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Commiphora molmol protects against methotrexate-induced nephrotoxicity by up-regulating Nrf2/ARE/HO-1 signaling

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ABSTRACT

Commiphora molmol possesses multiple therapeutic benefits against various diseases; however, its protective role against methotrexate (MTX) renal toxicity has not been previously investigated. MTX is a dihydrofolate reductase inhibitor that can induce acute kidney injury (AKI). This study evaluated the *in vitro* antioxidant activity and the protective effect of *C. molmol* resin extract against MTX-induced oxidative stress, inflammation and renal injury. Male Wistar rats received 125 and 250 mg/kg *C. molmol* resin extract for 15 days and a single injection of MTX at day 16. *C. molmol* showed a radical scavenging activity against DPPH, superoxide and nitric oxide (NO) radicals. Rats received MTX showed renal injury evidenced by the significantly elevated serum creatinine and urea, and the histological alterations. The kidney of MTX-induced rats exhibited increased lipid peroxidation, NO, NF-κB and pro-inflammatory cytokines. Pre-treatment with *C. molmol* prevented MTX-induced kidney injury and attenuated oxidative stress and inflammation. *C. molmol* down-regulated Bax and enhanced the activity and expression of the antioxidant defenses. Furthermore, the expression of Bcl-2, Nrf2, NQO-1 and HO-1 was down-regulated in the kidney of MTX-induced rats. Pre-treatment with *C. molmol* resin up-regulated Bcl-2 and activated Nrf2/HO-1 signaling in the kidney of MTX-induced rats. In conclusion, *C. molmol* resin provided protection against MTX-induced AKI *via* activation of Nrf2 signaling and mitigation of oxidative stress.

1. Introduction

The kidneys maintain homeostasis and perform an essential role in the metabolism and excretion of toxins and drugs [1]. Acute kidney injury (AKI) is an alarming problem with untoward economic and health consequences [2]. Nephrotoxic drugs, the main culprit behind AKI, are therapeutic agents that cause adverse effects and compromise renal function [3]. Although drug development has produced several life-saving therapeutic agents for cancer patients, the use of these medications was complicated by nephrotoxicity. Drug-induced AKI among hospitalized patients has been estimated to account for 19% to 26% of cases [4]. The nephrotoxic potential of drugs relates to their molecular characteristics, metabolites, and tendency to crystalize and precipitate within tubular lumens [5]. Methotrexate (MTX) is a dihydrofolate reductase inhibitor known to cause crystalline nephropathy due to its crystallization, precipitation and direct toxic effects on the renal tubules [6]. MTX is a disease-modifying drug used for the treatment of neoplastic diseases, psoriasis, rheumatoid arthritis and lupus erythematosus [7–9]. The therapeutic applications of MTX are often limited by its adverse effects [10,11]. High-dose MTX causes kidney damages varying from subclinical tubulopathy to AKI [12].

Numerous studies have demonstrated the role of oxidative stress and inflammation in MTX toxicity [13–16]. MTX increases the production of reactive oxygen species (ROS) *via* diminishing the intracellular levels of nicotinamide adenine dinucleotide phosphate (NADPH) [17], stimulation of neutrophils [18], and decreasing the remethylation of homocysteine [19]. Additionally, MTX can up-regulate the expression of nuclear factor-kappaB (NF-κB) and the production of pro-inflammatory cytokines [20], and activate the mitochondrial pathway of apoptosis [21]. Hence, agents with antioxidant/anti-

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inflammatory potential can attenuate MTX-induced nephrotoxicity.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a basic leucine zipper protein that regulates the expression of protective and antioxidant genes, including NAD(P)H quinone dehydrogenase 1 (NQO-1) and heme oxygenase 1 (HO-1) [22–24]. Under unstressed conditions, Nrf2 is quickly degraded by a cluster of proteins in the cytoplasm. The substrate adaptor protein Kelch like-ECH-associated protein 1 (KEAP1) facilitates the ubiquitination of Nrf2 by Cullin 3. Ubiquitinated Nrf2 is degraded by proteasomes and its components are then recycled [25]. By disrupting critical cysteine residues in Keap1, oxidative stress reduces the ubiquitination of Nrf2 which translocate into the nucleus and activate the transcription of genes that protect against oxidative damage and inflammation [26].

Medicinal plants and their bioactive constituents have been well-acknowledged in counteracting drug-induced toxicity. Myrrh is a resinous exudate of a number of small trees of the genus *Commiphora* (Family *Burseraceae*). *Commiphora molmol* is a shrub resembling tropical tree that thrive in arid/semi-arid regions [27]. The oleo-gum resin of *C. molmol* possesses therapeutic beneficial effects and has been used throughout history in the treatment of various diseases. Recently, we have reported that myrrh attenuate hepatocarcinogenesis [28] and hyperammonemia [29] by activating Nrf2/antioxidant response element (ARE)/HO-1 signaling. To date, nothing has yet been reported on the potential of myrrh to protect against MTX nephrotoxicity. Therefore, the aim of this study was to investigate the preventive effect of *C. molmol* resin extract on MTX-induced oxidative stress, inflammation, apoptosis and kidney injury in rats. We assumed that *C. molmol* can attenuate MTX-induced nephrotoxicity by up-regulating Nrf2 signaling.

2. Materials and methods

2.1. Chemicals and reagents

MTX was supplied by Shanxi PUDE Pharmaceutical Company (Shanxi, China). Trichloroacetic acid (TBA), nitro blue tetrazolium (NBT), thiobarbituric acid (TCA), pyrogallol, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 2,2-diphenyl-1-picrylhydrazyl (DPPH), phenazine methosulfate (PMS), 1,1,3,3-tetramethoxypropane, sodium nitroprusside (SNP), sodium dodecyl sulfate (SDS), reduced glutathione (GSH), hydrogen peroxide, and Griess reagent were purchased from Sigma-Aldrich (USA). Creatinine and urea assay kits were supplied by Spinreact (Spain). Rat NF-κB, tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) ELISA kits were purchased from MyBioSource (CA, USA). Antibodies for Nrf2 and β-actin were purchased from Santa Cruz Biotechnology (USA). All other chemicals and reagents were supplied by Sigma-Aldrich or other standard suppliers.

2.2. Preparation of C. molmol extract

The oleo-gum resin of *C. molmol* was purchased from Harraz Medicinal Plant Company (Cairo, Egypt), ground into a fine powder and extracted with 90% ethanol. The mixture was kept at 4 °C with daily shaking for 3 days [30]. The extract was filtered and the solvent was evaporated under vacuum at a temperature not exceeding 45 °C. The extract was stored frozen till use.

2.3. Assay of radical scavenging activity

2.3.1. DPPH radical scavenging activity

A methanolic solution of 0.1 mM DPPH was prepared and mixed with different concentrations of *C. molmol* resin extract dissolved in methanol. The reaction mixture was shaken and kept protected from light at room temperature for 30 min. The absorbance was measured at 517 nm using methanol as a blank [31]. Ascorbic acid was used as a standard antioxidant.

