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.

1 THE MODULATORY EFFECT OF *MORINGA OLEIFERA* LEAF EXTRACT ON

2 ENDOGENOUS ANTIOXIDANT SYSTEMS AND INFLAMMATORY MARKERS IN

3 ACETAMINOPHEN-INDUCED NEPHROTOXIC MICE MODEL

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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

50   effectiveness against APAP-induced nephrotoxicity through enhancement of endogenous

51   antioxidant system and modulatory effect on specific inflammatory cytokines in kidney tissues.

52   **Abbreviations: MO** - *Moringa oliefera*; **APAP** - acetaminophen; **GSH-Px**- glutathione

53   peroxidase; **SOD** - superoxide dismutase; **CAT** - catalase; **MDA** - Malondialdehyde; **NAPQI** -

54   N-acetyl-p-benzoquinoneimine; **TNF-α -** Tumor necrosis factor- α; **IL –** Interleukin; **NAC**-N-

55   acetylcysteine.

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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.

91   N-acetylcysteine (NAC), a precursor of GSH, is well known for its hepato-protective nature

92   against APAP-induced hepatotoxicity in both humans and animals; however, it has a limited

93   function towards APAP-induced renal toxicity (Eguia & Materson 1997, Mazer & Perrone

94   2008). Thus, the hunt for alternative, safe and therapeutically effective compounds against

95   APAP-induced renal toxicity is essential.

96   *Moringa oleifera* Lam (MO) is a wide-spread tropical and subtropical species belongs to

97   Moringaceae family. It is well known for its remarkable nutritional value and elite therapeutic

98   potential against extensive clinical conditions. *Moringa oleifera* Lam is commonly known as

99   "drumstick tree" or "horseradish tree" and almost all parts of this plant, including the root, bark,

100 stem, leaves, flowers and pods possess huge amounts of micro- and macronutrients. It provides,

101 both animal and human nutritional supplements (Siddhuraju & Becker 2003, Anwar et al. 2007).

102 It possesses a rich and rare combination of therapeutically-active candidates such as kaempferol,

103 rhamnetin, quercitin, chlorogenic acid, rutin, and apigenin, and is also enriched with an

104 exogenous supply of ascorbic acid and carotenoids, which are renowned antioxidant candidates

105 (Anwar et al. 2007, Karthivashan et al. 2013). MO has been utilized for ages as traditional

106 medicine in the treatment of numerous disorders as an antiseptic, anti-diabetic, antiepileptic,

107 antiparalytic, antiviral, anti-inflammatory effect. Additionally, numerous scientific reports on

108 various parts of the plant have reported on its medicinal value, among which its leaves has been

109 extensively studied in a wide variety of clinical conditions for antimicrobial, anti-inflammatory,

110 anti-cancer, and anti-diabetic effects (Anwar et al. 2007). Our research team has recently

111 identified that flavonoids such as kaempferol, quercitin and apigenin were likely involved in the

112 enhanced antioxidant effect of MO leaves extract, and further established its hepatoprotective

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

114 Karthivashan et al. 2013).

115   In, recent years, it has been established that the existence of trace elements in MO leaf

116 extract also contributes to improvising human health and combating various health disorders

117 (Gowrishankar et al. 2010, Prashanth et al. 2015). Thus, in this study, we evaluated several

118 essential / non-essential trace elements of MO leaf extract to investigate their possible

119 involvement against APAP toxicity. The pathophysiology of APAP-induced hepatotoxicity is

120 proposed to be similar to that of APAP nephrotoxicity; thus, here we extended our investigation

121 on the potential nephro-protective mechanism of MO leaf extract against APAP-induced

122 nephrotoxicity. Furthermore, silymarin has been selected as the positive control for this study,

123 based on previous study reports due to its enhanced hepato- and renal-protective properties

124 against APAP toxicity in mice due to its enriched antioxidative and anti-inflammatory nature (He

125 et al. 2004, Bektur et al. 2013). This would pave way for further investigation on the

126 advancement of MO leaf extract as an effective therapy for both APAP-induced nephro- and

127 hepato-toxicity in the field of clinical / translational medication.

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129 **2.   MATERIALS AND METHODS**

130 **2.1   Chemicals**

131   Acetaminophen and silymarin were procured from Sigma (St. Louis, MO, USA). All

132 kidney function markers kits, malondialdehyde (MDA) and antioxidant enzyme assay kits were

133 purchased from Roche Diagnostics (Germany), Biovision Research kits (CA, USA) and Cayman

134 chemical company (Ann Arbor, MI, USA) respectively. HEPES buffer was obtained from

135 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

159   after 4 hours and the process continued until whitish grey ash residue was attained. The residue

160   was dissolved in 5 ml of nitric oxide and increased to 10 mL volume in appropriate volumetric

161   flask.

162   **2.4.2   Determination of trace elements**

163   In this study, we evaluated three essential trace elements; Copper (Cu), Manganese (Mn), Nickel

164   (Ni), and three toxic trace elements; Cromium (Cr), Lead (Pb) and Cadmium (Cd) present in the

165   MO leaf extract. Working standard solutions of appropriate elements were prepared from stock

166   standard solution (1000 mg/L) and absorbance values were obtained for various working

167   standards for each element in the samples, using an atomic absorption spectrometer (AAS). The

168   absorbance values were plotted against concentration, whereby the formed linear calibration

169   curves revealed the actual concentration of the sample. A blank reading was also taken and

170   essential correction was made during the calculation of concentration of various elements.

171   **2.5   Animals and experimental design**

172   Male Balb/c mice of 25 - 30 g weight (10-12 weeks old) were handled at the Animal House Unit,

173   Faculty of Medicine, Universiti Putra Malaysia (UPM). Animals were acclimatized for a week at

174   26 ± 2 °C with a 12 h light/dark cycle. Free access to food and water was allowed at all times.

175   During the acclimatization period, five mice were housed per cage in plastic cages using

176   homogenized wood shavings as bedding. All experimental protocols used on the animals were

177   done with the approval (UPM/IACUC/AUP-17/2013) and standard ethical guidelines of the

178   Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Malaysia, IACUC

179   (Institutional Animal Care and Use Committee) were followed.

180   A single-dose acute oral toxicity design was performed on Balb/c mice in this study. On

181   Day 8, following acclimatization, the mice were randomly assigned into five groups ($n = 6$ per

182   group) with same housing setup. The APAP was dissolved in an appropriate concentration in

183   warm saline. Group 2, 3, 4 and 5 received a toxic dose of APAP (400 mg/kg of bw, i.p) followed

184   by administration of saline (10 mL/kg), silymarin (100 mg/kg of bw, i.p), MO leaf extract (100

185   mg/kg of bw, i.p) and MO leaf extract (200 mg/kg bw, i.p) respectively, an hour after the

186   administration of APAP lethal dose. Group 1 was administered saline (10 mL/kg) during the two

187   sessions. The animals were sacrificed using diethyl ether about 24h after induction of APAP

188   toxicity and subsequent treatment with the respective doses of silymarin /MO extract. Blood

189   samples were rapidly obtained by cardiac puncture, and serum was prepared and stored at -20

190   °C. The kidney was collected, snap-frozen and stored immediately at - 80 °C and a portion of

191   them was fixed at 10 % buffered formalin.

