Research Article

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Hepatoprotective effects of safranal on acetaminophen-induced hepatotoxicity in rats

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Abstract: This research aimed to explore the protective and therapeutic properties of safranal in mitigating inflammation and oxidative stress induced by elevated acetaminophen (APAP) doses in a rat model. The protective and therapeutic effects of safranal were determined by histopathologically and examining some biochemical parameters such as aspartate transaminase (AST), alanine transaminase (ALT), glutathione, glutathione peroxidase, catalase, malondialdehyde, interleukin-6, tumor necrosis factor-α, and interleukin-1β. Male Wistar–Albino rats were subject to random allocation, forming five groups, each comprising seven rats (n = 7) in the study. Group 1 was the control group. APAP was administered in Group 2 to induce hepatotoxicity. Rats in Groups 3, 4, and 5 received intraperitoneal injections of safranal at doses of 0.025, 0.05, and 0.1 mL/kg/day for 14 days, respectively. On the 15th day, to induce APAP-induced hepatotoxicity, four groups (Groups 2, 3, 4, and 5) acquired a single intraperitoneal injection of 600 mg/kg APAP. The presence of APAP-induced hepatotoxic effect was proven by elevated AST and ALT levels, which are typical biomarkers of liver function in addition to the demonstration of histopathological changes. The findings suggest that pre-treatment with safranal may offer a protective effect against hepatotoxicity by attenuating oxidative stress and the inflammatory response.

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1 Introduction

The acute liver injury might be due to metabolic diseases, Wilson's disease, acute Budd–Chiari syndrome, neoplastic infiltration, mushroom poisoning, and heat stroke in addition to the administration of paracetamol (PCM; acetaminophen (APAP); *N*-acetyl-aminophenol) that is frequently used in analgesic and antipyretic drugs. In 58% of the cases in the literature, acute liver injury has been demonstrated to originate from drugs, among which APAP has a significant place with 46% [1]. Suicidal or accidental ingestion of APAP, which is an over-the-counter drug that has been broadly used, might lead to death. Liver toxicity occurs frequently although APAP-induced kidney failure can also develop [2]. Large amounts of APAP in a single dose might cause damage to multiple organs such as the liver, kidney, and testicles. Especially, alcoholics often present with simultaneous liver failure and renal failure [2,3].

About 10–15 g single dose of (150–250 mg/kg) APAP may lead to hepato-toxicity, whereas doses of 20–25 g or more are considered fatal. A very small amount of APAP at therapeutic doses is excreted in the urine unchanged [4]. Approximately 97% of the compound is detoxified after conversion to water-soluble glucuronide and sulfate conjugates in the hepatocytes and excreted partially in the bile and blood circulation. The residual, minimal quantity of APAP undergoes oxidation within the liver through the cytochrome P450 enzyme system, forming a harmful metabolite referred to as *N*-acetyl-*p*-benzoquinone imine (NAPQI), which subsequently binds to cellular macromolecules. The highly reactive NAPQI is rapidly converted to non-toxic cysteine or mercapturic acid conjugates by joining with hepatic glutathione (GSH) and excreted in the urine [5,6]. However, it is suggested that large doses of APAP increase the over-production of reactive oxygen species (ROS) along with oxidative stress by increasing the production of excess NAPQI and decreasing the amount of GSH and GSH-related

enzymes, thus inhibiting the cellular antioxidant system [7–9]. Therefore, there is a critical balance between the antioxidant system and liver injury. After liver injury, plant extracts such as safranal supplementation rich in natural antioxidants may be useful in increasing tissue regeneration, reducing oxidative damage, and preventing APAP toxicities.

In studies with plant extracts, the composition of the plant extract is primarily determined, and then, studies are carried out on various experimental animal models to determine the possible therapeutic effects of the extract [10]. However, in recent years, the evaluation of active ingredients in the extract rather than the plant extract has taken precedence [11].

Saffron (*Crocus sativus*) is a medicinal plant with many therapeutic effects, and the healing role of *Crocus sativus* petal extract in acute liver damage is known [12]. However, there is a need for studies on which ingredients in saffron may be responsible for this effect. Phytochemical investigations have unveiled that saffron comprises a minimum of four active constituents, namely, safranal, picrocrocin, crocetin, and crocin. Safranal, an organic compound derived from saffron flowers, serves as the principal contributor to saffron's distinctive aroma. Safranal has been reported to be produced through picrocrocin following the cleavage of the carotenoid named zeaxanthin [13,14].

Recent studies have shown that safranal, an active component of *Crocus sativus*, has many pharmacological effects such as antidiabetic [15], antioxidant [16], anti-cancerogenic [17], antimicrobial [16], anti-inflammatory, cardioprotective, gastrointestinal protective, nephroprotective and neuroprotective properties due to its high radical scavenging activity [18]. Also, the use of safranal in drug-induced toxicities [19] and osteoarthritis [20] can be given as an example of its use for therapeutic purposes. The fact that safranal especially strengthens the antioxidant defense system and modulates oxidative stress has increased these effects [21]. Furthermore, saffron and its active components have been shown to exhibit minimal toxicity when administered at therapeutic doses [22,23]. All these properties may suggest that safranal may be protective or curative on tissues against toxic exposures.

This study aimed to assess the defensive and remedial properties of safranal in a rat model, particularly in response to the toxicity, inflammatory reactions, and oxidative stress triggered by substantial APAP doses.

