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*Original article*

# Betulinic acid mitigates methotrexate-induced hepatic injury via suppression of oxidative stress and NLRP3/caspase-1 inflammasome activation

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*NLRP3 inflammasome*
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## Abstract

**Objective**: Methotrexate (MTX) is a highly effective chemotherapeutic drug, but its therapeutic utility is limited by dose-limiting hepatotoxicity. We designed this study to assess the protective efficacy of betulinic acid (BA), a natural pentacyclic triterpenoid, on MTX-induced liver injury, focusing on the NLRP3/caspase-1 inflammasome signaling pathway.

**Materials and methods:** Male mice were divided into four groups including control, BA (25 mg/kg, intraperitoneally; i.p.), MTX (20 mg/kg, i.p.), and co-administration of BA 25mg/kg and MTX 20mg/kg. After the experimental period, mice were euthanized, and blood and hepatic tissues were collected for biochemical and histopathological investigations.

**Results:** Compared to controls, MTX significantly increased serum liver enzymes, interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), lipid peroxidation, nitric oxide, and hepatic NLRP3/caspase-1 expression. Conversely, MTX decreased total thiols, glutathione peroxidase, superoxide dismutase (SOD), and catalase (CAT) activities. BA pretreatment significantly reduced liver enzymes suppressed lipid peroxidation, IL-1β, and TNF-α levels, restored thiol content and antioxidant enzyme activities including SOD and CAT, and downregulated NLRP3 and caspase-1 expression. Histological analysis confirmed that BA alleviated MTX-induced liver injury.

**Conclusion:** BA showed potent attenuation of MTX-mediated hepatic damage by modulating oxidative stress and inhibiting the NLRP3/caspase-1 inflammasome activation. These data suggest that BA administration could be an appropriate supportive strategy to minimize liver injury induced by MTX treatment.

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## Introduction

Methotrexate (MTX) is an antifolate widely used as an anticancer and immunosuppressive drug for treating cancer, psoriasis, and rheumatoid arthritis (Bedoui et al. 2019). While effective, MTX frequently causes hepatotoxicity which manifests as elevated liver enzymes, oxidative damage, and histopathological alterations (Duman et al. 2013). MTX has been linked to chronic drug-induced liver injury, namely liver fibrosis, limiting long-term use. MTX undergoes hepatic metabolism where it inhibits dihydrofolate reductase (DHFR), disrupting folate metabolism, nucleotide synthesis, and cellular proliferation (Schmidt et al. 2022). In parallel, MTX metabolism generates reactive oxygen species (ROS), initiating oxidative damage, lipid peroxidation, mitochondrial dysfunction, and DNA damage in hepatocytes (Ezhilarasan 2021). MTX also triggers inflammatory cascades, increasing proinflammatory mediators like tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, which amplify hepatic inflammation (Zhao et al. 2023).

Recent evidence implicates activation of the NLRP3 (NOD-like receptor family pyrin domain-containing protein 3) inflammasome in cellular damage. NLRP3 is a cytosolic pattern recognition receptor that senses cellular danger signals, including ROS, mitochondrial dysfunction, and damage-associated molecular patterns (Kelley et al. 2019). Activation of NLRP3 results in its oligomerization and recruitment of the adaptor protein ASC, which subsequently activates caspase-1(CASP1). Subsequently, CASP1 cleaves pro-IL-1β and pro-IL-18, yielding their mature, pro-inflammatory cytokines that exacerbate hepatic inflammation and cell death through pyroptosis (Swanson et al. 2019). Persistent NLRP3 inflammasome activation has been linked to chronic liver injury, fibrosis, and progression of liver disease (Wree et al. 2014).

Betulinic acid (BA) is a natural triterpenoid compound found in several plant species, particularly in the bark of the white birch tree; *Betula pubescens* (Yogeeswari and Sriram 2005). BA exhibits a diverse pharmacological profile, encompassing immunomodulatory, redox-regulating, and antineoplastic activities. The anti-inflammatory and antioxidant effects of BA have been extensively studied in various experimental models, demonstrating its ability to suppress proinflammatory cytokines, inhibit oxidative injury, and attenuate tissue damage (Hordyjewska et al. 2019). Evidence suggests BA can modulate inflammasome signaling, making it a candidate for mitigating MTX hepatotoxicity (Dokumacioğlu et al. 2021; Yi et al. 2014).

The objective of the current study was to examine the protective potential of BA against MTX-induced hepatic injury in mice, specifically evaluating its role in mitigating oxidative stress, inhibiting inflammatory cytokines, and modulating the NLRP3/ CASP1 inflammasome pathway. Understanding these mechanisms could facilitate the development of BA as an adjunct therapy to improve MTX safety and efficacy.

