

# Evacuation the Effect of Single- and Multi-dose Administration of Ethanolic Extract of *Pinus eldarica* Pollen Against Acetaminophen-induced Rat Liver Injury

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## Abstract

**Introduction:** Acetaminophen (APAP)-induced liver injury is one of the main causes of acute liver failure in the world. *Pinus eldarica* is specially distributed in the north of Iran and has been used for decades to treat wounds, pain, infection, fever, bronchitis and inflammation in many countries. This study was undertaken to evaluate the role of pine pollen extract (PE) on APAP-induced hepatotoxicity.

**Methods:** This study was conducted in two separate parts: single- and multi- dose administration of PE. In a multiple dosing regimen, different doses of extract (10, 20, 30, 40 and 50 mg/kg) were orally administrated for 2 weeks and then on the 15<sup>th</sup> day, the animals received a single dose of APAP (600 mg/kg). In the second part, the highest dose of PE (50 mg/kg) was orally administrated half an hour after receiving the APAP at dose of 600 mg/kg. The negative and positive control groups were treated with normal saline and N-acetylcysteine (NAC), respectively. At the end of procedure, the biochemical parameters including alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) as well as pathological findings were evaluated.

**Results:** A single and multiple oral dose of APAP increased the serum level of ALT, AST and ALP that were significantly attenuated by PE administration in both model. Indeed, exposure to APAP caused extensive necrosis and lymphocytic inflammation that were completely prevented by single- and multi-dose administration of PE, specially at a high dose.

**Conclusion:** Finally, the present results indicate that PE could reverse the reduction of aminotransferases and improve histological changes of APAP-induced liver toxicity. Further research is needed to confirm this finding and reveal the exact mechanisms.

**Keywords:** Acetaminophen, Hepatotoxicity, N-acetylcysteine, Paracetamol, *Pinus eldarica*, Toxicity.

## Introduction

Liver is an important vital organ for nutrition metabolism and has a critical role in detoxification, biotransformation of toxins and drugs.<sup>1</sup> Acetaminophen

(N-acetyl-p-aminophenol, APAP) is known as a non-narcotic analgesic drug with low side effects in therapeutic dose. High dose of APAP can induce liver necrosis in human and various animal species.<sup>2,3</sup> APAP

in therapeutic doses is mainly metabolized into non-toxic metabolites while at high doses is converted to toxic metabolite (N-acetyl-p-benzoquinoneimine, NAPQI) in liver. However NAPQI conjugates with glutathione (GSH) and eliminates via kidneys in low doses, it can not be detoxified and induces liver toxicity in large doses.<sup>4-6</sup> N-acetylcysteine (NAC) is the first treatment for APAP-induced hepatotoxicity through promoting the hepatic GSH synthesis.<sup>7</sup> Although NAC is still the best treatment in APAP overdose, it will be effective when administrated early.<sup>8</sup>

The family of Pinaceae (pine trees) with more than 220 species is spread in tropical regions of the northern hemisphere.<sup>9</sup> *Pinus eldarica* (Iranian pine) belongs to the Pinaceae family with specially distribution in the north of Iran.<sup>10</sup> Different parts of this tree including leaves, fruit and seeds have been used for decades to treat wounds, itching, rash, pain, infection, fever, bronchitis and inflammation in many countries.<sup>11,12</sup> They are rich in flavonoids, alkaloids, lipo-polysaccharides and phenolic compounds such as interleukins.<sup>12-14</sup>

In this study, we evaluated the hepatoprotective effect of pine pollen extract (PE) of *P. eldarica* against acetaminophen induced liver injury in rats.

## Materials and Methods

### Preparation of Ethanolic Extract of *Pinus eldarica* Pollen

The *P. eldarica* pollen (pine pollen) was collected from Mashhad forest area at the spring season 2016. The plant specimen was identified by Plant Research Center of Ferdowsy University, Mashhad, Iran and one sample of plant was kept there (No. 11945). After gathering the pollens they were completely dried and purified by sieving. In order to prepare the PE, the pollen was exposed to thermal shock in liquid Nitrogen and then broken down for 10 minutes by ultrasonic homogenizer (Bandelin Sonopuls, the Netherland). At the next step, 2.5 g of pollens was macerated in 140 mL of %70 methanol for 48 hours at controlled room temperature. The assay was repeated a total of three times and the resulting extracts were mixed together. The resulting extract was concentrated by rotary evaporator (Heidolph, Germany) at 45°C, freeze dried and then kept at -20°C for future experiments. The yield of the dried extract related to the weight of the dried seeds was 29%.

