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Original Article

Combination of N-acetyl Cysteine and Thymoquinone Alleviates Hepatocellular Toxicities by Radiation and CCl_4 Intoxication in SD Rats

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ABSTRACT

Radiation and chemicals were the major clinical toxicants known to cause cellular damage during prognostic interventions *in vivo*. Cellular and molecular damages in the liver were the major causes for the hepatocellular injury due to various toxicities. Though many antioxidants alleviate various types of hepatotoxicities, protection exerted by the combination of N-acetyl cysteine (NA) and Thymoquinone (TQ) in the combined toxicities of radiation and carbon tetrachloride (CCLR) in Sprague-Dawley (SD) rats were unknown. Current research was focused on the protective efficacy of combination of NA with optimized dose of TQ (NATQ) in radiation/CCl_4 (CCLR)-induced hepatotoxicities. At the end of the experimental period (6 weeks), body weight, liver weight, serum and liver tissues were analyzed for marker enzymes (AST, ALT, LDH), oxidative stress level (MDA, GSSG), antioxidant status (GSH, Vitamin E, Vitamin C), activities of enzymatic antioxidants (SOD, GPx, CAT, GST), liver histopathology and studies for the hepatic levels of NfkB, IL-6, TNF-α, MMP-3, MMP-12, Nrf2 and HO-1 were done. Significant ($p \leq 0.05$) toxicological alterations in the above parameters were recovered to normal in the treatment of NATQ combinations in SD rats. In conclusion, we provide evidence of protective efficacy of NATQ combination in alleviating the hepatotoxicities produced by CCLR in SD rats.

Keywords: N-acetyl cysteine, Thymoquinone, Carbon tetrachloride, Radiation toxicity, NfkB, MMP.

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INTRODUCTION

Hepatocellular toxicity is becoming major worldwide health issue because of increased use of chemicals, certain medications like antifungal agents and exposure to radiation. Liver is the primary organ for xenobiotic metabolism. Due to oxidative stress and inflammation hepatocytes are damaged in this process of detoxification. Cases of Drug Induced Liver Injury (DILI) is found to be more in China, 23.80 per 10000 compared to other countries which were studied.[1] Drugs such as antitubercular, non-steroidal anti-inflammatory, anti-hyperlipidemic, anti-rheumatic and antiepileptic drugs cause DILI, depending on dosage, time, age and sex.[2] Another major cause of liver toxicity is radiation. Exposure to radiation from electrical appliances, communicating device has been significantly increased.[3] Exposure to radiation in cancer patients on radiotherapy causes extensive damage.[4] Radiation Induced Liver Injury (RILD), due to radio therapy in cancer patients is need to be addressed, that too in case of patients with deranged liver function.[5] No effective treatment for the hepatocellular toxicity caused by xenobiotics and radiation were documented till date.

Carbon Tetrachloride (CCl_4) has been used to induce reversible liver toxicity in animal models for research. Dimethyl nitrosamine, thioacetamide, D-galactosamine also reported to cause hepatocellular toxicity.[6] The pathology of liver disease of CCl_4 induced toxicity is similar to human cirrhosis.[7] CCl_4 causes increase in serum AST, ALT and GSH.[8] It also causes

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increase in lipid peroxidation which eventually leads to elevated MDA and also causes changes in ECM proteins.[9] Exposure to radiation affects serum enzyme markers, antioxidant levels and ECM proteins.[10] Prominent changes in hepatic cells were reported in CCl_4 and radiation induced hepatocellular toxicity.[8] Hepatocellular toxicity induced by drugs and radiation leads to fatal health issues which needs to be addressed with development of new therapy. Many plant extracts, phytochemicals and food supplements are proved to be promising in alleviating effects of liver toxicity. Silibinin, matrine, oxymatrine, periplocoside A, glycyrrhizin, saikasaponoin C/b2, baicalin, baicalein are studied for its liver protective property.[11] Thymoquinone is phytochemical, a monoterpenoid found in the plant *Nigella sativa*. The use of seed and its oil in Chinese, Indian and Arabic traditional medicine is a known fact. Thymoquinone is well studied for its antimicrobial, cardioprotective, anticancer, hepatoprotective properties. It is also being studied for any promising effects for COVID-19 treatment.[12] Thymoquinone can effectively restore the liver function in CCl_4 toxicity.[13] The studies on effect of many plant extracts and individual phytochemicals gave insight for the investigation of thymoquinone for its hepatoprotective activity.

N-acetyl Cysteine (NAC) is FDA approved drug known for its use in medication induced poisoning, a positive regulator of redox homeostasis[14] and as mucolytic agent.[15] This acetylated form of L-cysteine is a proven chelating agent in metal poisoning.[14] NAC acts as nucleophile and also-SH donor, this makes it to detoxifying agent. It also promotes glutathione synthesis.[16] NAC alleviate CCl_4 induced toxicity by its antioxidant and anti-inflammatory property.[17] It is also found to be effective in attenuating X-ray induced liver injury.[18] Comparative study of NAC (100mg/kg body weight for 25 days) and TQ (20mg/kg body weight for 25 days) in alleviating uranium induced toxicity proves TQ is more effective than NAC.[19] Although hepatoprotective properties of N-acetyl cysteine and thymoquinone were reported separately, this is the first study which projects the combining efficacy of N-acetyl cysteine and thymoquinone in hepatotoxicity induced by the combination of CCl_4 and Radiation (CCLR).

MATERIALS AND METHODS

Chemicals and kits

The following chemicals were procured from Sigma Aldrich, USA: CCl_4, N-acetyl cysteine, thymoquinone, hematoxylin and eosin (H&E) staining dye, phosphate-buffered saline (PBS). Commercial kits for AST, ALT, LDH, Vitamin E, Vitamin C, catalase, SOD, GPx, GSH, GSSG and MDA were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. GST kit was purchased from Abcam, China. ELISA kits for analysis of cellular signaling molecules (TNF-α, NFkB, IL-6, Nrf2, HO-1, MMP3 and MMP12) were purchased from MyBioSource, Inc., USA. All other chemicals used for this study were procured from reputed companies with highest purity and quality.

Experimental design

For this study, 32 Sprague-Dawley male rats, 9 weeks of age and 200±10 g of body weight were used. After acclimatization in our laboratory, these animals were divided into 4 groups of 8rats in each group. The study was conducted for 6 weeks, during this period rats were maintained in cage with 12 hr day/light cycles. Rats were given full access to fresh drinking water and food. The grouping of animals for study was as followed:

Group 1 [C]-Control animals were injected with normal saline for 6 weeks.

