

Fresh Bitter Melon Fruit (*Momordica charantia*) Attenuated Oxidative Stress, Fibrosis and Renal Injury in Carbon Tetrachloride Treated Rats

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ABSTRACT: Chemical or drug-induced kidney damage is increasing every year and the end-stage renal disease is becoming a burden for health care system of many countries. Oxidative stress may be a crucial pathway for the development of end-stage renal disease. Thus, natural antioxidant or plant-based therapy would be a better alternative to protect renal function against chemical-induced renal damage. To determine these aspects we evaluated renoprotective effects of *M. charantia* in carbon tetrachloride administered rats. A 10% w/w mixture of fresh fruits of *M. charantia* was given with the chow food every day to CCl₄ treated rats. After fourteen days, all animals were sacrificed and the kidneys were examined to observe the possible protective effects of *M. charantia* against CCl₄ induced toxicity. The CCl₄ treated rats showed increased oxidative stress parameters and decreased antioxidant enzymes activities. Supplementation of 10% w/w *M. charantia* fruits in CCl₄ administered rats prevented the oxidative stress and restored the antioxidant enzymes activities. *M. charantia* fruits supplementation also prevented the rise of uric acid and creatinine concentration in plasma of CCl₄ treated rats. Furthermore, histological studies showed that supplementation of 10% w/w *M. charantia* fruits prevented the collagen deposition, immune cell migration and iron deposition in kidney sections of CCl₄ treated rats. The results of this study revealed that the fruits of *M. charantia* may protect oxidative stress-mediated damage in kidneys due to CCl₄ administration, which is mediated probably via the restoration of anti-oxidant enzyme functions.

Key words: Renal dysfunction, carbon tetrachloride, malondialdehyde, superoxide dismutase, Oxidative stress, *Momordica charantia*.

INTRODUCTION

Diabetes, obesity, insulin resistance and hypertension have been increasing very fast and these are becoming an epidemic which leads to kidney dysfunctions like nephritis, hypertrophy proteinuria and podocyte injury as well as apoptosis.¹ These factors increase mortality rate and put the extra burden on the patient and thereby increasing the overall cost of the treatment.² Carbon tetrachloride (CCl₄), a synthetic halogenated and the highly lipid soluble molecule, is applied on various laboratory animal models to study the potential roles of an

active molecule, plant or and microbe.³ CCl₄ is not only known as a hepatotoxin⁴ but it also significantly affects the heart,⁵ brain,⁶ lung,⁷ reproductive organs and most importantly in kidney.⁸ The actual mechanism of CCl₄ in the kidney is yet not clear but it is described that the pathogenic pathway is depending onto free radical-mediated oxidative stress.⁹ However, the study described that CCl₄ undergoes biotransformation through CYP-2E₁ and converts to a highly reactive molecule named trichloro-chloride radical (*OCCl₃)¹⁰ which further produces lipid peroxidation by oxidizing membrane lipid resulting cellular damage.¹¹ A recent study focused that metabolites of CCl₄ lowered the amount of natural anti-oxidation capacity i.e-SOD, GSH,

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GPX, catalase, ascorbate and hemeoxygenase.¹² Our previous study revealed that free radicals generated from CCl₄ invites various immune cells like macrophages, monocytes, mast cell, T-cells and neutrophil which further recruit various pro-inflammatory cytokines such as tumor necrosis factor- α , interleukins-1 β , nuclear factor- β , macrophage inflammatory protein and many more. Furthermore, oxidative stress induces pro-fibrogenic molecules like matrix metalloproteinase, transforming growth factor- β and F₂-Isoprostane to activate local fibroblastic tissue which secret extra cellular matrix and collagen around the injured tissue. Taken together, free radicals often disturb heme oxygenase which regulates iron metabolism.¹³

Modern medicines focus not only towards active drug constituents but also traditional medicines including various plants, herbs, animals, microorganisms and marine creatures in disease prevention.¹⁴ The study also suggested that traditional medicines which contain anti-oxidant property can reduce free radical generation.¹⁵ *M. charantia* is a well-investigated plant which was used as anti-malarial, analgesic and anti-inflammatory, anti-obesity, anti-hyperlipidemic and anti-diabetic agents. This plant possesses several groups of bioactive molecules like polyphenols, flavonoids, glycosides, triterpenes, tannins and fixed oils.¹⁶ Our previous study found out that 10% w/w fruit supplementation of *M. charantia* with food ameliorated hepatic damage, oxidative stress and fibrosis.¹⁷ Interestingly, the renoprotective activity of this plant has not been focused well on CCl₄ induced animal model. Therefore, this study will reveal renoprotective activities of *M. charantia* by reducing oxidative stress-mediated kidney damage, inflammation and fibrosis.