### Animals and Drugs

Seventy-eight Male Wistar rats, weighting 180-200 g, were obtained from the animal house, Toxicology Department of Buali Research Institute, Mashhad, Iran. The animals were kept in the separated cages not more than six rats per cage and acclimatized to standard laboratory conditions (temperature: 22-24°C and a cycle of light: darkness of 12:12 hours) for a week before experiments. They were permitted free access to pellet diet and water. Animal experiments were approved by the Animal Care

Committee of Mashhad University of Medical Sciences. APAP and NAC were prepared from SIGMA-ALDRICH (Germany).

### Liver Injury Model

APAP was administrated at single dose of 600 mg/kg and elevated transaminase activities as well as histopathological changes were considered as an index marker of hepatotoxicity.

### Multi-dose Administration of Ethanolic Pine PE in APAP-Induced Rat Liver Injury

Animals were divided into nine groups of six rats. Six group of animals were treated by different dose of PE (10, 20, 30, 40 and 50 mg/kg) or NAC (450 mg/kg, positive control group). All substances were administered orally for 2 weeks. At the end of the procedure, single dose of APAP at dose of 600 mg/kg was gavaged. Additional groups, received normal saline (negative control) or PE at dose of 50 mg/kg, by the same route and in the same time period.

### Single-Dose Administration of Ethanolic Pine PE in APAP-Induced Rat Liver Injury

The animals were randomly divided into four groups (n=6). The negative control group received a single dose of normal saline alone. Other groups were orally administered with the highest dose of PE (50 mg/kg) or NAC (700 mg/kg, positive control), half an hour after receiving a dose of 600 mg/kg APAP.

## Experimental Protocols

### Biochemical Parameters

The day after treatment, animals were sacrificed under ketamine and xylazine anesthesia. For evaluation of serum alkaline phosphatase (ALP), aspartate transaminase (AST) and alanine transaminase (ALT), the blood samples were taken through cardiac puncture. The biochemical tests were performed by laboratory of Imam Reza hospital in Mashhad (Iran).

### Histopathological Examination

24 hours after drug administration, the liver of animals was removed after euthanasia and fixed in 10% neutral buffered formalin. The right lobe of the liver was dissected and processed for paraffin embedding. They cut at a thickness of approximately 5 µm and stained with hematoxylin and eosin.

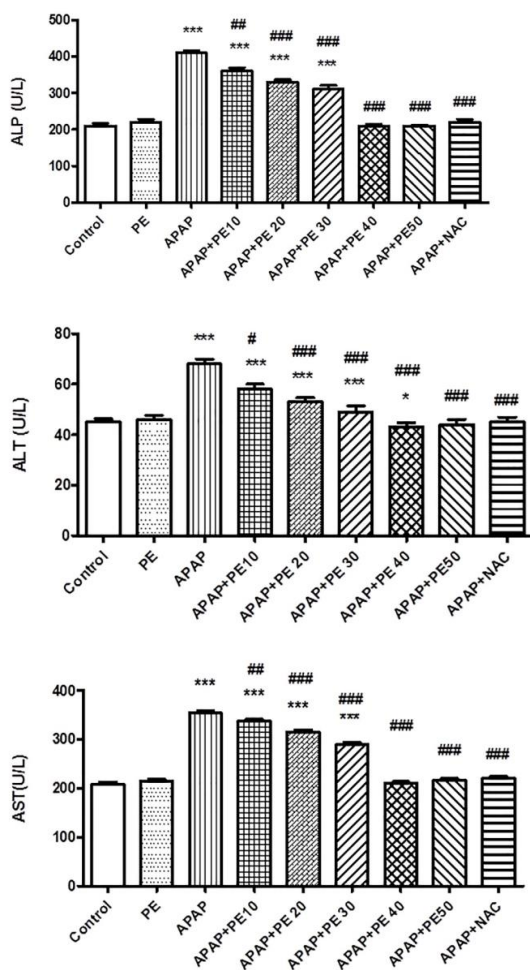
### Statistical Analysis

The data were expressed as mean ± standard deviation (SD). Statistical differences between groups were performed with one-way analysis of variance (ANOVA) followed by a Tukey-Kramer test.  $P < 0.05$  was considered statistically significant. Data analysis was performed using SPSS version 21.

