# *Eryngium Bungei* Boiss Extract Has Hepatoprotective Effect Against Liver Damage Induced by Acetaminophen in Rats: Novel Antioxidant and Anti-Inflammatory Effects

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A B S T R A C T

## Background:

Acetaminophen-induced toxicity is a common cause of acute liver failure. This study investigated the hepatoprotective effects of *Eryngium bungei B*oiss (EB) extract against the hepatotoxicity in rats.

## Methods:

Thirty adult male rats were randomly assigned to five groups, with free access to water and food. They were treated as follows: *Group 1* (negative controls): distilled water (DW); *Group 2* (positive controls): DW for 7 days and a single dose of 500 mg/kg BW acetaminophen orally on day 8th; *Groups 3, 4* and *5* (experimental groups): received EB extract mixed in DW at 100, 200 and 400 mg/kg/day orally for 7 days plus 500 mg/kg acetaminophen on the 8th day. On the 9thday, blood and liver samples were collected from all rats.

## Results:

The EB extract improved the adverse histological changes in the rats' livers and resulted in reduced serum ALT and ALP enzymes. Oxidative stress was noted in the liver tissue in *Group 2*. Pretreatment with EB extract reduced MDA concentration and increased GSH levels, compared to that for *Group 2*. The extract at 200 and 400 mg/kg/day significantly increased SOD activity, compared to that for *Group 2*. The IL-1β and TNF-α levels increased significantly in *Group 2*, compared to those in *Group 1*. Administration of EB extract in Groups 3, 4 and 5 significantly decreased the IL-1β and TNF-α parameters.

## Conclusions:

The hepatoprotective effects of EB extract appears to be linked to its glutathione-mediated detoxification, free radical scavenging and anti-inflammatory properties.

## Keywords:

*Acetaminophen Hepatotoxicity; Eryngium Bungei Extract; Inflammation; Oxidative Stress; Rats*

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## INTRODUCTION

Liver is a multi-functional organ with diverse tasks, ranging from detoxification to contribution to food digestion. Hepatic disorders include a wide spectrum of pathogenic mechanisms, ranging from toxic insults to infectious and auto immune conditions. Environmental chemical exposure and the use of drugs, even at therapeutic doses, can be associated with varying degrees of hepatic injuries. Acetaminophen is an antipyretic and analgesic medication, which is widely used singly or in combination with other drugs (1). Acetaminophen-induced hepatotoxicity is still the most common cause of acute liver failure in the USA and UK. Although administration of N-acetyl cysteine is beneficial in the early stages of acute acetaminophen toxicity, in situations where hepatic injury progresses to advance stages, liver transplantation remains the only treatment option (2).

The toxicity is initiated by production of N-acetyl-p-benzoquinone imine (NAPQI) (3), which is a highly reactive metabolite and readily interacts with sulfhydryl groups of proteins, especially mitochondrial ones. Its high production depletes glutathione reserves in hepatocytes, leading to reduced liver ability to fight against oxidative stressors, mitochondrial dysfunction, and hepatocytes necrosis (4, 5). The end result of acetaminophen overdose is the hepatic sterile inflammation followed by mitochondrial damage and

activation of Kupffer cells, which prepare the grounds for the ultimate liver failure. Recruitment of neutrophils and monocytes in response to cytokine release by activated Kupffer cells can trigger more cellular injury although the inflammation may help remove the debris and facilitate the subsequent repair processes (6).

In acetaminophen-induced liver pathologies, the identification of agents with the potential to prevent or restore the various aspects of cellular injury is a much needed clinical demand. Medicinal plants containing certain phytochemicals with strong antioxidant and anti-inflammatory properties have stimulated numerous studies (7).

