

Nephroprotective effect of aqueous acetonc extract of *Morus alba* and its underlying mechanisms against glyphosate-induced toxicity - *in vivo* model

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SUMMARY

Glyphosate, the active substance in Roundup®, is the most widely used pesticide in the world and may be present as a residue in derived foods and drinking water. Previous reports have confirmed that extracts from leaves of *Morus alba* exert many pharmacological activities. However, renoprotective effects of *M. alba* extract and its underling molecular mechanism is still unknown.

Wistar rats (180-200 g) were used in this study (n=5-6). A control group received 0.2 ml normal saline intraperitoneally (i.p) once daily for two weeks. Control animals received standard diet. Treated groups received either polyphenolic extract (100 mg/kg,i.p) or glyphosate (100 mg/kg, i.p), or co-administration (extract $\mu\text{g ml}^{-1}$ kg b.w. and glyphosate 100 mg kg⁻¹ b.w, i.p), daily until the 15th day of treatment. Lactate deshydrogenase LDH, serum concentrations of blood urea, creatinine and nitric oxide were measured using standard coloromertic methods.

Renal oxidative stress, evidenced by increased malondialdehyde (MDA) and protein carbonyl levels and decline in superoxide dismutase (SOD) activity, was significantly alleviated by mulberry leaves extract (MLE) administration. MLE also appears to be able to modulate altered biochemical parametres by maintaining free iron and Ca₂⁺ homeostasis, and regulate the endogenous antioxidant enzymes system. It seems that concurrent use of the aqueous acetonc fraction of *M. alba*, rich in chlorogenic acid and its isomeres, can protect kidneys from glyphosate-induced nephrotoxicity. Overall, MLE may possess protective activity against glyphosate-induced toxicity, which may be attributed to chlorogenic acid and its isomers, the most abundant phenolic acids present in its extracts.

Mulberry leaves are a source of phenolic compounds and can be a good start towards discovering a new chemical compound which may lead to a new drug. A mulberry extract supplement could serve as a candidate for developing a safe, and promising nutraceutical product for the management of nephrotoxicity.

Keywords: glyphosate, oxidative stress, nephrotoxicity, mulberry, nephroprotective effect

INTRODUCTION

Glyphosate is one of the most commonly used herbicides in the world, including Tunisia. Residual amounts of glyphosate have been detected in soil, vegetables, grains and other food products. A recent study revealed that glyphosate residues can be accumulated in chicken organs and muscles after consuming glyphosate in their feed (Shehata et al., 2014). Furthermore, detection of glyphosate residues in human urine has indicated that many diseases currently on the rise, such as hepatotoxicity, gastrointestinal, cardiovascular and respiratory disorders, are associated with glyphosate use as the cause of multi-organ toxicity. However, the toxic effect of glyphosate or its surfactant in renal function has not been well established. Kidney damage is also frequent and usually reflects reduced organ perfusion. Renal failure requiring haemodialysis, metabolic acidosis and hyperkalaemia may supervene in severe cases (Bradberry et al., 2004).

Since kidney may also be an organ for the excretion of glyphosate components (Sribanditmongkol et al., 2012), there is an early evidence of kidney damage that could be used to predict the risk of fatal outcome in glyphosate toxicity. The plant protection product glyphosate was used in the current study, and the surfactant used in Roundup® may contribute to herbicide nephrotoxicity (Seok et al., 2011). Renal dysfunctions have been reported in cases of acute intoxication. Recent studies have reported that the mechanism of pesticide nephrotoxicity might be related to oxidative stress, apoptosis or an inflammatory response, and therefore several chemical and natural compounds with antioxidant and/or anti-inflammatory activity have been examined for their protective effects against pesticide-induced nephrotoxicity. Intoxication with glyphosate has been found to induce early kidney damage, i.e. acute tubular, glomerular necrosis and apoptosis (Naqvi, 2017). These modes of cell death occurred in tubules and glomeruli during the acute stages of Roundup® toxicity. Wunnapuk et al. (2014) investigated a panel of kidney injury biomarkers in terms of suitability to detect acute kidney injury (AKI) as a major renal disease associated with high mortality rate and increasing prevalence (Wunnapuk et al., 2014). After oral administration of glyphosate to rats, suitable biomarkers are able to detect the early stages of kidney injury, and glyphosate has been shown to be a causative agent of vasculitic neuropathy, while exposure to a large quantity of glyphosate-based herbicide over a short

time through transcutaneous or inhalation pathways without protection may cause vasculitis, not only in the peripheral nervous system but also in other organs (Kawagashira et al., 2017).

Recently, much attention has been focused on the protective effects of antioxidants and naturally occurring substances against oxidative stress damage. *Morus alba* L. or white mulberry (family Moraceae) is widely distributed and cultivated in Iran, India, China, southern Europe and North America (Yang et al., 2010). Several pharmacological, biological and clinical properties, including antibacterial, antiviral (Wang et al., 2008), antitussive, hypoglycemic (Naowaboot et al., 2004), hypotensive (Emami et al., 2004), antiatherogenic, antihyperlipidemic (Nickavar & Mosazadeh, 2009), diuretic, astringent and antioxidant (Yang et al., 2010; Kobayashi et al., 2011), have been reported for *M. alba* leaves.

In folk medicine *M. alba* has been used for treatments of urinary incontinence due to its strong diuretic property (Yeung, 1985). Its leaves in the forms of infusion and decoction are well-known in different parts of the world and used for preventing or treating urinary disorders (Yang et al., 2010). They are also reported to have diuretic, antiviral, and bacteriostatic properties (Chu et al., 2006). Mulberry fruits also have a tonic effect on kidneys (Duke & Ayensu, 1985). Various phenolic compounds have been identified from mulberry leaves, such as flavonoids and other derivatives; these compounds are responsible for most of the potential health benefits of mulberry leaves and help maintain the body against cellular injuries.

