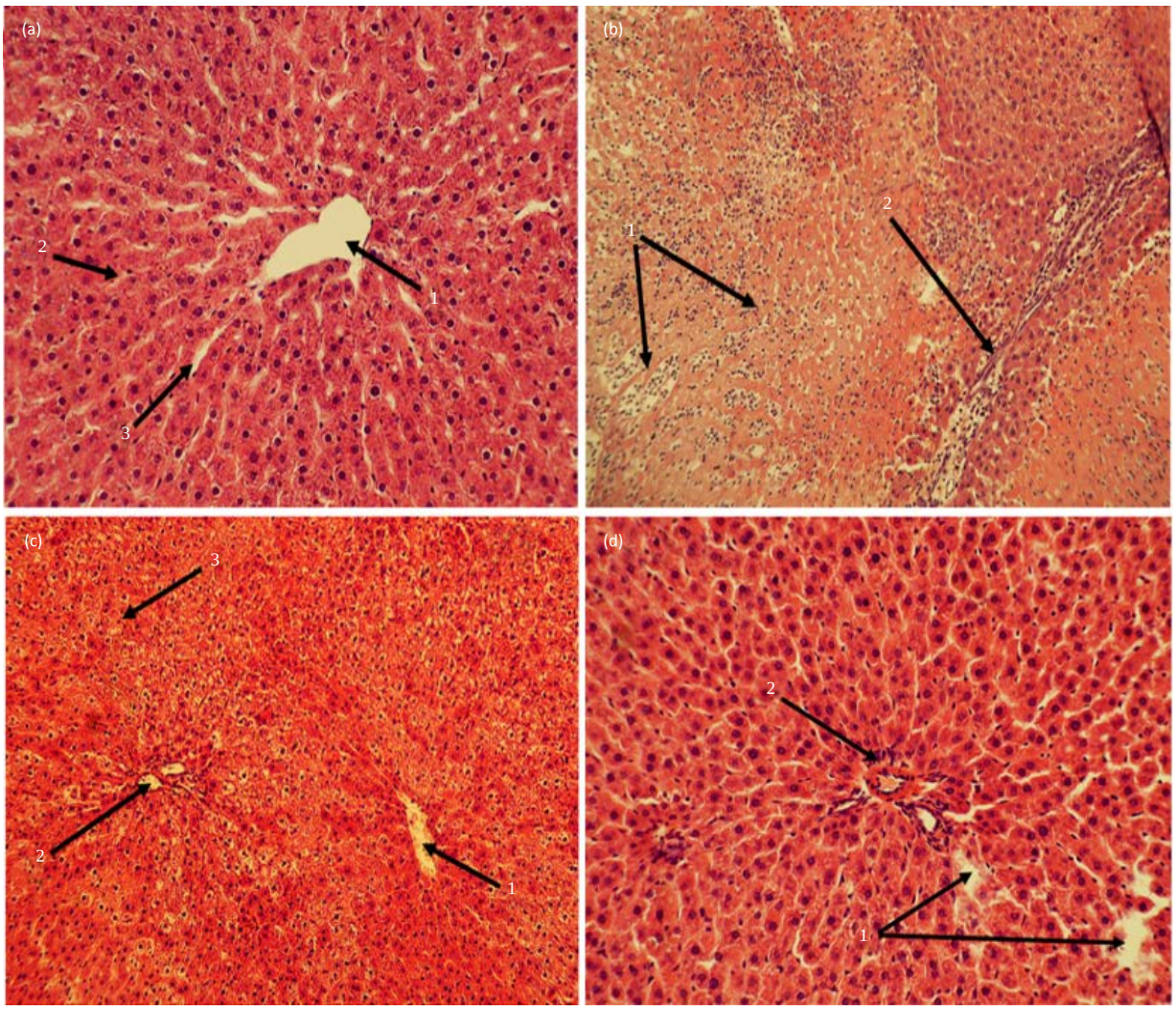

Fig. 1(a-d): (a) Photomicrograph showing histology of the liver tissue of control group rats, 1 branch of a centrilobular vein, 2 small capillaries sinusoids, 3 large sinusoids capillaries and portal area (see black arrows), (b) Liver histology of rat intoxicated with glyphosate, Notice the visibly focal necrosis of hepatocytes (cell degeneration), the infiltration of immune cells into the portal, (c) Liver histology of rat treated with *Morus alba* Notice the comparable hepatic structure with the liver of a normal animal and (d) Histology of the liver tissue of rat co-treated with glyphosate and *M. alba*, 1 appearance of the worn space, some small necrotic areas, Notice the equivalent hepatic structure with liver of rat intoxicated with glyphosate in Fig c and d. Notice the prominent dilated capillaries sinusoïdes in Fig. c and d liver of rats A and D×400, B and C ×100

influence of Glyphosate and *Morus alba* on the activity of Superoxide dismutase, we note a decrease in the activity of this enzyme in the group that received only Glyphosate 0.09659 U/min/mg protein ($p>0.9999$), while this activity is clearly and significantly stimulated both in the group that received the phenolic extract or the double treated group always comparing with the control group. The assessment of the activity of antioxidant enzymes, SOD, CAT and peroxidases proves that glyphosate induces the alteration of the antioxidant balance. The re-establishment of normal antioxidant enzymes levels indicates that MA protects hepatic cells.