*Eryngium bungei Boiss* (EB) is a perennial, glabrous herb that grows 5-30 cm high, and has its upper stem branched with oblong or lanceolate leaves (8). This plant is rich in tannins, saponins, alkaloids, and flavonoids (9). In previous studies, it has been shown that the methanolic, ethanolic, or aqueous extracts of *Eryngium* genus have anti-inflammatory, anti-scorpion venoms, anti-hyperglycaemic, and anti-convulsant effects (10, 11). Other pharmacological properties of this genus include: potent inflammation modulatory, analgesic and antioxidant, diuretic and laxative properties (11, 12). In traditional medicine, the leaves are used as remedy for cold, asthma, coughs, sinusitis, and rheumatic condition (11, 12). The objective of this study was to investigate the hepatoprotective, antioxidant and anti-inflammatory properties of the hydro-alcoholic extract of EB against acetaminophen-induced liver injury in rats.

## MATERIALS AND METHODS

***Preparing the Extract*:** The fresh aerial parts of the EB plant (mostly leaves and thin branches) were collected from the hillsides of Mount Lalehzar in Kerman, Iran, in April 2018. The plant was authenticated by a botanist at Shahid Bahonar University of Kerman and the herbarium *code KF1145* was assigned to the specimens. The collected plant materials were washed thoroughly with tap water, dried in shade, and powdered. Fifty grams of the dried, coarse powder of this material was extracted, using 500 ml mixture of distilled water and ethanol (70/30,v/v) by cold maceration process. The last trace of solvent was removed by reduced pressure distillation with the extraction yield being 10%. The extract was then freeze-dried. The liquid extract was subsequently subjected to the preliminary phytochemical analyses, as will be reported later.

***Determination of Free Radical Scavenging Activity:*** The radical scavenging activity of the extract was determined by the method described by Brand-Williams *et al*., 1995 (13). The method was based on the reduction of the free radical DPPH (2,2- diphenyl-1-pycrilhidrazin) and the decrease in absorbance at 517nm. The $IC_{50}$ value, i.e., the concentration of the extract that reduces 50% of the free-radical concentration, was then calculated as mg/ml.

***Determination of Total Phenolic Content:*** The total phenolic content of the extract was determined, using folin-ciocalteu reagent. Gallic acid was used as a reference for plotting the calibration curve. Briefly, 0.5 ml of the extract (0.01g/10 ml, methanol 60%) was mixed with 0.5 ml of the folin-ciocalteu reagent and 0.4 ml bicarbonate solution (7.5% w/v) was added after 3-5 minutes, and the reaction mixture was incubated at room temperature for 30 min. The absorbance was read at 756nm, using a UV-Vis spectrophotometer. The total phenolic content was determined, using a standard curve prepared with Gallic acid and the content of total phenolic compounds was expressed as mg/g gallic acid equivalent of the dry extract (14).

***Determination of Total Flavonoid Content:*** The total flavonoid content of the extract was determined by the aluminium chloride colorimetric method. Briefly, 0.5 ml of the extract (0.01 g/10 ml of methanol 60%) was mixed with 0.5 ml $AlCl_3$ solution (2%); then, 0.3 ml of 5% $NaNO_2$ solution was added to the mixture. After a period of 40-minute incubation, the absorbance was read at 515nm. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg rutin equivalent per gram of dry extract (15).

***Acute Oral Toxicity Assay:*** The acute oral toxicity of the extract in rats was determined by using acute toxic class method following the guidelines set by OECD number 423 (16). Three animals (female Wistar rat) were tested at each step and since there was no information on the toxicity of the extract, the experiment was started at a dose of 300 mg/kg.

***Animals and Study Design*:** Thirty male albino Wistar rats weighing 200 ± 20g were obtained from the institute of experimental animal research, University of Medical Science, Shiraz, Iran. The animals were housed under standard conditions (12/12hr light/dark cycle at room temperature) and fed with commercially available rodent food and tap water *ad libitum*. After a week of adaptation, the rats were randomly assigned to five experimental groups (N=6, each), as follows:

- Group 1: Normal control (negative), received distilled water orally.
- Group 2: Acetaminophen hepatotoxicity control (positive). The rats received distilled water for 7 days, and on day 8, they were given a single dose of 500 mg/kg BW acetaminophen in warm distilled water by oral gavage. The dose selection was based on a previous report (17).
- Groups 3, 4 & 5: (treatment groups T1, T2 & T3) received the EB extract in distilled water at 100, 200 and 400 mg/kg/day by oral gavage for 7 days, respectively. On the 8th day, they were given 500 mg/kg BW acetaminophen in warm distilled water by oral gavage.