## Materials and Methods

### Animals

Male NMRI mice (23–27 g), aged four weeks, were procured from the laboratory animal house of Ahvaz Jundishapur University of Medical Sciences (Ahvaz, Iran). Mice were housed under a controlled environment (25 ± 1°C; 12-hr lighting cycle) and provided *ad libitum* access to a standard laboratory diet and water. All experimental procedures were performed in accordance with the ethical standards approved by the Animal Ethics Committee at Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.ABHC.REC.1401.025).

## Experimental design

Male NMRI mice were randomly assigned to four experimental groups (n=6 per group). Control group (normal saline intraperitoneally; i.p. daily for 7 days); BA group (25 mg/kg, i.p. for 7 days), MTX group received normal saline for 7 days, and a single dose of MTX (20mg/kg, i.p.) on the 4th day. Group 4 received BA 25 mg/kg, i.p. for 7 days, and a single dose of MTX (20mg/kg, i.p.) on the 4th day. The dose and route of administration of MTX and BA in mice were selected according to previously reported studies (Oriakhi et al. 2021; Sahindokuyucu-Kocasari et al. 2021). BA and MTX were purchased from Sigma-Aldrich (USA) and Nanoalvand Pharmaceuticals (Tehran, Iran), respectively.

Twenty-four hours following the final dose, mice underwent anesthesia with a ketamine/xylazine combination (80/8 mg/kg, i.p.). Serum was isolated from the intracardiac blood samples after centrifugation (3000 ×g, 10 min) and stored at −80°C pending biochemical evaluation. Liver tissues were divided into two portions: one was fixed in 10% phosphate-buffered formalin for histopathological evaluation, while the remainder was homogenized and centrifuged, and the resulting supernatant was collected and stored at −80°C until various analyses were performed.

## Biochemical analysis

Hepatic functional status was evaluated through quantification of key serum enzymes. Serum enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were determined using commercial assay kits (Pars Azmoon, Iran).

## Total thiol (TT) assay

Total thiol levels were determined using the Ellman method with DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) as the reagent. Briefly, the supernatant was mixed with 0.01 M DTNB and incubated for 20 min at room temperature, and the absorbance of the formed yellow complex was recorded at 412 nm (Ellman 1959).

## Lipid peroxidation assay (TBARS method)

Lipid peroxidation levels were determined through the thiobarbituric acid reactive substances (TBARS) assay. Briefly, the sample supernatant was treated with 30% trichloroacetic acid in phosphate buffer and subjected to centrifugation (3500 ×g, 15 min). Following this, the resulting supernatant was reacted with thiobarbituric acid in a boiling water-bath. The formation of a pink chromogen was quantified spectrophotometrically by measuring the absorbance at 532 nm(Mihara and Uchiyama 1978).

## Evaluation of hepatic antioxidant enzyme activities

Catalase (CAT) enzymatic activity was assessed as previously described (Shangari and O'Brien 2006). Briefly, supernatants were incubated with hydrogen peroxide at ambient temperature, followed by the addition of ammonium molybdate. After a 10-min reaction period, the absorbance was recorded at 410 nm using a microplate reader. Superoxide dismutase (SOD) activity and glutathione peroxidase (GPx)in liver tissue homogenates were determined using ELISA kits (ZellBio GmbH, Germany) in accordance with the manufacturer's instructions.

## Nitric oxide (NO) level measurement

Hepatic NO levels were quantified using a standardized commercial assay kit (ZellBio GmbH, Germany) according to the manufacturer's guidelines.

## Cytokine measurement

Hepatic TNF-α and IL-1β levels were determined via commercial ELISA kits (BT LAB, China), with absorbance measured at 450 nm using a microplate reader.

### Western blotting

The liver tissue samples were homogenized in radioimmunoprecipitation assay buffer (RIPA) buffer containing a protease inhibitor and centrifuged (12,000 ×g , 20 min, 4°C). The protein content of the resulting supernatant was measured via the bicinchoninic acid (BCA) assay. Equal amounts of protein (25 µg) were separated on 10% sodium dodecylsulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Matouk et al. 2022). Following overnight blocking at 4°C using 5% non-fat milk to suppress background signal, the membranes were probed with anti-NLRP3 and anti-CASP1 primary antibodies (Cell Signaling Technology, USA). After thorough washing, the blots were exposed to secondary antibodies for 2 hr at room temperature. Bands were visualized and quantified using ImageJ software (version 1.51), with glyceraldehyde-3-phosphatedehydrogenase (GAPDH) as the loading control.

### Histopathological assessment

A portion of the liver was fixed in 10% neutral buffered formalin. Then, it was dehydrated in graded concentrations of alcohol and embedded in paraffin. Tissue sections of 5 µm thickness were cut and subsequently subjected to hematoxylin and eosin staining and examined by a light microscope (Olympus, BX43, Japan). Semi-quantitative evaluation of histological changes was performed, including irregular structure, inflammatory infiltrate, sinus congestion, and pyknotic nuclei. The severity of the lesions was assessed by scoring as follows: normal, mild, moderate, and severe (0, 1, 2, and 3, respectively) (Mohammadian et al. 2018). An average of six fields was calculated for each slide.

