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Therapeutic potential of resveratrol, a polyphenol in the prevention of liver injury induced by diethylnitrosamine (DEN) through the regulation of inflammation and oxidative stress

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ABSTRACT

Background: Resveratrol (RES) is an essential polyphenolic compound found in various foods, including vegetables, fruits, and chocolate. Its antioxidants and anti-inflammatory potential have attracted significant interest in the management of pathogenesis, including the treatment of hepatocellular carcinoma.

Objectives: This study was designed to measure RES hepatoprotective potential against Diethylnitrosamine (DEN)-induced liver injury.

Methods: The hepatoprotective potential of RES (20 mg/kg b.w) was evaluated by measuring the liver function enzymes, oxidative stress, and inflammation against DEN (200 mg/kg i.p)-induced liver injury. The liver tissue architecture and fibrosis were determined using hematoxylin and eosin, Masson trichrome, and Sirius Red staining. Moreover, the expression pattern of Cyclooxygense-2 (Cox-2) protein was evaluated by immunohistochemistry staining.

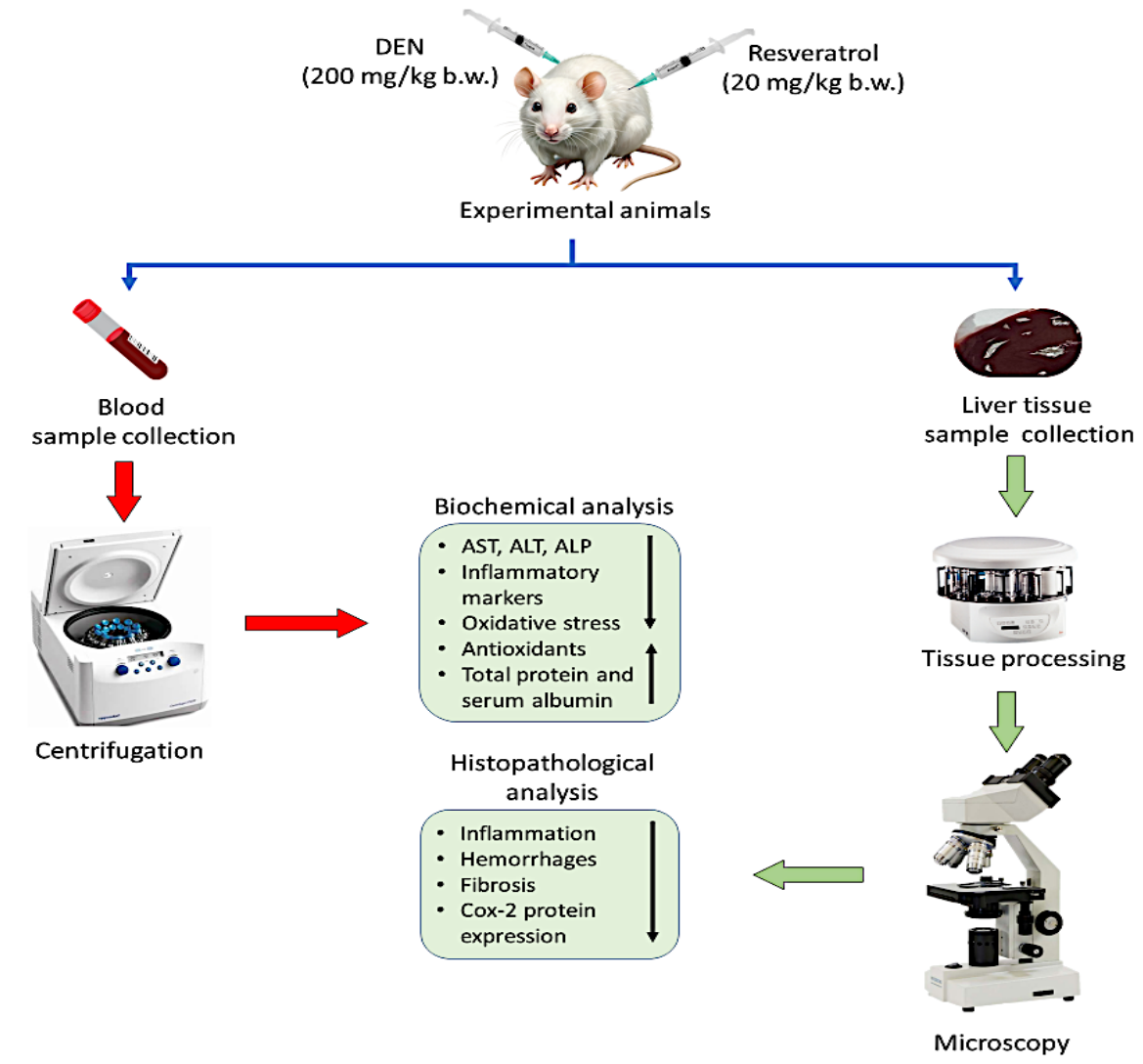
Results: Seven weeks of RES treatment after exposure to toxic DEN led to a significant reduction in liver function enzymes (AST, ALT, and ALP) and lipid peroxidation compared to rats treated by DEN only. Further, results showed that RES treatment reduced the pro-inflammatory cytokines (TNF-α, CRP, IL-1β, and IL-6) and increased the antioxidant enzyme

(Catalase, SOD, GST, and GPx) levels compared to rats treated by DEN only. The DEN-induced group of rats showed various liver tissue alterations, increased fibrosis, and high Cox-2 protein expression. However, the group treated with RES showed hepatoprotective potential with reduced liver tissue alterations and fibrosis. Moreover, RES treatment decreased the Cox-2 protein expression compared to rats treated by DEN only, suggesting its anti-inflammatory potential.

Conclusion: This study revealed that RES treatment reduced DEN-induced hepatic injury and liver cancer by protecting liver cells by reducing oxidative stress, inflammation, and liver function enzymes. Therefore, the results indicate that RES could be a possible therapeutic approach for liver diseases, including drug and alcohol-related problems.

Keywords: Resveratrol, Hepatoprotective, Oxidative stress, Inflammation and cancer, Alcohol-related problems, Fibrosis

Graphical Abstract: Therapeutic potential of resveratrol, a polyphenol in the prevention of liver injury induced by diethylnitrosamine (DEN) through the regulation of inflammation and oxidative stress



INTRODUCTION

The liver is a chief organ of the human body, it is involved in nutrient metabolism, growth signaling control, the breakdown of drugs and toxins, and the metabolism of proteins and amino acids [1]. It is an important organ for metabolizing nutrients, drugs, and xenobiotics chiefly by a family of enzymes called cytochrome P-450 [2]. Nitrosamines are highly toxic chemical compounds recognized as potent human and animal carcinogens [3]. N-nitroso alkyl compounds, such as diethylnitrosamine (DEN), can initiate various types and stages of malignancies in different organs and are commonly used as inducer chemicals to promote cancer in experimental animals, most frequently in rats [4]. The mechanism of action of DEN involves its potential to form adducts. After certain CYP450 enzymes bioactivate it, it transforms into a potent alkylating agent that forms an adduct in the DNA, resulting in a direct carcinogenic effect [5].

Current treatment approaches for liver diseases can lead to various side effects, which further complicate the therapy of these conditions [6]. Hence, there is an urgent need to explore alternative therapeutic approaches that offer high efficacy and a safe profile for the treatment of liver diseases [6-8]. In this context, natural compounds have been recognized as promising for preventing liver pathogenesis due to their antioxidant potential [9]. For instance, olive fruit has been found to have a protective effect on the liver by increasing the levels of antioxidant enzymes, reducing inflammation, and maintaining the structure of liver cells [10]. Additionally, using thymoquinone has been shown to improve the biochemical and histopathological changes in the liver [11].

Resveratrol (RES) is a well-known polyphenolic molecule [Figure 1] in various foods such as vegetables, fruits, and chocolate. Due to its antioxidant and anti-inflammatory properties, it has gathered significant attention in chronic disease management. A study found that high glucose levels can increase the production of reactive oxygen species (ROS), malondialdehyde (MDA) levels, apoptotic cell percentage, and Bax expression while decreasing Bcl-2 expression and superoxide dismutase in cardiac microvascular endothelial cells. However, the administration of resveratrol reversed these effects [12]. Many studies have indicated that resveratrol is crucial in regulating the inflammatory response through various signaling pathways. The role of resveratrol in cancer has been reported through the modulation of different cell signaling pathways [13]. Additionally, resveratrol's role in managing childhood obesity has been reported through various mechanisms [14]. The main goal of this study was to investigate the potential liver-protective benefits of RES by examining liver injury markers, oxidative stress, and inflammation. Additionally, the study used histopathological and immunohistochemical staining to examine the structure of liver tissue and the pattern of Cox-2 protein expression.

