HEPATOPROTECTIVE EFFECT *OF CORNUS MAS* FRUITS EXTRACT ON SERUM BIOMARKERS IN METHOTREXATE-INDUCED LIVER INJURY IN MALE RATS

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Abstract

Methotrexate (MTX) is an antineoplastic drug. It is used in treatment of cancer and autoimmune diseases. Some of the best-known side effects of MTX are hepatotoxicity and kidney failure. Therefore, the current study was designed to investigate the probable therapeutic effects of *Cornus mas* fruit extract (CMFE) in MTX-induced acute toxicity in liver of rats. Male wistar rats weighing [200-250 g] were divided into six groups; Control, CMFE, MTX (single dose 20mg/kg) and three MTX (20mg/kg) + CMFE (300, 700, 1400mg/kg) groups. After termination of experimental days, liver tissue dissected to measure activity of some antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (MDA) by spectrophotometer. The levels of Total- Direct and indirect bilirubin, Aspartate transaminase (AST), Alanine transaminase (ALT), alkaline phosphatase (ALP), Lactate dehydrogenase (LDH) were measured photometrically in a biochemistry auto analyzer. This study revealed that administration of CMFE (700 and 1400mg/kg) significantly prevented MTX-induced alterations in these biochemical parameters, that is, AST, ALT, ALP, Direct bilirubin and LDH activity (P<0.05) and liver lipid peroxidation level was significantly decreased compared to MTX group (P<0.05). There were no significant differences in SOD and CAT parameter among the groups. The present study indicated the hepatoprotective effect of CMFE against methotrexate induced liver injury.

Keywords: *Cornus mas,* hepatoprotective, methotrexate, rat

Introduction

Methotrexate, abbreviated MTX and formerly known as amethopterin, is an antineoplastic, antimetabolite and antifulate drug [1] [2]. Methotrexate began to replace the more toxic antifulate aminopterin starting in the 1950s. It is on the World Health Organization's List of Essential Medicines, a list of the most important medications needed in a basic health system [3]. It is used in treatment of cancer, autoimmune diseases, ectopic pregnancy, and for the induction of medical abortions. MTX has a wide range of therapeutic effects for example, at high doses in many malignancies and at low doses in autoimmune diseases [4]. However, the use of the drug is limited from time to time due to its adverse effects. Some of the best-known side effects of MTX are hepatotoxicity (liver damage), ulcerative stomatitis, low white blood cell count and thus predisposition to infection, nausea, abdominal pain, fatigue, fever, dizziness, acute pneumonitis, rarely pulmonary fibrosis and kidney failure [5].

Methotrexate is thought to affect cancer and rheumatoid arthritis by two different pathways. For cancer, methotrexate competitively inhibits dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis [6] [7]. The affinity of methotrexate for DHFR is about one thousand-fold that of folate. DHFR catalyses the conversion of dihydrofolate to the active tetrahydrofolate. Folic acid is needed for the de novo synthesis of the nucleoside thymidine, required for DNA synthesis. Also, folate is essential for purine and pyrimidine base biosynthesis, so synthesis will be inhibited. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins. [6] Together, these enzymes are responsible for the production of the main source of nicotinamide adenine dinucleotide phosphate (NADPH) in proliferating cells. Therefore, the use of MTX might cause decrease in the intracellular NADPH levels [8]. Thus, the decreased NADPH levels due to MTX administration might cause a decrease in the level of glutathione, which sensitizes the hepatocytes against reactive oxygen radicals (such as hydrogen peroxide, superoxide anions, hydroxyl radicals and hypochlorite radicals) and the consequent hepatocyte damage [9].

mechanisms of MTX-mediated hepatotoxicity are a direct toxic effect of MTX and the development of reactive oxygen species (ROS) [10,11]. Increased levels of ROS caused an imbalanced state between oxidants and antioxidants, described by the well-known term 'oxidative stresses. Therefore, it is recommended that its usage be combined with antioxidant agents in order to prevent MTX toxicity.