2 Materials and methods

2.1 Experimental animals

An experimental Wistar–Albino rat model was created to develop new treatment strategies against liver injury that may occur in humans. This investigation involved a cohort of 35 male Wistar–Albino rats aged 10–12 weeks and weighing about 220 ± 30 g. The rats were distributed randomly into five groups, each consisting of 7 individuals (as depicted in Table 1). They were housed in rat cages in a well-ventilated house and were allowed to acclimatize for 10 days before the experiment. Each rat was housed individually under a 12 h light/dark cycle and supplied with unrestricted access to both standard pellet diet and water. All procedures conducted on animals throughout the study were performed with the approval of the Animal Research Local Ethics Committee at Fırat University (Approval number for animal experiments: 2017/38, Decision number: 82). The animals were provided care at Fırat University Experimental Animal Research and Application Center.

2.2 Drugs and reagents

For this study, APAP powder was procured from Sigma-Aldrich (A5000; Kappel-weg, Germany), and Safranal was acquired from Sigma-Aldrich (Chemical Co., St. Louis, USA). The hematoxylin and eosin (H&E) staining kits, as well as cytoplasmic and nuclear extraction kits, were sourced from Nanjing Jian Cheng Bioengineering Research Institute (Nanjing, China).

Table 1: Experimental protocol

Groups	
Group 1	(Control): 14 day 0.1 mL/kg/day % 0.9 NaCI i.p.
Group 2	(APAP): 14 day 0.1 mL/kg/day % 0.9 NaCI i.p. + 15th day single dose 600 mg/kg i.p. APAP
Group 3	(Safranal + APAP): 14 day safranal 0.025 mL/kg/day i.p. + 15th day single dose 600 mg/kg i.p. APAP
Group 4	(Safranal + APAP): 14 day safranal 0.05 mL/kg/day i.p. + 15th day single dose 600 mg/kg i.p. APAP
Group 5	(Safranal + APAP): 14 day safranal 0.1 mL/kg/day i.p. + 15th day single dose 600 mg/kg i.p. APAP

2.3 Experimental design

Crocus sativus, derived from the Arabic word "Zafaran" meaning yellow, has a rich history. Safranal is among more than 150 volatile and flavoring compounds that contribute to the color, taste, and aroma characteristics of this plant.

In this study, the safranal component (Sigma-Aldrich, Chemical Co., St. Louis, USA) of commercially purchased *Crocus sativus* extract was evaluated in male rats. The current literature was reviewed to determine the dose of safranal for the experiment [24]. The experimental scheme of acute liver failure (ALF) and safranal application is shown in Figure 1. Safranal was dissolved in liquid paraffin and was intraperitoneally injected in daily doses of 0.025, 0.05, and 0.1 mL/kg/day in groups 3, 4, and 5, respectively, for 14 days [25]. The intraperitoneal ID50 values for safranal were documented as follows: 1.48 mL/kg in male mice, 1.88 mL/kg in female mice, and 1.50 mL/kg in male rats [26]. For the formation of APAP-induced hepatotoxicity, rats in all groups besides the control group were injected with a single dose of 600 mg/kg i.p. APAP after 12 h of a fasting period. On the 15th day, the APAP powder was dissolved freshly at 55°C with 0.9% NaCl to 20 mg/mL in a water bath before the application and was then cooled to 37°C. The appropriate dose was decided according to the previous studies [27,28]. Rats were euthanized 24 h after APAP injection (Day 16) by intraperitoneal injection of 50 mg/kg ketamine and 10 mg/kg xylazine for anesthesia. After intracardiac blood collection, the serum was separated by centrifugation at 4,000 × *g* for 15 min at 4°C and stored at 20°C [29]. The liver tissues were quickly removed and fixed in a 10% formalin solution and then embedded in paraffin.

2.4 Serum biochemical analysis

The serums separated in anticoagulant-free tubes were thawed, and then, aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were studied by the Siemens ADVIA 2400 Autoanalyzer using commercially available kits provided for each sample by the procedures based on spectrophotometric methods identified by The International Federation of Clinical Chemistry and Laboratory Medicine. Also, the pre-experimental values of the rats used were within normal limits.

2.5 Determination of serum inflammatory marker levels

Among the inflammatory markers, serum levels of tumor necrosis factor-α (TNF-α) (Catalog no: ER1393, Fine Bio-Tech Co., Wuhan, China), interleukin-1β (IL-1β) (Catalog no: ER1094, Fine Bio-Tech Co., Wuhan, China), and interleukin 6 (IL-6) (Catalog no: ER0042, Fine Bio-Tech Co., Wuhan, China) were determined in the serum samples that had been separated earlier. All samples were thawed to room temperature and mixed before assaying. All samples were analyzed on the same day to avoid possible inter-assay variation. This measurement was made using enzyme-linked immunosorbent

