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Original Research Article

Ameliorative Effect of FerulaAsafoetida on Formaldehyde-Induced Liver Damage in Male Rats: Biochemical, Histological, and Immunohistochemical Perspectives of Ki-67 Expression

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ABSTRACT

Background: Formaldehyde is a common environment and industrial toxicant that is known to cause oxidative stress-mediated hepatotoxicity. Antioxidant and cytoprotective properties of natural compounds like Ferula asafoetida have been explored to alleviate such toxic effects. This study sought to compare the hepatoprotective and anti-proliferative properties of Ferula asafoetida on formaldehyde-induced liver damage in male albino rats, using biochemical, histological, and immunohistochemical methods.

Materials and Methods: Thirty six adult male albino rats were randomly categorized into six groups (n = 6). Animals were given oral formaldehyde (10 mg/kg) and/or Ferula asafoetida extract (25 mg/kg) over a period of 42 days, after which they were allowed a 42 days recovery period in respective groups. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in serum at Days 15, 29, 43, and 85. The weight of the liver was measured at the conclusion of the experiment period. Hematoxylin and eosin staining were used to evaluate histopathological and Ki-67 immunohistochemistry was used to evaluate cellular proliferation. One-way ANOVA with Tukey post hoc test were used to conduct statistical analysis.

Results: Formaldehyde exposure led to a substantial increase in the levels of AST, ALT, and ALP suggestive of progressive hepatocellular and hepatobiliary damage. Histological examination showed that the inflammation, fibrosis and hepatocellular apoptosis were significantly increased with a rise in Ki-67 expression, which indicated an improvement in cell proliferation. A partial response was on the co-administration of Ferula asafoetida to inhibit biochemical changes, histopathological injury and decrease Ki-67. The differences in liver weights of recovery groups were not statistically significant. Although there was some improvement, after the exposure was stopped, hepatic architecture and function could not fully recover.

Conclusion: Ferula asafoetida shows moderate hepatoprotective and anti-proliferative effects against liver toxicity induced by formaldehyde, but does not completely reverse hepatic homeostasis.

KEYWORDS: Formaldehyde,Ferula asafoetida, hepatotoxicity, Ki-67, immunohistochemistry, liver weight.

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INTRODUCTION

Formaldehyde is a very common chemical in industries, environment, and biomedical industries, such as its applications in disinfection, textile processing and preservation of biological specimens. Formaldehyde is a strong toxicant due to its high reactive property that can cause profound cellular and molecular damages. The long-term or chronic exposure has been linked to systemic toxicity especially in metabolically active organs like liver. Formaldehyde is particularly toxic to the liver, which is the main location of xenobiotic metabolism and detoxification. Formaldehyde exposure is reported to cause hepatocellular degeneration, inflammatory infiltration and alterations to normal hepatic architecture which eventually causes liver dysfunction [1].

Oxidative stress and inflammation are the main mediators of the pathogenesis of the hepatotoxicity caused by formaldehyde. Exposure to formaldehyde stimulates the overproduction of reactive oxygen species (ROS), which cause lipid peroxidation, protein peroxidation as well as DNA damage. This oxidative imbalance causes cellular homeostasis and triggers inflammatory and apoptotic signaling pathways. Hepatocellular injury is, therefore, indicated by high serum biomarkers of hepatic dysfunction, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) which is sensitive markers of hepatic dysfunction [2,3]. Continued exposure also leads to hepatobiliary dysfunction and morphological changes of the liver. Other experimental models such as cyclophosphamide- or acetaminophen-induced hepatotoxicity have also been reported to have similar mechanisms of liver injury mediated by oxidative stress [4,5].

Histopathological analysis offers important information on structural changes of hepatic injury. The toxicity of formaldehyde is marked with periportal inflammation, fibrosis, hepatocellular degeneration, and apoptosis, causing the derailment of the normal hepatic structure [1]. These morphological alterations are a supplement to the biochemical results and are the direct evidence of the tissue damage. Besides structural changes, organ weight analysis, especially liver weight, is a significant dose of toxicological effect, which indicates underlying cellular hypertrophy, degeneration or tissue remodeling after exposure to the chemicals. Immunohistochemistry Immunohistochemical markers (e.g. Ki-67, a nuclear protein expressed during active cell cycle phases) can be used to measure cellular proliferation in response to injury and regeneration. Ki-67 expression is increased with an increased turnover in cells and typically serves as an indicator of regenerative and pathological proliferative responses to toxic injury [5]. Thus, the examination of histopathological alterations and proliferative activity at the same time enables a more comprehensive insight into the processes of hepatic injury and repair.

Natural compounds with antioxidant and anti-inflammatory effects have been of interest in recent years as potential therapeutic agents to counteract the toxicity caused by chemicals. These molecules have been known to react with free radicals, regulate inflammatory mechanisms, and reestablish cellular homeostasis. Ferula asafoetida, oleo-gum-resin of the genus Ferula, is one of them with bioactive constituents like ferulic acid, coumarins, and sulfur-containing sesquiterpenes, which are involved in the pharmacological action. It has been shown that Ferula asafoetida is an important antioxidant, anti-inflammatory and hepatoprotective agent [1]. On the same note, other natural compounds like ginger extract, baicalin, berberine and Zataria multiflora have demonstrated protective properties in experimental models of hepatic injury by alleviating oxidative stress, pro-inflammatory cytokine generation, and improving biochemical and histological indices [6–9].

The dysfunction of mitochondria and the impairment of cell cycle control are strongly associated with impaired hepatic regeneration after toxic injury. There is evidence that oxidative stress, activation of DNA damage response, and changes in cell cycle checkpoints play a role in the impaired proliferation of hepatocytes and slowed tissue recovery [5,10]. As such, it is necessary to assess both the

structural damage and proliferative activity to comprehend the degree of damage and recovery in the hepatic injury.

Despite the hepatoprotective effects of Ferula asafoetida being described in previous studies, its effect in regulating formaldehyde induced hepatic damage especially in cellular proliferation and regeneration as measured by the expression of Ki-67 has not been well studied. In addition, there is a dearth of research that combines biochemical, histopathological, organ weight, and immunohistochemical parameters into one experimental study. Thus, the aim of the current study was to test the hepatoprotective and anti-proliferative properties of Ferula asafoetida on a rat model of formaldehyde-induced toxicity. This was done with a combined methodology of using biochemical markers of liver functioning (AST, ALT, ALP), liver weight, histopathological examination of liver architecture and immunohistochemical staining of Ki-67 protein expression. The results of this paper should give a detailed insight into the protective effect of Ferula asafoetida in relation to chemical-induced hepatic injury.

MATERIALS AND METHODS

Sample Collection and Experimental Design.

