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ANTI-INFLAMMATORY ACTIVITY OF LECTIN PURIFIED FROM *MORUS NIGRA* AGAINST LIPOPOLYSACCHARIDE (LPS) INDUCED RENAL STRESS IN RATS

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ABSTRACT

The study was designed to investigate the possible protective role of lectin of *Morus nigra* in lipopolysaccharide renal inflammatory, by using biochemical approaches. The effects of lectin of *Morus nigra* on LPS induced oxidative and renal stress were evaluated by serum creatinine, urea and uric acid levels, kidney tissue lipid peroxidation, GSH levels, SOD, GSH-Px , GST and catalase activities. Administration of LPS induced significant increase in serum: creatinine, urea and uric acid concentration showing renal inflammatory. LPS also induced oxidative stress, as indicate by decreased kidney tissue of GSH level, SOD, GSH-Px, GST and catalase activities along with increase the level of lipid peroxidation. Furthermore, treatment with LPS caused a marked elevation of kidney weight and decreased body weight. lectin of *Morus nigra* treatment markedly reduced elevated serum: creatinine, urea and uric acid levels and conteracted the deterious effects of LPS on oxidative stress markers and attenuated histological changes caused by LPS in kidney. Our results indicate that lectin of *Morus nigra* could have a beneficial role against LPS induced nephrotoxicity and oxidative stress in rat.

KEYWORDS: Antioxidant enzymes, LPS, lectin, *Morus nigra*, renal inflammatory.

1. INTRODUCTION

Lipopolysaccharide (LPS), an endotoxin, is a major glycolipid component of the outer cell wall of gram-negative bacteria, made up of a polysaccharide O-chain and a biologically active lipid-A moiety, embedded within the bacterial membrane (Larrosa *et al*., 2011).

Endotoxemia-induced toxicity is characterized by injury to various organs, including liver, kidney and the brain, and it has been implicated as a contributing factor to bacterial infection resulting in sepsis, which is one of the major causes of morbidity and mortality in intensive care units (Sebai *et al*., 2009). Most of the toxicities observed in LPS-induced injury in the kidney and systemic circulation has been attributed to toxic mediators produced by activated macrophages, including cytokines, such as tumor necrosis factor-α (TNF-α), interleukins (IL-1, IL-6, IL-8, and IL-12), other proinflammatory molecules, including platelet-activating factor, prostaglandins, as well as reactive oxygen and nitrogen species (RONS), such as nitric oxide (NO) and superoxide radical. The systemic inflammatory response triggered in the host by LPS is characterized by fever, leucocytosis, thrombocytopenia, changed metabolic responses and redox status impairment (Sebai *et al*., 2009) . The consequences of impaired intracellular redox balance includes the generation of excessive RONS, induction of lipid peroxidation, DNA and protein damage, depletion of intracellular stores of endogenous antioxidants and inhibition of antioxidant enzymes (Sebai *et al*., 2008). The involvement of oxidative stress in injury associated with LPS suggests that dietary antioxidants may enhance the efficacy of treatment protocols designed to mitigate LPS-induced endotoxemia. Medicinal plants, fruits, vegetables, spices and teas are drawing a lot of attention because of their demonstrated health benefits, with scientific evidence demonstrating that phytochemicals in fruits, vegetables, spices and teas possess a high number of protective biological properties, including antioxidant, anti-inflammatory and other beneficial effects (Krzyzanowska *et al*., 2010). Lectin or glycoprotein's are carbohydrate binding proteins that interact with specific sugars and induce several biological activities. Lectin have attracted great research due to their various biological activities like cell agglutination, antitumor, immunomodulatory, antifungal, antiproliferative and antiviral activities These proteins were powerful antioxidants which could inhibit lipid peroxidation and scavenge free radicals (Azevedo *et al*., 2001). In the present work we describe the protective effect of a new lectin purified from the roots of *Morus nigra* collected from Algeria against LPS induced inflammatory in rat's kidney.

2. MATERIALS AND METHODS

All chemicals used in this work were purchased from sigma chemical company. Laboratory animals, *Albino wistar* male rats, were brought from the Algiers Pasteur institute at the age of 4 weeks, with an average live weight of 160g. They were located in a room with an ambient

temperature of 21±1°C and up to 12h of light daily. The rats were divided into six experimental groups; each consists of six rats.

