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RESEARCH ARTICLE

Nephroprotective and Antioxidant Effects of *Moringa Oleifera* (Sohanjna) in Paracetamol Induced Nephrotoxic Albino Rabbits

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ABSTRACT

In present study nephroprotective and antioxidant effects of *Moringa oleifera* were investigated in paracetamol induced nephrotoxic albino rabbits. Thirty-six healthy adult albino rabbits randomly divided into six equal groups were provided with normal routine feed and drinking water. Except untreated control group-1, the rest of the groups administered orally with 500 mg/kg paracetamol as nephrotoxic drug for 0-15 days. Group-II served as untreated control on paracetamol only and group-III as treated control on synthetic nephroprotective drug, silymarin 150 mg/kg orally. *M. oleifera* seed powder was given orally to treated groups-IV, V and VI at dose rate of 200, 400 and 600 mg/kg body weight, respectively. Blood samples were collected at 0 and 15 days. At the termination of experiment, rabbits were slaughtered and kidney tissues were excised for histopathological examination. Data were compared statistically by Duncan's DMR test at 5% level of significance. The results suggested that paracetamol induced renal damage significantly ($P<0.05$) increased the levels of serum creatinine (Cr), blood urea nitrogen (BUN), total oxidant status (TOS) and malondialdehyde (MDA). However, catalase (CAT) and total antioxidant capacity (TAC) were significantly ($P<0.05$) decreased along with histopathological necrotic damage of renal tissues in nephrotoxic rabbits. *M. oleifera* seed powder, at the dose rate of 600 mg/kg, exhibited nephroprotective and antioxidant effects through biochemical and histological protections against paracetamol induced renal damage in albino rabbits. This indicates that *M. oleifera* seed powder at the dose rate of 600 mg/kg is as efficacious as silymarin in exerting nephroprotective and antioxidant effects.

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INTRODUCTION

Kidneys being essential organs are required for normal functioning of urinary system and regulation of homeostasis, electrolytes, acid-base balance, and blood pressure (Cotran *et al.*, 2005). Exposure of any toxic substance (chemicals or medication) to kidneys either accidently or intentionally, exert toxic effects which are documented as nephrotoxicity. Different drugs produce the nephrotoxic effects by different pathological mechanisms (Jackson and Morrow, 2001).

Paracetamol (acetaminophen) belongs to para-aminophenol, a class of NSAIDs and being used safely in wide range of treatments having analgesic and antipyretic activity at therapeutic dose, 1-3 g, three to four times a day (Galley, 2000). Renal- hepatic necrosis and apoptosis has been resulted when paracetamol ingestion exceeded than 150 mg/kg body weight in adults (Zed and Krenzelok, 1999). The accumulation of paracetamol in renal tissues was thought to initiate a chain of biochemical reactions those encountered acute or chronic nephropathies (Schnellman, 2001). Glutathione disappearance was examined by paracetamol overdose. Due to glutathione removal, paracetamol metabolites were not eliminated from the body rather accumulated intracellular and made covalent bonds with cellular proteins. Lipid peroxidation

was initiated in renal tubular cells which disturbed body's natural antioxidants system and ultimately death of renal tubular cells (Bessems and Vermuelen, 2001). The reactive oxygen species (ROS) or free radicals are important mediators of paracetamol induced nephrotoxicity. Global estimates show that about 80% of 4 billion world population depends upon the traditional medicines derived from plant origin. Side effects of allopathic drugs and development of resistance to these drugs led to an increased use of plants as source of medicines in variety of diseases (Manjula *et al*., 2009). *Moringa oleifera* of family *Moringaceae*, commonly known as Sohanjna, is a small sized tree which is native to India, Sri Lanka, Pakistan and Burma (Mbikay, 2012; Razis *et al*., 2014)and contains many phytochemicals such as carotenoids, amino acids, glycosides, alkaloids, sterols, flavonoids, vitamins, minerals and phenolic compounds (Moyo *et al*., 2011; Teixeira *et al*., 2014; Razis *et al*., 2014). It has been proved that *M. oleifera* has iron concentration more than spinach and potassium concentration more than banana (Siddhuraju and Becker, 2003). *M. oleifera* has well documented activity against Inflammation, infectious diseases, cardiovascular, hematological, gastrointestinal, and hepatic disorders (Razis *et al*., 2014). Seeds of *M. oleifera* have antihyperlipidemic and antitumor activities (Mbikay, 2012). Moreover, its leave are widely used as a basic food because of their high nutrition content and have remarkable antihyperglycemic and anti-inflammatory effects (Mbikay, 2012; Razis *et al*., 2014)while seeds are used in liver dysfunction, cardiovascular and hematological disorders having significant antioxidant and diuretic activities (Stohs and Hartman, 2015).

