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The modulatory effect of Moringa oleifera leaf extract on endogenous antioxidant systems and inflammatory markers in acetaminophen-induced nephrotoxic mice model

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N-Acetyl-p-Aminophenol (APAP), aka acetaminophen, is the most commonly used over-the counter analgesic and antipyretic medication. However, its overdose leads to both liver and kidney damage. APAP-induced toxicity is considered as one of the primary causes of acute liver failure; numerous scientific reports have focused majorly on APAP hepatotoxicity. Alternatively, not many works approach APAP nephrotoxicity focusing on both its mechanisms of action and therapeutic exploration. *Moringa oleifera* (MO) is pervasive in nature and is reported to possess surplus amount of nutrients, and is enriched with several bioactive candidates including trace elements that act as curatives for various clinical conditions. In this study, we evaluated the nephro-protective potential of MO leaf extract against APAP nephrotoxicity in male Balb/c mice. A single-dose acute oral toxicity design was implemented in this study. Group 2, 3, 4 and 5 received a toxic dose of APAP (400 mg/kg of bw, i.p) and after an hour, these groups were administered with saline (10 mL/kg), silymarin - positive control (100 mg/kg of bw, i.p), MO leaf extract (100 mg/kg of bw, i.p), and MO leaf extract (200 mg/kg bw, i.p) respectively. Group 1 was administered saline (10 mL/kg) during both the sessions. APAP-treated mice exhibited a significant elevation of serum creatinine, blood urea nitrogen, sodium, potassium and chloride levels. A remarkable depletion of antioxidant enzymes such as SOD, CAT and GSH-Px with elevated MDA levels has been observed in APAP treated kidney tissues. They also exhibited a significant raise in pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and decreased anti-inflammatory (IL-10) cytokine level in the kidney tissues. Disorganized glomerulus and dilated tubules with inflammatory cell infiltration was clearly observed in the histology of APAP treated mice kidneys. All these pathological changes were reversed in a dose dependent manner after MO leaf extract treatment. Therefore, MO leaf extract has demonstrated some therapeutic effectiveness against APAP-induced nephrotoxicity through enhancement of endogenous antioxidant system and modulatory effect on specific inflammatory cytokines in kidney tissues.

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THE MODULATORY EFFECT OF *MORINGA OLEIFERA* LEAF EXTRACT ON ENDOGENOUS ANTIOXIDANT SYSTEMS AND INFLAMMATORY MARKERS IN ACETAMINOPHEN-INDUCED NEPHROTOXIC MICE MODEL

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Short Title*: Nephroprotective role of Moringa leaves*

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ABSTRACT

N-Acetyl-p-Aminophenol (APAP), aka acetaminophen, is the most commonly used over-the counter analgesic and antipyretic medication. However, its overdose leads to both liver and kidney damage. APAP-induced toxicity is considered as one of the primary causes of acute liver failure; numerous scientific reports have focused majorly on APAP hepatotoxicity. Alternatively, not many works approach APAP nephrotoxicity focusing on both its mechanisms of action and therapeutic exploration. *Moringa oleifera* (MO) is pervasive in nature and is reported to possess surplus amount of nutrients, and is enriched with several bioactive candidates including trace elements that act as curatives for various clinical conditions. In this study, we evaluated the nephro-protective potential of MO leaf extract against APAP nephrotoxicity in male Balb/c mice. A single-dose acute oral toxicity design was implemented in this study. Group 2, 3, 4 and 5 received a toxic dose of APAP (400 mg/kg of bw, i.p) and after an hour, these groups were administered with saline (10 mL/kg), silymarin - positive control (100 mg/kg of bw, i.p), MO leaf extract (100 mg/kg of bw, i.p), and MO leaf extract (200 mg/kg bw, i.p) respectively. Group 1 was administered saline (10 mL/kg) during both the sessions. APAP-treated mice exhibited a significant elevation of serum creatinine, blood urea nitrogen, sodium, potassium and chloride levels. A remarkable depletion of antioxidant enzymes such as SOD, CAT and GSH-Px with elevated MDA levels has been observed in APAP treated kidney tissues. They also exhibited a significant raise in pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and decreased anti-inflammatory (IL-10) cytokine level in the kidney tissues. Disorganized glomerulus and dilated tubules with inflammatory cell infiltration was clearly observed in the histology of APAP treated mice kidneys. All these pathological changes were reversed in a dose dependent manner after MO leaf extract treatment. Therefore, MO leaf extract has demonstrated some therapeutic

effectiveness against APAP-induced nephrotoxicity through enhancement of endogenous antioxidant system and modulatory effect on specific inflammatory cytokines in kidney tissues.

Abbreviations: **MO** - *Moringa oliefera*; **APAP** - acetaminophen; **GSH-Px**- glutathione peroxidase; **SOD** - superoxide dismutase; **CAT** - catalase; **MDA** - Malondialdehyde; **NAPQI** - N-acetyl-p-benzoquinoneimine; **TNF-α -** Tumor necrosis factor- α; **IL –** Interleukin; **NAC**-N-acetylcysteine.

1. INTRODUCTION

Acetaminophen (APAP) is a readily available over-the-counter medication as an effective painkiller and fever suppressor. APAP retains a virtuous safety profile at therapeutic doses. However, when its therapeutic index is breached, it results in acute / chronic hepato-renal damage in both human and experimental animals (Ghosh et al. 2010, Karthivashan et al. 2015a, Karthivashan et al. 2015b). Though the incident rate of APAP hepatotoxicity is higher than the renal toxicity, the latter leads to 1-2 % of acute renal failure in patients with APAP overdose and can be fatal (Eguia & Materson 1997). The pathophysiology of APAP-induced nephrotoxicity is not much explored compared to APAP hepatotoxicity. Based on previous literature they both allegedly expressed a similar kind of pathophysiology, yet some subtle differences were observed, and remain indistinct (Li et al. 2003, Cekmen et al. 2009, Aycan et al. 2015).

The most probable mechanism of APAP nephrotoxicity involves the metabolic activation of the reactive toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI). At therapeutic doses, only a few percent of APAP gets converted to the reactive toxic metabolite NAPQI, which is further reduced by glutathione and subsequently excreted as glucuronidated and sulfated (non-toxic) hydrophilic metabolites through the renal system. In an APAP overdose, the supply of sulfate and glutathione get exhausted, thus more NAPQI is generated via CYP450 metabolism. This electrophilic intermediary binds with available cellular proteins and initiate lipid peroxidation, mediated reactive oxygen species (ROS) and other free radical formation, thereby inducing oxidative stress and inflicting renal tissue damage (Isik et al. 2006, Ahmad et al. 2012). This cascade furthermore provokes inflammatory signals and extended the injury, resulting in tubular cell-death / acute renal failure (Möller-Hartmann & Siegers 1991). Due to its fatal nature, the requirement of an antidote / therapeutic agent against APAP renal toxicity becomes crucial.

N-acetylcysteine (NAC), a precursor of GSH, is well known for its hepato-protective nature against APAP-induced hepatotoxicity in both humans and animals; however, it has a limited function towards APAP-induced renal toxicity (Eguia & Materson 1997, Mazer & Perrone 2008). Thus, the hunt for alternative, safe and therapeutically effective compounds against APAP-induced renal toxicity is essential.

