Evaluation of antioxidant and antiapoptogenic effects of Sumach seed extract on liver injury induced by amethopterin in male rat model

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Background and objective
Amethopterin (AM), like most chemotherapy medications, causes cellular generation of oxidative stress. In Iranian traditional medicine, Sumach is widely used owing to its antioxidant properties. In this study, the authors aimed to assess the probable antioxidant, antiapoptogenic, and therapeutic effects of *Sumach* extract on liver tissue and hepatocytes against hepatic degeneration induced by AM as a chemotherapy drug.
Materials and methods
A total of 64 rats were grouped (n=8), including (1) control, (2) AM (20 mg/kg), (3–5) Sumach (75, 100, 150 μg/ml), and (6–8)- AM+Sumach. Hydroalcoholic extract of Sumach seeds was prepared. Treatments with Sumach extract were applied intraperitoneally daily for 28 consecutive days. Nitrite oxide was measured by Griess assay. Ferric reducing ability of plasma (FRAP) and malonaldehyde test were used to measure total antioxidant and lipid peroxidation levels, respectively. Hepatocyte apoptotic index was analyzed via tunnel test. The expression level of p53 and Bax genes was measured by real-time PCR. Furthermore, liver enzymatic function, histopathological changes, and morphometric alterations were examined.
Results and conclusion
Antioxidant values, p53 and Bax genes' expression, nitrite oxide, enzymes, and morphometrical features were increased significantly (except FRAP levels, decreased) in AM group as compared with the control group ($P<0.01$). Besides, the evaluated parameters were all significantly reduced (except insulin and FRAP levels and Bcl2 gene expression, which were increased) in SMGC and diabetic +SMGC groups in comparison with the diabetic group ($P<0.05$). Moreover, evaluated parameters were significantly reduced in Sumach and Sumach+AM groups (except FRAP level, which is increased) compared with the AM group ($P<0.01$). According to the acquired data, Sumach through its antiapoptogenic and antioxidant features is able to eliminate hepatotoxicity induced by AM administration, which leads to restoration of histopathological liver changes.

Keywords:
amethopterin, antioxidant, apoptogenic, liver, Sumach

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Introduction

Growing industries, environmental pollutions, and human exposure to carcinogenic agents are three main aspects of carcinogenesis in humans. Today, besides the increasing rate of cancer incidence, discovery of synthetic anticancer drugs has attracted great scientific considerations by scholars [1]. Anticancer drugs have two effects on body tissues at the same time: therapeutics effects and adverse effects [2]. Amethopterin (AM) is a vital chemotherapy agent with various therapeutic application in the field of malignant tumor treatment (such as leukemia, lymphoma, and breast cancers) and non-cancerous diseases (such as rheumatoid arthritis and skin-related autoimmune disorders) [3,4]. Based on the chemical structure, AM precipitates in some various tissues like kidney, gall-bladder, and liver [5]. Chronic accumulation of AM in hepatocytes can lead to the generation of active oxygen radicals [6]. AM inhibits synthesis of folic acid, purines, and pyrimidines, which causes the formation of impaired DNA, as well as arrests cell division [7]. AM was initially distributed commercially as a foliate antagonist [8]. Then, it was found as an enhancer for induction of oxidative stress. This cytotoxic phenomenon causes direct destruction of renal tubules in urinary system [9]. Cellular lipid peroxidation as a result of oxidative stress is involved in

damages to the membranes of cell and organelles, lipoproteins, and other lipid constructions in intracellular environment or even in extracellular spaces. Following lipid peroxidation, the peroxides and hydroxides are activated within the cells, which lead to organ toxicity and decreased level of antioxidant enzyme [10]. To withstand the aforementioned pathological conditions, the improvement of antioxidant system of body seems necessary.

Plant-based antioxidant substances have less detrimental adverse effects with more fabulous therapeutic features, like Sumach, which belongs to the Anacardiaceae family. Sumach grows widely in the northern mountainous areas of Iran [11]. In traditional medicine, the aqueous solution of Sumach is widely used for relief of some diseases like hemorrhoids, diarrhea, and insect bite pain [12]. In the published biochemical literature, Sumach has been found as a rich source of tannins and flavonoids [13]. Flavonoids are an active antioxidant agent with hypoglycemic, antitumor, and free radical scavenging features [14]. Thus, the hydroalcoholic extract of Sumach initiates the activity of intracellular antioxidant enzymes (catalase and superoxide dismutase) [15]. Based on the cellular toxicity and oxidative stress conditions following AM administration, and extensive use of AM in cancer treatment, the anti-oxidative properties of Sumach following AM administration was assessed biochemically and histologically. To the best of our knowledge, no previous reports have been published on antiapoptogenic and anti-oxidative effects of Sumach. Therefore, the aim of this study was to assess/determine the possible therapeutic properties of Sumach extracts on liver injury, induced by AM.

Materials and methods

Animals

The animal house of Kermanshah University of Medical Sciences provided the male Wistar rats needed for this research (n=64). All animals were mature with the age of 8 weeks and weight of 200–250 g. The animals were kept in standard conditions, including 12 : 12 h light/dark cycle, and 23±2°C of temperature. All ethical and humane principles of research were followed, and the study was approved and accepted by the Ethics Committee of Medical Sciences (IR.KUMS. REC.1396.425 Ethics Certificate) [16].

