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Thymoquinone, an Active Constituent of Black Seed Attenuates CCl4 Induced Liver Injury in Mice via Modulation of Antioxidant Enzymes, PTEN, P53 and VEGF Protein

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Keywords: CCl4; Hepatoprotective agents; PTEN; P53; Immunohistochemistry; Antioxidant enzymes; Aminotransferases

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Abstract

AIM: The present study was undertaken to evaluate the possible protective role of thymoquinone on CCl4-induced hepatotoxicity.

METHODS: The activities of liver function enzymes and antioxidant enzymes were measured. Haematoxylin-Eosin staining was performed to analyze the live tissue alterations. Additionally, expression pattern of different proteins was evaluated through immunohistochemistry staining.

RESULTS: The antioxidants enzymes activities were decreased significantly in the CCl4 induced group whereas recovery/increase of antioxidant enzymes was observed when thymoquinone was given to the mice. Moreover, thymoquinone administration significantly decrease the serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and serum aspartate aminotransferase (AST). Liver tissue alterations were noted in CCl4 treated group whereas treatment with thymoquinone significantly prevented the CCl4-induced histological alteration. The expression of PTEN protein was high in CCl4 plus thymoquinone treated group while the loss of PTEN protein expression was observed in CCl4 treated group. Moreover, high expression of P53 protein was noticed in CCl4 treated the group as compared to CCl4 plus thymoquinone group. Difference in expression pattern of PTEN and p53 protein in CCl4 group and thymoquinone plus CCl4 treated group was statically significant ($p < 0.05$). Besides, expression of VEGF was high in CCl4 treated group as well as thymoquinone plus CCl4 treated group and difference in expression pattern was statically insignificant ($p > 0.05$).

CONCLUSION: Our results suggest that thymoquinone can protect CCl4 induced liver damage and could be a preventive drug in the development of novel therapeutic agents for liver diseases.

Introduction

Liver disease is one of the major global health problems regarding morbidity and mortality. In this regard, several factors including viruses and alcohol abuse show a role in the pathogenesis, but exact molecular mechanism involved in this respect is not fully explained. Moreover, the molecular routes causal the pathogenesis of acute liver injury is recognised to involve a complex interplay of oxidative stress, apoptosis, inflammation and another process [1], [2].

Carbon tetrachloride (CCl_4) is a well-known hepatotoxin that is usually used to induce liver injury in a large variety of laboratory animals [3], [4]. The overdoses of CCl_4 either orally or intraperitoneally, induce hepatic damage, including loss of architecture of hepatocytes, inflammation, congestion, degeneration and necrosis. Furthermore, exposure of CCl_4 shows role in the reactive oxygen species generation, which in turn decreases the antioxidant enzymes that show role in the detoxification of toxic materials. Additionally, CCl_4 reduces antioxidant enzymes which catalyse the decomposition of hydrogen peroxide to water and oxygen and which neutralise the reactive superoxide radical activity.

Intake of medicinal plants and its active component with antioxidant phytochemicals is confirmed to enhance the antioxidant level and inhibit the pathogenesis. In this view, medicinal plant or their constituents have proven their role in the inhibition of pathogenesis due to the rich source of antioxidant [5],

[6]. Thymoquinone is the principal component of *Nigella sativa* which is familiar as black cumin or black seed [7], [8] and its use in the cure of diseases, as well as inhibition of pathogenesis, has been described. Moreover, Ayurveda, Unani, Arabic and Chinese medicine have shown its importance in health management. Also, its role in cancer prevention has been noted through modulation of cell signalling pathways including angiogenesis, apoptosis and tumour suppressor gene [9]. However, its anti-tumour activity has been proven as it shows role in the cell death and tumour growth inhibitory activities and has been found to be associated with other tumorigenic processes [10], [11].

This study was undertaken to examine whether TQ protects against CCl_4-induced hepatotoxicity in mice.

