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REGULAR ARTICLE

Addition of mulberry leaf (*Morus Alba* L.) to a diet formula impeded its hypoglycemic effect and exacerbated dyslipidemia in high-fructose- and high-fat-induced CD-1 mice

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ABSTRACT

Morus alba L. (mulberry leaf) is frequently used for therapeutic purposes in China. This study investigated the hypoglycemic and hypolipidemic effects of a reported antidiabetic herbal formula (PLCP) supplemented with mulberry leaves (MPLCP) in high-fructose- and high-fat- (HFF) fed mice. Six-week-old CD-1 male mice were fed on normal standard diet for 10 weeks. Fat emulsion with or without aqueous/ethanol extracts of PLCP/MPLCP was administrated by gavage daily for HFF groups. The normal group only received vehicle. 15% Fructose drinking water was supplied for HFF groups. MPLCP was less effective than PLCP in hypoglycemic effect. Furthermore, the addition of mulberry leaves impeded the regulation effects of PLCP on insulin resistance, serum free fatty acid content, and spleen index. The ethanol extract of MPLCP caused remarkable increase in low-density lipoprotein levels ($p < 0.01$) and significant increase in total cholesterol level ($p < 0.05$) compared with model group. Thus the effect of MPLCP was detrimental to abnormal serum lipid levels. The addition of mulberry leaves to PLCP weakened the hypoglycemic effects of the original formula and exacerbated dyslipidemia.

Keywords: Dyslipidemia; Glucose regulation; Insulin resistance; Mulberry leaf

INTRODUCTION

Traditional Chinese medicines have been extensively used to prevent and treat human diseases in East Asia. *Morus alba* L. (mulberry) is mainly found in China, Japan, and Korea, where it is frequently used as a therapeutic agent for fever, protecting the liver, and blood pressure (Venkatesh et al., 2008). It has also been implicated in anti-inflammatory, anti-atherosclerosis, and anti-cancer activities (Naowaratwattana et al., 2010; Padilha et al., 2010; Sharma et al., 2010). Various food products containing mulberry leaves, such as teas and porridges, are commercially available in China and Japan.

Mulberry leaves contain high amounts of iminosugars, such as the glucose analogue 1-deoxynojirimycin, which is a strong α-glucosidase inhibitor (Kimura et al., 1995). Thus, mulberry leaves may be used to reduce hyperglycemia in the pathogenesis of diabetes. Ethanol-soluble fractions of mulberry leaves scavenge reactive oxygen species and suppress glucose absorption in mice (Tao et al., 2010). In diabetic and hypercholesterolemic subjects, the supplementation of mulberry leaves can significantly reduce the levels of blood glucose, total cholesterol (TC), total triglycerides (TG), low-density lipoprotein (LDL) cholesterol, and very low-density lipoprotein cholesterol; this supplementation can also significantly increase the levels of high-density lipoprotein (HDL) cholesterol (Andallu et al., 2003). Furthermore, other bioactive ingredients of mulberry leaves have been identified including polyphenolic compounds, such as rutin, quercetin, chlorogenic acid, apigenin, and luteolin (Chu et al., 2006; Zhishen et al.1999). This finding indicated possible antioxidant property of mulberry leaves (Arabshahi-Delouee and Urooj, 2007).

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Given the potential regulatory activity on diabetes-related disorders, mulberry leaf was added to an antidiabetic formula (Liu et al., 2014), composed of *Puerariae radix, Lycium barbarum, Crataegus pinnatifida,* and *Polygonati rhizoma* (PLCP) to see whether it could work synergistically with PLCP and enhance the effects. The new formula is designated as MPLCP. The effects of PLCP and MPLCP on blood glucose, blood lipid, and NAFLD were investigated using the same mouse model.

MATERIALS AND METHODS

Chemicals and reagents

Cholesterol, pig bile salt, and Tween 80 were purchased from Dingguo Biotech Co. (Beijing, China). Fructose was supplied from Archer Daniels Midland Company (Shanghai, China). All the enzymatic or quantification kits used in the study were purchased from Beijing Zhongsheng Hightech Bioengineering Company (Beijing, China). Other reagents used were all analytical grade.