Moringa oleifera Lam (MO) is a wide-spread tropical and subtropical species belongs to Moringaceae family. It is well known for its remarkable nutritional value and elite therapeutic potential against extensive clinical conditions. *Moringa oleifera* Lam is commonly known as "drumstick tree" or "horseradish tree" and almost all parts of this plant, including the root, bark, stem, leaves, flowers and pods possess huge amounts of micro- and macronutrients. It provides, both animal and human nutritional supplements (Siddhuraju & Becker 2003, Anwar et al. 2007). It possesses a rich and rare combination of therapeutically-active candidates such as kaempferol, rhamnetin, quercitin, chlorogenic acid, rutin, and apigenin, and is also enriched with an exogenous supply of ascorbic acid and carotenoids, which are renowned antioxidant candidates (Anwar et al. 2007, Karthivashan et al. 2013). MO has been utilized for ages as traditional medicine in the treatment of numerous disorders as an antiseptic, anti-diabetic, antiepileptic, antiparalytic, antiviral, anti-inflammatory effect. Additionally, numerous scientific reports on various parts of the plant have reported on its medicinal value, among which its leaves has been extensively studied in a wide variety of clinical conditions for antimicrobial, anti-inflammatory, anti-cancer, and anti-diabetic effects (Anwar et al. 2007). Our research team has recently identified that flavonoids such as kaempferol, quercitin and apigenin were likely involved in the enhanced antioxidant effect of MO leaves extract, and further established its hepatoprotective

mechanism of action against APAP-induced hepatotoxicity (Karthivashan et al. 2015b, Karthivashan et al. 2013).

In, recent years, it has been established that the existence of trace elements in MO leaf extract also contributes to improvising human health and combating various health disorders (Gowrishankar et al. 2010, Prashanth et al. 2015). Thus, in this study, we evaluated several essential / non-essential trace elements of MO leaf extract to investigate their possible involvement against APAP toxicity. The pathophysiology of APAP-induced hepatotoxicity is proposed to be similar to that of APAP nephrotoxicity; thus, here we extended our investigation on the potential nephro-protective mechanism of MO leaf extract against APAP-induced nephrotoxicity. Furthermore, silymarin has been selected as the positive control for this study, based on previous study reports due to its enhanced hepato- and renal-protective properties against APAP toxicity in mice due to its enriched antioxidative and anti-inflammatory nature (He et al. 2004, Bektur et al. 2013). This would pave way for further investigation on the advancement of MO leaf extract as an effective therapy for both APAP-induced nephro- and hepato-toxicity in the field of clinical / translational medication.

2. MATERIALS AND METHODS

2.1 Chemicals

Acetaminophen and silymarin were procured from Sigma (St. Louis, MO, USA). All kidney function markers kits, malondialdehyde (MDA) and antioxidant enzyme assay kits were purchased from Roche Diagnostics (Germany), Biovision Research kits (CA, USA) and Cayman chemical company (Ann Arbor, MI, USA) respectively. HEPES buffer was obtained from Nacalai Tesque (Kyoto, Japan). Porcelain crucible, analytical balance (OHAUS, made in

136 Switzerland), oven (Genlab, UK), type 1500 furnace, desiccators, and Solaar M atomic
137 absorption spectrometer (AAS) (Thermo Elemental, USA) were used for AAS analysis. All
138 glassware used was rinsed and soaked in 10 % (v/v) HNO_3 overnight. They were rinsed with de-
139 ionized water and dried before use. All other chemicals and reagents used were obtained from
140 Sigma (St. Louis, MO, USA) unless indicated otherwise

141 **2.2 Plant materials**

142 Fresh mature leaves from the *Moringa oliefera* tree were harvested from Garden-2, Universiti
143 Putra Malaysia (UPM) and have been confirmed similar to the voucher specimen (SK 1561/08)
144 previously deposited in the Institute of Bioscience, UPM (IBS) Herbarium unit. The whole plant
145 leaves were collected, washed in running tap water, air dried at room temperature (24 °C) for a
146 day and oven dried for two consecutive days at 45 °C. The dried plant material was ground using
147 a mechanical blender and stored in an airtight container after processing.

148 **2.3 Preparation of leaf extract**

149 The *Moringa oleifera* leaf powder was macerated exhaustively with 90% ethanol (ethanol:
150 distilled water, 90:10) in aspirator bottle for 3 consecutive days at room temperature with
151 continuous shaking. The residue was strained and the filtrate was condensed using a rotary
152 evaporator at 40 °C. The condensed residue was of slurry nature and dark green in color, which
153 were further freeze-dried. The obtained freeze-dried extracts were weighed, kept in a capped
154 container, labeled appropriately and stored at -20 °C.

155 **2.4 Preliminary analysis of trace elements**

156 **2.4.1 Sample digestion - Dry ashing method**

157 One gram of MO leaf extract was placed in a porcelain crucible in a furnace. The furnace
158 temperature was steadily increased from room temperature to 350 ˚C. The sample turned to ash

after 4 hours and the process continued until whitish grey ash residue was attained. The residue was dissolved in 5 ml of nitric oxide and increased to 10 mL volume in appropriate volumetric flask.

2.4.2 Determination of trace elements

In this study, we evaluated three essential trace elements; Copper (Cu), Manganese (Mn), Nickel (Ni), and three toxic trace elements; Cromium (Cr), Lead (Pb) and Cadmium (Cd) present in the MO leaf extract. Working standard solutions of appropriate elements were prepared from stock standard solution (1000 mg/L) and absorbance values were obtained for various working standards for each element in the samples, using an atomic absorption spectrometer (AAS). The absorbance values were plotted against concentration, whereby the formed linear calibration curves revealed the actual concentration of the sample. A blank reading was also taken and essential correction was made during the calculation of concentration of various elements.

2.5 Animals and experimental design

Male Balb/c mice of 25 - 30 g weight (10-12 weeks old) were handled at the Animal House Unit, Faculty of Medicine, Universiti Putra Malaysia (UPM). Animals were acclimatized for a week at 26 ± 2 °C with a 12 h light/dark cycle. Free access to food and water was allowed at all times. During the acclimatization period, five mice were housed per cage in plastic cages using homogenized wood shavings as bedding. All experimental protocols used on the animals were done with the approval (UPM/IACUC/AUP-17/2013) and standard ethical guidelines of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Malaysia, IACUC (Institutional Animal Care and Use Committee) were followed.

A single-dose acute oral toxicity design was performed on Balb/c mice in this study. On Day 8, following acclimatization, the mice were randomly assigned into five groups (n = 6 per

group) with same housing setup. The APAP was dissolved in an appropriate concentration in warm saline. Group 2, 3, 4 and 5 received a toxic dose of APAP (400 mg/kg of bw, i.p) followed by administration of saline (10 mL/kg), silymarin (100 mg/kg of bw, i.p), MO leaf extract (100 mg/kg of bw, i.p) and MO leaf extract (200 mg/kg bw, i.p) respectively, an hour after the administration of APAP lethal dose. Group 1 was administered saline (10 mL/kg) during the two sessions. The animals were sacrificed using diethyl ether about 24h after induction of APAP toxicity and subsequent treatment with the respective doses of silymarin /MO extract. Blood samples were rapidly obtained by cardiac puncture, and serum was prepared and stored at -20 °C. The kidney was collected, snap-frozen and stored immediately at - 80 °C and a portion of them was fixed at 10 % buffered formalin.

2.6 Biochemical parameters

All biochemical assays were performed spectrophotometrically using a Hitachi-912 Autoanalyser (Mannheim, Germany) with kits supplied by Roche Diagnostics (Mannheim, Germany). Indicators of kidney function, including serum creatinine, urea, sodium (Na^+), potassium (K^+), and chloride (Cl^-) levels were measured. In order to obtain data with good sensitivity and validity, serum samples were analyzed blindly and in triplicate.

2.7 Histopathological examination

Renal tissues from each group were fixed in 10 % formalin and fixed samples were embedded in paraffin, sectioned in 5 µm-thick sections and stained with hematoxylin-eosin (H&E) stain. All the pathological changes in renal tissues were examined and photographed using an Olympus microscope (BX-51; Olympus, Tokyo, Japan).