Natural antioxidants such as fruits and vegetables, which provide protection against free radicals, can decrease the incidence and mortality rates of cancer and heart diseases, in addition to their other health benefits [12]. Recently, the consumption of herbs such as cornelian cherry (*Cornus mas*), with high levels of antioxidants and anthocyanins, has been increased. C. mas fruits are prescribed for gastrointestinal and excretory disorders [13], besides improving liver and kidney functions [14] [15]. This plant was used to treat diarrhea, intestinal inflammation, fever, malaria, kidney stones and kidney and bladder infections in traditional medicine. *Cornus mas* fruits has anthocyanins, flavonoids, and plenty of oxalic acid content [16]. It also contains antioxidant substances including butyrate hydroxyanisole and butylated hydroxytoluene, and has the potential to fight cancer [17] [18].

To our knowledge, there is no report regarding the protective effect of *Cornus mas* fruit extract administration on MTX-induced oxidative damage in the liver of rats. Therefore, the current study was designed to determine the possible therapeutic effects of *Cornus mas* fruit extract in MTX-induced acute hepatotoxicity.

Materials and methods

Animals and Treatment

Forty-two male albino wistar rats weighing [200-250 g] were used in this study. The rats were procured from the Pastor institute Tehran, Iran. The animals were kept in plastic cages under standard humidity, light (12h light/12h darkness) and temperature (22±2°C) condition during the experiment. The rats were provided unlimited access (*ad libitum*) to water and food. (Ethic code of commitment of animal ethic and rights of university).

Plant materials

Cornus mas fruits were obtained from suburbs of Kaleibar (East Azerbaijan, Iran) at the end of summer 2014. The fruits were air-dried and then ground into powder; in all the steps, the components were protected from direct sunlight. The powder was kept at 8°C.

Extraction

The air-dried *C. mas* fruits were ground into a coarse powder, 500 g of which was mixed with a methanol: water (7:1) solution at 25 ± 2°C. The solvent was completely re-moved by rotary vacuum evaporator at 50°C. Afterwards, CMFE was frozen at -20°C until

using time.

Experiment protocol

Rats were randomly divided into six groups, two as a healthy and CMFE groups consisting of seven rats and the remaining fore groups consist of ten rats each.

Group I (Control group, n = 7): Physiologic saline (PS) (0.09%NaCl) solution was administered orally in approximately the same volume as the drugs given to the other animals on the same day for seven days starting from the first day;

Group II (CMFE group, n = 7): CMFE (*Cornus mas* fruit extract) was administered in a dose of 700 mg/kg orally for seven days starting from the first day.

Group III (MTX group, n = 10): MTX (Methotrexate®10 mg/ml, Ebetrex Pharma) was administered in a single dose of 20 mg/kg (i.p.) on the first day of the study. On the other days with no drug administration, the solution was administered orally in approximately the same volume as the drugs given to other animals on the same day;

Group IV (MTX + CMFE group, n = 10): CMFE (*Cornus mas* fruit extract) was administered in a dose of 300 mg/kg orally for seven days starting from the first day. MTX was administered only on the first day, as in Group III.

Group V (MTX + CMFE group, n = 10): CMFE (*Cornus mas* fruit extract) was administered in a dose of 700 mg/kg orally for seven days starting from the first day. MTX was administered only on the first day, as in Group III.

Group VI (MTX + CMFE group, n = 10): CMFE (*Cornus mas* fruit extract) was administered in a dose of 1400 mg/kg orally for seven days starting from the first day. MTX was administered only on the first day, as in Group III.

Diarrhea was observed in some of the MTX-administered rats. But we immediately supported them with liquid by oral gavages.

Sample collection and preparation

At the end of the experimental days, anaesthesia was produced by means of a cocktail prepared by using ketamine hydrochloride (90 mg/kg) and xylazine hydrochloride (10 mg/kg) i.p. Following anaesthesia, all rats were sacrificed, and intracardiac blood samples and tissue samples were obtained. Portions of the tissue samples obtained were placed in 10% neutral formalin solution after sectioning into pieces of 3–5 cm thickness for histopathological investigations. The rest of the tissue samples were stored in a freezer at −80◦C for biochemical analyses. Blood samples obtained for the biochemical analyses were analyzed on the same day.

