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Original Article

Ameliorative effects of umbelliferone against acetaminophen-induced hepatic oxidative stress and inflammation in mice

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Abstract

Background and purpose: Acetaminophen (APAP) is a commonly used antipyretic and pain reliever that its overdose causes acute liver toxicity. Umbelliferone (UMB) has many pharmacological effects. In this study, the hepatoprotective effect of UMB on acute hepatotoxicity induced by APAP was investigated.

Experimental approach: Forty-nine male mice were separated into seven groups. The control received vehicle (i.p.), UMB group received UMB (120 mg/kg, i.p.), APAP group was treated with a single dose of APAP (350 mg/kg, i.p.), and pretreated groups received N-acetylcysteine (NAC, 200 mg/kg, i.p.) or different doses of UMB (30, 60, and 120 mg/kg, i.p.), respectively before APAP. Twenty-four hours after APAP injection, mice were sacrificed and blood and liver samples were collected. Then, serum and tissue samples were investigated for biochemical and histological studies.

Findings/Results: A single dose of APAP caused elevation in the serum liver enzymes, including alanine aminotransferase, aspartate transaminase, and alkaline phosphatase. The amounts of thiobarbituric acid reactive substances, tumor necrosis factor-alpha, and nitric oxide increased in the mice's liver tissue. Moreover, the amount of total thiol and the activity of antioxidant enzymes (catalase, superoxide dismutase, and glutathione peroxidase) significantly diminished in the APAP group. Histological results confirmed the hepatotoxicity induced by APAP. However, UMB (more effective at 60 and 120 mg/kg) lessened APAP-induced hepatic injuries, which is comparable with NAC effects.

Conclusion and implications: The findings of this study provided evidence that UMB ameliorates liver injury induced by APAP through its antioxidant and anti-inflammatory effects.

Keywords: Acetaminophen; Hepatotoxicity; Inflammation; Oxidative stress; Umbelliferone.

INTRODUCTION

The liver is the most important organ responsible for drug catabolism and also the main sensitive site for toxicity induced by drugs (1). Acetaminophen (N-acetyl-para-aminophenol; APAP) is the most commonly used and popular antipyretic and pain reliever drug (2). At therapeutic doses (4 g/day or less), it can be considered effective and safe, but the overdose of APAP accidentally or intentionally may cause severe liver injury, liver failure, and even death (3-5). Approximately 50% of all cases of liver damage in the United States are correlated with APAP (5).

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After consumption, about 90% of the drug is converted to non-toxic sulfated or glucuronidated metabolites by the liver and subsequently excreted in the urine. About 2% is excreted in the urine unchanged, and 5-10% of the drug is metabolized by cytochrome P450 enzymes and converted to N-acetyl-p-benzoquinoneimine (NAPQI), a highly toxic metabolite of APAP, which is detoxified through conjugation with glutathione (GSH) (6). At toxic doses of APAP, sulfation and glucuronidation pathways become insufficient and hepatic GSH is depleted due to excessive NAPQI. As a result, it binds to cellular proteins in hepatocytes and causes oxidative stress, which ultimately leads to mitochondrial dysfunction, liver inflammation, and liver necrosis (6,7). N-acetylcysteine (NAC), a precursor of GSH, is currently the only antidote used for APAP hepatotoxicity. However, it is only effective in the early stages of APAP hepatotoxicity. In addition, several side effects, such as anaphylactic and gastrointestinal reactions have been reported, which limit its clinical use (8,9). Hence, it is important to find safe and effective compounds for APAP-hepatotoxicity therapy.