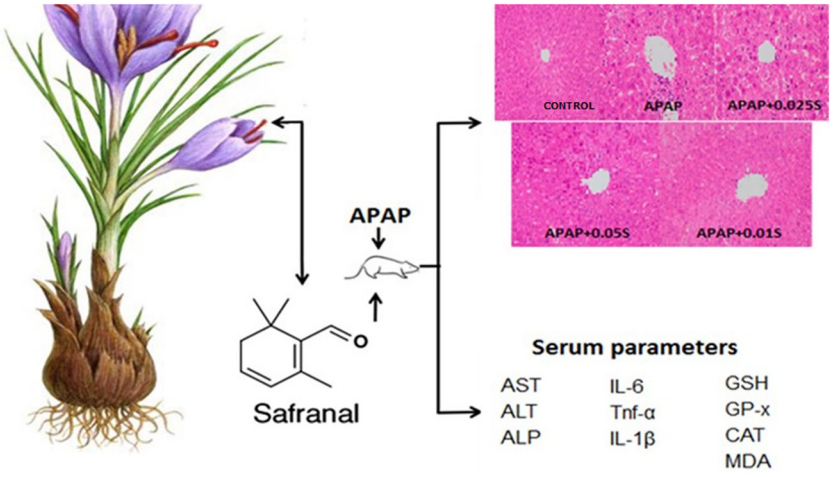

Figure 1: Experimental scheme of ALF and safranal application.

assay on a 96-well plate, following the protocols provided with the commercial kits, using a Biotek ELx800 instrument.

2.6 Serum oxidative stress and antioxidant capacity determination of malondialdehyde (MDA)

MDA which is one of the final products formed by free radical-mediated peroxidation of unsaturated fatty acids, reacts with thiobarbituric acid to form a colored compound. The quantification of MDA levels was carried out employing a commercial colorimetric assay kit (MDA assay kit; ZellBio GmbH, Ulm, Germany), and the resulting colored compound was measured on the spectrophotometer (Shimadzu 1700 UV/VIS) at the wavelengths of 532 and 600 nm.

2.7 Determination of GSH

Reduced GSH, a yellow-colored final product resulting from the reaction of –SH groups with DTNB (5,5′-2-nitrobenzoic acid) in the serum, was measured for absorbance at the wavelength of 412 nm using the Randox-Ransod enzyme kit on a spectrophotometer.

2.8 Determination of glutathione peroxidase (GP-x)

GP-x catalyzes the reaction that oxidizes reduced GSH with cumene hydroxide. In the presence of GSH reductase and nicotinamide adenine dinucleotide phosphate (NADPH) in the same milieu, oxidized glutathione (GSSG) undergoes reduction to its reduced form, GSH, facilitated by the oxidation of NADPH to NADP. The GP-x activity was measured in the spectrophotometer using the Randox-Ransod enzyme kit.

2.9 Determination of catalase (CAT)

Catalase activity was assessed using an enzymatic assay kit (ZellBio GmbH CAT Colorimetric Assay Kit). All reagents and samples were added as described in the kit catalog and were then incubated at 37°C for 1 min. The absorbance of the final product that turned into a chromogenic color by the addition of the last reagents was read colorimetrically, and CAT activity was calculated consistent with the manufacturer's formula.

2.10 Histopathological examination

Upon euthanizing the rats, liver tissue sections were promptly submerged in 10% neutral buffered formalin, subjected to dehydration through a series of graded ethanol solutions, and subsequently cleared using xylene. Following these processes, the sections were embedded in paraffin and sliced into five µm thick sections. The H&E staining was then conducted on these sections to facilitate histopathological examination using an optical microscope, adhering to the instructions outlined in previous reports [30]. Morphological changes of the stained liver tissue were observed under an optical microscope (BX43, Olympus, Tokyo, Japan) and obtained a colored image at a magnification of 40×.

2.11 Statistical analysis

SPPS statistical package program (IBM SPSS Version 22.0, Armonk, 2013; NY: USA) was used for the evaluation of data. The Kruskal–Wallis test was used to decide the variations between groups, and Dunn's multiple comparisons were used as a post hoc analysis for binary comparisons. Data were presented as the mean and standard error for the groups. Throughout the study, p values <0.05 were considered statistically significant.

3 Results

3.1 Treatment of APAP-induced liver injury with safranal in rats

Serum AST and ALT levels, as well as histopathological findings, serve as sensitive indicators for discerning the presence of hepatotoxicity in the liver tissue induced by APAP. We examined the serum AST, ALT, and ALP levels and the structure of hepatic tissue cells to investigate the therapeutic effects of safranal on liver injury. As shown in Figure 2, the levels of AST, ALT, and ALP were found to be significantly increased in Group 2 compared to the control group ($p < 0.001$). Nonetheless, the safranal-treated groups demonstrated a dose-dependent reduction, and the therapeutic interventions with safranal resulted in decreased AST, ALT, and ALP levels in Groups 4 and 5 (0.05 and