Numerous studies have focused on the nephroprotective activity of *M. alba*. A study has investigated the nephroprotective effect of hydroalcoholic extract and flavonoid fraction of *M. alba* leaves on cisplatin-induced nephrotoxicity in rats. Flavonoids from *M. alba* could also prevent cisplatin-induced pathological damage of the kidney. Cisplatin as an important anti-tumor drug causes nephrotoxicity mainly by oxidative stress and renin-angiotensin system (RAS). *M. alba* leaf extracts have been reported to have protective effects on cisplatin-induced nephrotoxicity in rats (Nematbakhsh et al., 2013) and prevent renal functioning alterations expected with the use of gentamicin, the most effective bactericidal drug against a wide range of Gram negative micro-organisms, and its nephrotoxic side effects (Ullah et al., 2016). Moreover, nephroprotective effects of hydroalcoholic extracts of *M. alba* L. against isoniazid (INH) have been studied in albino rabbits. Isoniazid is the first line

drug for the treatment of tuberculosis and can cause nephrotoxicity in humans and animals (Faqir et al., 2014). The effect of mulberry tea supplement on the cellular damage of kidney induced by supratherapeutic acetaminophen administration was also investigated (Salih et al., 2015). Acetaminophen, also known as paracetamol, is widely used as an analgesic and antipyretic, prescribed as pain reliever and fever reducer. Mulberry tea extract supplement did help to maintain kidneys closer to normal and served to protect them from severe damage due to nephrotoxicity, compared to animals that received no such supplement (Salih et al., 2015).

Positive correlation between glyphosate exposure and health deterioration has been increasingly recognized, and chronic exposure to glyphosate may be the cause of widespread nephrotoxicity. Previous studies have demonstrated that extracts from *M. alba* had renoprotective properties but the effects of MLE on glyphosate-induced kidney injury remained unclear. The aim of this study was to examine the effects of MLE on glyphosate-induced kidney injury and elucidate its molecular mechanisms.

MATERIALS AND METHODS

Glyphosate [N-(phosphonomethyl) glycine] (GLP), Roundup® plus 450g/L(H.029-11), is a commercial formulation, purchased from ATLAS AGRICOLE, which has been registered with the Tunisian Ministry of Agriculture. Thiobarbituric acid (TBA), 2,6-di-tert-butyl-4-hydroxytoluene (BHT), trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), 2-methoxyphenol (gaialcol), bovine catalase 4-(1-hydroxy- 2-methylamino-ethyl)-benzene-1,2-diol (epinephrine), and 2,4-dinitrophenyl hydrazine (DNPH) were obtained from Sigma-Aldrich (Germany). Buffer salts (KCl, $NaHCO_3$, Na_2HPO_4 , NaH_2PO_4 , K_2HPO_4 , and KH_2PO_4) were purchased from Baker Inc. (Phillipsburg, USA).

M. alba leaf extract preparation

Mulberry (*Morus alba* L.) leaves were collected in May-June in the north of Tunisia where the species grows wildly. The leaves were then washed with distilled water and extracted with 70% cold acetone ($-20^\circ C$). The supernatant was collected and pooled, then concentrated to dryness under vacuum, using a rotary evaporator ($60^\circ C$), to obtain a final volume of 3 ml (Rebai et al.,

2017). Then, the aqueous extract was lyophilized to obtain MLE, which was then stored at $-20^\circ C$ before use. Phenolic compounds from the mulberry leaves were assayed by the Folin-Ciocalteu method, and MLE ($100\ \mu g\ ml^{-1}\ kg^{-1}\ b.w.$) was used at the concentration of $100\ \mu g/ml$.

HPLC-DAD analysis and LC-electrospray ionization (ESI)-MS

Phenolic compounds were separated by reverse phase HPLC analysis under conditions previously described (Fattouch et al., 2008) with UV or DAD detection. Analytical RP-HPLC analysis was performed with a C18 column. The mobile phase consisted of 1% aqueous formic acid (solvent A) and methanol (solvent B). The elution was allowed to run with 95% A and 5% B, 75% A for 10 min, 65% A for 3 min, 55% A for 35 min, 40% A for 40 min, 50% A for 45 min, 45% A for 50 min, 30% A for 53 min, 25% A for 56 min, 20% A for 60 min and 95% A for 95 min for 10 min. The flow rate was 1 ml/min. Polyphenols in the eluted fractions were detected at 280 nm and 350 nm with a diode array detector. The LC-ESI-MS system consisted of an Agilent LC 1100 series (Agilent Technologies, Inc., CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000b (Bruker Daltonics, GmbH, Germany) mass spectrometer, equipped with an ESI source and ion trap mass analyzer. The ESI was operated in the positive mode with ESI source probe at $250^\circ C$; CDL at $250^\circ C$, block at $240^\circ C$, flow gas (N_2) at 4.5 l/min, probe voltage 4.5 kV, fragmentor voltage 20 V, and a nominal mass range up to m/z 800. Compounds were identified by comparing their retention times and spectra to those of standards, when available. Quantification was then confirmed by comparison with the calibration curves obtained with standards, i.e. reference solution of phenolic compounds. Unknown chromatographic peaks were tentatively identified via their spectral features in comparison with literature data.

Animals

Wistar rats (180–240 g) were purchased from the Society of Pharmaceutical Industries Tunisia (SIPHAT), allowed to acclimatize in the laboratory environment for 1 week at room temperature $22 \pm 1^\circ C$, and supplied with standard diet and tap water *ad libitum*. Procedures with the laboratory animals and their care were in accordance with the NIH guidelines.

Experimental procedure and treatment

The animals were randomly divided into four groups of six animals each: group 1 received standard diet (control), group 2 was injected (i.p.) with glyphosate (100 mg kg⁻¹ b.w.), group 3 was injected with MLE (100 µg ml⁻¹ kg⁻¹ b.w.) (i.p.), and finally group 4 was injected with both glyphosate and MLE according to Rebai et al. (2017). The rats were observed daily for mortality and signs of toxicity. At the end of the experimental period (15 days), the animals were anesthetized with urethane (40 mg ml⁻¹) and then sacrificed. Blood samples were collected and allowed to clot at room temperature before centrifuging at approximately 3000 rpm for 15 minutes. The serum was stored at -20 °C until assaying for biochemical parameters. Kidneys were removed and dissected free from the surrounding fat and connective tissue, then homogenized in PBS buffer of pH 7.4 with an Ultrathurax T25 homogenizer, and centrifuged (10 at 10,000 g, 4 °C) and the supernatant was used for determination of markers for oxidative stress and biochemical parameters.

Measurement of lactate dehydrogenase (LDH) released

The amount of LDH released is measured with an enzymatic reaction which converts iodonitrotetrazolium or INT (a tetrazolium salt) into a red color formazan. When LDH is present in the cell culture, it reduces NAD⁺ to NADH and H⁺ through the oxidation of lactate to pyruvate. The catalyst (diaphorase) then transfers H/H⁺ from NADH + H⁺ to the tetrazolium salt INT to form a colored formazan salt. The amount of color produced is measured at 490 nm by standard spectroscopy and is proportional to the amount of damaged cells in the culture.

