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RESEARCH ARTICLE

Nitrate Provoked Kidney Toxicity and DNA Impairment in Adult Rats: Alleviation by *Hyparrhenia hirta*

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ABSTRACT

Our research explored the effects of *Hyparrhenia hirta* (*H. hirta*) against kidney impairment caused by nitrate. Experimentation was performed on rats separated in three groups. Sodium nitrate ($NaNO_3$) was supplied everyday by gavage to treated groups (400 mg/kg BW) either alone, or co-administered with methanolic extract of *H. hirta* (200 mg/kg BW). Nephrotoxicity caused by sodium nitrate was objectified by an increase in plasma and a reduction in urine concentrations of urea and creatinine, while uric acid level decreased in plasma and increased in urine when compared to controls. Our results showed also, a significant decrease in clearance of creatinine in $NaNO_3$-treated group. In addition, sodium nitrate produced oxidative stress in the kidney characterized by enhanced lipid peroxidation, reduced total glutathione level and catalase, superoxide dismutase and glutathione peroxidase activities. Sodium nitrate provoked fragmentation of DNA as reflected by smear and no formation of ladder. Renal damages were characterized histologically after nitrate treatment by degeneration of renal tubule cells and mononuclear cells infiltration. Co-administration of *H. hirta* improved biomarkers of kidney toxicity, malondialdehyde level, activities of antioxidant enzymes and histological damages. We concluded that *H. hirta* had a significant role in animal protection from nitrate-induced kidney dysfunction.

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INTRODUCTION

Changing in motive of farming practice, food treatment and industrial enterprise induce environmental accretion of nitrates. Moreover, this pollutant is amply utilized as an antibiotic and for favoring in several agricultural products (Chow and Hong, 2002). $NaNO_3$ is used as chemical fertilizers. Its excess has an impact on both human and animals (Nabiuni *et al.,* 2014). A chronic exhibition across drinking water became a growing worldwide worry of public health. Gupta *et al.* (2000) have shown that nitrates may be transformed into nitrites in stomach, then in nitrosamine compounds considered as gastric carcinogens. After their absorption by the intestinal tract, they are transferred to blood and could cause methemoglobinemia. Nitrates and nitrites are the oxidized products and the willing origins of nitric oxide. Peroxynitrite and superoxide, metabolites of nitrite, can affect biological membranes causing lipid peroxidation (Salvemini and Cuzzocrea, 2002).

To counteract the oxidative stress, medicinal plants have been used (Zengin *et al.,* 2011). In fact, herbal drugs prevent and treat various diseases and are constantly used throughout the world (Atta *et al.,* 2013). Natural antioxidants are the aim of previous investigations showing potentially safe origins, efficient and inexpensive antioxidants (Mundhe *et al.,* 2011).

In our study, *H. hirta* is considered as a plant providing antioxidant natural sources. It is distributed in Mediterranean countries belonging to the African continent. This plant is renowned for its diuretic action, and has an

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antioxidant property due to its richness in flavonoids as it has been published in our precedent paper (Bouaziz *et al.,* 2014). A number of extracts of natural products and dietary antioxidants have been reported to study the protective effects against nephrotoxicity. While there were no reports describing nephroprotective effects of *H. hirta* in literature. The aim of our work was to detail the toxicity of $NaNO_3$ renal related injury and its protection by *H. hirta* in adult rats.

MATERIALS AND METHODS

The powdered *H. hirta* aerial parts were charged into Soxhlet extractor. Removal of chlorophyll was achieved by a pre-extraction with dichloromethane. The remaining was then extracted with methanol for 48 h, filtered and condensed to remove the methanol using rotary evaporation (Büchi Rotavapor; Büchi Laboratories, Switzerland) at 40°C within vacuum.

