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Hepatoprotective effect of parthenolide in rat model of nonalcoholic fatty liver disease

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ABSTRACT

Context: The active ingredients of traditional medical herbs have been the focus of scientific interests.

Objective: This study was designed to explore the mechanisms of actions of parthenolide on nonalcoholic fatty liver disease (NAFLD).

Materials and methods: Thirty-five male Wistar rats were fed high-fat diet (HFD) for eight weeks with or without an intraperitoneal injection of parthenolide to develop NAFLD. Liver triacylglycerol (TG), total antioxidant capacity (TAC), total oxidative status (TOS), thiobarbituric acid reactant substances (TBARs), total thiol groups and tumor necrosis factor alpha (TNF-α) and cytochrome P4502E1 (CYP2E1) levels as well as liver ALT, AST and catalase activities were determined. In addition, quantitative real-time PCR was performed to obtain hepatic gene expression levels of TNF-α, CYP2E1 and nuclear factor-kB (NF-κB).

Results: HFD caused a significant weight gain and increased liver TG content as well as alteration in ALT and AST activities, which were attenuated after administration of parthenolide (p < .05). Weakened liver antioxidant system (TAC, total thiol groups and catalase activity) and increased oxidative stress markers (TBARs and TOS) were mainly ameliorated by parthenolide treatment (p < .05). Increased hepatic TNF-α, NF-κB and CYP2E1 at the both gene expression and protein levels were found associated with necroinflammatory changes in histopathological observations and were abrogated almost completely after parthenolide treatment. Oxidative and inflammatory changes observed in HFD fed rats were indicative of NAFLD, which were suppressed with parthenolide treatment.

Conclusions: Based on these results, parthenolide might be a candidate agent for preventing NAFLD due to its anti-inflammatory and anti-oxidative potency.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a term referred to a group of the conditions in which excess fat accumulates in the liver. It is a common disease especially in developed countries and is associated with obesity, dyslipidemia, diabetes mellitus and insulin resistance. Although the accumulation of fat may not lead to liver damage, but develops the progressive form of the disease, which is consequently converted to liver cell inflammation. This inflammatory process leads to the hepatocellular ballooning (nonalcoholic steatohepatitis, NASH) and causes different degrees of scarring that can ultimately progress to cirrhosis and hepatocellular carcinoma. Fatty infiltration during NAFLD sensitizes hepatocytes to oxidative stress (OS) and proinflammatory cytokines.

Progression of NAFLD during high-fat diet (HFD) consumption results in the increased oxidation of free fatty acids which trigger the formation of reactive oxygen species (ROS). An imbalance between production and scavenging of ROS generates deleterious processes, changes cell membrane function and accelerates the inflammatory processes through redox-sensitive transcription factors such as nuclear factor κB (NF-κB). In inflammatory processes and oxidized conditions, inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) is secreted by Kupffer cells under the regulation of NF-κB and in a vicious cycle leads to the further lipid peroxidation and ROS production. Along with the inflammatory condition, destructive accumulation of fat which is metabolized by cytochrome P4502E1 (CYP2E1) fosters production of thiobarbituric acid reactant substances (TBARs) via peroxidation of polyunsaturated fatty acids.

There are compelling evidences for the major roles of fatty acids and oxidative stress in the development and progression of NAFLD. Thus, several lines of studies have considered the treatment of fatty liver based on dietary recommendations and pharmacotherapy. Many studies have been conducted in the use of chemical drugs during the last decades. Unfortunately, the use of chemicals has unpleasant side effects such as re-accumulation of fat in the liver after drug discontinuation, adverse drug effects and the potential
of drug abuse. Therefore, it is proposed that the use of plant-derived agents with less adverse effects would be beneficial and worthy to be considered.

A sesquiterpene lactone, parthenolide, derived from the feverfew leaf extract (Tanacetum parthenium), has previously been used as an anti-migraine headache compound, anti-arthritis and fever reducer in traditional medicine. This compound is also known for its anti-tumorigenic and anti-inflammatory activities. Its anti-inflammatory effect has been shown via the inhibition of NF-κB and blockage of pro-inflammatory cytokines such as TNF-α. It is believed that the anti-tumor activity of this agent in the redox status of tumor cells is in part mediated through inducing of oxidative stress. Interestingly, parthenolide exhibits a dual effect by increasing of GSH in normal cells while reducing its level in hepatoma cancer cells. The antioxidant potency of parthenolide in HT22 neural cells has also been reported. Additionally, the oxidative effect of parthenolide in hepatic stellate cells through increasing intracellular ROS, depletion of intracellular GSH level and amelioration of hepatic fibrosis in a thioacetamide-induced rat model has also been reported. This study aimed to evaluate the effect of parthenolide against the oxidative stress and inflammation induced by NAFLD and to determine its potential to attenuate progression of NAFLD.