192   **2.6   Biochemical parameters**

193   All biochemical assays were performed spectrophotometrically using a Hitachi-912

194   Autoanalyser (Mannheim, Germany) with kits supplied by Roche Diagnostics (Mannheim,

195   Germany). Indicators of kidney function, including serum creatinine, urea, sodium (Na^+),

196   potassium (K^+), and chloride (Cl^-) levels were measured. In order to obtain data with good

197   sensitivity and validity, serum samples were analyzed blindly and in triplicate.

198   **2.7   Histopathological examination**

199   Renal tissues from each group were fixed in 10 % formalin and fixed samples were embedded in

200   paraffin, sectioned in 5 μm-thick sections and stained with hematoxylin-eosin (H&E) stain. All

201   the pathological changes in renal tissues were examined and photographed using an Olympus

202   microscope (BX-51; Olympus, Tokyo, Japan).

203   **2.8   Measurement of kidney oxidative stress and inflammatory markers**

204 The frozen renal tissue was thawed and homogenized in 10 % (w/v) with ice-cold 0.1M

205 phosphate buffer saline and centrifuged at 9000 rpm for 20 min at 4 ˚C, and the supernatants

206 were assayed according to the instructions provided by the manufacturer. BioVision Research

207 kits (CA, USA) were used to determine the MDA level, and the activity of anti-oxidant enzymes

208 were determined using SOD, CAT and GPx kits obtained from the Cayman Chemical Company

209 (Ann Arbor, U.S.A). The levels of pro-inflammatory (TNF-α, IL-1β, IL-6) and anti-

210 inflammatory (IL-10) cytokines in renal tissue homogenates were determined using

211 commercially available ELISA kits, in accordance with manufacturers' instructions (R&D,

212 Mannheim, Germany).

213 **2.9   Statistical analysis**

214 The results are expressed as mean ± SEM. The normal distribution of the data was confirmed by

215 Shapiro–Wilk test using GraphPad software 5.0 (GraphPad, La Jolla, CA). Statistical analyses,

216 such as one-way ANOVAs and associated Student t-tests were performed for the biochemical,

217 oxidative stress and inflammatory parameters using Excel software (Microsoft, Redmond, WA).

218 A p-value less than 0.05 considered as statistically significant.

219 **3.   RESULTS**

220 **3.1 Trace elements of MO leaf extract**

221 The MO leaf extract were found to contain various trace elements, which aid numerous

222 biochemical processes in the human body. In the present study, six trace elements, namely Cu,

223 Mn, Ni, Cr, Pb and Cd were determined with substantial accuracy. The concentration of each

224 element was determined, and the corresponding linear calibration curves were obtained and are

225 reported in Table 1, with their biochemical functions. The dried MO leaf extract contain high

226   amount of Manganese (Mn) and Copper (Cu) with the value of 36.157 ± 0.037 and 12.323 ±

227   0.098 mg/kg of dry leaf extract, respectively compare to Nickel (Ni) with the value of 1.657 ±

228   0.008 mg/kg of dry leaf extract. Toxic trace elements such as Chromium (Cr), Cadmium (Cd)

229   and Lead (Pb) were expressed in a negligible amount, which was found to be less than 0.005

230   mg/kg of dry leaf extract.

231   **3.2 MO leaf extract minimizes APAP-induced nephrotoxicity in mice**

232   The serum obtained from mice treated with toxic dose of APAP revealed significant ($p < 0.05$)

233   elevation in creatinine (0.51 ± 0.02 mg/dL), blood urea nitrogen (42.0 ± 2.0 mg/dL), sodium

234   (Na^+; 142.20 ± 0.35 mEq/L) potassium (K^+; 12.30 ± 0.24 mEq/L), and chloride (Cl^-; 106.80 ±

235   0.84 mEq/L) levels, compare to the control group which displayed the following values:

236   creatinine (0.25 ± 0.01 mg/dL), blood urea nitrogen (19.60 ± 1.4 mg/dL), sodium (Na^+; 138.10 ±

237   0.45 mEq/L) potassium (K^+; 11.10 ± 0.16 mEq/L) and chloride (Cl^-; 103.70 ± 1.08 mEq/L).

238   Mice treated with MO extract had lowered levels of serum creatinine, blood urea nitrogen, Na^+,

239   K^+, and Cl^- compared to the groups that were not treated with MO, and the reduction was found

240   to be dose-dependent. Among the adapted two doses of MO leaf extract, mice treated with 200

241   mg/kg of bw displayed significant ($p < 0.05$) decrease in serum kidney biomarkers with the

242   values of creatinine (0.29 ± 0.04 mg/dL), blood urea nitrogen (30.8 ± 1.0 mg/dL), sodium

243   (Na^+;140.20 ± 0.18 mEq/L), potassium (K^+; 11.30 ± 0.12 mEq/L and chloride (Cl^-; 104.0 ± 0.42

244   mEq/L and it proximate the effects of the silymarin treated (positive control) group (Fig. 1A-E).

245   The histological micrographs of APAP intoxicated mice kidney sections portrayed

246   further, renal tissue damage by exhibiting severely disorganized glomerulus, dilated tubules,

247   presence of granular casts and inflammatory cell infiltrates (Fig. 4B1). Histological analysis of

248   MO (100 mg/kg bw) treated mice kidney sections showed some sparsely disorganized

249 glomerulus, tubular dilation with moderate tubular casting and inflammation (Fig. 4C1).

250 However at a higher dose of 200 mg/kg of MO (Fig. 4D1), the glomerular and tubular

251 architecture were well preserved. They showed negligible amount of granular casting in the renal

252 tubules, similar to that observed in the positive control (Fig. 4E1) group and closely resembles

253 the untreated sham group (Fig. 4A1). Subsequently, scores were awarded to the histology

254 images. Compared to the control group, a substantial elevation in the scores was observed in

255 APAP-treated group (negative control). In contrast, MO (100 mg/kg bw) treated mice scored

256 lower than the APAP-treated group. At 200 mg/kg bw, MO treated group equaled the score of

257 positive control group (Fig. 4F).