MA and enzymatic hepatic markers: Figure 4a and b showed that administration of glyphosate solution disrupted liver balance by inducing a significant increase in transaminases for

ALT and AST, the group's AST and ALT levels are remarkably elevated by 235.8 U L^{-1} plasma (p = 0. 0373) for AST (Fig. 4a) and 77.44 U L^{-1} plasma (p = 0.0160) for ALT (Fig. 4b) compared with the untreated group, this increase was corrected nonsignificantly in the group that received *Morus alba* extract with glyphosate. Glyphosate administration induced increases in the liver enzymes AST and ALT. Treatment with MA counteracted glyphosate-induced liver damage.

Glyphosate and liver lipoperoxidation: This study focused on assessing the content of MDA as it represents an ideal indicator of lipid peroxidation in the liver in Fig. 5. Current results indicated that the administration of Glyphosate induces a highly significant (****) increase in MDA level of 1,713 nmol mg^{-1} protein (p<0.0001). The protection by the phenolic extract was noted highly and significantly in the double-treated group through the decrease of the level of MDA by 0.7515 nmol mg^{-1} protein in liver tissue to a rate not far from normal. The injection of phenolic extract alone

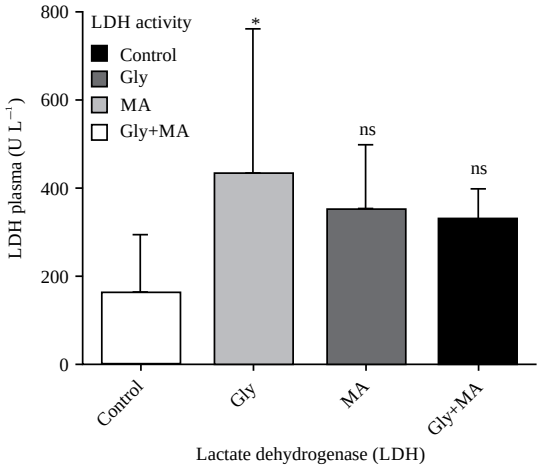

Fig. 2: Effect of MALE on glyphosate-induced lactate dehydrogenase release

Results are represented as percentages of lactate dehydrogenase released, Results were expressed as mean ± SEM (n = 6), Asterisk p<0.05 compared with control, numerical signs p<0.01 compared with pesticide-treated