Kidney protein quantification

Total soluble proteins were determined according to Ohnishi and Barr (1978) using the Biuret method. At acidic pH, a blue-colored complex of soluble proteins with copper was spectrophotometrically measured at 546 nm using NanoSpec Double UV (Germany) (Ohnishi & Barr, 1978).

Urea and creatinine assessment in plasma

Urea and creatinine in plasma were measured using a commercially available spectrophotometric enzymatic kit

(commercial kit from Biomaghreb, Tunisia) according to Larsen (1972).

Antioxidant enzyme activity

Catalase (CAT) assay

Catalase activity was assayed by measuring the initial rate of H₂O₂ disappearance at 240 nm. The reaction mixture contained 33 mM H₂O₂ in 50 mM phosphate buffer pH 7.0 and 5 µl of sample. CAT activity was calculated using the extinction coefficient of 40 mM⁻¹cm⁻¹ for H₂O₂. One unit of catalase activity is defined as the amount of enzyme catalyzing the degradation of 1 mmol of H₂O₂ per minute at 37 °C and specific activity corresponding to transformation of substrate (in mmol) (H₂O₂) per minute per milligram protein (Aebi, 1984).

Peroxidase (POD) assay

Peroxidase activity was measured at 25 °C using guaiacol as hydrogen donor. The reaction mixture contained 9 mM guaiacol, 19 mM H₂O₂ in 50 mM phosphate buffer pH 7, and 10 µl samples in 1 ml final volume. The reaction was initiated by the addition of H₂O₂ and monitored by measuring the increase in absorbance at 470 nm each 30 s for 3 min. Peroxidase activity was expressed as nanomoles of guaiacol oxidized per minute with a molecular extinction coefficient of 26.2 mM⁻¹ for calculation.

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was determined by using the modified epinephrine assay. At alkaline pH, superoxide anion (O₂⁻) causes the auto-oxidation of epinephrine to adrenochrome. One unit of SOD is defined as the amount of extract that inhibits the rate of adrenochrome formation by 50%. Samples were added to 2 ml reaction mixture containing 10 µl bovine catalase (0.4 U µl⁻¹), 20 µl epinephrine (5 mg ml⁻¹), and 62.5 mM sodium carbonate/sodium bicarbonate buffer pH 10.2. Changes in absorbance were recorded at 480 nm each 30 s for 3 min (Misra & Fridovich, 1972).

Calcium determination

Extracellular ionizable calcium was determined using a commercially available kit from Biomaghreb, Tunisia. At basic pH, calcium was constituted with

cresolphthalein, a purple-colored complex measurable at 570 nm. Briefly, 50 μ l of sample was added to the reaction mixture containing 2-amino-2-methyl 1-propanol buffer (500 mmol L⁻¹), cresolphthalein (0.62 mmol L⁻¹), and hydroxy-8 quinoleine (69 mmol L⁻¹). Incubation was carried out at room temperature for 5 min assuming the complex was stable during 1 h (Stern & Lewis, 1957).

Free iron determination

Free iron was determined according to Leardi et al. (1998) using a commercially available kit from Biomaghreb (Ariana, Tunisia). Briefly, at acidic pH 4.8, all Fe³⁺ released from transferrin were reduced by ascorbic acid into Fe²⁺, which constitutes with ferrozine a colourful purple complex measurable at 560 nm. Heart extract was added to 250 μ l of reaction mixture containing ascorbic acid (5 g/L) and ferrozine (40 mM), and incubation was performed at 37°C for 10 min (Leardi et al., 1998).

Lipid peroxidation determination

Lipid peroxidation was carried out using the method of MDA measurement according to Draper and Hadley (1990). An aliquot of brain homogenate was mixed with BHT-TCA solution containing 1% BHT and 20% TCA. After centrifugation, the supernatant was mixed with a second solution containing 0.5 N HCL and TBA (120 mmol L⁻¹) and then heated at 80 °C for 10 min. After cooling, the absorbance of the resulted chromophore was measured at 532 nm using a Nanolytik® NanoSpec Double UV-visible spectrophotometer (Nanolytik, Quality from Germany). Malondialdehyde (MDA) contents were expressed as millimoles MD per milligram protein with an extinction coefficient of 1.56105 mol L⁻¹ cm⁻¹ (Draper & Hadley, 1990).

Protein carbonylation

The most commonly used marker of protein oxidation was used to quantify protein carbonyls (PC) in the brain homogenate by the reaction between 2,4-dinitrophenylhydrazine and DNPH, and protein carbonyls. After the precipitation of proteins in the sample with 20% TCA, and centrifugation at 10,000 g

for 10 min at 4 °C, DNPH-containing buffer reacted with protein carbonyls and dissolved in 20 mmol L⁻¹ potassium phosphate (pH 2.3) containing guanidine HCL 6 mol L⁻¹. The amount of protein carbonyls produced is quantified spectrophotometrically at an absorbance of 366 nm using the molar extinction coefficient of 22,000 mol L⁻¹ cm⁻¹. Carbonyl content can be standardized to protein concentration and expressed as nanomoles of carbonyl residues per milligram of protein.

Statistical analysis

Data are presented as the mean \pm SEM from three independent experiments performed in quadruplicate. Statistical analysis of the data was performed by using Student's t-test and ANOVA, followed by Bonferroni's test. 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.

RESULTS

Evaluation of body and kidney weights

The animals were checked for both body and kidney weight gain or loss. The results show that weight variations were time-dependent effects of glyphosate in the exposed rat group during glyphosate treatment period at a dose of 100 ppm. The final body weight of rats moderately decreased in the glyphosate-exposed groups, compared to controls, as shown in Figure 1. Weight loss was observed when rats were administered daily glyphosate dose (100 μ g kg⁻¹ b.w.), while co-treatment with both MLE and glyphosate induced a significant gain in body weight, compared with the glyphosate-treated group. On the other hand, no statistically significant difference in absolute kidney weight was observed between the control and MLE-treated group (P>0.05). However, absolute kidney weight showed a significant increase in glyphosate-treated groups, compared to control group. Moreover, the absolute kidney weight of rats submitted to glyphosate co-treatment and to MLE-treatment was compared to the control group, and no statistically significant changes were observed (P>0.05).