Measurement of biochemical parameters in the serum

Rat's blood samples were collected in biochemistry clot activator tubes without preservatives, and they were centrifuged at 4000 rpm for 10 min (Hettich, Germany). The levels of Total- Direct and indirect bilirubin, Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Lactate dehydrogenase (LDH) were measured photometrically in a biochemistry auto analyzer (Mindray BS 800 M, China).

Preparation of liver homogenate

After treatment days, animals were scarified. The liver tissues were homogenized in KCl (10 mM) phosphate buffer (1.15%) with EDETA (pH= 7.4) and centrifuged at 3000 rpm for 30 minutes. The supernatant was collected to be used for measurement of malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD). The total protein content was determined based on Lowry's method [19].

Designation of lipid peroxidation

Lipid peroxidation was measured based on formation of Thiobarbituric acid reactive substance (TBARS) from liver homogenate. For preparation of Thiobarbituric acid reagent, 2ml (15% w/v TCA, 0.375% w/v TBA, and 0.25 M Hcl) was added to 2ml of the supernatant. The dilution was heated for 15 minutes in boiling water. After cooling, it was centrifuged at 1000g for 10 minutes and the precipitate was removed. MDA forms were mixed with TBA, which was measured by spectrophotometer at 532 nm. The concentration of MDA was computed based on the absorbance coefficient of the TBA-MDA complex (ε = 1.56 × 105/M/cm), and presented as nmol/mg of protein [20].

Antioxidant enzymes activity

For the measurement of catalase activity, one unit of catalase was required to decompose 1 µM of H_2O_2 in 1 minute. By adding 1.0 mL of 20 mM H_2O_2 (freshly prepared), the reaction was inaugurated. Decomposition level of H_2O_2 was determined by spectrophotometer at 240 nm for 2 minutes. The enzyme activity was presented as U/mg of protein [21].

The SOD activity was assayed as described by

(Winterbourn et al, 1975). The reaction mixture contained 3 mL of 0.067M potassium phosphate buffer (pH = 7.8), 0.2 mL of 0.1 MEDTA, 0.3 mM sodium cyanide (NaCN) and 0.1 mL of 1.5mM nitroblue tetrazolium (NBT). One unit of enzyme activity (as amount of SOD) was determined to produce a 50% inhibition of NBT reduction and the specific enzyme activity was presented as units per milligram of total protein.

Statistical analysis
The statistical analysis was performed by using a Windows compatible SPSS®16.0 program. The groups were compared using one of the non-parametric tests, the Kruskal–Wallis test, in the analysis of the biochemical and histochemical findings obtained in the blood samples. The Mann–Whitney *U*-test was used in the comparisons of measurements between the two groups. The level of significance was accepted as $p < 0.05$. The values were expressed as Mean ±SEM. In the analysis of the findings obtained from the tissue samples, the groups were compared using a nonparametric test, one-way ANOVA, and the significance among the groups were identified using the least significant difference among the post-hoc tests. The level of significance was accepted as P<0.05.

Result
CMFE reverses MTX-induced hepatotoxicity
Our data revealed that MTX administration caused a nine-fold increase of LDH level in the serum, which was decreased fourfold in the MTX+CMFE group (Figure 1). MTX-treated group also showed significant increase in the hepatotoxicity markers AST (76.36%), ALT (44.76%), serum ALP (46.66%) and Direct Bilirubin levels (84.52%) when compared to control (P<0.05), that indicates MTX-induced hepatotoxicity (Table 2 and 3). In CMFE-administered groups ALT, AST, ALP and Direct bilirubin showed no significant changes. Administration of CMFE significantly prevented MTX-induced alterations in these biochemical parameters, that is, AST (P<0.05), ALT (P<0.05), ALP (P<0.05), Direct bilirubin (P<0.05) and LDH activity (P<0.05).