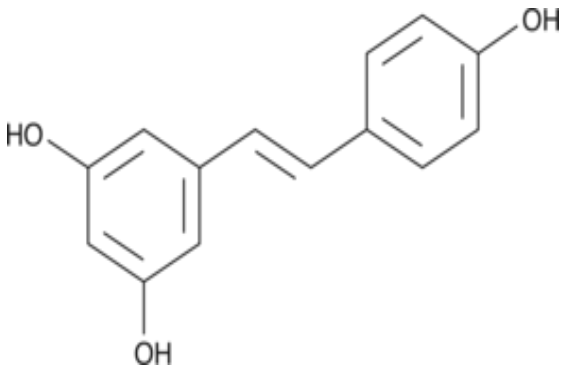


Figure 1: Chemical structure of resveratrol

MATERIALS AND METHODS

Chemicals and reagents: Catalase (Cat), Glutathione S transferase (GST), Superoxide dismutase (SOD), and Glutathione peroxidase (GPx) kits were bought from Abcam. The kits for estimating liver function enzymes were purchased from Abcam (Cambridge, UK). C-reactive protein (CRP), interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α) were obtained from Abcam. The Masson's trichrome, Sirius red stains kits, TUNEL assay and the antibody (Cox-2) were also purchased from Abcam (Cambridge, UK). The lipid peroxidation assay for the malondialdehyde (MDA) kit was also sourced from Abcam (Cambridge, UK). Diethyl nitrosamine and Resveratrol were procured from Sigma/Aldrich, USA, while all other chemicals and reagents utilized in this research were of analytical grade and were sourced from authorized suppliers in Saudi Arabia.

Selection of animals for in vivo experiments: Male albino Wistar rats weighing 180-200 g, sourced from King Saud University, Saudi Arabia, were used for this study. The rats were housed in standard conditions with free access to food and water under a 12-hour light/dark cycle at a room temperature of 25±2 °C. The study commenced in the animal house after a seven-day acclimatization period. All experimental procedures were permitted by the animal ethical guidelines of Qassim University, Saudi Arabia (Approval of the Standing Committee for Scientific Research Ethics Number: 24-04-04).

Animal grouping and experimental design: In this study, eight animals were included in each experimental group because this sample size is statistically significant for this type of research. The animals were simply randomized into four groups, and the naming of each group was done by writing the names of different groups on paper slips and were selected for each group. The treatment regimen was extended to seven weeks. Group 1 (normal control, NC) was administered normal saline as a vehicle. Group 2 (DEN treatment) served as the disease control and received a single intraperitoneal injection of 200 mg/kg of DEN on the first day of the treatment plan [15]. Group 3 (co-treatment group) (DEN +RES) received DEN and RES at a dosage of 20 mg/kg body weight (b.w.) twice weekly by oral gavage/oral treatment. Group 4 (RES treatment) rats were given only RES at 20 mg/kg b.w. [16] twice weekly by oral gavage for seven weeks.

Table 1. The plan is to induce liver injury and administer the hepatoprotective compound, given as RES, as explained in Table 1 and Figure 2.

Animal group name	Group abbreviation	Treatment strategy
Normal Control	NC	Rats have free access to standard rat chow and water and are orally given normal saline as a placebo.
Disease Control	DEN	The animal received an intraperitoneal injection of DEN (200 mg/kg b.w) [15].
Disease Control + Resveratrol	DEN+RES	The groups with liver injury were orally administered with RES at a dosage of 20 mg/kg b.w. [16].
Resveratrol only	RES	The rats were given only RES by oral gavage (20 mg/kg b.w.) [16]

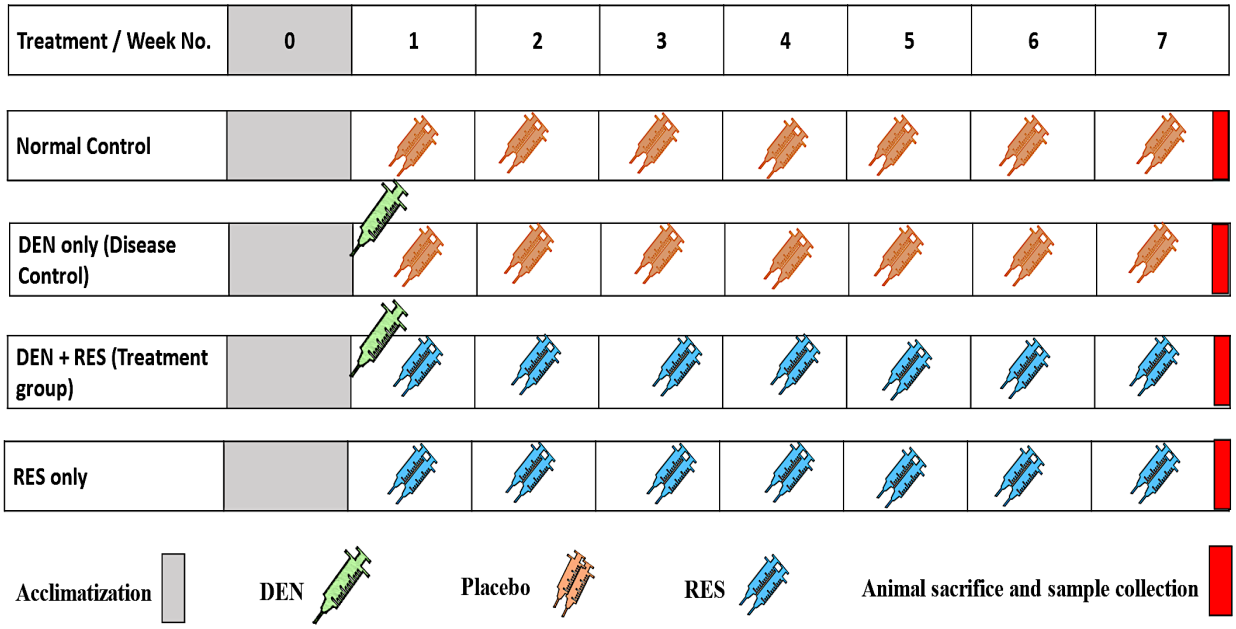


Figure 2: Treatment plan of the study

After the 7-week treatment regimen, all animals were anesthetized using chloroform, and blood and tissue samples were gathered to assess the hepatoprotective potential of RES [Figure 3]. Each experiment for biochemical studies was performed in triplicate, and the data was analyzed using proper statistical analysis.

Measurement of liver function enzymes (ALT, ALP, AST), serum albumin and total protein levels: Serum liver enzymes are routinely measured clinical markers representing different liver dysfunction dimensions [17]. Elevations in serum enzyme levels are considered a biomarker of liver toxicity. In this study, liver function enzymes were measured to evaluate the hepatoprotective potential of RES. Blood samples were taken from the animals in each experimental group. After clotting, the samples were centrifuged at 3000 rpm for 10 minutes, and the serum was separated and stored at -20°C to measure liver function enzymes. Enzyme levels were determined using kits to assess RES liver protective role against DEN-induced liver injury. Total protein and albumin levels were also evaluated accordingly.

Measurements of lipid peroxidation: Lipid peroxidation states that the oxidative degradation of lipids and quantification of lipid peroxidation are essential to determine oxidative stress. The products of lipid peroxidation chain reactions display high biological action [18]. They can damage enzymes, DNA, and protein activity, and serve as molecular signals to trigger pathways that initiate cell death [19]. In this study, we measured lipid peroxidation to assess the hepatoprotective potential of RES by its ability to reduce lipid peroxidation. The liver tissues were homogenized using cold lysis buffer. The liver homogenate was centrifuged for 10 min at 12,000 rpm. In the lipid peroxidation assay, malondialdehyde (MDA)-thiobarbituric acid (TBA) adduct was checked calorimetrically at 532 nm, and the data was interpreted as nmol of MDA equivalents/g of tissue.

Assessment of antioxidant enzymes: The enzymatic antioxidants efficiently defend against oxidative attack due to their capability to crumble ROS [20]. The potential of antioxidant enzymes (Catalase, SOD, GST, and GPx) in liver tissue/serum was measured using ELISA. The

intensity of the color in each well was measured at different absorbances according to the kit instructions.

Measurements of proinflammatory marker levels: Cellular and vascular events, the general components of the inflammatory process, are initiated by chemicals called inflammatory mediators, molecules such as chemokines, vasoactive amines, cytokines, and proteolytic cascade products [21]. Acute and chronic exposure to DEN produces numerous inflammatory mediators, which can lead to pathogenesis. In this study, proinflammatory mediators were evaluated to assess the hepatoprotective potential of RES by its ability to reduce inflammation.

Liver tissues/serum were utilized to evaluate the levels of pro-inflammatory markers (TNF-α, CRP, IL-6, and IL-1β) through ELISA-based kits obtained from Abcam, Cambridge and the findings were interpreted accordingly.

Histopathological assessment using Hematoxylin and eosin (H&E) staining: The mechanism of DEN-induced liver injury has been associated with changes in liver function enzymes, enhancement of lipid peroxidation, inflammation, and reduction of antioxidant enzymes, and all these factors lead to liver injury. For histopathological studies, the liver samples were taken from each animal from all different groups to evaluate the hepatoprotective role of RES. The tissues were taken, cleaned with phosphate-buffered saline (PBS), and then fixed for 48 hours in 10% neutral buffered formalin. An automated tissue processor processed all tissue samples and subsequently made paraffin-embedded blocks. Hematoxylin and eosin staining were performed on all tissue sections to assess the liver tissue architecture. The slides were examined under the microscope and photographed.

Evaluation of collagen fiber using Masson trichrome staining: Chronic liver disease (CLD) is a significant global health concern caused by various factors, including alcohol abuse, obesity or metabolic diseases, and viral hepatitis [22]. This study is based on a 7-week exposure to DEN. Therefore, we evaluated the fibrosis pattern in different groups of animals to assess the anti-fibrotic potential of resveratrol. A special stain (Masson's trichrome) was carried out to determine the amount of collagen fiber deposition in different groups of animals.