Preparation of aqueous and ethanol extract

All the plant materials were purchased from Beijing TongRenTang Pharmacy Store (China). Kudzu root (*Puerariae radix*), goji berry (*L. barbarum*), hawthorn (*C. pinnatifida*), and Huangjing (*Polygonati rhizoma*) were formulated with a weight ratio of 4:3:3:4 to get the formula of PLCP; the addition of mulberry leaves (*M. alba*) to PLCP at 20% (w/w) obtained the MPLCP formula. Both aqueous and ethanol extracts were prepared as previously described (Liu et al., 2014). Briefly, the materials were cleaned, combined, ground, and passed through a 60-mesh sieve to produce the powder form of each formula. The ethanol extract was achieved by extracting the powder in 70% ethanol in a 1:8 ratio (w/v), with 30 min sonication at 50 °C and the resulting mixture was filtered. The pellet was extracted twice using the same procedure, and the filtrates were pooled and concentrated with a vacuum rotary evaporator to obtain the extract. The same procedure was performed when aqueous extract was prepared (w/v, 1:8). Both extracts were diluted with water to yield 2 g crude PLCP/mL or 2.4 g crude MPLCP/mL as stock solutions and kept at −20 °C. The aqueous and ethanol extracts of PLCP were referred to as AP and EP, respectively, whereas the aqueous and ethanol extracts of MPLCP were referred to as AM and EM, respectively.

Experimental animals and diets

Six-week-old CD-1 male mice (Beijing Vital River Laboratory Animal Center) were maintained in a humidity-, temperature-, and 12-h light/dark-controlled animal facilities. The animals were randomized into 8 groups (n = 10 per group) as follows: normal standard diet (ND), high-fructose and high-fat diet (HFF), and HFF diet supplemented with a medium dose (20 g crude PLCP/kg) of AP or EP (APM and EPM, respectively), medium dose (25 g crude MPLCP/kg) of AM or EM (AMM and EMM, respectively), and high dose of EP (30 g crude PLCP/kg) or EM (37.5 g crude MPLCP/kg) (EPH and EMH, respectively). The HFF diet contained high levels of fat emulsion which was administered by gavage (20 mL/kg). The fat emulsion was prepared in water (100 mL) that contained 50 g lard, 1.5 g cholesterol, 0.3 g pig bile salt, and 7 mL Tween 80, as compared to vehicle (7% Tween 80) in the ND diet. Animals were given *ad libitum* access to standard laboratory chow (Experiment Animal Center of Beijing, China), and all groups except ND received 15% fructose in their drinking water. All supplementations were given once daily for 10 weeks. All the animals were humanely treated in accordance with in accordance with the approval and guidelines under national and international laws and policies, and the protocols were approved by the Ethics Committee of the Beijing Key Laboratory of Functional Food from Plant Resources (Permit number: A330–5).

Oral glucose tolerance test (OGTT) and HOMA-IR index

To determine the effect of each extract on glucose tolerance, OGTT was carried out by oral administration of 2 g glucose/kg body weight to overnight-fasted mice. Plasma glucose levels were measured immediately before, 30, 60, 90, and 120 min after the glucose challenge using a calibrated One Touch Ultra® glucometer (Lifescan, CA, USA). The mean values of the total areas under the curves (AUC) for serum glucose were calculated using the trapezoidal rule. Insulin levels were measured in plasma samples through the radioimmunoassay method (Insulin Radioimmunoassay Kit, X-Y Biotechnology Co. Ltd, Shanghai, China). Insulin resistance (IR) was calculated using the homeostasis model assessment equation:

HOMA-IR = [fasting insulin (mmol/L) × fasting glucose (mU/L)/22.5] (Matthews et al., 1985).

Insulin tolerance test (ITT)

In the last week of the experiment, ITT was performed on all animals. Mice were deprived of food for 4 h and then administered an intraperitoneal injection of insulin (0.6 U/kg). The concentrations of glucose were measured in the blood collected from the tail vein at 0, 30, 50, 70, and 90 min.