Plant collection

The fresh seeds of Sumach (Anacardiaceae or cashew family) were collected in August 2019 from local areas of Javanrood (Kermanshah, Iran). The accuracy of plant was authenticated by Dr F. Firozian (Department of Botany, H.U.M Institute, Hamedan, Iran), and the voucher specimen code was given to Sumach plant (Pharm-PCT-2037).

Preparation of Sumach seed extract

The seeds were processed as follows: the plant was dried under shade, the powder was prepared (by abrasion), and the herbal extract solution was provided (300 g of powder in 1300 ml of water-ethanol solution in the ratio of 50 : 50). Two days later, the solution was filtered and centrifuged at 3000 rpm for 10 min. Finally, the supernatant was separated and dried at 38°C. The prepared extract was stored in a refrigerator for future administration [17].

Experimental design

A total of 64 animals were categorized into eight groups (n=8): the first group (control) received I.P injection of normal saline (as the same to the volume of treatment groups). The second group (AM group) received a single dose of AM (20 mg/kg) I.P. Group 3–5 (Sumach group) received respective doses of 75, 100, and 150 µg/ml of Sumach I.P (for 28 days). Groups 6–8 (AM+Sumach groups) received a single dose of AM (20 mg/kg) to induce liver damage followed by administration of 75, 100, 150 µg/ml of Sumach (I.P for 28 days), correspondingly [14,18].

Sample preparation

A day after the last injection, the rats were killed by ether inhalation. Overall, 5 ml of the blood sample was aspirated from the right ventricle. After keeping the blood for 20 min at 37°C, the formation of blot clot was seen, and it was centrifuged (4000 rpm) for 10 min. The obtained serum was reserved in a −70°C refrigerator for future hepatic biochemical analysis. A fragment of the liver (1×13) was fixed in 10% formalin solution for morphometric and histological examinations [10].

Determination of NO level

The Griess method based on colorimetry approaches was employed to measure the NO levels. Overall, 500 µl of serum was deproteinized by adding zinc sulfate (10 mg), and then it was followed by centrifugation (3000 rpm for 10 min). Equal amounts of Griess reagent (1% sulfanilamide and 0.1% naphtylethylenediimide in 2.5% phosphoric acid) were also added to the supernatant in 96-well ELISA plates (incubated for 10 min at 37°C). The absorbance was measured at 450 nm by the use of a

microplate reader. Nitrite concentrations were considered by sodium nitrite standard curve [16].

Lipid peroxidation levels

The lipid peroxidation levels were measured according to the dye generated based on the reaction between thiobarbituric acid (TBA) and malondialdehyde (MDA). The frozen samples of the liver were homogenated by an ultrasonic homogenizer machine. The liver was washed with cold phosphate-buffered saline (pH 7.4) containing EDTA. Overall, 60 µl of tissue was mixed with 5 µl of butylated hydroxytoluene, 100 µl of phosphoric acid (10 M), and 100 µl of TBA solution, respectively. The tubes were incubated (30 min, 50 °C) and then centrifuged (3000 rpm, 15 min). Then, 75 µl of the supernatant was poured into the spectrophotometer tubes, and the dye generated by the molecular reaction in the commercial kit was read at 532 nm. The MDA levels were stated in nmol/mg unit [17].

Determination of FRAP level

The total antioxidant potential of liver homogenate was measured based on the modification of ferric reducing ability of plasma (FRAP). A mixture of 10 ml of 50 mmol/l 2,4,6-tripyridyl-s-triazine in 100 ml of 0.5 M acetate buffer (pH 5), 10 ml of 10 mmol/l ferric chloride, and 30 mmol/l of hydrochloric acid was considered as FRAP reagent (with mixture ratio of 25 : 2.5 : 2.5). The FRAP test was done by reagent preheated to 37°C for 20 min, followed by 100 µl of a liver homogenate sample mixed with 100 µl of substance. The mixture was centrifuged (10 000 rpm, 20 min), and the absorbance of supernatant was read at 540 nm, in comparison with standard curve [10].

Measurement of hepatic enzymes

The liver homogenate was centrifuged twice (12 000 rpm, 10 min) and the supernatant was separated for hepatic enzymes assessment, including aminotransferase, aspartate alanine aminotransferase, and alkaline phosphatase. The alanine aminotransferase and aspartate aminotransferase were tested based on Reitman and Frankel biochemical methods. The alkaline phosphatase protocol was also determined according to the technique, which was set out in the practical research laboratory [16].

Morphometrical and histopathological alterations

A part of the right lobe of liver was fixed in 10% formalin for morphometrical assessments. Following the routine histological process using tissue processors machine, the tissues were embedded in paraffin, and thin sections (4 µm) were prepared using a microtome (Leica RM 2125, Nussloch, Germany). These slices were stained by hematoxylin and eosin technique. Some morphological assessments were measured by light microscopy (×40 magnifications), including full cellular area, hepatocyte outline, maximum and minimum axis, mean axis, and central hepatic vein. At least 50 cells from the separate region were measured to remove the probable measurement bias. All procedures were applied with a microscope linked to a DP12 Camera (3.34 million-pixel resolution). Finally, the morphometrical assessments were done using the Olysia Bio-software (Olympus Optical Co. Ltd, Tokyo, Japan) [10].