Experimental animals were used in the study to create six groups (G1-G6) in control, treatment, and recovery conditions. At certain intervals (Days 15, 29, 43, and 85), animals were anaesthetised, and blood samples were collected from the retroorbital venous plexus and analysed biochemically. After sacrifice, liver tissues were thoroughly removed and washed in ice-cold normal saline to eliminate extraneous blood, followed by handling through histological and immunohistochemical analyses.

Experimental Animals

The current study involved the use of thirty-six healthy adult male albino rats with the weight of 200 -220 g. The animals were acquired at an approved laboratory animal facility and kept in clean polypropylene cagesunder ideal laboratory conditions, such as temperature of 22 + or -2 C, relative humidity of 50-60 and 12 hour light/dark cycle. The basic pellet feed and water were given to the animals at ad libitum during the experiment. The researchers maintained all animals in acclimatization period of one week before the study started, where they monitored the health condition, behavior, and signs of stress in the animals daily. All laboratory experiments were in line with institutional policies regarding laboratory animal research.

Grouping and Experimental Design.

The animals were then randomly grouped into six (n=6) groups after acclimatization. The sample used was decided upon on the basis of past experimental studies and considerations. No formal a priori power calculations were done. This model is widely used in animal studies and is informed by ethical principles of reduction to minimize the use of animals but yield scientific validity [11,12]. Group 1 (G1) was used as the negative control and distilled water was applied to them over 42 days.. Group 2 (G2) was used as a positive control and was exposed to formaldehyde at 10 mg/kg body weight/day during 42-days consecutive exposure. Group 3 (G3) was administered Ferula asafoetida extract at a rate of 25 mg/kg of body weight per day, 42 days in a row. Group 4 (G4) was treated with Ferula asafoetida (25 mg / kg body weight per day) and formaldehyde (10 mg / kg body weight per day) after 42 days. All treatments were done in a single day through oral gavage using the right vehicle. The dosing volumes were altered based on the body weight so as to deliver the respective treatments accurately. To test the recovery after exposure, group 5 (G5) was used as negative control recovery group, where distilled water was applied to the group after 42 days, and the untreated period was also 42 days to test recovery. Group 6 (G6) was the formaldehyde recovery group and was administered formaldehyde (10 mg/kg body weight per day) over 42 days, and then a 42 day untreated recovery period to determine spontaneous recovery following the termination of exposure. Notably, no Ferulaasafetida therapy was followed in these groups in their recovery phase. At the end of

the treatment period (day 42), animals in Groups 1 to 4 were humanely euthanized, but animals in Groups 5 and 6 were humanely euthanized at the end of the recovery period (day 84) to be compared.

Extract Preparation

The oleo-gum resin in Ferula asafoetida was extracted by use of Soxhlet extraction. About 25 g of the sample was placed in the Soxhlet apparatus and it was extracted within a period of 10 h using the right solvent. A rotary evaporator was used to concentrate the extract at 50 o C to ensure that all the solvents were removed off the sample. The researchers prepared a 100 mg/mL stock solution by reconstituting the concentrated extract and keeping it in a refrigerator until required. Our formulations were made on a daily basis and administered to the patients.

Standardisation and Composition of Sample.

Ferula asafoetida used in the current study was acquired in the form of commercially available oleo-gum-resin (hing) in crystalline form. A qualified botanist was used to authenticate the sample to guarantee its botanical identity. Three large fractions in asafoetida oleo-gum-resin have been known, the largest of which is resin (40-64 percent), followed by gum (about 25 percent), and volatile essential oil (10-17 percent). The ferulic acid and its derivatives, especially the esters, the coumarins, and the sesquiterpene derivatives are mainly contained in the resin fraction and are polysaccharides, i.e., glucose, galactose, rhamnose and glucuronic acid are commonly found in the gum fraction. This volatile fraction contains a lot of sulfurcompounds and monoterpenes that cause its typical smell. No high-level chromatographic standardization (HPLC or GC-MS profiling) was carried out in the current study. Nonetheless, extraction was done under controlled conditions and used 70% ethanol through Soxhlet extraction in order to be consistent. The absence of standardization of compounds is recognized as a weakness of the study.

Formaldehyde Formulation Preparation

Formaldehyde dilution A 37 percent w/v solution of formaldehyde in a commercially available product was diluted in distilled water to 2 mg/mL. In short, the stock solution was combined with 185 mL of distilled water, with a final volume depending on the daily dosing needs but with the same concentration. The formulation was not kept but prepared afresh every day and well mixed before the administration. The dosing schedule was 10 mg/kg body weight/day of formaldehyde (dose volume was 5 mL/kg). This dosage of 10 mg/kg body weight was chosen by considering the earlier experimental research showing that this dosage could cause predictable reproductive and testicular toxicity, including oxidative stress and histopathological changes, without causing excessive death rates [13].

Vehicle Used for Dosing

Making a 0.5% carboxymethyl cellulose sodium (Na-CMC) solution involved dissolving 5g of Na-CMC in distilled water to a final volume of 1L. Na-CMC was chosen as the oral delivery system by the researchers due to the water-insolubility of the test compound in preliminary formulation studies and complete suspension in Na-CMC.

Preparation and Dose Administration Preparation Formulation Preparation and Dose Administration.

The appropriate amount of Ferula asafoetida extract was weighed properly using an analytical balance and triturated in a mortar with a suitable vehicle to a fine suspension. The vehicle was added in small portions to make sure that it had been completely dispersed, and then the preparation was transferred to a measuring cylinder. The last volume was diluted to achieve a concentration of 2.5 mg/mL, which is equivalent to the dose of 25 mg/kg of body weight at a dosing volume of 10 mL/kg. The dose of Ferula asafoetida was 25 mg/kg body weight, which was chosen according to the published literature that showed the safety and pharmacological effects of Ferula asafoetida and especially its antioxidant and cytoprotective effects in experimental models [14,15]. The dose of 25mg/kg was chosen because literature reports suggested that Ferula asafoetida is safe at a higher dose. Each of the assigned treatments

was given everyday over 42 days as per the different group allocation. Animals in Groups 1 (G1), 2 (G2), 3 (G3) and 4 (G4) were humanely euthanized after the treatment period to evaluate them, but animals in Groups 5 (G5) and 6 (G6) were not treated further after 42 days as part of the evaluation to assess spontaneous recovery following discontinuation of formaldehyde exposure. Notably, Groups 5 and 6 did not get Ferula asafoetida on the recovery phase and thus, were not treatment-induced reversal groups but untreated recovery controls.

Biochemical Analysis and Liver Weight.

