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Antiobese and hypocholesterolaemic effects of an *Adenophora triphylla* extract in HepG2 cells and high fat diet-induced obese mice

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ABSTRACT

The ethyl acetate extract from *Adenophora triphylla* root (ATea) had strong antioxidant effect. We hypothesised that a high fat (HF) diet might induce oxidative stress and so, dietary antioxidant may have beneficial effects on hypercholesterolaemia, but the underlying mechanisms involved are not fully understood. To test this hypothesis, C57BL/6 mice were fed with HF diet for 9 weeks. In the last 4 weeks, the HF diet was supplemented with 0, 25 or 75 mg/kg ATea. ATea decreased body weight gain and both ATea doses significantly reduced the plasma and hepatic cholesterol levels of the obese mice. Analysis of the hepatic expression of proteins known to play important roles in cholesterol metabolism indicated that ATea significantly enhanced low density lipoprotein receptor (LDL receptor) and cholesterol 7α-hydroxylase (CYP7A1) expression but inhibited the 3-hydroxy-3-methylglutaryl–CoA reductase (HMG–CoA reductase) expression in HepG2 cells and mice. No mutagenic activity was observed at high doses of ATea.

1. Introduction

Hypercholesterolaemia is a dominant risk factor that contributes to the development and progression of atherosclerosis and subsequent cardiovascular disease, which is one of the most serious diseases in humans (Deepa & Varalakshmi, 2005; Prasad & Kalra, 1993). In particular, epidemiological, clinical, genetic, and experimental studies have shown that high serum levels of low density lipoprotein (LDL) cholesterol are associated with atherosclerosis and an increased risk of coronary heart disease (Ballantyne, 1998; Levine, Keaney, & Vita, 1995).

It has been shown that some phytochemicals in traditional medicinal plants have hypolipidaemic effects because they modulate the expression of genes involved in lipid and lipoprotein metabolism (Chung et al., 2007, 2008). It has also been demonstrated that antioxidant supplements can effectively reduce the atherogenic lipoprotein profile in patients with hyperlipidaemia and atherosclerosis (Diaz, Frei, Vita, & Keaney, 1997). These observations suggest that the antioxidant activities and cholesterol-lowering effects of various plant extracts could reduce the development and/or progression of atherosclerosis and cardiovascular diseases (Chung et al., 2007, 2008). We were interested in whether extracts of the traditional oriental medicinal plant *Adenophora triphylla* (Korean name: *Jan-dae*, English name: *Three-leaf ladybell*) could also prevent hyperlipidaemia.

A. triphylla water extracts have been shown to exert hepatoprotective effects (Gum, Lee, & Cho, 2007). Moreover, *A. triphylla* extract has been found to suppress *in vitro* tumour cell growth and *in vivo* gastric epithelial proliferation (Lee et al., 2000). *A. triphylla* root (AT) and Platycodi Radix which is the root of *Platycodon grandiflorum* A. De (Campanulaceae) have been eaten as food for preventing obesity in Korea and saponins are the primary constituents of Platycodi Radix and AT (Cho, 1985; Zhao et al., 2005). Zhao et al. (2005) reported that Platycodi Radix extract and its saponins prevented increases in body weight, adipose tissue weight and liver triacylglycerol in mice fed a high fat diet. The ethyl acetate soluble fraction of medicinal plants contains saponins (Martinez-Vazquez, Gonzalez-Esquinca, Cazares Luna, Moreno Gutierrez, & Garcia-Argaez, 1999). Thus, the possibility exists that ethyl acetate extract from *A. triphylla* root (ATea) have antiobese properties.

We recently reported that *A. triphylla* extract up-regulate antioxidant genes (Choi et al., 2008). Oxidative stress plays an important role in the etiology of atherosclerosis, thus their antioxidative properties is believed to improve hepatic lipid metabolism partly by reducing oxysterol formation (Chung et al., 2008). Therefore, we speculated that these phytochemicals in *A. triphylla* could also prevent hyperlipidaemia. However, the effects of *A. triphylla* extracts on lipid metabolism have not yet been examined.

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The aim of the present study was to determine the potential of ATea as antiobesity and a lipid-lowering product and its mechanism of action. We hypothesised that the ATea may contain dietary antioxidant to improve blood lipids and the effects on the expression of key genes in cholesterol metabolism. In addition, the ATea may prevent the obesity induced by feeding a high fat diet to mice and the antiobesity effect of the ATea in mice fed a high fat diet may be due in part to the inhibition of intestinal absorption of dietary fat by the saponins of ATea (Martinez-Vazquez et al., 1999; Zhao et al., 2005). To test this hypothesis, we first studied the *in vitro* antioxidant effects of various extracts from *A. triphylla*. After finding that the ethyl acetate extract from *A. triphylla* root (ATea) had the highest antioxidant extract of all the extracts, we then evaluated its lipid-lowering capacity *in vitro* and *in vivo*. Since obesity is associated with a number of complications, including not only coronary heart disease, diabetes, and hypertension but also hyperlipidaemia (Tripathy et al., 2003; Woods, Seeley, Rushing, D'Alessio, & Tso, 2003; Zhao et al., 2005), we found that obesity was induced by feeding a high fat diet containing 40% beef tallow for 5 weeks to male mice (data not shown) and so we used the diet-induced obese mice model to assess the effect of ATea on obesity and hyperlipidaemia. Antiobese effect and the lipid-regulating function of ATea in high fat diet-induced obese mice and HepG2 cells has not been investigated on a molecular level, thus we also examined the effect of ATea on the expression of key cholesterol metabolism protein in the model obese mice livers and hepatic cell HepG2. Finally, we evaluated its *in vitro* mutagenic potential by using the Ames test for the safe use of ATea.

2. Materials and methods

2.1. Chemicals and reagents

PowerScript reverse transcriptase was obtained from Clontech (Palo Alto, CA, USA). The oligo$(dT)_{15}$ primers and GoTaq® Green Master Mix were obtained from Promega (Madison, WI, USA). Kits for measuring plasma total cholesterol, triglyceride and high density lipoprotein (HDL) cholesterol levels were purchased from Asan Chemical (Asanpharm, Korea). LDL cholesterol, glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) were purchased from Stanbio Laboratory (Stanbio, TX, USA). Anti-HMG–CoA reductase (goat polyclonal antibody), anti-LDL receptor (goat polyclonal antibody), CYP7A1 (rabbit polyclonal antibody), monoclonal anti-alpha tubulin, horseradish peroxidase-conjugated goat–anti-rabbit and donkey anti-goat secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nutrient broth and Bacto agar were obtained from Difco Laboratories Co. (Detroit, MI, USA). Folin–Ciocalteu reagent, caffeic acid, and all other reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of A. triphylla extracts

A. triphylla root was purchased from Ku-Ryong Pharmaceutical Co., Ltd. (Seoul, Korea), cleaned, dried, and ground to fine power before being extracted three times with 10 vol of 70% ethanol for 8 h at 80 °C.