Umbelliferone (UMB) is a coumarin compound that is abundant in many members of the Apiaceae family, including important and economic plants such as alexanders, asafoetida, angelica, celery, cumin, parsley, and fennel (10). Studies have shown that UMB has antioxidant (11), anti-inflammatory (12), antimicrobial (13), and anticancer (14) effects. Also, its protective effects against various models of liver damage have been reported in different studies (15-18). According to the studies, the hepatoprotective effects of UMB may be due to the increase of antioxidant defense and reduction of inflammation in liver tissue (19). However, to our knowledge, its effect on liver injury caused by APAP has not been studied. Considering the antioxidant and anti-inflammatory effect of UMB, this study aimed to evaluate the effects of pretreatment of mice with UMB against APAP-induced acute hepatotoxicity.

MATERIALS AND METHODS

Chemicals

APAP, UMB, dithiobis-2-nitrobenzoic acid (DTNB), and thiobarbituric acid (TBA) were provided by Sigma Chemical Co. (St Louis, Missouri, USA). Dimethyl sulfoxide (DMSO) was obtained from Merck Co., Germany. Alanine aminotransferase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) kits were purchased from Pars Azmoon Co. (Tehran, Iran). Catalase (CAT), superoxide dismutase (SOD), and GSH peroxidase (GPx) were provided by ZellBio GmbH Co. (Ulm, Germany). Tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) kits were obtained from Abcam Co. (Cambridge, UK).

Animals and ethical approval

In this research, 49 adult male NMRI mice (6-8 weeks old, weighing 20-25 g) were provided from the laboratory animal breeding center of Ahvaz Jundishapur University of Medical Sciences (AJUMS), Iran. The mice were retained in polycarbonate cages at room temperature (25 ± 2 °C), humidity (40-50%) with a 12/12-h light/dark cycle, and free access to standard food and drinking water. Before starting the experiments, the mice were acclimated to the laboratory environment for one week. This study was confirmed by the Ethical Committee Acts of AJUMS on the use and care of laboratory animals (Ethics code: IR.AJUMS.ABHC.REC.1398.020).

Study design

The mice were accidentally selected and put into seven groups as follows:

The control group received the vehicle for five days; the UMB group received UMB (120 mg/kg) for five days; animals subjected to the APAP group were injected with APAP (350 mg/kg) on the fifth day of the study to induce hepatotoxicity; the NAC group received NAC (200 mg/kg) for five days + a single dose of APAP (350 mg/kg) on the fifth day of the study; the three remaining groups were pretreated with UMB at 30, 60, and 120 mg/kg for five days, respectively, + a single dose of APAP (350 mg/kg) on the fifth day of the study.

One day before administration of APAP, mice were fasted and APAP was administered 30 min after receiving the last dose of vehicle or drugs. APAP (20), NAC (21), and UMB (22) doses were selected based on the previous studies and injected intraperitoneally. APAP was dissolved in warm saline, NAC was diluted

with normal saline, and UMB was dissolved in 10% DMSO. One day after the APAP injection, animals were anesthetized with a ketamine/xylazine (90/10 mg/kg) cocktail, and their blood samples were collected *via* cardiac puncture, and centrifuged (3,000 rpm, 15 min) to gain sera, and stored at -70 °C to measure the activity of liver enzymes. The mice livers were separated into two sections, one section was placed in 10% formalin for histological tests, and the other was placed at -70 °C for tissue factors examination.

Measurement of serum activity of liver enzymes

The activity of ALT, AST**,** and ALP enzymes was measured based on the manufacturer's instructions for standard diagnostic kits.

Tissue homogenizing and measurement of protein content

A portion of liver tissue from each mouse was homogenized with phosphate-buffered saline (ratio: 1:10 w/v, pH = 7.4) using a homogenizer at 740 rpm for 2 min and then centrifuged. To assess the protein concentration of the supernatants, the Bradford method was applied (23).

Measurement of total thiol

For this purpose, DTNB was added to the supernatant samples, and after 10 min, the absorbance of the yellow color created as a result of the reaction of DTNB with free thiol groups was recorded at a wavelength of 412 nm by a microplate reader (Tecan Austria GmbH, Sunrise-Basic Tecan) (24).

