Consumption of dried fruit of *Crataegus pinnatifida* (hawthorn) suppresses high cholesterol diet-induced hypercholesterolemia in rats

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Short title: Hypocholesterolemic effects of hawthorn

ABSTRACT:

The hypocholesterolemic and atheroscleroprotective potentials of dietary consumption of hawthorn (dried fruit of *Crataegus pinnatifida*, Shan Zha) were investigated by monitoring plasma lipid profiles and aortic relaxation in Sprague-Dawley rats fed with either normal diet, high-cholesterol diet (HCD) or HCD supplemented with hawthorn powder (2%, w/w) (4 weeks). In HCD-fed rats, an increased plasma total cholesterol and LDL-cholesterol with a decreased HDL-cholesterol was observed, and consumption of hawthorn markedly suppressed the elevated total cholesterol and LDL-lipoprotein levels plus an increased HDL-cholesterol level. The blunted acetylcholine-induced, endothelium-dependent relaxation of isolated aortas of HCD-fed rats was improved by hawthorn. The development of fatty liver, an increased nitric oxide synthase activity and an elevated oxidative stress (as estimated by the attenuated levels of anti-oxidant enzymes) associated with HCD were attenuated by hawthorn. Thus, the results demonstrated that hawthorn consumption provides overall beneficial effects on reversing HCD associated detrimental changes.

Key words: cholesterol; *Crataegus pinnatifida*; dietary supplement; hawthorn; high fat diet; rat

1. Introduction

Hypercholesterolemia is a major risk factor for the development and progression of atherosclerosis and related cardiovascular diseases (Prasad & Kalra, 1993; Deepa & Varalakshmi, 2005). A high-cholesterol diet (HCD) is a major environmental contributor to an unbalanced lipoprotein metabolism. It is associated with an increased prevalence of atherosclerosis which is the major source of morbidity and mortality in the developed world, and it claims more lives than all types of cancer combined (Stocker & Keaney, 2004). Previous reports have clearly indicated a positive correlation between serum cholesterol level and the risk of cardiovascular disease (Leys et al., 2002; Yu-Poth et al., 2004). Despite the fact that there are drugs available clinically for treating hypercholesterolemia, the consumption of functional foods/dietary supplements in lowering/controlling serum cholesterol levels and risk of cardiovascular diseases (Roberfroid, 1999) has gained enormous global acceptance over the years by the general public.

Hawthorn (also known as Shan Zha which is the fruit of *Crataegus* species) is commonly used in Chinese dishes to reduce risk of cardiovascular diseases such as heart failure (Schwinger et al., 2000; Tauchert, 2002), arrhythmias (Makdessi, 1999) and hypertension (Walker et al., 2002). Apart from the fruit, *Crataegus* leaves have

been shown to possess beneficial effects on cardiovascular diseases. *Crataegus* leave with flower extract was shown to reduce the incidence of sudden cardiac death in certain patients (Holubarsch et al., 2008) and be effective in treating chronic heart failure (Pittler et al., 2008). *Crataegus* is a member of the *Rosaceae* family and there are more than 280 species worldwide (Upton, 1999), and *Crataegus pinnatifida* and *Crataegus cuneata* are the two common species cultivated and used in PR China (Zhang et al., 2001); whereas *Crataegus monogyna* and *Crataegus laevigata* are found in Europe (Wang *et al,* 1999).

Consumption of hawthorn (*Crataegus pinnatifida*) altered the digestive enzymes of the stomach and cholesterol metabolism of the liver (Zhu, 1998), and a reduction in blood lipid and cholesterol levels has been reported (Shanthi et al., 1994). In addition, hawthorn contains abundant amount of antioxidants such as chlorogenic acid, epicatechin, hyperoside and quercetin (Liu et al., 2010) which may be useful in alleviating the adverse effects associated with low-density lipoprotein (LDL)-cholesterol oxidation in atherosclerosis (Schwinger et al., 2000; Stocker & Keaney, 2004).