Methods and materials

Parthenolide (Tocris Biosciences, Bristol, UK) was dissolved in dimethyl sulfoxide (DMSO) to obtain a concentration of 100 mM, stored at −30 °C and was diluted in normal saline (0.9%) to prepare working solution. CYP2E1 ELISA kit was obtained from Neobiolab (NeoScientific, London, UK) whereas ELISA kit to determine rat liver TNF-α was purchased from eBioscience (eBioscience Inc., San Diego, CA) and Sigma-Aldrich (Sigma-Aldrich Co. Ltd., Dorset, UK) supplied ELISA kit for rat insulin determination.

Animals

Thirty-five male Wistar rats weighing 140–160 g were purchased from the Animal care center at Hamadan University of Medical Sciences (Hamadan-Iran) and were housed under controlled condition (21 ± 2 °C, 50 ± 5% relative humidity and a 12 h light/dark cycle). Animals received commercial rat chow diet and tap water ad libitum for one week to acclimatize. Rats were then randomly divided (n = 5 per group) to seven experimental groups and received chow diet or high-fat diet and treated with a given dose of parthenolide by intraperitoneal (i.p.) injection based on animal body weight (e.g., 4 mg/kg). Experimental groups were assigned as: (N) normal control rats; (D) normal rats which received 200 μl of normal saline (i.p.) containing 1.5% (v/v) DMSO as vehicle three times a week; (NP2) normal rats fed chow diet and received parthenolide (2 mg/kg) three times a week; (NP4) normal rats fed chow diet and received parthenolide (4 mg/kg) three times a week; (F) rats fed high-fat diet to induce NAFLD; (FP2) rats fed high-fat diet and received parthenolide (2 mg/kg) in normal saline (i.p.) three times a week; and finally (FP4) rats fed high-fat diet and received parthenolide (4 mg/kg) in normal saline (i.p.) three times a week. Rats were treated for eight weeks and weighted at the first day of grouping and at the end of the experiment. High-fat diet was prepared using fat (62% kcal), carbohydrates (20% kcal) and proteins (18% kcal) as well as vitamins and minerals, as previously reported by Lieber et al. The research was approved by the local ethics committee of our faculty, state and national medical board and was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

Blood and tissue sample collection

At the end of the experiment, rats were sacrificed after overnight fasting and blood samples from abdominal aorta were collected for serum separation. Liver tissue samples were dissected and washed with phosphate buffer saline (PBS). Some parts of liver tissue samples were transferred into cryotubes, kept in liquid nitrogen for 1 h and finally stored at −80 °C for further analysis, while the remaining parts of liver tissues were fixed in 10% neutral buffer formalin for histopathological examination.

Analysis of biochemical parameters

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) as well as serum triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured using commercially available kits. The atherogenic index was calculated as: [Atherogenic Index = TC/(HDL-C)] and the percentage of weight gain was calculated as [(final weight − initial weight)/initial weight × 100] and the liver index was obtained as [(liver weight/body weight) × 100].

Fasting blood sugar (FBS) was determined by a conventional enzymatic method using commercially available kit while serum insulin was measured using an ELISA kit and a Bio-Tek ELX 808 TM plate reader (Bio-Tek Instruments, VT). All assays were performed in duplicate. Insulin resistance was determined by homeostasis model assessment for insulin secretion (HOMA-IS) based on the following formula:

\[ \text{HOMA-IS} = \frac{\text{fasting insulin (μU/ml) } \times \text{ FBS (mmol/L)}}{22.5} \]

To confirm the extent of hepatic steatosis, TG content of liver tissue samples was determined according to the method described by Folch et al. and expressed in mg per gram of tissue. Hepatic total cholesterol was also determined by routine commercial cholesterol oxidase assay kit and expressed as mg per gram of tissue.

Determination of oxidative stress biomarkers

Frozen liver tissue samples were thawed and homogenized in RIPA lysis buffer (Santa Cruz Biotechnology Inc., CA).
After 30 min of incubation on ice, samples were centrifuged for 20 min at 15,000 × g. supernatants were separated and protein content was measured by bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as standard. Thiobarbituric acid reactant substances (TBARs), as biomarkers of oxidative damage to polyunsaturated fatty acids were determined in liver tissue samples and expressed as nmol.mg⁻¹ of protein²⁶. Total antioxidant capacity (TAC) was assessed as a previously modified method²² by Benzi and Strain in 1996. The assay is on the basis of the conversion (reduction) of ferric ion (Fe³⁺) to the ferrous ion (Fe²⁺). The changes in the absorbance were determined using a spectrophotometer and expressed as nmol.mg⁻¹ of protein. Total oxidant status (TOS) of liver samples was determined by the Ferric-xenol orange 1 (FOX1) reagent, according to the method described by Erel²³. The color intensity related to the oxidant molecules was measured by spectrophotometry at 560 nm after 30 min incubation and the results were expressed in nmol.mg⁻¹ of protein comparing to a solution of H₂O₂ as standard. The TOS/TAC ratio was accepted as oxidative stress index (OSI) representing the degree of oxidative stress²⁴. Catalase activity was measured in liver samples at 374 nm by an enzymatic method in the presence of H₂O₂ and enzyme activity was expressed as KU.g⁻¹ of the protein²⁵. Total thiol (SH) groups of proteins in liver tissue samples were quantified by spectrophotometry using 5,5'-dithiobisnitrobenzoic acid (DTNB), as described in review by Rudyk and Eaton and expressed as nmol.mg⁻¹ of protein²⁶. The level of advanced protein oxidation products (APOP) in plasma was measured according to the previously described method by Witko-Sarsat et al. and expressed as nmol.ml⁻¹ of chloramine-T equivalents²⁷.