Thirty seven Wistar adult rats (200 g) were maintained at standard conditions of temperature and humidity. 24 rats were randomly divided into 3 groups of 8 each as follows: a control group was given distilled water; $NaNO_3$-groups were treated by gavage everyday either by $NaNO_3$ alone (400 mg/kg BW) or co-administered by *H. hirta* methalonic extract administered through drinking water (200 mg/kg BW). The dose of $NaNO_3$ was used according to a previous study of our laboratory (Bouaziz *et al.,* 2014) and corresponded to 1/12 of LD_{50} (4800 mg/kg BW). The plant extract lethal dose (LD_{50}) was established by the Lorke method (1983). The experimental procedure was performed according to the local institute ethical committee guidelines and the principles of laboratory animal care published by the Council of European Communities (1986).

After cervical decapitation, blood samples were collected and centrifuged for 10 min at 2200×g. Plasma specimens were stored at -20°C until analysis.

Urinary samples were obtained from each rat housed 24 hours in a specially designed metabolic cage in order to eliminate feces. All samples were recorded and centrifuged for 5 min at 3000×g. Kidneys were prelevated and weighed. Some of them were cleaned and homogenized in a suitable buffer (pH=7.4) and centrifuged.

Urea, creatinine and uric acid concentrations in plasma and urine were estimated using commercial diagnostic kits, (Refs respectively. 20151, 20091 and 20143), taken up from Biomagreb (Ariana, Tunisia). The clearance of creatinine, was measured by UV/P equation where V was the urinary sample volume collected within 24 h, U was the level of urinary creatinine, and P the concentration of plasma creatinine.

The level of malondialdehyde (MDA) in tissues was measured spectrophotometrically by Draper and Hadley method (1990). Lipid peroxidation was expressed as nmoles of thiobarbituric acid reactive substances/mg of protein. GSH in kidney was estimated according to Jollow *et al*. technic (1974). The content of total GSH was calculated in terms of µg /mg of protein. The activity of superoxide dismutase (SOD) was assayed as stated by Beauchamp and Fridovich (1971). The SOD activity was calculated in terms of units/mg of protein. The activity of catalase (CAT) was determined according to Aebi (1984). CAT activity was expressed as µmoles H_2O_2 consumed/ min/mg of protein. Glutathione peroxidase (GPx) activity was assayed as stated by Flohe and Gunzler (1984). The enzyme activity was calculated in terms of nM of GSH oxidized/min/mg protein. The extent of DNA fragmentation in kidney was determined by the method described by Kanno *et al*. (2004).

Some kidney specimens, planned for histological study by light microscopy, were embedded in buffered formalin solution (10%) and treated with graded ethanol solutions. Then they were fixed in paraffin, sectioned and colored with hematoxylin–eosin.

Statistical analysis was performed by using the SPSS for Windows statistical package (version 20). Results were expressed as mean±SD, for eight rats in each group. Statistical data were analyzed using one-way analysis of variance (one-way ANOVA) followed by Tukey-Kramer as post-hoc test for multiple comparisons with statistical significance of $P<0.05$.

RESULTS

The present study showed a significant decline in the rat body weight when compared to controls. While body weights of rat treated with both $NaNO_3$ and *H. hirta* were increased compared to those of rats treated only with $NaNO_3$. Results presented in table 1 indicated that the relative kidney weights were increased in $NaNO_3$ treated group and were similar to those of controls in $NaNO_3$ and *H. hirta* treated rats. In $NaNO_3$ and ($NaNO_3$+HH)-treated groups, water consumption was similar to controls. In $NaNO_3$ treated-group, there was a significant reduction in food intake.

A little degree of toxicity was observed in plant extracts. The acute toxicity LD_{50} of *H. hirta* methanolic extract in rats was established at a dose higher than 5000 mg kg^{-1}.

$NaNO_3$-treated animals revealed a renal dysfunction evidenced by enhance of urine output and the changes in levels of creatinine, urea and uric acid. In fact, the daily urine volume in intoxicated rats was higher than in controls (Fig. 1).