Despite of vast pharmacological activities of *M. oleifera*, literature regarding its efficacy against paracetamol induced nephrotoxicity is scanty. Hence nephroprotective and antioxidant effects of *Moringa oleifera* were investigated in paracetamol induced nephrotoxic albino rabbits.

MATERIALS AND METHODS

Experimental Design: The seeds of *Moringa oleifera* were purchased from local herbal market of Faisalabad. The plant material was taxonomically identified and authenticated by the Department of Botany, University of Agriculture, Faisalabad. The seeds were washed thoroughly with distilled water to remove dust or any other extraneous material and then dried in shade. Dried seeds were powdered with electrical grinder. Thirty six healthy adult male albino rabbits of 1.5-2.5 kg of body weight were purchased from local market of Faisalabad. The rabbits were housed in individual iron cages at room temperature (22±2ºC) with 12/12 h light/dark period. Prior to the experimentation, the rabbits were acclimatized for one week. The experiment was conducted with the prior approval by the Directorate of Research and Advanced Studies and with the consent of the Society of Ethics of Animals, University of Agriculture, Faisalabad, Pakistan. Rabbits were randomly divided into six equal groups.

Feeding schedule of normal routine feed, paracetamol, silymarin and powdered seed of *M. oleifera* in adult albino rabbits during the experimental period of 0 to 15 days has been designed by keeping Group I as normal control group without any treatment while Group II, III, IV, V and VI were induced nephrotoxicity with Paracetamol 150 mg/kg in 5 ml distilled water. Nothing is used as nephroprotective agent in Group II but silymarin 150 mg/kg orally, *M. oleifera* seed powder 200 mg/kg, 400 mg/kg and 600 mg/kg orally were given as nephroprotective agent in Group III, IV, V and VI respectively. Blood samples were collected from jugular vein in each experiment at 0 and 15 days, post medication. For serum separation, samples were allowed to clot for 20 minutes at refrigeration temperature and then centrifuged at 4000 rpm for 5 minutes. Serum was stored at freezing temperature till analysis.

Biochemical parameter analysis: Serum was analyzed for creatinine (Cr) by alkaline picrate method using commercially available kit (Ecoline® S+) and blood urea nitrogen by 'Urease – GLDH': enzymatic UV test method using commercially available kit (Breurer & Breuer Diagnostic kit, Germany). Serum was also used for determination of biomarkers by measuring the level of total antioxidant capacity (TAC), total oxidant status (TOS), catalase (CAT) and malondialdehyde (MDA) using commercially available kits. For TAC and TOS [Product Code # RL0017 (TAC) & RL0024 (TOS), Rel Assay Diagnostics kit, Mega Tıp, Gaziantep, Turkey] according to methods as reported by Erel (2004; 2005), for MDA [Kit # ab118970 Lipid Peroxidation (MDA) Assay Kit, abcam®, USA] and for CAT (Kit #ab83464, Catalase Assay Kit, Abcam®, USA) were used.

Histopathological Examination: All the rabbits were sacrificed for kidney tissue sampling. These tissues were immersion-fixed in 10% buffered formalin and were further processed with graded series of ethanol. Samples were then impregnated with molten paraffin wax and then embedded. Paraffin sections (3-4 µm) were mounted and affixed to slides. Sections were stained as a routine in Harris's alum hematoxylin and eosin. Mounted and stained slides were examined for its morphometric measurements on Olympus PM – 10ADS automatic light microscope (Olympus optical Co., Tokyo, Japan) with a 40X objective and calibrated ocular micrometer (Afshar *et al*., 2008).