### Statistical analysis

Data analysis was conducted in GraphPad Prism (version 9.0.0), and the data are presented as mean ± SEM. To determine differences between groups, a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was utilized. Statistical significance was defined as $p<0.05$.

## Results

### Impact of BA on serum markers of hepatic function

According to the statistical data, MTX treatment led to substantial increases in serum activity of AST, ALT, and ALP relative to the control group (Figure 1A, B, and C). However, a significant decrease in serum AST, ALT, and ALP activities was displayed in BA-pretreated animals relative to the MTX group ($p<0.01$, $p<0.001$, and $p<0.001$, respectively).

### Impact of BA on oxidative damage and antioxidant activity

Figure 2 demonstrates that MTX exposure induced oxidative stress in liver tissue, manifested by elevated TBARS levels (Figure 2B, $p<0.01$), and significantly suppressed the antioxidant defense system including decreased activities of CAT (Figure 2C, $p<0.01$), GPX (Figure 2E, $p<0.01$), and SOD (Figure 2D, $p<0.05$), and decreased total thiol content (Figure 2A, $p<0.01$) relative to control. BA treatment significantly improved oxidative stress by reducing TBARS levels ($p<0.01$ vs. MTX) and restoring antioxidant capacity, as demonstrated by significant increases in the activities of SOD ($p<0.01$), GPX ($p<0.001$), CAT ($p<0.05$), and total thiol content ($p<0.001$) in comparison to the MTX group.

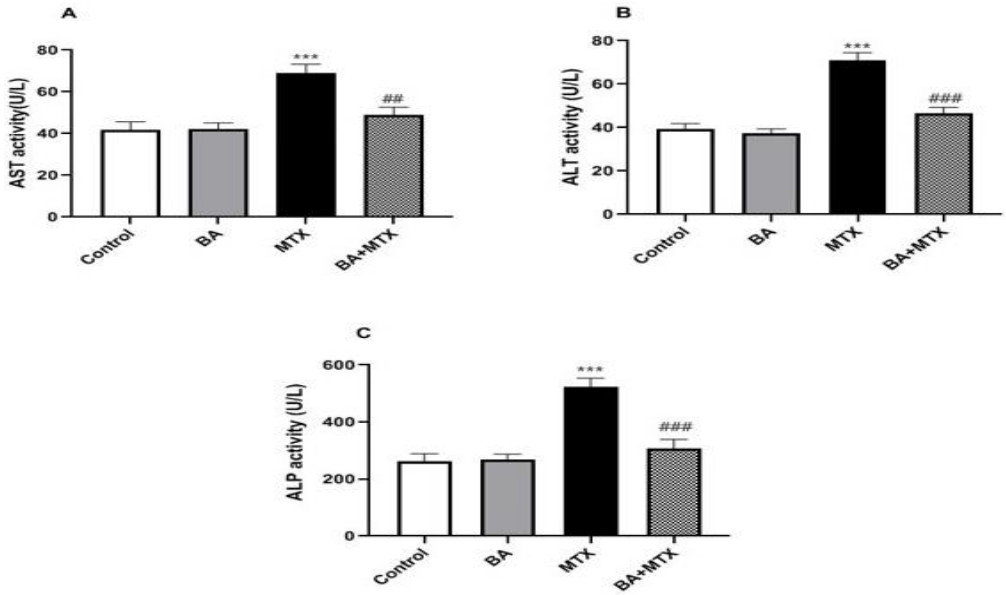


Figure 1. The effect of BA on serum activity of liver function enzymes in liver damage caused by MTX. Data are expressed as mean ± SEM (n=6). BA; betulinic acid, MTX; methotrexate, AST; aspartate aminotransferase , ALT; alanine aminotransferase , ALP; alkaline phosphatase. * Significant difference with the control group (***p<0.001).# Significant difference with the MTX group (##p<0.01 and###p<0.001).

