

SUPPRESSION OF HEPATIC TNF- α AND TGF- β GENE EXPRESSIONS IN RATS WITH INDUCED NONALCOHOLIC STEATOHEPATITIS

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Summary

The aim of this study was to investigate the effect of ethyl acetate extract of *Teucrium polium*, with high anti-oxidant activity, on experimental nonalcoholic steatohepatitis (NASH). Male N-Mary rats were divided into three groups: control group was fed a normal diet, MCD group received a methionine and choline deficient (MCD) diet and MCD+E group was fed a MCD diet plus ethyl acetate extract of *T. polium* orally (0.5 g/kg/day). After 8 weeks, all rats were sacrificed. Hepatic tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), reduced Glutathione (GSH), malondialdehyde (MDA) and liver histopathology were examined. MCD diet led to grade 1 liver steatosis, lobular inflammation and ballooning degeneration. These factors decreased significantly among the rats of MCD+E group. Hepatic MDA level in the MCD+E group was lower than those of the MCD group. The GSH level in the MCD+E group was significantly higher than those in the MCD group. Western blot analyses and Real-Time PCR showed that the levels of hepatic TNF- α and TGF- β have markedly decreased among MCD+E group relative to MCD group. These results demonstrate that the ethyl acetate extract of *T. polium* has effectively prevented the progress of NASH, most probably through its strong antioxidant capability.

Keywords: *Teucrium polium*, NASH, Methionine and choline deficient diet, TNF- α , TGF- β

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a metabolic disorder associated with a wide spectrum of liver abnormalities ranging from simple steatosis (fatty liver) to nonalcoholic steatohepatitis (NASH), hepatocellular carcinoma and, in progressive states, cirrhosis (1). NASH is histologically characterized by diffused fatty infiltration, lobular inflammation, ballooning degeneration and fibrosis in the liver. The recognized NASH clinical features mainly include insulin resistance, obesity, hyperlipidemia and hypertension (2,3).

Although the exact mechanisms that mediate transition from steatosis to NASH remain unknown, oxidative stress and cytokine-mediated injuries are believed to play key roles in NASH pathogenesis (4,5). Increased generation of reactive oxygen species (ROS), under oxidative stress conditions, is known to lead to membrane lipid peroxidation, inflammatory responses and stimulation of stellate cells followed by fibrosis (6). One of the cytokines involved in hepatocyte injuries is tumor necrosis factor- α (TNF- α). It has been shown that this proinflammatory cytokine plays major roles in almost all steps of NASH development (7,8). Increased levels of serum TNF- α and elevated hepatic TNF- α mRNA content have been reported in patients with NASH (9). Using experimental animal models, it has been shown that therapies against TNF- α has attenuated the extent of liver necrosis, inflammation and fibrosis (10). The other cytokine is transforming growth factor- β (TGF- β). This profibrogenic cytokine released by the activated kupffer cells and the hepatocytes plays a pivotal role in hepatic fibrogenesis by activation of hepatic stellate cells (11). It has been reported that plasma TGF- β level increases in patients with NASH and the measurement of this factor may be useful in differentiating between NASH and fatty liver (12).

Methionine and choline deficient (MCD) diet is commonly used to induce NASH in experimental animals (13). The proposed biochemical basis for steatosis induction in this approach is the impaired phosphatidylcholine synthesis. Phosphatidylcholine is essential for VLDL processing, packaging and secretion from hepatocytes (14). Consequently, triglycerides build up in hepatocytes leading to high fatty liver incidences. Rodents on a MCD diet have developed NASH associated with higher than normal levels of oxidative stress, TNF- α and TGF- β (15,16).

Although, there is yet no effective drug therapy, the involvement of oxidative stress in incidence of NASH has provided the opportunity of evaluating synthetic and/or natural plant antioxidants for NASH clinical treatments. Yalniz and colleagues have recently shown that genistein, a strong antioxidant agent is capable of decreasing the plasma TNF- α level and attenuating the incidence of NASH due to its antioxidant potency (17). Lima and coworkers have also reported on the health beneficial effects of Yo Jyo Hen Shi ko (YHK), a Chinese herbal complex of four different botanicals (*Panax pseudoginseng*, *Eucommia ulmoides*, *Polygonati rhizome* and *Licorice root*), on marked reduction in macrosteatosis among the MCD and high fat (HF) diet-fed experimental animals along with significant improvements in histological markers of inflammation (18). These effects have also been associated with marked reduction in serum aminotransferases and hepatic lipoperoxide and glutathione contents, implying that the YHK herbal complex has noticeable antioxidant activity. *Teucrium polium* L. (Lamiaceae) is a medicinal plant used for various purposes such as anti-inflammatory, anti-nociceptive, anti-bacterial, anti-hypertensive, anti-heperlipidaemia, anti-rheumatoid and anti-hyperglycemia (19-22). The antioxidative activity effects of *T. polium* extract has been evaluated and published by several independent groups (23-25). Moreover, the extract has been found to diminish oxidative stress in streptozotocin-induced diabetic rats and to inhibit lipid peroxidation in rat liver microsomes (26,27).