2.8 Measurement of kidney oxidative stress and inflammatory markers

The frozen renal tissue was thawed and homogenized in 10 % (w/v) with ice-cold 0.1M phosphate buffer saline and centrifuged at 9000 rpm for 20 min at 4 ˚C, and the supernatants were assayed according to the instructions provided by the manufacturer. BioVision Research kits (CA, USA) were used to determine the MDA level, and the activity of anti-oxidant enzymes were determined using SOD, CAT and GPx kits obtained from the Cayman Chemical Company (Ann Arbor, U.S.A). The levels of pro-inflammatory (TNF-α, IL-1β, IL-6) and anti-inflammatory (IL-10) cytokines in renal tissue homogenates were determined using commercially available ELISA kits, in accordance with manufacturers' instructions (R&D, Mannheim, Germany).

2.9 Statistical analysis

The results are expressed as mean ± SEM. The normal distribution of the data was confirmed by Shapiro–Wilk test using GraphPad software 5.0 (GraphPad, La Jolla, CA). Statistical analyses, such as one-way ANOVAs and associated Student *t*-tests were performed for the biochemical, oxidative stress and inflammatory parameters using Excel software (Microsoft, Redmond, WA). A *p*-value less than 0.05 considered as statistically significant.

3. RESULTS

3.1 Trace elements of MO leaf extract

The MO leaf extract were found to contain various trace elements, which aid numerous biochemical processes in the human body. In the present study, six trace elements, namely Cu, Mn, Ni, Cr, Pb and Cd were determined with substantial accuracy. The concentration of each element was determined, and the corresponding linear calibration curves were obtained and are reported in Table 1, with their biochemical functions. The dried MO leaf extract contain high

amount of Manganese (Mn) and Copper (Cu) with the value of 36.157 ± 0.037 and 12.323 ± 0.098 mg/kg of dry leaf extract, respectively compare to Nickel (Ni) with the value of 1.657 ± 0.008 mg/kg of dry leaf extract. Toxic trace elements such as Chromium (Cr), Cadmium (Cd) and Lead (Pb) were expressed in a negligible amount, which was found to be less than 0.005 mg/kg of dry leaf extract.

3.2 MO leaf extract minimizes APAP-induced nephrotoxicity in mice

The serum obtained from mice treated with toxic dose of APAP revealed significant ($p < 0.05$) elevation in creatinine (0.51 ± 0.02 mg/dL), blood urea nitrogen (42.0 ± 2.0 mg/dL), sodium (Na^+; 142.20 ± 0.35 mEq/L) potassium (K^+; 12.30 ± 0.24 mEq/L), and chloride (Cl^-; 106.80 ± 0.84 mEq/L) levels, compare to the control group which displayed the following values: creatinine (0.25 ± 0.01 mg/dL), blood urea nitrogen (19.60 ± 1.4 mg/dL), sodium (Na^+; 138.10 ± 0.45 mEq/L) potassium (K^+; 11.10 ± 0.16 mEq/L) and chloride (Cl^-; 103.70 ± 1.08 mEq/L). Mice treated with MO extract had lowered levels of serum creatinine, blood urea nitrogen, Na^+, K^+, and Cl^- compared to the groups that were not treated with MO, and the reduction was found to be dose-dependent. Among the adapted two doses of MO leaf extract, mice treated with 200 mg/kg of bw displayed significant ($p < 0.05$) decrease in serum kidney biomarkers with the values of creatinine (0.29 ± 0.04 mg/dL), blood urea nitrogen (30.8 ± 1.0 mg/dL), sodium (Na^+;140.20 ± 0.18 mEq/L), potassium (K^+; 11.30 ± 0.12 mEq/L and chloride (Cl^-; 104.0 ± 0.42 mEq/L and it proximate the effects of the silymarin treated (positive control) group (Fig. 1A-E).

The histological micrographs of APAP intoxicated mice kidney sections portrayed further, renal tissue damage by exhibiting severely disorganized glomerulus, dilated tubules, presence of granular casts and inflammatory cell infiltrates (Fig. 4B1). Histological analysis of MO (100 mg/kg bw) treated mice kidney sections showed some sparsely disorganized

glomerulus, tubular dilation with moderate tubular casting and inflammation (Fig. 4C1). However at a higher dose of 200 mg/kg of MO (Fig. 4D1), the glomerular and tubular architecture were well preserved. They showed negligible amount of granular casting in the renal tubules, similar to that observed in the positive control (Fig. 4E1) group and closely resembles the untreated sham group (Fig. 4A1). Subsequently, scores were awarded to the histology images. Compared to the control group, a substantial elevation in the scores was observed in APAP-treated group (negative control). In contrast, MO (100 mg/kg bw) treated mice scored lower than the APAP-treated group. At 200 mg/kg bw, MO treated group equaled the score of positive control group (Fig. 4F).

3.3 MO leaf extract regulates and restores the antioxidant status, in APAP-induced nephrotoxic mice

The level of MDA, SOD, CAT and GPx activities in the renal samples are presented in Fig 2. The renal MDA level of APAP group increased significantly ($F = 31.63$; $p < 0.05$) to 1.44 ± 0.17 nmol/mg of tissue compared with the control group, which was 0.76 ± 0.13 nmol/mg of tissue, whereas, the MO (200 mg/kg of bw) and silymarin treated groups showed a significant ($F = 30.29, 41.6$; $p < 0.05$) decrease in the level of MDA with the values around 0.80 ± 0.11 nmol/mg of tissue (Fig. 2A). Kidney obtained from the mice intoxicated with APAP showed significant decrease in the SOD (47.17 ± 5.05 U/mg), CAT (65.83 ± 5.54 nmol/ min/mg of protein) and GPx (2.55 ± 1.12 nmol/min/mg of protein) when compared with control groups showing values of 63.83 ± 6.00 U/mg, 104.27 ± 5.54 and 4.25 ± 0.26 nmol/min/mg of protein respectively ($F = 8.26, 96.29, 6.24$; $p < 0.05$). On the contrary, SOD, CAT and GPx activities of the groups treated with MO leaf extract demonstrated a dose-dependent increase, as shown in Fig. 2B-D. At the higher dose (200 mg/kg of bw), MO leaves extract exhibited values of 93.33 ±

272 5.01 U/mg, 92.33 ± 9.20 and 7.64 ± 0.33 nmol/ min/mg of protein of SOD, CAT and GPx
273 activities respectively, which were significantly (F = 112.84, 18.73, 12.85; $p < 0.05$) higher than
274 the APAP-intoxicated mice kidney. The silymarin - (positive control) treated group exhibited
275 SOD, CAT and GPx activity of 63.83 ± 4.01 U/mg, 104.27 ± 3.41 and 6.79 ± 0.16 nmol/min/mg
276 of protein respectively. However, from Figures 2B and 2D, it was clear that treatment with MO
277 leaf extract greatly exceeded the level of SOD and GPx activity compared to the silymarin-
278 treated group. These results indicate that MO leaf extracts effectively restore the antioxidant
279 status of APAP-intoxicated mice kidney.