**TNF-α and CYP2E1 protein assay**

TNF-α and CYP2E1 were determined in liver tissue samples using eBioscience (eBioscience Inc., San Diego, CA) and Neobiolab (NeoScientific, London, UK) ELISA kits, respectively according to the manufacturer’s instructions.

**Quantitative PCR for gene expression assay**

Hepatic CYP2E1, NF-κB and TNF-α gene expression levels were determined by quantitative real-time PCR using SYBR premix Ex Taq™ II (Takara Biotechnology, Shiga-Japan) on a Roche LightCycler® 96 System (Roche Diagnostics Corporation, Indianapolis, IN). Total RNA was extracted from liver tissue samples using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, CA)²⁸. Complimentary DNA (cDNA) was synthesized through reverse transcription of 500 ng of total RNA using PrimeScript RT reagent kit (Takara Biotechnology, Shiga, Japan). Forward and reverse primer sequences were as; CYP2E1 (forward: 5′-ACCTTTCCTTCTTCCATC-3′ and reverse: 5′-CCGTCCAGAAAAACTCATT-3′); NF-κB (forward: 5′-CTCTCAATGGCAGACGAC-3′ and reverse: 5′-CTCTTGTTT CCGTTGCTC-3′); TNF-α (forward: 5′-TGTTATCGCTTCTTACCAC-3′ and reverse: 5′-ACTACTTCCAGCGTCTCGT-3′); GAPDH (forward: 5′-AAGGTCGGTGTAACCGATTGTTG-3′ and reverse: 5′-TCCTGGAAGATGGTGATGGT-3′). Relative gene expressions (fold changes) were calculated as 2⁻ΔΔCt compared with the expression of GAPDH as housekeeping gene.

**Histopathological analysis of liver tissues**

For histopathological evaluation, formalin-fixed liver tissue samples were embedded in paraffin, sectioned at 5 μm thicknesses and stained with Masson’s trichrome and hematoxylin-eosin for light microscopic examination. Description and scoring of steatosis and hepatic necro-inflammatory activity were performed according to the method of Brunt et al. (1999) and Knodell histological activity index (HAI) system, respectively²⁹,³⁰.

The severity of macrovesicular steatosis was graded on the basis of the extent of parenchyma involved. Grade 1(−): <33% of hepatocytes were involved, Grade 2(++): 33% to 66% of hepatocytes were involved, Grade 3(+++): >66% of hepatocytes were involved and Normal (−): no hepatocytes were involved. Hepatocellular ballooning and disarray were evaluated for zonal location and an estimate of the severity (mild or marked) was made based on numbers of hepatocytes showing this change. Hepatic necro-inflammatory activity scored by the severity of portal inflammation (P), intralobular inflammation (I), piecemeal necrosis (PN) and bridging necrosis (BN). The score from 1 to 4 was given in accordance with the severity of lesions and the total score was calculated as: [score = P + I + 2 × (PN + BN)].

Although, the zone 3 perisinusoidal fibrosis is unique form that is characteristically seen in steatohepatitis, but three categories were separately evaluated: (i) perisinusoidal fibrosis was scored 0–3 based on the percent of zone 3 foci involved (0 is none, 1 is up to 33%; 2 is 33–66%; and 3 is >66%); (ii) portal fibrosis was scored as 0–4 (0 is none; 1 is expanded portal tracts; 2 is periportal fibrosis; 3 is bridging fibrosis with nodular architecture; and 4 is cirrhosis); (iii) any form of bridging fibrosis was scored 0–4 (0 is none; 1 is 1 focus; 2 is >1 focus with no nodularity; 3 is bridging fibrosis with nodular remodeling; and 4 is cirrhosis).

**Statistical analysis**

Statistical analysis was carried out using the Statistical Package for Social Sciences version 16 (SPSS Inc., Chicago, IL) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). Values were presented as mean ± SD and statistical significance was defined as p values less than .05 (p < .05). The one-way ANOVA with post hoc Tukey test was used for comparison between groups. For histopathological evaluation, differences among the groups were evaluated using the non-parametric Mann-Whitney U test (two-tailed) and Kruskal-Wallis test.