Measurement of fiber using Sirius red staining: Liver tissue, with a thickness of 5 µm, was taken, and fibrosis was assessed using Sirius red staining kits (Abcam, Cambridge). The slides were examined under a light microscope and photographed with a camera (Olympus, Hamburg, Germany), and the results were taken consequently.

Cox-2 protein determined using immunohistochemistry staining: Due to its critical role in both inflammatory and pathological processes, COX-2 has become a noteworthy target for therapeutic intervention [23]. Furthermore, expression of COX-2 has been detected in several liver pathologies [24]. Chronic exposure to DEN causes various types of tissue destruction. Therefore, we evaluated the liver tissue status pattern in different groups of animals to assess the liver tissue architecture maintainer potential of RES. The expression of the protein (Cox-2) was evaluated in all groups of rats using previously described procedures with some modifications [24, 25]. In summary, liver tissues were deparaffinized using xylene and various grades of alcohol. Antigen retrieval was done using the pressure cooker method. A blocking agent was then used to block unwanted sites. Next, a Cox-2 primary antibody was applied, and the tissues were incubated for one hour. Then, a secondary biotinylated antibody was added for 30 minutes and incubated with streptavidin peroxidase for 30 minutes. Diaminobenzidine (DAB) chromogen was added, and the counterstained was made using hematoxylin. Analysis of Cox-2 protein was performed by

assessing cytoplasmic positivity. Approximately 400-500 cells were counted in four to five fields for the expression pattern of inflammatory markers, and a mean percentage of positivity was obtained. Images were captured, and results were analyzed accordingly.

TUNEL assay for apoptosis determination: The apoptosis level in all liver tissue sections was evaluated using the TUNEL assay. The staining process followed the manufacturer's instructions, which included quenching, equilibration, labeling reaction, termination of labeling reaction, blocking, and development with DAB and counterstaining. After staining, nuclei were examined under a light microscope to detect DNA fragmentation. The number of TUNEL-positive nuclei was calculated, and the results were analyzed after capturing photographs.

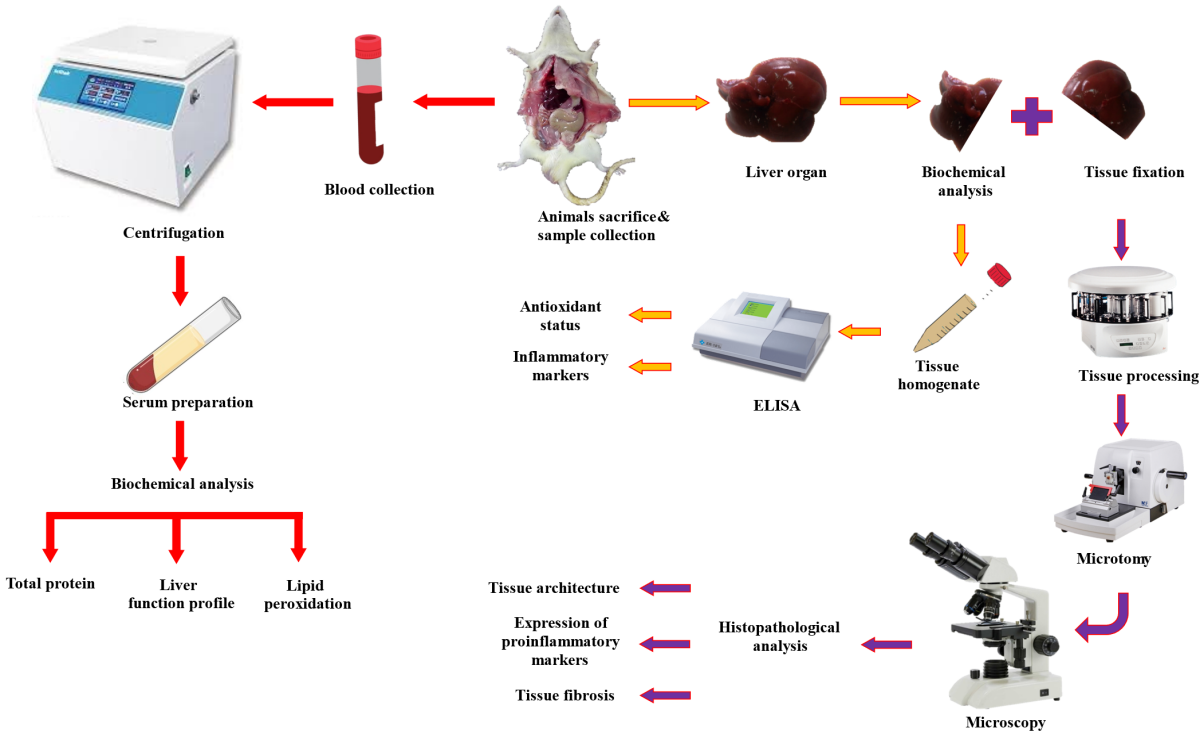


Figure 3: Sample collection and processing of blood and tissues

Molecular Docking Studies: The present study selected antioxidant enzymes (catalase and superoxide dismutase) and molecules implicated in inflammation (tyrosine kinase, TNF-α, and nitric oxide synthase) to predict a possible interaction with resveratrol. Three-dimensional structures of enzymes and other molecules were downloaded from the Protein Databank (https://www.rcsb.org/, accessed on 12 December 2023) in PDB format.

Resveratrol's three-dimensional structure was downloaded from the NCBI PubChem compounds database (https://pubchem.ncbi.nlm.nih.gov/, accessed on 13 December 2023) in 3D structure data file (pdf) format. These files were converted into Protein Databank (PDB) using the Open Babel program (https://openbabel.org/wiki/Main_Page, accessed on 13 December 2023).

The interaction between the substrate and enzyme was identified through molecular docking. Docking was carried out using AutoDockvina v.1.2.0 (https://vina.scripps.edu/, accessed on 14 to 20 December 2023) [26], and the protein was prepared in BIOVIA Discovery Studio Visualizer (https://discover.3ds.com/discovery-studio-visualizer-download/, accessed on 14 to 20 December 2023).

The workspace was cleared of all water molecules that could interact with the receptors. Catalase (1DGH)

Sphere object attributes for resveratrol are xyz = 27.128875, 50.969500, and 79.699042, with a radius of 20 for. Superoxide Dismutase (5YTU) sphere object attributes for resveratrol are xyz = -76.426000, 6.217538, and -3.453154, with a radius of 20 for. The sphere object attributes of TNF-α (2AZ5) are xyz = -19.409600, 74.650750, and 33.849550 with a radius of 20 each. The sphere object attributes of NOs (5UO1) for resveratrol are xyz = 120.067875, 249.064833, 358.515083, with a radius of 20 for each. Tyrosine Kinase (3SXR) sphere object attributes for resveratrol are xyz = 8.774909, - 5.036939, and 24.291848 with a radius of 20 for each.

Statistical Analysis: The mean ± standard deviation was calculated for all the data collected, and the differences were analyzed using a one-way analysis of variance (ANOVA) test. SPSS software was used to check the statistical analysis. Statistical significance was represented as $p < 0.05$.

Results of the study are presented in a stepwise manner as:

The potential role of RES on liver function enzymes and protein levels: The serum liver function enzymes were used as biochemical markers to evaluate the liver functions in the experimental liver injury. During the progression of liver diseases, when hepatocyte necrosis or liver cell membrane damage occurs, the serum levels of liver function enzymes increase [27]. The DEN-treated rats exhibited a substantial increase in ALP, ALT, and AST compared to the control group ($p < 0.05$). However, rats treated with 20 mg/kg b.w. of RES meaningfully reduced AST, ALP, and ALT levels when compared to the DEN-administered (Disease control) group ($p < 0.05$) [Figure 4]. This result confirms that RES can protect the liver by reducing liver function enzymes.

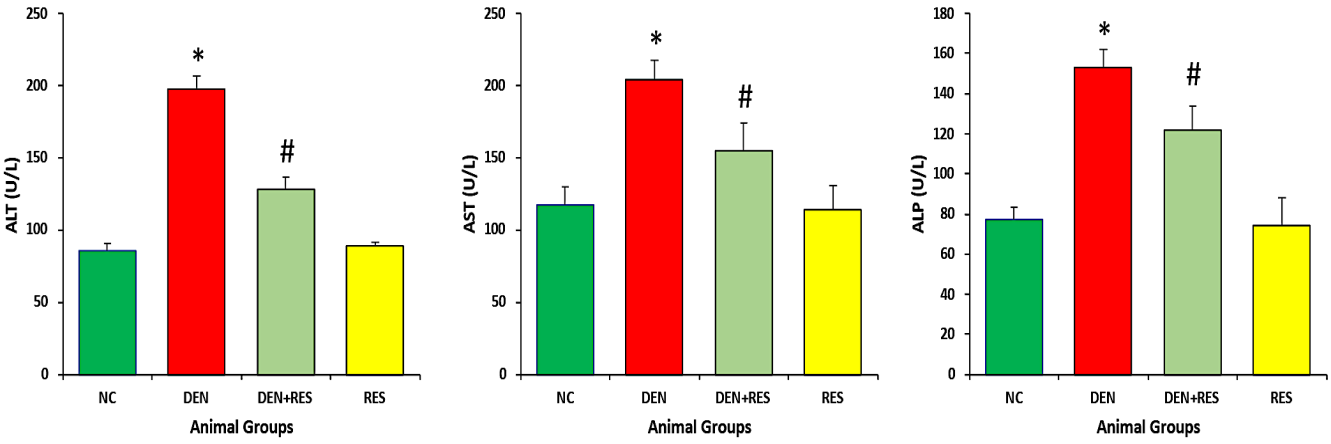


Figure 4. The liver function enzyme levels in treated and untreated animals.