Group 2 [NATQ]-NA and TQ were dissolved in DMSO and diluted with normal saline, administered for 6 weeks.

Group 3 [CCLR]-CCl_4 and Gy irradiation (Whole body).

Group 4 [CCLR+NATQ]-CCl_4 and Gy irradiation administered and NATQ treated.

The group 1 rats were injected intraperitonially with normal saline. The group 2 animals were injected intraperitonially with thymoquinone 40mg/kg body weight and N acetyl cysteine 150 mg/kg body weight twice a week for 6 weeks. Group 3 animals were injected intraperitonially with 50% CCl_4 at dosage of 1mL/kg body weight twice a week for 6 weeks. Whole body irradiation was administered by anesthetizing rats using pentobarbital sodium (50mg/kg body weight) and irradiation done once a week for 6 weeks with 6Gy dosage each time with Gamma irradiator (≈0.4Gy per minute). The animals were allowed to recover senses naturally. The group 4 animals were treated similar to group 3 and given NA and TQ at same dosage as that of group 2 after a day of CCl_4 and irradiation. All the above protocols were submitted to institutional animal ethics committee and the approval for the same was obtained before the initiation of the animal research (Approval number: 202110-64).

Sample collection

Blood samples from all the experimental animals were collected from retro-orbital plexus of the eye socket vein. Samples were centrifuged at 3000 rpm for 10 min, and the serum samples obtained were used for the analysis or stored under appropriate condition for later use. The liver tissues for histopathological studies were taken by sacrificing the animals and were fixed in 10% formalin for further analysis.

Measurement of body weight and liver weight

The body weights of all the experimental animals were measured before and after experimental period. Changes in the body weight were compared at the end of experimental period. The liver weight was measured after 6 weeks of study upon sacrificing the animals. The percentage ratio of liver weight to body weight gives relative liver weight.

Serum marker enzymes

Serum collected was quantified for AST, ALT, and LDH using kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China. (C010-3-1, C009-3-1, A020-1-2respectively), provided by manufacturer and the same protocol was followed.

Cellular oxidative stress markers

All sample preparations were done as per manufacture's instruction. MDA was assayed in liver and serum using kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China (A003-1-1) in the presence of MDA. Thiobarbutric Acid (TBA) forms a red complex that has an absorbance at 532 nm. On comparing with standards supplied in the kit the concentration of MDA was calculated as per instruction in the kit and expressed as nmol/g for liver; nmol/L for serum. GSH was quantified in liver and serum using kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China (A006-1-1), expressed as nmol/g for liver; nmol/L for serum, based on the principle of oxidation of GSH by 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5'-thio-2-nitrobenzoic acid (TNB), and absorbance was read at 412 nm. GSSG was measured in liver tissue using kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China (A061-2-1). The enzymatic recycling method used to determine GSSG. The sample treated with 2-vinylpyridine to eliminate the GSH, then GSH assay done to determine GSSG. The end product was read at 405nm. Calculation done using formula given in the instruction, quantity of GSSG expressed as nmol/g.

Vitamin C and Vitamin E analysis

Vitamin C and E were analyzed using kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China. A009-1-1, A008-1-1 respectively). These assays were based on the reduction of Fe^{3+} to Fe^{2+} by Vitamin C or E, which reacts with phenanthroline and the colored product with absorbance maxima at 533nm. The Vitamin concentrations were determined by comparing O.D of sample with standard values and were expressed as micromole/L using formula given in kit manual.

Protein assay

Liver tissue total protein assay performed by Lowry's method[20] using Folin's reagent, under alkaline condition copper ion reduced on reacting with peptide bond this reacts with Folin's reagent to give a product, its absorbance read at 650nm in spectrophotometer. BSA was used as standard. Protein concentration was calculated by comparing with O.D values of standard.

Enzymatic antioxidant assay in liver tissue

All the commercial kits procured by Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The Samples were prepared according to manufacturer's protocol. Catalase activity was determined in liver tissue using kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China, A007-1-1) and expressed as µmole H_2O_2 decomposed/min/mg protein. Ammonium molybdate can pause H_2O_2 decomposing reaction Catalyzed by Catalase (CAT) immediately, residual H_2O_2 can react with ammonium molybdate to produce a yellowish complex. CAT activity was calculated by measuring absorbance at 405nm. Glutathione-S-Transferase (GST) activity determined in liver tissue using kit (Abcam, China. ab65326) and expressed as nmol CDNB conjugated/min/mg protein. The GST activity calculated using conjugated CDNB, which can be detected by spectrophotometry reading absorbance at 340 nm. Superoxide Dismutase (SOD), activity measured by Xanthine oxidase method using kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China (A001-1-1). SOD has inhibitory effect on super oxide anion, which is produced by xanthine and xanthine oxidase system can oxidize hydroxylamine to form nitrite which appear purplish red after chromogenic reaction, absorbance measured at 550nm. Calculation was done as given in the manufacture's instruction and SOD value mentioned as U/mg protein. Glutathione Peroxidase (GPx) were assayed using commercial kits as per manufacture protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China (A005-1-1). The reaction of GPx with H_2O_2 was done in the presence of GSH. The unutilized GSH reacts with DTNB forms the product which was read at 412nm. Calculation was done as given in the manufacture's instruction and GPx activity was expressed as µmole GSH oxidized/min/mg protein.

Liver tissue histological studies

Liver tissue was kept it 10% formalin solution, dehydration was done using graded ethanol, tissues was embedded in paraffin and sliced into 4µm thickness, followed by Hematoxylin-Eosin staining and were studied under microscope. Images were recorded for further analysis.

ELISA for Nfkb, Nrf2 and HO-1

In order to quantify the cell signaling mediator Nfkb, Nrf2, HO-1 in the liver tissue, experimental protocol was followed as per manufacturer's instruction and ELISA was done using the commercially available kits (MyBioSource, Inc., USA). For Nrf2 quantification, liver tissue was rinsed in ice cold PBS, minced, homogenized, centrifuge at 1000xg for 20min at 2 to 8°C, supernatant collected for assay. The test is based on sandwich ELISA, pre-coated biotin conjugated Nrf2 antibody binds to sample Nrf2, then addition enzyme conjugated avidin followed by chromogen A and B produce color change, then enzymatic reaction is terminated by adding stopping solution and read at 450nm in ELISA reader. Concentration of Nrf2 was calculated by comparing O.D value of sample to standard curve. The results expressed as ng/mg protein. ELISA assay for Nfkb and HO-1, implies competitive enzyme immunoassay. The sample preparation is same as that of Nrf2. The Nfkb and Nfkb HRP conjugate competes for binding site in anti-Nfkb antibody. The

Table 1: Hepatoprotective activity of NATQ on body and liver weight in CCLR induced toxicity.