On the 9th day, i.e., 24 hours after the acetaminophen administration, blood samples were collected from all animals by cardiocentesis under ether anesthesia. All the animals were then sacrificed by deepening anesthesia. Liver samples were immediately removed

and rinsed in cold saline, weighed and stored at minus 70ºC until further laboratory analyses. Moreover, liver samples were set aside in formalin for histopathological evaluations.

All animal experiments were approved by the State Committee on Animal Ethics, Shiraz University, Shiraz, Iran. The guidelines were based on the European Council Directive 2010/63/EU, regarding the standards in the protection of animals used for experimental purposes.

***Determination of Serum Parameters:*** Blood samples were centrifuged at 750g for 15 minutes and the sera were harvested for the determination of the following biochemical parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) activities, using standard methods and commercial kits (Pars Azmoon Co., Tehran, Iran), and an auto-analyzer (Alpha Classic AT, Sanjesh, Iran).

***Histopathological Evaluation of Liver Tissue:*** For the histopathological examinations, the liver tissue samples were fixed in buffered 10% formalin and processed routinely. The samples were then embedded in paraffin wax and 5µm thick sections were stained with haematoxylin and eosin, and were examined under light microscopy.

***Determination of Oxidative Stress Parameters and Inflammatory Cytokines in Liver Homogenates:*** Liver samples were manually homogenized in PBS at pH 7.4 (100mg tissue per 1 ml PBS). The samples were then centrifuged at 4000-6000g for approximately 20 minutes, and the supernatants were collected and frozen at -80ºC for further analysis. The protein content of the homogenates was assayed by the Bradford method (18).

The Malondialdehyde (MDA) content was measured, using Zell Bio GmbH kit (Germany), based on the reaction with thiobarbituric acid under acidic condition and high temperature, and the color complex was measured calorimetrically at 535nm. The liver glutathione (GSH) concentration and superoxide dismutase (SOD) activities, as the hepatic antioxidant defense enzymes, were assayed; using a commercially available kit (Zell Bio Company, Germany) based on the manufacturer's instructions.

The concentration of inflammatory cytokines (IL-1β and TNF-α) were assayed by ELISA method, which included rat interleukin1β ELISA kit (Bioassay Technology Laboratory, China) with a sensitivity of 2.34 pg/ml and an assay range of 5-1500 pg/ml; and rat TNF-α ELISA kit (Bioassay Technology Laboratory, China) with a sensitivity of 0.01 ng/L and an assay range of 0.03-9 ng/L. The intra-and inter-assays were CV<8% and CV<10% for both kits.

***Statistical Analyses*:** The data were expressed as means ± standard deviations (SD), subjected to normality test, and analyzed by one-way analysis of variance (ANOVA) and Tukey's test. The significance level was considered as P<0.05.

## RESULTS

***Phytochemical Parameters:*** The $IC_{50}$ value of the extract was 157µg/ml, the total phenolic content was 17.34mg gallic acid per gram of dry extract, and the flavonoid content was 7.28 mg rutin/g of the dry extract.

***Acute Oral Toxicity of the Extract:*** The acute oral toxicity of the extract started at 300 mg/kg. No death was observed during the two steps at this stage and; therefore, the extract was tested at 2000 mg/kg. Finally, the $LD_{50}$ cutoff point of the extract was determined to be 2500 mg/kg based on the results at this stage.