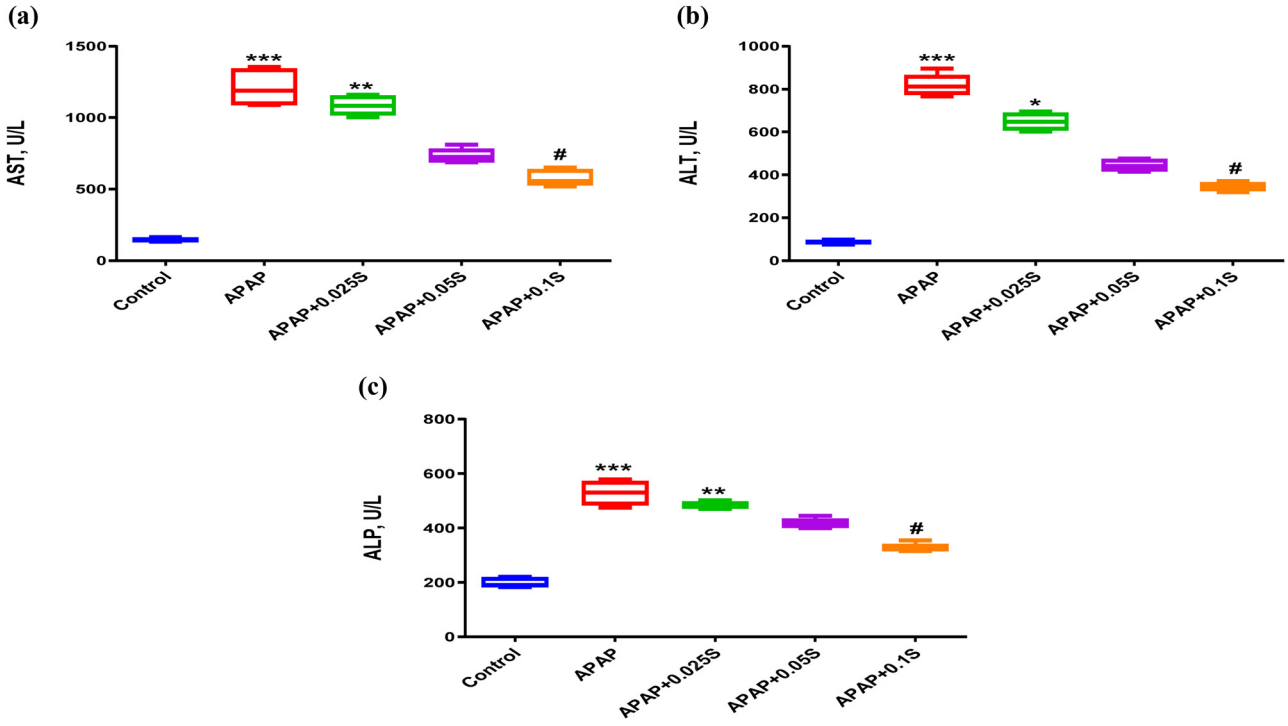

Figure 2: Effect of APAP treatment on blood serum levels of AST, (Panel a), ALT, (Panel b), and ALP, (Panel c) in rats. Data are presented as mean and standard error. Data were evaluated by Kruskal–Wallis and Dunn's multiple comparisons tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control group; #$p < 0.05$ compared to APAP group.

0.1 mL/kg), as presented in Table 2. In Group 5, the levels of AST, ALT, and ALP decreased significantly compared to the APAP group ($p < 0.05$).

As shown in Figure 3, the hepatic tissue structures in the control group were normal and the central vein was stained separately and clearly. In rats in the APAP group, the structure of the hepatocytes was heterogeneous (black arrows), the central vein (blue arrow) was irregular and fuzzy, and the structure of the cells was mostly disrupted by many inflammatory cells (arrowhead). Safranal alleviates the structural liver damage in a dose-dependent manner in APAP-induced liver injury and exhibits a noticeable effect at the dose of 0.1 mL/kg (Group 5). Safranal improved liver injury and histological changes in hepatocytes in rats.

3.2 Pre-treatment with safranal reduced oxidative stress in rats receiving APAP

APAP-mediated hepatotoxicity induces cellular mitochondrial oxidative stress and depletes the antioxidant reserve. After the induction of hepatotoxicity by APAP in Group 2, a noteworthy reduction was observed in the serum levels of GSH, GP-x, and CAT in comparison with the control group ($p < 0.001$), as visually represented in Figure 4. Conversely, there was a substantial increase in MDA levels, an indicator of lipid peroxidation, within Group 2 following APAP administration when compared to the control group ($p < 0.001$), as depicted in Figure 4.

However, pre-treatment with safranal significantly increased GSH, GP-x, and CAT levels in a dose-dependent

Table 2: Serum levels of AST (U/L), ALT (U/L), and ALP (U/L) in rats

	Control (Group 1)	APAP (Group 2)	APAP + 0.025S (Group 3)	APAP + 0.05S (Group 4)	APAP + 0.1S (Group 5)
AST (U/L)	145.15 ± 5.78	1210.76 ± 56.04***	1084.52 ± 30.24**	733.41 ± 22.46	577.47 ± 24.83#
ALT (U/L)	85.9 ± 3.74	817.61 ± 22.32***	648.38 ± 18.04*	444.55 ± 12.23	345.27 ± 8.81#
ALP (U/L)	198.6 ± 7.73	528.6 ± 19.57***	484 ± 5.44**	416.8 ± 7.8	326.8 ± 7.24#

Data are represented in terms of the mean value and standard error. Statistical analysis was performed using the Kruskal–Wallis test, followed by Dunn's multiple comparisons tests. Significance levels are denoted as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to the control group, and #$p < 0.05$ when compared to the APAP group.