The values designate the mean ± SEM, with eight rats per group. The rats treated with DEN showed significantly higher ALP, ALT, and AST levels than the control group. The DEN meaningly decreased the ALP, ALT, as well as AST compared to DEN-administered group rats. Statistical differences or changes are indicated by an asterisk (*) to denote significance at $p < 0.05$ compared to the control group and a hashtag (#) to point to $p < 0.05$ compared to the DEN-treated group.

Moreover, total protein and albumin were measured, and it was observed that decreased levels of serum total protein and albumin in DEN-treated rats in comparison to the control group ($p < 0.05$). However, the rats treated with RES 20 mg/kg b.w. significantly increase the albumin and total protein levels when compared to DEN-administered group rats ($p < 0.05$) [Figure 5].

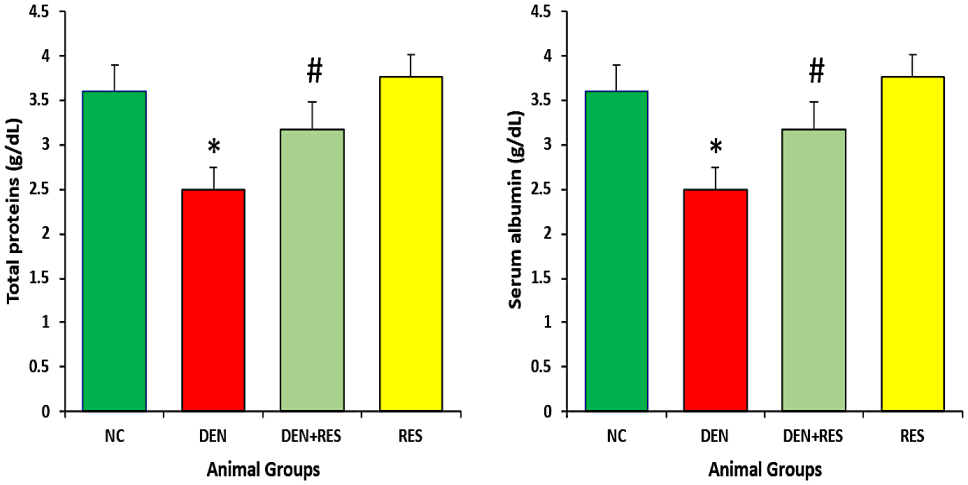


Figure 5. Assessment of total protein and albumin levels in treated and untreated animal groups.

The rats treated with DEN only (disease control) showed reduced total protein and albumin levels as compared to the control group. The RES administration increased the serum albumin and total protein levels as compared to the DEN-treated animals. The statistical variances are selected using an asterisk (*), showing significance at $p < 0.05$ in comparison with the control group, and a hashtag (#), suggesting $p < 0.05$ in contrast with the DEN-treated group.

Effect of RES on MDA in liver tissue: The effects of RES on MDA in liver tissue of different groups were measured. In DEN-induced rats, the malondialdehyde (MDA) level was significantly increased ($p < 0.05$). In comparison to the DEN-treated (disease control) group, the rats treated with RES (20 mg/kg b.w.), pointedly diminished the MDA level ($p < 0.05$) [Figure 6].

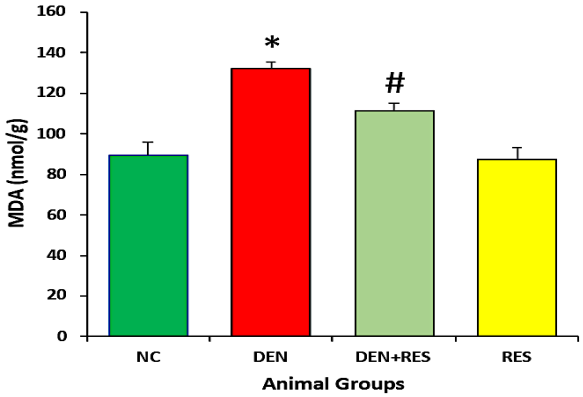


Figure 6. The measurement of MDA level in treated and untreated animal groups.

In the rats treated with DEN, MDA levels were significantly elevated, and RES treatment pointedly decreased the MDA level compared to DEN-treated rats. The statistical differences or changes are signified using an asterisk (*), showing significance at $p < 0.05$ in comparison with the control group, and a hashtag (#), signifying $p < 0.05$ in comparison with the DEN-treated (Disease control) Group.

Effect of RES on inflammatory markers: After seven weeks of continuous treatment of DEN, a significant increase in the TNF-α, CRP, IL-6, and IL-1β was observed as compared to the standard control group and compared to the DEN-treated rats (disease control) group, RES (20 mg/kg b.w.) meaningfully reduced the level of pro-inflammatory cytokines *(*$p < 0.05$*)* [Figure 7].

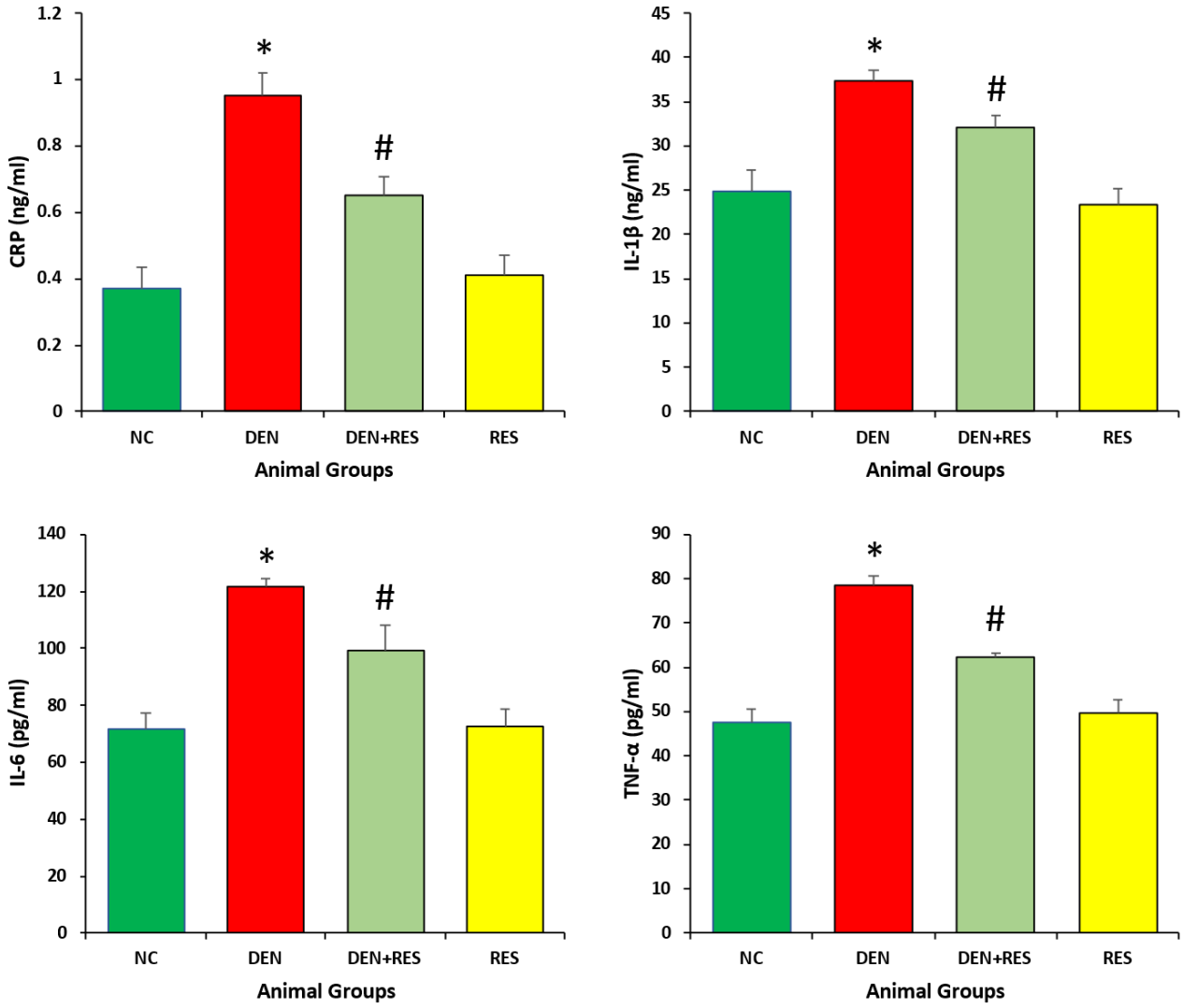


Figure 7. The inflammatory marker levels were measured in treated and untreated animal groups.

In the rats treated with DEN inflammatory markers (TNF-α, CRP, IL-6, and IL-1β), levels were meaningfully increased compared to the control group. The RES (20 mg/kg b.w.) significantly decreased these cytokines compared to DEN-treated rats. The statistical variances/changes are symbolized using an asterisk (*), showing significance at $p < 0.05$ compared the control group, and a hashtag (#), signifying $p < 0.05$ in comparison with the DEN-treated (Disease control) Group.