Material and Methods

This study was conducted by the guidance of the ethical committee for animal handling at Qassim University. The experimental procedure was approved by the Bioethics Review Committee of the College of Applied Medical Science, Qassim University.

A total number of 24 young adult male albino mice were included in this study. The mice were housed in the animal house of the College of Applied Medical Science, Qassim University. The mice age was between the six and seven weeks and weighed between 23-28 g were included in the study. All mice were fed in the laboratory maintained at approximately 22°C with a 12-h light–dark cycle with free access to food and water. An acclimation period of 1 week was employed earlier to the experiments. Animal grouping and treatment plan are described in Table1.

Table 1: Grouping of animals and treatment plan

Group number	Experimental group	Treatment	Number of animal per group
1	Control	Normal mice administered with vehicle solution	8
2	Disease group	CCl_4 treatment group	8
3	Treatment groups	CCl_4 (0.04 cc of a 40 per cent solution of CCl_4) plus thymoquinone (10 mg/kg/day dissolved in a DMSO) treatment	8

The mice were randomly divided into 3 groups as follows:

Group 1: The first group is the untreated control group and was administered with olive oil (orally by gavage twice in a week until the last day of the experiment) which was used as a vehicle, and fed normal diet and water for 12 weeks.

Group 2: (Diseases control: CCl_4 control group): Mice were treated with CCl_4 (0.04 cc of a 40 per cent solution of CCl_4) in olive oil [12] orally by gavage three times in a week for 12 weeks.

Group 3: Thymoquinone plus CCl_4 treated group. Thymoquinone-CCL_4 treated group, mice received thymoquinone (10 mg/kg body weight/day) [13] starting one week before CCl_4 administration and continued throughout the experiment for 12 weeks.

Blood samples were collected and allowed to clot for 30 minutes and centrifuged to separate clear serum. The activities of enzymes such as alanine aminotransferases (ALT), aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Catalase, Superoxide dismutase (SOD) Glutathione peroxidase (GPx) and total antioxidant capacity activity was measured through ELISA kits, and the results were interpreted accordingly (Figure 1).

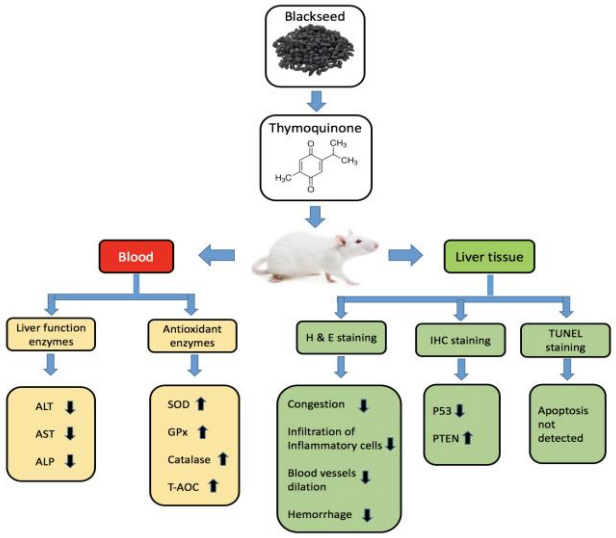

Figure 1: Implication of thymoquinone in the prevention of liver damage through modulation of biological activities

Liver tissues were collected from mice of each group and fixed in 10% buffered formalin. These tissues were processed through tissue processor to make a paraffin-embedded block. Sections were made from each tissue block and Hematoxylin-Eosin (H & E) staining was performed to analyse the live tissue alterations, and observation was noted under a light microscope.