258 **3.3 MO leaf extract regulates and restores the antioxidant status, in APAP-induced**

259 **nephrotoxic mice**

260 The level of MDA, SOD, CAT and GPx activities in the renal samples are presented in Fig 2.

261 The renal MDA level of APAP group increased significantly ($F = 31.63$; $p < 0.05$) to 1.44 ±

262 0.17 nmol/mg of tissue compared with the control group, which was 0.76 ± 0.13 nmol/mg of

263 tissue, whereas, the MO (200 mg/kg of bw) and silymarin treated groups showed a significant (F

264 $= 30.29$, 41.6; $p < 0.05$) decrease in the level of MDA with the values around 0.80 ± 0.11

265 nmol/mg of tissue (Fig. 2A). Kidney obtained from the mice intoxicated with APAP showed

266 significant decrease in the SOD (47.17 ± 5.05 U/mg), CAT (65.83 ± 5.54 nmol/ min/mg of

267 protein) and GPx (2.55 ± 1.12 nmol/min/mg of protein) when compared with control groups

268 showing values of 63.83 ± 6.00 U/mg, 104.27 ± 5.54 and 4.25 ± 0.26 nmol/min/mg of protein

269 respectively ($F = 8.26$, 96.29, 6.24; $p < 0.05$). On the contrary, SOD, CAT and GPx activities of

270 the groups treated with MO leaf extract demonstrated a dose-dependent increase, as shown in

271 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

318 essential trace element" has also been found in the MO leaf extract. Recent reports indicated that

319 Ni functions either as a cofactor facilitating the intestinal absorption of the Fe^{3+} ion, or alters

320 membrane properties and influences oxidation/reduction systems (Samal & Mishra 2011,

321 Prashanth et al. 2015). In this study, the elemental analysis results of MO leaves show high

322 amounts of Manganese (Mn) and Copper (Cu) and considerable amounts of nickel (Ni) which

323 might involve assisting the enhancement of endogenous antioxidant system to combat APAP

324 nephrotoxicity. The expression of negligible amount of toxic trace elements in MO leaves

325 suggests its safety aspects in biological systems.

326   Acetaminophen (APAP), as the most common and established pain relieving and

327 antipyretic medication on the market, disclose safety breach during overdose and results in

328 hepato-renal damage. The incident rate of APAP hepatotoxicity is higher than renal toxicity,

329 however, recent reports highlighted that renal impairment can be lethal (Eguia & Materson 1997,

330 Ghosh et al. 2010, Karthivashan et al. 2015b). During APAP overdose, there is saturation of

331 hepatic metabolic pathways and reduced liver clearance of APAP, allowing for higher amounts

332 of the unmetabolized toxic intermediate, N-acetyl-para-amino-benzoquinoneimine (NAPQI), to

333 come into contact with the kidneys. These toxic metabolites are involved in protein arylation,

334 precisely in the S3 segment of the proximal tubule, thereby initiating renal tubular cell death

335 (Tarloff & Kinter 1997, Bjorck et al. 1988).

336   Elevations in serum creatinine, blood urea nitrogen, sodium, potassium and chloride

337 levels are the most occurring changes seen in APAP-induced nephrotoxicity (Pradhan et al.

338 2013, Sebastian et al. 2007). During renal damage, accumulation of serum urea occurs when the

339 rate of serum urea production exceeds the rate of its clearance; whereas endogenous breakdown

340 of tissue creatine leads to elevation of serum creatinine levels and other electrolytes (Palani et al.

341 2010). Previous study reports showed that APAP-induced renal damage leads to serum

342 osmolality of Na^+, K^+, and Cl^-, and was significantly increased at 12 h and further increased at

343 24 h, which was supposedly due to renal hemodynamic compromise and tubular function

344 impairment (Goddard et al. 2003, Pakravan et al. 2015). Thus the serum concentration of these

345 parameters serves as the most reliable biomarkers of renal dysfunction. In accordance, the results

346 of this study indicated that administration of APAP-inflicted substantial renal damage as

347 evidenced by the elevated levels of serum creatinine, blood urea nitrogen, Na^+, K^+, and Cl^-.

348 However, mice treated with varying doses of MO leaves extract presented with significantly

349 reduced levels of serum creatinine, blood urea nitrogen, Na^+, K^+, and Cl^-. The values for these

350 were equivalent or even less than the silymarin (positive control) treated group.

351 This was similar to a previous study report, whereby ethanol extract of Citrus macroptera

352 (EECM) effectively restored the serum biomarkers and electrolytes level, thereby curbing the

353 deterioration caused by alterations of serum Na^+, K^+, and Cl^- levels in APAP-inflicted renal

354 impairment (Paul et al. 2016). The renal histological results correlate well with the alterations

355 noted in biochemical parameters. The APAP-intoxicated mice revealed severely disorganized

356 glomerulus, dilated tubules, and inflammatory casting, which is in agreement with the previous

357 studies (Hamid et al. 2012, Ahmad et al. 2012), whereas MO leaf extract at a higher dose

358 (200mg/kg) preserved glomerulus and tubular architecture with insignificant tubular casting,

359 similar to the positive control/sham group. However, the histological findings in APAP-

360 intoxicated mice were at variance with that of Sharifudin et al., 2013 (Sharifudin et al. 2013),

361 where male Sprague-Dawley rats administered 7g/kg body weight of APAP did not reveal any

362 significant changes in kidney histology. The observed variance could be attributed to differences

363 in the doses administered, and animal models (Hook 1993).

364   To analyze the NAPQI, mediated intracellular reactive oxygen species (ROS) production

365   in the kidney tissues, lipid peroxidation (MDA) levels, and activities of the antioxidant enzymes

366   (SOD, CAT and GPx) were measured. During APAP overdose, an imbalance occurs between the

367   formations of ROS and its scavenging mechanism through the endogenous antioxidant system

368   (Hook 1993, Ozbek 2012). This causes an oxidative stress environment, leading to cellular

369   damage via peroxyl radical formation, which is further reorganized through a cyclization process

370   to endoperoxides, and produces malondialdehyde (MDA) as the final product (Yin et al. 2011).

371   In this study the APAP-treated mice showed a significant elevation in MDA levels and

372   substantial decrease in SOD, CAT, and GPx activities, when compared to normal control groups.

373   However, administration of MO leaf extract significantly decreased the levels of MDA and

374   efficiently elevated SOD, CAT, and GPx activities, compared to the APAP-treated group. Our

375   research team has previously reported the existence of flavonoids such as kaempferol, apigenin,

376   quercetin, and multiflorin in the MO leaf extract, which are likely responsible for advancing

377   antioxidant potential.