Group 1: served as the control, treated with normal saline.
Group 2: treated with reference drug diclofénac at a dose of 3mg/kg for 14 days.
Group 3: treated with purified lectin only at a dose of 15mg/kg by intraperetoneal injection for 14 days.
Group 4: treated with lipopolysaccharide at a dose of 200µg/kg by intraperetoneal injection for 14 days.
Group 5: treated with reference drug diclofénac at a dose of 3mg/kg 30 min before lipopolysaccharide injection for
14 days.
Group 6: treated with purified lectin at a dose of 15mg/kg by intraperitoneal injection 30 min before lipopolysaccharide injection for 14 days.

Twenty four hours after the last administration the blood was collected by retro- orbital sinus punction from each anesthetized rats. After centrifugation at 3000 rpm for 10min, the serum was separated immediately and stored at−20°c until determination of: urea, creatinine and uric acid. Subsequently, rats were decapitated and kidneys were removed.

Tissue preparation

About 500mg of kidney was homogenized in 4ml of buffer solution of phosphate buffered saline (w/v: 500mg tissue with 4ml PBS, PH 7.4) homogenates were centrifuged at 10.000xg for 15min at 4°c. And the resultant supernatant was used for determination of: reduced glutathione (GSH), Thiobarbituric acid- reactive substance (TBARS) levels, and superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) glutathione –S-transferase (GST) and catalase activities.

Determination of Biochemical parameters

Serum urea, creatinine and uric acid were determined using automate analyses.

Determination of lipid peroxidation (LPO)

Lipid peroxidation level in the liver was measured by the method of Buege and Aust (1978). 125µl of supernatant were homogenized by sonication with 50 µl of PBS, 125 µl of 20% TCA + BHT 1% (TCA-BHT) in order to precipitate proteins, and centrifuged (1000xg,

10min, 4°c), afterwards, 200µl of supernatant were mixed with 40µl of HCl (0,6M) and 160µl of TBA dissolved in tris (120 mM), and then the mixture was heated at 80°c for 10min, the absorbance of the resultant supernatant was obtained at 530nm. The amount of TBARS was calculated using a molar extinction coeffient of $1.56x10^5$ M/Cm.

Determination of reduced glutathione (GSH)

GSH content in liver was measured spectrophotometrically by using Ellman's reagent (DTNB) as a colouring reagent, following the method described by Weeckbekeretcory (1988).

Superoxide dismutase activity (SOD)

The superoxide dismutase (SOD) (E.C.1.15.1.1) activity was determined using a method of Asada et al (1974) SOD activity was evaluated by measuring of its ability to inhibit the photo reduction of nitro-blue tetrazolium (NBT). One millilitre of homogenate's supernatant was combined 50mM phosphate buffer (pH 7.8), 39 mM methionine, 2.6 mM NBT and 2.7 mM EDTA-Riboflavin, as to obtain a final concentration of 0.26 mM, was added as the last and switching on the light started the reaction, changes in absorbance at 560nm were recorded after 20min. In this assay, one unit of SOD is defined as the amount that inhibits the NBT reaction by 50%. Specific activity was defined as units/mg of protein.

Determination of glutathione-S-transferase (GST) (EC2.5.1.18)

The cytosolic glutathione-S-transferase activity was determined spectrophotometrically at 37°c by method of Habig *et al* (1974). The reaction mixture (1ml) contained 0.334ml of 100mM phosphate buffer (PH 6.5), 0.033ml of 30mM CDNB and 0.33ml of reduced Glutathione. After pre-incubating the reaction mixture for 2min the reaction was started by adding 0.01ml of diluted cytosol and the absorbance was followed for 3min at 340 nm. The specific activity of GST is expressed as µmole of GSH-CDNB conjugate formed/ min /mg protein using extinction coefficient of 9.6 Mm^1 cm^{-1}.

