

Reishi Mushroom Attenuates Hepatic Inflammation and Fibrosis Induced by Irradiation Enhanced Carbon Tetrachloride in Rat Model

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Abstract

This work was undertaken to establish a new experimental model of hepatic fibrosis by gamma irradiation and CCl₄ and to study the hepatoprotective effect of Reishi Mushroom (RM) against hepatic fibrosis induced in that model. Our results revealed that oral co-administration of 110 mg/kg RM by gavage to fibrotic rats offered an obvious hepatic protection as assured by the significant decrement in ALT and AST, HP content, MDA and NO levels with elevation of the antioxidant enzymes activities. The levels of TGF- β , TNF- α , HO-1 and type-1 collagen and their m-RNA expression were markedly declined as compared with those of fibrotic rats. Microscopical examination revealed that the exposure of rats to radiation aggravated the effect of CCl₄ causing extensive collagen deposition and marked pseudolobulation of the hepatic parenchyma indicative of bridging fibrosis. While, oral co-administration of RM obviously improved the state of steatosis and apparently suppressed hepatic fibrogenesis.

Keywords

Carbon Tetrachloride, Gamma Irradiation, Hepatic Fibrosis, Reishi Mushroom, Rats

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1. Introduction

Liver is the key organ of metabolism and excretion of many substances; hence it is often exposed to variety of xenobiotics and therapeutic agents. Hepatic fibrosis induced by chronic liver injuries as a result of hepatitis viruses, chronic alcohol intake, lipidperoxidative products and various drugs could end by cirrhosis [1]. Chronic liver diseases are usually associated with inflammatory reaction that is considered a key contributor of hepatocellular damage with a resultant progression to liver fibrosis and may be to hepatocellular carcinoma [2].

Radiation-induced liver disease is mainly due to oxidative damage, leading to liver inflammation and fibrosis [3].

CCl_4 has been widely reported to induce acute and chronic tissue injuries. A single dose of CCl_4 can result in centrilobular necrosis and steatosis [4]. While, prolonged administration of CCl_4 can cause hepatic fibrosis caused by its highly active metabolite trichloromethyl (CCl_3) that is produced by Cytochrome p450 during CCl_4 metabolism in hepatocytes [5]. Those trichloromethyl radicals are known to trigger a cascade of events that result in hepatic inflammation and fibrosis [6] through activation of hepatic stellate cells (HSCs) [7]. Marked changes in the extracellular matrix (ECM) take place as hepatic fibrogenesis progresses with more expression of type-1 collagen which is the most abundant ECM protein in hepatic fibrosis [8]. The activation of HSCs is mediated by reactive oxygen species and various cytokines, including transforming growth factor (TGF)- β , tumor necrosis factor (TNF)- α [9] and interleukin-6 (IL-6), which are able to stimulate collagen synthesis [10].

Mushroom is widely used everywhere all over the world as an important source of nutrition and therapy [11]. Reishi Mushroom (RM), also known as *Ganoderma lucidum* (GL) is a traditional oriental medicinal mushroom. Its role in the treatment of chronic hepatopathy with little or no side effects is widely known [12]. Generally, many constituents in Mushrooms are known to have varieties of biological activities including antioxidant [13], anti-inflammatory [14] and hepatoprotective properties [15]. Furthermore, a protective action of water or ethanol extracts of *G. lucidum* against acute hepatitis in rats and mice have been recorded [16]. In addition, among many constituents present in *G. lucidum*, triterpenoids and polysaccharides have proved to inhibit hepatitis and liver fibrosis [17] [18].

Aim of work: The main objective of the present study was to establish a fibrosis model using gamma irradiation and CCl_4 and to demonstrate the protective effect of RM aqueous solution against the induced hepatic fibrosis with focusing on the precise cellular and molecular mechanisms.

2. Materials and Methods

2.1. Chemicals

CCl_4 (C25630) was obtained from Sigma Chemical Co. (St. Louis, MO) USA. The kits used were purchased from Bio-diagnostic, Cairo, Egypt. Reishi Mushroom (RM) powder was provided by DXN marketing SD.BHD (283904-P), Malaysia.

2.2. Animals

Fifty-five male albino rats with an average weight of 145 - 160 gm were obtained from the animal house belonging to Research Institute of Ophthalmology, Giza, Egypt. Rats were housed in regular designed cages and maintained in good ventilation, at a temperature of $25^\circ\text{C} \pm 5^\circ\text{C}$, 60% humidity, with suitable illumination conditions (light/dark cycle) and were allowed standard pellet diet and fresh water *ad libitum*. Animals were left one week for acclimatization on lab environment before starting the onset of the experiment. Animal care and the protocol of animal treatment were approved by the Animal Care Committee of the National Centre for Radiation Research and Technology (NCRRT), Cairo, Egypt, and in accordance with the recommendations of the proper care and use of laboratory animals.

2.3. Irradiation Facilities

Irradiation was performed through the use of a Canadian Gamma Cell-40 (^{137}Cs) at the National Centre for Radiation Research and Technology (NCRRT), Cairo, Egypt. The dose rate was 0.675 Gy/minute.

2.4. Induction Models of Liver Fibrosis in Rats

Two models of hepatic fibrosis were established in the current study. The 1st model was established using CCl_4

alone as; CCl₄ in olive oil (50% V/V) at a dose of 2 ml/Kg body weight was delivered subcutaneously three times/week for six weeks. While in the 2nd model, gamma irradiation was used to promote and enhance the CCl₄ induced hepatic fibrosis. Rats were exposed to six fractions (each of 2 Gy) of gamma irradiation once/week up to cumulative dose of 12 Gy concurrently with CCl₄ as in the 1st model.