In this study, we tested the hypothesis that consumption of hawthorn provides an overall improvement/benefit to alleviate the detrimental outcomes associated with HCD of the liver and the cardiovascular systems.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (~550 g, 8 month-old) were supplied by the Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University (Hong Kong SAR, PR China). Rats were housed under standard conditions (temperature 20 ± 1 °C, humidity 60 ± 10%, light from 6 a.m. to 6 p.m.) with free access to water. 21 rats were randomly divided into 3 groups (i.e. 7 rats per group): Control with normal rat chow, which was obtained from Guangdong Provincial Medical Laboratory Animal Center (Guangzhou, PR China) [composition: protein (~14%), fat (~10%) and carbohydrate (~76%)]. Hypercholesterolemia diet (HCD) contained normal rat chow with the supplement of cholic acid (1%), cholesterol (2%) and cooking oil (5.5%) as described elsewhere (Yan et al., 2006; Li et al., 2009). Hawthorn-treated group received HCD plus dried hawthorn powder (2%). The dried hawthorn herb was purchased from Hip Shing Hong Ltd. (Hong Kong SAR, China). After removal of the seeds, the dry fruit flesh was ground into powder (particles pass a sieve with a mesh size of 1 mm) using a coffee grinder. Rats were fed with different diets (as mentioned above) for 4 weeks before they were sacrificed for experiments. The voucher sample of hawthorn herb was stored in the laboratory of Dr. Peter H.F.

Yu (Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong). At the end of a 4-week treatment period, rats were fasted overnight, sacrificed by cervical dislocation and tissue samples (blood, livers and aortas) were collected for different experiments. The experimental protocol was conducted under the animal license issued by the Health Department of the Government of the Hong Kong SAR and the Animal Subjects Ethics Sub-committee (ASESC No. 05/21) of The Hong Kong Polytechnic University. All procedures were consistent with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health and the principles outlined in the Declaration of Helsinki. Every effort was made to limit animal suffering and to limit the number of animals used in this study.

2.2. High performance liquid chromatography (HPLC) analysis on dried hawthorn sample

Dried hawthorn fruit powder (10 g) was weighed and mixed with 100 ml of 80% ethanol and shaken in a horizontal shaker at 37 °C, 300 rpm for 1 hr. After 1 hr, the solution was centrifuged at 2735 ×g for 3 min. The supernatant was collected and the residue was re-extracted for one more times with same volume of 80% ethanol. Finally, all of the supernatant was filtered using filter paper (Whatman no. 4). The

filtered solution was treated in the rotary evaporator (Laborota4000, Heidolph, Germany) to remove excess extraction solvent before lyophilizing in freeze dryer (Labconco, Freezone 6, MO, USA) for about 1 week. Dried extract (0.1 mg) was dissolved in 1 ml of 0.2% H_3PO_4 (pH= 2). The solution was diluted to 0.01 mg/ml and filtered by a 0.45µm filter before HPLC analysis.

HPLC analyses were performed using an Agilent 1100 HPLC system (Milford, MA, USA) coupled to a photodiode array detector. The samples were separated on a reversed-phase C_{18} column (250 mm × 4.6 mm i.d.) from Alltech Associate Inc. (Lokeren, Belgium) with a C_{18} guard column (7.5 mm × 4.6 mm i.d.) (Alltech, Lokeren, Belgium). The mobile phase consisted of acetonitrile (solvent A) and 0.2% H_3PO_4 pH= 2 (solvent B). At time = 0, the solvent A and B were in 1:9 (v/v) ratio. The gradient mobile phase was changed gradually to 89:11 (v/v) A to B ratio in 20 min consisted of 24% acetonitrile and 76% acetic acid (5%). The eluent was monitored by a UV detector. The detection wavelength was set at 360 nm. An autosampler was utilized for sample injection with injection volume of 10 µl.

2.3. Chemicals

Phenylephrine hydrochloride, heparin, indomethacin, acetylcholine hydrochloride (ACh), chlorogenic acid, quercetin, hyperoside and neostigmine bromide were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). The kit for the determination of nitric oxide (NO) levels was provided by Nanjing Jiancheng Bioengineering Company (Nanjing, PR China). Other chemicals used were analytical grade.

2.4. Analysis of blood lipoprotein-cholesterol levels

Blood samples from the 12-h fasted rats were collected on day 28 of this study. The 100 unit/ml heparin-containing blood was centrifuged at 1500 ×g for 15 min. Plasma was collected and stored at -20 °C before analysis. Plasma concentrations of total cholesterol, triacylglycerol, HDL-cholesterol and LDL-cholesterol were analyzed and compared as per instructions of the manufacturer (Biosino Bio-technology and Science Inc., Beijing, PR China).