Sample collection and biochemical analysis

At 4 and 7 weeks, fasting blood samples were drawn from the retro-orbital plexus of anesthetized mice. Plasma was separated by centrifugation at 1,500 × *g* for 10 min at 4 °C and stored at −20 °C for lipid analysis. At the end of the experiment (10 weeks), overnight-fasted

animals were sacrificed, and their blood separated using the above mentioned centrifugation procedure. The liver, kidney, spleen, and abdominal adipose tissue were excised after dissection and weighed. TC, TG, HDL, LDL, superoxide dismutase (SOD), malondialdehyde (MDA), total antioxidant capacity (TAC), and plasma free fatty acid (FFA) levels in serum, as well as the hepatic glycogen content in liver were measured using standard kits. The hepatic glucokinase activity was determined using the spectrophotometric continuous assay and correlated with total hepatic protein content based on a previous report (Davidson & Arion, 1987). In addition, the hepatic fat deposition was analyzed from frozen liver sections stained with Oil Red O, which was used to measure cellular neutral lipid droplet accumulation.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD) of at least three individual experiments. Analysis was performed using Origin version 8.5 by one-way analysis of variance followed by Tukey's tests. A p value less than 0.05 was considered to be statistically significant, and a p value less than 0.01 was considered to be highly significant.

RESULTS AND DISCUSSION

Effects of AM, EM, AP, and EP on body weight and relevant tissue index

Although no significant differences in body weight were observed at week 10 (Table 1), the adipose index was significantly increased ($p < 0.05$) in HFF mice compared to ND mice. Treatment of AMM significantly decreased the adipose index by approximately 22% compared to the HFF group. In addition, high fructose- and high fat-induced reductions in the spleen index were significantly elevated by EMH, APM, EPM, and EPH treatments ($p < 0.05$).

Table 1: Effects of AM, EM, AP and EP on body mass and relevant tissue weight index

Groups	Weight (g)	Tissue index (g/100 g body weight)			
		Liver	Spleen	Kidney	Adipose
ND[a]	34.3±2.7	3.58±0.23	0.24±0.06	1.48±0.15	1.43±0.80
HFF[a]	33.3±5.0	3.73±0.35	0.19±0.06#	1.36±0.22	2.38±1.42#
AMM	34.2±3.0	3.64±0.44	0.17±0.03	1.31±0.12*	1.86±0.50*
EMM	32.7±3.6	3.85±0.16	0.19±0.05	1.36±0.14	2.22±0.78
EMH	34.5±2.1	3.89±0.42	0.21±0.06*	1.37±0.31	2.03±0.57
APM[a]	34.9±4.3	3.58±0.29	0.21±0.02*	1.39±0.23	2.37±1.02
EPM[a]	35.4±4.4	3.48±0.31	0.26±0.10*	1.47±0.08	2.38±0.71
EPH[a]	34.9±3.2	3.84±0.57	0.24±0.05*	1.43±0.20	2.30±0.73

Values are means±SD (n=10), *p<0.05 compared with HFF; #p<0.05 compared with ND; ND: normal diet; HFF: high-fructose and high-fat diet; AMM: medium dose of AM; EMM: medium dose of EM; EMH: high dose of EM; APM: medium dose of AP; EPM: medium dose of EP; EPH: high dose of EP, [a]Data of weight, liver index, and adipose index for ND, HFF, APM, EPM, and EPH were cited from previous study (Liu et al., 2014)

Spleen is an important antibody-producing organ and a cross-point for antigenic information transported by the blood and the immune system (Timens & Leemans, 1992). Spleen index of the HFF-treated group was significantly lower than that of the ND mice, which may represent the deterioration of spleen function. EMH, APM, EPM, and EPH treatments prevented the spleen from shriveling, suggesting an enhancement of immune function (Wang et al., 2010). However, in some reported diabetic mice models, no significant changes were observed in spleen index compared to normal mice (Chen et al., 2010; Zhang et al., 2010). Thus, it was therefore speculated that HFF not only impaired glucose regulation but also weakened the immune system.