Parameter	Control	NATQ	CCLR	NATQ+CCLR
Initial BW (g)	203.25±6.61	204.25±8.41	205.88±8.36[a*]	209.5±8.88[b*]
Final BW (g)	218.5±6.57	219.75±6.07	179.75±9.53[a*]	203.25±7.29[b*]
Liver Wt (g)	6.01±0.26	6.64±0.39	5.05±0.41[a*]	5.61±0.45[b*]
LW/BW ratio(g)	2.75±0.05	3.02±0.11	2.81±0.11[a*]	2.76±0.14[b*]

(NATQ-N-acetyl cysteine and thymoquinone, CCLR-CCl_4 and Gy irradiation treated, CCLR+NATQ-CCl_4 and Gy irradiation treated and NATQ treated. BW-body weight, Wt-weight, LW-liver weight)Average of each group was calculated, expressed as mean±SD. Comparison were made as follows:"a"-compared with control; "b"-compared with CCLR; "*"-significance $P<0.05$. Statistical analysis was calculated by one way ANOVA with an appropriate *post-hoc* analysis. Activities of marker enzymes

concentration of Nfkb calculated as per manufacture's instruction and expressed as ng/mg protein. The assay HO-1 is same as that of Nfkb, HO-1 and HO-1 HRP conjugate competes for binding site in anti-HO-1 antibody. The concentration of HO-1 calculated as per manufacture's instruction and expressed as ng/mg protein.

ELISA for pro-inflammatory cytokines (IL-6 and TNF-α)

IL-6 was measured using ELISA kit (MyBioSource, Inc., USA (Catalogue no-MBS2021530) and TNF-α using (Rat TNF-α PicoKine ELISA Kit, MBS175904). All procedures followed as per manufacture's instruction. Liver tissue was rinsed in ice cold PBS, minced, homogenized in lysis buffer (MyBioSource, Inc., USA, Catalogue no-MBS2090451), sonicated till solution was clarified, centrifuged at 1500xg for 10 min at 2 to 8°C, supernatant collected for assay. The test was based on sandwich ELISA, pre-coated biotin conjugated IL-6 antibody binds to sample IL-6, followed by the addition of enzyme conjugated avidin followed by TBM to produce color change, and the enzymatic reaction was terminated by adding sulphuric acid and read at 450nm in ELISA reader. Concentration of IL-6 was calculated by comparing O.D value of sample to standard curve. The results were expressed as ng/mg protein. The assay principle was same for TNF-α, except the plate coated with biotin conjugated TNF-α antibody.

ELISA for MMP-3 and MMP-12

The assay of MMP-3 and MMP-12 was done using kit procured from MyBioSource, Inc., USA (MBS704398 AND MBS2023016 respectively). Tissue sample were prepared as per manufacture's instruction. The test is based on sandwich ELISA, pre-coated biotin conjugated MMP antibody binds to sample MMP, then addition enzyme conjugated avidin followed by TBM produce color change, then enzymatic reaction is terminated by adding sulphuric acid and read at 450nm in ELISA reader. Concentration of MMP was calculated by comparing O.D value of sample to standard curve.

Statistical analysis

The data recorded were used for statistical analysis using SPSS 20 software. The results were expressed as mean±SD. One-way ANOVA was performed to compare the differences between the groups with an appropriate *post-hoc* analysis. A $p<0.05$ value between the groups was considered statistically significant.

RESULTS

Changes in body weight and liver weight

The body weight and liver weight of experimental animals were given in Table 1. Liver weight/body weight ratio indicates the relative liver weight. Significant decrease in body weight was observed after induced toxicity (Group 3) as compare to control (Group1). After treatment with NATQ (Group 4) increase in body weight suggested positive effect of NATQ. There was a slight increase in the liver weight in Group 4 compared to Group3. Table 1 showed that the positive effect of treatment with NATQ and was statistically significant ($p<0.05$).

Serum biochemical analysis recorded and data were represented in Figure 1(A-C). Increased levels serum AST, ALT and LDH observed in group 3 compared to that of group 1. Treatment with NATQ showed notable decrease of AST, ALT and LDH in group 4. This showed that NATQ has positive effects. Comparison between group 1 and group 3; group 3 and group 4 showed that decrease in the level hepatotoxicity biomarkers was statistically significant ($p<0.05$).

Cellular oxidative stress markers

Serum and liver MDA levels (Figure 2A and Figure 3A respectively) in group 1, was at basal level and also in group 2. Compared to control in toxicity induced animals (group 3), there was nearly three-fold increase in MDA, which proves induction of oxidative stress. Group 4 animal showed decrease in MDA compared to group 3. The comparison of group 1 and group 3; group3 and group4 shows statistical significance ($p<0.05$) of the data. The levels of GSH were shown in Figure 2B and 3B respectively. Decrease in serum and liver GSH level in group 3 was observed and restoration of the same was seen in group 4. The levels of liver GSSG (Figure 3C), was highly increased in group 3 and decreased notably in group 4, basal level was found in both group1 and 2.