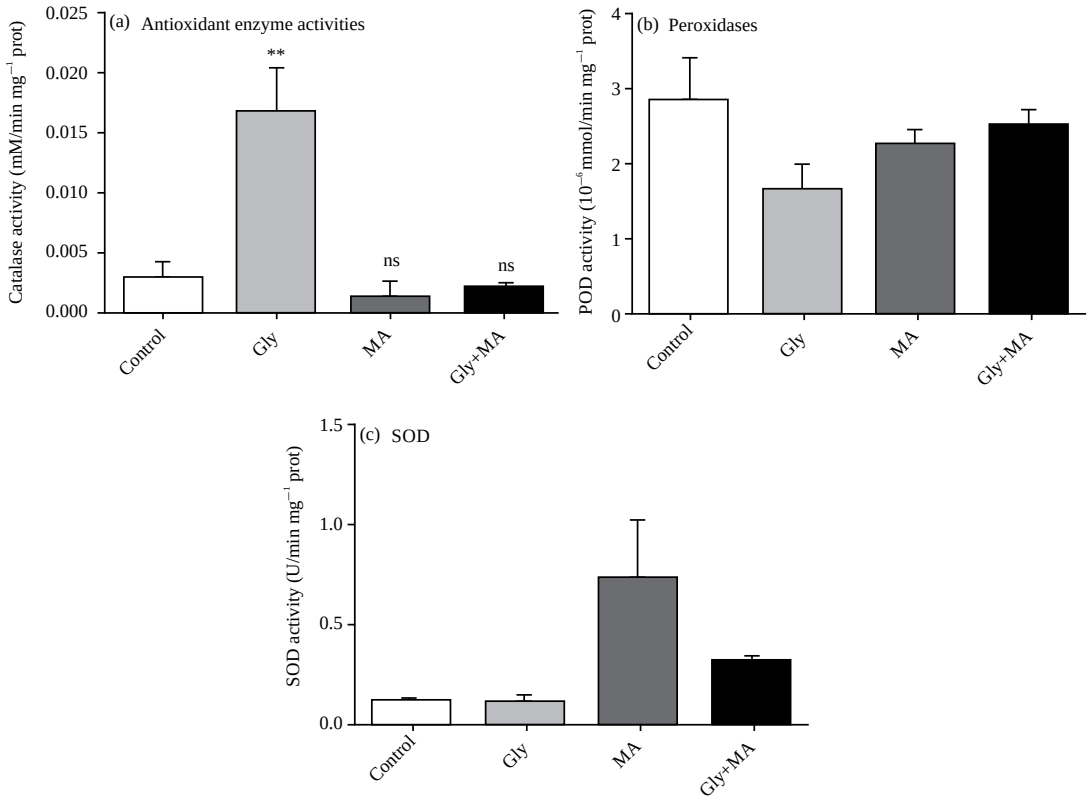

Fig. 3(a-c): Incidence of glyphosate and MALE treatment on catalase activity, (b) Incidence of glyphosate and MALE treatment on Peroxidase activity and (c) Incidence of glyphosate and MALE treatment on SOD activity

Wistar rats were administrated intraperitoneal with *Morus alba* leaf extract (MALE), glyphosate 100 mg kg^{-1} b.wt., (glyph), or glyphosate plus MA (MA+Glyph). Liver catalase, peroxydases and superoxide dismutase activities were determined, Results were expressed as mean ± SEM (n = 6), One asterisk p<0.05 vs control, two asterisks p<0.05 vs control, one number sign p<0.05, two number signs p<0.01 vs pesticide-treated rats