2.3.2. Superoxide radical ($O_2\cdot^-$) scavenging assay

The scavenging activity of *C. molmol* resin extract against $O_2\cdot^-$ was determined as previously described [32]. Briefly, various concentrations of *C. molmol* resin extract were mixed with 120 μM PMS, 936 μM NADH, 0.1 M Tris − HCl and 300 μM NBT. The mixture was incubated for 5 min at room temperature and the absorbance was read at 560 nm. Ascorbic acid was used as a standard antioxidant.

2.3.3. Nitric oxide radical (NO•) scavenging activity

In this assay, *C. molmol* resin extract was mixed with 10 mM SNP in phosphate buffered saline (PBS; pH 7.4) and the mixture was incubated for 2 h at room temperature. Griess reagent was added and the absorbance was read at 546 nm [33]. Ascorbic acid was used as a standard antioxidant.

2.4. Experimental animals and treatments

Adult male Wistar rats weighing 140–160 g, obtained from the National Research Centre (NRC, Giza, Egypt), were included in the present study. The animals were maintained at normal atmospheric temperature (23 ± 2 °C) and relative humidity of 50–60% on a 12 h light/dark cycle. The rats were supplied a standard laboratory diet of known composition and water *ad libitum*. All animal procedures were approved by the Institutional Research Ethics Committee, Beni-Suef University (Egypt).

The rats were randomly divided into 4 groups, each comprising 8 as following:

Group I (Control): rats received the vehicle 0.5% carboxymethyl cellulose (CMC) *via* oral gavage for 15 days and a single intraperitoneal (i.p.) dose of saline at day 16.

Group II (MTX): rats received 0.5% CMC *via* oral gavage for 15 days and a single i.p. injection of MTX (20 mg/kg body weight) dissolved in saline [15] at day 16.

Group III (MTX + 125 mg/kg *C. molmol*): rats received 125 mg/kg *C. molmol* resin extract [28] dissolved in 0.5% CMC *via* oral gavage for 15 days and a single i.p. injection of 20 mg/kg MTX at day 16.

Group III (MTX + 250 mg/kg *C. molmol*): rats received 250 mg/kg *C. molmol* resin extract [28] dissolved in 0.5% CMC *via* oral gavage for 15 days and a single i.p. injection of MTX at day 16.

At the end of the experiment (Day 19), rats of all experimental groups were sacrificed, and blood was collected. The blood was left to coagulate and centrifuged to separate serum. The rats were then dissected, and both kidneys were excised and washed with ice cold saline. Samples from the kidneys were fixed in 10% neutral buffered formalin for histological processing while others were homogenized (10% w/v) in PBS (pH 7.4). The homogenate was centrifuged, and the clear supernatant was collected and stored at −80 °C for biochemical assays. Other samples from the kidneys were kept at −80 °C for RNA isolation and western blotting.

2.5. Biochemical assays

2.5.1. Determination of serum urea and creatinine

Serum urea and creatinine levels were determined using reagent kits purchased from Spinreact (Spain), following the methods of Coulombe and Favreau [34] and Larsen [35], respectively.

2.5.2. Determination of NF-κB, TNF-α and IL-1β

NF-κB, TNF-α and IL-1β were determined in the kidney homogenate using specific ELISA kits purchased from MyBioSource (CA, USA), according to the manufacturers' instructions.

2.5.3. Determination of lipid peroxidation, nitric oxide (NO) and antioxidant defenses

Lipid peroxidation was determined in the kidney homogenate as malondialdehyde (MDA) [36]. NO was assayed as nitrite according to

Green et al. [37] using Griess reagent. GSH, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were determined in the kidney homogenates using the methods of Beutler et al. [38], Marklund and Marklund [39], Matkovics et al. [40] and Cohen et al. [41], respectively.

2.6. Histological examination

Samples from the kidney were fixed in 10% neutral buffered formalin for 24 h. The fixed samples were proceeded and 5-μm sections were cut using a rotatory microtome. The sections were then stained with hematoxylin and eosin (H&E) and examined using a light microscope.

2.7. Gene expression study

The effect of *C. molmol* resin on the gene expression levels of Nrf2, NQO-1, HO-1, SOD, CAT, GPx, Bcl-2 (B-cell lymphoma 2) and Bax (Bcl-2-associated X protein) was studied using qRT-PCR as we previously described [42]. Total RNA was isolated from frozen kidney samples using TRIzol reagent (Invitrogen, USA). The isolated RNA was purified using RNeasy purification kit (Qiagen, Germany), and quantified at 260 nm using a nanodrop. cDNA was synthesized from 2 μg RNA using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, USA). The cDNAs were amplified using SYBR Green master mix (Fermentas, USA) and the primers set listed in Table 1. The amplification data were analyzed using the $2^{-\Delta\Delta Ct}$ method [43] and the results were normalized to β-actin.

2.8. Western blotting

Total protein was isolated from the frozen kidney samples using RIPA buffer with proteinase inhibitors, and their concentration was determined using Bradford reagent. The protein expression of Nrf2 in the kidney samples were determined using Western blotting as previously described [44]. Fifty μg protein was separated on SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked for 1 h in 5% milk/Tris-buffered saline-tween (TBST) incubated with primary antibodies for Nrf2 and β-actin overnight at 4 °C. The membranes were washed with TBST and probed with the secondary antibody. The blots were developed using enhanced chemiluminescence kit (BIO-RAD, USA). ImageJ (version 1.32j, NIH, http://rsb.info.nih/ij/) was used to quantify intensity of the bands and the results were presented as percent of control.

Table 1
Primers used for qRT-PCR.

Gene	GenBank Accession number	Sequence (5'-3')
NRF2	NM_031789	F: TTGTAGATGACCATGAGTCGC R: TGTCCTGCTGTATGCTGCTT
NQO-1	NM_017000.3	F: GGCCATCATTTGGGCAAGTC R: TCCTTGTGGAACAAAGGCGA
HO-1	NM_012580	F: GTAAATGCAGTGTTGGCCCC R: ATGTGCCAGGCATCTCCTTC
SOD	NM_012880.1	F: ACACCTATGCACTCCACAGAC R: ACATTCGACCTCTGGGGGTA
CAT	M25670.1	F: GCGGGAACCCAATAGGAGAT R: CAGGTTAGGTGTGAGGGACA
GPX	NM_183403.2	F: GCATGGCTTACATCGCCAAG R: AGTCCCGGGTAGTTGTTCCT
BAX	NM_017059.2	F: AGGACGCATCCACCAAGAAG R: CAGTTGAAGTTGCCGTCTGC
BCL-2	NM_016993.1	F: ACTCTTCAGGGATGGGGTGA R: TGACATCTCCCTGTTGACGC
Actb	NM_031144.3	F: AGGAGTACGATGAGTCCGGC R: CGCAGCTCAGTAACAGTCCG

Actb: β-actin gene.

2.9. Statistical analysis

Data analysis was performed using Graphpad Prism 5 (La Jolla, CA, USA). The results were expressed as means ± standard error of the mean (SEM) and were compared using the one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. In vitro radical scavenging activity of C. molmol resin extract

The antioxidant and radical scavenging activity of *C. molmol* resin extract was tested using the DPPH·, $O_2\cdot^-$ and NO· scavenging assays as shown in Fig. 1. *C. molmol* resin extract showed a concentration-dependent increase in its scavenging activity against DPPH· (Fig. 1A), $O_2\cdot^-$ (Fig. 1B) and NO· (Fig. 1C).