Measurement of thiobarbituric acid reactive substances

The Buege and Aust method (25) was used to determine the amount of thiobarbituric acid reactive substances (TBARS). The absorbance of the colored complex formed due to the reaction of TBA with lipid peroxides was recorded at 535 nm.

Measurement of the activity of antioxidant enzymes

CAT, SOD, and GPx enzyme activities were determined using commercial colorimetric kits based on the manufacturer's instructions.

Measurement of inflammatory markers

TNF-α and NO levels in liver tissues of mice were determined by the technique of enzyme-linked immunosorbent assay (ELISA) and commercial colorimetric kit, respectively based on the kit manufacturer's instructions.

Histopathological studies

Twenty-four hours after fixing the tissues in 10% formalin, liver tissues were embedded in paraffin. Then, a section of 5 µm was obtained and stained with hematoxylin and eosin (H&E) dye. For each mouse, six microscopy slides were investigated to evaluate histological alterations (magnification: ×300). Histopathological alterations, including degeneration of hepatocytes, necrosis, infiltration of inflammatory cells, and dilation of sinusoids, and semiquantitative estimation and scoring were examined by a light microscope (Olympus, BH2-RFCA, Japan) (26).

Statistical analysis

Data analysis was conducted using GraphPad Prism (version 9.5.0) software. The average of data from each group was presented as mean ± SEM. For comparing the means of the groups, one-way ANOVA was used followed by the Tukey post-hoc test. *P*-values < 0.05 were considered significant.

RESULTS

UMB effects on serum markers of liver injury

As shown in Fig. 1A-C, serum levels of ALT, AST, and ALP enzymes significantly increased after the injection of a single dose of APAP. The increase of these enzymes indicated the induction of hepatotoxicity by APAP. However, pretreatment with UMB (60 and 120 mg/kg) significantly decreased the serum levels of ALT and AST, and at a dose of 60 mg/kg, decreased the level of ALP compared to the APAP group. In this study, NAC, as a standard treatment improved the activity of ALT and AST enzymes compared to the APAP group.