378   It was well established that APAP overdose-induced nephrotoxicity occurred via the

379   formation of NAPQI, but some recent reports strongly suggested the role of inflammatory

380   responses in the progression of renal injury (Ghosh et al. 2010, Samal & Mishra 2011, Ozbek

381   2012). APAP intoxication induces oxidative stress mediated renal damage, which further triggers

382   a secondary inflammatory cascade associated with cytokine release from Kupffer cells. Pro-

383   inflammatory cytokines like TNF-α, IL-1β and IL-6 are prominently reported in APAP-induced

384   hepato-renal toxicity. They are engaged in massive tubular infiltration of leukocytes, thereby

385   inducing a sterile inflammatory environment and further exaggerating the renal damage (Hörl

386   2010, Sanz et al. 2008). A recent study reported N-acetylcysteine and ozone therapy exhibited

387 effective anti-inflammatory activity based on its inhibitory activities against the expression of

388 TNF-α in APAP-intoxicated mice kidney (Ucar et al. 2013). In another study, bazhen decoction

389 was reported to possess some protective role against APAP toxicity through suppression of

390 various pro-inflammatory cytokines, notably TNF-α, IL-1β and IL-6 (Song et al. 2014). The

391 results of our study showed elevated levels of TNF-α, IL-1β, IL-6 in APAP-administered mice

392 kidney, which was significantly suppressed by the MO leaves extract. Anti-inflammatory

393 cytokines such as IL-10 were also produced by the Kupffer cells at the site of tubular

394 inflammation to check this detrimental influence.

395   Ucar et al. (2013) reported that n-acetylcysteine and ozone therapy also played a renal-

396 protective role in APAP toxicity by significantly elevating IL-10 cytokine level (Ucar et al.

397 2013). Our results consistently indicated significant suppression of IL-10 cytokine in APAP-

398 intoxicated mice, which was effectually restored by MO leaf extract in a dose-dependent manner.

399 Thus MO leaf extract protects the inflammatory-mediated exacerbation of renal damage in

400 APAP-intoxicated mice by modulation of both pro and anti-inflammatory cytokine level. Thus,

401 the postulated overall mechanism of action of MO leaf extract against APAP-induced nephro-

402 toxicity pathway has been clearly elucidated in this study (Fig. 5).

403

404 **5.   CONCLUSION**

405 The MO leaf extracts shielded kidney from APAP toxicity through enhancement of the

406 endogenous antioxidant system/ enzymatic level to counteract the oxidative stress environment

407 (ROS). Certain naturally incorporated bioactive constituents, and highly accessible essential

408 trace elements present in MO leaves aided the renal protective activity. The MO leaf extract also

409 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.

417 **REFERENCES**

418 Ahmad ST, Arjumand W, Nafees S, Seth A, Ali N, Rashid S, and Sultana S. 2012. Hesperidin
419   alleviates acetaminophen induced toxicity in wistar rats by abrogation of oxidative stress,
420   apoptosis and inflammation. *Toxicology Letters* 208:149-161.
421   http://dx.doi.org/10.1016/j.toxlet.2011.10.023

422 Anwar F, Latif S, Ashraf M, and Gilani AH. 2007. Moringa oleifera: a food plant with multiple
423   medicinal uses. *Phytother Res* 21:17-25. 10.1002/ptr.2023

424 Aycan IO, Tokgoz O, Tufek A, Alabalik U, Evliyaoglu O, Turgut H, Celik F, and Guzel A.
425   2015. The use of thymoquinone in nephrotoxicity related to acetaminophen. *Int J Surg*
426   13:33-37. 10.1016/j.ijsu.2014.11.020

427 Bektur NE, Sahin E, Baycu C, and Unver G. 2013. Protective effects of silymarin against
428   acetaminophen-induced hepatotoxicity and nephrotoxicity in mice. *Toxicol Ind Health*.
429   10.1177/0748233713502841

430 Bjorck S, Svalander CT, and Aurell M. 1988. Acute renal failure after analgesic drugs including
431   paracetamol (acetaminophen). *Nephron* 49:45-53.

432 Cekmen M, Ilbey YO, Ozbek E, Simsek A, Somay A, and Ersoz C. 2009. Curcumin prevents
433   oxidative renal damage induced by acetaminophen in rats. *Food Chem Toxicol* 47:1480-
434   1484. 10.1016/j.fct.2009.03.034

435 Dichi I, Breganó JW, and Cecchini R. 2014. *Role of Oxidative Stress in Chronic Diseases*: CRC
436   Press.

437   Eguia L, and Materson BJ. 1997. Acetaminophen-related acute renal failure without fulminant
438   liver failure. *Pharmacotherapy* 17:363-370.
439   Ghosh J, Das J, Manna P, and Sil PC. 2010. Acetaminophen induced renal injury via oxidative
440   stress and TNF-α production: Therapeutic potential of arjunolic acid. *Toxicology* 268:8-
441   18. http://dx.doi.org/10.1016/j.tox.2009.11.011
442   Goddard J, Strachan FE, and Bateman DN. 2003. Urinary sodium and potassium excretion as
443   measures of ibuprofen nephro-toxicity. *Journal of Toxicology: Clinical Toxicology*
444   41:747.

445   Gowrishankar R, Kumar M, Menon V, Divi SM, Saravanan M, Magudapathy P, Panigrahi B,
446   Nair K, and Venkataramaniah K. 2010. Trace element studies on Tinospora
447   cordifolia(Menispermaceae), Ocimum sanctum (Lamiaceae), Moringa oleifera
448   (Moringaceae), and Phyllanthus niruri (Euphorbiaceae) using PIXE. *Biological trace*
449   *element research* 133:357-363.
450   Hamid ZA, Budin SB, Jie NW, Hamid A, Husain K, and Mohamed J. 2012. Nephroprotective
451   effects of Zingiber zerumbet Smith ethyl acetate extract against paracetamol-induced
452   nephrotoxicity and oxidative stress in rats. *Journal of Zhejiang University Science B*
453   13:176-185.
454   Harris ED. 1992. Copper as a cofactor and regulator of copper,zinc superoxide dismutase. *J Nutr*
455   122:636-640.
456   He Q, Kim J, and Sharma RP. 2004. Silymarin Protects Against Liver Damage in BALB/c Mice
457   Exposed to Fumonisin B1 Despite Increasing Accumulation of Free Sphingoid Bases.
458   *Toxicological Sciences* 80:335-342. 10.1093/toxsci/kfh148
459   Hook JB. 1993. *Toxicology of the Kidney*: CRC Press.
460   Hörl WH. 2010. Nonsteroidal Anti-Inflammatory Drugs and the Kidney. *Pharmaceuticals*
461   3:2291.
462   Isik B, Bayrak R, Akcay A, and Sogut S. 2006. Erdosteine Against Acetaminophen Induced
463   Renal Toxicity. *Molecular and Cellular Biochemistry* 287:185-191. 10.1007/s11010-005-
464   9110-6
465   Jarapala SR, Kandlakunta B, and Thingnganing L. 2014. Evaluation of Trace Metal Content by
466   ICP-MS Using Closed Vessel Microwave Digestion in Fresh Water Fish. *Journal of*
467   *Environmental and Public Health* 2014:8. 10.1155/2014/201506