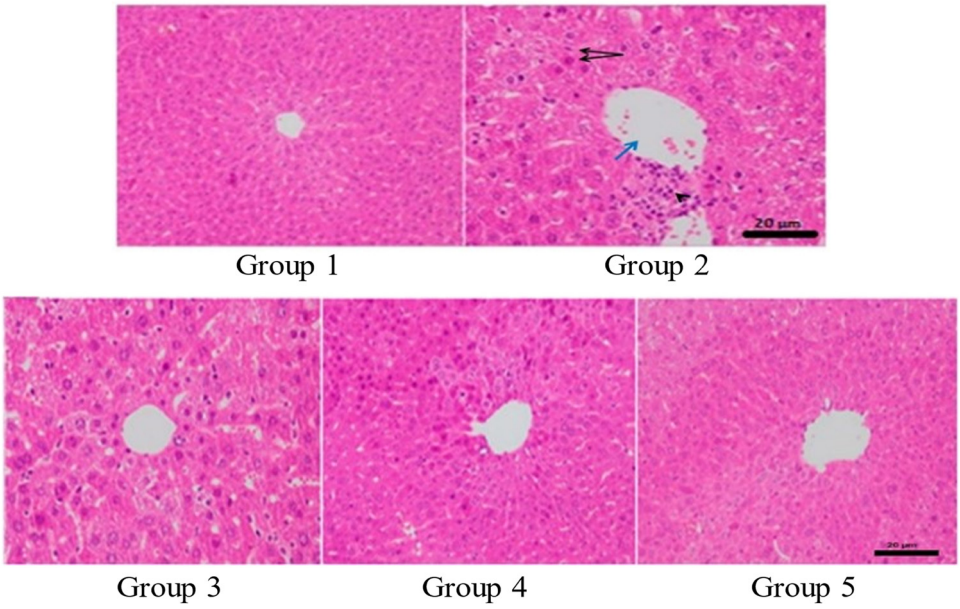

Figure 3: Morphological observation of hepatic tissues in all groups (Group 1 X10, other groups X40). Safranal attenuated APAP-induced liver damage in a dose-dependent manner, and this effect was most pronounced at a dose of 0.1 mL/kg (Group 5).

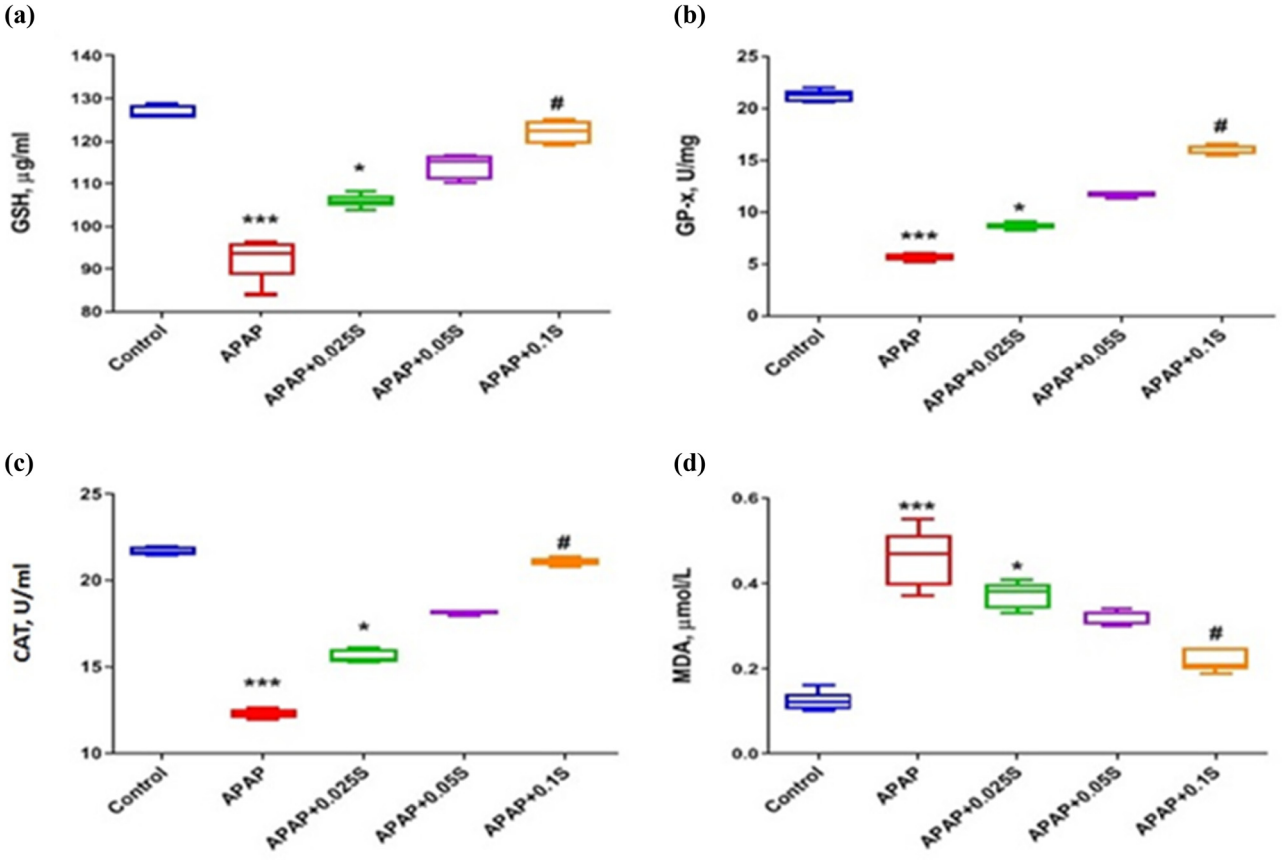

Figure 4: Impact of APAP treatment on blood serum levels of (GSH, Panel a), GP-x, (Panel b), CAT, (Panel c), and MDA, (Panel d) in rats was assessed. The data are depicted as the mean values along with the standard error. Statistical analysis was conducted using the Kruskal–Wallis test followed by Dunn's multiple comparisons tests. Significance levels are indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the control group; #$p < 0.05$ compared to the APAP group.