Blood samples collected were left to clot at room temperature and were then centrifuged at 3000 rpm within 10 minutes to get serum. The serum was separated and the serum harvested very carefully and kept at -20 C awaiting further biochemical examination. Asptate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) serum levels were estimated through a standard enzyme colorimetric technique with commercially prepared diagnostic enzyme kit according to the manufacturer instructions. A semi-automated biochemical analyzer was used to measure the enzyme activities in units per liter (U / L). During the sacrifice, liver tissues were removed, blotted thoroughly to remove any surplus blood and weighed with a calibrated digital analytical balance. The liver weight was measured in grams and served as another parameter to determine the changes in hepatic status due to the treatment. All biochemical parameters and liver weight changes were analyzed at specific experimental intervals (Days 15, 29, 43, and 85) to measure the development of the changes in the liver caused by the exposure of the liver to formaldehyde and the possible protective action of Ferula asafoetida.

Histological Staining and Hematoxylin and Eosin.

To maintain tissue architecture, liver tissues were immobilized by fixing in 10% neutral buffered formalin at least 24-48 hours. After fixation, tissues were dehydrated using graduated series of alcohol then cleared with xylene followed by embedding in paraffin wax. Blocks (paraffin-embedded) were cut at 4-5 µm thickness with a rotary microtome. These sections were placed on fresh glass slides and were stained with the stains of hematoxylin and eosin (H&E) through the routine staining methods. Stained parts were observed under light microscope to assess the histomorphological changes and representative photomicrographs were taken to document them.

Histopathological Analysis and Semi-Quantitative Scoring.

To assess the hepatic architecture, inflammatory changes, fibrosis, hepatocellular degeneration, and apoptosis, the histological evaluation of liver sections was carried out. The extent of histopathological changes was determined by a semi-qualitative score system in terms of severity. The parameters were rated out of 0 to 3 where 0 meant that there was no lesion, 1 meant slight changes, 2 meant moderate changes, and 3 meant severe changes. Inflammation, fibrosis and apoptosis were scored separately. The scoring was done in various fields within each section to achieve consistency and reliability of observations.

Ki-67 Expression Immunohistochemical Analysis.

Immunohistochemistry was carried out to assess the proliferative activity of cells using Ki-67 as a marker. Liver sections (4–5 µm thick, paraffin embedded) were placed on the poly-L-lysine-coated slides. Section deparaffinization was followed by rehydration with alcohol graded series. The heat-induced conditions were performed in citrate buffer (pH 6.0) to obtain antigen retrieval. The activity of endogenous peroxidase was inhibited by incubation with 3 percent hydrogen peroxide. Then each section was incubated with primary anti-Ki-67 antibody (Anti Ki 67 Antibody AB9260, Sigma Aldrich, Chemicon) at optimized concentration as recommended by the manufacturer. After incubation with primary antibody, sections were incubated with a suitable secondary antibody conjugated with horseradish peroxidase (HRP) detection system.The antigen-antibody reaction was then visualized with the help of 3,3-diaminobenzidine (DAB) as a chromogen,

which formed a brown precipitate at the locations of Ki-67 expression. The sections were counterstained with hematoxylin, and then dried with graded alcohols, cleared in xylene, and then mounted in a cover slip.

Ki-67 Immunoreactivity and Scoring.

The expression of ki-67 was determined using a light microscope with brown nuclear and/or cytoplasmic staining with negative cells painted blue with the help of hematoxylin. These were assessed as hepatocytes, sinuoidal endothelial cells, Kupffer cells, inflammatory cells, cholangiocytes and vascular endothelial cells. Semi-quantitative scoring was done on the percentage of positively stained cells per high-power field, with a 0 value (no staining) to 4 value (>75% positive cells).

Statistical Analysis

Mean and standard deviation (SD) were used to express all data. One-way analysis of variance (ANOVA) was used to ascertain the difference between groups, and subsequently ,the Tukey post hoc test was used to make multiple comparisons. A p-value below 0.05 was taken to be significant. The magnitude of treatment effects was determined with the use of the effect size (η 2). Statistical work was performed with the help of the SPSS software (Version 27.00).

RESULTS

Liver Weight

At the termination of the experiment (24 weeks) analysis of liver weight (Groups 5 and 6) showed that the control Group 5 (recovery) had a mean liver weight of 11.62 ± 0.39 g, but Group 6 (formaldehyde recovery) had a mean weight of 10.68 ± 1.64 g (Figure 1A). Though Group 5 had higher average liver weight, Group 6 had more variation amongst animals. One-way ANOVA statistical analysis of the groups showed that there was no significant change between the groups ($F = 1.8706$, $p = 0.2014$) and therefore, the change was not statistically significant. The effect size obtained was calculated to have a moderate effect (η 2=0.16) which implied that it was possible that about 16 percent of the difference in the liver weight was due to the effect of groups.

The post hoc analysis also confirmed that there was no statistical significance between the Groups 5 and 6 since the mean difference (0.9434) had a non-significant p-value and confidence intervals covering the mean of 0.00 (Figure 1A).

Aspartate Aminotransferase (AST)

Serum AST was measured to assess the hepatocellular injury at various time points (Days 15, 29, 43 and 85) (Figure 1B). At Day 15, the levels of formaldehyde were slightly higher in the formaldehyde-treated group (G2) and the combination group (G4) compared to the control group (G1), but these were not statistically significant ($F(3,20) = 3.045$, $p = 0.052$), indicating that there was little early hepatic injury. By Day 29, AST levels increased significantly among groups ($F(3,20) = 16.58$, $p < 0.001$). Group 2 showed much more AST than Groups 1 and 3, which is a confirmation of the hepatotoxicity caused by formaldehyde. Group 4 also exhibited high levels of AST in comparison with controls but there was no significant difference in Groups 2 and 4 and this implies that there is limited protective efficacy at this stage. The peak of AST levels was observed at Day 43, and the difference between intergroups was extremely significant ($F(3,20) = 23.33$, $p < 0.001$). Group 2 was still considerably high in comparison to Groups 1 and 3, and Group 4 also exhibited higher levels in comparison with controls, once again suggesting the incomplete hepatoprotection. In the recovery phase (Day 85), AST levels were still highly elevated in the formaldehyde recovery group (G6) as compared to the recovery control group (G5) ($F(1,10) = 19.62$, $p = 0.001$). These results show long-term hepatocellular injury even after exposure is stopped and that Ferula asafoetida only partially alleviates the injury (Figure 1B).