2.5. Endothelium-dependent vasorelaxation of thoracic aortas

The relaxation effects of isolated aorta were measured as described previously (Yan et al., 2006). Briefly, the thoracic aortas were harvested and the fat and the surrounding

connective tissues were carefully cleared under a dissecting microscope. The aortic rings (about 4 mm in length) were prepared and mounted between two stainless steel hooks in 5-ml, water-jacketed organ baths (37 °C) containing Krebs'-Ringer bicarbonate solution (gassed with 95% O_2/5% CO_2 (pH = 7.4)) of the following composition (mM): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2 and glucose, 11.0. An optimal load of 15 ± 1 mN was applied to the rings, and changes in tension were recorded by isometric force-displacement transducers connected to the PowerLab Data Acquisition system (AD Instruments, Sydney, NSW, Australia). Tissues were allowed to equilibrate for 60 min under the resting tension, and were washed with drug-free Krebs'-Ringer solution every 20 min, and the resting tension was readjusted, if necessary, before commencing the experiments. After equilibration, the aortic rings were sensitized with 40 mM KCl until two consecutive contractile responses were reproducible. To investigate the relaxant effects of acetylcholine, the preparations (in the presence of indomethacin (1 μM) and neostigmine (1 μM)) were precontracted with phenylephrine (1 μM, a concentration which elicits approximate 85% of maximum effective contraction (EC_{85}) of the tissues). After a steady-state contraction was established, cumulative concentrations (1 nM - 100 μM) of acetylcholine were added. Concentration-response curves were plotted as percentage relaxation of phenylephrine induced-contraction

versus the logarithmic concentration (in the organ bath) of acetylcholine added.

2.6. Histological change of the livers

At the end of the study, the whole liver was harvested from rats and rinsed in saline (3 - 4 times) to remove as much blood as possible. The gross appearance of livers of individual rat was photographed for comparison. Sample of liver (~5 g) was dissected and immersed in formalin (10 %) until sectioning. Wax specimens of individual liver sample were prepared and stained using Hematoxylin and Eosin methods, as described previously (Ratcliffe, 1982).

2.7. Determination of the antioxidant enzyme activities in the livers and kidneys

To monitor the activities of antioxidant enzymes in both livers and kidneys, isolated liver (~1 g) and kidneys (~0.5 g) of each rat were weighted and kept at -80 °C immediately until homogenization procedures. Each frozen sample was homogenized in ice-cold saline (1:1, w/v) using an ulteraturax T-25 homogenizer for 10 bursts of 10 s each, separated by a pause of 15 s. Then, the homogenates were centrifuged at 150 ×g for 5 min (4 °C), and the supernatants were collected and stored at -20 °C until enzyme activities analysis. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were measured using commercial kits

(Nanjing Jiancheng Bioengineering Institute, Nanjing, PR China). SOD, CAT and GPx activities are expressed as U/mg protein. The protein content was measured by Bradford method (Bio-Rad, Hercules, CA).

2.8. Evaluation of NOS activities in the liver and kidneys

The frozen samples were homogenized in ice-cold saline and the homogenates were centrifuged at 1500 ×g for 10 min (4 °C). The supernatant was used for the determinations of NOS activity (expressed in U/mg protein). It was determined spectrophotometrically by measuring NO synthesis using the oxidation of oxyhaemoglobin to methaemoglobin by NO. The incubations contained 1.6 mM oxyhaemoglobin, 200 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM L-arginine, 100 mM NADPH, 50 mM L-valine (to inhibit arginase), 40 mM potassium phosphate pH 7.2 and up to 20% v/v tissue supernatant. This method was described previously (Knowles et al., 1990). The protein content was measured by Bradford method (Bio-Rad, Hercules, CA, USA).

2.9. Statistical analysis

All data was expressed as means ± standard deviations (S.D.), and n denotes the

number of replications for each data point. Relaxations (%) were expressed as the percentage of the phenylephrine (1 μM)-induced contraction. GraphPad Prism 4.02 (San Diego, California, USA) was used for curve fitting and the estimation of the magnitude of maximum relaxation (R_{manx}). After validation of each parameter collected for homogeneity of variance, differences between groups were analyzed using one-way analysis of variance (SPSS, Version 15 for Windows; Chicago, IL, USA). *Post hoc* tests were performed for inter-group comparisons using the least significance difference test, and a *P* value of < 0.05 was considered significant.