MATERIALS AND METHODS

Chemicals and reagents. Thiobarbituric acid (TBA) was purchased from Sigma Chemical Company (USA). Reduced glutathione (GSH) and trichloroacetic acid (TCA) were purchased from J.I. Baker (USA). Uric acid (UA), creatinine assay kits

were obtained from DCI diagnostics (Budapest, Hungary), 5, 5-dithiobis-2-nitrobenzoate (Ellman's reagent) from Sigma (USA) and sodium hydroxide from Merck (Germany). All other chemicals and reagents used were of analytical grade.

Animals used and experimental protocol. Eight to ten weeks old, 28 Long Evans female rats (160–170 gram each) were obtained from Animal Production Unit of Animal House at Department of Pharmaceutical Sciences, North South University and were kept in individual cages in an air-conditioned room where the temperature was maintained at 22 \pm 3°C with a 12 hours dark/light cycles. Rats had access to standard laboratory feed (Pellet foods) and mineral water, according to the study protocol which was approved by Ethical Committee of Department of Pharmaceutical Sciences, North South University for animal care and experimentation. To observe the reno-protective effects of *M. charantia*, rats were divided into four groups (6-8 rats in each group) named as Control (Group A), Control + *M. charantia* (Group B), CCl₄ (Group C) and CCl₄ + *M. charantia* (Group D). Only rats of group B and D were provided *M. charantia* supplement. All animals were checked for body weight, food and water intake on a daily basis. After 14 days of grouping, all animals were weighed, sacrificed, and all internal organs such as heart, kidney and spleen were taken. Immediately after collection of the organs, they were weighted and stored in neutral buffered formalin (pH 7.4) to be processed for histology. Rat kidney tissue was homogenized and centrifuged in 8000 rpm for 10 minutes then the clear solution was taken. Immediately after collecting solutions, tissue were stored in -20° C for further biochemical analysis to observe the possible oxidative mediated renal dysfunctions.

Administration of CCl₄ and treatment with *M. charantia*. Two types of bitter melon are available in the market. Smaller fruits were used in this study instead of larger. Fresh *M. charantia* was purchased from local market, then washed properly and dried at room temperature. Later, the fruits have compressed a mixture of a blender and 10 % w/w given every day

with the chow food. *Momordica charantia* has been identified by Mr. Sarker Nasir Uddin, Senior Scientific Officer, of National Herbarium, Mirpur, Dhaka Bangladesh and a receipt was deposited (Accn. No. 40566) for future reference.

Assessment of biochemical parameters. Kidney markers uric acids (UA) and creatinine were estimated in kidney tissue by using Diatec diagnostic kits (Hungary) according to the manufacturer's protocol.

Table 1. Different animal groups and their treatment strategies used in this study.

Group A	Group B	Group C	Group D
Number: 6	Number: 6	Number: 8	Number: 8
Duration: 14 days	Duration: 14 days	Duration: 14 days	Duration: 14 days
Treatment: given only oral olive oil 3 ml/kg bw	Treatment: 10% w/w <i>M. charantia</i> with food alone with oral olive oil 3 ml/kg bw	Treatment: given oral olive oil 3ml/kg bw + CCl ₄ 1ml/kg bw twice a week	Treatment: given oral olive oil 3ml/kg bw + CCl ₄ 1 ml/kg bw twice a week with 10% w/w <i>M. charantia</i>

Determination of lipid peroxidation (LPO) as malondialdehyde (MDA). Lipid peroxidation in kidney was estimated calorimetrically measuring malondialdehyde followed by previously described protocol.¹⁸ Samples (1 ml) were mixed with 1 ml of 0.67% thiobarbituric acid and placed in a boiling water bath for 10 min. The mixture was cooled and diluted with 1 ml distilled water. The absorbance of the solution was then read using spectrophotometer at 532 nm. The content of malondialdehyde (MDA) (nmol/ml) was then calculated, by reference to a standard curve of MDA solution.