3.2. C. molmol prevents MTX-induced kidney injury in rats

To evaluate the protective potential of *C. molmol* resin extract on kidney injury induced by MTX, the kidney function markers creatinine and urea were assayed, and histopathological examination was performed.

Rats received a single i.p. injection of MTX showed a significant ($P < 0.001$) increase in serum creatinine levels when compared with the control rats. On the other hand, MTX-induced rats pre-treated with either 125 or 250 mg/kg *C. molmol* resin extract showed a significant ($P < 0.001$) decrease in serum creatinine levels (Fig. 2A). Similarly, circulating urea levels were significantly ($P < 0.001$) increased in the MTX-induced rats when compared with the control group as depicted in Fig. 2B. Pre-treatment of the MTX-induced rats for 15 days with *C. molmol* resin extract produced a marked improvement in serum urea levels (Fig. 2B).

To further confirm the protective effect of *C. molmol* resin extract, H&E-stained sections in the kidney of control and treated rats were prepared and examined. Control rats showed normal histological structure of the kidney with normal glomeruli and renal tubules (Fig. 3A). MTX-induced rats exhibited several histological alterations, including leukocyte infiltration, interstitial hemorrhage and degenerative changes of tubular epithelial cells (Fig. 3B & C). Rats received 125 mg/kg (Fig. 3D) and 250 mg/kg (Fig. 3E) showed improved structure of the glomeruli and renal tubules.

3.3. C. molmol mitigates MTX-induced oxidative stress in the kidney of rats

The lipid peroxidation marker MDA was significantly ($P < 0.001$) increased in the kidney of MTX-induced rats when compared with the control group as represented in Fig. 4A. Rats treated with 125 and 250 mg/kg *C. molmol* resin extract for 15 days before the administration of MTX showed a significant ($P < 0.001$) improvement in kidney MDA levels (Fig. 4A). Similar to the MDA results, MTX-induced rats exhibited a significant ($P < 0.001$) increase in kidney NO levels when compared with the control rats (Fig. 4B). Pre-treatment with either dose of *C. molmol* resin extract markedly ($P < 0.001$) diminished NO levels in the kidney of MTX-induced rats (Fig. 4B).

The protective effect of *C. molmol* resin extract against MTX-induced oxidative stress was supported by data of the enzymatic and non-enzymatic antioxidant defenses. GSH showed a significant ($P < 0.001$) decrease in the kidney of MTX-induced rats when compared with the control group as depicted in Fig. 4C. MTX-induced rats pre-treated with 125 mg/kg *C. molmol* resin extract showed a significant ($P < 0.01$) improvement in kidney GSH levels. The higher dose of *C. molmol* resin extract significantly ($P < 0.001$) alleviated GSH levels in the kidney of MTX-induced rats (Fig. 4C). Activity of the antioxidant enzymes SOD (Fig. 4D), CAT (Fig. 4E) and GPx (Fig. 4F) was significantly

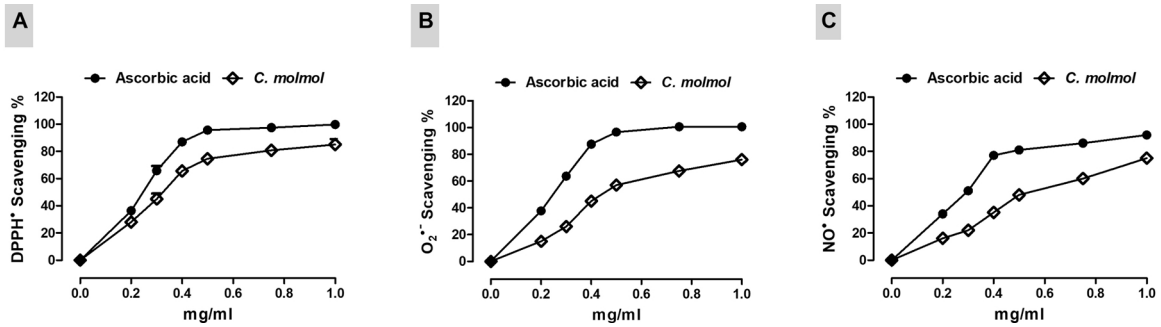

Fig. 1. DPPH (A), superoxide (B) and nitric oxide (C) radical scavenging activity of different concentrations of *C. molmol* resin extract. All assays were carried out in triplicate (n = 3). Data are expressed as Mean ± SEM.

($P < 0.001$) declined in the kidney of MTX-induced rats when compared with the control group. The 125 mg/kg *C. molmol* resin extract produced a significant amelioration of SOD ($P < 0.001$), CAT ($P < 0.01$) and GPx ($P < 0.01$) activity in the kidney of MTX-induced rats. The higher *C. molmol* resin extract dose markedly ($P < 0.001$) boosted the activity of SOD, CAT and GPx in the kidney of MTX-induced rats (Fig. 4D-F).

3.4. *C. molmol up-regulates the expression of antioxidant enzymes in the kidney of MTX-induced rats*

The gene expression analysis showed a significant ($P < 0.01$) decrease in SOD mRNA expression in the kidney of MTX-induced rats when compared with the control group (Fig. 5A). MTX-induced rats pre-treated with 125 mg/kg *C. molmol* resin extract showed a significant ($P < 0.01$) up-regulation of kidney SOD mRNA abundance. The higher *C. molmol* resin extract dose significantly ($P < 0.001$) increased SOD mRNA abundance in the kidney of MTX-induced rats (Fig. 5A).

CAT mRNA expression was significantly ($P < 0.001$) down-regulated in the kidney of MTX-induced rats when compared with the control rats (Fig. 5B). Pre-treatment with either dose of *C. molmol* resin extract markedly alleviated CAT mRNA expression in the kidney of MTX-induced rats (Fig. 5B).

GPx mRNA exhibited a similar pattern where its expression was significantly ($P < 0.001$) reduced in the kidney of MTX-induced rats when compared with the control group (Fig. 5C). Pre-treatment with either dose of *C. molmol* resin extract significantly ($P < 0.001$) alleviated GPx mRNA expression in the kidney of MTX-induced rats (Fig. 5C).

3.5. *C. molmol attenuates inflammation and apoptosis in the kidney of MTX-induced rats*

NF-κB is a transcription factor that activates the expression of inflammatory mediators. Therefore, we determined the levels of NF-κB, TNF-α and IL-1β in the kidney of MTX-induced rats to investigate the anti-inflammatory effect of *C. molmol* resin extract.

MTX administration induced a significant ($P < 0.001$) increase in NF-κB levels in the kidney when compared with the control rats (Fig. 6A). On the contrary, MTX-induced rats pre-treated with 125 mg/kg and 250 mg/kg *C. molmol* resin extract showed a significant decrease in kidney NF-κB levels (Fig. 6A). The pro-inflammatory cytokines TNF-α and IL-1β showed a significant ($P < 0.001$) increase in the kidney of MTX-induced rats when compared with the control rats. Pre-treatment with either 125 or 250 mg/kg *C. molmol* resin extract produced a significant ($P < 0.001$) decrease in the levels of TNF-α (Fig. 6B) and IL-1β (Fig. 6C) in the kidney of MTX-induced rats.