Alanine Aminotransferase (ALT)

Another sensitive indicator of hepatocellular damage was serum ALT levels, which had a significant change across groups (Figure 1C). By Day 15, ALT levels were significantly high ($F(3,20) = 10.88$, $p < 0.001$), and the Groups 2 and 4 were higher than controls, which was due to early hepatic injury. On Day 29, the ALT levels also rose considerably more

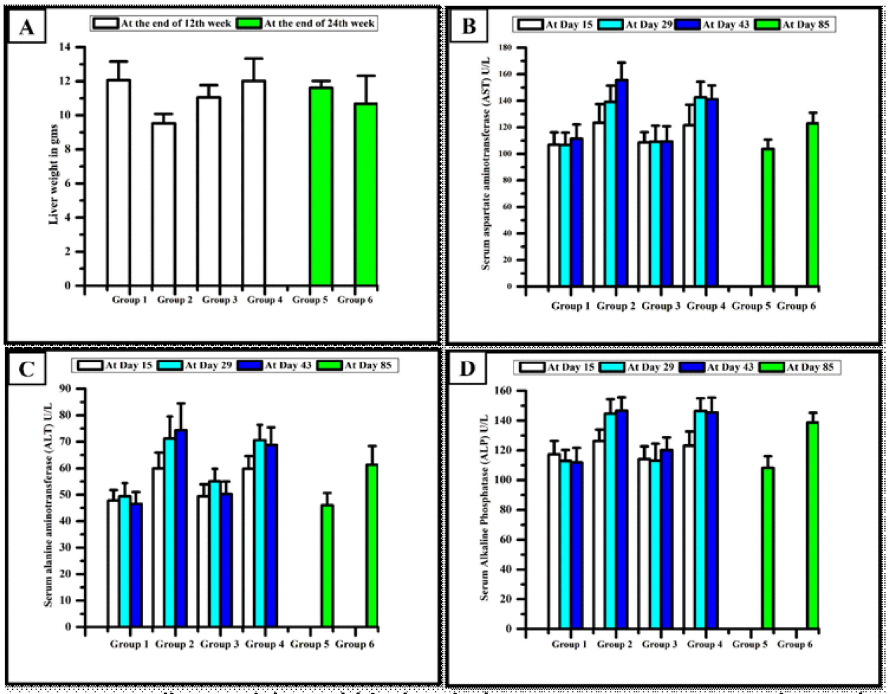

Fig. 1:Effect of treatments on liver weight and biochemical parameters across experimental groups. (A) Liver weight (g) measured at the end of the experimental period (12th and 24th weeks). (B) Serum aspartate aminotransferase (AST) levels. (C) Serum alanine aminotransferase (ALT) levels. (D) Serum alkaline phosphatase (ALP) levels. Measurements in panels (B–D) were recorded at Days 15, 29, 43, and 85 (recovery phase). Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.

($F(3,20) = 19.38$, $p < 0.001$) with Groups 2 and 4 still being significantly higher compared to Groups 1 and 3. There was no important difference in Groups 2 and 4 which indicated low protection at this point. The maximum of ALT levels was reached on Day 43 ($F(3,20) = 23.62$, $p < 0.001$) with the highest values being observed in Group 2 compared to all other groups. Group 4, though marginally less, did not significantly differ with Group 2 suggesting that hepatotoxicity was not attenuated to a significant extent. The ALT levels were still significantly higher in Group 6 than Group 5 in the recovery phase (Day 85) ($F(1,10) = 19.69$, $p = 0.001$) indicating continued hepatocellular injury and lack of full recovery after the exposure of formaldehyde (Figure 1C).

Alkaline Phosphatase (ALP)

ALP in serum was evaluated to determine hepatobiliary involvement (Figure 1D). At Day 15, there were no foundable differences between groups ($F(3,20) = 2.37$, $p = 0.101$), which means that there is no early biliary dysfunction. But, on Day 29, ALP levels were much higher ($F(3,20) = 24.04$, $p = 0.001$) withGroups 2 and 4 having higher values than Groups 1 and 3. This continued until Day 43 when ALP levels were still high ($F(3,20) = 21.25$, $p < 0.001$) indicating progressive hepatobiliary injury. On Day 85, the ALP level in Group 6 was significantly greater than that in Group 5 ($F(1,10) = 53.48$, $p < 0.001$) reflecting ongoing hepatobiliary dysfunction despite formaldehyde exposure being stopped (Figure 1D).

The biochemical data subsequently indicates that exposure to formaldehyde causes progressive hepatocellular and hepatobiliary damage, as was indicated by the significant increases in levels of AST, ALT and ALP over time (Figure 1B–D). The trend is that there is early hepatocellular injury and subsequent hepatobiliary injury. Ferula asafoetida had some protective effects, but failed to fully inhibit biochemical changes. Additionally, the continual presence of high levels of enzymes in the recovery phase suggests that hepatic functioning is not totally restored after toxic exposure.

Histopathological Results and the Quantitative Analysis.

Hepatological observations of liver sections, stained with hematoxylin and eosin (H&E) had shown that the experimental groups had specific morphological changes (Figure 2).

Semi-quantitative scoring of inflammation, fibrosis and apoptosis, and statistical analysis further corroborated these qualitative findings.Group 1 (Negative control) had a normal hepatic architecture with properly laid hepatocytes in cords extending around the central vein (Figure 2A–D). There was normal cytoplasmic and nuclear morphology of hepatocytes in portal, periportal, and centrilobular areas. There were no signs of inflammation, degeneration, fibrosis or apoptosis and the presence of sinusoids and portal triads.

Group 2 (Formaldehyde-treated) showed significant histopathological changes (Figure 2E–H) with multifocal periportal and peribiliary inflammation with intense inflammatory cell infiltration. There was also increased growth of prominent fibrous connective tissue and hepatocellular degeneration, which resulted in the derailment of normal hepatic architecture, which is a sign of severe hepatic injury. Group 3 (treated with ferula asafoetida) showed normal histological appearance when compared to the control group (Figure 2I–L). The hepatocytes were intact and their architecture was preserved, and no notable pathological changes were seen, which validated the safety of the extract.

Group 4 (Ferula asafoetida + Formaldehyde) showed multifocal hepatocellular apoptosis that was scattered across the liver parenchyma, and inflammatory cells infiltrated it (Figure 2M–P). They also had mild periportal and peribiliary fibrosis. Nevertheless, foci of hepatocyte preservation and partial hepatic architecture renewal could also be observed, which is indicative of a protective influence on formaldehyde-induced toxicity.Group 5 (Recovery control) evidence of mild to moderate periportal and peribiliary inflammation, including inflammatory cell infiltration and fibrous connective tissue proliferation (Figure 2Q–T) indicated partial recovery.Group 6 (Formaldehyde recovery) had significant hepatic architecture restoration (Figure 2U-X). In portal, periportal, and centrilobular areas, hepatocyte appearance was mostly normal, and there were few signs of inflammation, indicating some spontaneous recovery after the exposure was discontinued.