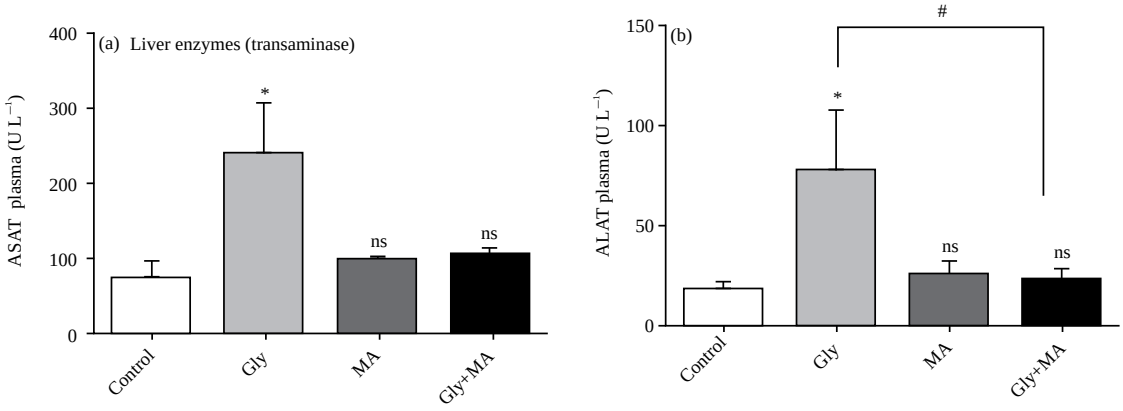

Fig. 4(a-b): Effect of MA extract and glyphosate on hepatic enzymatic markers, (a) ASAT effect and (b) ALAT effect

Wistar rats received intraperitoneal injection of *M. alba* leaf extract (MA), glyphosate 100 mg kg^{-1} b.wt., (Glyph), or of 100 µg mL^{-1} daily, Results were expressed as mean±SEM (n = 6), Asterisk p<0.05 vs control, number signs p<0.01 vs pesticide-treated rats number signs p<0.01 vs pesticide-treated rats

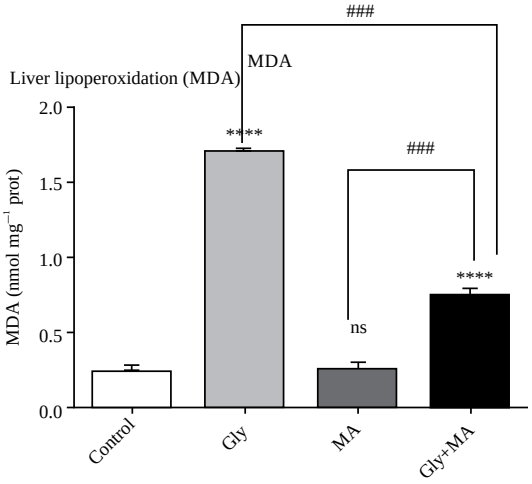

Fig. 5: Protective effect of male against glyphosate-induced liver lipoperoxidation

Animals were treated with a single/day dose of glyphosate (100 mg kg^{-1} b.wt.) for 15 days, Male was administered at a dose of 100 µg mL^{-1}, Results were represented as mean±SEM (n = 6), Asterisk p<0.05 vs control, numerical signs p<0.01 vs pesticide-treated rats

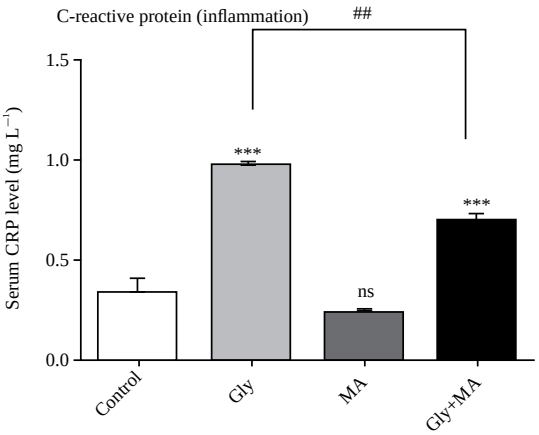

Fig. 6: Serum CRP level, Protective effect of MALE against glyphosate-induced inflammation