Determination of GSH-Px (E.C.1.11.1.9)

Glutathione peroxidase (EC 1.11.1.9) activity was modified from the method of Flohe and Gunzler (1984). for the enzyme reaction , 0.2ml of the supernatant was placed into a tube and mixed with 0.4ml GSH (reduced glutathione, sigma product, analytical grade), and the mixture was put into an ice bath for 30min. then the mixture was centrifuged for 10min at 3000rpm, 0.48ml of the supernatant was placed into a cuvette, and 2.2ml of 0.32M Na_2HPO_4

and 0.32ml of 1m mol/l 5,5'-dithio-bis(2-nitrobenzoic acid)(DTNB, sigma) were added for color development. The absorbance at wavelength 412nm was measured with a UV spectrophotometer after 5min. The enzyme activity was calculated as a decrease in GSH within the reaction time as compared to that in the non-enzyme reaction.

Catalase activity (CAT)

The activity of catalase (CAT) (E.C.1.11.1.6) was measured according to the method of Aebi (1984) The reaction mixture 1ml contained a 100mM phosphate buffer (pH 7), 500mM H_2O_2 and liver supernatants. The reaction started by adding H_2O_2 and its decomposition was monitored by following the decreased in absorbance at 240nm for 1 min. The enzyme activity was calculated by using an extinction coefficient of 0.043mM-1cm-1.

Protein quantification

Protein was measured by the method of Bradford (1976) using bovine serum albumin as the standard.

Histopathological examination

kidney from autopsied animals were excised out and fixed in formalin (10%). five micron think section were prepared by using microtome and these section were stained with hematoxyline and eosin. For histological alterations these slides were observed under light microscope.

Statistical analysis

The data were subjected to student *t* test for comparison between groups. The values are expressed as mean ± SEM. Significance level was set at $P<0.05$, $P<0.01$, $P<0.001$.

3. RESULTS

Effects of treatments on body, absolute and relative kidney weights

Table 1 shows the effect of lipopolysaccharide, purified of new lectin from *Morus nigra*, reference drug (diclofenac) and combined treatment with (a new lectin and lipopolysaccharide), (diclofenac and lipopolysaccharide) . The merked increased in rats body gain was observed in lipolysaccharide treated rats and a new lectin + lipopolysaccharide and diclofenac + lipopolysaccharide groups, but the result was not significant as compared to control. Along a new lectin and diclofenac showed decreased body gain but result was not significant. Lipopolysaccharide treated rats showed a no significant increased kidney weight

and relative kidney weight as compared to control. Combined treatment with purified lectin or diclofenac showed no significant increased relative kidney weight, while alone purified lectin or diclofenac treatment had showed no significant effect.

Effects of treatment on serum biochemical parameters

A highly significant ($P \leq 0.001$) elevation in serum urea, creatinine and uric acid levels was observed in lipopolysaccharide intoxicated rats. Only purified lectin or diclofenac treatment did not show any significant alteration. However, the combined treatment of lectin or diclofenac with lipopolysaccharide show no significant decline in serum urea, creatinine and uric acid levels was noticed respect to controls (table 2).

Effects of treatments on renal oxidative stress parameters

Lipopolysaccharide exposure a highly significant depleted in reduced glutathione level, SOD, GSH-Px GST and catalase activities. And no significant increase in kidney lipid peroxidation level in lipolysaccharide intoxicated rats was noticed. Diclofenac or purified lectin alone treatment did not show any significant decline. In combined treatment of lipopolysaccharide with diclofenac or purified lectin no significant increase in reduced glutathione level, SOD, GSH-Px GST and catalae activities. And no significant depletion in lipid peroxidation level was recorded with respect to the control (Fig.1 and 2).

Histological studies

The histological changes in Kidney are presented in Fig.3. LPS induced various pathological alterations in kidney of rats. These alterations were characterized by renal tubular damage, indicating by tubular necrosis (Fig 3.D). In combination group were lectin or diclofenac was administration with LPS showed reparative changes. Kidney showed promiment recovery in the form of normal renal tubular and very less tubular necrosis (Fig 3. E, F). kidney of the control group had a regular histological structure (Fig.3. A). furthermore, no histological alterations were observed in the kidney of lectin or diclofenac treated group (Fig.3. B, C).'

Table 1: Changes in body and absolute and relative kidney weights of control and rats treated with lectin, diclofenac, lipopolysaccharide (LPS) and combined treatment of LPS with lectin or Diclofenac after 14 days of treatment.