3. Results

3.1. HPLC analysis on dried hawthorn sample

A representative HPLC chromatogram was shown in Figure 1. The 80% ethanol hawthorn extract (1 mg) was found to contain chlorogenic acid (0.81 ± 0.027 μg), hyperoside (0.43 ± 0.021 μg) and trace amount of quercetin.

3.2. Plasma cholesterol and lipoprotein profiles

The plasma lipid profiles: total cholesterol, triacylglycerols, HDL-cholesterol and LDL-cholesterol levels of various treatment groups of rats are summarized in Figure 1. An elevated total plasma cholesterol and LDL-cholesterol with a decreased HDL-cholesterol was observed in rats fed with high cholesterol diet (HCD) (HDL-cholesterol/LDL-cholesterol ratio: control, 3.41; HCD-fed, 0.67). Supplementation of the HCD with hawthorn powder (2%, w/w) markedly altered the lipid profiles: a suppression of total cholesterol (HCD-fed, 6.06 ± 0.19 mM; HCD + hawthorn-fed, 4.00 ± 0.48 mM) and LDL-lipoprotein levels with an increase in HDL-lipoprotein (Table 1) was recorded. There was an increase in serum HDL-lipoprotein/LDL-lipoprotein ratio (HCD-fed, 0.67; HCD + hawthorn-fed, 2.16). No apparent effect on triacylglycerol levels was observed in three groups of rats

(Table 1).

The atherogenic index [(total serum cholesterol minus HDL) / HDL] (which estimates the relative risk of coronary heart disease) of HCD-fed rats was significantly higher (~ 100-fold) than controls (10.7 ± 2.56 versus 0.98 ± 0.24), and hawthorn supplement in HCD-fed rats restored the atherogenic index towards the control level (1.87 ± 0.46) (Table 1).

3.3. Evaluation of relaxation responses

After phenylephrine (1 μM)-induced contraction reached the steady-state condition, acetylcholine (with 1 μM neostigmine, an anti-cholinesterase) was cumulatively added to the aortic preparations. Acetylcholine elicited a concentration-dependent (1 nM - 100 μM) aortic relaxation of the Normal, HCD-fed and HCD + SZ-fed rats with the maximum relaxation (R_{max}) (100 μM acetylcholine) of ~110, ~80 and ~90%, respectively (Fig. 3). In HCD-fed rat, the magnitude of acetylcholine-induced (0.3 to 100 μM) relaxation was attenuated compared to controls, and the blunted relaxation was restored, to a certain extent, in HCD + hawthorn-fed rats (Fig. 2).

3.4. Histological examination of the liver

The gross appearance of the liver from rats fed with Normal diet, HCD and HCD +

hawthorn is depicted in Fig. 3. The liver of control (Normal) rats has a relatively dark-red colour whereas the HCD-fed rats have an enlarged liver (Weight of liver in Normal diet-fed rats: 13.08 ± 0.50 g; HCD-fed: 21.49 ± 0.74 g; whereas HCD + hawthorn-fed: 17.15 ± 0.36 g) with a yellowish colour. In rats fed with HCD + hawthorn, there was not as much enlargement of liver (with a relatively "lesser yellowish" colour) as that was observed in HCD-fed rat. Histological examination of the livers of both control rats and HCD + hawthorn-fed rats revealed an intact cell architecture (Figs. 3A and 3C). In contrast, the liver of HCD-fed rats illustrated poor cellularity with extensive lipid depositions and enlarged hepatocytes (Fig. 3B). In HCD + hawthorn-fed rats (Fig. 3C), a lesser degree of lipid deposition and hepatocytes enlargement was observed.

3.5. Antioxidant enzyme activities in liver and kidney

Table 2 depicts the activities of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the liver and kidneys of rats fed with either normal diet, HCD or HCD + hawthorn. The actvities of anti-oxidant enzymes (i.e. SOD, CAT and GPx) measured in the kidneys are generally lower than that measured in the liver (Table 2). In HCD-fed rats, a marked decrease of SOD activity (~47% decrease) was recorded, compared to control rats (Table 2). Hawthorn

supplement managed to prevent HCD-induced reduction of SOD activity of the liver (~13 % decrease) (Table 2). Similar to SOD activity, CAT activity of the liver was suppressed (~49% decrease) in HCD-fed rats (Fig. 5B), and the consumption of hawthorn restored, to a certain extent, the suppressed CAT activity (~12% decrease) (Figure 5B). Unlike SOD and CAT activities, neither HCD nor HCD + hawthorn treatments altered the GPx activity of the liver (Table 2).

