

## Protective Role of Ursolic Acid Extracted from *Zizyphus spina-christi* Against Methotrexate-Induced Hepatotoxicity in Balb/C Mice

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**Abstract:** Methotrexate (MTX), is an effective anticancer and immunosuppressive agent. However, hepatotoxicity is one of the complications of its use for which several hypotheses have been put forward, including oxidative stress. The aim of this study was to evaluate the possible protective effect of ursolic acid (UA) extracted from *Zizyphus spina-christi*, a novel antioxidant, against methotrexate-induced hepatic oxidative stress in Balb/c mice. Forty mice were assigned randomly into four groups, three being control groups [group 1 (control), group 2 (UA control), group 3 (MTX control) and group 4, an experimental group, MU]. Mice of groups MTX and MU were treated with MTX (10mg/kg b.w., i.p.) for 2 weeks. Mice of groups UAC and MU were treated with UA (30mg/kg b.w. orally, daily). After two weeks all mice were decapitated and their livers were removed and perfused in Hanks buffer and blood was obtained immediately. Our results showed significant increment in the levels of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), gamma-glutamyl transaminase (GGT) and nitric oxide (NO) in the sera of MTX treated mice. It was also shows that MTX administration increased the hepatic level of malondialdehyde (MDA), protein carbonyl contents (PCC), DNA protein cross link, reactive oxygen species (ROS) levels and decreased myeloperoxidase (MPO) activity and the level of glutathione (GSH) in liver homogenate of mice treated with MTX. These alterations were reversed in UA-treated groups.

**Key words:** Methotrexate • Ursolic Acid • *Zizyphus spina-christi* • Antioxidants

### INTRODUCTION

Methotrexate is antimetabolite and antifolate drug. It is a cytotoxic chemotherapeutic agent widely used in the treatment of acute lymphoblastic leukemia, lymphoma, osteosarcoma and some types of cancers and also, in the therapy of non-oncologic disorders such as rheumatic diseases and psoriasis [1, 2]. It was found by others [3,4] that oxidative stress contributes in the pathogenesis of MTX-induced damage in various organs.

The folic acid antagonist MTX is used clinically to inhibit the synthesis of purines and pyrimidines [5] and commonly used at high doses to limit the growth of malignancies. It may also be used at low doses in inflammatory diseases including psoriasis and rheumatoid arthritis, to inhibit the proliferation of inflammatory leukocytes [6,7].

It was demonstrated that MTX-induces hepatotoxicity has a potent inhibitory effect on several enzymes related to DNA synthesis [8], as well as an

enhancing effect on the production of reactive oxygen species (ROS). It has been established that MTX inhibits cytosolic nicotinamide adenosine diphosphate (NADP)-dependent dehydrogenase and NADP malic enzyme, resulting in decreased availability of NADPH in cells [9]. NADPH is normally used by enzyme glutathione reductase (GSH-Rd) to maintain cellular reduced glutathione (GSH), which is an important protective agent against ROS [10, 11]. Antioxidant defense mechanisms can effectively protect cells and tissues from free radicals mediated deleterious effects [12]. Thus, the significant reduction in GSH levels induced by MTX therapy leads to suppression of the antioxidant enzyme defense system, sensitizing the cells to ROS [13].

Dihydrofolate reductase (DHFR) which catalyses the reaction of 7,8-dihydrofolate and NADPH to form 5,6,7,8-tetrahydrofolate and NADPH<sup>+</sup> and its inhibition leads to depletion of the intracellular tetrahydrofolic acid pool [14].

Ursolic acid (3 $\beta$ -hydroxy-urs-12-en-28oic acid) is a pentacyclic triterpenoid which exists in natural plants in the form of free acid or aglycones [15]. It is present in fruits, flowers and leaves of many kinds of medicinal plants such as olive, zizyphus and japonica [16]. Due to its antioxidant, antitumor and anti-inflammatory activities, UA has been studied at depth by others as chemopreventive agent in several cancer models [17]. The present study was, hence, aimed to carry out a systematic investigation of the protective role of ursolic acid on hepatocytes by estimating biomarkers in serum and liver homogenate along with assessment of redox status, as these parameters are considered for maintenance of biochemical homeostasis within the cell.

## MATERIAL AND METHODS

**Chemicals:** Chemicals used in this study were of analytical grade. All standard kits, reagents and other chemical were purchased from Sigma Co. USA.

**Extraction of UA:** Ripe and healthy fruits of *Zizyphus spina-christi* were cleaned 3 times in tap water then soaked in phosphate buffer (pH 7.4) for four hours, then air dried, homogenized in phosphate buffer (pH 7.4) and then lyophilized. UA was extracted according to the method reported by Sultana *et al.* [18].