To scrutinize the role of *C. molmol* resin extract in mitigating MTX-induced apoptosis in the kidney of rats, the expression levels of Bax and Bcl-2, and the Bax/Bcl-2 ratio were determined. As represented in Fig. 6D, MTX-induced rats showed a significant ($P < 0.001$) increase in kidney Bax mRNA abundance when compared with the control rats. MTX-induced rats pre-treated with 125 or 250 mg/kg *C. molmol* resin extract showed a significant ($P < 0.01$) down-regulation of Bax mRNA expression (Fig. 6D). The expression of Bcl-2 was significantly ($P < 0.001$) down-regulated in the kidney of MTX-induced rats (Fig. 6E). Pre-treatment with the 125 mg/kg *C. molmol* resin extract produced a significant ($P < 0.05$) up-regulation of Bcl-2 mRNA expression. The higher *C. molmol* resin extract dose significantly up-

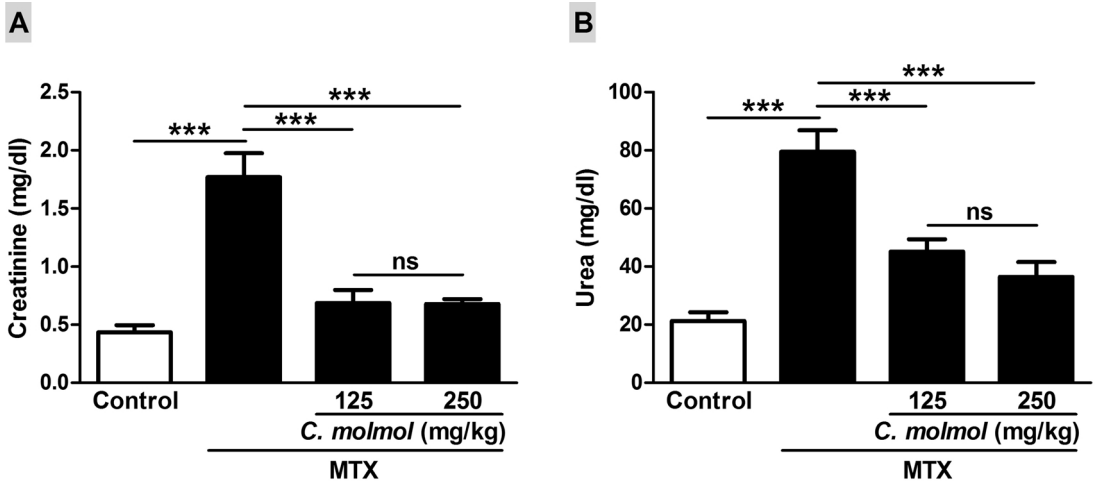

Fig. 2. Effect of *C. molmol* on serum (A) creatinine and (B) urea levels in MTX-induced rats. Data are expressed as Mean ± SEM, n = 8. ***$P < 0.001$. MTX, methotrexate; *C. molmol*, *Commiphora molmol*; ns, non-significant.

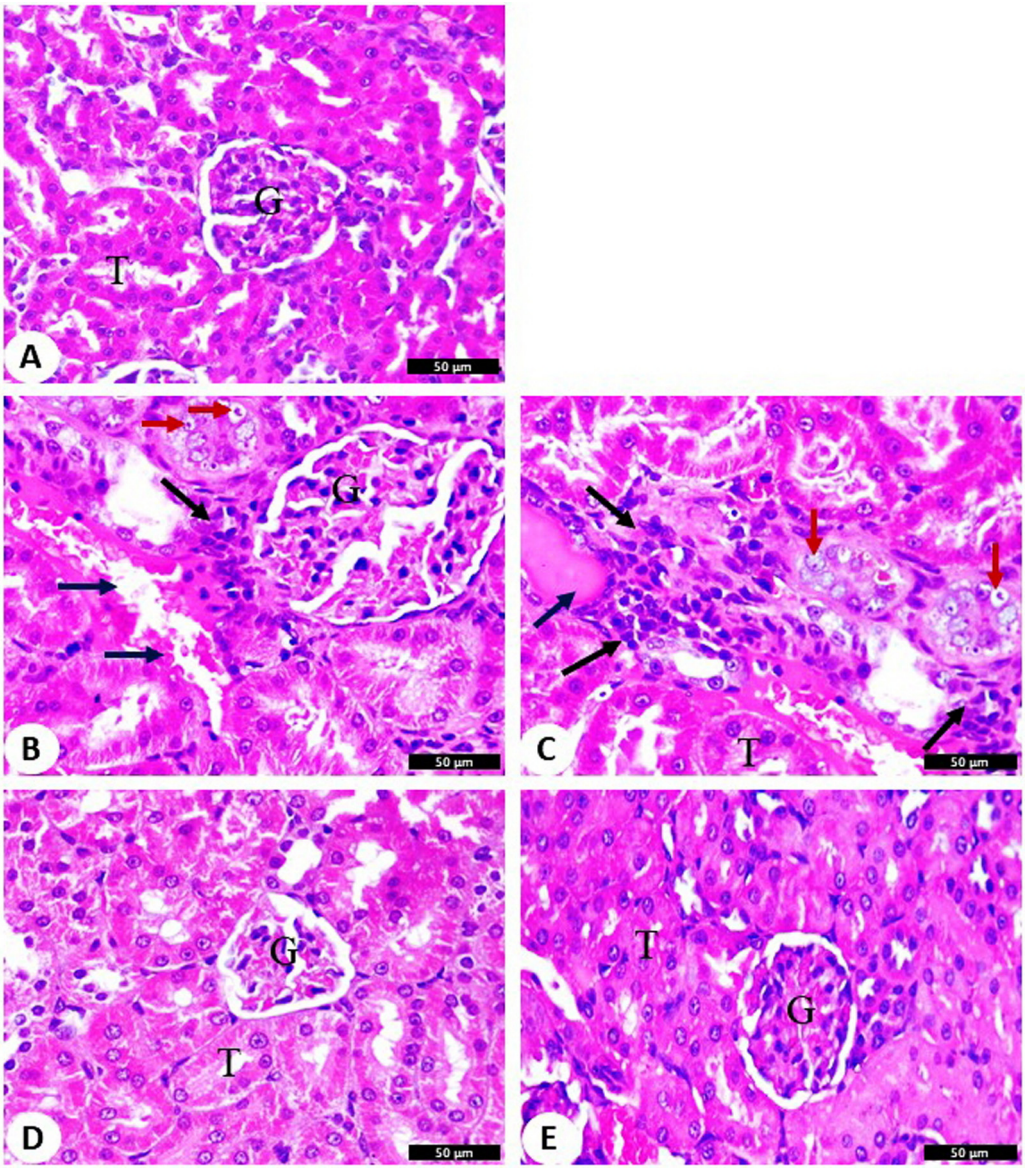

Fig. 3. H&E-stained sections in the kidney of (A) control rats showing normal histological structure of the kidney with normal glomeruli (G) and renal tubules (T), (B & C) MTX-induced rats showing inflammatory cells infiltration (black arrow), interstitial hemorrhage (blue arrow) and degenerated nuclei and chromatin (red arrow), (D) MTX-induced rats pre-treated with 125 mg/kg *C. molmol,* and (D) MTX-induced rats pre-treated with 250 mg/kg *C. molmol* resin extract showing improved structure of the kidney. Scale bar = 50 µm.

regulated Bcl-2 mRNA expression in the kidney of MTX-induced rats when compared with the MTX control ($P < 0.001$) and with the lower dose supplemented rats ($P < 0.05$). The Bax/Bcl-2 ratio was significantly ($P < 0.001$) increased in the kidney of MTX-induced rats, an effect that was markedly prevented in the rats pre-treated with 125 mg/kg ($P < 0.01$) or 250 mg/kg ($P < 0.001$) *C. molmol* resin extract (Fig. 6F).