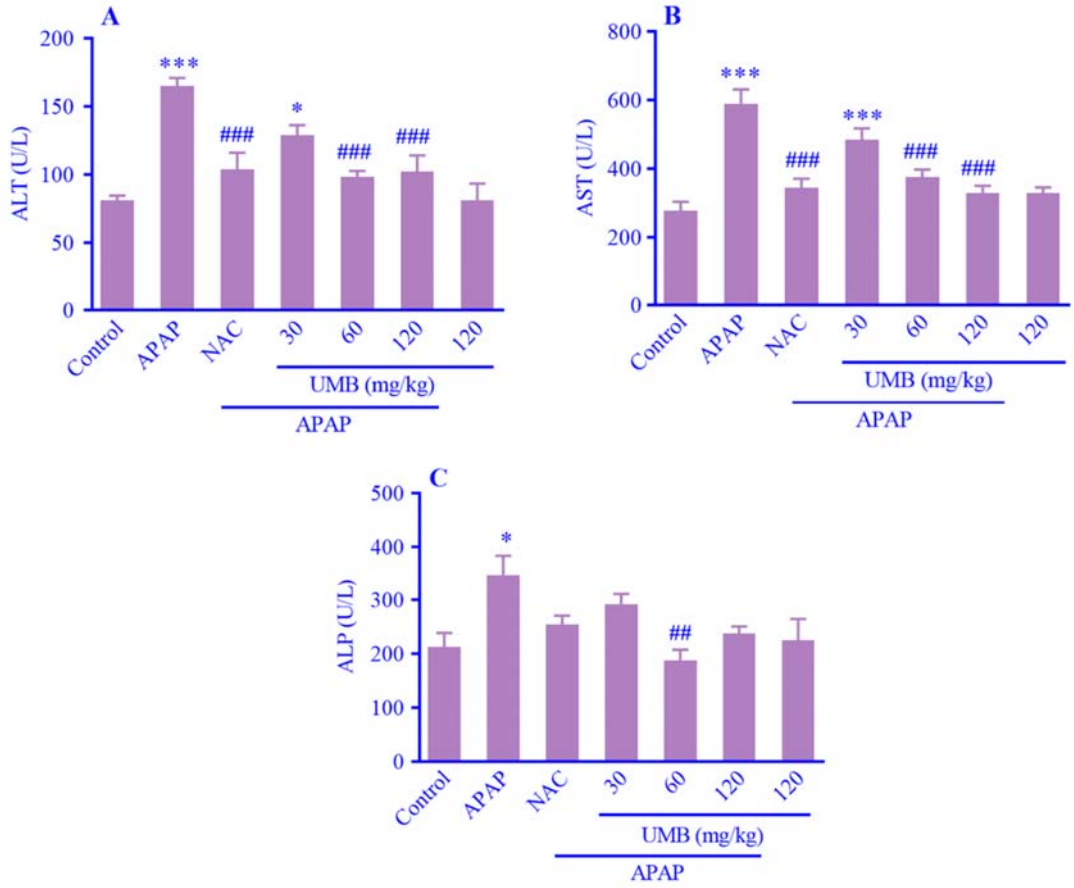

Fig. 1. Effect of UMB on serum levels of (A) *ALT*, (B) *AST*, and (C) *ALP* in the APAP (350 mg/kg) model of hepatotoxicity in mice. The data are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate significant differences in comparison with the control group; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus the APAP group. UMB, Umbelliferone; ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; APAP, acetaminophen; NAC, N-acetylcysteine.

UMB effects on total thiol and TBARS levels in liver tissue

In the APAP group, the amount of total thiol was significantly reduced (Fig. 2A) and TBARS levels significantly increased (Fig. 2B) compared to the control group. Pretreatment with UMB (30, 60, and 120 mg/kg) significantly increased total thiol levels compared to the APAP group. In addition, pretreatment with UMB at doses of 60 and 120 mg/kg significantly diminished the amount of TBARS compared to the APAP group. Also, NAC significantly enhanced total thiol and decreased TBARS levels compared to the APAP group.

UMB effects on the activity of antioxidant enzymes in liver tissue

The enzymatic activities of CAT, SOD, and GPx (Fig. 3A-C) in the APAP group were significantly diminished compared with the control group. UMB-pretreated group (60 mg/kg) significantly elevated the activity of GPx enzyme, and at 60 and 120 mg/kg, dose-dependently increased CAT and SOD activity compared with the APAP group. Also, NAC significantly elevated the activity of these enzymes compared to the APAP group.