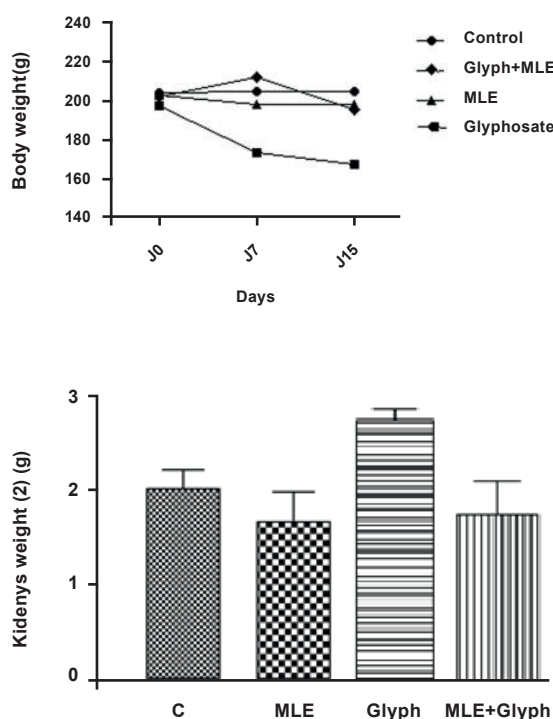


Figure 1. Body weight evolution and kidney weight variations during treatment. Glyphosate and MLE were administered in a single dose i.p. to rats daily for 15 days. Data are expressed as mean \pm SEM

MLE protects kidneys from toxicity induced by glyphosate exposure

Lactate dehydrogenase (LDH), an important intracellular enzyme used as a sensitive parameter that reflects oxidative stress and altering of the enzymatic system in tissues, was used to evaluate the nephroprotective effect of MLE against toxicity induced by glyphosate. As shown in Figure 2A, administration of a single dose of glyphosate at 100 ppm $100 \text{ g}^{-1} \text{ b.w}$ for two weeks induced cell death by increased LDH release from kidney tissue, about +50% compared to the non-treated control group. Moreover, the co-administration of glyphosate and the phenolic extract MLE attenuated the toxic effect of the pesticide and decreased LDH levels in kidney homogenate in rats.

Then, focusing on renal specific biomarkers toxicity using creatinine, and urea levels, our results show that both creatinine and urea levels decreased in the glyphosate and MLE co-treated group (Figure 2B,C) to correct the deleterious effect of glyphosate in the renal function of rats exposed only to glyphosate.

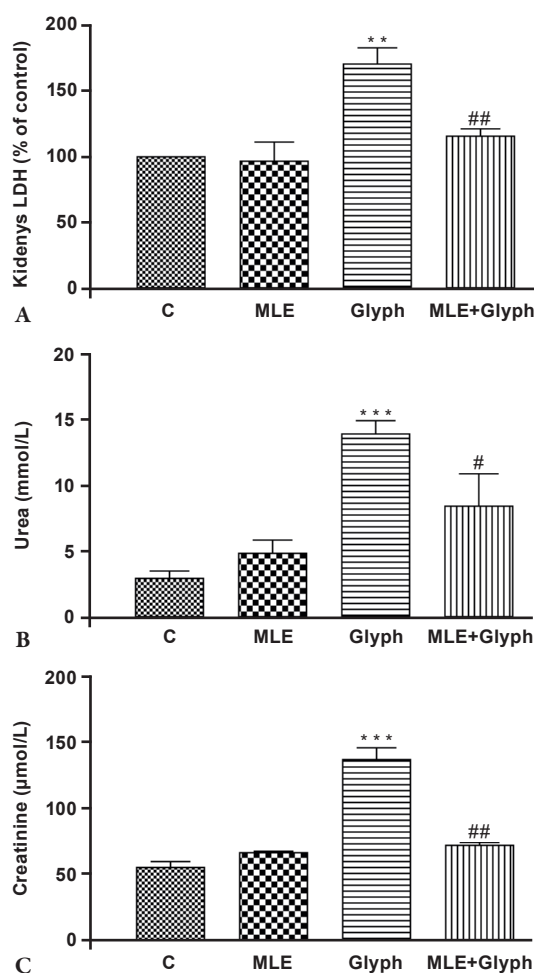


Figure 2. Nephroprotective Effect of MLE against on glyphosate-induced renal toxicity.

- (A) Glyphosate-induced release LDH from kidneys. The results are expressed as percentages of lactate dehydrogenase (LDH) released, quantified by using an LDH activity kit assay.
- (B, C) MLE restores renal toxicity biomarkers (urea and creatinine) induced by glyphosate. Results are expressed as mean \pm SD ($n = 5$). Asterisk $P < 0.05$ vs control, number signs $P < 0.01$ vs pesticide treated rats

Enzymatic and antioxidant status in kidney

The results showed that oxidative stress induced by glyphosate altering the enzymatic system affects antioxidant enzyme activities. Catalase (CAT) and peroxidase (POD) activities increased significantly in the exposed groups, compared with control groups, but the levels of superoxide dismutase (SOD) activity decreased markedly in kidneys by -87.5% in the exposed groups, compared to controls. MLE administered in co-treatment

with glyphosate decreased both CAT and POD activities near to the level in non-treated group. Total SOD activity was corrected by MLE administration, bringing it to near control levels (Figure 3).