After filtration, the solvent was removed by using a rotary evaporator, thereby yielding a 70% ethanol residue. The ethanol extract was suspended in water and extracted with hexane three times. The ensuing aqueous layer was then extracted sequentially with first chloroform, then ethyl acetate, and finally butanol. Each extraction was performed three times. The hexane, chloroform, ethyl acetate, and butanol extracts and the final aqueous layer were concentrated by using a vacuum evaporator (EYELA: Rotary evaporater NE-SERIES, Tokyo, Japan) and then freeze-dried in a lyophiliser (Ilshin Freezer, FD5508, Yang Ju Si, Korea). The freeze-dried product was stored at −40 °C until use.

2.3. Determination of total phenolic levels

The concentration of phenolics in the six extracts, namely, the crude extract, the hexane, chloroform, ethyl acetate, and butanol extracts, and the final water layer, was determined by using the method described by Folin and Denis (1915). For this, 5 mL of extract (0.2 mg/5 mL) was mixed with 0.5 mL of Foline-Ciocalteau's phenol regent. After 3 min, 1 mL of saturated Na_2CO_3 solution was added to the mixture. The reaction was kept in the dark for 1 h, after which the absorbance was read at 700 nm (VERSA max microplate reader, CA, USA). The total phenol content of the extract was estimated by comparison with a standard curve generated by analysing caffeic acid.

2.4. DPPH radical scavenging activity

The DPPH radical scavenging activity of the six extracts was measured according to the procedure described by Hatano, Kagawa, Yasuhara, and Okuda (1988) with slight modifications. Different concentrations of the six extracts (0, 100, 250, 500, and 1000 mg/mL) and the ascorbic acid standard (0, 5, 10, 50, and 100 mg/mL) were placed in different test tubes. The various extracts or ascorbic acid (180 μL) were mixed with 120 μL of ethanolic solution containing the DPPH radical (1.5×10^{-4} M). The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. The reduction of the DPPH radicals was measured by an ELISA reader at 517 nm. Radical scavenging activity was measured as the decrease in DPPH absorbance and the inhibition percentage was calculated by using the following equation:

$$\text{Scavenging activity } (\%) = (1 - A_{\text{Sample}(517\,\text{nm})}/A_{\text{Control}(517\,\text{nm})}) \times 100.$$

2.5. Hydroxyl radical scavenging activity

Hydroxyl radicals were measured by the deoxyribose method and hydroxyl radical scavenging activity of the extracts was determined as described by Halliwell, Gutteridge, and Aruoma (1987) with minor modifications. Briefly, 100 mM sodium phosphate buffer (250 μL), 1 mM EDTA (100 μL), 36 mM deoxyribose (100 μL), 1 mM $FeCl_3 \cdot 6H_2O$ (100 μL), 1 mM L-ascorbic acid (100 μL), 10 mM H_2O_2 (100 μL), distilled water (100 μL) and 100 μL of the extract, or the standards (ascorbic acid and vitamin E) (0, 100, 250, 500, and 1000 mg/mL) were placed in a tube and mixed by vortexing. In this system, hydroxyl radicals (·OH) were generated by mixing Fe^{3+}–EDTA, hydrogen peroxide, and ascorbic acid. The mixture was incubated at 38 °C for 1 h after which 1 mL of 1.0% thiobarbituric acid (TBA) in 0.05 M NaOH and 1 mL of 10% trichloroacetic acid (TCA) were added. After mixing, the tube was heated at 100 °C for 10 min and then immediately cooled on ice. The absorbance of the resulting solution was measured at 532 nm. The presence of hydroxyl radicals degrades the sugar deoxyribose into fragments; these fragments can be detected after being heated with TBA at a low pH because they generate a pink chromogen. The percent inhibition of the hydroxyl radical levels by the extracts or standards was calculated as follows: % Inhib = $[(A_{\text{control}(532\,\text{nm})} - A_{\text{sample}(532\,\text{nm})}/A_{\text{control}(532\,\text{nm})}] \times 100$.

2.6. Cell culture

HepG2 cells were seeded in 6-well Falcon plates at 1×10^6 cells/mL in MEM supplemented with 10% FBS, and 1% PEST. The

cells were cultured at 37 °C in a humid atmosphere containing 5% CO^2 until 60–80% confluent and were then used for the RT–PCR. The HepG2 cells were incubated in fresh MEM with or without ATea (0, 0.05, and 0.1 mg/mL) for 24 h.

2.7. Animals and feeding protocol

Male of 5-week-old C57BL/6J mice (25 ± 2.5 g) were purchased from Samtaco (n = 40) (Kyung Gi-do, Korea), housed individually in stainless steel cages, and maintained in a room at 22 ± 2 °C with a 12 h light: 12 h dark cycle (08:00–20:00 h). Animal care and handing were performed under protocols approved by the Committee on Animal Experimentation of the Hae-Eun Biotech Research Committee. All mice were provided with a commercial diet and tap water ad libitum for 1 week prior to their division into five groups. The animals were then fed with either a high fat (HF) diet [fat accounted for 40% of the diet (w/w); n = 32] or normal chow (HF-free diet group, n = 8) for 5 weeks. After 5 weeks, the HF animals were divided into four groups of eight. One group continued to receive the HF diet alone (control) whilst the other three groups were fed for four weeks with the HF diet supplemented with 1 mg/kg of the positive control Simvastatin (HF + Simvastatin group) or 25 mg/kg or 75 mg/kg ATea (HF + ATea 1 and HF + ATea 2 groups). The HF-free diet group continued to receive normal chow. During the experimental period, the food intake of mice was measured twice a week, and their body weights were measured every 10 days but at the end of the 4-week feeding period, the body weights were measured after 8 days.