Statistical analysis: The two way analysis of variance (ANOVA) was applied to observe the difference between control and experimental groups at different time intervals. Statistical differences among different treatment groups were determined by Duncan's Multiple Range test (DMR) at 5% level of significance.

RESULTS

Biochemical Analysis: Table 1 suggests that paracetamol induced renal damage by significantly ($P<0.05$) increasing the levels of serum Cr, BUN, TOS and MDA and decreasing CAT and TAC contents. However, silymarin significantly ($P<0.05$) changed these values which differed non-significantly ($P>0.05$) to their respective values in control group. *M oleifera* seed powder at-least at dose rate of600 mg/kg exerted its nephroprotective and antioxidant role by inducing significant ($P<0.05$) changes in values of paracetamol treated animals. These resultant values

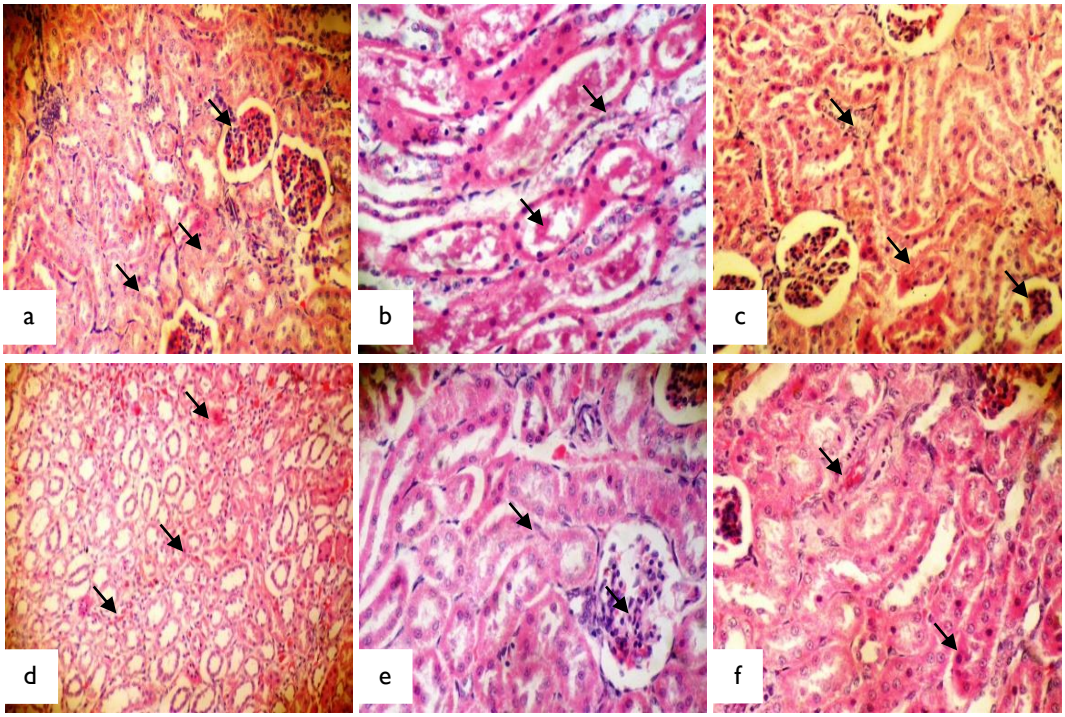

Fig. 1: Histopathological slides of each group are shown; a) Photomicrograph of kidneys of control group showing normal renal parenchyma (H & E Staining 400X). b) Photomicrograph of kidneys of toxic group (paracetamol 500 mg/kg) showing pyknotic nuclei of tubular epithelial cells and congestion. c) Photomicrograph of kidneys of synthetic nephroprotective group (silymarin 150 mg/kg) showing normal renal parenchyma (H & E Staining 200X). d) Photomicrograph of kidneys of paracetamol 500 mg/kg and *Moringa oleifera* 200 mg/kg treated group showing condensed and normal nuclei in tubular epithelial cells (H & E Staining 200X). e) Photomicrograph of kidneys of paracetamol 500 mg/kg and *Moringa oleifera* 400 mg/kg treated group showing condensed and normal nuclei in tubular epithelial cells (H & E Staining 400X). f) Photomicrograph of kidneys of paracetamol 500 mg/kg and *Moringa oleifera* 600 mg/kg treated group showing normal nuclei in tubular epithelial cells (H & E Staining 400X).