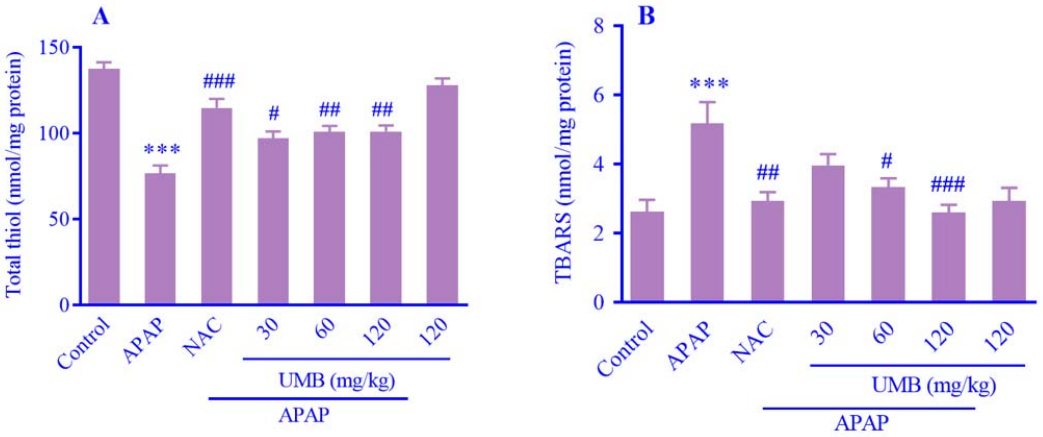

Fig. 2. Effect of UMB on (A) total thiol and (B) TBARS in the APAP (350 mg/kg) model of hepatotoxicity in mice. The data are expressed as mean ± SEM. ***$P < 0.001$ Indicates significant differences in comparison with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the APAP group. UMB, Umbelliferone; TBARS, thiobarbituric acid reactive substances; APAP, acetaminophen; NAC, N-acetylcysteine

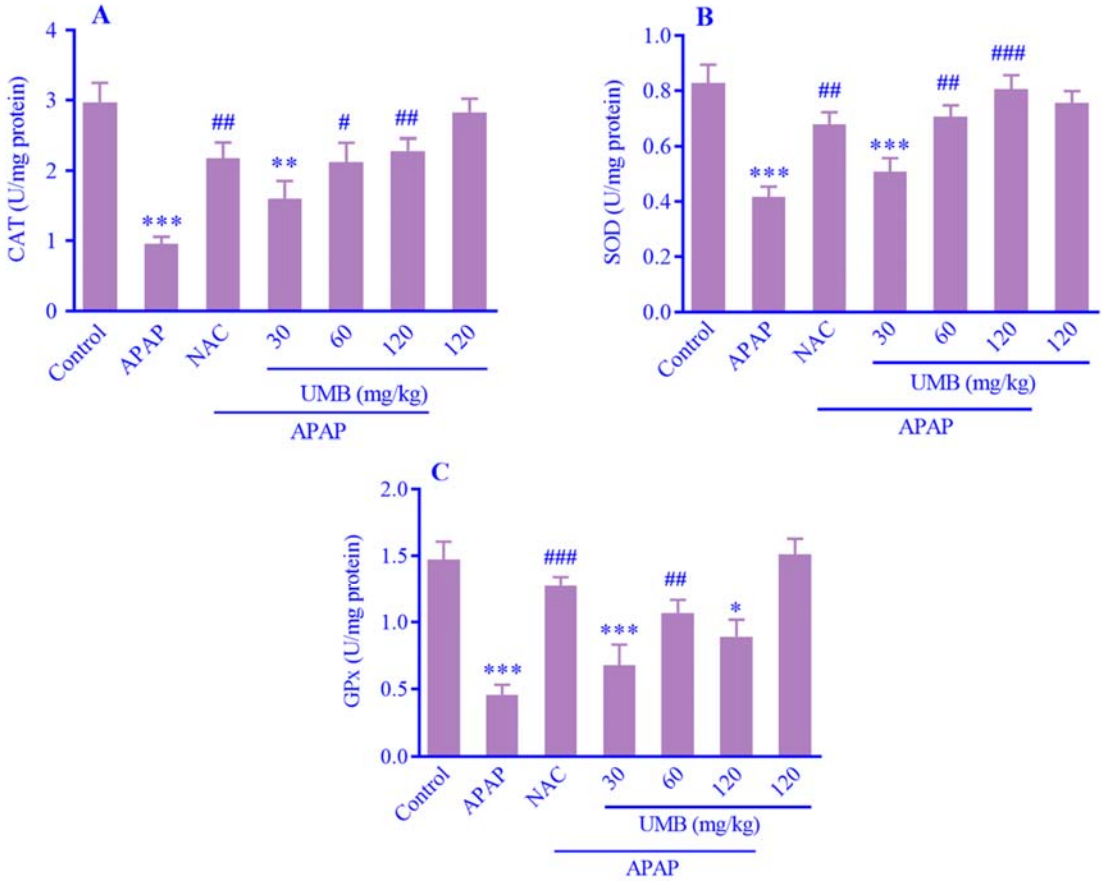

Fig. 3. Effect of UMB on (A) CAT, (B) SOD, and (C) GPx enzyme activity in the APAP (350 mg/kg) model of hepatotoxicity in mice. The data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences in comparison with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the APAP group. UMB, Umbelliferone; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; APAP, acetaminophen; NAC, N-acetylcysteine.

