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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