*Original Article*

# Acute Toxicity and Protective Effect against Diabetic Nephropathy of the Combination Extract of *Magifera indica* and *Polygonum odorontum* in Streptozotocin-diabetic Rats

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***Background:*** The prevention against diabetic nephropathy is still required due to the limitation of the current therapeutic strategy. Based on the effect of antioxidants on the restoration of renal dysfunction in diabetic nephropathy, we hypothesized that the combination extract of seed of *Mangifera indica* or mango var. Namdokmai and aerial part of *Polygonum odoratum* or Vietnamese coriander (MPO) which possessed antioxidant activity might prevent diabetic nephropathy and be safe for consumption.

***Objective:*** To test this hypothesis, we aimed to determined acute toxicity and renoprotective effect of MPO in STZ-diabetic rats.

***Materials and Methods:*** Acute toxicity was assessed in Wistar rats. Single administration of MPO at dose of 5,000 $mg.kg^{-1}$ BW failed to produce mortality and related toxicity signs. To test renoprotective effect, diabetic rats were induced by a single injection of streptozotocin (STZ). STZ-diabetic rats were orally given MPO at doses of 2, 10 and 50 $mg.kg^{-1}$ BW for 10 weeks. Then, they were determined body weight, kidney weight, urine volume, urine specific gravity and blood biochemical parameters at the end of experiment. In addition, kidneys were collected and determined histomorphology, nitric oxide synthase (NO), NF-kappa B (NF-kB), vascular endothelial growth factor (VEGF), aldose reductase (AR) and oxidative stress status.

***Results:*** Low dose of MPO significantly improved creatinine clearance while medium and high doses of MPO significantly improved albuminuria. MPO also improved Bowman space, glomerular tuft, eNOS, oxidative stress status and NF-kB but no significant change of VEGF and AR were observed in kidney. Therefore, the possible underlying mechanism of MPO might be partly associated with the improvement of kidney structures, oxidative stress status, eNOS and NF-kB in kidney.

***Conclusion:*** Our data suggest that MPO is the potential candidate of renoprotectant against diabetic nephropathy but sub chronic toxicity and clinical trial are still essential.

***Keywords:*** Acute toxicity, Diabetic nephropathy, Renoprotective, *Mangifera indica*, *Polygonum odoratum*

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Diabetic nephropathy, a major microvascular complication of diabetes mellitus, has been recognized as an important cause of morbidity and mortality of diabetic patients[1,2]. It is characterized by persistent elevated albuminuria and declined glomerular filtration rate (GFR). The kidney damage in diabetic nephropathy is manifested by tubulointerstitial injury and glomerulosclerosis[3]. Recent studies have demonstrated that podocyte derived vascular endothelial growth factor (VEGF) plays the crucial role on the increased protein filtration[4,5]. In addition, several lines of evidence also reveal that both inflammatory cytokine such as tumor necrosis factor-a (TNF-a) and oxidative stress also play the essential roles on the pathophysiology of diabetic nephropathy[6,7]. Currently, it has been shown that oxidative stress is the key factor to regulate the expression of both VEGF[7] and TNF-a[8]. Since most of the current therapeutic strategies against diabetic nephropathy often produce side effects and toxicity. Therefore, the developments of the novel therapeutic strategies which are interrelated with diabetic nephropathy related pathophysiology are still necessary.

During the last decade, antioxidant has been proposed to be the potential therapeutic strategy for diabetic nephropathy[9]. It has been reported that vitamin E, probucol, alpha-lipoic acid, or taurine could restore diabetes-induced not only renal dysfunction such as albuminuria and glomerular

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hypertension but also glomerular pathologies[9]. Nowadays, agricultural waste especially non-edible part and vegetables are considered as valuable resources due to their abundance in polyphenolic compounds. Our pilot study demonstrated that the hydro-alcoholic extract of seed of *Mangifera indica* or mango var. Namdokmai and aerial part of *Polygonum odoratum* or Vietnamese coriander (MPO) possess potent antioxidant activity (unpublished data). Based on the synergistic concept according to traditional folklore and aforementioned information, the health benefit to protect against nephropathy in diabetic condition induced by streptozotocin (STZ) of MPO had gained attention. Since the MPO had been categorized as the novel product and no data concerning safety and renoprotective effect were available, the present study aimed to determine the acute toxicity and renoprotective against diabetic nephropathy of the combined extract of mango seed and aerial parts of Vietnamese coriander in streptozotocin-diabetic rats.

## Material and Methods

### *Plant material and extract preparation*

Mango or *Mangifera indica var.* Namdokmai and Vietnamese coriander or *Polygonum odoratum* L. were harvested during May 2015. The voucher specimens (voucher specimen 2015001 and 2015002) were kept at the Integrative Complementary Alternative Medicine Research and Development Center, Khon Kaen University. The seed of *M. indica* and aerial part *P. odoratum* were prepared as 50% hydro-alcoholic extract by maceration technique and prepared as the combination extract (MPO) (The detail was under petty patent registration). The extract contained total phenolic compounds at the concentration of 81.96±2.42 mg/L GAE/mg extract. In addition, quercetin and gallic acid were observed at the concentrations of 2.286 mg Quercetin equivalent (QE)/100 mg MPO extract and 0.636 mg Gallic acid/100 mg MPO, respectively[10].