Determination of nitric oxide (NO). Nitric oxide (NO) was determined according to the method described previously as nitrate.¹⁹ In the study, Griess-Ilosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 ml) containing tissue sample (2 ml) and phosphate buffer saline (0.5 ml) was incubated at 25°C for 150 min. A pink colored chromophore was formed in diffused light after addition of modified Griess-Ilosvoy reagent. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. NO level was measured by using the standard curve and expressed as nmol/mL.

Determination of advanced protein oxidation products (APOP) assay. Determination of APOP levels was performed by modification of the

previously described method elsewhere.^{20,21} Two mL of tissue sample was diluted 1:5 in PBS: 0.1 ml of 1.16 M potassium iodide was then added to each tube, followed by 0.2 ml acetic acid after 2 min. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 2 ml of PBS, 0.1 ml of KI, and 0.2 ml of acetic acid. The chloramine-T absorbance at 340 nm was found linear within the range of 0 to 100 nmol/ml, APOP concentrations were expressed as nmol/ml chloramine-T equivalents.

Determination of catalase (CAT) activity. CAT activities were determined using previously described method elsewhere.^{22,23} The reaction solution of CAT activities contained 2.5 ml of 50 mmol phosphate buffer (pH 5.0), 0.4 ml of 5.9 mmol H₂O₂ and 0.1 ml enzyme extract. Changes in absorbance of the reaction solution at 240 nm were determined after one minute. One unit of CAT activity was defined as an absorbance change of 0.01 as units/min.

Determination of reduced glutathione assay (GSH). Reduced glutathione was estimated by previously described method.²⁴ One (1.0) ml of 10% tissue sample was precipitated with 1.0 ml of (4%) sulfosalicylic acid. The samples were kept at 4°C for 1 hr and then centrifuged at 1200 × g for 20 min at 4°C. The total volume of 3.0 ml assay mixture composed of 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (5,5-

dithiobis-2-nitrobenzoic acid), (100 mM). The absorbance of the yellow color mixture was read immediately at 412 nm on a Smart Spec TM plus Spectrophotometer and expressed as ng/mg protein.

Determination of superoxide dismutase (SOD). The determination of SOD was performed by a modified procedure previously described elsewhere.²⁵ Briefly, each 300 μ l reaction mixture contained 50 mM sodium phosphate (pH 7.8), 13 mM methionine, 75 mM nitrobluetetrazolium (NBT), 2 mM riboflavin, 100 mM EDTA, and 2 ml of tissue sample. The change in absorbance of each sample was then recorded at 560 nm for the production of blue formazan. Results were expressed as percentage activity of SOD enzyme. The equation for measuring the activity of SOD was = [(Initial absorption - subsequent absorption at 30 s interval)/Initial absorption] x 100.

Estimation of total protein concentration. Total protein in tissue was determined by BCA protein acid kit (Thermo Scientific) according to the manufacturer's protocol.

Histopathological determination of inflammation, fibrosis and iron overload. For microscopic evaluation, kidney tissue was fixed in neutral buffered formalin and embedded in histology graded paraffin, sectioned at 5 μ m with the help of Microtome and subsequently stained with hematoxylin and eosin to evaluate inflammatory cell migration. Sirius red staining was also performed to evaluate the fibrosis in kidney sections. Moreover, prussian blue staining was also performed to determine the iron overload in kidney tissues. The slides were basically examined for pathomorphological changes. Sections were studied under a light microscope at 40 \times magnifications.

Statistical analysis. The values are expressed as a mean \pm standard error of the mean (SEM). The findings were analyzed by using the one way ANOVA and with Bonferroni post hoc test using Graph Pad Prism software, version 6.0 (USA). Statistical significance was considered $p < 0.05$ in all cases.

RESULTS AND DISCUSSION

Effect of CCl₄ and *M. charantia* on body weight, food and water intake. Body weight of each rat was recorded every day during the experiment, and percentage change was calculated for all groups. It was found that the body weight increased consistently to only Group-A, on the other hand body weight of all groups was decreased gradually. Food and water intake was also found to decrease except for control group. Gradual weight losing as well as losing food habit might be due to CCl₄ intoxication and bitter test of fruits.