Rats were treated with a single/day dose of glyphosate (100 mg kg^{-1} b.wt.) for 15 days. AM was administered intraperitoneally at a dose of 100 µg mL^{-1} per day, Results were represented as mean±SEM (n = 6). Asterisk p<0.05 vs control, numerical signs p<0.01 vs pesticide-treated rats

Table 2: Body weights and organ weights of rats treated with glyphosate and *M. Alba* for 15 days

	Control	Glyphosate (100 mg kg^{-1} b.wt.)	*Morus alba* (100 µg kg^{-1} b.wt.)	Gly+MA (100 µg kg^{-1} b.wt.)
Number of animals	6	6	6	6
Initial body weight (g)	245.8000±3.371	288.3000±8.736	210.2000±4.679	258.7000±2.092
Body weight (g) (the day of sacrifice)	258.2000±4.339	277.3000±12.07	206.2000±13.37	247.3000±6.280
Weight gain (%)	5.0440±0.776	-3.8150±0.723	-1.9000±0.3499	-4.4060±0.3331
Liver (g)	8.5270±0.305	10.1400±0.536	9.5820±0.2470	7.7520±0.341
Relative liver (%)	3.3000±0.001	3.6000±0.001930	4.6000±0.001195	3.1000±0.001
Kidney (g)	1.7150±0.041	1.6020±0.05471	1.5300±0.1755	1.6750±0.059
Relative kidney (g)	0.6583±0.0001	1.1780±0.005	0.5950±0.0001	1.0400±0.004
Brain	1.7150±0.064	1.8080±0.016	1.7580±0.049	1.7730±0.024
Relative brain (%)	0.6483±0.0002	0.6833±8.819	0.6767±0.0001	0.6817±9.458
Heart	0.8133±0.0420	0.9933±0.153	0.8117±0.0332	0.8283±0.046
Relative heart (%)	0.3100±0.0001	0.3533±0.0005	0.3883±0.0001	0.3300±0.0001

Values shown are the mean±SEM of 6 animals per group, Compared to control, *p<0.05, **p<0.01

Table 3: Biochemical parameters in rats liver homogenate

Groups	Ca^{2+} (mmol mg^{-1} prot)	H_2O_2 (mmol mg^{-1} prot)	Free iron (nmol mg^{-1} prot)
Control	0.00213511±0.000188	8.90±0.27	2.30±0.50
Glyphosate (100 mg kg^{-1} b.wt.)	0.00743584±0.0009963	11.80±0.65	7.80±1.75
MA (100 ug mL^{-1})	0.00300855±0.0003617	9.01±0.25	5.00±0.57
MA+Glyphosate (100 mg kg^{-1} b.wt.)	0.00393117±0.0004072	10.23±0.42	4.20±0.72

Data are mean±SEM, (n = 3 replicates), a: p<0.05, when compared with non treated group and b: p<0.05 when compared with glyphosate treated group