3.6. *C. molmol up-regulates Nrf2/ARE/HO-1 signaling in the kidney of MTX-induced rats*

Rats received MTX injection showed a significant ($P < 0.001$) down-regulation of Nrf2 mRNA expression in the kidney when compared with the control rats as depicted in Fig. 7A. On the contrary, MTX-induced rats pre-treated with 125 or 250 mg/kg *C. molmol* resin extract exhibited a marked up-regulation of kidney Nrf2 mRNA expression ($P < 0.001$). Similarly, Nrf2 protein expression was significantly ($P < 0.001$) reduced in the kidney of MTX-induced rats when compared with the control group (Fig. 7B). Pre-treatment of the MTX-induced rats with 125 mg/kg *C. molmol* resin extract produced a marked ($P < 0.01$) increase in kidney Nrf2 protein expression. Administration of the higher dose of *C. molmol* resin extract before MTX administration significantly ($P < 0.001$) protected against MTX-induced down-regulation of Nrf2 protein expression in the kidney of rats (Fig. 7B).

To confirm the positive impact of *C. molmol* resin extract on Nrf2 signaling in the kidney of MTX-induced rats, we determined the gene expression levels of NQO-1 and HO-1, two enzymes directly induced *via* Nrf2 activation [22–24]. The kidney of MTX-induced rats exhibited a significant decrease in NQO-1 ($P < 0.001$; Fig. 7C) and HO-1 ($P < 0.01$; Fig. 7D) mRNA expression when compared with the control rats. Pre-treatment with 125 mg/kg *C. molmol* resin extract increased the expression of NQO-1 and HO-1 significantly ($P < 0.01$) in the kidney of MTX-induced rats. Rats received 250 mg/kg *C. molmol* resin extract a significant improvement in kidney NQO-1 ($P < 0.001$) and HO-1 ($P < 0.01$) mRNA expression.

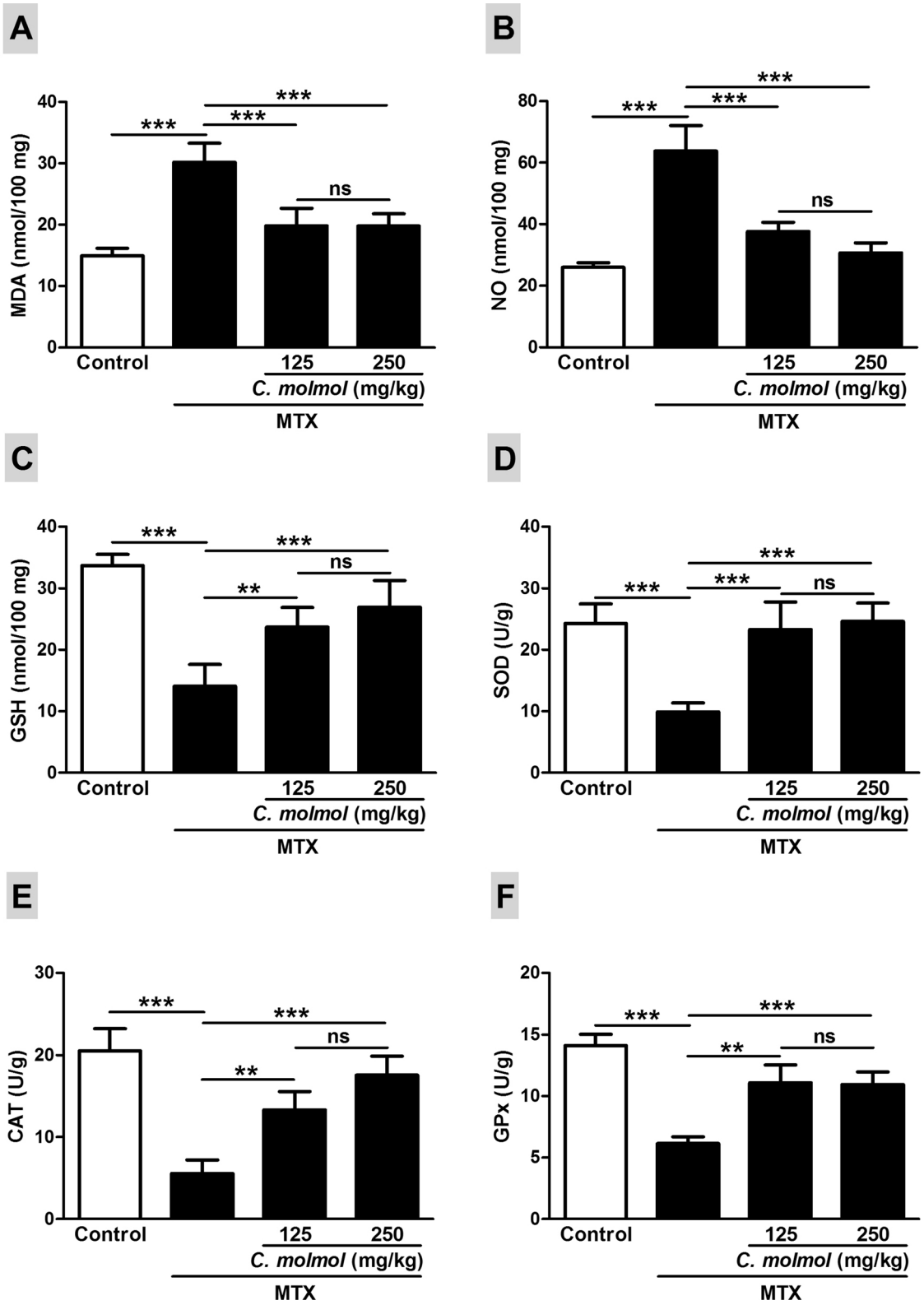

Fig. 4. *C. molmol* decreases (A) lipid peroxidation and (B) nitric oxide, and increases (C) GSH, (D) SOD, (E) CAT and (F) GPx in the kidney of MTX-induced rats. Data are expressed as Mean ± SEM, n = 8. **P < 0.01 and ***P < 0.001. MTX, methotrexate; *C. molmol*, *Commiphora molmol*; MDA, malondialdehyde; NO, nitric oxide; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; ns, non-significant.