2.5. Experimental Design

Rats were randomly divided into five groups. Group (Gp) 1; control rats were s/c injected with the vehicle olive oil (0.2 ml). Gp2; rats in this group were treated with RM aqueous solution (1100 mg/kg) by gavage three times/week. Gp3; represented the 1st model of fibrosis; rats were subcutaneously injected with CCl₄ in olive oil (2 ml/kg) 3 times/week for six weeks. Gp4; represented the 2nd model of fibrosis; rats of this group were subjected to gamma irradiation and CCl₄ as described previously. Gp5; (RM + IRR + CCl₄ treated group) rats were treated with RM as in group 2 and were exposed to both IRR + CCl₄ as in group 4. The time interval between CCl₄ injection and RM administration was taken into account to be at least 5 hours to avoid the interference of the metabolic substances. Careful observation was carried out for all animals along the experimental period and body weights were recorded weekly. At the end of the experimental period, rats were sacrificed under gentle diethyl ether anesthesia prior to which blood samples were collected into heparinized test tubes for plasma separation for biochemical analysis. Through PM examination was carried out and livers were dissected out, washed with saline, dried on a filter paper and weighted. Each liver was divided into two parts; one was fixed in 10% buffered neutral formalin for histopathological examination while the second part was kept at -20°C till used for biochemical analysis.

2.6. Biochemical Parameters Investigated in Blood Plasma

ALT and AST activities, total Protein (TP) and albumin (Alb) in plasma were determined using the available commercial kits purchased from Bio-Diagnostic Co., Cairo, Egypt. Plasma TGF- β and TNF- α were measured by ELISA kit immunoassay supplied by R & D Quantikine USA (Catalog Number: RTA00, MB100B) according to manufacturer's instructions.

2.7. Biochemical Parameters Investigated in Liver Homogenate

2.7.1. Hydroxyproline (HP) Assay

HP; an indicator for hepatic collagen amount was colorimetrically assayed in liver tissue homogenate as previously described [19].

2.7.2. Evaluation of Antioxidant Status in Hepatic Tissue

Malondialdehyde (MDA); the end product of lipid peroxidation [20], nitric oxide (NO) [21], glutathione (GSH) content [22], the activities of super oxide dismutase (SOD) [23] and catalase (CAT) [24] were assayed in the hepatic tissue homogenates of rats of all groups.

2.7.3. Heme Oxygenase-1 (HO-1) Activity Assay

Liver tissues were homogenized with 2.5 volume Tris-HCl buffer (10 m mol/L, pH 7.6) containing 250 mmol /L sucrose and 0.4 mmol/L phenyl methylsulfonyl fluoride. The homogenates were centrifuged at 800 g for 10 minutes and then centrifuged at 13.500 g for 20 minutes to produce the mitochondrial pellet. The supernatant was withdrawn. The protein content in liver homogenate was determined [25]. The activity of HO-1 in the supernatant was determined as previously described [26]. Billirubin formed was determined by calculation of the difference in absorbance between 464 nm and 530 nm. The HO-1 activity was expressed as picomoles of billirubin per milligram of protein per hour using standard billirubin curve.

2.7.4. RNA Extraction and RT-PCR Analysis for TGF- β 1, TNF- α , HO-1 and Type-1 Collagen

Total RNA was isolated from liver tissue homogenate using RN easy Purification Reagent (Qiagen, Valencia, Callifornia) according to the manufacturer's protocol. Extracted RNA was quantified by spectrophotometer at 260 nm. Reverse transcription was carried out on 5 μ g RNA from each liver sample using MMuLV reverse transcriptase in a 50 μ L reaction volume. Mixtures of the reverse transcription were used for amplification of fragments specific for TGF- β 1, TNF- α , HO-1 and Type-1 collagen by PCR using the primer pairs listed in **Table 1**.

Table 1. Primer sequences used for real time PCR.

| primer | sequence |
|-----------------|---|
| TGF- β 1 | Forward primer: 5'-AGGGCTACCATGCCACTTC-3' Reverse primer: 5'-GCGGCACGCAGCACGGTGAT-3' |
| TNF- α | Forward primer: 5'-GAAAAGCAAGCAGCCAACCA-3' Reverse primer: 5'-CGGATCATGCTTTCTGTGCTC-3' |
| HO-1 | Forward primer: 5'-TTTCAAAGGGTCAGGTGTC-3' Reverse primer: 5'-CCTTCTGCGGCAATCTTTC-3' |
| Type-1 collagen | Forward primer: 5'-AATTGGAGCTGTTGGTAACGC-3' Reverse primer: 5'-CACCAAGTAAGCCGTTTGC-3' |
| GADPH | Forward primer: 5'-CTCCATTCTTCCACCTTTG-3' Reverse primer: 5'-CTTGCTCTCAGTATCCTTGC-3' |

The levels of expression of all transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same tissue sample. The real time PCR was performed using the QuantiTect SYBR green PCR Kit (Qiagen, Germany) according to the manufacturer's instructions, by Applied Biosystems 7500 Instrument, USA. The PCR reaction mix was carried out in a total volume of 25 μ L, containing 2 \times QuantiTect SYBR green PCR master mix, 20 pmol/ μ L specific primer. Subsequently, cDNA was synthesized from purified RNA. The protocol consisted of 45 amplification cycles, each conducted as follows; 10 min at 95°C (holding stage), 15 sec for denaturation at 95°C, 30 sec for annealing at 60°C and another 15 sec for elongation at 60°C. RT-PCR was carried out as has been described previously [27].

2.8. Histopathological Studies

Formalin fixed liver specimens were routinely processed using conventional paraffin embedding technique. Paraffin blocks were serially sectioned at 4 - 5 μ m thickness and stained with H & E [28]. Azan stain was used to demonstrate collagen fibers and the progress of fibrosis [28].

2.9. Immunohistochemistry

Labeled streptavidin-biotin method was used for immunohistochemical detection of α -SMA using a Histostatinplus bulk kit (Zymed Laboratories Ins., San Francisco, CA, USA). The primary antibody used was monoclonal anti-smooth muscle actin, at a dilution of 1:800 (clone 1A4, Sigma Co., St. Lois, MO, USA).

2.10. Statistical Analysis

Data were analyzed using SPSS Version 20.0. Differences between experimental groups were analyzed using one-way analysis of variance. All differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Clinical and Postmortem Examination

Rats of CCl₄ and IRR + CCl₄ treated groups appeared depressed with lusterless fur and decreased appetite. Livers of rats of CCl₄ were swollen with rounded borders, irregular surface and fatty appearance. While that of IRR + CCl₄ treated rats were severely distorted with fatty appearance.