468 Karthivashan G, Arulselvan P, and Fakurazi S. 2015a. Pathways involved in acetaminophen
469   hepatotoxicity with specific targets for inhibition/downregulation. *RSC Advances* 5:62040-
470   62051. 10.1039/c5ra07838e

471 Karthivashan G, Arulselvan P, Tan SW, and Fakurazi S. 2015b. The molecular mechanism
472   underlying the hepatoprotective potential of Moringa oleifera leaves extract against
473   acetaminophen induced hepatotoxicity in mice. *Journal of Functional Foods* 17:115-126.
474   http://dx.doi.org/10.1016/j.jff.2015.05.007

475 Karthivashan G, Tangestani Fard M, Arulselvan P, Abas F, and Fakurazi S. 2013. Identification
476   of Bioactive Candidate Compounds Responsible for Oxidative Challenge from Hydro-
477   Ethanolic Extract of Moringa oleifera Leaves. *Journal of Food Science* 78:C1368-C1375.
478   10.1111/1750-3841.12233

479 Li C, Liu J, Saavedra JE, Keefer LK, and Waalkes MP. 2003. The nitric oxide donor, V-
480   PYRRO/NO, protects against acetaminophen-induced nephrotoxicity in mice. *Toxicology*
481   189:173-180. http://dx.doi.org/10.1016/S0300-483X(03)00129-X

482 Mazer M, and Perrone J. 2008. Acetaminophen-induced nephrotoxicity: pathophysiology,
483   clinical manifestations, and management. *J Med Toxicol* 4:2-6.

484 Möller-Hartmann W, and Siegers CP. 1991. Nephrotoxicity of paracetamol in the rat—
485   mechanistic and therapeutic aspects. *Journal of Applied Toxicology* 11:141-146.
486   10.1002/jat.2550110213

487 Ozbek E. 2012. Induction of Oxidative Stress in Kidney. *International Journal of Nephrology*
488   2012:465897. 10.1155/2012/465897

489 Pakravan N, Shokrzadeh M, Akbari F, and Shadboorestan A. 2015. Effect of a Toxic Dose of
490   Acetaminophen on Electrolytes and Histopathological Changes in the Kidney.
491   *International Journal of Clinical Toxicology* 2:64-70.

492 Palani S, Raja S, Naresh R, and Kumar BS. 2010. Evaluation of nephroprotective, diuretic, and
493   antioxidant activities of plectranthus amboinicus on acetaminophen-induced nephrotoxic
494   rats. *Toxicol Mech Methods* 20:213-221. 10.3109/15376511003736787

495 Paul S, Islam MA, Tanvir EM, Ahmed R, Das S, Rumpa N-E, Hossen MS, Parvez M, Gan SH,
496   and Khalil MI. 2016. Satkara (Citrus macroptera) Fruit Protects against Acetaminophen-

497   Induced Hepatorenal Toxicity in Rats. *Evidence-Based Complementary and Alternative*
498   *Medicine* 2016.

499   Pradhan S, Mandal S, Roy S, Mandal A, Das K, and Nandi DK. 2013. Attenuation of uremia by
500   orally feeding alpha-lipoic acid on acetaminophen induced uremic rats. *Saudi*
501   *Pharmaceutical Journal* 21:187-192. http://dx.doi.org/10.1016/j.jsps.2012.03.003

502   Prashanth L, Kattapagari KK, Chitturi RT, Baddam VRR, and Prasad LK. 2015. A review on
503   role of essential trace elements in health and disease. *Journal of Dr NTR University of*
504   *Health Sciences* 4:75.

505   Samal L, and Mishra C. 2011. Significance of Nickel in Livestock Health and Production.
506   *IJAVMS* 5:349-361.

507   Sanz AB, Santamaria B, Ruiz-Ortega M, Egido J, and Ortiz A. 2008. Mechanisms of renal
508   apoptosis in health and disease. *J Am Soc Nephrol* 19:1634-1642.
509   10.1681/asn.2007121336

510   Sebastian MM, Baskin S, and Czerwinski SE. 2007. Chapter 11 - Renal toxicity. In: Gupta RC,
511   ed. *Veterinary Toxicology*. Oxford: Academic Press, 161-I.

512   Sharifudin SA, Fakurazi S, Hidayat MT, Hairuszah I, Aris Mohd Moklas M, and Arulselvan P.
513   2013. Therapeutic potential of Moringa oleifera extracts against acetaminophen-induced
514   hepatotoxicity in rats. *Pharmaceutical Biology* 51:279-288.
515   10.3109/13880209.2012.720993

516   Siddhuraju P, and Becker K. 2003. Antioxidant Properties of Various Solvent Extracts of Total
517   Phenolic Constituents from Three Different Agroclimatic Origins of Drumstick Tree
518   (Moringa oleifera Lam.) Leaves. *Journal of Agricultural and Food Chemistry* 51:2144-
519   2155. 10.1021/jf020444+

520   Song E, Fu J, Xia X, Su C, and Song Y. 2014. Bazhen Decoction Protects against
521   Acetaminophen Induced Acute Liver Injury by Inhibiting Oxidative Stress, Inflammation
522   and Apoptosis in Mice. *PLoS ONE* 9:e107405. 10.1371/journal.pone.0107405

523   Tarloff J, and Kinter L. 1997. In vivo methodologies used to assess renal function.
524   *Comprehensive Toxicology* 7:99-120.

525   Ucar F, Taslipinar MY, Alp BF, Aydin I, Aydin FN, Agilli M, Toygar M, Ozkan E, Macit E,
526   Oztosun M, Cayci T, and Ozcan A. 2013. The effects of N-acetylcysteine and ozone

527   therapy on oxidative stress and inflammation in acetaminophen-induced nephrotoxicity

528   model. *Ren Fail* 35:640-647. 10.3109/0886022x.2013.780530

529 Yin H, Xu L, and Porter NA. 2011. Free radical lipid peroxidation: mechanisms and analysis.

530   *Chem Rev* 111:5944-5972. 10.1021/cr200084z.