4. Discussion

Myrrh is the common name of the resin collected from the knots that form on the trunk of several members of the genus *Commiphora*. It has several beneficial effects and has been used in the traditional treatment of various human diseases. Our recent work demonstrated the anticarcinogenic, hepatoprotective [28], antioxidant and anti-hyperammonemic [29] effects of *C. molmol* resin extract. Other previous

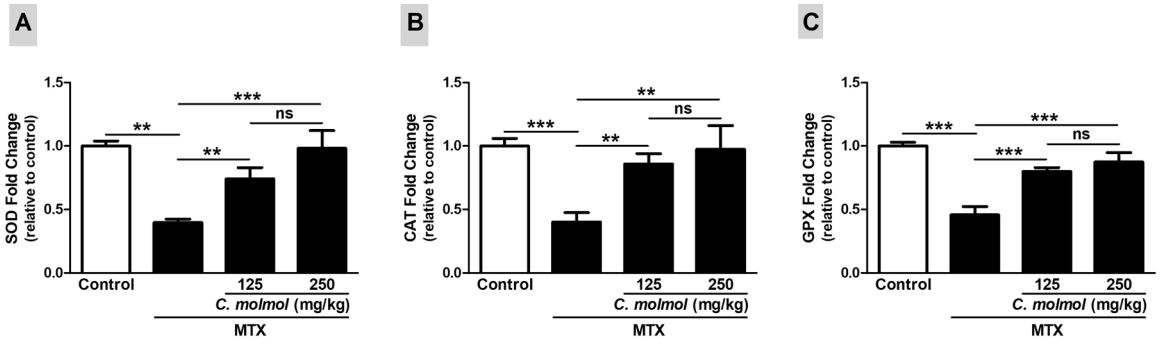

Fig. 5. *C. molmol* up-regulates the expression of (A) SOD, (B) CAT) and (C) GPx in the kidney of MTX-induced rats. Data are expressed as Mean ± SEM, n = 8. **P < 0.01 and ***P < 0.001. MTX, methotrexate; *C. molmol*, *Commiphora molmol*; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; ns, non-significant.

studies have reported the hypoglycemic [45], anti-inflammatory [46], anti-bacterial [47] and cardioprotective [48] activities of myrrh. However, its protective effect against MTX nephrotoxicity has not been previously reported. In this study, we investigated the *in vitro* radical scavenging activity of *C. molmol* resin extract and its potential to protect the kidney against MTX-induced oxidative stress, inflammation and apoptosis, pointing to the role of Nrf2/ARE/antioxidant signaling.

The *in vitro* DPPH radical scavenging assay showed the efficacy of *C. molmol* resin extract to scavenge free radicals. Recently, Mahboubi and Kazempour [49] demonstrated the radical scavenging activity of *C. molmol* resin ethanol extract and the IC_{50} was 625 µg/ml in the DPPH assay. In the same context, we have recently tested the radical scavenging activity of *C. molmol* resin ethanol extract [29] using DPPH assay. Using the same assay, Mohamed et al [50] showed the radical scavenging activity of the methanol and ethyl acetate extracts of *C. molmol* resin. In addition, our results showed that *C. molmol* resin extract can scavenge $O_2^{\bullet -}$ and $NO^{\bullet}$. These findings point to the potent antioxidant efficacy of *C. molmol* resin.

The kidneys have a central role in the metabolism and excretion of toxins and drugs; therefore, receive a large volume of the cardiac output. Due to their high capacity for the uptake of drugs, the proximal convoluted tubules are particularly vulnerable to toxicity. The drug-induced injury results from excessive production of ROS during the increased intracellular concentration and subsequent metabolism of the drugs [5]. Given that *C. molmol* resin showed a potent radical scavenging activity, we demonstrated its protective effect against MTX-induced oxidative stress and nephrotoxicity. MTX-induced rats in the present study showed a significant increase in serum levels of creatinine and urea, demonstrating kidney injury. The nephrotoxicity in MTX-induced rats was further confirmed by the histological findings, including leukocyte infiltration, interstitial hemorrhage and degenerative changes of tubular epithelial cells. These findings were supported by our previous findings where we reported increased circulating kidney function markers and many histopathological alterations in the kidney of rats received 20 mg/kg MTX [13]. MTX is a weak organic acid that is filtered and secreted by the kidneys and is poorly soluble in acidic urine

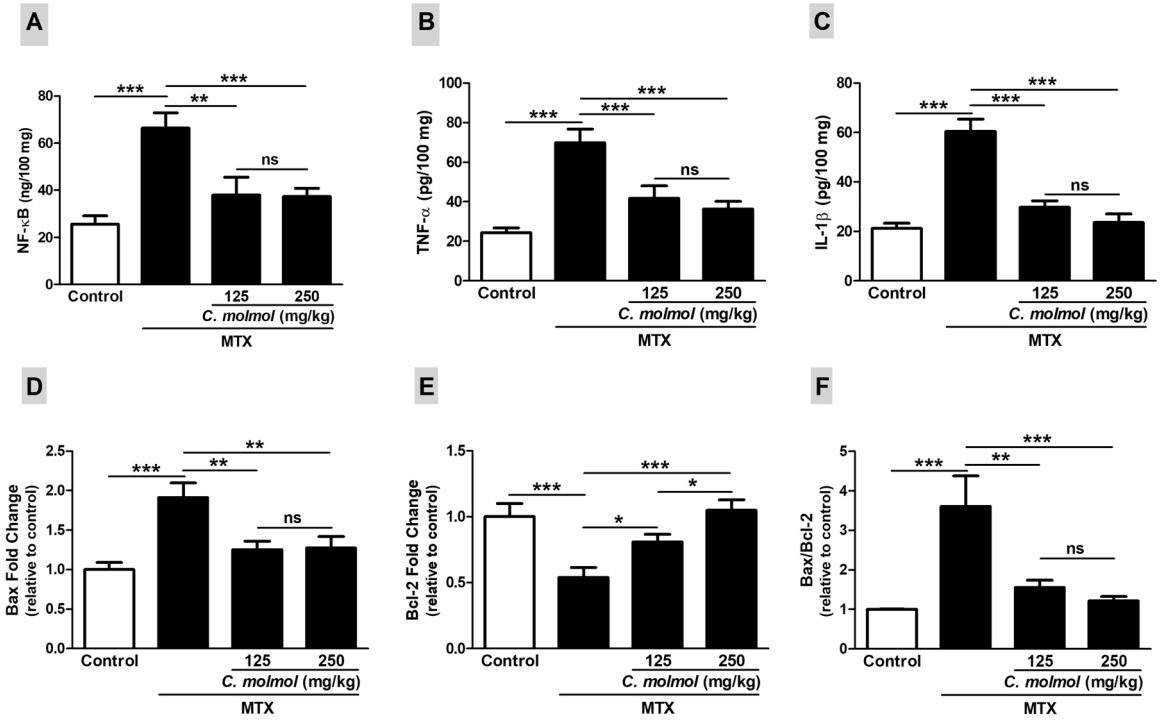

Fig. 6. *C. molmol* attenuates inflammation and apoptosis in the kidney of MTX-induced rats. C. molmol decreases kidney (A) NF-κB, (B) TNF-α and (C) IL-1β levels. C. molmol decreases Bax expression (D) and Bax/Bcl-2 ratio (F) and increases Bcl-2 expression (E). Data are expressed as Mean ± SEM, n = 8. *P < 0.05, **P < 0.01 and ***P < 0.001. MTX, methotrexate; *C. molmol*, *Commiphora molmol*; NF-κB, nuclear factor-kappaB; TNF, tumor necrosis factor; IL, interleukin; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; ns, non-significant.