UMB effects on inflammatory factors of liver tissue

To evaluate the involvement of inflammation in the hepatoprotective effects of UMB on liver injury induced by APAP, hepatic TNF-α and NO were measured. As indicated in Fig. 4A and B, the levels of TNF-α and NO significantly increased in the APAP group compared with the control group. UMB (30, 60, and 120 mg/kg) significantly decreased TNF-α level compared with the APAP group, and at doses of 60 and 120 mg/kg significantly decreased APAP-induced elevation of NO level. Additionally, NAC significantly improved the levels of inflammatory factors compared to the APAP group.

UMB effects on histopathological factors

In the control group and the group receiving UMB alone at 120 mg/kg, the appearance of the liver tissue was normal. Centrilobular necrosis, hepatocyte degeneration, dilation of sinusoids, and infiltration of the inflammatory cells were observed in the APAP group. UMB pretreated groups (60 and 120 mg/kg) reduced APAP-induced hepatic injuries. This reduction was more evident in the group receiving UMB at 120 mg/kg (Fig. 5 and Table 1). Moreover, in the NAC pretreated group, a small number of inflammatory and necrotic cells were observed, and no significant pathological damage was indicated.

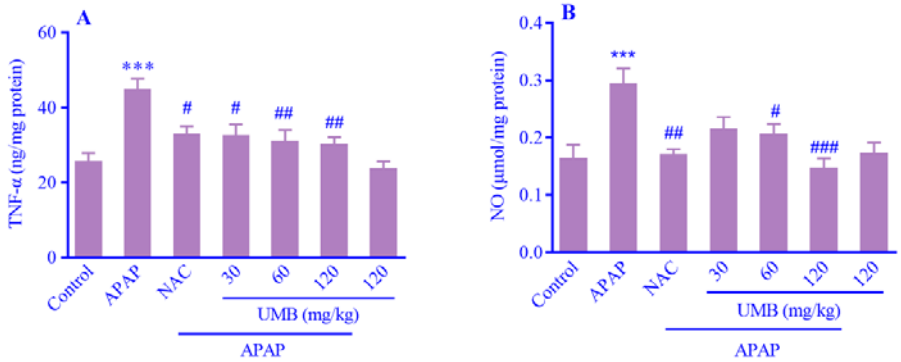

Fig. 4. Effect of UMB on (A) TNF-α and (B) NO in the APAP (350 mg/kg) model of hepatotoxicity in mice. The data are expressed as mean ± SEM. ***$P < 0.001$ Indicates significant differences in comparison with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the APAP group. UMB, Umbelliferone; TNF-α, tumor necrosis factor alpha; NO, nitric oxide; APAP, acetaminophen; NAC, N-acetylcysteine.