Just before the diet was changed to include ATea (0 weeks), the mice were fasted for 16–19 h overnight and the mice were anesthetised with Avertin (Lee et al., 2007). The blood samples were collected in EDTA tubes. This process was repeated 2 and 4 weeks later. Plasma was obtained by centrifugation of the blood at 4 °C and 3000 rpm for 15 min. After the last bleed on week 4, the mice were euthanised with an intraperitoneal injection of Avertin (Lee et al., 2007) and their livers were removed, rinsed with phosphate–buffered saline, and quick-frozen in liquid nitrogen. The plasma and liver samples were stored at −80 °C until they were analysed.

2.8. Analysis of plasma, hepatic and fecal lipid levels

The plasma was separated from the blood by centrifugation at 3000 rpm for 15 min. Two or three mice were housed per cage, and faeces were collected for a 5-day period before the end of treatment from three cages of mice per group. Faeces were separated, freeze-dried, and weighed. Dried faeces (0.1 g) were used to extract fecal lipids. The hepatic and fecal lipids were measured according to the method with a slight modification of Hsu and Huang (2007) and Folch, Lees, and Sloan-Stanley (1956), respectively.

The total cholesterol, triglyceride and HDL-cholesterol levels in plasma, hepatic and fecal lipids were measured enzymatically (Asan Chemical, Seoul, Korea). Plasma LDL cholesterol, GPT and GOT were measured with an automatic chemistry analyser (BT-1000, Rome, Italy).

2.9. Isolation of total RNA, reverse transcription–polymerase chain reaction (RT–PCR) and western blot analysis

Isolated liver tissues were homogenised and total RNA in liver and cells was extracted by using Trizol reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instruction for the determining mRNA expression levels. The RNAs were then reverse-transcribed by using a Superscript II kit (Invitrogen) with oligo$(dT)_{15}$ primer, according to the manufacturer's recommendations. The PCR was performed with a GoTaq® Green Master Mix PCR kit (Promega, Madison, WI, USA) in 20 μL of total reaction mixture containing 1 μL of the RT-reaction mixture and 0.5 μL of each primer (forward and reverse, 15 μM).

In vitro PCR primers were designed using published nucleotide sequences for the human LDL receptor, HMG–CoA reductase and CYP7A1 and 18 S rRNA (Chung et al., 2008b). The following primers were used: for the LDL receptor, F, 5′-CAATGTCTCACCAAGCTCTG and R, 5′-TCTGTCTCGAGGGGTAGCTG; for HMG–CoA reductase: F, 5′-TACCATGTCAGGGGTACGTC and R, 5′-CAAGCCTAGAGACATAAT CATC; and for CYP7A1, F, 5′-GCATCATAGCTCTTTACCCAC and R, 5′-GGTGTTCTGCAGCAGTCCTGTAAT. The 18 S rRNA (F, 5′-CGGCTACCACATCCAAGGAA and R, 5′-GCTGGAATTACCGCGGCTGC) transcripts were used as internal controls.

PCR using the LDL receptor and HMG–CoA reductase primers were performed with an initial cycle of 4 min at 94 °C; followed by 30 cycles of 30 s at 94 °C, 30 s at 62 °C, and 30 s at 72 °C; and a final extension for 5 min at 72 °C. PCR using the CYP7A1, and 18 S primers were performed similarly, with the exception of the annealing temperature (CYP7A1, 57 °C; 18 S, 60 °C) and number of cycles (CYP7A1, 32 cycles; 18 S, 15 cycles). The 18 S transcripts were used as internal controls.

The PCR primers were designed on the basis of published nucleotide sequences for the LDL receptor (Hanaka, Abe, Itakura, & Matsumoto, 2000), HMG–CoA reductase and CYP7A1 (Chung et al., 2008), and β-actin (Wood, Hunter, & Trayhurn, 2003). The sequences of the primers were as follows: LDL receptor-F-5′-ACT CAGGCAGCAGGAACGAG-3′ and R-5′-GTCATTTTCACAGTCTACCT-3′; HMG–CoA reductase-F-5′-GTTCTTTCCGTGCTGTGTTCTGGA-3′ and R-5′-CTGATATCTTTAGTGCAGAGTGTGGCAC-3′; CYP7A1-F-5′-CCTTGGACGTTTTCTCGCT-3′ and R-5′-GCGCTCTTTGATTTAGGAAG-3′; β-actin-F-5′-TGCTGTCCCTGTATGCCTCT-3′ and R-5′-AGGTCTT TACGGATGTCAACG. The PCR with the LDL receptor primers was performed with an initial cycle of 4 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 42 °C, and 30 s at 72 °C, and a final extension for 5 min at 72 °C. The PCRs using the HMG–CoA reductase, CYP7A1, and β-actin primers were performed similarly except that the annealing temperature was 57 °C, 52 °C, and 50 °C, respectively, and the number of cycles was 29, 30, and 23, respectively. The PCR products were analysed by 1.5% agarose gel electrophoresis. The β-actin transcripts served as internal controls.

Western blotting was performed as described previously (Chung et al., 2008) with minor modifications. Briefly, proteins were separated using SDS–PAGE and then transferred to nitrocellulose membranes. Membranes were blocked for 18 h at 4 °C in 5% non-fat dried milk and then incubated with the polyclonal anti-HMG–CoA reductase, LDL receptor, CYP7A1 and α-tubulin antibody (1:1000 dilution) for 1 h at room temperature on an orbital shaker. Detection was achieved using horseradish peroxidase-conjugated secondary antibodies and ECL reagents (GE Healthcare, Bucks, UK).

2.10. Mutagenicity test

The crude extract, the hexane, chloroform, ethyl acetate, butanol, water extracts of AT was assayed for mutagenic potential by using the preincubation Ames test (Maron & Ames, 1983).

2.11. Statistical analysis

The data are expressed as means ± SD (standard deviation) and are the average values of three to five values per experiment. The data were analysed by using the SPSS package (Version 10.0, SPSS, Chicago, USA). The experiments were repeated at least three times to confirm the results. Analysis of variance (ANOVA) was conducted, and Duncan's multiple range tests were used to determine

the significance of differences between groups. The level of statistical significance was set to $P < 0.05$. Linear regression was carried out by least square fitting and the fit to a straight line expressed as the Pearson product-moment correlation coefficient.