### *Animals*

Healthy male Wistar rats weighing between 180 to 220 grams were used as experimental anima in this study. All rats were given access to food and water *ad labium* and housed in group of 5 per cage in standard metal cages at 23±2°C on 12: 12 h light-dark cycle. The Ethical Committee on Animals Experiments of Khon Kaen University (AEKKU-NELAC 8/2558) was used to maintain and treat all animals

### *Acute oral toxicity assessment*

Acute oral toxicity was carried out by using fixed-dose procedure according to the Organization for Economic Co-operation and Development (OECD) Guideline 420[11]. Rats of both sexes were fasted overnight. Experimental rats were administered a single dose of MPO at dose of 5g.kg$^{-1}$ BW whereas control rats were administered vehicle (distilled water) via oral route. Food and water were provided 2 hours later. Animals were observed individually at least once during the first 30 min and periodically during the first 24 hours with special attention at the first 4 hours. Then, they were observed twice daily for 14 days in order to assess changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. At the end of study, blood samples were collected for biochemical and hematological analyses. The organs were excised, weighed and subjected to gross necropsy. Principal vital organs were fixed in10% formalin buffer, trimmed, processed, embedded in paraffin wax, sectioned at a thick-ness of 5 µm, stained by Hematoxylin and Eosin and observed under light microscope for histological changes.

### *Determination of nephroprotective effect*

All rats (n = 10 per group) were divided into various groups as follows:

Group 1) Control group, all rats were administered citrate buffer, a vehicle of streptozotocin (STZ).

Group 2) DM + vehicle group, the rats were induced diabetes mellitus via single injection of STZ and received distilled water or vehicle of the extract.

Group 3-4) DM+ the combination extract of *M. indica var.* Namdokmai and *P. odoratum* L. (MPO) at doses of 2, 10 and 50 mg.kg$^{-1}$ BW respectively.

All rats in DM group were fasted for 12 hours before being subjected to a single injection of streptozotocin (STZ) which was dissolved in citrate buffer (pH 4.5) at dose of 55 mg.kg$^{-1}$ BW in order to induce diabetic nephropathy. Only rats which showed the blood sugar levels more than 250 mg.dL$^{-1}$ were recruited for further study. They were treated with the assigned interventions once daily at 3 days after injection of STZ and maintained for 10 weeks. Body weight of each animal was determined at the initiation and every week throughout of the study. At the end of experiment, fasting blood and 24 h urine samples were collected to determine urine volume, urine specific gravity and blood biochemical parameters. Moreover, kidneys were collected and determined histomorphology, endothelial nitric oxide synthase (eNOS), NF-kappa B (NF-kB), vascular endothelial growth factor (VEGF), aldose reductase (AR) and oxidative stress status including malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes in kidney.

### *Blood preparation and assessments*

Blood samples were collected from the overnight-fasted rats and mixed with EDTA as anticoagulant at the end of experiment. Then, the samples were subjected to a 3,000 g-centrifugation at 4°C for 1 hour and the plasma likes a supernatant was harvested and used for the determination of hematological and biochemical changes. In this study, hematological and biochemical changes were determined at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

### *Kidney function assessment*

A 24-hour urine output was collected daily in a graduated cylinder and its volume was measured. The urine

samples were subjected to the determination of volume, specific gravity, and albumin. Creatinine assessment was performed by using Creatinine Assay Kit (Diaa Sys Dianotic System, USA). In brief, 50 µl of sample or standard was mixed with sodium hydroxide (reagent 1), and incubated 5 minutes Then, each well was added 250 µl of picric acid (reagent 2), mixed and read absorbance at 490 nm after 60 and 120 seconds and regarded as A1 and A2.Both plasma and urine creatinine (mg/dL) were calculated by using the following formulas. Plasma creatinine = [(A2-A1 sample)/(A2-A1 Standard)]/[Cal x Conc. Standard/ Cal (mg/dL)] whereas urine creatinine = [(A2-A1 sample)/(A2-A1 Standard)]/[Cal x Conc. Standard/ Cal (mg/dL)] x 50.
(Conc = concentration Cal = calibrator)

Creatinine clearance (CCr) was calculated according to the following formula. CCr = urinary creatinine (UCr) (mg/ml) x urine volume (ml/kg)/creatinine in plasma (mg/ml)[12].

Albumin level was determined by using Albumin Assay Kit (Diaa Sys Dianotic System, USA). In brief, 2 µl of sample or standard was mixed with 200 µl of reagent solution (ready to used) consisting of 30 mmol/L of citrate buffer; pH = 4.2 and 0.26 mmol/L of bromocresol green. After mixing, it was incubated at room temperature for 10 minutes. An absorbance at 595 nm was recorded. Albumin (g/dL) was calculated according to the following formula. [(A sample)/(A Standard)/Cal] x [Conc. Standard/Cal (g/dL)].

Urea Assay Kit (DiaaSys Dianotic System, USA) was used for determining urea level. In brief, 5 µl of sample or standard was mixed with 100 µl of reagent 1 solution (ready to used) consisting of 150 mmol/L of TRIS; pH = 7.8, 9 mmol/L of 2-Oxoglutarate, 0.75 mmol/L of ADP, Urease and lutamate dehydrogenase. Then, the absorbance at 340 nm was recorded after the incubation at 60 and 120 seconds as A1 and A2. Then urea was calculated according to the formula below.

Urea level = [(A1-A2 sample)/(A1-A2 Standard/Cal)] x [Conc. Standard/Cal (mg/dL)].

### *Oxidative stress status assessment*

Kidney was collected and homogenized in 1 ml of 0.1 M phosphate buffer, pH 7.4, adjusted to 10% w/v. The homogenate was centrifuged at 12,000 rpm at 4°C for 30 min and the supernatant was separated for the estimation of oxidative stress status.

Malondialdehyde (MDA) level, a lipid peroxidation product, was monitored by using thiobarbituric acid reacting substances (TBARS) assay. In brief, 100 µl of sample was mixed with the solution containing 100 µl of 8.1% (w/v) sodium dodecyl sulphate, 750 µl 20% (v/v) acetic acid (pH 3.5), and 750 µl of 0.8% thiobarbituric acid (TBA). The solution was heated at 95°C for one hour and cooled immediately under running tap water. Then, 500 µl of chilled water and 2,500 µl of butanol and pyridine [15: 1 v/v] were added into each tube admixed thoroughly with vortex. Then, the solution was centrifuged at 800 x g for 20 min. The upper layer was separated and measured absorbance at 532 nm. 1,3,3-tetra ethoxy propane (TEP) was used as the reference[13]. The level of MDA was expressed as U/mg. protein.