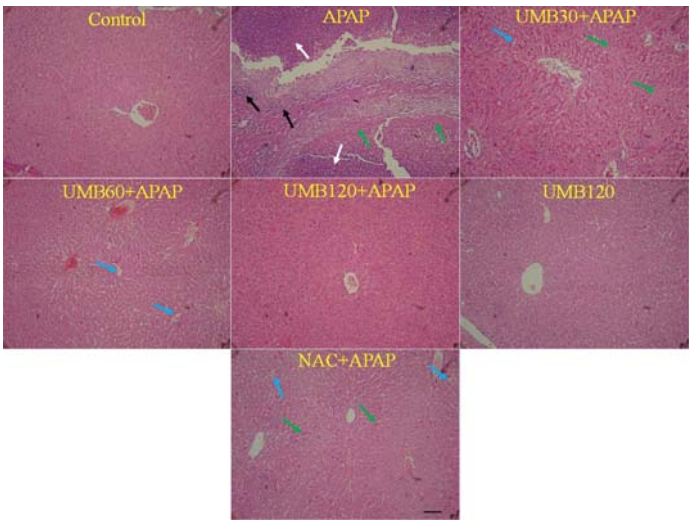

Fig. 5. Evaluation of the effect of UMB (mg/kg) on liver tissue changes in the APAP (350 mg/kg) model of hepatotoxicity in mice using hematoxylin and eosin staining; magnification: ×300, scale bar: 100 µm. Green arrows: necrosis, black arrows: infiltration of inflammatory cells, white arrows: degeneration of hepatocytes, and blue arrows: dilation of sinusoids. UMB, Umbelliferone; APAP, acetaminophen; NAC, N-acetylcysteine.

Table 1. Effect of UMB on histopathological parameters of liver tissue in the APAP (350 mg/kg) model of hepatotoxicity in mice. Signs indicate normal (-), mild (+), moderate (++), severe (+++), and very severe (++++) injury.

Histological criteria	Control	APAP	NAC (200 mg/kg) + APAP	UMB (30 mg/kg) + APAP	UMB (60 mg/kg) + APAP	UMB (120 mg/kg) + APAP	UMB (120 mg/kg)
Degeneration of hepatocytes	-	++++	-	++++	++	+	-
Necrosis	-	++++	++	++++	+	-	-
Infiltration of inflammatory cells	-	++++	++	++++	++	-	-
Dilation of sinusoids	-	++++	++	++++	+++	+	-

DISCUSSION

Drug toxicity is one of the main reasons for acute liver failure. APAP is one of the drugs that causes acute hepatotoxicity when taken in high doses. Various mechanisms have been suggested for APAP hepatotoxicity, including oxidative stress, inflammation, and the role of the apoptotic pathway (27).

Many phytochemicals have been scientifically studied due to their different pharmacological properties in APAP hepatotoxicity (28). UMB is a derivative of coumarin, also known as 7-hydroxycoumarin and has received much attention in various studies due to its antioxidant and anti-inflammatory features (19,29). Thus, in the present study, we investigated the possible hepatoprotective effects of UMB on APAP-induced mouse hepatotoxicity.

In the present study, UMB as a hepatoprotective agent (more effective at 60 and 120 mg/kg) lessened the APAP-induced oxidative stress and inflammation and increased the antioxidant defense in the liver tissue of mice (Fig. 6). This research demonstrated that a single dose of APAP (350 mg/kg) caused hepatotoxicity. Evidence for this claim was a significant increase in the activity of serum liver enzymes (ALT, AST, and ALP), degeneration of hepatocytes, necrosis, dilation of sinusoids, and infiltration of inflammatory cells in the liver tissue. Consistent with our study, these hepatotoxic effects of APAP were also observed in the other studies (30-33). Our results indicated that UMB reduced liver enzymes and improved histological changes induced by APAP. In a similar study, Yin *et al*. showed that UMB decreased liver enzymes and histological changes in a diabetic mice liver injury model (16). In another study conducted by Mahmoud *et al*. UMB was able to reduce cyclophosphamide-induced liver histopathological changes, which is consistent with our study (15).

One of the main mechanisms of hepatotoxicity induced by APAP has been reported to be oxidative stress and the production of reactive oxygen species (ROS) (34). The active metabolite of APAP, NAPQI, binds to thiol groups of GSH and other cysteine-containing proteins in the hepatocytes. Therefore, depletion of GSH is considered one of the major biomarkers for the hepatotoxicity caused by APAP. In addition, the depletion of GSH causes endogenous ROS, such as superoxide and hydroxyl radicals, to bind to cellular macromolecules resulting in lipid peroxidation, membrane breakdown, and cell death (35,36). Moreover, studies have shown that the activity of antioxidant enzymes reduces as a result of APAP-induced liver damage (37). Our study showed that APAP increased the levels of TBARS and diminished the amount of total thiol and antioxidant enzyme activities (CAT, SOD, and GPx) in the mice's liver. Based on the results, UMB reduced TBARS and increased antioxidant defense by increasing the amount of total thiol and the activity of the mentioned enzymes, which is consistent with a previous study by Ramesh and Pugalendi (11). Also, another study investigated the neuroprotective effects of UMB on the Parkinson's disease model and showed that UMB prevented neuronal damage against MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine)-induced toxicity due to its antioxidant effects (38).