3.2. The Effect of RM on Body Weight and Liver Weight Index of Fibrotic Rats

As shown in **Table 2**, a significant ($P < 0.001$) decrease in body weight gain coupled with significant increase ($P < 0.001$) in liver weight index was observed in both models of fibrosis generated in this study as compared to the control set. Moreover, more pronounced changes in body weight ($P < 0.001$) and in liver index ($P < 0.01$) were noticed in IRR + CCl₄ treated rats than in those treated with the sole CCl₄ alone. On contrary, RM co-administration to IRR + CCl₄ treated rats caused significant restoration ($P < 0.001$) in body weight gain and liver weight index near to the normal values ($P > 0.05$).

Table 2. The effect of Reishi Mushroom (RM) on body weight and liver weight index in fibrotic rats.

| Treatments | Body Weight (g) | | Liver Index (%) |
|-----------------------------|---------------------------|------------------------------|-------------------------------|
| | Initial | Final | |
| CT | 154.4 ± 2.4 | 268.4 ± 3.5 | 2.48 ± 0.19 |
| RM | 154.8 ± 3.8 | 244.1 ± 6.8 [*] | 2.78 ± 0.02 |
| CCl ₄ | 145.6 ± 1.8 | 242.3 ± 4.7 ^{**} | 3.9 ± 0.21 ^{***} |
| IRR + CCl ₄ | 144.7 ± 1.3 ^{NS} | 218.8 ± 5.7 ^{***,a} | 4.76 ± 0.12 ^{***,##} |
| RM + IRR + CCl ₄ | 146.3 ± 0.6 ^{NS} | 239.8 ± 5.9 ^{**b} | 3.48 ± 0.11 [‡] |

Each value represents the mean ± SE of 5 variables/group. Significant difference versus corresponding CT group at ^{***} $P < 0.001$, ^{**} $P < 0.01$, ^{*} $P < 0.05$. Significance change of CCl₄ from IRR + CCl₄ at [#] $P < 0.05$, ^{##} $P < 0.01$. Significant difference of Gp5 (RM + IRR + CCl₄) from IRR + CCl₄ at [‡] $P < 0.001$.

3.3. Effect of RM on ALT and AST Activities, TP, Alb, TNF- α and TGF- β Levels of Fibrotic Rats

It was observed that; RM treated rats did not show any significant ($P > 0.05$) changes of any of the investigated parameters in their blood plasma as well as in hepatic tissue compared to those of control rats.

A significant ($P < 0.001$) elevation in ALT and AST activities, TNF- α and TGF- β with a significant ($P < 0.001$) reduction in plasma TP and Alb concentration was noticed in plasma of CCl₄ and IRR+CCl₄ treated groups when compared with controls (Figures 1-3). The highly significant ($P < 0.05$) changes were noticed in IRR + CCl₄ treated rats compared with those of the sole CCl₄ treatment. On contrary, the administration of RM to IRR + CCl₄ treated rats resulted in significant ($P < 0.001$) improvement of all of the above altered parameters compared to those of model 2 rats.

3.4. Effect of RM on Hydroxyproline Content in Liver Homogenate of Fibrotic Rats

Hydroxyproline content was significantly ($P < 0.001$) increased following CCl₄ treatment and showed higher significant ($P < 0.01$) increase following IRR + CCl₄ treatment than its level in model 1. By contrast, oral administration of RM with IRR + CCl₄ significantly restored the hepatic hydroxyproline content compared with its level in model 2 rats (Figure 4).

3.5. Effect of RM on Antioxidant Status in Hepatic Tissue of Fibrotic Rats

Data in (Figure 5 and Figure 6) revealed significant ($P < 0.001$) elevation in MDA and NO levels accompanied with significant ($P < 0.001$) decrease in GHS content and SOD and CAT activities in liver homogenates of CCl₄ as well as IRR + CCl₄ treated rats. Nevertheless, the changes in the antioxidant markers were higher in IRR + CCl₄ treated group ($P < 0.01$) compared to the corresponding values in CCl₄ alone treated rats. Oral administration of RM significantly restored the altered levels of the investigated antioxidants in hepatic tissue of Gp5 rats compared with Gp4.

3.6. Effect of RM on Heme Oxygenase-1 (HO-1) Activity in Hepatic Tissue of Fibrotic Rats

The activity of HO-1 in hepatic tissue was significantly increased in the two models ($P < 0.001$). A highly significant increase in HO-1 activity was recorded in the 2nd model in comparison with the 1st one. Concurrent administration of RM along with IRR + CCl₄ could significantly ($P < 0.05$) reduced the HO-1 activity as compared to IRR+ CCl₄ treated rats (Figure 6).

3.7. Effect of RM on the mRNA Levels of TGF- β , TNF- α , HO-1, and Type-1 Collagen in Hepatic Tissue of Fibrotic Rats

As shown in (Figure 7(A) and Figure 7(B)) the mRNA levels of TGF- β , TNF- α , HO-1 and type-1 collagen were significantly ($P < 0.001$) increased in the two rats' models as compared with control set, with a more conspicuous ($P < 0.001$) increase in their mRNA expression in the 2nd model when compared to the 1st model.

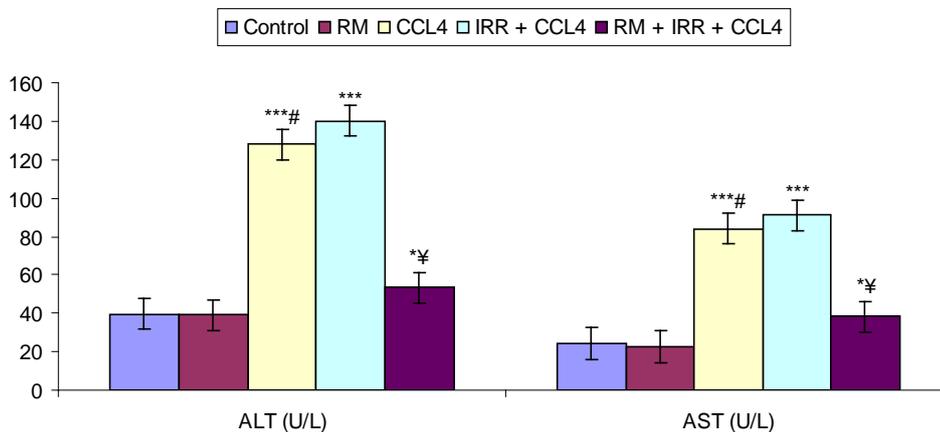


Figure 1. Effect of RM on ALT and AST activities in fibrogenic rats. Each value represents the mean \pm SE. Significant difference versus corresponding control group at ^{***} $P < 0.001$. Significant difference of IRR + CCL₄ versus corresponding CCL₄ group at [#] $P < 0.05$, ^{##} $P < 0.001$. Significant difference of RM + IRR + CCL₄ versus corresponding IRR + CCL₄ group at [¥] $P < 0.001$.