Effects of RES on oxidative stress: Oxidative stress, regarding ROS, plays a vital role in depleting antioxidant enzyme levels and initiates pathogenesis. The onset of liver diseases can be seen as triggered by a change in the redox balance, leading to oxidative stress [28]. The hepatoprotective effects of RES were evaluated by determining antioxidant enzymes. In rats treated with DEN, the levels of antioxidant enzymes showed a significant decrease compared to the control group ($p < 0.05$). In contrast to the DEN-administered (disease control) group, the rats treated with 20 mg/kg of RES b.w they have significantly enhanced the antioxidant enzymes (Catalase, SOD, GST, and GPx) levels ($p < 0.05$) [Figure 8]. These findings advocate that RES has hepato-protective potential by decreasing oxidative stress or increasing antioxidant enzyme levels.

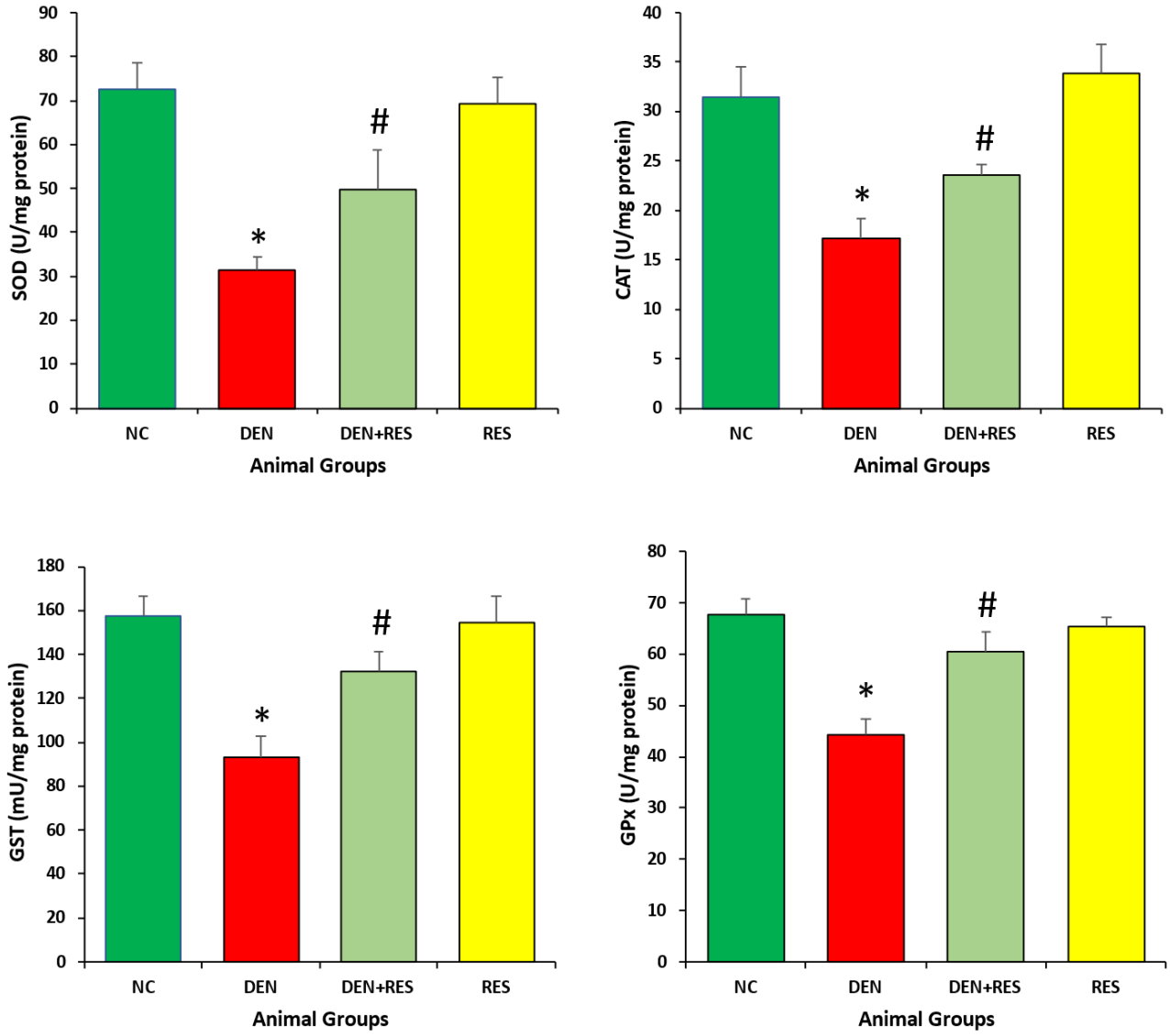


Figure 8. The measurement of (Catalase, SOD, GST, and GPx) levels. In the rats treated with DEN, the antioxidant enzyme level meaningfully decreased. The RES (20 mg/kg b.w.) increased the levels as compared to DEN-administered rats. The statistical variances/changes are denoted as an asterisk (*), representative significance at $p < 0.05$ in comparison with the normal control group, and a hashtag (#), demonstrating $p < 0.05$ as compared with the DEN-treated group.

Effect of the RES on liver tissue architectures: DEN is usually accepted as one of the most toxic drugs due to its potential to prompt different forms of necrosis, at last resulting in fibrosis [29]. Research has demonstrated that DEN can lead to substantial liver damage by destructive DNA and elevating the generation of reactive oxygen species (ROS) [30]. In the current study, no histological changes or normal architecture of hepatocytes were seen in the control group. In the DEN-treated group, various types of histological changes, including hemorrhage, congestion, infiltration of inflammatory cells, and fibrosis, were observed. However, the DEN-administered liver tissue changes were lessened by the treatment of RES [Figure 9].

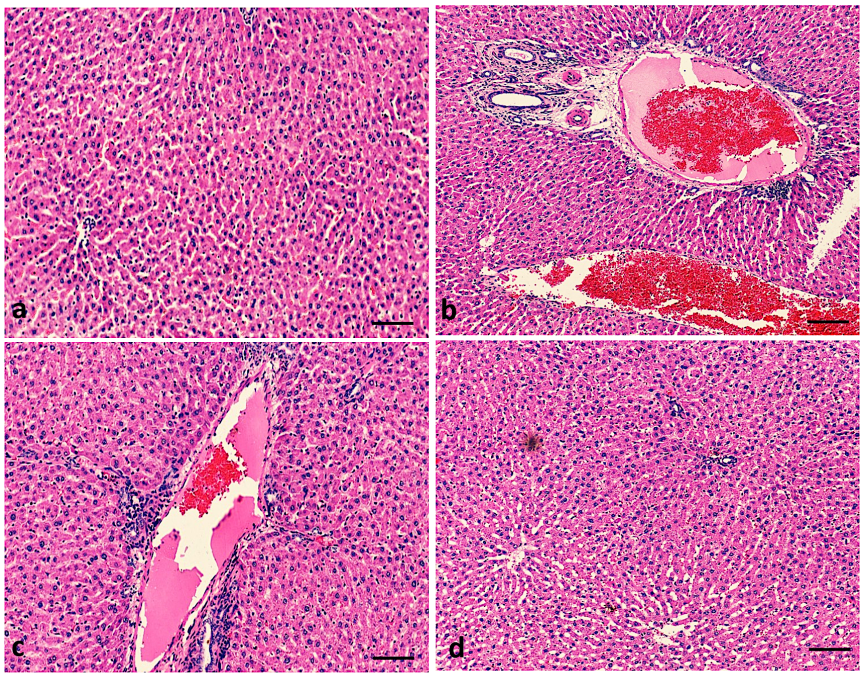


Figure 9. Liver tissue histological analysis in experimental groups animal were evaluated: (**a**) control animals displayed normal liver tissue architecture; (**b**) DEN-administered rats exhibited changes as congestion inflammatory cell infiltration as well as fibrosis; (**c**) the tissue changes were decreased in the RES treated group; (**d**) in the only RES-given group, normal liver tissue architecture was noticed.

The impact of RES on liver fibrosis: The liver tissue sections of different experimental groups were stained using Masson trichrome stain. The control group observed a normal distribution of collagen fibers. As compared to normal control group, the DEN-induced group showed severe collagen deposition. The DEN-induced fibrosis was reduced by pretreatment with RES (20 mg/kg b.w) [Figure 10].

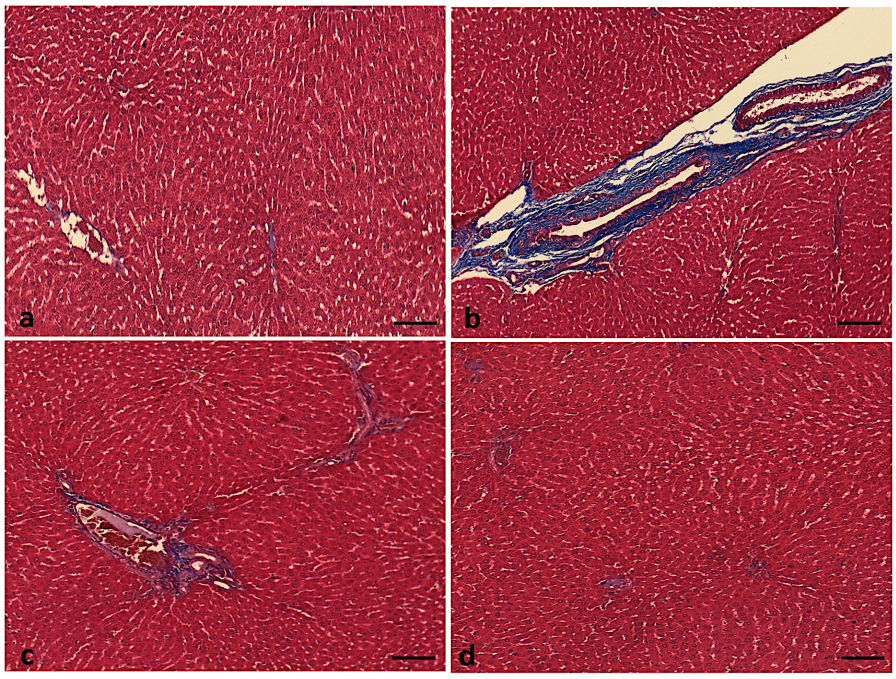


Figure 10. Anti-fibrotic effect RES. (**a**) In the rats from the control group, normal fiber was observed (**b**) DEN- group showed severe collagen deposition (**c**) RES treatment displays a decrease of DEN- given fibrosis (**d**) In RES treated group only, normal collagen was detected.