***Liver Enzymes Activity:*** As outlined in Table 1, the results indicated that all biochemical parameters (serum ALT, AST, ALP) increased significantly (p<0.001) in positive controls (Group 2) compared to the negative controls (Group 1). The increase was more than 3 folds for all of the assayed enzymes. The extract administration at the 3 doses resulted in a significant decrease in ALT and ALP activities compared to those for positive controls (Group 2) without a significant change noted in the AST activity level (p<0.001). Moreover, ALP activity in sera of rats that received the extract at 200 mg/kg (Group T2) was significantly lower than that for Groupg T1 (p=0.001) and T3 (p=0.015).

***Liver Tissue Histopathology*:** The liver specimens showed typical architecture with normal appearing hepatocytes in the control group (Figure 1A). The specimens from rats that received acetaminophen showed various toxic changes including fatty droplets, hepatocyte degeneration and necrosis accompanied by varying degrees of the infiltration of mononuclear inflammatory cells particularly in portal spaces (portal hepatitis), and congestion (Figure 1B & C). The EB extract attenuated the extent and intensity of acetaminophen-induced liver injuries (Figure 1D, E & F).

***Oxidative Stress in Liver Tissue:*** As shown in Figure 2, administration of acetaminophen to rats in positive controls (Group 2) resulted in an obvious oxidative stress in the liver tissue, as demonstrated by a significant increase in MDA level, a decrease in SOD activity, and GSH concentration compared to those noted in Group 1 (negative controls; p<0.001). Pretreatment of rats with EB extract at the three doses caused a significant reduction in MDA concentration compared to that seen in Group 2 (positive controls; p<0.001 for Groups T1, T2, T3 vs. Group 2). Likewise, pretreatment with the extract significantly increased the GSH levels in the treatment groups compared to that noted for Group 2 (p<0.001). Group T3 showed a significantly higher GSH concentration compared to those for Groups T1 and T2 (p< 0.001). Only the two high doses of the extract could significantly increase SOD activity compared to Group 2 (p<0.001). Rats in Group T3 showed appreciably higher levels of SOD activity compared to that noted in Group T2 (p<0.001).

***Inflammatory Cytokine Levels in Liver Tissue:*** Both inflammatory cytokines increased significantly in Group 2 compared to the negative controls, Group 1 ($p<0.001$). The administration of the extract at the three doses was associated with significant decreases in IL-1β and TNF-α levels compared to those noted for rats in Group 2 ($p<0.001$). Rats in Group T2 showed significantly lower levels of IL-1β compared to those for Groups T1 and T3 ($p<0.001$ & $p<0.05$), respectively. The level of TNF-α in Group T3 was significantly lower than those for Groups T1 and T3 ($p<0.001$ and $p< 0.01$), respectively (Figure 3).

**Table 1.** Serum activities of AST, ALT, and ALP in various rat groups.

| Parameters (U/L) / Groups | AST | ALT | ALP |
|---|---|---|---|
| Negative control (distilled water) | 49± 3.03 [a] | 25.3±3.82 [a] | 191±5.45 [a] |
| Positive control (acetaminophen) | 290 ± 55.8 [b] | 76.7± 10.9 [b] | 856± 5.45 [b] |
| T1: 100 mg/kg extract+acetaminophen | 273±55.3 [b] | 60.8± 11.3 [c] | 697± 49 [c] |
| T2: 200 mg/kg extract+acetaminophen | 248± 65.7 [b] | 57.3± 7.87 [c] | 513±91 [d] |
| T3:400 mg/kg extract+acetaminophen | 223±46.6 [b] | 53.5± 9.97 [c] | 641± 56.7 [c] |

Values in a column with different superscript letters (a-d) are significantly different at $p<0.05$, while the presence of a common superscript letter shows insignificant difference of values in a column ($p>0.05$).