The main goal of this study was to investigate whether the ethyl acetate extract of *T. polium*, with strong antioxidant activity and high polyphenolic content, is capable of attenuating the progress of steatosis to steatohepatitis by alternations of hepatic oxidative stress and cytokine levels among the experimental animal models.

Methods

Material:

Reduced glutathione (GSH) was obtained from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA) was obtained from Sigma Chemical Co. (MO, USA). 5, 5'-dithiobisnitro benzoic acid (DTNB), thiobarbituric acid (TBA) and Bovine serum albumin (BSA) were obtained from Merck Co. (Germany). Sulphosalicylic acid was obtained from Carlo Erba (Milan, Italy). TNF- α and TGF- β antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All primers and probes were obtained from Bioneer (Chungwon, Korea). All other chemicals used were analytical grade.

Plant extract preparation:

The aerial parts of *T.polium* L. were collected from Yasouj province, Iran during spring. A voucher herbarium specimen (No. 570) was deposited in the herbarium of the school of pharmacy, Shaheed Beheshti University of medical sciences, Tehran, Iran. The plant aerial parts were air-dried, protected from direct sunlight, and then powdered. The powdered plant material (300 g) was extracted three times with ethanol (EtOH 80%), at room temperature (RT) overnight. The EtOH extracts were combined and concentrated under reduced pressure on a rotary evaporator and the volume was adjusted to 300 ml. The EtOH extract was then reextracted four times with ethyl acetate. The extract was evaporated to dryness to give the ethyl acetate residue. This residue (0.75 g) was used for in vivo studies after dissolution in distilled water at a concentration of 0.25 mg/ml.

Animals and experimental protocols:

Male N-Mary rats, weighing 175-220 g, were housed in cages with controlled light/dark cycle and free access to food and water ad libitum. Animals were divided into three groups: Control group (n=5) was fed a normal diet (28), MCD group (n=5) received a MCD diet (28) and MCD+E group (n=5) was fed a MCD diet plus ethyl acetate extract of *T.polium* orally (equivalent to 0.5 g of plant leaves powder/kg/day). All experiments were carried out according to the guidelines for the care and use of experimental animals approved by the state veterinary administration of University of Tehran. After 8 weeks, rats were sacrificed and the blood and the livers were taken for biochemical, histopathological and molecular examinations.

Sera biochemical analyses:

Alkaline phosphatase (ALP), Aspartate aminotransfrase (AST) and Alanine aminotransfrase (ALT) were assayed using the corresponding commercial kits (Pars Azmoon, Iran).

Preparation of liver homogenate:

The liver samples were cut into small pieces and homogenized in Tris-HCl buffer (25 mM, pH 7.5) with a homogenizer to give a 10% (w/v) liver homogenate. The homogenates were then centrifuged at 12,000 rpm for 15 min at 4° C (Beckman). The supernatant obtained was used for MDA and GSH analyses. The protein concentration of each extract was determined by the method of lowry using Bovine serum albumin (BSA) as the standard (29).

MDA assay:

The malondialdehyde (MDA) level of each liver sample was determined by the double heating method (30). The method is based on spectrophotometric measurement of the purple color generated by the reaction of thiobarbituric acid (TBA) with MDA. Briefly, 0.5 mL of the liver homogenate was mixed with 2.5 mL of Trichloroacetic acid (TCA, 10%, w/v) solution followed by boiling in a water bath for 15 min. After cooling to room temperature, the sample was centrifuged at 3000 rpm for 10 min and 2 mL of each sample supernatant was transferred to a test tube containing 1mL of TBA solution (0.67%, w/v). Each tube was then placed in a boiling water

bath for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm with respect to the blank solution. The concentration of MDA was calculated based on the absorbance coefficient of the TBA–MDA complex ($\epsilon = 1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$) and it was expressed as nmol/mg protein.