**Results**

**Effect of parthenolide on body weight and biochemical parameters**

In order to investigate the impact of parthenolide on serum lipid profile and therefore on the formation of fatty liver,
serum TC, TG, HDL-C and LDL-C levels were determined. As summarized in Table 1, the increased serum TC, TG, LDL-C and atherogenic index in group F due to the intake of high-fat diet were partially modulated after administration of parthenolide, being more attenuated at 4 mg/kg compared with 2 mg/kg. Interestingly, administration of 4 mg/kg parthenolide significantly prevented the enhancement of serum LDL-C (p < .001) and markedly compensated high-fat diet induced reduction in HDL-C (p < .02). In contrast to the treatment with 4 mg/kg parthenolide, the rats received 2 mg/kg parthenolide did not show normalized LDL-C and HDL-C levels in respect to the normal rats (group N) or to the high-fat diet fed rats (group F). High-fat diet also increased serum liver enzymes activities (ALT and AST) in group F (p < .01) whereas parthenolide attenuated this enhancement in a dose-dependent manner but could not completely bring ALT and AST activities back to the normal values.

Since NAFLD is usually accompanied by increased body weight and enhanced liver weight, TG and cholesterol content together with an alteration in liver enzymes activities we assessed the effect of parthenolide administration at two different doses in high-fat diet fed rats. As indicated in Table 2, body weight increased in all rats after 8 weeks of experiment. While there was no difference in body weight between groups at the beginning of experiment with an average weight of 138.6 ± 21.9 g, rats weighed from 369.2 ± 4.81 g in group N to the mean of 433.6 ± 18.63 g in group F. As shown in the tabulated data (Table 2), feeding of rats with high-fat diet for eight weeks (group F) resulted in a significant (p < .01) weight gain and increased liver index compared with chow diet fed groups (groups N, D, NP2 and NP4) whereas treatment with parthenolide (4 mg/kg) significantly reduced body weight (p = .03) and liver index (p = .033) compared with untreated group F. In fact, with nearly 33% weight gain, rats showed some degrees of obesity in group F compared with normal rats (group N) and treatment with parthenolide prevented high-fat diet-induced obesity by nearly 50%. Nevertheless, administration of parthenolide at none of 2 or 4 mg/kg doses attenuated body weight and liver index to the normal levels observed in groups N, D, NP2 and NP4. There was also no significant difference in weight gain or liver index between the rats treated with 2 or 4 mg/kg parthenolide (FP2 and FP4 groups).

As shown in Table 2, the pronounced significant increment in liver TG content (approximately 28.8%) and incline in total cholesterol (over 50%) indicated the induction of NAFLD due to the high-fat diet feeding in group F. Administration of 4 mg/kg parthenolide (but not 2 mg/kg) abrogated almost completely the increased liver TG and total cholesterol contents (p < .01) induced by high-fat diet in group FP4.

To study the possible protective role of parthenolide against insulin resistance, the serum FBS and insulin levels as well as HOMA-IS were determined. As shown in Table 3, rats which received high-fat diet (group F) had a significantly elevated level of FBS compared with the normal groups fed with chow diet (p < .001). The enhancement in FBS was remarkably reversed by parthenolide and no significant difference was found between control rats and those treated with high-fat diet plus 4 mg/kg parthenolide (p > .05). In contrast, high-fat diet induced increase in insulin level (group F) compared with control normal rats was not alleviated by the administration of parthenolide (Table 3). The increase in insulin level was accompanied by a 96% enhancement in insulin resistance (HOMA-IS) in group F compared to the rats fed normal chow diet (N, D, NP2 and NP4; p < .001 for all). The observed enhancement in HOMA-IS was greatly prevented by 22% and 34% after the treatment of rats with 2 or 4 mg/kg of parthenolide, respectively (p = .024 and p < .001) and 4 mg/kg parthenolide (but not 2 mg/kg) statistically reduced FBS and HOMA-IS back to the normal value (Table 3).