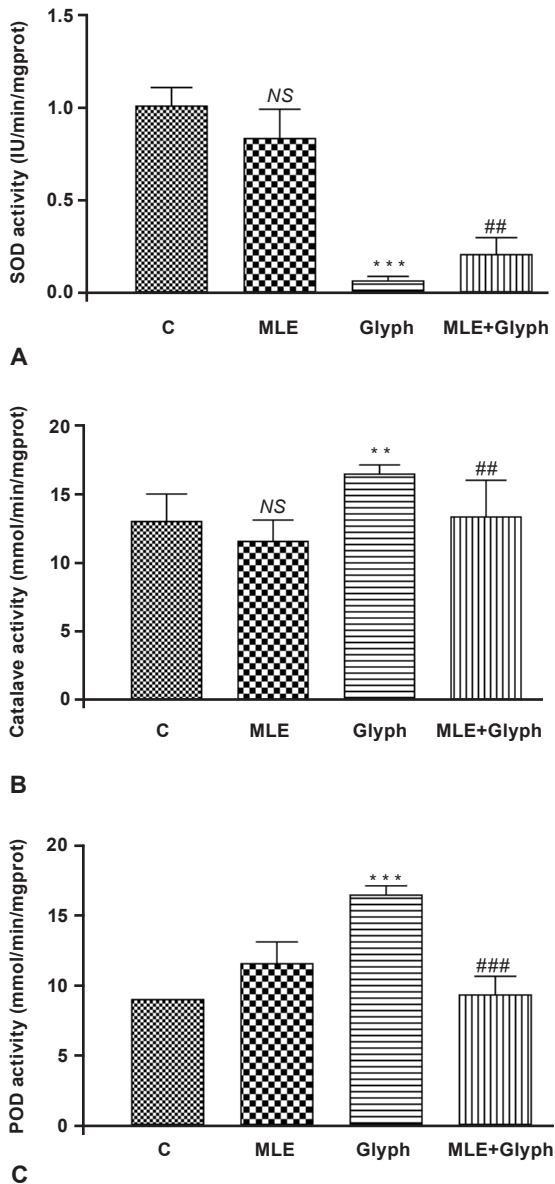


Figure 3. Effect of MLE extract and glyphosate on kidney antioxidant enzymatic system. Wistar rats were administered i.p. with *Morus alba* leaf extract (MLE), glyphosate 100 mg kg⁻¹ b.w. (Glyph), or glyphosate plus MLE (MLE + Glyph). (A) superoxide dismutase (SOD), (B) kidney catalase, and (C) peroxidase (POD) activities were determined. Results were expressed as means \pm SD (n = 5). 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.

Estimation of MDA and protein carbonyl levels in kidneys

Glyphosate exposure provoked lipid peroxidation, inferred from an almost double increase in MDA levels, and three-fold in protein carbonyls in the groups treated with 100 ppm glyphosate for two weeks, compared with those in control groups (Figure 4). Co-treatment with mulberry leaf extract abrogated the toxic effect of glyphosate, bringing it near to control group levels.

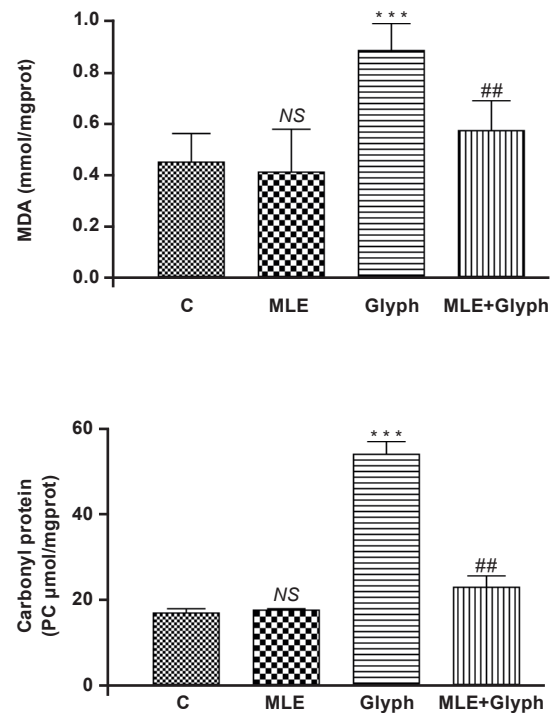


Figure 4. Renoprotective effect of MLE on glyphosate-induced lipoperoxidation and protein carbonylation. Rats were treated with glyphosate (100 mg kg⁻¹ b.w.). MLE was administered i.p. with the dose of 100 μ g ml⁻¹ daily for 15 days. Results are expressed as mean \pm SD (n = 5). Asterisk P < 0.05 vs control, number signs P < 0.01 vs pesticide-treated rats

Glyphosate and intracellular mediators in kidneys

Calcium pathway and free iron accumulation, the intracellular mediators involved in the molecular target of glyphosate-induced oxidative stress against which MLE could exert its neuroprotective effects, were investigated. Glyphosate provoked an increase in renal free iron (Figure 5A), and ionizable calcium (Figure 5B). Co-treatment of rats with glyphosate and MLE significantly decreased these mediators when compared to the control group.

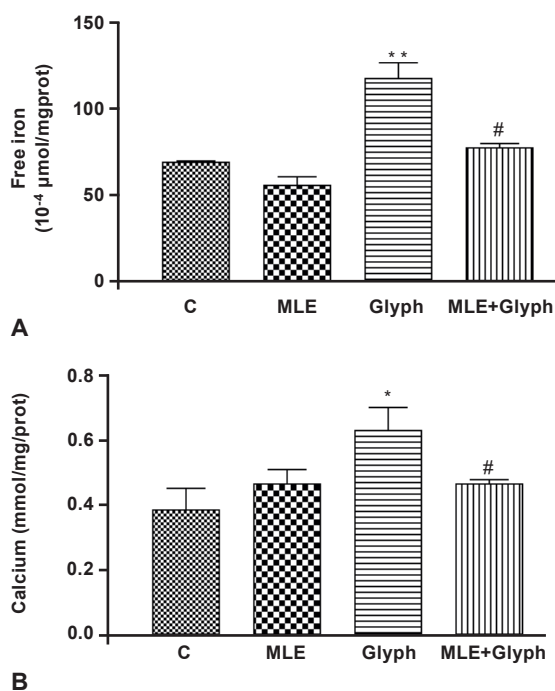


Figure 5. Protective effects of MLE on glyphosate-induced free iron (A) and Ca_2^+ (B), in kidneys. Rats were administered i.p. with *Morus alba* leaf extract (MLE), glyphosate 100 mg kg⁻¹ b.w. (Glyph), or glyphosate plus MLE (MLE + Glyph). Results are shown as means \pm SD (n = 5). Asterisk P < 0.05 vs control, number signs P < 0.01 vs pesticide-treated rats

DISCUSSION

Glyphosate [N-(phosphonomethyl) glycine] is an organophosphate and one of the most popular herbicides in the world and the active ingredient of the plant protection product Roundup®. As previous investigations performed on various organs have revealed, glyphosate causes hepatotoxicity (El-Shenawy, 2009), neurologic disorders (Negga et al., 2012; Rebai et al., 2017), and nephrotoxicity (Astiz et al., 2009). Importantly, detection of glyphosate residues in human urine has proposed that early evidence of kidney injury could be used to predict the risk of glyphosate toxicity (Valcke et al., 2017).