GSH assay:

Reduced glutathione (GSH) was assayed by the method of Jollow et al (31). An aliquot of 0.5 mL of each tissue homogenate was precipitated with 1mL of sulphosalicylic acid (4% w/v). The precipitate was removed by centrifugation. The filtered sample (0.5 mL) was mixed with 0.1 mL 5, 5'-dithiobisnitro benzoic acid (DTNB, 4 mg/mL) and 0.9 mL phosphate buffer (0.1 M, pH 7.4). The yellow color developed was read at 412 nm. Reduced glutathione was expressed as $\mu\text{g}/\text{mg}$ of protein.

Histopathological examination:

Liver tissue samples were kept in 10% formalin, and paraffin blocks were prepared. The sections from blocks were stained with hematoxylin-eosin (HE) and masson trichrom. The histopathological evaluation was performed blindly by an expert pathologist using a scoring system proposed by Kleiner et al (32): steatosis (0-3), lobular inflammation (0-3) and ballooning degeneration (0-2). Fibrosis was evaluated as absent/present.

Determination of mRNA level using Real-time PCR:

Total RNA was isolated from frozen liver tissues using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The RNA concentration and the quality were determined spectrophotometrically at 260 nm and by the A260/A280 ratio, respectively. Total RNA (4 μg) was reversed transcribed into cDNA with the use of 200 U of M-MuLV reverse transcriptase (Fermentas, Lithuania) and 0.2 μg of random hexamer (Fermentas) as the primer. The amplification reactions were performed on a Roche light cycler instrument (Roche diagnostics GmbH, Mannheim, GER) applying the following thermal cycling conditions: an initial activation step for 3 min at 95°C followed by 45 cycles including a denaturation step for 10 s at 95°C, annealing step for 15 s at 55°C and extension step for 20 s at 72°C. β -actin was used as a normalizer and the fold change in expression of each target mRNA relative to β -actin was calculated based on $2^{-\Delta\Delta\text{Ct}}$ comparative expression method. The primers and probes used are listed in Table I (33-35).

Western blot analysis:

The frozen liver tissue (10-20 mg) was homogenated with 0.2 ml lysis buffer containing 10 mM Tris (pH 7.4), 100 mM NaCl, 1mM EGTA, 1mM EDTA, 1 mM NaF, 20 mM sodium pyrophosphate, 2 mM Na_3VO_4 , 1% Triton X-100, 10% glycerol, 1mM DTT, 1mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin and 60 $\mu\text{g}/\text{ml}$ aprotinin.

Table 1. Sequences of primers and probes used for real-time RT-PCR.

Gene	primers/probes	sequence
TNF- α	Forward	CCAGGTTCTCTTCAAGGGACAA
	Reverse	CTCCTGGTATGAAATGGCAAATC
	Probe	Fam-CCC GACTATGTGCTCCTCACCCACA-Tamra
TGF- β	Forward	TAGCAACAATTCCTGGCGTTAC
	Reverse	GGACTGATCCCATTGATTTCC
	Probe	Fam-CAGTGGCTGAACCAAGGAGACGGAATACAG-Tamra
β -ACTIN	Forward	CGTGAAAAGATGACCCAGATCA
	Reverse	CACAGCCTGGATGGCTACGT
	Probe	Fam-TTTGAGACCTTCAACACCCAGCCA-Tamra

After 30 min, the liver homogenate was centrifuged at 14000 rpm for 15 min at 4°C. Protein concentration of each sample was determined using Lowry's procedure (29). Equal quantities of protein (70 µg/lane) were separated by SDS-PAGE and electroblotted to polyvinylidene difluoride (PVDF) membrane. Each Membrane was blocked in Tris-buffered saline pH 7.6 plus 0.1% Tween-20, 0.05% sodium azid and 4% non-fat dry milk overnight at 4°C. Blocked blots were incubated with primary antibodies for 2 h at room temperature using antibody dilutions as in Tris-buffered saline pH 7.6, containg 0.05% Tween-20, and 1% non-fat dry milk, followed by 1 h incubation at room temperature with anti-mouse or anti-goat horseradish peroxidase-conjugated secondary antibodies (Biosource, Belgium). The protein bonds were detected by an enhanced chemiluminescence (ECL) detection system (Amersham-Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. The signals were revealed by autoradiography. In all experiments, the equal protein loadings have been confirmed relative to β-tubulin protein content.