531

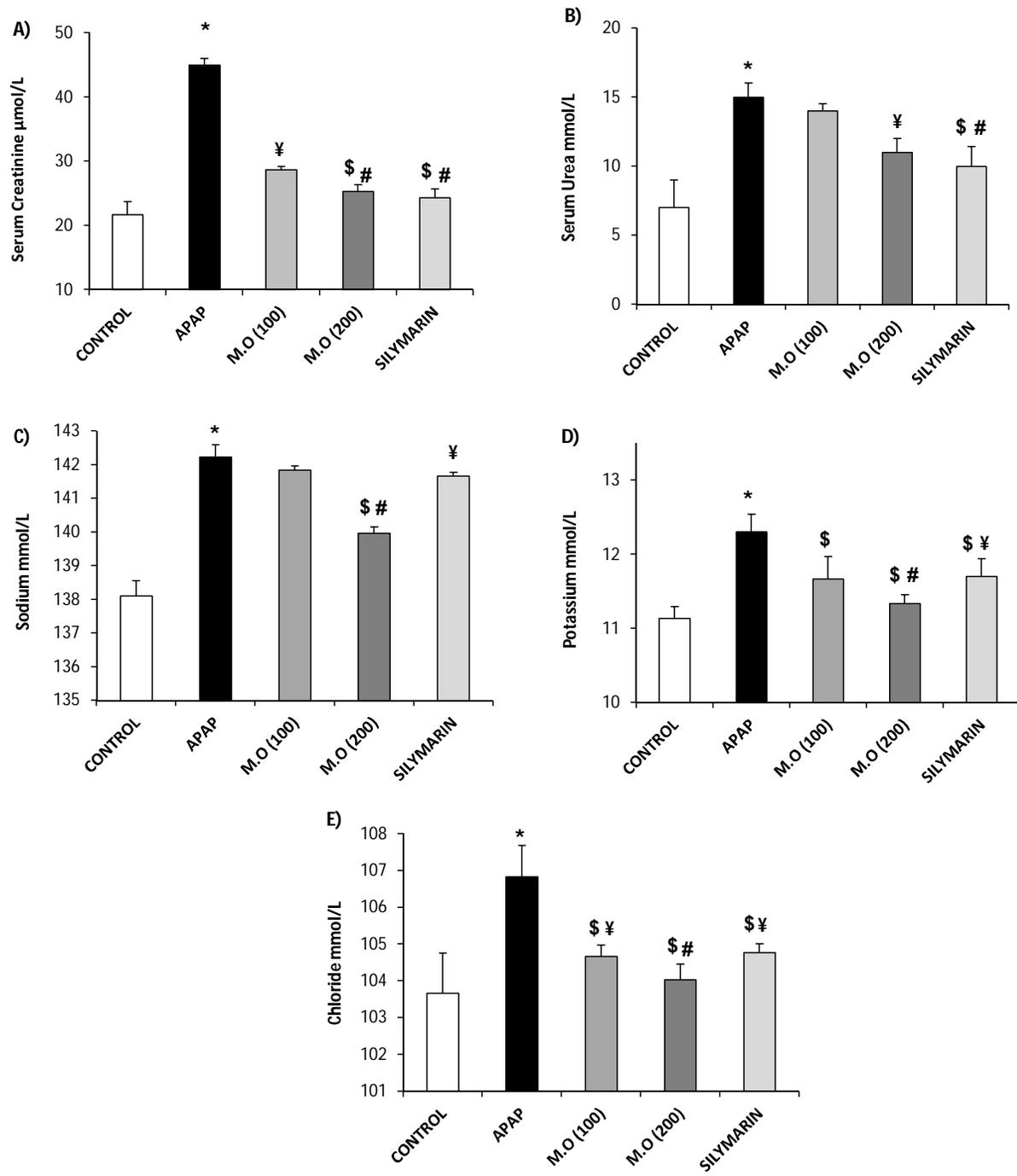
Modulation of Serum biochemical markers and electrolytes level

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)

B)

C)

D)

E)

Level of antioxidant enzymes in kidney tissue

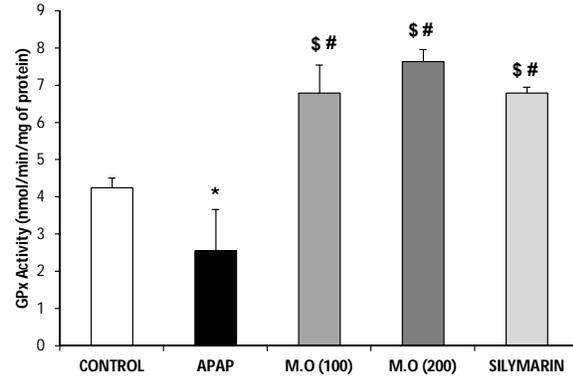
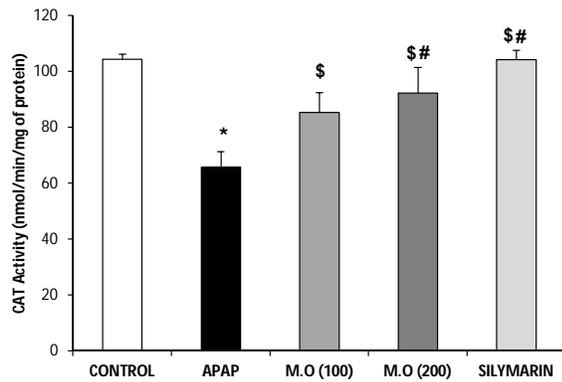
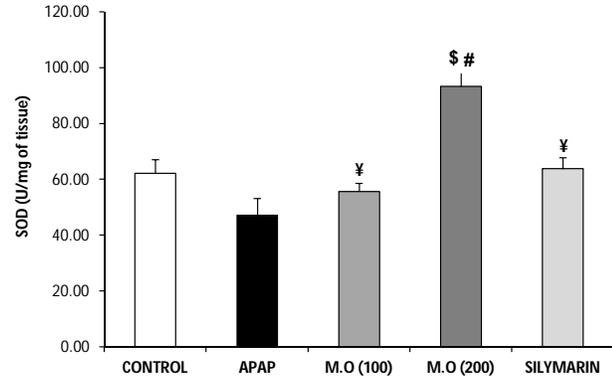
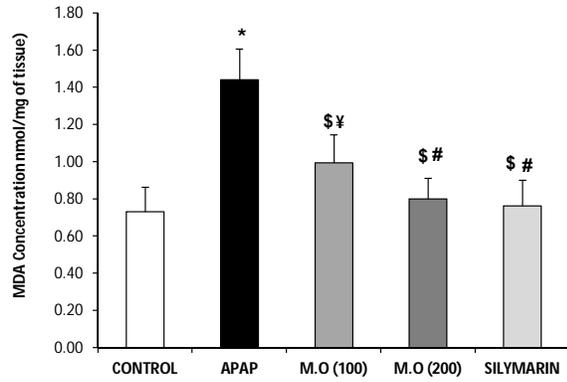
Figure 2: Dose dependent effect of *Moringa oleifera* (M.O) leaves extract against APAP intoxicated mice kidney via augmentation of endogenous antioxidant status: [A] lipid peroxidation activity (MDA); endogenous antioxidant enzyme levels ([B]-SOD, [C]-CAT & [D]-GPx). Values are expressed as the mean ± 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.