Parameters	treatment groups					
	Control	Lectin	Diclofenac	LPS	Lectin+LPS	Diclofenac+LPS
Initial body weight (g)	213±12	212.2±16	212.15±25	211.5±17	212.3±7.3	212.6± 6.2
Final body weight (g)	214.25±12	213±10	213±6.2	210.75±7.5	211.3±7.3	212.2± 6.2
kidney weight (g)	2.1±0.1	2.2±0.3	2.3±0.2	3.2±0.2	2.9±0.1	2.7±0.2
Rkw (g/100g b.w)	0.9±0.01	0.9±0.07	0.9±0.02	1.5±0.02	1.3±0.01	1.2±0.01

Table 2: Changes in biochemical parameters of control and rats treated with lectin, diclofenac, lipopolysaccharide (LPS) and combined treatment of LPS with lectin or Diclofenac after 14 days of treatment.

Parameters	treatment groups					
	Control	Lectin	Diclofenac	LPS	Lectin+LPS	Diclofenac+LPS
Urea (g/l)	0.37±0.005	0.38±0.002	0.36±0.006	0.47±0.002***	0.40±0.002	0.38±0.005
Creatinine (mg/l)	1.45±0.001	1.42±0.002	1.44±0.005	2.45±0.005***	1.58±0.002	1.56±0.001
Uric acid (mg/l)	11.13±0.52	11.2±0.58	10.1±0.58	17.33±0.3***	14.33 ±0.4	14.12±0.2

Values are given as mean ± SEM for group of 6 animals each. *P≤0.05, compared to controls. **P≤0.01, compared to controls. ***P≤0.001, compared to controls.

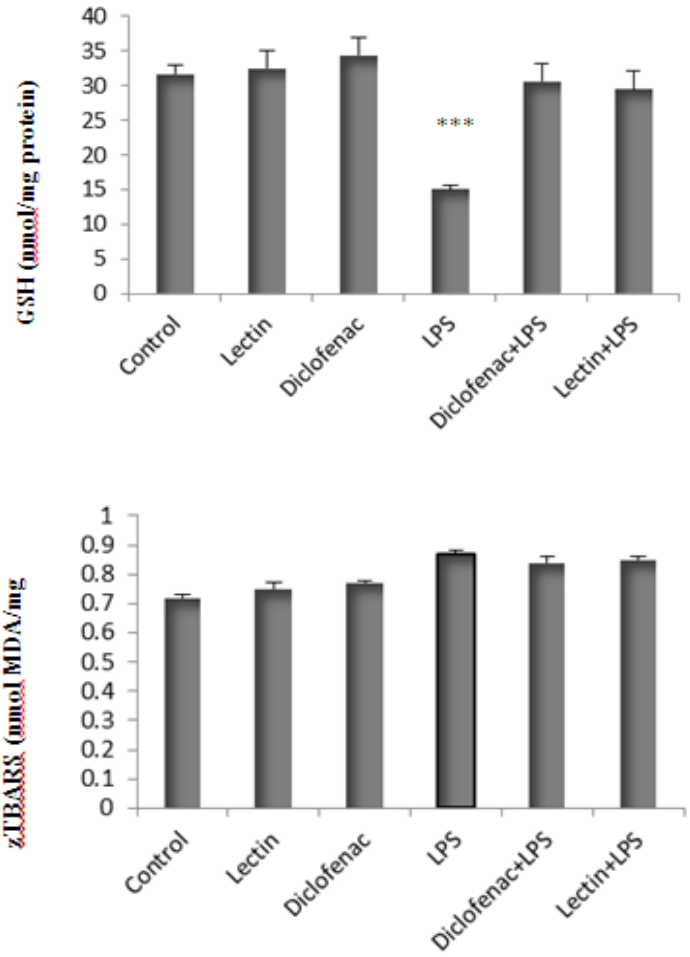

Fig. 1. Reduced glutathione (nmol/ mg protein) and TBARS (nmol MDA /mg protein) levels in kidney of control and rats treated with Lectin, Diclofenac, LPS and combined treatment of LPS with Diclofenac or Lectin after 14 days of treatment. Values are given as mean ± SEM for group of 6 animals each significant difference: * compared to controls (*P≤0.05; **P≤0.01; ***P≤0.001).