Spectrophotometric assay of superoxide dismutase (SOD) activity was measured based on the reduction of highly water-soluble tetrazolium salts by xanthine-*xanthine oxidase*. The reaction mixture contained 20 µl of sample and 200 µl of reaction mixture consisting of 57 mM phosphate buffer solution (KH2PO4), 0.1 mM EDTA, 10 mM cytochrome C solution and 50 µM of xanthine solution and 20 µl of xanthine oxidase solution (0.90 mU/ml) were prepared at 25°C. Then, an absorbance at 415 nm was monitored. A system devoid of enzyme served as the control and three parallel experiments were conducted[14]. SOD activity was expressed as U/mg.protein.

Measurement of catalase (CAT) was performed based on an ability of the enzyme to break down $H_2O_2$. Briefly, the reaction mixture containing 50 µl of 30 mM hydrogen peroxide (in 50 mM phosphate buffer, pH 7.0), 25 µl of $H_2SO_4$ and 150 µl of $KMnO_4$ was mixed with 10 µl of sample. A system without hydrogen peroxide was served as the control. An absorbance at 490 nm was recorded. The difference in absorbance per unit time was expressed as the activity. An amount of enzyme required to decompose 1.0 M of hydrogen peroxide per minute at pH 7.0 and 25° is regarded as one unit[15]. The value of CAT activity was expressed as U/mg.protein.

Glutathione peroxidase (GPx) activity was measured by using the DTNB-GSSG reductase recycling assay with minor modifications[10]. In brief, 20 µl of sample and the reaction mixture consisting of 10 µl of dithiothreitol (DTT) in 6.67 mM potassium phosphate buffer (pH 7), 100 µl of sodium azide in 6.67 mM potassium phosphate buffer (pH 7), 10 µl of glutathione solution and 100 µl of hydrogen peroxide, were mixed thoroughly and incubated at room temperature for 5 to 10 minutes. Then, 10 µl of DTNB (5,5-dithiobis-2-nitrobenzoic acid) was added and an absorbance at 412 nm was monitored at 25°C over a period of 5 minutes. Activities were expressed as nmoles/min/mg lens protein. GPx activity was expressed as U/mg.protein.

### *Assessment of aldose reductase enzyme activity*

Aldose reductase (AR) activity was assessed via spectrophotometric method. A reaction mixture consisting of 0.7 mL of phosphate buffer (0.067 mol), 0.1 mL of NADPH ($25x10^{-5}$ mol), 0.1 mL of DL-glyceraldehyde (substrate, $5x10^{-4}$ mol) and 0.1 mL of kidney sample were prepared and adjusted pH to 6.2. After adding DL-glyceraldehyde, absorbance at 390 nm was recorded against a reference cuvette containing all other components except DL-glyceraldehyde over a 4-minute period[16]. The enzyme activity was expressed as (nmol/min/mg).

### *Endothelial nitric oxide synthase (eNOS) , NF-kappa B p65 assessments*

Endothelial nitric oxide synthase (eNOS), an indirect indicator of nitric oxide, was determined by using colorimetric method of Abcam®. Antibody cocktail was freshly prepared by combining 3 ml of rat nitric oxide synthase 1 lyophilized

capture antibody and 3 ml of 10x Nitric oxide synthase 1 detector antibody. Then, an aliquot of the solution at the volume of 50 ml was added to the 96 well plate strips, sealed and incubated for 1 hour at room temperature with shaking at a rate of 400 round per minute (rpm). At the end of incubation, each well was washed 3 times with 350 ml 1x wash buffer. After the last wash, the plate was inverted and blot against clean paper towels to remove excess liquid. Then, 100 ml of TMB substrate was added and incubated in dark room on plate shaker which was set at 400 rpm for 15 minutes. After the incubation, 100 ml of stop solution were added to each well. The solution was shaken on plated shaker for 1 minute to provide thoroughly mixing and absorbance at 450 nm was recorded.

NF-kappa B p65was assessed with the similar protocol except that the antibody cocktail was prepared by combining 3 ml of 3 ml of 10x NF-kappa B p65 (total) capture antibody and 3 ml of 10x NF-kappa B p65 (total) detector antibody.

### *Assessment of vascular endothelial growth factor (VEGF)*

The determination of VEGF was also performed by colorimetric method of Abcam®. Aliquots of standards and samples at the volume of 100 ml each were added to a 96 well plate strips, sealed and incubated overnight at 4°C with gentle shaking. After the incubation, the solution of each well was discarded and washed 4 times with 300 µl 1x wash buffer. Then, the plate was inverted and blot against clean paper towels to remove excess liquid. Aliquot of 1x biotinylated VEGF detection antibody at the volume of 100 µl was added to each well and incubated at room temperature with gentle shaking for 1 hour. After the incubation, the solution was discarded and the wash was repeated. Following this process, 100 µl of 1x HRP-Streptavidin solution was added and incubated at room temperature in the dark with gentle shaking for 30 minutes. Then, 50 µl of stop solution was added and the optical density at 450 nm was read immediately.

### *Histomorphology evaluation*

Kidneys were removed after the scarification. Tissues were fixed in 10% buffered formalin, and embedded in paraffin. Sections at 5 mm thick were prepared, stained with Hematoxylin and Eosin (H&E), examined under light microscope and assessed histologically. Mean values of Bowman capsule's area, glomerulus tuft area and Bowman space area were measured in 10 non-overlapping fields for each group.