Expression of different types of proteins including PTEN, VEGF and P53 was evaluated through immunohistochemistry as by previously described method [14]. Concisely, deparaffinization of the sections was made through xylene and rehydration on each section was performed. A blocking agent such as hydrogen peroxidase was used to block the endogenous peroxidase activity (Abcam, USA). Furthermore, nonspecific binding was blocked by a protein blocking agent (Abcam, USA). Monoclonal antibodies of PTEN, VEGF and P53 (Abcam, USA) were used as primary antibodies. Secondary antibody and tertiary antibody were used on each section for 90 minutes respectively. Finally, diaminobenzidine (DAB) step was performed on section according to the manufacturer's instructions,

and the sections were counterstained with haematoxylin. The cases were considered as positive for each marker when more than 5% of the cells showed positive expression or less than 5% expression was considered as a negative expression.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling was performed to assist in the detection of apoptotic cells in tissue sections. Apoptoticaly fragmented cellular DNA was identified by TUNEL assay according to kits guidelines (Apoptosis Detection Kit, Abcam, USA). Counterstaining with haematoxylin was made to evaluate and characterise the normal and apoptotic cells.

All values are expressed as mean ± SD. A level of $p \leq 0.05$ was taken as statically significant. Chi-square χ^2 test was used to make the correlation of marker with histopathological findings.

Results

Oral administration of CCl_4 to mice showed 2 (25%) mortality in CCl_4 treated group (Diseases group), whereas no mortality was seen in the other groups such as control group and CCl_4 plus thymoquinone treated group.

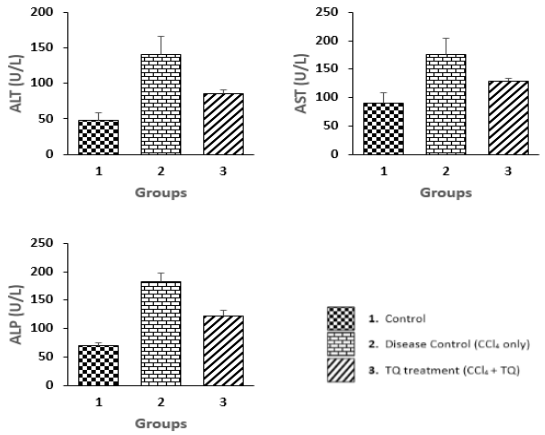

Figure 2: Effect of thymoquinone on serum liver function enzymes activity. Liver function enzymes were significantly reduced in CCl_4 plus thymoquinone treated group and was high in CCL_4 treated group ($p \leq 0.05$)

To study the therapeutic implication of thymoquinone (TQ) in liver toxicity, TQ was given to CCl_4-induced hepatotoxicity mice. The serum of ALT, AST and ALP enzymes activity was measured in different groups, and it was noticed that ALT, AST and ALP activity significantly increased in the CCl_4 treated group (Disease group) as compared to control group (Figure 2). Moreover, ALT, AST and ALP activity was significantly decreased in the group that received thymoquinone (CCl_4 plus thymoquinone treated mice group) ($p \leq 0.05$). This finding confirms that thymoquinone has a potential role in the liver protection through reduction of liver functions enzymes in CCl4 hepatotoxicity (Figure 2).

In this study, the activity of the antioxidant enzymes SOD, GPx, catalase activity and total antioxidant capacity were significantly decreased in the disease control group (group 2) as compared to the untreated control (Group 1) (Figure 3). Moreover, it was observed that thymoquinone significantly restore the antioxidant enzyme activity ($p \leq 0.05$) including SOD, GPx, catalase and total antioxidant capacity in CCl_4 plus thymoquinone group as compared to the disease control group (CCL_4 treated only) (Figure 3).

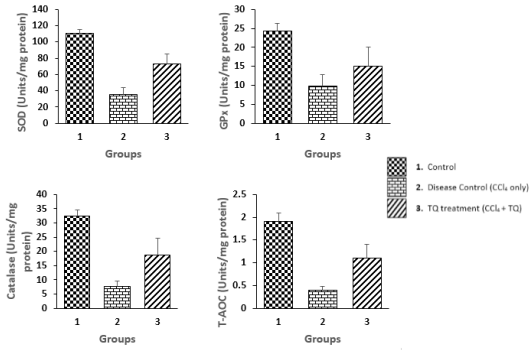

Figure 3: Effect of thymoquinone on SOD, GPx, Catalase and total antioxidant capacity (T-AOC) activity. Hepatic SOD, GPx, Catalase and total antioxidant capacity was high in thymoquinone plus CCl_4 treated groups as compared CCL_4 treated group only and this difference was statically significant) ($p \leq 0.05$)

Histopathological changes in liver tissues are presented in different groups of mice. The normal architecture of liver tissue was maintained in the control group (Figure 4a).