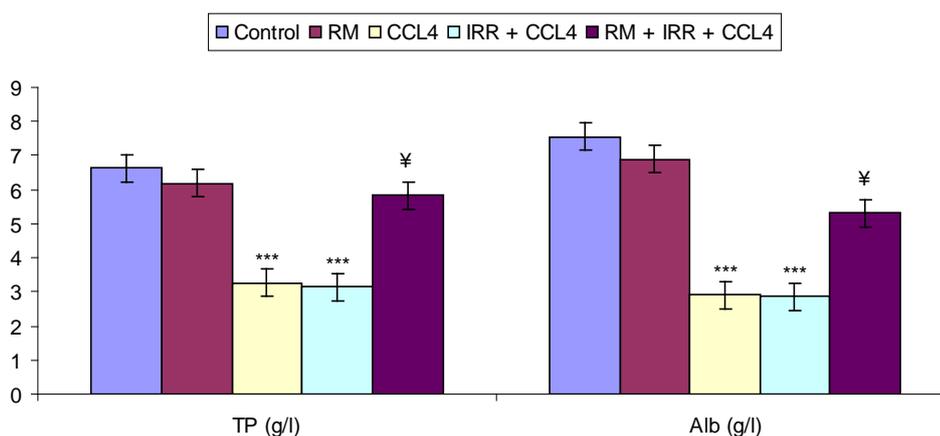


Figure 2. Effect of RM on total protein (TP) and albumin (Alb) in fibrogenic rats. Each value represents the mean \pm SE. N = 5/group. Significant difference versus corresponding control group at ^{***} $P < 0.001$. Significant difference of RM + IRR + CCL₄ versus corresponding IRR + CCL₄ group at [¥] $P < 0.001$.

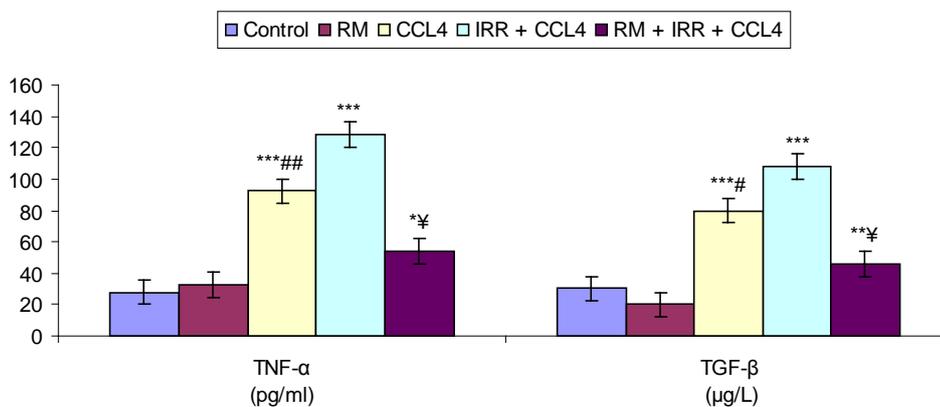


Figure 3. Effect of RM on levels of TNF- α and TGF- β in plasma of fibrogenic rats. Each value represents the mean \pm SEM. N = 5/group. Significant difference versus corresponding control group at ^{***} $P < 0.001$, ^{**} $P < 0.01$. Significant difference versus corresponding CCL₄ at ^{##} $P < 0.001$, [#] $P < 0.01$. Significant difference of RM + IRR + CCL₄ versus corresponding IRR + CCL₄ group at [¥] $P < 0.001$.

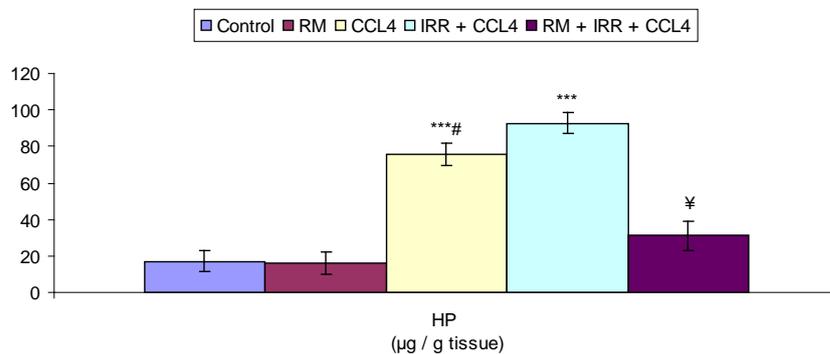


Figure 4. Effect of RM on hydroxyproline (HP) content in liver homogenate of fibrogenic rats. Each value represents the mean \pm SEM. N = 5/group. Significant difference versus corresponding control group at $***P < 0.001$, $**P < 0.01$. Significant difference versus corresponding CCL₄ at $#P < 0.01$. Significant difference of RM + IRR + CCL₄ versus corresponding IRR + CCL₄ group at $¥P < 0.001$.