### *Statistical analysis*

All data were presented as mean ± standard error mean (mean ± SEM). The analysis of data was performed by using one-way analysis of variance (ANOVA) followed by the *post hoc* test of LSD via SPSS version 15. Statistical differences were considered at $p$-value <0.05.

## Results

### *Acute toxicity*

Data obtained from acute toxicity of the combined extract of mango seed and aerial parts of Vietnamese coriander were shown in Table 1 to 3 and Figure 1. Our data showed no evidence of toxicity was observed in rats which were administered the combined extract of mango seed and aerial parts of Vietnamese coriander up to 5,000 $g.kg^{-1}$ BW.

### *Effect of MPO on body weight and kidney changes*

Effect on MPO on body weight and kidney was shown in Table 4. Diabetic rats showed the significant reduction of bodyweight but increased kidney weight, urine volume, creatinine clearance and albumin in urine ($p$-value <0.001, 0.001, 0.001, 0.05 and 0.001, respectively; compared to control group. No significant change in urine specific gravity was observed. All doses of MPO failed to restore body weight, kidney weight and urine volume in diabetic rat. It was found that low dose of MPO significantly decreased creatinine

**Table 1.** Effect MPO on neurobehaviors and toxicity signs

| Observed parameters | 1st hr | 2nd hr | 4th hr | 6th hr | D2-D14 |
|---|---|---|---|---|---|
| Alertness | N | N | N | N | N |
| Grooming | A | A | A | A | A |
| Hyperactivity | A | A | A | A | A |
| Tremors | A | A | A | A | A |
| Convulsion | A | A | A | A | A |
| Salivation | N | N | N | N | N |
| Fur | N | N | N | N | N |
| Eye | N | N | N | N | N |
| Diarrhea | A | A | A | A | A |
| Lethargy | A | A | A | A | A |
| Sleep and coma | N | N | N | N | N |
| Injury | A | A | A | A | A |
| Pain response | A | A | A | A | A |
| Signs of illness | A | A | A | A | A |

**Table 2.** Effect of MPO on organs weight. Data are expressed as mean ± SEM

| Organs (g)<br>Relative organ weight (%) | Male | | Female | |
|---|---|---|---|---|
| | Control | MPO (5 g.kg$^{-1}$ BW) | Control | MPO (5 g.kg$^{-1}$ BW) |
| Body weight | 333.50±2.92 | 325.33±2.32 | 218.33±1.20 | 227.33±4.12 |
| Brain | 2.14±0.03 | 2.04±0.04 | 1.87±0.03 | 1.91±0.03 |
| Liver | 10.31±0.28 | 9.14±0.22 | 6.68±0.27 | 6.692±0.41 |
| Lung | 1.86±0.06 | 1.65±0.11 | 1.22±0.06 | 1.33±0.05 |
| Heart | 1.14±0.02 | 1.11±0.03 | 0.78±0.01 | 0.83±0.04 |
| Kidney | | | | |
| Right side | 1.20±0.03 | 1.11±0.04 | 0.82±0.05 | 0.78±0.02 |
| Left side | 1.01±0.18 | 1.11±0.04 | 0.80±0.05 | 0.78±0.02 |
| Spleen | 0.81±0.04 | 0.83±0.04 | 0.65±0.03 | 0.74±0.05 |
| Pancreas | 1.61±0.11 | 1.27±0.17 | 1.30±0.09 | 1.27±0.15 |
| Testis/Ovary | | | | |
| Right side | 1.73±0.04 | 1.70±0.06 | 0.16±0.01 | 0.19±0.02 |
| Left side | 1.73±0.04 | 1.68±0.07 | 0.14±0.01 | 0.18±0.02 |
| Thymus | 0.66±0.04 | 0.66±0.14 | 0.40±0.02 | 0.41±0.03 |
| Salivary gland | | | | |
| Right side | 0.16±0.01 | 0.11±0.01 | 0.12±0.01 | 0.13±0.01 |
| Left side | 0.16±0.01 | 0.13±0.02 | 0.11±0.01 | 0.10±0.01 |
| Adrenal gland | | | | |
| Right side | 0.09±0.01 | 0.10±0.02 | 0.08±0.00 | 0.07±0.01 |
| Left side | 0.12±0.04 | 0.08±0.00 | 0.07±0.00 | 0.09±0.01 |
| Stomach | 2.39±0.25 | 1.90±0.18 | 1.68±0.10 | 1.50±0.05 |
| Urinary bladder | 0.10±0.04 | 0.17±0.02 | 0.09±0.00 | 0.09±0.01 |
| Intestinal | 9.26±0.53 | 8.07±0.71 | 6.35±1.08 | 6.64±0.23 |
| Food intake rate (g) | 143.50±0.77 | 133.50±2.47 | 81.93±0.71 | 96.07±2.13 |
| Water intake rate (mL) | 342.93±3.79 | 304.14±3.65 | 186.31±2.24 | 217.36±3.25 |