In contrast to the liver, only SOD (but not CAT and GPx) activity of the kidneys was suppressed in HCD-fed rats (but only a subtle suppression, ~16% decrease), and the supplement of hawthorn completely restored the reduced SOD caused by HCD (Table 2).

3.6. Determination of NOS activities

Comparing with the controls, the HCD-treated rats showed a marked increase of NOS activity in liver (110 % increase, $P < 0.01$ versus controls). No apparent change in NOS activity of the kidneys of all groups of rats was measured (Table 2). In animals fed with HCD + hawthorn, the elevated hepatic NOS activity caused by HCD was returned to the control/normal levels (Table 2).

4. Discussion

It is well known that diet plays an important role in the control of cholesterol homeostasis. The consumption of cholesterol-enriched diet is regarded as an important factor in the development of cardiovascular diseases as it leads to the development of hyperlipidemia, atherosclerosis and abnormal lipid oxidation/metabolism (Onody, 2003). Thus, natural products with hypocholesterolemic and hypolipidemic properties may be useful in reducing the risk of cardiovascular disease development. In this study, we evaluated the effects of supplementation of hawthorn (dried fruit of *Crataegus pinnatifida*) on the lipoprotein-cholesterol profiles and vascular response of high cholesterol diet (HCD)-fed rats. It has been reported that fresh and dried hawthorn fruit flesh containing different amount of phenolic antioxidants (Liu et al., 2009). For mimicking the usage of hawthorn in China, dried hawthorn was used in the current study. It has been reported that rats fed with cholesterol enriched diet for 4 weeks showed significant increase in total cholesterol and LDL (Yan et al., 2006; Li et al., 2009), which lead to development of secondary complications e.g. fatty liver and hypertension as observed clinically. In addition, diet supplemented with 2% hawthorn was shown to have hypolipidemic effect in rabbits fed a high cholesterol diet (Zhang

et al., 2002b). Therefore, similar dose regime was adopted in the current study. Similar to previous clinical studies (Kannel et al., 1971; Yu-Poth et al., 1999; Jeppesen et al., 2006), in our study HCD consumption (4 weeks) resulted in an increased total cholesterol and LDL-cholesterol (so-called the "bad" cholesterol/lipoprotein) with a concomitant decreased HDL-cholesterol (the "good" lipoprotein-cholesterol). Despite all these detrimental/unfavorable changes of cholesterol/lipoprotein levels after HCD consumption, supplementation of hawthorn (4 weeks) with the HCD resulted in a marked decrease in total cholesterol and LDL-lipoprotein, and more importantly with an increase in HDL-lipoprotein. In line with these observations, histological examination revealed that HCD-fed rats have enlarged livers (with a yellowish colouration), accumulation of lipid deposition, loss of hepatocytes integrity and hepatocyes enlargement. These changes are probably associated with or responsible for the aberrant changes of cholesterol/lipoprotein observed (i.e. a fatty liver) in rats fed with HCD.

In view of the overall anatomical changes of the liver after feeding with HCD, these biological changes may be considered, by rats (and even by humans), as a form of stress/inflammation (although we did not measure the inflammatory biomarkers, if any, present in blood). Thus, it is not surprising to observe an increase in the activity of NOS (presumably iNOS which participates in inflammation responses) of the liver

(but not kidneys) in HCD-fed rats, as described elsewhere (Kim et al., 2002). Perhaps, the inflammation associated with HCD is resulted from an attenuated level of hepatic antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) as clearly illustrated in our study. Our results are in fact consistent with previous reports in which a high-fat diet induces critical oxidative damage in the liver (Prasamthi et al., 2005; Schrauwen, 2007). It is interesting to point out that the liver seems to be more vulnerable to HCD insults than the kidneys as only SOD but not CAT or GPx levels was attenuated. The underlying mechanisms responsible for the "differential effects" of HCD on the levels of antioxidant enzymes of the liver and the kidneys are unknown. Perhaps, it is related to the physiological functions of these organs i.e. metabolism (liver) versus excretion (kidneys).