Effects of AM, EM, AP, and EP on OGTT at weeks 3, 6, and 9

The average drinking volume of mice did not significantly differ among the groups (data not shown). As shown in Table 2, mice fed HFF developed impaired glucose tolerance

Table 2: Plasma glucose and AUC responses of OGTT after 3, 6, and 9 weeks

Groups	Blood glucose (mmol/L)				AUC
	0 min	30 min	60 min	120 min	(mmol×h/L)
Week 3					
ND[a]	5.5±0.5	18.5±3.0	12.9±2.2	6.5±0.7	23.6±2.9
HFF[a]	5.7±0.8	21.4±1.8#	14.3±2.8	7.3±1.0	26.5±2.1#
AMM	5.9±1.3	18.6±2.3*	11.8±1.2*	6.8±1.2	23.1±1.8*
EMM	6.2±1.1	21.4±2.9	12.9±2.5	6.8±0.8	25.3±3.3
EMH	5.7±0.9	20.0±2.5	13.0±3.4	7.1±1.2	24.7±4.1
APM[a]	5.6±1.0	18.2±4.0*	12.3±1.5	6.9±1.3	23.2±2.0*
EPM[a]	6.5±1.2	20.6±1.8	14.6±1.8	7.8±2.1	26.7±2.7
EPH[a]	5.2±0.9	17.7±3.0**	11.5±1.8**	6.0±0.8*	21.7±2.7**
Week 6					
ND[a]	4.7±0.8	15.8±2.5	10.0±0.9	6.2±0.8	19.7±1.5
HFF[a]	4.9±0.7	19.5±3.9##	12.0±3.3#	6.8±1.2	23.4±4.4##
AMM	4.7±0.8	16.4±2.2*	12.6±3.3	7.2±1.6	22.4±3.7
EMM	5.2±1.5	15.9±3.2*	11.1±1.6	6.5±0.6	20.9±2.9
EMH	4.8±0.5	17.4±4.6	12.6±2.2	7.0±1.3	22.8±4.1
APM[a]	4.3±0.7	17.3±2.0	10.9±0.9	7.6±0.8	21.7±0.9
EPM[a]	5.0±0.7	18.6±3.4	11.8±2.4	6.2±1.2	22.5±4.0
EPH[a]	4.6±0.9	14.6±3.1**	11.0±1.9	6.4±1.0	19.9±1.8**
Week 9					
ND[a]	7.0±0.6	16.3±2.0	11.6±1.5	7.7±0.7	22.4±1.8
HFF[a]	9.0±0.9##	19.1±2.0#	12.8±1.4	8.8±1.2#	25.7±1.4##
AMM	8.0±1.2	18.3±1.6	12.6±2.2	9.0±1.4	25.0±2.5
EMM	6.7±1.2**	13.8±2.2**	10.7±2.0	7.7±1.2	20.5±2.9**
EMH	6.6±0.6**	15.7±2.1**	10.6±1.8	8.3±1.4	21.6±2.7**
APM[a]	7.9±1.5	16.9±3.7	13.1±3.0	9.2±1.7	24.9±4.8
EPM[a]	7.1±1.2**	15.1±2.3**	11.5±1.5	7.9±1.2	22.0±2.8**
EPH[a]	6.8±1.4**	13.5±2.9**	10.3±2.0*	7.1±1.6**	19.8±3.5**

Values are means±SD (n=10), **p<0.01, *p<0.05 compared with HFF; ##p<0.01, #p<0.05 compared with ND; ND: normal diet; HFF: high-fructose and high-fat diet; AMM: medium dose of AM; EMM: medium dose of EM; EMH: high dose of EM; APM: medium dose of AP; EPM: medium dose of EP; EPH: high dose of EP, [a]Data of ND, HFF, APM, EPM, and EPH were cited from previous study (Liu et al., 2014)