**Table 3.** Effect of MPO on hematological and biochemical parameters. Data are expressed as mean ± SEM

| Hematological parameters | Male | | Female | |
|---|---|---|---|---|
| | Control | MPO ($5\ g.kg^{-1}$ BW) | Control | MPO ($5\ g.kg^{-1}$ BW) |
| Red blood cell ($10^6$/uL) | 7.24±0.21 | 6.37±0.79 | 7.19±0.32 | 7.60±0.15 |
| Haemoglobin (g/dL) | 14.23±0.48 | 13.56±0.56 | 13.83±0.61 | 14.73±0.24 |
| Hematocrit (%) | 42.28±1.53 | 37.08±4.46 | 39.95±1.89 | 42.98±0.58 |
| White blood cells ($10^3$/Ul) | 4.20±0.55 | 3.02±0.41 | 4.35±0.68 | 2.03±0.23 |
| Platelet count ($10^3$/uL) | 762.00±66.10 | 894.00±67.30 | 803.00±6.36 | 800.50±8.50 |
| Mean platelet volume (fL) | 5.25±0.06 | 5.30±0.15 | 5.38±0.09 | 5.63±0.10 |
| Neutrophils (%) | 10.25±4.02 | 11.36±4.58 | 19.25±3.81 | 13.15±3.63 |
| Lymphocytes (%) | 84.55±5.69 | 86.94±4.96 | 78.65±4.18 | 77.03±6.25 |
| Monocytes (%) | 2.63±2.13 | 0.36±0.17 | 0.48±0.32 | 1.13±0.43 |
| Eosinophil (%) | 0.18±0.14 | 0.56±0.14 | 0.53±0.22 | 0.50±0.21 |
| Basophil (%) | 2.40±1.40 | 0.78±0.47 | 1.10±0.63 | 1.45±0.88 |
| Mean orpuscular volume (fL) | 58.40±0.69 | 58.34±0.52 | 55.45±0.61 | 56.55±0.47 |
| Mean corpuscular Hemoglobin (pg) | 19.68±0.25 | 22.82±3.12 | 19.25±0.27 | 19.35±0.23 |
| Mean corpuscular Hemoglobin concentration (g/dL) | 33.65±0.10 | 39.04±5.09 | 34.63±0.20 | 34.23±0.17 |
| Red blood cell distribution width (%) | 13.40±0.19 | 13.00±0.34 | 13.58±0.31 | 13.85±0.30 |
| Blood biochemical parameters | | | | |
| Albumin (g/dL) | 4.38±0.14 | 4.06±0.19 | 4.34±0.13 | 3.87±0.06 |
| ALAT (IU/L) | 10.79±0.70 | 11.34±1.01 | 9.66±0.50 | 10.74±0.27 |
| ASAT (IU/L) | 9.56±0.52 | 10.18±1.19 | 8.67±0.44 | 8.77±0.33 |
| Bilirubin (mg/dL) | 0.27±0.12 | 0.41±0.05 | 0.21±0.01 | 0.33±0.00 |
| CK-MB (U/L) | 0.37±0.01 | 0.40±0.05 | 0.35±0.02 | 0.35±0.01 |
| Creatinine (mg/dL) | 0.71±0.03 | 0.58±0.04 | 0.60□0.04 | 0.52±0.02 |
| HDLC (mg/dL) | 44.5±33.39 | 53.09±5.66 | 60.68±1.46 | 65.57±2.17 |
| LDH (U/I) | 290.16±8.04 | 292.03±18.00 | 252.68±17.47 | 289.31±7.03 |
| LDLC (mg/dL) | 55.69±7.20 | 67.22±18.85 | 44.56±14.42 | 43.33±0.33 |
| Urea (mg/dL) | 11.00±1.32 | 17.81±1.17 | 10.58±1.16 | 8.66±1.11 |

clearance ($p$-value <0.01; compared to DM + vehicle) while medium and high doses of MPO produced the significant reduction in albumin in urine ($p$-value <0.001 all; compared to control group). No significant change in urine specific gravity was observed in diabetic rats which received all doses of MPO.

The current data also demonstrated that diabetic rats showed the thickening of basement membrane, focal scaring of glomerulus, damages of proximal and distal tube and expansion of mesangial cells as shown in Figure 2. In addition, it was found that an area of glomerular tuft and Bowman space were also increased as shown in Figure 3 and Figure 4. The increase in glomerular tuft was attenuated in diabetic rats which received MPO at high dose ($p$-value <0.05; compared to DM + vehicle) whereas the increase in Bowman space was mitigated by all doses of MPO used in this study ($p$-value <0.001; compared to DM + vehicle).

### *Effect of MPO on vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS)*

Since glomerular filtration was also under the influences of vascular endothelial growth factor (VEGF) and nitric oxide, we also determined the effect of MPO on the alterations of VEGF and endothelial nitric oxide synthase (eNOS) in kidney and results were shown in Figure 5 and Figure 6. Diabetic rats which received vehicle showed the increase in VEGF but no significant change was observed. All doses of MPO also failed to induce the significant change in VEGF in kidney. It was found that diabetic rats which received vehicle showed the reduction of eNOS ($p$-value <0.05; compared to control group). Interestingly, the medium dose of MPO could counteract this change in diabetic rats ($p$-value <0.05; compared to DM + vehicle).

### *Effect of MPO on aldose reductase, oxidative stress status and NF-kB in kidney*

Figure 7 showed the effect of MPO on aldose reductase activity in kidney. Although the diabetic rats showed the increase in aldose reductase activity in kidney but no significant change was observed. All doses of MPO also failed to produce the significant change of this parameter in diabetic rats. The effect of MPO on oxidative stress status of kidney was shown in Figure 7 to 10. Diabetic rats showed the reduction of SOD, CAT and GPx ($p$-value <0.05 all; compared to control group) but increased MDA level ($p$-value <0.01; compared to control group). Medium dose of MPO could increase SOD, CAT and GPx but decreased MDA level in diabetic rats ($p$-value <0.001, 0.05, 0.05 and

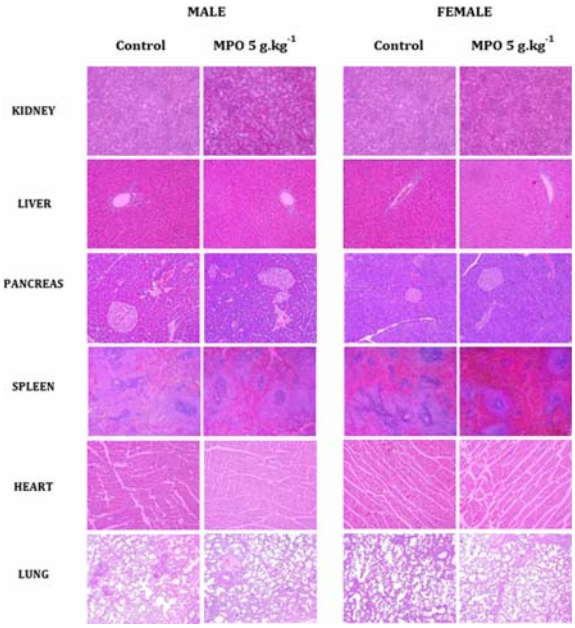


**Figure 1.** Histomorphological analysis of various organs of rats treated with single dose of MPO at dose of 5,000 mg.kg$^{-1}$ BW observed at 14 days after treatment.

