

Hawthorn ethanolic extracts with triterpenoids and flavonoids exert hepatoprotective effects and suppress the hypercholesterolemia-induced oxidative stress in rats

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ABSTRACT

Objective(s): The current study was aimed to determine the bioactive constituents and biological effects of the *Crataegus monogyna* ethanolic extracts from bark, leaves and berries on hypercholesterolemia.

Materials and Methods: Oleanolic acid, ursolic acid, quercetin and lupeol concentrations were quantified by HPLC. Total phenol content and radical scavenging activity of extracts were also measured. The hypocholesterolemic, antioxidant, and hepatoprotective effects of the extracts were examined in hypercholesterolemic rats and compared with orlistat.

Results: The highest phenol content, oleanolic acid, quercetin and lupeol levels and free radical scavenging potency were found in the bark extract, and the highest ursolic acid level was found in the berries extract. Orlistat and extracts significantly ($P < 0.05$) lowered the hypercholesterolemia-increased serum level of hepatic enzymes and lipid peroxidation level. Hawthorn's extracts protected from hepatic thiol depletion and improved the lipid profile and hepatic damages.

Conclusion: Data suggested that hawthorn's extracts are able to protect from hypercholesterolemia-induced oxidative stress and hepatic injuries. Moreover, the hypocholesterolemic effect of extracts was found comparable to orlistat.

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Introduction

Hawthorn (*Crataegus* spp.) is a member of Rosaceae family and has been recorded as a popular medicinal plant in most of the countries. Hawthorn extract is traditionally used for preventing and treating cardiovascular diseases including angina, hypertension, and congestive heart failure (1-3). During the last few years, hawthorn due to having other medical benefits such as lowering the plasma cholesterol and triglycerides attracted much attention (4, 5). Various constituents such as flavonoids, proanthocyanidins, and organic acids with remarkable biological activities including free radical scavenging, anti-lipoperoxidation and anti-inflammatory, have been isolated from different parts of hawthorn (6). Meanwhile, previous toxicological studies indicated that the consumption of hawthorn fruit exerts little side effects (7).

Hawthorn and hawthorn-derived products including its extract and dried fruit are used worldwide. There are various species of hawthorn, which is widely used as medicinal and food materials in the European countries, China and other countries. Among the countries, different species and diverse parts of hawthorn are used. For example, in most of the European countries, leaves and flowers of the grown hawthorn are used as herbal medicine, while in china hawthorn fruit is used for fresh consumption or for preparation of jam and juice (8). Many studies have characterized the European and Chinese hawthorn species and demonstrated their biological activities, while there is lack of knowledge about the most common hawthorn species in Iran and its biologically active compounds. Hence, in this study we aimed to identify some of the

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bioactive constituents of the *Crataegus. monogyna* ethanolic extract from bark, leaves and berries. Thereafter, the hypocholesterolemic, antioxidant, and hepatoprotective effects of the extracts were examined in experimentally hypercholesterolemic rats. Moreover, the biological activities of extracts were compared with routinely used hypocholesterolemic agent of Orlistat, which inhibits intestinal lipase activity.

Materials and Methods

Chemicals

Oleanolic acid (OA, O 5504), Ursolic acid (UA, U 6753), Quercetin (QCT, Q 4951), Lupeol (LPL, L 5632), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), butylated Hydroxytoluene (BHT) and 5.5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich (Germany). Thiobarbituric acid (TBA), phosphoric acid (85%), dimethyl sulfoxide (DMSO), gallic acid, and Folin-Ciocalteu's reagent were purchased from Merck (Darmstadt, Germany). N-butanol was obtained from Carl Roth, GmbH Co. (Germany).

Serum levels of hepatic functional enzymes, alanine transaminase (ALT), aspartate transaminase (AST) and gamma glutamyl transferase (GGT) were determined spectrophotometrically using commercially available standard kits (ZiestChem Inc., Tehran, Iran). Serum lipids and lipoproteins, total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides (TG) were measured using standard kits (ParsAzmun Inc., Tehran, Iran).

Plant material collection and Extraction

Hawthorn bark, leaves and berries were collected from east and west Azerbaijan, Iran during August to September 2012, and after identification by an expert botanist (Faculty of Basic Sciences, Department of Biology, Botanic Herbarium), the samples were air-dried. The seeds were removed from berries and different parts were individually ground using grinder for bark and leaves and a coffee grinder for dried berries. The individual powders from different parts of hawthorn were extracted using ethanol (70%), and after filtering through Whatman filter (No. 42), were concentrated using a vacuum evaporator (Heidelberg, Germany). In order to get fully dried products, the extracts were exposed to gentle stream of N₂ gas.

Analysis and quantification of the triterpenic acids, quercetin and lupeol in the ethanolic extracts of bark, leaves and berries of hawthorn

The triterpenic acids level (Oleanolic acid and Ursolic acid) was analyzed using reversed-phase high performance liquid chromatography (HPLC) according to previously described method with minor modification (5). The HPLC analysis was performed on an Agilent 1200 HPLC system (Milford,

MA, USA) equipped with a Quartz pump (G 1311 A), an auto-sampler (G 1329 A), and a UV detector (VWD, G 1314 B). The extracts were dissolved in mobile phase and 20 µl of the dissolved solution was injected onto the column (Eclipse XDB-C 18, 5 µm, 4.6 mm × 15 cm) by using an auto-sampler. The mobile phase consisted of a mixture of 15% H₂O, 85% methanol and 0.15% acetic acid. The eluent was eluted with a flow rate of 1 ml/min and was detected by a UV detector at 210 nm.

To determine the quercetin concentration of the extracts, previously validated method was used (9). In brief, the separation of quercetin was made using an Eclipse XDB-C 18 column (5 µm, 4.6 mm × 15 cm). Elution by mobile phase consisting of methanol-distilled water-trifluoroacetic acid (700 + 300 + 1, v/v/v) was performed with a flow rate of 0.8 mL/min and the eluent was detected by a UV detector at 254 nm wavelength.