Table 3: Serum oxidant–antioxidant parameters

	Control (Group 1)	APAP (Group 2)	APAP + 0.025S (Group 3)	APAP + 0.05S (Group 4)	APAP + 0.1S (Group 5)
GSH (μg/mL)	126.71 ± 0.74	92.57 ± 2.23***	105.89 ± 0.71*	114.17 ± 1.33	122.1 ± 1.22#
Gp-x (U/mg)	21.2 ± 0.26	5.62 ± 0.16***	8.66 ± 0.15*	11.71 ± 0.14	16.08 ± 0.22#
CAT (U/mL)	21.72 ± 0.1	12.31 ± 0.13***	15.67 ± 0.17*	18.15 ± 0.06	21.11 ± 0.11#
MDA (μmol/L)	0.12 ± 0.01	0.46 ± 0.03***	0.37 ± 0.01*	0.32 ± 0.01	0.22 ± 0.01#

Data are presented as mean and standard error. Data were evaluated by Kruskal–Wallis and Dunn's multiple comparisons tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control group; #$p < 0.05$ compared to APAP group.

manner and reduced MDA levels when compared with Group 2 (Table 3). It was determined that the administration of safranal at the doses of 0.05 and 0.1 mL/kg increased the levels of GSH, GP-x, and CAT, but there was no considerable difference between those and levels in the control group. Groups 4 and 5 were therefore seen to approach the levels in the control group in response to safranal administration. Also, these groups showed a significant increase compared to Group 2. In addition, MDA was found to be decreased in Groups 4 and 5 with no significant difference compared to the control group. The results indicate that safranal inhibits APAP-induced oxidative liver injury (Table 3).

3.3 Safranal reduced the inflammatory response caused by APAP hepatotoxicity

APAP-induced liver injury presents with inflammatory reactions, which subsequently exacerbate [31]. We examined the serum cytokine levels in rats after APAP-induced hepatotoxicity. Elevated levels of IL-6, TNF-α, and IL-1β were observed in the APAP group when compared to the control group. These findings are indicative of acute liver inflammation. The groups treated with safranal exhibited a decrease in cytokine levels in a dose-dependent manner, compared to the APAP group (Table 4).

There was no significant difference in IL-6, TNF-α, and IL-1β levels between the control group and Groups 4 and 5, in which safranal was administered at the doses of 0.05 and 0.1 mL/kg, respectively ($p > 0.05$). Group 5 (0.1 mL/kg), one of the safranal groups, revealed decreased cytokine levels with a significant difference compared to the APAP group exhibiting the therapeutic effect of safranal ($p < 0.05$). The anti-inflammatory effect of safranal was more significant at the dose of 0.1 mL/kg compared to the dose of 0.05 mL/kg (Figure 5).

4 Discussion

The primary cause of drug-induced liver injury, which remains a significant issue in Western medicine, particularly in industrialized nations, is the overuse of APAP [32]. The administration of *N*-acetyl cysteine (NAC) is an effective antidote for APAP toxicity, which is the major cause of ALF [33]. Although NAC is an effective drug when used as an early intervention, new drugs are needed to alleviate APAP-induced ALF in the late stages. In this study, we examined the mechanisms of liver injury induced by APAP in rats, with particular emphasis on the involvement of safranal, particularly in relation to oxidative stress and inflammation.

Overdose of APAP causes ALF and severe hepatotoxic effects, which can result in death [34]. The presence of APAP-induced hepatotoxicity was observed as significant elevations in AST, ALT, and ALP levels, which are typical biomarkers of liver function and with histopathological changes (Figures 2 and 3). The toxic metabolite NAPQI that is developed secondary to APAP hepatotoxicity and

Table 4: Serum cytokine parameters

	Control (Group 1)	APAP (Group 2)	APAP + 0.025S (Group 3)	APAP + 0.05S (Group 4)	APAP + 0.1S (Group 5)
IL-6 (pg/mL)	39.51 ± 1.12	64.67 ± 4.35**	51.4 ± 1.63	44.47 ± 1.39	42.21 ± 2.47#
TNF-α (pg/mL)	276.17 ± 4.9	404.79 ± 6.97***	336.01 ± 6.5*	316.26 ± 4.46	285.82 ± 8.37#
IL-1β (pg/mL)	170.94 ± 1.33	1696.24 ± 7.99***	1300.56 ± 13.92*	984.96 ± 9.17	762.47 ± 12.84#

Data are presented as the mean value along with the standard error. Data were evaluated by Kruskal–Wallis and Dunn's multiple comparisons tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control group; #$p < 0.05$ compared to APAP group.

is capable of binding to macromolecules disrupts the structure and function of the hepatocyte membrane leading to the release of AST, ALT, and ALP into the plasma [35]. Elevated AST and ALT levels are critical indexes for assessing the severity of hepatocyte injury [36]. Elevated AST and ALT levels and APAP-induced histological changes in liver tissue in APAP-induced ALF in BALB/c mice supporting the presence of the hepatotoxic effect similar to this present study have been reported in many studies [37].