Table 1: Serum Cr, BUN, TAC, TOS, CAT and MDA after oral administration of *Moringa oleifera* seed powder, paracetamol and silymarin in male adult albino rabbits

Groups / Tests	I	II	III	IV	V	VI
	Control on routine feed	Treated with Paracetamol	Treated with Silymarin + Paracetamol	Treated with *M. oleifera* 200mg/kg + Paracetamol	Treated with *M. oleifera* 400mg/kg + paracetamol	Treated with *M. oleifera* 600mg/kg + Paracetamol
Serum Cr (mg/dl)	0.85±0.04^{b}	1.41±0.17^{a}	0.97±0.05^{b}	1.05±0.06^{b}	0.96±0.05^{b}	0.89±0.03^{b}
BUN (mg/dl)	21.4±1.17^{b}	33.6±4.30^{a}	26.1±1.51^{b}	27.0±1.43^{b}	24.9±1.50^{b}	21.9±1.25^{b}
TAC (mmol/L)	1.94±0.10^{a}	0.84±0.35^{b}	1.57±0.18^{ab}	1.23±0.27^{b}	1.36±0.20^{ab}	1.52±0.12^{ab}
TOS (µmol/L)	2.93±0.25^{c}	6.54±2.30^{a}	3.41±0.24^{bc}	4.33±0.96^{ab}	3.78±0.43^{ab}	3.43±0.19^{bc}
CAT (Kilo U/L)	48.2±3.06^{a}	25.3±9.08^{c}	41.4±3.36^{ab}	33.2±6.12^{bc}	38.9±6.89^{ab}	40.2±3.77^{ab}
MDA (nmol/L)	5.92±0.14^{c}	24.5±13.0^{a}	9.67±3.21^{bc}	16.7±8.00^{ab}	12.5±4.92^{ab}	10.3±3.36^{bc}

Mean±SE sharing similar letter(s) in a row are statistically non-significant (P>0.05).

remained non-significantly (P>0.05) different than their respective values in silymarin treated nephrotoxic rabbits.

Histopathological examination: The biochemical results were also confirmed by the histological studies. Tubular epithelial cells of normal kidney were normal in appearance with nucleus having nucleolus with fine mesh work of chromatin (Fig.1a). Kidney cells of paracetamol treated rabbits showed that moderate to severe degree of congestion was present in renal parenchyma. Tubular epithelial cells showed moderate degree of necrotic changes indicating pyknotic nuclei in tubular epithelial cells (Fig.1b). Kidney cells of silymarin treated rabbits showed that nuclei of tubular epithelial cells were normal in appearance. Pyknotic nuclei were present that indicated mild degree of amelioration by silymarin. At few places, mild degree of congestion was also present (Fig.1c). Kidney cells of *M. oleifera* seed powder treated animals at dose rate of 200 mg/kg body weight along with paracetamol showed pyknotic nuclei in tubular epithelial cells. Mild to moderate degree of congestion was also present (Fig.1d). Kidney cells of *M. oleifera* seed powder treated rabbits at dose rate of 400 mg/kg body weight showed tubular epithelial cells normal with fine chromatin material. At few places mild to moderate degree of congestion was also present. Tubular epithelial cells were also having pyknotic nuclei (Fig.1e). *M. oleifera* seed powder at dose rate of 600mg/kg body weight was given orally along with paracetamol. The renal parenchyma of this group was like normal animals. Tubular epithelial cells were normal with fine chromatin material. At few places mild degree of congestion was also present. Urinary spaces were clear and dilated indicating complete amelioration by the plant (Fig.1f).