The liver tissue sections of different experimental groups were stained using Sirius red staining. The control group observed a normal distribution of collagen fibers. The DEN-induced group showed severe collagen deposition compared to the control groups. In comparison to the DEN-treated (disease control) group, the rats treated RES meaningfully lessened the fibrosis [Figure 9].

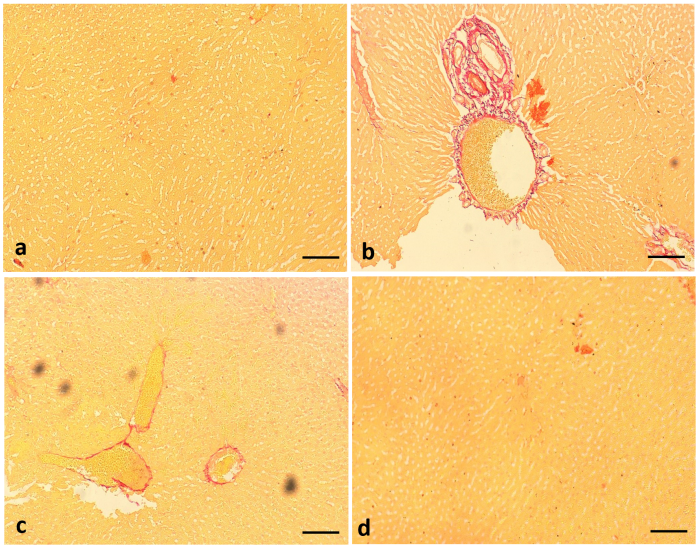


Figure 11: Sirius red staining for collagen fibers Anti-fibrotic effect RES. (**a**) In the control group rats, normal fiber was detected; (**b**) DEN- the given group showed severe collagen deposition (**c**) RES treatment displays a decrease of DEN-given fibrosis (**d**) In the treated group only, normal collagen was seen.

The impact of RES on Cox-2 protein expression: Immunohistochemistry staining was conducted to judge the expression of Cox-2 protein. In the control group, the expression of Cox-2 was not noticed, whereas the DEN-induced group exhibited high cytoplasmic expression of this protein. The expression of Cox-2 protein was reduced in the RES-treated group compared to the DEN-treated group [Figure 12].

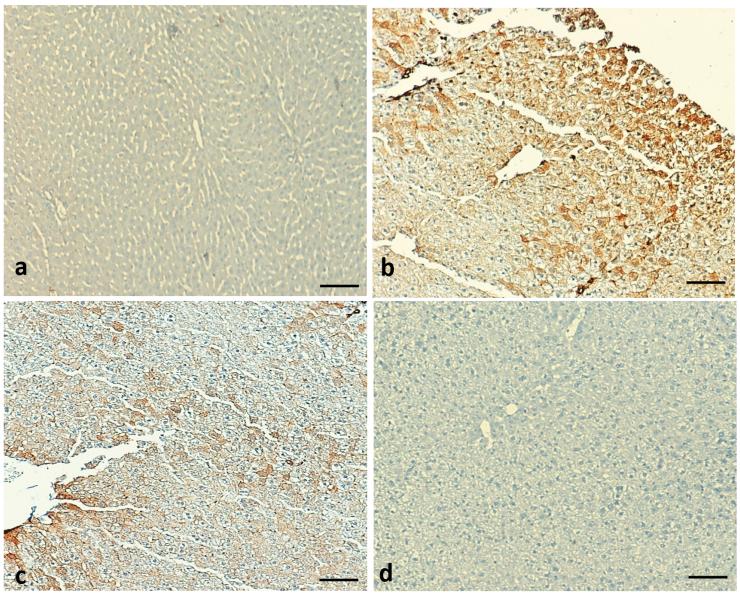


Figure 12: Expression of Cox-2 protein was measured using immunohistochemistry (**a**) Expression Cox-2 protein was not detected in control group rats; (**b**) In DEN induced group, high Cox-2 protein expression was noticed (**c**) RES shows low Cox-2 protein expression in the DEN-induced positivity (**d**) No expression of Cox-2 protein was observed in animals treated with RES alone (Scale bar = 100 µm).

Effects of RES on apoptosis: TUNEL assay was conducted on all experimental groups. No TUNEL-positive cells were observed in the control sections. However, there was a significant increase in TUNEL positivity in the DEN-treated group compared to the control group. Interestingly, the RES-treated group showed a lower number of TUNEL-positive cells compared to the DEN-treated group. This finding indicates that RES plays a significant role in attenuating apoptotic cell death [Figure 13].

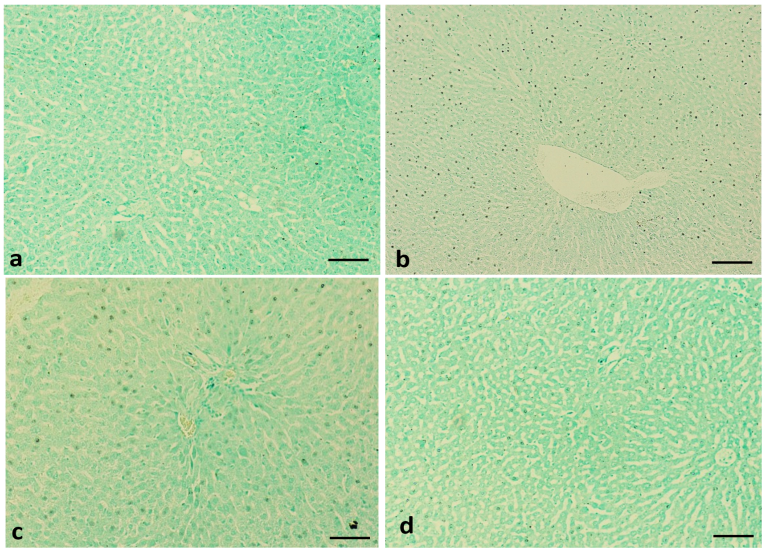


Figure 13: Apoptosis was measured using TUNEL assay (**a**) No apoptosis was not detected in control group rats; (**b**) In the DEN induced group, apoptosis was noticed (**c**) RES shows low less apoptosis in the DEN-induced positivity (**d**) No apoptosis was observed in animals treated with RES alone (Scale bar = 100 µm).

Interaction of antioxidant enzymes as well as inflammatory markers with resveratrol: The investigation delved into the molecular interaction of important antioxidants such as catalase, superoxide dismutase, and proinflammatory molecules like tyrosine kinase, TNF-α, and NOs with bioactive resveratrol. Table 2 presents the binding energy and the amino acid residues involved in the interaction of both antioxidants and anti-inflammatory agents responsible for activity. The active residues chiefly found in these interactions are Asn149, Thr150, Phe198, Ser201, Arg203, Val450, for catalase, and Asp11, Thr39, Glu121, Leu144, in superoxide dismutase respectively. The main active residues found in these interactions of tyrosine kinase included Ser425, Val431, Lys445, and TNF-α are Tyr59, Tyr151 (Table 2**)** and NOs are Trp414, Ala,417, Arg419, Met575, Phe589, Trp683, Tyr711 (Table 2**).** Figures 14 a-e show the results of different molecular docking investigations.