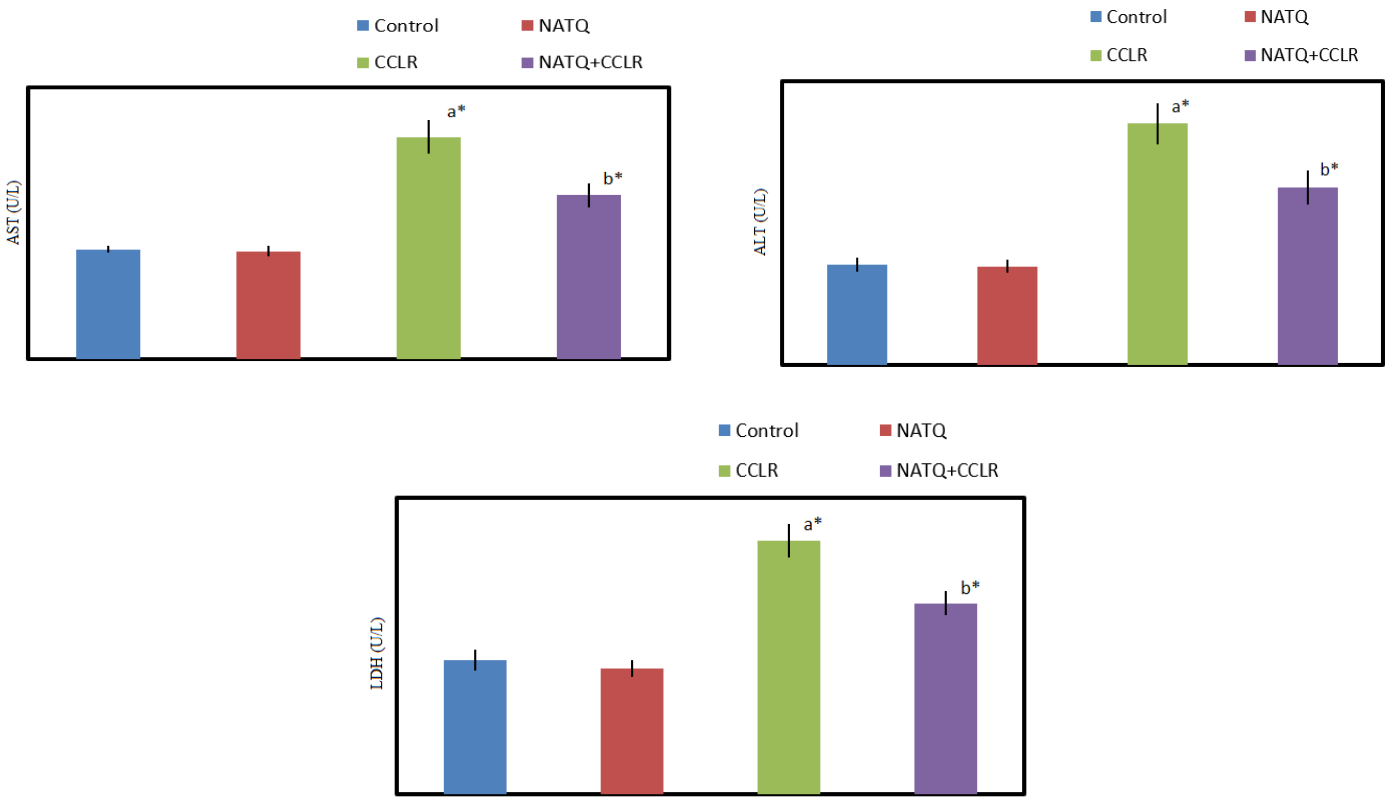

Figure 1: A-C: Hepatoprotective activity of NATQ on Serum marker enzymes in CCLR induced toxicity. (NATQ-N-acetyl cysteine and thymoquinone, CCLR-CCl_4 and Gy irradiation treated, CCLR+NATQ-CCl_4 and Gy irradiation treated and NATQ treated) Average of each group was calculated, expressed as mean±SD. Comparison were made as follows: "a"-compared with control; "b"-compared with CCLR; "*"-significance $p<0.05$. Statistical analysis was calculated by one way ANOVA with an appropriate *post-hoc* analysis.

Levels of Vitamin C and Vitamin E

Figure 4A and 4B shows average levels of Vitamin C and E. In group 1 and group 2 the levels of Vitamin E and C were similar. The levels of Vitamin E and Vitamin C had increased slightly in group 2 compared to group 1. Decreased levels of Vitamin C and E in group 3 were seen in hepatocellular toxicity, on treatment with NATQ, it was also increased in group 4. Comparison between group 1 and group 3; group 3 and group 4 showed data obtained is statistically significant ($p<0.05$).

Activities of enzymatic antioxidants

The activities of enzymatic antioxidants were given in Figure 5(A-D). SOD, CAT, GPx and GST activity were decreased in group 3, and in group 4 their levels were restored close to control. The comparison of group 1 and group 3; group3 and group 4 showed statistical significance ($p<0.05$) of the data. However, a mild increase in enzymatic antioxidants activities were noticed without statistical significance in the NATQ group, which proved the healthier effect of NATQ.

Liver histopathological analysis

The liver histology analysis (Figure 6) showed that liver cells were normal in case of group 1 and 2. The group 3 sections revealed the infiltration of cells, fibrosis, edema and necrosis. Striking difference was observed in group 4 tissue sections, which showed the restoration of normal hepatocytes architecture. This showed decrease infiltration of cells, fibrosis, edema and necrosis during NATQ treatment.

Analysis of cellular signaling molecules

The NFkB (Figure 7A) levels were increased in group3 animals and levels were restored close to control in group 4. Nrf2 (Figure 7B) and HO-1 (Figure 7C) decrease in group 3 and it was restored close to control in group 4. The ELISA assay for the cellular signaling molecules in liver tissues was done. Increased levels of TNF-α (Figure 8A) and IL-6 (Figure 8B) toxicity induced subjects (Group 3), observed in our study. There was a restoration of the same in near to that of control among the treated animals (Group 4). MMP3 (Figure 9A) and MMP12 (Figure 9B) were significantly increased in toxicity induced animals (Group 3), and restored in Group 4. Comparison between the groups had shown the difference was statistically significant ($p<0.05$), and thus NATQ was efficient in prevention of CCLR toxicities.

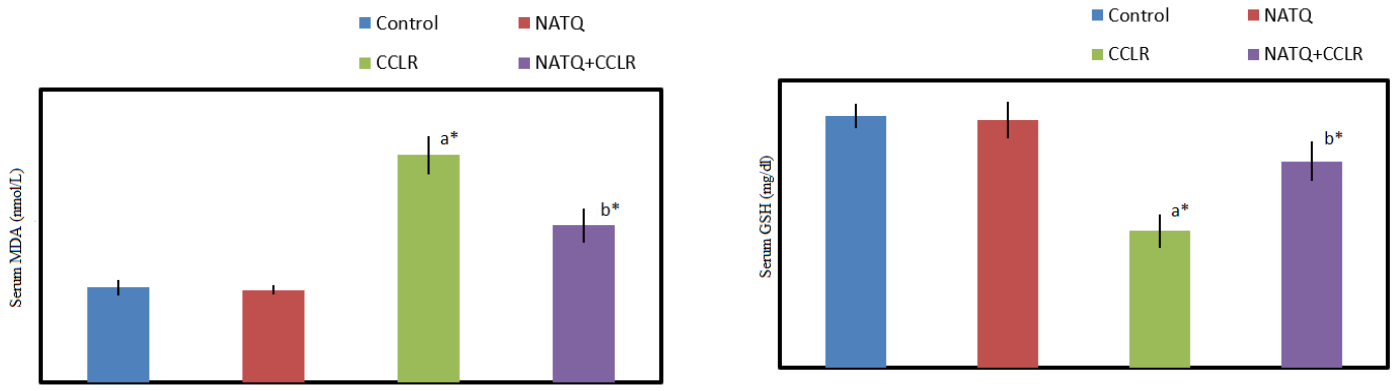

Figure 2: A-B: Hepatoprotective activity of NATQ on serum oxidative stress markers in CCLR induced toxicity. (NATQ-N-acetyl cysteine and thymoquinone, CCLR-CCl_4 and Gy irradiation treated, CCLR+NATQ-CCl_4 and Gy irradiation treated and NATQ treated) Average of each group was calculated, expressed as mean±SD. Comparison were made as follows: "a"-compared with control; "b"-compared with CCLR; "*"-significance $p<0.05$. Statistical analysis was calculated by one way ANOVA with an appropriate *post-hoc* analysis.