Effect of CCl₄ and *M. charantia* on organ wet weight. Table 2 shows various effects of CCl₄ and *M. charantia* on rat's organs wet weight. The wet weight of heart and kidney was decreased immediately after collection in CCl₄ treated rats when compared with control rats. However, spleen wet weight was increased in CCl₄ treated group as compared to control rats.

Effects on CCl₄ and *M. charantia* on kidney markers. From our biochemical assay, it was found that tissue uric acid and creatinine concentration was quite high due to CCl₄ administration and activation of free radicals pathogenic pathways. In all cases the kidney markers (uric acid and creatinine) values were found to be significant (Table 3).

Effects of CCl₄ and *M. charantia* on natural anti-oxidant capacity system. Glutathione, which is considered as a potent anti-oxidant was found (19.40 \pm 1.68 ng/mg) in control kidney, while in CCl₄ treated kidney it was almost half of the control group. Interestingly, treatment with *M. charantia* improved very well and the level was found more than the control group (23.91 \pm 1.31). In case of SOD activity, CCl₄ treated kidney (13.57 \pm 1.70) lost less than half of control kidney (29.19 \pm 1.64) and treatment (24.92 \pm 3.10) improved almost near to control group. Surprisingly, catalase was found much more in the treated group (11.79 \pm 0.74) than the control rats (9.03 \pm 0.83). In all cases, treatment with *M. charantia* was found to be safe when other groups were compared with Group B (Table 3).

Table 2. Effect of *M. charantia* on various parameters in different groups of CCl₄ treated rats.

Parameters	Control (Group A)	Control + <i>M. charantia</i> (Group B)	CCl ₄ (Group C)	CCl ₄ + <i>M. charantia</i> (Group D)
Initial bw (g)	164.23 ± 2.66	172.15 ± 1.26	168.76 ± 2.36	166.45 ± 2.30
Final bw (g)	177.82 ± 3.27	162.50 ± 1.68	162.71 ± 3.01	160.67 ± 1.98
Food intake (g/day)	19.16 ± 2.10	13.48 ± 1.97	12.92 ± 1.40	12.86 ± 1.47
Water intake (ml/day)	20.05 ± 2.09	19.18 ± 1.82	14.83 ± 1.78	14.98 ± 2.34
Both kidney wet weight (g/100 g of bw)	0.67 ± 0.04	0.63 ± 0.02	0.55 ± 0.01	0.60 ± 0.01
Heart wet weight (g/100 g of bw)	0.37 ± 0.02	0.35 ± 0.01	0.32 ± 0.01	0.34 ± 0.01
LV wet weight (g/100 g of bw)	0.28 ± 0.02	0.27 ± 0.01	0.26 ± 0.01	0.27 ± 0.01
RV wet weight (g/100 g of bw)	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Spleen wet weight (g/100 g of bw)	0.38 ± 0.02	0.39 ± 0.01	0.43 ± 0.03	0.39 ± 0.01

Values are presented as mean ± SEM, where n=6. One way ANOVA with Bonferoni tests were done as post hoc test. Values are considered significance at p<0.05.

Table 3. Effect of *M. charantia* on oxidative stress parameters in kidney of CCl₄ treated rats.

Parameters	Control	Control + <i>M. charantia</i>	CCl ₄	CCl ₄ + <i>M. charantia</i>
Kidney				
Uric acid (mg/dL)	13.09 ± 0.71a	10.46 ± 0.83a	20.96 ± 1.28b	16.79 ± 0.57b,c
Creatinine (mg/dL)	15.76 ± 0.84a	14.82 ± 1.27a	26.57 ± 1.38b	23.02 ± 2.21b
APOP (nmol/mL)	190.26 ± 9.34a	183.83 ± 12.73a	714.08 ± 45.45b	534.77 ± 43.30b,c
MDA (nmol/mL)	56.49 ± 1.82a	50.52 ± 2.87a	86.87 ± 3.55b	68.07 ± 4.12a
NO (nmol/mL)	16.53 ± 1.28a	15.11 ± 1.63a	35.70 ± 2.02b	29.30 ± 0.93b,c
Catalase (U/Min/mg protein)	9.03 ± 0.83a	9.70 ± 0.82a	7.68 ± 0.91a	11.79 ± 0.74a
GSH (ng/mg protein)	19.40 ± 1.68a	20.46 ± 3.12a	10.28 ± 0.74b	23.91 ± 1.31a
SOD (% of activity)	29.19 ± 1.64a	32.13 ± 4.44a	13.57 ± 1.70b	24.92 ± 3.10a