after 3 weeks, indicating that glucose level was significantly elevated at 30 min and AUC was significantly increased when compared to ND ($p < 0.05$, Table 1). Supplementation with AMM, APM, and EPM had hypoglycemic effects, with significant ($p < 0.05$ or $p < 0.01$ vs. HFF group) reductions in AUC, whereas hypoglycemic effects were not detected in EMM and EMH groups. In addition, the hypoglycemic effect of EPH was sustained from 30 min to 120 min, with the plasma glucose eventually returning to basal level compared to ND. By the sixth week, no significant differences ($p > 0.05$) in AUC were observed among the groups fed with the extracts from mulberry diet compared to HFF. Moreover, the hypoglycemic effect of APM was diminished. Only the group receiving EPH supplementation showed 15.0% inhibition of AUC. After exposure to high-fructose and high-fat diet for 9 weeks, the fasting plasma glucose was significantly ($p < 0.01$) higher compared to that of NC mice, and the groups receiving ethanol extracts exhibited significantly ($p < 0.01$) lower fasting plasma glucose compared with the HFF group. In addition, significant ($p < 0.01$) reductions in AUC values were observed among all the ethanol extract, but not in the aqueous extract groups, and treatment of ethanol extract of PLCP was shown to induce a dose-dependent decrease in AUC, with significant hypoglycemic effects of EPH at all three time points. Ethanol extracts of PLCP appeared to exert prolonged and better effect than aqueous extracts. This difference may be attributed to the strong extraction capacity of 70% ethanol, which enabled extraction of alcohol-soluble, as well as some water-soluble active components that possessed excellent blood glucose-lowering activity. These results suggested that ethanol extracts of PLCP showed continuous hypoglycemic effect (from week 3 to week 9), while MPLCP was significantly effective only at week 9. Thus, the lost efficacy of ethanol extracts of MPLCP on glycemia during the first 6 weeks might be due to the addition of mulberry leaves. In the following three weeks, the antagonistic effect gradually diminished and was not apparent by week 9, indicating that the same active compounds existed in PLCP might have finally showed its property in MPLCP on hyperglycemia. Finally, both ethanol extracts of the two different diets could significantly improve the impaired glucose tolerance caused by HFF feeding. However, the underlying mechanism is still unknown.

Effects of AM, EM, AP, and EP on ITT at week 10

Insulin injection with 4 h fasted mice caused significant reduction of blood glucose levels ($p < 0.05$, Fig. 1). ITT revealed remarkable insulin resistance in the HFF group ($p < 0.05$) with a 1.67-fold increase in glucose level at 90 min compared to ND, whereas significant improvement in insulin resistance was observed in the ethanol extract groups. Compared to HFF mice, the plasma glucose at 90 min was significantly lowered by 45.7%, 51.4%, 48.6%, and 62.9% for EMM, EMH, EPM, and EPH mice, respectively ($p < 0.01$). Moreover, ethanol extracts of both two diets worked in a dose-dependent manner, with significant ($p < 0.05$) efficacy at 70 min. Fasting blood glucose levels in ITT test at week 10 were mostly higher than those in the OGTT test at week 9, which was likely due to fewer hours of food deprivation. As shown in OGTT at week 9 (Table 2), all the ethanol extracts of both diets improved glucose tolerance, and the data from ITT indicated their attenuation of peripheral insulin resistance as well. In all, the inhibition effect of mulberry on hypoglycemia could be diminished with time in MPLCP diet.

Effect of AM, EM, AP, and EP on HOMA-IR, glucokinase, and serum antioxidant status at week 10

Data in Table 3 indicated that the HOMA-IR was significantly ($p < 0.05$) elevated in HFF-treated mice and inhibited by EPM and EPH treatment ($p < 0.05$). However, when PLCP was added with mulberry leaf, the inhibitory effect was weakened ($p > 0.05$). In addition, the liver glucokinase level was significantly reduced by approximately 38.6% by HFF feeding, and this alteration was normalized by EMH and EPH treatments ($p < 0.05$). The increased glucokinase activity might be attributed to improved hepatic insulin sensitivity, because glucose uptake induced by insulin could stimulate glucokinase and glycogen synthesis (Tahrani et al., 2011).

Treatment with HFF exhibited a significant decrease ($p < 0.05$) in TAC level and SOD activities, and an increase in MDA level ($p < 0.01$), compared to ND (Table 3). After feeding with AMM, EMM, APM, EPM, and EPH, TAC levels were elevated by 29.2%, 59.2%, 21.7%, 25.0%, and 38.3%, respectively, and MDA levels were reduced by 29.4%, 40.3%, 36.1%, 40.3%, and 27.7%, respectively. The activities of SOD were only significantly increased (19.0%; $p < 0.05$) by EMM. MPLCP diet did not significantly improve antioxidant abilities when it was applied in high doses, probably because of the binding or antagonistic effect of mulberry leaves with antioxidant components in PLCP diet. However, further investigations are needed to understand the mechanisms.