Lupeol was separated by using a phenomenex C18 column (250 × 4.6 I.D., 5 µm particle size) based on previously described method (10). The extracts were dissolved in mobile phase consisting of acetonitrile and water (90:10, V/V), and then 20 µl of the dissolved solution was injected onto the column. The eluent was eluted with flow rate of 1 ml/min and was detected by a UV detector at 230 nm.

To identify the triterpenoids (OA and UA), quercetin and lupeol, we used pure standards to prepare calibration curves (0.01 to 5 mg/ml) and compare the peaks of the samples with those of the external standards at the same conditions.

Total Phenol measurement

The total phenol content of hawthorn extracts was measured based on the Folin-Ciocalteu method as described previously (11). Absorbance of each individual concentrations of extracts and the absorbance of gallic acid standard at concentrations of 0, 31.12, 62.5, 125, 250, 500, 1000 and 2000 mg/l, were determined at 765 nm against the blank. The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per mg of extract, using a standard curve range of 0 to 2000 mg of gallic acid/l, giving an equation as:

$$\text{Absorbance} = 0.0117 \text{ gallic acid (mg/l)} + 0.0229 \\ (R^2 = 0.9997)$$

Determination of radical scavenging activity

To determine the radical scavenging activity of various concentrations of hawthorn extracts (0.2, 2, and 20 mg/ml), the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay was conducted as described previously (12). DPPH in form of radical is dark purple-colored and has maximum absorbance at 515 nm; however, when it is reduced by antioxidant agents, the purple color changes to yellow and the absorption

disappears. The change in absorption is proportional to the radical scavenging power of the test compound. Briefly, 50 µl of various concentrations of extracts was added to 4 ml of DPPH (0.04 g/l) in methanol. The reaction was carried out in closed eppendorf tubes (5 ml) at room temperature for 30 min. The absorbance was measured at 515 nm using a Pharmacia LKB- Novaspec II spectrophotometer (Biochrom, England). The effect of butylated hydroxyanisole (BHA) at different concentrations (10, 100 and 1000 µg/ml) on DPPH was also measured to compare with that of extracts. The average of three times measurements was expressed as the percentage of antioxidant activity (AA) and calculated by using the following formula:

$$\%DPPH = [(Ab_{515 \text{ control}} - Ab_{515 \text{ sample}}) / Ab_{515 \text{ control}}] \times 100$$

Where $Ab_{515 \text{ control}}$ is the absorbance of the control reaction, which contains all reagents except extract, and $Ab_{515 \text{ sample}}$ is the absorbance of various concentrations of extracts.

Animals and experimental design

Forty eight adult male Wistar rats (200-220 g) were obtained from the animal resource of the Faculty of Veterinary Medicine, Urmia University, Iran. The rats were in good health condition and were acclimatized for one week in animal house at 25 °C with a 12 hr light-dark cycle and had free access to food (normal chow diet containing 11% fat, 15% protein and 74% carbohydrate) and water. The high cholesterol diet (HCD) contained normal chow diet, supplemented with 4% cholesterol (13). The experimental protocols were approved by the ethical committee of Urmia University in accordance with principles of laboratory animal care (AECUU/123/2011).

Animals were randomly assigned into six groups (n=8) including: control (C), non-treated hypercholesterolemic group (HC) and treated hypercholesterolemic groups. The later were divided into 4 subgroups and along with high cholesterol diet received bark (T1), leaves (T2), berries (T3) extracts (100 mg/kg/day, orally) (14), and orlistat (T4) (10 mg/kg/day, orally) (15).

All the control and test groups received the normal saline (5 ml/kg) and test compounds for 4 weeks (28 consecutive days). Before the experimental procedures, all animals were weighed individually and this procedure was repeated at the end of the study to evaluate any treatment-related changes in body weight gain.

Serum preparation and tissue samples collection

On day 29, after an overnight fasting, blood samples were collected via cardiac puncture under light anesthesia provided with diethyl ether. After 30 min at room temperature, the samples were centrifuged at 3000 ×g for 10 min to obtain the serum. The serum samples were then stored at -20 °C until further analyses.

The anesthetized animals were ultimately euthanized by using CO₂ gas. The liver specimen were immediately removed and rinsed with chilled normal saline. One half of the liver samples were fixed in 10% buffered formaldehyde solution until histopathological examinations, and the second half was snap frozen in liquid nitrogen and kept in -70 °C until further biochemical analyses.

Determination of the serum level of hepatic functional enzymes

The serum levels of ALT, AST and GGT were measured using commercially available standard kits according to manufacturer's instructions.

Analyses of lipid profile

Serum samples were subjected to lipid profile analyses; therefore, the serum concentrations of total cholesterol, triacylglycerol, HDL-cholesterol and LDL-cholesterol were analyzed according to manufacturer's instructions.

Measurement of total thiol molecules (TTM)

Total sulfhydryl levels in the liver samples were measured as described previously (16). Briefly, 0.3 to 0.4 g of the liver samples were homogenized in ice-cold KCl (150 mM), and the mixture was centrifuged at 3000×g for 10 min. Then 0.2 ml of the supernatant of the tissue homogenate was added to 0.6 ml Tris-EDTA buffer (Tris base 0.25 M, EDTA 20 mM, pH 8.2), and thereafter 40 µl 5,5'-Dithiobis-2-nitrobenzoic acid (10 mM in pure methanol) was added to the 10 ml glass test tube. The final volume of this mixture was made up to 4.0 ml by an extra addition of methanol. After 15 min incubation at room temperature, the samples were centrifuged (Roter-Uni II, BHG, Germany), at 3000 × g for 10 min, and ultimately the absorbance of supernatant was measured at 412 nm. The TTM capacity was expressed as nmol per mg of protein in samples. The protein content of the samples was measured according to the Lowry *et al* method (17).