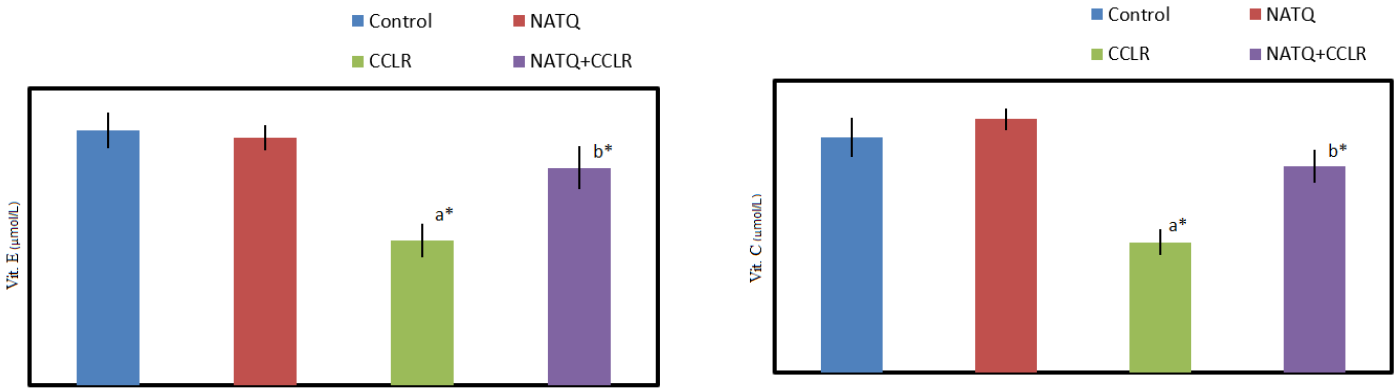

Figure 3: A-B: Hepatoprotective activity of NATQ on serum non enzymatic antioxidants in CCLR induced toxicity. (NATQ-N-acetyl cysteine and thymoquinone, CCLR-CCl_4 and Gy irradiation treated, CCLR+NATQ-CCl_4 and Gy irradiation treated and NATQ treated) Average of each group was calculated, expressed as mean±SD. Comparison were made as follows:"a"-compared with control; "b"-compared with CCLR; "*"-significance $p<0.05$. Statistical analysis was calculated by one way ANOVA with an appropriate *post-hoc* analysis.

DISCUSSION

N-acetyl cysteine was reported for its hepatoprotective activity.[16-18] Thymoquinone found in *Nigella sativa*, was extensively studied for its hepatoprotective activity.[12,13,19] This study aimed at revealing the combined efficacy of these two components on CCLR toxicity induced liver damage. Reduction in body weight has been reported in case of liver disease.[8] Significant decrease in body weight in CCLR toxicity supported the studies of body weight loss in liver diseases.[8] Thus, toxic effects CCl_4 and radiation was correlated well as reported earlier.[4,13] The notable increase in body weight in NATQ treated animals proved the efficacy of the same in treating liver diseases. In liver, toxicity due to radiation and oxidative stress causes the release of cytosolic hepatocellular enzymes AST and ALT, which eventually release into circulation causing serum levels to increase.[21] In our study, CCl_4 and radiation caused hepatotoxicity that was proved by the elevated levels of AST, ALT and correlated well with earlier studies.[21] Also, CCl_4 causes increase in LDH, which is useful in the prognosis of acute liver disease.[22] LDH levels in our study supported the same as we can see tremendous increase in the toxicity induced animals (group 3). Treatment with NATQ alone doesn't show much variation in LDH levels. However, in treatment group, decreased LDH levels suggested that NATQ alleviated the toxicity. Collectively, tissue damage released of the cellular enzymes AST, ALT and LDH, which was prevented in NATQ treatment.

Many studies projected the depletion of serum Vitamin E and Vitamin C levels in hepatotoxicity.[8] In this study, we reported that oxidative stress resulted due to CCLR toxicity and caused the decrease in levels of antioxidant Vitamins, as they were utilized by cells to combat the free radicals. On treatment with NATQ, notable increase in Vitamin E and C showed the cellular protective effects of NATQ in combination. In line with this earlier report, administration of NATQ resulted in increase of Vitamin E and C that were depleted during CCLR toxicities. The redox homeostasis of liver was maintained by enzymatic antioxidants and non-enzymatic antioxidants. The ROS produced due to