Creatinine and urea levels increased in plasma and decreased in urine, but uric acid level decreased in plasma and increased in urine after $NaNO_3$ treatment (Table 2). Thus we have observed a decrease in creatinine clearance, an index of glomerular failure (Fig. 1). Supplementation of *H. hirta* in drinking water of $NaNO_3$-treated group improved all the precited parameters.

The present study showed a significant rise in the level of lipid peroxidation in kidney of $NaNO_3$-treated rats, as evidenced by the improved MDA concentrations (Table 3). Supplementation of *H. hirta* decreased MDA content in the kidney and restored the levels of MDA to near normal values. A significant reduction of GSH kidney levels was found in rats exposed to $NaNO_3$ (Table 3). Treatment with *H. hirta* restored the GSH level to normal values. $NaNO_3$ administration to adult rats induced a significant reduction in all antioxidant enzyme activities (SOD, CAT and GPx) when compared to those of control group. Supplementation of *H. hirta* in ($NaNO_3$+HH)-group ameliorated enzymatic antioxidant activities when compared to $NaNO_3$ group.

Table 1: Body and relative kidney weights, daily water and food consumption by control, $NaNO_3$-treated and ($NaNO_3$+HH)-treated rats

Parameters and treatments	Controls (n=8)	$NaNO_3$ (n=8)	$NaNO_3$+HH (n=8)
Final body weight (g)	274.42±17.01	236.61±14.06**	261.33±9.83†
Relative kidney weight (mg/g BW)	3.03±0.11	3.41±0.17**	3.16±0.12†
Water consumption (ml/day/rat)	29.56±2.84	29.97±2.76	30.87±2.06
Food consumption (g/day/rat)	18.13±2.35	15.52±1.64**	16.95±1.84††*

Values are mean±SD. Comparisons between 2 groups: $NaNO_3$, $NaNO_3$+HH-treated group *vs* control group: *P<0.05; **P<0.01; $NaNO_3$+HH-treated group *vs* $NaNO_3$ treated group: †P<0.05; †† P<0.01.

Table 2: Plasma and urinary levels of creatinine, urea and uric acid in control, $NaNO_3$-treated and ($NaNO_3$+HH)-treated rats

Parameters and treatments		Controls (n=8)	$NaNO_3$ (n=8)	$NaNO_3$+HH (n=8)
Creatinine (µmol/L)	Plasma	30.30±2.40	37.46±1.77**	35.23±2.69†*
	Urine	5094.2±327.1	1608.4±118.1**	2797.5±238.6††**
Urea (mmol/L)	Plasma	7.91±0.55	9.57±0.81*	8.17±0.87††
	Urine	123.04±4.12	78.26±4.61**	107.6±2.62††**
Uric acid (µmol/L)	Plasma	138.67±11.94	72.28±8.88*	92.72±4.05††*
	Urine	690.54±50.99	966.74±79.93**	798.95±48.20†*

Values are mean±SD. Comparisons between 2 groups: $NaNO_3$, $NaNO_3$+HH-treated group *vs* control group: *P<0.05; **P<0.01; $NaNO_3$+HH-treated group *vs* $NaNO_3$ treated group: †P<0.05; †† P<0.01.

Table 3: Kidney MDA and GSH levels, SOD, CAT and GPx activities in control, $NaNO_3$-treated and ($NaNO_3$+HH)-treated rats

Parameters and treatments	Controls (n=8)	$NaNO_3$ (n=8)	$NaNO_3$+HH (n=8)
MDA (nmoles/mg protein)	0.58±0.02	1.81±0.09**	0.69±0.03††
GSH (µg/mg protein)	1.12±0.01	0.98±0.01*	1.11±0.01†
SOD (units/mg protein)	130.31±4.36	104.26±6.62*	122.39±4.32†*
CAT (µmoles H_2O_2 degraded/min/mg protein)	19.94±0.86	13.36±1.54**	16.69±0.61†*
GPx (nmoles of GSH/min/mg protein)	9.51±0.15	7.60±0.12**	8.02±0.06††*

Values are mean±SD. Comparisons between 2 groups: $NaNO_3$, $NaNO_3$+HH-treated group *vs* control group: *P<0.05; **P<0.01; $NaNO_3$+HH-treated group *vs* $NaNO_3$ treated group: †P<0.05; ††P<0.01; MDA: Malondialdehyde, GSH: Glutathione, SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione peroxidase.