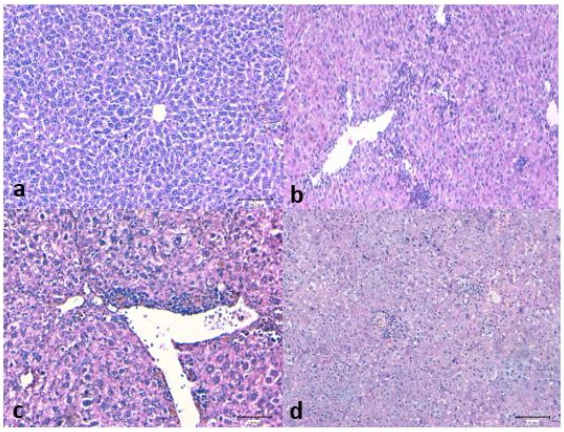

Figure 4 (a-d): a: Liver histology of control group showing the normal architecture of hepatocytes b: CCl_4 treated mice showing severe infiltration of inflammatory cells c: CCl_4 treated mice showing infiltration of inflammatory cells, and blood vessel dilation d: CCl_4 plus Thymoquinone treated mice showing less inflammatory cells and less congestion (Orig.MagX40)

Different types of liver tissue alterations were seen in CCl_4 treated group, and it was observed as severe congestion, infiltration of inflammatory cells, haemorrhages, fatty degeneration and blood vessel dilation (Figures 4b and c). Though these consequences were also noticed in the CCl_4 plus thymoquinone group, the incidence and severity of alterations were less than those in CCl_4 treated group (Figure 4d).

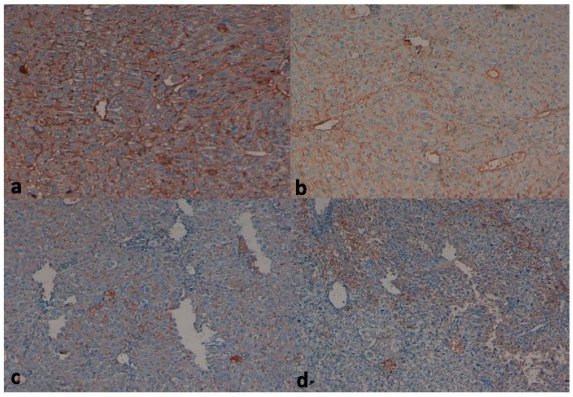

Figure 5: a) PTEN protein expression was detected in control cases and intensity of expression was high; b) PTEN protein expression was detected in CCl_4 plus thymoquinone treated group (Orig. X mag40); c) PTEN protein expression was detected in CCl_4 treated group, and the intensity of expression was low; d) PTEN protein expression was detected in CCl_4 treated group and intensity of expression was low (Orig. X mag40)

PTEN protein expression was examined in all the groups of mice and results were interpreted based on the expressional patterns. The loss of PTEN protein was noticed in CCl_4 treated group. The intensity of expression was high in the control group (Group 1) as well as in CCl_4 plus thymoquinone treated group (Group 3) as compared to CCl_4 treated group (Group 2) (Figure 5 a, b, and c).