Nonetheless, our novel results demonstrate that consumption of hawthorn provided protective effects for the liver to overcome the detrimental biological effects associated with HCD. For instance, the suppressed levels of the hepatic SOD, CAT and GPx as well as SOD level in kidneys were normalized to levels as observed in rats fed with normal diet. In addition, there was a reduction of the elevated cholesterol (total) and LDL-cholesterol, an increased of HDL-cholesterol with a lesser degree of lipid deposition in the liver in rats fed with HCD plus hawthorn supplement. It is in agreement with previous findings in which hawthorn consumption prevented the

decrease of antioxidants levels like glutathione and α-tocopherol and maintained the level of antioxidant enzyme activities in liver, aorta and heart of rats on atherogenic diet (Shanthi et al., 1994). Furthermore, the elevated NOS activity of the liver (HCD-fed rats) and the increased atherogenic index were reversed to the level as observed in the normal rats. It remains to be determined whether the hypocholesterolemic effects of hawthorn consumption is associated with either up-regulation of the hepatic LDL receptors (Rajendran et al., 1996; Zhang et al., 2002a), reduction of the absorption of dietary cholesterol from intestine by down-regulation of intestinal acyl CoA: cholesterol acyltransferase activity (Zhang et al., 2002a) or alteration of the hepatic cholesterol metabolism (Zhang et al., 2002b). Taken together, our results suggest that hawthorn consumption carries atherosclerosis protection properties.

Hypercholesterolemia and atherosclerosis have a close association with vascular dysfunction (Kuchan & Frango, 1994). In this study, our results clearly illustrated a blunted acetylcholine-induced vascular relaxation (an endothelium-dependent process) which represents an endothelial/nitric oxide dysfunction in HCD-fed rats which is consistent with reports previously described in humans (Sorensen et al., 1994) and animals (Matsumoto et al., 2004). Interestingly, hawthorn supplementation restored, only to a certain extent, the blunted acetylcholine-induced endothelium relaxation. It

is well known that components found in hawthorn such as quercetin, hyperoside and isoquercitrin possess relaxation effects through the endothelium-dependent cascade (Zhang et al., 2001; Taubert et al., 2002) and procyanidins (one of the agents found in hawthorn) has been suggested to be responsible for the endothelium-dependent NO-mediated relaxation in isolated rat aorta (Kim et al., 2000).

Hawthorn (dried fruit of *Crataegus pinnatifida*) is commonly consumed in China; while *Crataegus* leaves with flowers are mainly use in Europe. The extracts of both parts showed beneficial effect on the cardiovascular system (Walker et al., 2002; Pittler et al., 2008). The similar pharmacological use of fruit and leave can be explained by the close chemical compositions for both parts of the herbs (Long et al., 2006).

In conclusion, our results suggest that consumption of hawthorn (2 % w/v., 4 weeks) has significant hypocholesterolemic and vasoprotective activities in rats fed with hypercholesterolemic diet (4 weeks). In addition, hawthorn supplement seems to protect the liver in response to oxidative stress (probably via the elevation of anti-oxidant enzymes levels) as well as alleviate the magnitude of fatty liver development in response to HCD. In line with these observations, the elevated activity of NOS (presumably the inducible isoform of NOS (iNOS) which is up-regulated in response to stress/inflammation challenge) in the liver after HCD regiment was

normalized, as observed for the first time, by hawthorn consumption. In addition, the blunted NO/endothelium-mediated aortic relaxation (presumably related to the suppressed endothelial isoform of NOS (eNOS)-mediated effects) in rats fed with HCD was restored, only to a certain extent, after hawthorn consumption. Nonetheless, our novel results clearly illustrate that consumption of hawthorn powder provides an overall improvement of the hepatic and cardiovascular systems that may be important in treating hypercholesterolemia-related cardiovascular complications.

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Figure legends

Fig. 1 - Chromatogram of hawthorn sample used in this study.

Fig. 2 - Cumulative concentration-response curves of acetylcholine constructed in the pre-constructed thoracic aorta of Normal, HCD and HCD + hawthorn groups.

Data (a decrease in steady-state tension elicited by phenylephrine (1 μM)) are expressed as means ± SEM, n = 4. *$P < 0.05$ compared to Control (Normal diet) group.