Quantitative Histopathological Scoring

These were objectively observed and scored semi-quantitatively as the changes in the histology against the inflammation, fibrosis and apoptosis (Table 1). The extent of hepatic injuries and healing was reflected in high deviation of the score of inflammation in the groups. The significance of the difference between the groups was observed to be very high ($F(5,30) = 48.62$, $p = 0.001$) and high effects size ($f=1.27$) and the total variance explained by effects of treatment were found to be 89 percent. The Tukey posthoc test showed that the percentage rate of inflammation in Group 2 was very high compared to Group 1 and Group 3 ($p < 0.001$). The Group 4 was significantly lower than Group 2 ($p = 0.004$) which was protective. The response in Group 5 ($p = 0.021$ relative to G2) was somewhat improved, but much higher than control ($p < 0.05$). The recovery of Group 6 was much worse with a comparatively low score than Group 2 ($p = 0.002$) and not significantly different than control ($p = 0.118$).

Treatment and recovery scores increased and decreased respectively because of being exposed to formaldehyde and treatment respectively. One-way ANOVA demonstrated a significant difference among groups ($F(5,30) = 36.45$, $p < 0.001$), with a large effect size ($f = 1.10$) and $ç^2 = 0.86$. The post hoc ($p < 0.001$) was applied to Group 2 which revealed that Group 2 already had a significantly higher fibrosis than Group 1 and Group 3. Such an antifibrotic reaction was promoted by a significant reduction in Group 4 compared to Group 2 ($p = 0.006$). Moderate fibrosis ($p = 0.031$ vs G2), significant improvement ($p = 0.008$ vs G2) and no significant difference ($p = 0.094$) against control were found in Group 5, Group 6, and control respectively.

Apoptosis scores also differed significantly among groups ($F(5,30) = 29.18$, $p < 0.001$), with a large effect size ($f = 0.98$) and $\eta^2 = 0.83$. Apoptotic rates were considerably higher in Group 2 compared to Group 1 and Group 3 ($p < 0.001$). Group 4 reduced compared to Group 2, though not significantly different ($p = 0.072$) that provided some partial protection. Group

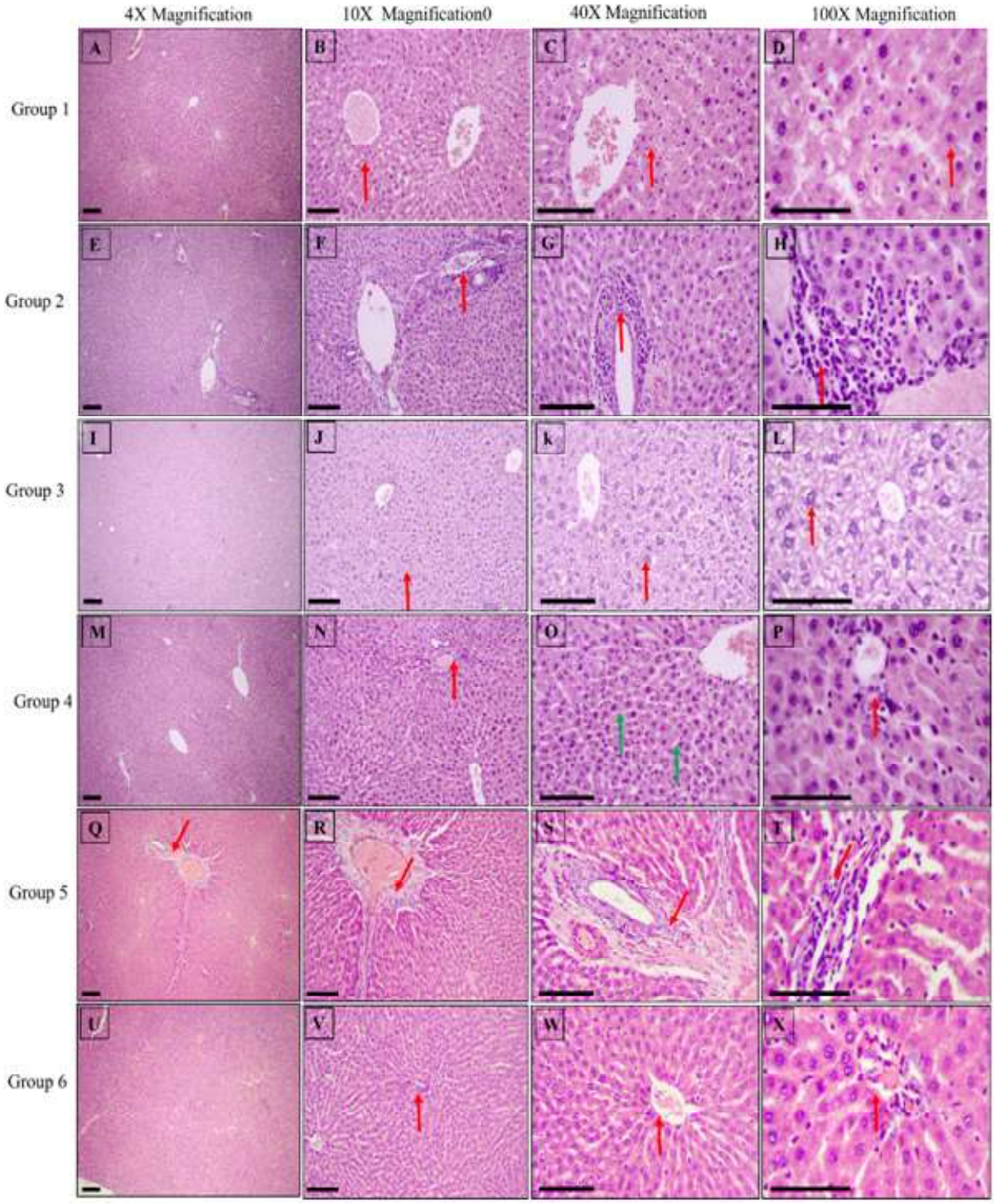

Fig. 2: Typical hematoxylin and eosin (H&E) histopathological sections of liver tissue in the control, treatment and recovery groups indicate morphological changes at different conditions of the experiment. Group 1 (G1, Negative control): (A -D) Healthy hepatic architecture with intact hepatocytes in portal, periportal and centrilobular regions. No histopathological abnormalities (red arrows). Group 2 (G2, Formaldehyde-treated): (E-H) Multifocal periportal and peribiliary inflammatory changes with massive inflammatory cell and fibrous connective tissue hyperplasia (red arrows). Group 3 (G3, Ferula asafoetida treated): (I–L) The hepatocytes have a normal morphology containing intact hepatic architecture in portal, periportal and centrolobular regions; no abnormalities were seen (red arrows). Group 4 (G4, Ferula asafoetida + Formaldehyde): Multifocal hepatocellular apoptosis scattered in liver parenchyma, and associated with inflammatory cell infiltration (red arrows). It had mild peribiliary fibrosis/ connective tissue proliferation. Areas of preserved hepatocytes that imply some protection are shown (green arrows). Group 5 (G5, Recovery control): (Q-T) Pale to moderate periportal/peribiliary inflammation with inflammatory cell inflammation and the growth of fibrous connective tissue (red arrows). Group 6 (G6, Formaldehyde recovery): (U-X) Recovery of hepatic architecture with hepatocytes observed to be nearly normal in portal, periportal and centrolobular regions and no significant pathological changes (red arrows). Red arrows (inflammation, apoptosis, and fibrosis) and green arrows (preserved or regenerating hepatocytes) do indicate pathological or regenerative changes in the hepatocytes. A, E, I, M, Q and U scale bars = 200 µm; B, F, J, N, R and V scale bars = 100 µm; C, G, K, O, S and W scale bars = 50 µm; D, H, L, P, T and X scale bars = 8 µm.