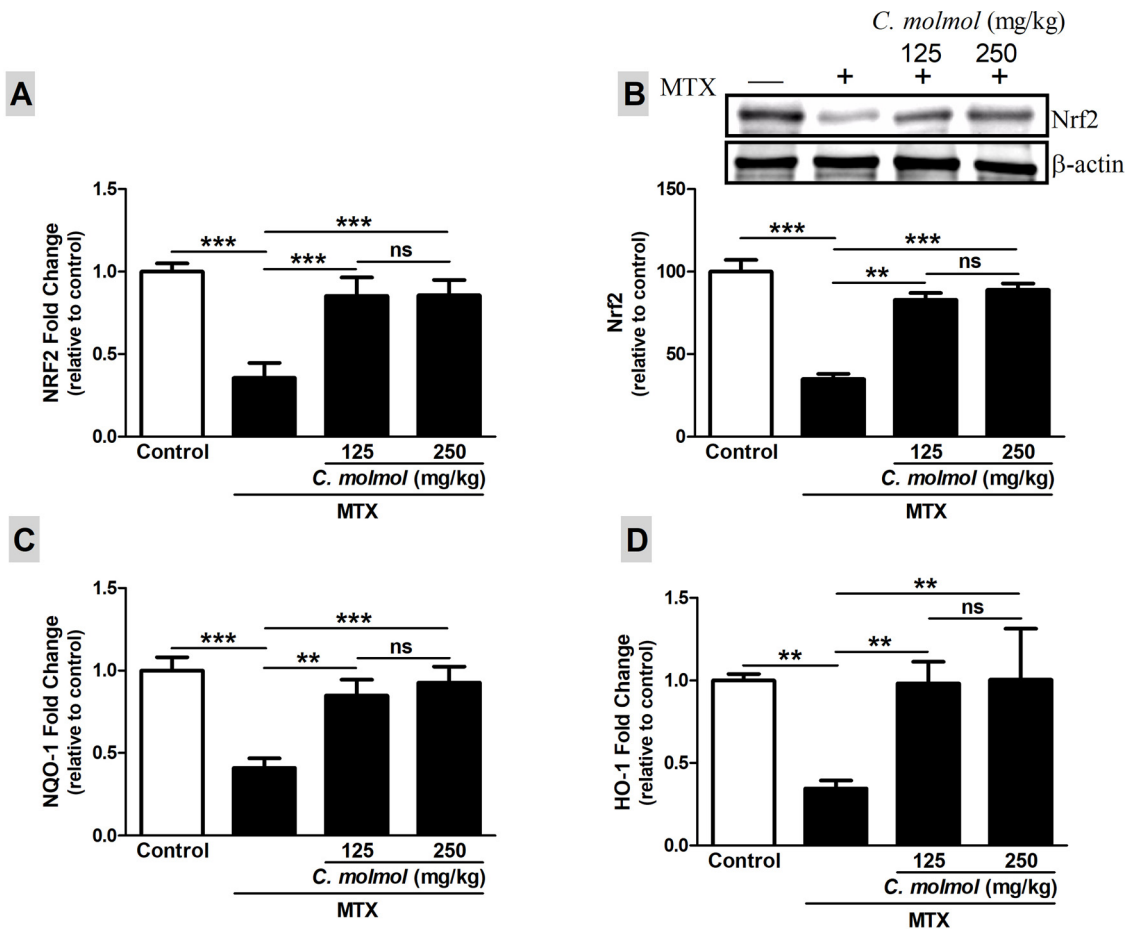

Fig. 7. *C. molmol* up-regulates Nrf2 mRNA, (A) Nrf2 protein, (C) NQO-1 mRNA and (D) HO-1 mRNA in the kidney of MTX-induced rats. Data are expressed as Mean ± SEM, n = 8. **P < 0.01 and ***P < 0.001. MTX, methotrexate; *C. molmol*, *Commiphora molmol*; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NQO-1; NAD(P)H quinone dehydrogenase 1 (NQO-1); HO-1, hemeoxygenase-1; ns, non-significant.

[51]. The nephrotoxic effect of MTX is pretended to be due to its precipitation and the direct toxic effects on the renal tubules [6]. MTX and its metabolites can accumulate in the renal tubules, leading to obstruction and diminished clearance [52]. The crystallization and accumulation are enhanced by acidic urine pH, low urine volume and high urinary concentration of MTX [53]. Interestingly, pre-treatment of the rats with 125 or 250 mg/kg *C. molmol* extract prevented MTX-induced renal dysfunction and injury. The protective effect of Mirazid®, a drug containing purified *C. molmol* resin extract, against the nephrotoxic effect of carbon tetrachloride (CCl_4) has been reported by Alm-Eldeen et al. [54]. In this study, the administration of 200 mg/kg body weight *C. molmol* resin extract for 7 consecutive days conferred protection against CCl_4-induced kidney injury in mice [54].

Oxidative stress is a hallmark of tissue damage caused by MTX administration [13,19,55]. We assumed that the nephroprotective effect of *C. molmol* was attributed to its antioxidant potential. Therefore, we investigated its effect on lipid peroxidation, NO production and antioxidant defenses in the kidney of MTX-induced rats. MTX administration induced a significant increase in MDA and NO, and reduced GSH, SOD, CAT and GPx in the kidney of rats. The antioxidants GSH, SOD, CAT and GPx protect against ROS-induced damage; therefore, diminished antioxidants provoke mitochondrial dysfunction, necrosis and impaired renal function [56]. Accordingly, we have demonstrated that MTX induces oxidative stress and reduces the enzymatic and non-enzymatic antioxidants in the kidney [13], liver [15,16] and cerebrum of rats [14]. Studies have shown that MTX increases ROS production by hampering the remethylation of homocysteine [19], stimulation of neutrophils [18], and depletion of NADPH [17]. MTX-induced rats treated with *C. molmol* resin extract showed decreased lipid peroxidation and NO along with enhanced activity and expression of antioxidant defenses in the kidney. These findings were in agreement with multiple studies demonstrating the antioxidant potential of *C. molmol* resin. In rodent models of hyperammonemia [29], hepatocarcinogenesis [28], lead- [57] and ethanol-induced hepatotoxicity [58] and ulcerative colitis [59], supplementation of *C. molmol* resin diminished lipid peroxides and NO, and boosted antioxidant defenses. These *in vivo* results were in positive correlation with the *in vitro* antioxidant and radical scavenging activity of *C. molmol* resin extract. The antioxidant potential of *C. molmol* resin could be explained in terms of its active constituents, such as, eugenol, pinene, terpenoids, commiphoric acids, limonene, m-cresol, furanosesquiterpenes [60,61] and phenolics [49].