Statistical analyses:

Data are expressed as mean ± SD of three independent experiments and statistically analyzed using Student's t-test. Values of $p < 0.05$ were considered significant.

Results

Biochemical analysis:

According to Table II, the plasma levels of ALP, ALT and AST significantly increased in the MCD group compared to the control group ($P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively). Treatment with the ethyl acetate extract of *T.polium* significantly reduced activity of these enzymes compared to animals of MCD group ($p < 0.001$, $p < 0.03$ and $p < 0.02$, respectively). Accordingly, it can be concluded that the plant extract has protective effects against the MCD diet-induced liver injuries.

Liver oxidative stress status:

The MCD diet has caused a severe decrease in the GSH content of liver by 58% relative to control group ($p < 0.01$) (Fig. 1A). The depletion of GSH by MCD diet was associated by an increased in the extent of lipid peroxidation by almost 113% as measured by the level of MDA in the liver homogenates (Fig. 1B). However, administration of ethyl acetate extract of *T.polium* significantly increased the GSH level and decreased the MDA level by 196% and 57%, respectively, compared to MCD group ($p < 0.001$ and $p < 0.05$, respectively).

Table 2. Changes in hepatic enzyme levels in rats fed normal, MCD or MCD + E diets.

parameter	Control	MCD	MCD+E
ALP (IU/L)	223.6 ± 8.1	499.3 ± 20.0*	218 ± 4.3**
ALT (IU/L)	16.6 ± 2.5	34.8 ± 5.3*	24 ± 3.0**
AST (IU/L)	34.6 ± 2.5	43 ± 5.2*	29.6 ± 4.7**

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Data represent means ± SD (n=5).

* Significantly different from control group ($p < 0.05$).

** Significantly different from MCD group ($p < 0.05$).

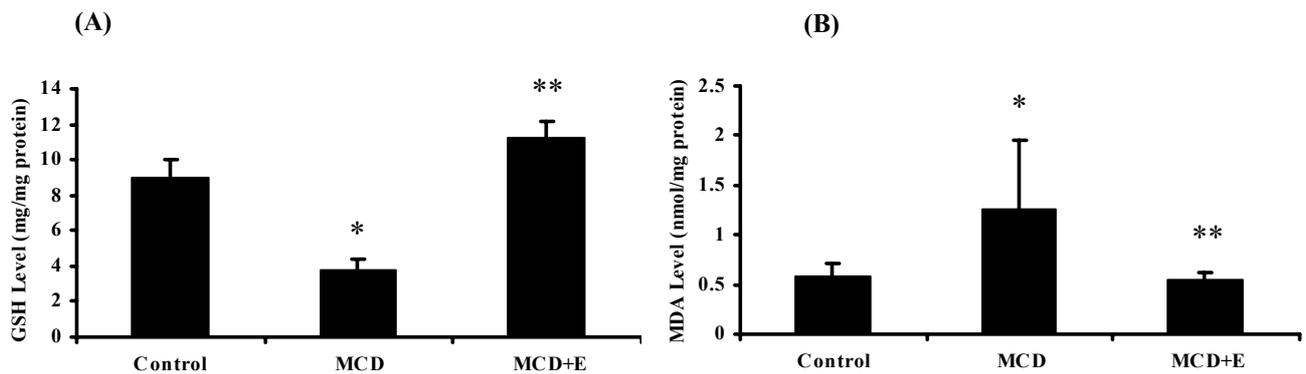


Fig 1. Hepatic levels of GSH and MDA in rats fed normal, MCD or MCD + E diets. Data represent means \pm SD (n=5). * Significantly different from control group ($p < 0.05$). ** Significantly different from MCD group ($p < 0.05$).

Histopathological findings:

In the control group no pathological changes were observed and histologically the livers appeared normal. However, the MCD diet led to grade 1 liver steatosis, inflammation and ballooning degeneration. In the MCD+E group, these factors abated to grade 0 in 80% of the rats while grade 1 was found in the remaining 20% of rats. Masson trichrom staining did not confirm the incidence of fibrosis in either group. The histopathological findings are presented in Fig. 2.