Values are presented as mean ± SEM. One way ANOVA with Bonferoni tests were done as post hoc test. Values are considered significance at p<0.05. APOP-Advanced protein oxidation product, expressed as nmol/mL equivalent to Chloramine-T. a vs b, control vs CCl₄; b vs c, CCl₄ vs treatment

Effects of CCl₄ and *M. charantia* on oxidative stress markers. AOPP, NO and MDA are the 3 important markers which have been evaluated in the present study and found very high due to CCl₄ administration. This indicates high oxidative stress inside disease group. Our biochemical assay found that control kidney contained (190.26 ± 9.34) nmol/mL AOPP, on the other hand CCl₄ treated kidney showed almost three times (714.08 ± 45.45) nmol/mL AOPP more than the control kidney. So the treated group significantly reduced AOPP level in the

kidney. NO, which is a vasodilator as well as signaling molecules observed high (35.70 ± 2.02) in CCl₄ group but *M. charantia* treatment lowered that concentration of nitric oxide (29.30 ± 0.93). We have also evaluated the MDA which is a product of damaged cell membrane. CCl₄ treated rats produced an increased concentration of MDA (86.87 ± 3.55) as compared to the control rats (56.49 ± 1.82). However, treatment with bitter melon lowered this level (68.07 ± 4.12 nmol/ml) (Table 3).

Evaluation of fibrosis, inflammation and iron deposition in kidney. In each and every case of kidneys from CCl₄ administrated rats (Figures 1C, 2C and 3C), we found huge fibrosis, inflammatory cell accumulation and iron overload around the glomerulus which was not found in control group (Figures 1A, 2A and 3A). Although treatment with

10% w/w *M. charantia* (Figures 1D, 2D and 3D) almost normalized collagen deposition, immune cell migration and iron accumulation when it was compared with control group. Treatment with *M. charantia* in control rats (Group B) did not show any pathological sign of liver damage as well as alteration in biochemical parameters (Figures 1B, 2B and 3B).

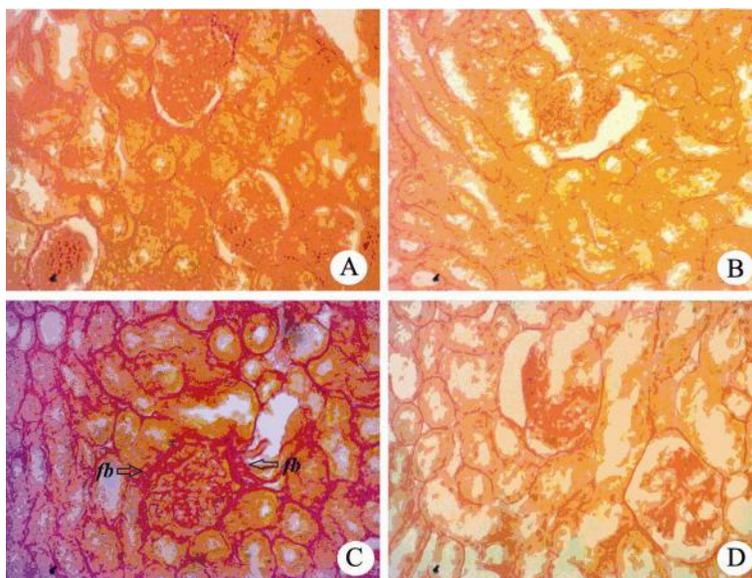


Figure 1. Sirius red staining showed fibrosis (Red color) in kidney section of various groups. A represents control kidney, B represents control + *M. charantia* kidney, C represents CCl₄ treated kidney and D represents CCl₄ + *M. charantia* kidney. Magnification 40X was used. *fb*- fibrosis.