Many studies have reported that accidental or intentional ingestion of glyphosate or other surfactant-based herbicide, such as Roundup®, leads to nephrotoxicity and severe renal function disorders. More recently, it has been reported that exposure to glyphosate-based herbicides in transcutaneous or inhalation pathways without protection may cause vasculitis neuropathy, not only in the peripheral nervous

system but also in other organs (Kawagashira et al., 2017). Moreover, other studies have reported that glyphosate is able to trigger dysregulation of numerous metabolism pathways, which may contribute to oxidative damage and generation of reactive oxygen and nitrogen species (ROS, RNS) (Astiz et al., 2009), and to cause mitochondrial oxidative phosphorylation by damaging the mitochondrial membrane (Bradberry et al., 2004), to mediate toxicity by lipid peroxidation and protein carbonyl formation (Mensah et al., 2012; Rebai et al., 2017), to enhance DNA damage (Navarro & Martinez, 2014) and to inhibit RNA transcription (Marc et al., 2005). Glyphosate may also induce apoptosis by inducing caspase 3 in *in vitro* studies (Culbreth et al., 2012).

Since kidneys are the main site of xenobiotic biotransformation and elimination, and are considered a major homeostatic organ, while it may also be an organ for the excretion of glyphosate components (Sribanditmongkol et al. 2012), several *in vitro* and *in vivo* research studies have been performed and a relatively large number of chemicals and natural compounds tested for their potential protection against pesticides-induced nephrotoxicity. Çavuşoğlu et al. (2011) showed that treatment of Swiss albino mice with *Ginkgo biloba* L. leaf extract (150 mg/kg b.w) produced an improvement in indices of nephrotoxicity, lipid peroxidation, and genotoxicity induced by glyphosate (50 mg/kg body weight). These *in vivo* results showed that *G. biloba* may present a significant protective effect against toxicity induced by glyphosate (Çavuşoğlu et al., 2011).

Based on these observations, changes in renal biochemical parameters in the kidney in our experimental study were examined in rats after applying sub-lethal doses of glyphosate (100 mg/kg b.w.) to identify suitable biomarkers able to detect the early stages of kidney injury and molecular mechanism involved in the nephroprotective effect of polyphenolic extract of *M. alba* leaves. The *M. alba* plant is reported to have an anti-inflammatory potential (Chen et al., 2013) and possess strong antioxidant properties (Sadighara & Barin. 2010). Several studies have also demonstrated that *M. alba* extract has significant beneficial kidney protection effect in drug-induced nephropathy. It has been reported to have a protective effect on cisplatin-induced nephrotoxicity in rats, and to prevent renal functioning alterations and nephrotoxicity caused by therapeutic treatment drugs, e.g gentamicin as a bactericide against Gram negative micro-organisms (Nematbakhsh et al., 2013), isoniazid (INH) as a tuberculosis drug (Faqr et al., 2014), supratherapeutic acetaminophen as a paracetamol analgesic and anti-pyretic (Salih et al., 2015).

To produce nephrotoxicity in experimental animals, different doses of glyphosate have been employed in different studies, and administration of large doses of glyphosate were reported to produce alteration in kidney functioning. A dose of 50 mg/kg causes renal tubular damage and glomerular filtration impairment and significant rise in serum creatinine (Çavuşoğlu et al., 2011). A dose of 126 mg/kg causes peritubular inflammatory reaction and nephrose characterized by cellular vacuolation and limited tubular necrosis. El-Shenawy. (2009) used a sub-lethal concentration of glyphosate alone (134.95 mg/kg) to induce nephrotoxicity, and thus to produce nephrotoxicity in experimental animals, while approximately 100 mg/kg has been employed in different other research experiments. Therefore, a daily dose of 100 mg of glyphosate/kg was selected in the current study to produce significant nephrotoxic effects in experimental animals (El-Shenawy, 2009).

Therefore, a reduction in kidney weight was observed in the group of rats treated with glyphosate and this loss of weight was ameliorated when rats received a daily dose of *M. alba* extract during the experimental period (Figure 1). This reduction in kidney weights can be attributed to oxidative and cellular damage caused by glyphosate. This renal atrophy may be the consequence of renal fibrosis and renal healing due to kidney inflammation, cellular phenotype transformation and renal parenchyma deficiency. In accordance with these observations, morphological changes in kidney following pesticide exposure were also reported by Hamdaoui et al. (2016), demonstrating that albino rats intraperitoneally treated with a sub-lethal concentration of glyphosate alone (126 mg/kg) led to significant reduction in kidney weight. However, it was not the case with El-Shenawy (2009), who demonstrated an increase in kidney weight between control and Roundup-treated groups when rats were treated with a sub-lethal concentration of glyphosate (134.95 mg/kg) at 2-day intervals during two weeks.

Generally, intracellular enzymes were used as important biomarkers for detection of hepatotoxic and nephrotoxic effects of pesticides. Benedetti et al. (2004) showed that glyphosate causes liver damage in rats by intracellular enzymes leakage in liver. Herein, kidney excretion of lactate dehydrogenase was studied to investigate the nephroprotective properties of *M. alba* extract. Lactate dehydrogenase (LDH), an intracellular enzyme, is recognized as a potential marker for assessing the toxicity of chemical products (Agrahari et al., 2007). An increase in LDH can reflect damage to a number of different tissues (skeletal or cardiac muscles, kidney or liver). LDH levels may rise whenever there is cell necrosis or when neoplastic

proliferation of cells causes an increased LDH production (Dzoyem et al., 2014). In this our study, LDH increased in animals treated with glyphosate. This can be explained to be the result of oxidative stress induced by LDH leaking from kidneys in the experimental model, which showed early detection of renal damage. However, it decreased to the normal range with MLE administration (Figure 2).

Therefore serum creatinine and blood urea have usually been used as an early and sensitive indicators, or biomarkers, for kidney injury in human pesticide intoxication. Serum Cr is the most commonly studied biomarker in glyphosate-induced clinical nephrotoxicity (Mohamed et al., 2016). In our study, a significant increase in serum urea and creatinine levels was observed after exposure to glyphosate. Renal tubular damage and glomerular filtration impairment were observed in the kidneys of mice exposed to glyphosate and these forms of damage may account for the increase in serum urea and creatinine levels of the animals receiving glyphosate (Çavuşoğlu et al., 2011; Mohamed et al., 2016). Our results clearly showed that the polyphenolic fraction of *M. alba* inhibited the glyphosate-induced increase in kidney damage biomarkers (urea and creatinine) as an earlier stage of nephrotoxicity (Figure 2). Also, Mansour and Mossa (2010) reported that pesticides can alter plasma urea, uric acid and creatinine levels as a result of impairment of the glomerular function and tubular damage in kidneys (Mansour & Mossa, 2010). Since creatinine is eliminated throughout glomerular filtration and tubular secretion in the proximal tube, the creatinine level is considered as a good risk marker for chronic renal insufficiency. Urea is the ultimate end product of protein catabolism in the body. The elevation of blood urea is also an indicator for renal failure and kidney dysfunction (Baudin et al., 2013). These results were in accordance with the results of Zhang et al. (2017) and Mesnage et al. (2015) who reported significant changes in kidney hemato-biochemical indices, including statistically increased levels of creatinine and urea in rats treated with glyphosate (Mesnage et al., 2015).