Malondialdehyde (MDA) determination

To determine the lipid peroxidation rate in the control and test groups, the MDA content of the liver samples was measured using the thiobarbituric acid (TBA) reaction as described previously (18). Briefly, 0.2 to 0.3 g of the samples were homogenized in ice-cooled KCl (150 mM), and then the mixture was centrifuged at 3000 g for 10 min. Subsequently, 0.5 ml of the supernatant was mixed with 3 ml phosphoric acid (1% V/V) and then after vortex mixing, 2 ml of 6.7 g L⁻¹ TBA was added to the samples. The samples were heated at 100 °C for 45 min, and then chilled on ice. After adding of 3 ml N-butanol, the samples were centrifuged at 3000 g for 10 min. The absorbance of supernatant was measured spectrophotometrically (Pharmacia, Novaspec II, Biochrom, England) at 532 nm, and the

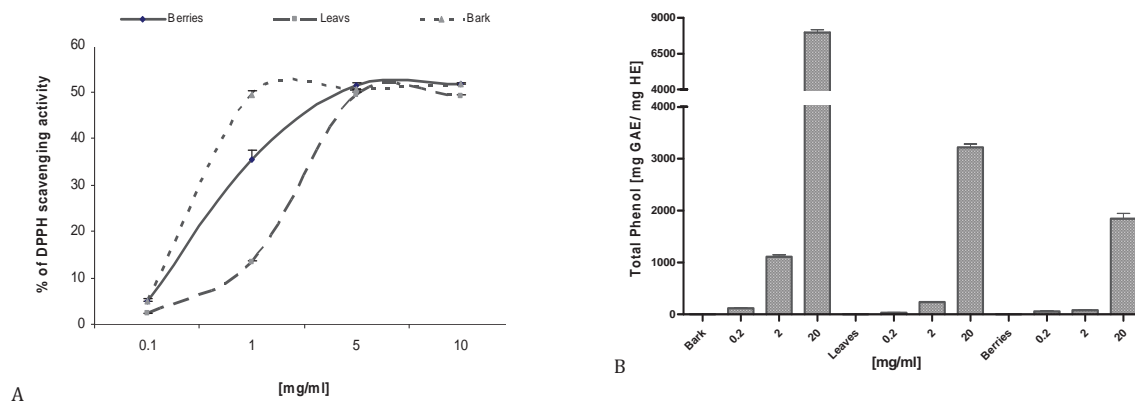


Figure 1. Diphenyl-1-picrylhydrazyl scavenging activities (A) and total phenol content (B) of different concentrations of hawthorn extracts

MDA amount was calculated according to the simultaneously prepared calibration curve using MDA standards. The amount of MDA was expressed as nmol per mg of protein. The protein content of the samples was measured based on Lowery *et al* method (17).

Histopathological examinations

The liver samples fixed in 10% buffered formaldehyde, were dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin wax. Six µm thick sections were prepared using a rotary microtome and stained with Hematoxylin and Eosin for histopathological examinations under light microscope. To evaluate the level of damages followed feeding a HCD, indices such as congestion, thickening of vascular wall, bile duct hyperplasia, and vacuolation of hepatocytes in the liver were scored numerically. The evaluation criteria were as follow: 0: indicating no detectable lesion, 1: mild changes, 2: moderate changes and 3: severe changes. For each animal in the control and test groups at least three slides were prepared and scored. The histopathological studies were conducted by a pathologist who was unaware of the study purposes.

Statistical analyses

The mean and standard deviation of the measured parameters were calculated. The results were analyzed using Graph Pad Prism software (version 2.01. Graph Pad Software Inc. San Diego, California, USA). The comparisons between groups were made by analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. For histopathological data, a non-parametric Kruskal-Wallis test was used. $P < 0.05$ was considered statistically significant.

Results

In vitro antioxidant capacity analyses of hawthorn extracts

To evaluate the antioxidant potency of hawthorn extracts, the DPPH scavenging potency and total phenol content of various ethanolic extracts were measured. We found that the free radical scavenging potency of bark extract was higher than that of leaves extract followed by berries extract (Figure 1-A). The total phenol content determination revealed that the highest phenol content belongs to the bark extract followed by leaves and berries extracts (Figure 1-B).

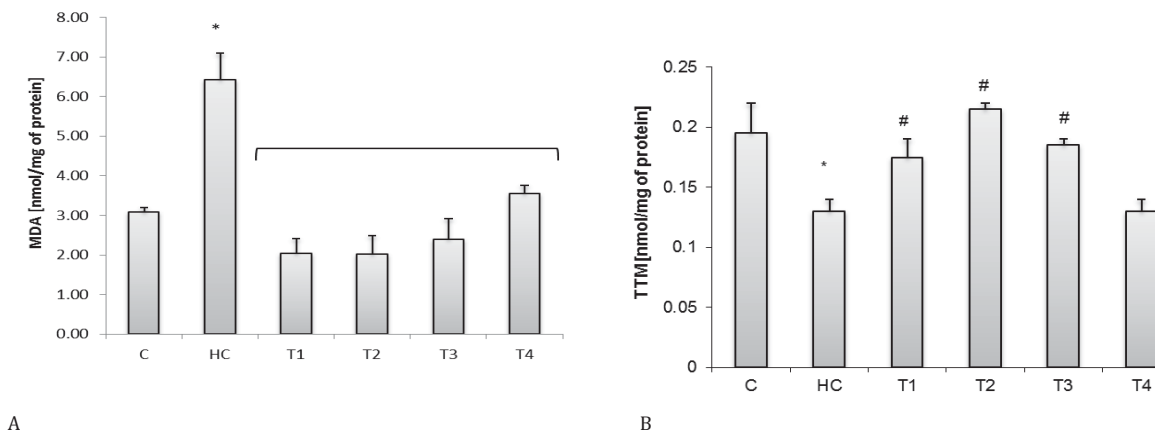


Figure 2. Effect of hawthorn extracts on: (A) hypercholesterolemia-elevated malondialdehyde content and (B) total thiol molecules concentration in the liver; bars represent mean ± SD; n = 8 in each group. Stars indicate significant differences ($P < 0.05$) between the control and untreated hypercholesterolemic groups and #s indicate significant differences between untreated and treated groups