CMFE reduces MTX-induced oxidative stress by modulating the activities of antioxidant defense enzymes
MTX was found to impede the antioxidant defense machinery of cells (Table 4). In the in vivo rat model, CMFE administration prevented this reduction in antioxidant activities. It was observed

that MTX administration significantly increased the MDA content in the liver cells (55% change) formed as a result of increased LPO, indicating the presence of oxidative stress, which was markedly suppressed by CMFE pretreatment (33% change). No significant difference of MDA level was observed between CMFE and control groups. In the cellular context, SOD, catalyzes the conversion of O_2 to a less harmful product, H_2O_2, which is then converted to water by CAT. MTX treatment also impaired the activities of CAT (0.8 nmoles of H_2O_2 consumed/min/mg of protein) and SOD (16 units/mg of protein). Treatment with CMFE maintained the activities of CAT and SOD close to normal (1.278 nmoles of H2O2 consumed/min/mg of protein and 28.04 units/mg of protein, respectively). Modulation of the activities of these enzymes by CMFE possibly prevents the mitochondria generated reactive radicals from causing oxidative stress and cellular damage.

Discussion
MTX-induced damage has been documented in several articles, including different types of tissues [22]. According to a search of the literature, until now, there has been no study performed on the effects of CMFE on MTX-induced liver tissue damage of rats. Hence, we aimed to determine whether CMFE have any protective effect on MTX-induced damage of a rat model. However, the present study demonstrated that CMFE have protective effects against acute MTX toxicity in the liver of rats.

As the liver is the major detoxifying organ in the body, it targets an enormous variety of drugs and chemicals. The conversion of MTX to one of its major components, 7-hydroxymethotrexate, occurs in the liver. Drugs used for chemotherapy are well known to trigger oxidative stress in multiple ways in various systems. It has been reported that MTX caused a significant decrease in antioxidant levels [22]. Antioxidant agents have been used to prevent the oxidative damage produced by MTX in several studies and these have achieved significant success [23, 24]. In another study, SOD and CAT enzyme activities increased in acute phase damage [25]. On the other hand, in a relatively longer term study compared to the others, antioxidant enzyme activities (SOD, CAT, and others) decreased in drug-mediated oxidative renal damage in rats. Moreover, MTX strongly limits folic acid conversion into tetrahydrofolate, which is necessary for DNA synthesis in S-phase. This results in cell death [26].

SOD and CAT are the enzymes used as detoxifying agents, and they are the most powerful enzymes against ROS products. In a previous study, increased

levels of SOD and CAT activities were observed in MTX-mediated renal damage [26]. In another study, SOD and CAT enzyme activities increased in acute phase damage [27]. On the other hand, in a relatively longer term study compared to the others, antioxidant enzyme activities (SOD, CAT, and others) decreased in drug-mediated oxidative renal damage in rats, while CMFE treatment provided an increase in those levels [28]. Increased SOD and CAT activities were determined in CMFE-administered groups (doses 700 and 1400 mg/kg). These results suggest that renal tissue is affected by MTX toxicity even in the acute phase, and the enzyme activities consequently reduced. CAT is the common enzyme in liver and kidney tissue, which increases in the acute phase. We also determined increased CAT activity in the acute phase of MTX administration. Accordingly, this enzyme not significantly affected by the damage in the acute phase. But, SOD activity may show changed activity due to damage, depending on the end organ tissue. CAT activities were increased in liver tissue.

Cells commonly attempt to store MTX in poly glutamated form. The principal toxicity of MTX is believed to result from its being kept in this form for a long time period under cytosolic conditions. Moreover, MTX is also responsible for inhibiting NADP-dependent dehydrogenases and NADP malic enzymes [29]. In normal physiological conditions, NADPH is used by glutathione reductase for maintaining cellular glutathione (GSH) levels. By using this pathway, MTX causes serious membrane damage [30]. In our study we determined significantly increased MDA levels in the liver tissues in MTX-administered rats. However, CMFE administration significantly decreased MDA levels. Higer dose of CMFE seems to be more efficient in counteracting increased MDA levels. According to another study, ALA (alpha lipoic acid) was also successful in reducing the MDA and NO levels in a rat model of drug-mediated hepatotoxicity [31]. Moreover, ALA was administered for treatment in a drug-mediated hepatotoxicity rat model and significantly decreased MDA levels [32]. Antioxidant components of CMFE may cause membrane stabilization and reverse the normalization of fluctuated biochemical profiles induced by methotrexate exposure. Therefore, plant extract compounds affect the liver and kidney by maintaining its normal function and decreasing the derangements of cell membrane. Purification of CMFE active components for determining their exact protective effects on nephrons and hepatocytes is recommended for further studies.