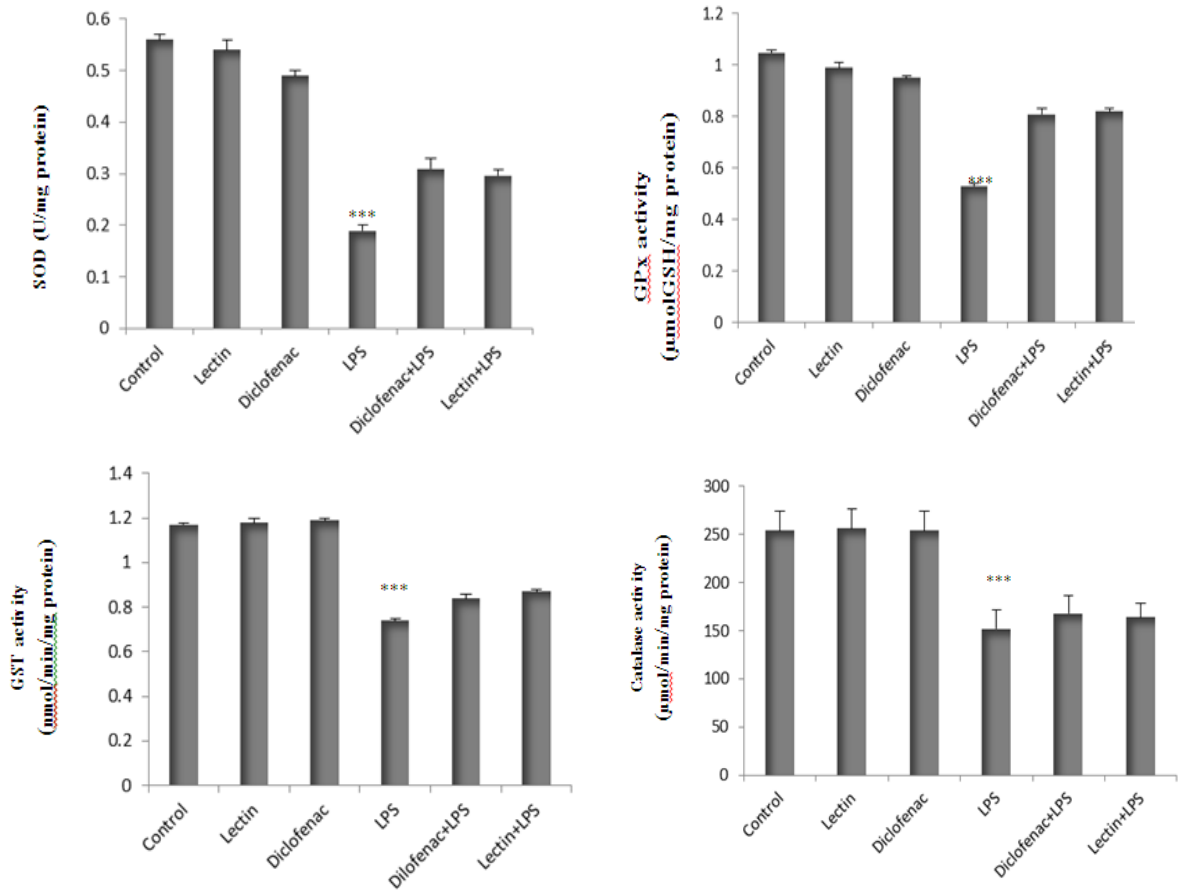

Fig.2. Enzyme activities of SOD (U/mg protein), GPx (µmol GSH/ mg protein), GST (nmol /min/mg protein) and Catalase (µmol/min/mg protein) in kidney of control and rats treated with Lectin, Diclofenac, LPS and combined treatment of LPS with Diclofenac or Lectin after 14 days of treatment. Values are given as mean ± SEM for group of 6 animals each significant difference: * compared to controls (*P≤0.05; **P≤0.01; ***P≤0.001).