Effects of AM and EM on liver steatosis

Histological evaluation revealed significant hepatic storage of lipids in the HFF-fed mice (Fig. 2), as indicated by increased area of Oil Red O staining of liver section. As shown in Fig. 2, TG accumulation was clearly alleviated by daily intake of AM and EM. Evidently, EM was more effective than AM in preventing liver steatosis in a dose-dependent manner. Hepatic steatosis could reduce the inhibitory effect of insulin on glucose production in

Table 3: Effects of AM, EM, AP, and EP on HOMA-IR index, hepatic glucokinase, serum FFA, and serum antioxidant status in mice after treatment for 10 weeks

Groups	HOMA-IR[a]	Hepatic glucokinase (nmol/min/mg pro)	FFA (mmol/L)	TAC (U/mL)	SOD (U/mL)	MDA (nmol/mL)
ND[b]	2.05±0.45	4.53±0.87	0.51±0.14	15.2±2.4	182.3±23.6	5.6±1.7
HFF[b]	3.32±0.66#	2.78±0.78#	0.73±0.26#	12.0±2.2##	152.9±19.4#	11.9±3.5##
AMM	2.47±0.77	2.82±0.55	0.71±0.14	15.5±1.2**	174.9±8.0	8.4±2.7**
EMM	2.68±0.84	3.86±0.65	0.62±0.18	19.1±7.4**	181.9±17.1*	7.1±0.9**
EMH	2.64±0.75	4.22±0.88*	0.54±0.16	13.4±2.1	165.5±14.7	9.9±2.6
APM[b]	2.74±0.64	2.62±0.20	0.67±0.18	14.6±1.8*	153.1±23.0	7.6±2.6**
EPM[b]	2.05±0.54*	3.01±0.85	0.46±0.16*	15.0±1.3*	166.2±24.9	7.1±1.8**
EPH[b]	2.09±0.70*	4.31±0.53*	0.48±0.19*	16.6±2.1**	170.5±12.4	8.6±2.8**

Values are means±SD (*n*=10). ***p*<0.01, **p*<0.05 compared with HFF; ##*p*<0.01, #*p*<0.05 compared with ND; ND: normal diet; HFF: high-fructose and high-fat diet; AMM: medium dose of AM; EMM: medium dose of EM; EMH: high dose of EM; APM: medium dose of AP; EPM: medium dose of EP; EPH: high dose of EP, [a]HOMA-IR=fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5, [b]Data of ND, HFF, APM, EPM, and EPH were cited from previous study (Liu et al., 2014)

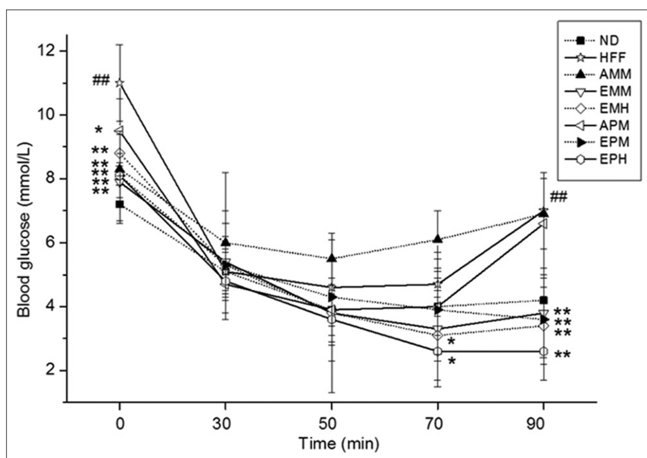

Fig 1. Blood glucose concentration of mice in ITT after treatment for 10 weeks. Values are means ± SD (n = 10). ***p*< 0.01, **p*< 0.05 compared with HFF; ##*p*< 0.01, #p < 0.05 compared with ND; ND: normal diet; HFF: high-fructose and high-fat diet; AMM: medium dose of AM; EMM: medium dose of EM; EMH: high dose of EM; APM: medium dose of AP; EPM: medium dose of EP; EPH: high dose of EP.