Determination of apoptotic genes' expression (p53 and Bax) using real-time PCR analysis: real-time quantitative PCR (RT-PCR) was performed to quantitatively determine mRNA expression of p53 and Bax in liver tissues. Total RNA extraction was conducted using RNeasy mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol (since the RNeasy procedure enriches for RNA species >200 nt, RNA yield does not include tRNAs, 5S rRNA, or other low-molecular-weight RNAs), and treated by RNase-free DNase set (Qiagen) to eliminate the genomic DNA contamination. They were then transferred to a 2 ml RNase-free polypropylene centrifuge tube. It is notable that there is no RNA isolation procedure that produces RNA completely free of genomic DNA contamination; therefore, the RNA samples were treated with DNase. Preparing of RNA free from DNA is a vital step before performing RT-PCR. DNase is an endonuclease that digests single-stranded and double-stranded DNA into oligonucleotides and mononucleotides and is appropriate for removing DNA from RNA. A simple 15-min digestion at room temperature removed the DNA contamination. The DNase can be inactivated either by adding the stop solution provided or heating. Heating also denatures the RNA, so that RNA can directly be used for reverse transcription. The concentration of total RNA was determined using a biophotometer (Eppendorf). To test the integrity of extracted RNAs, 2–3 µg of RNA was run on an agarose gel electrophoresis. Total RNA (100 ng) was reverse-transcribed to cDNA by the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania), according to the manufacturer's instructions. The cDNA was used for the validation of the custom PCR mix. The Maxima SYBR Green Rox qPCR master mix kit (Fermentas)

was used for real-time RT-PCR to ensure cDNA template. The qPCR primers employed in this study are listed in Table 1. The house-keeping gene glyceraldehydes-3-phosphate dehydrogenase was used as an endogenous control. Real-time PCR reactions were performed using Step one plus (Applied Biosystem, Foster City, CA, USA). The PCR amplification conditions consisted of 10 min at 95°C followed by 40 cycles of denaturation step at 95°C for 15 s and annealing and extension for 1 min at 60°C. Data were analyzed using the comparative C*t* ($^{\Delta\Delta}$C*t*) method. The relative expression level of p53 and Bax was calculated by determining a ratio between the amount of p53 and Bax and that of endogenous control. Melting curve analysis (60°C→95°C increment of 0.3°C) was used to determine the melting temperature of specific amplification products and primer dimers. These experiments were carried out in triplicate and were independently repeated at least 3 times [1].

Tunnel assay

The tunnel test was performed according to the manufacture's procedure (Roche, Mannheim, Germany). Paraffin-embedded blocks were prepared using an automatic tissue processor. Overall, 5-μm histological slices were cut through a microtome (Leica, Germany), and five slices per rat were selected. The tissues were deparaffinized and the tunnel-positive cells were counted with light microscopy [17].

Data analysis

The statistical analyses were done by one-way analysis of variance. Tukey post-hoc test was also used to determination the statistical alterations among the studied groups. SPSS (version 16.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis, the outcomes were expressed as mean±SE, and *P* less than 0.05 was considered as significant.

Results

Levels of FRAP, lipid peroxidation, and serum NO of liver

In AM group, the levels of FRAP were lower significantly ($P<0.01$) than the control group. Moreover, in whole groups of Sumach and AM +Sumach, the FRAP levels were elevated significantly ($P<0.01$) as compared with the AM group following Sumach administration. AM, owing to its detrimental effects, showed significantly increased levels of nitrite oxide and lipid peroxidation ($P<0.01$) in AM group in comparison with the control group. It is also found that all doses of Sumach extract significantly reduced the mean levels of serum nitrite oxide and lipid peroxidation in Sumach and AM+Sumach groups as compared with the AM group ($P<0.01$) (Table 2).

Table 1 Primers used in real-time PCR

Primer ID	Primer sequences
GAPDH	F: 50-AAGCTCATTTCCTGGTATG-30
	R: 50-CTGCCACAAGAACTAGAGA-30
p53	F: 50-AGAGACCGCCGTACAGAAGA-30
	R:5 0-GCATGGGCATCCTTTAACTC-30
Bax	F: 50-CCGGCGAATTGGAGATGAACT-30
	R: 50-CCAGCCCATGATGGTTCTGAT-30

Levels of liver enzymes activity

AM administration can lead to significant increase ($P<0.01$) in levels of liver enzymes in comparison with the control group. No significant differences ($P>0.05$) were also found in the mean concentration of hepatic enzymes in all Sumach groups compared with the control group. Moreover, the entire various doses of Sumach in Sumach and AM+Sumach groups expressed a significant decline ($P<0.01$) in the mean concentration of hepatic enzymes compared with the AM group (Table 3).

Morphometric examination

In the experimental groups, significant incremental alteration was detected in the mean diameter of hepatocytes and CHV among control and AM groups ($P<0.01$). Moreover, in mean diameter and CHV, no significant variations were seen histologically in all Sumach groups than the control group ($P>0.05$).