3. DISCUSSION

In the present study, oxidative stress induced by LPS was evidenced in kidney of rats by increase in lipid peroxidation level and the inhibition of SOD, GSH-Px, GST and catalase activities. As consequence of lipid peroxidation biological membranes are affected causing cellular damage. In the present study, serum urea, creatinine, uric acid levels were significantly increased after 14 days LPS (200µg/kg), showing insufficiency of renal function. Studies in animals have established that tubular injury plays a central role in the reduction of glomerular filtration rate in acute tubular necrosis (Necib *et al.*, 2013) . Two major tubular abnormalities could be involved in the decrease in glomerular function in LPS treated rats: obstruction and backleak of glomerular Filtrate. The alterations in glomerular

function in LPS treated rats may also be secondary to ROS(reactive oxygen species), which induce mesangial cells contraction, altering the filtration surface area and modifying the ultrafiltration coefficient factors that decrease the glomerular filtration rat . The activity of SOD, GSH-Px, GST and Catalase that can clear to protect the cells from being injured represents the competence of clearing free radicals from the organism. MDA content manifests the level of lipid peroxidation, and then indirectly represents the level of damage of the cell of renal mitochondria. Evaluating from GSH, MDA levels and SOD, GSH-Px, GST and Catalase activities in kidney of rats. LPS alone significantly decreased GSH level, SOD, GSH-Px, GST and Catalase activities and increased MDA content along with histological damage in kidney.

The focus of this study was to investigate the acute effects of LPS-induced renal oxidative stress and inflammatory responses, and the possible protection offered by administration of purified lectin of *Morus nigra*.

Oxidative stress is a well-known mechanism of LPS induced renal injury, and the redox imbalance produced may result in depletion of endogenous antioxidants such as the antioxidant enzymes and alteration of GSH redox status. Thus, augmenting the antioxidant defense system becomes necessary, especially during infections or periods of chronic oxidative insult. Whole extracts or isolated compounds from plants are popular applications to reverse and/or prevent renal toxicity and oxidative stress produced by noxious agents, such as LPS and these beneficial effects may be attributed to their antioxidant and anti-inflammatory properties. In this study, it was observed that injection of LPS did not have a negative effect on the body weight gain, absolute kidney weight and relative kidney weight of the rats. However, LPS injection resulted in renal injury as indicated by an elevation in the levels of serum urea, creatinine and uric acid, all circulating markers of renal injury (Kaur *et al*. 2006; Kaur *et al*. 2006; Sebai *et al*., 2010).

Results from the current study showed that supplementation with the purified lectin of *Morus nigra* for 30 min prior to the LPS injection, reversed the induced damage in the kidney.

The protective effect of purified lectin of *Morus nigra* observed in our study may be due to the ability of the proteins to stabilize and maintain the integrity of the renal membrane, as well as repair damaged renal tissues by stimulating renal cells regeneration and protein synthesis. LPS-induced lipid peroxidation is an index of oxidative stress, and several

previous studies have reported enhanced lipid peroxidation in many tissues (including liver, heart, brain, small intestine and stomach) of rats (Kaur *et al*., 2006; Kaur *et al*., 2006; Sebai *et al.*, 2010; Depboylu *et al*., 2013). Under conditions of oxidative stress, reactive oxygen and nitrogen species (RONS) attack the polyunsaturated fatty acids (PUFAs) of cell membranes causing destabilization, disintegration and alteration in membrane fluidity and permeability, all events which increase the rate.