Table 2. Binding energy as well as interacting amino acid residue of both inflammatory and antioxidant activity

Resveratrol		**DB02709**	
Protein	PDB id	Binding energy (kcal/mol)	Interacting amino acids
TNF-α	2AZ5	-7.0	Tyr59, Tyr151
NOs	5UO1	-8.4	Trp414, Ala,417, Arg419, Met575, Phe589, Trp683, Tyr711
Superoxide Dismutase	5YTU	-6.0	Asp11, Thr39, Glu121, Leu144
Tyrosine kinase	3SXR	-5.8	Ser425, Val431, Lys445
Catalase	1DGH	-8.0	Asn149, Thr150, Phe198, Ser201, Arg203, Val450

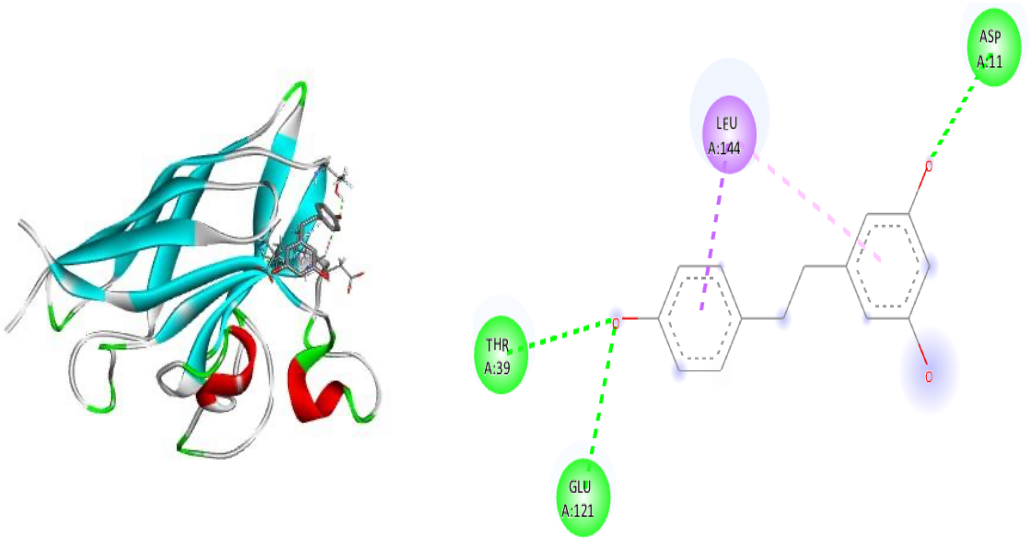


Figure 14a. Superoxide dismutase and resveratrol

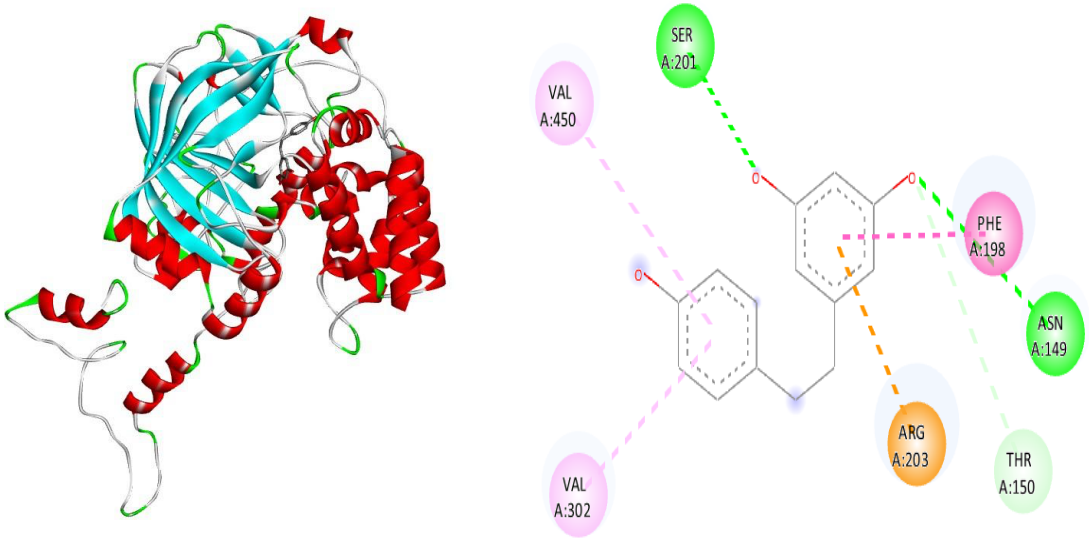


Figure 14b. Catalase with resveratrol

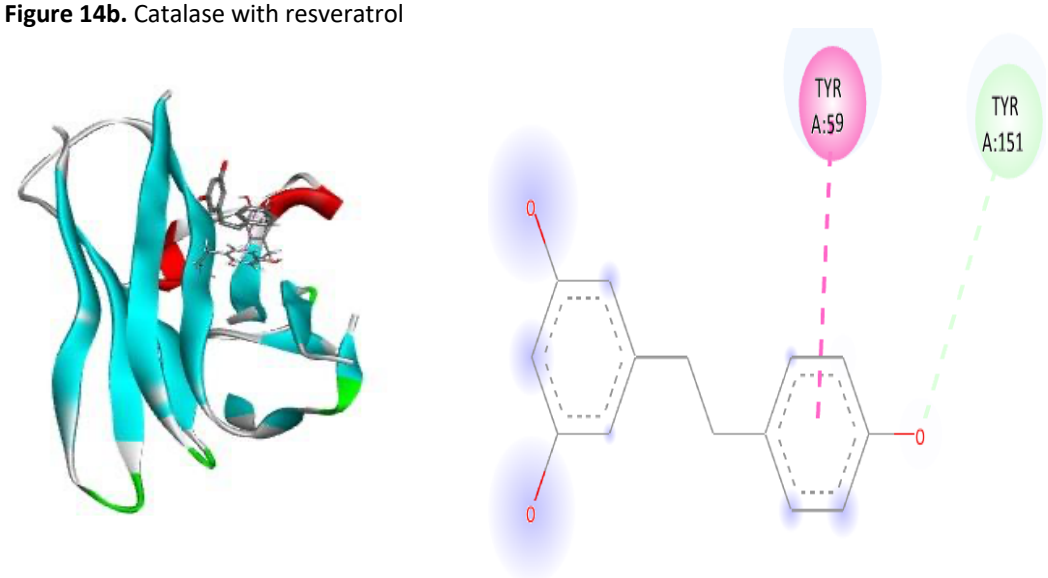


Fig 14 c. TNFα with resveratrol

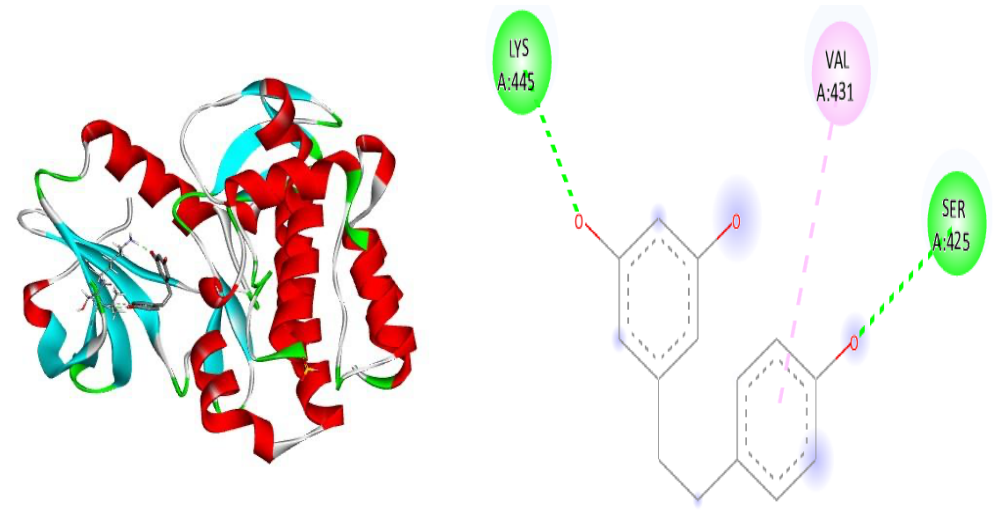


Fig 14d. Tyrosine kinase with resveratrol

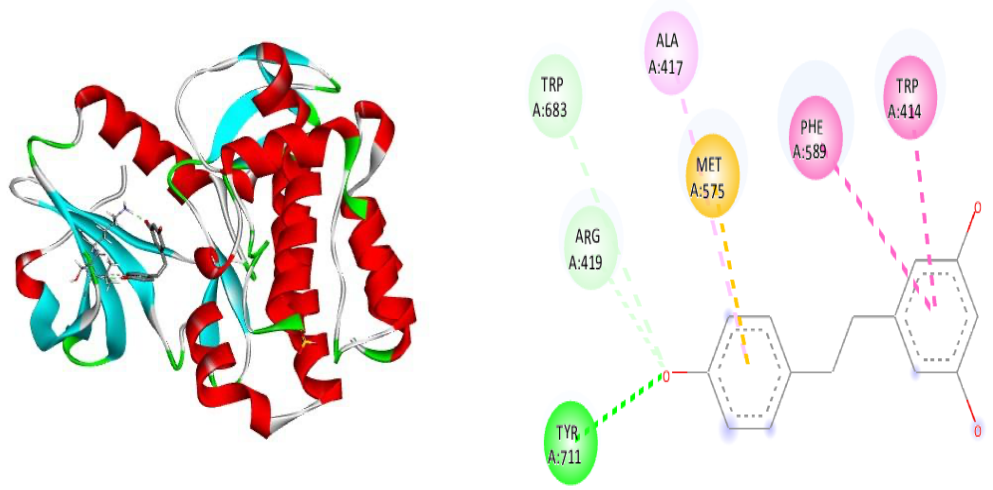


Fig 14e. NOs with resveratrol

Figures 14 a-e. Show the results of different molecular docking investigations.

DISCUSSION

Toxins cause liver injury through oxidative stress, inflammation, and necrosis [31]. In the laboratory, DEN is one of the commonly used experimental models to cause liver pathogenesis, and prolonged administration causes liver fibrosis. Moreover, these carcinogenic materials cause various cell alterations, imbalances of ROS, antioxidants, and inflammation. Due to this circumstance, they can generate oxidative stress and have been exploited to cause liver injury in experimental animals [32]. Enhanced serum levels of liver function enzymes indicate hepatic damage or injury resulting from the loss of liver cell membrane integrity, which releases ALT and AST enzymes into the serum [33]. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are important biomarkers for evaluating liver functions [34]. Understanding the role of serum enzyme levels in diagnosis provides valuable information for managing liver health and associated conditions.