3. Results

3.1. Antioxidant properties of six extracts from A. triphylla root (AT)

The extract yield (amount of total extractable compounds) of the crude extract, the hexane, chloroform, ethyl acetate, and butanol extracts, and the final water layer were 45.64 g/100 g, 0.53 g/100 g, 0.52 g/100 g, 0.55 g/100 g, 15.40 g/100 g, and 28.01 g/100 g root powder, respectively. The highest total phenolic amounts were present in the butanol extract (9.97 ± 0.73 mg/mL), followed by the ethyl acetate extract (8.01 ± 0.1 mg/mL). The remaining extracts had relatively low total phenol contents that ranged from 2.81 ± 0.05 mg/mL to 1.84 ± 0.09 mg/mL with the following order: hexane (2.81 ± 0.05 mg/mL) > crude extract (2.49 ± 0.09 mg/mL) > chloroform (2.25 ± 0.05 mg/mL) > water (1.84 ± 0.09 mg/mL).

The free radical scavenger activity of the six extracts was evaluated by reacting different concentrations of the extracts with DPPH. The ability of a sample to reduce DPPH absorbance is indicative of its capacity to scavenge free radicals (Ozsoy, Can, Yanardag, & Akev, 2008). All six extracts scavenged DPPH radicals in a dose-dependent manner (Table 1). At all concentrations tested, the ethyl acetate extract had the highest scavenging activity and its maximal activity (at 1000 μg/mL) was 92.58 ± 0.22%. At 1000 μg/mL, the remaining extracts had activities (from highest to lowest) of 83.46 ± 1.32 (chloroform), 82.74 ± 0.65 (butanol), 58.30 ± 1.19 (crude extract), 57.65 ± 2.98 (hexane), and 31.86 ± 2.95% (water) (Table 1).

The hydroxyl radical scavenging activity of the six extracts was then determined (Table 1). All six extracts scavenged hydroxyl radicals in a dose-dependent manner. At nearly all concentrations tested, the ethyl acetate extract exhibited the highest hydroxyl radical scavenging activity. The remaining extracts also showed relatively good hydroxyl radical scavenging activity at the maximal dose. However, the scavenging activities of all extracts were significantly lower than those of the ascorbic acid and vitamin E positive controls.

Thus, the ethyl acetate extract had one of the highest phenolic contents of the six extracts and also exhibited the highest antioxidant activity. Thus we conducted *in vitro* and *in vivo* experiments with this extract to determine its hypocholesterolaemic activity.

3.2. Antiobese effects of ethyl acetate extract from A. triphylla root (ATea) in high fat diet-induced obese mice

As shown in Fig. 1A, there was no significant difference in body weight gain amongst the three groups until 20 days of treatment. After 4 weeks, mice fed an HF diet containing 25 mg/kg or 75 mg/kg ATea exhibited a significant reduction in the body weight compared to the HF controls ($P < 0.05$). There was no difference in food intake (data not shown).

3.3. Hypolipidaemic effects of ethyl acetate extract from A. triphylla root (ATea) in high fat diet-induced obese mice

The four groups of HF diet-fed mice were treated with the hypocholesterolaemic drug Simvastatin (1 mg/kg), 25 mg/kg ATea 1 or 75 mg/kg ATea 2 as described in Section 2 and their plasma lipid were determined on weeks 0, 2, and 4 of treatment; their plasma GOT and GPT levels, fecal lipid levels and hepatic lipid levels were determined on week 4 (Table 2; Fig. 1B–E).

Table 1
DPPH radical and hydroxyl radical scavenging activities of *Adenophora triphylla* extracts.

Sample	Concentration (mg/L)	Inhibition %[A]	
		DPPH radical scavenging activity	Hydroxyl radical scavenging activity
70% ethanol	100	2.93 ± 1.94^{a}	14.91 ± 0.98^{a}
	250	16.14 ± 1.61^{de}	24.18 ± 0.82^{d}
	500	35.41 ± 3.70^{i}	39.77 ± 1.52^{hi}
	1000	58.30 ± 1.19^{kl}	49.52 ± 1.13^{kl}
Hexane	100	18.89 ± 2.24^{e}	13.73 ± 3.81^{a}
	250	31.37 ± 2.54^{g}	23.73 ± 0.38^{d}
	500	41.10 ± 1.61^{j}	41.35 ± 0.52^{ni}
	1000	57.65 ± 2.98^{k}	47.84 ± 0.28^{jk}
Chloroform	100	8.69 ± 0.75^{b}	18.17 ± 1.08^{b}
	250	23.63 ± 2.73^{f}	29.06 ± 1.97^{ef}
	500	62.20 ± 2.35^{m}	42.19 ± 0.42^{i}
	1000	83.46 ± 1.32^{n}	45.41 ± 0.42^{j}
Ethyl acetate	100	34.88 ± 2.05^{hi}	27.91 ± 0.99^{e}
	250	61.02 ± 2.77^{lm}	40.69 ± 1.73^{hi}
	500	89.66 ± 0.39^{o}	50.45 ± 0.53^{l}
	1000	92.58 ± 0.22^{op}	56.40 ± 1.68^{m}
Butanol	100	13.53 ± 1.51^{cd}	21.03 ± 2.65^{c}
	250	25.11 ± 1.92^{f}	35.65 ± 1.90^{g}
	500	44.17 ± 0.24^{j}	46.66 ± 0.92^{j}
	1000	82.74 ± 0.65^{n}	56.23 ± 1.24^{m}
Aqueous	100	2.12 ± 0.57^{a}	15.21 ± 2.06^{a}
	250	12.69 ± 1.42^{c}	25.10 ± 1.31^{d}
	500	18.02 ± 1.55^{e}	31.29 ± 0.30^{f}
	1000	31.86 ± 2.95^{gh}	39.30 ± 1.86^{h}
Vitamin E	100	–	40.67 ± 0.26^{hi}
	250	–	55.76 ± 0.32^{m}
	500	–	63.33 ± 0.19^{n}
	1000	–	70.68 ± 0.12^{o}
Ascorbic acid	5	43.55 ± 0.06^{j}	–
	10	94.19 ± 0.17^{pq}	–
	50	96.55 ± 0.22^{q}	–
	100	97.14 ± 0.28^{q}	34.69 ± 0.01^{g}
	250	97.11 ± 0.52^{q}	47.75 ± 0.22^{jk}
	500	96.91 ± 0.42^{q}	61.46 ± 0.21^{n}
	1000	96.52 ± 0.32^{q}	72.75 ± 0.07^{o}

[A] Values are means ± SD of four groups. Means with the different letters in the same column are significantly different ($P < 0.05$) by Duncan's multiple range test.