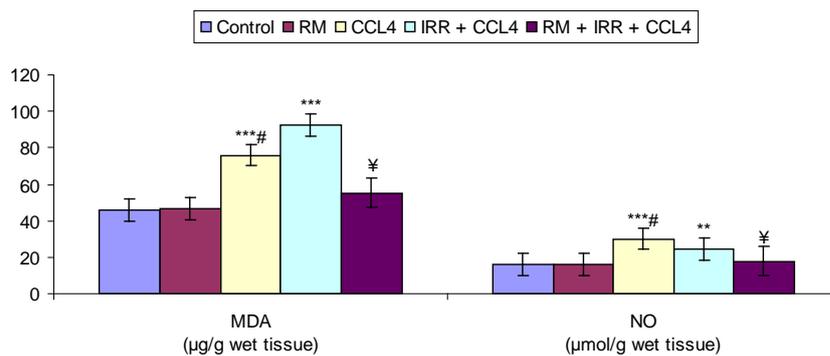


Figure 5. Effect of RM on MDA and NO in liver homogenate of fibrogenic rats. Each value represents the mean \pm SEM. N = 5/group. Significant difference versus corresponding control group at $***P < 0.001$, $**P < 0.01$. Significant difference versus corresponding CCL₄ at $#P < 0.01$. Significant difference of RM + IRR + CCL₄ versus corresponding IRR + CCL₄ group at $¥P < 0.001$.

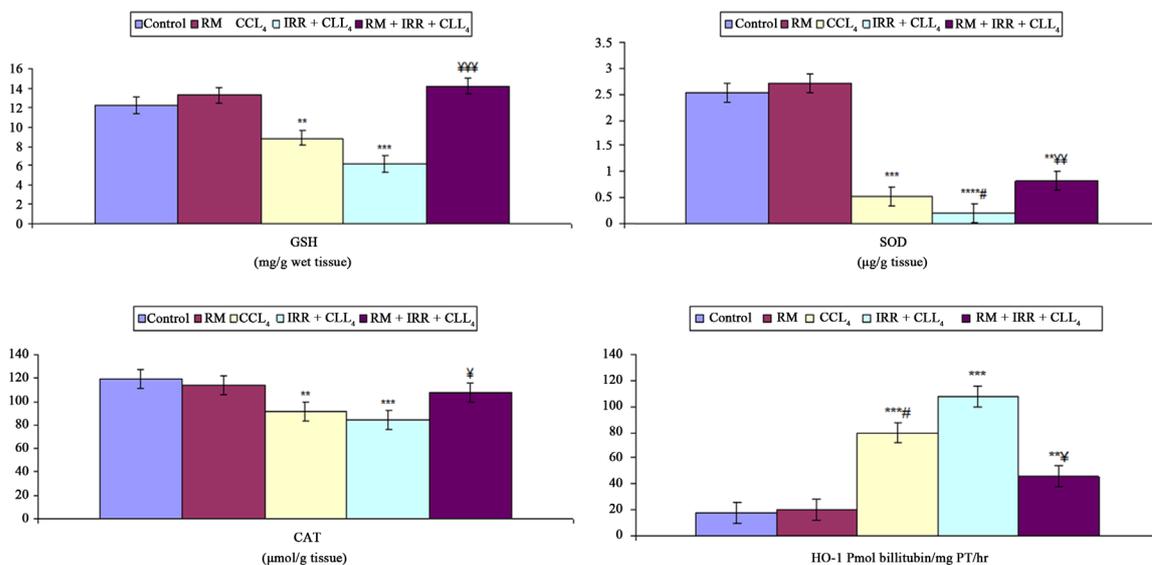


Figure 6. Effect of RM on GSH content, SOD and CAT activities and HO-1 in liver homogenates of fibrogenic rats. Each value represents the mean \pm SEM. Significant difference versus corresponding control group at $***P < 0.001$, $**P < 0.01$. Significant difference of IRR + CCL₄ versus corresponding CCL₄ group at $#P < 0.05$. Significant difference of RM + IRR + CCL₄ versus corresponding IRR + CCL₄ group at $¥P < 0.05$, $¥¥P < 0.01$, $¥¥¥P < 0.001$.

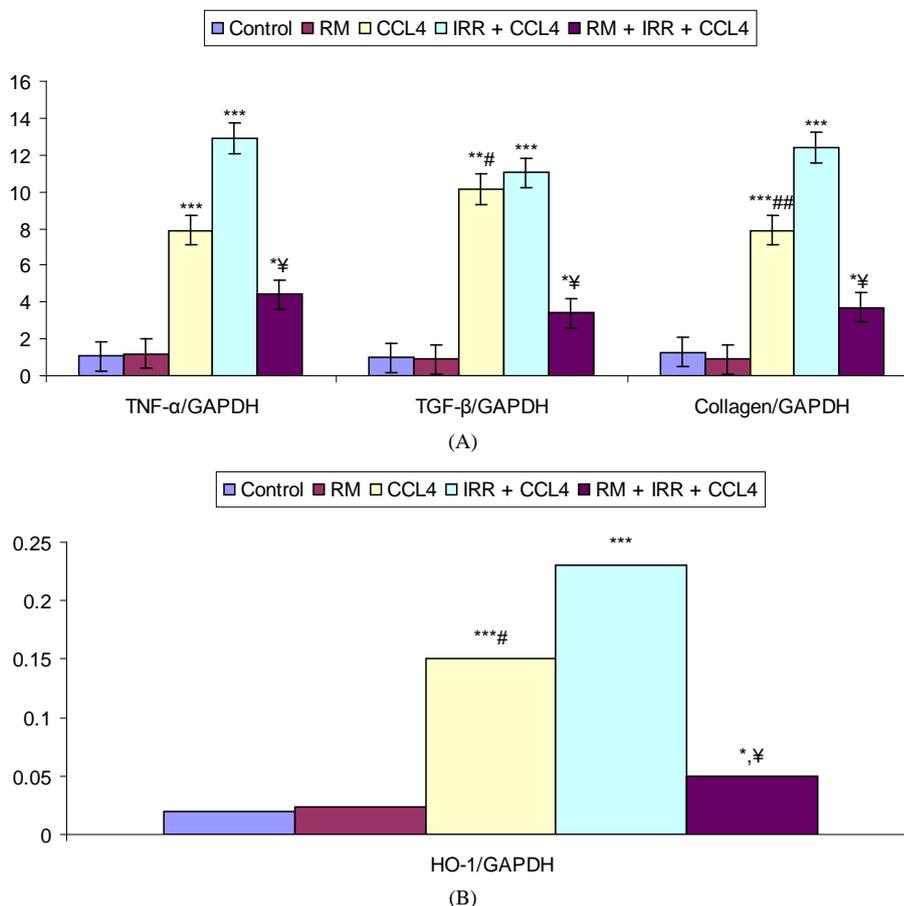


Figure 7. Effect of RM on (A): mRNA levels of TNF- α , TGF- β and collagen type-1; (B): HO-1 of fibrotic rats as analyzed by RT-PCR in liver homogenates. Each value represents the mean \pm SEM. Significant difference versus corresponding control group at *** $P < 0.001$, ** $P < 0.01$. Significant difference of CCl₄ versus corresponding IRR + CCl₄ group at # $P < 0.01$, ### $P < 0.001$. Significant difference of RM + IRR + CCl₄ versus corresponding IRR + CCl₄ group at ¥ $P < 0.001$.