In this study, AST, ALT, and ALP levels were found to be elevated upon the administration of APAP, and the best therapeutic effect following safranal administration was observed in Groups 4 and 5. The pretreatment of safranal probably exhibits a dose-dependent hepatoprotective effect reducing AST, ALT, and ALP levels significantly and protects the hepatocytes against injury. This effect was also supported by the histopathological findings in the liver in the groups treated with safranal. As shown in Figure 2, in rats in the APAP group, the structure of the hepatocytes was heterogeneous, and the central vein was irregular and fuzzy; the structure of the cells was mostly disrupted, and a large number of inflammatory cells was present. It is noted that safranal used in the treatment of APAP-induced liver injury in rats attenuates the structural damage in liver tissues in a dose-dependent manner and improves histological changes in the hepatocytes (Figure 3).

It was reported that lipid peroxidation was increased, whereas the effect of antioxidant defense mechanisms was decreased in APAP-induced ALF. Oxidative stress plays a significant function in APAP toxicity and the excess NAPQI formed due to the overdose triggers the formation of the superoxide radical (O2–) and hydrogen peroxide (H_2O_2). In addition, NAPQI causes thiol oxidation, which is one of the underlying causes of liver toxicity. GSH is a critical antioxidant in the excretion of NAPQI, ROS, and peroxide arising from hepatotoxicity [38]. The NRF2/ARE (nuclear factor erythroid 2-related factor 2/antioxidant response element) pathway has been recognized as a protective mechanism against liver injury and plays a pivotal role in mitigating hepatotoxicity induced by toxic substances. Activation of the NRF2/ARE signaling pathway has been documented to shield animals from liver damage resulting from APAP and various other hepatotoxic agents [39]. Significant changes in the GSH cycle may direct the NRF2/ARE pathway. A plausible explanation for the prevention of NAPQI-induced depletion of GSH is the promotion of GSH biosynthesis.

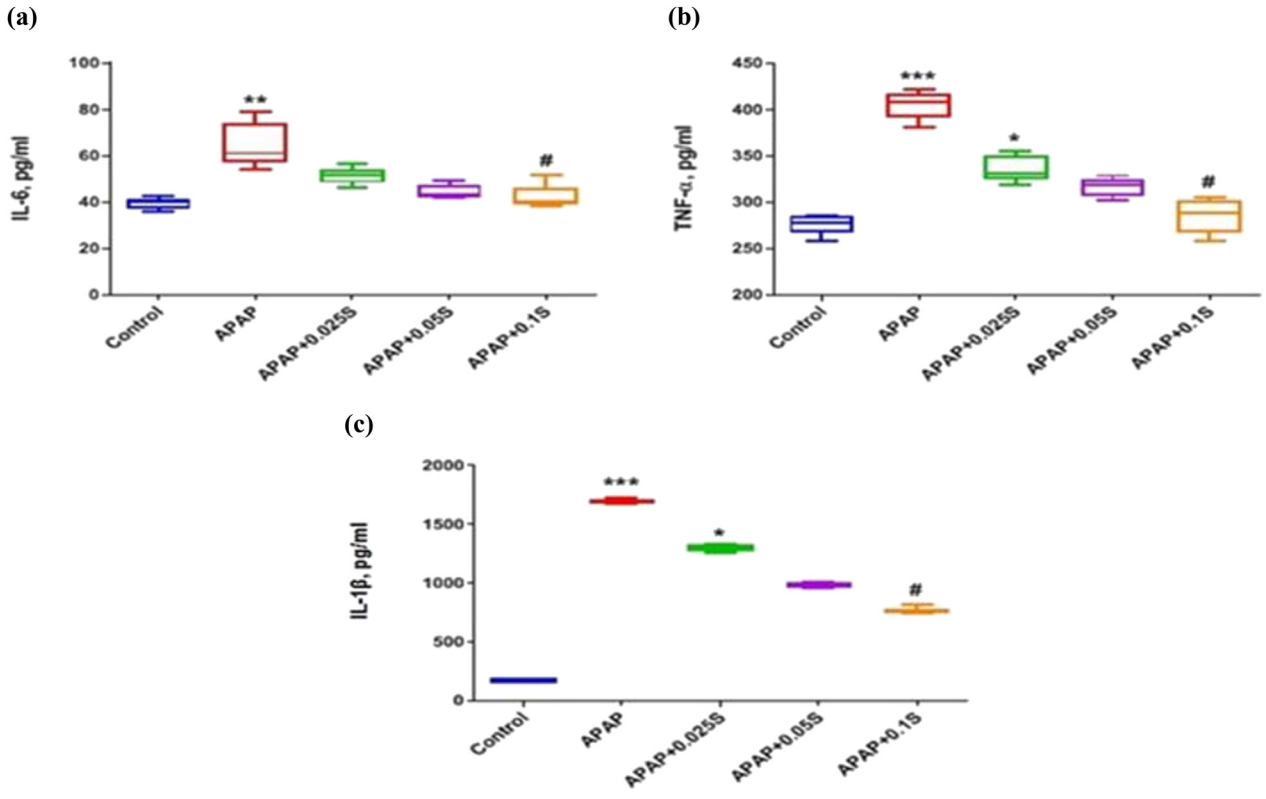

Figure 5: Effect of APAP treatment on blood serum levels of IL-6 (Panel a), TNF-α (Panel b), and IL-1β (Panel c) in rats. Data are presented as mean and standard error. Data were evaluated by Kruskal–Wallis and Dunn's multiple comparisons tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control group; #$p < 0.05$ compared to APAP group.

NRF2-targeted genes activate rate-limiting enzymes in GSH synthesis [40].