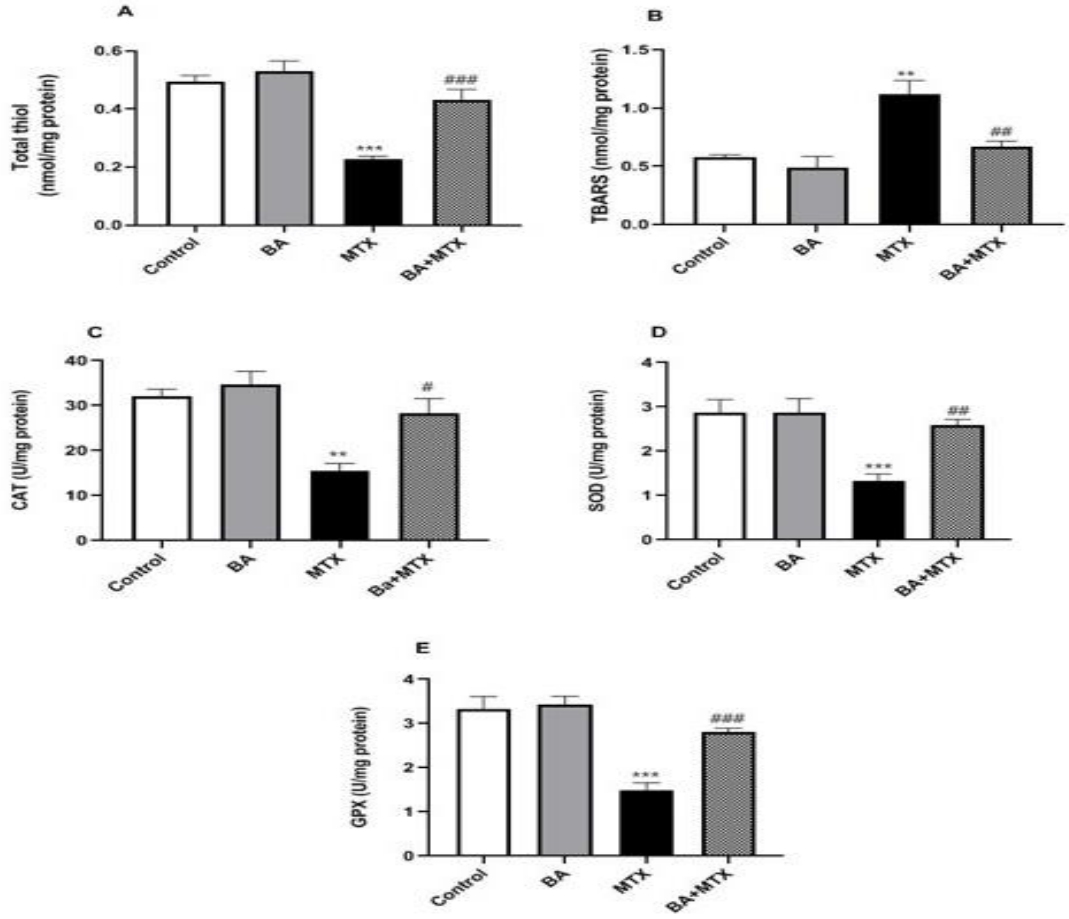


Figure 2. The effect of BA on oxidative stress markers in liver damage caused by MTX. (A) Total thiol, (B) Thiobarbituric acid reactive substances (TBARS), (C) Catalase (CAT), (D) Superoxide dismutase (SOD), and (E) Glutathione peroxidase (GPx). Data are expressed as mean ± SEM (n=6). BA; betulinic acid, MTX; methotrexate.* Significant difference with the control group (**p<0.01 and***p<0.001).# Significant difference with the MTX group (#p<0.05, ##p<0.01, and ###p<0.001).

## Impact of BA on NO level

As shown in Figure 3, hepatic NO levels increased significantly following MTX administration relative to the controls ($p<0.001$). Conversely, BA + MTX co-treatment significantly attenuated MTX-induced NO elevation ($p<0.001$).

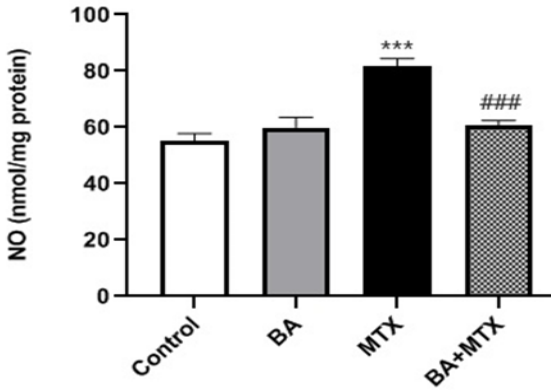


Figure 3. The effect of BA on the hepatic level of nitric oxide (NO) in liver damage caused by MTX. Data are expressed as mean ± SEM (n=6). BA; betulinic acid, MTX; methotrexate. * Significant difference with the control group (***p<0.001).# Significant difference with the MTX group (###p<0.001).

## Impact of BA on cytokine level of TNF-α and IL-1β

The results revealed that TNF-α (Figure 4A) and IL-1β levels (Figure 4B). were significantly increased in the MTX group in comparison with controls ($p<0.001$). Conversely, the BA + MTX group showed a significant decrease in these cytokine levels ($p<0.001$).

## Impact of BA on the expression of NLRP3 and CASP1 proteins

Figure 5A illustrates the hepatic expression levels of NLRP3 and CASP1proteins across the experimental groups. Relative to controls, MTX-treated mice showed a significant upregulation in both NLRP3 and CASP1 protein expression ($p<0.001$ and $p<0.01$; Figure 5B and C). Pretreatment with BA significantly attenuated the MTX-induced elevation of these proteins ($p<0.001$ and $p<0.01$, respectively).