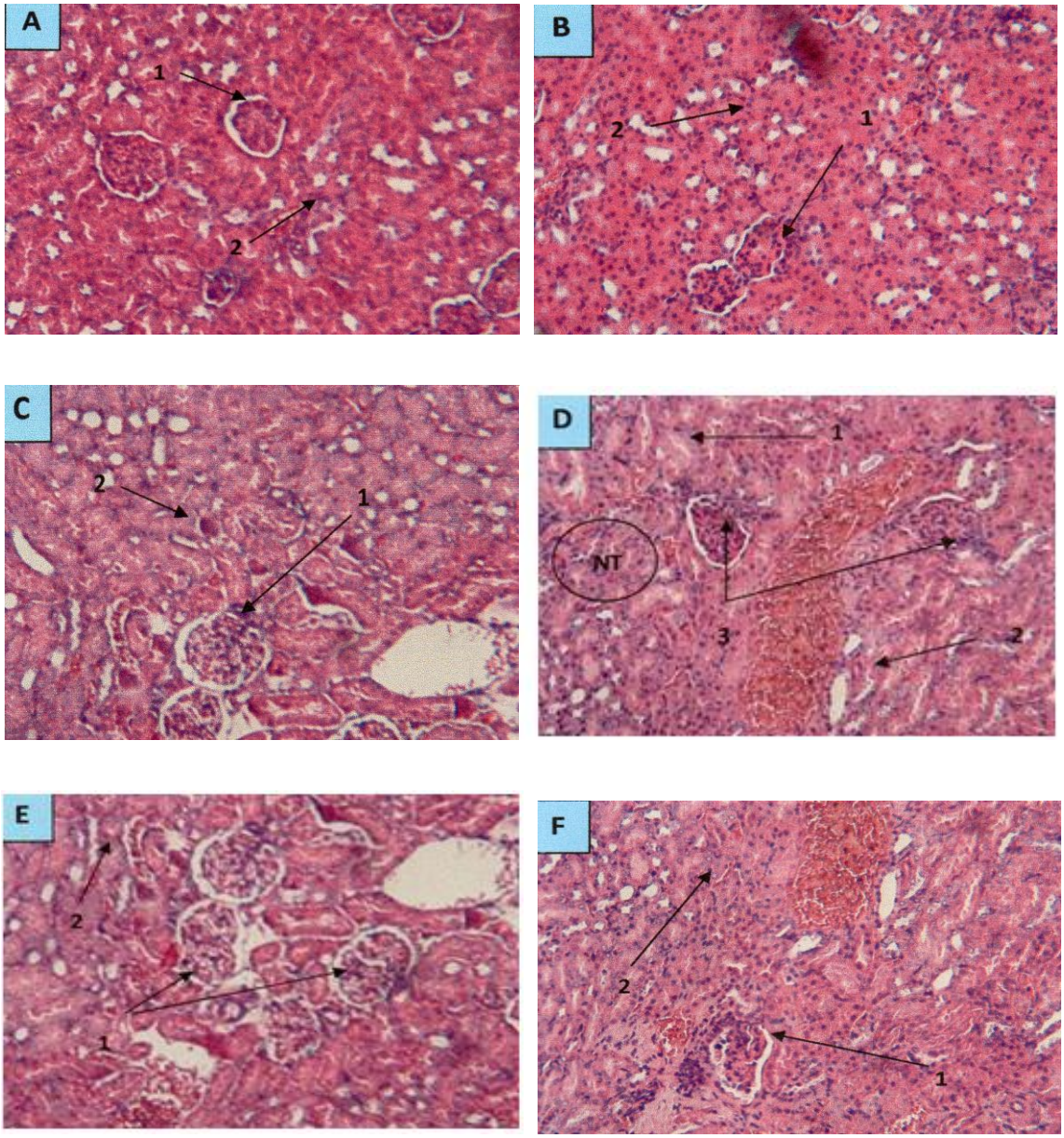

Fig. 3.T.S. of kidney of male rat treated with Lipopolysaccharide (LPS) alone, and in combination with Diclofenac or Lectin . (A) control (H&E400X): showing well develop glomerulus (1), with normal tubular cells; (B, C) Lectin , Diclofenac alone treatment (H&E 400X): showing normal glomerulus(1), and normal tubular cells; (D) LPS treatment (H&E100X): showing degeneration of tubular cells (1), loss of nuclus (2), degeneration of glomerulus (3); (E, F) combined treatment of LPS with Diclofenac or lectin (H&E400X): showing normal glomerulus (1), normal tubular cells (2).