DISCUSSION

Non-steroidal anti-inflammatory drugs inhibit cyclooxygenase enzymes affecting the synthesis of prostaglandins and resultantly affect liver, kidney and

gastrointestinal tract (Yousef *et al*., 2010). Paracetamol at toxic dose level has been reportedly responsible for glutathione diminution and ultimately lipid peroxidation and starts its intracellular accumulation where its reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), after making covalent bond with renal tissues, causes deterioration and death of cells which in turn is associated with electrolyte imbalance and creatinine and blood urea nitrogen instabilities (Alderman *et al*., 2002). It has been documented that elevation in serum Cr and BUN were found to be more reliable and well reported parameters for scrutinizing the drug induced renal damage (Schnellman, 2001).

In current study, administration of paracetamol significantly (P<0.05) elevated serum Cr and BUN levels as compared to their respective levels in control group. *M. oleifera* seed powder at oral doses of 200 mg/kg, 400 mg/kg and 600 mg/kg significantly (P<0.05) lowered acute elevation in the serum Cr and BUN. These values were non-significantly different than the respective values in silymarin treated nephrotoxic rabbits (Table 1). Earlier studies have shown that renal tissues are targeted by paracetamol overdose and resulted in high serum level of Cr and BUN (Dasilva *et al*., 2006). These results of present study were in agreement with previous findings where gentamicin and acetaminophen were used to induce nephrotoxicity in rats and aqueous and seed extracts of *Phyllanthus amarus* were used as nephroprotective remedy. The extracts significantly (P<0.05) lowered acute elevation in the serum Cr and BUN (Adeneye and Benebo, 2008). Further, the extract of *Curcuma longa* at the dose rate of 1000 mg/kg was found to reduce Cr and BUN significantly (P<0.05) in paracetamol treated group. The reported nephroprotective activity of turmeric was due to the presence of curcumin which possessed strong antioxidant activity (Cekmen *et al*., 2009). Similarly, *Harungana madagascariensi*, *Pimpinellas tirupatiensis*, *Cardiospermum halicacabum*, *Canarium schweinfurthi*, *Tapinanthus globiferus* and *Spathodea camanulata* also reduced the Cr and BUN levels in paracetamol induced nephrotoxic experimental animals (Siddiq *et al*., 2012).

In present study TAC declined and TOS elevated significantly (P<0.05) after oral administration of paracetamol in rabbits which may be due to inactivation of enzymes through cross-linking and/or collapse of the antioxidant enzymes and resultant amplified lipid peroxidation (Somani *et al*., 2000). Further, elevation in TOS level may also be attributed to the over production and afterwards accumulation of paracetamol metabolite, NAPQI, which promotes the formation of free radicals that are liable to induce oxidative stress on renal tissue and hence leads to cellular damage and nephrotoxicity (Yousef *et al*., 2010). These finding were supported by earlier reports where animals showed higher level of TOS and lower level of TAC after paracetamol overdose (Alderman *et al*., 2002).However, *M. oleifera* showed antioxidant action against lipid peroxidation by significantly (P<0.05) lowering the TOS and elevating TAC levels may be based on protecting antioxidant enzymes (Kumar and Pari, 2003).

In present investigations, CAT level decreased in paracetamol treated rabbits which are well associated with enhanced lipid peroxidation and disabling of antioxidant defense system of body owing paracetamol overdose (Yousef *et al*., 2010). The CAT is essential for detoxification of hydrogen peroxide (H_2O_2) and oxygen derived free radicals in the cells (Linares *et al*., 2006). Treatment with *M. oleifera* at 400mg/kg and 600mg/kg dose levels significantly (P<0.05) improved CAT activity. These findings were in agreement with earlier reports in which Isoniazid was used as antitubucular drugs to induce lipid peroxidation in rats.

In present study, administration of paracetamol resulted in significantly (P<0.05) higher level of MDA in rabbits. Elevated level of MDA is an indicator of oxidative stress in renal tissues. As end product of lipid peroxidation, it is responsible to exaggerate the damaging effect of reactive species on DNA and cellular membrane. Former studies have clearly verified that paracetamol overdose increases lipid peroxidation evidenced by high level of MDA and enhanced oxidative stress in renal tissues (Rodrigo and Bosco, 2006). Paracetamol induced lipid peroxidation reduces membrane fluidity, deactivates membrane bound receptors and enhances seepage of substances from membrane that do not pass normally (Gosh and Sil, 2007). In contrast, on the administration of 600mg/kg seed powder of *M. oleifera*, the level of MDA was significantly (P<0.05) decreased when compared to paracetamol treated rabbits. The ability of *M. oleifera* in prevention of lipid peroxidation may be due to its glucosinolate and phenolic contents being reductive in nature and lowering the level of MDA in nephrotoxic experimental animals (Bennet *et al*., 2003).