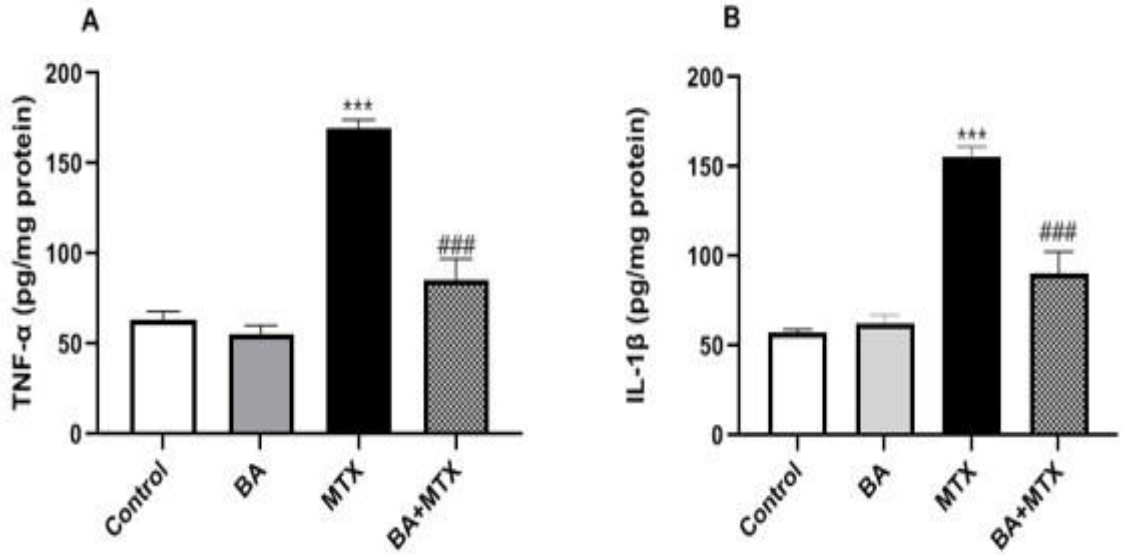


Figure 4. The effect of BA on hepatic levels of (A) tumor necrosis factor-alpha (TNF-α) and (B) interleukin-1beta (IL-1β) in liver damage caused by MTX. Data are expressed as mean ± SEM (n=6). BA; betulinic acid, MTX; methotrexate.* Significant difference with the control group (***p<0.001).# Significant difference with the MTX group (###p<0.001).

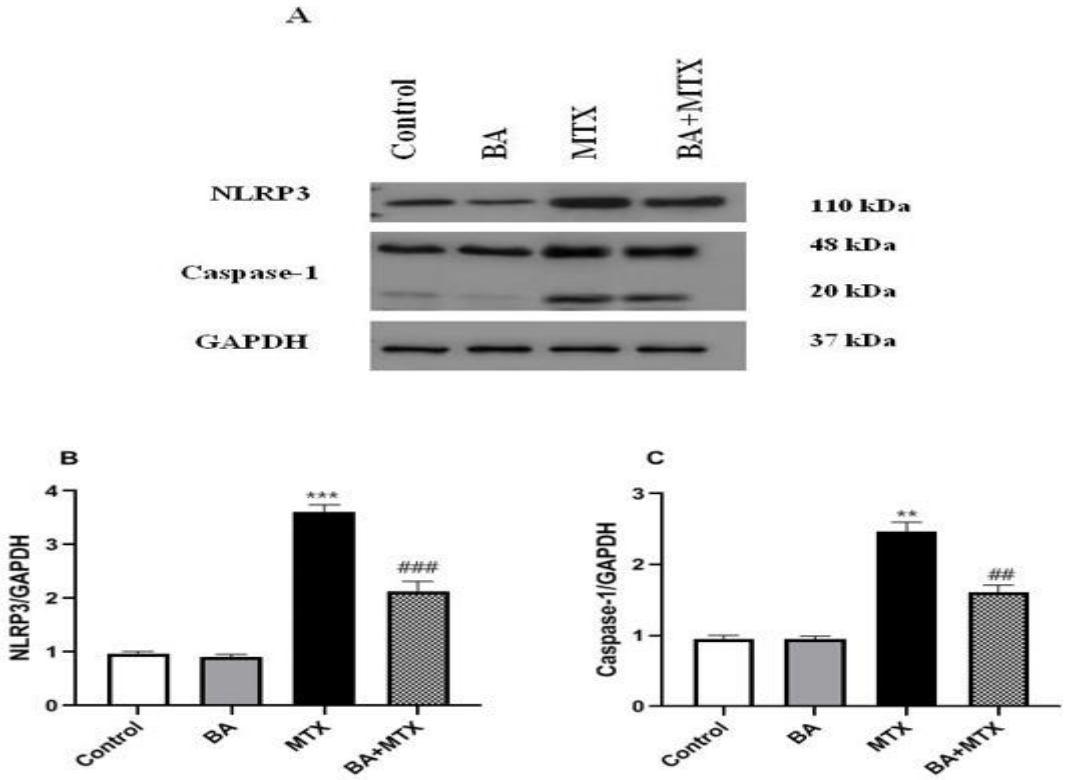


Figure 5. The effect of BA on protein expression of NLRP3 and Caspase-1 in liver damage caused by MTX. Data are expressed as mean ± SEM. BA; betulinic acid, MTX; methotrexate. * Significant difference with the control group (*p<0.01 and***p<0.001).# Significant difference with the MTX group (##p<0.01 and ###p<0.001).