Table 2 Effects of AM and Sumach on antioxidant parameters and AI in male rats (*n*=8 for each group)

Groups	NO (mmol/ml)	FRAP (mmol/ml)	LP (nmol/ mg)	AI
Control	75.45±3.2	115.35±5.3	0.66 ±00.4	1.3 ±0.02
AM	230.36 ±7.1*	24.45±2. 2*	0.23 ±0.05*	17.4 ±2.12*
Sumach 75 μg/ ml	74.33 ±2.3†	118.72±6.8†	0.67 ±0.02†	1.3 ±0.02†
Sumach 100 μg/ ml	72.07 ±4.3†	120.34±7.3†	0.62 ±0.06†	1.6 ±0.03†
Sumach 150 μg/ ml	76.58 ±5.7†	121.07±3.2†	0.64 ±0.04†	1.7 ±0.05†
Sumach+AM 75 μg/ml	170.44 ±8.3$	61.85±5.1$	0.43 ±0.01$	3.5 ±0.04$
Sumach+AM 100 μg/ml	132.37 ±6.2$	73.46±6.6$	0.46 ±0.01$	2.4 ±0.07$
Sumach+AM 150 μg/ml	133.55 ±4.1$	73.34±8.1$	0.43 ±0.02$	2.1 ±0.03$

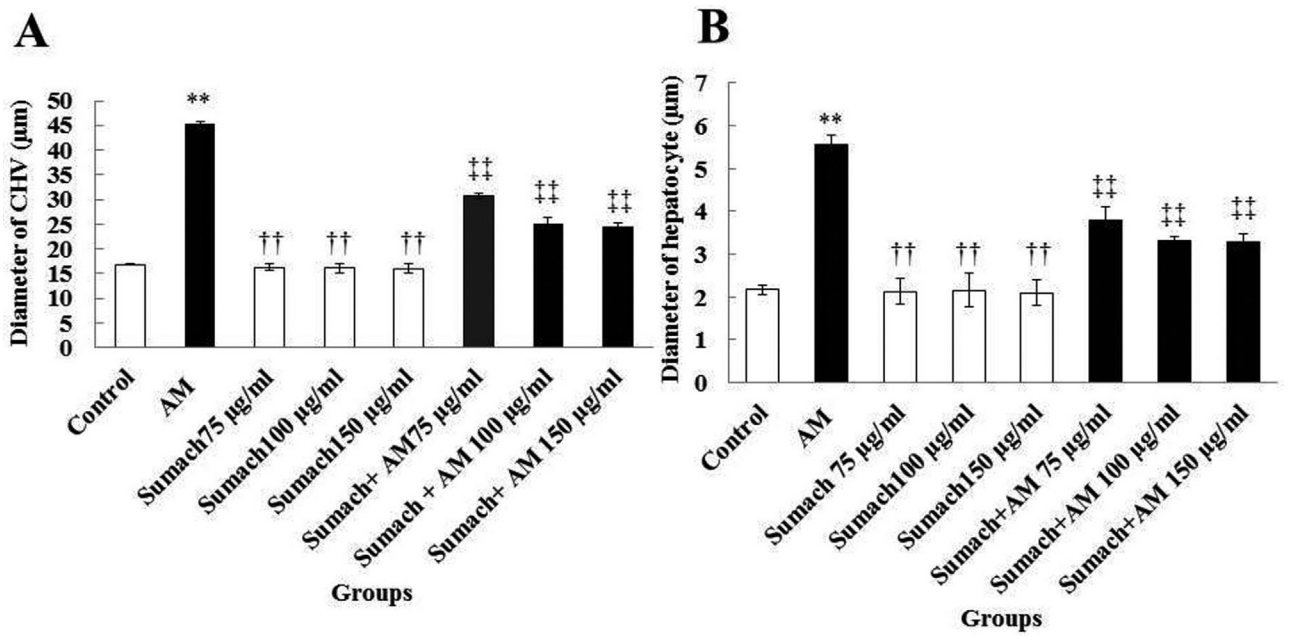
Data are presented as mean±SEM. *$P<0.01$ compared to the control group. †$P<0.01$ compared to AM group. $$P<0.01$ compared to the AM group. AI, apoptotic index; AM, amethopterin, FRAP, ferric reducing the ability of plasma; LP, lipid peroxidation; NO, nitrite oxide.

Table 3 Effects of AM and Sumach on liver enzyms in male rats (*n*=8 for each group)

Groups	AST (ng/ml)	ALT (ng/ml)	ALP (ng/ml)
Control	264.48±4.3	43.35±2.7	311.62±8.5
AM	412.25±6.6*	105.25±6.1*	602.03±6.1*
Sumach 75 μg/ml	264.75±3.4†	42.22±3.4†	310.61±7.2†
Sumach 100 μg/ml	260.36±5.7†	40.04±2.5†	315.12±7.1†
Sumach 150 μg/ml	261.08±6.1†	43.47±1.7†	312.61±5.4†
Sumach+AM 75 μg/ml	328.37±7.2$	73.86±6.1$	497.42±4.6$
Sumach+AM 100 μg/ml	305.82±5.3$	71.41±4.7$	475.16±6.3$
Sumach+AM 150 μg/ml	304.21±6.3$	71.37±3.1$	471.73±8.2$

Data are presented as mean±SEM.*P<0.01 compared with the control group. †P<0.01 compared with AM group.$P<0.01 compared with the AM group. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AM, amethopterin; AST, aspartate aminotransferase.

Figure 1



Morphometric alteration assessments of AM, Sumach, and Sumach+AM administration regarding CHV (a) and hepatocyte diameter (b) in the right lobe of the liver stained with hematoxylin and eosin by a light microscope at 100× magnifications with a ADPMEPD12 Camera (3.34-million-pixel resolution) and using the Olysia Bio-software. **Significant difference than the control group ($P<0.01$). ††Significant difference than the AM group ($P<0.01$). ‡‡Significant difference than the AM group ($P<0.01$). AM, amethopterin; CHV, central hepatic vein.