### Table 1. The serum lipid profiles in experimental and control groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I; N</th>
<th>Group II; D</th>
<th>Group III; NP2</th>
<th>Group IV; NP4</th>
<th>Group V; F</th>
<th>Group VI; FP2</th>
<th>Group VII; FP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>1.37 ± 0.19&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>1.45 ± 0.14</td>
<td>1.45 ± 0.13</td>
<td>1.41 ± 0.18</td>
<td>2.64 ± 0.12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.31 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.11 ± 0.28&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.96 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.99 ± 0.06</td>
<td>0.94 ± 0.06</td>
<td>0.93 ± 0.06</td>
<td>1.59 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.46 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.45 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.07 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.06 ± 0.10</td>
<td>1.04 ± 0.07</td>
<td>1.04 ± 0.06</td>
<td>0.77 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.87 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.94 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>0.51 ± 0.04&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>0.51 ± 0.07</td>
<td>0.50 ± 0.04</td>
<td>0.51 ± 0.04</td>
<td>0.75 ± 0.08&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>0.71 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.61 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Atrogenic index</td>
<td>1.28 ± 0.02&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>1.38 ± 0.24</td>
<td>1.40 ± 0.21</td>
<td>1.35 ± 0.20</td>
<td>3.41 ± 0.29&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.67 ± 0.41&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>2.23 ± 0.26&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>27.40 ± 5.00&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>28.80 ± 5.30</td>
<td>27.80 ± 6.30</td>
<td>24.80 ± 5.00</td>
<td>96.40 ± 6.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.80 ± 12.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>67.80 ± 7.90&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>15.40 ± 4.40&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>18.20 ± 1.90</td>
<td>23.20 ± 3.90</td>
<td>24.80 ± 1.90</td>
<td>66.00 ± 4.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.60 ± 6.40&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>34.80 ± 3.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SD. In each row, groups with similar alphabetic letters a, b, c, d, e or f represent significant difference (p < .05). For simplicity differences among main groups have been shown, only.

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IU: international unit per liter.

### Table 2. Body weight, liver index, liver TG, liver total cholesterol, ALT and AST in experimental and control groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I; N</th>
<th>Group II; D</th>
<th>Group III; NP2</th>
<th>Group IV; NP4</th>
<th>Group V; F</th>
<th>Group VI; FP2</th>
<th>Group VII; FP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>139.4 ± 2.7</td>
<td>139 ± 1.58</td>
<td>139.8 ± 1.92</td>
<td>136.4 ± 9.23</td>
<td>139.4 ± 1.14</td>
<td>138.2 ± 1.48</td>
<td>138.6 ± 2.3</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>369.2 ± 4.81&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>372 ± 2.73</td>
<td>359.8 ± 8.78</td>
<td>348.4 ± 2.5</td>
<td>433.6 ± 18.63&lt;sup&gt;d&lt;/sup&gt;</td>
<td>414.2 ± 10.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>398.2 ± 17.16&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight gain (%)</td>
<td>164.95 ± 7.6&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>167.65 ± 3.72</td>
<td>157.47 ± 9.69</td>
<td>156.44 ± 18.98</td>
<td>210.99 ± 11.59&lt;sup&gt;d&lt;/sup&gt;</td>
<td>199.69 ± 5.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>187.33 ± 12.49&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver TG (mg/g tissue)</td>
<td>13.25 ± 1.22&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>13.38 ± 0.1</td>
<td>14.16 ± 0.73</td>
<td>13.38 ± 0.83</td>
<td>17.45 ± 1.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.23 ± 0.68&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.63 ± 0.70&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver TC (mg/g tissue)</td>
<td>2.09 ± 0.09&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>2.07 ± 0.05</td>
<td>2.07 ± 0.04</td>
<td>2.02 ± 0.05</td>
<td>3.16 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.84 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.55 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD. In each row, groups with similar alphabetic letter (a, b, c, d, e or f) represent significant difference (p < .05). For simplicity differences among main groups have been shown, only.
Effect of parthenolide on histopathological changes

The histopathological examination of the liver tissue samples confirmed the results obtained from the biochemical tests. Liver tissues from all rats in D, NP2 and NP4 groups showed normal lobular architecture with central veins, radiating hepatic cords and little collagen deposition while a mild, moderate and severe tissue changes were observed in liver tissues of those received high-fat diet (F) and high-fat diet + parthenolide (FP2 and FP4).

In 80% of samples from group F, the main histopathological findings were as moderate to severe macrovesicular steatosis, marked hepatocellular ballooning, portal and intra-lobular inflammation with chronic inflammatory cells accompanied with focal necrosis, whereas in the remaining 20% of the samples from group F the main histopathologic finding was piecemeal necrosis (Figure 1).

Table 4. Effects of parthenolide treatment on fibrosis and HAI scores in rat model of nonalcoholic fatty liver.

<table>
<thead>
<tr>
<th>Groups</th>
<th>HAI system median (range)</th>
<th>HAI score</th>
<th>Fibrosis median (range)</th>
<th>Fibrosis score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group D, NP2, NP4</td>
<td>0 (0–1)</td>
<td>0.3 ± 0.15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group F</td>
<td>3.5 (3–4)</td>
<td>3.5 ± 0.16</td>
<td>3 (2–3)</td>
<td>3.1 ± 0.27</td>
</tr>
<tr>
<td>Group FP2</td>
<td>2 (1–3)</td>
<td>2.2 ± 0.2</td>
<td>2 (1–3)</td>
<td>2.1 ± 0.27</td>
</tr>
<tr>
<td>Group FP4</td>
<td>2 (1–3)</td>
<td>2.2 ± 0.22 ± 0.2</td>
<td>2 (1–2)</td>
<td>1.7 ± 0.15</td>
</tr>
</tbody>
</table>

aData are represented as means ± SE.