Alteration in the level of serum inflammatory markers

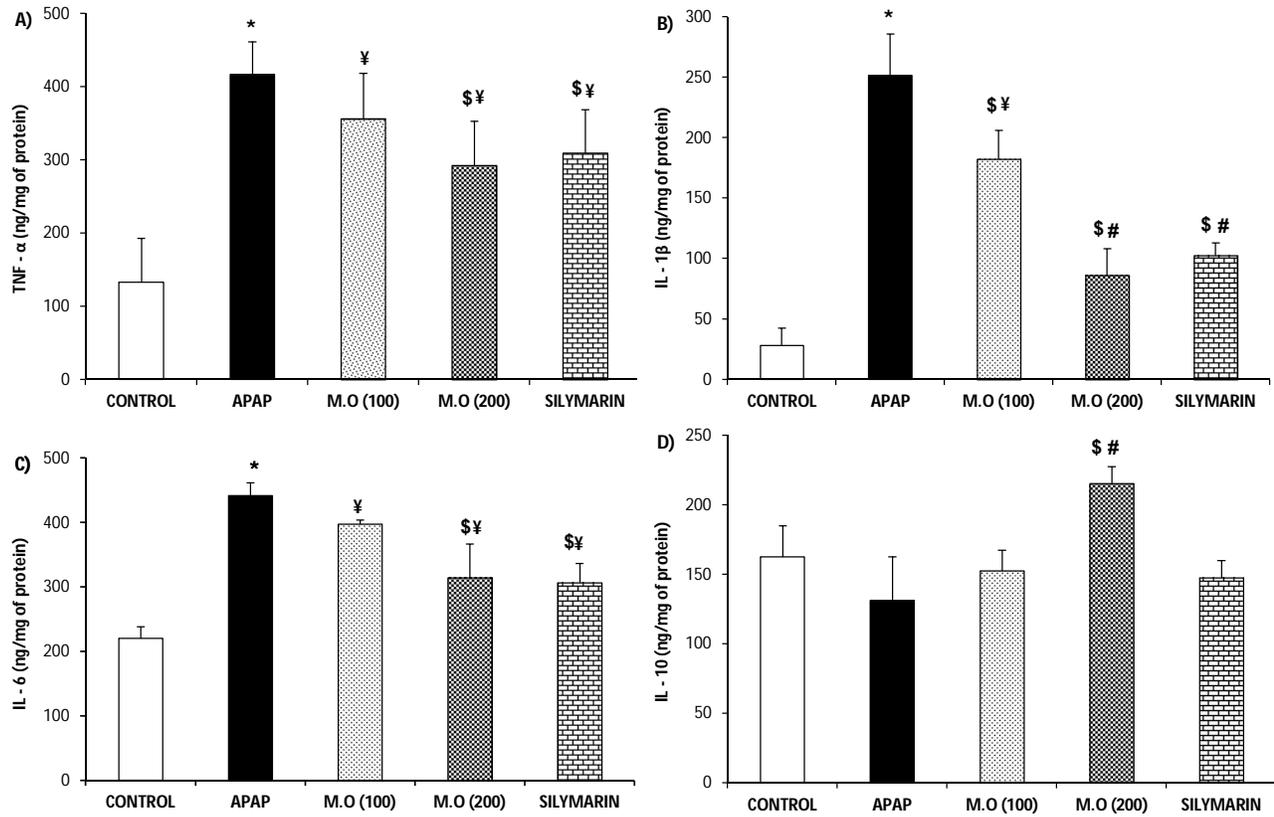
Figure 3: Modulatory effect of *Moringa oleifera* (M.O) leaves extract against APAP intoxicated kidney inflammatory cytokines – [A] TNF-α; [B] IL-1β; [C] IL-6; [D] IL-10. Values are expressed as the mean ± SEM of n=6 mice in each group. The normality distribution of the data has been confirmed by Shapiro-Wilk 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.

Photographs of Histopathological modification in kidney tissue

Figure 4: Photographic sections (H&E 20X – **1**; 40X - **2**) of the mice kidney, A_1: Control group showing normal histological architecture of tubules (T) and Glomerulus (G) A_2: Flawless tubules with striated border (T), intact glomerulus (arrow) with surrounding Bowman's capsule (line). B_1: Kidney of mice treated with APAP showing disorganized glomerulus (DG), dilated tubules (DT) with tubular casting (C) and inflammation (I) B_2: severely disorganized glomerulus (dotted arrow), tubular dilation (arrow head), and inflammatory casting were observed 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) C_2: few disorganized glomerulus (dotted arrow) and tubular dilation (arrow head) with tubular casting (C) and inflammation (I) are noticed. 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) D_2: mild tubular dilation (arrow head) with tubular casting (C) and inflammation (I) are noticed. E_1: Kidney of mice treated with APAP and silymarin (positive control) showing preserved glomerulus (G) and tubules (T) architecture 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.