**Measurement of Free Radical Scavenging Activity (FRSA):** The free radical scavenging activities of UA was measured by the adverse in the absorbance of methanol solution of 1,1-diphenyl-2-picryl-hydrazyl (DPPH). Freshly extracted antioxidant (0.1 ml) was mixed with 0.9 ml of Tris-HCl buffer (100mM, pH 7.4) to which 1 ml of 500  $\mu$ M DPPH was added. After 30 min, O.D. was measured spectrophotometrically at 340nm. The antioxidant activity was calculated as in the following:

$$\text{FRSA \%} = \text{O.D. sample (340nm)} / \text{O.D. control (340 nm} \times 100).$$

**DPPH Free Radical-Scavenging Activity:** The DPPH free radical scavenging assay was carried out, as previously reported [19]. The antioxidant activity was calculated as an inhibitory effect (IE %) of the DPPH radical formation as in the following:

$$\text{IE\%} = 100 \times \left( \frac{A_{517\text{control}} - A_{517\text{sample}}}{A_{517\text{control}}} \right)$$

and expressed as IC<sub>50</sub>, the value of which is defined as the concentration (in  $\mu$ g/ml) of the compound required to scavenge the DPPH radical by 50%.

**Biochemical Studies:** Forty healthy male balb/c mice (6-7 weeks old, 29-30g weight), were housed individually in stainless steel cages in a temperature-controlled (24 $\pm$ 1 $^{\circ}$ C) and 12h dark/light cycle room free from any source of chemical contamination. Mice were maintained on standard laboratory diet and water *ad libitum* and randomly assigned into four groups (10 mice each); group 1 was control (C); mice were supplemented with solvent vehicle control (NaCl) for two weeks. Second group was MTX control (MC); mice were given MTX (8mg/kg, b.w.) daily (i.p.) for two weeks. The third group was UA control group (UC); mice were supplemented with UA (30mg/kg b.w.) orally daily for two weeks. Experimental group (UM) mice were treated by giving UA (30mg/kg b.w. orally and MTX (8mg) as i.p. daily) for two weeks. All mice were sacrificed by cervical dislocation after 24h of the last injection. Livers were immediately removed and perfused with Hanks buffered-saline to remove excess blood and homogenized with phosphate buffer saline (pH 7.2) using Ultra Turax homogenizer, centrifuged at 3000g for 30 min. The supernatant was removed and stored at -80 $^{\circ}$ C. Blood was immediately collected and serum was isolated and kept at -70 $^{\circ}$ C. The assessment of the liver toxicity was performed by using serum enzymes levels as a biomarkers; SGOT, SGPT and GGT activities were measured by standard methods reported before (20, 21 and 22 respectively). In liver homogenate, LPO level was measured as malondialdehyde (MDA) according to the method reported in the literatures [23]; protein carbonyl contents were assayed using standard method reported before [24]. ROS was determined in hepatocyte homogenate and quantified by the dichlorofluorescein diacetate (DCFH-DA) assay using microplate reader [25]. The extent of DNA-protein cross-links %, were assayed as reported before [26]. MPO activity was measured using a procedure recommended before [27].

**Statistical Analysis:** All results were expressed as the mean  $\pm$  S.E.M. from ten mice per group. One way analysis of variance (ANOVA) followed by Tokay test was used to determine the significance of the differences between the groups. Statistical significance was declared when *P* value was equal to or less than 0.05. The statistical analysis was performed using the Sigma State Statistical Software version 3.5.

## RESULTS

The present results revealed the final bodyweight of MTX administered animals was significantly (*P*=0.05) lower than that of control group, while the liver weight

Table 1: Effects of MTX and UA on the body weight and, liver weight of Balb/c mice

Particular	Group 1 (C)	Group 2 (MC)	Group 3 (UC)	Group 4 (UM)
Body weight (g)	30.71±1.77	26.33±1.08	31.61±1.78	29.83±1.47
Liver weight (g)	1.68±0.04	2.49±0.09	1.62±0.07	1.82±0.06

Table 2: Effects of MTX and UA on the levels of enzymes studied in the serum of Balb/c mice

Particulars	Group 1 (C)	Group 2 (M)	Group 3 (U)	Group 4 (UM)
AHH(μmol/min/mg protein)	0.44±0.01	1.72±0.03	0.41±0.01	0.71±0.02
SGOT (U/L)	17.11±2.08	54.61±7.33	17.08±1.78	21.14±2.11
SGPT (U/L)	18.24±2.05	97.45±9.91	18.76±2.64	27.19±2.77
GGT (nmol/min/mg protein)	1.82±0.06	3.08±0.68	1.64±0.11	2.02±0.06