Activation of Nrf2 signaling is another mechanism we hypothesized mediating the protective effect of *C. molmol* resin against MTX-nephrotoxicity. Nrf2 is a redox-sensitive transcription factor that activates antioxidant and cytoprotective genes [22–24]. Nrf2 is kept deactivated in the cytosol through ubiquitination by KEAP1 and Cullin 3 [25]. ROS disrupts the Keap1-Cullin 3 ubiquitination system and Nrf2 builds up in the cytosol [26]. Nrf2 translocates into the nucleus and forms a dimer with a small MAF protein, and the dimer activates the transcription of antioxidant genes through binding to ARE [62]. Herein, the gene and protein expression of Nrf2 was significantly declined in the kidney of MTX-induced rats. The down-regulation of Nrf2 signaling has been confirmed by the reduced expression of NQO-1 and HO-1. Recently, we have demonstrated the impact of MTX on Nrf2 signaling in the kidney [13] and liver of rats [14,15]. MTX-induced rats pre-treated with *C. molmol* resin extract showed a significant up-regulation of Nrf2

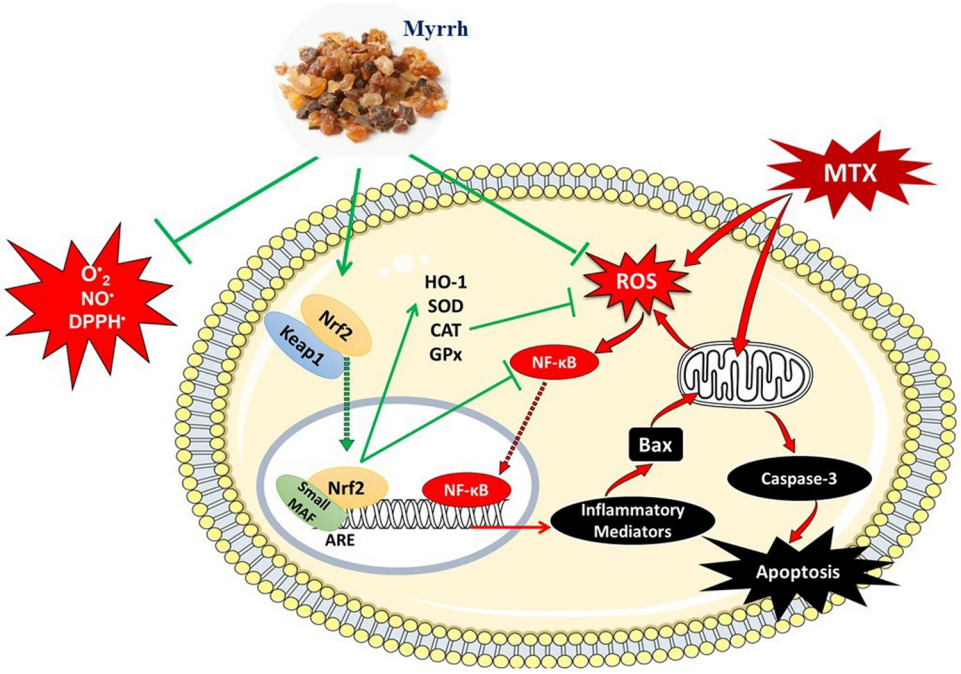

Fig. 8. A proposed schematic diagram illustrating the protective mechanism of *Commiphora molmol* against MTX-induced nephrotoxicity. MTX, methotrexate; ROS, reactive oxygen species; NF-κB, nuclear factor-kappaB; HO-1, hemeoxygenase-1; Bax, Bcl-2-associated X protein; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; NO·, nitric oxide radical; $O_2^{\bullet -}$, superoxide radical; Nrf, Nuclear factor (erythroid-derived 2)-like 2; Keap1, Kelch like-ECH-associated protein 1; ARE, antioxidant response element.

signaling evidenced by the increased expression of Nrf2, NQO-1, HO-1, SOD, CAT, and GPx. The current study added support to our previous findings where the administration of *C. molmol* resin up-regulated Nrf2 signaling in the liver and cerebrum in rat models of hepatocarcinogenesis [28] and hyperammonemia [29], respectively. Although activated by ROS, the declined Nrf2 signaling in the kidney of MTX-induced rats could be a direct consequence of excessive ROS generation. Our previous work showing down-regulated Nrf2 signaling in oxidative stress conditions added support to this notion [13,15,16,28,29,44,63–68].

MTX toxicity has been associated with inflammation and apoptosis [15,16]. In this study, MTX administration induced a significant increase in NF-κB, TNF-α and IL1β in the kidney of rats. Activation of NF-κB in the kidney of MTX-induced rats is a direct result of increased production of ROS. Multiple studies have demonstrated an inflammatory response following MTX administration in rodents [15,16]. In AKI, studies have reported increased expression of NF-κB in human [20] and experimental animals [19]. In renal disease, the inflammatory cytokines can activate neutrophils and macrophages, provoke inflammatory cell infiltration, up-regulate adhesion molecules on endothelial cell and increase ROS production [69,70]. In this context, Kolli et al have reported increased infiltration of neutrophils and generation of ROS in MTX-induced renal damage [71].

Inflammation and oxidative stress work in concert to induce cellular damage and apoptosis. The kidney of MTX-induced rats in our investigation showed increased expression of Bax and decreased Bcl-2, demonstrating apoptosis. The susceptibility of cells to apoptosis is controlled by the Bcl-2 gene family [72]. Bcl-2 is an anti-apoptotic protein that block the release of mitochondrial cytochrome C [73] and apoptosis is initiated upon its down-regulation [74]. Several studies have shown increased apoptosis in the kidney after MTX administration [75–78]. MTX-mediated apoptosis is believed to be induced by excessive ROS production that results in DNA damage. Oxidative stress can suppress the expression of Bcl-2, up-regulate Bax and activate the mitochondrial pathway of apoptosis [21]. Accordingly, we have reported increased Bax and decreased Bcl-2 expression in the liver of rats following MTX injection [15]. Pre-treatment with *C. molmol* resin prevented apoptosis by up-regulating Bcl-2 and decreasing Bax expression in the kidney of MTX-induced rats. The anti-apoptotic effect of *C. molmol* resin is a direct result of its antioxidant/anti-inflammatory potential. Mahmoud and colleagues have recently reported the anti-inflammatory effect of *C. molmol* in hepatocarcinogenesis [28] and hyperammonemia conditions [29]. Other studies demonstrated the ability of *C. molmol* resin to attenuate inflammation [30,59,79]. Activation of Nrf2 might have a role in mediating the anti-inflammatory efficacy of *C. molmol* resin. In addition to attenuation of oxidative stress, activation of Nrf2 was associated with suppression of NF-κB, iNOS and pro-inflammatory cytokines [15,28,29,63,64,66].

In conclusion, this study confers novel information on the protective effect of *C. molmol* resin against MTX nephrotoxicity in rats. *C. molmol* resin showed a potent *in vitro* radical scavenging activity. *C. molmol* prevented oxidative/nitrosative stress, enhanced antioxidant defenses, and attenuated inflammation and apoptosis in the kidney of MTX-induced rats *via* up-regulation of Nrf2/ARE signaling (Fig. 8). Therefore, *C. molmol* resin may be an effective nephroprotective agent, pending further detailed mechanistic studies.

Authors' contribution

AMM conceived the study, designed and performed all animal experiments, biochemical and molecular assays, analyzed the data and wrote the manuscript. MOG performed extraction and *in vitro* experiments. KMA, AHM, MF and AAA acquired the funding, performed *in vitro* experiments and read the histology slides. All authors revised and approved the manuscript for publication.

Conflicts of interest

There are no conflicts of interest to declare.

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