280 **3.4 MO leaves extract modulates pro/anti-inflammatory cytokines in APAP-induced**
281 **nephrotoxic mice**

282 To further understand the mechanism of action of MO leaf extract, we evaluated its role in
283 altering the inflammatory cytokines level as displayed in Fig. 3A-D. We also compared these
284 inflammatory changes with the microscopic evidence of granular cast and inflammatory cell
285 infiltrate into the renal tissues (Fig. 4A2–E2). The kidneys of APAP-intoxicated mice showed a
286 significant (F = 24.20, 89.71, 112.95; $p < 0.05$) rise in the level of the pro inflammatory
287 cytokines TNF-α (416.67 ± 44.93), IL-1β (251.50 ± 34.18), and IL-6 (441.76 ± 19.98) ng/mg of
288 protein, compared to the control group with the values of 132.91 ± 60.16, 28.16 ± 14.24, 219.94
289 ± 18.68 ng/mg of protein respectively. At the same time, treatment with APAP suppressed the
290 level of anti-inflammatory cytokine IL-10 (131.03 ± 31.81 ng/mg of protein) compared to the
291 control (162.77 ± 22.46 ng/mg of protein). Contrarily, MO leaf extract showed a dose-dependent
292 modulation in the level of these inflammatory markers. Specifically, at 200 mg/kg of bw, MO
293 leaf extract significantly (F = 15.74, 112.73, 15.58; $p < 0.05$) suppressed the level of TNF-α
294 (292.50 ± 60.71), IL-1β (86.22 ± 21.75), and IL-6 (314.18 ± 52.76) ng/mg of protein, and

295 enhanced the level of IL-10 (215.63 ± 12.34 ng/mg of protein). From Fig. 5A–D, it was clear that
296 the reduction of inflammatory markers in MO treated group exceeded the effects seen in
297 silymarin (positive control) treatment, where the silymarin-treated group exhibited levels of
298 TNF-α (309.58 ± 59.61), IL-1β (102.61 ± 10.13), and IL-6 (306.60 ± 29.82) ng/mg of protein,
299 and the level of anti-inflammatory cytokine IL-10 with a value of 147.69 ± 12.34 ng/mg of
300 protein. This was supported by the histology micrographs (Fig. 4B2) of APAP-treated mice,
301 where significant inflammatory cell infiltrate and tubular casting were observed. In the case of
302 MO (200 mg/kg of bw) and silymarin (positive control) treated mice kidney meager/negligible
303 inflammatory cell infiltrate was observed.

304 **4. DISCUSSION**

305 Human and animal bodies contain a certain quantity of trace elements, mostly located in the
306 liver, bones and blood. Enzymes like arginase, mitochondrial superoxide dismutase,
307 cholinesterase, phosphoglucomutase, pyruvate carboxylase and several phosphates, peptidases
308 and glycosyltransferases function with aid of these elements as co-factors (Jarapala et al. 2014).
309 Trace elements are minute in quantity, yet play a vital role in biochemical processes. It has been
310 well established that copper (Cu) and manganese (Mn) are highly known catalytic co-factors for
311 Cu/Zn-SOD and Mn-SOD antioxidant enzymes that enhances the free-radical scavenging
312 activity, thereby ameliorating the effects of oxidative metabolism. Copper is also necessary for
313 both Fe and energy metabolism, it also acts as a reductant in the enzymes lysil oxidase,
314 cytochrome oxidase, dopamine hydroxylase, superoxide dismutase (Harris 1992; Dichi et al.
315 2014). In this study, both Cu and Mn were found to be highly present in MO leaf extract
316 compared to the other notable trace elements. However, negligible amounts of toxic elements
317 were found in the MO leaf extract. Nickel (Ni) which has been categorized as a “probable

essential trace element" has also been found in the MO leaf extract. Recent reports indicated that Ni functions either as a cofactor facilitating the intestinal absorption of the Fe^{3+} ion, or alters membrane properties and influences oxidation/reduction systems (Samal & Mishra 2011, Prashanth et al. 2015). In this study, the elemental analysis results of MO leaves show high amounts of Manganese (Mn) and Copper (Cu) and considerable amounts of nickel (Ni) which might involve assisting the enhancement of endogenous antioxidant system to combat APAP nephrotoxicity. The expression of negligible amount of toxic trace elements in MO leaves suggests its safety aspects in biological systems.

Acetaminophen (APAP), as the most common and established pain relieving and antipyretic medication on the market, disclose safety breach during overdose and results in hepato-renal damage. The incident rate of APAP hepatotoxicity is higher than renal toxicity, however, recent reports highlighted that renal impairment can be lethal (Eguia & Materson 1997, Ghosh et al. 2010, Karthivashan et al. 2015b). During APAP overdose, there is saturation of hepatic metabolic pathways and reduced liver clearance of APAP, allowing for higher amounts of the unmetabolized toxic intermediate, N-acetyl-para-amino-benzoquinoneimine (NAPQI), to come into contact with the kidneys. These toxic metabolites are involved in protein arylation, precisely in the S3 segment of the proximal tubule, thereby initiating renal tubular cell death (Tarloff & Kinter 1997, Bjorck et al. 1988).

Elevations in serum creatinine, blood urea nitrogen, sodium, potassium and chloride levels are the most occurring changes seen in APAP-induced nephrotoxicity (Pradhan et al. 2013, Sebastian et al. 2007). During renal damage, accumulation of serum urea occurs when the rate of serum urea production exceeds the rate of its clearance; whereas endogenous breakdown of tissue creatine leads to elevation of serum creatinine levels and other electrolytes (Palani et al.

2010). Previous study reports showed that APAP-induced renal damage leads to serum osmolality of Na^+, K^+, and Cl^-, and was significantly increased at 12 h and further increased at 24 h, which was supposedly due to renal hemodynamic compromise and tubular function impairment (Goddard et al. 2003, Pakravan et al. 2015). Thus the serum concentration of these parameters serves as the most reliable biomarkers of renal dysfunction. In accordance, the results of this study indicated that administration of APAP-inflicted substantial renal damage as evidenced by the elevated levels of serum creatinine, blood urea nitrogen, Na^+, K^+, and Cl^-. However, mice treated with varying doses of MO leaves extract presented with significantly reduced levels of serum creatinine, blood urea nitrogen, Na^+, K^+, and Cl^-. The values for these were equivalent or even less than the silymarin (positive control) treated group.

This was similar to a previous study report, whereby ethanol extract of Citrus macroptera (EECM) effectively restored the serum biomarkers and electrolytes level, thereby curbing the deterioration caused by alterations of serum Na^+, K^+, and Cl^- levels in APAP-inflicted renal impairment (Paul et al. 2016). The renal histological results correlate well with the alterations noted in biochemical parameters. The APAP-intoxicated mice revealed severely disorganized glomerulus, dilated tubules, and inflammatory casting, which is in agreement with the previous studies (Hamid et al. 2012, Ahmad et al. 2012), whereas MO leaf extract at a higher dose (200mg/kg) preserved glomerulus and tubular architecture with insignificant tubular casting, similar to the positive control/sham group. However, the histological findings in APAP-intoxicated mice were at variance with that of Sharifudin et al., 2013 (Sharifudin et al. 2013), where male Sprague-Dawley rats administered 7g/kg body weight of APAP did not reveal any significant changes in kidney histology. The observed variance could be attributed to differences in the doses administered, and animal models (Hook 1993).

To analyze the NAPQI, mediated intracellular reactive oxygen species (ROS) production in the kidney tissues, lipid peroxidation (MDA) levels, and activities of the antioxidant enzymes (SOD, CAT and GPx) were measured. During APAP overdose, an imbalance occurs between the formations of ROS and its scavenging mechanism through the endogenous antioxidant system (Hook 1993, Ozbek 2012). This causes an oxidative stress environment, leading to cellular damage via peroxyl radical formation, which is further reorganized through a cyclization process to endoperoxides, and produces malondialdehyde (MDA) as the final product (Yin et al. 2011). In this study the APAP-treated mice showed a significant elevation in MDA levels and substantial decrease in SOD, CAT, and GPx activities, when compared to normal control groups. However, administration of MO leaf extract significantly decreased the levels of MDA and efficiently elevated SOD, CAT, and GPx activities, compared to the APAP-treated group. Our research team has previously reported the existence of flavonoids such as kaempferol, apigenin, quercetin, and multiflorin in the MO leaf extract, which are likely responsible for advancing antioxidant potential.