At all time points examined, the HF alone mice had higher total cholesterol, triglyceride, LDL cholesterol, GPT and GOT levels than the normal chow-fed mice, whilst no change in HDL-cholesterol levels was observed (Table 2; Fig. 1B–E). Simvastatin significantly reduced the plasma total cholesterol and triglyceride levels of the HF diet-fed mice by weeks 2 and 4; by week 4, Simvastatin had reduced the total cholesterol and triglyceride levels (by 16.7% and 11.5%, respectively) to close to the levels seen in normal chow-fed mice. By week 4, Simvastatin had also significantly reduced the LDL cholesterol levels by 27.8% (Fig. 1C). GPT and GOT levels were also reduced significantly by week 4 by 19.1% and 27.3%, respectively. Indeed, the GPT levels were reduced to the levels seen in normal chow-fed mice (Table 2). However, Simvastatin had no effect on HDL levels (Fig. 1D). Thus, this drug exerted strong hypocholesterolaemic effects on the HF diet-fed mice.

Both concentrations of ATea also significantly reduced the total cholesterol levels of HF diet-fed mice by weeks 2 and 4; their effects were greater by the fourth week than by the second week. By week 4, the lower and higher concentrations had reduced total cholesterol levels by 12.5% and 19.4%, respectively (Fig. 1B). Both ATea concentrations also reduced the LDL cholesterol levels of the HF diet-fed mice on week 4 by 27.8% and 40.8%, respectively (Fig. 1C). In addition, by week 4, the two ATea concentrations reduced the GPT levels by 17.3% and 19.1%, respectively, and GOT

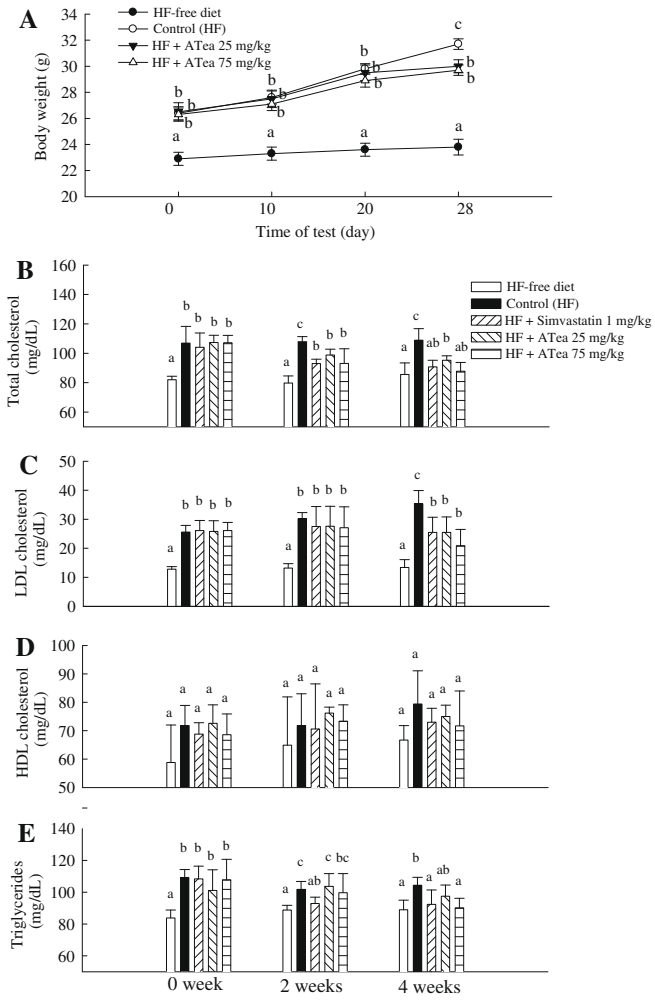

Fig. 1. Effect of *Adenophora triphylla* root ethyl acetate extract (ATea) on body weight change (A), plasma total cholesterol (B), LDL cholesterol (C), HDL-cholesterol (D) and triglyceride (E) levels in high fat (HF) diet-fed obese mice. Four groups of eight mice were fed with a HF diet (40% of the diet consisted of fat) and one group was fed with normal chow (HF-free diet group, $n = 8$) for 5 weeks. After 5 weeks, the HF diet-fed mice were fed with the HF diet alone (control) or the HF diet supplemented with 1 mg/kg Simvastatin or 25 mg/kg or 75 mg/kg of ATea for 4 weeks. The HF-free diet group was fed normal chow for 4 weeks. Blood samples were collected on weeks 0, 2, and 4 after commencing treatment. In image (A), the values shown are means ± SEM ($n = 8$) and means with the different letters in the same concentration are significantly different ($P < 0.05$). In images (B)–(E), the values shown are means ± SD ($n = 8$) and means with different letters differ significantly from the same week ($P < 0.05$), as determined by Duncan's multiple range test.

levels by 26.5% and 27.3%, respectively. Indeed, both ATea doses reduced the GOT and GPT levels better than Simvastatin. Whilst the higher ATea concentration had a greater effect on total cholesterol, LDL cholesterol, triglyceride, GPT and GOT levels than the lower ATea concentration, these differences did not achieve statistical significance. However, with regard to the triglyceride levels in HF diet-fed mice, only the higher ATea concentration was able to reduce them significantly, and it only did so only by week 4 (a 13.6% reduction was observed) (Fig. 1E). Neither of the ATea doses altered the HDL-cholesterol levels in HF diet-fed mice ($P > 0.05$).

Feeding the high fat diet caused with hepatic accumulation of triglyceride and total cholesterol (Table 2). The hepatic triglyceride and total cholesterol levels were significantly lower in the ATea groups than in the control group (Table 2). The ATea resulted in a significant increase in fecal triglyceride level but ATea had no effect on fecal total cholesterol level (Table 2).

3.4. Effect of ethyl acetate extract from A. triphylla (ATea) root on the mRNA and protein levels of the hepatic LDL receptor, HMG–CoA reductase, and CYP7A1 in vitro and in vivo

To study the molecular mechanism by which ATea influenced the lipid profile of the HepG2 cells and the mice, especially their cholesterol levels, we used RT–PCR and western blotting to measure the expression levels of three hepatic proteins known to be involved in lipid metabolism.

HMG–CoA reductase levels decreased dose-dependently in ATea-treated cells but LDL receptor and CYP7A1 mRNA levels in 100 mg/L ATea-treated cells increased (Fig. 2B). HMG–CoA reductase protein levels were also decreased at 50 and 100 μg/mL of ATea-treated HepG2 cells (Fig. 2A and C). CYP7A1 protein levels in 50 and 100 μg/mL ATea-treated HepG2 cells increased and LDL receptor protein levels were significantly up-regulated in the 100 μg/mL ATea-treated HepG2 cells (Fig. 2A and C).