Table 1: Semi-Quantitative Histopathological Scores of Liver Tissue Across Experimental Groups.

Group	Description	Inflammation (G) Mean ± SD	Fibrosis (F) Mean ± SD	Apoptosis (A) Mean ± SD
G1	Negative Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
G2	Formaldehyde	3.00 ± 0.00***	2.50 ± 0.50***	2.00 ± 0.00***
G3	Ferula asafoetida	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
G4	FA + Formaldehyde	2.00 ± 0.50††	1.00 ± 0.00††	2.00 ± 0.50
G5	Recovery Control	1.50 ± 0.50†	1.50 ± 0.50†	1.00 ± 0.00†
G6	Formaldehyde Recovery	0.50 ± 0.50††	0.50 ± 0.50††	0.50 0.50††

Data expressed as Mean ± SD, Statistical test: One-way ANOVA followed by Tukey's post hoc test, *p < 0.001 vs G1 (control), †p < 0.05 vs G2 (formaldehyde group), ††p < 0.01 vs G2 (formaldehyde group)

5 had mild apoptosis and considerable decrease (p = 0.041 vs G2) and Group 6 had near normal levels (p = 0.009 vs G2; p = 0.221 vs G1).

The qualitative and quantitative data presented in Figure X enable concluding that the most severe type of hepatic damage caused by the exposure to formaldehyde i.e.the rise in the inflammations, fibrosis and apoptosis. Fertula asafoetida, too, has a high level of hepatoprotective effect particularly in the subsiding inflammation and fibrosis. The withdrawal in exposure results in the partial reversal of the hepatic damage and recovery groups also show the near normal recovery.

Immunohistochemistry and Semi-Quantitative Expression of Ki-67.

The Ki-67 immunohistochemical staining of liver tissues revealed that the activity of cellular proliferation in each of the experimental groups had definite differences (Figure 3A–X). Ki-67 positivity with the counter stain species being blue with hematoxylin and negative cells being blue with hematoxylin was defined as brown to dark brown nuclear and / or cytoplasmic stain. This term was quantified in hepatocytes, sinuoidal endothelial and Kupffer cells, inflammatory cells in periportal and centrilobular zones, cholangiocytes in the bile ducts and vascular endothelial cells.

A section of the liver of the negative control group (Group 1; Figure 3A -D) was found to have a normal structure of the liver architecture and no Ki-67 was observed in all the cell types studied. The infrequent or absent positive stained cells in each of the high-power fields were observed, which was related to a score of 0 and indicated the occurrence of low basal proliferative activity. On the other hand, in the formaldehyde-treated group (Group 2; Figure 3E -H), there was a great degree of Ki-67 immunoreactivity with strong expression in sinuoidal endothelial and Kupffer cells and moderate expression in hepatocytes and portal structures. This proportion of stained cells ranged at 5075 percent per high power field having a score of 3 which is a severe proliferative activity of formaldehyde-induced hepatic injury.

Group 3 to which Ferula asafoetida was added (Figure 3I-L) was under control and showed no apparent hepatic architecture to control group but Ki-67 was evidently expressed in no cell component. All these combine to make a score of 0 and confirm the fact that the extract does not stimulate proliferation. The combined treatment group (Group 4; Figure 3M P) showed mild Ki-67 staining, predominately in sinuoidal endothelial and Kupffer cells and small amounts in hepatocytes and portalareas. The proportion of positive cells was never greater than 25 percent per high-power field, which corresponds to a score of 1, i.e. the activity of proliferation was significantly reduced with respect to the formaldehyde-treated group, which is indicative of the protective effect of Ferula asafoetida.The liver sections of the recovery control group (Group 5; Figure 3Q-T) were normal in the