It was well established that APAP overdose-induced nephrotoxicity occurred via the formation of NAPQI, but some recent reports strongly suggested the role of inflammatory responses in the progression of renal injury (Ghosh et al. 2010, Samal & Mishra 2011, Ozbek 2012). APAP intoxication induces oxidative stress mediated renal damage, which further triggers a secondary inflammatory cascade associated with cytokine release from Kupffer cells. Pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 are prominently reported in APAP-induced hepato-renal toxicity. They are engaged in massive tubular infiltration of leukocytes, thereby inducing a sterile inflammatory environment and further exaggerating the renal damage (Hörl 2010, Sanz et al. 2008). A recent study reported N-acetylcysteine and ozone therapy exhibited

effective anti-inflammatory activity based on its inhibitory activities against the expression of TNF-α in APAP-intoxicated mice kidney (Ucar et al. 2013). In another study, bazhen decoction was reported to possess some protective role against APAP toxicity through suppression of various pro-inflammatory cytokines, notably TNF-α, IL-1β and IL-6 (Song et al. 2014). The results of our study showed elevated levels of TNF-α, IL-1β, IL-6 in APAP-administered mice kidney, which was significantly suppressed by the MO leaves extract. Anti-inflammatory cytokines such as IL-10 were also produced by the Kupffer cells at the site of tubular inflammation to check this detrimental influence.

Ucar et al. (2013) reported that n-acetylcysteine and ozone therapy also played a renal-protective role in APAP toxicity by significantly elevating IL-10 cytokine level (Ucar et al. 2013). Our results consistently indicated significant suppression of IL-10 cytokine in APAP-intoxicated mice, which was effectually restored by MO leaf extract in a dose-dependent manner. Thus MO leaf extract protects the inflammatory-mediated exacerbation of renal damage in APAP-intoxicated mice by modulation of both pro and anti-inflammatory cytokine level. Thus, the postulated overall mechanism of action of MO leaf extract against APAP-induced nephro-toxicity pathway has been clearly elucidated in this study (Fig. 5).

5. CONCLUSION

The MO leaf extracts shielded kidney from APAP toxicity through enhancement of the endogenous antioxidant system/ enzymatic level to counteract the oxidative stress environment (ROS). Certain naturally incorporated bioactive constituents, and highly accessible essential trace elements present in MO leaves aided the renal protective activity. The MO leaf extract also exhibited modulatory effect on specific inflammatory cytokines, and aided in combating the

410 inflammatory cascade associated renal damage seen in APAP toxicity. In the light of
411 biochemical results and histological findings, MO leaf extract can be suggested as a convincing
412 remedy against APAP-induced nephrotoxicity. Thus, further broad translational investigation of
413 these promising protective effects of MO leaves against APAP-induced renal injury may have a
414 substantial influence on developing clinically-feasible strategies to treat patients with renal
415 impairment, or as a supplemental treatment to aid several nephrotoxic drugs on widening of their
416 therapeutic index.

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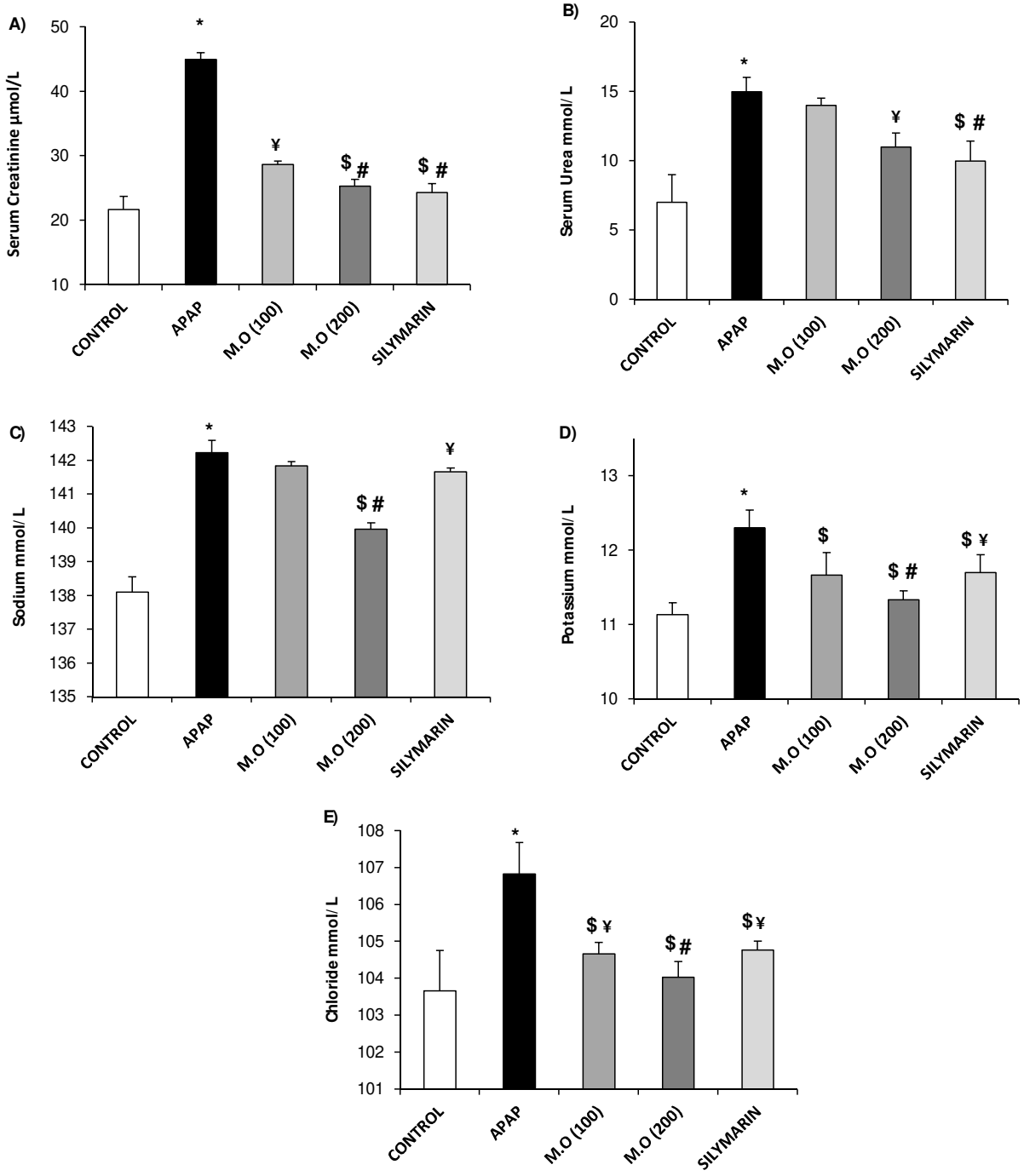
Figure 1(on next page)

Modulation of Serum biochemical markers and electrolytes level

Figure 1: *Moringa oliefera* leaves suppress the detrimental effect of APAP induced nephrotoxicity. A - E represents the level of serum biochemical markers such as creatinine, urea and electolytes such as sodium, potassium and chloride. Values are expressed as the mean ± SEM of n=6 mice in each group. The normality distribution of the data has been confirmed by Shapiro-Wilk test and statistical analysis was performed using one-way ANOVA associated student t-test.*P<0.05 compared to the control group, $ P<0.05 compared to APAP administered group. Among the treatment groups, when the protection is total (i.e., different from acetaminophen group and relevant to control group) or partial (i.e., different from acetaminophen and control groups) were represented as # or ¥ respectively.