Additionally, the Sumach significantly decreased the mean diameter of cells and CHV in all treated members of Sumach and AM+Sumach groups compared with the AM group ($P<0.01$) (Fig. 1).

Histopathological alterations

According to the findings of histological assessments, the normal liver structure was changed in AM control and Sumach treatment groups. Following AM administration in AM group, the liver displayed significant histological alterations and liver damages, including the increase in white blood cells (indicating inflammation process), an increase in irregularities rate, dilatation of hepatic sinusoidal space, and vacuolization of hepatocytes (indicating necrosis). Treatment with AM+Sumach in all doses reduced hepatic injury caused by AM administration (Fig. 2).

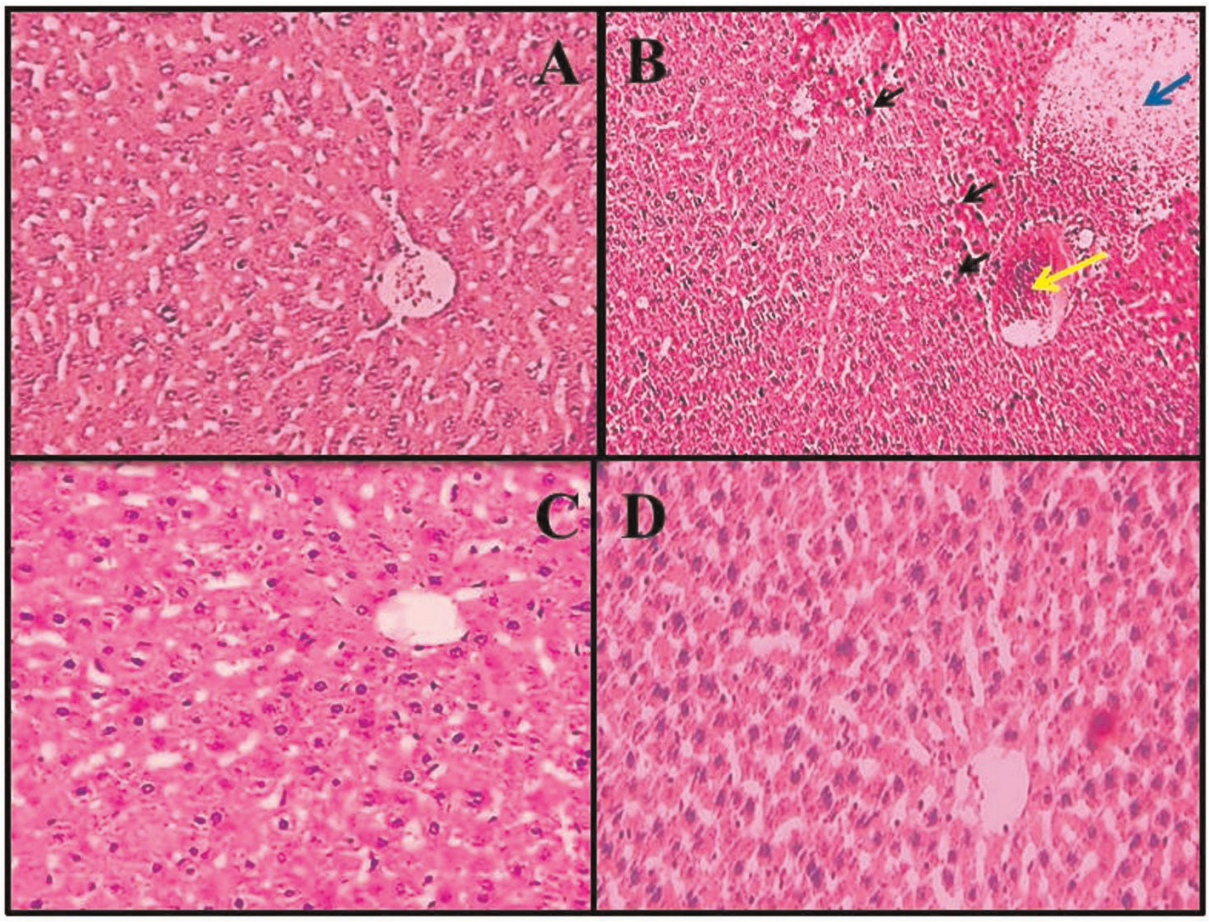
Levels of gene expression

The statistical analyses including one-sample *t*-test and analysis of variance were applied for assessments of AM and control group and also for Sumach and AM +Sumach groups, respectively. According to the statistical analysis of gene expression, upregulation of the apoptotic genes' expression (p53 and Bax) was detected significantly in the AM-treated group compared with the control group ($P<0.01$). Similarly, significant downregulation of these apoptotic genes was distinguished with all doses of Sumach and AM+Sumach groups compared with the AM group (Fig. 3).

Apoptotic index

The apoptotic index was significantly higher in AM group compared with the control group ($P<0.01$). No

Figure 2



Microscopic captured images in various groups (4 μm thin sections, H&E staining, 100×) by histopathological method. Control group (a) having normal liver structure. AM group (20 mg/kg) (b): an increased number of white blood and macrophage cells (inflammation) (black arrows), CVH dilatation (blue arrow) and hyperemia (yellow arrow), owing to the oxidative stress, were recognized in AM group. Sumach group (150 μg/ml) (c) in normal liver. Sumach (150 μg/ml)+AM (20 mg/kg) group (d) in normal liver. AM, amethopterin; CHV, central hepatic vein; H&E, hematoxylin and eosin.