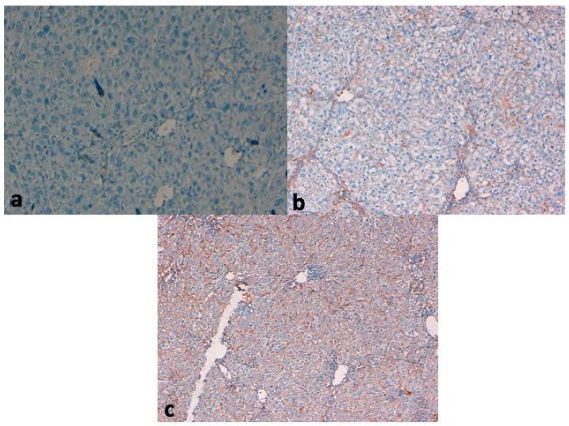

Figure 6: a) P53 protein expression was not detected in control cases; b) P53 protein expression was detected in CCl_4 plus thymoquinone group but expression intensity was low; c) P53 protein expression was detected in CCl_4 group, and intensity of expression was high (Orig. X mag40)

P53 expression was evaluated in all group of mice, and it was noticed that all 8 cases of the control group did not show any expression (Figure 6a). A higher expression of P53 protein was noticed in CCl_4 treated group (diseases control) (Figure 6b) as compared to CCl_4 plus thymoquinone group (Figure 6c). The difference in expression pattern among CCl_4 treated group and thymoquinone plus CCl_4 treated group was statically significant ($p < 0.05$)

The control group showed weak cytoplasmic VEGF expression (Figure7-a) whereas expression was high in CCl_4 treated group (Figure 7-b). Moreover, VEGF expression was also noted in thymoquinone plus CCl4 treated group (Figure 7-c). The difference in expression pattern between CCl_4 treated group and CCl_4 plus thymoquinone treated group was statically insignificant ($p > 0.05$)

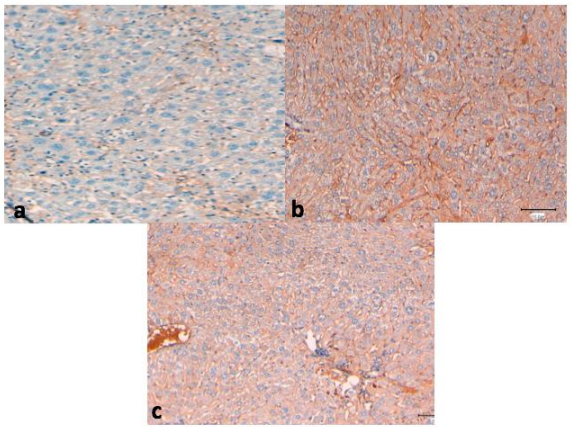

Figure 7: a) Weak VEGF protein expression was noted in control group; b) VEGF protein expression was detected in CCl_4 group; c) VEGF protein expression was detected in CCl_4 plus thymoquinone group (Orig. X mag40)

Apoptosis was not detected in any group including CCl_4 treated group (Figure 8a) as well as CCl_4 plus thymoquinone treated group (Figure 8b).

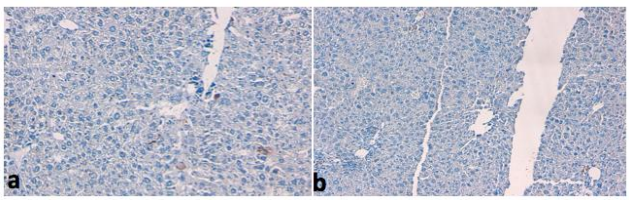

Figure 8: a) Apoptosis was not detected in CCl_4-treated group; b) Apoptosis was not detected in CCl_4 plus thymoquinone treated group (Orig. mag×40)