Table 1. Concentrations (mg/20 g) of oleanolic acid, ursolic acid, quercetin and lupeol in the dried extracts of hawthorn

Compound	Bark	Leaves	Berries
Hawthorn Powder (g)	20	20	20
Dry Extract Yield (g)	2.4 (12%)	2.26 (11.3%)	4.1 (20.5 %)
OA	0.25	0.048	0.18
UA	0.218	0.122	0.56
QCN	3.07	2.39	0.92
LPL	13.7	3.09	0.87

OA, UA, QCN and LPL are abbreviation for oleanolic acid, ursolic acid, quercetin and lupeol, respectively

Hawthorn ethanolic extracts contains high level of lupeol

The chemical compositions and the level of dry extracts yield of various extracts are depicted in Table 1. HPLC analyses showed that except UA, which was found at higher concentration in berries, the bark extract contains higher amount of OA, QCN and LPL. Among the analyzed components, we found the highest level of LPL in the bark extract and the lowest concentration of OA in the leaves extract.

Hawthorn extracts and Orlistat lowered the HC-increased hepatic functional enzymes activities and the HC-increased ratio of hepatic/total body weight

Exposing the rats to HCD for 28 days resulted in a remarkable elevation of the liver weight and consequently elevation of hepatic/total body weight ratio. Comparing the total body weight between the control group and the HC group revealed that body weight gain in a time-dependent manner was declined in the HC group. At the same time, the serum level of hepatic functional enzymes in the HC group increased significantly ($P<0.05$). While administration of various hawthorn extracts and orlistat for 28 days in the rats both lowered the hepatic/total body weight ratio and also declined all three functional enzymes level in serum (Table 2). There is no significant difference between the potency of hawthorn extracts and orlistat.

In vivo antioxidant effects of hawthorn ethanolic extracts

Two antioxidant biomarkers of lipid peroxidation rate and total thiol molecules level in the liver tissue were examined following 28 days either only exposure to HCD or exposure to HCD, and simultaneously administration of hawthorn extracts and/or orlistat. A significant ($P<0.05$) elevation in the MDA level of liver tissue in the HC group was obtained, while all treated groups showed a

remarkable and statistically significant ($P<0.05$) reduction in the MDA level. There were no significant differences between those groups, which received hawthorn various extracts (Figure 2A). Orlistat administration although could lower the level of HCD-induced MDA production in the liver; however, hawthorn extracts were able to reduce the MDA level statistically more than orlistat. At the same hepatic tissue samples, the level of thiol molecules as an indicator of antioxidant index was measured and the results indicated that TTM level was lowered in the HC group, while hawthorn extracts and not orlistat could protect from the HCD-induced TTM depletion in the liver tissue. The leaves extract exerted the strongest protective effect regarding the TTM level (Figure 2B).

Hypocholesterolemic effects of hawthorn ethanolic extracts

Lipid profile assays indicated a significant elevation in TC, LDL and TG and reduction in HDL levels in the rats that received HCD, while concurrent administration of hawthorn extracts and/or orlistat with HCD could remarkably prevent from TC, LDL and TG elevation (Table 3). By contrast, both hawthorn extracts and the orlistat-treated animals showed an increase in HDL level albeit with differences. Hawthorn extracts administration resulted in a non-significant ($P<0.05$) HDL increase compared to the HC group.

Ethanolic extracts of hawthorn improved the hypercholesterolemia-induced hepatic damages

The normal histological feature of the liver from the control (C) rats are depicted in Figure 3-A and 3-B. Histopathological findings in the liver of HC animals represented congested blood vessels, bile duct hyperplasia, inflammatory cells infiltration (perivascular cuffing), highly vacuolated hepatocytes

Table 2. Serum level of hepatic functional enzymes and the hepatic/total body weight ratio, n = 8 and mean values \pm SD are given

Groups	C	HC	T1	T2	T3	T4
H/TBW	0.030 \pm 0.003	0.038 \pm 0.001*	0.033 \pm 0.001#	0.033 \pm 0.002#	0.031 \pm 0.001#	0.032 \pm 0.003#
ALT (U/L)	36.3 \pm 2.7	55.3 \pm 3.8*	41.8 \pm 1.7##	47.0 \pm 3.3#	42.8 \pm 3.1##	40.9 \pm 4.1##
AST (U/L)	133.2 \pm 5.5	193.1 \pm 9.1*	147.6 \pm 4.0##	161.9 \pm 3.6#	151.4 \pm 8.9##	143.9 \pm 4.7##
GGT (U/L)	15.6 \pm 2.8	23.2 \pm 2.0*	19.2 \pm 1.0#	19.8 \pm 0.8#	18.0 \pm 1.7##	18.4 \pm 1.1#

Stars indicate significant differences ($P<0.05$) between the control and HC groups and # and ## represent significant differences ($P<0.05$ and $P<0.01$, respectively) between the HC and treatment groups at the same column. H/TBW: hepatic/total body weight, ALT: alanine aminotransferase, AST: aspartate transaminase and GGT: gamma glutamyltransferase

Table 3. Effect of hawthorn ethanolic extracts and/ or listat on lipid profile in experimentally-induced hypercholesterolemic rats