## Impact of BA on histopathological alterations of liver

After the histopathological examinations, no damage was observed in the control group, and the normal structure of hepatic cells was preserved (Figure 6). In contrast, MTX-group mice exhibited significant hepatic injury relative to controls, characterized by irregular architecture, moderate-to-severe inflammatory cell infiltration, pronounced sinusoidal dilation, and extensive pyknotic nuclei (p<0.01), as confirmed by both semi-quantitative scoring (Table 1) and microscopic examination (Figure 6). All histopathological lesions were significantly attenuated following BA + MTX administration. Notably, the BA + MTX groups displayed preserved structural integrity, with an absence of inflammatory infiltration and sinusoidal dilation, as well as a significant reduction in pyknotic nuclei in comparison with the MTX group (p<0.01 for all comparisons; Table 1). Collectively, these findings demonstrate that BA significantly ameliorates MTX-induced histopathological damage in the liver.

Table 1. The level of damage in the liver tissue of the experimental groups was divided into 4 levels: intense (3), moderate (2), weak (1), and normal (0) based on the histological features.

| Group | Irregular architecture | Infiltration of inflammatory cells | Sinusoid dilation | Pyknotic nuclei |
|---|---|---|---|---|
| Control | 0 | 0 | 0 | 0 |
| BA | 0 | 0 | 0 | 0 |
| MTX | 3** | 2.5 ± 0.5** | 3** | 3** |
| BA+MTX | 0 ## | 0 ## | 0.3 ± 0.5 ## | 0.5±0.7 ## |

#Significant difference with the MTX group (##p<0.01 and ###p<0.001). *Significant difference with the control group (**p<0.01). BA; betulinic acid, MTX; methotrexate