Discussion

The prevalence of liver pathogenesis is comparatively increasing, and the mortality and morbidity rates are significantly increasing in Saudi Arabia [15], [16]. The current study was based on a

mice model performed to examine the hepatoprotective effects of thymoquinone in repeated CCl_4 administration induced hepatotoxicity. CCl_4-induced acute liver injury in a murine model has been used for screening the hepatoprotective activities of drugs [17]. Thymoquinone (TQ) is an active constituent present in the black seed and broadly studied over the decades for its role in diseases cure without promoting any severe side effects. TQ has proven its role in the protection of organs against oxidative damage induced by a free radical generating agents [18]. TQ and a synthetic structurally-related TBHQ strongly inhibited iron-dependent microsomal lipid peroxidation in a concentration-dependent manner [19]. TQ importance has been discussed by earlier investigator [20], and TQ has proven role through modulation of diverse oncogenic transcription factors [21]. Moreover, another study was to investigate the effects of TQ on head and neck squamous cell carcinoma (HNSCC) cell lines. The result of the study revealed that TQ exhibited dose-dependent cytotoxicity via apoptosis in the investigated cell lines [22].

In the present study, TQ treatment showed significant protection against CCl_4-induced liver injury, which was noticed by the decrease of serum ALT, ALP and AST levels. Whereas serum activity of ALT, AST and ALP were significantly increased in the CCl_4 treated group (Disease control). This finding confirms that thymoquinone has a potential role in the liver protection through a reduction in liver functions enzymes. This result agrees with an earlier study [23] reported that serum ALT, AST levels, and SOD activity, as well as the serum and tissue MDA levels, were found to be higher in the acetaminophen group than in the control group. Whereas in the acetaminophen + TQ group, serum ALT, AST levels, SOD activity and the serum and tissue MDA levels were found to be lower as compared to that of the APAP group and such difference was statistically significant [23]. This result is consistent with the previous study, and it was reported that of exposure to sodium fluoride resulted in a change in liver function as designated by a significant increase in the activity of AST, ALT, ALP, LDH and the concentration of total bilirubin. Moreover, administration of TQ at a dose of 10 mg/kg protected the liver against sodium fluoride toxicity and improved its functioning as proven via the noteworthy decrease in these liver function biomarkers compared to the sodium fluoride group [24].

SOD, GPx is the major antioxidant enzyme-reducing superoxide and this way antioxidant enzyme prevent the pathogenesis. In this study, it was observed that thymoquinone, significantly restore the antioxidant enzyme activity including SOD, GPx and catalase in CCl_4 plus thymoquinone group as compared to the CCl_4 treated (disease control group). The activity of the antioxidant enzymes SOD, GPx and catalase were significantly decreased in the disease control group (Group 2) as compared to the untreated control (Group 1) (Figure 3). An interesting study reported that lead exposure significantly decreased reduced glutathione level and superoxide dismutase, glutathione peroxidase, catalase, and glutathione reductase activities in the renal tissue. Remarkably, supplementation with TQ significantly improved the affected antioxidant parameters [25]. Another finding result revealed that in the acetaminophen + TQ group, the GPx activity was found to be significantly higher compared to the acetaminophen group [23].

Liver tissue alterations were noted in CCl_4 treated group, and it was severe congestion, infiltration of inflammatory cells, degeneration and blood vessel dilation. Though these consequences were also noticed in the CCl_4 plus thymoquinone group, the severity of liver tissue alterations was less than those in the CCl_4 treated group. Earlier findings demonstrated that subchronic ethanol exposure caused severe steatosis, central vein congestion, and infiltration of inflammatory factors in focal portal space in the liver [26]. In this regard, other results demonstrated that the gentamicin-induced liver histological alterations, such as hydropic degeneration of hepatocytes, fatty changes, inflammatory cell infiltration and congestion of portal vein were successfully recovered by thymoquinone and curcumin treatment [27]. This finding revealed that TQ has potential effect in normalisation of architecture of liver tissue damage by CCl_4 induced hepatotoxicity.