A)
Serum Creatinine µmol/L
50
40
30
20
10
*
¥
$ #
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CONTROL
APAP
M.O (100)
M.O (200)
SILYMARIN
B)
Serum Urea mmol/ L
20
15
10
5
0
*
¥
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CONTROL
APAP
M.O (100)
M.O (200)
SILYMARIN
C)
Sodium mmol/ L
143
142
141
140
139
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137
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CONTROL
APAP
M.O (100)
M.O (200)
SILYMARIN
D)
Potassium mmol/ L
13
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10
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$ #
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CONTROL
APAP
M.O (100)
M.O (200)
SILYMARIN
E)
Chloride mmol/ L
108
107
106
105
104
103
102
101
*
$ ¥
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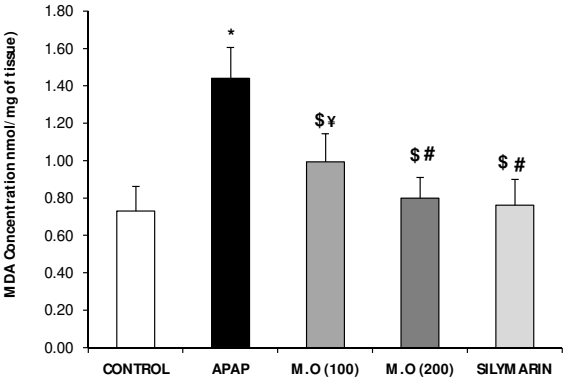
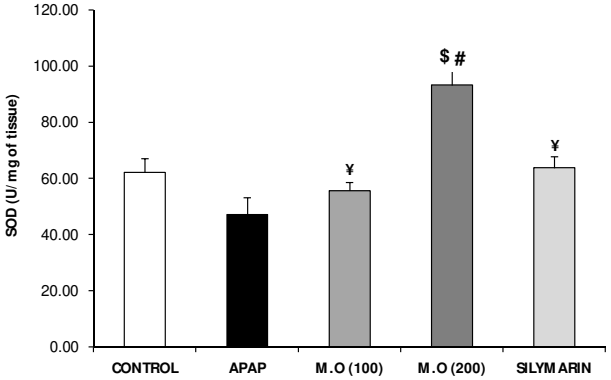
$ ¥
CONTROL
APAP
M.O (100)
M.O (200)
SILYMARIN

Figure 2(on next page)

Level of antioxidant enzymes in kidney tissue

Figure 2: Dose dependent effect of *Moringa oleifera* (M.O) leaves extract against APAP intoxicated mice kidney via augmentation of endogenous antioxidant status: [A] lipid peroxidation activity (MDA); endogenous antioxidant enzyme levels ([B]-SOD, [C]-CAT & [D]-GPx). Values are expressed as the mean ± SEM of n=6 mice in each group. The normality distribution of the data has been confirmed by Shapiro-Wilk test and statistical analysis was performed using one-way ANOVA associated student t-test. * $P<0.05$ compared to control group, $ $P<0.05$ compared to APAP group. Among the treatment groups, when the protection is total (different from acetaminophen group and relevant to control group) or partial (different from acetaminophen and control groups) were represented as # or ¥ respectively.


MDA Concentration nmol/ mg of tissue)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
$¥
$ #
$ #
CONTROL
APAP
M.O (100)
M.O (200)
SILYMARIN


SOD (U/ mg of tissue)
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80.00
60.00
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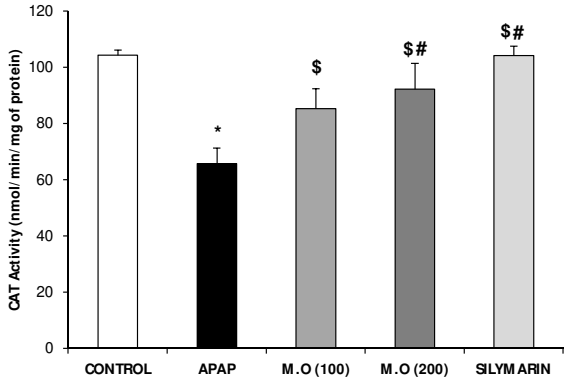
CONTROL
APAP
M.O (100)
M.O (200)
SILYMARIN


CAT Activity (nmol/ min/ mg of protein)
120
100
80
60
40
20
0
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$#
CONTROL
APAP
M.O (100)
M.O (200)
SILYMARIN

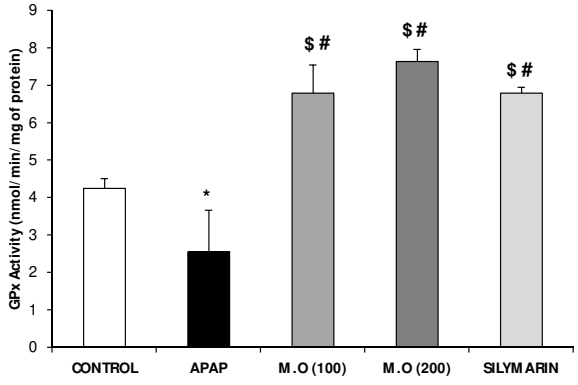

GPx Activity (nmol/ min/ mg of protein)
9
8
7
6
5
4
3
2
1
0
*
$ #
$ #
$ #
CONTROL
APAP
M.O (100)
M.O (200)
SILYMARIN

Figure 3(on next page)

Alteration in the level of serum inflammatory markers

Figure 3: Modulatory effect of *Moringa oleifera* (M.O) leaves extract against APAP intoxicated kidney inflammatory cytokines – [A] TNF-α; [B] IL-1β; [C] IL-6; [D] IL-10. Values are expressed as the mean ± SEM of n=6 mice in each group. The normality distribution of the data has been confirmed by Shapiro-Wilk test and statistical analysis was performed using one-way ANOVA associated student t-test. Results are shown as the mean ± SEM;* $P<0.05$ compared to control group, $ $P<0.05$ compared to PCM group. Among the treatment groups, when the protection is total (different from acetaminophen group and relevant to control group) or partial (different from acetaminophen and control groups) were represented as # or ¥ respectively.

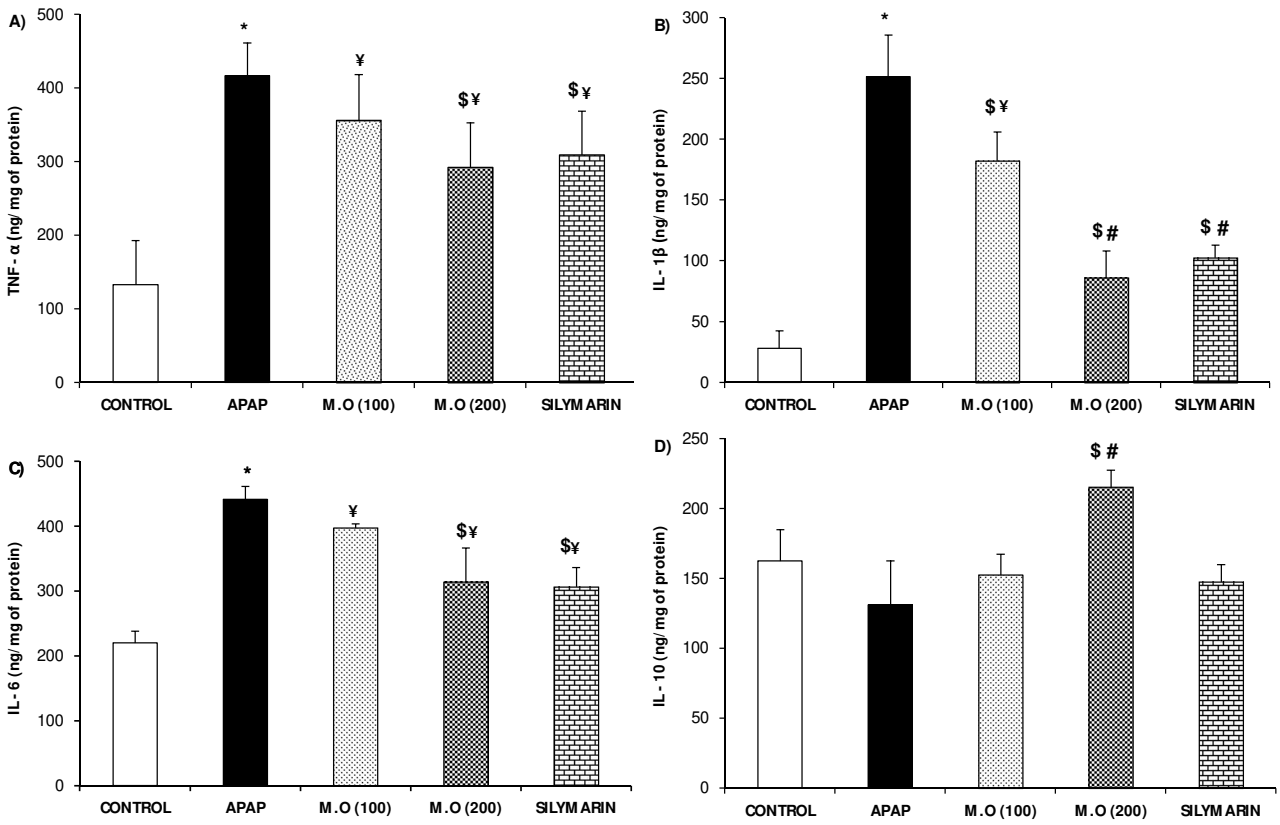
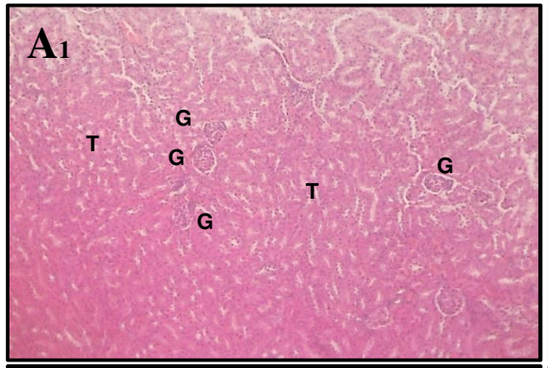
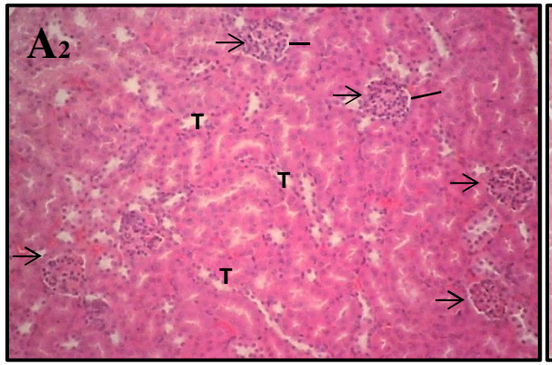