Effect of parthenolide on histopathological changes

The histopathological examination of the liver tissue samples confirmed the results obtained from the biochemical tests. Liver tissues from all rats in D, NP2 and NP4 groups showed normal lobular architecture with central veins, radiating hepatic cords and little collagen deposition while a mild, moderate and severe tissue changes were observed in liver tissues of those received high-fat diet (F) and high-fat diet + parthenolide (FP2 and FP4).

In 80% of samples from group F, the main histopathological findings were as moderate to severe macrovesicular steatosis, marked hepatocellular ballooning, portal and intra-lobular inflammation with chronic inflammatory cells accompanied with focal necrosis, whereas in the remaining 20% of the samples from group F the main histopathologic finding was piecemeal necrosis (Figure 1).

As shown in Table 4, the score of histological activity index (HAI) in group F was significantly higher than that of the control group (3.5 ± 0.16 versus 0.3 ± 0.15, p < .001). Histopathological examination of FP2 and FP4 groups showed mild macrovesicular steatosis (Grade 1) and mild hepatocellular ballooning (Figure 2). The score of HAI in FP2 (2.2 ± 0.2) and FP4 (2 ± 0.21) groups were significantly lower than that of group F (p = .001 and p < .001, respectively) but these scores did not reach the normal level in control group (2.2 ± 0.2 and 2 ± 0.21 versus 3.5 ± 0.16, p < .001). The HAI score also did not significantly differ between FP2 and FP4 groups (p = .49).

According to the observations made on the Masson’s trichrome stained sections, fibrosis score was markedly increased in rats received high-fat diet (3.1 ± 0.27), but treatment of rats with 2 and 4 mg/kg parthenolide almost completely abrogated high-fat diet induced fibrosis score (2.1 ± 0.27, p = .03 and 1.7 ± 0.15, p = .001, respectively) as shown in Figure 3.

Effect of parthenolide on liver TNF-α, NF-κB and CYP2E1 gene expression

The gene expression levels of liver TNF-α and NF-κB (as sensitive proinflammatory biomarkers) and expression level of
cytochrome P450 2E1 (CYP2E1) as an indicator of oxidative damage were assessed by real-time PCR. As shown in Figure 4(A–C), the expression of TNF-α, NF-κB and CYP2E1 were significantly increased in group F (p < .01) after eight weeks of receiving high-fat diet. Although administration of 2 and 4 mg/kg of parthenolide could not completely normalize the expression levels of TNF-α, NF-κB and CYP2E1 in parthenolide-treated rats to the level observed in normal rats (group N), it significantly down-regulated the gene expressions in a dose-dependent manner compared with the group F (Figure 4(A–C)), being more reduced in rats received 4 mg/kg of parthenolide. TNF-α, NF-κB and CYP2E1 gene expression levels slightly increased in normal rats received parthenolide (NP2 and NP4) or carrier (D) compared with control rats (N) but did not reach statistical significance. The lack of significant difference in gene expression levels between normal rats of NP4 group and high-fat diet plus parthenolide fed rats (FP2 and FP4) indicated that parthenolide almost completely suppressed diet induced gene expression.

Effect of parthenolide on serum APOP and hepatic biomarkers of oxidative stress

To analyze the anti-oxidant effect of parthenolide against the NAFLD-related oxidative stress, hepatic TBARs, TAC, TOS, OSI, total thiol groups (–SH) and catalase activity as well as serum advanced protein oxidation products (APOP) concentration were evaluated. As is summarized in Table 5, receiving of high-fat diet for 8 weeks strongly caused oxidative stress and significantly increased liver TBARs, TOS, OSI and serum APOP concentration whereas markedly decreased the hepatic catalase activity, total antioxidant capacity (TAC) and total thiol group content (p < .001 for all). The alterations in TBARs, APOP and TAC were mostly ameliorated after treatment of rats with parthenolide (p < .01 for all). Although receiving of 4 mg/kg parthenolide suppressed the incline in TBARs by 50%, the TBARs level did not decline to the normal level. Similarly, the 4-fold enhancement in APOP level in high-fat diet fed rats (group F) was significantly suppressed after
Effect of parthenolide on liver TNF-α and CYP2E1 protein level

ELISA assay was performed to determine protein levels of TNF-α and CYP2E1 in order to confirm the effect of parthenolide on their corresponding gene expression levels in liver tissues. Apart from the increase in gene expression levels, an enhancement in protein levels of TNF-α and CYP2E1 was also observed due to the high-fat diet feeding (Figure 5(A) and Figure 5(B)). High-fat diet in group F resulted in a 4-fold increase in liver TNF-α and a 2-fold enhancement in CYP2E1 protein levels. Although administration of parthenolide at the both doses of 2 or 4 mg/kg partly reduced TNF-α protein level (Figure 5(A)), only treatment with 4 mg/kg of parthenolide (group FP4) showed no significant difference \( (p > .05) \) between parthenolide-treated and untreated normal rats (group N). Likely, the 2-fold increased CYP2E1 protein level induced by high-fat diet (group F), was dose dependently reduced by parthenolide and eventually reached to a level that did not statistically differ \( (p > .05) \) from untreated normal rats (group N).