PTEN is located on chromosome 10 and tumour suppressor gene is located in the 10q23 region encoding for a 403-amino acid multifunctional protein, which possesses lipid and protein phosphatase activities [28]. Additionally, the loss of PTEN gene expression causes abnormal activation of the PI3K/Akt and ERK pathways and, accordingly, leads to cancer cell proliferation and, finally, stimulates tumourigenesis [29]. In the current study, expression of PTEN protein was noted in all the animal groups. The intensity of the expression was high in the control group as well as in CCl_4 plus thymoquinone treated group as compared to CCl_4 treated group. Additionally, loss of PTEN protein expression was noticed in CCl_4 treated group as compared to control group as well as CCl_4 plus thymoquinone group. An interesting study based on TQ reported that TQ induces apoptosis in doxorubicin-resistant breast cancer cells through up-regulation of PTEN at transcription level and its treatment increased cellular levels of PTEN proteins, resulting in a significant decrease of phosphorylated Akt, a known regulator of cell survival [30].

P53 remains the most frequently mutated gene in several common human cancers, with mutations estimated to occur in 50% of all types of cancers [31]. Altered expression of P53 has been noticed in several types of tumors. P53 expression was evaluated in all groups of mice and it was noticed

that all cases of control group did not show any expression. A higher expression of P53 protein was noticed in CCl_4 treated group as compared to CCl_4 plus thymoquinone group. The difference in expression pattern among CCl_4 treated group and thymoquinone plus CCl_4 treated group was statically significant ($p < 0.05$). A recent study based on MCF-7 breast cancer cells confirms that thymoquinone can induce apoptosis in MCF-7 breast cancer cells through the up-regulation of P53 expression [32]. Another study reported that P53 higher expression was noticed in CCl_4 treated group as compared to control group and green tea extract (GTE) group. A supplementation of GTE with CCl_4 induced a significant reduction of P53 level [33]. Another study based on curcumin reported that STZ exposure significantly increased P53 protein levels and CUR attenuated this activation [34].

Vascular endothelial growth factor (*VEGF*) is a signalling protein that promotes the growth of new blood vessels. It is produced by several cell types including tumour cells [35], [36], macrophages [37] and its altered expression has been noticed in many tumours. In the current study, the control group showed weak cytoplasmic expression whereas expression was high in CCl_4 treated group. Moreover, VEGF expression was also noted in thymoquinone plus CCl_4 treated group. The difference in expression pattern between CCl_4 treated group and CCl_4 plus thymoquinone treated group was statically insignificant. Moreover, a finding demonstrated that administration of NAC (N-acetylcysteine) and ALA (α-Lipoic acid) and THQ (Thymoquinone) either alone or in combination along with acetaminophen down regulates flat-1 (VEGFR1) expression [38]. The potential anticancer activity of the combination of thymoquinone (TQ) and resveratrol (RES) against breast cancer in mice was evaluated, and the results demonstrated that combination therapy enhanced apoptosis, and decreased VEGF expression [39].

Understanding the mechanisms of apoptosis is crucial and helps in the understanding the pathogenesis of conditions as a result of disordered apoptosis [40]. Apoptotic bodies were not detected in any group including control, CCl_4 treated as well as CCl_4 plus thymoquinone treated group. In this regards, the previous study confirmed that apoptotic bodies were observed in cancer cases, while control cases did not show apoptosis [14].

In this study, a hepatotoxic agent such as CCl_4 causes liver hepatotoxicity as evidenced by the increase of liver function enzymes and also causes liver tissue alteration including infiltration of inflammatory cells, fatty degeneration and blood vessel dilation. The mice treated with CCl_4 plus thymoquinone shows role in the protection of the liver structure as the severity of fatty degeneration and blood vessel dilation and infiltration of inflammatory was less. Also, thymoquinone might play a role in restoring the liver antioxidant enzyme activity including SOD, GPx and Catalase. The intensity of PTEN expression was high well as in CCl_4 plus thymoquinone treated the group as compared to CCl_4 treated group. A higher expression of P53 protein was noticed in CCl_4 treated the group as compared to CCl_4 plus thymoquinone group and difference in expression pattern was statically significant. The protective effect of thymoquinone signifies a potentially preventive drug in the development of novel therapeutic agents for liver tissue alteration in the CCl_4 hepatotoxicity.

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