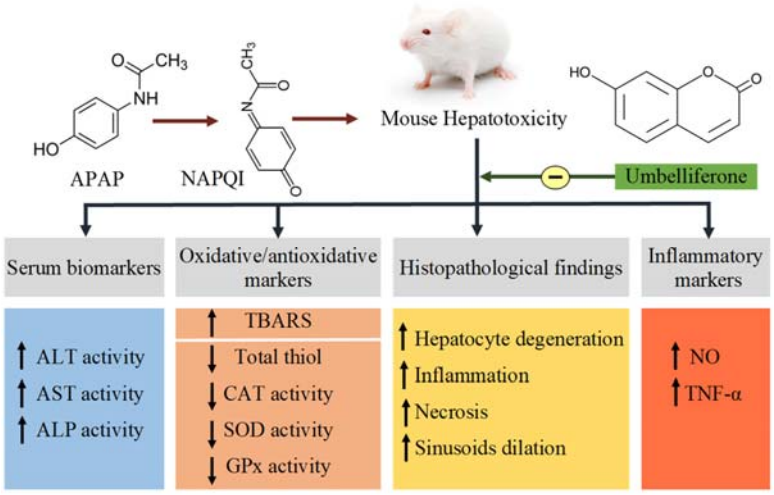

Fig. 6. The hepatoprotective effects of umbelliferone on acute hepatotoxicity caused by APAP in mice. APAP, acetaminophen; NAPQI, N-acetyl-p-benzoquinoneimine; ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; TBARS, thiobarbituric acid reactive substances; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; NO, nitric oxide; TNF-α, tumor necrosis factor-alpha.

It has been demonstrated that the oxidative stress caused by APAP leads to an increase in the release of pro-inflammatory cytokines, such as TNF-α, which is involved in APAP hepatotoxicity (39). It has been known that TNF-α induces neutrophil accumulation in the liver, macrophage stimulation, and NO production in hepatocytes, which further increase hepatotoxicity caused by APAP (40). In this study, APAP significantly improved the levels of TNF-α and NO in the liver tissues of mice compared to the control group. Our results showed that UMB suppresses APAP-induced elevation in TNF-α and NO levels, which are consistent with the findings of previous studies (12,41-43).

CONCLUSION

The findings of this study revealed that APAP causes toxicity in the mice's liver. Also, oxidative stress and inflammation played an important role in activating this toxicity. Our results showed that UMB improved the hepatic injury caused by APAP. The findings of the present study provided evidence that UMB, with its antioxidant properties, alleviates oxidative stress, suppresses APAP-induced inflammation with its anti-inflammatory effect, and protects mice's liver, which is comparable with NAC effects. Thus, UMB may serve as a complementary or alternative therapeutic factor for NAC to prevent APAP-induced acute hepatotoxicity.

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Conflicts of interest statement

The authors declared that no conflict of interest in this study.

Authors' contributions

S. Sadeghinejad contributed to the experimental studies, data acquisition, and manuscript preparation. M. Moosavi contributed to conceptualization and supervision. L. Zeidooni contributed to methodology, statistical analysis, and manuscript preparation. E. Mansouri interpreted the pathology-related data. Sh. Mohtadi analyzed the data and wrote and revised the manuscript. M.J. Khodayar contributed to the conceptualization, supervision, validation, review, and editing of the manuscript. The finalized article was read and approved by all the authors.

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