Hepatic TNF- α and TGF- β levels:

Western blot analyses indicated that in MCD group the TNF- α and TGF- β protein levels were increased relative to the control group. However, following administration of the ethyl acetate extract, the TNF- α and TGF- β protein levels were significantly decreased relative to MCD group (Fig. 3A and 4A). Consistent with the increased TNF- α and TGF- β protein levels in MCD group, the real time RT-PCR also showed that TNF- α and TGF- β mRNA levels have increased by 1.7 and 2 fold, respectively, compared to normal healthy rats. Treatment with ethyl acetate extract of *T. polium* significantly decreased the TNF- α and TGF- β gene expressions by 51% and 41%, respectively compared to animals of MCD group (Fig. 3B and 4B).

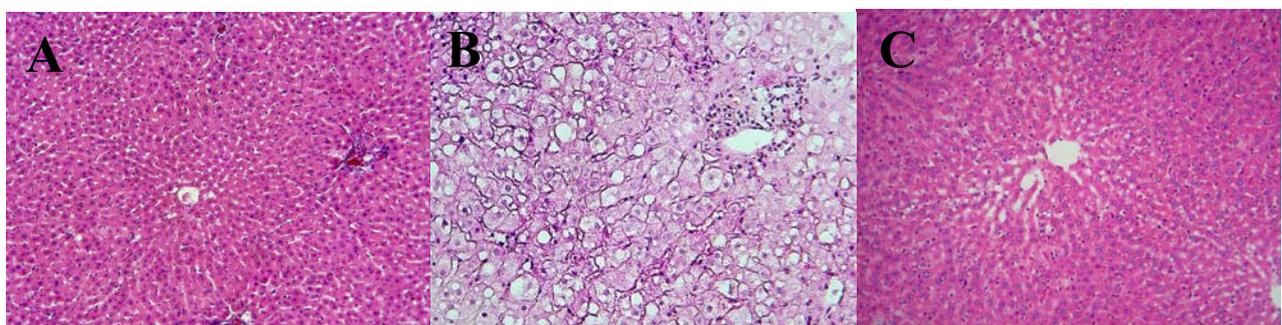


Fig 2. Histological examination of liver samples of rats fed normal, MCD or MCD + E diets. (A) Control group: normal liver histology. (B) MCD group: macro and microsteatosis, ballooning degeneration and lobular inflammation. (C) MCD+E group: marked reduction in steatosis, ballooning degeneration and lobular inflammation.