However, treatment of fibrotic rats (IRR + CCl₄) with RM was able to significantly ($P < 0.001$) down regulate the mRNA expression of the previous parameters in hepatic tissue when compared to model 2.

3.8. Histopathological Examination

Liver of control as well as RM treated rats' revealed normal histological appearance. While examination of different liver sections of CCl₄ treated rats revealed massive destruction and alteration in the normal hepatic histology. Centrilobular congestion was evident with disorganization of the hepatic cords and massive fatty change that reached to marked fat steatosis. Necrosis of the hepatocytes as small groups of cells and as single cell necrosis (apoptosis) (Figure 8(A)) was clearly observed in most cases. The portal triads showed inflammatory cells infiltration and marked oval cell hyperplasia (Figure 8(B)) that begins to insinuate among the hepatic parenchymal cells with an obvious abundant collagen fibers accumulation (Figure 8(C)). The hepatic normal architecture was unclear with expansion of fibrosis in portal area and peripherally. The expanded fibrosis formed slender fibrous septa that led to conspicuous bridging fibrosis (that demonstrated by Azan stain) (Figure 8(D) and Figure 8(E)) at which the neighboring portal areas were bridged by fibrous septa with beginning of pseudolobulation formation of the hepatic parenchyma. That septa contained inflammatory cells, fat steatotic cells, hyperplastic oval cells and fibroblasts (Figure 8(F)). On the other hand, microscopical examination of livers' sections of IRR + CCl₄ treated rats revealed that the exposure of rats to irradiation aggravated the effect of CCl₄ in fibrosis induction. The arrangement of the hepatic plate was completely distorted with remarkable fat steatosis. The hepatocytes showed necrosis in fragments with appearance of remnants of degenerated and necrotic

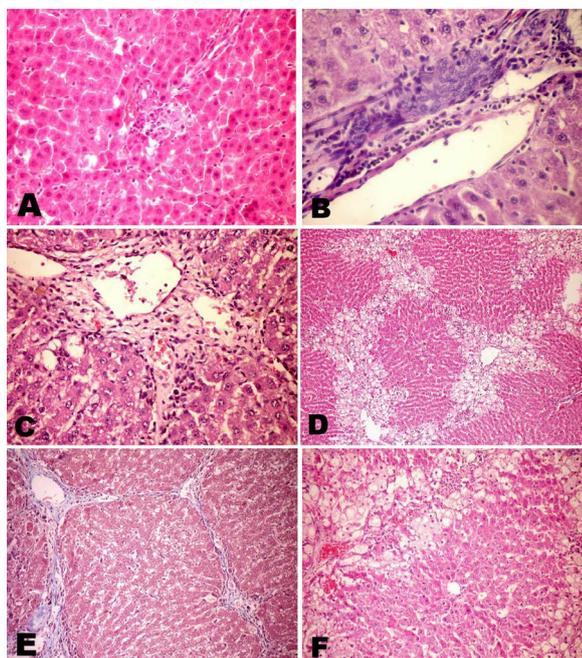


Figure 8. Livers of CCl₄ treated rats showing: (A) area of hepatocellular necrosis with multiple apoptotic bodies (arrow); (B) Portal triads with inflammatory cells infiltration and marked oval cell hyperplasia; (C) Abundant collagen fibers accumulation in the portal area and its extension peripherally; (D) Bridging fibrosis between the neighboring portal areas with apparent pseudolobulation formation; (E) Bridging fibrosis (Azan stain); (F) Fibrous septa contained inflammatory cells and fat steatotic cells with hyperplastic oval cells and fibroblasts. (H & E ×400, 100 and 200).

hepatocytes containing acidophilic hyaline globules (**Figure 9(A)**).

Extensive collagenous formation was observed that was even showed along the hepatic sinusoids and surrounded the necrotic hepatocytes (**Figure 9(B)** and **Figure 9(C)**). Bridging fibrosis was marked and the collagenous septa were much thicker than those of the sole CCl₄ treated group causing evident pseudolobuli formation.

It was noticed that RM co-administration to IRR + CCl₄ treated rats could undoubtedly improve the state of steatosis (**Figure 9(D)**) and increased number of apoptotic bodies as well as apparently suppressed hepatic fibrogenesis. The later was evidenced either by absence of fibrotic septa or in few cases by reducing the thickness of bridging fibrotic septa although collagen fibers accumulation and proliferation in the portal triad was still existed (**Figure 9(E)**). No obvious pseudolobulation (**Figure 9(F)**) was observed in most cases.

3.9. Results of Immunohistochemistry

Control and RM treated rats showed positive α -SMA staining around central vein and portal vein indicating normal expression of myofibroblasts. While, in CCl₄ and IRR + CCl₄ treated groups, α -SMA positive cells were markedly increased in the portal area, around the central vein and along the bridging fibrosis, exhibited the spread of collagen fibers from portal area (**Figure 10(A)**). Stronger pattern of positivity were observed in IRR + CCl₄ treated rats that even extended in the perisinusoidal spaces (**Figure 10(B)**). However, the expression of α -SMA positive cells was decreased in RM + IRR + CCl₄ treated rats and only observed in the portal area along the accumulated collagen fibers and around the central vein.