	C	HC	T1	T2	T3	T4
congestion	0.0 ^a	2.8 ± 0.4 ^b	1.1 ± 0.3 ^c	1.4 ± 0.5 ^c	2.1 ± 0.3 ^c	0.9 ± 0.6 ^d
vacuolation of hepatocytes	0.0 ^a	2.8 ± 0.4 ^b	1.7 ± 0.5 ^c	2.2 ± 0.4 ^c	1.2 ± 0.4 ^c	0.6 ± 0.4 ^d
BDH	0.0 ^a	2.7 ± 0.5 ^b	0.6 ± 0.4 ^c	1.2 ± 0.4 ^c	2.2 ± 0.4 ^c	0.9 ± 0.6 ^c
ICI	0.0	2.4 ± 0.5 ^b	0.9 ± 0.3 ^c	0.8 ± 0.4 ^c	1.3 ± 0.4 ^c	1.3 ± 0.4 ^c
Pyknosis	0.0	2.9 ± 0.3 ^b	1.9 ± 0.6 ^c	2.1 ± 0.6 ^c	1.6 ± 0.5 ^c	0.9 ± 0.6 ^d

Stars indicate significant differences ($P < 0.05$) between the control and sham groups and # and ## represent significant differences ($P < 0.05$ and $P < 0.01$, respectively) between the sham and test groups at the same column

and pyknotic nuclei (Figure 3C, and 3D). Administration of bark and leaves extracts although reduced the HCD-induced damages in the liver, there are however symptoms of congestion, perivascular cuffing and vacuolated hepatocytes (Figure 3E, 3F, 3G and 3H, respectively). We found that 4 weeks administration of hawthorn berries extract could fairly improve the HCD-induced hepatic damages, and the liver looks normal (Figure 3-I and 3-J). The orlistat-treated group showed remarkable reduction in the density of vacuolated hepatocytes, and other injuries compared to the HCD group (Figure 3K and 3L). The observed histopathological changes in various experimental groups were scored in at least 3 slides for each individual animal, and the final score was calculated (Table 4).

Discussion

This study showed that hawthorn ethanolic extracts exert antioxidant and hypolipidemic effects in both *in vivo* and *in vitro* systems. Our analytical studies revealed that all parts of hawthorn including bark, leaves and berries albeit with differences contain triterpenoids including oleanolic acid, ursolic acid and lupeol. Moreover, our results showed that the hawthorn extracts contains one of the important flavonoids namely quercetin. The hepatoprotective, antioxidant and hypolipidemic effects of hawthorn extracts were compared with orlistat as a chemical drug, which is used to reduce the cholesterol level.

In this study, we showed that hawthorn extracts have considerable phenol content, and interestingly a positive and strong association between phenol content and free radical scavenging potency of extracts was found. The concentration-dependent antioxidant capacity of hawthorn berries from *Crateagus pinnatifida* (*C. pinnatifida*) have been recently demonstrated in superoxide anion scavenging assay (19). Another analytical study identified the major phenolic compounds of ideain,

chlorogenic acid, procyanidin B₂, epicatechin, hyperoside and isoquercitrin in Chinese hawthorn (8). Polyphenolic compounds due to having the capacity to act as electron donor are suitable substances in converting free radicals to stable chemicals. Therefore, hawthorn ethanolic extracts with high phenolic content could be powerful natural scavengers. Here we report the existence of polyphenol compounds not only in hawthorn berries, which already have been reported from the Chinese and European hawthorns, but also in the leaves and bark of *C. monogyna* collected from the northwest area of Iran with strong antioxidant effects characterized with DPPH scavenging capacity.

To demonstrate any implication of *in vitro* antioxidant capacity of extracts, an *in vivo* experiment was conducted and beside the histopathological examinations, and hepatic functional enzymes measurement, two oxidative stress monitoring biomarkers were analyzed in the liver. Our findings indicated that hawthorn extracts albeit with minor differences all could reduce the HCD-induced lipid peroxidation, which measured with MDA level in the liver. This finding is in agreement with *in vitro* antioxidant capacity as the bark extract exerted the strongest anti-lipid peroxidation effect, suggesting a positive correlation between the polyphenol content of extracts and their *in vivo* antioxidant capacity. Orlistat also lowered the MDA production in the liver, suggesting that the cholesterol lowering approach also may result in anti-lipid peroxidation effect. It seems that the hawthorn extracts-induced antioxidant effect directly affected the liver, while orlistat indirectly attenuates the MDA production via reduction of cholesterol absorption. At the same series of experiment, the TTM level of liver was measured and results showed that hawthorn extracts and not orlistat protected from the HCD-induced TTM depletion. This finding may partly explain the

Table 4. Effect of Hawthorn's extracts on histological changes in the liver of hypercholesterolemic rats; mean values ± SD are given

Groups	C	HC	T1	T2	T3	T4
TC (mg/dl)	72.8 ± 2.7	100.1 ± 2.1 [*]	87.5 ± 2.1 [#]	90.5 ± 2.3 [#]	80.7 ± 3.1 ^{##}	75.2 ± 3.1 ^{##}
LDL (mg/dl)	50.1 ± 2.2	81.2 ± 6.2 [*]	68.5 ± 1.2 [#]	70.8 ± 2.4 [#]	60.7 ± 3.0 ^{##}	54.3 ± 3.7 ^{##}
TG (mg/dl)	35.2 ± 2.2	46.5 ± 2.4 [*]	42.8 ± 2.2	44.7 ± 1.3	41.1 ± 1.7 [#]	37.4 ± 1.7 ^{##}
HDL (mg/dl)	15.7 ± 2.6	9.5 ± 1.2 [*]	10.3 ± 1.3	10.6 ± 2.5	11.7 ± 1.8	13.4 ± 2.3 [#]