Discussion

The accumulation of fat in the liver, termed nonalcoholic fatty liver disease (NAFLD), is the most common liver disorder with the globally increasing prevalence. It is more common in developed countries and becomes the predominant cause of chronic liver diseases worldwide. Herbal plants and plant derived chemicals have so far been used for treatment of diseases due to their anti-diabetic, antimicrobial, hypolipidemic, anticancer, and anti-inflammatory properties or because of their effectiveness on memory and learning. Parthenolide, a feverfew plant (Tanacetum Parthenium) derived sesquiterpene lactone, has been used for years as a prominent candidate herbal medicine due to its anti-inflammatory effects via regulation of TNF-α and NF-kB and its anticancer properties. In addition, countered antioxidant effects in normal cells and oxidative properties in cancerous cells have also been reported for parthenolide. Since the antioxidant potential of parthenolide has already been investigated in various experimental models, its therapeutic potential warrants further exploration.
documented, the effects of parthenolide against NAFLD and NAFLD-related oxidative stress were examined in this study with a special consideration on the effects of parthenolide on TNF-α and NF-κB expression levels in rats. High-fat diet (62% kcal) was used to induce NAFLD and the effects of 2 and 4 mg/kg parthenolide were evaluated at the different transcriptional and translational levels as well as histopathological examination in rat liver tissues. High-fat diet induced a remarkable 28% weight gain in group F which was mostly prevented with 4 mg/kg parthenolide treatment. Since overweight and obesity are clearly associated with NAFLD and the likelihood of developing NAFLD increases with the degree of obesity3, it is postulated that the rats fed high-fat diet in group F are at the higher risk of NAFLD. In addition to the weight gain, an increased liver index and higher liver TG and total cholesterol contents were also observed in high-fat diet fed rats. Together, these observations are the indicators of the development of NAFLD. Interestingly, this finding was confirmed at the next step by our histopathological observations showing microvesicular steatosis and hepatocellular ballooning of liver tissue samples as well as elevated serum lipid profile in the rats fed high-fat diet. Therefore, it can be concluded that the administration of high-fat diet for 8 weeks may lead to NAFLD and the use of parthenolide may preclude body weight gain, prevents deposition of TG and total cholesterol in the liver, normalizes serum lipid profile and almost completely abrogates high-fat diet induced fibrosis, according to the observations made on the Masson's trichrome stained sections.

The most common impairments in lipid metabolism in NAFLD are hypertriglyceridemia, hypercholesterolemia, low HDL-C, high LDL-C and high atherogenic index37. In accordance with previous reports, administration of high-fat diet for 8 weeks resulted in elevated serum lipid profile in group F. Interestingly, our findings showed that parthenolide partly abrogated high-fat diet induced increase in TC, LDL-C and atherogenic index and abolished reduction in HDL-C. Therefore, we showed for the first time that parthenolide treatment strongly reduced NAFLD severity by remarkably attenuating the hepatic steatosis and significantly preventing accumulation of TG deposits.

CYP2E1 is a member of oxidoreductase cytochrome family and its stimulatory function on fat accumulation and generation of oxidative stress has previously been reported39. Here, we observed that CYP2E1 gene expression up-regulated and its protein level increased in high-fat diet fed rats (group F). This finding again explains the presence of TG deposits and hepatic steatosis in liver tissue and therefore confirms the induction of NAFLD in group F.

It is believed that the inflammatory processes resulted from dyslipidemia during the development of NAFLD leads to the insulin resistance, increased lipolysis and free fatty acids output40. Subsequently, the alteration of fat metabolism and influx of free fatty acids into the liver leads to the triglyceride accumulation in hepatocytes40, as observed in our study. In addition, over-expression of CYP2E1 promotes liver injury, activates lipid peroxidation processes and induces insulin resistance via impaired hepatic insulin signaling41. These data once again support our finding showing increased insulin resistance (HOMA-IS) in high-fat diet induced NAFLD rats (group F).