Furthermore, controlling the antioxidant enzyme system, MLE can modulate the increased levels of CAT and POD activities. Our results are in accordance with the findings of Peluso et al. (1998), who detected the induced formation of DNA and enhanced hepatic CAT activity in rats treated with glyphosate. Among the antioxidant enzymes, SOD is the primary step of the defense mechanism against oxidative stress by catalyzing dismutation of superoxide radicals (O_2^-) into molecular oxygen (O_2) and H_2O_2 (McCord et al., 1971). H_2O_2 is neutralized by the combined action of CAT in all vertebrates. SOD enzymes play critical

roles in the regulation of cellular oxidative stress. The important SOD decline in glyphosate-induced toxicity was significantly restored by MLE (Figure 3). However, the interaction between glyphosate and the activities of SOD enzymes is not yet understood. An hypothesis is that glyphosate exposure leads to a SOD decline in kidneys, which results from the loss of copper and zinc, which directly binds to sulfhydryl groups on cysteine. Further studies should specify whether the cytoplasmic Cu/Zn SOD or the mitochondrial Mn-SOD were most affected. Another hypothesis is that modulation of antioxidant enzymes could correspond to post-translational modification as oxidative phosphorylation. Furthermore, glyoxylate, a breakdown product of glyphosate, is a potent glyating agent and would cause DNA damage by attacking Cu and Zn-SOD (Kaneta et al., 1994).

Generally, it is well established that elevated intracellular MDA and the protein carbonyl (PC) affect membrane integrity which is correlated to oxidative stress and pathological conditions (Rizvi & Maurya, 2007). Especially renal cells are highly susceptible to oxidative damage because of the high polyunsaturated fatty acid content of their membrane. It has already been reported that glyphosate induces lipid peroxidation (El-Shenawy, 2009) and its administration generates overproduction of free radicals, causing oxidative damage, and increases lipid peroxidation levels in kidney tissues even at lower doses (10 mg kg^{-1}). Our results show that MLE reduces glyphosate-induced intracellular lipid peroxidation by decreasing levels of MDA as a final product of peroxidation. (Figure 4). This protective effect can be due to scavenging MDA molecules by the active ingredient content of MLE or by inhibiting mitochondrial chain reactions. Furthermore, a significant increase in protein carbonyl levels suggests that the oxidative protein damage might be one of the explanations of glyphosate toxicity that is restored by the administration of daily dose of MLE.

In fact, the kidney is the major organ involved in the regulation of calcium and phosphate homeostasis (Wei et al., 2016). We found that glyphosate alters intracellular mediators in kidneys as well as in brain, as demonstrated by Rebai et al. (2017) under the same experimental conditions. MLE is able also to cancel increases in the levels of both calcium and iron triggered by the toxic effect of glyphosate (Figure 5).

Indeed, as glyphosate increased free iron levels, it could also increase hydroxyl radicals, toxic radicals that may in turn modify calcium homeostasis. Several studies have demonstrated that glyphosate promotes calcium

uptake by L-type voltage-activated channels leading to calcium-overload cell death (De Liz Oliveira et al., 2013). Moreover, Rebai et al (2017) demonstrated that *in vivo* exposure to the same dose of glyphosate induced Ca_2^+ uptake in brain homogenates. Further experimentation should investigate the putative involvement of calcium channels in the mechanism of action of glyphosate in kidney tissue using calcium channel blockers.

Another possible target that can be involved in the protective effect of MLE against glyphosate-induced toxicity in the kidneys is its ability to reduce the levels of free iron. It is already known that free iron acts like a catalyst of auto-oxidation and there is an evidence that increase in free iron is correlated with oxidative stress status. Its role in disease processes seems to be a common theme of cellular injury. Thus, it is of interest that experimental evidence exists for the role of antioxidant molecules in contrast-induced injury kidneys, in particular AKI (Bakris et al., 1990).

Importantly, MLE counteracted glyphosate-induced renal damage and this nephroprotective activity could result from synergism between various *M. alba* extract-containing phenolic compounds. The phenolic constituents of the investigated MLE were analyzed by RP-HPLC, and monitored by UV or diode-array detector and mass spectrometry (ESI-MS) analysis (Figure 6 and Table 1). Considering the elution profile of chlorogenic acid isomers from plant foods reported in the literature on C18 HPLC columns (Fang et al., 2002), chlorogenic acid (5-CQA) and its isomers, as members of cinnamic acid, are the most abundant constituent in acetic-aqueous mulberry leaves extract. This finding is in agreement with previous reports suggesting that 5-CQA is the major constituent of mulberry leaves (Thabti et al., 2012; Sánchez-Salcedo et al., 2015). The findings of many researches have confirmed that these compounds are responsible for useful effects in oxidative stress. Thus, antioxidant compounds from mulberry (*M. alba*) leaf extract are absorbed in the small intestine and then pass into blood circulation and retain their antioxidant activity in the animal (Lee et al., 2007). Chlorogenic acid (5-CQA) is a class of cinnamic acid, which possesses different isomeric forms, and it is the predominant phenolic compound in coffee and berries. Pharmacological effects of chlorogenic acid in kidneys have been demonstrated and it appears to exhibit renoprotective activity. A dose of CGA (100 mg/kg body weight) was given to mice for 8 days and it was reported to reverse lipid peroxidation, cause inactivation of cytochrome P450 and increase cellular defense (Kapil et al., 1995).