^{abc}: values in same row with different superscripts differ significantly ($P < 0.05$). BDH: Bile duct hyperplasia, ICI: Inflammatory cells Infiltration,

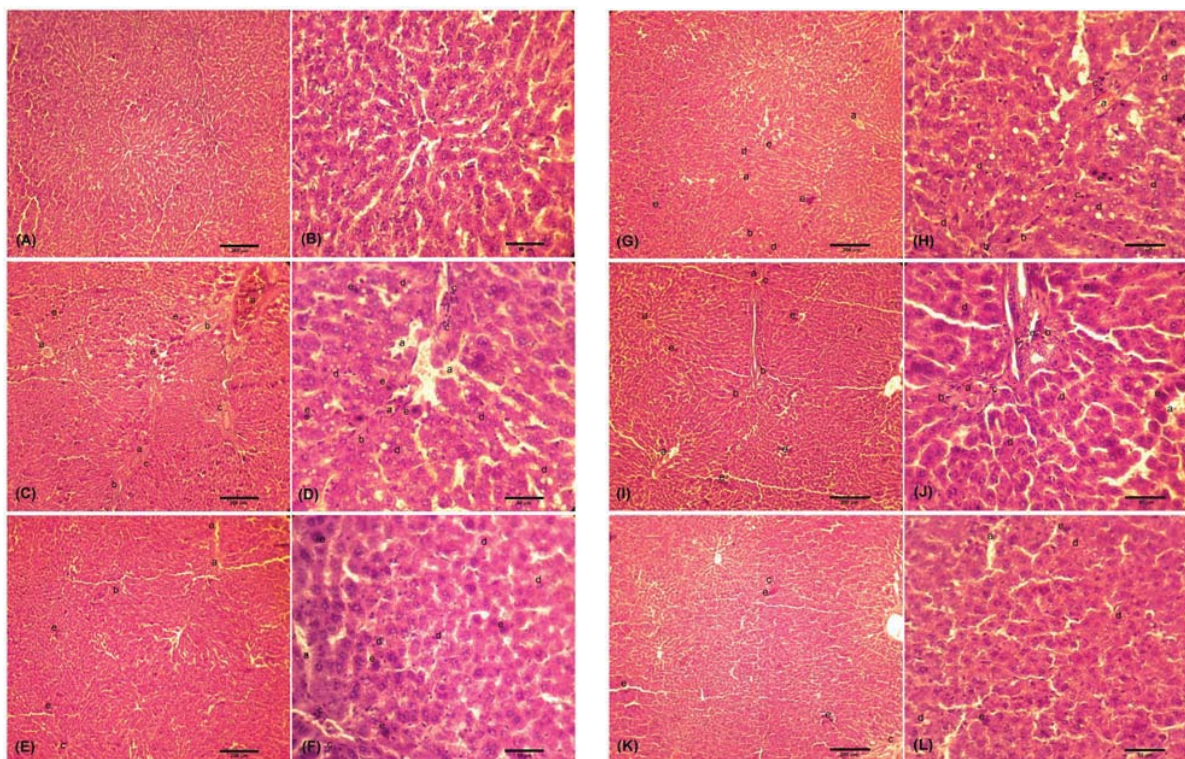


Figure 3. Histopathological findings of rat livers from different treatment groups. (A and B): Control, (C and D): hypercholesterolemic group (HC), (E and F): T1, (G and H): T2, (I and J): T3, (K and L): T4. a: congestion, b: bile ducts hyperplasia, c: inflammatory cells infiltration, d: vacuolation and e: pyknotic nuclei. [H&E staining; (A, C, E, G, I and K $\times 100$) and (B, D, F, H, J and L $\times 400$)]

stronger effect of extracts on MDA production than that of orlistat. The later analyses of serum lipid profile confirmed that the higher antioxidant potency of extracts in comparison to orlistat, related to dual action of extracts as hawthorn extracts both contain polyphenol compounds and flavonoids with strong antioxidant property and are able to reduce the TC, LDL and TG, while orlistat only improves the lipid profile. The exact mechanism based on which hawthorn extracts are able to reduce the TC, LDL and TG, and enhance the HDL remains to be clarified; however, alteration of digestive enzymes of the stomach and cholesterol metabolism in the liver following hawthorn consumption have been reported (20). Orlistat as a lipase inhibitor also reduces intestinal absorption of saturated fat by 30% (21). There are enormous published data indicating that hyperlipidemia or high cholesterol diet results in oxidative stress in various tissues or entire body as reported in this study. Therefore, hawthorn extracts with high antioxidant capacity and hypocholesterolemic effect, which relate to flavonoids and triterpenoids content of extracts, would be novel therapeutic compound for the treatment of patients with hyperlipidemia.

In vitro and *in vivo* antioxidant properties of hawthorn extracts led us to analyze the components

of the extracts by HPLC. Among the others, the dominant constituent of all three extract was found to be lupeol with different percentages in various extracts. Lupeol, a pentacyclic triterpene has been isolated from different parts of various plants and trees such as *Himatanthus drasticus*, stem bark of *Crataeva nurvala* and *Calotropis gigantean* (22-24). Lupeol is found in common fruits such as mango, olive and fig, and due to its biological activities including anti-inflammatory, cardioprotective, cytoprotective, antimutagenic, antiarthritic, and antioxidant properties has received much attention (25-27). Our analytical studies indicated that all three parts of hawthorn have lupeol with maximum percentage in bark extract. At the same time, our *in vivo* studies showed that the strongest anti-lipid peroxidation effect was found for bark extract. This finding may suggest a positive correlation between the high percentages of lupeol content in bark extract with strong anti-lipid peroxidation effect. The protective effect of lupeol on cadmium-induced lipid peroxidation has been previously reported (25). The anti-hyperlipidemic effects of lupeol have also been reported by previous studies in rats fed with high fat diet and also in cell culture model (28, 29). Another compound, which has been isolated from extracts, was UA, with the highest level in the berries extract.