4. Discussion

In the current study, two models of hepatic fibrosis were generated experimentally in rats along six weeks. It was observed that the exposure to gamma irradiation in the 2nd model could promote and enhance CCl₄ induced fibrosis more than the sole use of CCl₄ in the 1st model. Moreover, the pathological extent of two models of fibrosis

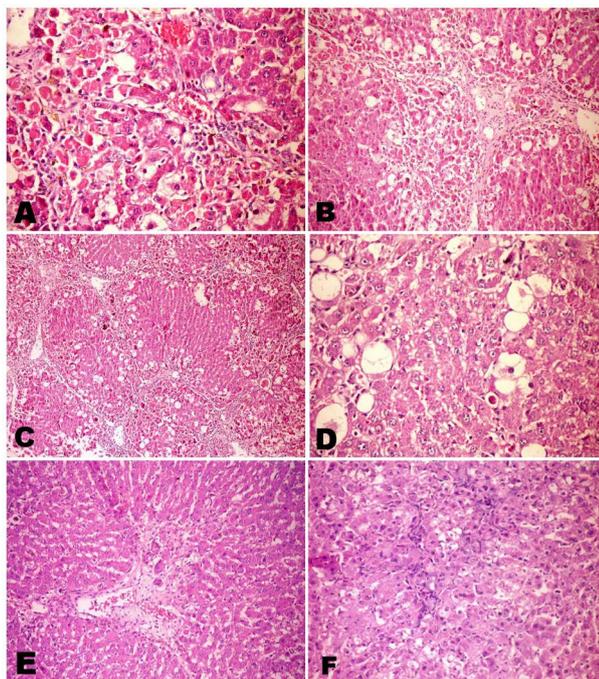


Figure 9. (A), (B), (C) Livers of IRR+ CCl₄ treated rats showing: (A): Complete distortion of the hepatic plate with necrosis of hepatocytes in fragments; (B) and (C): Extensive collagenous formation with its interpolation along the hepatic sinusoids and surrounded the necrotic hepatocytes; (D)-(F): Livers of RM + IRR + CCl₄ treated rats showing; (D): Improvement of the state of steatosis with marked apoptotic bodies (arrow); (E): Collagen fibers accumulation and proliferation only in the portal triad. (F): Inhibition of hepatic fibrogenesis with no obvious pseudolobulation. (H & E ×400, 100 and 200).

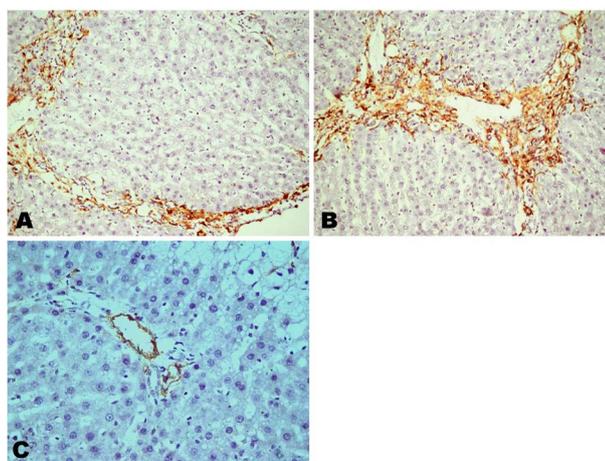


Figure 10. (A) Liver of CCl₄ treated rat showing positive α -SMA along the fibrous septa; (B) Liver of IRR + CCl₄ treated rat showing strong positivity of alpha-SMA in the portal areas and extending along the pseudolobulation; (C) Livers of RM + IRR + CCl₄ treated rats showing expression of α -SMA only in the portal area around portal vein.

and the protective effect of RM against the 2nd model were further evaluated by biochemical and histopathological analysis in this study.

It is well known that: 1) CCl₄ is metabolized by cytochrome P450 in liver into the highly reactive trichloromethyl radicals (CCl₃[•]) and trichloromethylperoxy radicals (CCl₃O₂[•]) resulting in initiation of cascade of lipid peroxidation, cell necrosis, steatosis, inflammation; 2) and this compound further promotes progression of hepatic

[29] [30] which is largely mimics hepatic fibrosis in human diseases [31]. Moreover, the exposure to ionizing radiation can lead to an increase in the generation of ROS leading to lipid peroxidation and oxidative stress [32]. The involvement of ROS and lipid peroxidation in hepatic fibrosis has been reported [33]. Accordingly, in the present work aggravation of fibrosis in the 2nd model was expected due to excessive production of ROS and lipid peroxidation by gamma irradiation [32].

There was a significant decrease in body weight gain of animals of both fibrotic models, which could be contributed to anorexia. Similar reduction in body weight caused by IRR as well as CCl₄ has been well documented [34]. Moreover, the increase in liver weight index in the two models follows the same pattern of previous researchers [35] who reported that the increase or decrease in either absolute or index of an organ weight after administration of a chemical or drug could be contributed to the toxic effect of that chemical. The co-treatment of RM to IRR + CCl₄ treated rats caused significant restoration in weight gain and significantly reduced the increase in liver weight index, which provided an evidence of the hepato-protective of RM.

The present study showed that the levels of ALT and AST, TP, Alb, TNF- α , TGF- β were significantly increased in the rats of the two models compared to control. The increased levels of hepatic function markers have been attributed to the liver injury and the release of these enzymes into the blood circulation after the administration of hepatotoxine; such as CCl₄ [36]. In addition, the damaged effect of hepatocytes could be due to liberation of large amount of free radicals that induced peroxidative degeneration of membrane lipids and formation of peroxides which probably caused membrane damages [37] and thus cellular alteration.

Total protein and albumin are clinically useful markers of hepatic synthetic function [38]. In agreement with other reports [39], our data indicated that induction of liver fibrosis in rats caused a significant diminution in total protein and albumin which was a further indication of liver damage. However, simultaneous administration of RM with IRR + CCl₄ contributes a hepatoprotective mechanism which stimulated protein synthesis and subsequently accelerates the process of regeneration and production of new hepatic cells [40].