In the DEN-treated group of rats, it was noticed that DEN exposure leads to lipid peroxidation in cells, hepatocyte injury, and increased levels of liver function enzymes. Moreover, RES-treated group rats pointedly decreased the levels of liver function enzymes. It was noticed that RES could maintain the hepatocytes by reducing the level of ROS, which could decrease the release of the level of liver function enzymes. Following this finding, a previous study reported that DEN-treated

animals showed high levels of liver function enzymes [35, 36]. Similar findings on the hepatoprotective potential of RES were reported as in α-Amanitin (α-AMA)-administered animals, the activities of serum AST and ALT levels evidently increased, and treatment with RES significantly decreased these enzyme activities [37]. In another study, it was found that the group consuming alcohol showed high levels of AST and ALT, which increased steadily, indicating continuing liver damage. On the other hand, the resveratrol supplementation group had significantly lower levels of liver enzymes (ALT and AST) than the alcohol group [38].

Oxidative stress plays a substantial role in triggering many signaling pathways that lead to inflammatory diseases and tissue damage [39]. It is also associated with the development of different metabolic and chronic disorders [40, 41]. Moreover, clinical circumstances and constant xenobiotic exposure also cause tissue injury and necrosis, often resulting in inflammation [42].

In this regard, the natural compound shows effects on oxidative stress, greatly enhances the antioxidant system, protects rats from liver damage [43], and reduces cell cytotoxicity [44]. The study was designed to assess the hepatoprotective effects of RES by examining lipid peroxidation and antioxidant enzymes in different groups of animals. In rats intoxicated with DEN, levels of monoaldehyde (MDA) increased, while antioxidant enzyme levels significantly decreased compared to the control group. Rats treated with RES, however, showed decreased MDA levels and enhanced antioxidant enzyme activities compared to the DEN-treated rats. These findings suggest that RES has the potential to protect the liver by reducing oxidative stress and increasing antioxidant enzyme levels. Hong and his colleagues observed the antioxidant effects of RES in rats with toxin-induced liver fibrosis. They reported that RES increased the levels of antioxidant enzymes and reduced the level of MDA, which strongly supports our outcomes [45]. Another study [46] has shown that RES remarkably prevented the dimethylnitrosamine-induced rise of serum bilirubin, ALP, ALT, and AST levels. RES also increased serum hepatic GSH and albumin levels and reduced the level of MDA due to its antioxidant effect. RES on liver fibrosis brought by CCl_4 in rats was examined. Rats fed with 50 and 100 mg/kg of RES significantly reduced levels of the serum liver hydroxyproline, ALT, and MDA and relieved liver fibrogenesis compared with the control group [47].

Inflammation plays a substantial role in liver pathogenesis, and cytokine has been linked to acute and chronic liver damage [48-50]. Furthermore, many xenobiotic drugs showed a role in the damage of the liver and trigger the release of pro-inflammatory cytokines [51-53]. In the present investigation, the DEN-induced liver damage model was distinguished by increased secretion of pro-inflammatory cytokines. This study indicates that RES mitigated liver injury, reducing IL-6, CRP, and TNF-α. Previous studies have suggested that pre-treatment with resveratrol improved the pathological effects of ConA-caused autoimmune hepatitis and significantly inhibited inflammatory markers [54]. This finding is consistent with the current study. A previous study showed that administering RES plays a role in inhibiting liver injury in rats by decreasing inflammation [55]. Another study showed a significant increase in TNF-α, IL-6, and INF-γ in TAA-intoxicated rats. In the meantime, the treatment of resveratrol reduced TNF-α, IL-6, and INF-γ [56]. Notably, it was already established that RES inhibits TNF-α- and decreases pro-inflammatory NF-κB levels [57]. Moreover, other natural compounds, such as curcumin, have anti-inflammatory potential in the inhibition of pathogenesis [58]. However, this study precisely delivers an understanding of one of the potential mechanisms of RES in protecting the liver.

Many clinical as well as experimental data indicate that DEN acts as a hepatotoxicant, leading to alteration in liver tissue architecture, including severe destruction of hepatocytes, inflammation, hemorrhages, and fibrosis. In

the DEN-treated group, histopathological studies showed the various changes observed in the liver, such as lymphocyte infiltration, fibrosis, congestion, and hemorrhages. In contrast, RES treatment reduced such changes as reduced lymphocyte infiltration, fibrosis, congestion, and hemorrhages. Previous studies were in accordance with current findings, and it was reported that DNE causes various types of alteration in liver tissue architecture [35, 36]. Moreover, it was demonstrated in the alcohol group that changes were noticed as the ballooning of hepatocytes, with necrosis, lymphocyte and neutrophil infiltration, and large numbers of bundles of collagen fibers. The resveratrol group remarkably revealed the absence of collagen fiber bundles and significantly reduced alcohol-induced changes [38]. Further study was in accordance with current findings as RES acts as protective effects, and it was revealed that tissue changes were less evident in the RES-treated rats than in disease control [59].

Chronic liver damage causes liver fibrosis, which results in the abnormal accumulation of extracellular matrix proteins, primarily fibrillar collagens. This can, at last, lead to cirrhosis, sideways with complications such as hepatocellular carcinoma, and liver failure [60]. DEN leads to substantial oxidative stress and DNA mutations, accelerates the progression of fibrosis and cirrhosis, and increases lipotoxicity. It has long been used in liver cancer models [61]. Ding et al. [62] injected DEN into rats at 30 mg/kg body weight. It was reported that the resulting model was characterized by three stages: the inflammation stage, the fibrosis stage, and the HCC stage, based on the duration of exposure [62]. In the current study, Masson's trichrome and Sirius red staining showed that the DEN-treated group caused severe fibrosis. In contrast, RES treatment reduced and acted as anti-fibrotic potential. Previous findings were consistent with the current findings, as chronic exposure to DEN causes fibrosis, as noted by special staining [35]. Earlier findings as per the current study revealed that the DEN group exhibited increased collagen fibers surrounding the lobules. In contrast, collagen expression was almost absent in the DEN plus Res group [45]. Resveratrol also showed hepatoprotective and anti-fibrogenic properties against DMN-caused liver injury, demonstrating its ability to prevent hepatic fibrosis development [63].

Inflammation is linked with the catalysis of two essential inflammatory mediators as well as cytotoxic factors: Cox-2 and NOS [64]. Cox-2, the inducible form of cyclooxygenase, plays a multifaceted role in developing liver diseases [65]. In the current study, the level of Cox-2 protein was explored by using immunohistochemistry, and it was noted that cox-2 expression was significantly higher in the DEN-induced group. However, in the treated group with RES, the pattern of Cox-2 protein positivity was lower compared to the DEN-induced group. Previous research indicated that administering kolaviron orally before DEN treatment notably reduced the levels of COX-2 proteins. Pretreatment with kolaviron suggestively inhibited epidermal COX-2 positive cells compared to DMN treatment alone [66]. Another study on different compounds reported that medicinal plants' bioactive compounds decrease inflammatory protein expression [67-69]. Moreover, food bioactive compounds in functional food science have been described [70-72]. The present study selected enzymes with cellular antioxidant properties and molecules implicated in inflammation to predict possible interaction with resveratrol. The results of the docking studies demonstrated that RES interacts with the inflammatory targets and suppresses inflammation. Molecular docking results also indicated strong binding with high energy at different sites between resveratrol and pro-inflammatory cytokines, demonstrating the compound's efficiency in liver protection via anti-inflammatory potential.

Resveratrol is a well-known polyphenolic compound commonly found in vegetables and fruits. Its role in liver disease prevention and management has

been reported through the modulation of oxidative stress and inflammation. In this study, we have examined this compound's potential role in liver pathogenesis by investigating liver function enzymes, oxidative stress, lipid peroxidation, and inflammatory markers. In addition, hepatocyte architecture was examined through histopathological analysis. Furthermore, the novelty of this study was further added by the evaluation of this compound's antifibrotic and anti-apoptotic potential. Besides, the in-silico studies of resveratrol through the docking studies demonstrated that this compound interacts with the inflammatory targets and suppresses inflammation, demonstrating the compound's efficiency in liver protection via anti-inflammatory potential.

CONCLUSION

The flavonoid RES has the potential to promote health due to its antioxidant and anti-inflammatory properties. The results of this study demonstrate that increased levels of liver function enzymes, as well as pro-inflammatory mediators, can lead to liver injury. RES exhibits significant antioxidant potential, which may help prevent oxidative stress and liver damage. Furthermore, these findings suggest that RES may aid in maintaining the structure of liver cells due to its anti-inflammatory and anti-fibrotic properties. Therefore, our results indicate that RES could be a potential therapeutic approach for liver diseases.

Abbreviations: Resveratrol: RES; alanine aminotransferase: ALT; aspartate aminotransferase: AST; Interlukin-6: IL-6; Diethylnitrosamine: (DEN); Superoxide dismutase: SOD; Catalase: Cat, Cyclooxygenase-2: COX-2; C-Reactive Protein: CRP

Author Contributions: Conceptualization, A.H.R.; data collection; Methodology and interpretation of the data, A.H.R, A.K, H.A.A, A.Y.B; manuscript writing, A.H.R, A.K, H.A.A, A.Y.B.; critical revision of the manuscript, A.H.R, A.K, H.A.A, A.Y.B and M.M.A.R

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