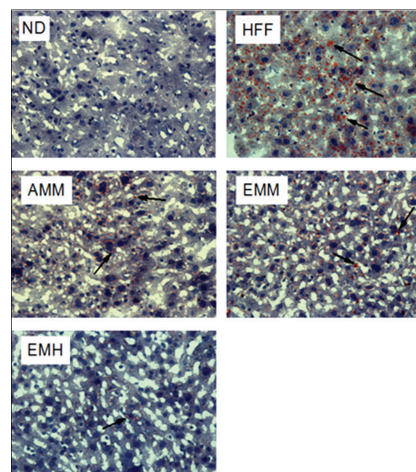

Fig 2. Histological changes in the liver (stain: Oil Red O and hematoxylin; original magnification: 200×). Lipid droplets stained by Oil Red O (arrows). ND: normal diet; HFF: high-fructose and high-fat diet; AMM: medium dose of AM; EMM: medium dose of EM; EMH: high dose of EM. The photos of ND and HFF were cited from previous study (Liu et al., 2014).

the liver, and intervene with insulin-induced activation of glucokinase (Samuel et al., 2004). In agreement with the histological findings, the decreased lipogenesis in the EMM- and EMH-treated groups might be responsible for the normalized fasting glucose and enhanced glucokinase activity, as indicated in Tables 2 and 3.

Effect of AM, EM, AP, and EP on serum lipids at weeks 4, 7, and 10

Changes in serum lipid profile during the study are summarized in Table 4. At week 4, HFF mice showed significant ($p < 0.01$) increase in plasma LDL (0.59 ± 0.15 vs. 0.37 ± 0.08) and TC (14.21 ± 2.75 vs. 6.81 ± 1.32), but a reduction in TG (1.81 ± 0.49 vs. 3.14 ± 0.97) compared to ND mice. The altered LDL and TC levels were significantly ($p < 0.05$ for LDL, $p < 0.01$ for TC) controlled by APM and EPH, and TC was inhibited by EPM supplementation. The LDL levels were dramatically ($p < 0.01$) elevated by EMM and EMH treatments. Meanwhile, the TC level was increased by EMM treatment, indicating that the diet with mulberry leaves aggravated serum lipid abnormality. Similar results were found at week 7. EPM and EPH diet supplementation resulted in significant attenuation of LDL and TC levels ($p < 0.05$ and $p < 0.01$, vs. HFF group), and both aqueous extracts of the two diets significantly reduced TC level in mice. However, mice under EMM treatment sustained higher levels of LDL and TC ($p < 0.05$, vs. HFF group). Likewise, the detrimental effect of EMM on lipid profile was noted at week 10. A significant ($p < 0.05$) increase in TC of 1.3-fold compared to HFF group and a modest increase in LDL were observed. In comparison, the TC increase caused by HFF was greatly suppressed by EPH by 19.0%. As shown in Table 3, plasma FFA level was significantly increased 1.4-fold in HFF-fed mice. EPM and EPH treatments significantly ($p < 0.05$) suppressed FFA level to a normal level. However, the addition of mulberry leaves prevented the suppression effect ($p > 0.05$, vs. HFF group). In all, data of EMM group showed higher TC level compared to HFF group throughout the experiment. In