Moreover, the observed increase in plasma levels of TNF- α and TGF- β in rats of the two models was accompanied with increased their hepatic mRNA expression. Accumulating evidence supports the concept that CCl₄ causes lipid peroxidation that leads to hepatocellular membrane damage and followed by the release of pro-inflammatory mediators, which are thought to potentiate CCl₄-induced hepatic damage [41]. Others demonstrated that irradiation induced chronic inflammatory mediators such as rapid activation of TGF- β [42] and TNF- α [43] resulted in fibrosis [44]. RM administration to IRR + CCl₄ treated rats significantly reduced the elevated levels of the two cytokines in plasma associated with reduction in their mRNA expression in liver tissue. This result is in close relationship with early results which suggested that the suppression that suppression of inflammation and reduced TGF- β level have been proposed as a molecular mechanism involved in protection against hepatic fibrosis [45].

Furthermore, our study indicated high HP content in the two established fibrosis models indicated the progression of fibrosis which was assured by the observed up regulation of collagen expression especially in the 2nd model. Hydroxyproline, is an amino acid and a characteristic product of collagen metabolism, its content indicated the total collagen present in liver. In consistent with our findings Fu *et al.* reported an elevated level of HP in response to CCl₄ induced fibrogenesis [46]. This result could be due to activation of the pro-fibrogenic cytokine TGF- β and promotion of fibrosis via activation of HSCs [47] and expression of collagen. In addition, gamma irradiation was demonstrated to cause rapid activation of TGF- β [42]. However, oral administration of RM could reduce HP concentration and consequently reduce collagen deposition and suppress the fibrogenic effect induced by CCl₄ alone or by combined IRR + CCl₄ treatments.

In the current investigation, a significant elevation in MDA and NO levels was recorded associated with significant reduction in GSH concentration, SOD and CAT activities in both fibrotic groups. This is an indication of fibrosis induced oxidative stress in liver. In agreement with our results, early reports demonstrated enhancement of lipid peroxidation after whole body irradiation [48] and CCl₄ treatment [49]. The increase in MDA and NO levels could be attributed to high degree of oxidative stress and over production of free radicals and ROS which attack macromolecules such as lipids and proteins initiating lipid peroxidation, thereby, causing depletion of antioxidants.

However, administration of RM to model 2 rats significantly lowered MDA and NO levels, prevented the depletion of GSH and enhanced the activity of antioxidant enzymes as well. This result could be due to the strong free radical scavenger and antioxidant activity of RM which endorsed by the presence of active compounds that are responsible for the hepatoprotective activity as well as the reduction of the free radicals that induce

oxidative damage to the liver [50].

Regarding, heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme catabolism that is induced by a variety of stimuli including oxidative stress and pro inflammatory cytokines. Our current investigation revealed significant elevation in HO-1 activity and its mRNA expression in hepatic tissue of fibrotic rats. Early reports recorded an elevation in HO activity and its mRNA expression in hepatic tissue caused by whole body gamma irradiation [51]. Moreover, in a recent study, an abnormal activity of HO-1 after CCl₄ treatment has been evidenced [52]. That elevation may be probably due to oxidative stress and inflammation induced by both CCl₄ and gamma irradiation. However, concurrent treatment of RM along with IRR + CCl₄ brought down the HO-1 activity and reduced its mRNA expression in hepatic tissue. The protective effect of RM could be due to its strong antioxidant activity.

Our histopathological results revealed marked hepatic tissue alterations as a result of CCl₄ and IRR + CCl₄ treatments. In addition, the induction of fibrosis models by using composite factors both of IRR and CCl₄ was succeeded as observed by more clear bridging fibrosis and pseudolobulation of livers of rats exposed to IRR + CCl₄ which encouraged the role of IRR in aggravation of hepatic fibrogenesis induced by CCl₄. The later effect could be related to the production of large amount of free radicals by both CCl₄ and gamma irradiation which by their direct toxic effect could lead to lipid peroxidation and activation of an immune-inflammatory mechanisms that could result in functional and morphological alterations and even cell death [53] [54].

Our results are in agreement with those of [55] [56] who noted that CCl₄ administration to mice could induce portal fibrosis and bridging fibrosis as well as regenerating nodules. The observed bridging fibrosis and pseudo lobulation in IRR + CCl₄ treated rats were accompanied by increased positivity of α -SMA wherever the collagen fibers deposited. That immunopositivity of α -SMA indicates activation of HSCs which were responsible for the fibrosis occurrence in CCl₄ and IRR + CCl₄ treated rats. The later attribution is in agreement with that of [57] who mentioned that; at HSCs activation, the levels of α -SMA and desmin increase with decreased GFAP expression due to their transformation into myofibroblast-like cells. Moreover, it has been reported that hepatocytes which undergoing oxidative stress and release of ROS stimulate HSC activation. Prior studies have demonstrated that chronic hepatic fibrosis and inflammation accompanied with Kupffer cell accumulation, HSC activation and collagen deposition [58] [59]. The observed amount of eosinophilic hyaline globules of variable sizes in fibrotic lesions of IRR + CCl₄ treated rats was correlated with increased collagen deposition and the degree of liver fibrosis. Such bodies or globules could be seen in the hepatocytes in alcoholic and nonalcoholic liver disorders [60]. Those bodies represent abnormal protein aggregates that could be reverted to normal state by molecular chaperones or degraded by proteasomes [61]. However, if reparation or degradation processes fail, abnormal proteins became segregated in the cytoplasm as inclusion bodies. On the other hand, RM administration to IRR + CCl₄ treated rats' revealed great ameliorative action of RM on the deleterious effects of both CCl₄ and IRR as evidenced by clear absence of bridging fibrosis and pseudolobulation. Such hepato protective and fibrosis-inhibition effects of RM have been recorded [17] [18]. This effect per se may be related to the antioxidant effect of RM' constituents and its scavenging activity of free radicals that reflected on saving the integrity of cellular membranes thus suppressing the inflammatory response and attenuated the fibrosis action.

5. Conclusion

In summary, this study provided evidence for experimental model of hepatic fibrosis using gamma irradiation and CCl₄. The present study also suggests that RM aqueous solution showed a considerable hepatoprotective activity against IRR + CCl₄ induced hepatic fibrosis and injury in rats. This protective effect could be due to its membrane cellular protection, free radicals scavenging activity, enhancing the endogenous antioxidant system, suppressing the inflammatory responses and attenuation of fibrogenesis. The histopathological study confirmed the biochemical findings.

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