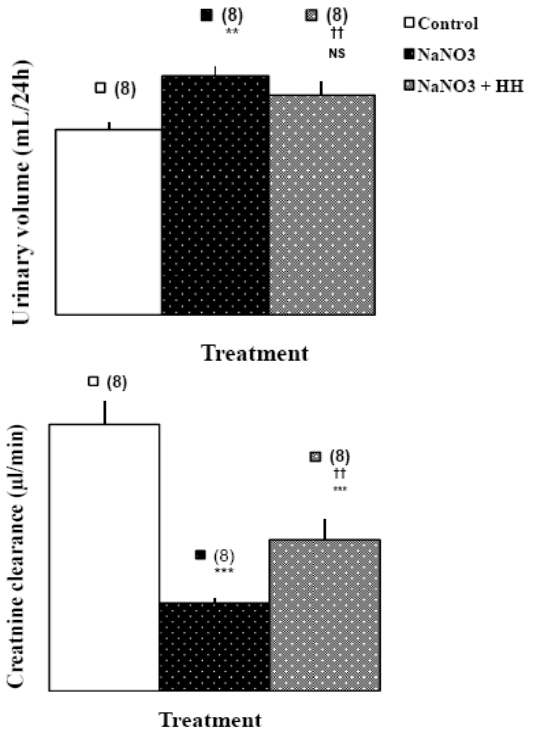

Fig. 1: Urinary volume and creatinine clearance in control, $NaNO_3$-treated and ($NaNO_3$+HH)-treated rats. Values (mean±SD) for eight rats in each group. $NaNO_3$ treated group versus control group: **P<0.01; ***P<0.001; $NaNO_3$+HH-treated group versus $NaNO_3$ treated group: ††P<0.01; $NaNO_3$+HH-treated group versus control group: ***P<0.001; NSNon significant difference.

A smear on agarose gel without ladder formation, proving random DNA damage, was showed across the lane of DNA kidney in $NaNO_3$-treated rats (Fig. 2). *H. hirta* treatment applied a corrective effect against $NaNO_3$ by decreasing the smear formation.

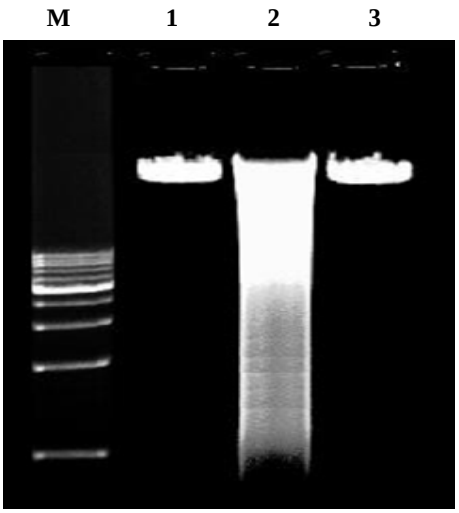

Fig. 2: Agarose gel electrophoresis of DNA fragmentation in kidneys of rats. M: marker (1 kb DNA ladder); Lane 1: DNA isolated from kidney of controls; Lane 2: DNA isolated from kidney of $NaNO_3$-treated rats; Lane 3: DNA isolated from kidney of ($NaNO_3$+HH)-treated rats.