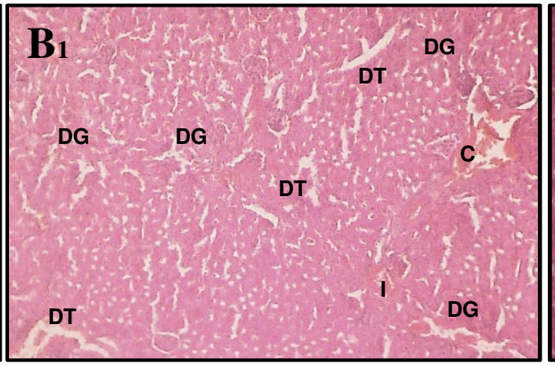
Figure 4(on next page)

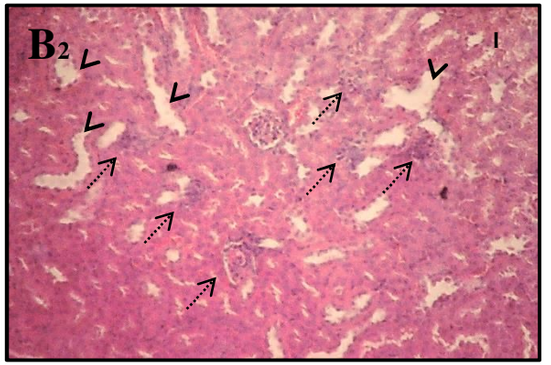
Photographs of Histopathological modification in kidney tissue

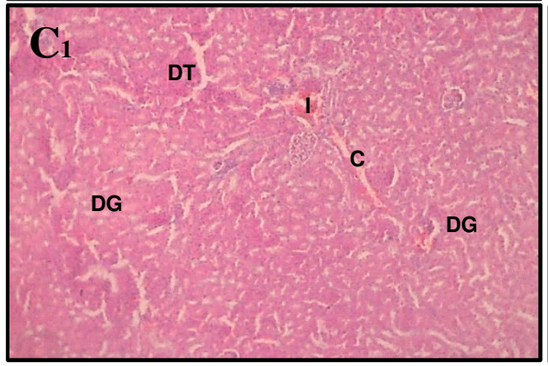
Figure 4: Photographic sections (H&E 20X – **1**; 40X - **2**) of the mice kidney, $\mathbf{A_1}$: Control group showing normal histological architecture of tubules (T) and Glomerulus (G) $\mathbf{A_2}$: Flawless tubules with striated border (T), intact glomerulus (arrow) with surrounding Bowman's capsule (line). $\mathbf{B_1}$: Kidney of mice treated with APAP showing disorganized glomerulus (DG), dilated tubules (DT) with tubular casting (C) and inflammation (I) $\mathbf{B_2}$: severely disorganized glomerulus (dotted arrow), tubular dilation (arrow head), and inflammatory casting were observed $\mathbf{C_1}$: Kidney of mice treated with APAP and M.O 100mg/kg showing sparsely disorganized glomerulus (DG), dilated tubules (DT) with moderate tubular casting (C) and inflammation (I) $\mathbf{C_2}$: few disorganized glomerulus (dotted arrow) and tubular dilation (arrow head) with tubular casting (C) and inflammation (I) are noticed. $\mathbf{D_1}$: Kidney of mice treated with APAP and M.O 200mg/kg showing preserved glomerulus (G) and tubules (T) architecture with mild tubular casting (C) and inflammation (I) $\mathbf{D_2}$: mild tubular dilation (arrow head) with tubular casting (C) and inflammation (I) are noticed. $\mathbf{E_1}$: Kidney of mice treated with APAP and silymarin (positive control) showing preserved glomerulus (G) and tubules (T) architecture $\mathbf{E_2}$: tubules with striated border (T), intact glomerulus (arrow) are noticed. **F**: The histological changes were graded as: (-) score (negative score): no any structural damage, (+) score (one positive score): marginal damage, (++) score (two positive score): moderate damage, (+++) score (three positive score): intense damage.