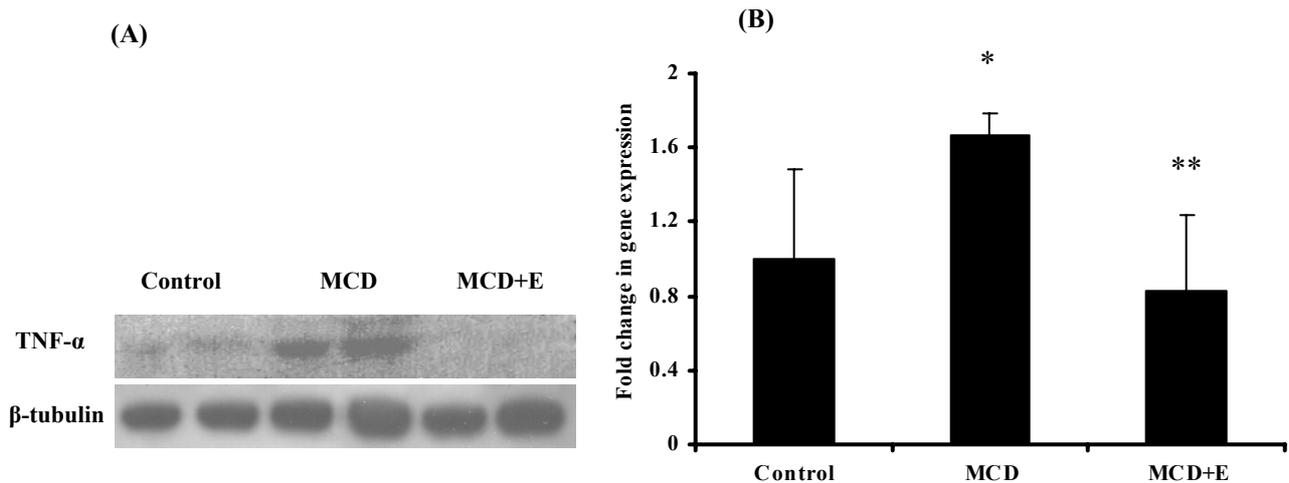


Fig 3. TNF- α expression in rats fed normal, MCD or MCD + E diets. (A) Liver homogenates (70 μ g/lane) were analyzed on a 15% SDS-polyacrylamide gel, transferred to PVDF membranes followed by incubation with the primary TNF- α antibody and then secondary conjugated antibody. Equal protein loading is confirmed by the β -tubulin protein content in each lane. (B) The relative mRNA expression of TNF- α was measured using real-time PCR after normalizing the cycle thresholds (Ct) of each sample against their corresponding β -actin. Data represent means \pm SD (n=4). * Significantly different from control group (p<0.05). ** Significantly different from MCD group (p<0.05).

Discussion

Nonalcoholic steatohepatitis is a stage of nonalcoholic fatty liver disease which might progress to liver cirrhosis. Thus, its prevention by pharmaceutical means is under the main focus of many labs worldwide. However, so far no ideal treatment for NASH has been found. Ethyl acetate extract of *Teucrium polium*, with strong antioxidant property and high polyphenolic content, exerted several favorable effects on experimental NASH induced by MCD diet. We found a significant decrease in serum AST, ALT, ALP and tissue TNF- α , TGF- β and MDA levels with accompanying improvements in the histopathological patterns regarding steatosis, ballooning degeneration and lobular inflammation.

The mechanisms that mediate the transition from steatosis to steatohepatitis (NASH) remain unknown. Day and James proposed that the development of NASH involves two pathophysiologic hits: an initial metabolic disturbance leads to accumulation of free fatty acids and triglycerides within the liver (steatosis, first hit). The progression of steatosis to NASH is associated with other factors (second hit) such as oxidative stress, mitochondrial injury and induction of proinflammatory cytokines (5). *T. polium* is a plant with antioxidant and anti-inflammatory activities. The antioxidative property of *T. polium* is certainly due to its chemical constituents. Phytochemical investigation of *T. polium* has demonstrated the presence of flavonoids (36). Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties. The isolation and structural elucidation of four flavonoids from the *T. polium* together with their antioxidant and free radical scavenging activity is also reported recently (24).

Ethyl acetate extract of *T. polium* not only effectively prevented the development of steatosis, ballooning degeneration and inflammation, but also considerably decreased markers of hepatic injury including the serum aminotransferases levels. In other words, *T. polium* successfully attenuated the development of steatohepatitis evident by histopathological and biochemical measurements.

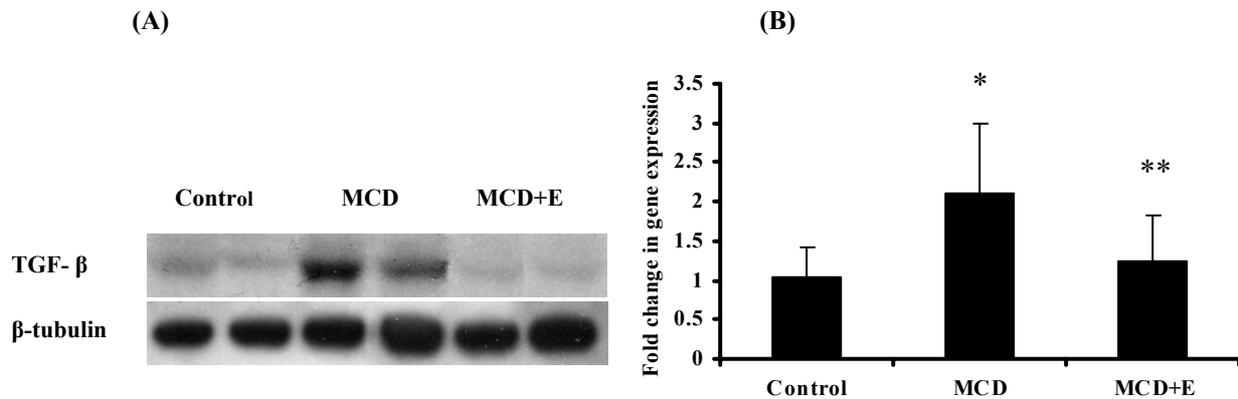


Fig 4. TGF- β expression in rats fed normal, MCD or MCD + E diets. (A) Liver homogenates (70 μ g/lane) were fractionated on a 15% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membranes followed by incubation with the primary TGF- β antibody and then secondary conjugated antibody. Equal protein loading is confirmed by the β -tubulin protein content in each lane. (B) The relative mRNA expression of TGF- β was measured using real-time PCR after normalizing the cycle thresholds (Ct) of each sample against their corresponding β -actin. Data represent means \pm SD (n=4). * Significantly different from control group (p<0.05). ** Significantly different from MCD group (p<0.05).