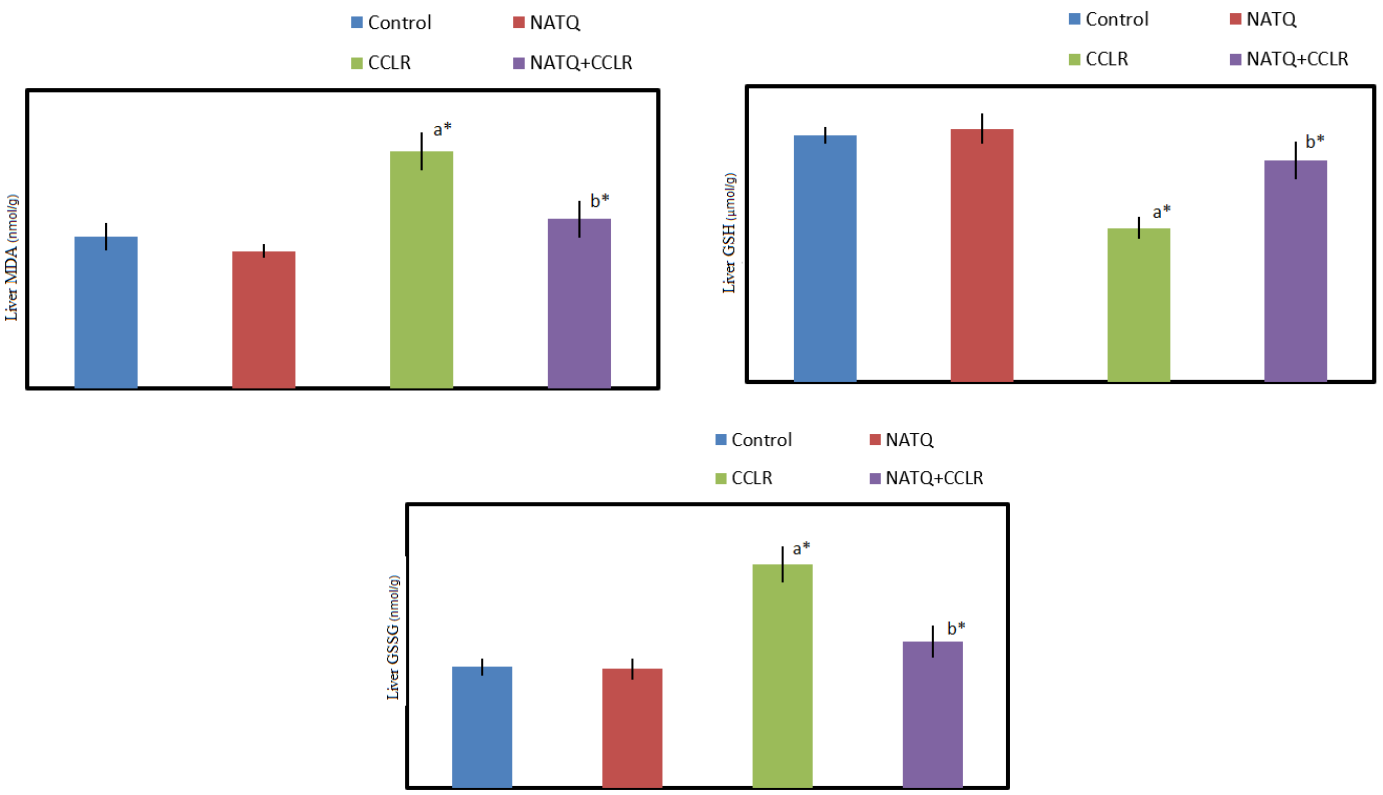

Figure 4: A-C: Hepatoprotective activity of NATQ on liver oxidative stress markers in CCLR induced toxicity. (NATQ-N-acetyl cysteine and thymoquinone, CCLR-CCl_4 and Gy irradiation treated, CCLR+NATQ-CCl_4 and Gy irradiation treated and NATQ treated) Average of each group was calculated, expressed as mean±SD. Comparison were made as follows: "a"-compared with control; "b"-compared with CCLR; "*"-significance $P<0.05$. Statistical analysis was calculated by one way ANOVA with an appropriate *post-hoc* analysis.

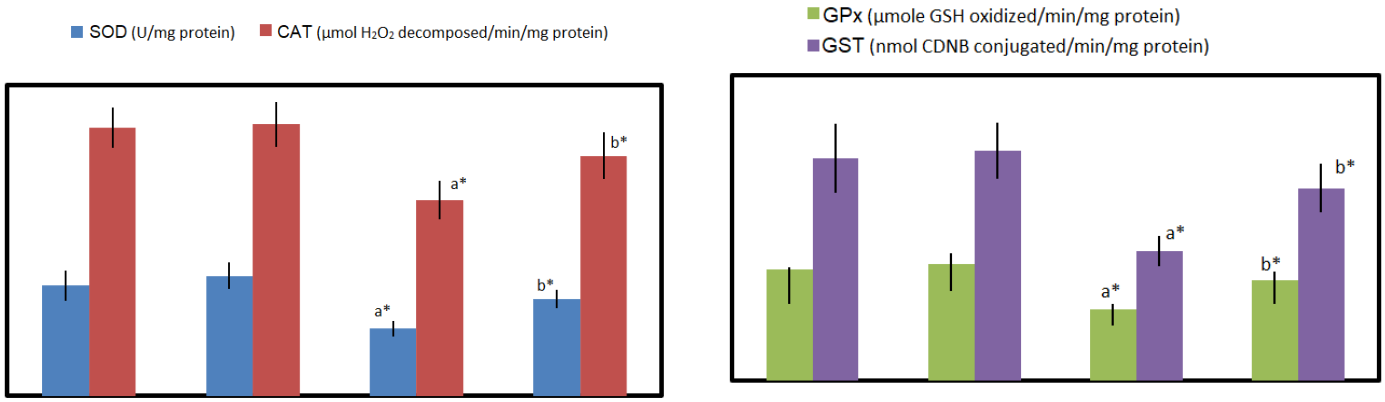

Figure 5: A-D: Hepatoprotective activity of NATQ on activity of liver enzymatic antioxidants in CCLR induced toxicity. (NATQ-N-acetyl cysteine and thymoquinone, CCLR-CCl_4 and Gy irradiation treated, CCLR+NATQ-CCl_4 and Gy irradiation treated and NATQ treated) Average of each group was calculated, expressed as mean±SD. Comparison were made as follows: "a"-compared with control; "b"-compared with CCLR; "*"-significance $p<0.05$. Statistical analysis was calculated by one way ANOVA with an appropriate *post-hoc* analysis.

drugs, radiation and many pathological conditions induces oxidative stress. Lipid peroxidation occurs because of oxidative stress, which results in production of malondialdehyde (MDA) causing its increase in the circulation.[22] MDA is a marker of oxidative stress.[23] Significant decrease in MDA on treatment with NATQ supported its efficacy to reduce lipid peroxidation. GSH, a tripeptide is primarily synthesized in liver tissue plays a major role in free radical scavenging, xenobiotic removal and signal transduction in cell.[23] Depletion in serum GSH and increase in oxidized glutathione (GSSG) is an indicator of liver toxicity primarily due to oxidative stress.[22] In our study, CCLR induced toxicity caused depletion in GSH, and its restoration by NATQ