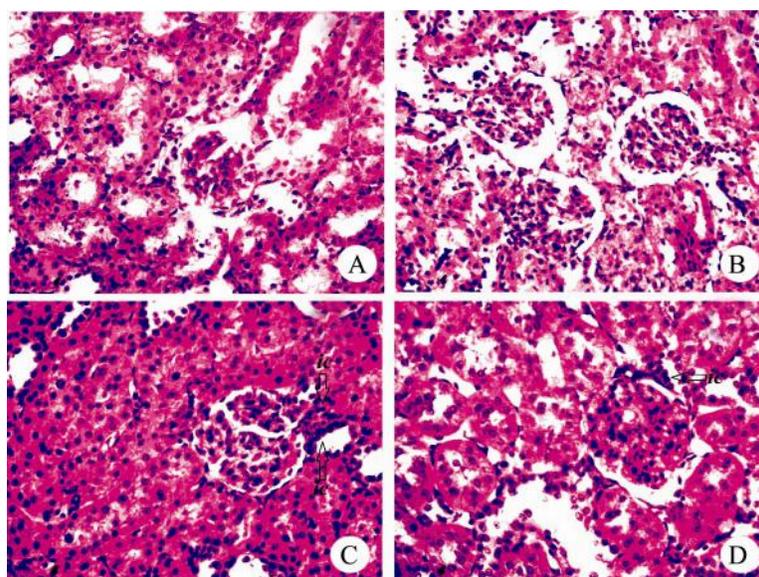


Figure 2. Hematoxylin and eosin staining showed inflammatory cell infiltration and necrosis in kidney of various groups. A represents control group, B represents control + *M. charantia* group, C represents CCl₄ treated group and D represents CCl₄ + *M. charantia* group. Magnification 40X was used. *ic*- inflammatory cells.

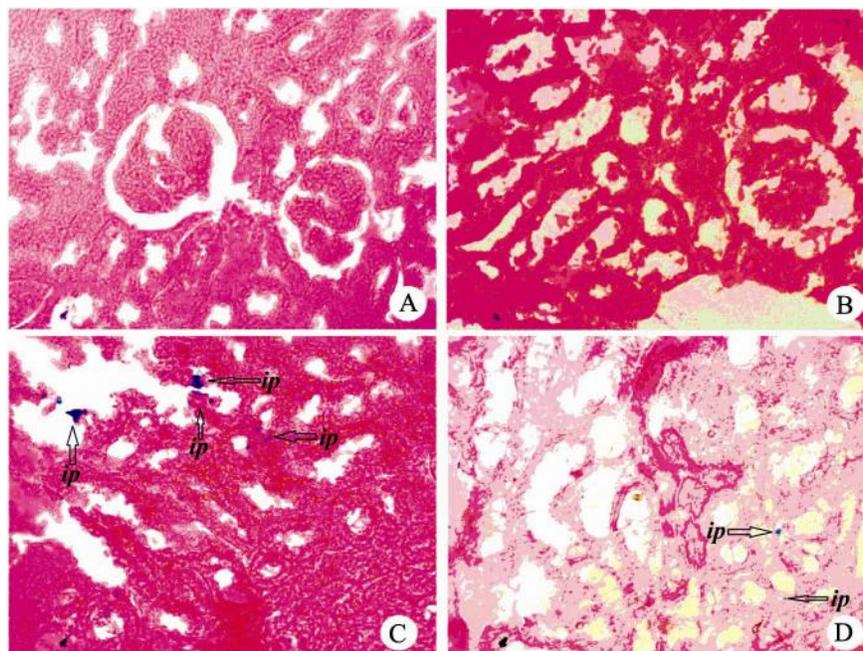


Figure 3. Prussian blue staining showed iron deposition in kidney section of various groups. A represents control group, B represents control + *Momordica charantia* group, C represents CCl_4 treated group and D represents CCl_4 + *Momordica charantia* group. Magnification 40X was used. *ip*- iron pigment.

The kidney plays several important roles to eliminate biological waste like dead cells, uric acid, unwanted muscle, several proteins via urine. It also produces several necessary hormones like vasopressin and erythropoietin, maintains pH of blood and other fluids, regulates blood pressure by renin and angiotensin system and most importantly it filters blood.²⁶ Several diseases are initiated by uric acid which is the ultimate end product of purine metabolism.²⁷ Unfortunately, uric acid acts as a potent oxidant and helps in the development of various pathogenesis such as hypertension and chronic kidney diseases to end-stage renal diseases.²⁸ In the same way creatinine is the waste product of muscle catabolism which justifies that high level of creatinine either in plasma or kidney indicates kidney dysfunctions.²⁹ Our study found that CCl_4 treated rats produced higher uric acid and creatinine level in kidney, which were considered as the ultimate feature of kidney dysfunction and our treatment group significantly lowered creatinine as well as uric acid level. Similar kind of outcome has been observed in