The expression levels of these three genes was also examined in livers taken from the mice fed with HF and then either left untreated or treated with 25 mg/kg or 75 mg/kg HF for 4 weeks. Administration of ATea dramatically changed the expression of all three genes. LDL receptor mRNA levels were significantly higher in the animals treated with either ATea concentration than in the untreated control mice ($P < 0.05$; Fig. 3B). This was also true for the CYP7A1 levels ($P < 0.05$; Fig. 3B); moreover, the higher concentration of ATea elevated CYP7A1 expression by 2.6-fold, which was significantly higher than the effect of the lower concentration (1.9-fold). However, both ATea doses significantly suppressed the gene expression level of HMG–CoA reductase (Fig. 3B). We also investigated their effect on LDL receptor, HMG–CoA reductase and CYP7A1 protein levels. LDL receptor and CYP7A1 protein levels also were significantly higher in ATea consumption but consumption of

Table 2
Effects of *Adenophora triphylla* ethyl acetate extract root (ATea) on plasma GPT and GOT, hepatic lipid and fecal lipid levels in high fat (HF) diet-fed obese mice.

Groups (4 weeks)[A]	HF-free diet	HF (control)	HF + S[B] (1 mg/kg)	HF + ATea 1 (25 mg/kg)	HF + ATea 2 (75 mg/kg)
Plasma GPT (U/L)	26.0 ± 2.6[aC]	33.6 ± 2.5[b]	29.0 ± 1.5[a]	27.8 ± 2.5[a]	27.2 ± 3.2[a]
Plasma GOT (U/L)	85.5 ± 4.1[a]	143.5 ± 3.8[d]	126.0 ± 4.8[c]	105.5 ± 5.5[b]	104.3 ± 9.8[b]
Hepatic lipids					
Triglyceride (mg/dL)	1.7 ± 0.1[a]	3.3 ± 0.8[b]	2.0 ± 0.4[a]	1.8 ± 0.1[a]	1.7 ± 0.1[a]
Cholesterol (mg/dL)	4.6 ± 1.7[a]	22.0 ± 2.2[d]	11.4 ± 1.1[b]	19.3 ± 2.4[c]	12.0 ± 3.3[b]
Fecal lipids					
Triglyceride (mg/dL)	4.7 ± 0.1[a]	4.4 ± 0.0[a]	4.3 ± 0.4[a]	6.0 ± 0.5[b]	12.4 ± 0.2[c]
Cholesterol (mg/dL)	1.2 ± 0.1[a]	1.7 ± 0.7[a]	4.2 ± 0.4[b]	1.4 ± 0.1[a]	1.6 ± 0.0[a]

[A] Plasma GOP and GPT, hepatic lipid and fecal lipid levels were determined from samples collected at sacrifice after 4 weeks of dietary treatment.
[B] S: Simvastain.
[C] Within each row, values with different superscripts are significantly different from each other ($P < 0.05$), as determined by Duncan's multiple range test, $n = 8$.

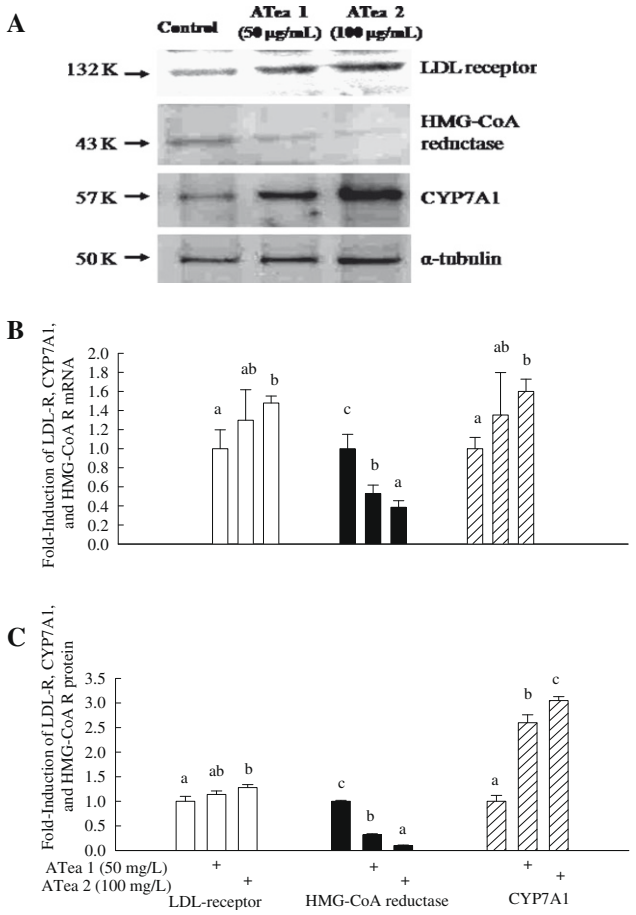

Fig. 2. Effects of *Adenophora triphylla* root ethyl acetate extract (ATea) on hepatic LDL receptor, HMG–CoA reductase and CYP7A1 mRNA (B) and protein (A and C) expression in HepG2 cells. The HepG2 cells were incubated in fresh MEM with or without ATea (0, 50, and 100 μg/mL) for 24 h. The LDL receptor, HMG–CoA reductase and CYP7A1 protein and mRNA levels in each sample were normalised to the α-tubulin or 18 S levels. (A and B) LDL receptor, HMG–CoA reductase and CYP7A1 protein and mRNA levels was measured by western blotting and RT–PCR. (C) Histograms illustrate densitometric analysis of protein levels shown in (A) based on α-tubulin expression. The density of each mRNA and protein band was quantified by using SigmaGel software (Jandel Scientific, San Rafael, CA) and the group data were averaged and plotted. Within each gene, means with different letters differ significantly from each other ($P < 0.05$), as determined by Duncan's multiple range test ($n = 4$).