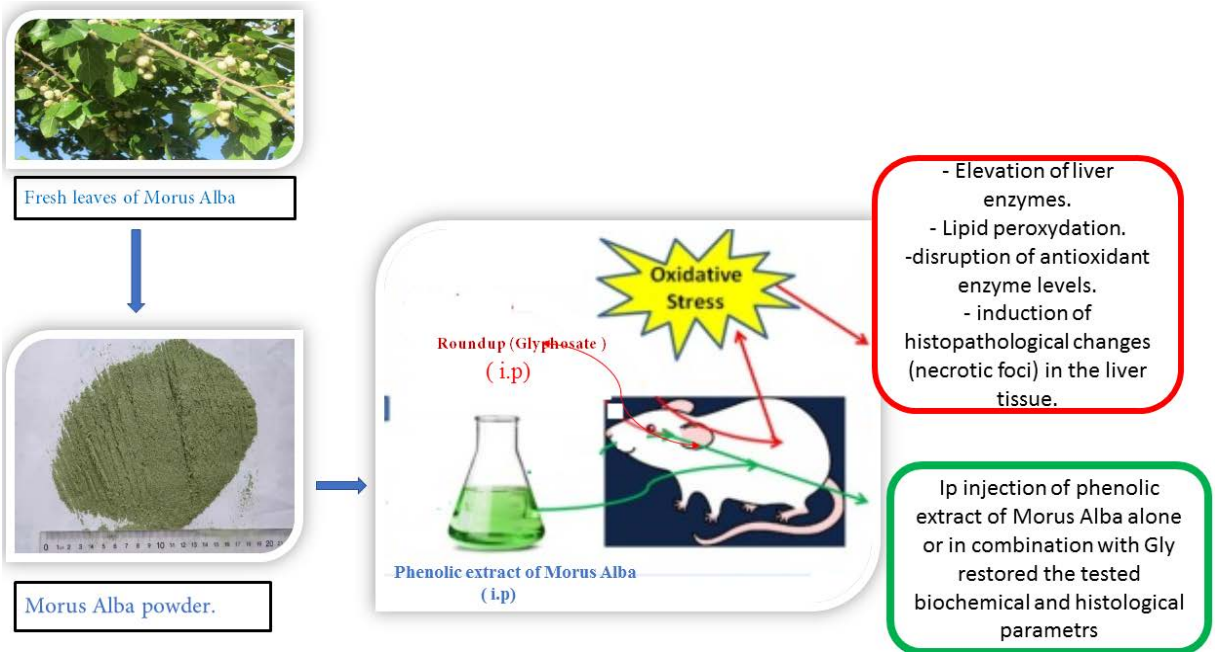

Fig. 7: Schematic abstract

maintained the MDA level to near control values. These results indicate that Glyphosate induces the increase of lipid peroxidation and that MA reduces cellular components oxidation.

Incidence of glyphosate on intracellular mediators: To investigate the intracellular mediators involved in the molecular target of the hepatoprotective effect of MALE against glyphosate-induced oxidative stress, ionizable calcium, H_2O_2 level and free iron mediators were estimated. Table 3 showed that glyphosate caused an increase in released levels of ionizable calcium with 0,00743584 mmol mg^{-1} protein (p<0.001), an increase in H_2O_2 level with 11.8 mmol mg^{-1} protein and an increase in free iron with 7.8 nmol mg^{-1}

protein. Glyphosate injection caused calcium and free iron overload and the excessive production of H_2O_2.

Co-treatment of rats with a once-daily dose of the phenolic extract decreased these mediators compared to the untreated control group very significantly for [Ca^{2+}] and not significantly for [H_2O_2] and free iron. MA can counteract the glyphosate-induced accumulation of calcium. Values are expressed in Table 3±SEM.

Glyphosate and inflammation markers (CRP): In Fig. 6, it is noted that the administration of Glyphosate induced a net increase in the released level of CRP protein in a highly significant way compared to the control group with 0.9758 mg L^{-1} ($p<0.0001$), while the double-treated group recorded a significant decrease in the expressed amount of this protein, this is due to the beneficial effect of the phenolic extract which has anti-inflammatory activity in addition to its antioxidant activity.

Figure 7 summarizes the work in a schematic abstract and highlighted the main aspects of the toxicity induced by Glyphosate and also the protective role of polyphenols extracted from this Tunisian variety of *Morus alba*.

DISCUSSION

Glyphosate [N-(phosphonomethyl) glycine] (Gly) is an organophosphate and the active ingredient in Roundup® and many other formulations. Recent research on various organs has revealed that glyphosate is likely to trigger numerous deregulations of metabolic pathways that can lead to oxidative damage and the generation of reactive oxygen and nitrogen species (ROS, RNS)[34].

In the present work, Subacute intoxication of rats with glyphosate did not cause mortality during the treatment period, however, behavioural signs of intoxication in rats that received the glyphosate solution as well as weight loss were recorded.