previous study in a renovascular hypertensive rat model.³⁰

Administration of CCl_4 generates huge free radicals which further helps to produce malondialdehyde (MDA) that oxidize membrane lipid and damage kidney cell membrane. Later, immune cell is attracted around the damaging area to protect from the injury.³¹ Another group of authors has also observed that MDA level damages kidney cells and increases serum AST, ALP and ALT concentration.³² We have found enormous MDA level in CCl_4 treated group which was normalized by bitter melon treatment through oral route. Nitric oxide, a signaling molecule that maintains vascular tone is generally induced by NOS but sometimes due to oxidative stress it may produce iNOS which is a dangerous molecule for damaging cell and recruit immune cell in an organ.³³ Inside kidney, NO often reacts with highly reactive free radicals and produce more harmful compound like peroxynitrite.³⁴ CCl_4 often travels to nucleus and interfere with genetic code like DNA, RNA, regulatory protein, genes and further produces faulty mRNA. Oxidizing genetic

materials produce advanced oxidative protein products (AOPP) in the kidney.³⁵ Our study found that treatment with only CCl₄ produced high amount of NO and AOPP level and 10% w/w bitter melon treatment significantly normalized concentration of both kidney tissue.

Carbon tetrachloride is well known toxicant which produces several oxidants and at the same time it reduces the amount of natural anti-oxidant defense capacity. CCl₄ also generates hydrogen peroxide anion, superoxide anion (O_2^-) and hydroxyl radical (OH^\bullet) etc.³⁶ Furthermore, oxidative stress also helps in the production of NAD(P)H oxidase which is again responsible for mitochondrial damage in the kidney.³⁷ SOD, GSH and catalase are known as natural anti-oxidants which are normally generated against any harmful stimuli. A previous study also described that kidney could be protected if the concentration of anti-oxidant is improved.³⁸ Our study has observed that the level of anti-oxidants found lower on the CCl₄ treated group and bitter melon treatment improved the level more than that of in the control group.

From the histological assessment, we observed that CCl₄ treated rats showed massive immune cell infiltration, collagen deposition and iron pigment accumulation in the liver. On the other hand treatment with bittermelon significantly reduced inflammation, fibrosis and iron deposition in the liver of CCl₄ administered rats. It is anticipated that the improvement of kidney histology as well as bio-assay proved that bitter melon treatment might be due to the significant anti-oxidant capacity. Our previous experiment also exposed that *M. charantia* served as hepatoprotective by reducing oxidative stress as well as free radicals.¹⁷ The previous study also explored that bitter melon regulates peroxisome proliferator-activated receptors (PPARs), monocyte chemoattractant protein-1 (MCP-1) and fatty acid synthesis (FAS) related genes.¹⁶ Bitter melon also reduced inflammatory, pro-inflammatory, fibrogenic, pro-fibrogenic and iron-mediated protein.¹⁶ Our study revealed that improvement of anti-oxidant component and reduction of oxidative stress markers could

protect from renal damage via anti-oxidant mediated mechanism. Recent literature focused that oxidative stress, inflammation and fibrosis may be produced by angiotensin-II mediated pathway.³⁹ One of our very recent studies found that angiotensin-converting enzyme inhibitor successfully normalized CCl₄ induced hepatic damage, oxidative stress and fibrosis.⁴⁰ Depending on that result we also propose that bitter melon might have the activity to suppress renin-angiotensin-system and thereby could regulate oxidative stress as well as down-regulate cytokines production. However, further study would be necessary to explore the exact molecular mechanism of *M. charantia* on renal protection.

AUTHOR'S CONTRIBUTIONS

Md. Ashraful Alam conceived the idea, designed the study and monitored all protocols for the study. Md Abu Taher Sagor and Nabila Tabassum carried out the animal study and performed the biochemical assay. Md Abu Taher Sagor and Md. Moshfequr Rahman carried out statistical analysis. Hasan Mahmud Reza and Md. Ashraful Alam evaluated the histology and wrote the manuscript. All the authors have checked and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest about this manuscript.

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