of protein degradation and eventually leads to cell lysis (Pari *et al*., 2010). Decomposition products of lipid hydroperoxides such as MDA and 4-HNE can also cause chaotic cross-linkage with protein and nucleic acids, leading to oxidative protein and DNA damage (Bharrhan *et al*., 2010). In this study, renal MDA, as markers of lipid peroxidation, was measured. elevated levels of renal MDA were observed. Pre-feeding lectin of Morus nigra for 14 days in the LPS-challenged rats inhibited the formation of MDA in the kidney. A large number of in vitro studies have established the excellent free radical scavenging ability of lectin purified from *Morus nigra* and this has been confirmed in many in vivo studies. Since lectin is a protein may be able to bind RONS directly and scavenge them or act as sacrificial antioxidants to inhibit the lipid peroxidation cascade as seen in this study. The impairment of the antioxidant defense system is a critical step in LPS-induced renal injury. Evidence has shown that a LPS insult is characterized by change in tissue and circulating antioxidant enzymes levels, as well as non-enzymatic antioxidants, including GSH (Kaur et al. 2006; Kaur et al. 2006). In our study, SOD, GSH-Px, GST and Catalase activities were significantly reduced by LPS treatment. In the kidney, the activity of SOD was inhibited by LPS treatment. This inhibition is not surprising since the O2•− has been implicated as one of the toxic mediators responsible for most toxicities observed in LPS-induced cellular injury, and SOD, a metalloprotein, is a key enzyme involved in the protection of cells by spontaneously dismutating O2•− to H_2O_2. The H_2O_2 produced by SOD is usually decomposed to water and oxygen by the hemoprotein CAT, localized in the peroxisomes. GPx, GST and catalase are important enzymes of the glutathione defense system. While GPx catalyses the reduction of H_2O_2 and lipid hydroperoxides using GSH as a co-substrate. In this study, the activities of both GPx, GST and catalase were significantly depleted by LPS treatment, an indication of their inactivation and failure of the antioxidant enzymes to overcome the influx of RONS after LPS exposure. Results from this study further showed that feeding lectin purified from Morus nigra for 30 min prior to the LPS injection for 14 days reversed the changes observed in the activities of SOD, GPx, GST and CAT in the kidney. The modulation of the antioxidant enzymes activities observed in the LPS-injection rats consuming lectin of *Morus nigra* could be ascribed to the direct quenching of RONS generated by LPS, since antioxidant activity of lectin is established free radical scavengers. Furthermore, the up-regulation and/or down regulation of the gene expression of the antioxidant enzymes may be an additional mechanism that should be elucidated in future studies. Reduced glutathione (GSH) is the major non-protein thiol in plant and animal cells. It is essential for the regulation of a variety of cellular functions, playing an important role in

intracellular protection against ROS and other free radicals (Kono *et al*., 1982). Because of its sulphydryl (–SH) group, it can function as a nucleophile, forming conjugates with many xenobiotics and/or their metabolites and also serve as a reductant in the metabolism of hydrogen peroxide and other organic peroxides (Naik *et al*., 2011).

During interaction with free radicals, the –SH group of GSH becomes oxidized, leading to the formation of corresponding disulfide compound (GSSG). Thus, a depletion of GSH is usually associated with an increase in GSSG concentration and a lowered GSH:GSSG redox ratio during conditions of oxidative stress (Suntres , 2011; Dickinson *et al.*, 2002). Results from the current study revealed a decrease in GSH in rats injection with LPS.

These events invariably resulted in a decrease in the GSH in both tissues of LPS-challenged rats. Feeding lectin to LPS-treated rats, restored the GSH to values comparable to those found in the negative control animals, indicating that lectin purified for *Morus nigra* is able to protect against LPS-induced glutathione imbalance, This result is supported with biochemical and histopathological findings which the effect of lectin on lipopolysaccharide induced oxidative stress in rats.

CONCLUSION

This study provides the first in vivo evidence of an anti-inflammatory effect of lectin purified from *Morus nigra* in LPS-induced renal injury in rats. LPS-induced the production of reactive oxygen and nitrogen species, resulting in lipid peroxidation as demonstrated in this study. Results from this study further demonstrates that lectin purified from Morus nigra is able to suppress LPS triggered oxidative stress and inflammatory responses in the kidney by attenuating liver damage, lipid peroxidation, and redox (GSH:GSSG) imbalance in a Wistar rat model. Lectin purified from Morus nigra is excellent antioxidant properties which may in part, explain this observed anti-inflammatory activity. This suggest that Lectin purified from Morus nigra may be of benefit in the prophylactic management of LPS-induced kidney injury, however, future studies are necessary to fully examine the specific mechanisms underlying the protective effects shown by this purified Lectin.

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