This weight loss was even recorded in a less severe way in the group that received the phenolic extract of *Morus alba*, which can be explained by the existence of chlorogenic acid as the majority molecule of the extract[35], this molecule has been attributed in several studies to its anti-obesity effect[36] as well. LDH, an important class of enzymes, was found to be effectively deregulated in the liver by glyphosate. The experimental results of this study revealed the direct involvement of glyphosate in inducing and exacerbating oxidative stress by disrupting the anti-oxidant-pro-oxidant balance in favour of the latter. Administration of a toxic dose of glyphosate 100 mg kg^{-1} b.wt., leads to marked liver damage increasing MDA levels by 1.713 nmol mg^{-1} protein ($p<0.0001$). These results are in line with those of Beuret *et al*.[37]. which demonstrate that glyphosate can increase the production of TABARS in the liver when pregnant rats and their fetuses received Gly during the gestational period and even at low doses (10 mg kg^{-1}) intraperitoneally. It also increased the plasma TABARS level. This protective effect is probably related to the scavenging of MDA molecules by the active ingredient of MALE or by the inhibition of mitochondrial chain reactions. Also, it was found that glyphosate can modify intracellular liver mediators. MALE counteracted the increase in calcium levels triggered by the toxic effect of glyphosate. Because glyphosate increased H_2O_2 and free iron levels, it could also increase hydroxyl radical, toxic radicals, which in turn could alter calcium homeostasis. On the other hand, free iron may act as a catalyst for auto-oxidation and cysteine residue-mediated oxidation represents a common mechanism by which H_2O_2 exerts its role as a second messenger in signal transduction pathways[38]. In addition, H_2O_2 by inducing oxidation of sulfhydryl residues can liberate intracellular calcium[39]. This oxidation may impact proteins such as calcium channels or antioxidant enzymes such as catalase[40] or the induction of SOD activity[41].

In this case, Glyphosate concomitantly increased free iron and H_2O_2 and MALE enhanced these deleterious effects in the liver. Therefore, Glyphosate affects the cellular antioxidant defence system such as SOD and CAT in the liver. SOD activity was inhibited by 0.09659 U $min^{-1}mg^{-1}$ protein ($p>0.9999$), while CAT activity was significantly increased by 0.01666 mM $min^{-1}mg^{-1}$ protein ($p = 0.0047$) with glyphosate treatment. These changes were significantly reduced when MA was administered to the rats. Thus, the increase in SOD activity levels by MA may be associated with superoxide scavenging properties in response to the attenuation of increased O^{-2} production and metabolizes the superoxide anion to hydrogen peroxide H_2O_2. The increase in hepatic CAT activity in the glyphosate-treated group could be due to the overproduction of ROS and CAT could act as a hydroxyl radical scavenger, which could be a response to the increased production of H_2O_2. Thus, MALE appears to be able to maintain the antioxidant enzyme system through its antioxidant property.

Since ALT and AST activities were used as the biochemical markers of liver injury to evaluate hepatic toxicity. A studied dose of 100 mg kg^{-1} can cause alterations of certain intracellular enzymes that suggested damage in hepatocytes. In glyphosate-treated rats, group levels of ALT and AST are remarkably elevated by 77.44 U L^{-1} plasma ($p = 0.0160$) for ALT and by 235.8 U L^{-1} plasma ($p = 0.0373$) for AST in

comparison with the non-treated group indicating the hepatic toxicity induced by glyphosate. The reduction in the activity of the main hepatic enzymes is probably an indicator of regeneration of the hepatocytes membranes.

These results are in agreement with the data of Djaber *et al.*[42] which showed that Roundup, with a dose of glyphosate equal to 269.9 mg kg^{-1} b.wt., was able to induce oxidative damage in adult rats. In response to these induced toxic effects and antioxidant activity will be established, reducing the toxicity of glyphosate by increasing lipid peroxidation and protein oxidation as well as a reduction in hepatic and renal glutathione content. This is following the results of Almeida *et al.*[43] and Milic *et al.*[44], who recorded changes in thiobarbituric acid reactive substances and a significant increase in ROS levels in the liver.