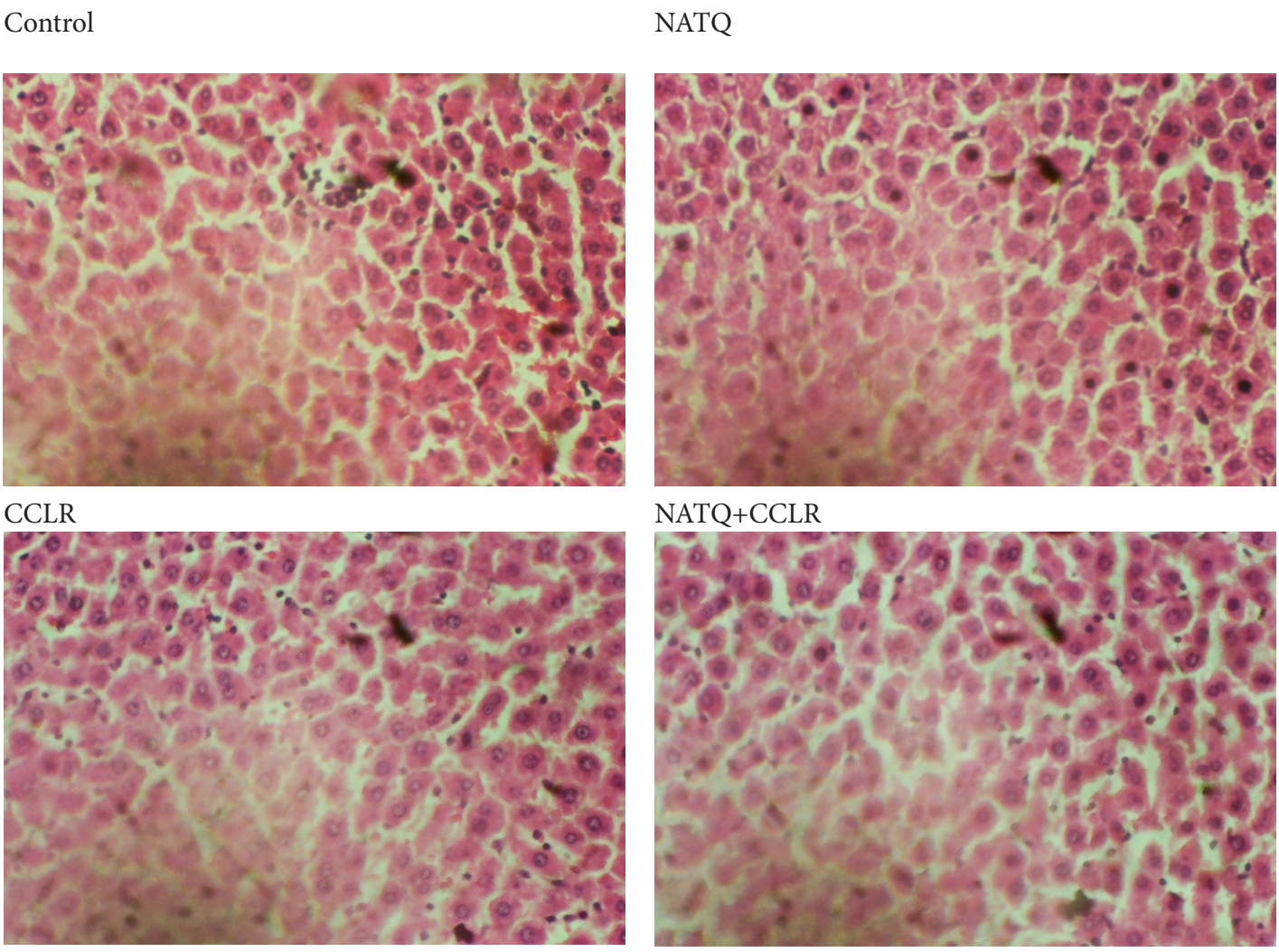

Figure 6: Hepatoprotective activity of NATQ effect on liver histopathology in CCLR induced toxicity. (NATQ-N-acetyl cysteine and thymoquinone, CCLR-CCl_4 and Gy irradiation treated, CCLR+NATQ-CCl_4 and Gy irradiation treated and NATQ treated.) H&E Staining of liver tissues showed notable liver injury characterized with infiltration of inflammatory cells, fibrosis, edema and necrosis in CCLR and restoration of normal histological features were seen on treatment with NATQ in CCLR toxicity induced rats.

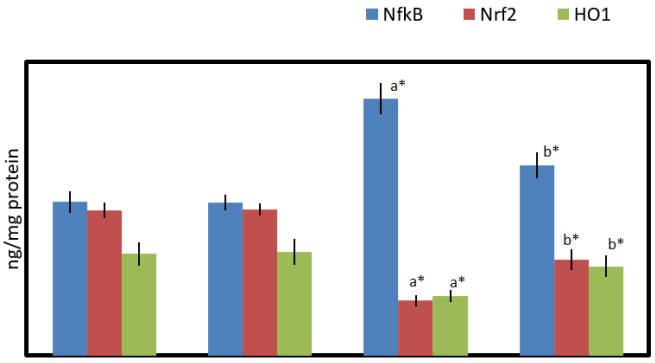

Figure 7: Hepatoprotective activity of NATQ on levels of liver NfkB, Nrf2, HO-1 in CCLR induced toxicity. (NATQ-N-acetyl cysteine and thymoquinone, CCLR-CCl_4 and Gy irradiation treated, CCLR+NATQ-CCl_4 and Gy irradiation treated and NATQ treated) Average of each group was calculated, expressed as mean±SD. Comparison were made as follows: "a"-compared with control; "b"-compared with CCLR; "*"-significance $p<0.05$. Statistical analysis was calculated by one way ANOVA with an appropriate *post-hoc* analysis.

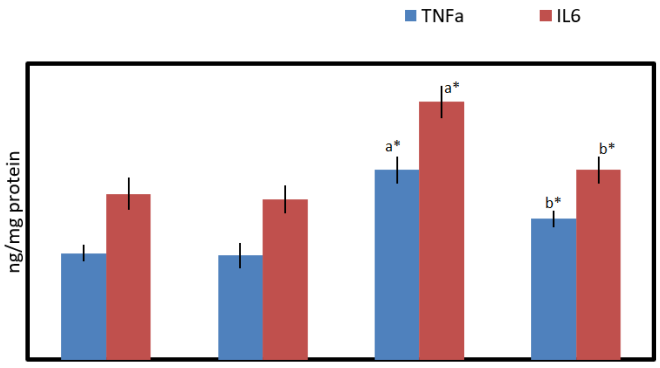

Figure 8: Hepatoprotective activity of NATQ on levels of liver TNF-α and IL6 in CCLR induced toxicity. (NATQ-N-acetyl cysteine and thymoquinone, CCLR-CCl_4 and Gy irradiation treated, CCLR+NATQ-CCl_4 and Gy irradiation treated and NATQ treated) Average of each group was calculated, expressed as mean±SD. Comparison were made as follows: "a"-compared with control; "b"-compared with CCLR; "*"-significance $P<0.05$. Statistical analysis was calculated by one way ANOVA with an appropriate *post-hoc* analysis.