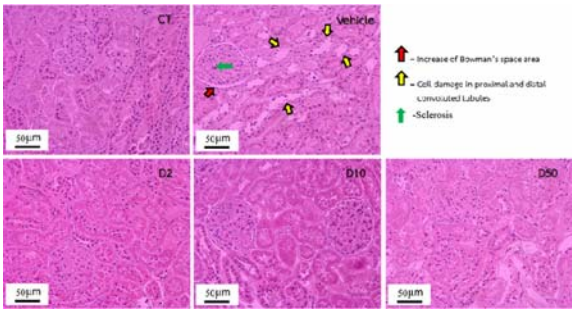


**Figure 2.** Photomicrograph of kidney subjected to hematoxylin & Eosin staining under a light microscope at 40x magnification. CT: control group, Vehicle: STZ-diabetic group + vehicle, D2: STZ-diabetic group + MPO at dose of 2 mg.kg$^{-1}$ BW, D10: STZ-diabetic group + MPO at dose of 2 mg.kg$^{-1}$ BW and D50: STZ-diabetic group + MPO at dose of 50 mg.kg$^{-1}$ BW.

0.05, respectively; compared to DM + vehicle). Only the high dose of MPO significantly increased SOD activity in diabetic rats ($p$-value <0.01; compared to DM + vehicle).

The effect of MPO on nuclear factor kappa-B (NF-kB) in kidney was also investigated and result was shown in Figure 11. It was found that diabetic rats showed the increase

**Table 4.** Effect of MPO on body weight and kidney changes including kidney weight, urine volume, urine sgpecificity, creatinine clearance and albumin in urine. Data are presented as mean ± SEM (n = 6/group). $^{###}p$-value <0.001; compared to control $^{**}p$-value <0.01; compared to DM + vehicle

| Group | Body weight (g) | % relative kidney weight | Urine volume (ml/24 hr) | Urine specific gravity | Creatinine Clearance (mL/min/1.73 m$^2$) | Albumin in urea |
|---|---|---|---|---|---|---|
| Control | 471.83±13.86 | 0.536±0.015 | 18.17±3.91 | 1.3470±0.00276 | 0.010±0.001 | 2.982±0.03 |
| DM+Vehicle | 225.17±8.70$^{###}$ | 1.462±0.040$^{###}$ | 81.92±2.45$^{###}$ | 1.3472±0.00032 | 0.002±0.000$^{\#}$ | 3.104±0.03$^{###}$ |
| DM+MPO (2 mg/kg.BW$^{-1}$) | 217.87±10.01$^{###}$ | 1.474±0.045$^{###}$ | 79.64±12.67$^{###}$ | 1.3463±0.00062 | 0.008±0.002$^{**}$ | 3.087±0.02 |
| DM+MPO (10 mg/kg.BW$^{-1}$) | 220.15±7.51$^{###}$ | 1.546±0.035$^{###}$ | 85.00±1.12$^{###}$ | 1.3460±0.00034 | 0.005±0.001 | 2.859±0.01$^{***}$ |
| DM+MPO (50 mg/kg.BW$^{-1}$) | 199.73±9.61$^{###}$ | 1.836±0.055$^{###}$ | 81.86±1.74$^{###}$ | 1.3467±0.00035 | 0.003±0.001 | 2.990±0.02$^{***}$ |

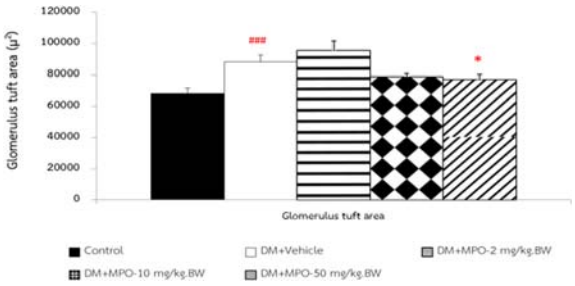


**Figure 3.** Effect of MPO on a glomerular tuft area. Data are expressed as mean ± SEM. ###*p*-value <0.001; compared to control. **p*-value <0.05; compared to DM + vehicle.

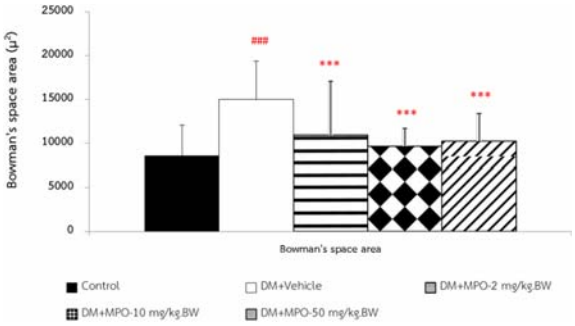


**Figure 4.** Effect of MPO on a Bowman's capsule space area. Data are expressed as mean ± SEM. ###*p*-value <0.001; compared to control. ***p*-value <0.01; compared to DM + vehicle.