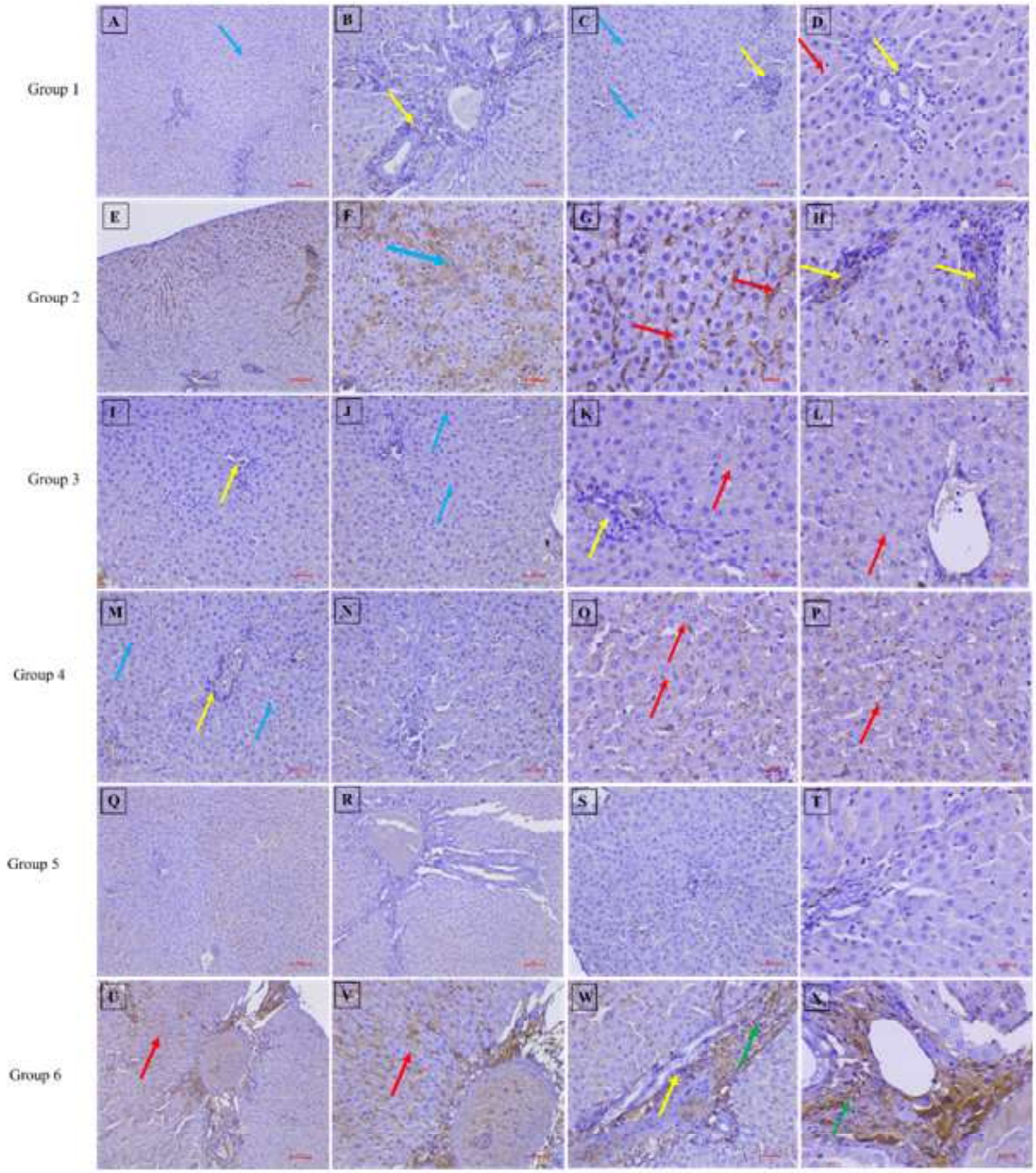

Fig. 3: Ki-67 immunohistochemical staining of liver tissue of study groups (A-X). (A1-D) Group 1: There is no Ki-67 present in hepatocytes (blue arrows), sinuoidal cells (red arrows) and portal structures (yellow arrows). Score: 0. With a good Ki-67 sinuoidal cell (red arrows), inflammatory cell (green arrows), moderate hepatocyte (blue arrows) and portal (yellow arrows) expression. Score: 3. (I-L) Group 3: No regions show any expression of Ki-67. Score: 0. (M-P) Group 4: Mild Ki-67 in cells of sinusoids, and minimal hepatocytic involvement. Score: 1. (QT) Group 5: No Ki-67 (such as control) expression. Score: 0. Group 6 (U6X): Perpetual high Ki-67 in sinuous and periporta regions, intermediate hepatocyte staining. Score: 3. arrow key: Red - Sinuoidal endothelial/Kupffer cells, Blue - Hepatocytes, Yellow - Cholangiocytes/portal endothelial cells, Green - Inflammatory/connective tissue cells. Scale bars: A, E, Q, R, U = 100 µm; B, C, F, I, J, M, N, S, V, W = 50 µm; D, G, H, K, L, O, P, T, X = 20 µm.

histoarchitecture, and lacking of Ki-67 expression, which is 0 and, thus, a steady cellular turnover during the recovery process. On the other hand, the formaldehyde recovery group (Group 6; Figure 3U–X) remained immunoreactive to Ki-67 particularly in sinuoidal endothelial and Kupffer cell and periportal inflammatory or connective tissue components and moderate hepatocyte staining. The proportion of positive cells was 50-75 per high power field that was a score of 3, which indicates the presence of persistent proliferative activity and an incomplete recovery following the termination of formaldehyde exposure.

The semi-quantitative analysis of Ki-67 expression (Figure 4) also supported these findings. The mean Ki-67 scores were 0.00 ± 0.00 in

Group 1, 2.83 ± 0.41 in Group 2, 0.00 ± 0.00 in Group 3, 1.17 ± 0.41 in Group 4, 0.17 ± 0.41 in Group 5, and 2.67 ± 0.52 in Group 6. The one-way analysis of variance was significant with a value of F (5,30) = 49.76, p = 0.001 and the effect size is very large (0.89). The Tukeyposthoc analysis revealed that the expression of Ki-67 was significantly high in Group 2 as compared to Groups 1 and 3 ($p < 0.001$). The significant reduction of Group 4 over Group 2 ($p = 0.002$) indicates that Ferula asafoetida possesses anti-proliferative properties. Group 5 did not differ significantly with the control group ($p > 0.05$) but Group 6 was significantly high compared to those of Group 1, 3 and 5 ($p < 0.001$), which suggested that the proliferative changes were persistent even when the exposure to formaldehyde was stopped. Overall, these findings suggest that formaldehyde exposure results in considerable proliferation of hepatic cells, and Ferula asafoetida exposure has a considerable inhibitory impact. Although recovery was partially observed when the exposure had been stopped, the fact that Ki-67 was still expressed in the formaldehyde recovery group illustrates that the hepatic cellular homeostasis had not completely been restored.

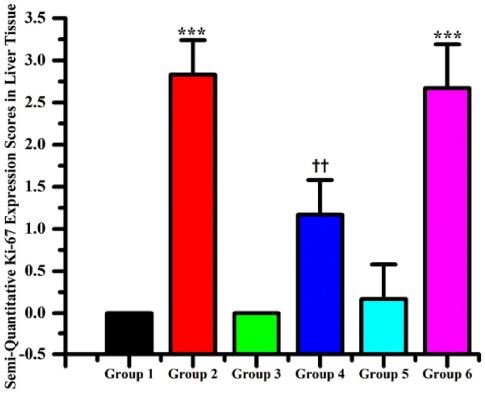

Fig. 4: Bar graph showing Ki-67 expression across experimental groups. Data are presented as mean ± SD. ***p < 0.001 vs G1; ††p < 0.01 vs G2.

DISCUSSION

The present research provides comprehensive details about the hepatic effects of formaldehyde exposure which has a progressive progression of hepatocellular and hepatobiliary injury with time, which is partially reversed by Ferula asafoetida administration. The study offers a multidimensional outlook of toxicant-induced liver damage and limitations of the therapeutic intervention by combining liver weight, serum biochemical alterations, histopathological alterations, and Ki-67 immunoexpression. The weight of the liver at the end of the study indicated a slight nonsignificant reduction in the formaldehyde recovery group versus the controls and with greater inter-individual variability. The findings suggest that formaldehyde does not produce gross morphological changes (such as hepatomegaly or atrophy) but causes more subtle and heterogeneous changes in liver mass. This observation is consistent with the previous observations that indicate that liver weight is insensitive to indicate hepatic injury and may fail to reflect underlying biochemical or cellular injury [1]. Therefore, the absence of great variations in the liver weight should not be interpreted as absence of toxicity.