Table 3: Effect of MTX and protective effect of UA on biomarkers of liver injury in Balb/c mice

Particular	Group 1(C)	Group 2(M)	Group 3(U)	Group 4 (UM)
MDA (nmol/mg of wet tissue)	0.58±0.02	7.21±0.22	0.51±0.01	1.24±0.06
GSH (μg/mg protein)	1.77±0.02	0.62±0.01	1.83±0.02	1.23±0.02
PCC (nmol/mg protein)	0.71±0.01	18.44±0.27	0.63±0.01	1.01±0.01
ROS % of control	100	161	72	117
DNA protein cross link %	0.88	16.41	0.81	3.96
MPO (U/g)	6.81±1.08	39.78±2.88	6.66±0.71	13.11±1.71

was significantly increased due to massive intra-hepatic haemorrhage and pooling of blood in the liver turning it darker in colour compared to other groups. Treatment of animals with antioxidant UA lead to maintenance of body weight in group UM, ameliorating the changes in body weight and liver weight, as well as liver colour to near normal (Table 1).

The activities of marker enzymes AHH, SGOT, SGPT and GGT were utilized to evaluate liver injury and found to be significantly ( $P=0.05$ ) increased in mice supplemented with MTX compared to those in control group (C) and were reversed to almost near normal in UA treated animals (Table 2). The levels of MDA, PCC, ROS, GSH and DNA protein cross link % were elevated significantly ( $P=0.05$ ) in hepatocytes homogenate of MTX treated mice; but these levels were reduced significantly ( $P=0.05$ ) in hepatocytes homogenates of experimental group, fed with UA, as presented in Table 3. The adverse changes reverted almost near to the control values.

MPO activity was measured in the liver homogenates as an indirect evidence of neutrophil infiltration. MTX treatment caused significant increase in the liver MPO activity (39.78±2.88 U/g), while after UA treatment it was 13.11±1.71 U/g.

## DISCUSSION

Although the underlying mechanism of MTX-induced hepatotoxicity remains to be fully elucidated [28], increases in oxidative stress caused by ROS, have been

linked to the effects of MTX as shown in the present study where it altered significantly the oxidant/antioxidant balance in Balb/c mice model system.

Analysis of serum marker enzymes serves as an indicator of the response of the hepatocytes to MTX toxicity. The high level of SGOT, SGPT, GGT and AHH activities reflect damage to hepatocytes and indicate the increased cellular permeability [29]. They are considered to be highly sensitive and fairly specific biochemical markers of hepatotoxicity [30].

Our results show that MTX increases lipid peroxidation leading to high level of MDA as a main product of lipid breakdown. But UA which has been proved to be a powerful antioxidant [31], reduced the formation of MDA significantly, probably due to its ability to scavenge free radicals.

MPO, is a hem-containing peroxidase, abundantly expressed in neutrophils and monocytes. MPO-derived oxidants affect various processes involved in cell signalling and cell-cell interactions and are, as such, capable of modulating inflammatory responses. Our results showed that MTX raised the activity of this enzyme significantly level which reflects severe damage to hepatocytes and cytoplasmic granules of neutrophils and monocytes.

Methotrexate, as a folic acid antagonist, blocks the synthesis of purines and pyrimidines by inhibiting several key enzymes [32]. Inhibition of dihydrofolate reductase (DHFR) decreases tetrahydrofolate (THF) levels, which results in attenuation of DNA/ protein/ lipid methylation,

inhibition of thymidylate synthase (TS), interference with DNA synthesis and inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase which blocks denovo purine synthesis [33]. The effect on purine and pyrimidine biosynthesis is also responsible for many toxic effects of methotrexate.

The mechanisms of MTX-induced hepatotoxicity have not been exactly established yet, but free radicals are expected to play a role. In this study we used MDA levels to show damage to the hepatocytes caused by lipid peroxidation. Elevated observed MDA levels suggest that lipid peroxidation, mediated by oxygen radicals which are believed to be an important cause of damage to cell membrane, was an important contributing factor to the development of MTX-mediated tissue damage [34]. However, MTX-induced lipid peroxidation was prevented by UA, indicating that UA played the role of a powerful antioxidant. It is proved by many authors [35] that UA controls and eliminates prooxidants and scavenges free radicals. It has been reported that UA increases the levels of antioxidant enzyme catalase and its mRNA expression in rats [36]. Thus UA may tend to increase the protein synthesis, which in turn leads to increase in the activity of the enzyme. Also, it was found by others that UA acts in biochemical restoration of thiol status, which in turn restores the biological activities of the cell. It is known that many enzymes have essential thiols which may be inactivated during oxidative stress. Restoration of thiol status, induced by UA, may be partly responsible for the favourable alterations in the homeostasis of the cell.

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