The correlation between insulin resistance (HOMA-IS) and oxidative stress has previously been reported42. Several lines of evidence suggested that insulin resistance disturbs lipid metabolism and fatty acid β-oxidation, generates excessive superoxide anion (or H2O2) and decreases the synthesis of catalase42. Similar results indicating increased insulin resistance accompanied with reduced catalase activity was also observed in our experiments in NAFLD rats, where treatment of the rats with parthenolide almost completely normalized alterations and restored anti-oxidant capacity of the cell (as an incline both in TAC and catalase activity). The other consequence of cellular oxidative damage is protein oxidation, a process that involves the production of cross-linked and aggregated metabolites resistant to proteolysis43 and generates a novel marker named as oxidant-mediated protein damage, APOP44. Apart from oxidant-mediated damages in proteins, the thiol groups which are the major portion of the reduced environment and the important part of glutathione, thioredoxin and other thiol-dependent antioxidants, are consumed during the oxidative stress, as the first antioxidant defense system45. We showed that high-fat diet increased oxidant-mediated protein damage (APOP) more than 3-fold.
and reduced free –SH groups of proteins to less than 50% in NAFLD rats. It seems that the reduction in antioxidant markers such as total thiol groups, TAC and catalase activity together with the increased unpleasant oxidative factors such as TBARs, TOS, OSI and APOP are responsible for the diminution of antioxidant defense in the liver of high-fat diet fed NAFLD rats.

In our study, the restoration of antioxidant enzymes and amelioration of oxidative damages, especially those represented by TOS, TBARs, APOP and oxidized thiol groups were observed as a result of parthenolide treatment. Parthenolide, particularly in higher dose, inhibited the high-fat diet induced oxidation of –SH groups and suppressed catalase activity to weaken the unpleasant effects of high-fat diet. These observations may explain the antioxidant effect of parthenolide, observed in present study. Recently, numerous investigations have focused on reactive oxygen species (ROS) as key players in the progression of fatty liver where cytochrome P450 2E1 (CYP2E1) has also emerged as a potentially important cause of ROS overproduction. Indeed, higher hepatic CYP2E1 expression and activity have been frequently observed in the context of NAFLD. Similarly, we showed an increased CYP2E1 gene expression together with a higher TOS level, as indicators of oxidative damage in the high-fat diet fed NAFLD rats (group F). Interestingly, in the present study the antioxidant effect of parthenolide was found associated with the down-regulation of CYP2E1 both at the mRNA and protein levels. This finding supports previous reports and confirms the role of CYP2E1 in the balancing of oxidant/antioxidant state. Moreover, it is believed that CYP2E1-mediated overproduction of ROS in NAFLD could promote hepatic insulin resistance, which can further aggravate fatty liver. Likewise, we observed enhanced CYP2E1 gene expression followed by insulin resistance and increased HOMA-IR in NAFLD rats (group F) while treatment with parthenolide mostly diminished these unpleasant alterations in FP4 group. Collectively, the results of the present study reasserted the previous reports on the correlation of hepatic steatosis with insulin resistance, oxidative stress, CYP2E1 and the inflammation.

The inflammatory response observed in the present study was a result of NAFLD due to the increase in liver ALT and AST activities, transcriptional up-regulation of TNF-α and NF-κB, and deleterious inflammatory changes in liver histopathology. These findings confirmed the previous report about the relationship between NF-κB activity and the expression of inflammatory cytokines followed by the promotion of inflammatory responses concurrent with insulin resistance. Additionally, our results about the inhibitory effects of parthenolide on TNF-α and NF-κB expression as well as its modulatory effect on necroinflammatory changes in hepatocytes are in line with previous reports reaffirming the anti-inflammatory properties of parthenolide.

Although some inflammatory cytokines such as IL-1β down-regulate CYP2E1 gene expression through inhibiting the function of transcription factors on the CYP2E1 upstream regulatory region, Hakkola et al. have reported that it is unlikely that TNF-α controls function of transcription factors in CYP2E1 gene expression in nonalcoholic steatohepatitis.

Likewise, we did not observe down-regulation of CYP2E1 by TNF-α in NAFLD rats (group F) and the expression of CYP2E1 gene was in correlation with the degree of steatosis. Similar to previous studies, our results showed the anti-inflammatory effect of parthenolide via the suppression of TNF-α and NF-κB gene expression, although the inhibitory effect of parthenolide on TNF-α at the protein level did not reach statistical significance.

Conclusions

In conclusion, our results showed that parthenolide inhibited high-fat diet induced oxidative stress, attenuated insulin resistance, reduced deleterious hepatic steatosis and suppressed production of TNF-α and NF-κB inflammatory cytokines in NAFLD rats. Therefore, parthenolide can be considered as a candidate therapeutic agent with potential anti-inflammatory and anti-oxidative properties. However, due to the limitations in the present study including low sample number, relatively shorter treatment period and resource limitation, it is supposed that determination of reactive oxygen species (ROS) and activity of antioxidant enzymes (e.g., superoxide dismutase and glutathione reductase) as well as determination of other cytokines and inflammatory mediators such as interleukins might lead to the more reliable results in further investigation.

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Disclosure statement

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