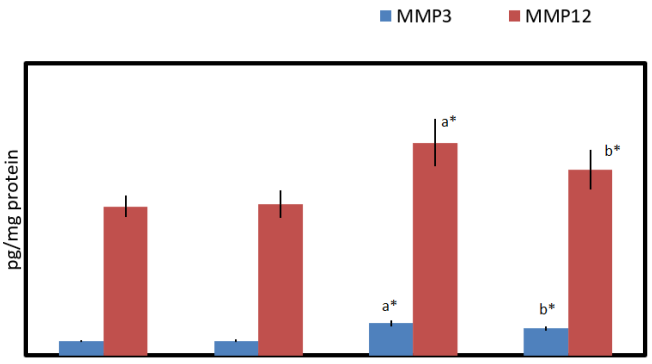

Figure 9: Hepatoprotective activity of NATQ on levels of liver MMP-3 and MMP-12 in CCLR induced toxicity.(NATQ-N-acetyl cysteine and thymoquinone, CCLR-CCl_4 and Gy irradiation treated, CCLR+NATQ-CCl_4 and Gy irradiation treated and NATQ treated) Average of each group was calculated, expressed as mean±SD. Comparison were made as follows: "a"-compared with control; "b"-compared with CCLR; "*"-significance $p<0.05$. Statistical analysis was calculated by one way ANOVA with an appropriate *post-hoc* analysis.

justifies its role in combating oxidative stress. GSSG is increased in oxidative stress, due to free radicals scavenging activity of GSH and get converted to GSSG. In this study restoration of GSH and decrease of GSSG levels suggested that NATQ has positive role in redox homeostasis.

Enzymatic antioxidants SOD, CAT, GST and GPx play a major role in sequestering free radicals, thus maintaining redox homeostasis and also provide information about liver disease.[24] SOD plays major role in sequestering superoxide radicals.[25] Hepatoprotective efficacy of phytochemicals against lipid peroxidation (LPO) is characterized by decreased MDA and increased SOD, CAT, GST and GPx activities.[22,23,25] Increased MDA combined with decreased SOD is found in liver disease.[23] In the present study, treatment of NATQ reported similar restoration of enzymatic antioxidants and decrease in LPO, that supported NATQ's efficiency in redox homeostasis.

Liver histopathological analysis in CCl_4 induced toxicity had shown infiltration of inflammatory cells, fibrosis, edema and necrosis.[13,22] RILD also has the similar pattern of liver histopathology.[4,5] N-acetyl cysteine was reported for restoration of affected hepatocytes to normal.[17,19] Thymoquinone treatment reduces the pathological condition of hepatocellular toxicity, and its efficacy is more comparable to N-acetyl cysteine.[19] Our present study supported the results of previous histopathological findings and the combination of NATQ ameliorated the hepatotoxicity by decreasing the infiltration inflammatory of cells, fibrosis, edema and necrosis in the liver of the rats.

Due to increased GSSG levels, cells secrete higher levels of TNF-α and could play a role in TNF-α mediated cell death, through the suppression of NF*k*B.[26] Also, liver inflammation or injury causes activation of TNF-α, which causes IL-6 levels to increase.[27,28] IL-6 also plays major role in liver regeneration.[28] There was a positive correlation between IL-6 and TNF-α levels that was observed in our study (Figure 8A and Figure 8B). An increase in IL-6, is a warning of liver tissue damage or toxicity,[27] which supported the rationale for the increased level of IL-6 in our study. Obviously, the decrease in levels indicated the reduced inflammation in our study due to protection exerted by NATQ. Increased TNF-α levels could activate NF*k*B.[28] Activated NFkB plays a major role in cell proliferation, inflammation, morphogenesis.[28] In support of these findings, increased level of NFkB in CCLR toxicity of our study evidenced a response towards the activation of NFkB expression and lead to further downstream gene expressions that were required for the damaged tissue repair. Oxidative stress is a major part of hepatoxicity, which causes reduced expression of Nuclear factor erythroid 2 Related Factor (Nrf2) that plays a major role in induction of transcription of drug metabolizing enzymes involved in antioxidant defense mechanism.[29] Nrf2 also induce stress response protein Heme Oxygenase-1 (HO-1).[30] HO-1 is maintained at basal rate under normal conditions.[31] Induction of HO-1 has shown a decrease in liver fibrosis due to its the free radical sequestering effect in xenobiotic toxicity.[32] Nrf2 and HO-1 decrease in CCLR group supported the data published in previous toxicity studies. Enzymatic antioxidants restoration in treatment group suggested that Nrf2 mediated defense mechanism activation in hepatocytes was prominent. Also, the positive correlation between Nrf2 and HO-1 supported the healthier effects of NATQ in antioxidant defense mechanism. However, NATQ had not suppressed these levels in control group and NATQ alone group, which supported the non-toxic nature of NATQ. Matrix Metalloproteinases (MMP) are the major Extra-Cellular Matrix (ECM) component that plays a major role in ECM mediated signaling, ECM-remodeling, wound healing, tissue regeneration, cell behavior regulation and more.[33] MMP-3 is a potential biomarker for liver injury and liver inflammation. MMP-3 can cleave ECM structural component and also activates few other MMPs. MMP-12 is a metalloelastase, highly expressed in case of liver toxicity and fibrosis. It also plays major role in ECM degradation and wound repair. In healthy hepatocytes, many secretary MMP are almost absent and they are expressed in active hepatic tissues involved in the development, tissue injury and repair.[34] Increased ECM turnover is an indicator of liver injury which is revealed by levels of some of MMPs.[33] An increase in levels of MMPs among toxicity group (CCLR) and its depletion in the treatment group of animals supported the protective role of NATQ in our study. Overall, all the above results suggested that NATQ combination helped to alleviate the hepatocellular toxicity induced by CCLR toxicities.

CONCLUSION

The present study has proved the protective effect of NATQ in hepatocellular toxicity induced by CCLR. The findings about the enzymatic markers, oxidative stress markers, histopathological findings and cell signaling molecules projected the hepatoprotective role of NATQ. Perhaps, the dose of NATQ used in the present study was also proved safe without any toxic effects in the experimental animals.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

NATQ: N-acetyl cysteine and thymoquinone; **CCLR:** Carbon tetrachloride and radiation; **SD:** Sprague-Dawley rats; **AST:** Aspartate transaminase; **ALT:** Alanine transaminase; **LDH:** Lactate dehydrogenase; **MDA:** Malondialdehyde; **GSSG:** Oxidized glutathione; **GSH:** Reduced glutathione; **Vit.E:** Vitamin E; **Vit.C:** Vitamin C; **SOD:** Superoxide dismutase; **CAT:** Catalase; **GPx:** Glutathione peroxidase; **GST:** Glutathione-S-transferase; **NfkB:** Nuclear factor kB; **IL-6:** Interleukin 6; **TNF-α:** Tumor necrosis factor-α; **MMP:** Matrix metalloprotein; **HO-1:** Heme oxygenase-1.

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