Table 4: Lipid profiles in serum of mice at 4, 7, and 10 weeks of treatment

Groups	LDL-C (mM)	HDL-C (mM)	TC (mM)	TG (mM)
Week 4				
ND	0.37±0.08	2.71±0.60	6.81±1.32	3.14±0.97
HFF	0.59±0.15##	3.59±1.43	14.21±2.75##	1.81±0.49##
AMM	0.51±0.12	3.89±1.19	12.08±3.19	1.68±0.33
EMM	0.83±0.18**	3.45±1.55	17.01±3.85*	1.77±0.48
EMH	0.80±0.18**	3.84±1.55	16.08±3.31	1.83±0.42
APM	0.46±0.15*	4.03±1.01	10.99±2.36**	1.80±0.71
EPM	0.48±0.09	2.48±0.89	9.93±1.68**	2.47±0.62*
EPH	0.47±0.11*	2.89±1.49	9.76±2.79**	2.16±0.86
Week 7				
ND	0.35±0.06	2.57±0.53	5.62±1.16	2.62±0.66
HFF	0.71±0.27##	3.89±0.81##	12.94±2.73##	2.20±1.94
AMM	0.57±0.18	3.63±0.61	10.04±2.95*	1.50±0.42
EMM	0.90±0.18*	3.37±0.56	15.44±2.03*	1.33±0.25*
EMH	0.78±0.19	3.93±0.49	13.15±2.88	1.55±0.35
APM	0.62±0.25	3.29±0.96	10.29±4.13*	1.10±0.22*
EPM	0.54±0.14*	3.04±0.70*	9.48±1.84**	1.57±0.71
EPH	0.49±0.09*	2.81±0.68*	8.33±1.50**	1.58±0.33
Week 10				
ND[a]	0.25±0.07	4.20±0.96	5.78±1.00	1.33±0.42
HFF[a]	0.38±0.11##	4.96±1.11	10.57±1.88##	1.49±0.78
AMM	0.32±0.07	4.50±0.97	9.70±1.59	0.99±0.26
EMM	0.51±0.10	4.48±1.61	13.48±1.53*	1.37±0.81
EMH	0.42±0.12	4.81±1.29	11.43±2.39	1.04±0.37
APM[a]	0.47±0.11	4.31±1.30	10.72±1.35	1.23±0.45
EPM[a]	0.38±0.09	3.83±0.92*	9.78±2.79	1.31±0.48
EPH[a]	0.37±0.08	3.84±0.96	8.56±1.98*	1.23±0.30

Values are means±SD (*n*=10). ***p*<0.01, **p*<0.05 compared with HFF; ##*p*<0.01, #*p*<0.05 compared with ND; ND: normal diet; HFF: high-fructose and high-fat diet; AMM: medium dose of AM; EMM: medium dose of EM; EMH: high dose of EM; APM: medium dose of AP; EPM: medium dose of EP; EPH: high dose of EP, [a]Data of ND, HFF, APM, EPM and EPH on week 10 were cited from previous study (Liu et al., 2014)

addition, EM exerted more severe negative effect on serum lipids than AM, and the toxic effect of mulberry leaves appeared to diminish with time. Moreover, the deleterious effect was reduced when EM was supplied at high doses, probably because the increase of some components in PLCP inhibited the toxic effect of mulberry leaves. HDL levels increased in the HFF group compared to ND group, especially at week 7, which showed a significant elevation ($p < 0.05$). However, as a risk marker for cardiovascular disease, the TC to HDL ratio in the HFF group was higher than that in the ND group throughout the experiment, indicating susceptibility to cardiovascular disease after HFF feeding (Siri-Tarino et al., 2010). Bile acids are essential constituents of bile that facilitate digestion and absorption of lipids. Compared to the ND group, the serum TG levels of the HFF group exhibited a significant reduction at week 4 ($p < 0.01$), probably because of the increase in bile acid secretion stimulated by high-fat diet. However, no significant difference in serum TG was observed between the HFF and ND groups ($p > 0.05$) since week 7, indicating a severe pathogenic condition or a deficiency in bile secretion.

Although diet with mulberry leaves had potent hypoglycemic and anti-NAFLD properties in high-fructose and high-fat induced mice, its deteriorating effect on lipid metabolism is noteworthy and warrants further investigation. This is one of the few studies to show the negative effect of mulberry leaves on lipid metabolism. It is not clear whether these results can be translated to humans, but the present study does not provide support for the widespread use of mulberry leaf extracts for treating dyslipidemia. Further work needs to be undertaken to clarify the precise mechanisms involved in its improvement of the cholesterol level and its antagonistic effects with other herbs.

CONCLUSIONS

Although addition of mulberry leaves to the antidiabetic formula partially attenuated the changes caused by high-fructose and high-fat feeding, its intervention or deterioration effect on lipid metabolism does not support the use of mulberry leaves to treat dyslipidemia.

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Authors' contributions

XiaoXuan Guo conducted the experiment and drafted the manuscript; Jia Liu analyzed the blood parameters; HongJuan Zhang helped with the animal experiment; BaoPing Ji interpreted the results; Feng Zhou designed the study.

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