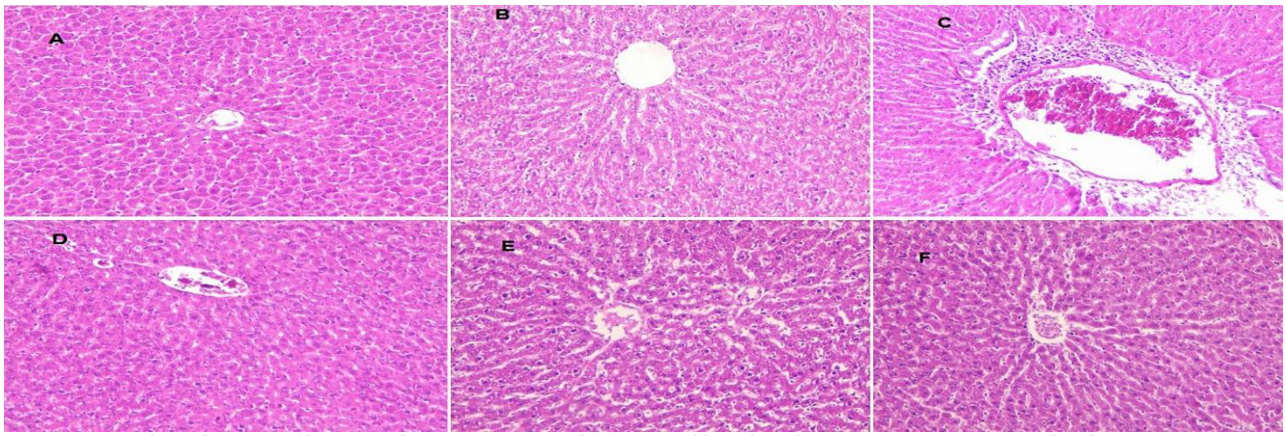


**Figure 1.** Histological images of liver samples. Negative control (A): Normal liver histology. Positive or acetaminophen hepatotoxicity control; (B, C): Inflammatory cells infiltration, centrilobular necrosis, fatty change, congestion, and portal hepatitis. Acetaminophen + EB extract at 100, 200, and 400 mg/ kg; (D, E, F): Reduced degeneration in hepatocytes and congestion.

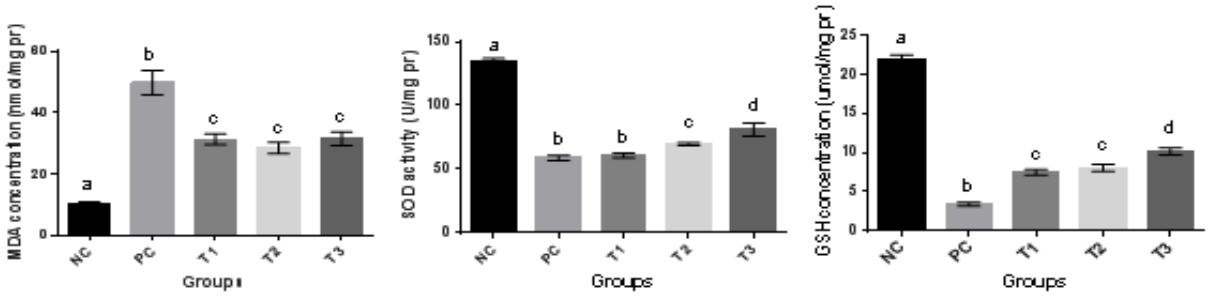


**Figure 2.** Oxidative stress parameters (means ± SD) in liver homogenates of rats in various groups. Each parameter group with different superscript letter (a-d) is significantly different at $p<0.05$, while the presence of a common superscript letter shows insignificant difference in values ($p>0.05$).