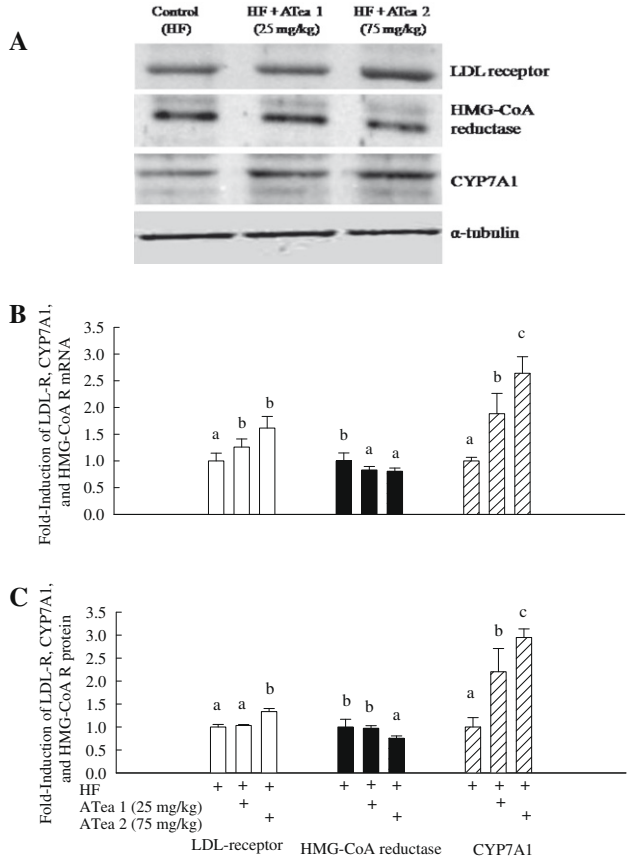

Fig. 3. Effects of *Adenophora triphylla* root ethyl acetate extract (ATea) on hepatic LDL receptor, HMG–CoA reductase and CYP7A1 mRNA (B) and protein (A and C) expression in high fat (HF) diet-fed obese mice. The mice were fed as described in Fig. 1 and liver samples were collected on week 4 after commencing treatment. The LDL receptor, HMG–CoA reductase and CYP7A1 mRNA and protein levels in each sample were normalised to the β-actin or α-tubulin levels. (A and B) LDL receptor, HMG–CoA reductase and CYP7A1 protein and mRNA levels was measured by western blotting and RT–PCR. (C) Histograms illustrate densitometric analysis of protein levels shown in (A) based on α-tubulin expression. The density of each mRNA and protein band was quantified by using SigmaGel software (Jandel Scientific, San Rafael, CA) and the group data were averaged and plotted. Within each gene, means with different letters differ significantly from each other ($P < 0.05$), as determined by Duncan's multiple range test ($n = 8$).

ATea significantly reduced HMG–CoA reductase protein (Fig. 3A and C).

3.5. Mutagenicity

Table 3 shows the effect of different AT extracts doses on the recovered colony numbers of the *Salmonella typhimurium* strains TA98 and TA100. The test concentration (0.5–10.0 mg/plate) of all extracts, in the absence of S9 mixture, had no significant effect on colony numbers as compared to the solvent control, which indicates that all extracts was not mutagenic for strains TA100 and TA98.

4. Discussion

Phenolic compounds are very important for plants due to the scavenging ability of their hydroxyl group (Hatano, Edamtsu, Mori, Fujita, & Yasuhara, 1989). In this study, we used the DPPH assay because it is one of the most accurate, sensitive and widely used assays for characterising the antioxidant capacity of plant materials or their extracts (Ozsoy et al., 2008). It has also been shown that the antioxidant activity of a sample, as measured by the DPPH assay, correlates well with the phenolic content of the sample (Ozsoy et al., 2008). The second method, we employed to determine the antioxidant capacity of the extracts was an assay measuring hydroxyl radical scavenging activity by the deoxyribose method (Halliwell et al., 1987). In this assay, deoxyribose is degraded into malonaldehyde on exposure to hydroxyl radicals generated by Fenton reaction using EDTA, iron(III) ions, ascorbic acid and hydrogen peroxide. Reducing agents, such as ascorbic acid, can accelerate hydroxyl radical formation by reducing Fe^{3+} ions to Fe^{2+} (Halliwell et al., 1987). If the extracts from *A. triphylla* roots have Fe^{2+}-chelating ability, the extracts are able to capture ferrous ion before the formation of hydrogen peroxide and may inhibit hydroxyl radical-mediated 2-deoxy-D-ribose degradation. Thus, the assay used may measure not only the reaction with ·OH, but also the chelation of iron (preventing ·OH formation in the system). Of the oxygen radicals, hydroxyl radicals are the most reactive and can severely damage adjacent biomolecules (Sakanaka, Tachibana, & Okada, 2005).

Here, we analysed the total phenolic contents and antioxidant activities of six extracts from *A. triphylla* root. The ethyl acetate

Table 3
Mutagenicity of the six extracts from *Adenophora triphylla* root on *S. typhimurium* TA98 and TA100.

Dose (mg/plate)	His$^+$ revertants/plate [A]		
		TA98	TA100
Spontaneous	0	28.16 ± 2.71[a]	150.00 ± 11.8[a]
70% ethanol	0.5	27.33 ± 1.52[a]	152.00 ± 7.54[a]
	1.0	25.00 ± 2.00[a]	136.33 ± 5.68[a]
	5.0	22.33 ± 2.51[a]	139.00 ± 7.00[a]
	10.0	21.33 ± 1.52[a]	134.66 ± 9.45[a]
Hexane	0.5	24.60 ± 1.13[a]	123.33 ± 4.16[a]
	1.0	25.00 ± 2.64[a]	128.00 ± 8.17[a]
	5.0	19.66 ± 1.15[a]	146.00 ± 7.21[a]
	10.0	21.66 ± 2.08[a]	154.00 ± 8.18[a]
Chloroform	0.5	23.33 ± 5.13[a]	137.33 ± 5.03[a]
	1.0	27.00 ± 1.73[a]	130.66 ± 3.51[a]
	5.0	22.66 ± 2.30[a]	143.00 ± 8.00[a]
	10.0	24.00 ± 2.08[a]	152.00 ± 13.45[a]
Ethyl acetate	0.5	23.66 ± 2.88[a]	138.66 ± 7.37[a]
	1.0	22.66 ± 3.78[a]	143.00 ± 6.24[a]
	5.0	24.66 ± 1.52[a]	136.00 ± 8.71[a]
	10.0	25.00 ± 4.35[a]	136.60 ± 4.13[a]
Butanol	0.5	23.00 ± 2.64[a]	150.66 ± 3.21[a]
	1.0	27.00 ± 2.64[a]	157.33 ± 4.16[a]
	5.0	25.33 ± 3.51[a]	154.00 ± 10.14[a]
	10.0	23.66 ± 1.52[a]	148.33 ± 4.16[a]
Aqueous	0.5	22.66 ± 3.51[a]	139.00 ± 6.55[a]
	1.0	27.33 ± 1.15[a]	137.33 ± 7.09[a]
	5.0	22.66 ± 3.05[a]	148.66 ± 8.02[a]
	10.0	24.00 ± 2.64[a]	146.33 ± 5.85[a]