In the kidney of $NaNO_3$-treated group**,** nitrate caused a significant injury in cortical and medullar regions of kidney structure. In fact, most glomeruli, showed congestion, a lobulation, a shrinkage, a large glomerular space and a peri-glomerular infiltration of mononuclear cells reflecting inflammatory activity (Fig. 3 B_1, $B_{1\text{-}1}$). There was also a tubular necrosis objectified either by a ballooning degeneration of renal tubule cells, or by a presence of tubules in the process of destruction, surrounded by inflammatory foci in the cortex (Fig. 3 B_1, $B_{1\text{-}2}$) and in the medulla (Fig. 3 B_2, $B_{2\text{-}1}$). Kidney damages were reduced when *H. hirta* was given in the drinking water of $NaNO_3$+HH-treated rats compared to $NaNO_3$-treated rats (Fig. 3C).

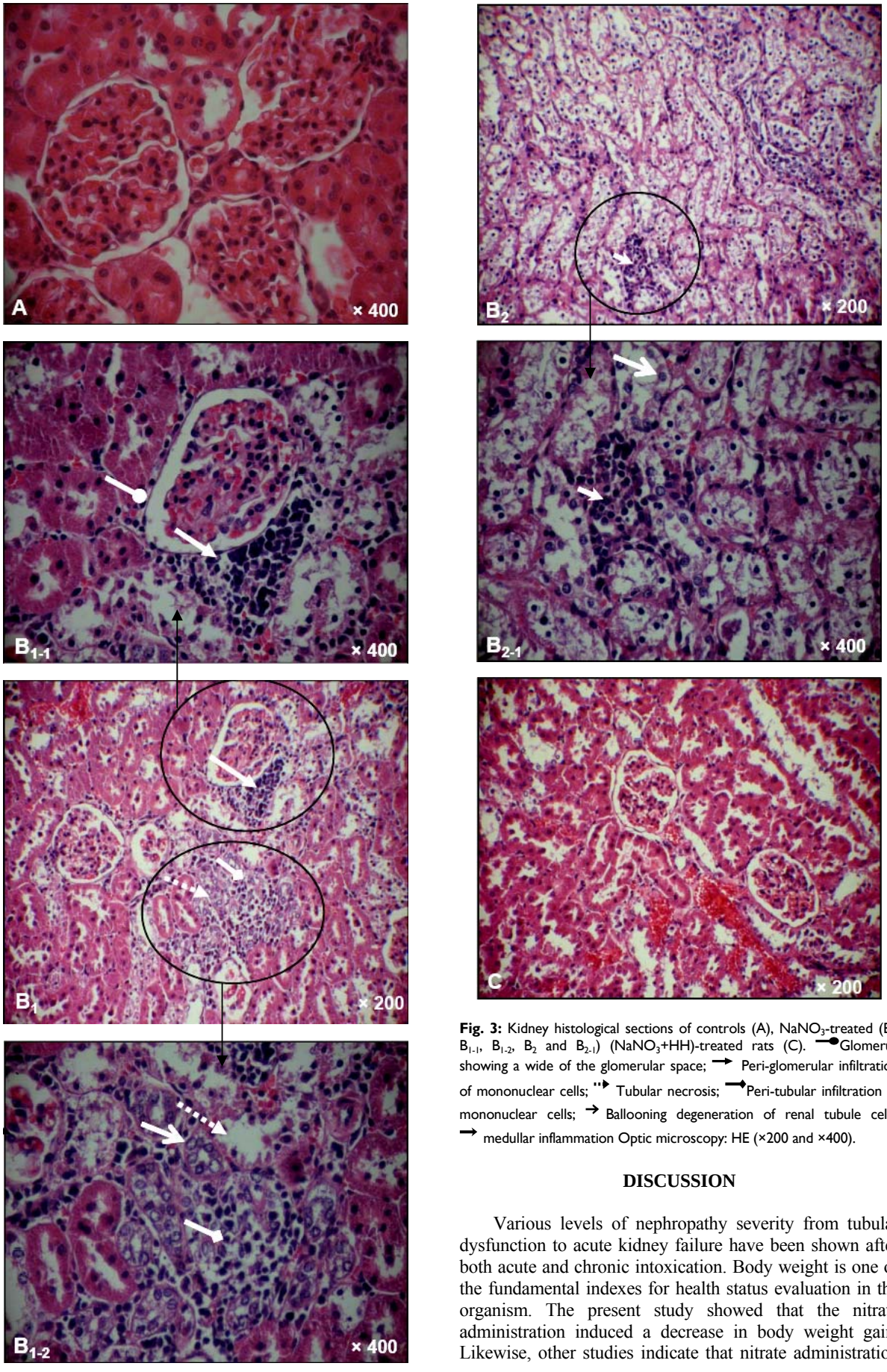

Fig. 3: Kidney histological sections of controls (A), $NaNO_3$-treated (B_1, B_{1-1}, B_{1-2}, B_2 and B_{2-1}) ($NaNO_3$+HH)-treated rats (C). Glomeruli showing a wide of the glomerular space; Peri-glomerular infiltration of mononuclear cells; Tubular necrosis; Peri-tubular infiltration of mononuclear cells; Ballooning degeneration of renal tubule cells; medullar inflammation Optic microscopy: HE (×200 and ×400).

DISCUSSION

Various levels of nephropathy severity from tubular dysfunction to acute kidney failure have been shown after both acute and chronic intoxication. Body weight is one of the fundamental indexes for health status evaluation in the organism. The present study showed that the nitrate administration induced a decrease in body weight gain. Likewise, other studies indicate that nitrate administration

induced a body weight decrease in in a dose-dependent manner which was higher in the offspring than in adult rats (El-Wakf *et al.,* 2011). Primary, a reduced body weight may be revealed by a decrease of food consumption as reported by us or by a rise in the protein catabolism explained by the reduced total protein plasmatic level (Al-Ayed, 2000).