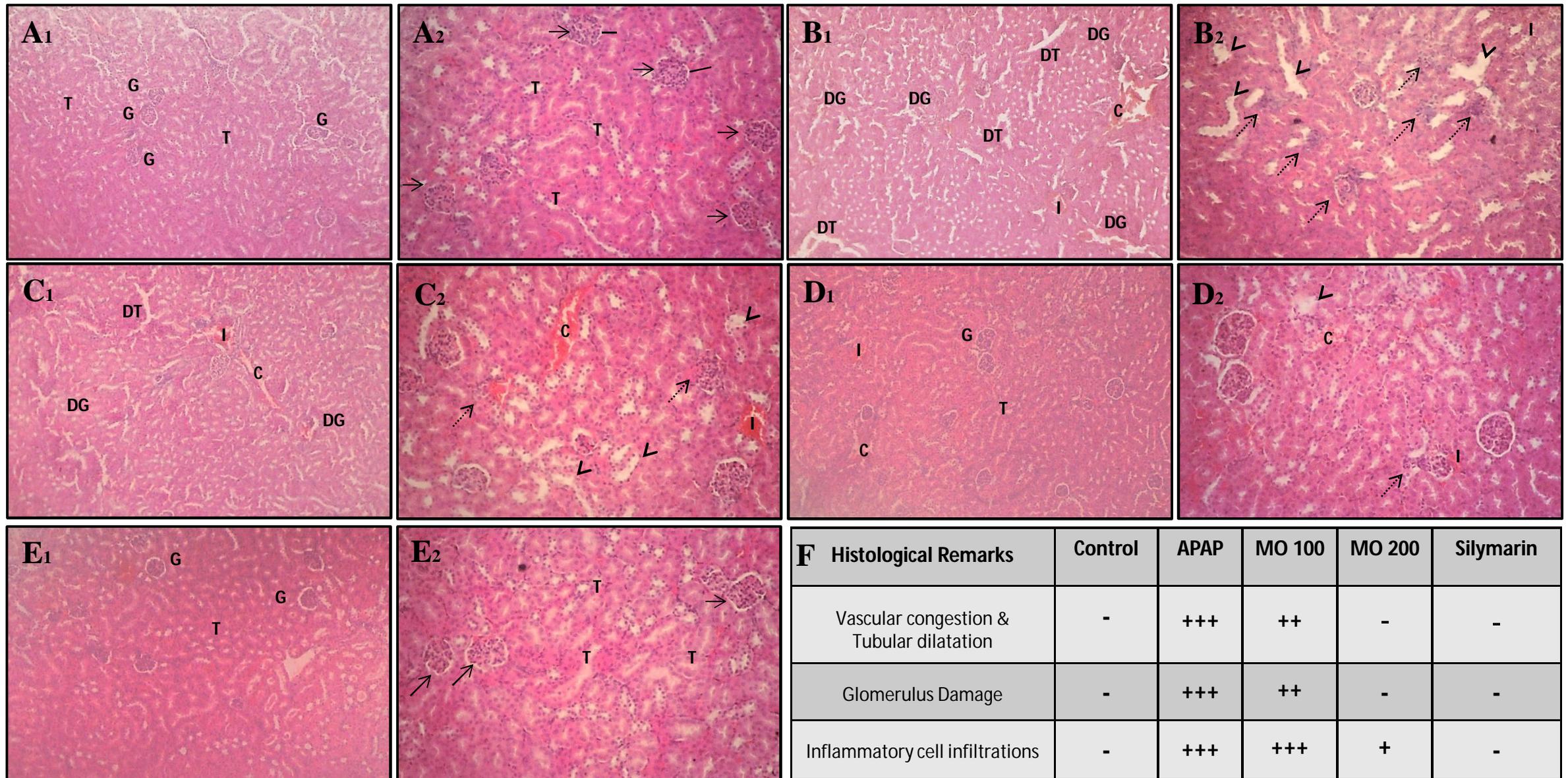
F	Histological Remarks	Control	APAP	MO 100	MO 200	Silymarin
	Vascular congestion & Tubular dilatation	-	+++	++	-	-
	Glomerulus Damage	-	+++	++	-	-
	Inflammatory cell infiltrations	-	+++	+++	+	-

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

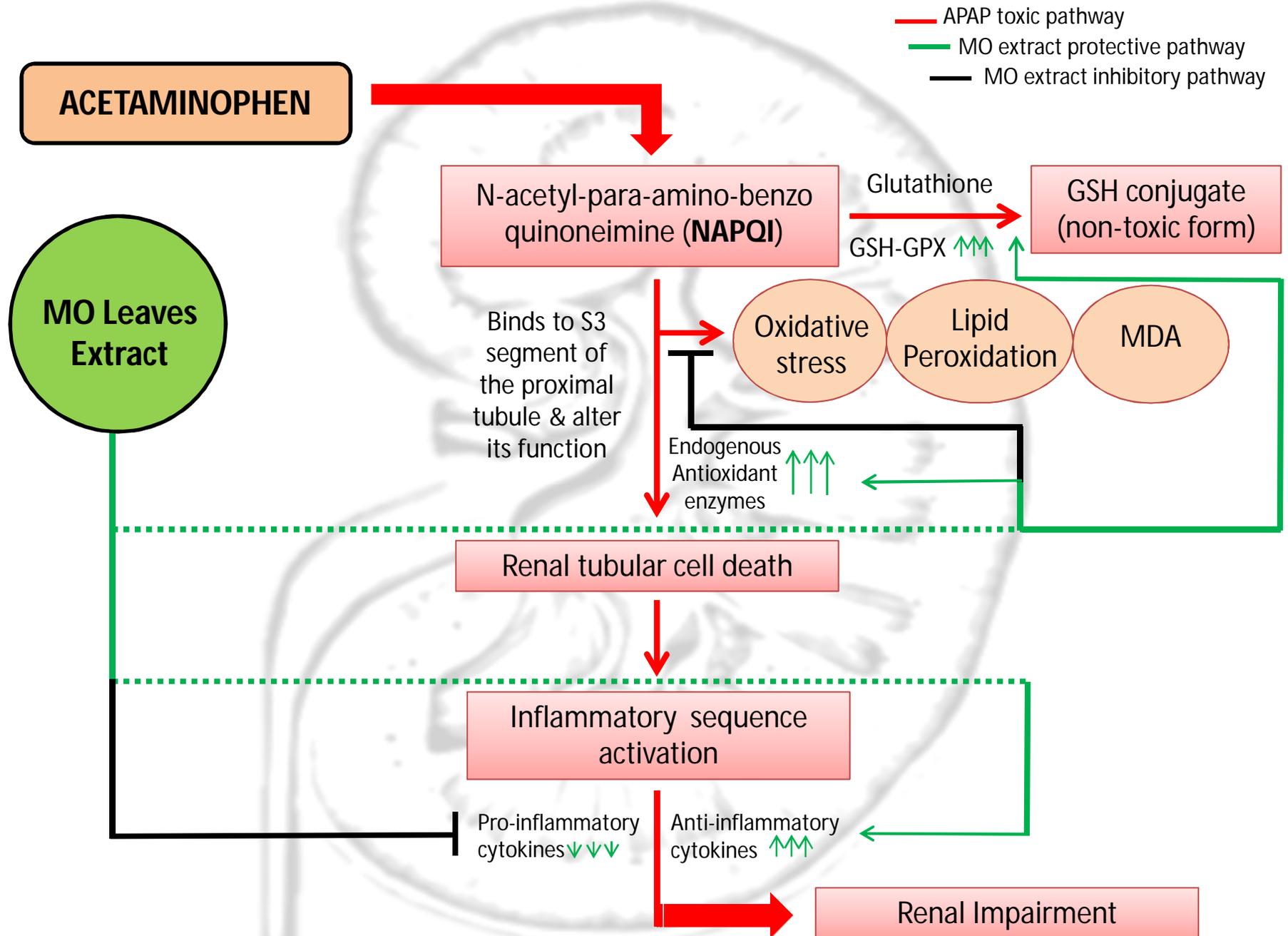
Figure 5: Mechanism of action of MO leaves extract against APAP induced renal toxicity pathway: - The active constituents and essential trace elements of MO leaves extract successfully enhance the GSH-GPx and endogenous antioxidant system thereby inhibit the oxidative stress mediated renal impairment, 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.

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.