We found that the anti-hyperlipidemic effect of berries extract was significantly dominant than two other extracts. This finding is supported by the recently reported antihyperlipidemic effect of ursolic acid (30, 31). Moreover, it has been demonstrated that ursolic acid and oleanolic acid, the triterpenoid components of *C. pinnatifida* reduce the intestinal absorption of cholesterol via inhibition of intestinal acyl-coenzyme A:cholesterol acyltransferase (ACAT) activity (5). It is likely that lupeol as a triterpenoid, due to similar chemical structure to ursolic acid and oleanolic acid could inhibit intestinal ACAT activity and contribute to hypolipidemic effects of hawthorn extracts.

The hepatic function and histological structure were examined through the assessment of hepatic functional enzymes in serum and the liver structural changes in histopathological studies. Our findings showed that the ratio of liver to entire body weight and also the serum levels of hepatic functional enzymes (ALT, AST and GGT) were significantly elevated in the animals that received HCD compared to the control group, suggesting a severe hepatomegaly and abnormal functioning of the liver. These abnormalities in the hepatic weight and functional enzymes were supported by structural disorders such as vacuolated hepatocytes with pyknotic nuclei in the liver of animals with high cholesterol diet. Many previous studies have shown that the high fat diet in rodent resulted in a hepatic steatosis, fibrosis and hepatic necrosis (32). The serum level of hepatic functional enzymes and in particular alanine aminotransferase are used as reliable biomarkers in clinical evaluation of non-alcoholic fatty liver diseases. Since ALT is produced in hepatocytes; therefore, its alteration can serve as an indicator of hepatocytes injuries. Serum levels of ALT, AST and GGT in the current study are positively associated with histopathological damages in the liver and the serum lipid profile as well. It is well known that there are positive correlation between an accumulation of free cholesterol and the endoplasmic reticulum stress induction, which eventually results in hepatocytes apoptosis (33, 34). The histopathological symptoms such as abundant hepatocytes with pyknotic nuclei, which are reported in this study, may support partly the HCD-induced hepatocyte apoptosis. On the other hand, the hepatic functional enzymes, serum lipid profile and the liver histopathological examinations in the animals that received various extracts, showed relatively to fully recover from the HCD-induced alterations. Our results revealed that the hawthorn extract possess considerable amount of quercetin, which its hepatoprotective effects on the CCL₄ and acrylonitrile-induced hepatotoxicity have been well documented (35, 36). The protective capacity of hawthorn bark extract is comparable with that of orlistat. These strongly related items indicate that likely hawthorn extracts hepatoprotective effects attribute to

their high level of polyphenol contents, their considerable free radical scavenging potency and their orlistat-comparable hypolipidemic capacity.

Conclusion

This study showed that hawthorn extracts with high level of polyphenol content, strong antioxidant capacity and having triterpenes such as lupeol, oleanolic acid, ursolic acid and flavonoids like quercetin exert hypolipidemic and hepatoprotective effects. Moreover, the hepatoprotective effects of extracts likely attribute to their antihyperlipidemic properties, which results in improvement of the HCD-induced damages and lowering the lipid peroxidation in the liver.

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References

- Petkov V. Plants with hypotensive, antiatheromatous and coronarodilatating action. *Am J Chin Med* 1979; 7:197-236.
- Rigelsky JM, Sweet BV. Hawthorn: pharmacology and therapeutic uses. *Am J Health Syst Pharm* 2002; 59:417-422.
- Tassell MC, Kingston R, Gilroy D, Lehane M, Furey A. Hawthorn (*Crataegus* spp.) in the treatment of cardiovascular disease. *Pharmacogn Rev* 2010; 4:32.
- Rajendran S, Deepalakshmi P, Parasakthy K, Devaraj H, Devaraj SN. Effect of tincture of *Crataegus* on the LDL-receptor activity of hepatic plasma membrane of rats fed an atherogenic diet. *Atherosclerosis* 1996; 123:235-241.
- Lin Y, Vermeer MA, Trautwein EA. Triterpenic acids present in hawthorn lower plasma cholesterol by inhibiting intestinal ACAT activity in hamsters. *Evid Based Complement Alternat Med* 2011; article ID 801272; doi: 10.1093/ecam/nep007
- Urbonavičiūtė A, Jakštas V, Kornysova O, Janulis V, Maruška A. Capillary electrophoretic analysis of flavonoids in single-styled hawthorn (*Crataegus monogyna* Jacq.) ethanolic extracts. *J Chromatogr A* 2006; 1112:339-344.
- Zhang Z, Chang Q, Zhu M, Huang Y, Ho WK, Chen Z-Y. Characterization of antioxidants present in hawthorn fruits. *J Nutr Biochem* 2001; 12:144-152.
- Liu P, Yang B, Kallio H. Characterization of phenolic compounds in Chinese hawthorn (*Crataegus pinnatifida* Bge. var. major) fruit by high performance liquid chromatography-electrospray ionization mass spectro-metry. *Food Chem* 2010; 121:1188-1197.
- D'Mello PM, Joshi UJ, Shetgiri PP, Dasgupta TK, Darji KK. A simple HPLC method for quantitation of quercetin in herbal extracts. *J AOAC Int* 2011; 94:100-105.
- Saboo S, Taapadiya G, Khadabadi S. Antioxidant and hepatoprotective potential of isolated fraction of *Rivea hypocrateriformis*. *J Biol Sci* 2011; 11:428-432.
- Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation

- substrates and antioxidants by means of folin-ciocalteu reagent. *Methods Enzymol* 1999; 299:152-178.
12. Mensor LL, Menezes FS, Leitão GG, Reis AS, Santos TCd, Coube CS, *et al.* Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytother Res* 2001; 15:127-130.
13. Lu LS, Wu CC, Hung LM, Chiang MT, Lin CT, Lin CW, *et al.* Apocynin alleviated hepatic oxidative burden and reduced liver injury in hypercholesterolaemia. *Liver Int* 2007; 27:529-537.
14. Hosseinimehr SJ, Azadbakht M, Abadi AJ. Protective effect of hawthorn extract against genotoxicity induced by cyclophosphamide in mouse bone marrow cells. *Environ Toxicol Pharmacol* 2008; 25:51-56.
15. Caner M, Dogruman H, Taskin E, Kandil A, Demirci C. Effects of orlistat and its relationship with nitric oxide in the small intestinal mucosa. *Chin J Physiol* 2005; 48:217-222.
16. Ranjbar A, Khorami S, Safarabadi M, Shahmoradi A, Malekiran AA, Vakilian K, *et al.* Antioxidant activity of Iranian *Echium amoenum* Fisch & CA Mey flower decoction in humans: a cross-sectional before/after clinical trial. *Evid Based Complement Alternat Med* 2006; 3:469-473.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193:265-275.
18. Niehaus W, Samuelsson B. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. *Eur J Biochem* 1968; 6:126-130.
19. Wang H, Zhang Z, Guo Y, Sun P, Lv X, Zuo Y. Hawthorn fruit increases the antioxidant capacity and reduces lipid peroxidation in senescence-accelerated mice. *Eur Food Res Technol* 2011; 232:743-751.
20. Kwok CY, Wong CNY, Yau MYC, Yu PHF, Au ALS, Poon CCW, *et al.* Consumption of dried fruit of *Crataegus pinnatifida* (hawthorn) suppresses high-cholesterol diet-induced hypercholesterolemia in rats. *J Funct Foods* 2010; 2:179-186.
21. Kushner R. Medical management of obesity. *Semin Gastrointest Dis* 2002; 13:123-132.
22. Silva JRdA, Rezende CM, Pinto ÂC, Pinheiro ML, Cordeiro MC, Tamborini E, *et al.* Triterpenic esters from *himatanthus sucuba* (Spruce) Woodson. *Quim Nova* 1998; 21:702-704.
23. Baskar R, Meenalakshmi Malini M, Varalakshmi P, Balakrishna K, Bhima Rao R. Effect of lupeol isolated from *Crataeva nurvala* stem bark against free radical-induced toxicity in experimental urolithiasis. *Fitoterapia* 1996; 67:121-125.
24. Saratha V, Subramanian SP. Lupeol, a triterpenoid isolated from *Calotropis gigantea* latex ameliorates the primary and secondary complications of FCA induced adjuvant disease in experimental rats. *Inflammopharmacology* 2012; 20:27-37.
25. Nagaraj M, Sunitha S, Varalakshmi P. Effect of lupeol, a pentacyclic triterpene, on the lipid peroxidation and antioxidant status in rat kidney after chronic cadmium exposure. *J Appl Toxicol* 2000; 20:413-417.
26. Saleem M, Afaq F, Adhami VM, Mukhtar H. Lupeol modulates NF- B and PI3K/Akt pathways and inhibits skin cancer in CD-1 mice. *Oncogene* 2004; 23:5203-5214.
27. Saleem M, Kaur S, Kweon M-H, Adhami VM, Afaq F, Mukhtar H. Lupeol, a fruit and vegetable based triterpene, induces apoptotic death of human pancreatic adenocarcinoma cells via inhibition of Ras signaling pathway. *Carcinogenesis* 2005; 26:1956-1964.
28. Sudhahar V, Kumar SA, Varalakshmi P. Role of lupeol and lupeol linoleate on lipemic-oxidative stress in experimental hypercholesterolemia. *Life Sci* 2006; 78:1329-1335.
29. Itoh M, Hiwatashi K, Abe Y, Kimura F, Toshima G, Takahashi J, *et al.* Lupeol reduces triglyceride and cholesterol synthesis in human hepatoma cells. *Phytochem Lett* 2009; 2:176-178.
30. Radhiga T, Rajamanickam C, Senthil S, Pugalendi KV. Effect of ursolic acid on cardiac marker enzymes, lipid profile and macroscopic enzyme mapping assay in isoproterenol-induced myocardial ischemic rats. *Food Chem Toxicol* 2012; 50:3971-3977.
31. Azevedo MF, Camsari Ç, Sá CM, Lima CF, Fernandes Ferreira M, Pereira Wilson C. Ursolic acid and luteolin 7 glucoside improve lipid profiles and increase liver glycogen content through glycogen synthase kinase 3. *Phytother Res* 2010; 24:220-224.
32. Jeong W, Jeong D-H, Do S-H, Kim Y-K, Park H-Y, Kwon O-D, *et al.* Mild hepatic fibrosis in cholesterol and sodium cholate diet-fed rats. *J Vet Med Sci* 2005; 67:235-242.
33. Beltroy EP, Liu B, Dietschy JM, Turley SD. Lysosomal unesterified cholesterol content correlates with liver cell death in murine Niemann-Pick type C disease. *J Lipid Res* 2007; 48:869-881.
34. Wang D, Wei Y, Pagliassotti MJ. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* 2006; 147:943-951.
35. Abo Salem OM, Abd Ellah MF, Ghonaim MM. Hepatoprotective activity of quercetin against acrylonitrile induced hepatotoxicity in rats. *J Biochem Mol Toxicol* 2011; 25:386-392.
36. Domitrović R, Jakovac H, Vasiljev Marchesi V, Vladimir-Knežević S, Cvijanović O, *et al.* Differential hepatoprotective mechanisms of rutin and quercetin in CCl4-intoxicated BALB/cN mice. *Acta pharmacol Sin* 2012; 33:1260-1270.