Consumption of nitrate by adult rats didn't affect only body weight, it affected also the soft organ weights including kidney. The relative weight was increased indicating a nephromegaly and its dysfunction. In fact, in the current study, in response to $NaNO_3$ treatment, urinary volume output was enhanced significantly. This was probably due to a blockage of resorption of salt and water in the proximal renal tubules and in the thick ascending limb of Henle's loop, by increasing the blood flow to the renal medulla, and by the reduced responsiveness of the collecting duct to antidiuretic hormone (Gaeggeler *et al.,* 2011). Polyuria could also be due to the blockage of vasopressin secretion, which normally provoked reabsorption of water through the collecting duct.

Additionally an increase of creatinine and urea plasma concentrations was also noted in $NaNO_3$-treated rats suggesting an impairment of kidney function. These results were in agreement with an increase of protein catabolism in mammals. Our results correlated with previous findings realized on nitrate sodium administered to adult rats (El-Wakf *et al.,* 2011) and ammonium nitrate (NH_4NO_3) administered by gavage to adult (Boukerche *et al.,* 2007).

Our study showed a decline in creatinine 24-hr urinary excretion and in clearance of creatinine indicated an alteration of glomerular filtration rate. These effects could also be attributed to the changes in the threshold of tubular reabsorption and renal blood flow.

Renal failure may also be due to kidney oxidative stress. A likely cause of tissue injury was the generation of reactive oxygen and nitrogen species destroying proteins, lipids and DNA by oxidation. Moreover, it is now recognized that nitrate was considered as an oxidant product and a ready source of nitric oxide. The latter reacted quickly with superoxide anion to obtain peroxynitrite (Chow and Hong, 2002). These radicals affect the cell membrane leading to disintegration and destabilization of cell membrane and to lipid peroxidation. Our findings confirmed previous results cited overhead. In fact, the increased lipid peroxidation shown by us after nitrate treatment implicated the oxidative stress in $NaNO_3$ produced kidney injury.