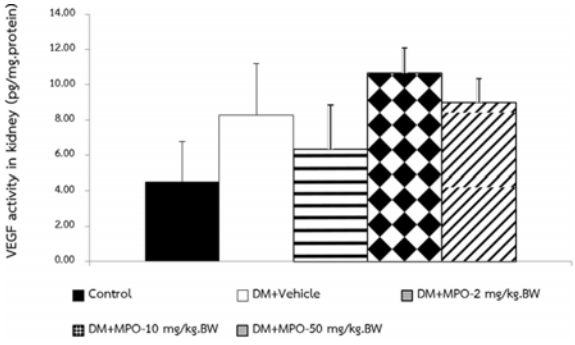


**Figure 5.** Effect of MPO on vascular endothelial growth factor (VEGF) activity in kidney. Data are expressed as mean ± SEM.

in NF-kB (*p*-value <0.001; compared to control group). This change was counteracted by the medium and high doses of MPO.

## Discussion

Acute toxicity analysis is recognized as the

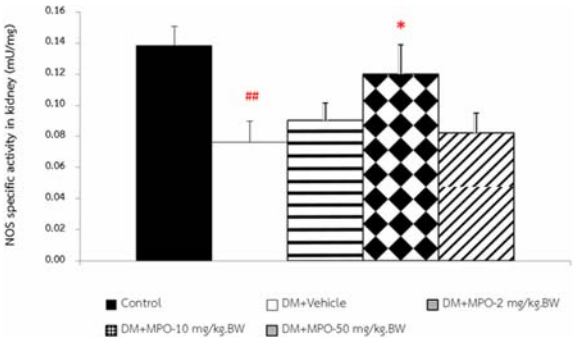


**Figure 6.** Effect of MPO on nitric oxide synthase (NOS) activity in kidney. Data are expressed as mean ± SEM. ##*p*-value <0.01; compared to control. **p*-value <0.05; compared to DM + vehicle.

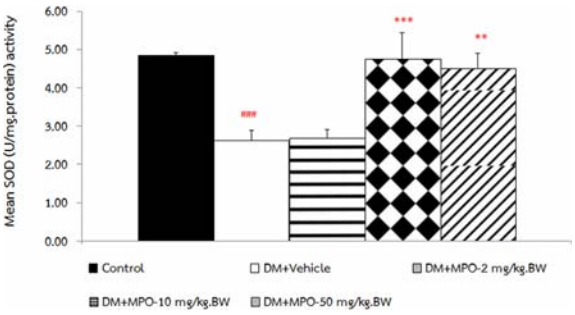


**Figure 7.** Effect of MPO on superoxide dismutase (SOD) enzyme activity in kidney. Data are expressed as mean ± SEM. ###*p*-value <0.001; compared to control. **,***p*-value <0.01 and 0.001 respectively; compared to DM + vehicle.

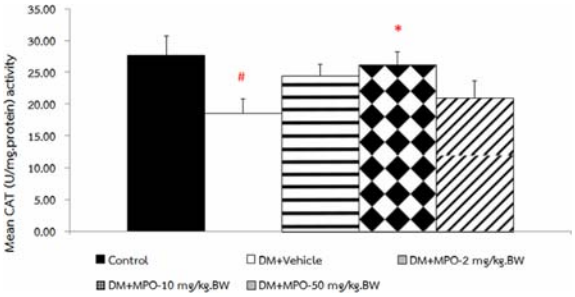


**Figure 8.** Effect of MPO on catalase (CAT) enzyme activity in kidney. Data are expressed as mean ± SEM. #*p*-value <0.05; compared to control. **p*-value <0.05; compared to DM + vehicle.

important toll for assessing the consumption safety of food, herb, chemical ingredients and food additive. Our data showed

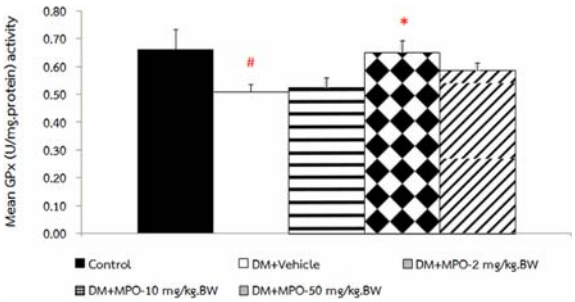


**Figure 9.** Effect of MPO on glutathione peroxidase (GPx) enzyme activity in kidney. Data are expressed as mean ± SEM. $^{\#}p$-value <0.05; compared to control. *$p$-value <0.05; compared to DM + vehicle.

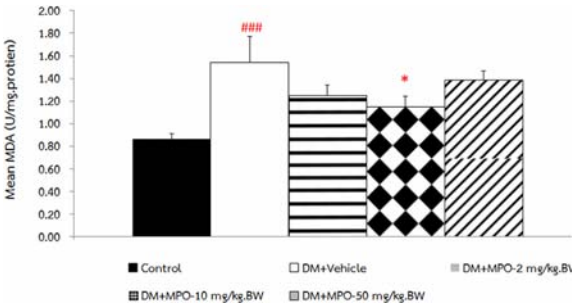


**Figure 10.** Effect of MPO on malondialdehyde (MDA) level in kidney. Data are expressed as mean ± SEM. $^{\#\#\#}p$-value <0.001; compared to control. *$p$-value <0.05; compared to DM + vehicle.

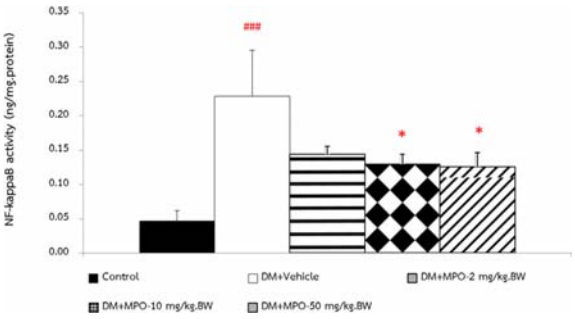


**Figure 11.** Effect of MPO on nuclear factor kappa-B (NF-kB) in kidney. Data are expressed as mean ± SEM. $^{\#\#\#}p$-value <0.001; compared to control. *$p$-value <0.05; compared to DM + vehicle.

that no mortality and toxicity signs were observed in rats treated with MPO at dose of 5000 mg.kg$^{-1}$ BW throughout the study period. This suggested that MPO was well tolerated and safe. LD50 of MPO was more than 5,000 mg.kg$^{-1}$ BW.