Glutathione, as a non-enzymatic antioxidant, effectively scavenges free radicals and other ROS directly and indirectly through enzymatic reactions (37). The decrease in GSH level in liver during NASH is probably due to its increased utilization by the hepatocytes to counteract the increased formation of lipids peroxides. In our study, a dramatic rise in liver GSH level was observed among the extract-treated rats. This probably indicates that the extract can either increase the biosynthesis of GSH or reduce the extent of oxidative stress leading to less GSH degradation, or it may have both effects. Also, based on our unpublished data, activity of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione reductase (GR) remarkably increased by ethyl-acetate extract of *T.polium*.

Lipid peroxidation is an important biological consequence of oxidative cellular damage which increases drastically in NASH. Lipid peroxidation end-products such as MDA are generated under high level of un-scavenged free radicals. MDA can cause formation of Mallory bodies; cytoplasmic filamentary aggregates containing cytokeratin material, during NASH (1). In addition, MDA can induce mitochondrial dysfunction by inhibiting mitochondrial respiration (38). Furthermore, it activates stellate cells, promoting collagen synthesis and initiating fibrogenesis (39). Several studies have shown that flavonoids can inhibit lipid peroxidation in animal models. For example, Kuzu and colleagues have reported that the increased plasma and liver tissue MDA levels in high-fat fed rats have decreased following administration of epigallocatechin gallate, a flavonoid of green tea (40). Our results demonstrated that ethyl acetate extract of *T.polium* prevented the MCD diet-induced elevation of MDA and resulted in a significant decrease in the MDA content of the liver.

In addition to triggering lipid peroxidation of cellular membranes, the excess ROS leads to over expression of TNF- α in hepatocytes, kupffer cells and adipose tissue via nuclear factor-kB (NF-kB) activation (2).

Crespo and co-workers have observed increased expression of TNF- α mRNA and its receptors in liver of patients with NASH and have also shown that higher serum TNF- α level is correlated with increased severity of NASH as manifested by more inflammation and fibrosis (9). It has been reported that TNF- α can induce insulin resistance, impair mitochondrial respiration and activate apoptosis in hepatocytes (1,4,7). In our study, ethyl acetate extract of *T.polium* significantly decreased TNF- α expression in the liver. Similarity, genistein, an isoflavone with strong antioxidant property, decreased plasma TNF- α level in rat model (17). Base on these results, it might be concluded that oxidative stress elevates the endogenous level of TNF- α . Regarding the reported role of TNF- α in liver fibrosis (41), it is expected that *T.polium* would be effective in attenuating the extent of fibrosis by lowering the TNF- α level.

In addition to growth regulating role, it is also proposed that TGF- β is involved in the pathogenesis of liver fibrosis. Enhanced hepatic production of TGF- β activates hepatic stellate cells as a key step in the process of liver fibrosis (42). It has been shown that the oxidative stress activates the nuclear factor kB/jun kinase pathway, generating nuclear c-jun that is known to have co-activator effect on TGF- β (11). It has been reported that antioxidants such as vitamin E and vitamin C can inhibit TGF- β production and attenuate hepatic fibrosis in patient with NASH (12,43). In our study, although no evidence on fibrosis was found among MCD group, however the TGF- β expression level has increased. This observation probably demonstrates the early activation of fibrosis process in rats and it seems that longer (>8weeks) administration of a MCD diet is required for development of fibrosis. Decreased TGF- β levels by administration of ethyl acetate extract might support the attenuation early phase of fibrogenesis.

In conclusion, ethyl acetate extract of *T.polium* effectively prevented the development of NASH among the experimental animals. This beneficial effect of *T.polium* might be attributed to its strong antioxidant and anti-inflammatory properties. Also, the extract might have favorable effects on liver fibrosis through decreasing TNF- α and TGF- β expression. However, complementary evaluations using the purified active constituents of *T.polium* are required to firmly confirm these predictions. These investigations are in progress in our lab.

Acknowledgment

The authors thank the Research Council of University of Tehran for the financial support of this investigation.

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