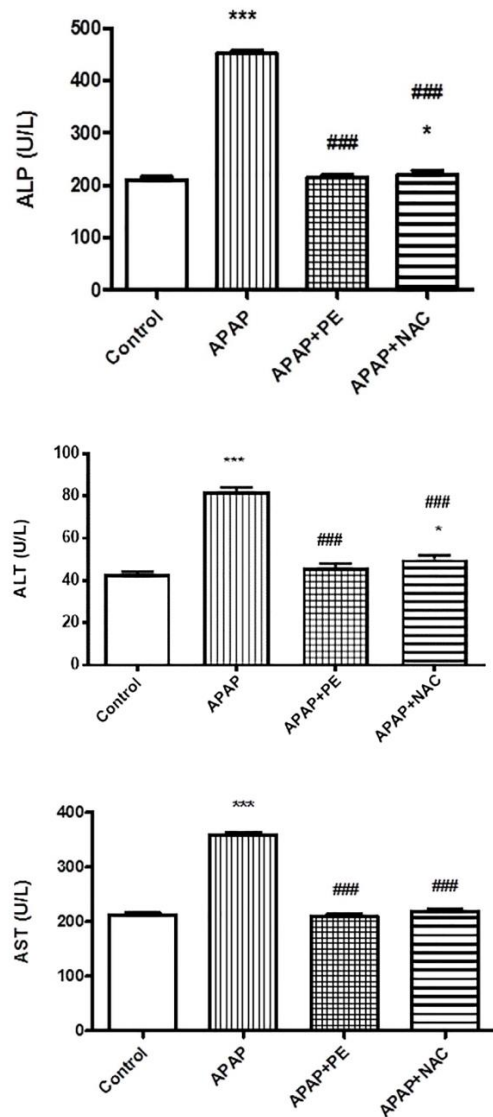
**Results**

**Biochemical Evaluation**

APAP at a single dose of 600 mg/kg, significantly increased the serum level of liver enzymes, in both experimental method, when compared to the control group (Figures 1 and 2). Multi dose administration of ethanolic PE extract attenuated the effect of APAP and significantly decreased the level of ALP, ALT and AST, compared to the APAP group, in a dose dependent manner (Figure 1). There was no difference between APAP+PE at dose of 50 mg/kg and control groups. Orally administration of NAC also decreased the amount of liver enzymes (Figure 1). At single dose method, high dose of extract or NAC significantly decreased aminotransferases activity compared to the APAP group (Figure 2).



**Figure 1.** The comparison of serum ALP, ALT and AST level in rats that were received normal saline, PE (10, 20, 30, 40 or 50 mg/kg) or NAC (450 mg/kg). All substances were administered orally for a duration of two weeks. At the end of the procedure, single dose of APAP at dose of 600 mg/kg was gavaged. Additional groups received normal saline (control) or PE (50 mg/kg) alone, by the same route and in the same time period. The results are reported as mean ± SD, \*\*\**P*<0.001 compared to the control group, #*P*<0.05, ##*P*<0.01 and ### *P*<0.001 compared to the APAP group. PE: Pine pollen extract, APAP: acetaminophen, NAC: N-acetyl cysteine. Tukey–Kramer test, n=6.



**Figure 2.** The comparison of serum ALP (A), ALT (B) and AST (C) level in rats that were orally administered with the highest dose of PE (50 mg/kg) or NAC (700 mg/kg, positive control), half an hour after receiving the APAP at dose of 600 mg/kg. Control group received a single dose of normal saline alone. The results are reported as mean ± SD, \**P*<0.05 and \*\*\**P*<0.001 compared to the control group, ###*P*<0.001 compared to the APAP group. PE: Pine pollen extract, APAP: acetaminophen, NAC: N-acetyl cysteine. Tukey–Kramer test, n=6.

**Histopathological Finding**

Histopathological changes of liver tissues are presented in Figures 3 and 4. All histological findings were normal in the control and PE receiving group. Exposure to APAP caused extensive necrosis and lymphocytic inflammation (Figure 3C and 4 B). At multi dose method, the administration of PE at lowest dose (10 mg/kg), in combination with APAP, attenuated the hepatocellular injury to the extent that moderate necrosis, congestion and lymphocyte aggregation were detectable (Figure 3D). The mild inflammation and necrosis were observed at group that received PE at dose of 20 or 30 mg/kg plus