The temporal pattern of serum enzymes demonstrates the presence of progressive hepatocellular injury. The modest and negligible early increases in AST were progressive to extremely high increases in AST and ALT at Days 29 and 43, showing a progressive hepatocyte damage following long-term exposure to formaldehyde. The peaks of enzymes on the Day 43 are linked to the peak of hepatocellular disruption that is likely to be caused by oxidative stress and lipid peroxidation and inflammatory responses [1]. Interestingly, that the levels of AST and ALT are both elevated during the recovery period suggests that hepatocells integrity is not completely recovered. This would suggest that formaldehyde provokes chronic cell damage, which could be attributed to mitochondrial dysfunction and chronic oxidative stress. Similar trends of ongoing apoptotic and inflammatory processes with enzyme rise despite the loss of the toxic insult were also reported in other models of chemical-induced liver injury [16,17].

Serum ALP, in contrast to hepatocellular enzymes, rose slowly but steadily and was significantly elevated at later times and remained elevated throughout recovery phase. The implication of this trend is that hepatobiliary dysfunction follows the initial

hepatocellular injury. The persistence of the high ALP means that there is a persistent cholestatic or biliary inflammation, which may be due to inflammation or structural destruction of the bile ducts. These findings are in accordance with the established toxicological paradigms in which secondary hepatobiliary damage is progressive to chronic harm of hepatocells[18]. These differences in the timing of AST, ALT and ALP alterations underline the importance of combination of the biochemical markers in full diagnosis of liver damage.

The co-administration of Ferula asafoetida resulted to some degree of attenuation of enzyme elevation though hepatoprotection was not as high as observed with the absence of significant differences between formaldehyde only and formaldehyde combination groups at different time points. Ferula asafoetida, despite the reported antioxidant and anti-inflammatory effects of the herb due to the presence of bioactive compounds, including ferulic acid and coumarins, did not fully protect in the present study [1,19]. Such factors as dose, the length of treatment and the degree of formaldehyde-induced oxidative stress that may be greater than the antioxidative capacity of the extract used can explain this decreased efficacy. The same has been demonstrated by other phytotherapeutic agents and they are also apt to provide partial, rather than complete, protection against toxic insults [19,20].

The histopathological findings also corroborated the biochemical data as severe hepatic injury was evident in the formaldehyde-treated group and was accompanied by periportal inflammation, extensive inflammatory infiltration, fibrosis, and hepatocellular apoptosis. These alterations of structure are evidence of the oxidative stress activation of inflammatory and fibrogenic processes, which is consistent with the processes in liver injury by toxicants [21,22]. Nevertheless, Ferula asafoetida alone in the other group resulted in a normal hepatic architecture, which demonstrated the safety of the latter. Co-treatment resulted in incomplete recovery, reduction of fibrosis and inflammation and persistence of apoptotic changes, which demonstrate that Ferula asafoetida reduces but does not prevent structural liver injury. During the recovery period, hepatic architecture would be partially recovered, but with a remaining hepatic inflammatory and fibrosis, spontaneous hepatic fibrosis repair would not be exhaustive over the period, as is anticipated of slow regression of hepatic fibrosis [22].

Further details of hepatic cellular processes were obtained by immunohistochemical analysis of Ki-67 expression. The high increase of Ki-67 expression in the formaldehyde treated group implies that there is a strong proliferative reaction which is composed of hepatocytes and non-parenchymal cells and most likely it is an adaptation to repair cellular damage. However, an increase in proliferation in this manner can also indicate continuous destruction and constant regenerative pressure in lieu of complete recovery [23,24]. The co-treatment of ferula asafoetida significantly decreased the levels of Ki-67, suggesting that the proliferation caused by injury was alleviated by the antioxidant and anti-inflammatory properties of ferula asafoetida[1,21]. The expression of Ki-67 in the recovery group is however persistent and this indicates that hepatic homeostasis is not completely restored and that regenerative activity is still going on. The expression of Ki-67 in Ferula asafoetida-only group as well is not observed, which once again confirms the fact that the extract does not induce abnormal cellular growth.

Overall, the findings of the current research paper describe a clear cascade of formaldehyde-induced hepatotoxicity, which begins with initial hepatocellular injury, progresses with hepatobiliary dysfunction, structural injury, and is not fully resolved even upon exposure ceasement. Ferula asafoetida partially has hepatoprotective properties that involve the reduction of oxidative stress and inflammation, but does not fully restore normal liver functions to normal in the conditions of this study. In conclusion, formaldehyde is an agent that leads to severe and chronic hepatic injury, but Ferula asafoetida is partially protective. The

recovery evidence shown so far suggests that there is need to further streamline the therapies and to investigate the possibilities of combination therapies, mechanisms of action, and long-term consequences of hepatic regeneration and functional recovery after toxic injury.

CONCLUSION

The current research shows that formaldehyde exposure causes severe hepaticdamage, as is observed by the high levels of serum biochemicals, the striking histopathologic changes and the increased proliferation of cells. An increase in the levels of AST, ALT, and ALP shows that hepatocytes and biliary structures are heavily damaged. Severe hepatic injury, which is manifested by inflammation, fibrosis, and apoptosis, is further confirmed by histological examination. Ferula asafoetida administration has a protective effect by reducing the severity of biochemical imbalances and the intensity of histopathological alterations, especially inflammation and fibrosis but full recovery does not occur. Immunostaining shows that exposure to formaldehyde increases the proliferation of cells, as evidenced by the higher expression of Ki-67, whereas Ferula asafoetida decreases the proliferation of the cells, implying that it has a role in regulating the abnormal cell growth. Although there is some progress, the fact that some of the biochemical and immunohistochemical markers remain changed demonstrates that normal hepatic functions have not yet been fully restored. All in all, the results indicate that Ferula asafoetida can provide a certain level of hepatoprotection against formaldehyde-induced toxicity, though the effects of long-term exposure to formaldehyde are irreversible tissue damage.

Ethics Approval: The experimental protocol involving animals was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) (TBPL/FART/009/24) on 17/08/2024. and conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All procedures were performed in compliance with national and institutional ethical standards for animal experimentation.

Availability of Data and Materials: The datasets generated and analyzed in the current study are available from the corresponding author upon reasonable request.

Conflicts of Interests: None

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Author Contributions

T.S.P., K.S., Y.D.P., and S.S. conceived and planned the experiments. T.S.P., K.S., and Y.D.P. conducted the experiments. T.S.P. and K.S. performed the histomorphometric analysis and statistical evaluation. T.S.P., Y.D.P., and S.S. contributed to sample collection, tissue processing, and histopathological preparation. T.S.P., K.S., Y.D.P., and S.S. contributed to the interpretation of the results. T.S.P. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and final manuscript.

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