The present study indicated the hepatoprotective effects of CMFE in methotrexate induced liver injury in rats.

Declaration of interest
The authors declared no potential conflicts of interest.

Ethical approval
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Table 1. Serum Parameters indicate the liver function

Groups	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)	Indirect Bilirubin (mg/dl)
Control	0.253±0.0103	0.026±0.008	0.215±0.010
CMFE	0.175±0.0137	0.028±0.009	0.155±0.0204
MTX	0.281±0.011[a]	0.168±0.063[a]	0.228±0.073
MTX+CMFE 300	0.301±0.014	0.036±0.005	0.25±0.094
MTX+CMFE 700	0.346±0.059	0.06±0.035	0.271±0.074
MTX+CMFE 1400	0.158±0.0172[b]	0.036±0.005[b]	0.123±0.089[b]

[a] Indicate the significance $P<0.05$ compared with the Control Group. [b] indicates the significance $P<0.05$ compared with the MTX group. Values are Mean±SEM (n=7 or n=10 in each group).

Table 2. Serum Parameters indicate the liver function

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)
Control	134.83±22.99	58±10.24	104.16±12.92
CMFE	160.33±34.81	89.16±20.6	110.15±15.98
MTX	567.83±180.01[a]	105.33±22.23[a]	195.83±15.49[a]
MTX+CMFE 300	395.33±98.20	106.83±18.04	173.16±13.31
MTX+CMFE 700	171.16±25.44[b]	56.16±5.72[b]	116±20.44[b]
MTX+CMFE 1400	149.00±20.60[b]	67.16±11.92[b]	149.16±20.13

[a] Indicate the significance $P<0.05$ compared with the Control Group. [b] indicates the significance $P<0.05$ compared with the MTX group. Values are Mean±SEM (n=7 or n=10 in each group).

Figure 1. [*] Indicate the significance $P<0.05$ compared with the Control Group. [#] indicate the significance $P<0.05$ compared with the MTX group. Values are Mean±SEM (n=7 or n=10 in each group).
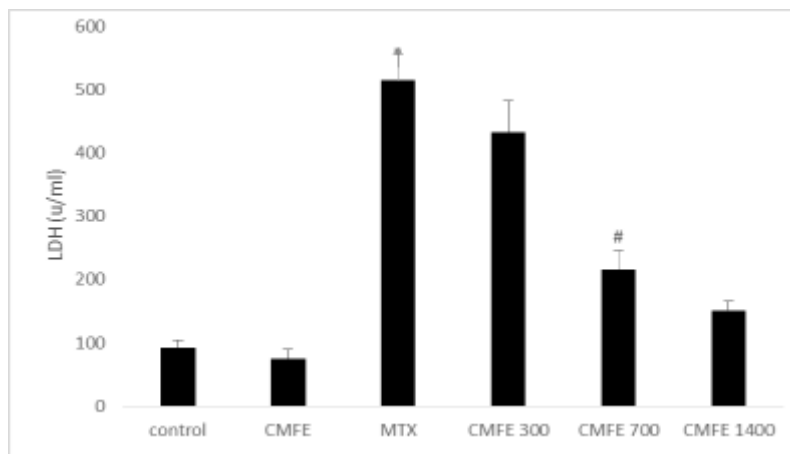
Table 3. Oxidative stress parameters indicate the liver function

Groups	SOD(U/mg protein)	CAT(nmol H2O2/min/mg protein)	MDA(nmol/mg protein)
Control	242.63 ± 29.39	189.22 ± 25.11	4.32 ± 0.89
CMFE	259.23 ± 32.25	174.34±36.09	4.06± 0.94
MTX	266.44 ± 36.45	259.87±81.23	10.87 ± 1.50[a]
MTX+CMFE 300	255.63 ± 14.85	236.41±52.22	7.70 ± 1.51
MTX+CMFE 700	245.22 ± 59.44	209.77±54.12	6.44 ± 1.73[b]
MTX+CMFE 1400	232.43± 62.36	195.44±46.57	5.23 ± 1.32[b]

[a] Indicates the significance P<0.05 compared with the Control Group. [b] indicates the significance P<0.05 compared with the MTX group. Values are Mean± SEM (n=7 or n=10 in each group).