Antioxidant enzymes are considered to be the primary defense in the body, which protect the biological molecules from oxidative stress and suppress peroxides, superoxide anion generated and free radicals inner the cell. Glutathione is a main constituent of the antioxidant defense mechanism and it functions as a reactive free-radical scavenger (Linder, 1995). Our result showed a reduction of renal GSH level in $NaNO_3$-treated rats which could be due to its increased utilization by the renal cells in order to scavenge toxic radicals. Furthermore, the significant reduction in the kidney antioxidant enzyme activities, found in our study, indicated the failure of antioxidant defense system in order to overcome of reactive oxygen species produced by $NaNO_3$ administration (Renugadevi and Milton Prabu, 2009). Our results supported previous studies of EL-Tahan *et al*. (2010). Thus the inhibition of enzymes leads to H_2O_2 accumulation, which promotes lipid peroxidation and DNA impairment and alters gene expression and cell death (Stohs and Bagchi, 1995).

Moreover free radical generation, after xenobiotic exposure, can cause DNA impairment leading to cell death and mutations. DNA kidney injury was assessed by electrophoresis of DNA. In general, DNA intact band appears to be concentrated near the application point without DNA smearing and no DNA fragmentation (El-Sharaky *et al*., 2009). $NaNO_3$ treatment induced DNA injury resulted in DNA shearing with no classical DNA ladder pattern seen in apoptosis (Cohen *et al.,* 1994).

Increase of a relative kidney weight observed in our investigation confirmed previous study of Yoshida *et al*. (1994) who have found an increase of the reno-somatic ratio in the rats treated by nitrate. Biochemical parameters confirmed histological data.

In kidney of $NaNO_3$-treated rats, there was a shrunken and degenerative renal tubular cells with a mononuclear cells infiltration. Also, most glomeruli demonstrated shrinkage, a large capsular space and a peri-glomerular infiltration of mononuclear cells. These results were in accordance with those of EL-Tahan *et al.* (2010). In fact, kidney of nitrate treated rats presented an increase of glomerular space, and an increase of the eosinophilic granules. These histological changes were due probably to the free radicals production and to the induction of lipid peroxidation.

The *H. hirta* treatment had a powerful preventive effect against oxidative stress and kidney injury in rats caused by $NaNO_3$, as revealed by an important reduction of noticeable reduction MDA level as well as an increase of antioxidant enzyme activities and of GSH level. The reversal of antioxidant enzymes and peroxidative damage in kidney by *H. hirta* extract spoke in favor of its antioxidant, anti-peroxidative homestead and its role as scavenger of free radicals, which could be due to flavonoids isolated from *H. hirta* by using HPLC/MS. Presence of apigenin, quercetin and luteolin derivatives in *H. hirta* was identified by HPLC analysis as it has been related in our precedent paper (Bouaziz *et al.,* 2014). The luteolin derivatives are the molecules capable to scavenge hydroxyl radicals and to remove reactive oxygen species produced by hydrogen peroxide. Moreover, the antioxidant capacity of quercetin and apigenin derivatives has been described in several studies (Azimova and Vinogradova, 2013).

In addition, *H. hirta* may improve nephrotoxicity to a great level, as shown by a decrease of plasma creatinine and urea levels and an increase of creatinine clearance. Furthermore, the administration of *H. hirta* extract in $NaNO_3$ treated group ameliorated the histological damages caused by nitrate. The kidney of ($NaNO_3$+HH)-group had nearly normal appearance. Thus confirmed the anti-inflammatory homestead of *H. hirta* as related by us in our previous study (Bouaziz *et al.,* 2014). In fact, quercetin and luteolin derivatives, present in the *H. hirta* extract, had in our study and in *Aegle marmelos* Seeds (Sharma *et al.,* 2011) a significant anti-inflammatory effect. *H. hirta* co-treatment was also shown to be efficient and to warn the $NaNO_3$-caused smear formation.

Conclusion: From the results achieved, it can be concluded that the administration of *H. hirta* has a beneficial role by

reversing some alterations in the kidney produced by nitrate, probably through its antioxidant properties.

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