Diabetic rats showed the increased kidney weight which reflected renal toxicity, tubular hypertrophy or chronic nephropathy[17] induced by STZ[18]. The increased glomerular tuft area which indicated glomerular hypertrophy was also observed. The glomerular hypertrophy appeared to occur as the result of expansion of mesangial matrix and thickening of basement membrane[19]. Although MPO at doses used in this study could decrease glomerular basement membrane thickness and the expansion of mesangial matrix the degree of changes might not be enough to produce a significant reduction in kidney weight.

The present study clearly demonstrated that diabetic rats showed a high blood glucose level and the reduction of creatinine clearance or glomerular filtration rate (GFR) which is in agreement with the previous study[20]. The thickening of basement membrane, focal scaring of glomerulus, and expansion of mesangial cells were also observed. It has been reported that glomerular basement membrane plays an important role on filtration[21] and there is an association between albuminurea and thickening of basement membrane[22]. Under normal condition, the small amount of albumin which escaped the healthy glomerulus was captured by the brush border. However, our data revealed that diabetic rats showed the damages of proximal tubule and distal tubule. Therefore, the presence of albuminurea might occur partly as the result of thickening of basement membrane and the damage of proximal tubule. Interestingly, albumiurea was mitigated by MPO at doses of 10 and 50 mg.kg$^{-1}$ BW. The possible underlying mechanism might be associated with the reduction of basement membrane thickness and the reduction of proximal tubule damage.

Our data also demonstrated the reduction of GFR which was previously reported to be associated with the formation of scar in glomerulus or glomerulosclerosis and expansion of mesangial cells[22]. In addition, GFR is maintained by ultrafiltration, which is defined by glomerular capillary pressure, plasma osmotic pressure, and Bowman capsule pressure[23,24]. The enlarged Bowman's space area observed in diabetic rats might induce the elevation of hydrostatic pressure in Bowman space which in turn decreased glomerular filtration rate. Based on aforementioned information, we did suggest that the effect of MPO at dose of 2 mg.kg$^{-1}$ BW to improve the reduction in GFR in diabetic rats might be partly associated with the improvement of glomerulosclerosis, the expansion of mesangial cells, and Bowman's space area. However, no closed relationship between the morphology changes mentioned earlier and the GFR was observed because the regulation of GFR involved multifactor. Not only the morphology changes of kidney but also the alterations of endothelial response also play the pivotal role on the regulation of GFR[25]. Many substances which can modify the vasoconstriction process of afferent and efferent arteries can also modify GFR. Therefore, the authors also determined the effect of MPO on vascular endothelial growth factor (VEGF) in kidney. VEGF plays an important role on neoangiogenesis promotion during an early phase of diabetic

nephropathy[26] so our study which was not in early stage failed to show the significant change of this parameter. In addition to VEGF, nitric oxide also contributes a role in the filtration process[27,28]. However, the present study failed to show a significant change of endothelial nitric oxide synthase (eNOS) which served as an indirect indicator of endothelial nitric oxide. Therefore, both VEGF and eNOS were less likely to play a pivotal role on the improvement of GFR induced by MPO especially at low doses.

The current data also revealed that STZ-diabetic rats decreased antioxidant enzyme activity giving rise to an elevation of oxidative stress which in turn attacked various components of cell including membrane leading to renal tubule damage. In addition, the elevation of oxidative stress can also increase NF-kB and enhanced damage of renal tubule. Moreover, oxidative stress also decreased NO bioavailability and enhanced the formation and stimulation of vasoconstrictive mediators resulting in the reduction of glomerular filtration[29]. MPO could enhance antioxidant enzymes giving rise to the reduction of oxidative stress status reflected by the reduction of MDA level. In addition, a reduction of oxidative stress also decreased NF-kB and improved renal tubule damage and glomerulosclerosis leading to the improvement in albuminurea and GFR. However, the effect of MPO on vascular response and the changes of vasoconstrictive agent still cannot be omitted but required further studies.

## Conclusion

The present study clearly has demonstrated that LD50 of MPO is more than 5,000 $mg.kg^{-1}$ BW so it is practically safe and can serve as the potential ingredient to prevent diabetic nephropathy. The possible underlying mechanism may involve the reduction of oxidative stress and inflammatory mediator giving rise to the reduction of damages of glomerular and renal tubules. The improvement of kidney structures in turn improves GFR and albuminurea. However, clinical trial study is essential to confirm the renoprotective effect of MPO.

## What is already known on this topic?

Antioxidants such as vitamin E, probucol, alpha-lipoic acid, or taurine could restore renal and glomerular functions for diabetic nephropathy. The hydro-alcoholic extract of seed of *Mangifera indica* or mango var. Namdokmai and aerial part of *Polygonum odoratum* or Vietnamese coriander (MPO) possess potent antioxidant activity.

## What this study adds?

The present study presents LD50 of MPO as more than 5,000 $mg.kg^{-1}$ BW so it is practically safe and can serve as the potential ingredient to prevent diabetic nephropathy.


## Acknowledgements

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## Data availability

All the data (tables and figures) used to support the findings of this study are included within the article and the detail will be provided on request due to the registration of petty patent and the technology transfer agreement.

## Potential conflicts of interest

The authors declare no conflict of interest.

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