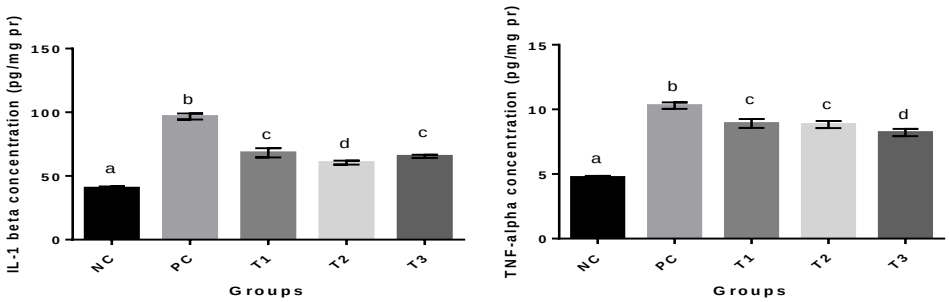


**Figure 3.** Inflammatory cytokines levels (mean ± SD) in liver homogenate of rats in various groups. Each parameter group with different superscript letter (a-d) is significantly different at $p<0.05$, while the presence of a common superscript letter shows insignificant difference in values ($p>0.05$).

## DISCUSSION

In this study, we demonstrated the hepatoprotective effect of EB extract against acetaminophen-induced liver injury. This effect was reflected by improvement of the detrimental changes detected upon histopathological evaluations and reduction in the serum activities of ALT and ALP enzymes.

Acetaminophen overdose is a popular model of liver injury in laboratory animals. This model is especially useful for the investigation of novel agents, such as herbal medicines and natural remedies (19). Two studies have suggested that antioxidant phytochemicals of medicinal plants provide protective effects against acetaminophen-induced hepatic injury (20, 21). The initial phase of acetaminophen-induced liver injury is mediated through the depletion of cellular GSH reservoirs. GSH is involved in the detoxification of peroxides by glutathione peroxidase. Thus, GSH depletion is associated with increased intracellular peroxide level and consequently oxidative stress, leading to cellular injury and necrosis (22). In this study, the oxidative stress was detected in the liver tissue of Group 2 rats (positive controls) in response to acetaminophen toxicity as shown by the subsequent decrease in SOD activity and GSH level accompanied by increased MDA level. The oxidative stress was appreciably diminished following the administration of EB extract, especially at high doses. It is well established that the *Eryngium* leaves and areal parts contain saponins and flavonoids (9). In our study, the EB extract contained relatively high contents of total phenolic and flavonoid compounds with low $IC_{50}$, reflecting high antioxidant activity.

Hepatocytes treated with acetaminophen, release a mediator that activates Kupffer cells (23). Subsequently, TNFα and IL-1β are released by the activated Kupffer cells and may have a pivotal role in the inflammatory response and the generation of reactive oxygen species. Both cytokines have diverse biological effects on cellular targets, such as neutrophil and endothelial cells activation, promotion of neutrophil recruitment, adhesion to endothelial cells, and stimulation of cytokines release and reactive oxygen species (24, 25). In this study, hepatotoxicity in the positive control rats (Group 2) was associated with an appreciable increase in IL-1β and TNFα concentrations in the liver tissue, which was significantly improved by the administration of the EB extract, especially at the two high doses (200 & 400 mg/kg/day). Our findings are consistent with those reported by earlier studies that demonstrated the anti-inflammatory properties of *Eryngium*s species (26, 27). In a study by Mekhora *et al*. (28), pretreatment of lipopolysaccharide-activated murine macrophages with the extract of *Eryngium foetidum* leaves inhibited the elevation of IL6 and TNFα in a dose-dependent manner. Similar effects have also been reported on IL8 and monocyte chemo-attractant protein-1 (MCP-1) due to prior incubation of Caco-2 cells with bioaccessible fraction of *Eryngium foetidum* leaves (29).

## CONCLUSIONS

In conclusion, the result of this study suggest that the hydro-alcoholic extract of EB has hepatoprotective properties that are related to its glutathione-mediated detoxification, free radical scavenging, antioxidant activities, and anti-inflammatory properties.

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## CONFLICT OF INTERESTS

The authors declare no conflict of interest throughout the conduction of this study.

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