In this research, the introduction of safranal led to a concurrent rise in GSH levels. This phenomenon might elucidate the mechanism by which co-treatment with safranal and APAP can elevate GSH levels, as well as how safranal on its own can induce an increase in GSH. These findings provide supporting evidence that the upregulation of GCLC and GSR by safranal through the NRF2/ARE pathway results in an enhanced synthesis of GSH. At the same time, it is known that decreased GSH levels after APAP-induced hepatotoxicity lead to a decrease in the activity of the GP-x enzyme [41]. MDA, a product of lipid peroxidation, is the major indicator of liver damage [42]. Measuring the levels of GSH, GP-x, SOD, CAT, and other antioxidants that constitute the first-line antioxidant defense system in mammals is useful in determining the oxidative stress caused by liver injury.

There are many studies in which the levels of SOD, CAT, and GSH-Px were found to be significantly decreased, whereas the MDA content was increased sharply as a result of liver injury [42–44]. In this study, the serum MDA levels increased following the APAP administration, and liver antioxidant capacity, which was characterized by the resulting oxidative stress, was found to be decreased along with the decreased levels of GSH, GP-x, and CAT (Table 3). The antioxidant defense system in the organisms is constantly renewed in response to oxidative stress and is often activated to protect cellular redox homeostasis. MDA levels in Groups 4 and 5, which were pre-treated with safranal, were found to be significantly decreased and the values were lowered down to the normal range, similar to those of the control group. Similarly, GSH, GP-x, and CAT levels in the group treated with safranal were found to be increased, similar to those of the control group. A study conducted by Farahmand et al. demonstrated that safranal improved the activity of antioxidative enzymes, inhibited lipid peroxidation, and reduced nitric oxide formation in the livers of aging male rats [45]. An additional study proposed that safranal significantly ameliorated the elevated levels of MDA, restored reduced GSH levels, and enhanced the activity of antioxidant enzymes in response to oxidative stress. Furthermore, this compound was found to have potential effectiveness against depressive-like symptoms induced by chronic stress, possibly by modulating the oxidative response in the brain [46]. It was also stated that safranal reduced the level of increased lipid peroxidation under oxidative stress in the old male rat brain and improved the decreased levels of GSH, SOD, and GST [47].

In our study, it is suggested that the therapeutic effect of safranal increases the antioxidant defenses in a dose-dependent manner, providing protection against oxidative stress and decreasing lipid peroxidation. There was no considerable difference between the control group and the results of Groups 4 and 5 in which safranal was administered ($p > 0.05$).

Hepatic macrophages, cytokines, chemokines, and ROS play a significant function in the pathogenesis of drug-induced liver injury. nuclear factor-kappa B (NF-κB) is a conserved family of transcription factors that remain inactive in the cytoplasm of various cell types. When activated, NF-κB is translocated to the nucleus, where it plays a pivotal role in processes involving inflammation, immunity, and apoptosis It is known that ROS-mediated inflammation plays a crucial role in the pathogenesis of APAP hepatotoxicity [48]. Overproduction of free radical species activates NF-κB at the source of inflammation, and consequently, proinflammatory gene expression such as TNF-α is induced and results in an increase in cytokine levels [49]. Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are the primary drivers of the acute phase response [50]. The levels of TNF-α and IL-1β in the circulation are significant hepatotrophic factors in rats with liver injury [51]. No study was encountered in the literature on saffron and cytokine levels in APAP-induced hepatic injury; however, there was a study conducted on curcumin also known as turmeric or Indian saffron, reporting a reduction in the increased expression activity of TNF-α, IL-1β, and IL-6 after PCM-induced liver injury and regulation of the inflammatory response by curcumin [52].

In this study, it was observed that safranal had a positive impact on the levels of pro-inflammatory cytokines in the context of APAP-induced liver injury. As seen in Table 4, inflammatory reactions occurred and were exacerbated after APAP-induced liver injury; however, they were found to be lowered down to a normal range, similar to those of the controls, in Groups 4 and 5, following safranal administration ($p > 0.05$). We suggest that NF-κB, the major mechanism for modulation of the inflammatory response, is suppressed with safranal. NF-κB plays a critical role in regulating the transcription of various inducible inflammatory mediators. In this study, safranal improved and modulated the APAP-induced changes in gene expression of cytokines by NF-κB inhibition, which suppressed the release of pro-inflammatory cytokines inclusive of TNF α, IL-6, and IL-1β.

In our study, the presence of injury was supported and proven in the light of the literature by the elevated liver enzymes and MDA levels, which can be used as a marker of APAP-induced hepatocyte damage, increased oxidation, deterioration of antioxidant defense mechanisms, and disrupted histopathological findings caused by tissue damage as well as inflammatory response. Reduced antioxidant activity of the liver and an augmented inflammatory response were recorded in the APAP group, and the antioxidant enzymes and cytokine

levels were restored following the administration of safranal. Histopathological findings suggest that APAP reduces the expression of antioxidant genes and increases the expression of acute-phase proteins inclusive of TNF α, IL-6, and IL-1β. Safranal administration improved APAP-induced changes in antioxidant and inflammatory cytokines.

5 Conclusion

This study demonstrates that safranal pre-treatment offers protection against APAP-induced hepatotoxicity by mitigating oxidative stress, histopathologic damage, and the inflammatory response. Also, safranal holds promise as a viable therapeutic agent for addressing APAP hepatotoxicity. Therefore, we believe this study's results will shed light on possible new treatment protocols and provide suggestions for individuals at risk for acute hepatotoxicity.

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