Fig. 3 - Histological examination of the liver obtained from rats fed with Normal diet, HCD or HCD + hawthorn groups. Photographs of the cross-section (400× magnification) of liver (top), and the gross appearance of the entire liver (bottom) are illustrated.

Table 1 - Serum lipid levels of rats fed with Normal diet (Control), HCD or HCD + hawthorn.

Animals groups	Control	HCD	HCD + hawthorn
Total cholesterol (mmol/L)	2.21 ± 0.06	6.06 ± 0.19$^{###}$	4.00 ± 0.18$^{###, ***}$
Triacylglycerols (mmol/L)	0.71 ± 0.04	0.73 ± 0.04	0.72 ± 0.05
LDL (mmol/L)	0.34 ± 0.04	0.96 ± 0.08$^{###}$	0.61 ± 0.03$^{###, ***}$
HDL (mmol/L)	1.16 ± 0.15	0.66 ± 0.11$^{##}$	1.32 ± 0.05***
Atherogenic index	0.98 ± 0.24	10.70 ± 2.56$^{###}$	1.87 ± 0.46***

Data are expressed as means ± S.D., n = 6 – 7.

$^{##}p < 0.01$ and $^{###}p < 0.001$ represent significant difference when compared with the Control group.

***$p < 0.001$ represents significant difference when compared with the HCD group.

Table 2 - Enzyme activities in liver and kidney from rats fed with Normal diet (Control), HCD or HCD + hawthorn.

Animals groups	Control	HCD	HCD + hawthorn
Liver			
SOD (U/mg)	542.36 ± 49.94	286.38 ± 54.06$^{\#}$	471.96 ± 0.18*
CAT (U/mg)	0.75 ± 0.07	0.38 ± 0.08$^{\#\#}$	0.66 ± 0.10*
GPx (U/mg)	148.04 ± 6.92	154.64 ± 12.82	139.05 ± 8.44
NOS (U/mg)	0.27 ± 0.09	0.57 ± 0.27$^{\#\#}$	0.25 ± 0.13**
Kidney			
SOD (U/mg)	154.02 ± 6.61	129.00 ± 5.65$^{\#}$	156.69 ± 11.27*
CAT (U/mg)	0.16 ± 0.02	0.15 ± 0.01	0.14 ± 0.01
GPx (U/mg)	86 ± 4.76	72.98 ± 5.58	85.07 ± 6.25
NOS (U/mg)	0.37 ± 0.08	0.37 ± 0.09	0.36 ± 0.11

Data are expressed as means ± S.D., n = 6 – 7.

$^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ represent significant difference when compared with the Control group.

$^{*}p < 0.05$ and $^{**}p < 0.01$ represent significant difference when compared with the HCD group.

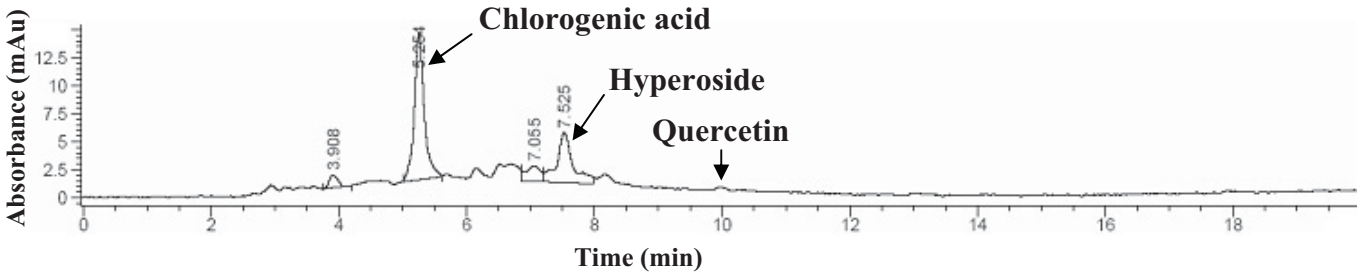
Chlorogenic acid
Hyperoside
Quercetin
Absorbance (mAu)
Time (min)
3.908
7.055
7.525