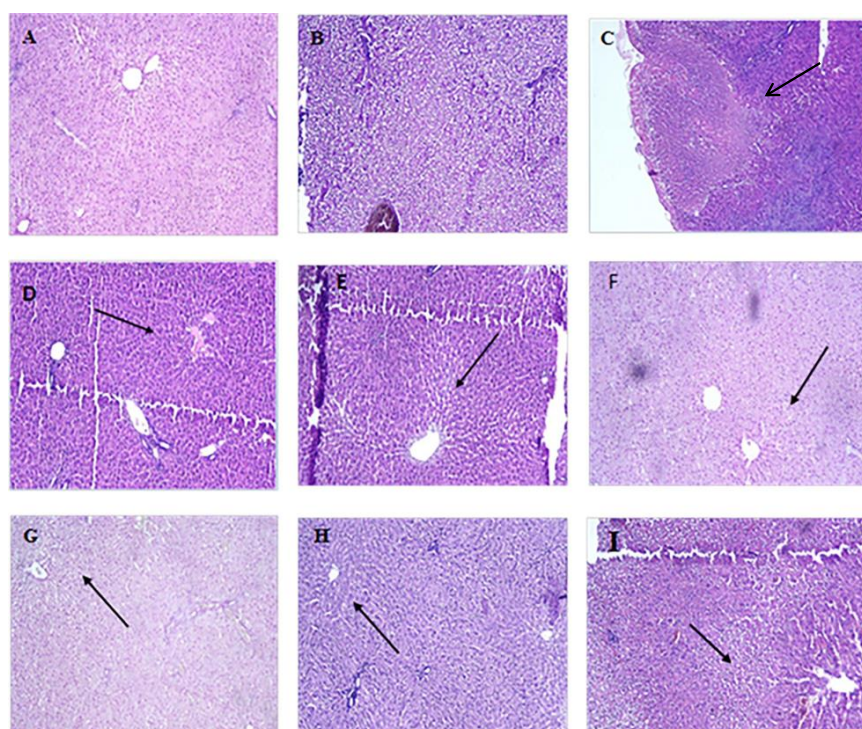
APAP (Figure 3E and 3F). Treatment with PE at higher doses (40 and 50 mg/kg) and NAC diminished the APAP-induced injury (Figure 3G, H and I, respectively). The APAP-induced hepatotoxicity was also prevented by single dose administration of PE (Figure 4C). The NAC at single dose could not completely abolish the injury and mild portal inflammation (Figure 4D).

### Discussion

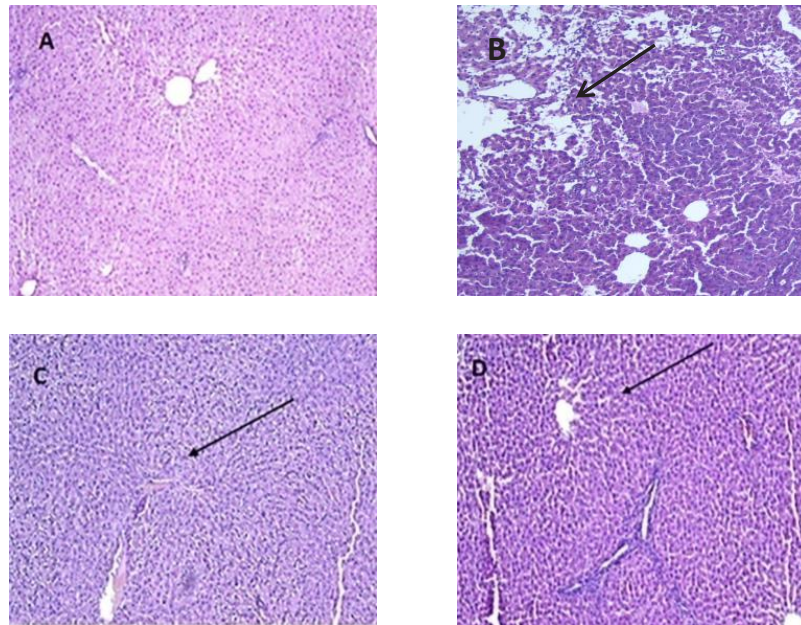
APAP, as a pain reliever, is widely used around the world. Unintentional APAP overdose is the most common cause of acute liver failure in the west world.<sup>15</sup> The intake of 10 g single dose or higher often leads to acute liver necrosis.<sup>16</sup> Serum levels of classical enzymes including ALP, ALT and AST are reliable indices of hepatocellular toxicity, specifically in detection of acute hepatitis.<sup>17</sup> In this study, APAP orally administration induced liver dysfunction and cell damage in association with increased ALT, AST and ALP levels. Single- and multiple-dose administration of pine decreased the enhanced level of serum AST, ALT and ALP, lessened the pathological changes and maintained the functional integrity of the liver cell, specially at high doses. The result of an *in vitro* study showed the protective effect of Masson pine pollen aqueous extract against CCl<sub>4</sub>-induced oxidative damage in human hepatic cells through

alleviating the morphology changes and inhibiting the abnormal rise in AST and ALT level in a dose dependent manner.<sup>18</sup> The pine PE also possesses analgesic and anti-inflammatory activities that may be related to the production of proinflammatory mediators.<sup>19</sup> Pine PE has also been found to be effective for the treatment of age-related conditions, diabetic nephropathy and chronic arthritis with reduction the level of inflammatory cytokine and increasing antioxidant activity.<sup>12,20,21</sup>