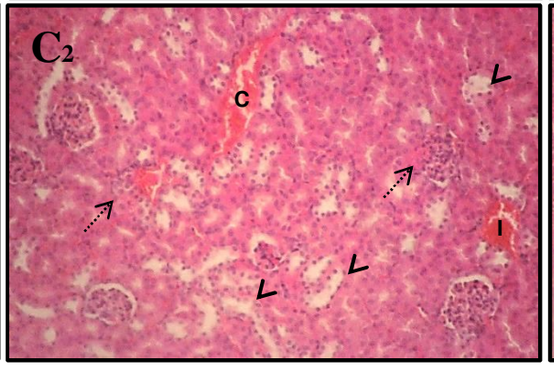


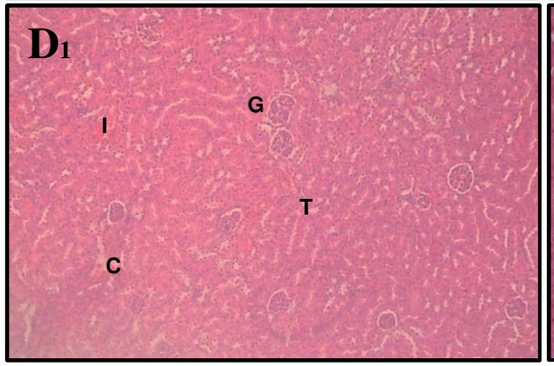


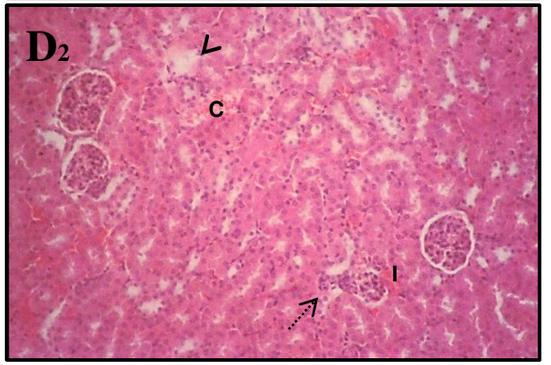


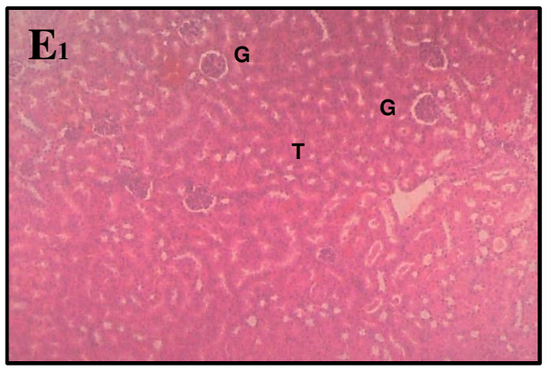


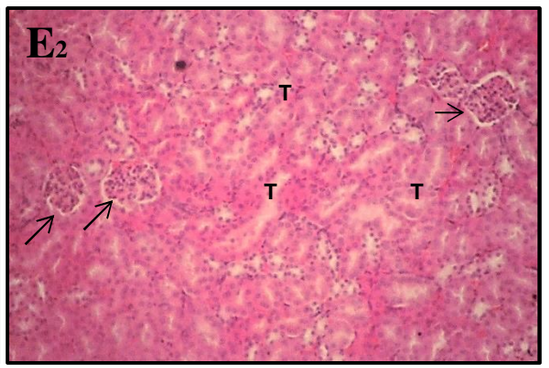












F Histological Remarks	Control	APAP	MO 100	MO 200	Silymarin
Vascular congestion & Tubular dilatation	-	+++	++	-	-
Glomerulus Damage	-	+++	++	-	-
Inflammatory cell infiltrations	-	+++	+++	+	-

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Figure 5(on next page)

Potential mechanism of action of MO leaves extract against APAP induced renal toxicity pathway

Figure 5: Mechanism of action of MO leaves extract against APAP induced renal toxicity pathway: - The active constituents and essential trace elements of MO leaves extract successfully enhance the GSH-GPx and endogenous antioxidant system thereby inhibit the oxidative stress mediated renal impairment, induced by APAP overdose. Despite, MO leaves extract also extensively inhibit the inflammatory cascade by effectual modulation of inflammatory cytokines. Thus curb the further exacerbation of renal injury mediated by inflammatory cytokines. These features evidently project MO leaves extract as a successful nephro-protective agent.

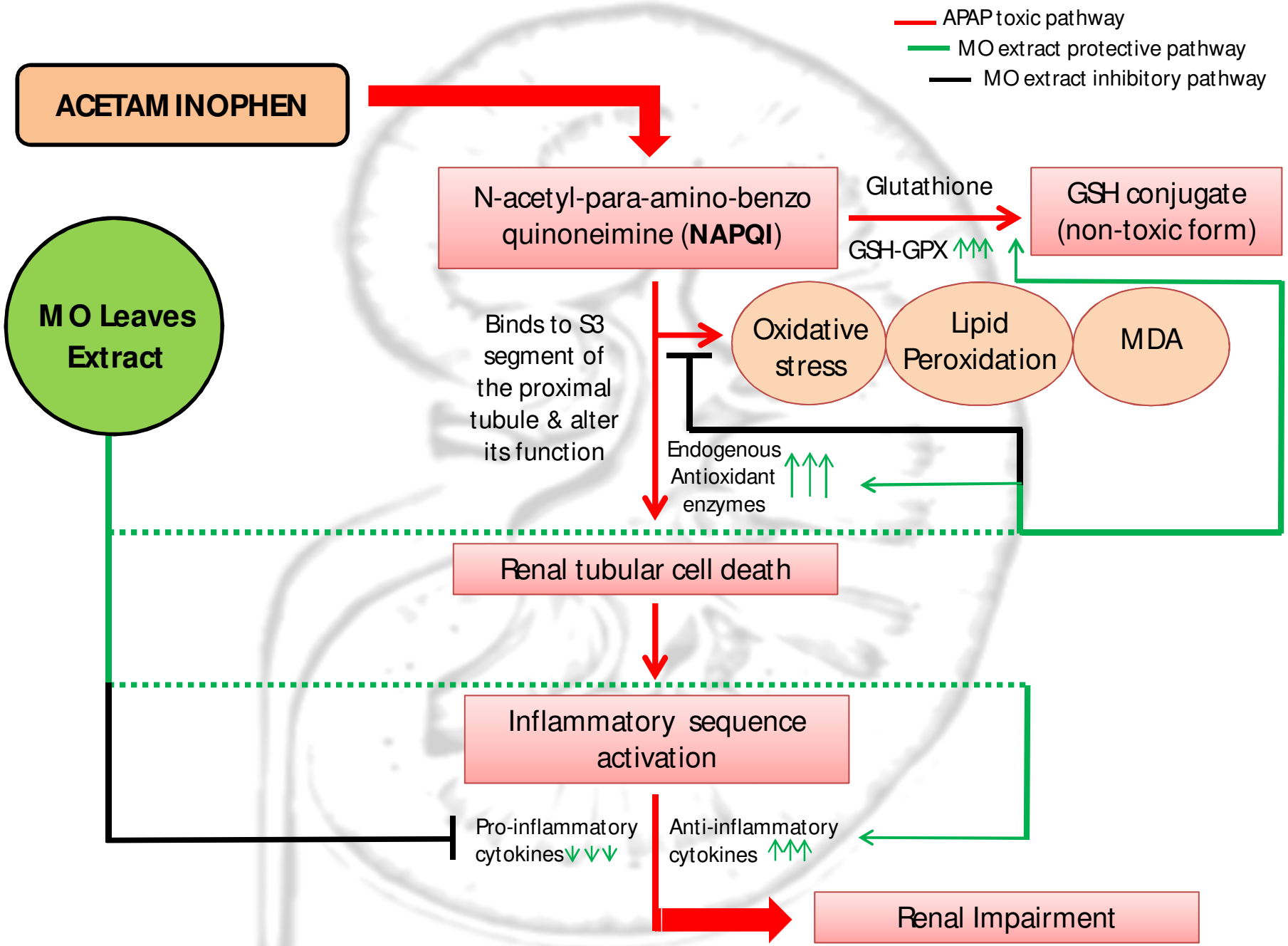

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Table 1(on next page)

Selective trace element composition of MO leaves

Table.1. Selective trace elemental composition of dried Moringa (M. oleifera Lam.) leaves.

1 Table.1. Selective trace elemental composition of dried Moringa (M. oleifera Lam.) leaves.

Trace elements	Concentration (mg/kg of dry leaf extract)	Function
Copper	12.323 ± 0.098	a catalytic cofactor in the redox chemistry of free radical scavenging
Manganese	36.157 ± 0.037	Activator of several manganese metalloenzymes and one form of antioxidant enzyme superoxide dismutase (SOD).
Nickel	1.657 ± 0.008	Aids in iron absorption, as well as adrenaline and glucose metabolism, hormones, lipid, cell membrane and improves bone strength
Chromium (VI)	< 0.005 ± 0.004	Causes gastrointestinal effects in humans and animals, including abdominal pain, vomiting, and hemorrhage.
Lead	< 0.005 ± 0.002	Lead has no known preferred function in the body, but accumulation of lead is highly toxic for human body.
Cadmium	< 0.005 ± 0.005	Cadmium is extremely toxic. It mainly affects the kidney, the cardiovascular system, and is related to cancer.

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