Figure 1

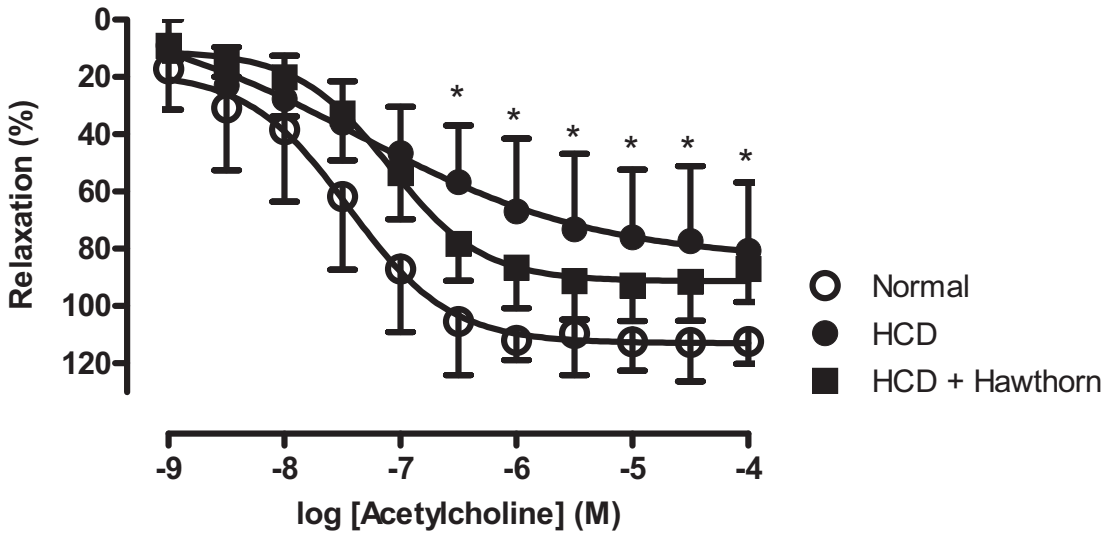
Relaxation (%)
0
20
40
60
80
100
120
*
*
*
*
*
*
-9
-8
-7
-6
-5
-4
log [Acetylcholine] (M)
Normal
HCD
HCD + Hawthorn

Figure 2

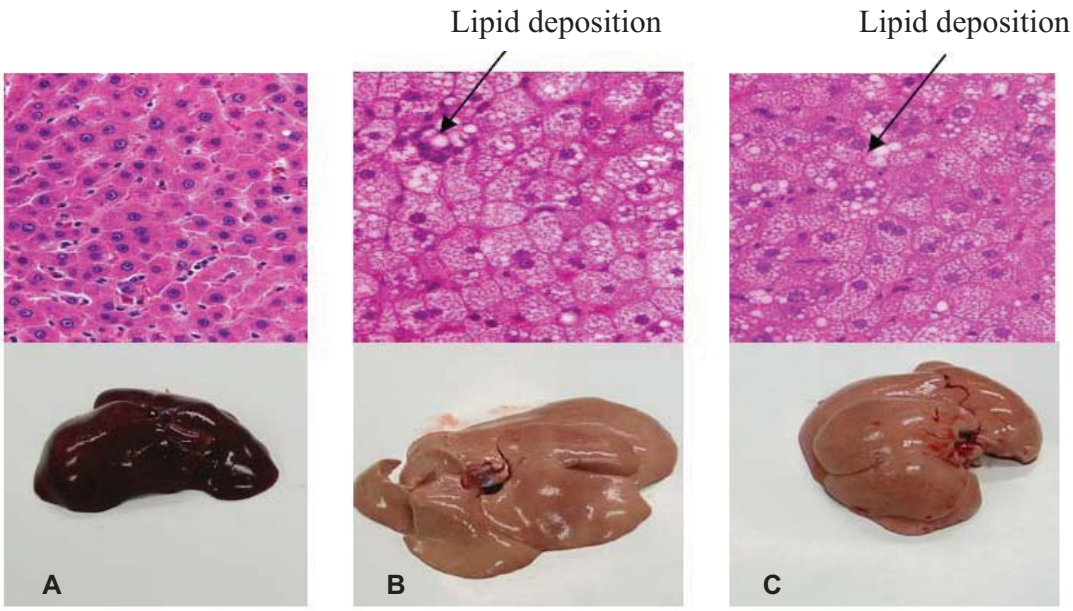

Figure 3