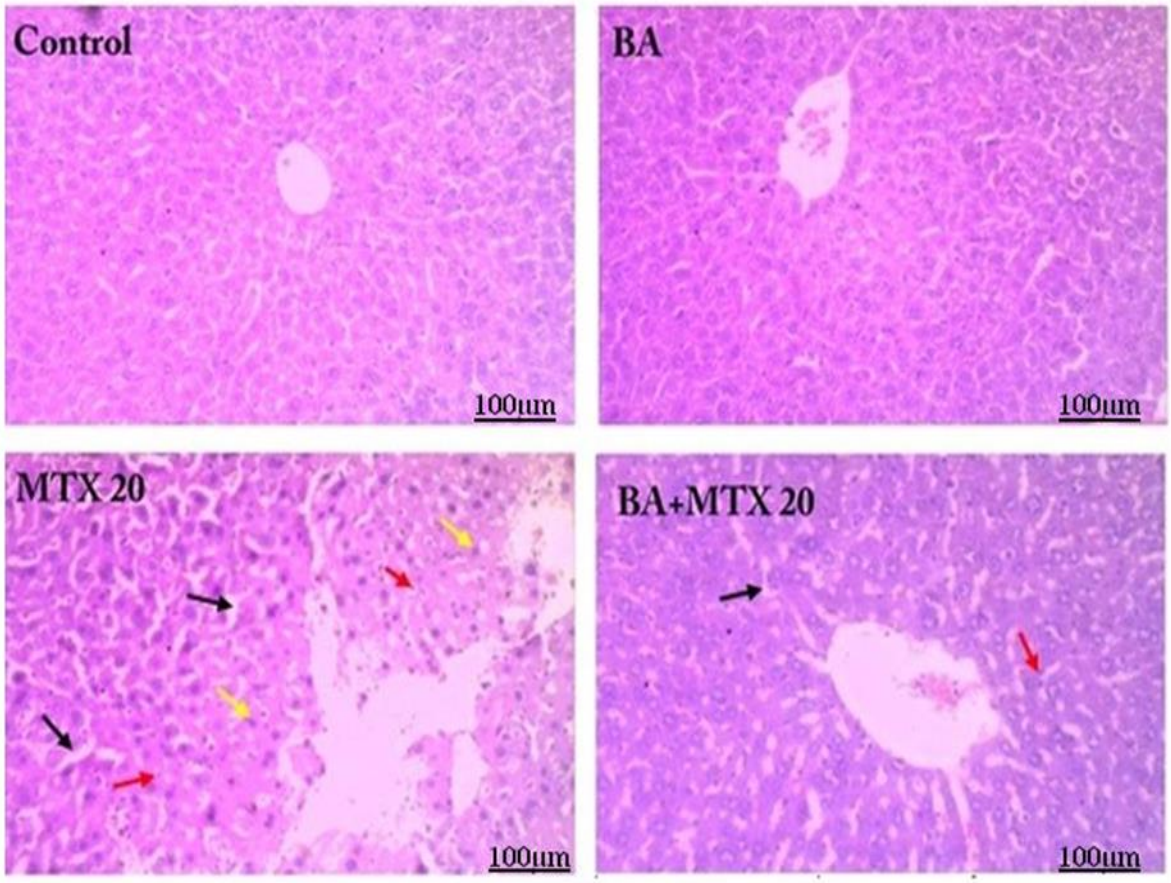


Figure6. The effects of betulinic acid (BA) on liver histopathology in methotrexate (MTX)-induced hepatotoxicity in mice with the H&E staining method. Black arrow: sinusoid dilation, yellow arrow: pyknotic nuclei, red arrow: infiltration of inflammatory cells. Magnification ×100.

## Discussion

Methotrexate is known to induce hepatotoxicity through multiple interrelated mechanisms including encompassing redox imbalance, mitochondrial impairment, and induction of proinflammatory signaling cascades. Although the exact molecular basis of MTX-induced liver injury is not completely elucidated, several mechanisms provide insight into its cytotoxic effects. MTX inhibits DHFR, an enzyme critical for the synthesis of purines, DNA, RNA, proteins, and adenosine triphosphate (ATP) (Ezhilarasan 2021). This inhibition disrupts nucleotide metabolism and cellular replication. Additionally, MTX decreases intracellular NADPH levels by inhibiting NAD-dependent mitochondrial dehydrogenases and NADP-dependent cytosolic dehydrogenases. NADPH is essential for maintaining glutathione in its reduced form, a key defense against ROS. Consequently, reduced NADPH levels impair glutathione regeneration, increasing hepatocyte susceptibility to oxidative damage (Ezhilarasan 2021). Consistent with previous studies, our results confirm that MTX elevates serum liver enzymes (ALT, AST, and ALP), reflecting hepatocellular injury and increased membrane permeability (Patel et al. 2014). Furthermore, MTX administration significantly reduced hepatic total thiol levels. The decline in total thiol is likely related to MTX-induced inhibition of glucose-6-phosphate dehydrogenase, which reduces NADPH availability and thereby limits glutathione reductase activity (Canevarolo et al. 2022). In this study, BA administration reversed many of these alterations. BA increased total thiol contents and enhanced the antioxidant

enzyme activities including GPx, SOD, and CAT. These effects are in line with previous reports demonstrating BA ability to scavenge ROS and improve endogenous antioxidant defenses (Zhu et al. 2020).

Lipid peroxidation, as indicated by increased thiobarbituric acid reactive substances levels, was significantly elevated in MTX-treated mice, consistent with excessive ROS generation and impaired antioxidant function. BA treatment significantly reduced TBARS levels, suggesting effective inhibition of oxidative membrane damage (Gaweł et al. 2004).

In addition to redox imbalance, MTX triggers proinflammatory reactions in the liver by increasing the levels of proinflammatory mediators, notably TNF-α. The elevation of TNF-α and other proinflammatory mediators contributes to liver damage and inflammation (Aladaileh et al. 2019). BA has demonstrated anti-inflammatory properties. It has been shown to reduce the levels of proinflammatory cytokine, TNF-α, which are elevated by MTX administration. This suggests that BA may help mitigate the inflammatory response triggered by MTX in the liver (Oliveira-Costa et al. 2022). Beyond oxidative stress, MTX-induced hepatotoxicity also involves inflammation. One of the central inflammatory pathways implicated is the NLRP3 inflammasome, an intracellular multi-protein assembly that detects cellular perturbations and danger-associated cues, including ROS and mitochondrial injury. NLRP3 activation leads to the recruitment and activation of CASP1, resulting in the cleavage of IL-1β and IL-18 into their active, proinflammatory forms (Guo et al. 2015; Kelley et al. 2019). CASP1 activation also induces pyroptotic cell death, exacerbating tissue injury (Broz and Dixit 2016).

Histopathological evaluation confirmed that BA mitigated structural liver damage, including sinusoidal dilatation, hepatocyte necrosis, nuclear pyknosis, and inflammatory cell infiltration (Zhao et al. 2022). Overall, these findings indicate that BA exerts hepatoprotective effects through a dual mechanism: attenuation of oxidative damage and suppression of NLRP3/CASP1-mediated inflammation.

In conclusion, our findings demonstrate that BA provides significant protection against MTX-induced hepatotoxicity in mice (Figure 7). BA restored antioxidant capacity, reduced lipid peroxidation, enhanced GPx, SOD, and CAT activities, and alleviated histopathological damage. Importantly, BA downregulated NLRP3 inflammasome and CASP1expression, indicating suppression of inflammasome-mediated inflammatory responses.