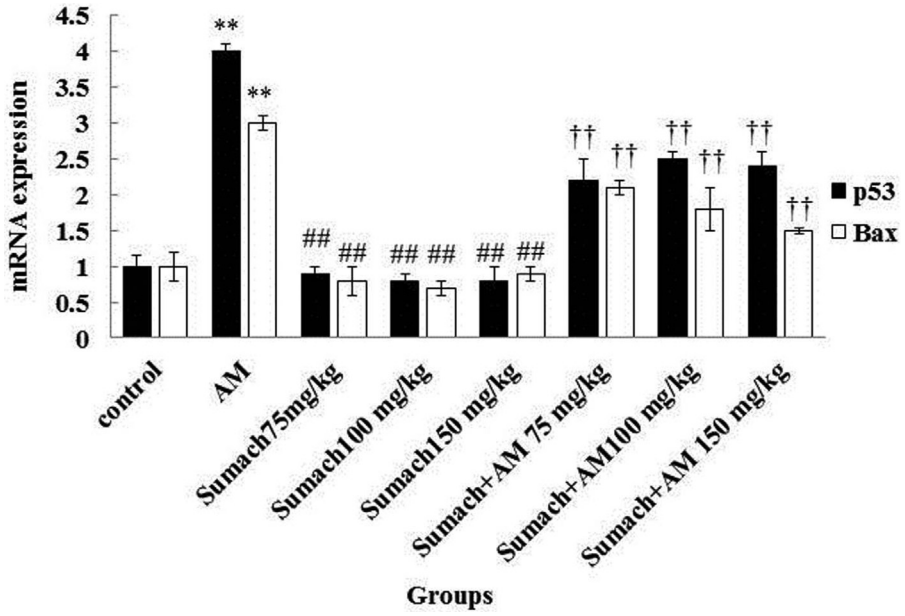
significant differences were found in the Sumach and AM+Sumach as compared with the control group ($P>0.05$). Furthermore, the entire several doses of Sumach in Sumach and AM+Sumach groups represented a significant decline in apoptotic index as compared with the AM group ($P<0.01$) (Fig. 4 and Table 2).

Discussion

The most important organ involved in the regulation of whole-body metabolism is the liver [10]. Thus, in this study, the toxic effects of AM on hepatocytes were assessed biochemically and histologically. Moreover, the therapeutic antioxidant features of Sumach on AM-related hepatic injuries were investigated.

AM is widely used in medical centers, but as a potential source of oxygen-radical production, it has various adverse effects on hepatocytes (owing to the involvement of the liver enzymes) [19]. This study confirmed the morphological changes following AM administration, including significant increase in hepatocytes diameter and the size of the central vein. Owing to the therapeutic effects of Sumach, in all animals that receiving AM+Sumach, a significant decline was seen on pathological changes. More differentiated microscopically features were also seen in the liver following AM administration like hyper-perfusion in Disse space of liver, aggregation of inflammatory cells (especially macrophages) around the central veins, central vein dilatation, and infiltration of the lymphoid cells within portal space. Based on histological principles, in any type of tissue inflammation or organ damages, macrophages are the main cells involved in inflammation, and secret chemical intermediates to adjust the tissue damage. Both altered dimensions of hepatic central vein and hepatocytes can be considered as the consequence of elevation in metabolic activity of detoxification to repel intracellular and extracellular toxins [20]. The metabolism of AM in detoxification process causes