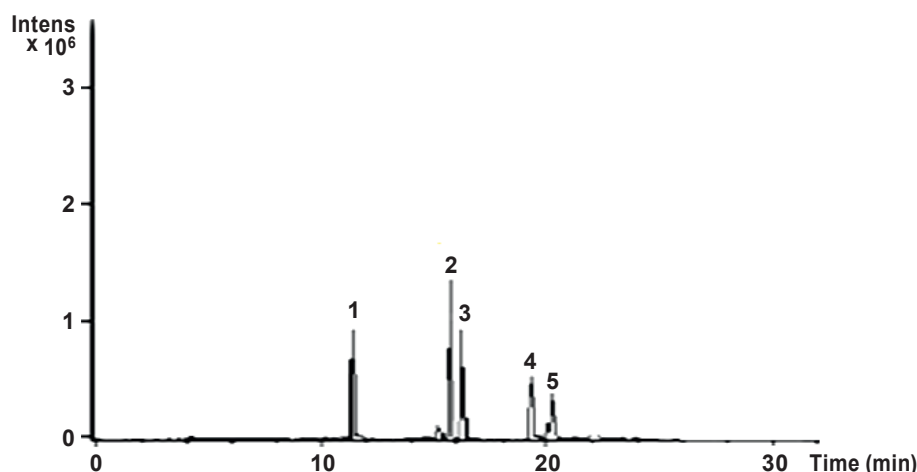


Figure 6. Typical liquid chromatography profile of *Morus alba* leaves extract detected by absorbance at 280 nm. Peaks were identified by LC-MS as shown in Table 1.

Table 1. RP-HPLC-DAD and LC-MS analyses of main phenolics in *Morus alba* leaf aqueous-acetone extracts

| Pic N° | tR (min) | m/z (M-H) ⁺ | Identity | Concentration (mg/100g fw) |
|--------|----------|------------------------|---|----------------------------|
| 1 | 11.5 | 353 | Neochlorogenic acid (3-CQA) | 5.12 |
| 2 | 15.5 | 353 | Chlorogénique acid (5-CQA) | 6.95 |
| 3 | 16.3 | 353 | 3,4 –Dicafeoly quinic (isochlorogenic acid) | 3.71 |
| 4 | 16.8 | 353 | Crypto chlorogenic acid (4-CQA) | 1.28 |

The renoprotective activity of caffeic acid (3, 4-dihydroxycinnamic acid), a major metabolite of chlorogenic acid, has been investigated through *in vitro* studies. Caffeic acid (CA) is already considered a potent antioxidant, and its function depends on its chemical structure. Coffee acid can improve chronic renal failure and reduce protein urea, blood urine nitrogen and blood creatinine, as well as oxidation stress in the kidney. CA administration alleviated glomerular sclerosis scores and tubulointerstitial injuries and this effect may be due to its anti-oxidation and inhibiting accumulation of extracellular matrix (Jingqiu et al., 2016). Several other studies have also demonstrated that CA has a significant beneficial kidney protection effect in drug-induced nephropathy (Domitrović et al., 2014) and diabetic nephropathy (Jin et al., 2015). The anti-hypertension effect of CA is well-established (Zhao et al., 2012), a property that may be a good marker to control progress of chronic kidney dysfunction (Suzuki et al., 2006). Also, it has been suggested that CA attenuates CP-induced kidney injury through suppression of oxidative stress, inflammation, apoptosis and autophagy, along with improvement in kidney regeneration which protects

the kidneys from nephrotoxicity by reducing the burden of tubular cells (Domitrović et al., 2014).

CONCLUSION

In this study we reported that glyphosate may induce oxidative stress leading to alterations in enzymatic redox, antioxidant endogenous system and scavengers, and other biochemical parameters which cause damage in the functional integrity of kidneys in rats. Our findings have shown that the use of mulberry leaves extract provides a protective effect to kidney tissue against nephrotoxicity caused by glyphosate, which indicates a significant improvement and reduction in tissue damage in the treated group. These findings suggested that mulberry's high phenolic levels, in particular chlorogenic acid and its isomers, have a potential to reduce or maintain renal toxicity. Finally, it suggests that mulberry leaves extract supplement may serve as a candidate for developing a safe and promising nutraceutical product for the management of renal toxicity induced by glyphosate residues in food of plant origin.

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Nefroprotektivno delovanje vodenog acetonskog ekstrakta *Morus alba* i mehanizam toksičnosti indukovane glifosatom - *in vivo* model.

REZIME

Glifosat, aktivna materija preparata Roundup®, je pesticid koji je u najširoj upotrebi u svetu i njegovi ostaci se mogu naći u prerađenoj hrani i pijaćoj vodi. Ranija istraživanja su potvrdila da ekstrakti lista *Morus alba* poseduju farmakološko dejstvo. Međutim, renoprotektivno delovanje ekstrakta *M. alba* i njihovog molekularnog mehanizma još uvek je nepoznato.

U ogledu su korišćeni laboratorijski beli pacovi (180–200 g) (n=5–6). Kontrolna grupa je intraperitonealno (i.p) dobijala 0.2 ml normalnog fiziološkog rastvora jednom u toku dana tokom dve nedelje. Kontrolnim životinjama je davana standardna hrana. Tretirane grupe su dobijale ili polifenolni ekstrakt (100 mg/kg, i.p) ili glifosat (100 mg/kg, i.p), ili pak njihovu smešu (ekstrakt $\mu\text{g ml}^{-1}$ kg t.m. i glifosat 100 mg kg^{-1} t.m, i.p), jednom dnevno do petnaestog dana tretmana. Merene su vrednosti laktat dehidrogenaze LDH, koncentracije uree, kreatitina i azot-monoksida u serumu, a korišćene su standardne kolorimetrijske metode.

Renalni oksidativni stress, konstatovan preko povišenih vrednosti malondialdehida (MDA) i nivoa proteinskih karbonila, kao i snižene aktivnosti superoksida dismutase (SOD), bio je značajno povišen delovanjem primenjenog ekstrakta lista duda (MLE). MLE je pokazao da može da modulira izmenjene biohemijske parameter održavanjem nivoa slobodnog gvožđa i homeostaze Ca_2^+ , kao i da reguliše endogeni sistem antioksidativnih enzima. Izgleda da istovremena primena vodenog acetonskog rastvora frakcije *M. alba* koji je bogat hlorogenom kiselinom i njenim izomerima može imati zaštitno delovanje na bubrege nakon nefrotoksične aktivnosti glifosata. Ukupno gledano, MLE može delovati zaštitno protiv toksičnosti indukovane glifosatom, što se može pripisati hlorogenoj kiselini i njenim izomerima, koji predstavljaju najprisutnije fenolne kiseline u ekstraktima.

List belog duda je izvor fenolnih jedinjenja i stoga može predstavljati osnovu za potragu za novim hemijskim jedinjenjem koje će omogućiti dobijanje novog lekovitog sredstva. Suplement od ekstrakta belog duda može biti kandidat za razvoj bezbednog i perspektivnog preparata kao dodatka ishrani namenjenog zaštititi od nefrotoksičnosti.

Keywords: glifosat, oksidativni stres, nefrotoksičnost, beli dud, nefroprotektivno delovanje