BA could be an effective hepatoprotective agent to prevent MTX-related liver injury, especially in patients requiring long-term MTX treatment. Nevertheless, additional investigations, including dose-response analyses, chronic toxicity evaluations, and controlled clinical trials, are warranted to establish the therapeutic efficacy and safety profile of BA in human populations.

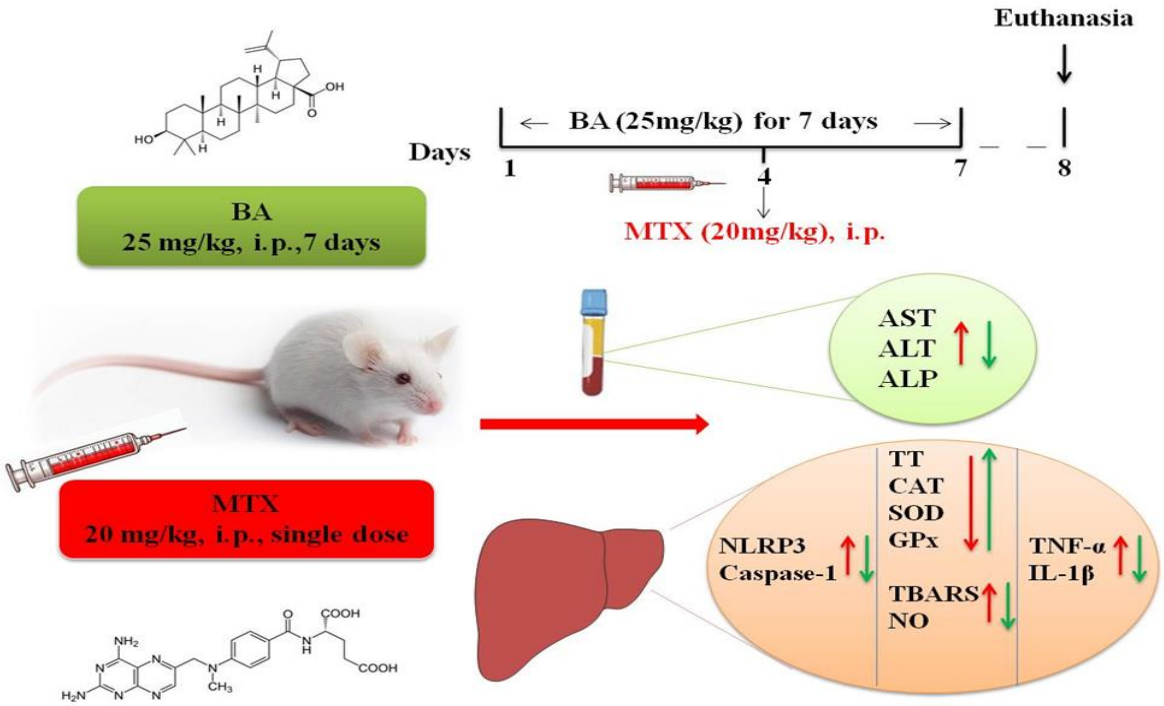


Figure7. Graphical abstract. The hepatoprotective effects of betulinic acid (BA) on methotrexate (MTX)-induced hepatic injury in mice. AST; aspartate aminotransferase, ALT; alanine aminotransferase, ALP; alkaline phosphatase, TNF-α; tumor necrosis factor-alpha , IL-1β; interleukin-1beta, NO; nitric oxide, TT; total thiol, TBARS; thiobarbituric acid reactive substances , CAT; catalase , SOD; superoxide dismutase , GPx; glutathione peroxidase . Parts of the graphical abstract were drawn by using pictures from Servier Medical Art *(https://smart.servier.com/)*. Servier Medical Art content is licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0). *( https://creativecommons.org/licenses/by/4.0/)*.

## Acknowledgment

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## Conflicts of interest

The authors had no competing interests.

## Funding

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## Ethical Considerations

The study was approved by the Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.ABHC.REC.1401.025).

## Code of Ethics

IR.AJUMS.ABHC.REC.1401.025

## Authors' Contributions

Y E Conceptualization, Investigation, Writing - Original Draft, Data Curation, M M Conceptualization, Methodology, Validation, Writing - Review & Editing, M J K Formal analysis, Visualization, M Ma re-analyzed the clinical and statistical data, E M Pathology. L Z re-analyzed. All authors read, edited and approved the final manuscript.

## Abbreviations

Methotrexate: MTX, Betulinic acid:BA, Aspartate aminotransferase: AST, Alanine aminotransferase: ALT, and Alkalinephosphatase: ALP, interleukin-1β: IL-1β, tumor necrosis factor alpha: TNF-α, Catalase: CAT, Superoxide dismutase: SOD, Glutathione Peroxidase: GPx, Thiobarbituric acid reactive substances: TBARS, Nitric oxide: NO

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