Different parts of pine have been used for medicine purpose: buds, seeds, needles, bark, resin, and of course, the pollen. Evaluation the effect of pine bark extract against cisplatin-induced hepatotoxicity showed that it could decrease the malondialdehyde concentration, serum level of aminotransferase and increase the reduced glutathione content, catalase, superoxide dismutase, and glutathione S-transferase activities. It was reported that the oral administration of pine bark extract for 10 days also prevented the histopathological alterations in liver tissue.<sup>22</sup> It has been well documented that the pine needle oil had antioxidant, antimicrobial and anticancer activities.<sup>23,24</sup> Pine needle oil treatment arrested the Hepg2 cycle cell and inhibited the proliferation of liver cancer cell line.<sup>25</sup> Other parts of the tree possess remarkable antioxidant, anti-inflammatory and antimicrobial



**Figure 3.** Light photomicrograph of hematoxylin and eosin– stained sections in the liver tissue of rats that were received normal saline, PE (10, 20, 30, 40 or 50 mg/kg) or NAC (450 mg/kg). All substances were administered orally for a duration of two weeks. At the end of the procedure, single dose of APAP at dose of 600 mg /kg was gavaged. Additional groups received normal saline (control) or PE (50 mg/kg) alone, by the same route and in the same time period. A and B: normal liver histology (control and PE received groups), C: extensive necrosis and lymphocytic inflammation (APAP received group), D: moderate necrosis, congestion and lymphocyte aggregation, E, F and G: mild inflammation and necrosis (D, E, F and G: received PE at different doses + APAP), H and I: normal liver histology (H: received PE at high dose and APAP, I: received NAC+APAP) (magnification:  $\times 100$ ). Arrows indicate necrosis or portal inflammatory infiltrates. PE: Pine pollen extract, APAP: acetaminophen, NAC: N-acetyl cysteine.



**Figure 4.** Light photomicrograph of hematoxylin and eosin– stained sections in the liver tissue of rats that were orally administered with the highest dose of PE (50 mg/kg) or NAC (700 mg/kg, positive control), half an hour after receiving the APAP at dose of 600 mg/kg. Control group received a single dose of normal saline alone. A: normal liver histology (control group), B: extensive necrosis and lymphocytic inflammation (APAP received group), C: normal liver histology (PE+APAP received group), D: mild portal inflammation (NAC + APAP received group) (magnification:  $\times 100$ ). Arrows indicate necrosis or portal inflammatory infiltrates. PE: Pine pollen extract, APAP: acetaminophen, NAC: N-acetyl cysteine.

activities.<sup>26,27</sup>

Plant-derived products with a wide range of phytochemicals and phenolic compounds have considerable as an antioxidant, antiradical, anti-inflammatory and anticarcinogenic activities. Phenolic compounds can act as metal chelators, reducing agents (free radical terminator/scavenger), and singlet oxygen and free-radical quenchers.<sup>28</sup> Pinus species are also natural source of phenolic compounds. In comparison between *P. pinaster*, *P. eldarica* and French *P. pinaster*, Iranian Pinus species bark extract have higher phenolic levels and antioxidant capacity making it suitable alternatives for food and medical applications.<sup>28,29</sup> The protective effect of pine PE against APAP-induced hepatotoxicity maybe related to the antioxidant and anti-inflammatory properties of phenolic compound.

### Conclusion

The present results indicate that PE had potential preventive and therapeutic effects on APAP-induced liver toxicity in rat. Single-dose administration of ethanolic pine PE could inhibit the abnormal rise in AST and ALT level and improved histological changes in acute hepatic damage by APAP. Multiple-dose administration of extract, especially at high doses, also reversed the reduction of aminotransferases and maintained the functional integrity of the liver cell, in a dose-dependent manner. However, further detailed research is needed to confirm this finding and reveal the exact mechanisms, as well as, compare the effectiveness and efficiency of pine

pollen and other plant components in the treatment of APAP-induced toxicity.

### Ethical Approval

All the experimental protocols were accepted by Ethical Committee of Mashhad University of Medical Sciences (IR.MUMS.REC.1395.91).

### Conflict of Interest Disclosure

There is no conflict of interest.

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### Authors' Contributions

Conception and design of study: LE, SAM, MoM. Acquisition of data: SA, NS, MuM, AG. Analysis and/or interpretation of data: LE, MoM. Drafting the manuscript: NAN, LE. Revising the manuscript critically for important intellectual content: LE, SA.

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