Paracetamol overdose may result in imbalance between oxidant-antioxidant equilibrium. Paracetamol induced renal damage was supported by biochemical and histopathological changes that correspond with observations recorded earlier (Sarumathy *et al*., 2011). Previous histopathological studies revealed that paracetamol treated group showed severe tubular necrosis, permeation of inflammatory cells, tubular deterioration, hemorrhage, distension of tubules and vacuolization (Pareta *et al*., 2011). Current investigations also produced similar histopathological findings in rabbits of paracetamol treated group. Kidney cells of paracetamol treated rabbits showed moderate to severe congestion present in renal parenchyma. Tubular epithelial cells showed moderate degree of necrotic changes indicated by condensed nuclei of tubular epithelial cells. It indicated moderate to severe degree of pathological changes induced by oral administration of paracetamol (Fig.1a).The present study showed that *M. oleifera* significantly (P<0.05) reduced the histopathological changes observed in paracetamol treated group. Treatment with different doses of *M. oleifera* along with paracetamol produced only mild degree of degeneration and absences of necrosis as compared to paracetamol treated group. *M. oleifera* seed powder at dose rate of 200 & 400 mg/kg showed tubular epithelial cells with mild condensed nuclei (Fig.1 d&e). However, *M. oleifera* seed powder at dose rate of 600 mg/kg showed tubular epithelial cells with normal and fine chromatin material indicating complete amelioration by seed powder of plant (Fig.1f).

The proposed mechanism of M. *oleifera* to reduce nephrotoxicity might be due to its antioxidant properties (Ndhlala *et al*., 2014). Antioxidant potential of this herb has been reported in many studies. The seeds of Moringa have contained many antioxidants like tocopherols, Vitamins C,

E and polyphenols possessing radical trapping ability (Siddhuraju and Becker, 2003). Previous studies showed that polyphenolic compounds which covered majority of constituents detected in *M. oleifera* have well documented antioxidant potential through inhibiting ROS and lipid peroxidation in renal tissues. Further, *M. oleifera* has been reported to restore depleted glutathione after toxic drug administration. Moringa seed and leaves has contained many active constituents as glucosinolates, isothiocyanates, thiocarbamates and various flavonoids which were responsible for antioxidant activity (Waterman *et al*., 2014). Falvonoids were reported to inhibit drug-induced nephrotoxicity due to its potent antioxidant activity (Bennet *et al*., 2003).

Besides antioxidant activity as mechanism for *M. oleifera* as nephroprotective plant, other mechanisms e.g. sympatholytic effect, modulation in nitric oxide synthesis and inactivation of renin–angiotensin system cannot be over ruled. Further studies are obligatory to interpret these possible mechanisms.

Conclusions: Based on the biochemical and histological findings of present investigations, it can safely be conceived that administration of *Moringa oleifera* seed powder decreased the damaging effects of paracetamol induced nephrotoxicity in rabbits; possibly by deterring lipid peroxidation process. Further inquiry of these promising protective effects of *M. oleifera* against paracetamol induced renal damage may have a substantial influence on developing clinical approaches for treatment of patients with renal failure and/or as an adjunct therapy aiming to improve the therapeutic index of some nephrotoxic agents.

Contribution of Authors: IJ, BA and JAK supervised this research project and built initial constructs and validated them in laboratory. AI designed and performed experiments, analyzed data and wrote the paper. IJ and MZK conducted the observations on microscope for histopathological samples. MAN and MMA helped in analyzing the biochemical parameters and health biomarkers. TK, ZI and ZUR discussed the methodology, results and discussion and commented on the manuscript at all stages. BA and JAK arranged the data and wrote results of the paper.

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