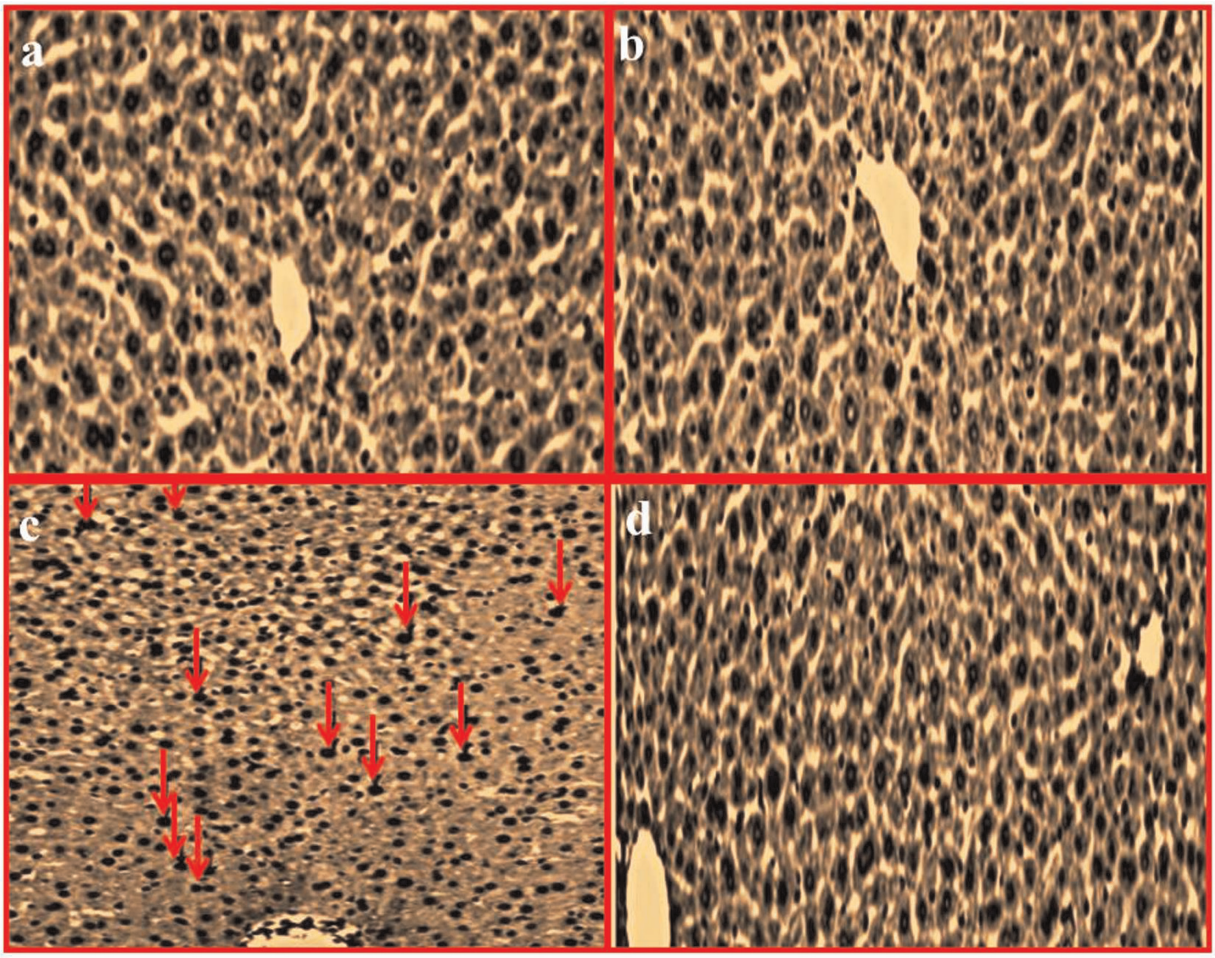
Figure 3



Results of real-time quantitative PCR of AM and Sumach at different doses on the p53 and Bax mRNA expression in liver tissue. Relative expression levels of each gene were obtained by using the comparative C*t* ($^{\Delta\Delta}$C*t*) method. **Statistically significant ($P<0.01$) between AM and control group. $^{\#\#}$Statistically significant ($P<0.05$) in AM and AM+Sumach groups. ††Significant modifications in Sumach groups compared with the AM group (P? 0.01). AM, amethopterin.

free radical accumulation in the cytoplasm, which leads to denaturation of membrane proteins, interference with DNA, lipid peroxidation, and finally, cell injury. The Kupffer cells located in sinusoidal space of the liver can be stimulated in answer to tissue damages. These cells secret *tumor necrosis factor*-α, interleukin-1, and NO, leading to hepatotoxicity and hepatocytes necrosis. AM is involved in extensive peroxidation of lipids in hepatocytes. Lipid peroxidation causes excessive cellular ROS generation and radical accumulation. ROS and free radicals attack the unsaturated fatty acids in cell membranes and induce protein alkylation process [10]. Based on the scientific finding including improved cellular levels of glutathione and loss of cytoplasm of hepatocyte, the AM seems to show hepatotoxic properties [19]. ROS in elevated non-physiological levels changes the three-dimension structure of the active site in hepatic enzymes and causes incomplete cell function, which eventually induces necrosis. Necrosis activates inflammatory responses in the liver, which is followed by infiltration of mononuclear inflammatory cells. Concurrently, the cells that die owing to necrosis release inflammatory chemical intermediates and induce systematic liver damage [17]. Conway and Carey [21] confirmed these pathological changes following AM administration. They found that the AM in an acute process can actively induce necrosis, induce liver hyper-perfusion in sinusoidal spaces, and increase the rate of *tumor necrosis factor*-α expression. AM is able to destroy the mitochondrial membrane in an indirect cellular pathway causing influx of mitochondrial enzymes into the cytoplasm and decreased total antioxidant action in cells [22]. Free radicals attack hepatocytes and induce necrosis in parenchymal cells, followed by inflammatory responses of other hepatocytes [16]. The induced liver injury is exacerbated by infiltration of inflammatory mononuclear cells into the impaired tissues. This inflammatory interaction could be exacerbated by proinflammatory mediators released from necrotic or damaged cells. These findings confirm the detrimental histological association among hepatocytes and AM administration. In a detailed theory, the histopathological variations can be related to a rise in NO levels which activates the oxidative stress process and rapid modifications in tissue such as hepatocytes atrophy, liver fibrosis, congestion, and steatosis. Although these histopathological variations can disappear in a few days, the diameters of central veins and hepatocytes still remain enhanced. Thus, NO has a critical role in AM-induced liver injury [17]. The outcomes of the current study are inconsistent with the results of the study carried out by Wu *et al.* [23], in which they presented AM with no effect on diameter of hepatocytes. The results of Fouad *et al.* [24] proved

Figure 4



Apoptotic induction (a–d) of 64 rats, which were equally divided into 8 groups, following AM and Sumach administration was examined using TUNEL assay. All experiments were carried out in triplicate. Apoptosis induction (a–d) (400× magnifications, TUNEL staining): control group (a), Sumach (150 μg/ml) group (b), AM (20 mg/kg) group (c) and Sumach (150 μg/ml)+AM (20 mg/kg) group (d). The red arrows referred to the apoptotic cells. AM, amethopterin.