Similarly, The results of Soudani *et al.*[45] indicate that even with a lower concentration of glyphosate with 50 mg kg^{-1} b.wt., hepatic oxidative stress was registered. This is evidenced by a very significant increase in malondialdehyde levels, hydrogen peroxide and protein carbonyls concentration. The author observed equally a significant decrease in antioxidant activities (superoxide dismutase, catalase, glutathione peroxidase) as well as a decrease in non-protein thiols, glutathione and vitamin C concentration. Blood indicators of hepatotoxicity (AST, ALT, ALP, γ-GT and albumin) were similarly modified. The toxicity of glyphosate likewise damaged DNA and increased the expression of metallothionein genes (MT I and MT-II). Finally, it should be noted that notable structural changes were observed in histological sections of the liver of rats treated with glyphosate. MA could significantly decrease the ALT and AST enzymes levels ($p<0.05$).

Taking together, Glyphosate may produce hepatotoxicity by direct effect through overproduction of ROS and protein damage. Glyphosate also causes depletion of the antioxidant enzymes both at the plasma level (SOD, CAT, peroxidase, etc) and at the tissue level (liver level). On the other hand, the protective effect of the bioactive substances of the phenolic extract against histomorphological damage is clear (the histological. sections of Fig. 1) as well as against the plasma level of inflammation triggered by glyphosate (CRP level, Fig. 6).

MALE appears to be able to abrogate this effect that could be attributed to the antioxidant activity of phytochemicals and phenolic content of tested aqueous fraction of white mulberry leaves. The results obtained following the treatment of rats with the phenolic extract are in concordance with many of the original scientific publications such as that of Kavitha[46], Teng-Gen Hu *et al.*[47] and others. However, current results regarding the protective effect of *Morus Alba* are partially in disagreement with the work of Kujawska *et al.*[48], who found that its treatment with mulberry leaf extract with a much higher dose than ours (1000 mg) in combination with N-nitrosodiethylamine (NDEA) did not affect the activity of antioxidant enzymes or glutathione content and subsequently KUJAWSKA concluded that his phenolic extract of *Morus alba* offers only partial protection.

CONCLUSION

The hepatoprotective effect of the aqueous fractions of *M. alba* leaves against glyphosate-induced toxicity in the liver can be ensured by the antioxidant and anti-inflammatory properties of the mulberry tree that protect against oxidation by the pathological accumulation of free radicals of molecules such as enzymes, proteins and lipids. Further studies focusing on the identification of the bioactive compounds in MALE responsible for this effect are underway. In conclusion, this work demonstrated that the glyphosate-based solution was responsible for oxidative damage in Wistar rats through its ability to induce and enhance lipid peroxidation, protein oxidation and a decrease in antioxidants protein oxidation. Moreover, the aggravation of inflammation and oxidative stress was confirmed by the determination of indicator proteins such as CRP and by the histological changes observed in the current work. On the other hand, the use of white mulberry extracts as a source of antioxidants was effective in attenuating oxidative stress, improving biochemical and histological parameters of the liver and preserving the integrity of the tissue structure. In summary, herbal medicine represents a very promising and feasible avenue of research in the fight against toxins and particular phytosanitary products such as pesticides.

SIGNIFICANCE STATEMENT

This study reinforces the scientific knowledge on phytotherapy in particular with the bioactive substances of the white mulberry of the Mediterranean zone and their beneficial effects in particular on the detoxifying organs like the liver against the xenobiotics like the pesticides, which can of an important utility for the pharmaceutical industry based on the natural resources. This work will therefore help researchers to better elucidate the risks of the arbitrary use of glyphosate-based products on the one hand and on the other hand to better valorize medicinal plants and their powerful protective molecules.

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