[A] Each value is the mean ± SD of five plates. Values in the same stain with same letter are statistically non-significant ($P > 0.05$), as determined by Duncan's multiple range test.

and butanol extracts had 3–4 times higher concentrations of total phenols than the crude, hexane, chloroform, and water extracts. The ethyl acetate extract also had the highest antioxidant activity of all six extracts at nearly all concentrations tested. This led us to choose the ethyl acetate extract for our cells and animal experiments. Dietary cholesterol is delivered to the liver cells, where substantial amount of reactive oxygen species are generated (Erdincler, Seven, Inci, Beger, & Candan, 1997). This process generates highly toxic products but natural antioxidants leads to inhibition of hypercholesteraemia and cell damage related to free radical and lipid peroxidation (Chung et al., 2007, 2008).

Additionally, we evaluated the molecular level of the hypocholesterolaemic effects of ATea on human liver cells (HepG2) and the liver of high fat diet-induced obese mice. The HepG2 cells line has been used as a model system in many studies on cholesterol metabolism (Chung et al., 2007, 2008). We also used male C57BL/6J mice as they are believed to be better models of spontaneous human obesity than other genetically altered animal models. This is because when C57BL/6J mice are fed HF diets, they become obese, hyperinsulinemic, and hyperlipidaemic, and hyperlipidaemia is widely recognised to be a major risk factor in the development of atherosclerosis (Gregoire et al., 2002; Schaefer, Lichtenstein, Lamon-Fava, McNamara, & Ordovas, 1995). The blood lipid levels are also probably a major determinant of cardiovascular disease. Many extensive studies have examined the ability of natural compounds to induce hypolipidaemia in normal animal models or diet-induced obese animal model without side effects (Chung et al., 2007, 2008; Zhao et al., 2005). In this study, we observed that, compared to untreated HF-fed obese mice, treatment with ATea significantly decreased the plasma total cholesterol, LDL cholesterol and triglyceride levels in HF diet-fed mice. It is known that plasma cholesterol levels can be reduced *via* three primary functions in the liver. First, the inhibition of HMG–CoA reductase directly inhibits the hepatic synthesis of cholesterol. Second, bile acids synthesis from cholesterol is a major metabolic pathway that degrades cholesterol; CYP7A1 is a rate-limiting enzyme of this pathway (Chiang, 2004). Thus, the induction of CYP7A1 promotes cholesterol degradation. Third, plasma cholesterol concentrations can be reduced by the expression of the LDL receptor, which removes cholesterol from the plasma.

We observed that ATea treatment significantly increased the mRNA and protein expression of LDL receptor and CYP7A1 in ATea-treated HepG2 cells and mice fed the high fat plus ATea. However, the mRNA and protein expression of HMG–CoA reductase was significantly reduced both *in vitro* and *in vivo*. Significant positive correlation was noted between the HMG–CoA reductase mRNA level and the plasma LDL cholesterol level ($r = 0.8329$, $P < 0.005$) by 75 mg/kg ATea supplementation. Furthermore, a significant and tight negative correlation existed between the CYP7A1 mRNA levels and the plasma LDL cholesterol level ($r = -0.7371$, $P < 0.005$) by 75 mg/kg ATea supplementation. The correlation between LDL receptor mRNA level and the plasma LDL cholesterol level in mice fed the high fat plus 75 mg/kg ATea was $r = -0.7020$ and highly significant, $P < 0.05$.

These results suggested that the ATea worked by inhibiting HMG–CoA reductase and by increasing CYP7A1 and this depletes the intracellular pool and leads to an up-regulation of cell surface LDL receptors. An increased hepatic LDL receptors expression by ATea results in improved clearance of plasma LDL. Thus, the decreased plasma LDL cholesterol level by ATea supplementation could result from various mechanisms such as increasing LDL uptake, reducing cholesterol biosynthesis, and enhancing cholesterol degradation. The dominant effect may be related to decrease liver cholesterol synthesis through the depression of HMG–CoA reductase.

The present study also shows that the lipid-lowering effects of ATea seed in hyperlipidaemic mouse is related to an increased excretion of triglyceride and a decrease in hepatic cholesterol and triglyceride content. The decreased liver cholesterol content by ATea supplementation might be a result of reduced cholesterol synthesis, and/or increased output from enhanced biosynthesis of bile acids.

Feeding the high fat diet caused fatty liver with accumulation of triglyceride. The accumulation of hepatic triglyceride was significantly decreased by feeding the high fat diets containing ATea compared with feeding the high fat diet alone. Mice fed the high fat plus ATea increased triglyceride excretion in faeces compared with the high fat diet group. These results might be due to the inhibitory effect of the ATea on lipid metabolism in mice fed a high fat diet. Therefore, the antiobesity effect of the ATea in mice fed a high fat diet may be due to the inhibition of lipid metabolism.

In addition, the plasma GOT and GPT levels were measured. Plasma GOP and GPT were used to reflect liver function. The mice fed with HF significantly ($P < 0.05$) elevated the levels of GOT and GPT compared with the mice fed with normal chow. ATea treatment significantly lowered the plasma GOP and GPT activities as compared with HF diet-fed mice. The results of the present study showed that ATea feeding had a markedly protective effect against high fat diet-induced hepatotoxicity.

The *Salmonella* mutagenicity assay is known to measure the mutation potential of chemical compound or natural foods. The all extracts of AT in the concentration range 0.5–10.0 mg/plate were checked for possible mutagenic effects in both TA98 and TA100 strains and no change in spontaneous revertant count indicated absence of any mutagenic effects in the tested dose range. Our finding provides scientific evidence for the safe use of AT extracts.

In conclusion, our observations indicate that ATea has antioxidant, antiobese and hypocholesterolaemic effects, and that these

hypocholesterolaemic effects may be achieved by multiple mechanisms, including reducing cholesterol, enhancing cholesterol degradation and biosynthesis increasing LDL uptake. In addition, no mutagenic activity was observed at high doses of ATea. Thus, the extracts of AT themselves were safe nutraceuticals. Further studies are needed to determine whether dietary supplementation with ATea could reduce plasma cholesterol levels in humans by similar mechanism showed in cells and animals. In addition, further investigations characterising the individual bioactive components in ATea are needed.

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