that the administration of AM shows no necrosis, which is also in contrast with the results of the present study. In oxidative stress onset and increased reactive oxygen and nitrogen species (ROS/RNS) condition, the mitochondrion is the first intracellular organelle that is damaged [16]. The decreased liver enzymes in AM group could be associated with the lack of outflow of intracellular enzymes because of maintaining the consistency and integrity of cell membrane or renewal of damaged hepatocytes owing to antioxidant properties and concentrated oxidative stress of Sumach. In this regard, it was approved that the Sumach can moderate lipid peroxidation membranes of cells and organelles and increase total antioxidant capacity of hepatocytes (indicating a reduction in the levels of oxidative stress) exactly like some other studies [14,15]. Thus, it is concluded that the Sumach due to its antioxidant features can moderate the level of lipid peroxidation to the normal state and increase the level of total antioxidant potential via inhibition of ROS production. As it was stated previously and based on histological assessments, the increase in central vein diameter is directly related to the cell necrosis after AM administration [25]. Sumach by antioxidant property can inhibit glutathione reduction. In conclusion, the activation of antioxidant enzymes owing to the Sumach administration enables the cells to cope with fatal effects of oxidative stress [15]. Thus, it can be concluded that the Sumach as an exogenous agent regulates inflammatory pathways and inhibits apoptosis process [26]. Pourahmad *et al.* [14] in an experimental study based on histological assessments concluded that the Sumach has anticytotoxicity effects against oxidative stress. The results of Pourahmad *et al.* [14] indicated that the consumption of Sumach could reduce liver fibrosis with no changes in the level of the liver enzymes in mice under ethanol treatment, which is in contrast with the findings of this study [14]. Other papers stated that the AM produces pathologic conditions of oxidative stress and nitrosative stress and also enhances the expression of NG-Kβ and

P38 pathways [27]. In confirmation to the results of this article, Salimi *et al.* [28] indicated that the Sumach reduces the MDA levels in the case of diazinon-induced toxicity in male rats.

In this study, the results showed a significant increase in liver enzyme activity in AM group. Furthermore, in all Sumach+AM groups, a reduction trend was seen in the levels of the liver enzymes in comparison with the AM group. Any disruption in the cell membrane causes the release of enzymes into the blood stream [17]. It appears that AM causes deliverance of receptors involved in hepatic damages as speckled droplet through down-regulation of RAR-α [29]. According to the previous statements, Sumach prevents enzymatic leakage and lipid peroxidation, leading to stability of cellular membranes [30]. AM can be metabolized into free radicals that invade hepatocytes and cause the necrosis of parenchymal cells. The induced liver damage is exacerbated by infiltration of inflammatory mononuclear cells releasing proinflammatory mediators which is accompanied by a rise in liver enzymes [31]. Pour *et al.* [32] in an experimental investigation that is in line with the consequences of the current study showed that the Sumach with membrane-stabilizing property could lead to a reduction of serum markers of damaged liver. Moreover, Sumach by endoplasmic reticulum recruitment can cause cellular defense and anti-apoptosis properties [15].The outcomes of this study revealed that the MA has potential to increase the serum level of NO. Moreover, owing to the beneficial effects of Sumach, the serum level of NO was reduced in comparison with the AM group, confirming two main features of Sumach on the cells: the anti-inflammatory and antioxidant properties. In the mammalian body, the NO is known as a free radical that interferes with the physiological molecular process [16]. Along with NO, the hydroxyl radical and superoxide anion can also cause hepatotoxicity [10]. The findings of Kuriyama *et al.* [33] displayed that the administration of genistein as a dominant antioxidant stimulates NO production in vascular endothelium, which is in contrast with the results of the present study. AM is capable of inducing amplified No construction via intracellular regulation of calcium and activation of calcium/calmodulin-dependent processes [34]. The hydroxyl radicals produced by NO and super-oxidation could interfere with pathogenesis process in liver [10]. It has been proven that low expression of iNOS can significantly reduce the NO production. Antioxidants disrupt the molecular process of enzyme activity involved in NO production (substrates, cofactors, and protein enzymes) [17]. The results of a research conducted by Anwar *et al.* [35] are in line with the results of this study, suggesting that Sumach causes the expression of HO-1 and calmodulin-calcium-dependent kinase-4 protein, leading to inhibition of lipopolysaccharide through NO. In this study, Sumach also showed decreased levels of apoptotic gene expression (Bax and p53) and apoptotic cell index. P53 makes the mitochondrial membrane permeable to the influx of cytochrome-C into the intracellular matrix. Accordingly, p53 adjusts the function of apoptotic elements like caspases and Bax [36]. As cell death is seen in hepatocytes, it is stated that the AM has up-regulatory effects on apoptotic factors [37]. Haas *et al.* [38] also found the apoptotic function of caspases and Bax. As cell death is seen in hepatocytes, it is stated that the AM has up-regulatory effects on apoptotic factors, and apoptotic genes are expressed significantly following AM administration.

Conclusion

In this study, Sumach reduced apoptotic cell index and apoptotic gene expression to prevent cell death. Based on the obtained results, it can be stated that Sumach has positive antioxidant effects on molecular function and histological construction of hepatocytes. This property causes diminished level of hepatic enzymes, boosts antioxidant capacity, and reduces NO levels.

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Conflicts of interest

There are no conflicts of interest.

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